# The *Aspergillus fumigatus* Transcriptional Regulator AfYap1 Represents the Major Regulator for Defense against Reactive Oxygen Intermediates but Is Dispensable for Pathogenicity in an Intranasal Mouse Infection Model[▽][†]

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**Macrophages and neutrophils kill the airborne fungal pathogen *Aspergillus fumigatus*. The dependency of this killing process on reactive oxygen intermediates (ROI) has been strongly suggested. Therefore, we investigated the enzymatic ROI detoxifying system by proteome analysis of *A. fumigatus* challenged by $H_2O_2$. Since many of the identified proteins and genes are apparently regulated by a putative *Saccharomyces cerevisiae* Yap1 homolog, the corresponding gene of *A. fumigatus* was identified and designated Af*yap1*. Nuclear localization of a functional AfYap1-eGFP fusion was stress dependent. Deletion of the Af*yap1* gene led to drastically increased sensitivity of the deletion mutant against $H_2O_2$ and menadione, but not against diamide and NO radicals. Proteome analysis of the ΔAf*yap1* mutant strain challenged with 2 mM $H_2O_2$ indicated that 29 proteins are controlled directly or indirectly by AfYap1, including catalase 2. Despite its importance for defense against reactive agents, the Af*yap1* deletion mutant did not show attenuated virulence in a murine model of *Aspergillus* infection. These data challenge the hypothesis that ROI such as superoxide anions and peroxides play a direct role in killing of *A. fumigatus* in an immunocompromised host. This conclusion was further supported by the finding that killing of *A. fumigatus* wild-type and ΔAf*yap1* mutant germlings by human neutrophilic granulocytes worked equally well irrespective of whether the ROI scavenger glutathione or an NADPH-oxidase inhibitor was added to the cells.**

In the last few decades *Aspergillus fumigatus* has become the most important airborne fungal pathogen of humans. Diseases caused by *A. fumigatus* can be divided into three categories: allergic reactions and colonization with restricted invasiveness are observed in immunocompetent individuals, while systemic infections with high mortality rates occur in immunocompromised patients. Due to the improvement in transplant medicine and the therapy of hematological malignancies, the number of cases of invasive aspergillosis has increased. Specific diagnostics are still limited, as are the possibilities of therapeutic intervention, leading to a high mortality rate of 30 to 98% for invasive aspergillosis (8). The genome of the *A. fumigatus* isolate Af293 was fully sequenced. It consists of a haploid set of eight chromosomes with a total size of 29.4 Mb, of which 9,926 protein-encoding sequences were identified (44). With the genome data available the regulation of genes and the expression profile of proteins of *A. fumigatus* can be analyzed on a global scale, including the conditions that are related to infection.

The infectious agent of *A. fumigatus* are conidia, which are inhaled during routine daily activities (8). Therefore, in immunocompromised patients, the lung is the site of infection of *A. fumigatus*. In immunocompetent individuals, mucociliary clearance and phagocytic defense normally prevent the disease. Alveolar macrophages are the major resident cells of the lung alveoli; they, along with neutrophils (which are actively recruited during inflammation), are the major cells in the phagocytosis of *A. fumigatus*. Macrophages were shown to kill conidia by producing reactive oxygen intermediates (ROI) (46). Conidia escaping alveolar macrophages grow out and are attacked by neutrophils. Neutrophils adhere to the surface of the hyphae, since hyphae are too large to be engulfed. However, polymorphonuclear leukocytes (PMN) are also able to kill resting or swollen conidia (reviewed in reference 8). Contact between neutrophils and hyphae triggers a respiratory burst, secretion of ROI and hypochlorous acid, and degranulation (16, 36, 40, 52).

Several genes involved in the defense against ROI of *A. fumigatus* have been characterized. Although deletion of these genes increased the sensitivity to ROI of the respective mutant strains, none of them showed reduced virulence in a mouse infection model, e.g., deletion of *catA* (conidial catalase) resulted in increased sensitivity against $H_2O_2$ but did not affect pathogenicity (45). Mn-superoxide dismutase (SOD) and Cu/

Zn-SOD were characterized but, thus far, the structural genes have not been deleted (25). Therefore, we analyzed here the proteomes of *A. fumigatus* wild-type strain grown without oxidative stress and in the presence of $H_2O_2$. The results obtained indicated an involvement of the key transcriptional regulator AfYap1, which was identified here, in the regulation of several defense genes against ROI. The molecular analysis of AfYap1 allowed us to study on a global scale the impact of enzymatic defense systems against ROI for virulence. Our results indicate that the AfYap1-regulated antioxidant proteins do not play a major role in the pathogenicity of *A. fumigatus*. Therefore, killing mechanisms other than the production of ROI by the host are presumably more important than previously thought.

## MATERIALS AND METHODS

**Fungal and bacterial strains, media, and growth conditions.** The *A. fumigatus* wild-type strain ATCC 46645 was used to generate the Af*yap1* knockout (ΔAf*yap1*) strain (Af*yap1::hph*; Hyg[r]) by using the hygromycin resistance (Hyg[r]) gene as the selectable marker gene. The ΔAf*yap1* strain was complemented with the Af*yap1* gene using the phleomycin resistance gene *ble* as the selectable marker gene to give strain ΔAf*yap1::*Af*yap1*. Strain Afyap1-eGFP (*otef*p-Af*yap1*-*eGFP*; Hyg[r]) was obtained by transformation of ATCC 46645 with an Af*yap1*-*eGFP* gene fusion controlled by the *otef* promoter as described below and in reference 54. As the selectable marker, the Hyg[r] gene was used. *A. fumigatus* strains were cultivated at 37°C in *Aspergillus* minimal medium (AMM) as previously described (37). As solid medium, malt extract (2% [wt/vol] malt extract, 0.2% [wt/vol] yeast extract, 1% [wt/vol] glucose, 5 mM ammonium chloride, 1 mM dipotassium hydrogen phosphate) or AMM containing 3% (wt/vol) agar was used. Hygromycin (200 μg/ml) or phleomycin (100 μg/ml) was added to the media when required. For transformation of *Escherichia coli*, the strains DH5α (Bethesda Research Laboratories), XL1-Blue (Stratagene), or INVαF′ or TOP10F′ (Invitrogen, The Netherlands) were used. *E. coli* strains were grown at 37°C in LB medium supplemented, when required, with 50 μg of ampicillin or kanamycin per ml.

**Measurement of sensitivity against reactive agents.** A total of $10^7$ conidia of the strains tested were mixed with 2% (wt/vol) AMM agar and poured in a petri dish. A hole 1 cm in diameter was punched in the middle of the agar plate. The well was filled with 150 μl of 3% (vol/vol) $H_2O_2$, 100 μl of 0.1 M diamide solution, 100 μl of 1 mM menadione, or 100 μl of 90 mM NO donor 2,2′-(hydroxynitrosohydrazono)bis-ethanimine (DETA NO) or the corresponding DEA base. Agar plates were incubated for 16 h at 37°C. The inhibition zone of four independently incubated agar plates was measured for each tested strain.

**Standard DNA techniques.** Standard techniques in the manipulation of DNA were carried out as described by Sambrook and Russell (51). Chromosomal DNA of *A. fumigatus* was prepared as previously described for *A. nidulans* (2). For Southern blot analysis, the chromosomal DNA of *A. fumigatus* was cut by different restriction enzymes. DNA fragments were separated on an agarose gel and blotted onto Hybond N+ nylon membranes (GE Healthcare Bio-Sciences, Germany). Labeling of the DNA probe, hybridization, and detection of DNA-DNA hybrids were performed by using the DIG HighPrime labeling and detection system (GE Healthcare Bio-Sciences) according to the manufacturer's recommendations.

**Sequence analysis.** DNA was sequenced on both strands by primer walking using the BigDye terminator cycle sequencing kit (Applied Biosystems, United Kingdom). Sequencing reactions were separated on an Applied Biosystems ABI 310 sequencer. DNA sequence data were edited by using the programs Sequence Navigator and Auto Assembler (Applied Biosystems). The analysis of sequences was carried out by using GeneWorks 2.2 (IntelliGenetics, Inc.). Amino acid sequence comparisons were performed by using the BLAST 2.0 server (www.ncbi.nlm.nih.gov/BLAST). The programs PepStat and CLUSTAL W (Husar 4.0; DKFZ, Heidelberg, Germany) were used for the prediction of protein molecular masses and amino acid sequence alignments, respectively. Shading of aligned amino acid sequences was performed with MacBoxShade 2.15 (www.ch.embnet.org/software/BOX_form.html). Features and amino acid motifs of proteins were predicted by using the ISREC server (http://hits.isb-sib.ch/cgi-bin/PFSCAN).

**Cloning of the *A. fumigatus* Af*yap1* gene.** To generate an eGFP fusion of AfYap1 under the control of the *otef* promoter (54), cDNA was used as the template for amplification of the Af*yap1* gene with the primers Yap10 (5′-

ATTGGATCCGACGCGACCCATGATGTCCTC) and Yap16 (5′-ATTGGAT CCCTTCGTTTTCAGTTGCCATGGCGG-3′). The PCR product generated was cloned into plasmid pCR2.1 (Invitrogen, The Netherlands). The DNA fragment encoding the Af*yap1* gene was reisolated by cutting the recombinant plasmid obtained with BamHI and cloning the DNA fragment into plasmid pUCGH (34) to give plasmid pOtef-Afyap1-eGFP.

**Generation of the Af*yap1* knockout plasmid.** To construct the Af*yap1* deletion plasmid pHyg-Δyap1, a 1.1-kbp PCR product consisting of the upstream region of the Af*yap1* gene was generated by using the oligonucleotides Yap1 (5′-CAC GGCCTGAGTGGCCCTAGGCCATAGGCGATTGC-3′) and Yap2 (5′-ATT GGATCCCGAAGCGGGGTATCG-3′). To amplify the 1-kbp downstream region of the Af*yap1* gene, oligonucleotides Yap3 (5′-ATTAGCTTGGACATCA TGGGTCGCG-3′) and Yap4 (5′-GTGGGCCATCTAGGCCCATTCGGTCCC CTTCGAC-3′) were used. Both flanking regions were cloned into plasmid pCR2.1 (Invitrogen, The Netherlands). The generated plasmid pCR2.1-right border was linearized with XhoI and XbaI. The Hyg[r] cassette was isolated by XhoI and MluI from the plasmid pANsCos.1. The left border of the knockout construct was isolated from the plasmid pCR2.1-left border (primer Yap1 and Yap2) with MluI and XbaI. Both fragments were ligated into the plasmid pCR2.1-right border to give plasmid pHyg-Δyap1. The DNA fragment used to replace the Af*yap1* gene was excised from plasmid pHyg-Δyap1 by using the restriction endonucleases SfiI and XbaI. It was used to transform the *A. fumigatus* wild-type strain ATCC 46645.

**Complementation of the Af*yap1* deletion mutant strain.** To complement the deletion of the Af*yap1* mutant strain, the Af*yap1* gene under the control of its own promoter was isolated from the plasmid pHyg-yap1-eGFP with PstI. The DNA fragment obtained was cloned into the plasmid pAN8.1 (accession no. Z32751), which consists of the *ble* gene from *Streptoalloteichus hindustanus* under the control of the *gpdA* promoter. The generated plasmid pAN8.1-yap1 was used to transform the *A. fumigatus* ΔAf*yap1* strain. Single integration of the plasmid was checked by Southern blot analysis (data not shown).

**Transformation of *A. fumigatus*.** Transformation of *A. fumigatus* was carried out using protoplasts as previously described (63).

**Northern blot analysis.** RNA isolation was performed with the RNeasy plant minikit according to the manufacturer's instructions (Qiagen). 10 μg of total RNA was blotted on a Hybond N+ membrane (GE Healthcare). Hybridization and probe labeling were performed with the Dig Easy Hyb kit according to the instructions of the manufacturer (Roche Applied Sciences). The probe for *cat1* (AFUA_2G00200) was amplified with the primers CAT1-F (5′-CTT CCTTGTTCCAGGGCG-3′) and CAT1-R (5′-GCGGCTGGTCATACTCC-3′); the probe for *cat2* (AFUA_8G01670) was amplified with the primers CAT2-R (5′-CGATCTCCTGATCCTCACC-3′) and CAT2-R (5′-CCAGGA CGATGAGATCAGC-3′).

**Catalase activity assay and determination of protein concentrations.** *A. fumigatus* strains were grown in AMM for 24 h at 37°C. After stress induction with 2 mM $H_2O_2$ for 45 min, mycelia were harvested, frozen in liquid nitrogen, and ground with pestle and mortar. Mycelia were suspended in extraction buffer (100 mM potassium phosphate buffer [pH 6.3]) and incubated on ice for 15 min. Samples were centrifuged at 4°C with $12,000 \times g$ for 10 min. The protein concentrations were determined according to the method of Bradford (7a). Then, 30 μg of protein extract was loaded onto an 8% polyacrylamide (wt/vol) Tris-glycine gel (Invitrogen). The catalase activity was determined directly in the gel according to the method of Goldberg and Hochman (21). The photometric determination of catalase activity was determined after addition of $H_2O_2$ by measuring the decrease in absorbance at 240 nm as described previously (7).

**Isolation of PMN from peripheral blood.** PMN were isolated from the blood of healthy volunteers using polymorph-prep gradient centrifugation as described previously (49). Remaining red blood cells were lysed with ACK lysis buffer (Sigma-Aldrich, Germany). PMN purity was checked by fluorescence-activated cell sorting using anti-CD66b monoclonal antibody (BD) and was determined to be >95%. The cell number was adjusted using a hemacytometer.

**Cytochrome *c* reduction assay.** A cytochrome *c* reduction assay was carried out according to the method of Babior et al. (6). A total of $10^6$ conidia of *A. fumigatus* strains were incubated for 9 h at 37°C in RPMI–5% (vol/vol) fetal calf serum (FCS; Gibco, Germany). Hyphae were harvested by centrifugation, coincubated with $10^7$ freshly isolated PMN, and resuspended in RPMI–5% (vol/vol) FCS in the presence of 75 μM cytochrome *c* from horse heart (Sigma-Aldrich). Samples were obtained every 30 min, cells were removed by centrifugation, and the absorbance of the supernatants was measured at 550 nm. A sample without hyphae was used as a reference control. The process is inhibited by adding horse heart SOD (Sigma-Aldrich). Three independent biological replicates were analyzed, and a standard deviation of the mean value was calculated for each time point.

**Quantification of oxidative burst.** Primary human neutrophils were incubated at a multiplicity of infection (MOI) of 10 with *A. fumigatus* hyphae in the presence of 2.5 $\mu$M dichlorfluoresceine diacetate (DCFH-DA; Sigma-Aldrich) for 145 min at 37°C. The generation of ROI was quantified in the presence or absence of 4 mM glutathione and 16 $\mu$M diphenyleneiodonium chloride (DPI) by measuring the conversion of DCFH-DA into green fluorescent dichlorfluoresceine (49). A fluorescence reader (GENios; Tecan, Germany) which measured the relative fluorescence units in 5-min intervals (excitation, 485 nm; emission, 520 nm) was used to monitor the production of ROI.

**Confrontation assay.** For plate-based inactivation assays, conidia of fungal strains were dispensed in RPMI 1640 plus 5% (vol/vol) FCS, followed by incubation overnight at room temperature in a shaker. Germ-tube formation was induced at 37°C for 4 h directly before the assay. A total of $8 \times 10^4$ PMN and $8 \times 10^4$ fungal cells were mixed in 40 $\mu$l of fresh RPMI 1640 plus 5% (vol/vol) FCS (MOI = 1), activated with 23 ng of phorbol myristate acetate/ml and incubated for different periods of time at 37°C (0, 120, 160, and 200 min). PMN were lysed by the addition of 2 ml of ice-cold distilled water. Dilutions of this suspension were plated on malt extract agar and incubated for 24 h at 37°C. When indicated, 4 mM glutathione was added to the assay to scavenge ROI or 16 $\mu$M DPI was used for the inhibition of NADPH oxidase activity. Each experiment was repeated independently with PMN from at least five different donors. Average values and standard deviations were calculated, and the significance was tested by using the Student *t* test.

**Animal infection model.** A murine low-dose model for invasive aspergillosis was applied (37) with modifications as previously described (31). In brief, 18- to 20-g female BALB/c mice were immunosuppressed with 150 mg of cyclophosphamide (Sigma-Aldrich)/kg on days −4, −1, 2, 5, 8, 11, and 14 prior to and after infection on day 0. A single dose of cortisone acetate (200 mg/kg; Sigma-Aldrich) was injected subcutaneously on day −1. *A. fumigatus* conidia suspensions were harvested with phosphate-buffered saline (PBS) containing 0.1% (vol/vol) Tween 80 (Merck, Germany) and filtered twice through Miracloth (Calbiochem, Germany). Mice were anesthetized by intraperitoneal injection of 100 mg of ketamine (WDTeG, Germany) and 10 mg of xylazine (RompunR; Bayer, Germany)/kg and intranasally infected with 25 $\mu$l of a fresh suspension containing $6 \times 10^4$ conidia. Survival was monitored daily, and moribund animals were killed humanely. A control group remained uninfected (inhalation of PBS) to monitor the influence of the immunosuppression procedure on vitality. Infections were performed with a group of 10 mice for each tested strain, and the whole experiment was carried out in duplicates. Mice were cared for in accordance with the principles outlined by the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (European Treaty Series, no. 123 [http://conventions.coe.int/treaty/Default.asp]).

**2D gel electrophoresis analysis.** Samples for two-dimensional (2D) polyacrylamide gel electrophoresis (2D-PAGE) were prepared with slight modifications as previously described (27). The pH of the samples was adjusted to 8.5 by the addition of 100 mM NaOH. Afterwards, the samples were labeled with CyDye minimal dyes according to the manufacturer's protocol (GE Healthcare Biosciences). Briefly, 50 $\mu$g of protein of either sample was labeled with 300 pmol of CyDye (dissolved in dimethyl formamide). Samples from stress-induced and noninduced conditions were labeled either with Cy3 or Cy5. A pool of two samples (induced and noninduced) was prepared, labeled with Cy2, and used as an internal standard. Samples were vortex mixed and incubated for 30 min in the dark on ice. The reaction was stopped by adding 1 $\mu$l of 10 mM L-lysine. An equal volume of 4× sample buffer (composition as for the lysis buffer mentioned above, plus 3.2% [vol/vol] ampholytes and 40 mM dithiothreitol) was added. Equal amounts of each of the three label preparations were mixed and applied via anodic cup loading onto a IEF-strip.

Strips of 24 cm covering a nonlinear pH range from pH 3 to 11 or a linear pH range from pH 4 to 7 (GE Healthcare Bio-Sciences), which had been rehydrated overnight (7 M urea, 2 M thiourea, 2% [wt/vol] CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}, 1% [wt/vol] Zwittergent 3-10, 0.002% [wt/vol] bromophenol blue, 0.5% [vol/vol] IPG buffer, 1.2% [vol/vol] De-Streak reagent [GE Healthcare Bio-Sciences]) were used for isoelectric focusing as previously described (27). Prior to the start of separation by the second dimension, the strips were equilibrated for 15 min in 10 ml of equilibration buffer containing 1% (wt/vol) dithiothreitol and subsequently for 15 min in 10 ml of equilibration buffer containing 2.5% (wt/vol) iodoacetamide. Electrophoresis was performed as described by Kniemeyer et al. (27). Proteins were visualized by analyzing the gels with a Typhoon 9410 scanner using a resolution of 100 $\mu$m. Spot detection of cropped images was performed with the DeCyder software package (version 5.0). The following parameters were applied: detection sensitivity, 2,500 spots (excluding filter set); slope, >2.1; area, <200; peak height, <600; and area, <10,000. Changes in the abundance of protein spots were

regarded as significant with a threshold of two standard deviation difference. Gels of five independent experiments were analyzed with the BVA software, and average ratios as well as *t* test values for each spot were calculated. Only spots with a *t* test value below 0.05 were regarded as significant. In order to analyze differently expressed proteins by mass spectrometry (MS), separate preparative gels were run and stained with colloidal Coomassie brilliant blue (27).

**MS identification.** Proteins were identified on a Bruker Ultraflex I matrix-assisted laser desorption ionization–time of flight (MALDI-TOF)/TOF device (Bruker Daltonics, Germany) operating in reflectron mode. MS spectra were subsequently identified by searching the NCBI database using the MASCOT interface (MASCOT 2.1.03; Matrix Science, United Kingdom) with the following parameters: Cys as a *S*-carbamidomethyl derivative and methionine in an oxidized form (variable), one missed cleavage site, and a peptide mass tolerance of 200 ppm. Hits were considered significant according to the MASCOT score ($P$ = 0.05). Database searches were improved by iterative recalibration and application of the peak rejection algorithm filter of the Score Booster tool implemented into the Proteinscape 1.3 database software (Protagen, Germany).

## RESULTS

**Proteome analysis of *A. fumigatus* grown under reactive oxygen stress.** To get a comprehensive picture of the defense of *A. fumigatus* against ROI, we analyzed the proteomes of *A. fumigatus* wild-type strain grown either without oxidative stress or challenged with 2 mM $H_2O_2$ for 45 min (Fig. 1). This time point was chosen, since in a preceding enzyme assay thioredoxin reductase showed maximum activity at between 30 and 60 min after addition of $H_2O_2$ (data not shown). Furthermore, the concentration of 2 mM $H_2O_2$ did not cause any growth inhibition and did not show any effect on spore germination (data not shown). $H_2O_2$ increases intracellular peroxide ($O_2^{2-}$) levels, which leads to the direct oxidation of the sulfur-containing amino acids and the generation of $OH^-$ radicals (22). The results of the proteome analysis are shown in Fig. 1 and Table 1. Reproducibly, 27 protein spots displayed an increase and 17 protein spots displayed a decrease in intensity of >1.5-fold (2-fold standard deviation) in extracts of $H_2O_2$ grown mycelia (see Table S1 in the supplemental material). Some proteins appeared in gels as more than one spot with the same apparent molecular mass, but with different pI values and abundance, presumably due to posttranslational modifications or isoenzyme variation, e.g., allergen AspF3 and the peroxiredoxin Prx1. Therefore, the identified 44 spots represented 28 different proteins (Table 1 and Fig. 1). The protein spot of each protein showing the highest change in abundance is depicted in Fig. 1. Several stress-associated proteins showed an increased level upon $H_2O_2$ stress (Table 1), including Cu/Zn-SOD, the mitochondrial peroxiredoxin Prx1, the allergen AspF3, and the spermidine synthase. AspF3 is a putative thioredoxin peroxidase and was the most highly induced protein after stimulation with $H_2O_2$. AspF3 is a known allergen in patients suffering from allergic bronchopulmonary aspergillosis (11). The protein could be extracted from the surface of conidia (4), although it was originally found in peroxisomal membranes (11). In *Saccharomyces cerevisiae*, the homolog Tsa1p functions as peroxidase, chaperone, and regulator for gene expression under stress conditions (14). It is therefore conceivable that AspF3 also functions in the detoxification of ROI. The peroxiredoxin Prx1 contains both a putative mitochondrial signal peptide and a conserved cysteine characteristic of peroxiredoxins. This finding makes it likely that this protein has thioredoxin peroxidase activity. The significant upregulation of the thioredoxin system in response to increased
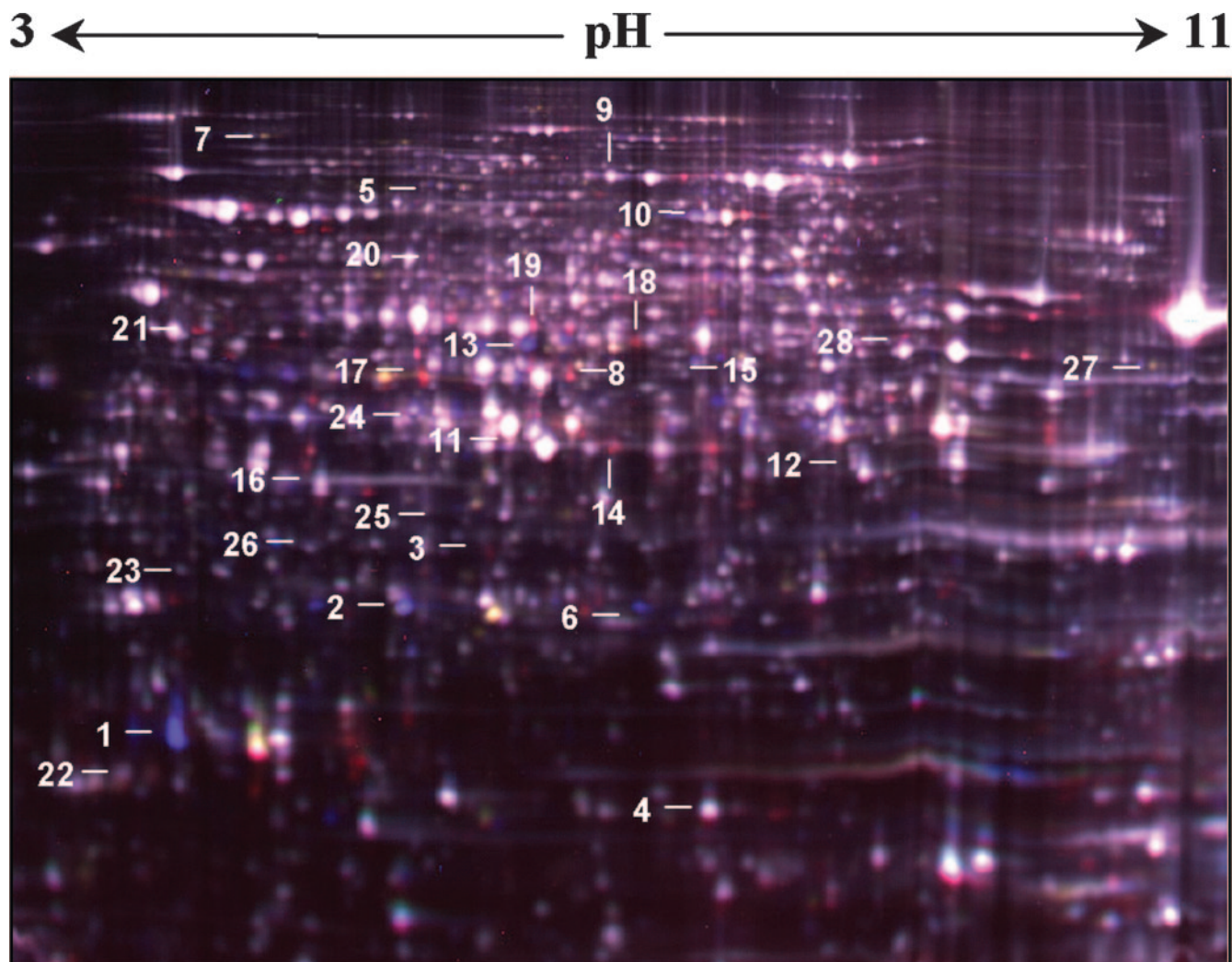
FIG. 1. 2D gel electrophoresis of protein extracts of wild type (Cy3-red) and wild type challenged with 2 mM $H_2O_2$ (Cy5-blue) for 45 min. Proteins were stained with the difference in gel electrophoresis (DIGE) labeling technique. The orientation of the IEF is indicated. The numbers refer to proteins whose levels changed significantly during growth with $H_2O_2$ (see Table 1).

levels of $H_2O_2$ indicates its importance for *A. fumigatus*. Spermidine synthases are involved in the biosynthesis of polyamines, which play a pivotal role in defense against environmental stresses. *Cryptococcus neoformans* mutants with the spermidine synthase gene *spe3* deleted showed reduced capsule formation, melanin production, and growth rate. A role of polyamine biosynthesis for virulence was reported (26). Several genes encoding the proteins shown in Table 1, which were upregulated under ROI stress, are known to be regulated in *S. cerevisiae* by the bZip-type transcription factor Yap1p. Therefore, it was likely that a homolog of Yap1 existed in *A. fumigatus* and, moreover, which could be of importance for defense against ROI. In the yeast *S. cerevisiae*, the transcriptional regulator Yap1p represents the key regulator of both the metabolic stress response and the acquisition of stress tolerance (5, 24).

**Identification and characterization of a Yap1 homolog of *A. fumigatus*.** We analyzed the *A. fumigatus* genome for the presence of a putative *S. cerevisiae yap1* homologue. On chromo-

some 6, a single gene was identified that we designated Af*yap1* (Fig. 2). Mutational analyses had established that a set of cysteine residues located in the carboxy-terminal cysteine-rich domain (c-CRD) region of both *S. cerevisiae* Yap1 and Cap1, the Yap1 homolog in *C. albicans* (1), is required for the export of Yap1 and Cap1 from the nucleus to the cytoplasm by the export protein Crm1 under nonstress conditions. Upon oxidant exposure, which changes the conformation of the c-CRD and masks the nuclear export signal (NES), Yap1 and Cap1 accumulate in the nucleus (10, 30, 64, 66). As shown in Fig. 2A, the five cysteine residues required for nuclear maintenance upon oxidation of Yap1p are conserved in AfYap1. In addition, putative homologs for the yeast gene *gpx3*, whose protein product was shown to oxidize Yap1 upon challenge with ROI (13), and the exportin gene *crm1*, were identified in the *A. fumigatus* genome as AFUA_3G12270 and AFUA_1G08790, respectively. The deduced *A. fumigatus* open reading frames show sequence similarity to the respective *S. cerevisiae* proteins of 59 and 54%, respectively.

TABLE 1. Differentially synthesized proteins of *A. fumigatus* after incubation with 2 mM $H_2O_2$

| Spot no.[c] | Putative function and protein name[a] | Ratio[b] 15 min | Ratio[b] 45 min |
|---|---|---|---|
| | **Proteins with antioxidant properties** | | |
| 1 | Allergen Asp F3 (AFUA_6G02280) | 4.3 | 10.3 |
| 2 | Mitochondrial peroxiredoxin Prx1 (AFUA_4G08580) | 2.1 | 3.7 |
| 3 | Cytochrome *c* peroxidase (AFUA_4G09110) | 2.0 | −2.0 |
| 4 | Cu/Zn-SOD (AFUA_5G09240) | 2.7 | 1.2 |
| 5 | Catalase Cat1 (AFUA_2G00200) | | −1.3 |
| | **Heat shock** | | |
| 6 | 30-kDa heat shock protein (AFUA_3G14540) | 1.8 | 2.5 |
| | **Proteases** | | |
| 7 | Zn dependent peptidase (AFUA_4G07910) | 1.2 | −2.0 |
| | **Protein translocation** | | |
| 8 | IDI2 (AFUA_4G05830) | 1.0 | −1.6 |
| 9 | Alanyl-tRNA synthetase (AFUA_8G03880) | 1.8 | 1.4 |
| | **Pentose phosphate pathway** | | |
| 10 | Transketolase TktA (AFUA_1G13500) | 2.1 | 2.1 |
| 11 | Transaldolase (AFUA_5G09230) | 2.3 | −1.0 |
| | **Glycolysis, pyruvate metabolism** | | |
| 12 | Glyceraldehyde-3-phosphate-DH (AFUA_5G01970) | 1.9 | 2.3 |
| 13 | Pyruvate-DH subunit α (AFUA_1G06960) | 1.7 | 3.2 |
| | **Fatty acid or lipid metabolism** | | |
| 14 | Zn-dependent alcohol-DH (AFUA_5G06240) | 1.0 | −2.1 |
| 15 | Acetyl coenzyme A acetyltransferase (AFUA_8G04000) | 1.2 | 1.6 |
| | **Polyamine pathway** | | |
| 16 | Spermidine synthase (AFUA_1G13490) | 2.9 | 1.3 |
| | **Amino acid metabolism** | | |
| 17 | Glutamine synthetase (AFUA_4G13120) | −2.0 | −2.0 |
| 18 | Carbamoylphosphate synthase (AFUA_5G06780) | −1.2 | −2.2 |
| 19 | Adenosylhomocysteinase (AFUA_1G10130) | 1.0 | 1.9 |
| | **Cytoskeleton** | | |
| 20 | Actin interacting protein 2 (AFUA_5G02230) | −3.1 | −1.3 |
| 21 | Beta-tubulin (AAL01593) | −1.8 | 1.1 |
| 22 | Topomyosin (AFUA_7G04210) | −1.7 | −1.5 |
| | **Regulatory protein** | | |
| 23 | 14-3-3 family protein ArtA (AFUA_2G03290) | 2.2 | 1.2 |
| | **Cofactor biosynthesis** | | |
| 24 | Thiamine biosynthesis protein Nmt1 (AFUA_5G02470) | 1.1 | 2.0 |
| | **Purine and pyrimidine synthesis** | | |
| 25 | Phosphoribosyltransferase (AFUA_4G04550) | 1.2 | 3.4 |
| | **Unclassified and proteins of unknown function** | | |
| 26 | GMC-oxidoreductase (AFUA_3G01580) | 2.3 | 6.2 |
| 27 | Conserved NAD-dependent DH (AFUA_7G00350) | −1.1 | −2.1 |
| 28 | UGP1 (AFUA_7G01830) | −2.1 | −2.3 |

[a] DH, dehydrogenase.
[b] Average ratios extracted from statistical analysis of DIGE gels by the Decyder software programs DIA and BVA.
[c] Spot number in Fig. 1.

**Deletion of the Af*yap1* gene and analysis of its importance for defense against $H_2O_2$, menadione, diamide, and NO radicals.** To analyze the importance of AfYap1 for the defense of *A. fumigatus* against reactive agents, we deleted the Af*yap1* gene in the *A. fumigatus* wild-type strain ATCC 46645. For this

purpose, plasmid Δyap1-Hyg-TOPO was generated (see Materials and Methods). As the selection marker, the hygromycin B resistance gene was used. After transformation, deletion of the Af*yap1* gene in the resulting transformants was detected by Southern blot analysis (Fig. 2C and D). The band characteristic for the Af*yap1* gene of the wild type (3 kbp) had disappeared in the ΔAf*yap1* mutant strains. Instead, the band characteristic of gene replacement of the Af*yap1* gene of 1.5 kbp was detected. The ΔAf*yap1* strain was viable and showed slightly reduced growth on malt agar plates (data not shown). As a control, the ΔAf*yap1* deletion mutant was complemented using the wild-type Af*yap1* gene under the control of its own promoter and using phleomycin as the selection marker gene (data not shown). To verify a single ectopic integration, the complementation was checked by Southern blotting with an internal Afyap1 probe.

To determine whether AfYap1 was required for the resistance of *A. fumigatus* against reactive agents, the wild type, the ΔAf*yap1* mutant strain, and a complemented mutant strain (ΔAf*yap1*/Af*yap1*) were placed on AMM agar plates containing a concentration gradient of $H_2O_2$, menadione, diamide, or the NO donor DETA NO. Among the stress-inducing agents selected, menadione generates superoxide anions ($O_2^-$) in a redox cycle, which inactivates 4Fe-4S cluster-containing proteins. Menadione may also affect the reduced glutathione (GSH) pool directly via a detoxification reaction catalyzed by glutathione *S*-transferase (48). Diamide is a thiol-oxidizing agent resulting in fast oxidation of GSH to oxidized glutathione (GSSG), resulting in a GSH/GSSG redox imbalance (57), and proteins with the possibility to form disulfide bonds become oxidized. DETA NO is a compound leading to the release of NO· (17). The NO radicals interact with, e.g., superoxide $O_2^-$ anions, and form the more reactive peroxynitrite. However, other oxidation states of NO· and the formation of other reactive nitrogen intermediates (RNI) are also possible. The MIC at which 50% of *C. albicans* isolates are inhibited by DETA NO was determined to be 0.25 to 1 mg/ml (39). RNI have been shown to inhibit mitochondrial respiration, causing DNA damage and leading to nitrosylation of thiol groups (17).

As shown in Fig. 3, the Af*yap1* deletion mutant was much more susceptible to $H_2O_2$ and menadione than was the wild type. Expression of the wild-type Af*yap1* gene complemented the hypersensitivity of the ΔAf*yap1* strain against $H_2O_2$ and menadione to an extent similar to that of the wild-type strain. Interestingly, the ΔAf*yap1* mutant strain did not exhibit increased sensitivity to diamide and DETA NO. The phenotypes observed suggest that AfYap1 is a key regulator of antioxidant defense of *A. fumigatus* directed toward peroxides and superoxide anions. In contrast, AfYap1 is not involved in the detoxification of NO radicals and diamide. Diamide oxidizes sulfhydryl groups of proteins and leads to Yap1p nuclear localization in *S. cerevisiae* (58). A deletion of *yap1* leads in contrast to *A. fumigatus* to an increased sensitivity to diamide (30).

**Identification of putative AfYap1 targets.** To analyze possible targets of AfYap1-dependent regulation, we examined the global effect of Af*yap1* deletion at the proteomic level. For this purpose, protein extracts of the wild-type and ΔAf*yap1* strains challenged with $H_2O_2$ were compared by 2D-PAGE. Reproducibly, 21 protein spots displayed an increase and 36 protein spots displayed a decrease in intensity of more than 1.3-fold
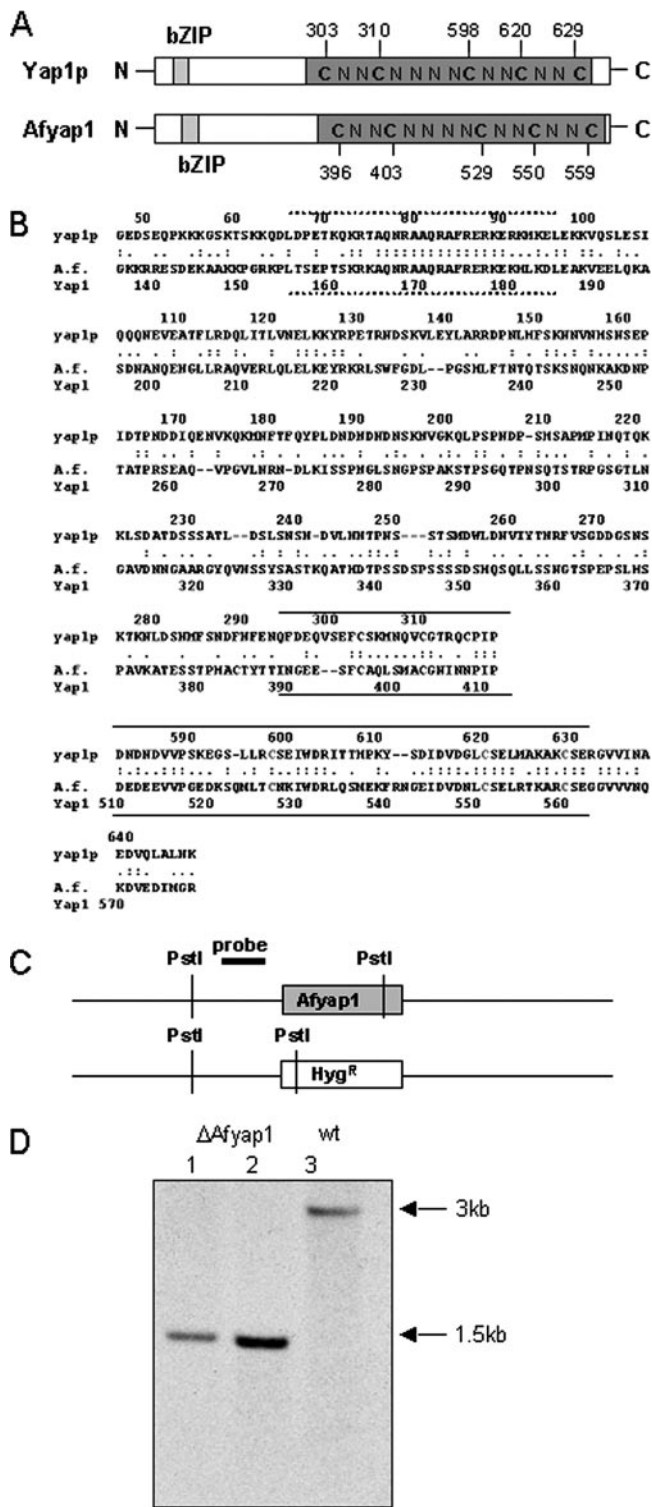
FIG. 2. *A. fumigatus* Yap1. (A) Schematic view of characteristic motifs of yeast protein Yap1p and *A. fumigatus* protein AfYap1. Numbers indicate the position of conserved cysteine residues in the NES. (B) Partial sequence alignment of AfYap1 from *A. fumigatus* (AFUA6G09930) with Yap1p of *S. cerevisiae* (YML007W) with CLUSTAL W. Identical amino acids between the different amino acid sequences at the same position are marked with colons. The numbers above the sequence indicate the positions of amino acids shown in the respective proteins. DNA interaction domain (bZIP domain) is marked with a dashed line, and t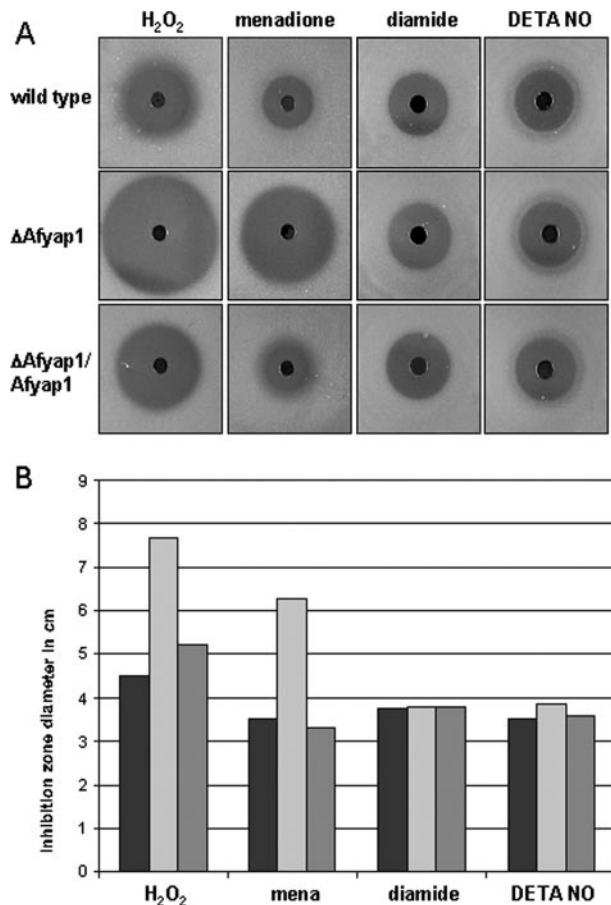he CRDs are marked with a contin-uous line. (C) Deletion of *A. fumigatus* Afyap1 gene. Schematic representation of the chromosomal Afyap1 locus of the wild type and the ΔAfyap1 deletion mutants. The PstI cleavage sites and the position to which the probe hybridizes are indicated. (D) Southern blot analysis of the Afyap1 deletion strains. Chromosomal DNA of the parental wild-type strain ATCC 46645 (lane 3) and the mutant ΔAfyap1 strain (lanes 1 and 2) was cut by PstI. An 830-bp Afyap1 flanking PCR fragment was used as a probe. In the ΔAfyap1 mutant strain, the band characteristic of the wild type (lane 3) had disappeared. Instead, bands characteristic for a gene replacement at the Afyap1 locus were detected.



FIG. 3. Sensitivity of *A. fumigatus* strains against different reactive agents. (A) Inhibition zone assay. A total number of $10^7$ conidia were mixed with 2% (wt/vol) AMM top agar. In the middle of the agar plate a well was filled either with $H_2O_2$, menadione, diamide, or DETA NO. The inhibition zones were measured 16 h after incubation at 37°C. (B) Schematic view of sensitivity assay. Inhibition zone diameters of the wild-type strain (black bar), ΔAfyap1 strain (light gray bar), and complemented ΔAfyap1/Afyap1 strain (dark gray bar) are indicated in centimeters. The ROI-producing agents $H_2O_2$, menadione (mena), diamide, and DETA NO are indicated on the left. The base DETA that corresponds to DETA NO was also tested, and no difference in sensitivity was observed for the ΔAfyap1 and the wild-type strain (data not shown).

(2-fold standard deviation) in ΔAfyap1 extracts (see Table S2 in the supplemental material). Some proteins appeared in gels as more than one spot with a different pI, e.g., methyltran-ferase, transketolase TktA, and UGP 1. In all, 29 protein spots,

TABLE 2. Decreased protein spots (and therefore putative AfYap1 targets) in *A. fumigatus* ΔAf*yap1* strain compared to wild type after exposure to 2 mM $H_2O_2$ for 45 min (pH gradient gel 4 to 7)

| Function and protein name[a] | Ratio[b] |
|---|---|
| **Proteins with antioxidant properties** | |
| Allergen Asp F3 (AFUA_6G02280) | −2.81 |
| Mitochondrial peroxiredoxin Prx1 (AFUA_8G07130) | −2.31 |
| Cytochrome *c* peroxidase (AFUA_4G09110) | −1.43 |
| Catalase Cat1 (AFUA_2G00200) | −2.01 |
| Catalase/peroxidase Cat2 (AFUA_8G01670) | −2.02 |
| **Heat shock** | |
| Chaperone HSP70 HscA (AFUA_8G03930) | −1.75 |
| HSP70 chaperone Hsp88 (AFUA_1G12610) | −1.68 |
| 30-kDa heat shock protein (AFUA_3G14540) | −1.67 |
| Heat shock protein class 1 (AFUA_6G06470) | −2.01 |
| **Proteases/protein degradation** | |
| Ubiquitin UbiA (AFUA_1G04040) | −2.46 |
| **Protein synthesis** | |
| Elongation factor EF-3 (AFUA_7G05660) | −2.22 |
| Elongation factor-1γ (AFUA_1G17120) | −4.79 |
| Eukaryotic initiation factor 5A (AFUA_1G04070) | −2.91 |
| **Glycolysis** | |
| Pyruvate-DH subunit α (AFUA_1G06960) | −1.71 |
| **Pentose phosphate pathway** | |
| Transketolase TktA (AFUA_1G13500) | −2.04 |
| Phosphoglucomutase PgmA (AFUA_3G11830) | −1.67 |
| **Purine and pyrimidine synthesis** | |
| GMP synthase (AFUA_3G01110) | −1.51 |
| **Unclassified and proteins of unknown function** | |
| Methionine synthase MetH/D (AFUA_4G07360) | −2.39 |
| Aminotransferase (AFUA_2G12470) | −1.82 |
| Peptidyl *cis-trans* isomerase (AFUA_3G07430) | −1.85 |
| Histone demethylase Aof2 (AFUA_4G13000) | −2.02 |
| Septin AspB (AFUA_7G05370) | −3.03 |
| Hypothetical protein AFUA_5G14680 | −2.01 |
| UGP 1 (AFUA_7G01830) | −2.11 |
| Hypothetical protein AFUA_3G00730 | −1.54 |
| *p*-Nitroreductase family protein (AFUA_5G09910) | −2.25 |
| PH domain protein (AFUA_4G12450) | −1.79 |
| Hypothetical protein AFUA_3G11400 | −1.57 |
| Hypothetical protein AFUA_1G09890 | −1.46 |

[a] Name and function are derived from GenBank (National Center for Biotechnology Information). DH, dehydrogenase.
[b] Average ratios are extracted from statistical tables of the Decyder software after DIA and BVA analysis.

including catalases, showed a decreased level in the ΔAf*yap1* mutant compared to the wild-type strain after exposure to 2 mM $H_2O_2$ and therefore present putative AfYap1 targets (Table 2).

**Catalases regulated by AfYap1.** The proteome analysis suggested that catalases are regulated by AfYap1. By measuring catalase activity, we examined which of the catalases was controlled by AfYap1. For this purpose, the wild-type strain and the Af*yap1* deletion strain were challenged with $H_2O_2$ for 45 min. Protein extracts were generated, and equal amounts of protein were loaded onto a native polyacrylamide gel. The observed, negative-stained bands were assigned according to Paris et al. (45). As shown in Fig. 4A, the activity of catalase
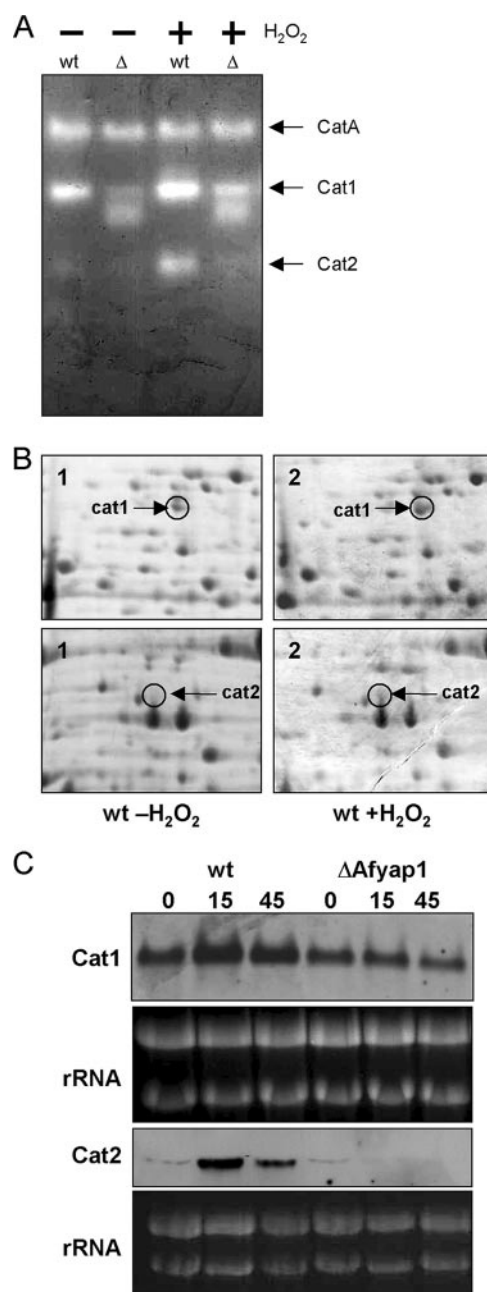


FIG. 4. Regulation of catalases 1 and 2 by oxidative stress. (A) Catalase activity staining modified according to the method of Goldberg and Hochman (21). Overnight cultures of wild type (wt) and ΔAf*yap1* mutant strain (Δ) were incubated with (+) or without (−) 2 mM $H_2O_2$ for 45 min before protein extracts were isolated. The negatively stained bands were allocated to the three different catalases CatA, Cat1, and Cat 2 as described previously (45). (B) Sections of Coomassie blue-stained 2D gels from *A. fumigatus* wild-type mycelium grown with (panels 1) or without (panels 2) 2 mM $H_2O_2$ for 45 min. Catalases 1 and 2 were identified by MALDI-TOF MS and marked with arrows. (C) Northern blot analysis of the Af*yap1*-dependent expression of catalase *cat1* and *cat2* under oxidative stress conditions. RNA was isolated from $H_2O_2$ stressed mycelia of *A. fumigatus* wild-type and ΔAf*yap1* strains after 0, 15, and 45 min of stress induction. rRNA bands are shown as controls.

Cat2 was completely absent in the ΔAf*yap1* mutant strain, and the activity of catalase Cat1 was reduced. Inspection of the putative promoter regions of the two catalase-encoding genes in *A. fumigatus* suggests the presence of two putative AfYap1 binding sites [TTA(G/C/T)TAA] in the Cat2 promoter and none in the Cat1 promoter (29). In addition, low activity of the conidial CatA was detected (Fig. 4). Catalase activity was also determined photometrically, and the decreased activity in the ΔAf*yap1* strain confirmed the results obtained by the activity staining after native PAGE. Protein extracts derived from wild-type mycelium showed an increase in catalase/peroxidase activity from 2.6 to 4.4 $\mu$mol min$^{-1}$ mg of protein after the addition of 2 mM $H_2O_2$, whereas in the ΔAf*yap1* strain the activity increased only from 0.6 to 2.6 $\mu$mol min$^{-1}$. 2D gels from proteins extracted from the cytosol showed a similar regulation of Cat1 and Cat2, as observed in the catalase activity assays (Fig. 4B). Both catalases were also detectable in supernatants of culture medium (data not shown). To confirm the data obtained by both 2D gel electrophoresis and activity staining on the transcriptional level, we carried out Northern blot analysis. The steady-state mRNA level of catalase *cat1* in *A. fumigatus* was only slightly $H_2O_2$ stress or AfYap1 dependent (Fig. 4C). The transcript level of catalase *cat2* was highly upregulated 15 min after the addition of $H_2O_2$ and slightly reduced after 45 min (Fig. 4C). In the Af*yap1* deletion strain no induction of *cat2* transcript levels was detectable, suggesting that the Yap1 response elements (YREs) at positions −140 and −605 in the promoter region of *cat2* are presumably functional.

**Nuclear localization of AfYap1 is stress dependent.** Previous work on both *S. cerevisiae* and *C. albicans* demonstrated that regulation of the Yap1 transcription factors involved oxidant-dependent nuclear localization of the protein (30, 66). As shown in Fig. 2A, the five cysteine residues required for nuclear maintenance upon oxidation of Yap1 are conserved in AfYap1. To analyze whether AfYap1 is also regulated by subcellular localization, the Af*yap1* gene was fused in frame with the gene encoding the enhanced green fluorescence protein (*egfp*) (Fig. 5). After transformation of *A. fumigatus* with plasmid *otef*p-Af*yap1-eGFP*, the cellular localization of the AfYap1-eGFP fusion was analyzed in the transformant strains. As shown in Fig. 5, in the absence of $H_2O_2$ the protein fusion exhibited diffuse cytoplasmic fluorescence. At 15 min after treatment with $H_2O_2$, the protein fusion began to localize in the nucleus, as seen by coincidence with the DAPI (4′,6′-diamidino-2-phenylindole) stain. After 60 min of $H_2O_2$ treatment, the AfYap1-eGFP signal fully accumulated in the nucleus. This accumulation was reversible, and after 120 min a diffuse cytoplasmic fluorescence was observed again. Hence, the cellular localization of the AfYap1-eGFP fusion protein is indicative of $H_2O_2$ stress to the fungal cell.

In the ΔAf*yap1* mutant, the presence of the GFP fusion protein containing wild-type AfYap1 exhibited $H_2O_2$ resistance that was indistinguishable from that of the wild-type strain (data not shown). Consistently, when the AfYap1-eGFP fusion protein was produced in the wild-type strain, the respective mutant strain showed elevated $H_2O_2$ tolerance above that of the wild-type strain, indicating the functionality of the protein fusion (data not shown).

**Despite the importance of AfYap1 for defense against ROI, deletion of Af*yap1* did not result in attenuated virulence in a mouse infection model.** To assess the role of AfYap1 in pathogenesis, the corresponding deletion mutant was tested in an animal infection model of invasive aspergillosis. Groups of 10 immunosuppressed, neutropenic mice were infected by inhalation with $6 \times 10^4$ conidia of the different strains. The results are shown in Fig. 6. In the groups infected with wild-type conidia (strain ATCC 46645), the mortality was 90% after 17 days. Similarly, when mice were infected with conidia of the ΔAf*yap1* mutant strain, the mortality was almost the same as that observed for the wild-type strain (Fig. 6). As a control, mice were infected with conidia of the complemented Af*yap1* mutant (ΔAf*yap1*/Af*yap1*), which showed the same virulence as wild-type conidia (Fig. 6). In the uninfected control group (PBS), mortality was 0%.

**Generation of ROI by PMN during contact with conidia of the different strains.** To study whether the generation of ROI by PMN depends on the different *A. fumigatus* mutant strains analyzed, a cytochrome *c* reduction assay (61) for measuring the presence of peroxides $O_2^-$ was applied. Thus, freshly isolated PMN from healthy human donors were coincubated with wild-type, ΔAf*yap1*, and ΔAf*yap1*/Af*yap1* hyphae, and the reduction of cytochrome *c* was measured over time at 550 nm (data not shown). No significant difference in the amount of cytochrome *c* reduction was observed for the tested strains, excluding that the amount of ROI generated by PMN differs between the strains analyzed.

**Survival of *A. fumigatus* wild type and ΔAf*yap1* mutant confronted with PMN.** To test whether ROIs are involved in the killing of hyphae by human PMN, *A. fumigatus* germ tubes of the wild-type strain, the ΔAf*yap1* mutant strain, and the complemented ΔAf*yap1*/Af*yap1* mutant strain (data not shown) were coincubated for 160 min with freshly isolated human PMN. In preliminary experiments with the wild-type strain, ca. 50% killing was achieved after 160 min of coincubation. No phagocytosis was seen, but attachment of PMN to the germ tubes was observed. As shown in Fig. 7, there was no significant difference in survival rate irrespective of whether the wild type or the Af*yap1* deletion mutant was tested. The same was also true for the complemented Af*yap1* deletion mutant (ΔAf*yap1*/Af*yap1*) (data not shown). The addition of glutathione to the assay did not display any effect on killing of *A. fumigatus* hyphae by PMN, further supporting that it is unlikely that ROI are important for killing of *A. fumigatus* hyphae by PMN under the conditions tested. At this concentration, glutathione was able to scavenge ca. 90% of ROI produced by human PMNs after stimulation with *A. fumigatus* hyphae in preliminary experiments using DCFH-DA as an indicator for ROI (data not shown). In control experiments, 4 mM glutathione did not affect fungal viability (J. Loeffler, unpublished data). To further substantiate these findings, NADPH oxidase activity in PMNs was inhibited by 5 $\mu$g of DPI/ml, which corresponds to a concentration of 16 $\mu$M. This concentration reduced the amount of ROI produced by PMN after contact with *A. fumigatus* hyphae to ca. 30% and did not significantly affect fungal viability (data not shown). In former studies, 10 $\mu$M DPI was shown to inhibit the rate of killing of the bacterial pathogen *Staphylococcus aureus* by 77% (23). Based on control experiments, higher concentrations of DPI were not used due
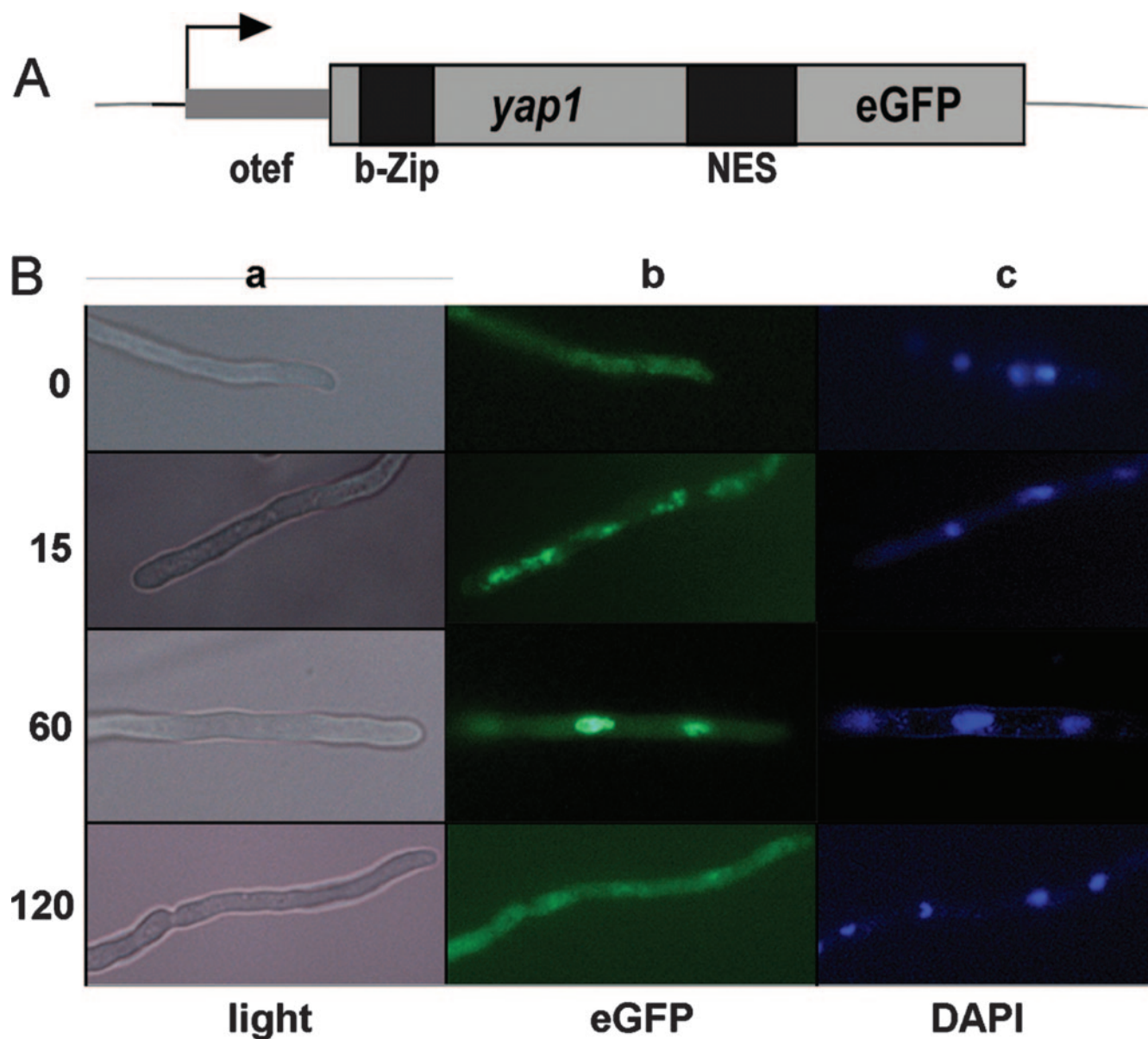
FIG. 5. Oxidative stress-regulated nuclear localization of AfYap1-eGFP. (A) Schematic representation of *otef*p-Af*yap1-eGFP* fusion construct. The putative bZIP domain and NES of AfYap1 are indicated. (B) Localization of AfYap1-eGFP. Overnight culture of strain otef-AfYap1-eGFP in AMM was challenged with 2 mM $H_2O_2$ for 0, 15, 60, and 120 min. Samples were analyzed by light microscopy (a) or fluorescence microscopy (b and c). Panel b shows the green fluorescent protein fluorescence signal. Nuclei were stained with DAPI (c).

to adverse effects on fungal growth. When added to the assay, the survival rate of hyphae of the wild type did not differ from that of the ΔAf*yap1* strain in the presence of PMN at any time point with or without ROI inhibitors (Fig. 7).

## DISCUSSION

A central aim of this study was the characterization of the stress response of *A. fumigatus* to ROI. The mechanisms of cellular redox homeostasis and the ROI metabolism are of particular interest, because ROI were shown to be important for macrophages in defense against the fungal pathogen *A. fumigatus* (46). We report here the identification of 27 proteins with increased levels and 17 protein spots with decreased levels upon exposure to 2 mM $H_2O_2$ by proteome analysis. In *S. cerevisiae*, this $H_2O_2$ concentration was found to induce stress defense at the molecular level (12). Consistently, as shown here, the Afyap1-eGFP was located in the nucleus at this $H_2O_2$ concentration, showing that AfYap1 was activated. Based on the proteome data a distinct picture of cellular processes involved in the adaptation to higher oxygen peroxide levels was obtained. Primarily affected are proteins required for antioxidant defense; heat shock proteins; the protein translation apparatus; proteins of central metabolic pathways such as glycolysis, the tricarboxylic acid cycle, and the pentose phosphate cycle; proteins of the amino acid and trehalose metabolism;
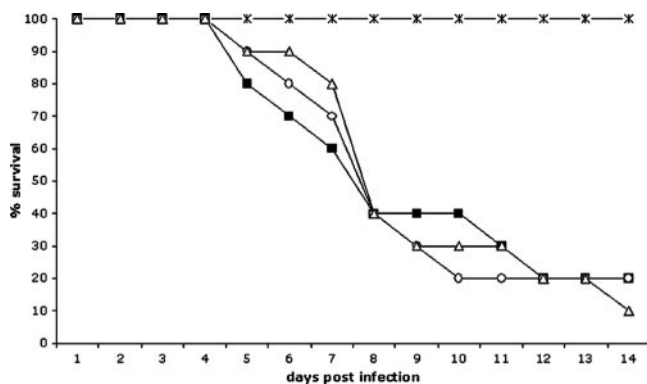
FIG. 6. Virulence of an Af*yap1* deletion strain. Virulence of *A. fumigatus* wild-type ATCC 46645 (■), ΔAf*yap1* (○), and complemented ΔAf*yap1* (△) strains in mice. Groups of 10 BALB/c mice were infected with $6 \times 10^4$ *A. fumigatus* conidia each, as indicated, by nasal inhalation. Survival was monitored for 14 days. A group of mice which inhaled PBS served as control (×).



FIG. 7. Survival rate of *A. fumigatus* hyphae of the wild-type (ATCC 46645) and ΔAf*yap1* strains after confrontation with human PMN. Freshly isolated human PMN were stimulated for 30 min with phorbol myristate acetate and coincubated for 160 min with young hyphae from the wild type (left) or the ΔAf*yap1* mutant strain (right) at an MOI of 1. Viable hyphae were quantified by plating after osmotic lysis of the PMN. White bars represent the percentage of viable wild-type or mutant hyphae in relation to controls without PMN (black bars). The addition of 4 mM GSH (dark gray bars) or 16 μM DPI (light gray bars) showed no effect on the survival of *A. fumigatus*.

and proteins of the cytoskeleton. Thus, the antioxidant response to ROI in *A. fumigatus* resembles the protection mechanism in *S. cerevisiae* (19, 20), *Schizosaccharomyces pombe* (62), and *Candida albicans* (32). However, as also found here, the response of proteins of the cytoskeleton to ROI seems to be stronger in filamentous fungi (47). Moreover, the upregulation of the thioredoxin system clearly shows its importance in the protection of *A. fumigatus* against $H_2O_2$, whereas the upregulation of catalases was only moderate and, interestingly, proteins of the glutathione system were not detected at all. The importance of the thioredoxin system for redox regulation has also been shown for *A. nidulans* (55).

In the present study, the Yap1 homologue of *A. fumigatus* was identified and designated AfYap1. The genes of several $H_2O_2$-induced proteins in *A. fumigatus* contained putative YREs in their promoter regions, such as allergen Asp F3, peroxiredoxin Prx1, and transaldolase. This finding suggests that the genes are direct targets of AfYap1, provided the identified YREs are functional. In contrast, it is also possible that the genes are only indirectly regulated by AfYap1 or that AfYap1 regulates the genes in cooperation with other transcription factors such as Skn7 and TFIIA, as known for genes in *S. cerevisiae* (28, 42). While the two transcription factors Yap1p and AfYap1 exhibit a high degree of sequence similarity, little information was available on the regulation of Yap1p homologs in filamentous fungi. We provide several lines of evidence that the bZip transcription factor AfYap1 functions in a similar way. First, the AfYap1-eGFP fusion protein showed nuclear localization under $H_2O_2$ stress (Fig. 5) similar to Yap1 accumulation in both *S. cerevisiae* and *C. albicans* (30, 66). Second, deletion strains of Af*yap1* in *A. fumigatus* exhibited increased sensitivity against $H_2O_2$ and menadione on agar plates similar to the *S. cerevisiae* Δ*yap1* and *C. albicans* Δ*cap1* mutants (60, 66). Third, complementation of the Af*yap1* deletion mutant by ectopic integration of the Af*yap1* gene restored the wild-type phenotype (Fig. 3). In contrast to *S. cerevisiae* (30), deletion of Af*yap1* did not lead to increased sensitivity to diamide. A similar phenotype has also been shown for *A. nidulans* (3). Because wild-type and Af*yap1* deletion strains did
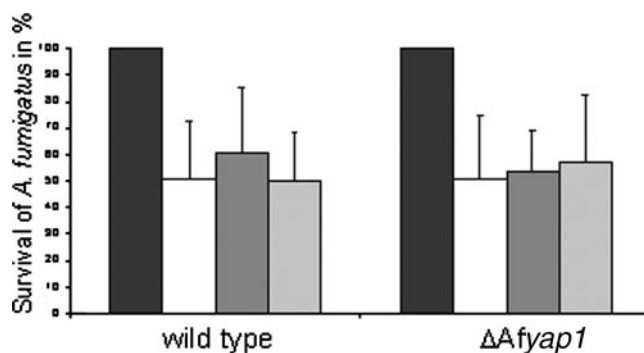
not exhibit increased sensitivity against the NO· donor agent DETA NO and the corresponding base DEA on agar plates, this led us to conclude that AfYap1 does not play a role in NO· detoxification in *A. fumigatus*. However, this finding does not exclude that an overlap between antioxidant and antinitrosative defense exists, because both ROI and RNI target thiols or iron-sulfur clusters (18). Consistently, a *Cryptococcus neoformans* mutant with a thiol peroxidase gene (*tsa1*) deleted was sensitive to both nitrosative and oxidative stress (41).

To characterize the targets of AfYap1 in the filamentous fungus *A. fumigatus*, we compared the proteome of the wild type and the ΔAf*yap1* deletion strain upon exposure to $H_2O_2$. The peroxiredoxin Prx1 did not show different levels in dependence of the Af*yap1* deletion upon challenge with $H_2O_2$ and is presumably not a target of AfYap1. Other known Yap1 targets followed characteristic AfYap regulation: thioredoxin peroxidase (AspF3), cytochrome peroxidase, and the protein AFUA_3G00730, which appears to belong to the glutathione *S*-transferase family. Proteins of the protein degradation pathway were regulated by AfYap1 as well, which is also known for yeast species (19, 60). In addition, a new putative AfYap1 target was identified with the *p*-nitroreductase family protein AFUA_5G09910. The members of this large family of eubacterial conserved proteins catalyze the reduction of nitro-substituted compounds using FMN or FAD as a prosthetic group and NAD(P)H as a reducing agent. The biological functions of these enzymes have not been elucidated, but a putative role in maintaining the oxidative and/or nitrosative balance within cells was discussed (15).

Because of the important function of catalases for scavenging ROI and their possible regulation by AfYap1, these enzymes were investigated in more detail. Three catalases were described in *A. fumigatus*: the conidial catalase CatA and the two mycelial catalases Cat1 and Cat2. Deletion of *cat1* in *A. fumigatus* increased the sensitivity of the deletion mutant to ROI but had no effect on pathogenicity (9, 45). Only a *cat1*/*cat2* double deletion in *A. fumigatus* increased the sensitivity of

the respective mutant to ROI and caused a delayed infection in a murine infection model (45). Here, we identified both mycelial catalases Cat1 and Cat2 by 2D gel analysis of $H_2O_2$ challenged mycelia and found that the activity of Cat2 but not of Cat1 clearly depends on the presence of ROI and AfYap1. We therefore conclude that catalase 2 is controlled by AfYap1. A Yap1-dependent regulation was also shown for catalase A in *S. cerevisiae* (19) and *C. albicans* (32). However, in *S. cerevisiae* in cooperation with the transcription factor Skn7, Yap1 controls several genes of the oxidative stress response, such as thioredoxin, thioredoxin reductase, and the catalase T (35). Such a regulation is also likely to occur for some genes in *A. fumigatus*, such as the catalase Cat 1. Also, an Skn7 ortholog is present in *A. fumigatus* (33).

The role of ROI for host resistance during microbial infections is a matter of debate. As reported here, the Af*yap1* deletion in *A. fumigatus* exhibited drastically increased sensitivity to $H_2O_2$ and menadione (Fig. 3) but did not show reduced pathogenicity in an in vitro killing assay with human PMN and in an infection model applying immunosuppressed, neutropenic mice (Fig. 6). The same was shown for a putative interaction partner of AfYap1, the response regulator Skn7. Deletion of the *skn7* homolog Af*SKN7* in *A. fumigatus* led to increased sensitivity against peroxides, but virulence was not affected. In contrast to the ΔAf*yap1* strain, the ΔAf*skn7* mutant exhibited no enhanced sensitivity against menadione (33). Therefore, the outcome of our study implies that superoxide and peroxide detoxification mechanisms of *A. fumigatus* do not play an important role during infection in immunocompromised mice or in the extracellular killing of *A. fumigatus* germtubes by neutrophils. Consequently, killing mechanisms other than the production of ROI by the host appear to be more relevant. Alternatively, it cannot be ruled out that the protective role of AfYap1 against ROI is dispensable in vivo because other mechanisms take over this task. Nevertheless, our results are in agreement with recent findings that the granule proteins in neutrophils play a major role in the killing process of microbes and ROI only function as activators of vacuolar enzymes (50, 53). This conclusion was also supported by the finding that knockout mice lacking the ability to produce cathepsin G and elastase were found to be susceptible to *Aspergillus* infection (56). Granulocytes are also able to produce RNI and hypochlorous acid to kill microbes (16, 17). The production of both RNI and ROI in granulocytes is regulated by gamma interferon. The production of ROI is therefore always coupled to the production of RNI. Hence, RNI may play a more relevant role in the killing of fungi than originally anticipated. However, NO synthase deficiency is not always detrimental (38), and macrophages generally produce considerably more RNI than neutrophils (43). In the same way, macrophages from iNO synthase-deficient mice did not show reduced in vitro killing of *A. fumigatus* conidia (46). Alternatively, the myeloperoxidase-$H_2O_2$-halide system may also be involved in the damage of hyphae by neutrophils (16), but a direct role for this system remains to be demonstrated. In addition, ROI are required as a substrate for the production of hypochlorous acid (53). Hence, the mechanism for how the innate immune system kills and digests spores and germlings of *A. fumigatus* needs to be further elucidated with a focus on non-ROI-mediated killing. One candidate for such non-ROI-

mediated killing is represented by the recently found extracellular fibers called neutrophil extracellular traps. They may mediate the extracellular killing of hyphae of *A. fumigatus*, since this has been shown for both the yeast-form and hyphal cells of *C. albicans* (59). The transferrin family protein lactoferrin with a high sequestration activity for iron is another recently found factor secreted by PMN, which mediates the inhibition of conidial growth of *A. fumigatus* independently of ROI production (65).

In summary, in the present study we show that the regulatory response upon oxidative stress of *A. fumigatus* is to a large extent regulated by AfYap1. For the first time, the ROI detoxifying system of *A. fumigatus* against $H_2O_2$ was characterized in detail. Taken as a whole, the present study strongly argues that the function and regulation during oxidative stress mediated by AfYap1 is not of significant importance for pathogenicity in an immunocompromised host. Consequently, our data challenge the idea that the innate immune system, in particular PMN, kills *A. fumigatus* mycelium during infection by the production of peroxides and superoxide anions.

## ACKNOWLEDGMENTS

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