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Authors
Tian, Chaoguang
Li, Jingyi
Glass, N Louise

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Exploring the bZIP transcription factor regulatory network in *Neurospora crassa*

Chaoguang Tian,[1,2] Jingyi Li[1]† and N. Louise Glass[1]

Correspondence
N. Louise Glass
Lglass@berkeley.edu

[1]Department of Plant and Microbial Biology, University of California, Berkeley, CA 94720-3102, USA

[2]Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin 300308, PR China

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Transcription factors (TFs) are key nodes of regulatory networks in eukaryotic organisms, including filamentous fungi such as *Neurospora crassa*. The 178 predicted DNA-binding TFs in *N. crassa* are distributed primarily among six gene families, which represent an ancient expansion in filamentous ascomycete genomes; 98 TF genes show detectable expression levels during vegetative growth of *N. crassa*, including 35 that show a significant difference in expression level between hyphae at the periphery versus hyphae in the interior of a colony. Regulatory networks within a species genome include paralogous TFs and their respective target genes (TF regulon). To investigate TF network evolution in *N. crassa*, we focused on the basic leucine zipper (bZIP) TF family, which contains nine members. We performed baseline transcriptional profiling during vegetative growth of the wild-type and seven isogenic, viable bZIP deletion mutants. We further characterized the regulatory network of one member of the bZIP family, NCU03905. NCU03905 encodes an Ap1-like protein (NcAp-1), which is involved in resistance to multiple stress responses, including oxidative and heavy metal stress. Relocalization of NcAp-1 from the cytoplasm to the nucleus was associated with exposure to stress. A comparison of the NcAp-1 regulon with Ap1-like regulons in *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Candida albicans* and *Aspergillus fumigatus* showed both conservation and divergence. These data indicate how *N. crassa* responds to stress and provide information on pathway evolution.

INTRODUCTION

The fungal kingdom is an excellent model for the study of transcription factor (TF) diversity and evolution, primarily due to the functional characterization and identification of direct target genes of TFs in the yeast *Saccharomyces cerevisiae*. The duplication of TFs (paralogues) and subsequent regulatory network divergence are major forces in the evolution of phenotypic complexity. In *S. cerevisiae*, the basic leucine zipper (bZIP) family of TFs is an excellent example of gene duplication and divergence of function. In *S. cerevisiae*, the bZIP TF family contains 14 genes and can be divided into three groups. The largest is the *YAP1* group, which includes eight members. Five *YAP1* family members (*YAPs 1*, *2*, *4*, *5* and *6*) have been implicated in the oxidative stress and DNA-damage responses (Tan *et al.*, 2008; Workman *et al.*, 2006). The differences in gene regulation among the Yap members can be attributed to two important mechanisms: sequence differences in the DNA-binding motifs that are targeted by the different Yap proteins (Cohen *et al.*, 2002), and variations in the regulation of the Yap proteins themselves, including post-translational modifications such as phosphorylation and disulfide bond formation (Kuge *et al.*, 2001). The integration of expression profiles and chromatin-binding studies of the Yap proteins in response to DNA damage have shown a significant overlap of Yap regulons, especially between Yap1 and Yap2 (Tan *et al.*, 2008). The second largest group of bZIP members in *S. cerevisiae* includes SKO1, ACA1 and CST6 (ACA2). Strains containing a mutation in SKO1 are affected in osmotic and oxidative stress, while mutations in ACA1 and CST6 result in strains that are affected in the utilization of alternative carbon sources (Rep *et al.*, 2001). The third group of bZIP TFs in

†Present address: Department of Molecular and Cellular Pharmacology, University of Miami, Miami, FL 33101, USA.

Abbreviations: 3-AT, 3-aminotriazole; bHLH, basic helix–loop–helix; bZIP, basic leucine zipper; ChIP data, chromatin-immunoprecipitation data; FunCat analysis, Functional Category analysis; HMM, hidden Markov model; β-ME, β-mercaptoethanol; qRT-PCR, quantitative real-time RT-PCR; TF, transcription factor; WT, wild-type.

The microarray data discussed in this paper are available from the *Neurospora* Functional Genomics Microarray database (experiment ID no. 141) (http://bioinfo.townsend.yale.edu/browse.jsp).

Five supplementary tables and two supplementary figures are available with the online version of this paper.

S. cerevisiae contains a single gene encoding Gcn4, which regulates genes in response to amino acid starvation (Hinnebusch, 2005).

Genome sequences of about 50 additional fungal genomes are currently available, and a number of these organisms, such as *Aspergillus nidulans*, *Magnaporthe grisea* and *Neurospora crassa*, have genetic tools available to assess the functions of predicted TF genes. In the filamentous fungus *N. crassa*, there are about 200 predicted general TFs (with and without DNA-binding domains) (Borkovich *et al.*, 2004; Galagan *et al.*, 2003; Shiu *et al.*, 2005). The predicted DNA-binding TFs in *N. crassa* mostly fall into six families, i.e. the basic helix–loop–helix (bHLH), bZIP, C2H2 zinc finger, GATA factor, Myb and Zn binuclear cluster families. Recently, whole-genome microarrays for *N. crassa* have been validated and have provided expression data associated with asexual spore germination and vegetative growth (Kasuga *et al.*, 2005; Kasuga & Glass, 2008). From a National Institutes of Health (NIH)-funded *Neurospora* program project, a large number of gene deletion strains have been constructed, including a viable deletion set of 103 predicted TF genes (Colot *et al.*, 2006; Dunlap *et al.*, 2007). The availability of such tools makes it possible to dissect TF networks and to compare regulatory networks that have evolved among paralogous TFs (homologues within one species) and between orthologous TFs (homologues between different species). Previously, we showed that the regulons of the bZIP TF Gcn4 of *S. cerevisiae*, which is involved in regulating genes in response to amino acid starvation (Hinnebusch, 2005), and its orthologue in *N. crassa*, CPC-1 (Ebbole *et al.*, 1991), show conservation in the functional categories of genes that respond to amino acid starvation and which are dependent on functional Gcn4 and CPC-1 (Tian *et al.*, 2007). Although the overall cellular response to amino acid starvation is similar between *S. cerevisiae* and *N. crassa*, there is substantial divergence in the direct target genes of Gcn4 in *S. cerevisiae* versus the predicted CPC-1 target genes in *N. crassa*.

In addition to *cpc-1*, there are eight genes in *N. crassa* with predicted bZIP DNA-binding domains, including the sulfur metabolism regulator *cys-3* (Fu & Marzluf, 1990). While this study was in progress, an additional bZIP family member, NCU03905 (*nap-1*; here named *NcAp-1*, see Results), was shown by Takahashi *et al.* (2010) to be involved in menadione resistance and was postulated to be a YAP1 orthologue. The functions of the remaining members of this family are currently unknown. In an effort to understand the bZIP regulatory network in *N. crassa*, we determined the transcriptional profiles of seven viable bZIP mutants during vegetative growth. This study revealed a genome-wide map of transcriptional regulation via members of the bZIP family in *N. crassa* and showed both cooperativity and specificity among the bZIP TF family in a filamentous ascomycete species. We subsequently assessed the sensitivity of the bZIP mutants to a variety of environmental stresses and also identified NCU03905 (*Neurospora crassa* AP 1-like TF, *NcAp-1*) as the functional orthologue of *S. cerevisiae YAP1*. By performing transcriptional profiling experiments of both the wild-type (WT) and a Δ*NcAp-1* mutant in response to oxidative stress, we identified the NcAp-1 regulon. Functional and transcriptional profiling comparisons of the *S. cerevisiae* Yap1, *Schizosaccharomyces pombe* Pap1, *C. albicans* Cap1 and *N. crassa* NcAP1 regulatory networks in response to oxidative stress (this study) and in response to exposure to menadione (Takahashi *et al.*, 2010) revealed both conservation and divergence of TF regulons.

METHODS

Strains and culture conditions. Strains containing deletion mutations in the predicted bZIP TF genes included FGSC 11121 (ΔNCU00499), FGSC 11343 (ΔNCU01994), FGSC 11131 (ΔNCU03905), FGSC 11133 (ΔNCU04211) and FGSC 11269 (ΔNCU08055) (Colot *et al.*, 2006), and were kindly provided by Dr K. Borkovich (University of California, Riverside), while the *cpc-1* mutant (FGSC 4264), *cys-3* mutant (FGSC 4028) and the WT sequenced strain (FGSC 2489) were obtained from the Fungal Genetics Stock Center (FGSC) (McCluskey, 2003). Strains were inoculated onto slants containing Vogel's minimal medium (VMM) (Vogel, 1956) and grown at 30 °C for 2 days, followed by incubation at 25 °C under constant light for 7 days. Conidia were harvested with water and inoculated as a horizontal strip onto 150 ml Bird's minimal medium (BMM) (Metzenberg, 2004) on a large (25 cm × 25 cm) Petri plate layered with a sheet of cellophane. Conidia were germinated under constant light at 25 °C for 24 h. A 4 mm wide mycelial section was subsequently inoculated as a horizontal strip onto fresh solid BMM. Twenty-four hours post-inoculation, a 1 cm wide section of mycelia at the periphery of the colony, which corresponded to hyphae 1–3 h old, and a 1 cm internal section of the colony (corresponding to hyphae 12–15 h old) were harvested, immediately plunged into liquid nitrogen and stored at −80 °C for RNA isolation.

For transcriptional profiling of the WT and the Δ*NcAp-1* strain in response to hydrogen peroxide (H_2O_2), strains were treated as above, with the exception that large (25 cm × 25 cm) Petri plates containing a solid physical barrier in the middle of the plate were used. One half of the plate contained BMM, while the other half contained BMM supplemented with 6 mM H_2O_2 (added when the medium was at about 40 °C). A preliminary comparison of expression data for the WT exposed to 2 and 6 mM H_2O_2 versus minimal medium revealed that there were no significant differences in expression when *N. crassa* was exposed to 2 mM H_2O_2, but significant differences were detected upon exposure to 6 mM H_2O_2. A horizontal strip containing hyphae was inoculated on the BMM, 3 cm from the barrier. After 12 h, the periphery of the colony reached the barrier and grew into the BMM + H_2O_2. After 6 h of growth, an ~1 cm strip of the colony periphery was isolated from the BMM + H_2O_2 plate, plunged into liquid nitrogen and stored at −80 °C until use.

For transcriptional profiling of the WT and Δ*NcAp-1* mutant in response to cadmium chloride ($CdCl_2$) treatment, conidia were harvested and inoculated in 100 ml liquid minimal medium for 24 h at 30 °C with shaking (225 r.p.m.). $CdCl_2$ was added to the cultures to a final concentration of 10 mM. After 30 min treatment, the mycelium-containing cultures were spun down, filtered through Whatman No. 1 filter paper and frozen immediately in liquid nitrogen.

RNA isolation and cDNA labelling. Total RNA from frozen samples was isolated using zirconia/silica beads (0.2 g, 0.5 mm diameter, Biospec) and a Mini-BeadBeater (Biospec) with 1 ml TRIzol reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. Total RNA (100 μg) was further purified using an RNeasy kit (Qiagen). RNA integrity was checked with a Nanodrop spectrophotometer and by agarose gel electrophoresis. cDNA synthesis and labelling followed the protocol of the ChipShot Indirect Labeling kit from Promega (catalogue no. Z4000). The dyes Cy3 and Cy5 (Amersham, catalogue no. RPN5661) were incorporated into cDNA by adding Cy3 or Cy5 monofunctional *N*-hydroxysuccinimide ester dye to the cDNA solution for 1 h at 22 °C. The cDNA was subsequently cleaned by using a ChipShot membrane column and dried under vacuum.

Microarray hybridization and data analysis. The methods for microarray hybridization and data analysis were as described in Tian *et al.* (2007). Briefly, 70-mer oligonucleotides representing all predicted *N. crassa* genes and additional intergenic regions were printed onto GAP slides (Corning, catalogue no. 40005). We used a closed loop for experimental design that increases statistical power (Townsend & Taylor, 2005). Each RNA sample was used in at least four individual hybridizations. Images from the Axon 4000B microarray scanner were analysed by Genepix 6.0 software; statistical support for differential expression levels was obtained by BAGEL analyses (Townsend & Hartl, 2002). *cis*-Element analysis was performed as previously described (Tian *et al.*, 2007). The 1000 bp region upstream of the predicted translational start site of nine common targets between H_2O_2 and $CdCl_2$ conditions was retrieved and evaluated by MDscan (Liu *et al.*, 2002), BioProspector (Liu *et al.*, 2001) and MEME (Bailey & Elkan, 1994) for motif prediction.

Quantitative real-time RT-PCR (qRT-PCR). qRT-PCR was performed on a 7300 Real-Time PCR system (Applied Biosystems) using a Qiagen SYBR kit (catalogue no. 204143). Amplification of mRNA from the actin gene was used as an internal control for normalization. Methods were as described previously (Dementhon *et al.*, 2006).

NcAp-1–GFP plasmid construction and *Neurospora* transformation. The ORF of NCU03905 was inserted into the pMF272 vector, which includes a GFP cassette (Freitag *et al.*, 2004). For the construct using the *NcAp-1* native promoter to drive expression, the *NcAp-1* upstream sequence and ORF were amplified using primers containing *Not*I and PacI sites (forward primer 5′-CTG CGG CCG CCC TTC CCT TCT CAG ATC TGA ATC G-3′, reverse primer 5′-CTT TAA TTA ACA GTG TGG CAG CTG CTT TTC G-3′). For the construct using the *ccg1* promoter, the *NcAP-1* gene was amplified using primers containing *Spe*I and PacI sites (forward primer 5′-CTA CTA GTA TGA CGT CGA CTC AGA ACC CTC-3′, reverse primer 5′-CTT TAA TTA ACA GTG TGG CAG CTG CTT TTC G-3′). The *NcAp-1–gfp* constructs were transformed to a ΔNCU03905 strain containing a *his-3* marker (R21-07) using *Neurospora* transformation methods (Margolin *et al.*, 1997). Verification of full-length *NcAp-1–gfp* insertion at the *his-3* locus was confirmed by PCR. To assess localization of NcAp-1–GFP upon oxidative stress, 16 h-old hyphae from the ΔNCU03905 (*NcAp-1–gfp*) strains (CT-C17 and CT-N9) were treated with 30 mm H_2O_2 and 10 mM $CdCl_2$. After 30 min of exposure, NcAp-1–GFP localization was assessed via fluorescence microscopy using a Zeiss Axioskop II microscope and GFP fluorescence filter. Photographs of hyphae were taken using a Zeiss Axioskop II microscope with a Hamamatsu digital charge-coupled device (CCD) camera, and further processed with Adobe Photoshop 7.0.

Stress screening of conidia. Fresh conidia were harvested from 7 day-old slants and suspended in 500 μl H_2O to a final concentration of 10^6 μl^{-1}. Seven microlitres of a conidial dilution series (10^5, 10^4, 10^3, 10^2 and 10^1 μl^{-1}) was spotted onto a BDES plate (Davis & De Serres, 1970) or BDES plates plus one of the following compounds: H_2O_2, 1 or 2 mM; $CdCl_2$, 50 or 100 μM; sorbitol, 0.8 M; β-mercaptoethanol (β-ME), 1 or 4 mM; 3-aminotriazole (3-AT), 1 or 2 mM; NaCl, 0.8 mM. Plates were incubated at 25 °C for 2 days and then growth was evaluated.

Identification of orthologues and regulogs in *N. crassa* and other fungi. The sequences of predicted ORFs in the *N. crassa* genome were downloaded from Broad Institute version 7 (http://www.broad.mit.edu/annotation/genome/neurospora/Downloads.html). *Neurospora discreta* and *Neurospora tetrasperma* genomic data were obtained from http://genome.jgi-psf.org/Neudi1/Neudi1.download.html and http://genome.jgi-psf.org/Neute1/Neute1.download.html, respectively. *S. cerevisiae* ORFs were downloaded from the SGD database (http://downloads.yeastgenome.org/sequence/genomic_sequence/orf_dna/). *Aspergillus fumigatus* genome data were downloaded from TIGR (ftp://ftp.tigr.org/pub/data/Eukaryotic_Projects/a_fumigatus/annotation_dbs/). *Candida albicans* genome data were downloaded from http://www.candidagenome.org/DownloadContents.shtml. *Sch. pombe* ORFs were downloaded from http://www.sanger.ac.uk/Projects/S_pombe/protein_download.shtml.

A. nidulans and *M. grisea* ORF protein sequences were downloaded from The Broad Institute (http://www.broad.mit.edu/annotation/fgi/). *Yarrowia lipolytica* ORF protein sequences were downloaded from http://www.genolevures.org/download.html. Orthologous genes were identified as best bidirectional hits using BLASTP, with a cut-off value of 1e-6. The bZIP member prediction was performed by hidden Markov model (HMM) searches; the bZIP TF HMM model (PF00170.10) was downloaded from Pfam (http://pfam.sanger.ac.uk/). The sequence was defined as a DNA-binding motif if the HMM search *P* value was $<1 \times 10^4$.

Transcriptional profiling data for *S. cerevisiae* in response to oxidative stress were obtained from Gasch *et al.* (2000), and the chromatin-immunoprecipitation (ChIP) data were downloaded from Harbison *et al.* (2004). *C. albicans* oxidant stress response data were downloaded from Wang *et al.* (2006). The Cap1 ChIP data were downloaded from Znaidi *et al.* (2009). *Sch. pombe* oxidant stress profiling data were downloaded from Chen *et al.* (2008). The *A. fumigatus* proteomics data for the H_2O_2 stress response were downloaded from Lessing *et al.* (2007). The *N. crassa* profiling data on menadione were downloaded from Takahashi *et al.* (2010).

RESULTS

TF gene expression in *N. crassa* during vegetative growth

In *N. crassa*, there are about 200 predicted general TFs (with and without a DNA-binding domain) (Borkovich *et al.*, 2004; Galagan *et al.*, 2003; Shiu *et al.*, 2005). The class of TFs with predicted DNA-binding domains comprises six families, i.e. the bHLH, bZIP, C_2H_2 zinc finger, Myb, GATA factor and Zn $(II)_2$ binuclear cluster groups, plus a group of miscellaneous TFs. We used an HMM to revise this list based on the sequence of the DNA-binding domain (see Methods). We identified 178 TFs (Supplementary Table S1, worksheet p1) with a clear DNA-binding motif; 166 of these belonged to one of the six defined gene families listed above. The remaining 12 genes encoded TFs classified as miscellaneous (various DNA-binding domains). These 178 TFs can be thought of as the core

set for *N. crassa*, although additional TF genes may be present that contain a non-typical DNA-binding motif(s).

Since TFs play a critical role in the regulation of gene expression patterns, we evaluated the expression pattern of the 178 predicted TFs during vegetative growth of *N. crassa* at two developmental time points. We evaluated the expression of predicted TFs using full-genome *Neurospora* microarrays (Kasuga *et al.*, 2005; Kasuga & Glass, 2008; Tian *et al.*, 2007) from hyphae at the periphery of a colony (a section of the colony that is 0–3 h old) and a second section from the interior of a 12–15 h-old colony. Different sections of a colony of *N. crassa* have been shown to vary in expression profile (Kasuga & Glass, 2008), and in addition the hyphae in these sections show morphological and developmental differences. For example, hyphae at the periphery exhibit foraging behaviour, while hyphae in the interior of a colony form asexual reproductive structures, including aerial hyphae and conidia. Expression patterns for 98 (55 %) of the predicted TFs were detected. With a minimum difference of 1.5-fold, 35 TFs showed a significantly different expression pattern between the periphery and the interior of a colony (Table 1). Most of these TFs (33/35) showed an increase in relative expression level in the colony periphery, while only two increased in expression level in the interior of a colony [NCU01459 and NCU08726; NCU08726 encodes *fl* (fluffy), a TF involved in asexual spore development] (Bailey & Ebbole, 1998). Strains containing mutations in 15 of the 35 predicted TFs showed phenotypes different from a WT strain (Table 1), including nine with vegetative growth defects

Table 1. Genes encoding TFs with significant differences in expression between the periphery and the interior of a colony

TF	Family	Tip/interior*	Growth (mm day^{-1})†	Annotation
NCU00749	BHLH	2.0	60–65	Predicted protein
NCU04731	BHLH	1.9	65–70	*sah-2*, short aerial hyphae-2
NCU02724	BHLH	1.6	70–75	Helix–loop–helix DNA-binding domain
NCU00499	bZIP	1.6	25–30	*ada-1*, all development altered-1
NCU01459	bZIP	0.6		*asl-2*, ascospore lethal-2
NCU00090	C2H2	6.0	60–65	Related to PacC protein
NCU00340	C2H2	3.5	20–30	*pp-1*, Ste12-like TF
NCU00694	C2H2	3.0	70–75	Putative protein
NCU02671	C2H2	2.4	20–25	Cutinase G-box binding protein
NCU03043	C2H2	2.0	60–65	Related to Krüppel protein
NCU03421	C2H2	7.7	60–65	Conserved hypothetical protein
NCU03699	C2H2	2.4	70–75	Conserved hypothetical protein
NCU06487	C2H2	2.1	75–80	Predicted protein
NCU06907	C2H2	1.8	70–75	Conserved hypothetical protein
NCU06503	C2H2	1.8	55–60	Related to DNA-binding protein
NCU08807	C2H2	1.7		*cre-1*, carbon repressor protein 1
NCU09252	C2H2	1.5	75–80	Conserved hypothetical protein
NCU01154	GATA	2.3		*sub1*, submerged protoperithecia-1
NCU09068	GATA	2.3	60–65	Conserved hypothetical protein
NCU05685	Myb	1.6		Conserved hypothetical protein
NCU00155	Zn2Cys6	2.0	70–75	Transcriptional activator Mut3p
NCU00289	Zn2Cys6	2.5	65–70	*tah-1*, tall aerial hyphae-1
NCU01097	Zn2Cys6	3.6	70–75	Related to NITA protein
NCU04866	Zn2Cys6	2.0	50–55	Related to FacB DNA-binding protein
NCU06799	Zn2Cys6	3.0	60–65	Hypothetical protein
NCU07139	Zn2Cys6	5.0	80–85	Conserved hypothetical protein
NCU07788	Zn2Cys6	2.7	55–60	*col-26*, colonial-26
NCU08063	Zn2Cys6	2.1	65–70	Putative protein
NCU08307	Zn2Cys6	3.0		Hypothetical protein
NCU02576	Zn2Cys6	1.9		Related to cutinase TF
NCU07392	Zn2Cys6	1.5	60–65	*adv-1*, all development altered
NCU06407	Zn2Cys6	1.5	55–60	*vad-3*, vegetative asexual development-3
NCU02307	Zn2Cys6	1.5	70–75	Conserved hypothetical protein
NCU08726	Zn2Cys6	0.2	20–25	*fl*, conidial development protein fluffy
NCU03593		1.7	10–15	Probable homeoprotein

*Significance cut-off (BAGEL) of at least 1.5-fold.

†Growth data from the Broad Institute (http://www.broadinstitute.org/annotation/genome/neurospora/MultiHome.html).

[Δ*pp-1* (NCU00340), ΔNCU02671, ΔNCU06503, Δ*cre-1* (NCU08807), Δ*ada-6* (NCU04866), Δ*col-26* (NCU07788), Δ*vad-3* (NCU06407), Δ*kal-1* (NCU03593) and the bZIP TF gene, Δ*ada-1* (NCU00499)] (Colot *et al.*, 2006).

Phylogenetic analysis of *N. crassa* bZIP family members

In *S. cerevisiae*, the bZIP TF family has been well studied and consists of 14 members. The *YAP* family of bZIP TFs, of which there are eight members, are thought to be a result of at least six duplication events from a common ancestor (*GCN4*) (Kellis *et al.*, 2004), and can be divided into four subfamilies: *YAP1* and *YAP2*, *YAP4* and *YAP6*, *YAP5* and *YAP7*, and *YAP3* and *YAP8* (Tan *et al.*, 2008) (Fig. 1). We performed phylogenetic analysis of predicted bZIP domain proteins from the hemiascomycete species *S. cerevisiae* (14 members) and *C. albicans* (seven members), a species at the base of the hemiascomycete clade, *Yarrowia lipolytica* (10 members), the archiascomycete species *Sch. pombe* (six members), and the euascomycete species *A. nidulans* (nine members), *M. grisea* (10 members) and *N. crassa* (nine members). The phylogenetic tree of the bZIP domains showed divergence into two separate clades (Fig. 1). The lower clade includes all known members of the *YAP* family (*YAP* clade), while the upper clade includes all other bZIP domain proteins, including *S. cerevisiae GCN4*, *SKO1*, *HAC1*, *MET28*, *ACA1* and *CST6* (Fig. 1) (*GCN4* clade). In *N. crassa*, the GCN4 clade bZIP TFs include *cpc-1* (NCU04050), *cys-3* (NCU03536), *ada-1* (NCU00499), *asl-1* (NCU01345) and NCU01459. While the *YAP* family clade includes eight members from *S. cerevisiae*, *Sch. pombe* contains only one homologue, *pap1* (Fig. 1). The four *YAP*-like TFs in *A. nidulans* and the three *YAP*-like TFs in *M. grisea* and *N. crassa* clustered within the *YAP* bZIP clade, although none of the predicted euascomycete *YAP*-like proteins clustered closely with the *S. cerevisiae* Yap proteins.

Although phylogenetic analysis clearly showed that gene duplication of bZIP genes occurred in *S. cerevisiae* (*ACA1* and *CST6*, and *YAP* members) and *Sch. pombe* (*ATF21* and *PCR1*), within the euascomycete species only a single recent gene duplication event was detected in *M. grisea* (MG02006.4 and MG04009.4), which clustered with a bZIP protein of unknown function (NCU01459) in *N. crassa*. Viable homokaryotic deletion mutations in NCU01459 are not recoverable (Colot *et al.*, 2006). A more ancient duplication within the GCN4 clade was identified in *A. nidulans* (AN8643.2 and AN6849.2); these bZIP domains do not cluster closely with any other bZIP proteins. No recent duplication of any bZIP domain gene was detected in *N. crassa*, consistent with the observation that gene duplications are not tolerated (Galagan & Selker, 2004). Thus, the major expansion of the bZIP gene family in fungi occurred before the divergence of euascomycete species from *Sch. pombe* and *S. cerevisiae*, approximately 300 million years ago.

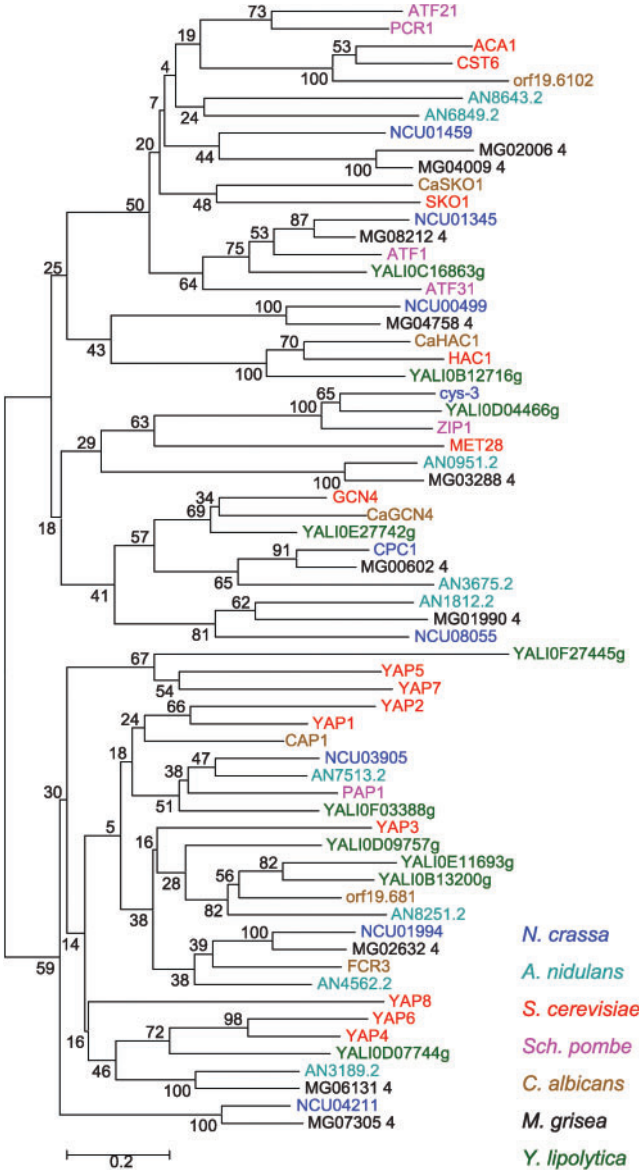

Fig. 1. Phylogenetic analysis of the bZIP TF gene family in ascomycete species. The bZIP member prediction was performed by HMM searches using the bZIP domain matrix downloaded from Pfam (PF00170.10). The tree was generated by the MEGA2 program using neighbour joining with bootstrap=100.

Transcriptional profiling of bZIP mutants

In *N. crassa*, two bZIP genes have been well characterized. The first encodes an orthologue of *GCN4*, called *cpc-1*, which is involved in regulating genes in response to amino acid starvation (Ebbole *et al.*, 1991; Hinnebusch, 2005; Tian *et al.*, 2007). The second bZIP gene encodes a transcriptional regulator, CYS-3, which regulates genes in response to sulfur sources (Paietta, 1992). While this study was in progress, a third member, NCU03905 (*nap-1*), was shown to be important for resistance to treatment with menadione and proposed to be a *YAP1* orthologue

(Takahashi *et al.*, 2010). Here we refer to NCU03905 as *NcAp-1*, as *nap* refers to a neutral amino acid permease locus in *N. crassa* (Perkins *et al.*, 2001; Rao & DeBusk, 1973). Homokaryotic mutant strains are available for seven of nine predicted bZIP genes [Δ*ada-1* (NCU00499), ΔNCU01994, Δ*NcAp-1* (NCU03905), ΔNCU04211, *cpc-1* (NCU04050), ΔNCU08055 and *cys-3* (NCU03536)]. The Δ*ada-1* mutant (all development altered; NCU00499), showed a reduced growth rate (2.5 cm day^{-1} compared with 7.5 cm day^{-1} for the WT) and very short aerial hyphae. Strains containing mutations in the other six bZIP TF genes (ΔNCU01994, Δ*NcAp-1*, ΔNCU04211, *cpc-1*, ΔNCU08055 and *cys-3*) showed growth and asexual reproduction phenotypes indistinguishable from those of the WT strain. Strains containing mutations in two remaining bZIP genes, *asl-1* (NCU01345) and *asl-2* (NCU01459), showed an ascospore lethality phenotype; homokaryotic strains containing mutations in these genes are not available (Colot *et al.*, 2006).

To identify putative target genes of the bZIP TF mutants, we compared transcriptional profiles of the seven bZIP mutants from hyphae at the colony periphery (0–3 h) with those from the interior of a colony (12–15 h). In the WT, Functional Category (FunCat) analysis (Ruepp *et al.*, 2004) of 509 genes that had higher expression levels in the colony periphery showed an enrichment for genes involved in cell type differentiation, cell wall proteins, carbon compound and polysaccharide metabolism, cell growth/morphogenesis (regulation of directional cell growth), cellular export and secretion, and cell signalling ($P<0.001$; Supplementary Table S2, worksheet p1). FunCat analysis of the 363 genes that had an increased expression level in the interior of the colony showed an enrichment for genes involved in stress response and detoxification, energy-related genes (glycolysis and pentose phosphate pathway), and amino acid and carbon metabolism ($P<0.001$; Supplementary Table S2, worksheet p4). In the colony periphery, the number of genes in the seven bZIP mutants that showed altered expression profiles compared with the WT ranged from 21 to 509 (Table 2 and Supplementary Table S3), including genes that increased in relative expression level (from three in Δ*NcAp-1* to 290 in Δ*ada-1*) and genes that showed a decrease in relative expression level (from five in Δ*cys-3* to 219 in Δ*ada-1*) (Table 2). Expression patterns of genes in the interior of a colony in the bZIP TF deletion strains also showed differences from the WT (Table 2 and Supplementary Table S4), ranging from three affected genes in Δ*NcAp-1* to 139 in Δ*cys-3*. The strain carrying a deletion of *ada-1* showed the greatest number of expression differences from the WT, consistent with its growth defect.

FunCat analysis of affected genes in the Δ*ada-1* mutant that showed reduced expression in the colony periphery showed enrichment for carbon compound and carbohydrate metabolism ($P=2.3\times10^{-7}$). Genes involved in carbon compound and carbohydrate metabolism also showed enrichment in other bZIP mutants, including ΔNCU01994, ΔNCU04211 and ΔNCU08055 ($P=0.001$ to 0.01).

Similar to data from the colony periphery, affected genes displaying decreased expression in the colony interior in the Δ*ada-1* mutant showed an enrichment for carbon compound and carbohydrate metabolism, and the pentose phosphate pathway ($P=1.03\times10^{-6}$). In the ΔNCU01994 and *cys-3* strains, genes involved in amino acid metabolism, carbon compound and carbohydrate metabolism, lipid, fatty acid and isoprenoid metabolism, and metabolism of vitamins, cofactors and prosthetic groups, were enriched ($P=0.002$ to 6.3×10^{-9}), while NAD/NADP binding was enriched in the ΔNCU04211 and ΔNCU08055 strains. Enriched categories of genes that showed an increased expression level in the colony interior included transport facility and cell defence genes in Δ*ada-1*, polysaccharide metabolism and cell wall genes in ΔNCU01994 and ΔNCU08055, and virulence and disease factor genes in ΔNCU04211.

Consistent with their phylogenetic relationships, strains containing mutations in bZIP TF genes that were more closely related showed an overlap in affected gene sets, with an overlap of up to 24 % in strains containing mutations in the closely related paralogues NCU08055 and *cys-3* (Supplementary Tables S3 and S4). By contrast, only a 1 % overlap in expression profiles was identified between strains containing deletions of distant bZIP paralogues, for example, *NcAp-1* (NCU03905) and *ada-1* (NCU00499). These data suggest that the regulation of some genes and processes has been conserved in the bZIP TF regulons

Table 2. Summary of expression profiling data for seven bZIP mutants

TF mutant	Genes downregulated in periphery	Genes upregulated in periphery	Genes downregulated in interior	Genes upregulated in interior
ΔNCU00499	219	290	80	56
ΔNCU01994	12	9	49	77
ΔNCU03905	47	3	6	3
ΔNCU04211	45	5	65	28
cpc-1	22	19	5	52
ΔNCU08055	32	6	50	107
cys-3	5	56	24	139

following duplication and divergence. Similarly, in *S. cerevisiae*, the number of genes co-regulated by YAP family members ranges between 9 and 15 %, and increases to 25 % upon exposure to DNA-damaging agents (Tan *et al.*, 2008).

NcAp-1 functional characterization and localization

To elucidate the molecular function of the bZIP TFs in *N. crassa*, we screened the bZIP TF mutants for sensitivity to a variety of chemical stresses, including exposure to sorbitol, NaCl, 3-AT, cadmium, *β*-ME and H_2O_2. The Δ*NcAp-1* mutant was sensitive to all the stresses tested except for *β*-ME (Supplementary Fig. S1). However, only slight sensitivity to H_2O_2, and no sensitivity to NaCl has been observed in an independently derived NCU03905 (*nap-1*) mutant during hyphal growth (Takahashi *et al.*, 2010). The Δ*cys-3* mutant showed a mild sensitivity to sorbose (Supplementary Fig. S1). The other bZIP TF deletion mutants did not show any obvious sensitivity under any of the conditions tested.

The predicted NcAp-1 protein (NCU03905) has a PAP1 domain (Fig. 2c), which is an important H_2O_2 response domain in *Sch. pombe* (Chen *et al.*, 2008; Cohen *et al.*, 2002; Vivancos *et al.*, 2006). In *N. crassa*, the closest paralogue to *NcAp-1* is NCU01994, although the predicted

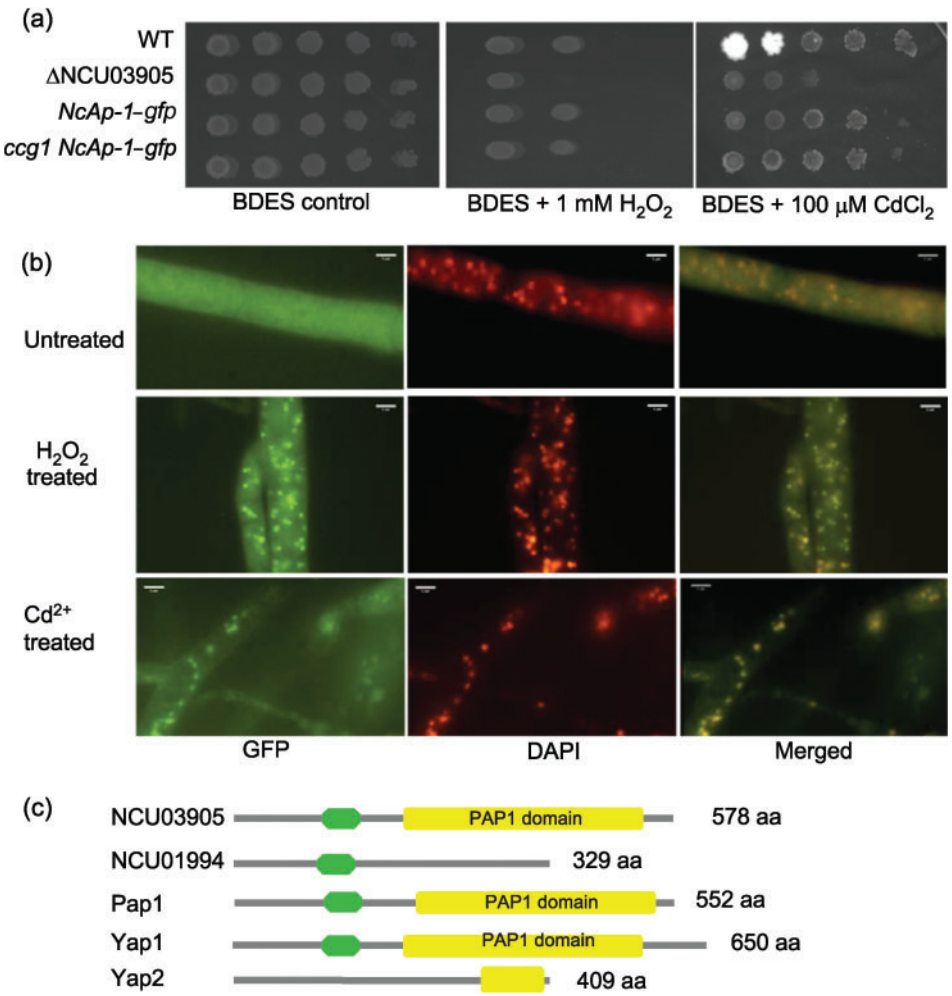

Fig. 2. Complementation of Δ*NcAp-1* and localization of NcAp-1–GFP. (a) Comparison of the phenotype of the WT (FGSC 2489), the *NcAp-1* mutant (ΔNCU03905) and Δ*NcAp-1* complemented strains (*NcAp-1–gfp* and *ccg1 NcAp-1–gfp*) exposed to 1 mM H_2O_2 or 100 μM Cd^{2+} using a dilution series of conidial suspensions (from left to right: 10^5, 10^4, 10^3, 10^2, 10^1). Plates were incubated at 25 °C for 2 days and then evaluated for growth. (b) NcAp-1–GFP localization in a Δ*NcAp-1* (*his-3*::*NcAp-1–gfp*) strain grown on minimal medium (untreated, upper panels), after treatment with 30 mM H_2O_2 (H_2O_2 treated, centre panels) or 10 mM $CdCl_2$ (Cd^{2+} treated, lower panels). The left-hand panels show GFP fluorescence (NCAp-1–GFP), while the centre panels show nuclear 4,6-diamidino-2-phenylindole (DAPI) staining. The right-hand panels show merged GFP and DAPI images. (c) Domain prediction of Yap1, Yap2, Pap1, NCU03905 (NcAp-1) and its closest paralogue NCU01994. Green, bZIP motif; yellow, PAP1 domain.

protein product of NCU01994 lacks a PAP1 domain (Fig. 2c). However, a strain containing both Δ*NcAp-1* and ΔNCU01994 deletions resembles the Δ*NcAp-1* mutant with respect to sensitivity to stress (Supplementary Fig. S1).

The redistribution of *S. cerevisiae* Yap1 from the cytoplasm to the nucleus in response to oxidative stress is a major regulatory mechanism (Kuge *et al.*, 1997), and also occurs in *Sch. pombe* (PAP1) (Vivancos *et al.*, 2004), *C. albicans* (CAP1) (Zhang *et al.*, 2000) and *A. fumigatus* (AfYap1) (Lessing *et al.*, 2007). In *N. crassa*, NcAp-1–GFP in a WT strain shows redistribution from the cytoplasm to the nucleus upon exposure to menadione (Takahashi *et al.*, 2010). To observe the behaviour of NcAp-1 during oxidative and heavy metal stress in *N. crassa*, we tagged NcAp-1 with GFP at the C terminus. The introduction of the *NcAp-1–gfp* construct into the Δ*NcAp-1* strain restored cadmium and H_2O_2 resistance, indicating that the construct encodes a functional NcAp-1 protein (Fig. 2a). Under conditions of no (or low) stress (minimal medium), NcAp-1 localized to the cytoplasm (Fig. 2b). However, when the Δ*NcAp-1* (*NcAp-1–gfp*) strain was exposed to stress (30 mM H_2O_2 for 5–30 min or 10 mM $CdCl_2$ for 5–10 min), NcAp-1–GFP accumulated in nuclei across the fungal colony (Fig. 2b). These data indicate that the redistribution of YAP-1-like proteins to the nucleus upon exposure to oxidants or heavy metals is a highly conserved mechanism of transcriptional regulation in ascomycete fungi in response to a variety of stresses.

Identification of the NcAp-1 regulon under oxidative stress

To determine the NcAp-1 stress regulon, we performed transcriptional profiling of the WT and the Δ*NcAp-1* mutant grown in minimal medium with and without oxidative stress imposed by treatment with 6 mM H_2O_2 (see Methods). There were 45 genes induced by H_2O_2 (Fig. 3a, sets C, D and E) that were dependent on *NcAp-1* for transcription. FunCat analysis (Ruepp *et al.*, 2004) of these 45 putative target genes showed that stress responses, oxygen detoxification and metabolism of peptide-derived compounds (such as thioredoxin) were enriched (Table 3). Of the 45 genes (Supplementary Table S5, worksheet P1), 11 have an annotated function in relation to the stress response, especially the oxidant stress response pathway, including two glutathione *S*-transferases, *gst-1* (NCU 05780) and *gst-2* (NCU04109); four NADP(H)-dependent dehydrogenase/flavin oxidoreductases, *mig-2* [*m*enadione-*i*nduced *g*ene (Takahashi *et al.*, 2010)] (NCU07452), *mig-3* (NCU04452), *mig-6* (NCU09285) and NCU08402; an oxidase (NCU05164); catalase-2 (NCU05770); a probable reductase (*mig-4*, NCU09040); a glutathione peroxidase (NCU09534); and a predicted ABC transporter (NCU 03776) (Table 4). We confirmed the dependence of expression on *NcAp-1* for four genes, *cat-2* (NCU05770), *gpx3* (NCU09534), NADH oxidase (*mig-3*, NCU04452) and glutathione transferase-1 (*gst-1*, NCU05780) by qRT-PCR (Supplementary Fig. S2). Five genes from this 11-gene oxidant stress set have been shown to be *NcAp-1*-dependent upon exposure to menadione (Takahashi *et al.*, 2010), including two predicted glutathione *S*-transferases [*gst-1* (NCU05780) and *gst-2* (NCU04109)] and three NADP(H)-dependent dehydrogenase/flavin oxidoreductases [*mig-2* (NCU07452), *mig-3* (NCU04452) and *mig-6* (NCU09285)]. These data indicate that the glutathione *S*-transferase and NADPH dehydrogenases are important for resistance to multiple types of oxidant stress in *N. crassa*.

Identification of the NcAp-1 regulon under cadmium stress

In addition to sensitivity to H_2O_2, the Δ*NcAp-1* mutant was also sensitive to other stress conditions, including exposure to heavy metals such as cadmium (Supplementary Fig. S1). We therefore compared expression profiles of the WT and Δ*NcAp-1* mutant upon exposure to $CdCl_2$ versus H_2O_2 (see Methods and Supplementary Table S1). A total of 34 genes induced by exposure to Cd^{2+} were dependent on *NcAp-1* for transcription (Fig. 3b, sets D and E, Supplementary Table S5, worksheet P2). Nine of these 34 genes overlapped with the 45 targets identified upon exposure to H_2O_2 and were dependent upon functional *NcAp-1*. These nine genes form the core targets of NcAp-1 (Table 4) and include the two glutathione *S*-transferase genes, *gst-1* and *gst-2* (NCU04109 and NCU05780, respectively), four NAD(P)H-dependent dehydrogenase/oxidoreductase genes, *mig-2*, *mig-3*, *mig-6* and NCU08402, a predicted methoxylase (NCU00847), an oxidase (NCU05164) and a glutathione peroxidase (NCU 09534). Six of these 34 genes are induced by menadione treatment (Takahashi *et al.*, 2010) (Supplementary Table S5, worksheet P2), including a predicted glutathione *S*-transferase, *gst-4* (NCU10521), plus five of the nine core genes identified above (*gst-1*, *gst-2*, *mig-2*, *mig-3* and *mig-6*). The 24 Cd^{2+} condition-specific target genes included several stress/oxidant stress-related genes, including a predicted thioredoxin (NCU06556) and uricase (NCU 07853). Interestingly, the bZIP TF *cys-3* and nitrogen and sulfur metabolism genes were also enriched in this list (Supplementary Table S5, worksheet P2), suggesting that sulfur metabolism regulated by CYS-3 may cross-talk with the metal response pathway regulated by NcAp-1.

cis-Element analysis of NcAp-1 targets

As indicated by FunCat analysis, the NcAp-1 target gene set (45 under H_2O_2 and 34 under Cd^{2+}) was enriched for genes involved in oxidative stress responses (Table 4). An earlier study evaluating promoter regions of genes that were induced upon treatment with menadione and which were NcAp-1-dependent failed to identify upstream regulatory elements (Takahashi *et al.*, 2010). To identify potential NcAp-1 *cis*-regulatory elements in this study, we analysed 1000 bp upstream of the nine common target genes identified between the H_2O_2 and Cd^{2+} responses

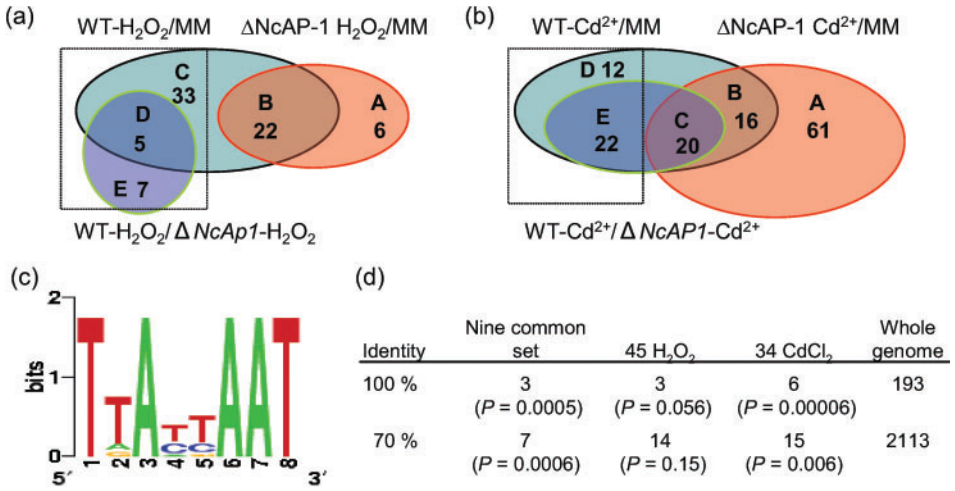

Fig. 3. The NcAp-1 regulon. (a) Venn diagram showing the overlap among the genes that showed statistically significantly increased expression levels in the WT (FGSC 2489) as compared with the Δ*NcAp-1* mutant when exposed to H_2O_2. Genes within C, D and E represent genes that were induced by exposure to H_2O_2 and were *NcAp-1*-dependent. (b) Venn diagram showing the overlap among the genes that showed statistically significantly increased expression levels in the WT (FGSC 2489) as compared with the Δ*NcAp-1* mutant when exposed to $CdCl_2$. Genes within D and E represent genes that were induced by exposure to $CdCl_2$ and were *NcAp-1*-dependent. (c) WebLogo of the *cis*-element that showed enrichment in the putative direct target genes of NcAp-1 (see Methods) (http://weblogo.berkeley.edu/logo.cgi). (d) Enrichment analysis of predicted *cis*-elements in the NcAp-1 target gene set. Using the predicted *cis*-element as an input matrix, the 1000 bp upstream of the predicted target genes was evaluated by PASTER (Hertz & Stormo, 1999). The perfect match and 70 % match to the *cis*-element are shown; statistical analysis (*P* values) was conducted by Fisher's exact test.

(see Methods). We identified the *N. crassa cis*-regulatory element TTA(t/c)(t/c)AAT, which was similar to *cis*-regulatory elements identified for Yap1 (TTACTAA), Cap1 (TTAc/gTAA), AfYap1 [TTA(G/C)TAA] and Pap1 (TTACGTAA), and which occurred in seven of the nine common target genes (P=0.0006), 14 of 45 H_2O_2-responsive target genes (P=0.15), and 15 of 34 cadmium-responsive target genes (P=0.006) (Fig. 3c, d). This *cis*-regulatory element was enriched in NcAp-1 putative target genes relative to its prevalence in the *N. crassa* genome. This set of genes is predicted to be directly regulated by NcAp-1. Of the nine common target genes, conservation of the NcAp-1 *cis* sequence was identified in the promoter regions of four orthologues in the related species *N. discreta* (NCU00847, NCU04452, NCU09285 and NCU05164) and in five orthologues in *N. tetrasperma* (NCU00847, NCU04452, NCU07452, NCU09285 and NCU09534).

Table 3. FunCat analysis of NcAp-1 targets

Enriched groups with P <0.001 are shown; groups enriched under both Cd^{2+} and H_2O_2 conditions are in bold type.

Functional category	Cd^{2+} *P* value	H_2O_2 *P* value
01 METABOLISM	3.99×10^{-6}	0.075951
01.02 Nitrogen, sulfur and selenium metabolism	1.13×10^{-5}	0.029173
01.02.07 Regulation of nitrogen, sulfur and selenium metabolism	7.41×10^{-5}	0.005066
01.20 Secondary metabolism	6.53×10^{-9}	0.000718
01.20.37 Metabolism of peptide-derived compounds	3.13×10^{-9}	6.50×10^{-5}
01.20.37.01 Metabolism of thioredoxin, glutaredoxin, glutathione	1.20×10^{-7}	0.001718
02 ENERGY	3.35×10^{-5}	0.085526
02.16 Fermentation	1.27×10^{-6}	0.025830
02.16.01 Alcohol fermentation	7.41×10^{-5}	0.005066
32 CELL RESCUE, DEFENCE AND VIRULENCE	0.000144	0.004342
32.07 Detoxification	0.000216	0.000716
32.07.07 Oxygen and radical detoxification	6.01×10^{-8}	1.00×10^{-5}
32.07.07.03 Glutathione conjugation reaction	2.53×10^{-8}	9.80×10^{-6}

Table 4. NcAp-1 common targets under H_2O_2 and Cd^{2+} exposure

Bold type indicates genes that have been shown to be a direct target of the AP-1 protein.

NcAp-1 target	Orthologue in *S. cerevisiae*	Orthologue in *Sch. pombe*	Orthologue in *C. albicans*	Orthologue in *A. fumigatus*	Annotation
NCU00847.2	NO	NO	NO	Afu3g15010	Methoxylase
NCU04109.2	YNL229C	**SPCC191.09c**	**orf19.2693**	Afu3g10830	Glutathione *S*-transferase
NCU04452.2	**OYE3**	SPAC5H10.10	orf19.3443	Afu2g04060	NADPH dehydrogenase chain OYE2
NCU05164.2	**YKL071W**	**SPCC24B10.20**	orf19.6502	**Afu4g00910**	Protoporphyrinogen oxidase
NCU05780.2	YNL229C	**SPCC965.07c**	NO	Afu4g14530	Glutathione *S*-transferase
NCU07452.2	**OYE2**	**SPBC23G7.10c**	**orf19.3131**	Afu5g01450	Flavin oxidoreductase
NCU08402.2	YCR105W	SPCC13B11.01	**orf19.5517**	Afu8g02430	Alcohol dehydrogenase
NCU09285.2	**ADH5**	**SPBC1773.06c**	orf19.5113	Afu4g08240	Alcohol dehydrogenase
NCU09534.2	Yir037w	**SPBC32F12.03c**	orf19.86	Afu3g12270	Peroxiredoxin

A common response gene set between *cpc-1* and *NcAp-1*

The bZIP TF CPC-1 regulates the response to amino acid starvation in *N. crassa* (Flint, 1985). Previously, we determined the *cpc-1* regulon and the response of *N. crassa* to amino acid starvation (Tian *et al.*, 2007). To determine whether a common set of genes is coordinately regulated under stress responses, we compared the *cpc-1* gene set (443 genes) with the *NcAp-1* H_2O_2/Cd^{2+} datasets (79 genes). There were 17 genes that were shared between the *cpc-1* and *NcAp-1* H_2O_2 or Cd^{2+} regulons, a significant enrichment ($P=0.02$), suggesting that crosstalk occurs between the H_2O_2, Cd^{2+} and 3-AT stress responses in *N. crassa*. Three of the 17 genes showed increased expression levels under all three conditions (Cd^{2+}, H_2O_2 and 3-AT), including NCU04109 (glutathione *S*-transferase), NCU 05164 (protoporphyrinogen oxidase) and NCU09285 (alcohol dehydrogenase). Four of 17 shared targets between the CPC-1 and NcAp-1 H_2O_2 regulons encode hypothetical proteins (NCU00995, NCU09507, NCU09874 and NCU10970), while the remaining three genes that overlap include *cat-2* (NCU05770), a predicted serine protease *spr-7* (NCU07159) and *mig-4* (NCU09040). Two of the seven genes shared between the CPC-1 and NcAp-1 Cd^{2+} regulons encode hypothetical proteins (NCU02888 and NCU07503), two encode predicted reductases (NCU04510 and NCU09821), one encodes a predicted pyruvate decarboxylase (NCU02397), one encodes a probable urate oxidase (NCU07853), and the last encodes a predicted epimerase/dehydratase (NCU00884).

DISCUSSION

In this study, we evaluated both the phenotype and the expression patterns associated with strains containing mutations in predicted bZIP TF genes in *N. crassa*. We identified a single bZIP mutant, containing a deletion of NCU03905, which showed sensitivity to multiple stresses. We further characterized the transcriptional response of strain Δ*NcAp-1* (NCU03905) upon exposure to oxidative and heavy metal stresses in comparison with the WT, and identified a number of core stress-response genes. We subsequently compared our data with those from a recent report of an independently derived Δ*nap-1* mutant and its effect on transcription in response to exposure to menadione (Takahashi *et al.*, 2010). We further compared our datasets with those obtained under different growth conditions and with expression profiling datasets from hemiascomycete species. These comparisons indicated that a small number of genes, which are primarily involved in an oxidant response, are regulated by AP1-like TFs and have been evolutionarily conserved.

Our screening showed that the Δ*NcAp-1* strain was sensitive to multiple stresses, including oxidant, heavy metal, osmotic and amino acid starvation stress, while strains containing mutations in six additional bZIP genes did not show increased sensitivity to the tested conditions. These data indicate that NcAp-1 is a critical stress response regulator in *N. crassa*. Our expression profiling results of the WT versus the *NcAp-1* mutant under H_2O_2 and Cd^{2+} exposure support this prediction, as multiple pathways were affected in the *NcAp-1* mutant, including, as expected, the oxidant response pathway, but also nitrogen metabolism and energy-related genes. In addition, *cys-3* (which is a sulfur metabolism regulator) and sulfur metabolism genes were induced by exposure to Cd^{2+} and were *NcAp-1*-dependent, suggesting crosstalk between the *NcAp-1* and *cys-3* regulons. In support of this hypothesis, genes involved in sulfate metabolism are also induced by Cd^{2+} exposure in *C. albicans* (Enjalbert *et al.*, 2006).

Genome-wide stress-response profiling data have been published for the genes orthologous to NcAp-1 in *S. cerevisiae* (Yap1) (Gasch *et al.*, 2000; Cohen *et al.*, 2002), *Sch. pombe* (Pap1) (Chen *et al.*, 2008) and *C. albicans* (Cap1) (Wang *et al.*, 2006). In addition, ChIP data are also available for Yap1 (Harbison *et al.*, 2004) and Cap1 (Znaidi *et al.*, 2009), and proteomics data are available for AfYap1 in *A. fumigatus* (Lessing *et al.*, 2007). The stress response, and in particular, the oxidant stress response, was enriched in the Yap1, Cap1, Pap1, NcAp-1 and AfYap1 datasets. Out

of the nine core NcAp-1-dependent stress response genes (Table 4), eight have orthologues in *S. cerevisiae*, and four of them (*OYE3*, YKLO71W, *OYE2* and *ADH5*) are regulated by Yap1 (Cohen *et al.*, 2002; Gasch *et al.*, 2000; Harbison *et al.*, 2004). Furthermore, YAP1 binds to the promoter region of *OYE2*, based on the ChIP data (Harbison *et al.*, 2004). Three of seven orthologues of the NcAp-1-dependent genes are bound and regulated by CAP1 in *C. albicans* (Wang *et al.*, 2006; Znaidi *et al.*, 2009), while in *Sch. pombe*, the regulation of six of the eight orthologues is dependent on PAP1 (Table 4). In *A. fumigatus*, the protein abundance of protoporphyrinogen oxidase (Afu4g00910) is dependent on AfYap1 (Lessing *et al.*, 2007) and the protein is orthologous to the NcAp-1 target NCU05164. In summary, eight of nine NcAp-1 core target genes (Table 4) have been shown to be regulated by Ap1-like proteins in at least one other ascomycete species. The ninth gene, NCU00847 (related to 7-alpha-cephem-methoxylase), has no clear orthologue in *S. cerevisiae*, *Sch. pombe* and *C. albicans*, and is possibly a filamentous fungal-specific oxidant stress response gene. In addition, genes encoding catalases are another group that show regulation under H_2O_2 conditions by Ap1-like TFs, including catalase-2 (NCU05770) (NcAp-1), catalase-2 (AfYap1), catalase-1 (Cap1) and catalase-T (Yap1). In a recent publication, Takahashi and co-workers found that catalase-3 (NCU00355) rather than catalase-2 is induced by menadione treatment in *N. crassa*, and is *nap-1* (*NcAp-1*)-dependent (Takahashi *et al.*, 2010). However, in our study, upon treatment with H_2O_2, NCU00355 was induced in both the WT (3.3-fold) and the Δ*NcAp1* mutant (2.5-fold).

The identification of the NcAp-1 regulon and pathway comparisons among ascomycete species suggest that the oxidant stress response pathway in fungi has been conserved over 300 million years of evolution and that AP1-like TFs are the main regulators of this response pathway. Both catalases and flavin oxidoreductases are regulated by AP1-like TFs in *S. cerevisiae*, *C. albicans*, *Sch. pombe* and *N. crassa*. We identified a common gene set that was induced by amino acid starvation, and oxidant and heavy metal stress, and whose predicted function was oxygen detoxification. These data suggest that reactive oxygen species (ROS) might be a general signal for stress. In all five ascomycete species (*S. cerevisiae*, *Sch. pombe*, *C. albicans*, *A. fumigatus* and *N. crassa*), the AP1-like protein localized to nuclei from the cytoplasm following exposure to a variety of stresses.

However, there is also evidence that the AP1 pathway has diverged among the ascomycete species. In *S. cerevisiae*, the functions of the duplicated Yap proteins are partially redundant, especially those of Yap1 and Yap2 (Cohen *et al.*, 2002). In *C. albicans*, the oxidant stress response has multiple distinct pathways: the Cap1 pathway and the Hog1 mitogen-activated protein kinase (MAP kinase) pathway (Enjalbert *et al.*, 2006). The Cap1 pathway is activated upon exposure to both low and high concentrations of H_2O_2, while the Hog1 pathway is only activated at higher concentrations of H_2O_2. In *Sch. pombe*, the Pap1 pathway is activated upon exposure to low H_2O_2 concentrations, while the Sty1 (Hog1 orthologue)–Atf1 (Sko1 orthologue) pathway is activated upon exposure to high concentrations of H_2O_2 (Chen *et al.*, 2008; Vivancos *et al.*, 2006). However, the Hog1 and Sko1 pathways in *S. cerevisiae* mainly regulate responses to osmotic stress (Rep *et al.*, 2001), although Skn7 is also involved in oxidative stress, independently of the Yap1 pathway (Estruch, 2000). In *N. crassa*, the *HOG1* orthologue is *os-2* (NCU07024) and the *SKN7* orthologue is *rrg-2* (NCU02413); both genes may be involved in the oxidant stress response pathway, as mutants are sensitive to H_2O_2 (Banno *et al.*, 2007). The Atf1/Sko1 orthologue in *N. crassa* is NCU01345, but is ascospore-lethal, and so homokaryotic strains are not available. Based on these data, in *N. crassa*, at least three oxidant stress response pathways may exist, the OS-2, RRG-2 and NcAp-1 pathways. In the future, it will be informative to study the relationships between various stresses and the pathways regulated by these three TFs.

Evolution of the bZIP TF family regulatory network

How an organism coordinates its transcriptional repertoire in response to developmental or environmental signals, and how evolution affects the topology of these TF networks, are still mostly unanswered questions. One way to study the regulatory networks is to focus on a single biological function. For example, over 30 TFs are involved in DNA damage in *S. cerevisiae* (Workman *et al.*, 2006). An alternative is to examine regulatory relationships using a phylogenetic framework, such as analysing the regulatory network within the Yap subfamily (Tan *et al.*, 2008). In our study, we analysed seven of the nine members of the bZIP TF family in *N. crassa*. Phylogenetic analyses indicated that the bZIP TF family is an ancient duplicated gene family, with no recent genome duplications in any fungal species, with the exception of *S. cerevisiae*. Although our data showed that the regulons of the bZIP TFs have diverged, up to 25 % of the target genes were shared between some TF pairs, for example, the affected gene sets from the colony interiors of the *cys-3* and ΔNCU08055 mutants. However, profile comparisons showed differences between the peripheral and interior sections of the colony among the different bZIP mutants. For example, although 25 % of the affected genes in the interior of a colony were identical between the ΔNCU08055 and *cys-3* mutants, only 6 % were identical between the ΔNCU08055 and *cys-3* datasets from the periphery of the colony. From our stress screening and phenotype analyses, most bZIP TF mutants did not show increased sensitivity to oxidant, metal or osmotic stress. In addition, none of the mutants showed a growth phenotype under minimal media conditions, with the exception of the Δ*ada-1* (NCU00499) mutant. Transcriptional profiling of four bZIP mutants (Δ*ada-1*, ΔNCU01994, ΔNCU04211 and ΔNCU08055) revealed a role for these TF genes in carbon compound and carbohydrate metabolism, provid-

ing a further testable hypothesis for metabolic processes regulated by these TFs. Three bZIP TF mutants could not be assayed, because homokaryotic mutants are not available, suggesting that these bZIP TFs regulate essential processes in *N. crassa*. Further genetic, phenotypic and molecular analyses to identify direct targets of the *N. crassa* bZIP TF family and epistasis relationships will reveal the commonalities and complexities of transcriptional networks regulated by this group of TFs. These data will also enable further comparative analyses to decipher mechanisms associated with the evolution of regulatory networks in *N. crassa* and other fungi.

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