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A Novel Motif in Fungal Class 1 Histone Deacetylases Is Essential for Growth and Development of *Aspergillus*

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Acetylation of the N-terminal tails of core histones is an important regulatory mechanism in eukaryotic organisms. In filamentous fungi, little is known about the enzymes that modify histone tails. However, it is increasingly evident that histone deacetylases and histone acetyltransferases are critical factors for the regulation of genes involved in fungal pathogenicity, stress response, and production of secondary metabolites such as antibiotics or fungal toxins. Here, we show that depletion of RpdA, an RPD3-type histone deacetylase of *Aspergillus nidulans*, leads to a pronounced reduction of growth and sporulation of the fungus. We demonstrate that a so far unnoticed motif in the C terminus of fungal RpdA histone deacetylases is required for the catalytic activity of the enzyme and consequently is essential for the viability of *A. nidulans*. Moreover, we provide evidence that this motif is also crucial for the survival of other, if not all, filamentous fungi, including pathogens such as *Aspergillus fumigatus* or *Cochliobolus carbonum*. Thus, the extended C terminus of RpdA-type enzymes represents a promising target for fungal-specific histone deacetylase-inhibitors that may have potential as novel antifungal compounds with medical and agricultural applications.

INTRODUCTION

The interaction of DNA and histones in chromatin is required for packaging the genetic information into the nucleus of the cell. A consequence of this compaction is the inaccessibility of DNA for factors that mediate DNA repair, replication, and transcription. Factors that induce a change in transcriptional activity first have to overcome this barrier (Wolffe and Kurumizaka, 1998; Li, 2002; Millar and Grunstein, 2006). However, the restricted accessibility of the genetic information as a consequence of chromatin formation enables cells to regulate transcription at a level beyond DNA sequence motifs. Eukaryotes have developed efficient mechanisms to remodel chromatin and modulate chromatin structure to regulate gene expression. Beside DNA methylation and the incorporation of histone primary structure variants into chromatin, two strategies have evolved to modulate chromatin structure. On one hand, ATP-dependent multiprotein remodeling complexes alter the structure of nucleosomes (Kingston and Narlikar, 1999; Vignali *et al.*, 2000; Narlikar *et al.*, 2002; Lusser and Kadonaga, 2003; Korber and Horz, 2004), and on the other hand, enzymes covalently modify core histones on residues primarily located within the N-terminal histone tails (Wu and Grunstein, 2000; Vaquero *et al.*, 2003). Among these histone modifications are acetylation, methylation, ubiquitination, sumoylation, and phosphorylation. Specific patterns of these modifications may generate a "histone code" that can be interpreted by specialized regulatory proteins (Strahl and Allis, 2000; Turner, 2007).

One of the best-studied posttranslational modifications is the acetylation of ε-amino groups of conserved lysine residues in the amino-terminal tails of the core histones H2A, H2B, H3, and H4. High levels of histone acetylation have been observed in actively transcribed genes, whereas histone deacetylation usually correlates with gene repression (Grunstein, 1997; Turner, 2000). Nevertheless, several exceptions from this basic rule have been described previously (De Nadal *et al.*, 2004; Robyr *et al.*, 2004; Hansen *et al.*, 2005).

Acetylation of histones is a dynamic process that depends on the concerted action of histone acetyltransferases and histone deacetylases (HDACs). Histone deacetylation is catalyzed by two unrelated groups of enzymes, the sirtuins and the classical HDACs. Sirtuins are NAD^+-dependent SIR2-type proteins (Shore, 2000). Classical HDACs are divided into at least three subclasses: 1) the RPD3-type enzymes, 2) the HDA1-type enzymes (Rundlett *et al.*, 1996), and 3) a group of enzymes that is absent in fungi and includes human HDAC11 and HDA2 of *Arabidopsis thaliana* (Gregoretti *et al.*, 2004).

Filamentous fungi show a wide range of morphological complexity with respect to growth, reproduction, and infection. As scavengers, they have developed an interconnected network of strictly regulated genes that enable these organisms to grow on remarkably different substrate resources (Casselton and Zolan, 2002). Some of these organisms are dreaded pathogens of humans, insects, and plants; others are producers of important secondary metabolites (SM), such as antibiotics or toxins.

Several HDACs were identified in filamentous fungi: 1) class 1 enzymes RpdA and HosA, two RPD3-type enzymes; 2) class 2 HDACs HdaA and HosB, two HDA1-type enzymes; and 3) several members of the sirtuins (class 4). Although thoroughly characterized at the biochemical level

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Table 1. Genotypes of *A. nidulans* strains used in this study

Strain	Origin	Genotype	Reference
FGSC A768		*pyrG89; riboB2; yA2; chaA1*	O'Hara and Timberlake (1989)
H7[a], H8[a]	A768	*pyrG; pyrG89; riboB2; yA2; chaA1*	Tribus *et al.* (2005)
H4[b]	A768	*hdaA*Δ*::pyrG; pyrG89; riboB2; yA2; chaA1*	Tribus *et al.* (2005)
A18	A768	*rpdA*p*::pyrG:alcA*p*:rpdA; pyrG89; riboB2; yA2; chaA1*	This study
X79	A768	*rpdA*p*::pyrG:xylP*p*:rpdA; pyrG89; riboB2; yA2; chaA1*	This study
FL	A18	*rpdA*p*::pyrG:alcA*p*:rpdA; xylP*p*:rpdA; riboB; pyrG89; riboB2; yA2; chaA1*	This study
TRU0	A18	*rpdA*p*::pyrG:alcA*p*:rpdA; xylP*p*:rpdAtru0; riboB; pyrG89; riboB2; yA2; chaA1*	This study
TRU1	A18	*rpdA*p*::pyrG:alcA*p*:rpdA; xylP*p*:rpdAtru1; riboB; pyrG89; riboB2; yA2; chaA1*	This study
TRU2	A18	*rpdA*p*::pyrG:alcA*p*:rpdA; xylP*p*:rpdAtru2; riboB; pyrG89; riboB2; yA2; chaA1*	This study
TRU3	A18	*rpdA*p*::pyrG:alcA*p*:rpdA; xylP*p*:rpdAtru3; riboB; pyrG89; riboB2; yA2; chaA1*	This study
B1	A18	*rpdA*p*::pyrG:alcA*p*:rpdA; xylP*p*:rpdAfum; riboB; pyrG89; riboB2; yA2; chaA1*	This study
N1	A18	*rpdA*p*::pyrG:alcA*p*:rpdA; xylP*p*:RPD3cer; riboB; pyrG89; riboB2; yA2; chaA1*	This study
9BH	H4$^{\chi}$FLhis	*hdaA*Δ*::pyrG; rpdA*p*::pyrG:alcA*p*:rpdA; xylP*p*:rpdAhis; riboB; pyrG89; riboB2; yA2; chaA1*	This study
23A2H	H4$^{\chi}$TRU3his	*hdaA*Δ*::pyrG; rpdA*p*::pyrG:alcA*p*:rpdA; xylP*p*:rpdAtru3his; riboB; pyrG89; riboB2; yA2; chaA1*	This study

Mutations of strain are given as follows: *rpdA*p*::pyrG:alcA*p*:rpdA* indicates the replacement of the endogenous *rpdA* promoter (*rpdA*p) by the *alcA* promoter (*alcA*p). For selection of the corresponding mutants, *pyrG* was used as auxotrophic marker. *xylP*p*:cri; riboB* (*cri* stands for *c*oding *r*egion of *i*nterest) denotes strains, with cri *(rpdA, rpdAtru0-3, rpdAfum*, and *RPD3cer)* under control of the *xylP* promoter (*xylP*p). For selection of the strains, the auxotrophic marker *riboB* was randomly integrated into the genome. All other mutations are named according to standard genetic nomenclature. For details, see *Materials and Methods* and Supplemental Figure 1. All experiments were done with at least three independent transformants of each genotype shown.

[a] Strains H7 and H8 have different single random integrations of the *pyrG* marker and were used in the experiments as wild-type controls.

[b] Strain H4 was used in this study for sexual crosses (χ) and as control strain (wt) for the chromatographic analysis.

(Graessle *et al.*, 2000; Trojer *et al.*, 2003; Tribus *et al.*, 2005), little is known about the biological functions of HDACs in filamentous fungi (for review, see Graessle *et al.*, 2001; Brosch *et al.*, 2008).

We have demonstrated previously that the class 2 enzyme HdaA, which represents the major HDAC activity in *Aspergillus nidulans*, contributes to cellular resistance against hydrogen peroxide and thus is involved in the oxidative stress response of filamentous fungi (Tribus *et al.*, 2005). Moreover, it affects the biosynthesis of SM such as penicillin or sterigmatocystin in *Aspergilli* (Yu and Keller, 2005; Shwab *et al.*, 2007; Lee *et al.*, 2009).

Whereas the structure of HdaA resembles that of typical class 2 HDACs of other organisms, class 1 (RpdA-type) enzymes of filamentous fungi are characterized by a remarkable extension of the C terminus of ~200 amino acids compared with homologous proteins of *Saccharomyces cerevisiae* and higher eukaryotes (Graessle *et al.*, 2000). Interestingly, this C-terminal extension comprises conserved motifs unique for HDACs of filamentous fungi. A closer characterization of these motifs and an investigation of their impact for HDAC activity is the subject of this study.

MATERIALS AND METHODS

Aspergillus Strains and Growth Media

Genotypes of *A. nidulans* strains generated in this study are shown in Table 1. All mutants derived from A768 (*pyrG89; riboB2; yA2; chaA1*) provided by the Fungal Genetics Stock Center (Kansas City, KS). Unless otherwise noted, strains were grown as described previously (Pontecorvo *et al.*, 1953). For induction of the alcohol dehydrogenase promoter (*alcA*p) of *A. nidulans*, spores were dotted onto solid medium with 3% lactose as the sole carbon source supplemented with 10 mM L-threonine. Repression was achieved by addition of 1% glucose. For Northern analysis, 50 mM 2-butanone, a strong inducer for *alcA*p, was added to the liquid medium (Felenbok *et al.*, 2001). For induction of the heterologous xylanase promoter (*xylP*p), media were supplemented with 1% (strong induction) or 0.05% (moderate induction) xylose. Repression was performed with 1% glucose (Zadra *et al.*, 2000). It is important to note that in contrast to *alcA*p, *xylP*p is induced when both repressor and inductor are present in the medium. Estimated promoter activities under appropriate growth conditions are summarized in Supplemental Table 1.

Generation of RpdA Knockdown Constructs and Expression Plasmids

Generation of RpdA knockdown strains was achieved by exchange of the endogenous regulatory sequence of *rpdA* with a controllable promoter by using targeted promoter replacement. Two inducible/repressible promoters were used for the generation of the knockdown constructs; the alcohol dehydrogenase promoter of *A. nidulans* (Felenbok *et al.*, 2001) and the heterologous xylanase promoter of *Penicillium chrysogenum* (Zadra *et al.*, 2000). For the generation of the *alcA*p*:rpdA* construct, a 0.7-kb region of the *alcA* promoter (Panozzo *et al.*, 1998) was fused to the *rpdA* coding sequence. For the generation of the *xylP*p*:rpdA* construct, a 1.6-kb fragment of the *xylP* promoter was used (Haas *et al.*, 1993). Both polymerase chain reaction (PCR)-amplified regulatory sequences were fused to an amplified 1.5-kb 3′-truncated *rpdA* fragment by using a PCR-mediated ligation technique as described previously (Graessle *et al.*, 1997). Resulting DNA fragments were cloned into a pGEM-T vector (Promega, Madison, WI), digested with XmaI and NotI, and ligated into a plasmid carrying the *pyrG* gene (selectable marker) following 1.2 kb of 5′ untranslated region (UTR) of *rpdA*. For transformation procedure of the recipient strain A768, a linear 5.1-kb NcoI-XbaI fragment (*alcA*p*:rpdA*) or a 6.0-kb NcoI-XbaI fragment (*xylP*p*:rpdA*) was used (Supplemental Figure 1A). Uridine/uracil prototrophic transformants (*pyrG* +) were recovered on media with 3% lactose as sole carbon source and 10 mM of L-threonine or 1% xylose to assure the expression of functional RpdA driven by the *alcA* or the *xylP* promoter.

For expression of truncated RpdA fragments or RPD3-type enzymes of other fungi, the knockdown construct *xylP*p:*rpdA* (see above) was used as origin for the generation of the expression plasmids. To this end, the coding sequence of *rpdA* was replaced by the PCR-amplified truncated *rpdA* fragments *rpdAtru0*, *-1*, *-2*, and *-3* (see Figure 4). For cloning of the truncated sequences, restriction sites XbaI and NotI (added to the 5′ end of the antisense primer) were used. In two of the expression constructs (full-length *rpdA* and *rpdAtru3*), the coding sequence of six histidines (also added to the 5′ end of the reverse primer) were fused in frame to the 3′ end of the sequence to be expressed.

For expression of the Rpd3-type proteins of *S. cerevisiae* or *Aspergillus fumigatus*, corresponding coding sequences were amplified by PCR and fused to an amplified 1.7-kb *xylP* promoter fragment by PCR-mediated ligation and cloned into a GEM T-vector. The fused PCR product was subsequently digested with SpeI and NotI (Supplemental Figure 1B) and ligated into the SpeI/NotI-digested expression construct *xylPp:rpdAtru2*.

All constructs were sequenced before transformation. Transformation procedure was carried out as cotransformation with pPL1, a vector containing the sequence complementing the mutated *riboB2* gene (Oakley *et al*., 1987) of the recipient strain A18 (Table 1). Transformants were recovered on media without riboflavin, but with 3% lactose as sole carbon source and 10 mM L-threonine to induce expression of full-length RpdA under control of the alcA promoter. To analyze the biological function of the truncated RpdA fragments and the RPD3-type enzymes of *A. fumigatus* and *S. cerevisiae*, recovered expression strains were grown in parallel on minimal medium (MM) plates under *alcA*p inductive/*xylP*p repressive (L-threonine) and *alcA*p repressive/*xylP*p inductive (glucose/xylose) conditions (Supplemental Table 1).

Transformation of A. nidulans and Screening of Transformants

The transformation of *Aspergillus* strains was done as described previously (Tilburn *et al*., 1995). Colonies deriving from homokaryotic spores of PCR-positive transformants were picked, and (single) genomic integration was confirmed by Southern blot analysis. Preparation of genomic DNA and Southern blot analysis were performed as described previously (Graessle *et al*., 2000).

Sexual Crosses of A. nidulans Strains

To generate RpdA expression strains with a Δ*hdaA* background, a *hdaA* minus mutant (H4) was crossed with strains FLhis and TRU3his, respectively. Crosses were done on agar plates containing MM with 3% glucose without riboflavin (Kafer, 1977). Double mutant strains 9BH and 23A2H (Table 1) were identified by PCR and Southern blot analysis.

Northern Blot Analysis

Transcription of all recombinant expression products was analyzed under inductive/repressive conditions of the respective promoter system. Strains were grown in MM for 18 h at 37°C in shake culture and for additional 3 h in the presence of the corresponding inducer/repressor as described. RNA preparation, blotting, and hybridization were done as described previously (Graessle *et al*., 2000). Poly(A) RNA was isolated from 100 μg of total RNA by using the Dynabeads Oligo(dT)25 kit (Invitrogen, Carlsbad, CA) following the manufacturer's instructions.

Microscopy and Determination of the Sporulation Rate

For morphological analysis, colonies of RpdA knockdown strains were inspected directly on agar plates with a stereomicroscope (MZ16; Leica, Wetzlar, Germany). Hyphae were examined on glass slides with a transmitted-light microscope (Axioplan' Carl Zeiss, Jena, Germany) at a magnification of 400×. To analyze the impact of RpdA suppression on sporulation, 10^4 conidia were point inoculated on MM supplemented as described and incubated at 37°C for 72 h. Determination of conidia produced by 1 cm^2 of the colonies was performed after 120 h by using a Neubauer counting chamber.

Extraction of Aspergillus Histones and Determination of H3 and H4 Acetylation

Frozen lyophilized mycelium (1 g) was ground to powder under liquid nitrogen and suspended in 50 ml of homogenization buffer (10 mM 1,4-piperazinediethanesulfonic acid, pH 6.9, 5 mM CaCl, 5 mM $MgSO_4$, 0.5 M sucrose, 1 mM phenylmethylsulfonyl fluoride, and 10 mM β-mercaptoethanol). Then, 300 nM trichostatin A was added to the buffer to avoid deacetylation during the extraction process of the core histones. The suspension was homogenized in a Potter-Elvehjem homogenizer and centrifuged for 10 min at 500 × *g*. The supernatant was centrifuged at 6000 × *g* in a swinging bucket rotor for 20 min to accumulate the nuclei. The pellet was resuspended in 1 ml of distilled water, and basic proteins were extracted by adding 5 volumes of 0.5 M hydrochloric acid and gentle stirring overnight. Subsequently, the extract was centrifuged at 14,000 × *g* for 20 min, and core histones in the supernatant were precipitated overnight at −20°C by addition of 5 volumes of precooled acetone. Precipitated proteins were spun down at 10,000 × *g* for 10 min, washed twice with 10 ml of acetone, air-dried, and dissolved in 600 μl of distilled water.

Aliquots of nuclear extracts were electrophoresed in 16% polyacrylamide gels and blotted onto nitrocellulose membrane as described previously (Trojer *et al*., 2003). Immunological detection was done using anti-acetyl histone H3, anti-histone H3 pan, anti-acetyl histone H4, anti-acetyl lysine, and anti-histone H4 pan antibodies (catalog nos. 06-599, 07-690, 06-598, 06-933, and 05-858; Millipore, Billerica, MA).

SourceQ Chromatography, Western Blot Analysis, and Histone Deacetylase Assay

Expression of recombinant products was confirmed by partial purification of the proteins from total protein extracts of the expression strains by using Source 15Q anion exchange chromatography as described previously (Tribus *et al*., 2005). Immunological identification of expressed (His-tagged) RpdA in protein fractions was done using an anti-6xHis antibody and an antibody against the C terminus of RpdA (Trojer *et al*., 2003). HDAC activity of the fraction was determined as described in Brosch *et al*. (2001).

Sequence Analysis

For sequence searches of (filamentous) fungi, the Broad Institute homepage (http://www.broad.mit.edu/annotation/fungi/), the J. Craig Venter Institute homepage (http://www.jcvi.org/cms/research/projects/msc/), and the homepage of the National Center for Biotechnology Information [http://www.fungalresearchtrust.org/] were used. Probability for the formation of alpha-helical coiled-coils in HDACs was estimated using the COILS program of Lupas *et al*. (1991); http://npsa-pbil. ibcp.fr/cgi-bin/npsa_automat.pl?page =/NPSA/npsa_server.html].

RESULTS

RpdA Is Essential for Growth and Development of A. nidulans

To identify the biological functions of HDACs in filamentous fungi, we deleted several genes coding for classical HDACs of *A. nidulans* and other filamentous fungi (Baidyaroy *et al*., 2001; Tribus *et al*., 2005; Shwab *et al*., 2007).

In contrast to deletions of HDA1-, HOS2-, or HOS3-type enzymes all efforts to generate RPD3-type null mutants (Δ*rpdA*) failed, suggesting that RpdA is essential for the recovery of transformed protoplasts. We therefore used a "knockdown" strategy to analyze effects of RpdA depletion in fungal hyphae (Supplemental Figure 1A). To this end, we replaced the endogenous *rpdA* promoter by either one of two inducible promoters: 1) the promoter of the alcohol dehydrogenase gene (*alcA*p) of *A. nidulans* (Felenbok *et al*., 2001) or 2) the promoter of the xylanase gene (*xylP*p) of *P. chrysogenum* (Zadra *et al*., 2000). Regeneration of transformed protoplasts under inductive conditions resulted in the recovery of several transformants. Subsequently, *rpdA* transcription was depleted by growing conidia of the derived strains on solid media containing 1% glucose, which represses both, the *alcA* and the *xylP* promoter. Repression resulted in a tremendous loss of radial growth of fungal colonies derived from *alcA*p:*rpdA* transformants (Figure 1A). Moreover, hyphae were distorted and hyperbranched (Figure 1B), and sporulation of these strains was decreased to <2% of the wild type (*wt*; Figure 1C). Although growth of *xylP*p:*rpdA* strains was also clearly inhibited, the phenotype was less severe than that of *alcAp:rpdA* strains. To assess, whether this could be due to differences in knockdown efficiencies between the two promoter systems, we performed Northern analysis. Indeed we found that repression of the *xylP* promoter was not complete under the conditions used (Figure 2 and Supplemental Table 1). However, RpdA induction fully restored the *wt* phenotypes in both types of knockdown strains. In liquid culture, the effect was less pronounced but still resulted in an ~60% decrease of mycelial mass compared with inductive conditions.

Together, our analysis demonstrates loss of viability, growth and sporulation of *A. nidulans* as a consequence of

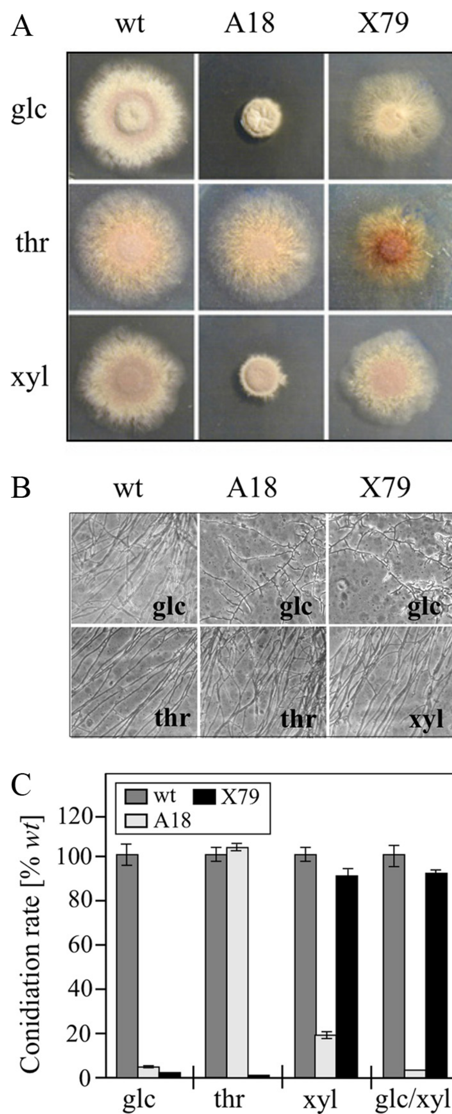

Figure 1. Growth of *alcAp:rpdA* (A18) and *xylPp:rpdA* (X79) strains under *rpdA* inductive/repressive conditions. Strains with different random integrations of the *pyrG* marker served as *wt* control. (A) Radial growth of the strains on agar plates with 3% lactose as carbon source, and 10 mM L-threonine (thr) or 1% xylose (xyl) for induction of the *alcA* or *xylP* promoter. Repression of both promoters was achieved by 1% glucose (glc). (B) Microscopic examination of hyphae grown under *rpdA* repressive (glc) or inductive (thr or xyl) conditions. (C) Conidiation rates of the strains under repressive (glc), inductive (thr or xyl), or repressive/inductive (glc/xyl) conditions. Error bars indicate the SD of the means of three independent determinations.

rpdA suppression and thus indicates that RpdA plays an essential role in fungal development.

A Conserved Region within the Fungal Specific C-Terminus Is Required for Physiological Function of RpdA

In addition to a highly conserved N-terminal catalytic core domain, eukaryotic class 1 HDACs contain very heterogeneous C-terminal tails (Grozinger and Schreiber, 2002). Despite low similarity between these C termini, they share distinct residues that are important for phosphorylation, acetylation, and other posttranslational modifications that are determining factors for stability, localization, activity, and protein–protein interaction of the enzymes (for review, see Brandl *et al.*, 2009). Compared with other eukaryotic RPD3-type enzymes, the C-terminal tail of RPD3 orthologues is considerably elongated (~200 amino acids) in filamentous fungi (Graessle *et al.*, 2000). In silico analysis revealed that it includes a few less conserved boxes and one highly conserved insertion from methionine 410 to lysine 427 (Figure 3).

To investigate the biological significance of these motifs, we expressed a truncated version of RpdA (RpdATru0) controlled by the *xylP* promoter in the *alcAp:rpdA* recipient strain (Table 1 and Supplemental Figure 1B). RpdATru0 lacks the fungal specific C-terminal extension of RpdA type enzymes but contains the highly conserved N-terminal HDAC domain essential for the catalytic activity of RPD3-type enzymes (Graessle *et al.*, 2000; Grozinger and Schreiber, 2002). We examined the ability of this truncated RpdA version to substitute for the full-length protein by growing transformant strains in the presence of xylose or glucose/xylose, which results in repression of full-length protein and simultaneous activation of the truncated enzyme. Although RpdATru0 was efficiently transcribed (Figure 2C), growth of the expressing strains was severely compromised (see Figure 5A). This result suggests that the C-terminal portion of RpdA essentially contributes to the RpdA's role in promoting viability of the fungus. For a more detailed examination of potentially critical sequence stretches within the C-terminal region, we analyzed this part of the protein for the presence of typical sequence motifs. We detected a highly negative charged insertion of ~20 amino acids that turned out to be conserved in all RpdA-type proteins of filamentous fungi (Figure 3). To test whether this or the less conserved motifs on the C-terminal end of the enzyme were responsible for the phenotype observed in the RpdATru0 expressing strain, we generated three expression constructs (TRU1–3) harboring different truncations of the C-terminal tail of RpdA under the control of the *xylP* promoter. These fragments were subsequently expressed in the *alcAp:rpdA* strain (A18). A detailed representation of the fragments tested for complementation of full-length RpdA is depicted in Figure 4. Although sufficiently expressed (see Figure 6B) full-length RpdA lacking the conserved motif did not restore the wt phenotype, whereas all other expressed *rpdA* fragments did (Figure 5A). These results clearly demonstrate the requirement of the conserved C-terminal motif for growth and development of *A. nidulans*.

A Conserved C-terminal Motif Is Required for Enzymatic Activity of RpdA

To examine, whether the conserved C-terminal motif affects HDAC activity of RpdA, we fractionated equal amounts of crude protein extracts from strains expressing either wild-type RpdA controlled by its endogenous promoter (strain H4), or His-tagged full-length RpdA with and without the motif controlled by the xylanase promoter (strain 9BH and 23A2H) by using SourceQ anion-exchange chromatography.

Eluted protein fractions were then further analyzed for HDAC activity. Because HdaA (a class 2 type HDAC) is known as the major source of total in vitro HDAC activity in *A. nidulans* (Trojer *et al.*, 2003), this analysis was performed in a Δ*hdaA*-background to increase the sensitivity of the assay. An overview of strains used in this study is given in Table 1.

As expected, HDAC activity of strains H4 and 9BH eluted at salt concentrations between 220 and 350 mM NaCl, which is consistent with earlier data (Trojer *et al.*, 2003; Tribus *et al.*, 2005). In contrast, no HDAC activity above background was detectable in the RpdATru3 expressing strain 23A2H—neither in protein fractions 21–39 nor in any other fraction of this strain (Figure 6A).

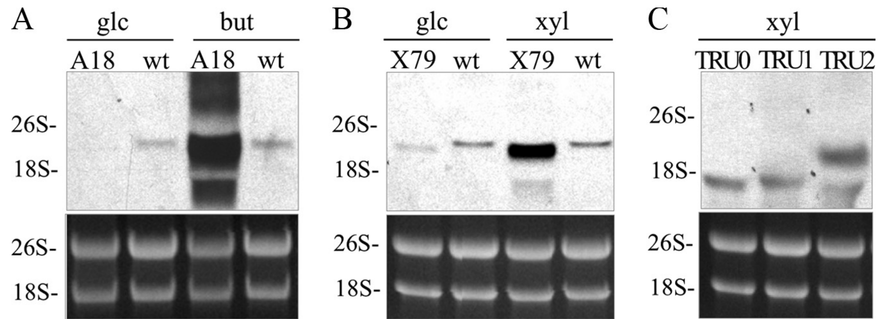

Figure 2. Determination of (truncated) *rpdA* transcription in *alcAp:rpdA* (A18), *xylPp:rpdA* (X79), *xylPp:rpdAtru0-2* (TRU0, TRU1, and TRU2), and *wt* strains under inductive/repressive conditions. Strains were grown for 18 h in minimal medium and for another 3 h in the presence of the corresponding inducer/repressor. For induction of the corresponding promoters, 50 mM 2-butanone (but) or 1% xylose (xyl) was added to the medium. For repressive conditions, 1% glucose (glc) was used. Northern analysis was performed with 10 μg of total RNA; 26S and 18S rRNA is shown as loading control. The shift of the *rpdA* fragment in *xylPp:rpdA* strains compared with that of the *wt* is due to the short 5′ UTR of the *xylP* promoter.

To confirm that the HDAC peak of 9BH and H4 is due to RpdA activity and to prove that loss of activity in strain 23A2H was not caused by inefficient expression of the RpdATru3 transgene, immunoblotting experiments were performed. Two antibodies were used for this analysis: 1) An RpdA antibody raised against the unique C terminus of *Aspergillus* RpdA (Trojer *et al*., 2003) and 2) an antibody specific for the 6xHis-tag fused to the C-terminal end of expressed RpdA. As expected, the bulk of RpdA protein eluted in the HDAC activity peak fractions 26–29 in 9BH and H4 (Figure 6B). In strain 23A2H, the expression profile of RpdATru3 was similar, although no catalytic activity was detected in the corresponding fractions. These results demonstrate that the conserved C-terminal motif of RpdA is absolutely required for its catalytic activity.

RpdA Is Responsible for the Deacetylation of H3 and H4

Despite of a continuously increasing number of nonhistone substrates identified (Glozak *et al*., 2005), core histones remain primary targets of HDACs. To explore whether RpdA is affecting the acetylation status of H3 and H4 in vivo,

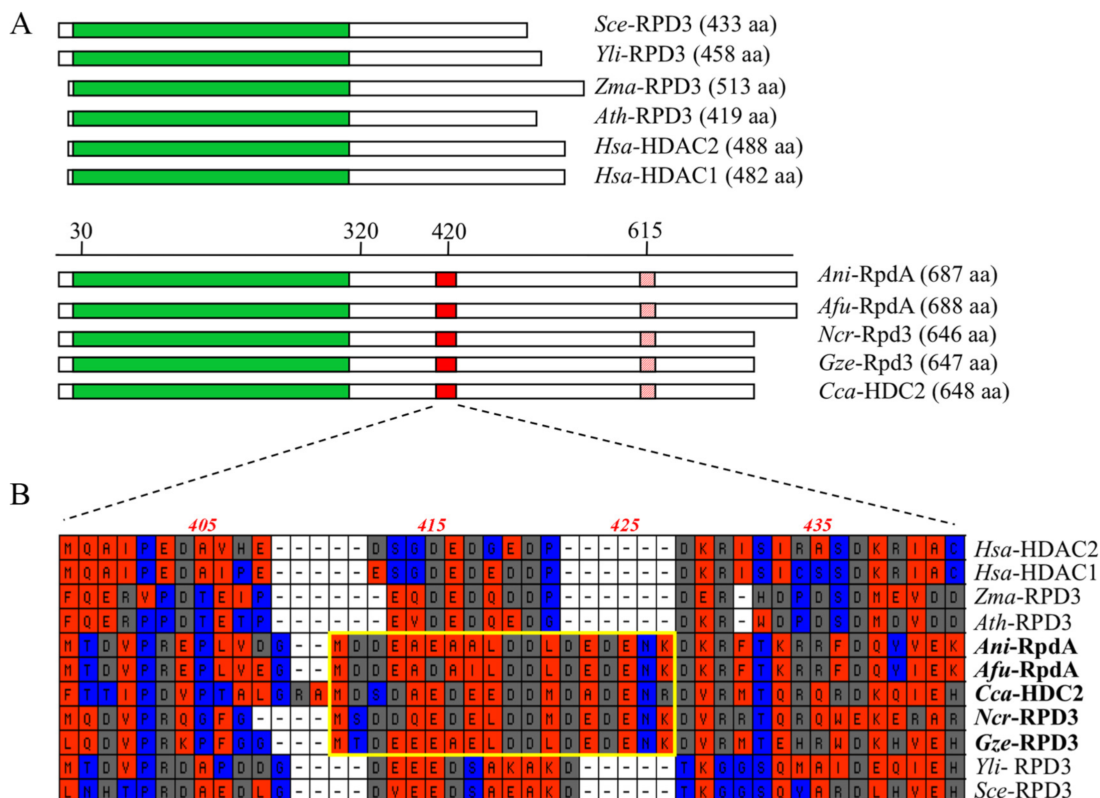

Figure 3. Schematic representation of amino acid sequences of RPD3-type HDACs in different eukaryotes. (A) Green bars indicate the highly conserved N-terminal domain of classical HDACs (Grozinger and Schreiber, 2002; Gregoretti *et al*., 2004). Regions of high and low similarities within the C-terminal tail of fungal RpdA-type enzymes are red or red striped. (B) The region of the highly conserved motif of filamentous fungi is boxed in yellow. The aa scale on the top of the figure refers to *A. nidulans* RpdA. Sequence names of filamentous fungi are in bold. Accession numbers are as follows: *A. nidulans*, *Ani*-RpdA (AAF80489); *A. fumigatus*, *Afu*-RpdA (XP_749474); *N. crassa*, *Ncr*-RPD3 (hypoth. protein, EAA35131); *G. zeae*, *Gze*-RPD3 (hypoth. protein, EAA70726); *C. carbonum Cca*-HDC2 (AAK35180); *S. cerevisiae*, Sce-RPD3 (NP_014069); *Y. lipolytica*, *Yli*-RPD3 (hypoth. protein, XP_504286); *Z. mays*, *Zma*-RPD3 (HDAC homologue, P56521); *A. thaliana Ath*-RPD3 (HDAC homologue; AAG28475); and *Homo sapiens*, Hsa-HDAC1 (NP_004955), Hsa-HDAC2 (Q92769).

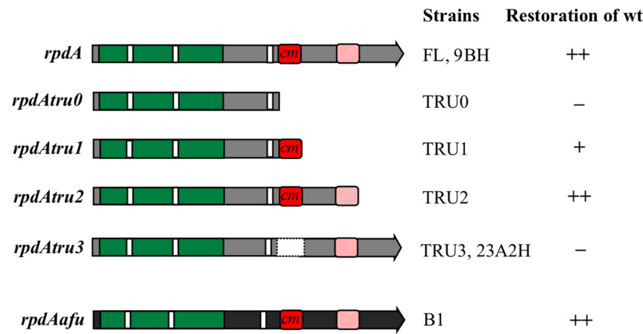

Figure 4. Schematic representation of *rpdA* fragments expressed in *A. nidulans* under the control of the *xylP* promoter. Bars illustrate the open reading frames, and introns are depicted in white. The conserved N-terminal region of classical HDACs is indicated in green, red boxes show the essential conserved motif (cm) or less conserved motifs (striped) in the C termini of RpdA-type proteins. The biological functionality of expressed fragments is indicated. ++, growth comparable with *wt*; +, growth slightly reduced; and −, growth inhibited.

nuclear extracts of strains 9BH (expressing full-length RpdA, +cm) and 23A2H (expressing RpdA without the conserverd

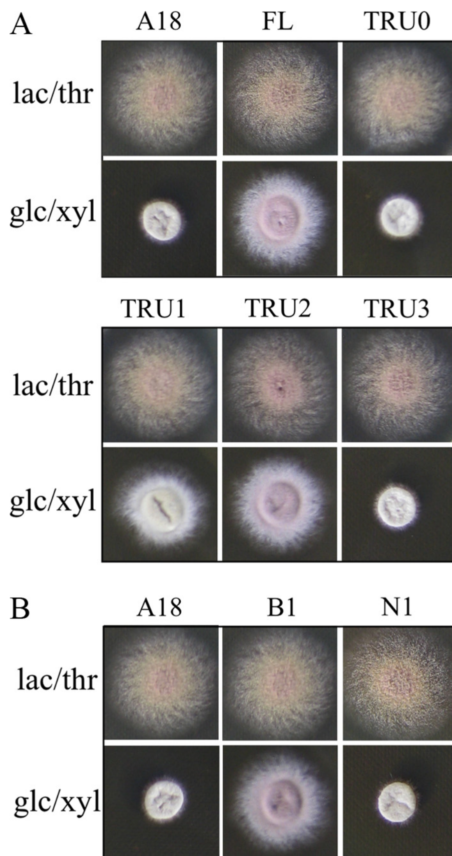

Figure 5. (A) Growth phenotype analysis of strains expressing different (truncated) HDACs under control of the xylanase promoter (*xylP*p). Strains were grown under induction of native *rpdA* (lac/thr), or under *rpdA* repression (glc/xyl) with simultaneously induced truncated RpdA proteins (TRU1–3) or induced full-length RpdA (FL). (B) Identical analyses were performed with strains expressing the RpdA homologous protein of *A. fumigatus* (B1) and RPD3 of *S. cerevisiae* (N1). In all analyses, an *alcAp:rpdA* recipient strain (A18) served as wild-type control.

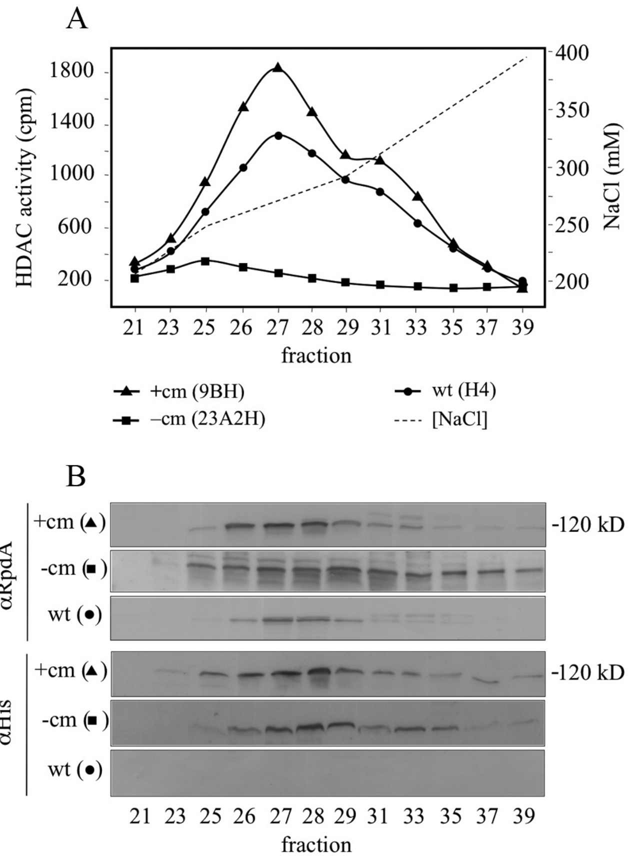

Figure 6. Purification of RpdA from protein extracts of *Aspergillus* strains. Protein extracts were loaded onto a Source 15Q anion-exchange column, and proteins were eluted with a linear gradient from 10 to 500 mM NaCl. Fractions of 2.5 ml were collected and aliquots were used for HDAC activity assays (A) and immunoblotting (B). (A) Protein fractions from Δ*hdaA* strains expressing RpdA under control of the endogenous promoter (wt) and 6xHis-tagged RpdA with (+cm) or without (−cm) the conserved motif under control of *xylP* promoter were measured with respect to HDAC activity. A dashed line shows the NaCl gradient applied for elution of proteins. (B) To proof sufficient expression of the recombinant RpdA fragments, fractions with HDAC activity were subjected to SDS-polyacrylamide gel electrophoresis with subsequent blotting onto nitrocellulose membranes. An antibody specific for the C-terminal tail of RpdA (top) and an anti-His antibody (bottom) were used for the immunological detection. The molecular mass of His-tagged full-length RpdA is indicated.

motif, −cm) were subjected to immunoblotting. Anti-acetyl H3, anti-acetyl H4, and anti-acetyl lysine-antibodies were used to estimate the acetylation status of core histones. Acetylation of both H3 and H4 was substantially increased in nuclear extracts of strain 23A2H expressing inactive RpdA compared with extract of 9BH expressing the active full-length enzyme (Figure 7). These data demonstrate that RpdA is responsible for a major part of bulk H3 and H4 deacetylation in a Δ*hdaA* background.

An RPD3-type Enzyme of A. fumigatus Is Able to Substitute for RpdA in A. nidulans

A. fumigatus is the most important pathogenic filamentous fungus in humans (Rhodes, 2006). The identification of potential targets for antifungal drugs that would kill the fungus but do not impair human cells is an important goal of biomedical research. Our findings that RpdA is an essential

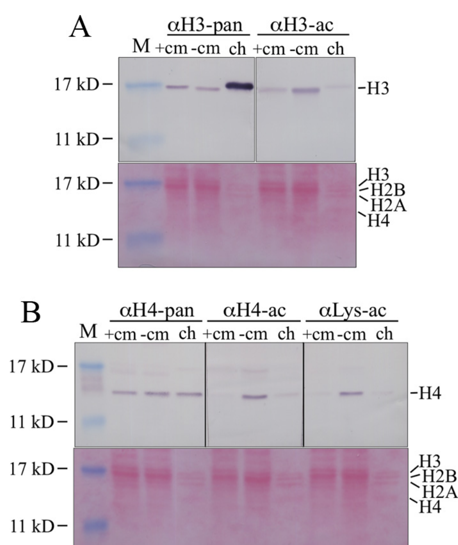

Figure 7. Histone acetylation status of *A. nidulans* Δ*hdaA* strains expressing RpdA with (+cm) or without (−cm) the conserved motif. Nuclear extracts were subjected to SDS-16% polyacrylamide gel electrophoresis, blotted onto nitrocellulose membranes, and probed with anti-histone H3 (A) and anti-histone H4 (B) antibodies. Chicken erythrocyte core histones (ch) were used as control. Acetylation of H3 was detected by an anti-acetyl H3 antibody (K9 and K14). Equal loading of H3 was determined with an anti-H3 pan antibody against the C-terminal tail of human H3. Acetylation of H4 was determined with an anti-acetyl H4 antibody (K5, K8, K12, and K16). H4 hyperacetylation assessed in the −cm strain was further confirmed with an anti-acetyl lysine antibody. Loading of H4 was determined with an anti-H4 pan antibody against amino acids 17–28 of H4. For all blots, consistent loading was confirmed by Ponceau red staining of the blots (bottom part of the figure), the positions of core histones are indicated.

enzyme in *A. nidulans* carrying essential fungal-specific sequence motifs prompted us to test whether the *A. fumigatus* RpdA is able to rescue *A. nidulans* strains with inactivated expression of the endogenous gene. Therefore, the RpdA homologous enzyme of *A. fumigatus* was expressed under the control of the *xylP* promoter in the *alcAp:rpdA A. nidulans* strain A18. In contrast to a strain expressing RPD3 of *S. cerevisiae*, recombinant RpdA of *A. fumigatus* was able to restore growth and sporulation of the RpdA knockdown mutant (Figure 5B).

Collectively, our data suggest that RpdA-type enzymes play an essential role in many, if not all, filamentous fungi and support the idea that these HDACs might substitute for each other also due to distinct features of a negatively charged C-terminal insertion that is conserved in these organisms.

DISCUSSION

During the last decade, it has become more and more evident that RPD3-type deacetylases affect numerous biological processes in eukaryotes. However, distinct phenotypes as a consequence of deletion of *RPD3* genes depend on the model organism used. In *Candida albicans*, RPD3 was found to play a key role in the regulation of phase-specific genes and to be involved in the suppression of switching between two morphological forms (Srikantha *et al*., 2001). In *S. cerevisiae*, disruption of *RPD3* influences transcription of genes involved in meiosis and sexual sporulation and leads to several pleiotropic effects (Vidal and Gaber, 1991; Bernstein *et al*., 2000). However, deletion is only lethal in the absence of other histone modifying enzymes such as the proposed arginine methyltransferase HSL7 (Ruault and Pillus, 2006).

In filamentous fungi, little is known about the biological functions or target genes of RPD3-type proteins (for review, see Brosch *et al*., 2008). In this study, we show that the depletion of RpdA, the RPD3-type HDAC of *A. nidulans*, leads to a significant retardation of growth and development of the fungus. High structural similarities of RpdA orthologous enzymes in *A. fumigatus*, *Neurospora crassa*, *Cochliobolus carbonum*, *Giberella zeae*, *P. chrysogenum* (Supplemental Table 1), and other *Pezizomycotina* strongly suggest that RpdA-type HDACs have similar crucial functions in many if not all filamentous fungi. This idea is supported by the facts that 1) the RpdA homologue of *A. fumigatus* can substitute for RpdA in *A. nidulans* and 2) several attempts to delete *rpdA*-homologous genes in *Cochliobolus* and other *Pezizomycotina* failed (Walton, personal communication; our unpublished data).

Although of vital importance, the biological role of RpdA-type enzymes in filamentous fungi remains unclear. In *S. cerevisiae*, chromatin immunoprecipitation and intergenic microarrays suggested a specific role for RPD3 as a gene repressor that primarily acts on promoter nucleosomes (Robyr *et al*., 2002). Recent analysis of the RPD3 homologous protein Clr6 in *S. pombe* confirmed this general function (Wiren *et al*., 2005). Consequently, lethality of RpdA-depleted *A. nidulans* strains due to a repressor upregulation of an essential factor is likely. In the plant pathogenic fungus *C. carbonum*, however, deletion of another class 1 HDAC, HDC1, drastically diminishes the virulence on maize plants as a consequence of a reduced expression of extracellular depolymerases. Interestingly, we could demonstrate evidence that these depolymerase genes might be directly activated by HDC1 (Baidyaroy *et al*., 2001). Consequently, members of (fungal) class 1 HDACs might also directly activate genes. Subsequent data from *S. cerevisiae* confirmed these suggestions (Wang *et al*., 2002; De Nadal *et al*., 2004). In the light of these findings, lack of activation of essential genes must also be considered as a possible reason for the growth retardation in RpdA-depleted strains.

With only a few exceptions, all class 1 HDACs act as catalytic subunits of multiprotein complexes. In *S. cerevisiae* and *Schizosaccharomyces pombe* two distinct RPD3 complexes with various complex-specific factors have been identified (Carrozza *et al*., 2005a,b; Nicolas *et al*., 2007). Complex I of *S. pombe* regulates promoters that drive sense transcription and is responsible for deacetylation of H3 at specific genomic locations, whereas complex II represses antisense transcription, protects DNA from genotoxic agents and deacetylates bulk histone H3 (for review, see Yang and Seto, 2008). Hyperacetylation of histone H3 was also found in our RpdA/HdaA mutants; moreover, H4-acetylation of these strains was significantly increased as well. The latter observation is consistent with recent in vivo data of *S. cerevisiae*, indicating H4-K5, -K8, -K12, and -K16 as typical target sites of RPD3 (for review, see Millar and Grunstein, 2006).

Irrespective of the biological impact of RpdA, a short negatively charged C-terminal insertion is required for its enzymatic activity and thus is essential for the viability of *A. nidulans*. Interestingly, this motif is conserved within the very heterogeneous C termini of RpdA-type HDACs of filamentous fungi (Supplemental Table 2). In RPD3-type enzymes of yeasts, however, this motif is missing. Consistently, *S. cerevisiae* RPD3 is unable to compensate the loss of the authentic RpdA in *Aspergillus*. Structural analysis of RPD3-type enzymes of higher eukaryotes shows an inconsis-

tent picture. The C termini of mammalian HDAC2 proteins contain a region predicted to form an α-helical coiled-coil, suggesting an involvement in protein–protein associations (for review, see Gregoretti *et al.*, 2004). However, the probability for a coiled-coil in mammalian HDAC1 enzymes and in the single *Drosophila* HDAC1/2 protein is much less pronounced. Moreover, distinct residues in the C-terminal tail of human class 1 HDACs were identified as targets for acetylation and phosphorylation (for review, see Brandl *et al.*, 2009). Most notably, phosphoserine residues 421 and 423 were reported to be required for corepressor interactions and enzymatic activity of HDAC1. Although also present in RpdA-type proteins of filamentous fungi, these C-terminal serine residues were abscised in the complementing TRU1 fragment. Consequently, phosphorylation of these serines obviously seems to be not required for the biological functionality of RpdA in *A. nidulans*.

Two ways are conceivable, by which the conserved fungal motif might affect the biological activity of RpdA. Either it stabilizes the protein and promotes its intrinsic activity, or it plays an essential role for the interaction with specific factors that are necessary for the formation of catalytically active RpdA complexes. Conceptual support for the latter assumption comes from recent studies and is also substantiated by our results: 1) In *S. pombe*, the RPD3-type protein Clr6 is only active as part of large multiprotein complexes. Interestingly, one of these complexes was recently found to be required for full viability of the fungus (Nicolas *et al.*, 2007). 2) Several subunits of the RPD3 complex of budding yeast (DEP1, SDS3, and RAF60) were shown to be critical for the formation of the RPD3 complex and/or are required for its catalytic activity (Carrozza *et al.*, 2005a; Colina and Young, 2005). 3) The expressed RpdA fragments RpdATRU3 comprises the entire catalytic HDAC-domain of RPD3-type proteins (Grozinger and Schreiber, 2002), however, is inactive and unable to substitute for the depleted full-length enzyme (Figure 5). 4) Fractions of chromatographically purified full-length RpdA eluting under high-salt conditions still contained considerable amounts of the enzyme without displaying significant catalytic activity most likely due to an inability of excess RpdA to recruit complex partners in a stoichiometric quantity (Figure 6).

In conclusion, we have reported the identification of a previously unrecognized motif in the class 1 HDAC RpdA of *A. nidulans* that is required for the catalytic activity of the enzyme. Moreover, we provide strong evidence that active RpdA is not only indispensable for growth and development of *A. nidulans* but may be essential for other filamentous fungi as well. Thus, RpdA represents a promising target for HDAC inhibitors with impact on the vitality of these organisms. Several HDAC inhibitors were recently approved (or are under evaluation) as therapeutic and chemopreventive agents against cancer, neurodegenerative disorders, and transplantation intolerance (for review, see Elaut *et al.*, 2007). Most of them, however, display only limited selectivity toward classical HDACs raising the question, which HDACs are actually affected by these inhibitors in patients. Fungal-specific motifs of important enzymes such as RpdA may serve as targets for novel and fungal-directed inhibitors. Because several filamentous fungi are not only well known for infection of food and crop plants (Tucker and Talbot, 2001) but also represent causative agents of infections in humans (Brakhage, 2005), the development of novel antifungal substances is highly desirable.

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