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Enzymatic Activity Associated with Class II HDACs Is Dependent on a Multiprotein Complex Containing HDAC3 and SMRT/N-CoR

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Summary

Histone deacetylases (HDACs) play a key role in regulating eukaryotic gene expression. The HDAC domain, homologous to the yeast repressors RPD3 and HDA1, is considered necessary and sufficient for enzymatic activity. Here, we show that the catalytic domain of HDAC4 interacts with HDAC3 via the transcriptional corepressor N-CoR/SMRT. All experimental conditions leading to the suppression of HDAC4 binding to SMRT/N-CoR and to HDAC3 result in the loss of enzymatic activity associated with HDAC4. In vitro reconstitution experiments indicate that HDAC4 and other class II HDACs are inactive in the context of the SMRT/N-CoR-HDAC3 complex and do not contribute to its enzymatic activity. These observations indicate that class II HDACs regulate transcription by bridging the enzymatically active SMRT/N-CoR-HDAC3 complex and select transcription factors independently of any intrinsic HDAC activity.

Introduction

The packaging of DNA into chromatin in eukaryotic cells restricts its accessibility to factors involved in fundamental cellular processes such as replication and transcription. The nucleosome is the fundamental organizing unit of chromatin and consists of an octamer of histone proteins, two copies each of H2A, H2B, H3, and H4, around which the DNA is tightly wrapped and bound via electrostatic interactions (Luger et al., 1997). Chromatin fibers are further compacted and organized into higher order structures establishing distinct functional domains (Wolffe, 1998; Kornberg and Lorch, 1999). Local or extended structural changes in chromatin are achieved by chromatin remodeling complexes and enzymes posttranslationally modifying histones (reviewed in Wu, 1997; Wolffe and Guschin, 2000; Jones and Kadonaga, 2000). Numerous studies have implicated a general repressive effect of chromatin on gene expression, and nucleosomal rearrangements accompany the activation of many genes (reviewed in Workman and Kingston, 1998; Tyler and Kadonaga, 1999).

The most abundant posttranslational modification of chromatin is the reversible acetylation of all four core histones (reviewed in Van Holde, 1988; Davie and Spencer, 1999; Wolffe and Hayes, 1999). Transcriptionally active regions in euchromatin are often associated with histone hyperacetylation (Wolffe, 1998). In contrast, histones in transcriptionally silent regions, such as heterochromatin and the inactivated mammalian X chromosome, have been found to be hypoacetylated (reviewed in Grunstein, 1997). Changes in the acetylation level of more discrete regions associated with certain promoters are directly linked to gene activation or repression (Rundlett et al., 1998; Kadosh and Struhl, 1998b). A major advance in our understanding of how histone acetylation is linked to transcriptional regulatory processes arose from the identification and characterization of transcriptional regulators containing histone acetyltransferase or histone deacetylase activities (reviewed in Struhl, 1998; Kornberg and Lorch, 1999; Cheung et al., 2000; Ng and Bird, 2000).

A growing number of HDACs has been identified in various systems and model organisms (reviewed in Ng and Bird, 2000; Cress and Seto, 2000; Fischle et al., 2001). These proteins are currently classified in three distinct families. The class I HDACs (HDAC1, 2, 3, and 8) are most closely related to the yeast transcriptional regulator RPD3 (Taunton et al., 1996; Rundlett et al., 1996). Class II HDACs (HDAC4, 5, 6, and 7) share domains with similarity to HDA1, another deacetylase in yeast (Rundlett et al., 1996; Carmen et al., 1996). Class III HDACs are related to the yeast silencing protein SIR2 and are dependent on NAD for enzymatic activity (Moazed, 2001). In contrast to the relative compact class I HDAC proteins, class II HDACs, HDAC4, 5, and 7, are comprised of two distinct domains (Grozinger et al., 1999; Fischle et al., 1999; Wang et al., 1999; Verdel and Khochbin, 1999; Miska et al., 1999; Fischle et al., 2001). The C-terminal domain shows homology to HDA1 and is associated with HDAC catalytic activity in vivo (Fischle et al., 1999). In addition, the N-terminal domain displays separate autonomous repressor activity (Miska et al., 1999; Sparrow et al., 1999; Zhou et al., 2000; Kao et al., 2000). HDAC6, another class II HDAC, contains two tandemly arranged HDA1-like domains (Verdel and Khochbin, 1999; Grozinger et al., 1999). HDACs of class I are expressed in most cell types, whereas the expression pattern of class II HDACs is far more restricted (Grozinger et al., 1999; Fischle et al., 1999), suggesting that they are involved in cellular differentiation and developmental processes. In contrast to class I HDACs,

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which are found exclusively in the cell nucleus, class II HDACs shuttle in and out of the nucleus and their subcellular distribution is influenced in part by their interaction with 14-3-3 proteins (Verdel et al., 2000; Wang et al., 2000; Grozinger and Schreiber, 2000).

HDACs exist in cells as part of large molecular weight complexes that are recruited to specific promoters via their interactions with sequence-specific DNA binding proteins (reviewed in Ng and Bird, 2000). These include the nuclear hormone receptors, the E box binding factors, and the methylcytosine binding protein MeCP2 (reviewed in Ng and Bird, 2000; Cress and Seto, 2000). Class I HDAC1 and HDAC2 are found in the SIN3 and NURD/Mi2 complexes (reviewed in Ng and Bird, 2000). HDAC3 is associated with corepressors N-CoR and SMRT mediating transcriptional repression by the thyroid hormone receptor (TR) and the oncoprotein v-ErbA (Li et al., 2000; Underhill et al., 2000; Urnov et al., 2000; Wen et al., 2000; Guenther et al., 2000). Class II HDACs 4, 5, and 7 can interact with N-CoR, SMRT, and BCoR, an additional corepressor that mediates repression by BCL-6 (Huynh et al., 2000; Kao et al., 2000; Huang et al., 2000).

Relatively little is known about the requirements and specificity of enzymatic activity displayed by histone deacetylases. Mutation analysis of RPD3 and HDAC1 indicates that the enzymatic activity is essential for the transcriptional regulatory functions of these proteins (Kadosh and Struhl, 1998a; Hassig et al., 1998). However, with few exceptions (Hassig et al., 1998; Carmen et al., 1999; Hu et al., 2000), it has been remarkably difficult to obtain HDACs demonstrating enzymatic activity in a recombinant form in vitro. When successful, these attempts have mainly yielded protein preparations with low specific activity especially if compared to the corresponding factor analyzed in vivo. Cumbersome transient transfection approaches followed by immunoprecipitation have been required to measure significant deacetylase activities associated to HDACs (Taunton et al., 1996; Kadosh and Struhl, 1998a; Hassig et al., 1998; Fischle et al., 1999). Biochemical fractionation of mammalian cell extracts has indicated that several additional proteins besides HDAC1 and HDAC2 might be necessary to build an enzymatically active HDAC1-HDAC2 core complex. The limitations observed in the expression of recombinant HDAC activity could therefore be due to limiting the presence or absence of crucial cofactors (Zhang et al., 1999).

In this report, we have addressed the mechanism of HDAC activity for members of the newly identified class II HDAC family, HDAC4 and HDAC5. We show that the HDAC domains of HDAC4 and HDAC5 do not possess intrinsic enzymatic activity as isolated polypeptides but associate with HDAC activity only in the context of SMRT/N-CoR-HDAC3 complexes.

Results

HDAC4 and HDAC5 Coimmunoprecipitate with HDAC3 via Their C-Terminal Domains

To define the domain of HDAC4 necessary for enzymatic activity in vivo, we generated stable cell lines expressing full-length HDAC4 (FLAG-tagged) and several deletion mutants. Epitope-tagged proteins were immunoprecipitated with an anti-FLAG antibody and assayed for HDAC activity (Figure 1A). Strong HDAC activity was associated with full-length HDAC4, and a fragment corresponding to the C-terminal domain (612–1084) exhibited comparable high deacetylase activity (Figure 1A). Further deletion of 50 amino acids (HDAC4 aa 662–1084) led to a complete loss of enzymatic activity (Figure 1A). These observations confirm our previous observations with transiently transfected HDAC4 (Fischle et al., 1999) and define a domain necessary for enzymatic activity corresponding to aa 612–1084.

Since HDAC3 has been previously reported to coimmunoprecipitate with HDAC4 (Grozinger et al., 1999), we tested the ability of both wild-type HDAC4 and its deletion mutants to interact with HDAC3. Analysis of HDAC4-bound proteins after immunoprecipitation from our stable cell lines showed that endogenous HDAC3 was associated with enzymatically active HDAC4 (wt and aa 612–1084) but not with the inactive mutant (662–1084) (Figure 1A). HDAC1, HDAC2, and endogenous HDAC4 did not interact with overexpressed HDAC4 (Figure 1A).

To generalize our findings, we tested other members of class II HDACs for interaction with HDAC3 in vivo. 293 cells were transiently transfected with FLAG-tagged HDAC4, 5, and 6 or with the HIV-1 Tat protein as a control. Western blot analysis verified that all epitope-tagged proteins were expressed and immunoprecipitated to the same degree (Figure 1B). When the immunoprecipitated material was examined with specific antisera for HDAC1, 2, or 3, HDAC4 and HDAC5 coimmunoprecipitated with endogenous HDAC3 but not with endogenous HDAC1 or HDAC2 (Figure 1B). In contrast, HDAC6 did not coimmunoprecipitate with any class I HDAC (Figure 1B). In the same experiment, HDAC1-FLAG associated with endogenous HDAC2 in agreement with previous work showing that both proteins are present in the same high molecular weight complexes (Zhang et al., 1997, 1999). Mapping experiments were performed with HDAC5 and confirmed that its interaction with HDAC3 was mediated via its C-terminal domain (data not shown). Similar observations were made in HeLa, Cos7, and NIH3T3 cells (data not shown).

To demonstrate the existence of an interaction between endogenous HDAC4 and HDAC3, we subjected HeLa cellular extracts to immunoprecipitation with an antiserum specific for HDAC4. The immunoprecipitated material was associated with significant HDAC activity that was completely inhibited by TSA (data not shown). Western blot analysis of the immunoprecipitated material showed a specific interaction of HDAC4 with HDAC3, whereas the control HDAC1 immunoprecipitation did not show associated HDAC3 protein (Figure 1C). Similar observations were made using 293 cell extracts (data not shown).

HDAC4 and HDAC3 Colocalize in Discrete Subnuclear Structures

We predicted that if HDAC4 and HDAC3 form a functional complex in vivo, they should colocalize within the cell nucleus. HDAC4-FLAG was transfected into HeLa cells, and its distribution was examined by confocal fluorescence microscopy. In the majority of cells,

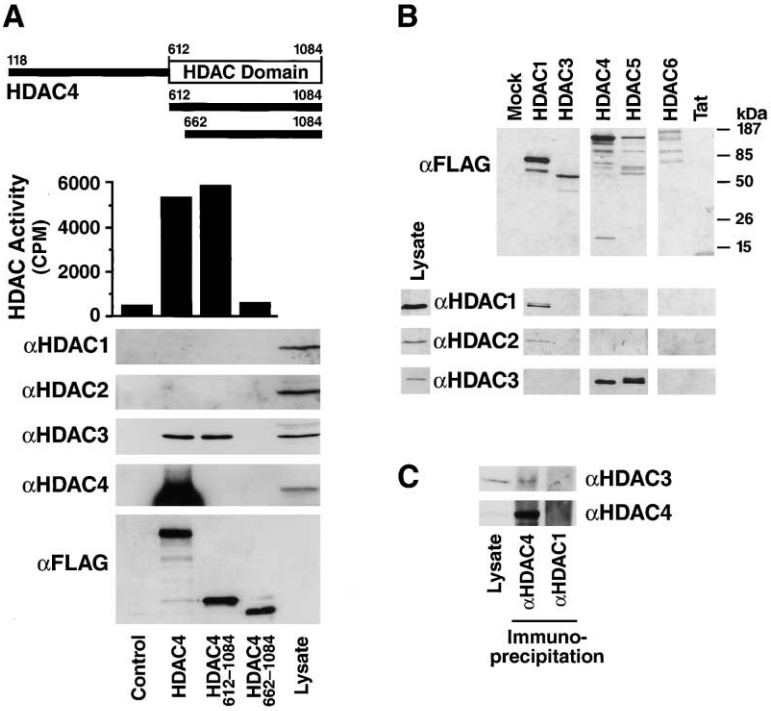

Figure 1. HDAC4 Coimmunoprecipitates with HDAC3 via Its C-Terminal Domain

(A) 293 cells were stably transfected with expression vectors encoding the indicated FLAG-tagged HDAC4 constructs and the empty vector as control. Immunoprecipitated HDAC4 proteins (anti-FLAG) were tested for HDAC activity and for the presence of HDAC1, 2, 3, and 4 by Western blotting.

(B) Vectors encoding HDAC1, 3, 4, 5, and 6 and the HIV Tat protein (all tagged with FLAG) were transiently transfected into 293 cells. Proteins were immunoprecipitated with an anti-FLAG antiserum and analyzed by Western blotting with specific antisera against HDAC1, 2, and 3 or the FLAG-epitope. (Note that the C-terminal FLAG-tag interferes with the epitopes recognized by the anti-HDAC3 antisera.)

(C) HeLa cell extracts were subjected to immunoprecipitation with antisera specific for HDAC1 and HDAC4. Immunoprecipitated material was analyzed by Western blotting using antisera directed against HDAC3 or HDAC4.

HDAC4 showed a micropunctate distribution (Figure 2A). A similar pattern with enrichment in numerous small foci is observed for endogenous HDAC 1, 3, 4, and 7 (Hendzel et al., 1998; M.J.H., unpublished data). The cell immediately to the left of the inset shows a distinct pattern of distribution with large intranuclear inclusions containing HDAC4. These inclusions have been reported previously for other overexpressed HDACs (Miska et al., 1999; Downes et al., 2000; Grozinger and Schreiber, 2000) but are never observed for endogenous proteins (Kruhlak et al., 2000). Consequently, we focused our analysis on cells where HDAC4 is present in smaller foci representative of structures found under physiological conditions. The inset shows that when the fluorescent signals for HDAC4 and HDAC3 are merged, a good correspondence between foci enriched in HDAC4-FLAG and endogenous HDAC3 is observed (Figure 2A).

To control for nonspecific association or for random overlap, we compared the distribution of HDAC4-FLAG with endogenous HDAC1 (Figure 2B). Very little overlap is observed, and upon careful examination, there is little evidence that individual foci are enriched in both HDAC4 and HDAC1. Similar observations were made with SKN-SH neuroblastoma and 10 T1/2 fibroblast cells (data not shown). This result supports the specific recruitment of HDAC4 and HDAC3, but not HDAC1, to similar regions of the nucleus.

HDAC4 Deletion and Mutation Analysis Indicates a Strict Correlation between HDAC3 Binding and HDAC4 Enzymatic Activity

To further study the relation between HDAC4-HDAC3 interaction and HDAC4 enzymatic activity, we transfected additional deletion mutants of the HDAC4 C terminus in 293 cells (Figure 3A) or Cos7 cells (data not shown). Interaction with HDAC3 was lost if aa 638–662 or aa 1002–1058 of HDAC4 was deleted. Additionally, an inactive internal deletion mutant of the most highly conserved region of the HDAC4 protein (aa 803–846) was not interacting with HDAC3 (Figure 3A). Each deletion mutant that showed a loss of interaction with HDAC3 was also inactive in terms of HDAC activity (Figure 3A). Importantly, all deletion mutants were expressed to similar levels as measured by Western blot (Figure 3A).

To further refine this analysis, we conducted an extensive mutation analysis of the HDAC4 C-terminal fragment (Figure 3B). Point mutations were selected based on the recently solved 3D structure of an HDAC-like protein, HDLP, and the relative sequence conservation of critical amino acids in the catalytic core of both enzymes (Finnin et al., 1999). The catalytic pocket of HDLP is delineated by seven loops that contain conserved aromatic and hydrophobic residues (Figure 3D, shown in yellow). These loops also contain several critical residues that have been implicated directly in the catalytic mechanism of deacetylation, mainly histidines (H131, H132, and H170), aspartic acids (D166, D168, D173, and D258) and a tyrosine (Y297) (shown in red, Figure 3D). As previously reported, mutagenesis of H803 and D840 into alanine residues abolished the enzymatic activity associated with HDAC4 (Miska et al., 1999). These two point mutations also abrogated the interaction with HDAC3 (Figure 3B). Other point mutants, H842A, N845D, and D934N, exhibited an identical phenotype: loss of HDAC activity and suppression of interaction with HDAC3. Surprisingly, mutation H802A, which affects residue predicted to be critical to HDAC activity based on the catalytic mechanism proposed for HDLP (Finnin et al., 1999), had no effect on enzymatic activity or on

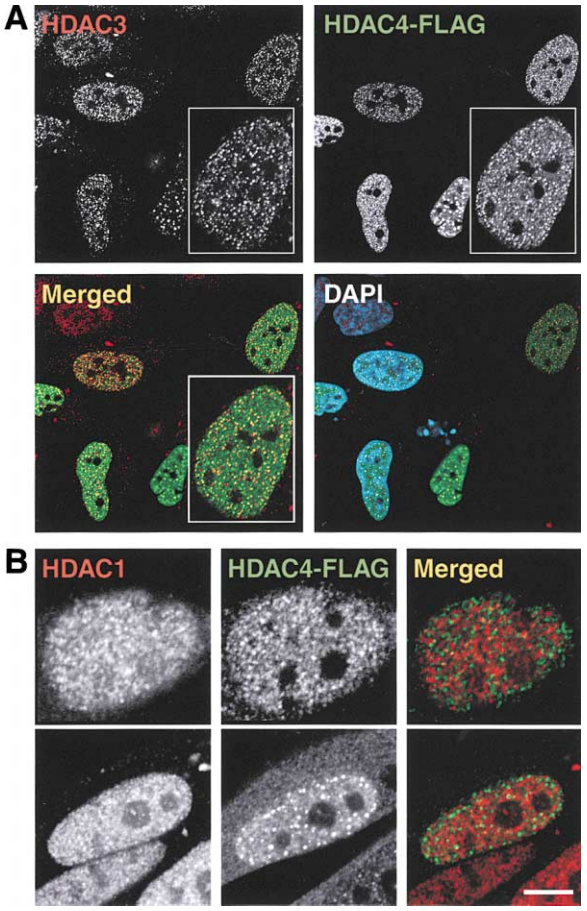

Figure 2. HDAC3 and HDAC4 Colocalize in Discrete Subnuclear Structures

(A) HeLa cells were transfected with an expression vector for HDAC4-FLAG. Endogenous HDAC3 was detected with an affinity-purified anti-HDAC3 antiserum combined with a Cy3-labeled secondary antibody (red). HDAC4 was visualized with M2 antibody in combination with an Alexa 488-tagged secondary antibody (green). Black and white images are shown for better visualization of expression intensities of individual HDACs. The inset represents magnification of an exemplary cell. The yellow dots in the merged image indicate colocalization of both factors. DAPI stains DNA in the cell nucleus.

(B) Similar analysis as in (A) with affinity-purified HDAC1 antibodies. Two cells expressing HDAC4-FLAG at different levels are shown. The scale bar represents 10 μm.

HDAC3 interaction. This is in contrast to the orthologous mutations in RPD3 (H150 in RPD3 corresponds to H802 in HDAC4), which abolished activity of RPD3 (Kadosh and Struhl, 1998a). Given these observations and the strict correlation observed between HDAC4 enzymatic activity and its interaction with HDAC3, we generated an additional 13 point mutants in the C-terminal region of HDAC4 and subjected them to the same functional assay. These mutations also targeted residues that are thought to participate in the building of the catalytic pocket or to participate in the catalytic mechanism but might not be conserved between all HDACs (Figure 3D). Functional analysis of these mutants demonstrated a perfect correlation between enzymatic activity and the ability to recruit HDAC3 (Figure 3C). These experiments therefore establish a tight functional connection between the ability of HDAC4 to interact with HDAC3 and its associated HDAC catalytic activity.

Affinity Purification of HDAC4-Bound Factors: SMRT/N-CoR and HDAC3

To further define the enzymatic activity associated with HDAC4, we expressed the active (612–1084) and the inactive fragments (662–1084) of HDAC4 as GST-fusion proteins in *Escherichia coli*. As previously shown for other HDACs, no deacetylase activity could be detected in the recombinant protein preparations (Figure 4A, − extract). To test whether HDAC4 might recruit cellular cofactor(s) necessary for activity, we incubated the GST-HDAC4 proteins with nuclear extracts from 293 or HeLa cells. High HDAC activity was found associated with the catalytic domain of HDAC4 (GST-HDAC4 [612–1084]) but not to the inactive fragment (GST-HDAC4 [662–1084]) (Figure 4A, + extract), suggesting that one or several cofactors present in 293 and HeLa extracts participate in the enzymatic activity associated with HDAC4. Western blot analysis of GST-HDAC4-bound material revealed that HDAC3 was highly enriched in the active fraction associated with GST-HDAC4 (612–1084) but not GST-HDAC4 (662–1084) or GST alone. Other HDACs such as HDAC1, HDAC2, or HDAC4 could not be detected (Figure 4A). These results are consistent with our coimmunoprecipitation experiments and the mutation analysis and indicate that HDAC4 enzymatic activity could be dependent on its association with HDAC3.

Several attempts to demonstrate direct binding between HDAC4 and HDAC3 were unsuccessful, suggesting that additional nuclear factors might be critical to establish a functional interaction (data not shown). To identify components of the enzymatically active HDAC4 complex, we took advantage of the GST-HDAC4 pull-down assays described above. We incubated in vivo ^{35}S-labeled extracts from 293 or HeLa cells with either active GST-HDAC4 (612–1084) or the inactive deletion mutant, GST-HDAC4 (662–1084). Three major polypeptides, p270, p62, and p32, were specifically recruited from 293 and HeLa cell extracts by active GST-HDAC4 but not by the inactive deletion mutant (Figure 4B). Upon longer exposure of the autoradiogram, several additional weaker bands were detected including a p50 polypeptide corresponding to HDAC3. The same polypeptides were coimmunoprecipitated from the cell line stably expressing HDAC4-FLAG with an anti-FLAG antiserum and importantly also with an anti-HDAC3 antiserum (data not shown). These experiments indicate that HDAC4 and HDAC3 could exist in cells as part of a multiprotein complex containing at least three additional polypeptides, p270, p62, and p32.

Recent reports have indicated that HDAC3 and HDAC4 individually interact with N-CoR and SMRT, two high molecular mass (270 kDa) proteins involved in transcriptional repression (Huang et al., 2000; Kao et al., 2000; Li et al., 2000; Underhill et al., 2000; Urnov et al., 2000; Wen et al., 2000; Guenther et al., 2000). These findings suggested that p270 might correspond to SMRT/N-CoR. Western blot analysis of material bound to GST-HDAC4 confirmed that SMRT and N-CoR inter-

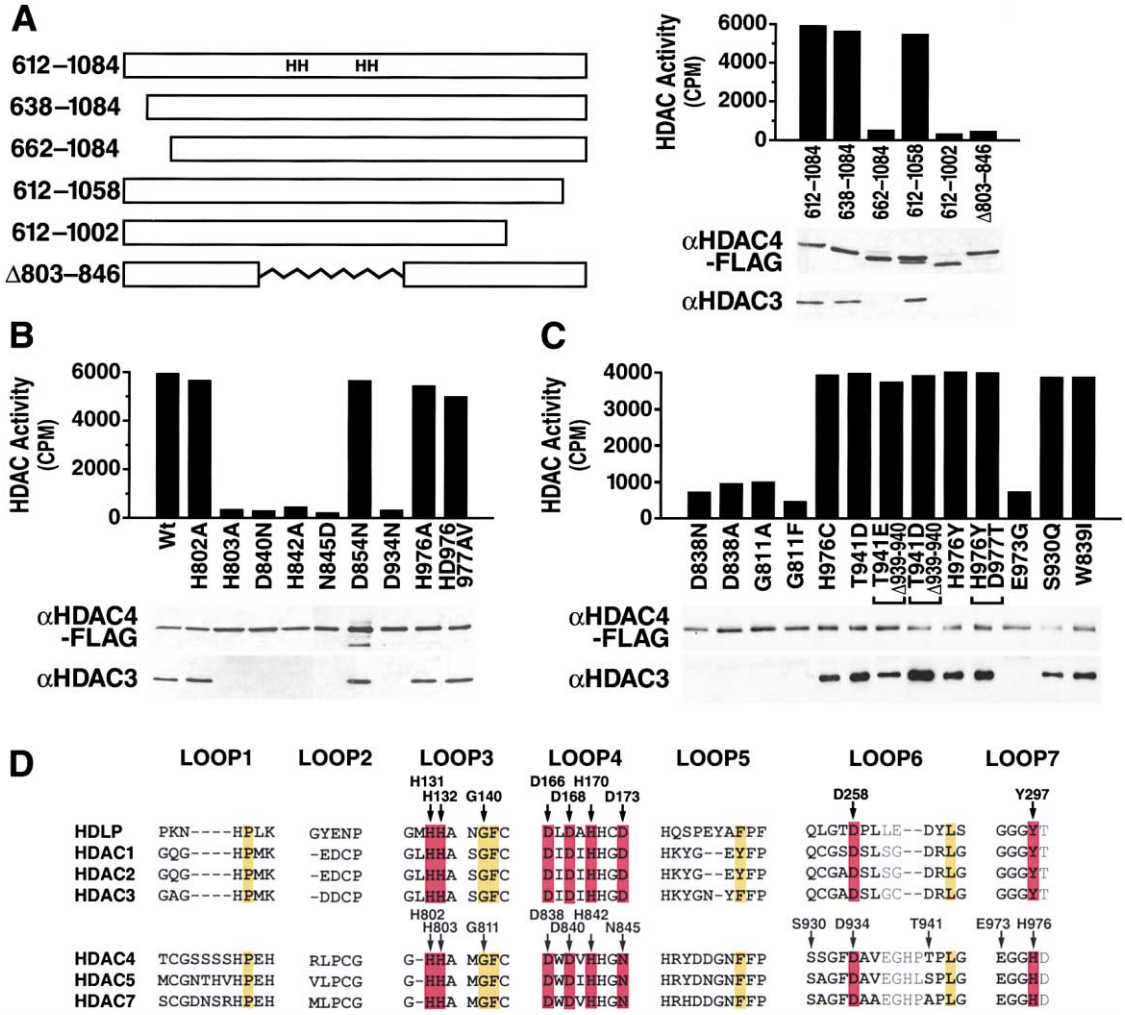

Figure 3. Mutation Analysis of HDAC4 Reveals a Strict Correlation between Recruitment of HDAC3 and HDAC4 Enzymatic Activity

(A) N-terminal, C-terminal, and internal deletions of the HDAC domain of HDAC4 were FLAG-tagged and transiently transfected into 293 cells. Immunoprecipitated proteins were analyzed for the presence of HDAC3 by Western blotting and assayed for HDAC activity.

(B) Conserved amino acids in the catalytic core of HDAC4 were mutated and the resulting FLAG-tagged constructs were tested for enzymatic activity and HDAC3 binding.

(C) Thirteen additional point mutations were introduced into the presumed catalytic domain of HDAC4 and tested for enzymatic activity and HDAC3 binding.

(D) Sequence alignment of HDLP and human HDAC1, 2, 3, 4, 5, and 7. Seven conserved loops that define the catalytic pocket of HDLP (Finnin et al., 1999) are shown aligned with the equivalent loops in other human HDACs. Conserved hydrophobic and aromatic residues that make up the wall of the catalytic pocket are shaded in yellow while residues directly involved in the catalytic mechanism are shaded in red. The relative position of residues is indicated for both HDLP and HDAC4.

act with HDAC4 via its C-terminal domain (aa 612–1084) but not with the deletion mutant containing aa 662–1084 (Figure 4C). In contrast, the corepressor SIN3 was not recruited by GST-HDAC4. These results suggest that N-CoR or SMRT could mediate the interaction between HDAC4 and HDAC3. In separate experiments, we also confirmed that endogenous HDAC4 and endogenous HDAC3 coimmunoprecipitated separately with N-CoR and with SMRT, but not with SIN3 (data not shown) (Huang et al., 2000; Guenther et al., 2000).

To test the formation of a complex between HDAC4, HDAC3, and N-CoR/SMRT, we subjected HeLa cellular extracts to affinity purification on GST-HDAC4 (612–1084). After elution, the purified material was subjected to immunoprecipitation with antisera directed either against N-CoR, SMRT, HDAC3, or against SIN3, HDAC1, and FLAG as controls. Significant HDAC activity was associated with the N-CoR, SMRT, and HDAC3, but not with the SIN3, HDAC1, or FLAG immunoprecipitates (Figure 4D). Western blot analysis revealed that material purified on GST-HDAC4 (612–1084) and further immunoprecipitated with anti-N-CoR and anti-SMRT antisera contained HDAC3 (Figure 4D). Conversely, the GST-HDAC4 affinity-purified material that was immunoprecipitated with an anti-HDAC3 antiserum contained N-CoR and SMRT (Figure 4D). These findings verify that the N-CoR/SMRT, HDAC4, and HDAC3 proteins can be part of a single complex with enzymatic activity. In pre-

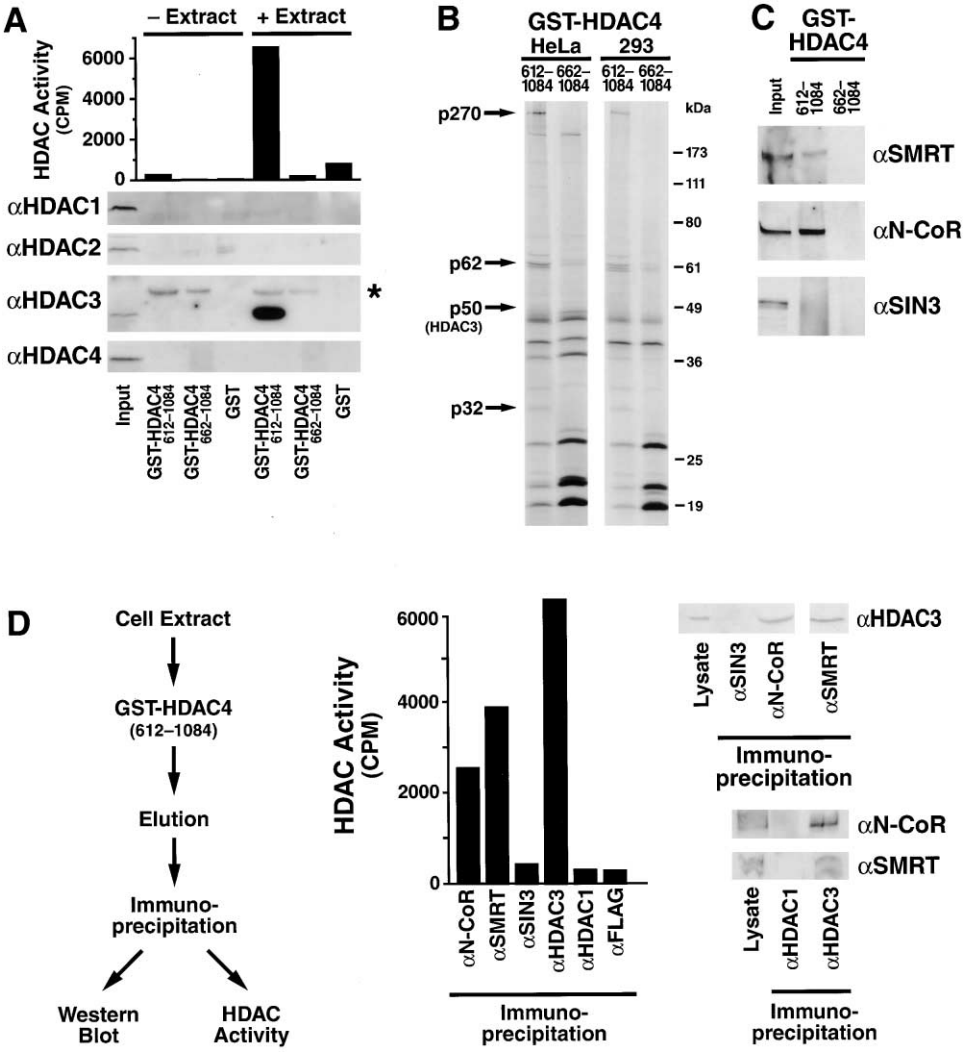

Figure 4. Affinity Purification of HDAC4 Binding Factors: HDAC3, SMRT, and N-CoR

(A) C-terminal fragments of HDAC4 were expressed as GST-fusion proteins and immobilized on agarose beads. HDAC activity was tested before (− extract) and after incubation of the fusion proteins with nuclear extract from HeLa cells (+ extract). GST-HDAC4 pull-down assays were analyzed by Western blotting with antisera specific for HDAC1, 2, 3, and 4. The asterisk indicates a bacterial protein crossreacting with the secondary antibody used to develop the HDAC3 Western blot. The lane indicated as "input" contains 10% of the lysate used in the pull-down.

(B) GST-fusion proteins of HDAC4 (612–1084) or HDAC4 (662–1084) were incubated with HeLa or 293 cell extracts metabolically labeled with ^{35}S-Methionine. Bound proteins were analyzed by SDS-PAGE followed by autoradiography.

(C) GST pull-downs with HDAC4 (612–1084) or HDAC4 (662–1084) were analyzed by Western blotting with antisera specific for N-CoR, SMRT, or SIN3. Input equaled 10% of cell lysate used in the reaction.

(D) Proteins from HeLa cell extracts bound to GST-HDAC4 (612–1084) were eluted and immunoprecipitated with antisera specific for N-CoR, SMRT, Sin3, HDAC3, HDAC1, and FLAG. The immunoprecipitated material was tested for HDAC activity (middle panel) and analyzed for the presence of HDAC3, N-CoR, and SMRT by Western blotting (right panel).

liminary experiments, chromatographic fractionation of HeLa extracts indeed showed that endogenous HDAC4, HDAC3, and SMRT/N-CoR proteins copurify as high molecular weight complexes (data not shown) similar to the enzymatically active complex described for HDAC4-FLAG (Figure 6A).

Binding of HDAC4 to N-CoR via RD3 Mediates Its Interaction with HDAC3

N-CoR contains three autonomous repression domains that repress transcription when tethered to sequence-specific DNA binding domains (Horlein et al., 1995; Huang et al., 2000). A previous report mapped the HDAC4 interaction domain of SMRT/N-CoR to RD3 (Huang et al., 2000). Using HDAC4 protein translated in vitro, we confirmed that HDAC4 only bound to GST-RD3, but not to GST-RD1 or GST-RD2 (Figure 5A) (Huang et al., 2000). The binding of HDAC4 to RD3 was also verified by incubating extracts from a cell line stably transfected with HDAC4 with GST-RD1, 2, or 3. Only GST-RD3 interacted specifically with HDAC4 (Figure 5A). Separate experiments have indicated that HDAC3 interacts with SMRT/N-CoR via other domains that are distinct from RD3 (Guenther et al., 2000, 2001; our unpublished data).

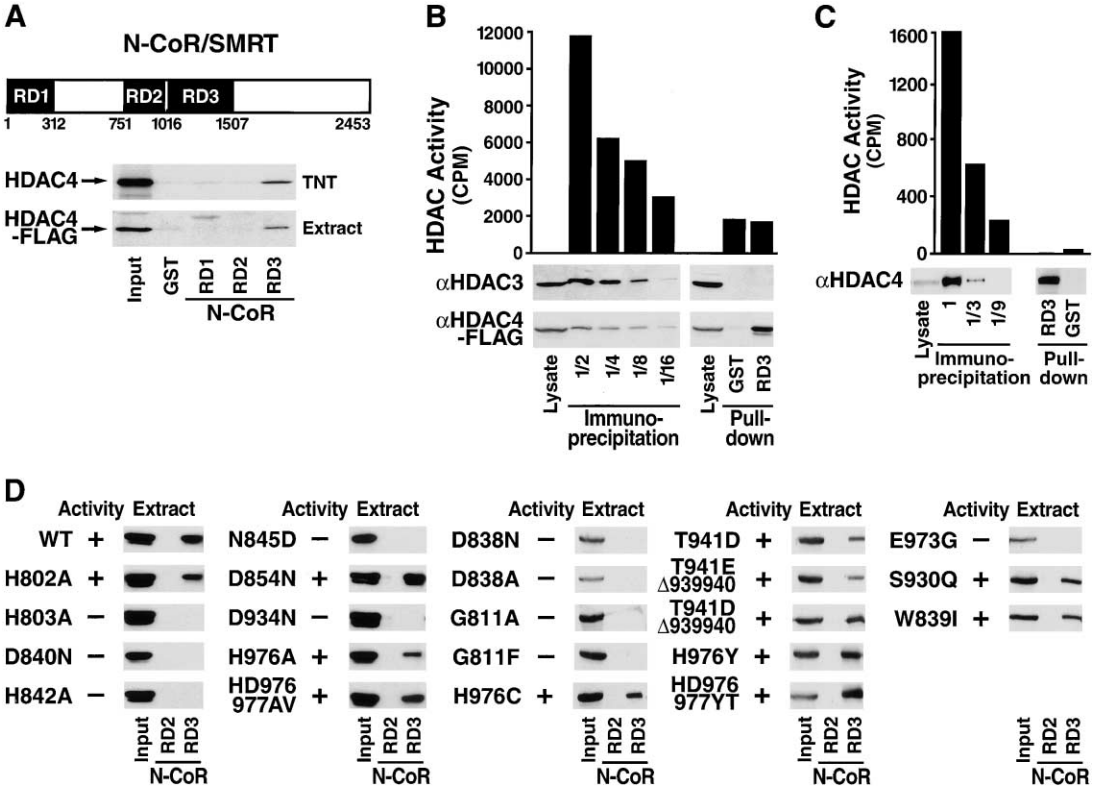

Figure 5. HDAC4 Binding to N-CoR via RD3 Is Critical for HDAC3 Recruitment

(A) Schematic representation of the N-CoR protein indicating distinct repression domains (RD). Binding of HDAC4 to recombinant GST-RD1, GST-RD2, and GST-RD3 of N-CoR was measured in pull-down assays with in vitro translated ^{35}S-labeled protein (TNT). Pull-down assays from extracts of a stable transfected cell line expressing HDAC4-FLAG were analyzed by Western blotting using anti-FLAG antiserum (extract). Input equals 10% of the material used for the pull-down.

(B) HDAC4-FLAG from a stable transfected cell line was pulled down with GST-RD3 or immunoprecipitated with anti-FLAG M2 agarose. Immunoprecipitated material was serially diluted as indicated. Equal portions of the immobilized material were analyzed by Western blotting (anti-FLAG and anti-HDAC3) and HDAC activity assays.

(C) Endogenous HDAC4 was pulled down from HeLa cell extracts with GST-RD3 or immunoprecipitated from the same extracts with anti-HDAC4 antiserum. Immunoprecipitated material was serially diluted as indicated. Material obtained from either procedure was tested for HDAC activity and for the presence of HDAC4 by Western blotting analysis.

(D) GST-RD2 and GST-RD3 were incubated with extracts obtained from cells transiently transfected with the corresponding HDAC4-FLAG point mutants. Pull-downs were analyzed by Western blotting with an anti-FLAG antiserum.

Since HDAC4 binds to RD3 independently of HDAC3, we tested whether binding of HDAC4 to N-CoR is sufficient for enzymatic activity. Overexpressed HDAC4 bound to GST-RD3 but was not associated with HDAC3 and showed no enzymatic activity (Figure 5B). In contrast, HDAC4 obtained after immunoprecipitation from the same cell extracts was bound to HDAC3 and exhibited high enzymatic activity (Figure 5B). We also confirmed these observations using endogenous HDAC4. Cellular extracts from 293 cells were incubated with GST or GST-RD3. In parallel, endogenous HDAC4 was immunoprecipitated with a specific antiserum. While significant HDAC activity was associated with the immunoprecipitated HDAC4, a similar amount of HDAC4 obtained by affinity purification on GST-RD3 was enzymatically inactive (Figure 5C).

Since point mutations of HDAC4 inhibited its ability to interact with HDAC3 (Figures 3B and 3C), we examined the effect of the same mutations on the ability of HDAC4 to interact with N-CoR. Fusion proteins GST-RD2 and GST-RD3 were incubated with extracts from cells transfected with the different HDAC4 mutants. All HDAC4 point mutants that are enzymatically inactive and fail to interact with HDAC3 in vivo (H803A, D840N, H842A, N845D, D934N, D838N, D838A, G811A, G811F, and E973G) also failed to bind to GST-RD3 (Figure 5D). In contrast, all enzymatically active mutants (H802A, D854N, H976A, HD976977AV, H976C, T941D, T941EΔ939-940, T941DΔ939-940, H976Y, HD976977YT, S930Q, and W839I) bound to GST-RD3 at levels comparable to wild-type HDAC4 (Figure 5D). Similar results were obtained in a direct interaction assay when GST-RD3 was incubated with HDAC4 mutants obtained by in vitro translation (data not shown). In addition, all HDAC4 mutants shown in Figure 3C were tested in cotransfection assays with N-CoR, and a strict correlation was observed between the ability of mutants to interact with RD3 in vitro and with full-length N-CoR in vivo (data not shown).

Depletion of HDAC3 from Cellular Extracts Abolishes HDAC4 Enzymatic Activity

Our observations suggest that HDAC4 could exist in cells in two forms: an enzymatically active fraction com-

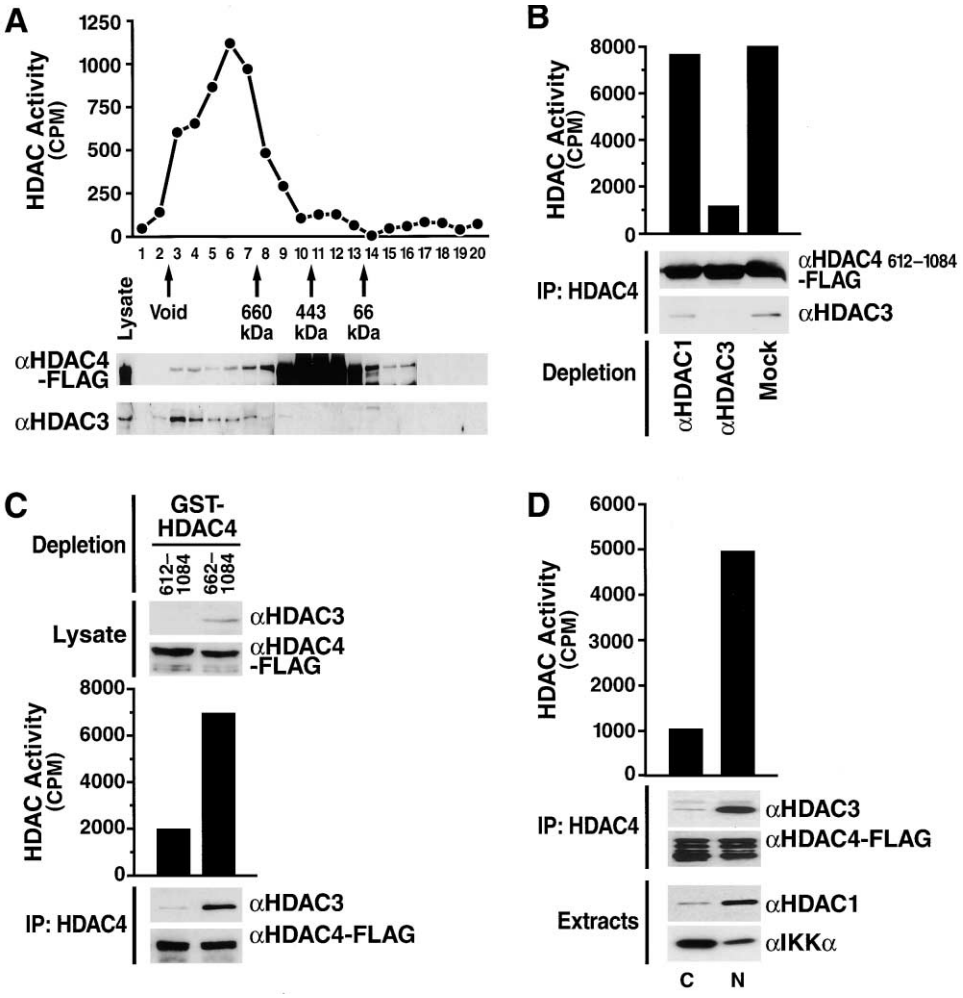

Figure 6. HDAC4 Exists in Two Different Forms: Nuclear, Complexed to HDAC3 and Enzymatically Active, and Cytoplasmic, Free of HDAC3 and Catalytically Inactive

(A) Cellular lysates from 293 cells stably expressing HDAC4-FLAG were separated on a Superose 6 gel filtration column. Fractions were analyzed for the presence of HDAC4-FLAG or HDAC3 by Western blotting and immunoprecipitated using the anti-FLAG antiserum. Immunoprecipitated fractions were analyzed for HDAC activity. Arrows indicate the elution positions of molecular weight standards and of dextran blue (void volume).

(B) Lysates from a stable cell line expressing HDAC4 (612–1084)-FLAG were subjected to three rounds of immunodepletion with antisera specific for HDAC1 and HDAC3 or subjected to a mock depletion using beads without antiserum. After depletion, HDAC4 was immunoprecipitated with anti-FLAG antiserum and analyzed for HDAC activity or by Western blotting using antisera specific for FLAG and HDAC3.

(C) Nuclear extracts from 293 cells stably transfected with HDAC4-FLAG were depleted on GST-HDAC4 (612–1084) or GST-HDAC4 (662–1084). Depleted lysates were analyzed by Western blotting with antisera specific for HDAC3 or FLAG (HDAC4-FLAG). HDAC4 was immunoprecipitated with the anti-FLAG antiserum and HDAC activity was measured. The HDAC4-immunoprecipitated material was tested for HDAC3 and HDAC4-FLAG by Western blotting.

(D) Nuclear (N) and cytoplasmic (C) extracts were prepared from 293 cells transiently expressing HDAC4-FLAG. Western blotting with antisera against the cytoplasmic protein IKKα and the nuclear protein HDAC1 confirmed the cytoplasmic and nuclear nature of extracts. Anti-FLAG immunoprecipitates from the extracts were either analyzed by Western blotting with antisera against HDAC3 and the FLAG-epitope or subjected to enzymatic activity assays.

plexed with HDAC3 and an enzymatically inactive form not bound to HDAC3. Fractionation of cellular extracts from 293 cells overexpressing HDAC4-FLAG on a Superose 6 column followed by immunoprecipitation with an anti-FLAG antiserum showed that HDAC4-associated enzymatic activity peaked at approximately 1 MDa (Figure 6A). In contrast, the majority of HDAC4 protein was migrating as a smaller molecular species (around 400 kDa). HDAC3 was only found in the fractions containing enzymatically active HDAC4 but was not detected in the lower molecular weight fractions containing inactive HDAC4 (Figure 6A).

If HDAC3 is essential to the enzymatic activity associated with HDAC4, depletion of HDAC3 from a cellular extract should reduce the enzymatic activity associated with HDAC4. To test this hypothesis, we subjected lysates prepared from a cell line stably expressing HDAC4 (aa 612–1084)-FLAG to three cycles of immunodepletion with antisera specific for either HDAC3 or for HDAC1. Western blotting analysis of the extracts documented

that the amount of HDAC3 was significantly reduced after three cycles of immunodepletion (data not shown). Similar amounts of HDAC4 proteins were immunoprecipitated from the HDAC1- and the HDAC3-depleted extracts (Figure 6B). This presumably reflects the fact that the HDAC4 protein is overexpressed in this clone in relation to HDAC3 (as shown in Figure 6A) and therefore only the small fraction of HDAC4 bound to HDAC3 was depleted. Depletion of HDAC3, but not of HDAC1, resulted in a significant decrease in the amount of enzymatic activity associated with HDAC4 (Figure 6B). As predicted, a strong reduction in the amount of HDAC3 protein coimmunoprecipitated with HDAC4 was noted from the HDAC3-depleted extract in comparison to the HDAC1-depleted extract (Figure 6B).

To further examine the effect of HDAC3 depletion on HDAC4 enzymatic activity, we performed a similar depletion experiment using either the active GST-HDAC4 (612–1084) fusion protein or its inactive counterpart, GST-HDAC4 (662–1084). GST-HDAC4 (612–1084) depleted HDAC3 from cell extracts while not affecting HDAC4 content (Figure 6C). HDAC4 immunoprecipitated from the extracts depleted on GST-HDAC4 (612–1084) contained significantly less HDAC activity than extracts depleted on GST-HDAC4 (662–1084) (Figure 6C). Similar amounts of HDAC4 were immunoprecipitated, but far less HDAC3 protein was associated with HDAC4 after depletion on GST-HDAC4 (612–1084) (Figure 6C).

Nuclear Localization of HDAC4 Is Necessary for HDAC Activity

HDAC4 shuttles between the cytoplasm and the nucleus (Miska et al., 1999; Wang et al., 2000; Grozinger and Schreiber, 2000). The fact that HDAC3 is exclusively nuclear (Emiliani et al., 1998) suggested that cellular HDAC4 could exist in two forms: enzymatically active in the nucleus when complexed with HDAC3 and SMRT/N-CoR and inactive in the cytoplasm when not bound to HDAC3 and SMRT/N-CoR. To test this possibility, we fractionated cells either transiently or stably (data not shown) transfected with HDAC4-FLAG into nuclear and cytoplasmic fractions. HDAC4 was immunoprecipitated from both fractions in equal amounts (Figure 6D). However, HDAC activity was five times higher in the nuclear fraction where HDAC4 was found associated with HDAC3 (Figure 6D). Western blot analysis of the nuclear and cytoplasmic fractions with antisera for endogenous HDAC1 (exclusively nuclear) and IKKα (exclusively cytoplasmic) showed that each fraction was approximately 80% pure (Figure 6D). Given the incomplete purity of these extracts, we conclude that only nuclear HDAC4 interacts with HDAC3 and exhibits significant HDAC activity.

HDAC3, but Not HDAC4, 5, or 7, Is Catalytically Activated after Binding to N-CoR

We have recently reported that HDAC3 becomes enzymatically activated when bound to N-CoR or SMRT (Guenther et al., 2001). A small domain of either protein, called the deacetylase activating domain (DAD), is required both for the catalytic activation of HDAC3 and for the major repressor function of SMRT (Guenther et al., 2001). These observations imply that HDAC3 can become enzymatically active when complexed to SMRT or N-CoR. Using the same experimental system, HDAC4 failed to become activated in the presence of N-CoR (Guenther et al., 2001). We used this system to test whether other class II HDACs could also become activated in the context of the full-length SMRT/N-CoR proteins. Myc-tagged HDAC3, 4, 5, and 7 proteins were translated in vitro in the presence or absence of N-CoR, immunoprecipitated with an anti-Myc antiserum, and tested for enzymatic activity. In the absence of N-CoR, none of the HDACs exhibited activity above background levels (Figure 7A). As recently reported (Guenther et al., 2001), the enzymatic activity of HDAC3 was activated upon binding to N-CoR (Figure 7A). However, using the same experimental conditions, none of the class II HDACs, including HDAC 4, 5, and 7, could be activated following binding to N-CoR (Figure 7A) or SMRT (data not shown). These results are in agreement with our findings that HDAC4 expressed in vivo (transfected or endogenous) is enzymatically inactive when bound to the RD3 domain of N-CoR (Figures 5B and 5C), a domain sufficient to mediate class II HDAC binding. Next, we tested whether class II HDACs might become activated in the context of the N-CoR/HDAC3 complex. Increasing amounts of HDAC4 were added to in vitro translated HDAC3/N-CoR. No increase in enzymatic activity was observed above the activity measured in the absence of HDAC4 (Figure 7B). Western blotting of the proteins coimmunoprecipitated with HDAC3 (α-Myc) confirmed the presence of HDAC4 (Figure 7B). We conclude that class II HDACs do not contribute to the activity of the corepressor complexes.

Discussion

In this report, we show that HDAC4, a class II HDAC, is not functionally active alone but only becomes associated with deacetylase activity when bound to HDAC3. In vivo, the formation of such complexes is limited to the nuclear compartment, where interaction with HDAC3 occurs (Emiliani et al., 1998). We provide evidence that the corepressors N-CoR and SMRT mediate the functional interaction of HDAC4 and HDAC3.

Class II HDAC Activity

HDAC4 is enzymatically inactive when not associated with HDAC3, either when overexpressed after transfection or as the native endogenous protein, or when produced in recombinant form. No HDAC activity is associated with GST-HDAC4 alone, with cytoplasmic HDAC4, with HDAC4 mutants that have lost their ability to bind to N-CoR, with HDAC4 affinity-purified on GST-RD3, or with in vitro translated HDAC4 even when bound to N-CoR.

Based on our observations, we favor the model that HDAC4 is not a functional histone deacetylase but serves to recruit preexisting, enzymatically active SMRT/N-CoR complexes that contain HDAC3 (Li et al., 2000; Underhill et al., 2000; Urnov et al., 2000; Wen et al., 2000; Guenther et al., 2000). In this context, the SMRT/N-CoR corepressors activate HDAC3 and provide a structural link between active HDAC3 and inactive

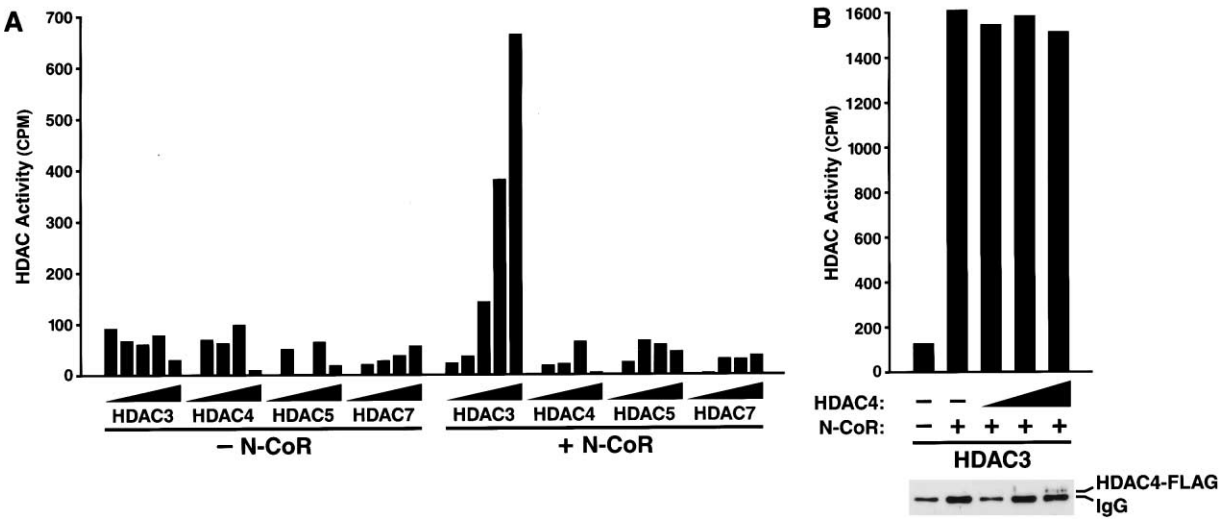

Figure 7. Activation of HDAC3, but Not of HDAC4, 5, and 7, by Binding to SMRT

(A) Myc-tagged HDAC3, 4, 5, and 7 were translated in vitro. Increasing amounts of each translation reactions (0, 2, 10, 50, or 200 μl of reticulocyte extract for each HDAC) were added to unprogrammed reticulocyte lysates (− N-CoR) or to translation reactions containing N-CoR (+ N-CoR). The resulting mixtures were immunoprecipitated using an anti-Myc-epitope antiserum and tested for HDAC activity.

(B) FLAG-tagged HDAC4, Myc-HDAC3, and FLAG-N-CoR were translated in vitro in separate reactions. They were mixed in various combinations as indicated on the *x* axis followed by immunoprecipitation with an anti-Myc-epitope antiserum and tested for HDAC activity. The presence of HDAC4 in the immunoprecipitated material was detected by Western blotting analysis using the anti-FLAG antiserum.

HDAC4. Accordingly, the totality of the enzymatic activity associated to that complex comes from the associated HDAC3 protein. While our recent observations clearly document that HDAC3 binding to SMRT/N-CoR results in the activation of its enzymatic activity (Guenther et al., 2001), no such activation could be demonstrated for either HDAC4, 5, or 7 despite many variations in experimental conditions (data not shown). Another factor arguing for a lack of intrinsic enzymatic activity of class II HDACs is our observation that mutations of some residues predicted to play a critical role in catalysis (for example, H802A) do not affect the enzymatic activity associated with HDAC4. One of these mutations (H802A) abolished the enzymatic activity associated with HDAC1 and RPD3 when introduced in that context (Hassig et al., 1998; Kadosh and Struhl, 1998b). In contrast, every mutation that suppressed the ability of HDAC4 to interact with SMRT/N-CoR resulted in a loss of HDAC3 binding and associated HDAC activity. We cannot completely exclude the possibility that class II HDACs become activated in the context of the SMRT/N-CoR complex in a manner that is not duplicated in reticulocyte system and cell extracts. An additional factor or a postranslational modification of either HDAC4 or SMRT/N-CoR could be missing in these experimental systems.

SMRT/N-CoR and Transcriptional Repression Involving Histone Deacetylation

The corepressors N-CoR and SMRT have been implicated in the repression mechanism mediated by several transcription factors (Glass and Rosenfeld, 2000). A first link between SMRT/N-CoR and histone deacetylation was established when corepression mediated by these factors was, at least in part, attributed to the Sin3/HDAC1/HDAC2 complex (reviewed in Xu et al., 1999; Ng and Bird, 2000). However, in other studies, it was observed that the majority of endogenous N-CoR and SMRT is stably associated with HDAC3 (Li et al., 2000) and a small fraction of cellular HDAC4 is bound to N-CoR and SMRT independently of the SIN3 complex (Huang et al., 2000). Our data indicate that N-CoR and SMRT can serve as a bridge between HDAC3 and HDAC4.

The nucleocytoplasmic transport of HDAC4, HDAC5, and HDAC7 is tightly regulated (Miska et al., 1999; McKinsey et al., 2000; Wang et al., 2000; Grozinger and Schreiber, 2000; Fischle et al., 2001). Our data suggest that the cytoplasmic sequestration of class II HDACs could play a significant role in keeping these factors in an inactive form by preventing them from interacting with the enzymatically active SMRT/N-CoR/HDAC3 complexes. Our ability to recruit the SMRT/N-CoR-HDAC3 complex to GST-HDAC4 suggests that SMRT/N-CoR-HDAC3 complexes devoid of class II HDAC preexist in cells. While such complexes can be enzymatically active, as our new observations show (Guenther et al., 2001), regulated binding of a class II HDAC and its associated factors (MEF2 factors, for example) might serve to recruit the complex to distinct promoters. The binding of a class II HDAC could therefore represent an additional targeting modality for the SMRT/N-CoR complex.

While SMRT and N-CoR are discussed interchangeably in this manuscript, these two proteins might be functionally equivalent and could differentially interact with different class II HDACs. Further, we have recently observed that HDAC7 also interacts with HDAC3 via SMRT/N-CoR and is dependent on this interaction for enzymatic activity (Fischle et al., 2001). HDAC4 and HDAC7 exist in separate and stable complexes sug-

gesting that another level of complexity and regulation might lie at the level of subunit composition of the SMRT/N-CoR-HDAC3-class II HDAC complex (Fischle et al., 2001). Further work will be necessary to dissect the exact composition and regulation of complexes containing different class II HDACs.

Our observations provide evidence that class II HDACs are strictly dependent on their ability to interact with the SMRT/N-CoR and HDAC3 proteins to associate with HDAC activity. Further studies will be necessary to define fully all the implications of our observations; in particular, the recruitment of the SMRT/N-CoR-HDAC3 complex to transcription factors known to bind to class II HDACs.

Experimental Procedures

Plasmids and Recombinant DNA

PCR-based strategies were used to generate C-terminal FLAG- or HA-tagged fusions of different HDACs in the pcDNA3.1 backbone (InVitrogen) (Fischle et al., 1999). HDAC5 cDNA was obtained as an EST clone (Id 1142916) from the I.M.A.G.E. Consortium, and the sequence corresponding to nt 394–3369 of the HDAC5 ORF was used to construct the fusion protein (Grozinger et al., 1999). Deletion constructs were generated by standard PCR and cloning procedures (Sambrook et al., 1989; Ausubel et al., 1995). Site-directed mutagenesis was performed with the Quickchange kit (Stratagene). The identity of mutations was verified by DNA sequencing. To construct GST-fusion proteins, EcoRI fragments from the pcDNA3.1 expression constructs were subcloned into the EcoRI or XhoI site of pGEX4T1 (Pharmacia), generating N-terminal GST-fusion proteins.

Immunoprecipitations

Immunoprecipitations were performed as previously described (Fischle et al., 1999). For FLAG-tagged proteins, M2 agarose (SIGMA) was used at 15 μl/ml. In the case of HA-epitope-tagged proteins, extracts were incubated with anti-HA antibody 3F10 (Roche) at 1 μg/ml. Anti-HDAC4 antibodies were used at 5 μg/ml. Immmuncomplexes were recovered by adding 20 μl of a preblocked (10 mg/ml BSA) 50% protein G-Sepharose slurry.

GST-Fusion Proteins: Expression and Pull-Downs

Fusion proteins were expressed in DH5α (Life Technologies) or BL21 RIP (Stratagene) and purified under standard conditions (Pharmacia). For pull-down reactions, either cellular or nuclear extracts from 293 and HeLa cells were used (Dignam et al., 1983; Fischle et al., 1999). After incubation for 1 hr at 4°C with rocking, reactions were washed three times in IPLS, three times in the same buffer but at 1 M NaCl, and two times in HD buffer (Fischle et al., 1999). Pull-downs were split and either processed for histone deacetylase activity assays or Western blotting with ECL (Amersham). For pull-down reactions with ^{35}S-labeled proteins, cDNAs were transcribed and translated by the TNT reticulocyte system (Promega). Binding of labeled factors to GST-fusion proteins was performed in GST buffer (20 mM Tris-HCl [pH 8.0], 150 mM KCl, 5 mM DTT, 2 mM EDTA, 10% glycerol, 0.5% nonfat dry milk, and 0.1% NP-40) for 1 hr at 4°C with rocking. Reactions were washed five times in the same buffer, and bound proteins were resolved on denaturing SDS-PAGE gels for analysis by autoradiography.

Antisera

Polyclonal anti-HDAC1 and anti-HDAC3 antisera have been described (Emiliani et al., 1998). Anti-SIN3, anti-HDAC2, anti-FLAG, and anti-HA antisera used were from Santa Cruz (Santa Cruz, CA). N-CoR and SMRT antibodies were obtained from Upstate Biotech (Lake Placid, NY) and ABR (Golden, CO). Polyclonal anti-HDAC4 antiserum was raised by immunizing rabbits with an *E. coli*-expressed N-terminal fragment of HDAC4 (GST-HDAC4 [118–662]) (Harlan Bioproducts).

Confocal Microscopy

Cells were plated onto glass coverslips and transfected the following day using Lipofecamine 2000 (Life Technologies). After incubation overnight, cells were fixed, permeabilized, and incubated with the appropriate antibodies at dilutions typically between 1:100 and 1:1000. Cells were mounted onto glass slides in PBS/90% glycerol containing 1 mM paraphenylenediammine as antifade reagent. Confocal microscopy was performed with a Zeiss LSM510 laser-scanning confocal microscope at 488 nm and 543 nm (Hendzel et al., 1998).

Histone Deacetylase Activity Assays

HDAC activity was measured as previously described (Fischle et al., 1999). Complexes immobilized on beads either from immunoprecipitations or GST pull-downs were incubated in 30 μl of HD buffer containing 50,000 cpm of substrate for 2 hr at 37°C.

Preparation of Nuclear and Cytoplasmic Extracts

Cell nuclei were prepared as described (Osborn et al., 1989) and buffer D (20 mM HEPES-NaOH [pH 7.9], 450 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5% NP-40, and 20% glycerol) was used for extraction. Cytoplasmic and nuclear extracts were diluted to 20 mM HEPES-NaOH (pH 7.9), 150 mM NaCl, 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5% NP-40, and 10% glycerol before immunoprecipitation.

Chromatography

Cellular lysates from 293 cells stably expressing HDAC4-FLAG were separated on a Superose 6 gel filtration column (Pharmacia, Superose 6 HR 10/30) with 50 mM Tris-HCl (pH 7.4), 300 mM NaCl, 0.5 mM EDTA, and 0.1% NP-40 as running buffer. Fractions (0.8 ml) were either concentrated for Western blotting by precipitation with 6% trichlorioacetic acid (TCA) and 0.01% deoxycholate or immunoprecipitated using M2 agarose for HDAC assays.

In Vitro Translation and Immunoprecipitation Assays

These assays were performed as recently reported (Guenther et al., 2001).

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References

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (1995) Current Protocols in Molecular Biology, First Edition (New York: John Wiley & Sons).

Carmen, A.A., Rundlett, S.E., and Grunstein, M. (1996). HDA1 and HDA3 are components of a yeast histone deacetylase (HDA) complex. J. Biol. Chem. *271*, 15837–15844.

Carmen, A.A., Griffin, P.R., Calaycay, J.R., Rundlett, S.E., Suka, Y., and Grunstein, M. (1999). Yeast HOS3 forms a novel trichostatin A-insensitive homodimer with intrinsic histone deacetylase activity. Proc. Natl. Acad. Sci. USA *96*, 12356–12361.

Cheung, W.L., Briggs, S.D., and Allis, C.D. (2000). Acetylation and chromosomal functions. Curr. Opin. Cell Biol. *12*, 326–333.

Cress, W.D., and Seto, E. (2000). Histone deacetylases, transcriptional control, and cancer. J. Cell. Physiol. *184*, 1–16.

Davie, J.R., and Spencer, V.A. (1999). Control of histone modifications. J. Cell. Biochem. Suppl. *32-33*, 141–148.

Dignam, J.D., Martin, P.L., Shastry, B.S., and Roeder, R.G. (1983). Eukaryotic gene transcription with purified components. Methods Enzymol. *101*, 582–598.

Downes, M., Ordentlich, P., Kao, H.Y., Alvarez, J.G., and Evans, R.M. (2000). Identification of a nuclear domain with deacetylase activity. Proc. Natl. Acad. Sci. USA *97*, 10330–10335.

Emiliani, S., Fischle, W., Van Lint, C., Al-Abed, Y., and Verdin, E. (1998). Characterization of a human RPD3 ortholog, HDAC3. Proc. Natl. Acad. Sci. USA *95*, 2795–2800.

Finnin, M.S., Donigian, J.R., Cohen, A., Richon, V.M., Rifkind, R.A., Marks, P.A., Breslow, R., and Pavletich, N.P. (1999). Structures of a histone deacetylase homologue bound to the TSA and SAHA inhibitors. Nature *401*, 188–193.

Fischle, W., Emiliani, S., Hendzel, M.J., Nagase, T., Nomura, N., Voelter, W., and Verdin, E. (1999). A new family of human histone deacetylases related to Saccharomyces cerevisiae HDA1p. J. Biol. Chem. *274*, 11713–11720.

Fischle, W., Dequiedt, F., Fillion, M., Hendzel, M.J., Voelter, W., and Verdin, E. (2001). Human HDAC7 histone deacetylase activity is associated with HDAC3 in vivo. J. Biol. Chem. *276*, 35826–35835.

Glass, C.K., and Rosenfeld, M.G. (2000). The coregulator exchange in transcriptional functions of nuclear receptors. Genes Dev. *14*, 121–141.

Grozinger, C.M., and Schreiber, S.L. (2000). Regulation of histone deacetylase 4 and 5 and transcriptional activity by 14-3-3-dependent cellular localization. Proc. Natl. Acad. Sci. USA *97*, 7835–7840.

Grozinger, C.M., Hassig, C.A., and Schreiber, S.L. (1999). Three proteins define a class of human histone deacetylases related to yeast Hda1p. Proc. Natl. Acad. Sci. USA *96*, 4868–4873.

Grunstein, M. (1997). Histone acetylation in chromatin structure and transcription. Nature *389*, 349–352.

Guenther, M.G., Lane, W.S., Fischle, W., Verdin, E., Lazar, M.A., and Shiekhattar, R. (2000). A core SMRT corepressor complex containing HDAC3 and TBL1, a WD40-repeat protein linked to deafness. Genes Dev. *14*, 1048–1057.

Guenther, M.G., Barak, O., and Lazar, M.A. (2001). The SMRT and N-CoR corepressors are activating cofactors for histone deacetylase 3. Mol. Cell. Biol. *21*, 6091–6101.

Hassig, C.A., Tong, J.K., Fleischer, T.C., Owa, T., Grable, P.G., Ayer, D.E., and Schreiber, S.L. (1998). A role for histone deacetylase activity in HDAC1-mediated transcriptional repression. Proc. Natl. Acad. Sci. USA *95*, 3519–3524.

Hendzel, M.J., Kruhlak, M.J., and Bazett-Jones, D.P. (1998). Organization of highly acetylated chromatin around sites of heterogeneous nuclear RNA accumulation. Mol. Biol. Cell *9*, 2491–2507.

Horlein, A.J., Naar, A.M., Heinzel, T., Torchia, J., Gloss, B., Kurokawa, R., Ryan, A., Ramei, Y., Soderstrom, M., Glass, C. K., et al. (1995). Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. Nature *377*, 397–404.

Hu, E., Chen, Z., Fredrickson, T., Zhu, Y., Kirkpatrick, R., Zhang, G.-F., Johanson, K., Sung, C.-M., Liu, R., and Winkler, J. (2000). Cloning and characterization of a novel human class I histone deacetylase that functions as a transcription repressor. J. Biol. Chem. *275*, 15254–15264.

Huang, E.Y., Zhang, J., Miska, E.A., Guenther, M.G., Kouzarides, T., and Lazar, M.A. (2000). Nuclear receptor corepressors partner with class II histone deacetylases in a Sin3-independent repression pathway. Genes Dev. *14*, 45–54.

Huynh, K.D., Fischle, W., Verdin, E., and Bardwell, V.J. (2000). BCoR, a novel corepressor involved in BCL-6 repression. Genes Dev. *14*, 1810–1823.

Jones, K.A., and Kadonaga, J.T. (2000). Exploring the transcription-chromatin interface. Genes Dev. *14*, 1992–1996.

Kadosh, D., and Struhl, K. (1998a). Histone deacetylase activity of Rpd3 is important for transcriptional repression in vivo. Genes Dev. *12*, 797–805.

Kadosh, D., and Struhl, K. (1998b). Targeted recruitment of the Sin3-Rpd3 histone deacetylase complex generates a highly localized domain of repressed chromatin in vivo. Mol. Cell. Biol. *18*, 5121–5127.

Kao, H.Y., Downes, M., Ordentlich, P., and Evans, R.M. (2000). Isolation of a novel histone deacetylase reveals that class I and class II deacetylases promote SMRT-mediated repression. Genes Dev. *14*, 55–66.

Kornberg, R.D., and Lorch, Y. (1999). Twenty-five years of the nucleosome, fundamental particle of the eukaryote chromosome. Cell *98*, 285–294.

Kruhlak, M.J., Lever, M.A., Fischle, W., Verdin, E., Bazett-Jones, D.P., and Hendzel, M.J. (2000). Reduced mobility of the alternate splicing factor (ASF) through the nucleoplasm and steady state speckle compartments. J. Cell Biol. *150*, 41–51.

Li, J., Wang, J., Wang, J., Nawaz, Z., Liu, J.M., Qin, J., and Wong, J. (2000). Both corepressor proteins SMRT and N-CoR exist in large protein complexes containing HDAC3. EMBO J. *19*, 4342–4350.

Luger, K., Mader, A.W., Richmond, R.K., Sargent, D.F., and Richmond, T.J. (1997). Crystal structure of the nucleosome core particle at 2.8 A resolution. Nature *389*, 251–260.

McKinsey, T.A., Zhang, C.-L., Lu, J., and Olson, E.N. (2000). Signal-dependent nuclear export of a histone deacetylase regulates muscle differentiation. Nature *408*, 106–111.

Miska, E.A., Karlsson, C., Langley, E., Nielsen, S.J., Pines, J., and Kouzarides, T. (1999). HDAC4 deacetylase associates with and represses the MEF2 transcription factor. EMBO J. *18*, 5099–5107.

Moazed, D. (2001). Enzymatic activities of Sir2 and chromatin silencing. Curr. Opin. Cell Biol. *13*, 232–238.

Ng, H.H., and Bird, A. (2000). Histone deacetylases: silencers for hire. Trends Biochem. Sci. *25*, 121–126.

Osborn, L., Kunkel, S., and Nabel, G.J. (1989). Tumor necrosis factor alpha and interleukin 1 stimulate the human immunodeficiency virus enhancer by activation of the nuclear factor kappa B. Proc. Natl. Acad. Sci. USA *86*, 2336–2340.

Rundlett, S.E., Carmen, A.A., Kobayashi, R., Bavykin, S., Turner, B.M., and Grunstein, M. (1996). HDA1 and RPD3 are members of distinct yeast histone deacetylase complexes that regulate silencing and transcription. Proc. Natl. Acad. Sci. USA *93*, 14503–14508.

Rundlett, S.E., Carmen, A.A., Suka, N., Turner, B.M., and Grunstein, M. (1998). Transcriptional repression by UME6 involves deacetylation of lysine 5 of histone H4 by RPD3. Nature *392*, 831–835.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, Second Edition (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Sparrow, D.B., Miska, E.A., Langley, E., Reynaud-Deonauth, S., Kotecha, S., Towers, N., Spohr, G., Kouzarides, T., and Mohun, T.J. (1999). MEF-2 function is modified by a novel co-repressor, MITR. EMBO J. *18*, 5085–5098.

Struhl, K. (1998). Histone acetylation and transcriptional regulatory mechanisms. Genes Dev. *12*, 599–606.

Taunton, J., Hassig, C.A., and Schreiber, S.L. (1996). A mammalian histone deacetylase related to the yeast transcriptional regulator Rpd3p. Science *272*, 408–411.

Tyler, J.K., and Kadonaga, J.T. (1999). The "dark side" of chromatin remodeling: repressive effects on transcription. Cell *99*, 443–446.

Underhill, C., Qutob, M.S., Yee, S.P., and Torchia, J. (2000). A novel nuclear receptor corepressor complex, N-CoR, contains components of the mammalian SWI/SNF complex and the corepressor KAP-1. J. Biol. Chem. *275*, 40463–40470.

Urnov, F.D., Yee, J., Sachs, L., Collingwood, T.N., Bauer, A., Beug, H., Shi, Y.B., and Wolffe, A.P. (2000). Targeting of N-CoR and histone deacetylase 3 by the oncoprotein v-erbA yields a chromatin infrastructure-dependent transcriptional repression pathway. EMBO J. *19*, 4074–4090.

Van Holde, K.E. (1988) Histone Modifications (New York: Springer).

Verdel, A., and Khochbin, S. (1999). Identification of a new family of higher eukaryotic histone deacetylases. Coordinate expression of differentiation-dependent chromatin modifiers. J. Biol. Chem. *274*, 2440–2445.

Verdel, A., Curtet, S., Brocard, M.P., Rousseaux, S., Lemercier, C., Yoshida, M., and Khochbin, S. (2000). Active maintenance of mHDA2/mHDAC6 histone-deacetylase in the cytoplasm. Curr. Biol. *10*, 747–749.

Wang, A.H., Bertos, N.R., Vezmar, M., Pelletier, N., Crosato, M., Heng, H.H., Th'ng, J., Han, J., and Yang, X.J. (1999). HDAC4, a human histone deacetylase related to yeast HDA1, is a transcriptional corepressor. Mol. Cell. Biol. *19*, 7816–7827.

Wang, A.H., Kruhlak, M.J., Wu, J., Bertos, N.R., Vezmar, M., Posner, B.I., Bazett-Jones, D.P., and Yang, X.J. (2000). Regulation of histone deacetylase 4 by binding of 14-3-3 proteins. Mol. Cell. Biol. *20*, 6904–6912.

Wen, Y.D., Perissi, V., Staszewski, L.M., Yang, W.M., Krones, A., Glass, C.K., Rosenfeld, M.G., and Seto, E. (2000). The histone deacetylase-3 complex contains nuclear receptor corepressors. Proc. Natl. Acad. Sci. USA *97*, 7202–7207.

Wolffe, A. (1998) Chromatin: Structure and Function, Third Edition (San Diego: Academic Press).

Wolffe, A.P., and Guschin, D. (2000). Review: chromatin structural features and targets that regulate transcription. J. Struct. Biol. *129*, 102–122.

Wolffe, A.P., and Hayes, J.J. (1999). Chromatin disruption and modification. Nucleic Acids Res. *27*, 711–720.

Workman, J.L., and Kingston, R.E. (1998). Alteration of nucleosome structure as a mechanism of transcriptional regulation. Annu. Rev. Biochem. *67*, 545–579.

Wu, C. (1997). Chromatin remodeling and the control of gene expression. J. Biol. Chem. *272*, 28171–28174.

Xu, L., Glass, C.K., and Rosenfeld, M.G. (1999). Coactivator and corepressor complexes in nuclear receptor function. Curr. Opin. Genet. Dev. *9*, 140–147.

Zhang, Y., Iratni, R., Erdjument-Bromage, H., Tempst, P., and Reinberg, D. (1997). Histone deacetylases and SAP18, a novel polypeptide, are components of a human Sin3 complex. Cell *89*, 357–364.

Zhang, Y., Ng, H.H., Erdjument-Bromage, H., Tempst, P., Bird, A., and Reinberg, D. (1999). Analysis of the NuRD subunits reveals a histone deacetylase core complex and a connection with DNA methylation. Genes Dev. *13*, 1924–1935.

Zhou, X., Richon, V.M., Rifkind, R.A., and Marks, P.A. (2000). Identification of a transcriptional repressor related to the noncatalytic domain of histone deacetylases 4 and 5. Proc. Natl. Acad. Sci. USA *97*, 1056–1061.