Histone Deacetylase 4 Is a Transcriptional Corepressor Regulated by Nucleocytoplasmic Shuttling

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June 2003

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

ABSTRACT

Histone acetylation plays an important role in regulating chromatin structure and thus gene expression. Analysis of histone deacetylase (HDAC) activity in *S. cerevisiae* revealed the presence of two deacetylase complexes, one containing Hda1 as its catalytic subunit, and the other possessing Rpd3. The three previously identified human HDAC proteins, HDAC1-3, were found to be homologs of Rpd3. This observation suggested that mammalian cells might contain an uncharacterized class of biochemically distinct Hda1-like proteins. The goal of my project has been to identify and characterize mammalian HDAC proteins which are similar to Hda1. I first identified the human histone deacetylase HDAC4, which contains a carboxy-terminal region significantly similar to the catalytic domain of yeast Hda1. When tethered to a promoter, HDAC4 functions as a transcription corepressor. Furthermore, HDAC4 interacts with the transcription factors MEF2 and RFXANK and represses transcription of their target genes, supporting the notion that HDAC4 is a transcription corepressor *in vivo*. Surprisingly, HDAC4 is localized mainly in the cytoplasmic region and shuttles between the nucleus and the cytoplasm. Nucleocytoplasmic shuttling of HDAC4 is controlled by multiple mechanisms. HDAC4 possesses a nuclear localization signal (NLS) and a nuclear export signal (NES) for its dynamic nucleocytoplasmic trafficking. Binding of 14-3-3 proteins exposes the NES of HDAC4, which then results in its nuclear export. From this work, I have identified HDAC4 and shown that it functions as a transcription corepressor whose activity is regulated by nucleocytoplasmic shuttling.

Résumé

L'acétylation des histones joue un rôle important dans le contrôle structural de la chromatine et, par conséquent, de l'expression des gènes. Chez *S. cerevisiae*, deux complexes enzymatique contenant séparement les histones déacétylases (HDAC) Hda1 et Rpd3 ont été identifiés et caractérisés. Originalement identifiées chez l'humain, HDAC1-3 ont été clonées grâce à leur homologie à la protéine de levure Rpd3. Cette observation a suggéré que les cellules de mammifères contiennent une nouvelle classe de protéine HDAC qui est biochimiquement similaire à Hda1. Le but de mon projet a été d'identifier et de caractériser les HDAC de mammifères démontrant une similarité à Hda1. J'ai initialement identifié l'histone déacétylase humaine HDAC4 qui contient une région carboxy-terminale similaire au domaine catalytique de Hda1. Une fois recrutée à un promoteur, HDAC4 agit comme un corépresseur de la transcription. De plus, j'ai démontré que HDAC4 interagit avec les facteurs de transcription MEF2 et RFXANK tout en réprimant la transcription de leurs gènes respectifs. Ces résultats confirment que HDAC4 est un corépresseur de la transcription *in vivo*. Etonnamment, HDAC4 est une protéine cytoplasmique et, par conséquent, doit être transportée dans le noyau pour exercer sa fonction de corépresseur. Le transport de HDAC4 du cytoplasme au noyau est régulé par plusieurs mécanismes. HDAC4 possède un signal de localisation nucléaire (NLS) et un signal d'exportation nucléaire (NES) qui contrôlent sa localisation cellulaire. La liaison de HDAC4 avec les protéines 14-3-3 permet l'exposition de son NES, ce qui lui permet d'être relocalisé dans le cytoplasme. Finalement, ce travail m'a

permis d'identifier et de démontrer que HDAC4 agit comme un corépresseur de la transcription dont l'activité est régulée par sa localisation cellulaire.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Xiang-Jiao Yang, for his intellectual and moral support for my scientific development. He has encouraged and guided me during the course of my studies.

Extended thanks go to all members of my thesis committee for their encouragement and help: Dr. Katherine Cianflone, Dr. Jean-Jacques Lebrun, Dr. Rongtuan Lin, and Dr. Alain Nepveu.

It would be impossible to complete my Ph.D project and thesis without the help of my husband, Yunghong Liao, who has constantly encouraged me. I am eternally grateful for the happiness and joy that he and our son have brought to me.

I would also like to thank my parents, Aizhen Wang and Weichu Yang, for their unconditional love, encouragement and support.

My appreciation to all the current and former members of Dr. Yang's laboratory for their stimulating discussion and friendship: Nick Bertos, Nadine Pelletier, Serge Gregoire, Lin Xiao, Siew-Lee Goh, Dr. Nathalie Champagne, Jianhong Liu, Jenny Tong, Henry Lim, Marco Vezmar, Junmei Jiang, and Hongwei Li.

Many thanks are given to Nick Bertos and Siew-Lee Goh for the correction of this thesis, and Nadine Pelletier and Serge Gregoire for the French translation.

Financial support for these studies was provided, in part, by a studentship from Royal Victoria Hospital research foundation and a Doctoral Research Award from the Canadian Institute of Health Research (CIHR).

Preface

"The Guidelines for Thesis preparation" issued by The Faculty of Graduate Studies and Research at McGill University reads as follows:

> "Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis.
>
> The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges between the different papers are mandatory.
>
> In addition to the manuscripts, the thesis must include the following: (a) a table of contents, (b) an abstract in English and French, (c) an introduction which clearly states the rational and objectives of the research, (d) a comprehensive review of the literature (in addition to that covered in the introduction to each paper), (e) a final conclusion and summary.
>
> As manuscripts for publication are frequently very concise documents, where appropriate, additional material must be provided (e.g., in appendices) in sufficient detail to allow a clear and precise judgment to be made of the importance and originality of the research reported in the thesis.
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> In general, when co-authored papers are included in a thesis, the candidate must have made a substantial contribution to all papers included in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled "Contributions of Authors" as a preface to the thesis. The supervisor must attest to the accuracy of this statement at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to clearly specify the responsibilities of all the authors of the coauthored papers. When previously published copyright material is presented in a thesis, the candidate must include signed waivers from the co-authors and publishers and submit these to the Thesis Office with the final deposition, if not submitted previously."

I have chosen to write my thesis according to these guidelines, with three published papers and two submitted manuscripts. The thesis is organized in seven chapters: (I) Literature review, (II – V) Manuscripts with their own abstracts, introduction, materials and methods, results, and discussion, (VI) General discussion, and (VII) contribution to original research.

Publications arising from the work of the thesis

First-author publications:

1. **Wang AH**, Bertos NR, Vezmar M, Pelletier N, Crosato M, Heng HH, Th'ng J, Han J, and Yang XJ. (1999) HDAC4, a human histone deacetylase related to yeast HDA1, is a transcriptional corepressor. *Mol. Cell. Biol.* 19: 7816-7827.

2. **Wang AH**, Kruhlak MJ, Wu J, Bertos NR, Vezmar M, Posner BI, Bazett-Jones DP, and Yang XJ. (2000) Regulation of the histone deacetylase HDAC4 by binding of 14-3-3 proteins. *Mol. Cell. Biol.* 20: 6904-6912.

3. **Wang AH**, and Yang XJ. (2001) Histone deacetylase 4 possesses intrinsic nuclear import and export signals. *Mol. Cell. Biol.* 21: 5992-6005.

4. **Wang AH**, Gregoire S, Xiao L and Yang XJ. (2003) Association of HDAC4 with ankyrin repeat proteins ANKRA2 and RFXANK. (to be submitted)

Other publications:

1. Champagne N, Bertos NR, Pelletier N, **Wang AH,** Vezmar M, Yang Y, Heng HH, and Yang XJ. (1999) Identification of a human histone acetyltransferase related to monocytic leukemia zinc finger protein. *J. Biol. Chem.* 274: 28528-36.

2. Zhou X, Richon VM, **Wang AH**, Yang XJ, Rifkind RA, Marks PA. (2000) Histone deacetylase 4 associates with extracellular signal-regulated kinases 1 and 2, and its cellular localization is regulated by oncogenic Ras. *Proc. Natl. Acad. Sci. USA* 97: 14329-33.

3. Bertos NR, **Wang AH**, and Yang XJ. (2001) Class II histone deacetylases: Stucture, function, and regulation. *Biochem. Cell Biol.* 79: 243-52.

Contributions of Authors

Bertos, NR

In manuscript #1, he performed HDAC activity assays of Figure 4. In manuscript #2, he performed immunofluorescence microscopy.

Crosato, M and Dr. John Th'ng

In manuscript #1, they prepared [^{3}H]-acetyl-histones for the HDAC activity assays.

Gregoire, S

In manuscript #4, he sequenced positive clones from yeast two-hybrid screen.

Heng, HH

In manuscript #1, he performed the fluorescence in situ hybridization (FISH) assay for Figure 3.

Kruhlak, MJ

In manuscript #2, he performed immunofluorescence microscopy.

Pelletier, N

In manuscript #1, she helped to set up DNA-binding assays.

Vezmar, M

In manuscript #1 and #2, he found that HDAC4 was localized in the cytoplasm.

Wu, J

In manuscript #2, he found the association of 14-3-3 proteins with HDAC4.

Xiao, L

In manuscript #4, she made the GFP-ANKRA2 and GFP-RFXANK constructs. She also helped to sequence positive clones from yeast two-hybrid screen.

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CHAPTER I

LITERATURE REVIEW

1 Chromatin structure

The genome of a cell contains in its DNA sequence the information to make many thousands of different protein and RNA molecules. A cell typically expresses only a fraction of its genes, and the different types of cells in a multicellular organism arise because different sets of genes are expressed. Moreover, cells are able to alter their patterns of gene expression in response to extracellular cues. Although all of the steps involved in expressing a gene can in principle be regulated, for most genes the initiation of RNA transcription is the most important point of control.

The eukaryotic genome is packaged into the compact state of chromatin from which the fundamental nuclear processes of transcription, replication and DNA repair occur. The fundamental packing unit of chromatin is the nucleosome; a nucleosome core particle comprises 146 base pairs of DNA wrapped around an octameric core containing two molecules each of core histones H2A, H2B, H3 and H4 to form the simple "beads-on-a-string" structure (203) (Figure 1). Each core histone contains a carboxy-terminal, highly helical globular domain that comprises about 75% of the amino-acid content and forms the interior core of the nucleosome core particle. The remaining amino-terminal portion of the core histone constitutes a flexible and highly basic tail region that is also highly conserved across various species. In nature, nucleosomes are usually packed together, with the aid of other non-histone proteins, into higher-order structures characteristic of chromatin.

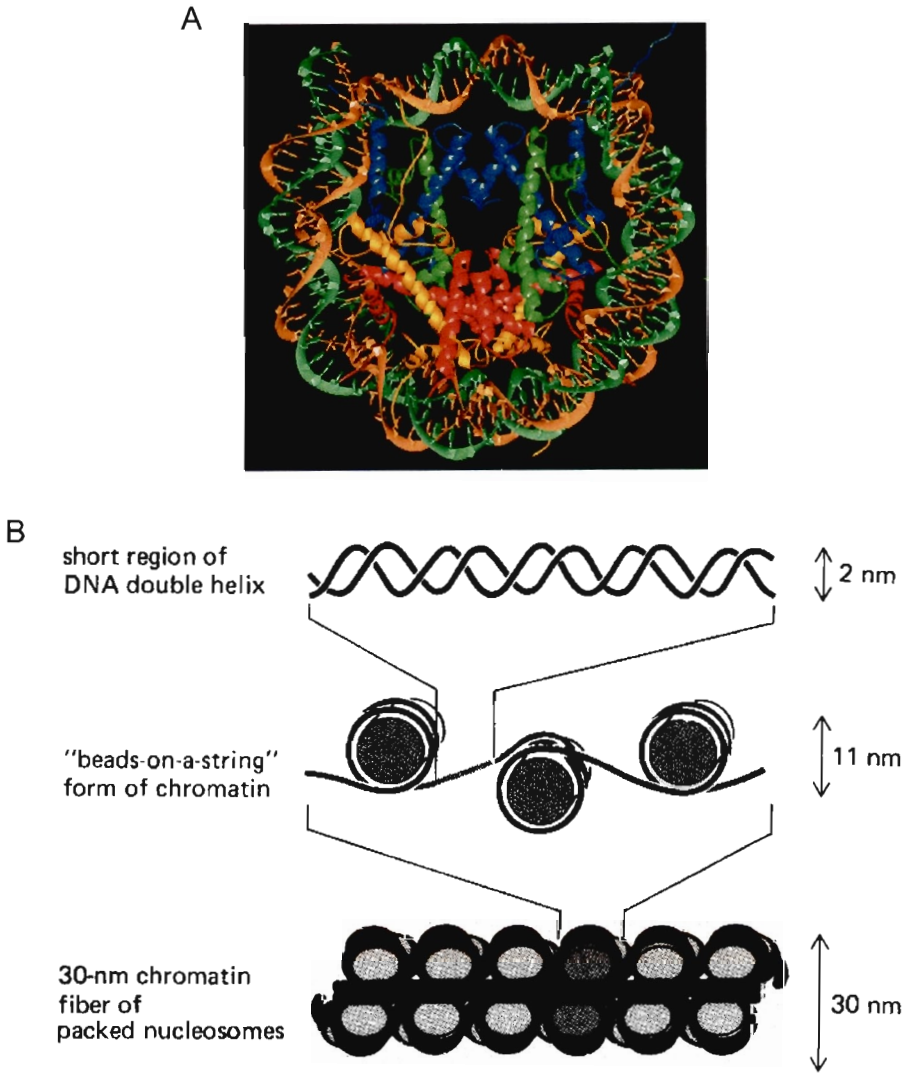

Figure 1 Chromatin structure

(A) Nucleosome core particle: ribbon traces for the 146 bp DNA phosphodiester backbones (brown and turquoise) and eight histone protein main chains (blue: H3; green: H4; yellow: H2A; red: H2B). Adapted from *Luger et al, (1997). Nature 389:251-260.*

(B) Model of chromatin packing. Adapted from *Molecular Biology of the Cell* (1994), Garland Publishing, Inc.

Chromatin is not uniform with respect to gene distribution and transcriptional activity. It is organized into different domains, such as heterochromatin and euchromatin, which have different chromosomal architecture, transcriptional activity and replication timing. Heterochromatin is a highly condensed form of chromatin that occurs at defined sites, such as silencer DNA elements or regions close to telomeres. This type of chromatin is transcriptionally inactive, and approximately 10% of the genome is packed into heterochromatic structure in a typical cell in interphase. Euchromatin generally contains decondensed, transcriptionally active regions of the genome. It exists in at least two forms: about 10% is in the form of active chromatin, which is the least condensed, while the rest is inactive euchromatin, which is more condensed than active chromatin but less condensed than heterochromatin. During mitosis, chromosomes are formed from chromatin in its most condensed state.

2 Regulation of chromatin structure

In addition to DNA packaging, chromatin also plays a regulatory role. It is now clear that modification of chromatin structure is critical for the regulation of gene expression. However, the decondensed form of chromatin is generally repressive for gene-specific transcriptional activation (174). The chromatin structure thus determines whether a gene can be activated because it is in an "open" chromatin state that is accessible to the transcription machinery, or whether it remains silent because its *cis*-regulatory elements are inaccessible.

Research in the past decade has identified a multitude of structural proteins and enzyme complexes which regulate chromatin structure and function. Generally speaking, there are two kinds of enzymatic activities involved in the regulation of chromatin structure. One type involves mainly ATP-hydrolyzing enzymes that can re-model chromatin by moving nucleosome positions and creating conformations where DNA is accessible on the surface of the histone octamer (15). The other includes a set of enzymes that are able to modify histones covalently at specific residues located most commonly at the amino-terminal histone tails (16). Such modification includes acetylation, phosphorylation, methylation, ubiquitination and ADP-ribosylation. It is also noteworthy that the non-protein component of chromatin, DNA, can be modified by cytosine methylation, which is catalyzed by DNA methyltransferases and also important for regulating chromatin structure and transcription (155).

2.1 ATP-dependent chromatin remodeling

ATP-dependent chromatin remodeling is mediated by the ATP-dependent complexes that use the energy of ATP hydrolysis to increase the accessibility of nucleosomal DNA (15), a fundamental step in transcriptional regulation. Based on the sequence similarity of their ATPase subunits, ATP-dependent chromatin remodeling complexes can be divided into three subfamilies: (i) the SWI/SNF subfamily, (ii) the imitation SWI (ISWI) subfamily, and (iii) the Mi-2/CHD subfamily (192). The Mi-2/CHD family members also show histone deacetylase activity (192). In addition to the helicase-like regions, the ATPase subunits of

ATP-dependent chromatin remodeling complexes contain other conserved motifs, such as bromodomain in SWI/SNF subfamily, SANT (swi3, ada2, N-CoR and TFIII B) domain in ISWI subfamily and chromodomain in Mi-2/CHD subfamily. Furthermore, each ATPase subunit forms complexes with different proteins. These complexes can expose or occlude DNA sequences by "sliding" nucleosomes --- transfering histone octamers from one region of a DNA fragment to another and generating a range of remodeled intermediates (52). However, recent studies suggest that sliding is not the sole mechanism for ATP-dependent chromatin remodeling. There is increasing evidence that ATP-dependent remodeling may involve changes in the topology of nucleosomal DNA (55), such as formation of DNA bulges and small loops (104, 138).

Accumulating evidence indicates that some transcription factors can bind directly to ATP-dependent chromatin remodeling complexes to target these activities to specific chromosome locations. For instance, SWI/SNF can be recruited to specific promoters by directly interacting with transcription activators, such as C/EBPβ (96), c-Myc (33), MyoD (37), the glucocorticoid receptor (GR) and the estrogen receptor (ER) (39, 70), to activate gene expression. In contrast, many ATP-dependent chromatin remodeling activities can repress transcription by interacting with transcription repressors. For example, hBRG1 represses transcription that is mediated by E2F (221) and c-fos (133). Isw2p has been shown to repress transcription of early mitotic genes by the transcriptional repressor Ume6p (56). The recruitment of Isw2p leads to the formation of inaccessible chromatin structure proximal to the Ume6p binding site and,

consequently, represses transcription. The NuRD complex, which contains an ATP-dependent chromatin remodeler, Mi-2, and histone deacetylases (182, 207, 224), can be recruited to heterochromatin by the DNA binding protein Ikaros (93). It has been proposed that this recruitment either maintains an inactive chromatin state or converts an accessible chromatin conformation to an inaccessible structure. The transcription repressor Kap-1 also targets NuRD to specific promoters to repress gene expression (165). The *Drosophila* dMi-2 protein associates with the hunchback protein to repress HOX gene transcription (91).

2.2 Post-translational modification of histones

While ATP-dependent chromatin-remodeling complexes use ATP as the energy source to modulate chromatin structure in a noncovalent manner, histone modifications covalently modify chromatin. The amino-terminal tail of each core histone contains a flexible and basic region, which is highly conserved across different species in the eukaryotic kingdom, and is subject to post-translational modifications, including acetylation, methylation, phosphorylation, ubiquitination, and ADP-ribosylation. Such modifications can modulate roles of the histone tails in chromatin compaction and have been correlated with different activities in chromatin assembly, DNA replication, and transcription.

2.2.1 Histone acetylation

Histone acetylation is the most characterized modification and is generally linked to transcriptional activation (63). Histone acetylation at certain lysine

residues was proposed to be involved in transcriptional activation almost 40 years ago (5). This reversible reaction is catalyzed by histone acetyltransferase (HAT) and histone deacetylase (HDAC) enzymes. HATs catalyze the transfer of acetyl groups from acetyl-coenzyme A (acetyl-CoA) to the ε-amino groups of specific lysine residues within histone N-terminal tails, whereas HDACs reverse the process by removing the acetyl group from acetyl lysine residues (Figure 2). Histone acetylation, thus, neutralizes part of a histone tail's positive charge, thereby weakening histone:DNA contacts, disrupting internucleosomal interactions, affecting the structure of individual nucleosomes and higher-order folding, and leading to a more open and permissive chromatin environment for transcription (159, 172). It has long been known that histone acetylation is associated with the states of transcription activation (72). Since the cloning of a nuclear HAT enzyme from *Tetrahymena* as the homologue of transcriptional co-activator Gcn5 from yeast (26), many studies have led to the discovery of HATs that were previously identified transcriptional co-activators, such as p300/CBP (9, 147), $TAF_{II}250$ (131), and SRC-1 (171). Several HDAC enzymes have been identified almost in parallel with the discovery of HATs and have been correlated with transcriptional repression. The enzymes involved in histone acetylation and deacetylation will be discussed in detail later.

2.2.2 Histone phosphorylation

Histone H3 phosphorylation is directly correlated with transcriptional induction of immediate early genes in mammalian cells, such as the *c-Fos* gene

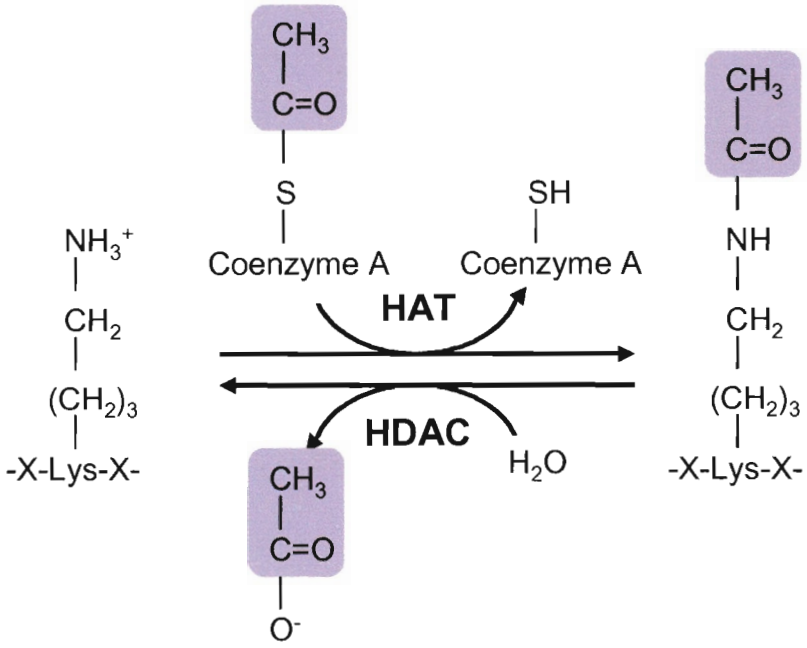

Figure 2 Histone acetylation

Histone acetyltransferases (HATs) transfer the acetyl moiety to the ε-NH_3^+ group of lysine residues of histone N-terminal domains. Reverse reaction is catalyzed by histone deacetylases (HDACs). Acetyl coenzyme A is the acetyl moiety donor for histone acetylation.

(122). Prior phosphorylation of Ser10 in histone H3 can promote the Gcn5-mediated acetylation at Lys14 (34, 114). Rsk-2 and MSK1, which are activated upon stimulation of the Ras-MAPK pathway, have been identified to phosphorylate this serine residue *in vitro* (161, 179). In *S. cerevisiae*, the Snf1 kinase is identified as a Ser10 kinase (113). Phosphorylation of histone H3 by Snf1 leads to Gcn5-mediated acetylation at the *INO1* promoter, which is required for full *INO1* transcriptional induction.

At least two serine residues, Ser10 and Ser28, on histone H3 are phosphorylated during mitosis (57, 200). Phosphorylation at Ser10 begins in early G2 in the pericentromeric heterochromatin of each chromosome and spreads throughout all chromosomes by metaphase (74). Mutation of H3 Ser10 to alanine in *Tetrahymena* leads to abnormal patterns of chromosome segregation and extensive chromosome loss (201). A direct link between H3 phosphorylation and condensin recruitment to chromosomes has been suggested by the colocalization of members of condensin complex with phosphorylated histone H3 during the early stages of mitotic chromosome cendensation (163). These observations indicate that phosphorylation of histone H3 plays an important role in mitotic chromosome condensation. Recently, the Ipl1/AIR-2 kinases and NIMA kinase have been identified as mitotic H3 kinase (38, 76). Mutations in Ipl1 and NIMA result in complete loss of H3 phosphorylation during mitosis. Moreover, in yeast, strains bearing temperature-sensitive mutations of *Ipl1* gene display defects in chromosome segregation.

2.2.3 Histone methylation

While histone acetylation and histone phosphorylation are highly dynamic processes with rapid turn-over rates, histone methylation appears to be a rather static process. Histone methylation can occur on either arginine (R) or lysine (K) residues. Several histone methyltransferases (HMT), which are either lysine or arginine specific, have been identified and characterized (226). The K-HMTs include an evolutionarily conserved sequence called a SET domain (for "Su(var), E(z), trithorax") as the catalytic core. The first identified member of this family is Suv39H that specifically methylates H3-K9 (153). Other members include G9a, SET1, MLL, EZH2, CLLL8, etc (226). The R-HMT superfamily (PRMT) does not contain a SET domain, but has a highly conserved S-adenosyl methionine (SAM) binding site. The PRMT protein family is composed of six proteins, PRMT1-6. These two families of HMTs have been shown to methylate specific residues within histone tails. Recently, another distinct class of K-HMT lacking a SET domain has been identified (49, 141, 186). The only known member of this K-HMT family is Dot1 and methylates histone H3 lysine 79, a conserved residue on the top and bottom of a nucleosomal core.

Methylation occurring on different lysines or arginines within histone tails is correlated with different transcriptional states. Within the histone H3 N-terminal tails, three lysines, K4, K9, and K27, are commonly methylated. Suv39h-mediated H3-K9 methylation sequentially leads to binding of HP1 (10, 98, 153). In cells derived from mice lacking Suv39h, the HP1 protein no longer localizes to heterochromatin (98), suggesting that the methyltransferase activity of Suv39h is

required for heterochromatin formation. Genetic analysis demonstrates that the Suv39h regulates K9 methylation at pericentric heterochromatin (149). Suv39h double null mice show severely impaired viability and chromosomal instability. On the other hand, the retinoblastoma (RB) protein recruits Suv39h/HP1 complex to repress the transcription of cell-cycle controlling genes such as cyclin E (142, 187), suggesting that local H3-K9 methylation is important for the transcriptional repession of cell-cycle regulatory genes. Conversely, methylation of H3-K4 is correlated with transcriptional activation (112, 144). Set7 (also called Set9), ALL-1 and MLL have been identified as H3-K4-specific methyltransferases which potentiate transcription activation (128, 136, 143, 197). Set9-mediated methylation on H3-K4 and methylation on H3-K9 by SUV39H, but not G9a, inhibit each other (143, 197). Moreover, it is methylation by Set9 at K4, not K9, that displaces the NuRD chromatin remodeling and histone deacetylase complex and activates transcription (143). Methylation of H3-K79 is associated with recombinationally active chromatin regions at the mouse IgH and TCRβ loci (140). However, hypomethylation of H3-K79 is restricted to heterochromatic regions and dependent on Sir proteins in *S. cerevisiae* (140), suggesting that Sir proteins prefer to interact with histone H3 that is unmethylated at K79. This is consistent with the finding that Dot1-mediated H3-K79 methylation limits telomeric silencing by preventing the binding of Sir proteins to the telomeric region (141, 186). Arginine methylation of histone is correlated with the active state of transcription. Arginine methyltransferase CARM1/PRMT4 interacts with GRIP1, a p160 family co-activator of nuclear hormone receptors, and methylates

specifically histone H3 in vitro (32). Moreover, by chromatin immunoprecipitation (ChIP) assays, methylation at H3-R17 by CARM1/PRMT4 has been shown to occur only when nuclear receptor-regulated promoters are active (14, 120). Taken together, histone methylation plays an important role in regulation of gene transcription.

2.2.4 Histone ubiquitination

In *S. cerevisiae*, K123 within the H2B carboxy-terminal tail is a substrate for the Rad6 ubiquitin (156). It has recently been shown that Rad6-mediated H2B-K123 ubiquitination is essential for methylation of both H3-K4 and H3-K79 (25, 43, 175).

2.2.5 Histone ADP-ribosylation

Poly(ADP-ribosyl)ation is mostly catalyzed by poly(ADP-ribose) polymerase-1 (PARP-1) and involved in DNA-base excision repair, DNA-damage signaling, regulation of genomic stability, and regulation of transcription and proteasomal function (29). It has long been postulated that poly(ADP-ribosyl)ation, as a regulator of chromatin, has an impact on gene expression. Recently, *Drosophila* PARP has been shown to be required for formation of puffs, an expanded chromatin state, and hsp70 expression after heat shock (184). This observation suggests that the activated PARP molecules modify nucleosomal histones with poly(ADP-ribose) chains to form a loose chromatin structure that may facilitate transcription.

2.3 Histone code and "cross-talk" of chromatin modification

The different and interdependent histone modifications led to the histone code hypothesis (173, 185), which suggests that distinct histone modification patterns act sequentially or in combination to form a histone code that is read by other proteins to elicit distinct downstream transcriptional events. Protein domains that are capable of interacting with specifically modified histone tails have been discovered. Bromodomains, which can be found in several HATs, have been shown to interact with acetylated lysines (40, 85). The chromodomain of HP1 protein specifically reads the H3-K9 methyl marker added by SUV39H (10, 98). Different modifications within the same histone tail can mutually affect each other. For example, phosphorylation of H3-S10 stimulates acetylation of H3-K14 by Gcn5, which, thus, leads to transcription activation (161, 179). Methylation of H4-R3 by PMRT1 facilitates the subsequent acetylation of H4-K8 and H4-K12 by p300, consequently activating transcription (198). H3-K4 methylation by Set9 is capable of blocking chromatin remodeling/deacetylation and methylation of H3-K9 by Suv39h (143). Furthermore, histone modifications can cross-talk between different histone tails. Rad6-mediated H2B-K123 ubiquitination specifically regulates methylation of both H3 K4 and K79 (25, 43, 175).

Chromatin remodeling and histone modifications cooperate together in the regulation of gene activity. Recent data strongly suggest that transcriptional activation linking via chromatin structure modifications is a time-dependent series of events. The best-characterized example in mammals is provided by studies of the human IFN-β gene. Upon virus infection, three transcription factors, NF-κB,

IRF, and ATF-2/cJun, bind cooperatively with the architectural protein HMGI(Y) to the enhancer and form the IFN-β enhanceosome. The enhanceosome targets the modification and repositioning of a nucleosome that blocks the formation of a transcriptional preinitiation complex on the IFN-β promoter (1, 2, 115). Initially, the Gcn5 HAT-containing complex is recruited to acetylate the nucleosome. This event is followed by the recruitment of the CBP-Pol II holoenzyme complex. Next, the SWI/SNF chromatin remodeling complex is recruited to the promoter via interaction with CBP and the acetylated histone tails. Specifically, acetylation of K8 of histone H4 is required for recruitment of the SWI/SNF complex through the bromodomain of the BRG1 subunit. SWI/SNF remodels nucleosomes and allows association of TBP with the TATA box. TBP binding induces bending of DNA and sliding of the SWI/SNF-modified nucleosome to a new position. Finally, this chromatin conformation of the IFN-β promoter allows TFIID recruitment, PIC assembly, and transcription initiation. Acetylation of K9 and K14 on histone H3 is critical for the recruitment of TFIID, and the double bromodomains in the $TAF_{II}250$ subunit are responsible for the binding of the K9/14 acetylated tail of histone H3.

3 Histone acetyltransferases and deacetylases

3.1 Structure and function of HATs

There are two major classes of histone acetyltransferases, the type A nuclear HATs and the type B cytoplasmic HATs (159, 172). The nuclear regulatory A-type HATs fall into several distinct families: GNAT (Gcn5-related N-

acetyltransferase) superfamily, MYST (MOZ, Ybf2/Sas3, Sas2, and Tip60) superfamily, p300/CBP, nuclear receptor co-activators, $TAF_{II}250$, and TFIIIC (Table 1). The GNAT and MYST families share a highly conserved motif containing an acetyl-CoA binding site. The catalytic domains of HATs are indispensable for their acetylation function. Besides these domains, there are other motifs in different families. For example, Gcn5, PCAF, p300/CBP and $TAF_{II}250$ contain bromodomains, which have been shown to bind acetylated H3 or H4 (40, 85). In the MYST family, there is a zinc finger motif in most members except yeast ESA1, human MOZ or its homologue MORF contains two PHD fingers, and yeast ESA1 or *Drosophila* MOF possesses a chromodomain. These additional domains appear to modulate their HAT activity. For instance, the bromodomain of human GCN5 interacts with the DNA-dependent protein kinase Ku/DNA-PKs, which in turn inhibits GCN5's HAT activity by phosphorylation (13). Deletion of the N-terminal region (including the PHD domain) of human MORF leads to increased *in vitro* HAT activity, suggesting that the N-terminal region of MORF inhibits its HAT activity (30).

In vitro, several HATs have been shown to have activity on histones, and display different histone specificities. Recombinant p300/CBP proteins can acetylate all four core histones either in the nucleosomal or free-histone form (9, 147), whereas recombinant GCN5 and PCAF proteins acetylate only H3 and H4 (97, 211). Proteins other than histones, such as transcription factors, HIV Tat protein, and importin, can also be substrates of HATs.

Table 1. Classification of HATs

HAT family	HAT	HAT complex
GNAT	Gcn5	SAGA, ADA, A2
	PCAF	PCAF
	Hat1	HatB
	Elp3	
	Hpa2	
MYST	Esa1	NuA4
	MOF	MSL
	Sas2	
	Sas3	NuA3
	MORF	
	MOZ	
	Tip60	Tip60
	Hbo1	ORC
p300/CBP	p300	
	CBP	
Basal transcription factors	TAFII250	
	TFIIIC	
Nuclear receptor cofactors	ACTR	
	SRC1	

HATs seem to act as part of large complexes *in vivo*, recruited to promoters by interaction with DNA-bound activator proteins. In yeast, four distinct complexes have been well characterized: SAGA, ADA, NuA3 and NuA4 complexes. Both SAGA and ADA complexes possess Gcn5 as a catalytic subunit (58), while NuA3 and NuA4 complexes contain Sas3 and Esa1, respectively, as their catalytic subunits (4, 87). Two human HAT complexes have also been purified: PCAF and Tip60 complexes, which have PCAF and Tip60 as their catalytic subunits, respectively (83, 146). These HAT complexes have been shown to play transcriptional roles. For example, the Tip60 complex has been shown to be recruited to the KAI1/CD82 promoter by NF-κB after IL-1β treatment, and the Tip60 HAT function is required for effective gene activation (8). Yeast SAGA complex is recruited by the Gal4 activation domain after galactose induction, and this recruitment is required for the recruitment of TBP to GAL gene promoters (18, 105). These findings indicate that SAGA functions as a coactivator, bridging interaction between basal transcription factors (e.g. TBP) and transcriptional activators. These complexes contain different subunit composition, suggesting that they are involved in distinct biological functions. For example, Spt16, a subunit of NuA3, and its mammalian homologs have been implicated in transcriptional elongation and DNA replication (159). Tip60 complex has been shown to have ATPase, DNA helicase, and structural DNA-binding activities, and to be involved in DNA repair and apoptosis (83).

HATs are also important in development and differentiation. PCAF and p300, which form a multimeric complex with MyoD, activate MyoD-dependent

transcription and promote myogenic differentiation (152). The HAT activity of PCAF, but not p300, is required for transcriptional activation and myogenic differentiation (152). MyoD was found to be acetylated by PCAF, but not p300, on three lysine residues, thereby increasing DNA binding, and nonacetylated MyoD can not stimulate transcription and induce muscle conversion (160). The genetic evidence that *p300* and *GCN5* knockout mice are embryonic lethal indicates that their HAT activity is essential for mammalian development (206, 212).

3.2 Structure and function of HDACs

As with HATs, the first histone deacetylase, HDAC1, was also identified in 1996 (178). In the subsequent years, additional HDACs were identified. These enzymes deacetylate acetylated histones, affect chromatin structure and result in transcriptional repression. For transcriptional repression, it is often necessary to assemble HDAC-dependent transcriptional repressor complexes *in vivo*. However, there are exceptions. For example, yeast histone deacetylase Hos2 has been shown to be preferentially associated with genes of high activity (193). Based on sequence homology, mammalian histone deacetylases can be divided into three classes (Table 2): (1) the class I PRD3-like, (2) the class II HDA1-like, and (3) the class III SIR2-like proteins.

Table 2. Classification of yeast and mammalian HDACs

Class	*S. cerevisiae* HDAC	Mammals HDAC	Complex
I	Rpd3 Hos1, -2	HDAC1, -2	Sin3, NuRD, CoREST
		HDAC3	N-CoR, SMRT
		HDAC8	
II	Hda1	HDAC4, -5, -7, -9	
		HDAC6	p97/PLAP
		HDAC10	
III	Sir2 Hst1-4	SIRT1-7	

3.2.1 Class I HDACs

This class contains HDAC1, 2, 3 and 8, which show sequence homology with yeast Rpd3. HDAC1 was isolated by affinity chromatography with trapoxin A, a known HDAC inhibitor (178). HDAC2 was identified as a transcription factor YY1-interacting protein by yeast two-hybrid screening (208). HDAC1 and HDAC2 are 85% identical in protein sequence. HDAC3 is less similar (48, 210). It shows 53% identity at the protein level compared with HDAC1. HDAC8 was cloned more recently (27, 77). All four HDACs have ubiquitous tissue distribution. HDAC1, 2, and 8 are exclusively localized to the nucleus, whereas HDAC3 is found in both the nucleus and the cytoplasm (209).

Among these four HDAC proteins, HDAC1 and HDAC2 are the best studied. They are generally found in stable protein complexes recruited by DNA-binding proteins. So far, three complexes containing HDAC1 and HDAC2 have been identified: the Sin3, NuRD, and CoREST complexes. The Sin3 and NuRD complexes share a "catalytic core" consisting of HDAC1, HDAC2 and the histone binding proteins RbAp46/48.

Sin3 complex. Sin3 complex purified from HeLa nuclear extracts contains seven polypeptides: mSin3, HDAC1/2, RbAp48/46, and two mSin3-associated proteins, SAP18 and SAP30 (71, 223). HDAC1 and HDAC2 are enzymatically active components of the complex. mSin3 was identified originally as the corepressor for the DNA-binding heterodimeric transcriptional repressor Mad-Max (7, 164). Both mSin3 isoforms are similar to the yeast global transcription repressor SIN3. mSin3 is proposed to act as a scaffold for the

complex. RbAp48 was shown to be dispensible for the enzymatic activity of the complex (178). RbAp48 is also a subunit of the chromatin assembly factor CAF-1. Since RbAp48 can interact with histone H4, it is believed that RbAp48 targets associated chromatin-altering enzymes to nucleosomes (191). The function of SAP18 and SAP30 is less clear. They can repress transcription when tethered to promoters (100, 223, 227). SAP18 can potentiate mSin3-HDAC1-mediated transcriptional repression (223). SAP30 has a structural and functional homolog in yeast. Deletion of yeast *SAP30* results in phenotypes similar to those associated with the deletion of *RPD3* and *SIN3* (227), strongly suggesting that Sap30 is required for the normal function of the Rpd3 complex.

The Sin3 complex has been shown to be recruited to promoters by interacting with DNA-binding transcription repressors or corepressors. For example, the enzymatic activity of the mSin3-associated HDACs is required for full repression by Mad-Max (101). Other transcription factors, such as p53 (134), REST/NRSF (79, 139, 157), AML-ETO (199), PML-RAR and PLZF-RAR fusion proteins (60, 111), also recruit the mSin3-HDAC complex to gene-specific promoters to repress transcription. The nuclear receptor corepressor N-CoR binds to SAP30 to target mSin3-HDAC complex for transcriptional repression (100). Another nuclear receptor corepressor SMRT directly interacts with mSin3A and recruits HDAC1 to repress retinoic acid responsive genes and inhibit differentiation of myeloid leukemia (HL-60) cells (135).

NuRD complex. In addition to its catalytic core, NuRD complex (nucleosomal remodeling and deacetylation) also contains Mi-2, MTA2 and

MBD3 (methyl CpG-binding domain-containing protein) (182, 207, 224). Mi-2 was originally identified as the dermatomyositis-specific autoantigen (166), and contains a SWI2/SNF2-like helicase/ATPase domain which has chromatin remodeling activity. The nucleosomal HDAC activity of the NuRD complex is stimulated by ATP, suggesting that chromatin remodeling by Mi-2 increases the accessibility of the histone tails to HDAC1 and 2 (182, 207). MTA2, which is highly related to the metastasis-associated MTA1, modulates the enzymatic activity of the catalytic core through interaction with MBD3 (225). NuRD complex contains two isoforms of MBD3 which directly interact with several subunits in the complex with the exception of Mi-2 (225). Neither MBD3 nor NuRD could bind to methylated DNA. However, the NuRD complex can be targeted to remodel and deacetylate nucleosomes containing methylated DNA via its interaction with MBD2, a homologue of MBD3 (225). In addition, NuRD complex is involved in several other repression phenomena in cells. For example, the lymphoid lineage-determining factors Ikaros and Aiolos recruit NuRD complex to regions of heterochromatin upon T cell activation (93). SATB1 (special AT-rich sequence binding 1), which is a protein found predominantly in thymocytes and important for T-cell development, can target NuRD complex to the IL-2Rα (interleukin-2 receptor α) gene transcribed in SATB1 null thymocytes (213). SATB1 recruits NuRD complex to a SATB1-bound site in the IL-2Rα locus, and mediates the specific deacetylation of histones in a large domain within the locus (213). These results suggest that NuRD complex may be involved in silencing of large chromosomal domains by a mechanism that requires its chromatin remodeling

activity. The transcription corepressor Kap-1 also targets NuRD to specific promoters to repress gene expression (165).

CoREST complex. This complex (81, 215) contains HDAC1 and HDAC2, but not RbAp48 and RbAp46. It also contains a protein termed CoREST, which was originally identified as a corepressor to the transcription factor REST (6). CoREST binds to HDAC1/2 through its SANT domain that is similar to that of MTA2. Besides CoREST, HDAC1 and HDAC2, this complex contains four additional polypeptides: p40, a Sox-like protein; p110b, with homology to polyamine oxidases; p110a, an eight-zinc finger protein ZNF217; and p80, a hypothetical protein of unknown function.

N-CoR/SMRT-HDAC3 complex. HDAC3 appears to be functionally distinct from HDAC1 and HDAC2. Biochemical studies have defined that HDAC3 is a subunit of stable complexes containing nuclear receptor corepressors SMRT/N-CoR and TBL1 (transducin β-like protein 1) (67, 108). These complexes also contain GPS2 (G protein pathway suppressor 2) and a TBL1-related protein TBLR1 (222). TBL1 has six WD40 repeats and possesses intrinsic repression activity (67). GPS2 and TBL1 interact cooperatively with repression domain 1 of N-CoR to form a heterotrimeric structure and stabilize the complex (222). SMRT/N-CoR not only serves as a platform for complex formation but also functions as a coactivator for HDAC3 enzymatic activity (66, 202, 222). The deacetylase activating domain (DAD) of SMRT/N-CoR contains an essential SANT domain and is sufficient for HDAC3 interaction and activation (67, 222). The HDAC3-containing SMRT and N-CoR complexes are involved in the

repression of transcription by unliganded thyroid hormone receptors (TRs) (108). Through the association with GPS2 subunit, N-CoR-HDAC3 complex inhibits JNK activation (222).

Although Class I HDACs are generally found in these stable protein complexes, many other proteins interact with HDACs either directly or through yet unidentified proteins. For example, the transcription factor YY1 directly interacts with HDAC2 to repress transcrtiption (208). The cell cycle G1 checkpoint controller Rb can recruit HDAC1 to E2F and cooperates with HDAC1 to repress gene expression (23, 119, 121).

Regulation of class I HDACs. The enzymatic activities of HDACs have been shown to be regulated by post-translational modification, especially phosphorylation. Casein kinase 2 (CK2) can phosphorylate HDAC1 and HDAC2 on S421 and S423 *in vitro* (151, 183). This phosphorylation seems to promote their enzymatic activities and complex formation. HDAC1 activity is also regulated by sumoylation (36). HDAC1 was identified as a substrate for SUMO-1. K444 and K476 of HDAC1 are sumoylated *in vivo*. Mutation of these lysine residues to arginine profoundly reduces HDAC1-mediated transcriptional repression in reporter assays, indicating that sumoylation potentiates its deacetylase activity. HDAC3 appears to shuttle between the nucleus and the cytoplasm (177, 209). HDAC3 requires the nuclear receptor corepressor SMRT for its enzymatic activity (66, 202, 222). The formation of SMRT-HDAC3 complex needs a preceding energy-requiring step, involving the TCP-1 ring complex (TRiC) (68), an ATP-dependent protein folding machine (46). HDAC3 forms a

complex in the cytoplasm with Hsc70-TRiC in an ATP-dependent manner. The primed enzyme then translocates into the nucleus. Upon SMRT binding, HDAC3 dissociates from TRiC to form a stable, enzymatically active deacetylase complex.

3.2.2 Class II HDACs

This class was identified based on sequence homology to yeast HDA1 and currently include HDAC4, 5, 6, 7, 9 and 10 (51, 61, 64, 88, 90, 129, 181, 190, 194, 229) (Figure 3). Compared to class I HDACs, (~50 kDa), class II HDACs are larger (about 120 kDa). Besides their deacetylase domains, HDAC4, 5, 7, and 9 show similarity to their extended N-terminal domains and small C-terminal tails (17). The HDAC9 gene is differentially spliced to encode multiple isoforms (150). One of them is called MITR (MEF2 interacting transcriptional repressor) or HDRP (HDAC-related protein), which lacks the catalytic domain of HDAC9 (170, 230). As a unique member of this family, HDAC6 contains two homologous deacetylase domains and an HUB domain at the carboxyl terminus (17). Unlike mouse HDAC6, human HDAC6 contains an SE14-repeat domain (190), indicating that this domain may have an important function for human HDAC6. The deacetylase domain of HDAC10 is more similar to those of HDAC6 (identity ~52%) than to those of HDAC4, 5, 7, and 9 (identity ~37%) (64, 89, 181). HDAC10 also contains a leucine-rich C-terminal domain. According to the sequence similarity, class II HDACs can be further divided into two subclasses: IIa (HDAC4, 5, 7 and 9) and IIb (HDAC6 and 10) (Figure 3).

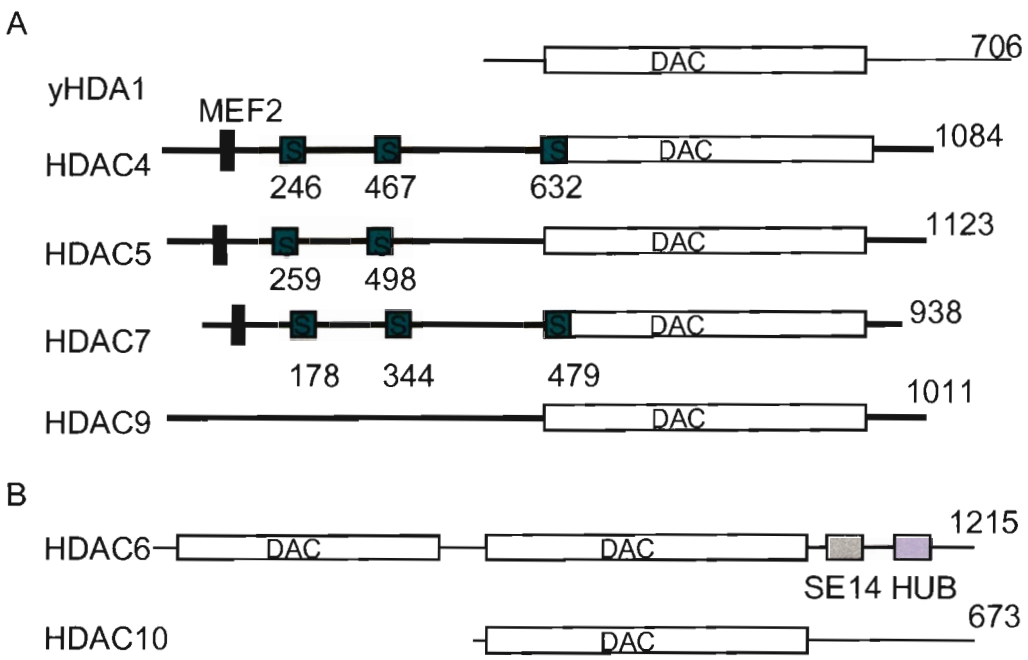

Figure 3 Class II HDACs

(A) Schematic representation of Hda1, HDAC4, 5, 7, and 9. Deacetylase domains are boxed and labeled with DAC. MEF2-binding motifs are depicted as solid small boxes. 14-3-3 binding sites are indicated with green boxes labeled with S.

(B) Schematic representation of HDAC6 and 10. The deacetylase domains are illustrated as in (A). SE14, SE-containing tetradecapeptide repeats; HUB, a zinc finger similar to those of USP3 and BRAP2.

All class II HDACs have been shown to have deacetylase activity. HDAC4, 5, and 6 deacetylate all four core histones equally, and both catalytic domains of HDAC6 are fully functional (61). HDAC9 has been shown to utilize histones H3 and H4 as substrates *in vitro* and *in vivo* (150).

While class I HDACs are ubiquitously expressed, class II HDACs display tissue-specific expression in humans and mice. HDAC4 is expressed in different human tissues: abundant in skeletal muscle, brain, and heart tissues, but very low in lung, liver, and placenta (51, 61, 194). Human HDAC5 has an expression pattern similar to that of HDAC4 (61). Mouse HDAC7 is only expressed in heart, lung, and skeletal muscle tissues (88). Similar to HDAC4, human HDAC9 has the highest expression levels in heart, brain, and skeletal muscle tissues (229). Human HDAC6 has the highest expression levels in heart, liver, kidney and pancreas, while mouse HDAC6 is highly expressed in testis (61). HDAC10 is much more widely expressed in adult human tissues, most abundant in liver, kidney, pancreas, and spleen (89, 181). These observations suggest that these deacetylases are not functionally redundant in vivo, but rather play distinct physiological roles.

Class II HDACs have different subcellular localization. Flag-HDAC4 and GFP-HDAC4 fusion proteins were found mainly in the cytoplasm in most cell lines (62, 129, 195). HDAC5 and HDAC7 are predominantly in the nucleus (62, 88, 126). Various isoforms of HDAC9 show different localization, with some in the nucleus, some in the cytoplasm (150). In the nucleus, HDAC5 and HDAC7 have been found to form a dot-like nuclear structure called MAD (matrix-associated

deacetylase) body (44). The formation and integrity of the MAD body, which appears to contain both class I and class II HDACs along with nuclear receptor corepressor SMRT/N-CoR, is dependent on deacetylase activity. While HDAC6 is mainly cytoplasmic (189), HDAC10 is localized to the nucleus and the cytoplasm (64, 89, 181).

3.2.2.1 Role of class II HDACs in transcription

Similar to class I HDACs, class II HDACs also function as transcriptional corepressors when tethered to promoters. In addition to their carboxy-terminal deacetylase domains, class II HDACs contain amino-terminal repression domains which are not dependent on deacetylase activities (88, 107, 129, 194). Several transcription factors and corepressors have been shown to recruit class II HDACs to repress transcription.

MEF2 (myocyte enhancer-binding factor 2). The four mammalian MEF2 proteins, MEF2A, MEF2B, MEF2C, and MEF2D, belong to the MADS (MCM1, Agamous, Deficiens, Serum response factor) box superfamily of DNA-binding transcription factors and bind A/T-rich sequences associated with muscle genes (19). MEF2s lack myogenic activity alone but cooperatively increase the activity of myogenic bHLH transcription factors like MyoD. Besides their established roles in myogenesis, MEF2 factors have been implicated in activation of non-muscle genes. MEF2 proteins are involved in the serum-dependent regulation of the *c-jun* promoter (69). MEF2D has been shown to mediate calcium-dependent transcription of *Nur77*, a key transcription factor involved in T

cell receptor (TCR)-mediated apoptosis of thymocytes (204). A yeast two-hybrid screen using *Xenopus* MEF2D as bait identified the *Xenopus* homologue of MITR/HDRP (170). Subsequently, HDAC4, 5, 7, and 9 were all shown to interact directly with MEF2 transcription factors (MEF2A, MEF2C, and MEF2D) and repress MEF2-dependent transcription (45, 90, 107, 116, 129, 194, 229), suggesting that the MEF2-interaction domain is conserved among these HDACs. Indeed, the interaction domain has been mapped to a very small region containing only ~20 residues, and point mutation analyses indicate several conserved residues are important for the binding of MEF2 (196). The region of MEF2 involved in interaction with HDACs is localized to the carboxy-terminal subregion of the MADS box and MEF2-specific domain, and interaction of MEF2 with class II HDACs does not interfere with binding of MEF2 to DNA (117). Calmodulin has been shown to bind to HDAC4 in the MEF2-binding region and compete for HDAC4 against MEF2 (216). Furthermore, interaction between MEF2C and HDAC5 is significantly diminished in the presence of activated CaMK (calcium/calmodulin-dependent kinase), and activated CaMK can restore transcriptional activity of MEF2C in the presence of HDAC5 in reporter gene assays (116). These results indicate that the transcriptional repression of MEF2 by class II HDAC is regulated by CaMK signaling pathway.

SMRT and N-CoR (nuclear receptor corepressor). These two related proteins function as corepressors not only for nuclear hormone receptors, but also for multiple classes of transcription factors including ETO, Pit-1, PLZF, BCL6/LAZ3, MyoD, and CBF-1/RBP-JK (28). SMRT and N-CoR are large

proteins (~270 kDa) containing three repression domains. SMRT and N-CoR interact indirectly with HDAC1 and HDAC2 by association with mSin3 via their repression domain 1 (3, 73, 135). These two nuclear receptor corepressors are subunits of multiple protein complexes which contain HDAC3 (67, 108, 202, 222), and interact with HDAC3 through their repression domains 2. A search for interacting partners for repression domains 3 of SMRT and N-CoR led to the isolation of class II HDACs including HDAC4, 5, and 7 (78, 88). The carboxy-terminal deacetylase domains of HDAC4, 5, and 7 are responsible for the interaction and deacetylase activities appear to be required for the interaction because point mutations in this domain that abolish the enzymatic activity of the class II HDACs can not bind to SMRT or N-CoR (44, 78).

CtBP (C-terminal binding protein). The transcriptional corepressor CtBP is a 48 kDa cellular phosphoprotein that binds to the C-terminal region of the human adenovirus E1A proteins via a conserved PxDLS-like motif, where x represents any amino acid (162). CtBP has been found to interact with different transcription factors, such as BKLF, ZEB, FOG, Pc2, BRCA-1, and E2F4/5, indicating that CtBP plays an important role in development and oncogenesis (35). CtBP has been reported to be associated with HDAC1/2 and Sin3 complex (176). Class II HDACs were found to interact with CtBP in a yeast two-hybrid screening using MITR/HDRP as bait (218). HDAC4, 5, 7, and 9 all interact directly with CtBP through the PxDLS motif located within their N-termini. However, it remains unclear whether HDAC activity is required for CtBP-dependent repression.

BCL-6 (B cell lymphomas 6) and BCoR (BCL-6 corepressor). The *BCL-6* gene is involved in chromosomal translocations associated with non-Hodgkin's lymphoma (NHL) (92, 214). BCL-6 is a transcriptional repressor that is required for germinal center formation and may influence apoptosis. It belongs to a subclass of zinc finger proteins, including PLZF, HIC-1, and APM-1, each of which contains a POZ domain (also called BTB or ZIN domain) at the amino terminus and C_2H_2 zinc fingers at the carboxyl terminus. The POZ domain and the zinc finger region of BCL-6 can mediate repression independently (31, 106, 168). The BCL-6 POZ domain interacts with N-CoR and SMRT and recruits Sin3-HDAC1/2 to promoters (41, 42). Another corepressor BCoR also interacts specifically with the POZ domain of BCL-6 and can potentiate the transcriptional repression by BCL-6 (82). Moreover, HDAC1, 3, 4 and 5 can associate with BCoR (82), suggesting that deacetylation is a mechanism for BCoR-mediated repression. Recently, HDAC4, 5, and 7 have been shown to interact directly with the C-terminal zinc finger domain of BCL-6 (106). The minimal BCL-6-interacting domain was defined in the most conserved region in the N-terminal domain of HDAC4, 5, and 7. This region is also present in HDAC9 and MITR/HDRP, suggesting that these proteins probably also interact with BCL-6. Therefore, class II HDACs may modulate the function of BCL-6 by both direct and indirect recruitment to BCL-6.

HP1 (heterochromatin protein 1). Originally identified as a heterochromatin-associated protein in *Drosophila*, HP1 is a highly conserved protein involved in gene regulation, DNA replication, and nuclear assembly (47).

It contains two conserved domains, the amino-terminal chromo domain and the carboxy-terminal chromo shadow domain separated by a less conserved hinge region. HP1 regulates gene expression at both heterochromatic and euchromatic domains. It has been identified as a partner for several transcription factors, such as Rb, TIF1β/KAP-1, TAFII130, and Dnmt3, which are involved in control of gene repression (109). Recent studies have shown that the chromodomain of HP1 specifically recognizes the methylated lysine 9 of histone H3 and represses transcription (11, 99). In yeast two-hybrid screens, HP1 was identified as an interacting protein of MITR/HDRP, HDAC4 and HDAC5 (219). HP1 and Suv39 repress MEF2-dependent transcription by interacting with HDAC4/5. MEF2 may recruit HP1 via HDAC4, HDAC5 or MITR/HDRP to promoters. Furthermore, during muscle differentiation, methylation of histone H3-K9 is decreased around a MEF2 responsive element in the myogenin gene promoter, suggesting that histone methylation may be involved in control of muscle differentiation.

3.2.2.2 Nucleocytoplasmic trafficking

As indicated above, class II members display different subcellular localization. While HDAC4 is found in either the nucleus or the cytoplasm, HDAC5 and HDAC7 are predominantly nuclear proteins. On the other hand, HDAC6 and HDAC10 are localized to both the nucleus and cytoplasm. Moreover, cytoplasmic HDAC4 relocates to the nucleus when cells are treated with leptomycin B (LMB), an inhibitor of the nuclear export receptor CRM1 (129). Nuclear HDAC5 and HDAC7 relocate to the cytoplasm during muscle

differentiation (45, 125). Together, these observations suggest that the subcellular localization of class II HDACs is controlled by active nuclear import and export. Indeed, HDAC4 possesses an intrinsic nuclear localization signal (NLS) and a nuclear export signal (NES), which are conserved among other class IIa HDACs, for its dynamic nucleocytoplasmic shuttling (196). The NLS of HDAC4 resides between residues 244 and 279, a region which contain three R/K-rich clusters. Mutational analysis of the HDAC4 NLS showed that the three R/K clusters are all necessary for its nuclear import activity. While the nuclear export receptor CRM1 usually recognizes a leucine-rich consensus sequence: LxxxLxxLxL (137), the leucine-rich sequences of HDAC4 do not have nuclear export activity. Instead, a hydrophobic motif (MxxLxVxV) located at the carboxy-terminal end functions as an NES for HDAC4 (196). This motif is sensitive to LMB treatment and can be recognized by CRM1, although it is slightly different from most leucine-rich export signals identified in other proteins (137). The NES of HDAC5, defined as VxxxxxLxV, is largely dependent on the CaMK signaling pathway (127). HDAC5 translocates to the cytoplasm in the presence of activated CaMK, whereas deletion and point mutants, in which the NES is deleted or impaired, remain nuclear in the presence of CaMK (127).

However, the intrinsic nuclear export activity alone is not sufficient for the nucleocytoplasmic trafficking of class IIa HDACs. Another regulatory mechanism involves 14-3-3 proteins, which bind to phospho-serine or phospho-threonine consensus motifs and play an important role in regulation of signal transduction, apoptotic, checkpoint control, and nutrient-sensing pathways (53). They often

alter the subcellular localization of their binding partners. 14-3-3 proteins have been shown to promote the cytoplasmic localization of many binding partners, including the pro-apoptotic protein BAD and the cell cycle regulatory phosphatase Cdc25C (53). HDAC4 associates with 14-3-3 proteins in mammalian cells (62, 195). In a yeast two-hybrid screen, the ε, η, τ, σ, and ξ isoforms of 14-3-3 have been identified using the amino-terminal region of HDAC4, but not that of HDAC5 (126). There are 5 putative 14-3-3 binding sites on HDAC4, but only three of them, Ser246, Ser467, and Ser632 are needed for binding of 14-3-3 proteins (195). These 14-3-3 binding sites are also conserved on HDAC5, HDAC7, and HDAC9. Actually, similar sites have been demonstrated for 14-3-3 binding: Ser259 and Ser498 of HDAC5; Ser178, Ser344 and Ser479 of HDAC7; Ser218 and Ser448 of MITR/HDRP, an alternatively spliced isoform of HDAC9 (90, 126, 220).

The 14-3-3 binding is dependent on the phosphorylation of class II HDACs and regulates their subcellular localization. Mutants defective in 14-3-3 binding are predominantly nuclear (62, 195). There is a decrease in 14-3-3 binding and an accumulation of HDAC4 in the nucleus after cells are treated with staurosporin, a protein kinase inhibitor, whereas an increase of 14-3-3 binding and cytoplasmic accumulation of HDAC4 are observed after cells are treated with calyculin A, a phosphatase inhibitor (62). Calcium/calmodulin-dependent protein kinases (CaMK) I and IV phosphorylate HDAC5 on Ser259 and Ser498 and thereby promote the 14-3-3 binding (125, 126). HDAC4, HDAC7 and MITR/HDRP all respond to CaMK signaling. Even though they are highly related,

class II HDACs display different subcellular localization, suggesting that they might be differentially regulated by 14-3-3 proteins. Several lines of evidence suggest that HDAC4 binds constitutively to 14-3-3 in yeast and mammalian cells, whereas HDAC5 binding to 14-3-3 is largely dependent on CaMK signaling. For example, HDAC4, but not HDAC5, interacts with 14-3-3 in yeast two-hybrid screening (126). Both HDAC4 and 14-3-3 are exclusively localized in the cytoplasm when they are co-overexpressed in the cell. While HDAC5 remains in the nucleus when it is co-overexpressed with 14-3-3, both HDAC5 and 14-3-3 are localized in the cytoplasm in the presence of CaMK I or IV (126). Therefore, phosphorylation may differentially control the subcellular localization of class II HDACs. The extracellular signal-regulated kinases 1 and 2 (ERK1/2) associate with HDAC4, and ERK2 phosphorylates HDAC4 *in vitro* (231). Moreover, oncogenic Ras or constitutively active MEK1, which activate ERK1/2, result in an increase in nuclear localization of HDAC4 in C2C12 myoblast cells.

Mechanistically, 14-3-3 binding inhibits the nuclear localization of HDAC4 by affecting its nuclear import and/or activation of its nuclear export. The NLS of HDAC4 is only two residues away from its S246 14-3-3 binding site (196), suggesting that 14-3-3 binding may mask the NLS and thereby inhibit its nuclear targeting activity. In agreement with this, 14-3-3 binding has been found to block the association of importin α with HDAC4 (62). 14-3-3 binding may also stimulate the nuclear export of class IIa HDACs. Since each 14-3-3 protein contains an NES (154), binding of dimeric 14-3-3 proteins to HDACs may provide active NES *in trans*. An HDAC4 mutant defective in 14-3-3 binding but containing the NES is

no longer localized in the cytoplasm (195). However, an HDAC4 mutant lacking its NES is able to bind to 14-3-3 but still resides in the nuclear compartment (196). Furthermore, 14-3-3 proteins associate with HDAC5 in the nucleus and stimulate its export to the cytoplasm in the presence of CaMK (126). MITR/HDRP does not contain the carboxy-terminal deacetylase domain and NES and is thus nuclear, although it binds to 14-3-3 in the presence of CaMK (220). These results suggest that 14-3-3 binding is necessary but not sufficient for the cytoplasmic localization of class IIa HDACs and that 14-3-3 binding sites and the NES are both required for redistribution of class IIa HDACs from the nucleus to the cytoplasm.

Besides 14-3-3 proteins, other proteins also contribute to controlling the subcellular localization of class II HDACs. Overexpression of MEF2 results in accumulation of HDAC4 in the nucleus (129, 196). MEF2 is able to direct an HDAC4 mutant lacking the NLS to the nucleus (196). Mutational analysis of the MEF2-binding site on HDAC4 further supports that direct binding of MEF2 promotes nuclear import of HDAC4 (196). Moreover, this effect is dependent on the NLS of MEF2 proteins since co-expression of a MEF2 NLS deletion mutant enhanced the cytoplasmic localization of HDAC4 (20). The nuclear receptor corepressor SMRT is also capable of directing HDAC4 from the cytoplasm to the nucleus (205). Furthermore, in the nucleus, class I and class II HDACs form a MAD body containing SMRT (44). The formation and integrity of the MAD body is dependent on deacetylase activity (44, 205). Accordingly, SMRT synergizes with class II HDACs to inhibit MEF2 transactivation of target promoters (205).

HDAC6 is localized mainly in the cytoplasm and is also capable of shuttling between the nucleus and the cytoplasm (189). Mouse HDAC6 has been shown to have an NES in its amino-terminus. The localization of this HDAC appears to be controlled by specific cellular signals, since a fraction of endogenous mHDAC6 translocates into the nucleus upon cell differentiation and the arrest of cell proliferation (189).

3.2.2.3 Control of myogenesis by class II HDACs

Differentiation of skeletal muscle cells involves an orchestrated pattern of gene expression, which is coordinated with terminal cell cycle exit. Activation of muscle gene expression by myogenic bHLH proteins, MyoD, myogenin, Myf5, and MRF4, is dependent on their association with MEF2 proteins. MEF2 factors lack myogenic activity alone but cooperatively increase the activity of myogenic bHLH transcription factors (19). Loss-of-function mutations in the single *Drosophila mef2* gene prevent myoblast differentiation (22, 110), and dominant-negative MEF2 mutants inhibit myoblast differentiation in cell culture (148), indicating an important role of MEF2 proteins in terminal muscle differentiation.

Control of myogenesis by class II HDACs is closely linked to their role in regulating the activity of transcription factor MEF2. It has been demonstrated that overexpression of HDAC4 and 5 inhibits C2 skeletal muscle cell differentiation, and also efficiently blocks MyoD-dependent conversion of fibroblasts into muscle through association with MEF2 (117). Both MEF2-binding and deacetylase domains of HDAC4 are required for inhibition of myotube formation (117, 130).

Repression of muscle differentiation by class II HDACs can be overcome by increasing the ratio of MyoD to HDAC (117). Moreover, CaMK signaling overcomes the inhibitory activity of HDAC4 and 5 on MyoD by preventing association of HDACs with MEF2 (116, 117). Signal-dependent nucleocytoplasmic trafficking of class II HDACs appears to play a key role in the control of myogenesis. HDAC5 and HDAC7 are localized in the nucleus of the myoblast and exported to the cytoplasm at the onset of differentiation (45, 125). Activated CaMK stimulates myogenesis and promotes the nuclear export of these HDACs (125). Phosphorylation of two conserved serines in HDAC5 by activated CaMK creates docking sites for 14-3-3, which disrupts HDAC5-MEF2 complexes (62, 125, 195). Binding of 14-3-3 masks the NLS (62) and activates the NES of HDAC5 (127, 196). As a result, HDAC5/14-3-3 complexes are exported from the nucleus to the cytoplasm, leaving MEF2 in the nucleus to recruit HATs and cooperate with MyoD to activate the expression of myogenic genes. However, HDAC4 is different from HDAC5 in its subcellular localization and responsiveness to myogenic signals. Endogenous HDAC4 in cycling C2C12 cells is found mainly in the cytoplasm, and is imported to the nucleus upon differentiation (130, 228). In contrast to HDAC5, the interaction of 14-3-3 and HDAC4 appears to be independent of CaMKIV, as co-expression of CaMKIV does not increase HDAC4 binding to 14-3-3 (228). The reason why HDAC4 translocates to the nucleus upon terminal differentiation is still unclear, and the significance of the difference between HDAC4 and HDAC5 needs to be investigated. Conceivably, the reciprocal localization of HDAC4 and HDAC5

during myoblast differentiation might suggest that HDAC4 and HDAC5 regulate a distinct subset of MEF2-dependent genes in the nucleus during certain stages of myogenesis to facilitate terminal differentiation.

Recently, it has been shown that a cardiac HDAC kinase other than CaMK activated by diverse hypertrophic signals phosphorylates the 14-3-3 binding sites that inactivate class IIa HDACs (217). HDAC mutants which lack 14-3-3 binding sites inhibit fetal gene expression and are resistant to hypertrophy stimuli. Furthermore, HDAC9 null mice are sensitive to stress signals that induce hypertrophy, suggesting that class II HDACs repress cardiac hypertrophy.

3.2.2.4 Tubulin deacetylation by HDAC6

Reversible acetylation of α-tubulin is important in regulating microtubule stability and function. Acetylated microtubules represent a more stable microtubule population. HDAC6 has been demonstrated to be responsible for the deacetylation of α-tubulin in cells (80, 124). HDAC6 co-localizes with microtubules at the leading edge, a highly dynamic structure devoid of stable microtubules and involved in cell motility. Overexpression of HDAC6, which deacetylates acetylated α-tubulin and therefore destabilizes microtubules, increases the chemotactic movement in NIH3T3 cells. Cells overexpressing catalytically inactive HDAC6 mutants show motility similar to that of control cells, further suggesting that HDAC6-mediated deacetylation regulates microtubule-dependent cell motility.

HDAC6 is a cytoplasmic protein in rapidly dividing cells and very abundant in testis tissue (167, 189). An HDAC6-containing complex, which contains two other proteins p97/VCP/Cdc48p and PLAP, has been purified from mouse testis cytoplasmic extracts (167). p97 and PLAP show striking sequence homology to yeast proteins involved in ubiquitin-dependent protein degradation, suggesting that HDAC6 may play a role in protein ubiquitination. HDAC6 itself possesses a ubiquitin carboxyl-terminal hydrolase-like zinc finger (ZnF-HUB) domain in its C-terminal region (17). It has been shown that HDAC6 binds to polyubiquitin through its zinc finger domain (75, 167). Interestingly, binding of ubiquitin to HDAC6 leads to dissociation of the mHDAC6 complex and release of p97 (167). Moreover, HDAC6 has been shown to associate with deubiquitinating enzyme activity (75). These results suggest that HDAC6 may provide a link between acetylation and ubiquitination.

3.2.3 Class III HDACs

This class contains SIRT1, 2, 3, 4, 5, 6, and 7, all of which show sequence homology to the yeast transcriptional repressor Sir2. There are four Sir2 homologs in *S. cerevisiae*, Hst1-4 (homolog of Sir two). The Sir2-like deacetylase domain contains a conserved 275 amino acids in size is unrelated to those of class I and class II HDACs. These sirtuins (Sir2-like proteins) deacetylate core histones in an NAD^+-dependent manner (84, 102, 169). The best-characterized sirtuin is yeast Sir2. It is involved in transcriptional silencing at telomeres, mating-type loci and ribosomal DNA loci (54). In addition, it has been linked to aging,

possibly by its NAD^+ dependence and suppression of rDNA recombination (65). Recently, *Drosophila* Sir2 (dSir2) has been shown to be involved in centromeric heterochromatic silencing, but apparently not telomeric silencing (158). Moreover, dSir2 interacts genetically and physically with the Hairy/E(Spl) bHLH repressors (158). The cellular function of mammalian sirtuins is poorly studied. SIRT1 is the closest homolog of yeast Sir2 and both proteins are localized to the nucleus. It has been shown that SIRT1 and mouse Sir2α interacts with p53 and deacetylates p53 at lysine 382 which is known to be acetylated by p300 and CBP (103, 118, 188). Consequently, this deacetylation attenuates the transcriptional activity of p53. Furthermore, hSir2 represses p53-dependent apoptosis in response to DNA damage and oxidative stress (118, 188) and antagonizes PML/p53-mediated premature cellular senescence (103).

4 Histone acetylation and cancer

Altered chromatin structure has been found to be associated with inappropriate expression of genes in cancer cells and aberrant acetylation of histone tails has been linked to carcinogenesis (86). Genetic abnormalities can cause improper targeting of HATs or HDACs to certain loci, functional inactivation of HATs, or overexpression of HDACs, which lead to tumor onset and progression (95).

Several lines of evidence suggest that HAT functions are linked to cancer. Translocation, amplification, overexpression and mutation in various cancers are found in genes that encode HATs. Mutations in the CBP gene that inactivate its

HAT activity are associated with leukemogenesis and the developmental disorder Rubinstein-Taybi syndrome, a condition that predisposes patients to cancer (132). Several translocations involving HATs are observed in leukemia. For example, a MOZ-CBP fusion resulting from a translocation between MOZ and CBP genes was reported in acute myeloid leukemia (AML) patients (21). The resulting hybrid protein retains the HAT domains from both proteins and has been shown to inhibit AML1-dependent transcription and differentiation of M1 cells into monocytes/macrophages in response to interleukin-6 (IL-6) (94). Fusions between MLL and CBP, MLL and p300, MORF and CBP, or MOZ and TIF2 have also been associated with AML (180). In addition, the interaction of the oncogenic adenovirus protein E1A with p300/CBP or PCAF can antagonize the expression of cellular genes that are normally activated by p300 and CBP (211). CBP also directly influence the activity of p53 tumor suppressor, because acetylation of p53 is required for the recruitment of co-activators/HATs and histone acetylation (12).

Alterations in the HDAC genes have not been discoverd in human cancers, but HDACs associate with several well-characterized onco-proteins and tumor suppressors. For example, c-Ski is a component of the HDAC-NCoR-mSin3 complex and is required for the Mad-mediated repression (145). The oncogenic v-Ski, which lacks the mSin3 interaction domain, is capable of transforming cells by abrogating Mad-induced repression in a dominant-negative fashion. Thus, the involvement of c-Ski in the HDAC complex indicates that the function of the HDAC complex is important for oncogenesis. The tumor suppressor Rb recruits

HDAC1 to E2F and cooperates with HDAC1 to repress gene expression (24, 119, 121). Given that deletions and mutations in the Rb gene are common in many cancers, HDAC1 is important for the Rb/E2F cell cycle regulatory pathway. The class III human HDAC SIRT1 and its mouse homolog Sir2α deacetylate p53 and consequently reduce its transcriptional activity, leading to a decrease of p53-dependent apoptosis in response to DNA damage and oxidative stress (118, 188). These findings suggests that overexpression of SIRT1 may contribute to the inactivation of tumor suppressor p53 and thus oncogenesis.

Acute promyelocytic leukemia (APL) is the best example to show the link between altered HDAC activity and tumorigenesis. The oncoproteins PML-RARα and PLZF-RARα encoded by the translocation-generated fusion gene in APL recruit theHDAC1 complex with high affinity to RAR-regulated genes, causing constitutive repression (59, 111). In the normal condition, RARα activates target genes in response to retinoic acid. However, in the PML-RARα and PLZF-RARα fusion proteins, RARα is no longer responsive to retinoic acid and becomes a constitutive transcriptional repressor that blocks normal differentiation of promyelocytes, leading to APL. HDAC-dependent aberrant transcriptional repression is also implicated in lymphoma and some types of acute myeloid leukemia (AML). In non-Hodgkin's lymphoma, the transcriptional repressor BCL6, which directly or indirectly recruits both class I and class II HDACs to repress transcription (41, 42, 82, 106), is overexpressed, leading to lymphoid oncogenic transformation. In AML, the fusion protein AML1-ETO converts AML1, a

transcriptional activator required for myeloid differentiation, to a transcriptional repressor by HDAC recruitment (199).

Histone deacetylase activity can be inhibited by a group of small compounds, termed HDAC inhibitors. By X-ray crystallographic studies, HDAC inhibitors TSA and SAHA have been shown to interact with the catalytic sites of HDACs, thereby blocking substrate access to active zinc ion at its base (50). Given that inappropriate transcriptional repression mediated by HDACs is a common molecular mechanism used by oncoproteins, HDAC inhibitors could be an exciting therapeutic approach to cancer. In the past fewer years, structurally diverse HDAC inhibitors have been identified that inhibit proliferation and induce differentiation and/or apoptosis of tumor cells in cell culture or animal models (123). More importantly, some of these inhibitors are currently in clinical trials with cancer patients (123).

5 Issues addressed in this thesis

Identification of a new class of HDAC and characterization of the function and regulatory modes of HDAC4 will be investigated in this thesis. Interaction studies and reporter gene assays will demonstrate that HDAC4 function as a transcription corepressor recruited to the promoters by transcription factor MEF2 or RFXANK. Green fluorescence microscopy and indirect immunofluorescence microscopy reveal that HDAC4 is mainly localized in the cytoplasm and subject to nucleocytoplasmic shuttling. Furthermore, investigation of regulation of

HDAC4 subcellular localization shows that shuttling of HDAC4 is controlled by multiple mechanisms involving 14-3-3 binding and the NLS/NES of HDAC4.

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CHAPTER II

HDAC4, a Human Histone Deacetylase Related to Yeast HDA1, Is a Transcriptional Corepressor

PREFACE

The most extensively studied form of posttranslational modifications of histones is acetylation that is conducted by histone acetyltransferase and histone deacetylase (HDAC) enzymes. The first histone deacetylase (HDAC1) was cloned from mammalian cells in 1996 and found to be highly homologous to the known yeast transcriptional coregulator Rpd3. Transcriptional repressors recruit HDAC1 and its two homologs HDAC2 and HDAC3 to downregulate transcription. The HDAC activities of these enzymes have been found to be important for transcriptional repression, suggesting that histone deacetylation directly leads to transcriptional repression. In yeast, two distinct histone deacetylase complexes have been characterized: one possesses Rpd3 as its catalytic subunit, while the other contains the histone deacetylase Hda1. Hda1 shows some sequence similarity to the catalytic domain of the Rpd3/HDAC1 family. However, its function was entirely unclear. The fact that homologs of yeast Rpd3 have been found in mammals encouraged us and others to identify the mammalian homologs of yeast Hda1. The following manuscript describes the identification and characterization of another class of human HDACs whose catalytic domains are similar to that of yeast Hda1.

ABSTRACT

Histone acetylation plays an important role in regulating chromatin structure and thus gene expression. Here we describe the functional characterization of HDAC4, a human histone deacetylase whose C terminal part displays significant sequence similarity to the deacetylase domain of yeast HDA1. HDAC4 is expressed in various adult human tissues, and its gene is located at chromosome band 2q37. HDAC4 possesses histone deacetylase activity intrinsic to its C terminal domain. When tethered to a promoter, HDAC4 represses transcription through two independent repression domains, with repression domain 1 consisting of the N terminal 208 residues and repression domain 2 containing the deacetylase domain. Through a small region located at its N terminal domain, HDAC4 interacts with the MADS-box transcription factor MEF2C. Furthermore, HDAC4 and MEF2C individually upregulate but together downmodulate *c-Jun* promoter activity. These results suggest that HDAC4 interacts with transcription factors such as MEF2C to negatively regulate gene expression.

INTRODUCTION

In eukaryotic cells, genetic information is packaged into chromatin, a highly organized DNA-protein complex which controls gene activities. A central question in studying eukaryotic gene regulation is how the generally repressive chromatin structure is regulated when necessary. In the past several years, three regulatory mechanisms have been recognized: DNA methylation, post-translational modifications of histones and ATP-dependent chromatin remodeling (53, 55, 57). The most extensively studied form of post-translational modifications of histones is acetylation of ε-amino groups of lysine residues located at the flexible N terminal tails of core histones (53, 55). The level of histone acetylation at a given region of chromatin correlates well with its transcriptional activity (38). Mechanistically, histone acetylation affects nucleosome stability and/or internucleosomal interaction (2, 29). The dynamic level of histone acetylation *in vivo* is maintained through opposing actions of histone acetyltransferases and deacetylases. Several known transcriptional coactivators possess intrinsic histone acetyltransferase activity (14, 27, 49, 57).

The first histone deacetylase, originally called HD1 (histone deacetylase 1) and later renamed HDAC1 (histone deacetylase 1), was cloned from mammalian cells (18, 50). HDAC1 was found to be highly homologous to the known yeast transcriptional coregulator RPD3 (50). Two HDAC1 homologs (HDAC2 and HDAC3) have been cloned from human cDNA libraries (10, 58, 59). Transcriptional repressors recruit RPD3 or HDAC1-3 to downregulate transcription (reviewed in 41, 56). The deacetylase activity of HDAC1 and RPD3

has been found to be important for transcriptional repression (18, 24), suggesting that histone deacetylation directly leads to transcriptional repression. Consistent with this contention, recruitment of RPD3 by the yeast repressor Ume6 leads to local histone deacetylation and formation of a highly localized domain of repressed chromatin *in vivo* (25).

Two distinct yeast histone deacetylase complexes have been characterized: one possesses RPD3 as its catalytic subunit while the other contains the histone deacetylase HDA1 (6, 43). The N terminal domain of HDA1 shows some sequence similarity to the catalytic domain of the RPD3/HDAC family (amino acid sequence identity, 26%; similarity, 49%), whereas its C terminal domain exhibits no sequence similarity to known proteins. A great deal of knowledge has been acquired about the function of the RPD3/HDAC family of histone deacetylases in transcriptional regulation (14, 27, 49, 57). In contrast, it is entirely unclear if and how HDA1 plays a role in transcriptional regulation.

In vertebrates, the MEF2 family of transcription factors, also called RSRFs (related to serum response factors), is composed of 4 isoforms, MEF2A, -B, -C and -D, all of which contain MADS-box DNA-binding domains at their N termini and adjacent MEF2-specific motifs (4, 36, 42). Although MEF2s were initially identified as myocyte enhancer-binding factors activating muscle-specific genes, their roles in non-muscle cells have also been demonstrated (7, 15, 16, 26, 44, 63). In non-muscle cells, MEF2s serve as nuclear targets of several signaling pathways (7, 9, 15, 26, 63). Moreover, it has been suggested that MEF2s are

involved in negative transcriptional regulation (40). How this occurs remains largely unexplored.

Here we report that HDAC4, a human histone deacetylase whose C terminal region is highly related to HDA1, physically and functionally interacts with the transcription factor MEF2C: through the N terminal domain of HDAC4, MEF2C recruits HDAC4 to repress transcription. Furthermore, MEF2C and HDAC4 individually upregulate but together downmodulate *c-Jun* promoter activity. These results suggest that like RPD3 and HDAC1-3, HDAC4 is recruited to promoters by target transcription factors to regulate transcription.

MATERIALS AND METHODS

Molecular cloning. Plasmid construction and DNA sequencing were performed following standard procedures. The cDNA clone KIAA0288 (GenBank accession No. AB006626) was kindly provided by T. Nagase (Kazusa DNA Research Institute, Chiba, Japan). This clone was used to construct expression plasmids for HDAC4 and its mutants except that the coding sequence for its N terminal 221 residues was obtained from a human bone marrow cDNA library (the KIAA0288 clone contains a C-to-T nonsense mutation at nucleotide 1135, kindly notified by T. Nagase). This mutation has also been identified by Grozinger et al. (13). The partial clone for HDAC7 was amplified from a human brain cDNA library by PCR with primers based on the sequences of 4 human BAC clones (GenBank accession No. AC002124, AC002088, AC002410 and AC002433). Northern analyses on polyA-RNA blots (Clontech) were carried out according to the manufacturer's instructions. The reporter tk-Luc was derived from pGL2 (Promega) by insertion of the thymidine kinase (tk) core promoter (-105/+52). Gal4-tk-Luc was constructed from tk-Luc by insertion of 5 copies of the Gal4-binding site upstream from the tk promoter. Gal4-SV40-Luc was constructed from pGL2-Control (Promega) by insertion of the Gal4-binding sites from Gal4-tk-Luc. Gal4-AdML-Luc and Gal4-CD4-Luc have been described elsewhere (34, 62). MEF2-E4-Luc was derived from the 3TP-Lux luciferase reporter (19), by replacement of its TRE region with an oligo duplex consisting of 5'-CTA GCT GGG CTA TTT TTA GG-3' and 5'-GAT CCC TAA AAA TAG CCC AG-3', where the MEF2-binding sites are underlined.

Fluorescence in situ hybridization (FISH). FISH was performed on human lymphocytes as described (21). The probe was a 5.5 kb HDAC4 cDNA fragment biotinylated with dATP using the BioNick labeling kit (Gibco).

Protein expression and purification. For expression in 293T cells, 10 μg of plasmid expressing HDAC4 or its mutants were used to transfect 1~1.5 x 10^6 cells (in a 10 cm dish) with 24 μl of SuperFect transfection reagent (Qiagen). After 48 hrs, cells were washed twice with PBS and collected in 1 ml of buffer B (20 mM Tris-HCl, pH 8.0, 10% glycerol, 5 mM $MgCl_2$, 0.1% NP-40 and protease inhibitors) containing 0.5 M KCl. The same buffer was used for washing M2 agarose beads immobilized with Flag-HDAC4; for elution, the concentration of KCl was reduced to 0.15 M.

For expression of HDAC4 mutants in Sf9 cells, recombinant baculoviruses were generated by the BaculoGold (Pharmingen) or Bac-to-Bac (Gibco) systems. HDAC4 mutants were affinity-purified as described above.

Deacetylase assay. [^{3}H]Acetyl-histones were prepared from HeLa cells. Briefly, after incubation for 2-6 h in media containing 50 μCi/ml [^{3}H] acetate (2.4 Ci/mmol; NEN Life Sciences) and 3 μM trichostatin A (Wako), HeLa cells were harvested and lysed in buffer N (10 mM Tris-HCl pH 8.0, 250 mM sucrose, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 1% Triton X-100, 1 mM PMSF and protease inhibitors), and nuclei were isolated as described (51). To isolate histones, the nuclei were extracted with 0.4 N H_2SO_4, and acid-extracted histones were precipitated with 9 volumes of acetone. After at least one hour on ice, histones were collected by centrifugation; the histone pellet was dissolved in 0.1 ml of 100 mM Tris-HCl pH

8.0 and precipitated with cold acetone 3-4 times. Histones were air-dried and dissolved in 2 mM HCl. Levels of histone acetylation were verified using Triton-acetic acid-urea gels (22).

[^{3}H]Acetyl-histones were also prepared by *in vitro* labeling: 50 μg of histones (Sigma) were incubated with 50 pmoles of [^{3}H]acetyl-CoA (4.7 Ci/mmol; Amersham) and 0.5 μg of Flag-PCAF in 100 μl of buffer A (50 mM Tris-HCl pH 8.0, 10% glycerol, 1 mM DTT, 0.1 mM EDTA and 1 mM PMSF) at 30°C for 30 min. The expression and purification of Flag-PCAF has been described (60). To remove unincorporated [^{3}H]acetyl-CoA, histones were precipitated by adding 2 μl of 5 M NaCl, 1 ml of cold acetone and 65 μg of BSA. The tube was left on dry ice for 2 h, and subsequently centrifuged at 14,000 rpm and 4°C for 5 min. The resulting pellet was washed with 1 ml of cold acetone, air-dried and dissolved in 100 μl of 2 mM HCl.

Deacetylase activity was determined by analysis of the release of [^{3}H]acetate from [^{3}H]acetyl-histones (20, 23). Assays were carried out in 0.2 ml of buffer H (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.1 mM EDTA and 0.1 mM PMSF) containing [^{3}H]acetyl-histones (25,000 dpm). The reaction was allowed to proceed at 37°C for 90 min and stopped by addition of 0.1 ml of 0.1M HCl/0.16M acetic acid. Released [^{3}H]acetate was extracted with 0.9 ml ethyl acetate. After centrifugation, 0.6 ml of the upper organic phase was quantified by liquid scintillation counting.

DNA-binding assay. A modified filter-binding assay was employed (17). Briefly, sheared fish sperm DNA (100 ng, Boehringer Mannheim) was labeled with [α-

^{32}P]-dCTP in a Ready-To-Go DNA labeling reaction tube (Pharmacia) and separated from free [α-^{32}P]-dCTP on a G-25 spin column. Flag-HDAC4 was immobilized on 10 μl of M2-agarose and incubated with 2 ng of [^{32}P]-labeled fish sperm DNA fragments. After extensive washing, bound DNA was quantified by liquid scintillation counting.

Protein-protein interaction. To examine the interaction between HDAC4 and MEF2C *in vivo*, HDAC4 (Flag-tagged) and/or MEF2C expression plasmids were cotransfected into 293T cells, and transfected cells were collected in buffer B/0.15 M KCl as described above. One third of the extract was used for immunoprecipitation with anti-Flag M2 agarose beads (Sigma). Beads with bound immunocomplexes were washed 4 times with buffer B/0.15M KCl and bound proteins were eluted with the Flag peptide or 0.1 M glycine-HCl pH 2.5. After separation by 8% SDS-PAGE, proteins were transferred to nitrocellulose membranes for Western blot analyses with anti-Flag and anti-MEF2C antibodies. Blots were developed with the Supersignal chemiluminescent substrate (Pierce). The same procedure was followed to examine the *in vivo* interaction between HDAC4 and MEF2D except that endogenous MEF2D was detected due to its reasonable expression level in 293T cells.

For *in vitro* MBP pull-down assays, the MEF2C fragment M178 was expressed as a fusion with maltose-binding protein (MBP) in *E. coli*, immobilized on amylose agarose beads and used to study the interaction with HDAC4 and its mutants, which were synthesized *in vitro* with the TNT-T7 coupled reticulocyte lysate system (Promega) in the presence of Redivue L-[^{35}S]methionine

(Amersham). After rotation for 30 min at 4°C, the complexes bound to agarose beads were washed three times with buffer B/0.15 M KCl and once with buffer B/0.5 M KCl, and boiled in 1xSDS sample buffer prior to separation by 8% SDS-PAGE and autoradiography.

Reporter gene assays. SuperFect transfection reagent (Qiagen) was used to transiently transfect a luciferase reporter plasmid (50-200 ng) and/or mammalian expression plasmids (50-200 ng) into NIH3T3 or 293T cells. pBluescript KSII(+) was used to normalize the total amount of plasmids used in each transfection and CMV-β-Gal (50 ng) was cotransfected for normalization of transfection efficiency. After 48 h, cells were lysed *in situ* and luciferase reporter activity was determined using D-(-)-luciferin (Boehringer Mannheim) as the substrate. Galactosidase activity was measured using Galacto-Light Plus™ (Tropix) as the substrate. The chemiluminescence from activated luciferin or Galacto-Light Plus™ was measured on a Luminometer Plate Reader (Dynex). As indicated, transfected cells were exposed to trichostatin A (TSA, 3 μM) for 16 h prior to reporter assays. Each transfection was performed at least 4 times.

RESULTS

A family of human histone deacetylases related to yeast HDA1. To identify new mammalian histone deacetylases, we performed sequence database searches with BLAST and PSI-BLAST (1). Using the amino acid sequence of yeast HDA1 as the bait, we found several human cDNA and genomic clones encoding polypeptides with significant sequence similarity to the catalytic domain of HDA1. Fig. 1A shows the schematic representation of these novel polypetides. Most of these clones were isolated in DNA sequencing projects, whereas HDAC5 was also isolated as a clone encoding a human colon cancer antigen recognized by an autologous antibody (37, 39, 45). Available sequence data indicated that HDAC4/5/7 are homologous, with their C terminal parts similar to the catalytic domain of HDA1 (Figs. 1A & 1B). Sequence alignment of the N terminal domains of HDAC4/5/7N is shown in Fig. 1C. HDAC6 possesses two homologous regions similar to the catalytic domain of HDA1, and a cysteine/histidine-rich domain located at its C terminal part (Figs. 1A and 1B). The putative catalytic domains of HDAC4-6 are more similar to yeast HDA1 than to human HDAC1-3 (sequence identity of 35% compared to 26%), suggesting that HDAC4-6 and probably HDAC7 constitute a new subfamily of human histone deacetylases, with HDAC4, HDAC5 and probably HDAC7 more similar to each other than to HDAC6. Since HDAC4 was identified first and its full-length cDNA was available, we chose to characterize it further.

To determine tissue distribution of HDAC4, Northern blot analyses were performed. These analyses indicated that HDAC4 is expressed in skeletal

muscle, brain, leukocyte, colon, small intestine and ovary, but not in liver, lung and placenta (Fig. 2). To map the chromosomal localization of the HDAC4 gene, FISH analyses were performed. These analyses revealed that the HDAC4 gene is located at chromosome band 2q37.2 (Fig. 3). Abnormalities in this region have been implicated in developmental delay and predisposition to certain cancers (8, 33). Moreover, this band has been found to contain a cellular senescence gene (52).

Histone deacetylase activity of HDAC4. To determine the histone deacetylase activity of HDAC4, Flag-tagged HDAC4 and deletion mutants dm1-3 (Fig. 4A) were expressed in 293T cells and subject to histone deacetylase assays. As shown in Fig. 4B, affinity-purified HDAC4 efficiently deacetylated [^{3}H]acetyl-histones. The mutant dm1 had activity 2.9-fold higher than full-length HDAC4. Whereas dm2 had minimal activity, dm3 was slightly more active than dm1, suggesting that dm3 contains a deacetylase domain. This is consistent with the observation that the HDA1-related domain of HDAC4 is located at its C terminal part (Fig. 1A).

To establish that the observed deacetylase activity is intrinsic to HDAC4 (but not due to any associated proteins), we prepared two point mutants with histidine 803 of HDAC4 replaced with leucine (H803L and dm1/H803L; Fig. 4A). Histidine residues at equivalent positions have been found to be important for the deacetylase activity of HDAC1 and RPD3 (18, 24). Compared with HDAC4 and dm1, both point mutants had much lower deacetylase activity (Fig. 4B),

suggesting that HDAC4 has intrinsic deacetylase activity and histidine 803 is important for this activity.

To examine the effects of deacetylase inhibitors, we determined the deacetylase activity of dm3 in the presence of various concentrations of trichostatin A (TSA) or sodium butyrate. As shown in Fig. 4C, TSA dramatically inhibited the activity of dm3, with an IC_{50} value of 5 nM, whereas sodium butyrate (up to 5 mM) had much smaller effects. Compared with HDAC4, HDAC1 and HDAC3 are more sensitive to sodium butyrate (10).

The mutants dm1 and dm3 were also expressed in Sf9 cells using the baculovirus expression system. Proteins prepared this way had activity inversely proportional to their expression levels. Even the most active preparations possessed much lower activity than those obtained from 293T cells (data not shown), suggesting that an elusive factor(s) required for deacetylase activity may not be present in sufficient quantities in insect cells.

Tethered HDAC4 functions as a repressor. The possession of intrinsic deacetylase activity by HDAC4 suggests that it may be involved in transcriptional regulation. To test this hypothesis, we first investigated if HDAC4 functions as a repressor when artificially tethered to a promoter. For this purpose, a mammalian vector was constructed to express HDAC4 fused to the Gal4 DNA-binding domain and tested by cotransfection assays with the Gal4-tk-Luc luciferase reporter (Fig. 5A) in NIH3T3 cells. As shown in Fig. 5B, while the Gal4 DNA-binding domain itself activated transcription by 2-fold, GAL4-HDAC4 repressed transcription by 14-fold. To delineate the repression domain(s), mammalian

vectors were constructed to express various HDAC4 mutants fused to the Gal4 DNA-binding domain. HDAC4 mutants tested include dm1-3 (Fig. 4A), dm4 (residues 1-208) and dm5 (residues 1-114). As shown in Fig. 5B, similar to Gal4-HDAC4, Gal4-dm1 repressed transcription by 11-fold. While Gal4-dm2 had minimal effects (~2-fold), Gal4-dm3 repressed transcription by 83-fold. In contrast, Gal4-dm3 had a much smaller repressive effect on the tk-Luc reporter (1.8-fold; data not shown). Western analyses with an anti-Gal4 antibody indicated that Gal4-HDAC4 and Gal4-dm1-5 were indeed expressed (Fig. 5C). All of these results suggest that dm3 contains an active, strong repression domain. Unexpectedly, Gal4-dm4 repressed transcription by 14-fold although both Gal4-dm2 and Gal4-dm5 had minimal effects (Fig. 5B), suggesting that residues 1-208 of HDAC4 constitute another repression domain.

The repression observed with dm3 is stronger than that reported for HDAC1-3 (59). To assess if the repression by Gal4-dm3 is cell line-dependent, we performed similar transfection assays in 293T cells. As shown in Fig. 5D, Gal4-dm3 repressed Gal4-tk-Luc reporter activity in these cells in a dose-dependent manner. Since repression mediated by HDAC1 was found to be promoter-dependent (30), we assessed if Gal4-dm3 is able to repress reporters containing other core promoters. For this purpose, transfection assays were performed with TATA-containing (Gal4-AdML-Luc and Gal4-SV40-Luc) as well as TATA-less (Gal4-CD4-Luc) reporters. As shown in Fig. 5D, Gal4-dm3 was able to repress transcription of all of these reporters. Taken together, these

results suggest that once tethered to a promoter, the deacetylase domain of HDAC4 functions as a transcriptional repressor.

Requirement of HDAC4 deacetylase activity for repression. The repression observed with HDAC4 could be due either to deacetylation mediated by HDAC4 and/or to association with a repressor(s). This prompted us to examine whether the intrinsic deacetylase activity of HDAC4 is important for the observed repression. Since TSA inhibited deacetylase activity of HDAC4 (Fig. 4C), we determined effects of TSA on HDAC4-mediated repression. TSA only partially relieved repression mediated by Gal4-HDAC4 and Gal4-dm1 (Fig. 5B). TSA had a much more dramatic effect on the repression mediated by Gal4-dm3 (Fig. 5B), suggesting that histone deacetylase activity is important for the repression observed with Gal4-dm3. The point mutation H803A reduced the repression by Gal4-dm1, and TSA had no effects on the residual repression observed with Gal4-dm1/H803A (Fig. 5B). By contrast, TSA did not relieve the repression mediated by Gal4-dm4 (Fig. 5B). Taken together, these results suggest that while the histone deacetylase activity of HDAC4 is important for its repression function, mechanisms independent of deacetylation are also involved.

HDAC4 does not directly bind to DNA. Promoter tethering of HDAC4 leads to transcriptional repression, so we next asked how HDAC4 is recruited to promoters *in vivo*. One possibility is that HDAC4 possesses intrinsic DNA-binding ability. Sequence-specific DNA-binding proteins can, although with lower affinity, bind to non-specific DNA. To address if HDAC4 directly binds to DNA, a DNA-binding assay was utilized to determine if HDAC4 could non-specifically bind to

fish sperm DNA (17). This assay revealed that Flag-HDAC4 immobilized on M2 agarose could not retain a significantly higher amount of DNA than M2-agarose itself (data not shown). Therefore, HDAC4 does not have intrinsic DNA-binding ability.

HDAC4 physically interacts with MEF2 transcription factors. Since HDAC4 does not bind to DNA by itself, we reasoned that other transcription factors might mediate the recruitment of HDAC4 to promoters. To identify such target transcription factors, we tested several active repressors, including human Groucho homolog TLE1 (12, 48), zinc-finger oncoprotein Evi1 (3), Polycomb-group protein EZH2 (28) and adenoviral protein E1B (61). Protein-protein interaction studies and reporter gene assays indicated that none of these repressors interact with HDAC4 (data not shown).

A novel *Xenopus laevis* repressor protein, termed MITR (GenBank accession No. Z97214; ref. 47), was identified as an interaction partner for the *Xenopus* myocyte enhancer-binding factors SL-1/2. *Xenopus* MITR is a homolog of HDAC7N (sequence identity, 59%; similarity, 67%). As illustrated in Fig. 1A, HDAC7N is composed of two regions, the N terminal part of which shows significant sequence similarity to HDAC4 (sequence identity, 46%; similarity, 58%). In light of these observations, we tested if HDAC4 interacts with human MEF2 transcription factors.

To examine *in vivo* interaction between HDAC4 and MEF2s, immunoprecipitation experiments were performed. For this purpose, HDAC4 (Flag-tagged) and/or MEF2C expression plasmids were cotransfected into 293T

cells, and extracts prepared from the transfected cells were subjected to immunoprecipitation with anti-Flag M2 agarose. Eluted immunocomplexes were subjected to Western blotting analyses with anti-Flag and anti-MEF2C antibodies. As shown in Fig. 6A, MEF2C specifically precipitated with Flag-tagged HDAC4 (lanes 1-4). Similar immunoprecipitation experiments revealed that HDAC4 precipitated with endogenous MEF2D (Fig. 6A, lanes 6-8). These results indicate that HDAC4 interacts with MEF2C and MEF2D *in vivo*.

These immunoprecipitation data also suggest that conserved regions of MEF2C and MEF2D mediate their interaction with HDAC4. The N terminal regions of MEF2C and MEF2D contain the MADS-box and MEF2-specific domains and are the most conserved, so we next asked if the MEF2C mutant M178 is able to interact with HDAC4 (Fig. 6B). For this, M178 was expressed in *E. coli* as a fusion with maltose-binding protein (MBP) and used for *in vitro* pull-down assays. As shown in Fig. 6C, M178 specifically interacted with HDAC4 (lanes 1-3). To delineate regions of HDAC4 required for such interaction, a series of HDAC4 mutants (Fig. 6E) was utilized. M178 interacted with dm1 (Fig. 6C, lanes 4-6) and less strongly with dm6 (lanes 7-9). By contrast, M178 did not interact with the mutants dm7-9 (lanes 10-18), suggesting that residues 118-188 of HDAC4 are essential for interaction with M178. Consistent with this contention, dm2 but not dm3 interacted with M178 (Fig. 6D, lanes 1-6). To further map the MEF2-interaction domain, dm4 and dm5 were tested. Unlike dm5, dm4 interacted with M178 (lanes 7-12), suggesting that residues 118-208 of HDAC4 are essential for interacting with M178. To address if these residues are sufficient,

dm10 was utilized (Fig. 6E). This mutant was found to interact with M178 (Fig. 6D, lanes 13-15), confirming that residues 118-208 of HDAC4 are sufficient for interaction with MEF2C. Furthermore, in immunoprecipitation experiments, dm4 was found to interact with MEF2C (Fig. 6A, lane 5) or MEF2D (lane 9) *in vivo*. Taken together, these results indicate that residues 118-208 of HDAC4 contain a MEF2-interacting domain (Fig. 6E).

HDAC4 represses MEF2C-dependent transcription. To explore the functional relevance of the observed physical interaction between HDAC4 and MEF2C, we constructed a luciferase reporter containing a MEF2-binding site (MEF2-E4-Luc; Fig. 7A). This reporter was transfected into NIH3T3 cells with or without expression plasmids for HDAC4 and/or MEF2C. As expected, MEF2C activated the reporter (Fig. 7B). While HDAC4 itself had minimal effects on the reporter activity in the absence of cotransfected MEF2C, HDAC4 repressed MEF2C-dependent transcription in a dose-dependent manner. The HDAC4 mutant dm7, which lacks a MEF2-binding site, had a much smaller effect. Since recruitment of HDAC4 by MEF2C repressed the reporter activity below the control level, HDAC4 may not be only inhibitory to the activation function of MEF2C. To substantiate this point, the MEF2C mutant M178 was tested. This mutant only weakly stimulated the reporter activity since it lacks the MEF2C activation domain located at its C terminal part (Fig. 7C). In a dose-dependent manner, HDAC4 repressed the reporter activity below the control level. On the other hand, dm7 had minimal effects. Western blotting analyses revealed that HDAC4 and dm7 were expressed at similar levels (data not shown). Taken

together, these results suggest that MEF2C recruits HDAC4 to repress transcription.

HDAC4 cooperates with MEF2C to inhibit c-Jun promoter activity. Next we wished to examine a native promoter containing a MEF2-binding site. In non-muscle cells, MEF2C regulates the expression of the proto-oncogene c-Jun (15, 26, 63). Therefore, we tested the reporter pJLuc (Fig. 8A), which contains the c-Jun promoter upstream from the luciferase gene (Fig. 8A; 16).

First, the expression plasmid for HDAC4 was cotransfected with this reporter to verify that HDAC4 does not regulate the promoter in the absence of cotransfected MEF2C. Unexpectedly, HDAC4 increased the reporter activity by 8-fold (Fig. 8B). To localize regions of HDAC4 involved in such activation, several deletion mutants were tested. While mutants dm2-5 had minimal effects, dm1 and dm7 activated the reporter by 4- and 10-fold, respectively. Since dm7 lacks MEF2C-binding ability (Fig. 6E), HDAC4-mediated activation of pJLuc may be independent of MEF2C. The point mutation H803A greatly diminished the activation ability of both HDAC4 and dm1 (compare the mutants H803L and dm1/H803L with HDAC4 and dm1, respectively), suggesting that the histone deacetylase activity of HDAC4 is important for activation of the c-Jun promoter.

We then investigated the effects of MEF2C on the reporter pJLuc. As expected, transfection of MEF2C activated the expression of this reporter by 15-fold (Fig. 8C). Cotransfection of HDAC4 repressed the activation mediated by MEF2C below the control level (Fig. 8C), raising an intriguing regulation scheme: transfected HDAC4 and MEF2C individually activate but together repress c-Jun

promoter activity. To determine which region of HDAC4 is required for this repression, HDAC4 deletion mutants were tested. The mutant dm1 repressed transcription by 28-fold, whereas dm2 and dm3 had minimal effects (Figs. 8C & 8D), suggesting that both the deacetylase domain and residues 118-626 are required for dm1 to repress MEF2C-dependent transcription. Compared with dm1, dm7 had a reduced ability to repress the reporter activity (Figs. 8C & 8D). Since dm7 lacks the MEF2C-binding domain (Fig. 6E), these results suggest that the MEF2C-interacting domain is important for dm1 to repress transcription of the reporter pJLuc.

The mutant dm4 repressed transcription by 49-fold, whereas dm5 had minimal effects (Figs. 8C & 8D). Western blotting analyses revealed that dm4 and dm5 were expressed at similar levels (data not shown). Therefore, HDAC4 represses MEF2C-dependent transcription through two repression domains. This may explain why the point mutation H803A had minimal effects on the ability of HDAC4 to repress MEF2C-dependent transcription (Fig. 8C). Surprisingly, the same mutation had minimal effects on the ability of dm1 to repress MEF2C-dependent transcription, implying the existence of additional repression mechanisms. Taken together, these results suggest that through an MEF2C-interaction domain and at least two repression domains, HDAC4 counteracts MEF2C-dependent activation of the c-Jun promoter.

DISCUSSION

HDAC4 has intrinsic histone deacetylase activity. Numerous studies have established that yeast RPD3 and human HDAC1-3 constitute one family of histone deacetylases (10, 50, 58, 59). The plant histone deacetylase HD2 may represent the first member of another family of deacetylases, which does not display any sequence similarity to RPD3 or HDAC1-3 (31). Human HDAC4-7 and yeast HDA1 constitute a third family of histone deacetylases which displays some sequence similarity to RPD3 and HDAC1-3 (Fig. 1). While this manuscript was under review, characterization of the histone deacetylase activity of human HDAC4-6 was reported (11, 13). Homologs of HDAC4-6 have been identified in mouse (54) and other organisms (GenBank accession No. Q20296 and P56523).

HDAC4 possesses intrinsic histone deacetylase activity (Fig. 4; ref. 35). The HDAC4 mutants dm1 and dm3 were found to be slightly more active than full-length HDAC4 (Fig. 4B). One explanation for this difference is that these proteins had differential post-translational modifications. Alternatively, the difference may suggest that the deacetylase activity of HDAC4 is subject to negative regulation by its N terminal domain. If so, this raises the intriguing possibility that other proteins may regulate the activity of HDAC4 by counteracting its auto-inhibitory function.

HDAC4 possesses at least two transcriptional repression domains. As implied by its deacetylase activity, HDAC4 repressed transcription when it was artificially tethered to promoters (Fig. 5). Intriguingly, we have found that HDAC4 possesses at least two repression domains: one composed of the N

terminal 208 residues and the other consisting of the HDA1-related deacetylase domain (Fig. 9). In contrast, HDAC1-3 do not appear to possess repression domains other than their deacetylase domains (10, 18, 59). The possession of redundant repression domains by HDAC4 reflects similar themes described for the histone acetyltransferases p300 and CBP, both of which possess transcriptional activation domains in addition to their acetyltransferase domains (14, 27, 49, 57).

Unlike its N terminal repression domain, the deacetylase domain of HDAC4 mediates TSA-sensitive repression. The mutation H803A greatly diminished the deacetylase activity of HDAC4 (Fig. 4), but its effects on the transcriptional ability of HDAC4 were somewhat mixed: (i) it reduced the repression function of Gal4-dm1 (Fig. 5B); (ii) it abolished the ability of HDAC4 and dm1 to activate the c-Jun promoter (Fig. 8B); and (iii) it had minimal effects on the ability of HDAC4 and dm1 to repress the activation function of MEF2C (Fig. 8C). There are several possible explanations for why the mutation had such varied effects. First, HDAC4 possesses at least one repression domain besides its deacetylase domain. Second, HDAC4 may homodimerize or heterodimerize with other HDACs. This is consistent with the recent finding that HDAC4 interacts with HDAC3 (13). Third, transiently transfected reporters may not possess standard chromatin structure. Further studies of integrated reporters or endogenous c-Jun promoter will certainly clarify this. From the current study, we conclude that the deacetylase activity of HDAC4 is important for repression, but additional mechanisms are also involved.

Recruitment of HDAC4 to promoters may lead to local deacetylation and thus transcriptional repression. Since histone acetyltransferases have been found to acetylate transcription factors, HDAC4 may also regulate acetylation levels of transcription factors. Therefore, the repression mediated by HDAC4 could be due either to deacetylation of hyperacetylated chromatin and subsequent formation of repressive chromatin structure, or to deacetylation of acetylated transcription factors. Further investigation is needed to elucidate how HDAC4 is involved in transcriptional repression.

HDAC4 physically and functionally interacts with MEF2C. How is HDAC4 recruited to promoters *in vivo*? HDAC4 does not have intrinsic DNA-binding ability, so it must be recruited by interaction with target transcription factors. Compared to HDA1, HDAC4 has a long N terminal domain (Fig. 1A). By immunoprecipitation experiments and *in vitro* binding assays, we have demonstrated that HDAC4 interacts with MEF2C and MEF2D and mapped the MEF2-interaction domain to residues 118-208 of HDAC4 (Fig. 6). This is consistent with a model in which the N terminal domain of HDAC4 mediates its interaction with target transcription factors such as MEF2C and MEF2D.

Using the luciferase reporter MEF2-E4-Luc, we have shown that HDAC4 is recruited by MEF2C to repress transcription (Fig. 7). Independently, it has been demonstrated that HDAC4 associates with MEF2A and represses MEF2A-dependent transcription (35). Furthermore, MITR interacts with MEF2 and negatively regulates MEF2-dependent transcription (47).

MEF2s are known transcriptional activators, so it is somewhat unexpected that MEF2s recruit HDAC4 or MITR to repress transcription. However, it has been suggested that MEF2s negatively regulate transcription by associating with a negatively acting accessory factor (40). These findings suggest that HDAC4 or MITR may be such an accessory factor. Interestingly, more and more transcription factors are being found to have dual function. For example, the transcriptional activator E2F binds to the tumor suppressor Rb and recruits the histone deacetylase HDAC1 to repress transcription (5, 30, 32). Therefore, it is tempting to propose that MEF2s play a dual role in transcriptional regulation.

HDAC4 and MEF2C cooperatively regulate c-Jun promoter activity. The proto-oncogene c-Jun is one of the immediate-early gene products whose expression is rapidly induced by treatment of cells with serum and many growth factors (7 and reference therein). c-Jun regulates cell-cycle progression in a p53-dependent manner (46). When cotransfected with MEF2C, HDAC4 repressed c-Jun promoter activity (Fig. 8C). Like HDAC4, both dm1 and dm4 repressed c-Jun promoter activity in the presence of transfected MEF2C (Fig. 8C). These results are consistent with a model that in the presence of transfected MEF2C, HDAC4 represses c-Jun promoter activity via at least two repression domains (Fig. 9).

Unexpectedly, in the absence of cotransfected MEF2C, HDAC4 activated the c-Jun promoter in NIH3T3 cells (Fig. 8B). The MEF2-interaction domain appears to be dispensable for this activation, suggesting that activation of the c-Jun promoter by HDAC4 operates through MEF2C-independent mechanisms. It is possible that HDAC4 activates the c-Jun promoter by regulating the function

and/or protein level of a required transcription factor(s). We favor the model in which HDAC4 downmodulates the expression of a repressor, whose function is required for repression of the c-Jun promoter, and thus leads to activation. In NIH3T3 cells, dependent on whether MEF2C is cotransfected, HDAC4 exerts opposing actions on the c-Jun promoter. In other types of cells, relative expression levels of HDAC4, MEF2C and the elusive repressor may dictate which action takes place. It is also possible that the actions of HDAC4 are subject to regulation by various signaling pathways. Therefore, we propose that HDAC4 regulates the c-Jun promoter in a context-dependent manner.

In summary, we have demonstrated that HDAC4, a human histone deacetylase related to HDA1, is composed of multiple functional domains: its N terminal part possesses repression domain 1 and a MEF2C-interacting region, whereas its C terminal part constitutes repression domain 2 and functions as the catalytic domain conducting deacetylation (Fig. 9). In NIH3T3 cells, dependent on the expression level of MEF2C, HDAC4 exerts opposing actions on the c-Jun promoter, suggesting that HDAC4 and probably its homologs HDAC5/7 cooperate with the MEF2 family of transcription factors to regulate their target genes such as c-Jun in a context-dependent manner. It will be interesting to determine if and how the interaction of HDAC4 with MEF2s is regulated to fulfill their roles in various types of cells.

ACKNOWLEDGEMENTS

We thank T. Nagase for the cDNA clone KIAA0288; R. Prywes for the pJLuc reporter and anti-MEF2D antibody; E.N. Olson for MEF2 expression plasmids; W.M. Yang and E. Seto for Gal4 expression plasmids; P. Haus-Seuffert and M. Meisterernst for Gal4-CD4-Luc; Y. Zhang and D. Reinberg for Gal4-AdML-luc; D. Sparrow, T.J. Mohun and T. Kouzarides for communicating results prior to publication; and A. Nepveu for helpful discussions.

This work was supported by funds from the National Cancer Institute of Canada (to X.Y.) and grants from the Medical Research Council (MRC) of Canada (to X.Y. & J. T.). X.Y. is an MRC scholar.

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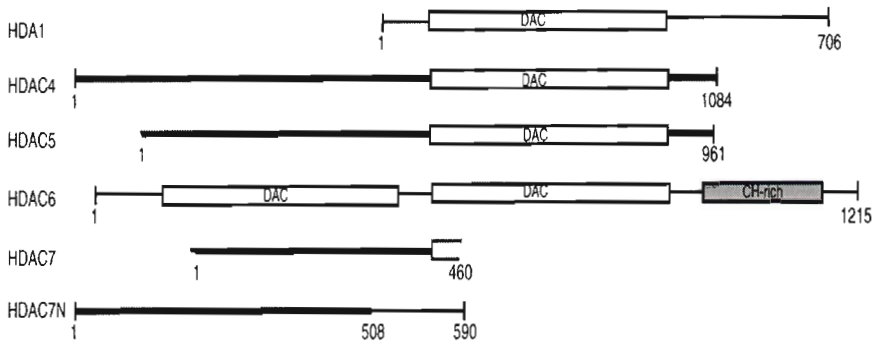
A



B

```
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HDAC5     584 SQKMYAVLPC GGIGVDSDTV WNEMHSSSAV RMAVGCLLEL AFKVAAGELK
HDAC6N    157 QYMNEGELR. .VLADTYDSV YLHPNSYSCA CLASGSVLRL VDAVLGAEIR
HDAC6C    552 EKMKTRELH. .RESSNFDSI YICPSTFACA QLATGAACRL VEAVLSGEVL
HDA1      146 EKMSREELL. .KETEKGDSV YFNNDSYASA RLPCGGAIEA CKAVVEGRVK

                      * *
HDAC4     792 NGFAVVRPPG HHAEESTPMG FCYFNSVAVA AKLLQQRL.. SVSKILIVDW
HDAC5     634 NGFAIIRPPG HHAEESTAMG FCFFNSVAIT AKLLQQKL.. NVGKVLIVDW
HDAC6N    205 NGMAIIRPPG HHAQHSLMDG YCMFNHVAVA ARY.AQQ.KH RIRRVLIVDW
HDAC6C    600 NGAAVVRPPG HHAEQDAACG FCFFNSVAVA ARH.AQTISG HALRILIVDW
HDA1      194 NSLAVVRPPG HHAEPQAAGG FCLFSNVAVA AKNILKNYPE SVRRIMILDW

HDAC4     840 DVHHGNGTQQ AFYSDPSVLY MSLHRYDDGN FFPG..SGAP DEVGTGPGVG
HDAC5     682 DIHHGNGTQQ AFYNDPSVLY ISLHRYDNGN FFPG..SGAP EEVGGGPGVG
HDAC6N    253 DVHHGQGTQF TFDQDPSVLY FSIHRYEQGR FWPHLKASNW STTGFGQGQG
HDAC6C    649 DVHHGNGTQH MFEDDPSVLY VSLHRYDHGT FFPMGDEGAS SQIGRAAGTG
HDA1      244 DIHHGNGTQK SFYQDDQVLY VSLHRFEMGK YYPGTIQGQY DQTGEGKGEG

HDAC4     888 FNVNMAFTGG LDPPMGDAEY LAAFRTVVMP IASEFAPDVV LVSSGFDAVE
hHDA5     730 YNVNVAWTGG VDPPIGDVEY LTAFRTVVMP IAHEFSPDVV LVSAGFDAVE
hHDA6N    303 YTINVPWNQ.  .VGMRDADY IAAFLHVLLP VALEFQPQLV LVAAGFDALQ
hHDA6C    699 FTVNVAWNG. ..PRMGDADY LAAWHRLVLP IAYEFNPELV LVSAGFDAAR
HDA1      294 FNCNITWPV. ..GGVGDAEY MWAFEQVVMP MGREFKPDLV IISSGFDAAD

HDAC4     938 GHPTPLGGYN LSARCFGYLT KQLMGLAGGR IVLALEGGHD LTAICDASEA
hHDA5     780 GHLSPLGGYS VTARCFGHLT RQLMTLAGGR VVLALEGGHD LTAICDASEA
hHDA6N    350 G..DPKGEMA ATPAGFAQLT HLLMGLAGGK LILSLEGGYN LRALAEGVSA
hHDA6C    746 G..DPLGGCQ VSPEGYAHLT HLLMGLASGR IILILEGGYN LTSISESMAA
HDA1      341 G..DTIGQCH VTPSCYGHMT HMLKSLARGN LCVVLEGGYN LDAIARSALS

HDAC4     988 CVSALLGNEL DPLPEKVLQQ RPNANAVRSM EKVMEIHSKY WRCLQRTTST
hHDA5     836 CVSALLSVKL QPLDEAVLQQ KPNINAVATL EKVIEIQSKH WSCVQKFAAG
hHDA6N    398 SLHTLLGDPC PMLESP...G APCRSAQASV SCALEALEPF WEVLVRSTET
hHDA6C    794 CTRSLLGDPP PLLTLP...R PPLSGALASI TETIQVHRRY WRSL.RVMKV
HDA1      389 VAKVLIGEPP DELPDPL..S DPKPEVIEMI DKVIRLQSKY WNCFRRRHAN
```
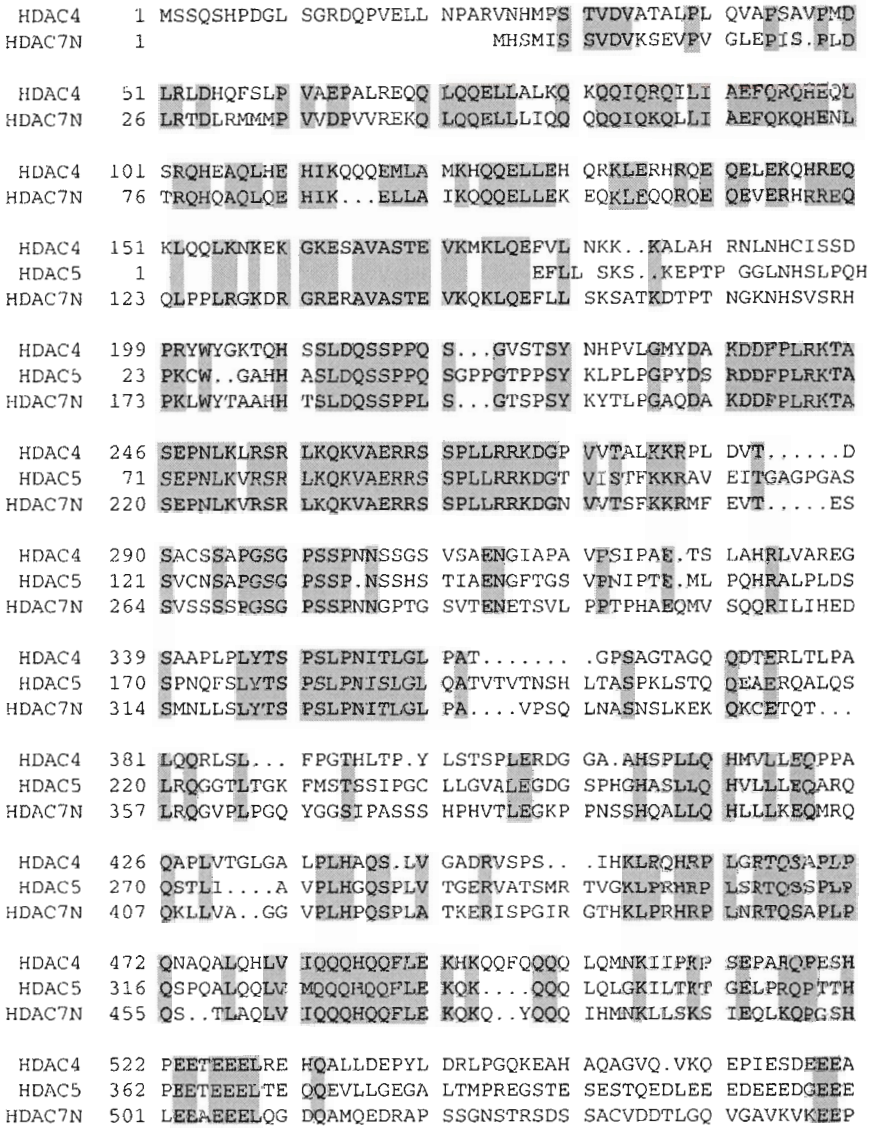

C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HDAC7N 1 MHSMIS SVDVKSEVPV GLEPIS.PLD
HDAC4 51 LRLDHQFSLP VAEPALREQQ LQQELLALKQ KQQIQRQILI AEFQRQHEQL
HDAC7N 26 LRTDLRMMMP VVDPVVREKQ LQQELLLIQQ QQQIQKQLLI AEFQKQHENL
HDAC4 101 SRQHEAQLHE HIKQQQEMLA MKHQQELLEH QRKLERHRQE QELEKQHREQ
HDAC7N 76 TRQHQAQLQE HIK...ELLA IKQQQELLEK EQKLEQQRQE QEVERHRREQ
HDAC4 151 KLQQLKNKEK GKESAVASTE VKMKLQEFVL NKK..KALAH RNLNHCISSD
HDAC5 1 EFLL SKS..KEPTP GGLNHSLPQH
HDAC7N 123 QLPPLRGKDR GRERAVASTE VKQKLQEFLL SKSATKDTPT NGKNHSVSRH
HDAC4 199 PRYWYGKTQH SSLDQSSPPQ S...GVSTSY NHPVLGMYDA KDDFPLRKTA
HDAC5 23 PKCW..GAHH ASLDQSSPPQ SGPPGTPPSY KLPLPGPYDS RDDFPLRKTA
HDAC7N 173 PKLWYTAAHH TSLDQSSPPL S...GTSPSY KYTLPGAQDA KDDFPLRKTA
HDAC4 246 SEPNLKLRSR LKQKVAERRS SPLLRRKDGP VVTALKKRPL DVT.....D
HDAC5 71 SEPNLKVRSR LKQKVAERRS SPLLRRKDGT VISTFKKRAV EITGAGPGAS
HDAC7N 220 SEPNLKVRSR LKQKVAERRS SPLLRRKDGN VVTSFKKRMF EVT.....ES
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HDAC5 121 SVCNSAPGSG PSSP.NSSHS TIAENGFTGS VPNIPTE.ML PQHRALPLDS
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HDAC7N 314 SMNLLSLYTS PSLPNITLGL PA....VPSQ LNASNSLKEK QKCETQT...
HDAC4 381 LQQRLSL... FPGTHLTP.Y LSTSPLERDG GA.AHSPLLQ HMVLLEQPPA
HDAC5 220 LRQGGTLTGK FMSTSSIPGC LLGVALEGDG SPHGHASLLQ HVLLLEQARQ
HDAC7N 357 LRQGVPLPGQ YGGSIPASSS HPHVTLEGKP PNSSHQALLQ HLLLKEQMRQ
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HDAC5 270 QSTLI....A VPLHGQSPLV TGERVATSMR TVGKLPRHRP LSRTQSSPLP
HDAC7N 407 QKLLVA..GG VPLHPQSPLA TKERISPGIR GTHKLPRHRP LNRTQSAPLP
HDAC4 472 QNAQALQHLV IQQQHQQFLE KHKQQFQQQQ LQMNKIIPRP SEPAQQPESH
HDAC5 316 QSPQALQQLV MQQQHQQFLE KQK....QQQ LQLGKILTRT GELPRQPTTH
HDAC7N 455 QS..TLAQLV IQQQHQQFLE KQKQ..YQQQ IHMNKLLSKS IEQLKQPGSH
HDAC4 522 PEETEEELRE HQALLDEPYL DRLPGQKEAH AQAGVQ.VKQ EPIESDEEEA
HDAC5 362 PEETEEELTE QQEVLLGEGA LTMPREGSTE SESTQEDLEE EDEEEDGEEE
HDAC7N 501 LEEAEEELQG DQAMQEDRAP SSGNSTRSDS SACVDDTLGQ VGAVKVKEEP

Fig. 1 Comparison of HDAC4-7 with HDA1.

(A) Schematic representation of HDA1 and HDAC4-7. The N terminus of HDAC5 is incomplete, as are both termini of HDAC7. HDAC7N may be an alternatively spliced variant of HDAC7. The conserved deacetylase domains are boxed and labeled with "DAC." Other domains shared by HDAC4/5/7 and HDAC7N are shown in bold lines. HDAC6 has a cysteine/histidine-rich domain (CH-rich, shaded box) at its C terminus. This diagram was generated based on BLAST search results. GenBank accession numbers for sequences referred to are: HDA1 (P53973), HDAC4 (AB006626), HDAC5 (AB011172 and AF039691), HDAC6 (AJ011972), HDAC7 (AF124924) and HDAC7N (AB018287). A genomic clone (GenBank accession no. AC004466) contains some coding sequences related to HDAC4/5/7 and may encode HDAC8.

(B) Sequence alignment of catalytic domains of HDAC4-6 and HDA1. Identical or highly conserved residues (4 out of 5 sequences) are shaded. For simplicity, only S/T, R/K and D/E are considered to be highly conserved. The asterisk (*) denotes histidine 803 of HDAC4, which may be important for its deacetylase activity.

(C) Sequence alignment of the N terminal domains of HDAC4/5/7N. Identical residues are shaded.

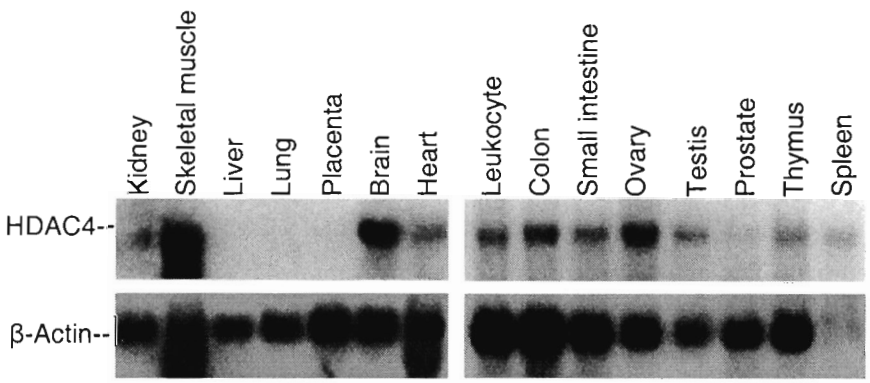
Kidney
Skeletal muscle
Liver
Lung
Placenta
Brain
Heart
Leukocyte
Colon
Small intestine
Ovary
Testis
Prostate
Thymus
Spleen
HDAC4--
β-Actin--

Fig. 2 Expression of HDAC4 in various adult human tissues.

PolyA-RNA blots (Clontech; 2 μg per lane) were probed with an HDAC4 cDNA fragment derived from the 3' untranslated region (*top*). As a loading control, the same blots were reprobed with a β-actin cDNA probe (*bottom*). Molecular size markers are shown at right.

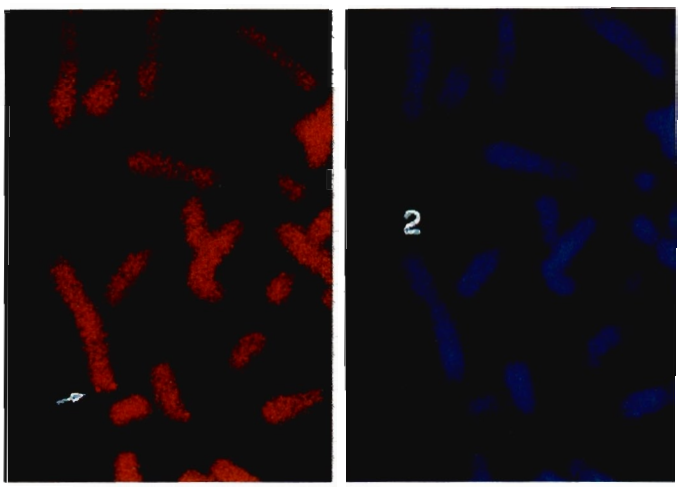
2

Fig. 3 Chromosomal localization of the HDAC4 gene.

Left panel shows FISH signals detected at chromosome band 2q37.2, indicated by an arrow, while right panel shows the same mitotic cell stained with DAPI to identify chromosomes. Human blood lymphocytes were used for FISH; the hybridization efficiency was 81%, i.e. 81 of 100 checked mitotic figures showed the indicated localization.

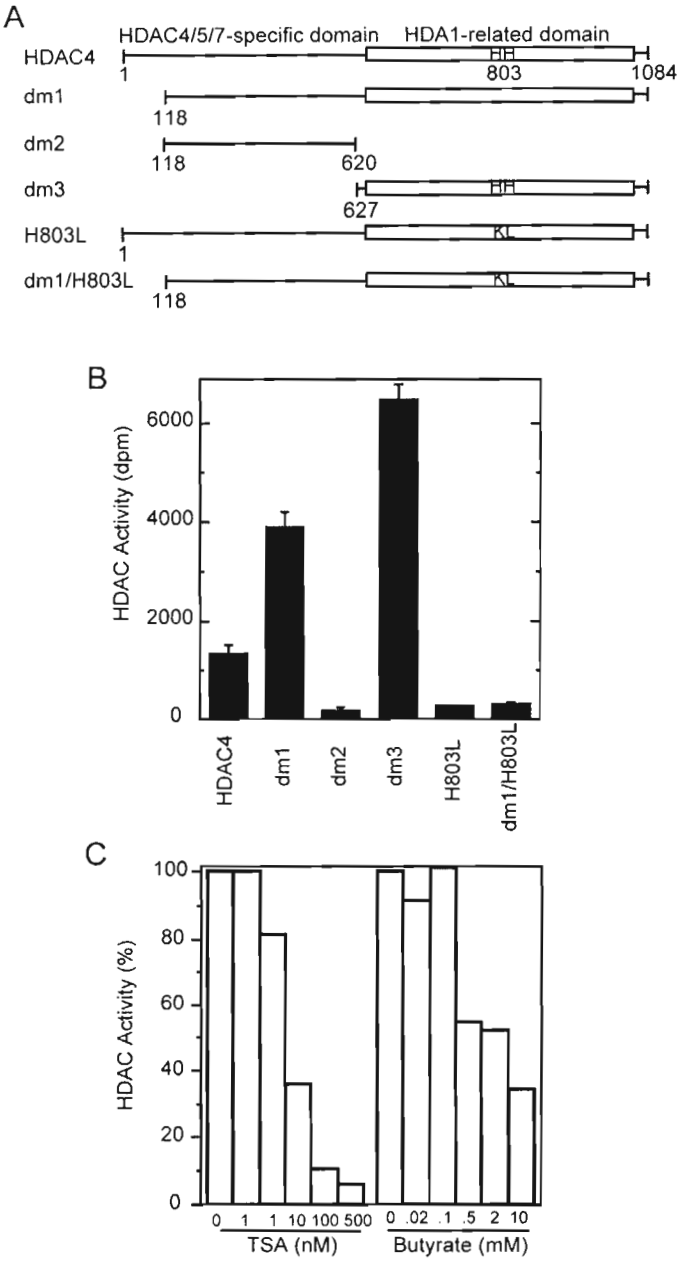
A
HDAC4/5/7-specific domain
HDA1-related domain
HDAC4
1
803
1084
dm1
118
dm2
118
620
dm3
627
H803L
1
KL
dm1/H803L
118
KL
B
HDAC Activity (dpm)
6000
4000
2000
0
HDAC4
dm1
dm2
dm3
H803L
dm1/H803L
C
HDAC Activity (%)
100
80
60
40
20
0
0 1 1 10 100 500
TSA (nM)
0 .02 .1 .5 2 10
Butyrate (mM)

Fig. 4 Characterization of histone deacetylase activity of HDAC4.

(A) Schematic representation of HDAC4 and its mutants used for deacetylase assays. Histidine 803 of HDAC4 is marked with the letter "H."

(B) Deacetylase activity of HDAC4 and its mutants. Deacetylase activity, measured as dpm of [^{3}H]acetate released from [^{3}H]acetyl-histones, was normalized to their relative protein concentration determined by Western analyses with an anti-Flag antibody. During purification of Flag-tagged proteins, a buffer containing 0.5 M KCl was used for extensive washing; under such conditions, with untransfected cell extracts, equivalent amounts of M2 agarose beads retained deacetylase activity close to background levels.

(C) Effects of TSA and sodium butyrate on deacetylase activity of dm3. The concentrations used are: 0, 1, 10, 100, 500 nM for TSA and 0, 0.02, 0.1, 0.5, 2, 10 mM for sodium butyrate.

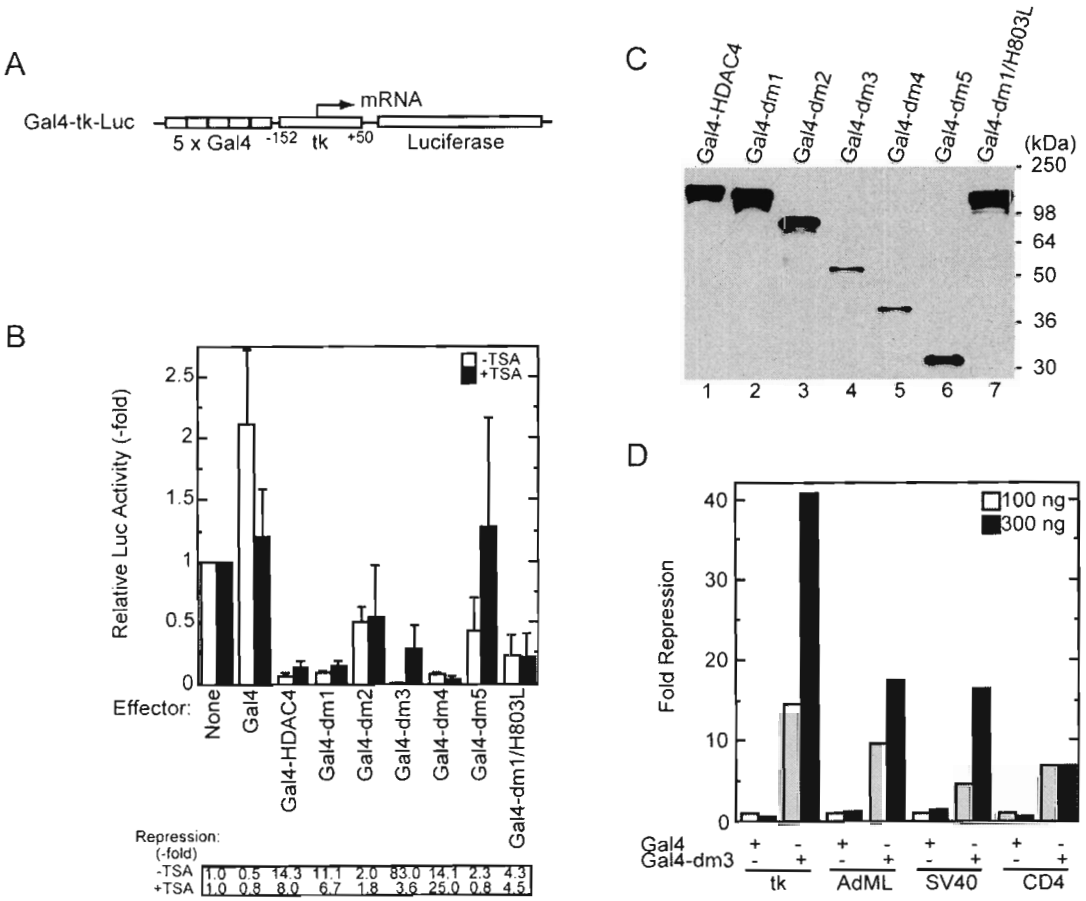
A
Gal4-tk-Luc
mRNA
5 x Gal4
-152
tk
+50
Luciferase
B
Relative Luc Activity (-fold)
-TSA
+TSA
Effector:
None
Gal4
Gal4-HDAC4
Gal4-dm1
Gal4-dm2
Gal4-dm3
Gal4-dm4
Gal4-dm5
Gal4-dm1/H803L
Repression: (-fold)
-TSA 1.0 0.5 14.3 11.1 2.0 83.0 14.1 2.3 4.3
+TSA 1.0 0.8 8.0 6.7 1.8 3.6 25.0 0.8 4.5
C
Gal4-HDAC4
Gal4-dm1
Gal4-dm2
Gal4-dm3
Gal4-dm4
Gal4-dm5
Gal4-dm1/H803L
(kDa)
250
98
64
50
36
30
1 2 3 4 5 6 7
D
Fold Repression
100 ng
300 ng
Gal4
Gal4-dm3
tk
AdML
SV40
CD4

Fig. 5 Tethered HDAC4 represses transcription.

(A) Schematic representation of the luciferase reporter Gal4-tk-Luc. Upstream from the thymidine kinase core promoter (-152 to +50) are 5 copies of the Gal4-binding site.

(B) Repression of Gal4-tk-Luc by HDAC4 and its mutants in NIH3T3 cells. The mutants dm1-3 and dm1/H803A are described in Fig. 4A; dm4 and dm5 contain the N terminal 208 and 114 residues of HDAC4, respectively. Mammalian constructs expressing HDAC4 and its mutants fused to the C terminus of Gal4(1-147) were transfected into NIH3T3 cells with the reporter Gal4-tk-Luc. Luciferase activities were normalized to the internal β-galactosidase control; the normalized luciferase activity from the transfection without any effector plasmid was arbitrarily set to 1.0.

(C) Expression of Gal4-HDAC4 and its mutants. Extracts (10 μg/lane), prepared from 293T cells transfected with expression plasmids for indicated fusion proteins, were subjected to Western blotting analyses using an anti-Gal4 antibody (Santa Cruz Biotech., RK5C1). Molecular size markers are shown at right.

(D) Repression of reporters with different core promoters by Gal4-dm3 in 293T cells. The reporters possess indicated core promoters replacing the tk region of Gal4-tk-Luc (*A*). 100 and 300 ng of expression plasmids were used as indicated.

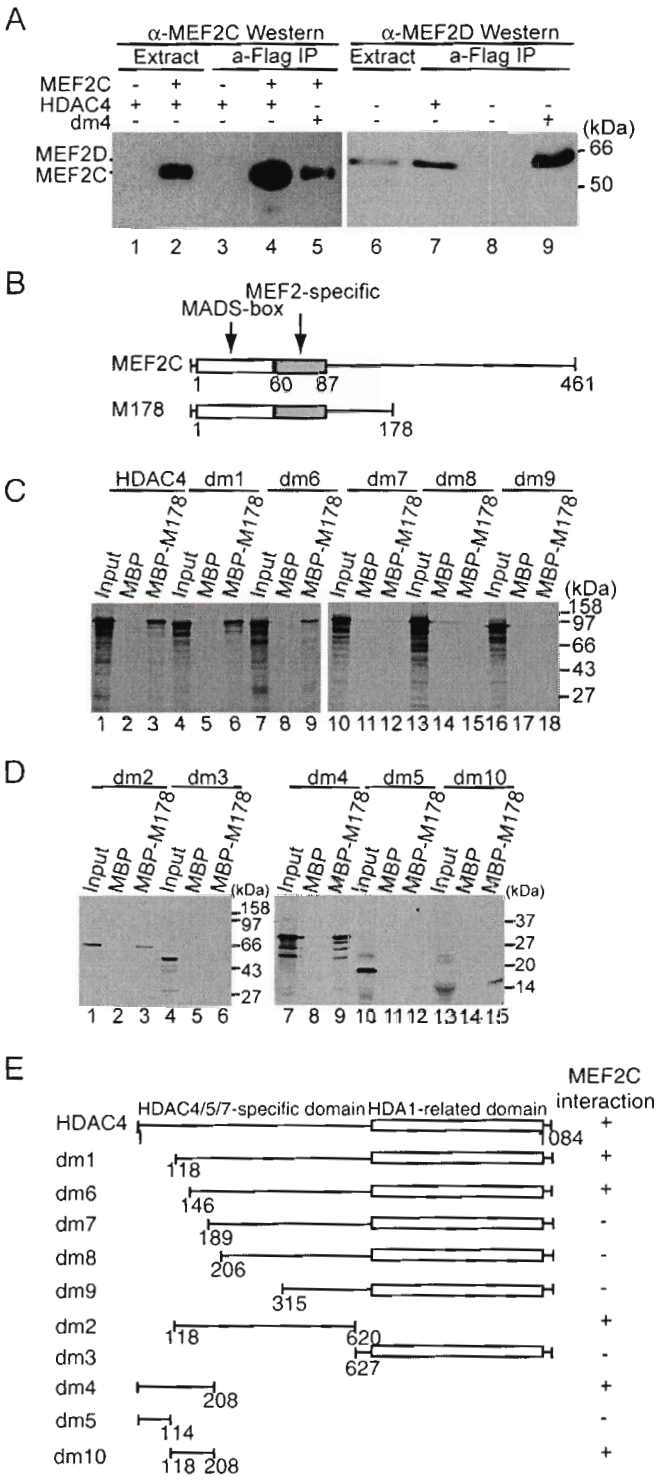
A
α-MEF2C Western
α-MEF2D Western
Extract
a-Flag IP
Extract
a-Flag IP
MEF2C
HDAC4
dm4
(kDa)
66
50
MEF2D
MEF2C
1 2 3 4 5 6 7 8 9
B
MEF2-specific
MADS-box
MEF2C
1
60
87
461
M178
1
178
C
HDAC4
dm1
dm6
dm7
dm8
dm9
Input
MBP
MBP-M178
(kDa)
158
97
66
43
27
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
D
dm2
dm3
dm4
dm5
dm10
Input
MBP
MBP-M178
(kDa)
158
97
66
43
27
37
27
20
14
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
E
MEF2C
interaction
HDAC4/5/7-specific domain
HDA1-related domain
HDAC4
1
1084
dm1
118
dm6
146
dm7
189
dm8
206
dm9
315
dm2
118
620
dm3
627
dm4
208
dm5
114
dm10
118
208
+
+
+
-
-
-
+
-
+
-
+

Fig. 6 HDAC4 interacts with MEF2 *in vivo* and *in vitro*.

(A) Immunoprecipitation of HDAC4 with MEF2C (lanes 1-5) or MEF2D (lanes 6-9). Flag-tagged HDAC4 (lanes 1-4 & 7) or dm4 (lanes 5 & 9) were expressed with (lanes 2, 4 & 5) or without (lanes 1, 3 & 6-9) MEF2C in 293T cells and immunoprecipitated with anti-Flag M2 agarose. Extracts (lanes 1, 2 & 6) and immunoprecipitated proteins eluted with Flag peptide (lanes 3-5 & 7-9) were subjected to Western blotting analyses with an anti-MEF2C (lanes 1-5) or anti-MEF2D (lanes 6-9) polyclonal antibody. The presence of Flag-tagged HDAC4 and dm4 was confirmed by Western blotting analyses of the same samples with an anti-Flag monoclonal antibody (data not shown).

(B) Schematic representation of MEF2C and its mutant M178 (consisting residues 1-178).

(C, D) Interaction of M178 with HDAC4 and its deletion mutants *in vitro*. MBP or MBP-M178 was immobilized on amylose agarose and tested for interaction with HDAC4 or its deletion mutants, synthesized *in vitro* in the presence of [^{35}S]methionine. Input lanes represent 20% of HDAC4 or its mutants used for interaction.

(E) Schematic representation of HDAC4 and its deletion mutants used in the interaction assays (A, C, D). The + symbol denotes that the protein shown at left interacts with MEF2C.

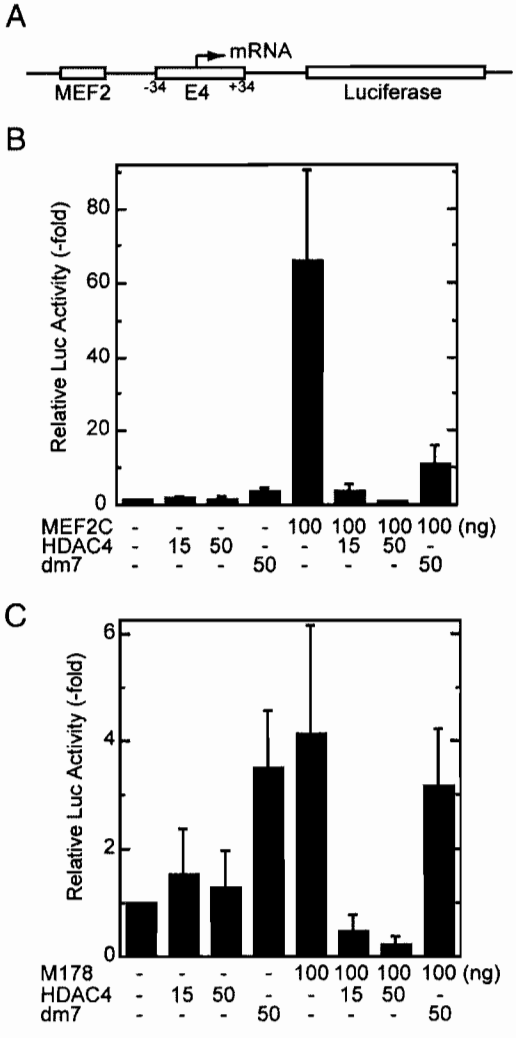
A
mRNA
MEF2
-34
E4
+34
Luciferase
B
Relative Luc Activity (-fold)
0
20
40
60
80
MEF2C - - - - 100 100 100 100 (ng)
HDAC4 - 15 50 - - 15 50 -
dm7 - - - 50 - - - 50
C
Relative Luc Activity (-fold)
0
2
4
6
M178 - - - - 100 100 100 100 (ng)
HDAC4 - 15 50 - - 15 50 -
dm7 - - - 50 - - - 50

Fig. 7 HDAC4 represses transcription in a MEF2C-dependent manner.

(A) Schematic representation of the reporter MEF2-E4-Luc, which contains one copy of the MEF2-binding site upstream from the adenovirus E4 core promoter (-34 to +34) and the luciferase coding sequence.

(B) HDAC4 represses MEF2C-dependent transcription. MEF2-E4-Luc was cotransfected into NIH3T3 cells with the expression plasmids at indicated amounts (ng). Luciferase activities were normalized to the internal β-galactosidase control; the normalized luciferase activity from the transfection without any effector plasmid was arbitrarily set to 1.0.

(C) Recruitment of HDAC4 by the MEF2C mutant M178 leads to repression. Reporter assays were performed as in (B) except that the expression plasmid for M178 was used instead.

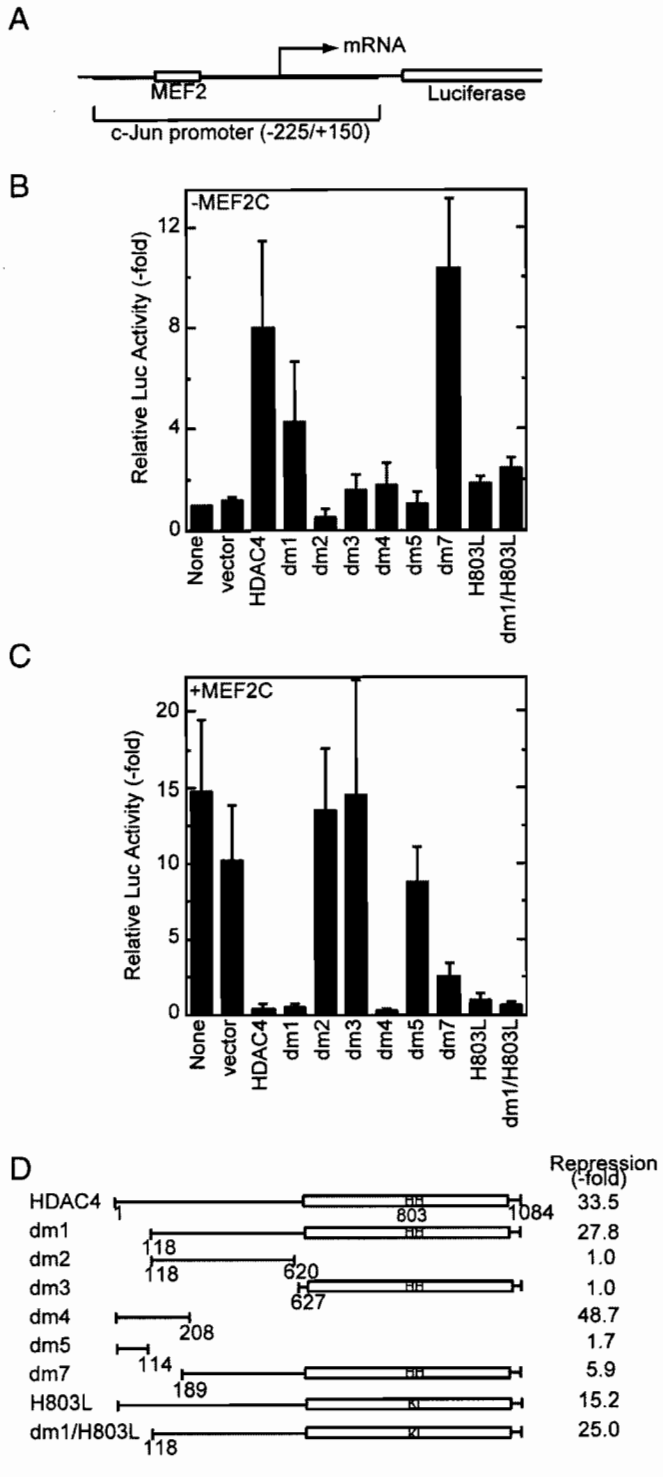
A
mRNA
MEF2
Luciferase
c-Jun promoter (-225/+150)
B
-MEF2C
Relative Luc Activity (-fold)
12
8
4
0
None
vector
HDAC4
dm1
dm2
dm3
dm4
dm5
dm7
H803L
dm1/H803L
C
+MEF2C
Relative Luc Activity (-fold)
20
15
10
5
0
None
vector
HDAC4
dm1
dm2
dm3
dm4
dm5
dm7
H803L
dm1/H803L
D
Repression (-fold)
HDAC4
1
803
1084
33.5
dm1
118
27.8
dm2
118
620
1.0
dm3
627
1.0
dm4
208
48.7
dm5
114
1.7
dm7
189
5.9
H803L
15.2
dm1/H803L
118
25.0

Fig. 8 HDAC4 and MEF2C cooperatively regulate c-Jun promoter activity.

(A) Schematic representation of the reporter pJLuc, which contains the -225/+150 region of the c-Jun promoter upstream of the luciferase coding sequence.

(B) HDAC4 activates c-Jun promoter activity in a MEF2C-independent manner. The pJLuc reporter and expression plasmids for HDAC4 or its mutants were cotransfected into NIH3T3 cells. Luciferase activities were normalized to the internal β-galactosidase control; the normalized luciferase activity from the transfection without any effector plasmid was arbitrarily set to 1.0.

(C) HDAC4 represses MEF2C-dependent transcription. Together with the MEF2C expression plasmid, pJLuc and indicated HDAC4 plasmids were cotransfected into NIH3T3 cells. Reporter assays were determined and calculated as in (B).

(D) Schematic representation of HDAC4 and its mutants used in (B, C). Repression of MEF2C-dependent transcription by each construct is shown at right.

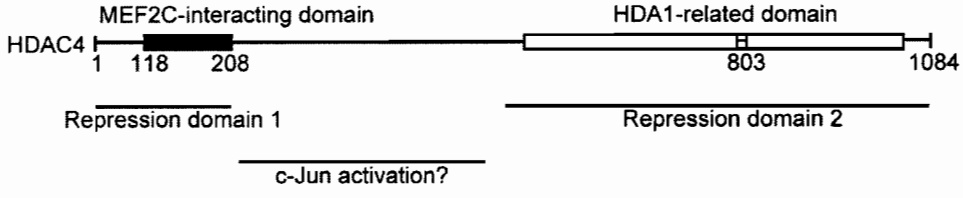
MEF2C-interacting domain
HDA1-related domain
HDAC4
1
118
208
803
1084
Repression domain 1
Repression domain 2
c-Jun activation?

Fig. 9 Functional domain organization of HDAC4.

HDAC4 possesses at least two repression domains, with repression domain 1 located at the N terminus and repression domain 2 at the C terminal part including the HDA1-related deacetylase domain. The MEF2C-interacting domain resides within the N terminal domain; the region C-terminal from the MEF2C-interacting domain may be involved in activation of the c-Jun promoter in the absence of transfected MEF2C.

CHAPTER III

Regulation of the histone deacetylase HDAC4 by binding of 14-3-3 proteins

Preface

In the previous chapter, HDAC4 was demonstrated to possess intrinsic HDAC activity. HDAC4 interacts with transcription factor MEF2 and represses MEF2-dependent transcription. Since transcriptional repression by HDAC4 is a nuclear event, it is expected that HDAC4 is localized in the nucleus to fulfill its work. However, we found that HDAC4 is mainly cytoplasmic, suggesting that the activity of HDAC4 may be regulated by a mechanism controlling its subcellular localization. In this manuscript, inhibition of the nuclear localization of HDAC4 by binding of 14-3-3 proteins is described.

ABSTRACT

Histone (de)acetylation is important for regulation of fundamental biological processes such as gene expression and DNA recombination. Distinct classes of histone deacetylases (HDACs) have been identified, but how they are regulated *in vivo* remains largely unexplored. Here we describe results demonstrating that HDAC4, a member of class II human HDACs, is localized in the cytoplasm and/or the nucleus. Moreover, we have found that HDAC4 interacts with the 14-3-3 family of proteins that are known to bind specifically to conserved phosphoserine-containing motifs. Deletion analyses suggested that S246, S467 and S632 of HDAC4 mediate this interaction. Consistent with this, alanine substitutions of these serine residues abrogated 14-3-3 binding. Although these substitutions had minimal effects on the deacetylase activity of HDAC4, they stimulated its nuclear localization and thus led to enhanced transcriptional repression. These results indicate that 14-3-3 proteins negatively regulate HDAC4 by preventing its nuclear localization and thereby uncover a novel regulatory mechanism for HDACs.

INTRODUCTION

Specific lysine acetylation of histones and non-histone proteins has been recently recognized as a major mechanism by which eukaryotic transcription is regulated (12, 23, 24, 44, 45, 56, 57). Such acetylation is reversible and dynamic *in vivo*, and its level is governed by the opposing actions of histone acetyltransferases (HATs) and deacetylases (HDACs). Distinct classes of HDACs have been identified in mammals (21, 36). Class I HDACs (HDAC1, -2, -3 and -8) are homologous to yeast Rpd3 (8, 16, 49, 60, 61). HDAC1 and HDAC2 interact with each other and form the catalytic core of Sin3 and NuRD complexes, both of which play important roles in transcriptional repression and gene silencing (26, 51, 53, 54, 58, 63-65). Various transcriptional repressors recruit these complexes to inhibit transcription (reviewed in 15, 45, 56). Class II HDACs (HDAC4, -5, -6, and -7) contain domains significantly similar to the catalytic domain of yeast Hda1 (9, 11, 20, 33, 41, 52, 55). HDAC4, HDAC5 and HDAC7 are homologous, whereas HDAC6 has two Hda1-related catalytic domains and a unique Cys/His-rich C-terminal domain. HDAC4 and -5 interact with the MEF2 transcription factors (28, 33, 55), and this interaction is regulated (30, 62). Related to this, MITR/HDRP, a protein related to the N-terminal part of HDAC4, -5 and -7, binds to MEF2s and represses transcription (43, 66). Moreover, HDAC4, -5 and -7 were found to interact with the nuclear receptor corepressors SMRT and N-CoR (13, 17, 20). These new findings suggest that like class I HDACs, some class II HDACs are recruited to promoters to inhibit transcription. One

interesting, but unaddressed, question is how the function of HDACs is regulated *in vivo*.

While HDAC1, -2 and -3 are nuclear, the plant deacetylase HD2 is a nucleolar protein (8, 31). Miska et al. reported that the HDAC4 protein lacking the N-terminal 117 residues is cytoplasmic or nuclear in HeLa cells, whereas Fischle et al. found this mutant predominantly nuclear in the same cell line (9, 33). Importantly, this same mutant is actively exported to the cytoplasm (33). We found that the same mutant is mainly cytoplasmic in NIH 3T3 cells (M.V. & X.J.Y., unpublished observation). Very recently, it was reported that HDAC5 and HDAC7 are nuclear in HeLa and CV-1 cells (20, 28). These findings suggest that the subcellular localization of HDAC4 and its homologs may be regulated in a cell context-dependent manner and that controlled subcellular localization may serve as a regulatory mechanism for these HDACs. However, how such regulation is achieved remains entirely unclear.

Emerging evidence indicates that 14-3-3 proteins function as cytoplasmic anchors for some binding partners (1, 38). For example, 14-3-3 proteins bind to and retain phosphorylated CDC25C, a phosphatase important for initiating the G2/M transition during cell cycle progression, in the cytoplasm (39). It has been recently shown that 14-3-3 proteins also regulate nuclear localization of transcription factors. Upon phosphorylation by the kinase Akt/PKB, the Forkhead transcription factor FKHRL1 interacts with 14-3-3 proteins and is sequestered in the cytoplasm (4). Such regulation may also control nuclear localization of two other transcription factors related to FKHRL1 (3, 22, 46) (reviewed in 6).

Furthermore, the yeast 14-3-3 protein, BMH2, interacts with the transcription factors MSN2 and MSN4 and may regulate their cytoplasmic retention in a TOR kinase-dependent manner (2). Intriguingly, 14-3-3 proteins were found to be part of a HAT1 complex purified from Xenopus oocytes (19). Here, we present evidence that 14-3-3 proteins bind to HDAC4 and sequester it in the cytoplasm, suggesting that 14-3-3 proteins negatively regulate HDAC4 and its homologs by excluding them from the nucleus.

MATERIALS AND METHODS

Molecular cloning. Expression plasmids for HDAC4 and some deletion mutants have been described previously (55). Additional HDAC4 mutants were generated by PCR with the Expand thermostable DNA polymerase (Roche) or by site-directed mutagenesis with single-stranded uracil-containing templates and T7 DNA polymerase. DNA sequencing was performed with T7 Sequenase 2.0 (Amersham Pharmacia Biotech) to confirm the mutations. GFP constructs were derived from pEGFP-C2 (Clontech). Luciferase reporters pJLuc, MEF2-E4-Luc and Gal4-tk-Luc have been described previously (5, 55).

Cytoplasmic and nuclear fractionation. A previously described procedure was used with modifications (5). Briefly, NIH 3T3 cells (~1 x 10^6) were washed twice with PBS and lysed *in situ* using 1 ml of ice-cold hypotonic lysis buffer (20 mM HEPES pH 7.6, 20% glycerol, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, 25 mM NaF, 25 mM β-glycerophosphate, 1 mM DTT and protease inhibitors). After 5 min on ice with occasional shaking, the cell lysate was harvested by scraping and centrifuged for 5 min on a benchtop centrifuge (1,300 *g*) at 4°C. The supernatant was collected, cleared by high-speed centrifugation (10 min @16,000 g) at 4°C, and saved as the cytoplasmic fraction. The nuclei pellet from the low-speed centrifugation was suspended in 0.2 ml of hypotonic lysis buffer containing 0.5 M NaCl and rotated for 20 min at 4°C. After high-speed centrifugation, the supernatant was collected as the nuclear extract.

Immunofluorescence microscopy. Subconfluent and cycling NIH 3T3, 293, COS1, or SKN (SK-N-SH; ATCC) cells growing on glass coverslips in complete

J-MEM medium (Gibco) were transfected using the Lipofectamine liposome reagent (Gibco). Briefly, 1 μg of Flag-tagged HDAC4 expression construct and 12 μl of Lipofactamine were used to transfect cells on a coverslip. Cells were incubated with the plasmid-liposome complex containing serum-free medium for 3 h prior to washing once with PBS and addition of complete medium. After 15 h, cells were fixed with PBS/1% paraformaldehyde at RT for 10 min. After being washed once with PBS, cells were permeabilized with PBS/0.5% Triton X-100 for 5 min at RT. Cells were again washed once with PBS and incubated with the α-FLAG M2 monoclonal antibody (Sigma) for 60 min at RT. Cells were washed once with PBS and incubated with goat anti-mouse IgG conjugated with Alexa 488 (Molecular Probes) for 60 min at RT. Cells were washed once with PBS and mounted on glass slides using a glycerol-based mounting medium containing the anti-fade agent para-phenylene-diamine (0.1 mg/ml; Sigma) and 4,6-diamidino-2-phenylindole (DAPI, 30 μg/ml; Sigma). Labeled cells were visualized using a digital deconvolution epifluorescence microscope (Leica); images were collected using a digital camera containing a 14-bit detector (Princeton Instruments) and further processed with Adobe Photoshop.

Alternatively, NIH 3T3 or 293 cells (2×10^4) were seeded on coverslips in a 12-well plate and transfected with 0.1 μg of a Flag- or GAl4-tagged HDAC4 expression plasmid using 2~5 μl of Superfect (Qiagen). 15-24 h later, cells were rinsed three times with PBS/1 mM $MgCl_2$/0.1 mM $CaCl_2$ and further processed for immunofluorescence microscopy with the α-Flag (1:300; Sigma) or α-Gal4

(RK5C1; Santa Cruz Biotechnology) antibody as described (32). For nuclear staining, either DAPI or Hoechst 33258 (20 ng/ml; Sigma) was used.

Live green fluorescence microscopy. Expression plasmids for GFP fusion proteins were transfected, with SuperFect, into NIH 3T3, 293 or SKN cells cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum and antibiotics. 16 h post transfection, transfected cells were subjected to live green fluorescence microscopy using a Zeiss Axiovert 135 microscope equipped with a temperature-adjustable platform and linked to a CCD camera (Princeton Instruments) controlled by a Hewlett Packard computer running Northern Eclipse (Empix Imaging). Images were taken and exported to a PowerMac computer for further processing with Adobe Photoshop.

To quantify transfected cells with different subcellular localization of GFP fusion proteins, transfected cells with green fluorescence were counted under the fluorescence microscope by eye. For each GFP fusion protein construct, 100-400 cells with green fluorescence were counted per experiment; at least three independent transfection experiments were performed to obtain consistent results.

Protein-protein interaction. To examine the interaction between HDAC4 and 14-3-3 proteins, the Flag-HDAC4 expression plasmid was cotransfected into 293 cells with or without an expression plasmid for HA-tagged human 14-3-3β. 3 μg of each plasmid was used to transfect 4 x 10^5 cells (in a 6 cm dish) with 9 μl of SuperFect transfection reagent. 48 h after transfection, cells were washed twice with PBS and collected in 0.5 ml of buffer B (20 mM Tris-HCl, pH 8.0, 10%

glycerol, 5 mM $MgCl_2$, 0.1% NP-40 and protease inhibitors) containing 0.15 or 0.5 M KCl. Cell extracts were prepared for affinity purification on M2 agarose beads (Sigma) or for immunoprecipitation with the mouse α-HA monoclonal antibody (Babco) and UltraLink™ Immobilized Protein A/G beads (Pierce). Beads with bound immunocomplexes were washed four times with buffer B supplemented with 0.15 or 0.5 M KCl, and bound proteins were eluted with the Flag peptide (Sigma) or SDS sample buffer. Eluted proteins were subsequently resolved on 10% SDS-PAGE and transferred to nitrocellulose membranes for Western analysis with the α-Flag or α-HA antibody. Blots were developed with Supersignal chemiluminescent substrates (Pierce).

To examine the interaction of Flag-HDAC4 with endogenous 14-3-3, Flag-HDAC4 was expressed in and purified from 293 cells as described above. Bound proteins were eluted and subjected to Western analysis with α-14-3-3 antibodies (K-19 and H-8; Santa Cruz Biotechnology). The interaction of Flag-tagged HDAC4 mutants with 14-3-3 proteins was similarly analyzed.

For interaction between endogenous HDAC4 and 14-3-3 proteins, NIH3T3 cell extracts (~1.5 mg in 0.4 ml of buffer B supplemented with 150 mM KCl and 50 mM NaF) were mixed with preimmune IgG or rabbit α-HDAC4 antibody and incubated at 4°C for 1 h. 20 μl (bed volume) of UltraLink™ Immobilized Protein A/G beads was added; after being rotated overnight at 4°C, the beads were washed extensively with buffer B supplemented with 150 mM KCl and 50 mM NaF. Bound immunocomplexes were eluted by boiling in the SDS smaple buffer

and subjected to Western analysis with the rabbit α-HDAC4 or the mouse α-14-3-3 antibody (H-8; Santa Cruz Biotechnology).

HDAC assays. Flag-tagged HDAC4 and mutant proteins were expressed in and purified from 293 or 293T cells as described above. HDAC assays were carried out using [^{3}H]acetyl-histones prepared from HeLa cells as described (55).

Reporter gene assays. Reporter assays were performed as described except that transfected cells were lysed for measurement of reporter activities 24 h post transfection (55).

RESULTS

Subcellular localization of HDAC4. For examination of subcellular localization of HDAC4, a rabbit polyclonal antibody was raised. This antibody detected Flag-HDAC4 expressed in and affinity-purified from 293 cells (Fig. 1A, lane 1). Western analyses of cytoplasmic and nuclear extracts of NIH 3T3 cells revealed that HDAC4 is mainly in the cytoplasmic fraction (lanes 2-3). As expected, α-14-3-3 and α-MEF2D antibodies detected 14-3-3 and MEF2D in the cytoplasmic and nuclear fractions, respectively (lanes 5-6). These results indicate that in NIH 3T3 cells, endogenous HDAC4 is mainly localized in the cytoplasm.

To examine the subcellular localization of HDAC4 in live cells, we performed green fluorescence microscopy. For this, a mammalian vector was constructed to express the fusion protein GFP-HDAC4, with HDAC4 fused to the carboxyl terminus of enhanced green fluorescent protein. This construct was transfected into NIH 3T3 cells, and live transfected cells were examined for green fluorescence. While GFP itself was pancellular, GFP-HDAC4 was predominantly cytoplasmic in ~90% of the NIH 3T3 cells transfected (Fig. 1B, left panel; data not shown). Similarly, unlike GFP, GFP-HDAC4 was cytoplasmic in most 293 cells transfected (Fig. 1B, middle panel). In a small portion of 293 cells transfected, GFP-HDAC4 was either pancellular or mainly in the nucleus, where it formed dot-like structures (Fig. 1B, right panel). Compared to NIH 3T3 and 293 cells, more SKN cells (~25%) expressed GFP-HDAC4 in the nucleus (Fig. 1C, cells a and b). Taken together, these results indicate that HDAC4 is localized in the cytoplasm and/or the nucleus in a manner dependent on cellular context.

The distinct subcellular localization of HDAC4 suggests that it may be actively shuttled between the cytoplasm and the nucleus. To address this, we treated transfected SKN cells with leptomycin B (LMB), a specific inhibitor of CRM1-mediated nuclear export (10, 25, 37). As shown in Fig. 1C, LMB elicited rapid nuclear translocation of GFP-HDAC4 in cell b, and after 40 min, GFP-HDAC4 was localized in nuclear dots. LMB treatment of NIH 3T3 and 293 cells also induced nuclear accumulation of GFP-HDAC4 in discrete dots (data not shown). Therefore, like the HDAC4 protein lacking its N-terminal 117 residues (33), full-length HDAC4 is actively exported to the cytoplasm in a CRM1-dependent manner.

HDAC4 interacts with 14-3-3 proteins. Subcellular compartmentation of HDAC4 may serve as a regulatory mechanism to control its repression function. We thus asked how HDAC4 might be retained in the cytoplasm. One possibility is that cytoplasmic anchor proteins are involved. 14-3-3 proteins have been shown to regulate the translocation of FKHRL1 and CDC25C from the nucleus to the cytoplasm (4, 39). 14-3-3 proteins bind to two types of consensus sites: R-[S/Ar]-[+/S]-pS-[L/E/A/M]-P and R-X-[Ar/S]-[+]-pS-[LEAM]-P, where Ar is an aromatic amino acid, pS is phosphoserine, + is a basic amino acid, and X is any amino acid (40, 59). However, atypical 14-3-3 binding sites have also been reported (29). Moreover, 14-3-3 proteins bind to two R-X-R-X-X-pS/T motifs of FKHRL1 (4). With these considerations, we inspected the HDAC4 sequence, and found that HDAC4 contains five potential 14-3-3 binding sites: 242-RKTASEP-248, 464-RTQSAP-469, 516-RQPESHP-522, 629-RAQSSP-632, and 703-

RGRKATL-709, where the conserved residues are underlined. This observation led us to postulate that HDAC4 may interact with 14-3-3 proteins.

To test this hypothesis, we performed immunoprecipitation. Expression plasmids for Flag-HDAC4 and HA-14-3-3β were transfected into 293 cells, and cell extracts were prepared for affinity purification on the α-Flag M2 agarose or for immunoprecipitation with an α-HA antibody. As shown in Fig. 2A (top), Flag-HDAC4 was specifically co-precipitated with HA-14-3-3β. Reciprocally, HA-14-3-3β was specifically co-precipitated with Flag-HDAC4 (Fig. 2A, bottom).

We also examined the interaction of endogenous HDAC4 and 14-3-3 proteins by using α-HDAC4 and α-14-3-3 antibodies. As shown in Fig. 2B (top), the α-HDAC4 antibody specifically precipitated endogenous HDAC4. Importantly, the same antibody also precipitated 14-3-3 proteins (Fig. 2B, bottom), further supporting that HDAC4 associates with 14-3-3 proteins.

S246, S467 and S632 of HDAC4 mediate the 14-3-3 binding. Next we mapped the 14-3-3 binding sites on HDAC4. We first utilized a series of HDAC4 deletion mutants that were already available in our lab. Some of these mutants have been described previously (55). These deletion mutants were expressed in 293 cells and affinity-purified on α-Flag M2 agarose, and their ability to copurify 14-3-3 proteins was assessed by immunoblotting. As demonstrated above, endogenous 14-3-3 proteins co-purified with Flag-HDAC4 (Fig. 3A, compare lanes 1 and 2). 14-3-3 isoforms have similar properties in binding to their partners (40). These results, therefore, confirm that HDAC4 physically interacts with 14-3-3 proteins. Like full-length HDAC4 (lanes 1-2), the mutants hm1-5 co-

precipitated 14-3-3 proteins (lanes 3-7). This suggests that residues 531-1084 of HDAC4 contain one or more 14-3-3 binding site (Fig. 3B). To test if S632 is essential for 14-3-3 binding, we replaced it with alanine to generate the mutant hm6 (Fig. 3B). This mutant was unable to bind to 14-3-3 proteins (Fig. 3A, lane 8), indicating that S632 but not T708 is important for 14-3-3 binding.

Unlike hm7, hm8 was able to bind to 14-3-3 proteins (Fig. 3A, lanes 9 and 10), indicating that there are 14-3-3 binding sites between amino acids 208-620 of HDAC4 (Fig. 3B). Additional deletion mutants (hm9-11) were analyzed and all found to bind 14-3-3 proteins (Fig. 3C & 3D, lanes 1-2). This led us to test if S246 of HDAC4 is important for 14-3-3 binding by replacing S246 with alanine to generate the mutant hm12 (Fig. 3B). This mutant was indeed defective in 14-3-3 binding (Fig. 3D, lane 3), indicating that S246 is important for 14-3-3 binding. To address if S467 is required for 14-3-3 binding, the mutants hm13 and hm14 were generated (Fig. 3B). Unlike hm13, hm14 was defective in 14-3-3 binding (Fig. 3D, lanes 4-5), indicating that S467 is important for 14-3-3 binding. To assess whether S520 is involved in 14-3-3 binding, the mutant hm15 was tested (Fig. 3B). This mutant was defective in 14-3-3 binding (Fig. 3D, lane 6). Taken together, these mapping results indicate that S246, S467 and S632 of HDAC4 mediate the binding of 14-3-3 proteins.

For verification of this conclusion and analysis of functional consequences of 14-3-3 binding, point mutations were introduced at S246, S467 and/or S632 of full-length HDAC4, generating the mutants S246A, S467A, S632A, S246/467A, S246/632A, S467/632A and S246/467/632A. Among these mutants, only

S246/467/632A was completely defective in 14-3-3 binding (Fig. 3E; data not shown). These results confirm that S246, S467 and S632 of HDAC4 are all involved in 14-3-3 binding.

14-3-3 binding inhibits nuclear localization of HDAC4. Next we wished to determine the functional consequences of 14-3-3 binding to HDAC4. The cytoplasmic localization of HDAC4 and its association with 14-3-3 proteins led us to test whether 14-3-3 binding regulates the subcellular localization of HDAC4. To this end, we constructed GFP expression plasmids for the full-length mutants S246A, S467A, S632A, S246/467A, S246/632A, S467/632A and S246/467/632A. Subcellular localization of these fusion proteins was examined by live cell fluorescence microscopy. Like the wild-type GFP-HDAC4, the mutants with single mutations were predominantly cytoplasmic in NIH 3T3 cells (Fig. 4A, B). For the mutants with two substitutions, GFP-S246/467A and -S246/632A were nuclear in a majority of transfected cells whereas fewer cells expressed GFP-S467/632A in the nucleus (Fig. 4A, B), suggesting that compared to S467 and S632, S246 plays a more important role in controlling the subcellular localization of HDAC4. The triple mutant GFP-S246/467/632A was nuclear in most transfected cells, and occupied discrete nuclear dots (Fig. 4A). Similar results were obtained with these mutants in 293 cells (Fig. 4C; data not shown). Since S246, S467 and S632 are important for 14-3-3 binding, these results suggest that 14-3-3 proteins bind to HDAC4 and sequester it in the cytoplasm.

14-3-3 binding does not affect the deacetylase activity of HDAC4. The 14-3-3 binding sites were mapped to the N-terminal half of HDAC4, whereas its

catalytic domain is located at the C-terminal part. The N-terminal truncations of HDAC4 lead to some activation of its deacetylase activity (9, 55). Moreover, 14-3-3 proteins are known to directly regulate the activity of several enzymes. We thus assessed effects of 14-3-3 binding on the enzymatic activity of HDAC4. As shown in Fig. 5, the mutant S246/467/632A was almost as active as wild-type HDAC4, suggesting that 14-3-3 binding has minimal effects on the deacetylase activity of HDAC4.

14-3-3 binding inhibits the repression potential of HDAC4. Since HDAC4 and its related proteins repress MEF2-dependent transcription (28, 30, 33, 43, 55, 66), we asked whether cytoplasmic retention of HDAC4 indirectly inhibits its repression function. To address this, we conducted reporter gene assays to compare the repression ability of HDAC4 and its mutant S246/467/632A. We first tested MEF2-E4-Luc, which contains a MEF2 consensus site upstream from the adenovirus E4 core promoter driving the Luc gene. As shown in Fig. 6A, 50 ng of the HDAC4 construct resulted in reduction of the MEF2C-stimulated reporter activity to the basal level, whereas 10 ng of the mutant construct achieved a similar level of repression. We also tested pJLuc, a Luc reporter driven by the c-Jun promoter (-225/+150) that is known to contain a MEF2 binding site (14). Compared to wild-type HDAC4, the mutant S246/467/632A was apparently more potent in repressing pJLuc reporter activity (Fig. 6B). To test whether expression of HDAC4 and its mutant leads to generalized repression, we cotransfected the reporter Gal4-tk-Luc with an expression plasmid for Gal4-VP16. As shown in Fig. 6C, HDAC4 and its triple

mutant had minimal effects on the activation mediated by Gal4-VP16, suggesting that expression of HDAC4 and its mutant does not lead to global repression.

We also assessed the apparent repression ability of HDAC4 and its mutant by artificially tethering them to a promoter. For this, HDAC4 and its mutant were expressed as proteins fused to the Gal4 DNA binding domain and tested for the ability to inhibit the reporter activity of Gal4-tk-Luc. As shown in Fig. 6D, Gal4-S246/467/632A was much more potent than Gal4-HDAC4 in repressing Gal4-tk-Luc reporter activity. Indirect immunofluorescence experiments with an α-Gal4 antibody revealed that unlike Gal4-HDAC4, Gal4-S246/467/632A was predominantly nuclear in NIH 3T3 cells (data not shown). Taken together, these results support that 14-3-3 proteins sequester HDAC4 away from its targets in the nucleus and thereby indirectly inhibit its repression function.

DISCUSSION

HDAC4 is localized in the cytoplasm and/or the nucleus. The results presented herein support that HDAC4 is localized in the cytoplasm and/or the nucleus. This is consistent with reports on the subcellular localization of the HDAC4 protein lacking the N-terminal 117 residues (9, 33). An interesting question is why even for the same cell line, HDAC4 is nuclear in some cells but cytoplasmic in the other (Fig. 1) (33). One possibility is that cell cycle progression may affect the subcellular localization. However, we did not find evidence that the subcellular localization of HDAC4 is regulated during cell cycle (data not shown). Other possibilities include growth conditions, extracellular signalling events, and heterogeneity of cells in the cell lines used. Clearly, these interesting issues merit further investigation. While HDAC4 was evenly distributed in the cytoplasm, it occupied dot-like patterns in the nucleus (Figs. 1 & 4). Such nuclear dots have been observed by others (20, 33), but their physiological significance remains to be established.

The cytoplasmic and nuclear localization of HDAC4 suggests that it may have functions in both compartments. Alternatively, such a subcellular localization may simply serve as a regulatory mechanism for HDAC4. Since HDAC4 is known to be involved in transcriptional regulation (30, 33, 55), its cytoplasmic localization may negatively regulate its function in the nucleus. Indeed, the nuclear localization of HDAC4 is negatively regulated by binding to 14-3-3 proteins (Figs. 2-6). This also suggests that in analogy with DNA-binding transcription factors, the control of nuclear localization is an important regulatory

mechanism for transcriptional coregulators. This is the case for at least two other coregulators, β-catenin and activated Notch (7, 42).

The distinct subcellular localization of HDAC4 also suggests that it is actively shuttled between the cytoplasm and the nucleus. Consistent with this suggestion, LMB treatment was found to elicit nuclear accumulation of GFP-HDAC4 (Fig. 1C). Since LMB specifically inhibits CRM1 (10, 25, 37), HDAC4 may be actively exported in a CRM1-dependent manner. Using known consensus nuclear import and export sequences (35), we inspected the amino acid sequence of HDAC4 and found that it contains three putative bipartite nuclear localization signals and three potential leucine-rich nuclear export signals. Therefore, HDAC4 possesses putative intrinsic nucleocytoplasmic trafficking signals. It is tempting to speculate that the subcellular localization of HDAC4 is dependent on its nuclear import as well as on its nuclear export (Fig. 7). If its nuclear import dominates, more HDAC4 molecules end up in the nucleus, or vice versa. Therefore, factors that alter its nuclear import, export or both will also affect the subcellular localization of HDAC4.

Physical association of HDAC4 with 14-3-3 proteins. Besides its putative nuclear localization and export signals, HDAC4 also contains five putative 14-3-3 binding sites (Fig. 3B). Importantly, we have found that HDAC4 interacts with 14-3-3 proteins (Figs. 2-3). Among the five putative 14-3-3 binding sites on HDAC4, only S246, S467 and S632 appeared to mediate the interaction (Fig. 3A-D). Consistent with this, the triple mutant S246/467/632A was completely incapable of binding to 14-3-3 proteins (Fig. 3E). These findings

indicate that HDAC4 possesses three functional 14-3-3 binding sites. By contrast, CDC25C contains only one 14-3-3 binding site (39). 14-3-3 proteins exist as homodimers in the cells (40, 59), so one molecule of HDAC4 may bind to two 14-3-3 homodimers with one of their four phosphoserine-binding pockets free. Interestingly, 14-3-3 proteins contain functional nuclear export signals within their binding pockets (40), raising the possibility that 14-3-3 proteins bind to HDAC4 and provide it with functional nuclear export signals (see below).

Regulation of HDAC4 by binding of 14-3-3 proteins. What is the functional consequence of 14-3-3 binding to HDAC4? The HDAC4 mutant S246/467/632A had deacetylase activity comparable to that of the wild-type protein (Fig. 5), suggesting that 14-3-3 binding does not to affect the deacetylase activity of HDAC4. Significantly, unlike the wild-type HDAC4 protein, the triple mutant S246/467/632A was predominantly nuclear (Fig. 4). This is consistent with the finding that this triple mutant was apparently more potent than the wild-type protein in reporter gene assays (Fig. 6D). Therefore, 14-3-3 binding negatively regulates the repression function of HDAC4 by interfering with its nuclear localization. Such a regulatory mode is similar to those reported for CDC25C (39) and Forkhead transcription factors (4, 6), but different from that reported for a homeodomain transcription factor (48).

How does 14-3-3 binding lead to the cytoplasmic accumulation of HDAC4? As discussed above, HDAC4 is actively shuttled between the cytoplasm and the nucleus, and any factors that alter its nuclear import, nuclear export or both also affect its subcellular localization. We speculate that without 14-3-3 binding, the

nuclear import of HDAC4 may prevail and lead to its nuclear localization. Consistent with this speculation, the triple mutant S246/467/632A was incapable of binding to 14-3-3 proteins and thus predominantly nuclear (Fig. 4). With 14-3-3 binding, the dynamic shuttling of HDAC4 may be shifted towards cytoplasmic accumulation. Therefore, 14-3-3 binding plays a contributing role in determining the subcellular localization of HDAC4. 14-3-3 binding may interfere with the nuclear import of HDAC4. Related to this, there are two putative nuclear localization signals close to the S246 14-3-3 binding site of HDAC4, and 14-3-3 binding to S246 of HDAC4 plays an important role in regulating the subcellular localization of HDAC4 (Fig. 4). Alternatively, association with 14-3-3 proteins may stimulate the nuclear export of HDAC4. Indeed, each 14-3-3 isoform contains a functional nuclear export signal (40). Therefore, we propose that 14-3-3 proteins sequester HDAC4 in the cytoplasm by directly hindering its nuclear import and/or facilitating its nuclear export (Fig. 7). A third possibility is that 14-3-3 proteins simply serve as cytoplasmic anchors for HDAC4. Further studies are needed to distinguish these possibilities.

Once in the nucleus, HDAC4 may initiate the assembly of fully functional repression complexes by association with DNA-binding transcription factors like MEF2s (28, 33, 55) and transcriptional corepressors such as HDAC3 (11) and SMRT/N-CoR (13, 17, 20). 14-3-3 binding to HDAC4 may serve as a switch that controls the assembly of these repression complexes. How is this switch turned on and off? As 14-3-3 proteins are known phosphoserine-binding adaptors (34, 40, 59), they may bind to HDAC4 in a phosphorylation-dependent manner. This

is supported by the finding that substitutions of S246, S467 and S632 of HDAC4 with the non-phosphorylable residue alanine abolished 14-3-3 binding (Fig. 3). Phosphorylation of these three serine residues may be controlled by known or unknown protein kinases and phosphatases. Consistent with this, we have found that Flag-HDAC4 is heavily phosphorylated in 293 cells (data not shown). How the interaction of HDAC4 with 14-3-3 proteins is regulated by phosphorylation is an interesting question merits further investigation.

Like HDAC4, HDAC5 and HDAC7 contain putative 14-3-3 binding sites (9, 11, 20, 33, 52, 55), so HDAC5 and HDAC7 may be subject to similar regulation by 14-3-3 proteins. On the other hand, HDAC6 and Hda1 possess no obvious 14-3-3 binding motifs (11, 33, 41, 52, 55). So, 14-3-3 proteins may regulate a subfamily of class II HDACs by affecting their subcellular localization. Interestingly, the subcellular localization of the recently-identified NAD-dependent deacetylase SIR2 may be also regulated (18, 27). Furthermore, it has been recently reported that chicken HDAC3 may be subject to active nuclear export (47). Therefore, controlled subcellular compartmentation may be one general regulatory mechanism for members of different classes of HDACs.

In summary, this study supports that HDAC4 is localized in the cytoplasm and/or the nucleus. Through S246, S467 and S632, HDAC4 interacts with the 14-3-3 family of proteins. Moreover, the binding of 14-3-3 proteins negatively regulates the function of HDAC4 by excluding it from the nucleus. Future experiments on how the association of 14-3-3 proteins with HDAC4 and perhaps

its homologs is regulated shall shed light on the molecular mechanisms by which deacetylation of acetylated histones and non-histone proteins is controlled *in vivo*.

ACKNOWLEDGEMENTS

We thank J. Th'ng for advice on isolation of [^{3}H]acetyl histones, M. Yoshida for leptomycin B, R. Prywes for α-MEF2D antibody, M. Park and her lab members for kind help with fluorescence microscopy, and V. Giguère for helpful discussions. This work was supported by funds from the National Cancer Institute of Canada and the Medical Research Council (MRC) of Canada (to X.J.Y.). A.H.W. is the recipient of an MRC doctoral research award. J.W. received support from the Lady Davis Medical Institute, Montreal, Quebec, Canada. X.J.Y. is an MRC scholar. A.H.W., M.J.K. and J. W. made equally significant contributions to this work.

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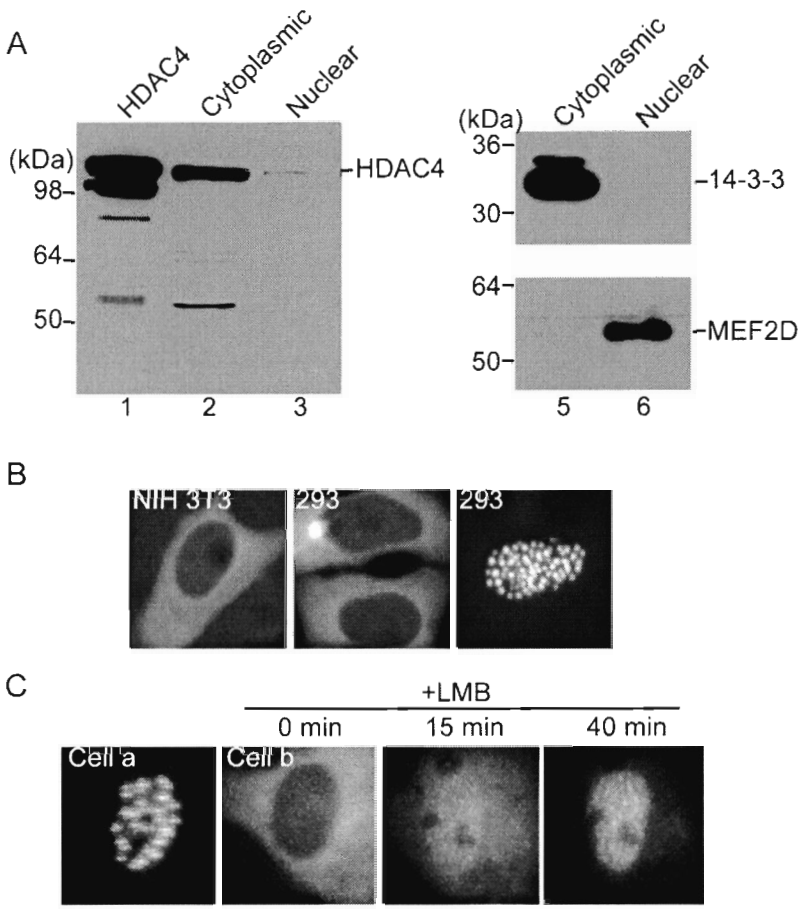

A
HDAC4
Cytoplasmic
Nuclear
(kDa)
98
64
50
HDAC4
1
2
3
(kDa)
Cytoplasmic
Nuclear
36
30
14-3-3
64
50
MEF2D
5
6
B
NIH 3T3
293
293
C
+LMB
0 min
15 min
40 min
Cell a
Cell b

Fig. 1 Cytoplasmic localization of HDAC4.

(A) Affinity-purified Flag-HDAC4 (lane 1), cytoplasmic (lanes 2 & 5) and nuclear (lanes 3 & 6) extracts of NIH 3T3 cells were subjected to immunoblotting with the α-HDAC4 (lanes 1-3), α-14-3-3 (lanes 5-6, *top*) or α-MEF2D (lanes 5-6, *bottom*) antibody. The amount of extracts was normalized according to cell numbers. The 55 kDa band on lane 2 may not be specific since it was not reproducibly detected by different bleeds of the α-HDAC4 antibody.

(B) Representative green fluorescence images of NIH 3T3 and 293 cells expressing GFP-HDAC4.

(C) Green fluorescence images of two SKN cells (cells a and b) expressing GFP-HDAC4. After initial examination for green fluorescence, LMB (10 ng/ml) was added to the medium and cell b was then analyzed for redistribution of green fluorescence at the indicated times. Under similar conditions, LMB had minimal effects on the pancellular localization of GFP itself (data not shown).

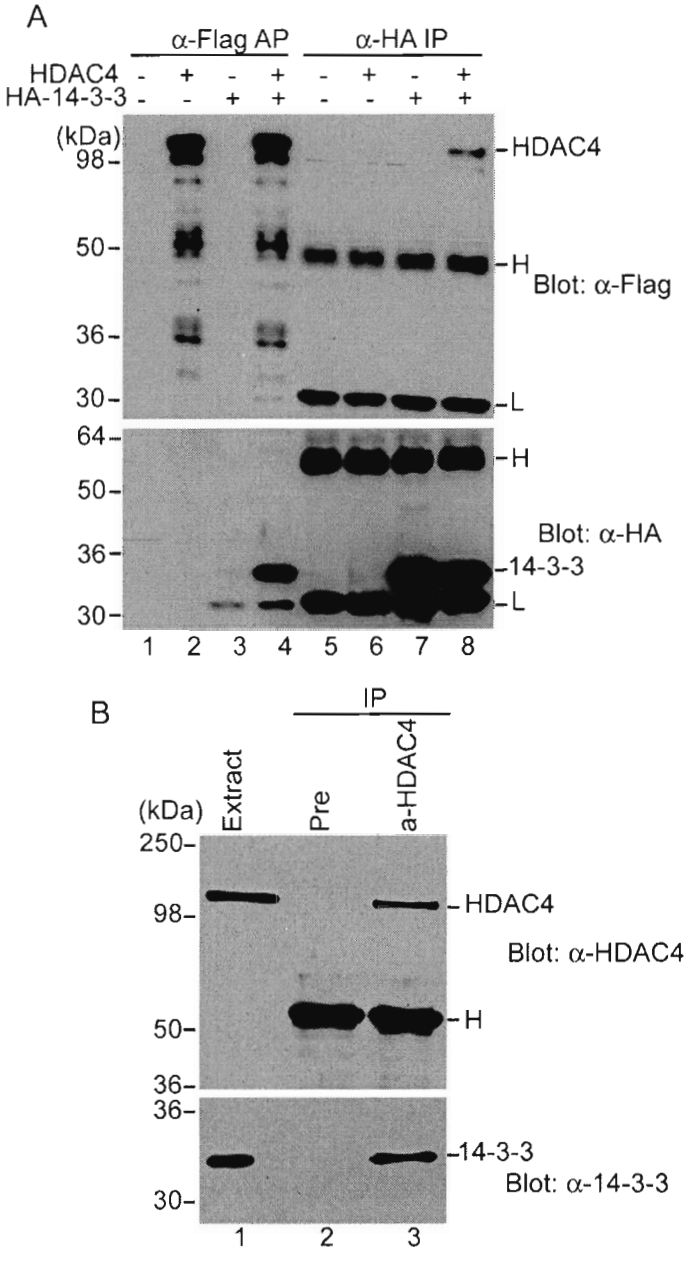

A
α-Flag AP
α-HA IP
HDAC4 - + - + - + - +
HA-14-3-3 - - + + - - + +
(kDa)
98
50
36
30
64
50
36
30
HDAC4
H
Blot: α-Flag
L
H
Blot: α-HA
14-3-3
L
1 2 3 4 5 6 7 8
B
IP
Extract
Pre
a-HDAC4
(kDa)
250
98
50
36
36
30
HDAC4
Blot: α-HDAC4
H
14-3-3
Blot: α-14-3-3
1 2 3

Fig. 2 HDAC4 interacts with 14-3-3.

(A) Expression plasmids for Flag-HDAC4 and HA-14-3-3β were cotransfected into 293 cells as indicated. 48 h after transfection, cell extracts were prepared for affinity-purification (AP) on M2 agarose beads (lanes 1-4) or immunoprecipitation (IP) with the α-HA monoclonal antibody (lanes 5-8). Bound proteins, eluted with Flag peptide (lanes 1-4) or the SDS sample buffer (lanes 5-8), were subjected to Western analyses with the α-Flag (*top*) or α-HA antibody (*bottom*). H, IgG heavy chain; L, light chain. Note that on lanes 1-4, no heavy and light chain bands are visible because the bound antigens were eluted with Flag peptide from M2 agarose beads, on which the α-Flag antibody is covalently crosslinked. Whether the bands at light chain position on lanes 3-4 (*bottom*) are due to light chains is unclear.

(B) NIH 3T3 extracts (lane 1) were subjected to immunoprecipitation with a rabbit preimmune IgG (lane 2) or the rabbit α-HDAC4 antibody (lane 3) and subsequent Western analysis with the rabbit α-HDAC4 antibody (*top*) or a mouse α-14-3-3 monoclonal antibody (*bottom*).

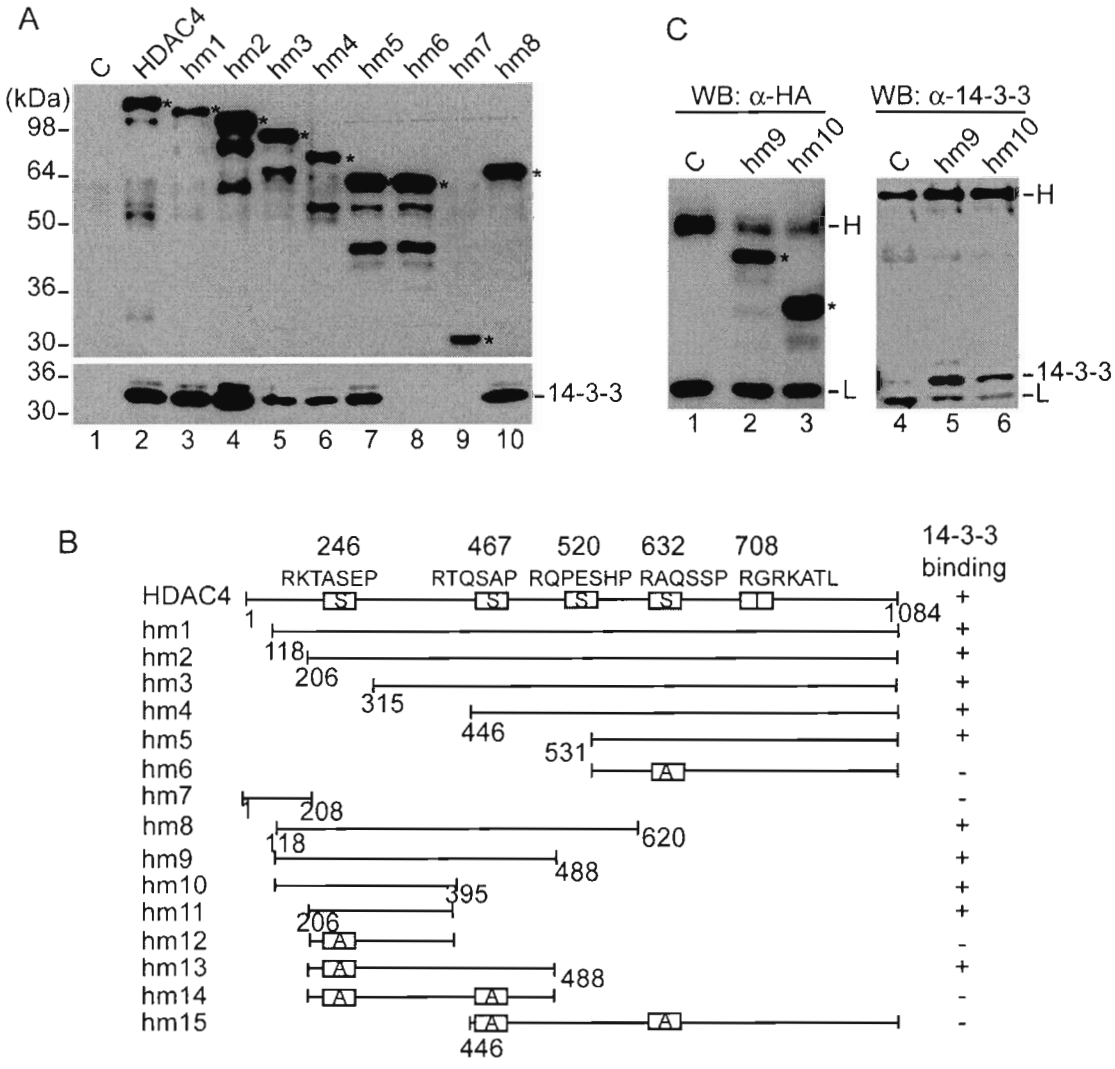

A
C
HDAC4
hm1
hm2
hm3
hm4
hm5
hm6
hm7
hm8
(kDa)
98-
64-
50-
36-
30-
36-
30-
14-3-3
1 2 3 4 5 6 7 8 9 10
WB: α-HA
WB: α-14-3-3
C
hm9
hm10
H
L
14-3-3
1 2 3 4 5 6
B
246
467
520
632
708
RKTASEP
RTQSAP
RQPESHP
RAQSSP
RGRKATL
14-3-3 binding
HDAC4
1
1084
hm1
hm2
118
hm3
206
hm4
315
hm5
446
hm6
531
hm7
hm8
208
620
hm9
488
hm10
395
hm11
206
hm12
hm13
488
hm14
hm15
446
D
(kDa)
C
hm11
hm12
hm13
hm14
hm15
64-
50-
36-
30-
14-3-3
1 2 3 4 5 6
E
HDAC4
S246/467A
S246/632A
S467/632A
S246/467/632A
(kDa)
98-
64-
50-
36-
30-
14-3-3
1 2 3 4 5

Fig. 3 Mapping of 14-3-3 binding sites.

(A) Expression plasmids for HDAC4 and its deletion mutants (all Flag-tagged) were transfected into 293 cells, and cell extracts prepared for affinity purification on M2 agarose. Bound proteins were eluted with the Flag peptide and subjected to Western analyses with the α-Flag (*top*) or α-14-3-3 (*bottom*) antibody. C (lane 1), control affinity-purification using non-transfected cells. For HDAC4 proteins, bands with expected molecular weights are indicated by asterisks.

(B) Schematic representation of HDAC4 and its mutants, with their 14-3-3 binding ability indicated at right.

(C) Expression plasmids for HA-tagged hm9 and hm10 were transfected into 293 cells and cell extracts were prepared for immunoprecipitation with the α-HA antibody. Immunocomplexes were subjected to immunoblotting with the α-HA (lanes 1-3) or α-14-3-3 antibody (lanes 4-6). H, IgG heavy chain; L, light chain.

(D, E) Interaction of Flag-tagged deletion mutants hm11-15 and full-length point mutants with 14-3-3 proteins. The migration difference between hm11 and hm12 may be due to differential phosphorylation. The Flag-tagged HDAC4 proteins were expressed, affinity-purified and analyzed as in (A).

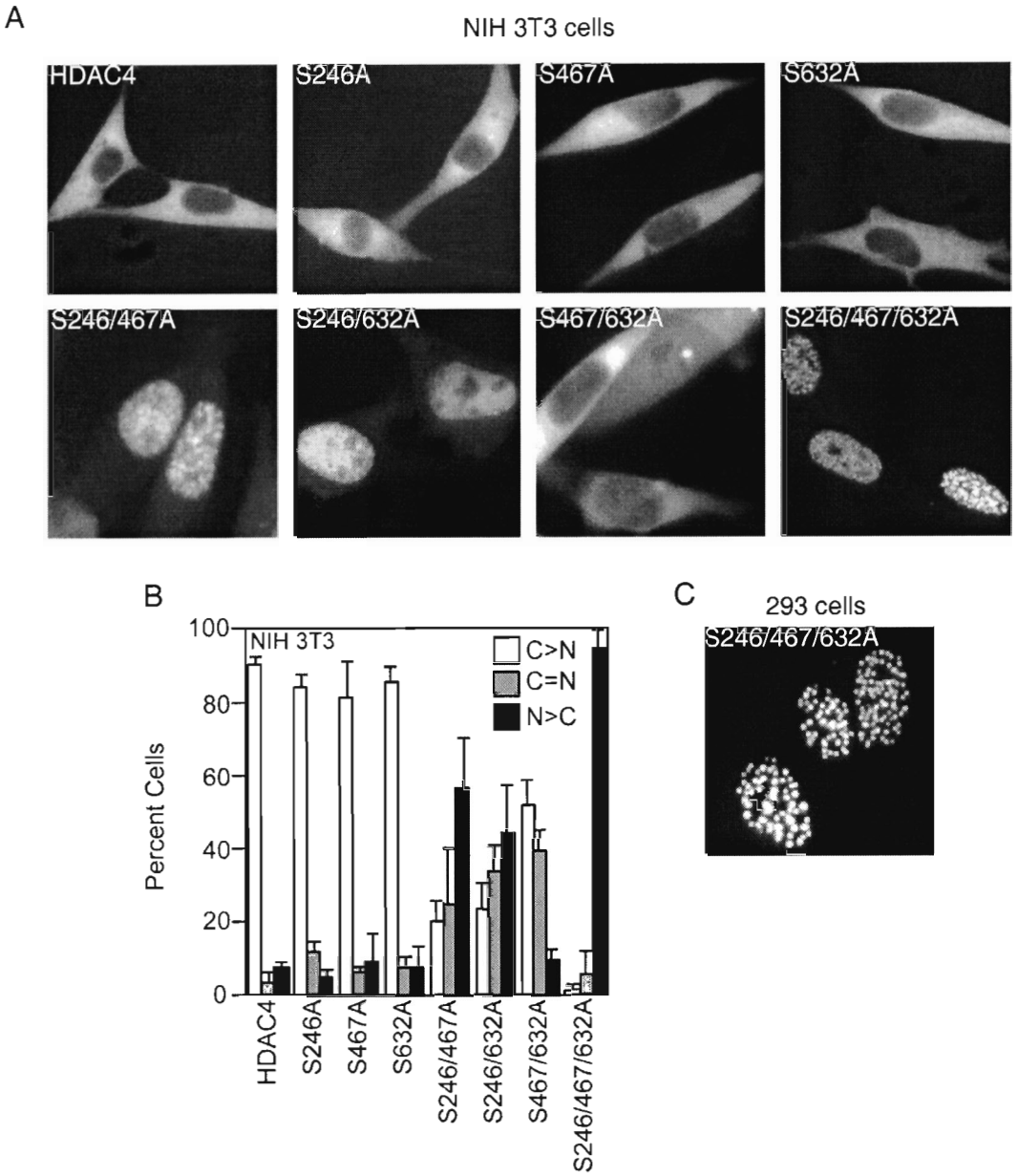

A
NIH 3T3 cells
HDAC4
S246A
S467A
S632A
S246/467A
S246/632A
S467/632A
S246/467/632A
B
NIH 3T3
C>N
C=N
N>C
Percent Cells
100
80
60
40
20
0
HDAC4
S246A
S467A
S632A
S246/467A
S246/632A
S467/632A
S246/467/632A
C
293 cells
S246/467/632A

Fig. 4 Effects of point mutations of S246, S467 and S632 of HDAC4 on its subcellular localization.

(A) Representative images of green fluorescence of NIH 3T3 cells expressing HDAC4 and its mutants fused to GFP.

(B) Quantitative representation of NIH 3T3 cells expressing HDAC4 or its mutants fused to GFP. Blank bar (C>N), more green fluorescence in the cytoplasm; shaded bar (C=N), equally in the cytoplasm and the nucleus; and filled bar (N>C), more in the nucleus. Average values of three independent experiments are shown with standard deviation.

(C) Representative images of green fluorescence of 293 cells expressing GFP-S246/467/632A.

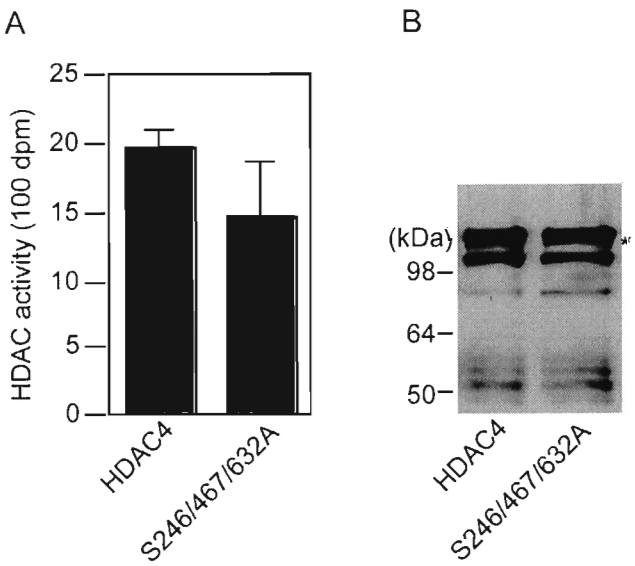

A
B
HDAC activity (100 dpm)
25
20
15
10
5
0
HDAC4
S246/467/632A
(kDa)
98
64
50
HDAC4
S246/467/632A

Fig. 5 Effects of point mutations of S246, S467 and S632 of HDAC4 on its deacetylase activity.

(A) Deacetylase activity of HDAC4 and its mutant S246/467/632A. Expression plasmids for Flag-tagged fusion proteins were transfected into 293 cells and cell extracts were prepared for affinity-purification on M2 agarose. Activities of eluted proteins (left) were determined by measuring release of [^{3}H]acetate from [^{3}H]acetyl histones.

(B) Amount of the eluted proteins was analyzed by immunoblotting with the α-Flag antibody. The migration position of full-length proteins is indicated by an asterisk.

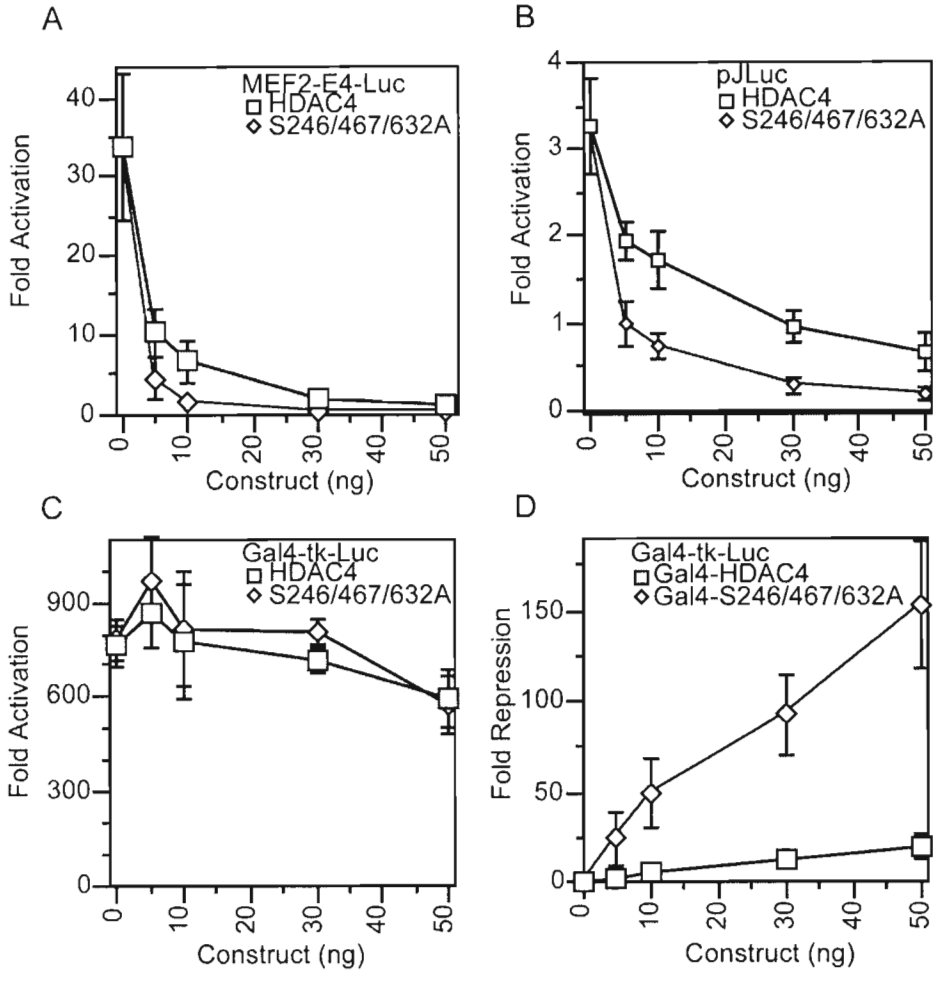

A
MEF2-E4-Luc
HDAC4
S246/467/632A
Fold Activation
Construct (ng)
B
pJLuc
HDAC4
S246/467/632A
Fold Activation
Construct (ng)
C
Gal4-tk-Luc
HDAC4
S246/467/632A
Fold Activation
Construct (ng)
D
Gal4-tk-Luc
Gal4-HDAC4
Gal4-S246/467/632A
Fold Repression
Construct (ng)

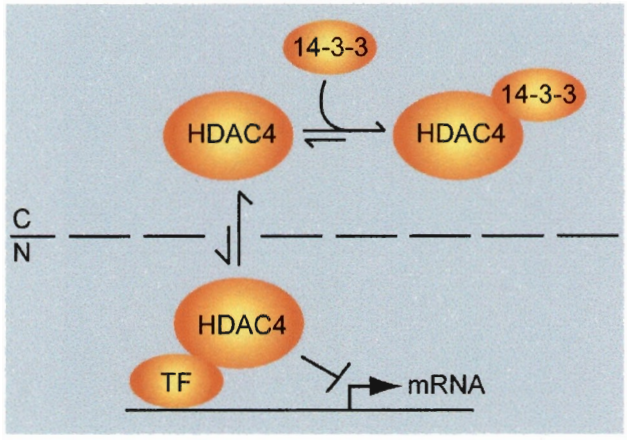
Fig. 6 Repression ability of HDAC4 and its mutant S246/467/632A.

(A, B) 200 ng of the reporter, MEF2-E4-Luc (A) or pJLuc (B), was transfected into NIH3T3 cells with a MEF2C expression plasmid (100 ng), an internal control plasmid (CMV-β-Gal; 50 ng), and the expression plasmid for Flag-tagged HDAC4 or S246/467/632A at the indicated amount. The normalized luciferase activity from the transfection without any effector plasmid was arbitrarily set to 1.0. Average values of at least three independent experiments are shown with standard deviation.

(C) 200 ng of the Gal4-tk-Luc reporter was transfected into NIH3T3 cells with a Gal4-VP16 expression plasmid (5 ng), the internal control plasmid CMV-β-Gal (50 ng), and the expression plasmid for Flag-tagged HDAC4 or S246/467/632A at the indicated amount. The reporter activities were measured as above.

(D) The Gal4-tk-Luc reporter was transfected into NIH3T3 cells along with an expression plasmid for Gal4-HDAC4 or -S246/467/632A. Normalized luciferase activities from transfection with effector plasmids at indicated amounts were compared with that from the reporter alone to calculate the relative repression. Average values of four independent experiments are shown with standard deviation.

A


14-3-3
HDAC4
14-3-3
HDAC4
C
N
HDAC4
TF
mRNA

B

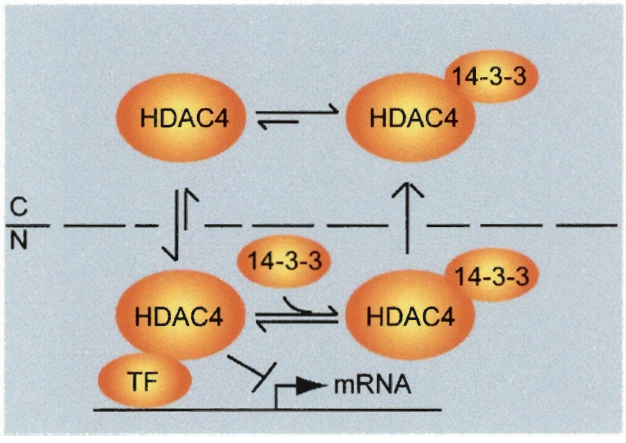

HDAC4
14-3-3
HDAC4
C
N
14-3-3
HDAC4
14-3-3
HDAC4
TF
mRNA

Fig. 7 Model depicting possible modes of regulation of HDAC4 by 14-3-3 proteins.

HDAC4 is actively shuttled between the cytoplasm (C) and the nucleus (N), and the relative rate of nuclear import and export may determine the subcellular localization. 14-3-3 binding may shift the distribution equilibrium of HDAC4 towards cytoplasmic accumulation by hindering its nuclear import (A) and/or facilitating its nuclear export (B). 14-3-3 proteins have been shown to be subject to active nuclear export (40), so they can interact with HDAC4 in the nucleus (B). Association of HDAC4 with other proteins may also affect its localization. In this study, we have investigated how 14-3-3 proteins regulate the functions of HDAC4. Theoretically, it is also possible that HDAC4 regulates the functions of 14-3-3 proteins such as their ability to regulate the function of their binding partners (1, 4, 19, 38, 39) and to bind to cruciform DNA molecules (19, 50).

CHAPTER IV

Histone deacetylase 4 possesses intrinsic nuclear import and export signals

Preface

The previous chapter described a novel regulatory mechanism for HDACs in which nucleocytoplasmic shuttling controls the activity of histone deacetylase. HDAC4 dynamically shuttles between the nucleus and cytoplasm. 14-3-3 binds and sequesters HDAC4 in the cytoplasm. However, the cytoplasmic HDAC4 moves into the nucleus after treatment of LMB, a specific inhibitor of nuclear export receptor CRM1, suggesting that CRM1 also contributes to the retention of HDAC4 in the cytoplasm. CRM1 usually recognizes a leucine-rich consensus sequence to export its target protein. The following manuscript identifies the nuclear import and export signals of HDAC4. Other factors that affect the subcellular localization of HDAC4 are also described. It is proposed that multiple mechanisms regulate the intracellular localization of HDAC4.

ABSTRACT

Nucleocytoplasmic trafficking of histone deacetylase 4 (HDAC4) plays an important role in regulating its function and binding of 14-3-3 proteins is necessary for its cytoplasmic retention. Here we report the identification of nuclear import and export sequences of HDAC4. While its N-terminal 118 residues modulate the nuclear localization, residues 244-279 constitute an authentic, strong nuclear localization signal. Mutational analysis of this signal revealed that three arginine/lysine clusters are necessary for its nuclear import activity. About nuclear export, leucine-rich sequences located in the middle part of HDAC4 do not function as nuclear export signals. By contrast, a hydrophobic motif (MxxLxVxV) located at the C-terminal end serves as a nuclear export signal that is necessary for cytoplasmic retention of HDAC4. This motif is required for CRM1-mediated nuclear export of HDAC4. Furthermore, binding of 14-3-3 proteins promotes cytoplasmic localization of HDAC4 by both inhibiting its nuclear import and stimulating its nuclear export. Unlike wild-type HDAC4, a point mutant with abrogated MEF2 binding ability remains cytoplasmic upon exogenous expression of MEF2C, supporting that direct MEF2 binding targets HDAC4 to the nucleus. Therefore, HDAC4 possesses intrinsic nuclear import and export signals for its dynamic nucleocytoplasmic shuttling, and association with 14-3-3 and MEF2 proteins affects such shuttling and thus directs HDAC4 to the cytoplasm and the nucleus, respectively.

INTRODUCTION

How protein functions are regulated *in vivo* is a fundamental issue relevant to various biological processes. Lysine acetylation has recently emerged as a major form of post-translational modification that regulates functions of histones, non-histone chromosomal proteins, and transcription factors (8, 21, 29, 52, 54). Acetylation of histones and other chromosomal proteins regulates chromatin activities in transcription, replication and recombination (3, 38, 42, 55, 62). Histone deacetylases (HDACs) are the enzymes responsible for reversing the acetylation of histones and other proteins. According to sequence homology and time of identification, mammalian HDACs can be divided into three classes. Class I HDACs (HDAC1, HDAC2, HDAC3 and HDAC8) show high similarity to the yeast deacetylase Rpd3 (4, 9, 12, 22, 56, 57, 65, 66). Class II HDACs (HDAC4, HDAC5, HDAC6, and HDAC7) possess catalytic domains significantly homologous to that of yeast Hda1 (13, 19, 27, 43, 48, 59, 60). Class III HDACs comprise of proteins with catalytic domains similar to that of the yeast NAD^+-dependent deacetylase Sir2 (15, 24, 31, 49).

Compared to class I deacetylases, much less is known about the second class (8). HDAC4, HDAC5 and HDAC7 are homologous, with their Hda1-related domains located in the C-terminal parts, whereas HDAC6 possesses tandem Hda1-related domains (13, 19, 27, 43, 59, 60). Like class I members, class II HDACs (except HDAC6) have been found to be corepressors recruited for transcriptional repression. The MEF2 transcription factors interact with HDAC4, HDAC5, HDAC7 and their related protein MITR/HDRP to repress transcription

(11, 32, 35, 43, 50, 60, 69). Moreover, this interaction is signal-dependent, and regulated during muscle differentiation (11, 35, 36, 67). HDAC4, HDAC5 and HDAC7 also interact with the nuclear receptor corepressors SMRT and N-CoR to repress transcription (23, 27).

How are functions of different deacetylases regulated *in vivo*? Emerging evidence suggests that cellular compartmentalization is one major regulatory mechanism for class II HDACs (8, 28). Active nucleocytoplasmic shuttling has been shown for HDAC4 (20, 43, 61), HDAC5 (40, 41), HDAC6 (58), and HDAC7 (11). Moreover, such shuttling is tightly controlled. 14-3-3 proteins directly bind to HDAC4 and HDAC5, and negatively regulate their roles in transcriptional repression (20, 40, 61). 14-3-3 binding to HDAC5 and perhaps to its homologs (i.e. HDAC4 and HDAC7) plays an important role in regulating functions of MEF2 during muscle differentiation (11, 36, 40, 41, 53). Three serine residues of HDAC4 (i.e. S246, S467 and S632) mediate its binding to 14-3-3 proteins (20, 61). Unlike wild-type HDAC4, S246/467/632A, the triple mutant that is completely defective in 14-3-3 binding, is localized to the nucleus (20, 61), indicating that 14-3-3 binding is necessary for retaining HDAC4 in the cytoplasm. However, it remains unclear whether 14-3-3 binding alone is sufficient for cytoplasmic retention of HDAC4.

During characterizing the interesting link between HDAC4 and 14-3-3 proteins, we unexpectedly found that 118-1084/S246/467/632A, the triple mutant lacking the N-terminal 118 residues of HDAC4, was mainly cytoplasmic or pancellular. To understand this intriguing finding, we engineered and analyzed

various HDAC4 mutants, which has led to the identification of sequence elements that are important for nucleocytoplasmic trafficking of HDAC4. While the N-terminal 118 residues and MEF2-binding site of HDAC4 modulate its nuclear localization, residues 244-279 constitute an authentic, tripartite nuclear localization signal (NLS) and a C-terminal hydrophobic motif functions as a functional nuclear export signal (NES). The NES is required for CRM1-mediated nuclear export of HDAC4. Furthermore, both 14-3-3 binding and the NES-mediated nuclear export are required for cytoplasmic retention of HDAC4. We propose that subcellular distribution of HDAC4 is controlled by multiple mechanisms *in vivo*. Such a regulatory scheme may provide flexibility for fine-tuning biological functions of HDAC4.

MATERIALS AND METHODS

Molecular cloning. Expression plasmids for HDAC4 and some deletion mutants have been described previously (60, 61). Additional HDAC4 mutants were generated by PCR with Expand (Roche) thermostable DNA polymerase or by site-directed mutagenesis with single-stranded uracil-containing templates and T7 DNA polymerase. DNA sequencing was performed with T7 Sequenase 2.0 (Amersham Pharmacia Biotech) for confirmation of mutations. GFP constructs were derived from pEGFP-C2 (Clontech).

Green fluorescence microscopy. NIH 3T3 and 293 cells were transfected with plasmids expressing GFP fusion proteins using SuperFect transfection reagent (Qiagen) (5, 60). 16 h after transfection, living cells were analyzed by GFP fluorescence microscopy as described (61). Fluorescence images were collected using a CCD camera (Q-imaging, Inc.) linked to a computer running Northern Eclipse 5.0 (Empix Imaging) and exported for further processing with Adobe Photoshop. Alternatively, cells were fixed with formaldelhyde and counter-stained with Hoechst 33528 to visualize the nuclei (61); Hoechst and green fluorescence images were subsequently collected.

Immunofluorescence microscopy. To assess effects of MEF2 binding on subcellular localization of HDAC4 and its mutants, MEF2C expression plasmid was transfected into NIH 3T3 cells along with mammalian expression plasmids for HDAC4 or its mutants fused to GFP. To detect the expression of MEF2C, cells were fixed with formaldelhyde 16 h after transfection, incubated with anti-MEF2C antibody and stained with Cy3 anti-rabbit IgG antibody (Jackson

Immunoresearch) as previously described (37, 61). Cells were counter-stained with Hoechst 33528 to visualize the nuclei. Expression of GFP fusion proteins was determined by green fluorescence microscopy. Similarly, effects of exogenous CRM1 on subcellular localization of HDAC4 mutants were determined.

Protein-protein interaction. To analyze interaction of the MEF2C mutant M178 with HDAC4 mutants, *in vitro* maltose-binding protein (MBP) binding assays were carried out as described (60). For analysis of intra- or inter-molecular interaction among HDAC4 molecules, its fragments were expressed as MBP fusion proteins in *E. coli*, immobilized on amylose agarose (New England Biolabs), and incubated with HDAC4 or its fragments synthesized *in vitro* by use of TNT-T7 coupled reticulocyte lysate system (Promega) in the presence of L-[^{35}S]methionine (Amersham Pharmacia Biotech). Agarose beads were washed three times with buffer B (20 mM Tris-HCl [pH 8.0], 10% glycerol, 5 mM $MgCl_2$, 0.1% NP-40, protease inhibitors) containing 0.15 M KCl and once with buffer B containing 0.5 M KCl. Bound proteins were then analyzed by reducing SDS-PAGE and autoradiography as previously described (60).

Western blotting analysis. Expression of GFP fusion proteins were also confirmed by Western blotting analysis of total cell extracts as previously described (6, 60). 293 cells were transfected with plasmids expressing GFP fusion proteins using SuperFect transfection reagent (Qiagen) (5, 60). 16 h after transfection, cells were washed twice with ice-cold phosphate-buffered saline (PBS) and collected in ice-cold buffer B containing 0.15 M KCl, or buffer H (20

mM HEPES [pH 7.6], 20% glycerol, 150 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, 25 mM NaF, 10 mM β-glycerophosphate, 1 mM dithiothreitol, protease inhibitors). After being rotated at 4°C for 20 min, the cell lysates were cleared by high-speed centrifugation at 4°C, and the supernatants were collected as total cell extracts. For immunoblotting, the total cell extracts (~10 μg/per lane) were resolved by reducing SDS-PAGE, electro-transferred to nitrocellulose membrane, and subsequently immunoblotted with anti-GFP antibody (Santa Cruz Biotechnology, sc-8334). For blocking and antibody incubation, PBS containing 20% of horse serum (GG-free, Gibco BRL) and 0.15% Tween 20 (Sigma) was used. For washing, PBS with 0.15% Tween 20 was used. Blots were developed with Supersignal chemiluminescent substrate (Pierce).

BLAST search. Amino acid sequence homology searches were performed at the NCBI BLAST server (http://www.ncbi.nlm.nih.gov/BLAST/) using ψ-BLAST with the matrix BLOSUM62 (1).

RESULTS

Compared to the yeast deacetylase Hda1, HDAC4 can be divided into three parts: an extended N-terminal region (residues 1-620), an Hda1-related deacetylase domain (residues 621-1039) and a small C-terminal module (residues 1040-1084) (Fig. 1A). The extended N-terminal region has been found to interact with MEF2 and 14-3-3 proteins (20, 43, 60, 61), whereas the function of the small C-terminal module remains elusive.

Role of the N-terminal 118 residues of HDAC4 in modulating its nuclear localization. We and others have previously shown that the HDAC4 triple mutant S246/467632A (TM1, Fig. 1A) is completely defective in 14-3-3 binding and thus predominantly nuclear (20, 61). As reported, GFP-HDAC4 and -TM1 were cytoplasmic and nuclear, respectively, whereas GFP itself was pancellular in NIH 3T3 cells (Fig. 1B-C). Also consistent with published reports (43, 61), the mutant 118-1084 was predominantly cytoplasmic (Fig. 1A-C and data not shown). Unexpectedly, we found that different from GFP-TM1, GFP-TM2 was either cytoplasmic or pancellular (Fig. 1A-C), suggesting that the N-terminal 118 residues are involved in regulating nuclear localization of HDAC4. BLAST searches revealed that residues 90-142 of HDAC4 show limited sequence similarity to the GTP-binding protein MAG1 (Fig. 1A) (7, 63). To address whether the MAG1-related region of HDAC4 is responsible for the observed difference between GFP-TM1 and -TM2, we engineered the mutants TM3 and TM4 fused to GFP (Fig. 1A). As shown in Fig. 1B-C, unlike GFP-TM3, GFP-TM4 was more similar to GFP-TM1, suggesting that the MAG1-homology

region may be important for controlling nuclear localization of HDAC4. As previously reported (61), GFP-TM1 was nuclear in most cells. By contrast, GFP-TM4 was found to be nuclear in 40-50% of the cells expressing this fusion protein (data not shown), suggesting that the N-terminal 85 residues are also important for nuclear localization of HDAC4. To determine whether GFP fusion proteins are expressed as expected, we performed Western blotting analysis. As shown in Fig. 1D, GFP fusion proteins with expected sizes were detected. Taken together, these results indicate that the N-terminal 118 residues of HDAC4 play an important role in modulating its nuclear localization.

How do the N-terminal 118 residues of HDAC4 modulate its nuclear localization? To promote its nuclear localization, the N-terminal 118 residues of HDAC4 may: (1) be involved in inter- or intra-molecular interaction with HDAC4 itself (such interaction may affect the exposure of potential nuclear import or export sequences); (2) interact with other nuclear proteins; or (3) be (part of) an NLS. To distinguish among these possibilities, we first investigated whether the N-terminal part of HDAC4 mediates inter- or intra-molecular interaction with HDAC4 itself. For this, we analyzed different HDAC4 deletion mutants (Fig. 2A) by *in vitro* binding assays using MBP or its fusion proteins immobilized on amylose agarose. As shown in Fig. 2B, no interaction was detectable between 315-1084 and MBP-1-326 (lanes 1-3). By contrast, 1-326 interacted with MBP-1-326 but not MBP itself (lanes 4-6). To test whether the N-terminal 118 residues are essential for this interaction, we tested the deletion mutants 1-208 and 1-114. As shown in Fig. 2C, neither mutant was retained by MBP-1-326, suggesting that

residues 118-326 may be responsible for the interaction. In agreement with this, MBP-1-326 interacted with HDAC4, 118-1084 and 118-326 (Fig. 2D), suggesting that residues 118-326 of HDAC4 constitute a dimerization domain. Taken together, these results indicate that the N-terminal 118 residues of HDAC4 do not appear to be involved in inter- or intra-molecular interaction with HDAC4 itself.

As discussed above, the N-terminal 118 residues may interact with other nuclear proteins and thereby stimulate nuclear localization of HDAC4. Two proteins are known to have the potential to interact with the N-terminal part of HDAC4. Although the exact binding site has not been mapped, HDAC1 has been shown to be associated with MITR, a corepressor with sequence similarity to the N-terminal part of HDAC4 (50). CtBP (adenovirus E1A C-terminal-binding protein) has also been shown to interact with HDAC4, and its N-terminal 118 residues possess a putative CtBP-binding site (68). To test whether HDAC1 or CtBP modulates subcellular localization of HDAC4, we examined effects of their overexpression on intracellular distribution of GFP-HDAC4 by fluorescence microscopy. The results indicated that overexpression of HDAC1 or CtBP had minimal effects on the cytoplasmic localization of GFP-HDAC4 (data not shown), suggesting that neither HDAC1 nor CtBP is involved in modulating intracellular localization of HDAC4. It still remains possible, however, that an unidentified protein may interact with the N-terminal 118 residues of HDAC4 and thereby modulate its intracellular distribution.

To investigate whether the N-terminal 118 residues of HDAC4 constitute (or are parts of) an NLS, we examined subcellular distribution of the HDAC4

deletion mutants 1-118 and 1-165 expressed as GFP fusion proteins (Fig. 3A). As shown in Fig. 3B, both mutants were partially enriched in the nucleus, suggesting that the N-terminal 118 residues of HDAC4 only possess weak nuclear targeting ability. This region does not show any sequence resemblance to classical arginine/lysine-rich nuclear import signals, raising the possibility that a yet unknown protein interacts with this region of HDAC4 and regulate its subcellular localization (see Discussion).

Identification of an authentic HDAC4 NLS. The distinct localization between GFP-TM1 (nuclear, Fig. 1) and GFP-1-165 (partially enriched in the nucleus, Fig. 3) further suggests that there is a strong NLS within residues 166-1084. Consistent with this, HDAC4 possesses two arginine/lysine-rich sequences (RK1 and RK2, Fig. 3A). To further understand how nucleocytoplasmic distribution of HDAC4 is controlled, we decided to map its NLS. To take a systematic approach, we first analyzed the deletion mutants 1-208, 1-266, 1-326, and 1-669 expressed as GFP fusion proteins (Fig. 3A). As shown in Fig. 3B, the localization of 1-208 was similar to 1-114 and 1-165, suggesting that RK1 is not an NLS. Distinct from 1-208, 1-266 was pancellular (Fig. 3B). One explanation for this is that 14-3-3 binding to S246 of 1-266 counteracts the weak nuclear targeting activity that the N-terminal 118 residues exhibit. Unlike 1-266, the mutants 1-326 and 1-669 were exclusively or predominantly nuclear, indicating that residues 267-326 are important for the nuclear localization activity. Consistent with this, the mutant 206-326 was exclusively nuclear. Together, these results suggest that RK2 may possess an authentic NLS. To further map

this NLS, we constructed and analyzed the mutants 206-279, 206-266, 244-326, and 263-326 (Fig. 3A). These mutants were designed according to the sequence of RK2 (residues 242-283, Fig. 4A). While 206-279 and 244-326 were nuclear, 206-266 and 263-326 were mainly pancellular (Fig. 3B), indicating that residues 244-279 are important for the nuclear localization activity. To test whether this region is sufficient, we examined the mutant 244-279 expressed as a GFP fusion protein (Fig. 3A). As shown in Fig. 3B, this mutant was exclusively nuclear, indicating that residues 244-279 of HDAC4 are capable of directing GFP to the nucleus. Western blotting analysis with anti-GFP antibody revealed that the deletion mutants used for mapping the NLS were correctly expressed (Fig. 3C). Taken together, these mapping data indicate that residues 244-279 of HDAC4 constitute an authentic NLS.

Point mutational analysis of the HDAC4 NLS. To further define the NLS, we sought to identify its critical residues. Residues 244-279 possess three clusters of arginine/lysine residues (Fig. 4A). To test whether these clusters are required for the nuclear targeting activity, we derived the point mutants PM1-4 from the deletion mutant 206-326 by mutating arginine/lysine residues. GFP fusion proteins were expressed and analyzed by green fluorescence microscopy. As shown in Fig. 4B, unlike 206-326, PM1-4 were pancellular, cytoplasmic, or partially enriched in the nucleus. Western blotting analysis revealed that these point mutants were correctly expressed (Fig. 4C). Therefore, the three clusters of arginine/lysine residues are all necessary for the nuclear localization of 206-326. This also implies that the NLS of HDAC4 is tripartite.

Nuclear export activity of leucine-rich sequences of HDAC4. We wondered why GFP-TM2 (Fig. 1) was not localized to the nucleus although it possesses the strong NLS just identified. Cytoplasmic localization of HDAC4 is sensitive to treatment with leptomycin B (LMB) (43, 61). LMB is a specific inhibitor of the nuclear export receptor CRM1 (14, 30, 46, 51), so HDAC4 is subject to active nuclear export. 14-3-3 binding to HDAC4 promotes its cytoplasmic retention (20, 43, 61). 14-3-3 proteins are dimeric, and each monomer is known to possess an active NES (34, 47). Therefore, one explanation for the active nuclear export of HDAC4 is that it binds to 14-3-3 proteins and is subsequently targeted to the cytoplasm through LMB-sensitive nuclear export. Alternatively, HDAC4 may possess an intrinsic NES that directs it to the cytoplasm. Since its 14-3-3 binding sites are impaired, the cytoplasmic localization of TM2 (Fig. 1) supports the latter possibility. However, this does not exclude the former. With this reasoning in mind, we dissected the underlying mechanisms by which HDAC4 is exported from the nucleus.

Some leucine-rich sequences are known export signals recognized by the nuclear export receptor CRM1 (45). HDAC4 possesses several leucine-rich sequences (Fig. 5A). In particular, residues 429-438 match exactly to the NES consensus sequence derived from various known export signals (2). In light of this observation, we expressed and analyzed the HDAC4 mutant 315-488 as a GFP fusion protein. As shown in Fig. 5B, this mutant was partially enriched in the cytoplasm. Since this mutant contains a 14-3-3 binding site, we decided to investigate whether its partial enrichment in the cytoplasm is due to 14-3-3

binding. For this, S467 was substituted with alanine to generate the mutant 315-488/S467A. This mutant was found to be pancellular (Fig. 5B). Without active nuclear import and export, such localization is expected since this mutant may be able to passively diffuse through nuclear pores (18). Western blotting analysis with anti-GFP antibody indicated that both 315-488 and 315-488/S467A were well expressed (Fig. 5C). Together, these results suggest that the enrichment of 315-488 in the cytoplasm is due to 14-3-3 binding, implying that the leucine-rich sequences of HDAC4 do not have nuclear export activity.

Mapping an NES to the C-terminal end of HDAC4. To investigate whether other sequences of HDAC4 may exhibit nuclear export activity, we examined subcellular localization of the deletion mutants 206-1084, 315-1084 and 621-1084 fused to GFP (Fig. 6A). As shown in Fig. 6B, these mutants were predominantly cytoplasmic. Western blotting analysis with anti-GFP antibody indicated that these deletion mutants were expressed as expected (Fig. 6C, lanes 1-3). To test whether the S632 14-3-3 binding site contributes to the cytoplasmic localization of 621-1084, we analyzed the mutants 531-1084 and 531-1084/S632A expressed as GFP fusion proteins. Both mutants were found to be cytoplasmic (data not shown), suggesting that 14-3-3 binding is not the major mechanism by which 621-1084 is sequestered to the cytoplasm. The cytoplasmic localization of 621-1084 could be either that it is not imported to the nucleus or that it is subject to active nuclear export. To distinguish between these two possibilities, we utilized the nuclear export inhibitor LMB since HDAC4 is known to be exported in an LMB-sensitive manner (43, 61). As shown in Fig. 6D, LMB

treatment inhibited the predominantly cytoplasmic localization of 621-1084, indicating that 621-1084 is subject to active nuclear export. The pancellular localization after LMB treatment is perhaps due to passive diffusion through nuclear pores (18). Therefore, residues 621-1084 possess an intrinsic NES.

To map the NES, we first considered whether it is located at the small C-terminal domain (residues 1040-1084) since this region is missing in Hda1 (Fig. 6A). This small domain was thus deleted to generate the mutant 1-1040 fused to GFP (Fig. 6A). This fusion protein was predominantly nuclear (Fig. 6E), indicating that the small C-terminal domain is indeed required for cytoplasmic localization of HDAC4. Since this mutant possesses intact 14-3-3 binding sites (20, 61), this exciting finding also indicates that 14-3-3 binding is not sufficient for retaining HDAC4 in the cytoplasm. To further define the small C-terminal domain, small deletions from the C-terminal end were engineered to express the mutants 1-1069, 1-1061 and 1-1055 as GFP fusion proteins (Fig. 6A). The mutant 1-1069 was predominantly cytoplasmic (Fig. 6E), suggesting that residues 1070-1084 of HDAC4 are dispensable for its cytoplasmic localization. The two mutants 1-1061 and 1-1055 were predominantly nuclear (Fig. 6E), indicating that residues 1062-1069 are essential for cytoplasmic retention of HDAC4. These results also imply that residues 1040-1069 of HDAC4 may constitute an NES. To test this hypothesis, we expressed the mutant 1044-1069 as a GFP fusion protein (Fig. 6A). As shown in Fig. 6E, this fusion protein was predominantly cytoplasmic. On the other hand, 621-1040 was pancellular (Fig. 6E). Western blotting analysis with anti-GFP antibody indicated that these deletion mutants were correctly

expressed (Fig. 6C, F). Together, these results indicate that residues 1044-1069 of HDAC4 function as an NES.

We also examined the mutants 315-1040 and 206-1040 expressed as GFP fusion proteins (Fig. 6A). Different from 315-1084 (Fig. 3B), 315-1040 was pancellular or cytoplasmic (Fig. 6D). Unlike 206-1084 (Fig. 3B), 206-1040 was predominantly nuclear (Fig. 6D). The distinct localization between the mutants 315-1040 and 206-1040 supports the aforementioned conclusion that residues 244-279 constitute an NLS (Fig. 3). The pancellular or cytoplasmic localization of 315-1040 is expected since this mutant may be able to passively diffuse into the nucleus through nuclear pores and 14-3-3 binding to S467 and S632 of 315-1040 may promote its active nuclear export. Western blotting analysis with anti-GFP antibody indicated 206-1040 and 315-1040 were correctly expressed (Fig. 6C, lanes 4-5). Together, these results underscore the importance of residues 1044-1069 for cytoplasmic retention of HDAC4.

To determine whether residues 1044-1069 retain HDAC4 in the cytoplasm by nuclear export, we treated NIH 3T3 cells expressing GFP-1044-1069 with LMB. This fusion protein is small (~28 kDa) and does not appear to contain an NLS, so it can passively diffuse through nuclear pores (18, 26). Therefore, this fusion protein would be expected to be pancellular if its nuclear export is inhibited by LMB. As shown in Fig. 6G, upon LMB treatment, 1044-1069 became pancellular within 15 minutes, indicating that nuclear export of 1044-1069 is sensitive to LMB. Therefore, residues 1044-1069 constitute an NES whose

activity is LMB-sensitive. Since LMB is a CRM1-specific inhibitor (14, 30, 46, 51), CRM1 may recognize this NES.

Point mutational analysis of the HDAC4 NES. To further define the NES, we sought to identify its critical residues. Sequence inspection revealed that residues 1056-1069 constitute a highly hydrophobic motif (Fig. 7A). Since CRM1 is known to recognize leucine-rich or other hydrophobic motifs (45), residues 1056-1069 may constitute a functional NES. To determine which residues are important, we performed alanine scanning mutagenesis to generate mutants with each non-alanine residue substituted with alanine (Fig. 7A). Fluorescence microscopic analysis of these GFP fusion proteins revealed that substitution of M1059, L1062, V1064 or V1066 of HDAC4 led to nuclear accumulation of the resulting mutants (Fig. 7B). Western blotting analysis with anti-GFP antibody indicated that all point mutants were correctly expressed (Fig. 7C). Together, these results suggest that M1059, L1062, V1064 and V1066 are important for nuclear export of HDAC4. These residues constitute a hydrophobic motif, MxxLxVxV, where x represents any amino acid residue.

CRM1 directs NES-mediated nuclear export of HDAC4. While the NES of HDAC4 is distinct from most known nuclear export sequences recognized by CRM1, its function appeared to be LMB-sensitive (Fig. 6). This suggests that the NES may be regulated by CRM1. To substantiate this, we sought to examine directly whether CRM1 can mediate nuclear export of HDAC4 and how the NES is involved. It has been demonstrated that overexpression of CRM1 leads to nuclear exclusion of two transcription factors (17, 71). Since wild-type HDAC4 is

mainly cytoplasmic, we tested whether overexpression of CRM1 can lead to nuclear exclusion of TM1 and S246/467A. While TM1 possesses no functional 14-3-3 binding sites, the double mutant S246/467A contains only one functional 14-3-3 binding site (S632, Fig. 1A). Both mutants have been found to be predominantly nuclear (61). As shown in Fig. 8, exogenous expression of CRM1 promoted cytoplasmic localization of both mutants, suggesting that CRM1 directs HDAC4 to the cytoplasm. To assess whether the NES of HDAC4 is involved, we examined the mutants 1-1040 and L1062A. 14-3-3 binding sites are intact in both mutants, but the NES is deleted in 1-1040 (Fig. 6) and impaired by point mutation in L1062A (Fig. 7). As shown in Fig. 8, CRM1 overexpression had minimal effects on the nuclear localization of these two mutants, indicating that the NES of HDAC4 is required for its CRM1-mediated nuclear export.

Direct MEF2 binding targets HDAC4 to the nucleus. Identification of intrinsic nuclear import and export signals of HDAC4 further supports that it is subject to dynamic nucleocytoplasmic shuttling. Binding of 14-3-3 proteins promotes cytoplasmic localization of HDAC4 by affecting such dynamic shuttling (20, 40, 61). This led us to ask whether association of other proteins also alter this shuttling. It has been shown that the HDAC4 mutant 118-1084 translocates to the nucleus upon exogenous expression of MEF2A in HeLa cells (43). It was not proven, however, whether direct MEF2 binding is required for this nuclear targeting. To further understand how MEF2 may affect intracellular localization of HDAC4, we first tested whether full-length HDAC4 is targeted to the nucleus upon exogenous expression of MEF2C in NIH 3T3 cells. As shown in Fig. 9, co-

expression of MEF2C led to nuclear accumulation of GFP-HDAC4. We then asked whether the nuclear targeting of HDAC4 requires its NLS and/or MEF2-binding site. To address this, we assessed whether co-expression of MEF2C affects subcellular localization of the HDAC4 mutants 1-208 and 206-1084. While 1-208 possesses the MEF2 binding site (32, 35, 43, 50, 60), 206-1084 contains the NLS described above. In the absence of exogenous MEF2C, 1-208 was partially enriched in the nucleus (Fig. 3), whereas 206-1084 was predominantly cytoplasmic (Fig. 6). As shown in Fig. 9, expression of MEF2C promoted nuclear accumulation of 1-208 but not 206-1084, suggesting that MEF2 directs HDAC4 to the nucleus in a manner dependent on its MEF2-binding site but not NLS.

To further investigate whether direct MEF2 binding is essential for the nuclear targeting, we sought to analyze an HDAC4 point mutant that is completely defective in MEF2 binding. For this, we first conducted mutational analysis of the MEF2-binding site of HDAC4 to test whether point mutations can abrogate the MEF2 binding and to identify residues critical for such binding. We and others have located the MEF2-binding site to a small motif conserved among HDAC4, HDAC5, HDAC7 and MITR (Fig. 10A) (32, 35, 43, 50, 60). Mutagenesis was thus performed to substitute potentially important residues of this motif with alanine, and *in vitro* binding assays were utilized to assess how well each mutant binds to MEF2. For binding assays, M178, a MEF2C mutant containing its N-terminal 178 residues (60), was expressed as a MBP fusion protein. As reported, HDAC4 interacted with MBP-M178 but not MBP itself (Fig. 10B, lanes 1-3). Like

wild-type HDAC4, the double mutant S168A/T169A interacted with M178 (lanes 4-6), indicating that S168 and T169 of HDAC4 are not critical for MEF2 binding. By contrast, the double mutant V171A/K172A was unable to interact with M178 (lanes 7-9), suggesting that V171 and/or K172 are important for MEF2 binding. Consistent with this, V171A weakly interacted with M178 (Fig. 10C, lanes 1-3), whereas K172A was completely defective in binding to M178 (lanes 7-9). Neither L175A (Fig. 10C, lanes 7-9) nor L175G (Fig. 10D, lanes 1-3) interacted with M178, indicating that L175 of HDAC4 is critical for MEF2 binding. In a similar fashion, V179, L180 and K182 of HDAC4 were found to be involved in interaction with MEF2 (Fig. 10D-E). Among the HDAC4 mutants analyzed, L175G is one whose MEF2 binding ability is completely abolished.

We next analyzed GFP-L175G by fluorescence microscopy. Like GFP-HDAC4, GFP-L175G was cytoplasmic (data not shown). As shown in Fig. 9, exogenous expression of MEF2C failed to target this point mutant to the nucleus, supporting that direct MEF2 binding is indeed responsible for nuclear targeting of HDAC4 by MEF2. Along with published reports (20, 41, 43, 61), these results indicate that direct binding of 14-3-3 and MEF2 proteins to HDAC4 affects its dynamic nucleocytoplasmic shuttling and thereby targets it to the cytoplasm and the nucleus, respectively.

DISCUSSION

The histone deacetylase HDAC4 is known to function as a transcriptional corepressor (32, 35, 43, 50, 60, 69). Its corepressor function is subject to regulation by active nucleocytoplasmic trafficking (20, 43, 61). 14-3-3 proteins bind to HDAC4, sequester it to the cytoplasm and thereby inhibit its corepressor function (20, 61). The results presented herein demonstrate that besides its 14-3-3 binding sites, HDAC4 possesses additional sequence elements that are also important for controlling its subcellular distribution (Fig. 11A).

The N-terminal 118 residues of HDAC4 modulate its nuclear localization. The N-terminal 118 residues play a contributing role in regulating nuclear localization of HDAC4 (Fig. 1). Residues 90-142 of HDAC4 show limited sequence similarity to MAG1 (Fig. 1A) (7, 63). The distinct subcellular localization between GFP-TM3 and -TM4 (Fig. 1) suggests that residues 85-105 are important for nuclear localization of HDAC4. GFP-TM4 was nuclear in a fraction of expressing cell (data not shown), the N-terminal 85 residues also modulate subcellular localization of HDAC4.

How do the N-terminal 118 residues modulate subcellular localization of HDAC4? This region may not be involved in intra- or inter-molecular interaction with HDAC4 molecules (Fig. 2). Moreover, neither HDAC1 nor CtBP appeared to modulate subcellular localization of HDAC4 (data not shown). Although the N-terminal 118 residues exhibited weak nuclear targeting activity (Fig. 3), this region does not appear to possess a classical arginine/lysine-rich NLS. This

region may contain a novel NLS. Alternatively, this region may interact with a protein that awaits to be identified (Fig. 11A).

In agreement with the latter possibility, a transcriptional repression domain has been mapped to this region (50, 60, 69). When tethered, the N-terminal 208 residues of HDAC4 function as a strong, active transcriptional repression domain. However, neither the N-terminal 118 residues nor residues 118-620 are able to repress transcription (60), suggesting that the N-terminal 118 residues are necessary but not sufficient for transcriptional repression. These findings suggest that this repression domain of HDAC4 may interact with an unidentified nuclear protein. Involvement of the N-terminal 118 residues in such binding may explain the role in modulating subcellular localization of HDAC4. It will be interesting to identify this elusive protein and study its role in regulating subcellular localization and function of HDAC4.

Tripartite nuclear import signal of HDAC4. Residues 244-279 of HDAC4 constitute a functional NLS (Fig. 11A). Mutational analysis of this NLS revealed that three clusters of arginine/lysine residues are necessary for its nuclear import activity (Fig. 4). Such a tripartite organization is distinct from known mono- or bi-partite nuclear import sequences (10, 26, 45). It is noteworthy that the HDAC4 mutant 206-326 was found to be nuclear although it still possesses an intact 14-3-3 binding site (S246, Fig. 3). Therefore, this NLS is unique and strong. Since it is arginine/lysine-rich, it can be recognized by importin α. Consistent with this, HDAC4 has been found to interact with importin α (20).

Shown in Fig. 11B is the sequence comparison of the HDAC4 NLS with the corresponding regions of HDAC5, MITR and HDAC7. The NLS of HDAC4 is highly conserved among these three proteins, suggesting that their corresponding regions may constitute authentic nuclear import signals. Consistent with this, an HDAC5 fragment containing the putative NLS has been very recently shown to possess strong nuclear localization activity (40). Further experiments are needed to verify the putative import signals of MITR and HDAC7.

Hydrophobic nuclear export signal of HDAC4. Deletion and point mutational analyses revealed that while leucine-rich sequences of HDAC4 do not exhibit nuclear export activity (Fig. 5), a hydrophobic motif (MxxLxxVxV) located at its C-terminal end functions as an NES (Fig. 11A). Alanine substitution of the four critical residue led to nuclear accumulation of the resulting mutants (Fig. 7). These mutants possess all three 14-3-3 binding sites (20, 61), so they are presumably able to interact with 14-3-3 proteins. Therefore, besides the three 14-3-3 binding sites, the NES is also required for cytoplasmic retention of HDAC4.

This NES is different from most leucine-rich export signals identified in other proteins (45). However, the NES of cyclin B contains only one leucine: LxxxFxxVxl, where x represents any amino acid residue (39). Although most known export signals are binding sites of CRM1 (45), CRM1-independent protein nuclear export pathways have also been found (25, 33). Cytoplasmic localization of HDAC4 is sensitive to LMB, a known CRM1-specific inhibitor (14, 30, 46, 51), so CRM1 may be involved in its nuclear export. The nuclear export function of residues 1040-1069 is LMB-sensitive (Fig. 6G), suggesting that CRM1

recognizes this NES. Consistent with this, we found that HDAC4 and CRM1 functionally interact *in vivo* and such interaction requires the NES of HDAC4 (Fig. 8). We also conducted pull-down and co-immunoprecipitation assays to analyze the physical interaction between HDAC4 and CRM1 *in vitro* or *in vivo*. Various efforts failed to verify this (data not shown), suggesting that the physical interaction may be transient or too weak to be easily detected.

Illustrated in Fig. 11C is the sequence comparison of the HDAC4 NES with the corresponding regions of HDAC5 and HDAC7. While HDAC7 possesses LxxLxVxI, HDAC5 contains MxxLxVxA. It has been reported that HDAC5 is mainly nuclear in several cell lines (27, 32, 40). We also found that unlike GFP-HDAC4, GFP-HDAC5 was mainly nuclear in NIH 3T3 cells (data not shown). Interestingly, A1096 of HDAC5 corresponds to V1066 of HDAC4, and the point mutant V1066A of HDAC4 was predominantly nuclear (Fig. 4). Besides these distinctions, there are other differences between HDAC4 and HDAC5. First, HDAC5 has been very recently reported to possess an NES within the deacetylase domain (40). The corresponding region of HDAC4 does not appear to be an NES since the mutant 621-1040 was pancellular (Fig. 6E). Second, although HDAC4 and HDAC5 are homologous (overall amino acid sequence identity, 62%; similarity, 69%), their sequences are quite divergent in some regions (13, 19, 43, 59, 60). Third, unlike S632 of HDAC4, S661 of HDAC5 does not mediate 14-3-3 binding (20, 41, 61). Finally, while 14-3-3 binding to HDAC4 is constitutive in most cells tested, 14-3-3 binding to HDAC5 is dependent on

activation of Ca^{2+}/calmodulin-dependent kinases (20, 41, 61). Therefore, nuclear export of HDAC4 and HDAC5 seems to be differentially regulated.

Both 14-3-3 and MEF2 proteins regulate intracellular localization of HDAC4. Mapping the NLS and NES of HDAC4 also shed light on how 14-3-3 proteins regulate its subcellular localization. First, the HDAC4 mutant 206-326 was exclusively nuclear although it has an intact 14-3-3 binding site (S246, Fig. 3). Second, while 315-488 was enriched in the cytoplasm, 315-488/S467A was pancellular (Fig. 5). Third, deletion of residues 1040-1084 or alanine substitution of the critical residues of the NES led to nuclear accumulation of the resulting mutants, although these mutants still contain all three 14-3-3 binding sites (Figs. 6-7). Finally, the triple mutant TM1 was found to be mainly nuclear (Fig. 1; 20, 61), although its NES remains intact. Taken together, these findings indicate both 14-3-3 binding and nuclear export mediated by the NES are required for cytoplasmic retention of HDAC4.

How does 14-3-3 binding promote cytoplasmic retention of HDAC4? The NLS of HDAC4 is only two residues away from the S246 14-3-3 binding site (Fig. 4A), so 14-3-3 binding to S246 may mask the NLS and thereby inhibit its nuclear targeting activity. Consistent with this, 14-3-3 binding has been found to interfere with the association of importin α with HDAC4 (20). 14-3-3 binding to S246 of HDAC4 may inhibit access of importin α to the NLS. Therefore, one mechanism by which 14-3-3 proteins negatively regulate nuclear localization of HDAC4 operates through direct inhibition of importin α binding to HDAC4. Substitution of S246 alone was found to be insufficient to alter cytoplasmic localization of

HDAC4 (20, 61), so additional mechanisms may be involved. The distinct localization of 315-488 and 315-488/S467A (Fig. 5) suggests that 14-3-3 binding also stimulates nuclear export of HDAC4. Since each 14-3-3 protein is known to contain an NES (34, 47), binding of dimeric 14-3-3 proteins to S467 of HDAC4 may provide an active NES. Therefore, as previously proposed (61), 14-3-3 binding may promote cytoplasmic retention of HDAC4 by both inhibiting its nuclear import and stimulating its nuclear export. Similar modes of action may also apply to some of other 14-3-3 binding partners (16, 44, 64).

Besides 14-3-3 proteins, MEF2 binds to HDAC4 and affect its subcellular localization (Fig. 9; 43). MEF2 was able to direct the mutant 1-208 to the nucleus although this mutant does not possess the NLS of HDAC4. Mutational analysis of the MEF2 binding site further supports that direct binding of MEF2 promotes nuclear import of HDAC4. Therefore, HDAC4 possesses multiple sequence elements controlling its subcellular localization (Fig. 11A). The intrinsic nuclear import and export signals of HDAC4 dictate its active shuttling between the cytoplasm and the nucleus. Such shuttling leads to a distribution equilibrium. Association of other proteins with HDAC4 then shifts this equilibrium towards the nucleus or the cytoplasm. Indeed, direct binding of 14-3-3 and MEF2 proteins to HDAC4 leads to its cytoplasmic and nuclear localization, respectively.

Cell signaling may regulate HDAC4 through controlling its interaction with 14-3-3 and MEF2 proteins. 14-3-3 binding motifs are putative phosphorylation sites of cAMP- or Ca^{2+}/calmodulin-dependent protein kinases, so these kinases may phosphorylate HDAC4, regulate its association with 14-3-3 proteins and

thereby affect its subcellular localization. Consistent with this, Ca^{2+}/calmodulin-dependent kinases have been shown to phosphorylate HDAC5, stimulate binding of 14-3-3 proteins and regulate its nuclear export (40). Ca^{2+}/calmodulin-dependent signaling has also been found to regulate MEF binding to HDAC4 (35, 40, 67). Since binding of MEF2 to HDAC4 leads to its nuclear localization (Fig. 9; 43), Ca^{2+}/calmodulin-dependent signaling may regulate subcellular localization of HDAC4 through modulating its interaction with MEF2. The recent finding that oncogenic Ras stimulates localization of HDAC4 to the nucleus also supports that its subcellular distribution is regulated by cell signaling (70).

In summary, HDAC4 possesses an NLS and an NES for its dynamic shuttling between the cytoplasm and the nucleus. Direct binding of 14-3-3 and MEF2 proteins alters such shuttling and targets HDAC4 to the cytoplasm and the nucleus, respectively. The N-terminal 118 residues of HDAC4 affect its intracellular localization perhaps through interaction with an unidentified nuclear protein. Further investigation of multiple mechanisms through which cell signaling pathways modulate subcellular localization of HDAC4 shall shed light on how different deacetylases are differentially regulated *in vivo*.

ACKNOWLEDGEMENTS

We wish to thank S. Khochbin and E.N. Olson for sharing results about subcellular localization of HDAC4 and HDAC5, C.M. Grozinger and S.L. Schreiber for HDAC5 expression plasmids, M. Yoshida for leptomycin B, C. Dargemont and R. Lin for CRM1 cDNA, D. Gorlich for RanQ69L expression plasmid, J. Han for anti-MEF2C antibody, J.J. LeBrun for use of a FluoChem imaging system, M. Park and S. Stifani for use of fluorescence microscopes. A.H.W. is the recipient of a Canadian Institutes of Health Research (CIHR) doctoral research award. This work was supported by grants from the National Cancer Institute of Canada and a scholarship from the CIHR (to X.J.Y.).

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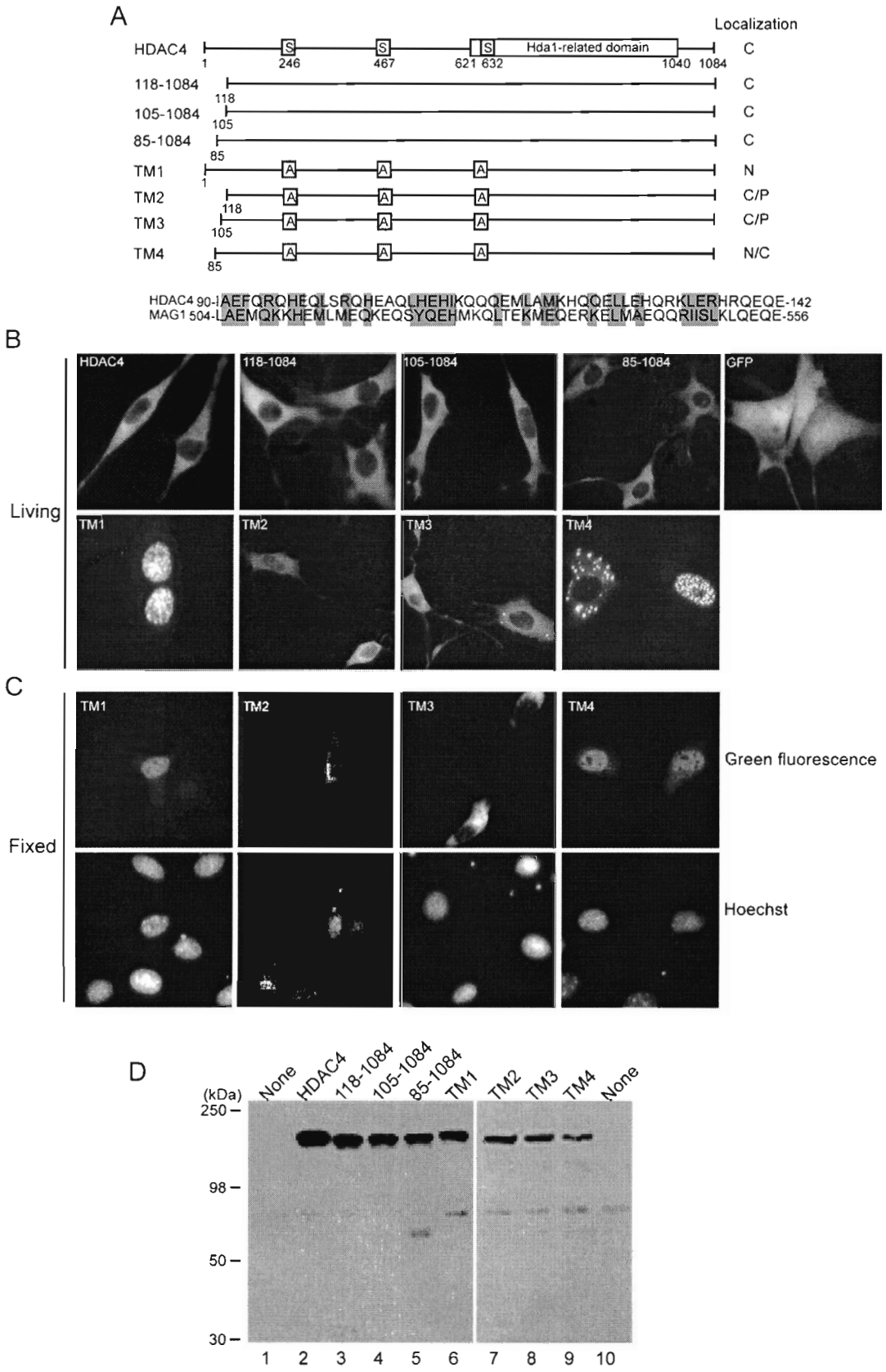

A
Localization
HDAC4
Hda1-related domain
1
246
467
621 632
1040 1084
C
118-1084
118
C
105-1084
105
C
85-1084
85
C
TM1
1
N
TM2
C/P
TM3
118
105
C/P
TM4
85
N/C
HDAC4 90-IAEFQRQHEQLSRQHEAQLHEHIKQQQEMLAMKHQQELLEHQRKLERHRQEQE-142
MAG1 504-LAEMQKKHEMLMEQKEQSYQEHMKQLTEKMEQERKELMAEQQRIISLKLQEQE-556
B
Living
HDAC4
118-1084
105-1084
85-1084
GFP
TM1
TM2
TM3
TM4
C
Fixed
TM1
TM2
TM3
TM4
Green fluorescence
Hoechst
D
(kDa)
250
98
50
30
None
HDAC4
118-1084
105-1084
85-1084
TM1
TM2
TM3
TM4
None
1 2 3 4 5 6 7 8 9 10

Fig. 1 Role of the N-terminal 118 residues of HDAC4 in regulating its nuclear localization.

(A) Schematic illustration of HDAC4 and mutants. For HDAC4, 14-3-3 binding sites (S246, S467 and S632) and the Hda1-homology domain are depicted by boxes. In the triple mutants TM1-4, the three serine residues critical for 14-3-3 binding are changed to alanine. Subcellular localization of HDAC4 and mutants is summarized at right: C, predominantly cytoplasmic; N, predominantly nuclear; and P, pancellular. Shown at the lower part of the panel is the sequence comparison between homologous regions of HDAC4 (residues 90-142) and MAG1 (residues 504-556), with identical or conserved residues shaded.

(B, C) Representative green fluorescence images of living (B) or fixed (C) NIH 3T3 cells expressing GFP or its fusion proteins. Cells were transfected with expression plasmids for GFP or its fusion proteins and subsequently analyzed by fluorescence microscopy 16 h after transfection. For (B), living cells were directly used for microscopic analysis. For (C), transfected cells were fixed with formaldehyde, counter-stained with Hoechst 33528 and analyzed by green fluorescence microscopy (*top*), with corresponding Hoechst fluorescence images also taken (*bottom*).

(D) Expression of GFP fusion proteins. 293 cells were transfected with expression plasmids for indicated GFP fusion proteins, and total cell extracts were analyzed by immunoblotting with anti-GFP antibody.

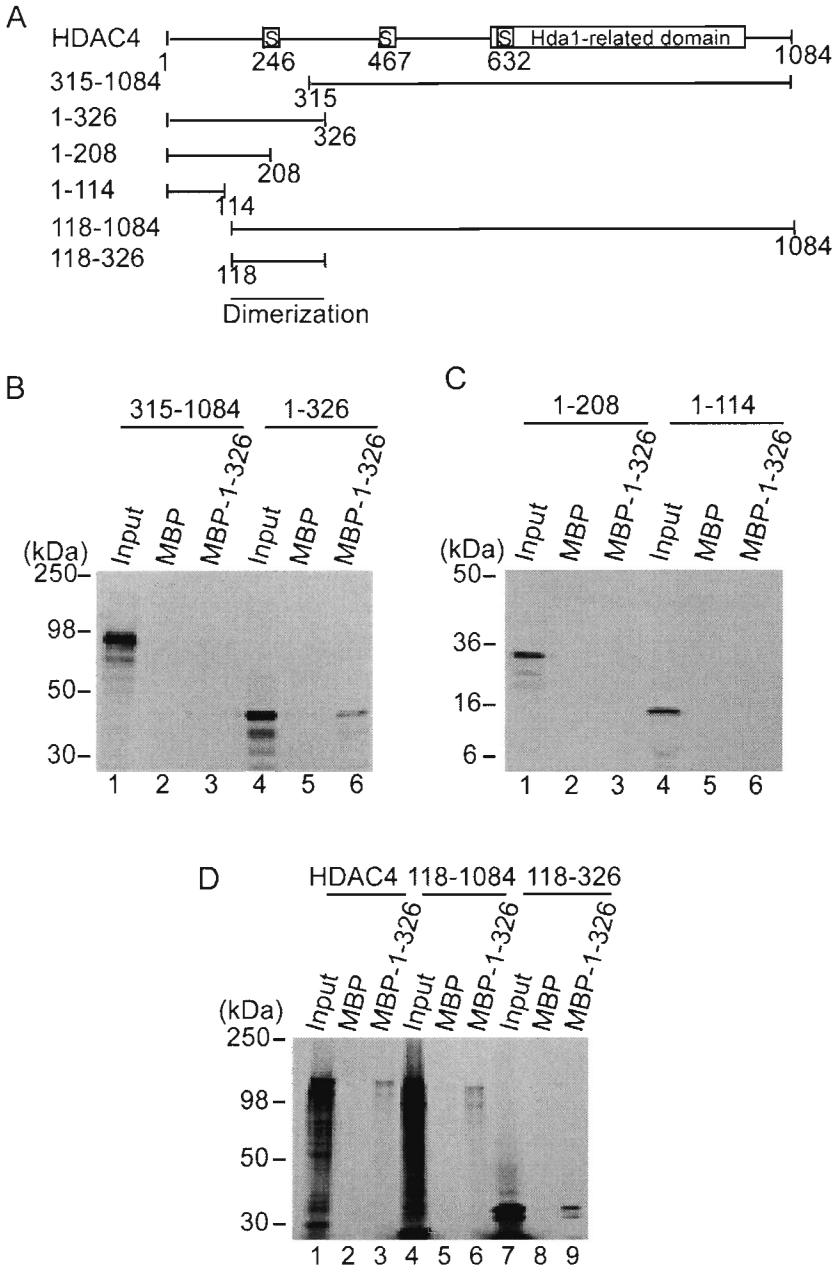
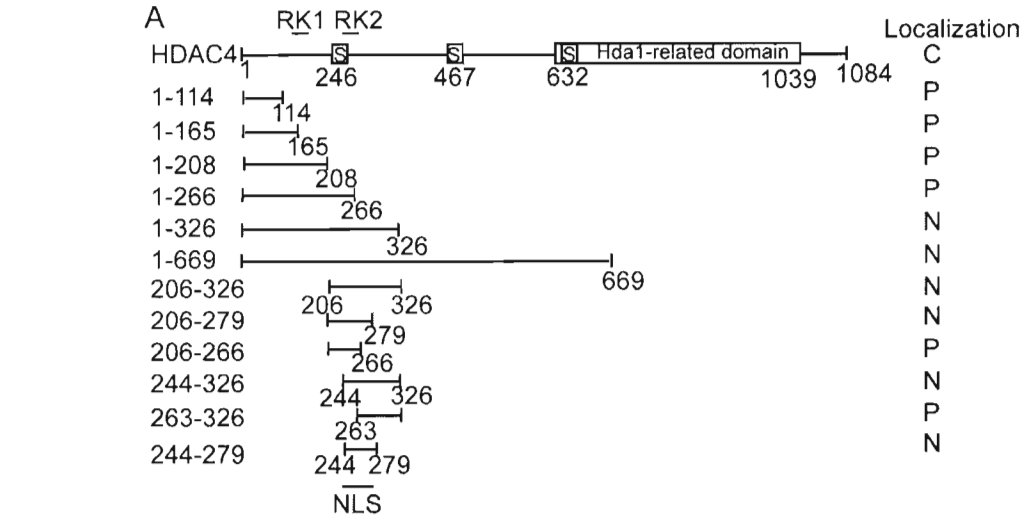

A
HDAC4
S
S
S
Hda1-related domain
1
246
467
632
1084
315-1084
315
1-326
326
1-208
208
1-114
114
118-1084
1084
118-326
118
Dimerization
B
315-1084
1-326
Input
MBP
MBP-1-326
Input
MBP
MBP-1-326
(kDa)
250–
98–
50–
30–
1 2 3 4 5 6
C
1-208
1-114
Input
MBP
MBP-1-326
Input
MBP
MBP-1-326
(kDa)
50–
36–
16–
6–
1 2 3 4 5 6
D
HDAC4
118-1084
118-326
Input
MBP
MBP-1-326
Input
MBP
MBP-1-326
Input
MBP
MBP-1-326
(kDa)
250–
98–
50–
30–
1 2 3 4 5 6 7 8 9

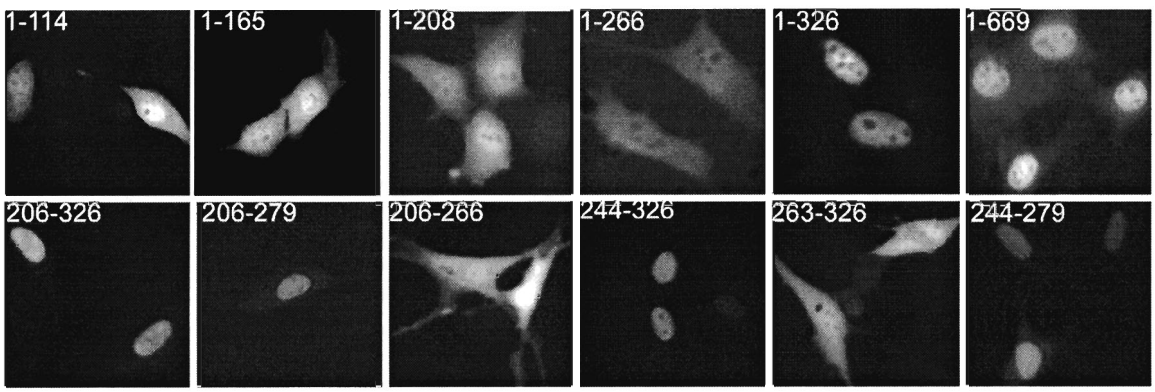
Fig. 2 Mapping the dimerization domain of HDAC4.

(A) Schematic representation of HDAC4 and deletion mutants. Motifs or domains are depicted by boxes as in Fig. 1A.

(B-D) Interaction among HDAC4 proteins. HDAC4 deletion mutants were expressed as MBP fusion proteins in *E. coli*, immobilized on amylose agarose and incubated with HDAC4 or deletion mutants synthesized *in vitro* in the presence of L-[^{35}S]methionine. Agarose beads were washed three times with buffer B-0.15 M KCl and once with buffer B-0.5 M KCl. Bound proteins were separated by SDS-PAGE and subsequently detected by autoradiography. Input represents 20% of the [^{35}S]-labeled protein used for each binding assay. Migrating positions of molecular markers are shown at the left of each panel.


A
RK1 RK2
Localization
HDAC4
S
S
S
Hda1-related domain
1
246
467
632
1039
1084
C
1-114
114
P
1-165
165
P
1-208
208
P
1-266
266
P
1-326
326
N
1-669
669
N
206-326
206
326
N
206-279
279
N
206-266
266
P
244-326
244
326
N
263-326
263
P
244-279
244
279
N
NLS

B


1-114
1-165
1-208
1-266
1-326
1-669
206-326
206-279
206-266
244-326
263-326
244-279

C

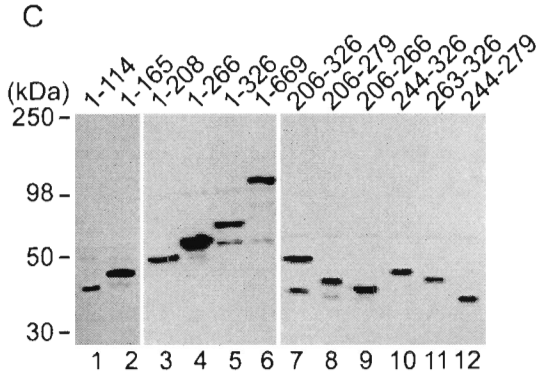

(kDa)
1-114
1-165
1-208
1-266
1-326
1-669
206-326
206-279
206-266
244-326
263-326
244-279
250
98
50
30
1 2 3 4 5 6 7 8 9 10 11 12

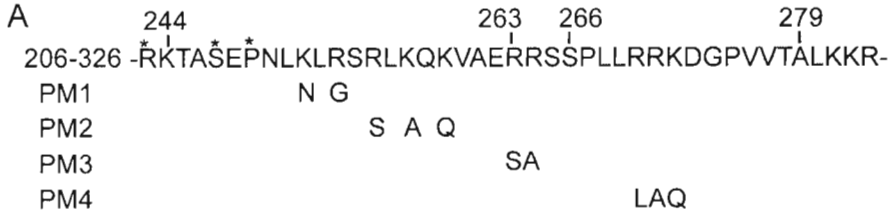
Fig. 3 Mapping the NLS of HDAC4.

(A) Schematic representation of HDAC4 and deletion mutants. Motifs or domains are depicted by boxes as in Fig. 1A. Also indicated are two arginine/lysine-rich regions: RK1 (residues 132-184) and RK2 (residues 242-283).

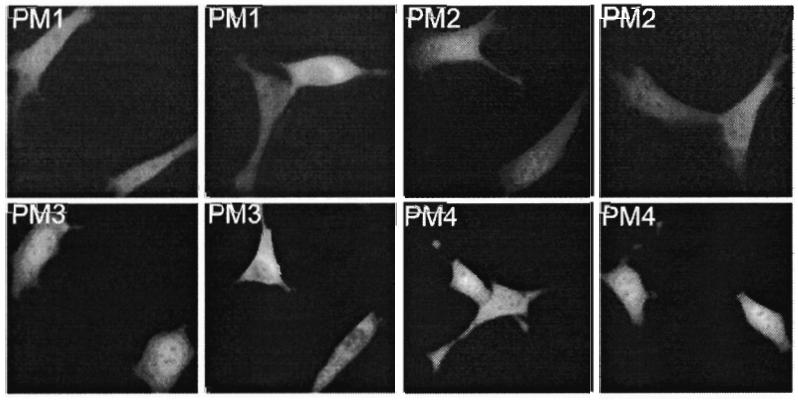
(B) Representative green fluorescence images of living cells expressing HDAC4 mutants fused to GFP. NIH 3T3 cells were transfected with expression plasmids for indicated GFP fusion proteins, and analyzed by live green fluorescence microscopy.

(C) Expression of GFP fusion proteins. 293 cells were transfected with expression plasmids for indicated GFP fusion proteins, and total cell extracts were analyzed by immunoblotting with anti-GFP antibody.

A


244
263
266
279
206-326 -RKTASEPNLKLRSRLKQKVAERRSSPLLRRKDGPVVTALKKR-
PM1 N G
PM2 S A Q
PM3 SA
PM4 LAQ

B


PM1
PM1
PM2
PM2
PM3
PM3
PM4
PM4

C

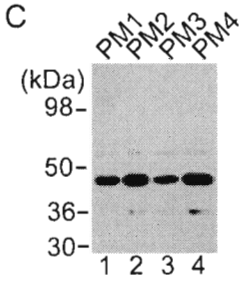

PM1
PM2
PM3
PM4
(kDa)
98-
50-
36-
30-
1 2 3 4

Fig. 4 Mutational analysis of the NLS.

(A) Illustration of 206-326 and its point mutants. Amino acid sequence of residues 242-283 of HDAC4 is listed, with potentially important arginine/lysine residues shown in bold. Residues important for 14-3-3 binding are labeled with asterisks. The point mutants PM1-4 were derived from the deletion mutant 206-326 by substitution of indicated arginine/lysine residues.

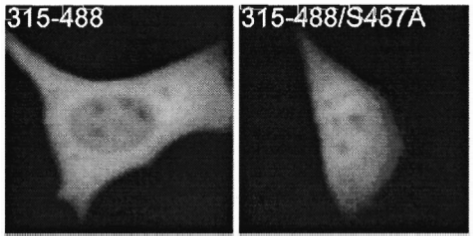
(B) Representative green fluorescence images of living NIH 3T3 cell expressing PM1-4 fused to GFP. Cells were transfected with expression plasmids for indicated GFP fusion proteins, and green fluorescence microscopy was performed with living cells. For each mutant, two images are shown to illustrate distinct localization in different cells.

(C) Expression of GFP fusion proteins. 293 cells were transfected with expression plasmids for indicated GFP fusion proteins, and cell extracts were analyzed by immunoblotting with anti-GFP antibody.

A

HDAC4376-LTLPALQQRLSL-387
413-LLQHMVLL-420
429-LVTGLGALPL-438
NES consensus: LxxxLxxLxL

B



C

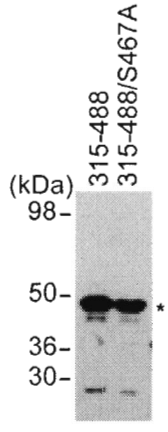
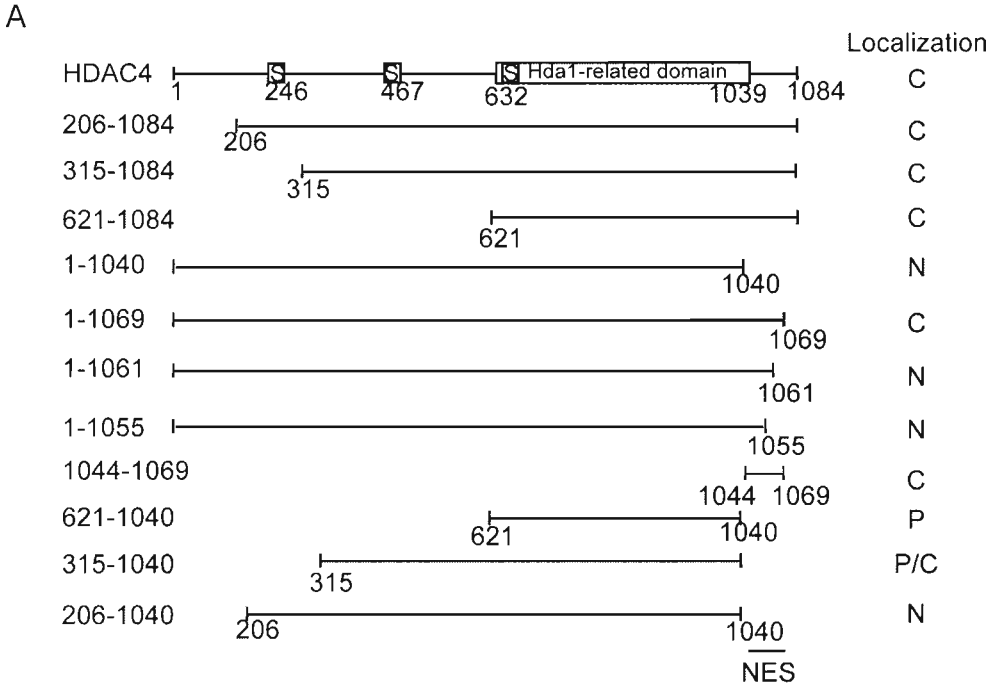


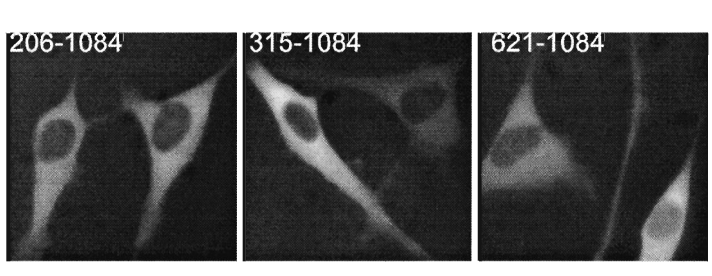
Fig. 5 Nuclear export activity of leucine-rich sequences of HDAC4.

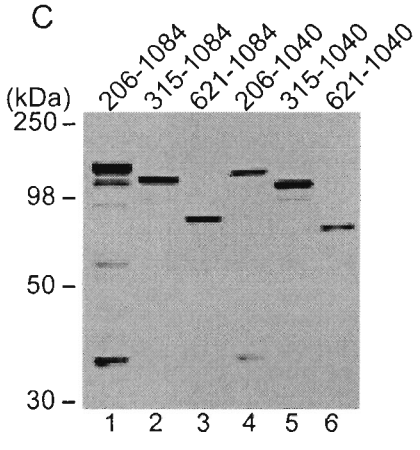
(A) Amino acid sequence of leucine–rich motifs of HDAC4, with leucine and methionine residues shown in bold. The consensus sequence of known leucine-rich export signals is also shown, with x denoting any amino acid residue.

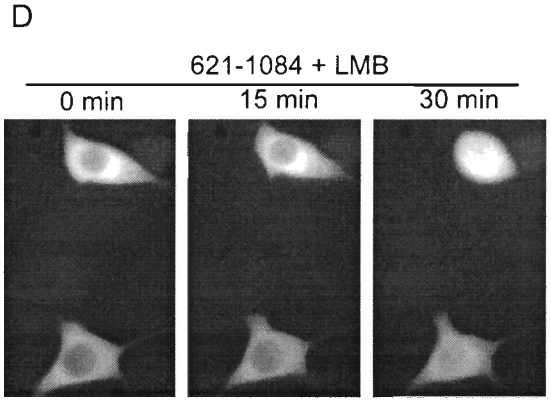
(B) Representative green fluorescence images of living cells expressing 315-488 and its point mutant fused to GFP. NIH 3T3 cells were transfected with expression plasmids for the mutants, and green fluorescence microscopy was performed with living cells.

(C) Expression of GFP-fusion proteins. 293 cells were transfected with expression plasmids for GFP-315-488 and -315-488/S467A, and total cell extracts were analyzed by immunoblotting with anti-GFP antibody.


A
Localization
HDAC4
S
S
S
Hda1-related domain
1
246
467
632
1039
1084
C
206-1084
206
C
315-1084
315
C
621-1084
621
C
1-1040
1040
N
1-1069
1069
C
1-1061
1061
N
1-1055
1055
N
1044-1069
1044
1069
C
621-1040
621
1040
P
315-1040
315
P/C
206-1040
206
1040
N
NES


B
206-1084
315-1084
621-1084


C
206-1084
315-1084
621-1084
206-1040
315-1040
621-1040
(kDa)
250
98
50
30
1
2
3
4
5
6


D
621-1084 + LMB
0 min
15 min
30 min

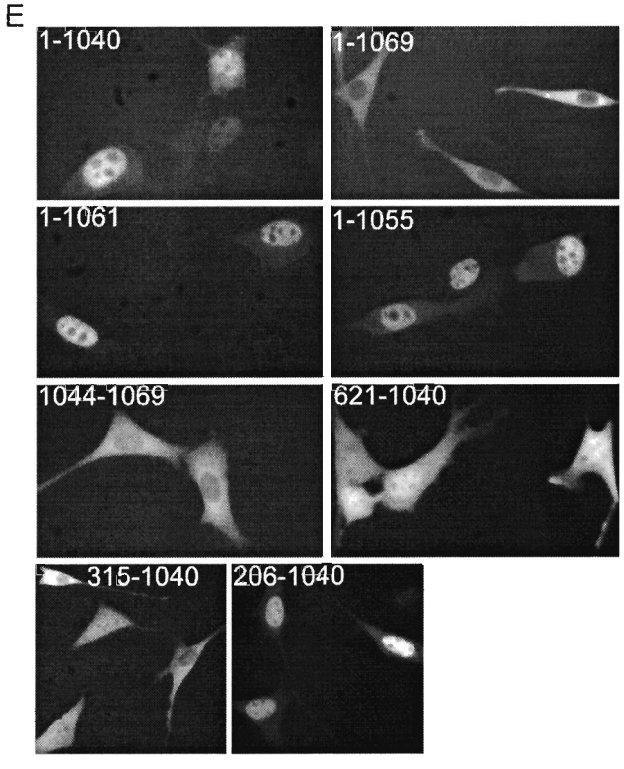

E
1-1040
1-1069
1-1061
1-1055
1044-1069
621-1040
315-1040
206-1040
F
(kDa)
1-1069
1-1061
1-1055
1-1040
1044-1069
250-
98-
50-
30-
*
*
1 2 3 4 5
G
1044-1069 + LMB
0 min
15 min
30 min

Fig. 6 Mapping intrinsic NES of HDAC4.

(A) Schematic illustration of HDAC4 and its deletion mutants. Motifs or domains are depicted by boxes as in Fig. 1A. Subcellular localization of HDAC4 and mutants is summarized at right.

(B, E) Representative green fluorescence images of living cells expressing HDAC4 and its deletion mutants fused to GFP. NIH 3T3 cells were transfected with expression plasmids for indicated GFP fusion proteins, and green fluorescence microscopy was performed with living cells.

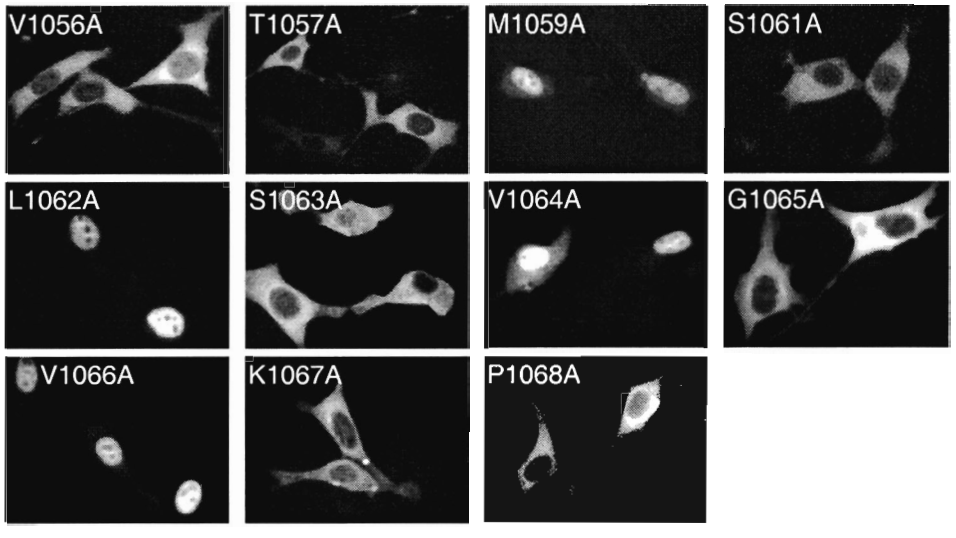
(C, F) Expression of GFP-fusion proteins. 293 cells were transfected with expression plasmids for indicated GFP fusion proteins, and total cell extracts were analyzed by immunoblotting with anti-GFP antibody.

(D, G) Effect of LMB on subcellular distribution of indicated GFP fusion proteins expressed in NIH 3T3 cells. After initial examination for green fluorescence, living cells expressing the indicated fusion proteins were treated with LMB (10 ng/ml) and their green fluorescence images were taken at indicated times. Under similar conditions, LMB had minimal effects on subcellular localization of GFP itself (data not shown).

A

		Localization
HDAC4	1056-VTAMASLSVGVKPA-1069	C
V1056A	A----------	C
T1057A	-A----------	C
M1059A	--A----------	N
S1061A	----A----------	C
L1062A	-----A----------	N
S1063A	------A----------	C
V1064A	-------A----------	N
G1065A	--------A----------	C
V1066A	---------A----------	N
K1067A	----------A----------	C
P1068A	-----------A----------	C

B



C

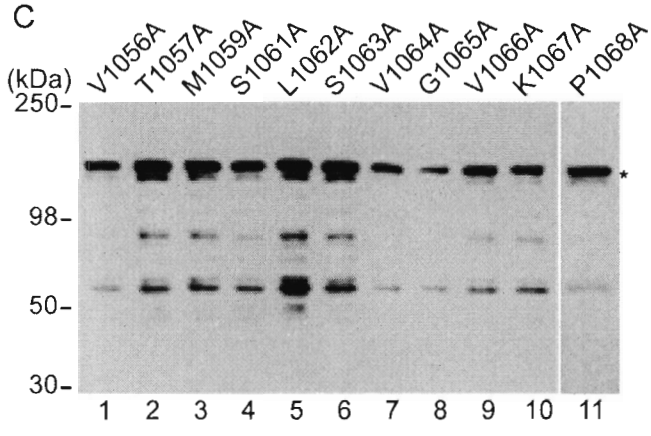


Fig. 7 Mutational analysis of the NES.

(A) Amino acid sequences of residues 1056-1069 of HDAC4 and mutants. M1059, L1062, V1064 and V1066 of HDAC4 are labeled with asterisks. For the point mutants, substituted and unchanged residues are indicated by the letter A (for alanine) and hyphens, respectively.

(B) Representative green fluorescence images of living cells expressing HDAC4 mutants fused to GFP. NIH 3T3 cells were transfected with expression plasmids for indicated GFP fusion proteins, and green fluorescence microscopy was performed with living cells.

(C) Expression of GFP-fusion proteins. 293 cells were transfected with expression plasmids for indicated GFP fusion proteins, and total cell extracts were analyzed by immunoblotting with anti-GFP antibody. The asterisk at right marks the expected migrating position.

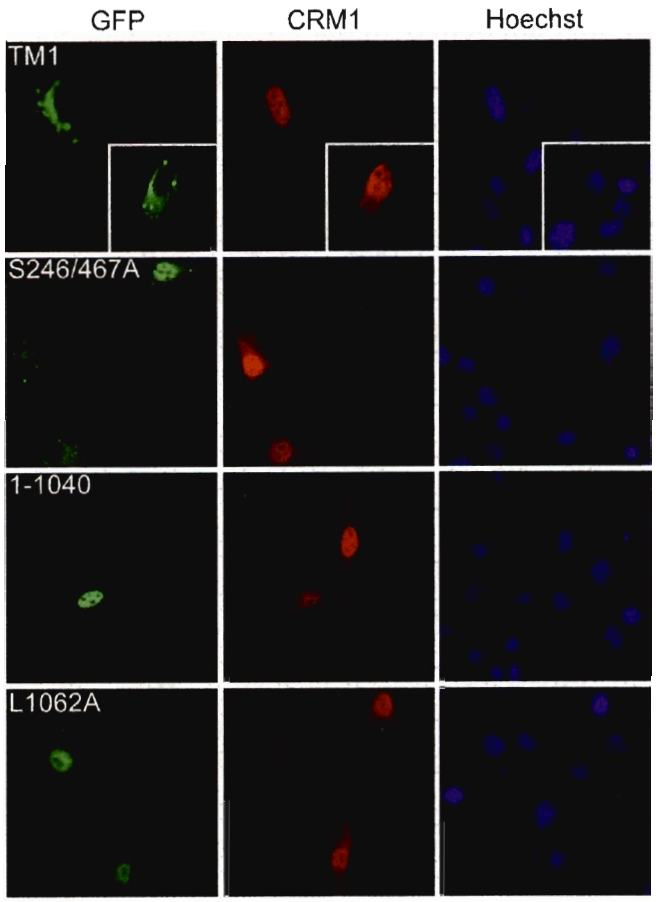

GFP
CRM1
Hoechst
TM1
S246/467A
1-1040
L1062A

Fig. 8 Effects of overexpressed CRM1 on subcellular localization of HDAC4 mutants.

An HA-CRM1 expression plasmid was transfected into NIH 3T3 cells along with mammalian expression plasmids for HDAC4 mutants fused to GFP. 16 h after transfection, cells were fixed and stained with anti-HA antibody to detect exogenous CRM1 (middle, red) by indirect immunofluorescence microscopy. Green fluorescence was used to determine subcellular localization of GFP fusion proteins (left, green). The cells were counter-stained with Hoechst 33528 to visualize nuclei (right, blue). While endogenous CRM1 is enriched around the nuclear envelope, overexpressed CRM1 has been found to be pancellular or nuclear (17, 71).

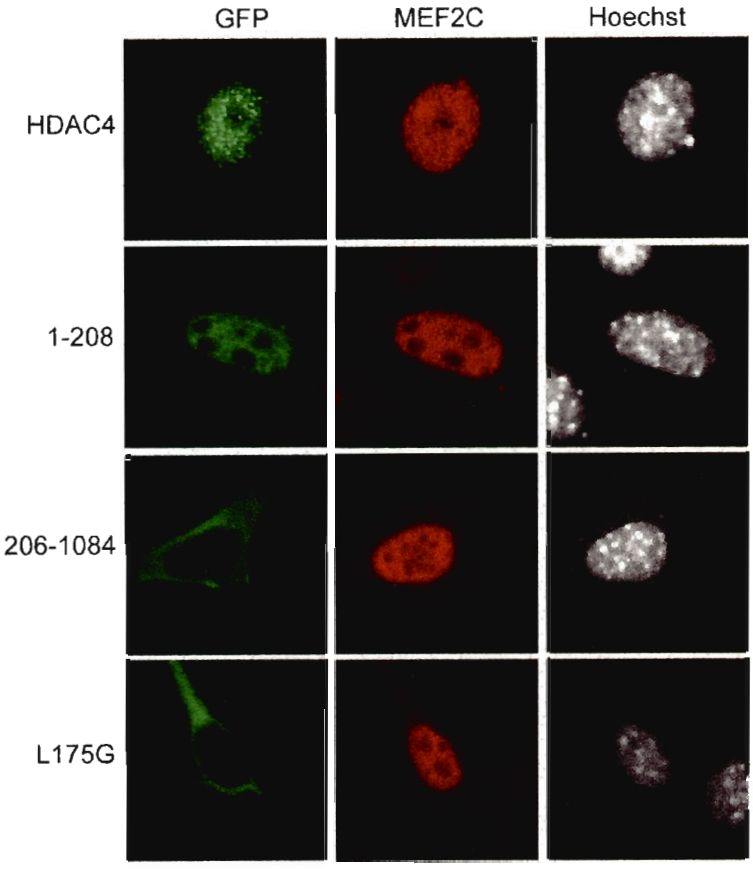

GFP
MEF2C
Hoechst
HDAC4
1-208
206-1084
L175G

Fig. 9 Effects of exogenous MEF2C on nuclear localization of HDAC4 and mutants.

The MEF2C expression plasmid was transfected into NIH 3T3 cells along with mammalian expression plasmids for GFP fusion proteins of HDAC4 or its mutants. 16 h after transfection, cells were fixed and stained with anti-MEF2C antibody to detect MEF2C by indirect immunofluorescence microscopy (middle, red). Green fluorescence was used to determine subcellular distribution of GFP fusion proteins (left, green). The cells were counter-stained with Hoechst 33528 to visualize nuclei (right, white).

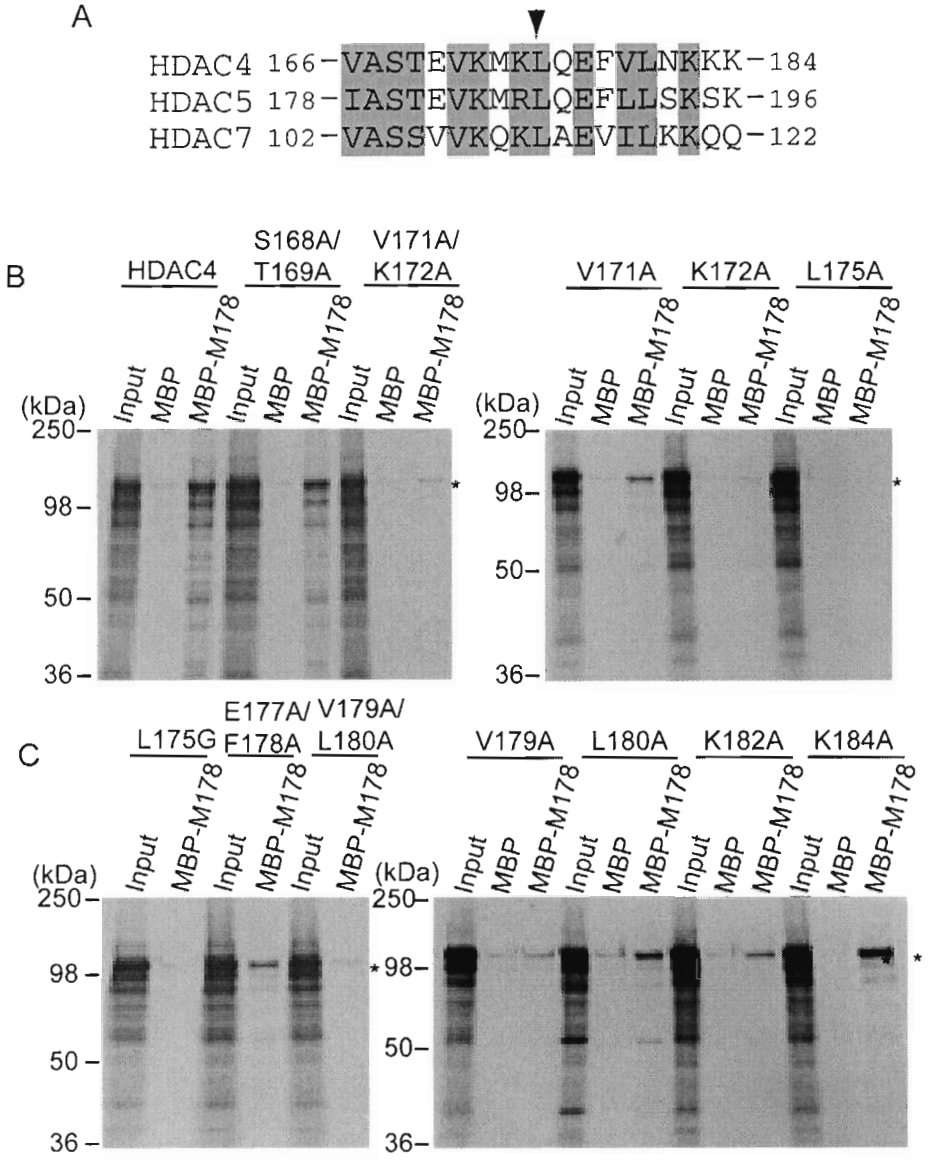
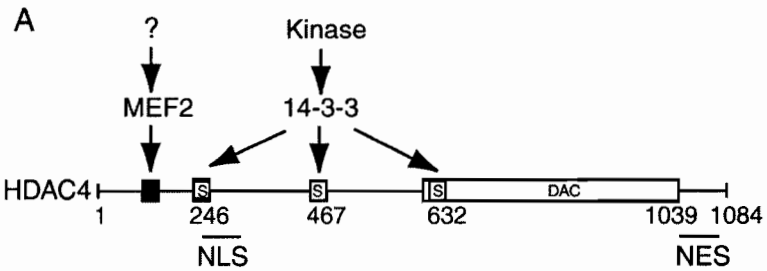

A
HDAC4 166-VASTEVKMKLQEFVLNKKK-184
HDAC5 178-IASTEVKMRLQEFLLSKSK-196
HDAC7 102-VASSVVKQKLAEVILKKQQ-122
B
HDAC4
S168A/ T169A
V171A/ K172A
V171A
K172A
L175A
Input
MBP
MBP-M178
(kDa)
250
98
50
36
C
L175G
E177A/ F178A
V179A/ L180A
V179A
L180A
K182A
K184A

Fig. 10 Mutational analysis of the MEF2-binding site of HDAC4.

(A) Sequence comparison of residues 166-184 of HDAC4 with the corresponding regions of HDAC5 and HDAC7. Identical or conserved residues are shaded, and L175 of HDAC4 is indicated by an asterisk.

(B-E) Interaction of the MEF2C mutant M178 with HDAC4 and point mutants. MBP or MBP-M178 was immobilized on amylose agarose and tested for interaction with HDAC4 or mutants synthesized *in vitro* in the presence of [^{35}S]methionine. Bound proteins were separated by SDS-PAGE and subsequent autoradiography. Input represents 20% of the [^{35}S]-labeled protein used for each binding assay. Migrating positions of molecular markers are shown at the left of each panel, whereas the positions of HDAC4 and its mutants are indicated by asterisks at right.



B

```
HDAC4  251-KLRSRLKQKVAERRSSPLLRRK-272
HDAC5  264-KVRSRLKQKVAERRSSPLLRRK-285
MITR   223-KVRSRLKQKVVERRSSPLLRRK-244
HDAC7  183-KLRYKPK.KSLERRKNPLLRKE-203
```

C

```
                MxxLxVxV
HDAC4  1056-VTAMASLSVGVKPA-1069
HDAC5  1086-VSAMALLSVGAEQA-1099
HDAC7   999-VTALASLSVGIL.A-1012
```

Fig. 11 (A) Model depicting how subcellular localization of HDAC4 is controlled.

HDAC4 possesses intrinsic nuclear import and export signals important for its dynamic nucleocytoplasmic shuttling. Association with 14-3-3, or MFE2, proteins modulates the shuttling. 14-3-3 binding promotes cytoplasmic localization of HDAC4 by both inhibiting its nuclear import and stimulating its nuclear export, whereas MEF2 interacts with HDAC4 and targets it to the nucleus. While phosphorylation of S246, S467 and S632 of HDAC4 stimulates the binding of 14-3-3 proteins, it remains less clear how the interaction between MEF2 and HDAC4 is regulated.

(B) Sequence comparison of the NLS of HDAC4 with the corresponding regions of HDAC5, MITR and HDAC7.

Critical residues of the HDAC4 NLS are boxed to the related residues of the other three proteins. (C) Sequence alignment of the NES of HDAC4 with the related regions of HDAC5 and HDAC7. Critical residues of the HDAC4 NES are boxed to the corresponding residues of HDAC5 and HDAC7.

CHAPTER V

Association of HDAC4 with the ankyrin-repeat proteins ANKRA2 and RFXANK

Preface

Class II HDACs have been shown to play an important role in regulating functions of MEF2 during muscle differentiation. In chapters II-IV, I have described that HDAC4 acts as a MEF2 corepressor whose function is regulated by nucleocytoplasmic shuttling. As shown in chapter II, HDAC4 is expressed in different human adult tissues, including skeletal muscle, brain, heart, thymus and spleen, suggesting that this deacetylase may have functions other than regulation of muscle differentiation. The amino-terminal domains of class II HDACs are unique and mediate interaction with transcription factors and corepressors. To further investigate the function of the amino-terminal extension of HDAC4, a yeast two-hybrid screen was performed. In this chapter, the binding of two ankyrin repeat proteins, ANKRA2 and RFXANK, to HDAC4 and the potential role of this interaction are described.

ABSTRACT

Histone acetylation plays an important role in regulating chromatin structure and thus gene expression. Class II histone deacetylases (HDACs) 4, 5, 7, and 9 share similar structural organization, with a carboxy-terminal catalytic comain and an amino-terminal extension. Association of these HDACs with some transcription factors and corepressors leads to transcriptional repression of target genes. Here we report that two ankyrin repeat-containing proteins, ANKRA2 and RFXANK, interact with the amino-terminal domain of HDAC4. The ankyrin repeats of ANKRA2 and RFXANK appear to mediate the association of HDAC4. HDAC4 and ANKRA2 or RFXANK colocalize both in the nucleus and in the cytoplasm. Furthermore, HDAC4 represses CIITA-mediated transactivation of MHC II genes. These results identify the transcription factor RFXANK as a new HDAC4-binding partner and suggest that HDAC4 may play a role in regulation of MHC II gene expression.

INTRODUCTION

Acetylation and deacetylation of histones, play a critical role in transcriptional regulation in eukaryotic cells and are controlled by histone acetyltransferase (HAT) and histone deacetylase (HDAC) enzymes (7). Mammalian HDACs can be divided into 3 classes (5). Class I HDACs, including HDAC1, 2, 3, and 8, show high similarity to the yeast deacetylase Rpd3. Class II contains HDAC4, 5, 6, 7, 9, and 10 which possess domains similar to the catalytic domain of yeast Hda1. Class III is composed of proteins that are homologous to yeast NAD^+-dependent deacetylase Sir2. Within class II, HDAC4, 5, 7 and 9 display sequence similarity in the regions outside of their catalytic domains, whereas HDAC6 and 10 are more similar to each other. Class IIa HDACs associate with myocyte enhancer factor 2 (MEF2) and play an important role in regulating functions of MEF2 during muscle differentiation (3, 11, 15, 17, 23, 33, 35, 41). Class II HDACs are recruited to repress gene transcription by other transcriptional factors or corepressors, such as BCL-6, B-CoR, N-CoR/SMRT, CtBP and HP1 (8-10, 14, 39, 40).

One of the unique and important characteristics of class II HDACs is that they are subjected to signal-dependent nucleocytoplasmic shuttling. Calcium/calmodulin-dependent protein kinases (CaMK) I and IV phosphorylate two conserved serines in HDAC4, 5, 7, and 9 (20), which then promotes the binding of 14-3-3 proteins and the dissociation of MEF2-HDAC complexes. The association with 14-3-3 proteins also masks the nuclear localization sequences

and exposes the nuclear export sequences of HDAC4, 5, 7, and 9 which leads to their export from the nucleus to the cytoplasm (6, 11, 21, 22, 36, 37).

To further characterize the function and regulation of class II HDACs, we used yeast two-hybrid screening to identify the HDAC4-interacting proteins. These efforts lead to the identification the ankyrin repeat proteins RFXANK and ANKRA2 as two new partners for HDAC4.

RFXANK, or RFX-B, was first isolated as a subunit of transcription factor RFX from the major histocompatibility complex (MHC) class II deficiency patients (19, 24). RFXANK associates with RFX5 and RFXAP to assemble into a trimeric RFX DNA-binding complex which binds specifically to the conserved X box of MHC class II gene promoters (31). All three subunits are required for binding of the complex. However, RFX binding is necessary but not sufficient for MHC II gene transcription. A transcriptional coactivator, class II trans-activator (CIITA), is a master switch that is recruited to the MHC II promoter via protein-protein interaction and triggers MHC II gene expression (34). Different mutations in these four *trans*-acting proteins, RFX5, RFXAP, RFXANK and CIITA, belong to four complementation groups of the bare lymphocyte syndrome (BLS) that is an autosomal and recessive severe combined immunodeficiency attributed to the lack of class II MHC expression on B cells (30). All three RFX subunits appear to be expressed ubiquitously and constitutively, whereas CIITA expression is tightly regulated. CIITA exhibits cell-specific, INF-γ-inducible, and differentiation-specific expression precisely parallel to that of MHC II synthesis (34).

Ankyrin-repeat family A protein 2 (ANKRA2) is a novel protein that was identified in yeast two-hybrid screening by using the cytoplasmic tail of megalin as a bait (28). ANKRA2 has three ankyrin repeats at its carboxyl terminus and show 61% overall homology to RFXANK in all regions except the amino-terminal domain. ANKRA2 interacts with meglin receptor through its ankyrin repeats. However, the function of ANKRA2 is still largely unknown.

Here we show that HDAC4 interacts with ANKRA2 and RFXANK. The ankyrin repeats of ANKRA2 and RFXANK mediate this interaction. Furthermore, HDAC4 can repress the MHC II gene expression through interaction with RFXANK.

MATERIALS AND METHODS

Yeast two-hybrid screening. A cDNA encoding the amino-terminal 666 residues of HDAC4 was fused in frame to the yeast GAL4 DNA-binding domain and used as bait to screen a human fetal brain cDNA library cloned into the yeast GAL4 activation domain vector (Clontech). The bait plasmids were transformed into yeast strain AH109 and mated with yeast strain Y187 which were pretransformed with the cDNA library. AH109 contains three reporters, *ADE2*, *HIS3*, and *MEL1* (or *lacZ*), under the control of distinct GAL4 upstream activating sequences and TATA boxes, while Y187 possesses one reporter, *lacZ*. Positive clones were isolated via growth on high stringent selection medium for all three reporters in AH109 and in the basis of β-galactosidase expression.

Those clones specific for interaction with bait were subjected to further sequencing. Liquid β-galactosidase assays were performed according to manufacturer's instrution (Clontech). Galactosidase activity was measured using Galacto-Light Plus™ (Tropix) as the substrate. The chemiluminescence from activated Galacto-Light Plus™ was measured on a Luminometer Plate Reader (Dynex).

Molecular cloning. The yeast two-hybrid bait was constructed in the pGBKT7 vector (Clontech). Mammalian expression plasmids for the epitope-tagged derivatives of human HDAC4, ANKRA2 and RFXANK were constructed into the pcDNA3.1 Vector (Invitrogen). Mammalian expression vectors for murine HDAC5 and murine HDAC7 were kindly provided by Drs S Khochbin and E Seto, repectively. Green fluorescent protein (GFP) constructs were derived from

pEGFP-C2 (Clontech). Mammalian expression plasmid CIITA and luciferase reporter DRA-Luc were kindly provided by Drs Kenneth Wright and Jenny Ting.

Cell culture and transfection. NIH3T3, 293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and penicillin-streptomycin. NIH3T3 and 293 cells were transfected using the SuperFect transfection reagent (Qiagen) according to the manufacturer's instruction.

Protein-protein interaction assays. To examine the interaction of HDAC4 with ANKRA2 or RFXANK *in vivo*, HDAC4 (Flag-tagged or HA-tagged) expression plasmid was transfected into 293 cells along with ANKRA2 (Flag-tagged) or RFXANK (HA-tagged) expression plasmids. 2.5 μg of each plasmid was used to transfect 4 x 10^5 cells (in a 6 cm dish) with 10 μl of SuperFect transfection reagent. 48 h after transfection, cells were washed twice with PBS and collected in 0.5 ml of buffer B (20 mM Tris-HCl (pH 8.0), 10% glycerol, 5 mM $MgCl_2$, 0.1% NP-40 and protease inhibitors) containing 0.15 M KCl. Cell extract was prepared for affinity purification on M2 agarose beads (Sigma). Beads with bound immunocomplexes were washed four times with buffer B supplemented with 0.15 M KCl, and bound proteins were eluted with Flag peptide (Sigma). Eluted proteins were subsequently resolved on 10% SDS-PAGE and transferred to nitrocellulose membrane for Western analysis with the α-Flag or α-HA antibody. Blots were developed with Supersignal chemiluminescent substrates (Pierce).

To examine the *in vitro* interaction of HDAC4 with ANKRA2 and its mutant, Flag-HDAC4 was expressed in SF9 cells, immobilized on M2 agarose beads.

ANKRA2 and its mutant were synthesized *in vitro* with the TNT-T7 coupled reticulocyte lysate system (Promega) in the presence of Redivue L-[^{35}S]methionine (Amersham). After rotation for an hour at 4°C, agarose beads were washed, three times with buffer B/0.15 M KCl and once with buffer B/0.5 M KCl, and boiled in SDS sample buffer prior to separation by 10% SDS-PAGE and autoradiography.

For MBP (maltose-binding protein) pull-down assays, ANKRA2, RFXANK, and different RFXANK mutants were expressed in *E. coli* as proteins fused to MBP and immobilized on amylose agarose beads. HDAC4 was synthesized *in vitro* with the TNT-T7 coupled reticulocyte lysate system (Promega) in the presence of Redivue L-[^{35}S]methionine (Amersham). After rotation for an hour at 4°C, the complexes bound to agarose beads were washed, three times with buffer B/0.15 M KCl and once with buffer B/0.5 M KCl, and boiled in SDS sample buffer prior to separation by 10% SDS-PAGE and autoradiography.

Immunofluorescence microscopy. NIH3T3 cells (4 x 10^4) were seeded on coverslips in a 12-well plate and transfected with HA-tagged HDAC4 and GFP-ANKRA2 or GFP-RFXANK expression plasmids using 5 µl of the Superfect transfection reagent (Qiagen). After 15-24 h, cells were rinsed three times with PBS/1 mM $MgCl_2$/0.1 mM $CaCl_2$, and fixed by incubation with PBS/2% paraformaldehyde at RT for 15 min. Cells were then rinsed three times with PBS, and free aldehyde groups were quenched with PBS/50 mM NH_4Cl for 10 min. After being washed twice with PBS, fixed cells were permeabilized with PBS/0.2% Triton X-100 for 5 min at RT, and blocking was performed with 1%

BSA in PBS for 10 min. Cells were further incubated with α-HA (1:500; Babco) antibody in blocking solution for 15 min. After being washed six times with PBS, cells were stained with Cy3 anti-mouse immunoglobulin G antibody (1:2000; Jackson Immunoresearch) and Hoechst 33258 (50 ng/ml; Sigma) for 20 min. Stained cells were washed seven times, and coverslips were then mounted in Immuno Fluore Mounting Medium (ICN Biodmedicals, Inc.). Expression of GFP fusion proteins was determined by green fluorescence microscopy. Fluorescence images were collected using a charge-coupled device camera (Q-imagine, Inc.) linked to a computer running Northern Eclipse (version 6.0; Empic Imagine) and exported for further processing with Adobe Photoshop.

Reporter gene assays. SuperFect transfection reagent (Qiagen) was used to transiently transfect a luciferase reporter plasmid (400 ng) into NIH3T3 cells, with or without mammalian expression plasmids (50-200 ng). pBluescript KSII(+) was included to normalize the total amount of plasmids used in each transfection and CMV-β-Gal (50 ng) was used for normalization of transfection efficiency. After 36-48 h, cells were lysed *in situ*, and luciferase reporter activity was determined using D-(-)-luciferin (Boehringer Mannheim) as the substrate. Galactosidase activity was measured using Galacto-Light Plus™ (Tropix) as the substrate. The chemiluminescence from activated luciferin or Galacto-Light Plus™ was measured on a Luminometer Plate Reader (Dynex).

RESULTS

Identification of ANKRA2 as an HDAC4 binding protein. To investigate the mechanisms involed in transcriptional repression by HDAC4, we performed a yeast two-hybrid screen with the amino-terminal 666 amino acids of HDAC4 as bait (Fig. 1A). Since HDAC4 was highly expressed in brain, a human fetal brain cDNA library was used. From the screen, 3 out of 13 positive clones encoded MEF2C and 14-3-3 that interacted with HDAC4 showed previously, suggesting that this screen was efficient. Two strong positive clones, clone #1 and #11, corresponded to ANKRA2 (Fig. 1B). While clone #1 encoded amino acids 49-313 of ANKRA2, clone #11 encoded amino acids 10-313. As shown in the liquid β-galactosidase assay (Fig. 1C), the ANKRA2 preys identified in the screen strongly activated β-galactosidase expression with the HDAC4 bait but not with GAL4-DNA binding domain alone, indicating that the association of two clones with the HDAC4 bait is specific.

To further test the specificity of the interaction between HDAC4 and ANKRA2, we expressed Flag-tagged HDAC4 in SF9 cells and produced ANKRA2 in an *in vitro* transcribed and translated system. As shown in Fig. 2A (lanes 1-3), HDAC4 interacted efficiently with [^{35}S]methionine-labeled ANKRA2. To examine if HDAC4 and ANKRA2 interact *in vivo*, coimmunoprecipitation assays were performed with protein lysates from transfected cells. Expression plasmids for Flag-ANKRA2 and HA-HDAC4 were transfected into 293 cells, and cell extracts were prepared for affinity purification on anti-Flag M2 agarose. Eluted immunocomplexes were subjected to Western blotting analyses with anti-

Flag and anti-HA antibodies. Consistent with *in vitro* pull-down experiments, Flag-tagged ANKRA2 was specifically coprecipitated with HA-tagged HDAC4 (Fig 2B). Therefore, three independent assays all support that ANKRA2 is capable of associating with HDAC4 *in vitro* and *in vivo*.

ANKRA2 is a novel protein that was identified in a yeast two-hybrid screen with the cytoplasmic tail of megalin as bait (28), but its function is barely known. It has three ankyrin repeats at its carboxy-terminus. Ankyrin repeats are protein-protein interaction motifs implicated in a wide range of biologically important regulatory events (32). The two clones isolated from our yeast two-hybrid screen both contained the carboxy-terminal ankyrin-repeat domain (Fig. 1B). Furthermore, a deletion mutant lacking ankyrin-repeat domain of ANKRA2 failed to interact with HDAC4 *in vitro* (Fig 2A, lanes 4-6), so the ankyrin-repeat domain of ANKRA2 mediates its association with HDAC4.

Interaction of RFXANK with HDAC4. The ankyrin repeat domain of ANKRA2 shows significant homology to RFXANK. As shown in Fig. 2C, amino acids 126 to 313 of ANKRA are 66% identical to amino acids 67 to 260 of RFXANK. Given that the ankyrin repeats of ANKRA2 mediate the interaction with HDAC4, we decided to test the interaction between HDAC4 and RFXANK.

To examine *in vivo* interaction between HDAC4 and RFXANK, we performed immunoprecipitation experiments in which Flag-tagged HDAC4 and/or HA-tagged RFXANK expression plasmids were cotransfected into 293 cells, and extracts prepared from the transfected cells were subjected to immunoprecipitation with anti-Flag M2 agarose. Eluted immunocomplexes were

subjected to Western blotting analyses with anti-Flag and anti-HA antibodies. As shown in Fig. 3A, RFXANK specifically precipitated with Flag-tagged HDAC4 (lanes 4-6). We also performed *in vitro* pull-down assays with a bacterially expressed MBP-RFXANK fusion protein and *in vitro* transcribed and translated HDAC4 (Fig. 3B, lanes 1-3). MBP-RFXANK interacted with [^{35}S]methionine-labeled HDAC4. The specificity of this binding was established because HDAC4 did not bind to MBP alone (Fig. 3B, lane 2).

To map the region of RFXANK that mediates the association with HDAC4, we initially generated a series of RFXANK deletion mutants and assessed their ability to bind with HDAC4. RFXANK contains four ankyrin repeats spanning the C-terminal part of the protein (25), homologous to that of ANKRA2 which may mediate the association with HDAC4. A series of deletion mutants were engineered to contain the N-terminal 248, 213, 180, 147, and 116 residues of RFXANK (Fig. 3C). These mutants have been used to map the interaction domain of CIITA on RFXANK (25). These deletion mutants were expressed in *E. coli* and incubated with *in vitro* transcribed and translated HDAC4 protein. RFXANK 1-248 and RFXANK 1-213 both bound to HDAC4 as efficiently as full length RFXANK (Fig. 3B, lanes 2, 7, and 8). Interestingly, when the ankyrin repeats of RFXANK were sequentially removed, the binding of HDAC4 decreased gradually (Fig. 3B, lanes 5 and 6) until it was completely abolished with RFXANK 1-116. From these data, it is clear that the first three ankyrin repeats of RFXANK are important for its binding to HDAC4.

Co-localization of HDAC4 and ANKRA2 or RFXANK. To examine the the subcelluar localization of ANKRA2 and RFXANK, we performed green fluorescence microscopy. NIH3T3 cells were transfected with GFP-ANKRA2 or GFP-RFXANK expression plasmids, and live transfected cells were examined for green fluorescence. While GFP itself was pancellular (data not shown), GFP-ANKRA2 and GFP-RFXANK were localized both in the nucleus and cytoplasm, partially enriched in the nucleus (Fig. 4A, b and c). Consistent with previous reports (36, 37), indirect immunofluorescence microscopy revealed that HA-HDAC4 was cytoplasmic in most transfected cells (Fig. 4A, a).

HDAC4 is subject to dynamic nucleocytoplasmic shuttling. Binding of 14-3-3 proteins promotes the cytoplasmic localization of HDAC4 (6, 20, 36), whereas association with nuclear protein MEF2 or SMRT results in the nuclear localization (23, 37, 38). Therefore, we asked whether binding of ANKRA2 or RFXANK also alters the shuttling of HDAC4. To address this question, we examined cells that were cotransfected with expression plasmids for HA-HDAC4 and GFP-ANKRA2 or GFP-RFXANK. As shown in Fig. 4B, coexpression of GFP-ANKRA2 or GFP-RFXANK led to nuclear accumulation of HA-HDAC4 (b and e). Besides nuclear colocalization, HDAC4 and ANKRA2 or RFXANK also appeared to colocalize in the cytoplasm (Fig. 4B, a, b, d and e). These observations confirm the interaction of ANKRA2 or RFXANK with HDAC4, and further suggest that ANKRA2 and RFXANK bind to HDAC4 and affect its dynamic nucleocytoplamic shuttling.

Repression of MHC II gene expression by HDAC4. RFXANK is required for assembling the RFX complex that binds to the X box of MHC II gene promoters and activates MHC II gene expression by recruiting the coactivator CIITA. Given the ability of RFXANK to associate with HDAC4, we investigated whether HDAC4 represses CIITA-mediated transactivation of MHC II genes. The role of HDAC4 in MHC II gene transcription was evaluated by transient transfection of the NIH3T3 cells with HLA-DRA-driven luciferase reporter construct and HDAC4. As shown in Fig. 5A, HDAC4 was able to repress the activity of DRA-Luc reporter in a dose-dependent manner. We then included CIITA in the transfection, since CIITA is not expressed in fibroblasts normally and is required for the transactivation of MHC II genes. In NIH3T3, CIITA was sufficient to induce MHC II transactivation (Fig. 5B). CIITA-mediated transactivation of MHC II was not enhanced by RFXANK or RFX5 (data not shown), indicating that the levels of RFX5 or RFXANK are not limiting in fibroblasts. As shown in Fig. 5B, CIITA-mediated MHC II transactivation was inhibited by HDAC4 in a dose-dependent manner. These results suggest that HDAC4 is recruited to RFX complex and represses CIITA-mediated transactivation of MHC II genes.

Discussion

The results of this study demonstrate that HDAC4 associates with two ankyrin-repeat proteins ANKRA2 and RFXANK. The interaction of HDAC4 with ANKRA2 and RFXANK is mediated by their ankyrin-repeat domains. The co-localization of HDAC4 with ANKRA2 and RFXANK confirms their association *in vivo*. Finally, HDAC4 is recruited to the MHC II promoters and represses CIITA-mediated transactivation of MHC II gene expression.

RFXANK, or RFX-B, was first isolated as a subunit of transcription factor RFX from the major histocompatibility complex (MHC) class II deficiency patients (19, 24). RFXANK contains ankyrin repeats at its C-terminal region (25). Ankyrin repeats are one of the most common protein sequence motifs, with each of them consisting of 33 residues (32). They have been found in proteins as different as Cdk inhibitors, signal transduction and transcriptional regulators, cytoskeletal organizers, developmental regulators, and toxins. These protein scaffolding modules mediate protein-protein interaction in a number of different biological systems. The ankyrin repeats of RFXANK have been suggested to provide an interaction platform to assemble the RFX complex (2, 26). Furthermore, RFXANK directly binds CIITA to its last three ankyrin repeats, of which the second ankyrin repeat seems to be the most critical one (25). Our results in this study showed that ankyrin repeats of RFXANK and ANKRA2 were required for the association with HDAC4. The ANKRA2 mutant lack of ankyrin repeat domain failed to interact with HDAC4 (Fig 2A). Further mapping of ankyrin repeat domain showed that the first three ankyrin repeats of RFXANK were important for its binding to

HDAC4 and the most critical repeat seemed to be the second one (Fig 3B). Since the binding sites for HDAC4 appear to overlap with those for CIITA on RFXANK (25), it is tempting to speculate that HDAC4 may compete with CIITA to bind to RFXANK and thereby inhibit the MHC II gene expression.

Histone acetylation has been shown to be involved in the activation of MHC II gene expression. Both CIITA and NF-Y can interact with HATs, p300/CBP and PCAF (4, 12). Moreover, CIITA itself has been shown to have intrinsic HAT activity (29). The role of HDACs has also been implicated in the control of MHC II gene expression. The HDAC inhibitor TSA rescues class II expression in tumor cells and mature dendritic cells (13, 18). HDAC1 has been shown to be recruited by transcripton factor YY1 to a YY1 binding element located in the first exon of the HLA-DRA promoter (27). Moreover, the recruitment of HDAC1 leads to repression of inducible HLA-DRA activation (27). IFN-γ regulates MHC II gene expression through the induction of CIITA transcription. ChIP (chromatin immunoprecipitation) assays have shown that CIITA binding to HLA-DRA promoter increases after IFN-γ treatment, as well as acetylation level of histone H3 and H4 (1). Removal of IFN-γ from cells results in a decrease in association of CIITA with HLA-DRA promoter and in acetylation level of histone H3 and H4 (1). The decrease of acetylation level of histone H3 and H4 must have resulted from the deacetylation of histones. From our results, HDAC4 directly bind to RFXANK, a subunit of RFX complex which binds to X box of all MHC II gene promoters. Therefore, after removal of IFN-γ from cells,

association of HDAC4 to the HLA-DRA promoter via RFXANK may increase, leading to deacetylation of histones and decrease of HLA-DRA expression.

RFXANK/Tvl-1 and ANKRA2 may define a novel family of scaffold proteins that promote the assembly of a variety of macromolecular complexes. ANKRA2 interacts with a membrane-associated receptor megalin (28). The ankyrin repeats of RFXANK provide an interaction platform to assemble the RFX complex (2, 26). RFXANK/Tvl-1has been shown to interact directly with Raf-1 through its ankyrin repeat domain and potentiate the activation of Raf-1 (16). We have shown here that HDAC4 also interacts with RFXANK via its ankyrin repeat domain. RFXANK may serve as a scaffold for assembling HDAC complex and/or affect the enzymatic activity of HDAC4.

In summary, the data presented in this study show that ANKRA2 and RFXANK both interact with HDAC4 in mammalian cells and that the ankyrin repeat domains of ANKRA2 and RFXANK are involved in mediating their association of HDAC4. Moreover, through RFXANK, HDAC4 represses transcription of MHC II genes.

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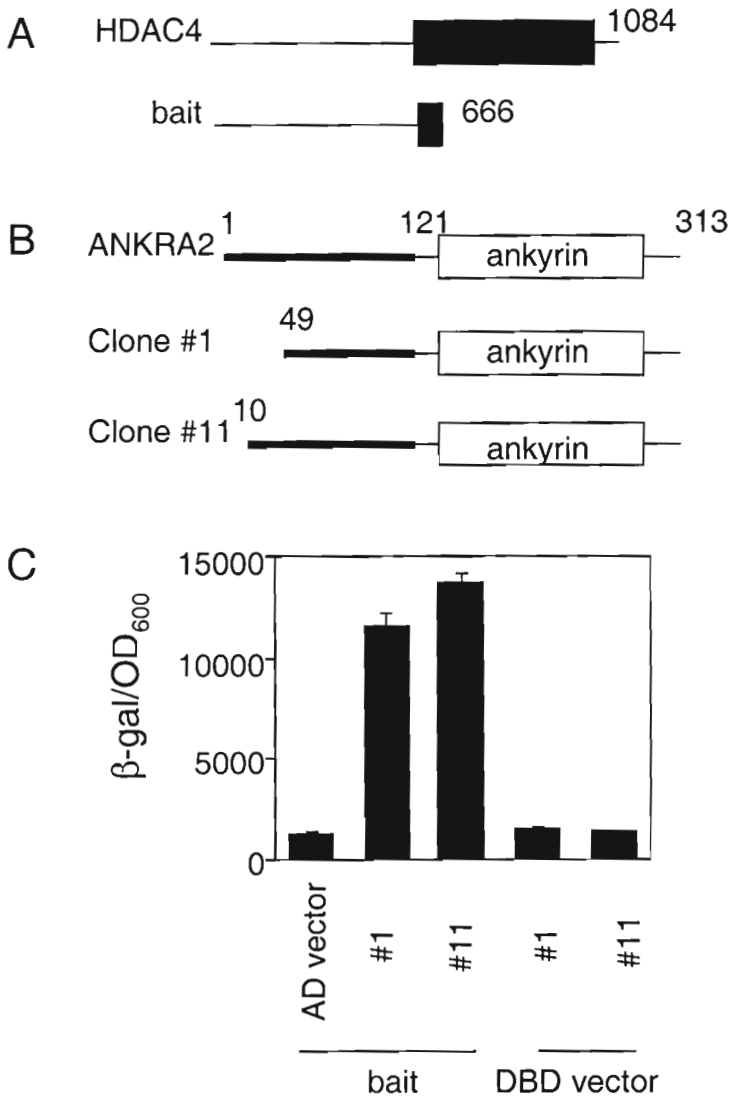
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HDAC4
1084
bait
666
B
ANKRA2
1
121
313
ankyrin
Clone #1
49
ankyrin
Clone #11
10
ankyrin
C
15000
10000
5000
0
β-gal/OD600
AD vector
#1
#11
#1
#11
bait
DBD vector

Figure 1. Interaction of HDAC4 and ANKRA2 in yeast.

(A) Schematic representation of HDAC4 and the amino-terminal region of HDAC4. HDAC4 1-666 was fused to the GAL4 DNA-binding domain (DBD) and used as bait in yeast two-hybrid screening.

(B) Schematic representation of ANKRA2 and two positive clones encoding ANKRA2 fragments isolated from the yeast two-hybrid screen.

(C) Interaction of ANKRA2 with HDAC4. The association of the two ANKRA2 clones isolated from screening with the HDAC4 bait was determined by measuring the β-galactosidase activity in liquid culture assays.

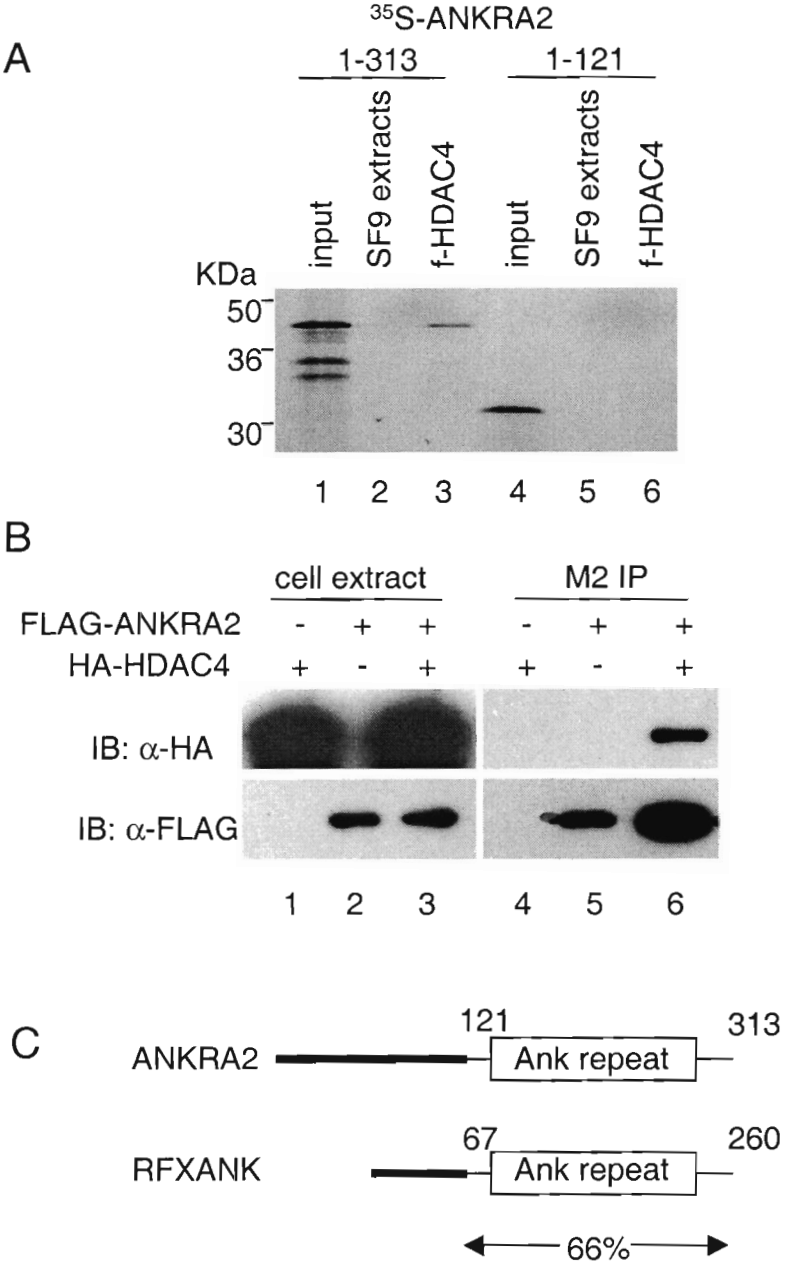
A
35S-ANKRA2
1-313
1-121
input
SF9 extracts
f-HDAC4
input
SF9 extracts
f-HDAC4
KDa
50
36
30
1 2 3 4 5 6
B
cell extract
M2 IP
FLAG-ANKRA2 - + + - + +
HA-HDAC4 + - + + - +
IB: α-HA
IB: α-FLAG
1 2 3 4 5 6
C
121
313
ANKRA2
Ank repeat
67
260
RFXANK
Ank repeat
66%

Figure 2. HDAC4 interacts with ANKRA2 *in vitro* and *in vivo*.

(A) Interaction of HDAC4 with ANKRA2 *in vitro*. Flag-HDAC4 was expressed in SF9 cells, immobilized on M2-agarose beads, and incubated with [^{35}S]-methionine-labeled full length and truncated ANKRA2. Associated proteins were resolved by SDS-PAGE and analyzed by autoradiography. Input lanes represent 20% of the [^{35}S]-methionine-labeled protein used for each assay.

(B) Interaction of HDAC4 with ANKRA2 *in vivo*. Flag-tagged ANKRA2 (lanes 2, 3, 5, and 6) was expressed with (lanes 1, 3, 4, and 6), or without (lanes 2 and 5) HA-tagged HDAC4 in 293 cells, and immunoprecipitated (IP) with anti-Flag M2 agarose. Immunoprecipitated proteins were eluted with Flag peptide (lanes 4-6), and extracts (lanes 1-3) were subjected to Western blotting analyses with anti-Flag or anti-HA antibody.

(C) Schematic representation of ANKRA2 and RFXANK. ANKRA2 and RFXANK are 66% identical within their C-terminal ankyrin-repeat domains.

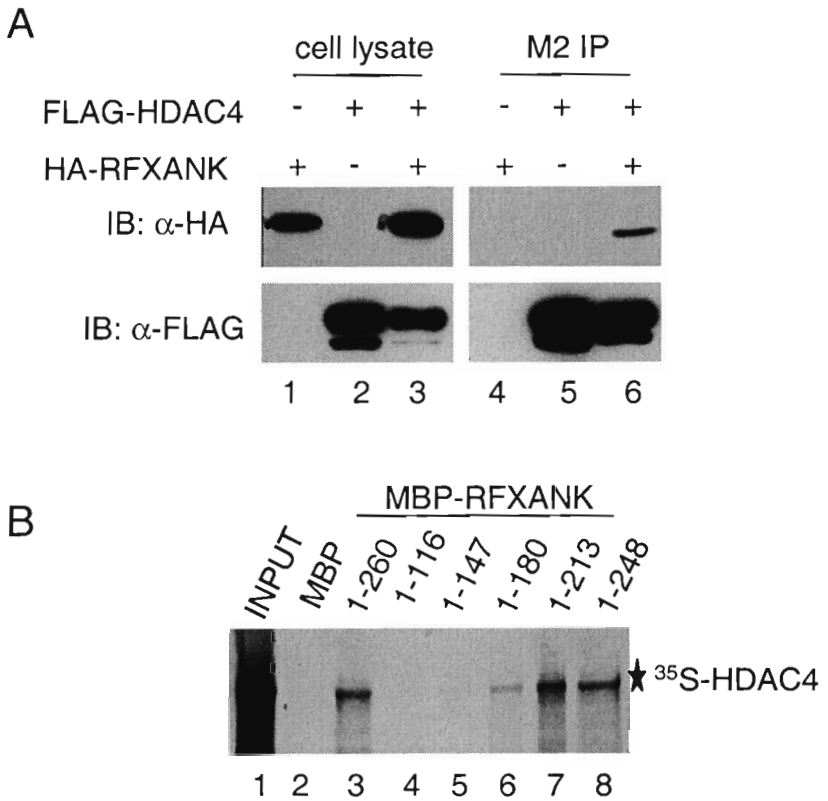
A
cell lysate
M2 IP
FLAG-HDAC4 - + + - + +
HA-RFXANK + - + + - +
IB: α-HA
IB: α-FLAG
1 2 3 4 5 6
B
MBP-RFXANK
INPUT
MBP
1-260
1-116
1-147
1-180
1-213
1-248
35S-HDAC4
1 2 3 4 5 6 7 8
C
RFXANK
1 2 3 4
260
1 2 3 4
248
1 2 3
213
1 2
180
1
147
116

Figure 3. HDAC4 interacts with RFXANK *in vitro* and *in vivo*.

(A) Immunoprecipitation of HDAC4 with RFXANK. Flag-tagged HDAC4 (lanes 2, 3, 5, and 6) was expressed with (lanes 1, 3, 4, and 6) or without (lanes 2 and 5) HA-tagged RFXANK in 293 cells and immunoprecipitated (IP) with anti-Flag M2 agarose. Immunoprecipitated proteins eluted with Flag peptide (lanes 4-6) and extracts (lanes 1-3) were subjected to Western blotting analyses with a α-Flag or α-HA antibody.

(B) Interaction of HDAC4 with RFXANK and its deletion mutants *in vitro*. MBP and MBP-RFXANK fusion proteins were expressed in E. coli, immobilized on amylase-agarose beads, and incubated with the [^{35}S]-methionine-labeled HDAC4. Associated proteins were resolved by SDS-PAGE and analyzed by autoradiography. Input lane represents 20% of the [^{35}S]-methionine-labeled protein used for each assay.

(C) Schematic representation of RFXANK and its deletion mutants.

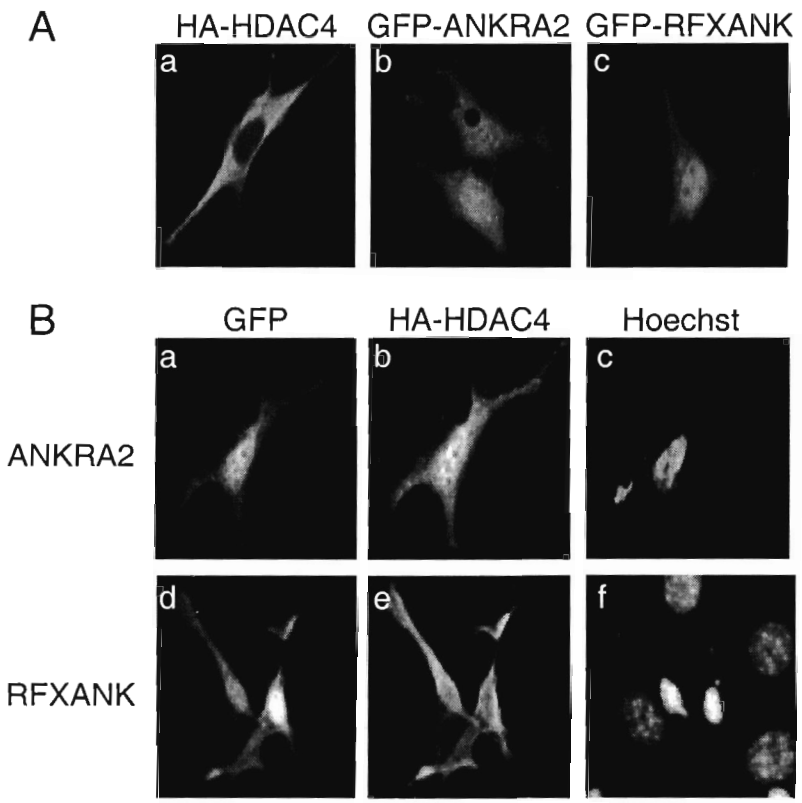
A
HA-HDAC4
GFP-ANKRA2
GFP-RFXANK
a
b
c
B
GFP
HA-HDAC4
Hoechst
ANKRA2
RFXANK
a
b
c
d
e
f

Figure 4. HDAC4 co-localizes with ANKRA2 or RFXANK.

(A) The HA-HDAC4, GFP-ANKRA2, and GFP-RFXANK expression plasmids were transfected into NIH 3T3 cells as indicated. 16h after transfection, cells were fixed and stained with anti-HA antibody to detect HDAC4 by indirect immunofluorescence microscopy (a). Green fluorescence was used to determine localization of GFP fusion proteins in live cells (b and c).

(B) The HA-HDAC4 expression plasmid was transfected into NIH 3T3 cells along with mammalian expression plasmids for GFP-ANKRA2 or GFP-RFXANK. 16h after transfection, cells were fixed and stained with anti-HA antibody to detect HDAC4 by indirect immunofluorescence microscopy (b and e). Green fluorescence was used to determine localization of GFP fusion proteins (a and d). The cells were counterstained with Hoechst 33528 to visualize their nuclei (c and f).

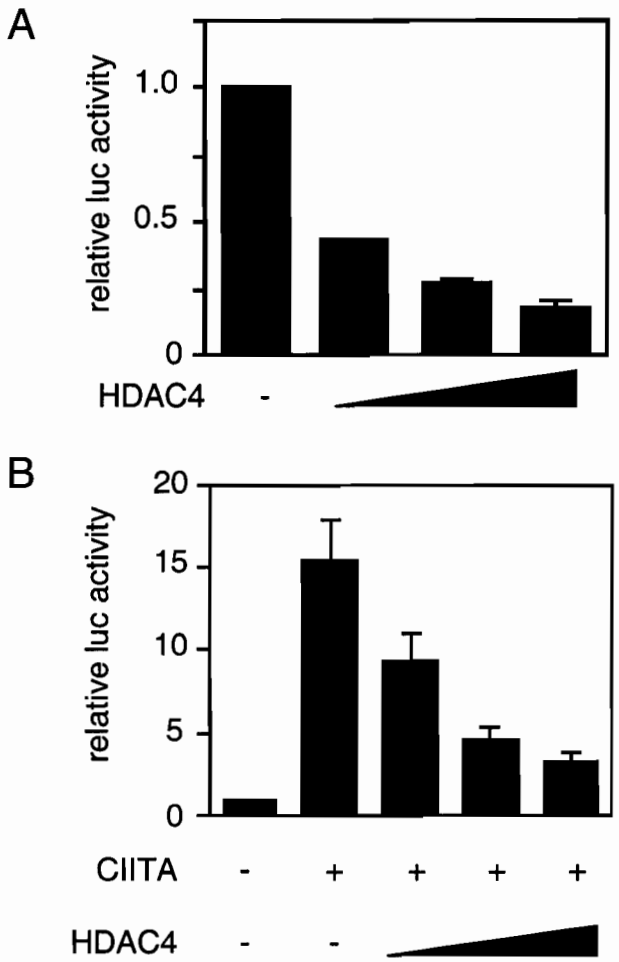
A
relative luc activity
1.0
0.5
0
HDAC4 -
B
relative luc activity
20
15
10
5
0
CIITA - + + + +
HDAC4 - -

Figure 5. HDAC4 represses MHC II gene expression.

(A) Inhibition of MHC II gene expression by HDAC4. A MHC II gene reporter plasmid (DRA-luciferase; 0.4 μg) was transfected into NIH3T3 cells with increased amounts of the HDAC4 expression vector (0.05 to 0.2 μg). A CMV-β-galactosidase reporter (0.05 μg) was also included for normalization of transfection efficiency and luciferase activity was determined as described in Materials and Methods. Values represent means ± standard deviations.

(B) Inhibition of CIITA transcriptional activity by HDAC4. The assay was performed as in (A) except a CIITA expression plasmid was also used as indicated.

CHAPTER VI

General Discussion

In chapters II-V, I have described that HDAC4 functions as an enzymatic transcriptional corepressor whose function is regulated by nucleocytoplasmic shuttling. Studies from other groups and ours have suggested that signal-dependent nucleocytoplasmic shuttling is a common mechanism regulating the activity of class II HDACs (1). Figure 1 summarizes the major findings of this thesis. Briefly, HDAC4 functions as a transcriptional corepressor recruited by transcription factors MEF2 and RFXANK. The activity of HDAC4 is regulated by nucleocytoplasmic shuttling. HDAC4 is localized mainly in the cytoplasm, and its cytoplasmic localization is controlled by the nuclear export signal (NES) and binding of 14-3-3 proteins. The balance of subcellular trafficking of HDAC4 can be further affected by association with MEF2 or RFXANK. In this section, I will discuss the significance of these findings in understanding the function and regulation of HDAC4 and related HDACs, and I will also raise questions that remain to be addressed.

Identification of a new class of HDAC family

Analysis of HDAC activity in *Saccharomyces cerevisiae* revealed the presence of two HDAC complexes, one containing Rpd3 as its catalytic subunit, and the other possessing Hda1 (14). Rpd3 and Hda1 are quite different in their molecular mass and primary structure, although both contain conserved deacetylase catalytic domains. The three human HDAC proteins, HDAC1-3, display greater homology to Rpd3 than to Hda1, suggesting that in mammalian cells, there may be an uncharacterized class of Hda1-like proteins biochemically

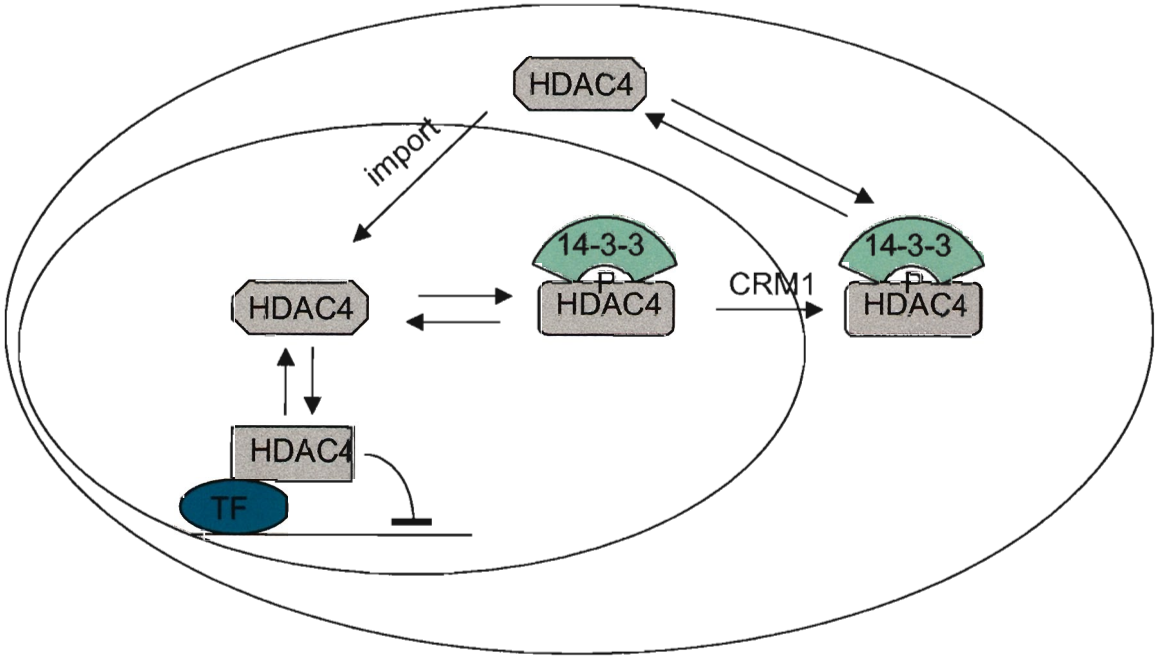

Figure 1. Regulation of the transcriptional repression activity of HDAC4 by nucleocytoplasmic shuttling.

distinct from HDAC1-3. Sequence database searches using BLAST and PSI-BLAST initially identified three putative human HDAC proteins, HDAC4, 5, and 6 (chapter II). Biochemical characterization of the recombinant enzymes confirms that these proteins possess HDAC activity. Therefore, human HDAC1-6 can be divided into two classes based on their primary structure and biochemical characteristics. The first class contains human HDAC1, 2 and 3, while the second class contains human HDAC4, 5 and 6. More recently, both families have expanded, with HDAC8 joining class I, and HDAC7, 9 and 10 identified as new class II members.

Different from yeast Hda1, HDAC4, 5, 7, and 9 contain deacetylase domains in their carboxy-terminal regions and amino-terminal extensions. They display significant sequence similarity not only in the catalytic domains, but also the amino-terminal extensions. According to sequence similarity, class II HDAC family can be further divided into two subclasses: IIa (HDAC4, 5, 7, and 9) and IIb (HDAC6 and 10). The amino-terminal regions are unique to class IIa proteins and may contribute to protein-protein interaction. Indeed, through the N-terminal region, HDAC4 interacts with MEF2 (chapter II), CtBP (19), HP1 (20), and RFXANK (chapter IV). However, more work is needed to further characterize roles of the N-terminal domains of class IIa HDACs. This will provide insight into how class IIa HDACs function *in vivo*.

Class II HDACs have been conserved in higher eukaryotes. Homologs have been identified in *C. elegans*, *Drosophila* and mouse. The evolutionary

conservation of these enzymes suggests that they also possess critical biological functions to those organisms.

Deacetylase activity and transcriptional repression potential of HDAC4

The crystal structure of the *A. aeolicus* HDAC homolog HDLP (HDAC-like protein) have been determined (3). The active site of HDLP consists of a tubular pocket, a zinc-binding site and two Asp-His charge-relay systems. The enzymatic activity requires Zn^{2+}, which is positioned in the bottom of the pocket and is coordinated by several histidine and aspartic acid residues. In agreement with this, mutations of the histidine and aspartic acid residues of the charge-relay systems abolish HDAC activity (5). A single point mutant of HDAC4 with the conserved histidine 803 replaced with leucine also abolished HDAC activity (chapter II).

It is well established that HDAC activities are important for transcriptional repression. In yeast, Rpd3 and Hda1 mediate gene-specific and global deacetylation. Rpd3 and Hda1 have different target genes, although there is a small degree of functional overlap (13). Human class I HDACs usually form different complexes with other proteins and can be recruited by DNA-binding proteins to repress transcription (4). Class II HDACs are also involved in transcriptional repression. As described in chapters II and V, HDAC4 directly interacts with transcription factors MEF2 and RFXANK to repress transcription of target genes.

By activating specific gene expression, the MEF2 family of transcription factors participates in diverse cellular processes, including muscle and neuronal differentiation (2). MEF2 proteins regulate transcription by recruiting HATs or HDACs. HDAC4 directly binds to MEF2 proteins and represses MEF2-dependent transcription. Interestingly, p300 and HDAC4 bind to the same domain of MEF2 (15). Binding of HDAC4 to MEF2 can be blocked by calmodulin because the MEF2-binding site on HDAC4 overlaps with the calmodulin-binding site (16). In the presence of calcium, HDAC4 binds to calmodulin and loses its interaction with MEF2, which then allows MEF2 to recruit p300. Therefore, altering its binding partners causes MEF2 to switch from silencing to activating gene expression. In addition, activated CaMK I and IV can dissociate HDACs from MEF2 and promote their nuclear export, resulting in derepression of MEF2-dependent transcription of muscle genes (6, 9). These data suggest that HDAC-mediated gene-specific repression of transcription is important for the control of gene expression by extracellular signals, in which genes are often maintained in an "off" state by repressor proteins until signal transduction pathways alleviate the repression.

MEF2 proteins also recruit another transcriptional corepressor, Cabin1 (17). Cabin 1 represses transcription by recruiting mSin3-HDAC1/2 complex. Thus, different HDAC activities are employed by MEF2. There are other cases in which transcription factors recruit multiple HDAC complexes. For example, BCL6 represses transcription by direct or indirect associations with both class I and class II HDACs (1). However, it remains unclear why different HDACs exist for

MEF2. It is possible that each HDAC is optimized for silencing a different region of the promoter when anchored to chromatin via interaction with MEF2. Alternatively, the maintenance of histone acetylation levels may be so critical to cell growth that multiple, functionally redundant enzymes are required.

HDAC activity can be inhibited by a group of small compounds, called HDAC inhibitors. Known HDAC inhibitors such as TSA and SAHA have been shown to interact with the catalytic site, thereby blocking substrate access to the active zinc ion at its base (3). These inhibitors are useful both for probing the biological functions of different HDACs and for therapeutic purposes such as inhibiting a specific HDAC that is associated with a particular disease. Although the catalytic domains of both class I and class II HDACs are relatively conserved, these deacetylases display different sensitivity to HDAC inhibitors. For example, HDAC6 is sensitive to TSA, but specifically resistant to trapoxin (TPX). Such a sensitivity difference has allowed the identification of α-tubulin as a substrate for HDAC6 (8). Since HDAC-mediated transcriptional repression is a common mechanism for repressing gene expression, isoform-specific HDAC inhibitors would be extremely valuable for research and therapeutic purposes.

Control of nucleocytoplasmic shuttling of HDAC4 by 14-3-3 proteins

Regulating the access of a transcriptional regulator to the nuclear compartment represents a common way to switch on or off transcription. This type of regulation has been well characterized for the class II HDACs. Phosphorylation of HDAC4 at three serine residues allows binding of 14-3-3 and

disrupts HDAC4-MEF2 interaction (chapter III, 6, 10). Binding of 14-3-3 masks the nuclear localization signal and exposes the nuclear export signal of HDAC4, resulting in its shuttling from the nucleus to the cytoplasm. After the release of HDAC proteins, MEF2 can activate transcription of genes required for muscle differentiation.

14-3-3 proteins have been shown to promote the cytoplasmic localization of many binding partners, including the pro-apoptotic protein BAD and the cell cycle regulatory phosphatase Cdc25C (12). They usually bind to phospho-serine or phospho-threonine consensus motifs. Thus, phosphorylation is the key to regulating the binding of 14-3-3 with its partner proteins. 14-3-3 binding sites are conserved among class IIa HDACs. However, HDAC4 and HDAC5 display different affinity to 14-3-3. While both HDAC4 and 14-3-3 are exclusively localized in the cytoplasm when they are co-expressed in the cells, HDAC5 remains in the nucleus when it is expressed with 14-3-3, but both HDAC5 and 14-3-3 are in the cytoplasm in the presence of activated CaMK I or IV (10). This suggests that HDAC4 is phosphorylated by one or more yet to be identified kinases and binds constitutively to 14-3-3, whereas HDAC5 binding to 14-3-3 is largely dependent on CaMK signaling. Different phosphorylation status may control the different subcellular localization of class II HDACs. Therefore, it is important to identify kinases that mediate constitutive interaction of HDAC4 and 14-3-3. Although CaMK signaling promotes the nuclear export of HDAC5, it remains possible that other signaling molecules can regulate its nuclear export in certain cellular contexts.

Biological function of HDAC4

The biological functions of HDAC4 are linked to its role in transcriptional regulation. It has been demonstrated that overexpression of HDAC4 and HDAC5 inhibits muscle differentiation through association with MEF2 (7). CaMK signaling overcomes this inhibitory activity by preventing association of HDACs with MEF2 and promoting nuclear export of HDACs (6, 7). Thus, the signal-dependent nucleocytoplasmic trafficking of class II HDACs appears to play a key role in the control of myogenesis. However, studies from two groups have shown that upon muscle differentiation HDAC4 is imported into the nucleus, while HDAC5 is exported to the cytoplasm (11, 21). This is not consistent with the simple model in which nuclear HDAC4 suppresses myogenesis. It is likely that HDAC5 represses MEF2-dependent transcription of muscle genes in myoblasts, whereas HDAC4 is needed to suppress the expression of certain genes required for terminal muscle differentiation to proceed normally. By ChIP assays, one could determine which muscle genes are regulated by HDAC4 or HDAC5 in the different stages of myogenesis. Besides regulation of skeletal muscle differentiation, class II HDACs have been suggested to repress cardiac hypertrophy. This is supported by the finding that HDAC9 null mice are sensitive to stress signals that induce cardiac hypertrophy and exhibit stress-dependent cardiomegaly (18). All studies to date have focused on the role of HDACs in muscle differentiation *in vitro*. It will be important to determine functions of these enzymes in muscle development *in vivo*. This is not easy because of the existence of multiple closely related class II HDACs with similar expression

patterns. Since both MEF2C and HDAC4 are highly expressed in brain, it will be interesting to determine whether the MEF2C-HDAC4 complex is involved in neuronal differentiation and development.

Two lines of evidence suggest that HDAC4 may participate in the regulation of the immune system. First, HDAC4 represses CIITA-mediated transactivation of MHC II genes through interaction with RFXANK (chapter V). MHC class II molecules are important for the development of a specific immune response to an extracellular pathogen by presenting peptides derived from the pathogens to T cells. Thus, repression of MHC II gene expression by HDAC4 is directly linked to the development of immune responses. Second, HDAC4 has also been shown to repress *Nur77* expression via association with MEF2 (16). Nur77 is a key transcription factor involved in TCR (T cell receptor)-mediated apoptosis of thymocytes. HDAC4 inhibits MEF2-mediated stimulation of the *Nur77* promoter in response to PMA and ionomycin, suggesting that HDAC4 might play an antiapoptotic role in thymocyte development.

It appears that transcriptional regulation by HDACs is a general mechanism for controlling a variety of cellular processes. To gain insight into *in vivo* biological functions of these HDACs, it is necessary to fully characterize their function and regulation. Elucidation of the biological functions of these enzymes will also refine our understanding of the roles of chromatin modulation.

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CHAPTER VII

Contribution to original research

1. Identify and characterize HDAC4 as a novel member of class II HDACs and a transcriptional corepressor. Demonstrate that HDAC4 directly interacts with MEF2 factors and represses MEF2-dependent transcription activity. These findings identify a new class of mammalian histone deacetylase and further support the notion that histone deacetylation regulates gene transcription.

2. Demonstrate that 14-3-3 proteins bind to three serine residues, 246, 467, and 632, of HDAC4 and indirectly inhibit the transcriptional repression activity of HDAC4 by sequestering HDAC4 in the cytoplasm. This provides insight into how nucleocytoplasmic shuttling of HDAC4 is regulated and suggests that the trafficking of HDAC4 is signal-dependent.

3. Identify the nuclear localization signal (NLS) and the nuclear export signal (NES) of HDAC4 and determine their roles in contributing to the nucleocytoplasmic shuttling. Demonstrate that both 14-3-3 binding and the NES are required for the nuclear export of HDAC4 and that direct MEF2 binding promotes its nuclear localization. These findings suggest that the nucleocytoplasmic shuttling of HDAC4 is regulated by multiple mechanisms.

4. Identify ankyrin repeat-containing proteins, ANKRA2 and RFXANK, as two new HDAC4-interacting partners. Demonstrate that HDAC4 interacts with

ANKRA2 and RFXANK in vitro, as well as in mammalian cells, and represses CIITA-mediated transcativation of MHC II genes. These findings suggest that HDAC4 may participate in the regulation of immune responses.