THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 280, No. 32, Issue of August 12, pp. 29117–29127, 2005
Printed in U.S.A.

Identification of the Ankyrin Repeat Proteins ANKRA and RFXANK as Novel Partners of Class IIa Histone Deacetylases*

Received for publication, January 10, 2005, and in revised form, June 9, 2005
Published, JBC Papers in Press, June 17, 2005, DOI 10.1074/jbc.M500295200

Audrey H. Wang‡, Serge Grégoire‡§, Eleni Zika§¶, Lin Xiao‡, Cathy S. Li‡, Hongwei Li‡, Kenneth L. Wright‖, Jenny P. Ting¶, and Xiang-Jiao Yang‡**

From the ‡Molecular Oncology Group, Department of Medicine, McGill University Health Centre, Montreal, Quebec H3A 1A1, Canada, ¶Lineberger Comprehensive Cancer Center and Department of Microbiology and Immunology, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, and ‖H. Lee Moffitt Cancer Center and Research Institute, University of South Florida, Tampa, Florida 33612

Eighteen human histone deacetylases (HDACs) have been identified, and according to their sequence similarity to yeast homologs, these enzymes are grouped into distinct classes. Within class II, HDAC4, HDAC5, HDAC7, and HDAC9 share similar domain organization both within the N-terminal extension and the C-terminal catalytic domain, thus forming a subclass known as class IIa. These HDACs function as signal-responsive transcriptional corepressors. To gain further insight into their function and regulation, we utilized an N-terminal fragment of HDAC4 as bait in yeast two-hybrid screens, which uncovered myocyte enhancer factor 2C, 14-3-3ζ, and ankyrin repeat family A protein (ANKRA). ANKRA is a poorly characterized protein with an ankyrin repeat domain similar to RFXANK, a subunit of the trimeric transcription factor RFX. Mutations on genes of the RFX subunits and the coactivator CIITA are responsible for the bare lymphocyte syndrome, an immunodeficiency disorder attributed to the lack of major histocompatibility complex class II (MHCII) antigens. Through its ankyrin repeat domain, RFXANK interacted with HDAC4. Two RFXANK-binding sites were found on HDAC4 with one located within residues 118–279 and another within residues 448–666. Interestingly, this deacetylase also interacted with CIITA. Consistent with the physical interaction with RFXANK and CIITA, HDAC4 and homologs repressed MHCII expression. These results identify ANKRA, RFXANK, and CIITA as novel targets of class IIa HDACs and suggest that these deacetylases play a role in regulating MHCII expression.

Lysine acetylation refers to the addition of an acetyl moiety to the ϵ-amino group of a lysine residue. Intensive research in the past decade has uncovered that this covalent modification occurs in over 50 transcription factors and about 30 other proteins that are not directly involved in transcriptional control (1–4). Because histones were considered to be major cellular proteins modified by lysine acetylation, many of the responsible enzymes were initially identified as histone acetyltransferases and histone deacetylases (HDACs).[1] It is now clear that some of them also modify other protein substrates. Known mammalian HDACs belong to distinct classes (5–8). Within class II, there are six members, HDAC4, -5, -6, -7, -9, and -10, whose catalytic domains exhibit significant sequence homology to yeast Hda1. According to sequence similarity, these six mammalian HDACs are further grouped into two subclasses: IIa (HDAC4, -5, -7, and -9) and IIb (HDAC6 and -10). Although little is known about biological functions of HDAC10, HDAC6 has been well characterized as a tubulin deacetylase (9–14).

Through interacting with DNA-binding transcription factors, class IIa HDACs play important roles in transcriptional regulation (6, 8, 15). They interact with myocyte enhancer factor 2 (MEF2) to control transcription during muscle differentiation, T cell apoptosis, and neuronal survival. They also serve as transcriptional corepressors for BCL6 and Runx proteins (16–18). These HDACs are subject to regulation by signal-dependent nucleocytoplasmic trafficking (7, 15). Protein kinases, such as calcium/calmodulin-dependent kinase and protein kinase D (also known as protein kinase C μ), phosphorylate class IIa HDACs and promote their 14-3-3 binding and cytoplasmic localization, thereby sequestering them away from their nuclear targets (19–27). Thus, these HDACs are signal-responsive transcriptional corepressors. To further understand their function and regulation, we sought to identify additional partners via yeast two-hybrid screening. One of the proteins uncovered is ankyrin repeat family A protein (ANKRA), which is known to interact with the cytoplasmic tail of megalin and possesses an ankyrin repeat domain with significant homology to regulatory factor for the X1 box with ankyrin repeats (RFXANK) (28). Although little is known about the function of ANKRA, an important role in transcriptional regulation has been well documented for RFXANK. Thus, a major focus of this report is on the effects of HDAC4 on RFXANK function.

Also known as RFX-B, RFXANK is a subunit of the trimeric transcription factor RFX isolated from major histocompatibility complex class II (MHCII)-deficient patients (29–31). RFXANK associates with RFX5 and RFXAP to form regulatory factor for the X1 box (RFX), a DNA-binding complex that in-

* This work was supported by funds from the Canadian Cancer Society through the National Cancer Institute of Canada and from the Canada Foundation for Innovation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Both authors contributed equally to this work.

** To whom correspondence should be addressed: McGill University Health Centre-RVH, Rm. H5.41, 687 Pine Ave. West, Montreal, Quebec H3A 1A1, Canada. Tel.: 514-934-1934 (ext. 34490); Fax: 514-843-1478; E-mail: xiang-jiao.yang@mcgill.ca.

[1] The abbreviations used are: HDAC, histone deacetylase; ANKRA, ankyrin repeat family A protein; CBP, cAMP-response element-binding protein (CREB)-binding protein; CIITA, class II transactivator; GFP, green fluorescent protein; HA, hemagglutinin; HEK, human embryonic kidney; IFNγ, interferon γ; Luc, luciferase; MBP, maltose-binding protein; MEF2, myocyte enhancer factor 2; MHCII, major histocompatibility complex class II; RFX, regulatory factor for the X1 box; RFXAP, RFX-associated protein; RFXANK, regulatory factor for the X1 box with ankyrin repeats (also known as RFX-B).

teracts specifically with the conserved X1 box of MHCII promoters. All three subunits are required for the optimal binding to the X1 box. RFX interacts with the transcriptional coactivator class II transactivator (CIITA) and recruits it to the X1 box for transcriptional activation (31, 32). Mutations on the genes of these four trans-acting proteins (*i.e.* RFX5, RFXAP, RFXANK, and CIITA) form the genetic basis for different complementation groups of the bare lymphocyte syndrome, an autosomal recessive immunodeficiency due to the lack of MHCII expression (30, 32). CIITA expression is tightly regulated and exhibits a cell-specific, IFNγ-inducible, and differentiation-specific pattern that roughly parallels the synthesis of MHCII (30–32). By contrast, RFX subunits are more ubiquitous and constitutively expressed, indicative of additional functions. Indeed RFX also functions as a repressor for collagen transcription (33). Here we show that class IIa HDACs interact with the ankyrin repeats of RFXANK as well as with CIITA to repress MHCII transcription, thus uncovering a novel and unexpected role of these deacetylases in regulating IFNγ-inducible gene expression.

EXPERIMENTAL PROCEDURES

Cell Culture—NIH3T3, 293, and HeLa cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% fetal bovine serum, penicillin, and streptomycin. Sf9 cells were maintained for baculovirus expression as described previously (34, 35).

Plasmid Constructs—Mammalian expression plasmids for epitope-tagged HDAC4 proteins have been described previously (20, 36, 37), whereas those for murine HDAC5, murine HDAC7, and human MEF2-interacting transcription repressor (MITR) were kindly provided by S. Khochbin (38), E. Seto (39, 40), and X. Zhou (41), respectively. The coding sequences of human ANKRA and RFXANK were cloned into pcDNA3.1 (Invitrogen). Green fluorescent protein (GFP) constructs were constructed from pEGFP-C2 (BD Biosciences). The CIITA mammalian expression plasmid and the DRA-Luc luciferase reporter have been described previously (42, 43).

Yeast Two-hybrid Screening—Matchmaker Gal4 Two-hybrid System 3 (BD Biosciences) was utilized. To minimize false positive clones, this system uses four reporters (ADE2, HIS3, MEL1, and LacZ) under the control of distinct Gal4 upstream activating sequences linked to different minimal promoters. Coding sequences for HDAC4 fragments were fused in-frame to the yeast Gal4 DNA-binding domain in the vector pGBKT7 to generate potential bait plasmids, which were transformed into the yeast strain AH109 and analyzed for protein expression by Western blotting. One bait plasmid (Fig. 1*A*, residues 1–666) expressed an expected product. The AH109 strain harboring this bait plasmid was thus used to mate with the yeast strain Y187 pretransformed with a human fetal brain cDNA library, constructed with the yeast Gal4 activation domain vector pACT2 (BD Biosciences, catalog number HY4028AH). After mating, about 1.0×10^7 diploid yeast cells were selected on plates with the dropout medium containing 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside (X-α-Gal) but lacking adenine, histidine, leucine, and tryptophan. This screen yielded about 800 blue colonies, which were further tested for 3-aminotriazole resistance (12.5 and 50 mM). 14 clones displayed resistance at both concentrations. Library plasmids were successfully recovered from 13 of them, and cDNA inserts were subcloned for sequencing. For testing the interaction specificity, liquid β-galactosidase assays were performed according to the manufacturer's instructions (BD Biosciences). 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).

Protein-Protein Interaction—For examining the interaction of HDAC4 with ANKRA *in vitro*, FLAG-HDAC4 was expressed in SF9 cells as described previously (34) and immobilized on M2 agarose beads (Sigma). ANKRA and deletion mutants were synthesized *in vitro* with the TNT T7 coupled reticulocyte lysate system (Promega) in the presence of Redivue L-[^{35}S]methionine (Amersham Biosciences). After rotation for an hour at 4 °C, agarose beads were washed three times with buffer B (20 mM Tris-HCl, pH 8.0, 10% glycerol, 0.15 M KCl, 5 mM $MgCl_2$, 0.1% Nonidet P-40, and protease inhibitors) and once with the same buffer containing 0.5 M KCl and then boiled in an SDS sample buffer prior to SDS-PAGE and autoradiography.

In addition, ANKRA and RFXANK were expressed *Escherichia coli* as proteins fused to maltose-binding protein (MBP). MBP and the fusion proteins were incubated with FLAG-HDAC4 immobilized on M2 agarose. After extensive washing, bound proteins were eluted for SDS-PAGE and staining with Coomassie Blue.

To compare their interaction with HDAC4, RFXANK and deletion mutants were expressed in *E. coli* as proteins fused to MBP and immobilized on amylose-agarose. HDAC4 was synthesized *in vitro* with the TNT T7 coupled reticulocyte lysate system (Promega) in the presence of Redivue L-[^{35}S]methionine (Amersham Biosciences). After rotation for an hour at 4 °C, agarose beads were washed three times with buffer B and once with the same buffer with 0.5 M KCl and then boiled in the SDS sample buffer prior to SDS-PAGE, Coomassie staining, and autoradiography.

To analyze the interaction of HDAC4 with RFXANK *in vivo*, expression plasmids for FLAG-tagged HDAC4, deletion mutants, and other class IIa HDACs were transfected into 293 cells along with HA-RFXANK expression plasmid as specified. 10 μg of plasmids was used to transfect 5–10 $\times$ 10^5 cells (in a 10-cm dish) with 24 μl of SuperFect (Qiagen) or TransIT (Mirus) transfection reagent. 48 h after transfection, cells were washed twice with phosphate-buffered saline and collected in 1 ml of buffer K (20 mM sodium phosphate, pH 7.0, 150 mM NaCl, 30 mM sodium pyrophosphate, 0.1% Nonidet P-40, 5 mM EDTA, 10 mM NaF, 0.1 mM Na_3VO_4, and protease inhibitors). Cell extracts (0.5 ml) were prepared for affinity purification on 15 μl of anti-FLAG M2 agarose (Sigma). After four washes with buffer K (0.3 ml each), bound proteins were eluted with the same buffer containing 0.2 mg/ml FLAG peptide (Sigma). Eluted proteins were subsequently resolved by 10% SDS-PAGE and transferred to nitrocellulose membrane for Western blotting analysis with anti-FLAG and anti-HA antibodies. Blots were developed with Supersignal substrates (Pierce). Interaction of RFX5, RFXAP, or CIITA with RFXANK *in vivo* was analyzed similarly.

Immunofluorescence Microscopy—Green fluorescence and indirect immunofluorescence microscopic analyses were performed as described previously (20, 37, 44) except that after incubation with secondary antibody, cells were stained with 2.5 μg/ml Hoechst 33258 (Sigma) for 60 s to locate nuclei.

Reporter Gene Assays—Reporter gene assays were performed as described previously (35, 36).

Reverse Transcription-PCR—HeLa cells ($\sim$1 $\times$ 10^6) were plated in 150-mm tissue culture plates and transfected 18 h later using FuGENE 6 (Roche Applied Science) according to the manufacturer's instructions. 24 h post-transfection, cells were harvested, and cDNA was synthesized as described previously (43, 45). Real time PCR was performed using the ABI Prism 7900 sequence detection system (PerkinElmer Life Sciences). The MHCII probe was labeled at the 5′ end with the reporter dye FAM and at 3′ end with the quencher dye TAMRA. The 18 S rRNA probe was labeled at the 5′ end with the reporter dye TET and at the 3′ end with the quencher dye TAMRA. Primer and probe sequences are as follows: MHCII probe, 5′-6 FAM-CTC CGA TCA CCA ATG TAC CTC CAG A-TAMRA-3′; sense primer, 5′-AAG CCA ACC TGG AAA TCA-3′; antisense primer, 5′-GGC TGT TCG TGA GCA CAG TT-3′; and 18 S rRNA probe, 5′-6 FAM-CAA ATT ACC CAC TCC CGA CCC G-TAMRA-3′; sense primer, 5′-GCT GCT GGC ACC AGA CTT-3′; antisense primer, 5′-CGG CTA CCA CAT CCA AGG-3′. Real time PCR analysis of cDNA samples was conducted as described previously (45).

RESULTS

Identification of New Binding Partners of HDAC4—To gain further insight into the function and regulation of class IIa members, we performed yeast two-hybrid screens with an N-terminal fragment of HDAC4 as bait (Fig. 1*A*). Because this deacetylase is highly expressed in the brain, a human fetal brain cDNA library was used. From the screen, 13 positive clones were obtained. 10 of them expressed the right protein products, including fragments of MEF2C (two clones), 14-3-3ζ (one clone), ANKRA (two clones), ATRX (one clone), REV3L (catalytic subunit of DNA polymerase ζ; two clones) and C19orf5 (chromosome 19 orf5, recently renamed MAP1S for microtubule-associated protein 1S; two clones). 14-3-3 proteins interact with HDAC4 in a phosphorylation-dependent manner (19, 20), so there could be a yeast kinase phosphorylating HDAC4 and promoting its association with 14-3-3ζ. Because MEF2C and 14-3-3ζ are known HDAC4 partners highly expressed in the brain, the screening was specific and efficient.

C1 and C11, two ANKRA fragments from the positive clones,

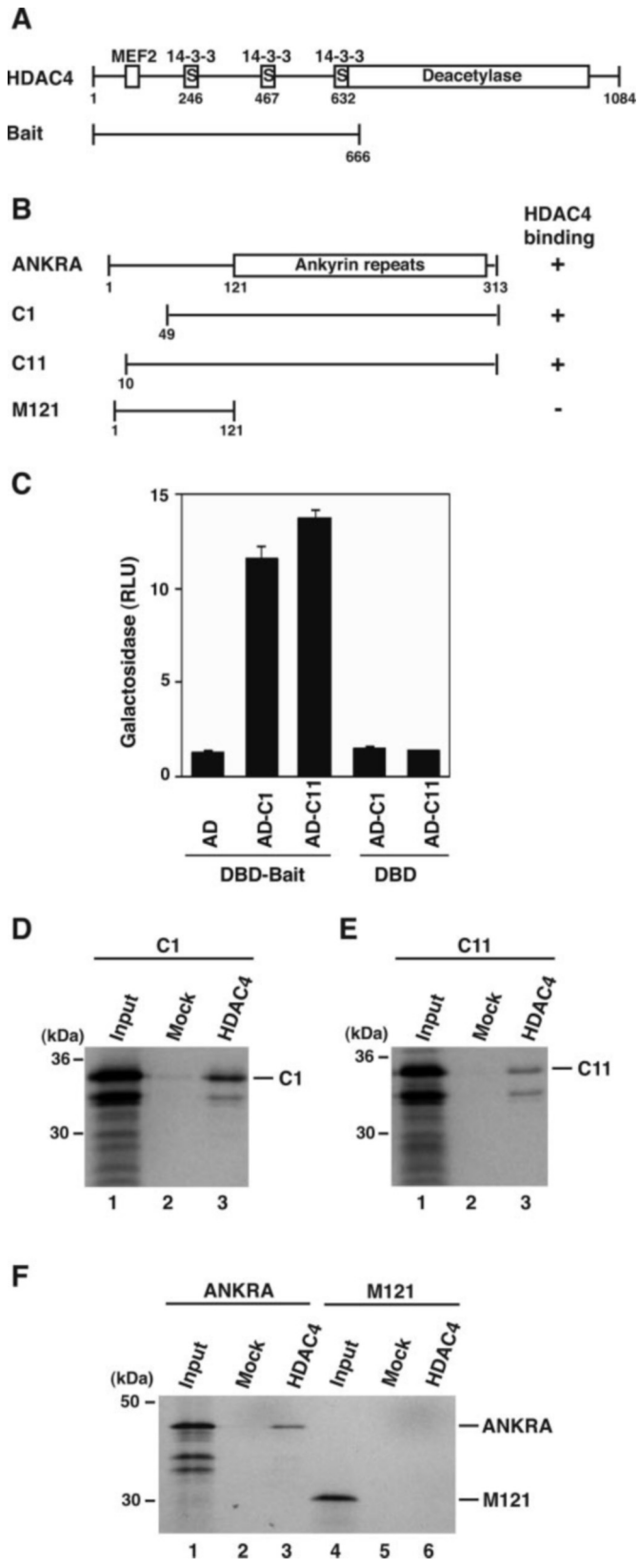

FIG. 1. **Interaction of ANKRA with HDAC4.** *A*, schematic representation of HDAC4. The deacetylase domain is depicted with a *rectangle*. The MEF2-binding motif is drawn as a *small box*, and 14-3-3-binding motifs are indicated with *small boxes* labeled with the letter "*S*" (for serine). Also shown is an HDAC4 fragment (residues 1–666) used as bait in yeast two-hybrid screens. *B*, schematic illustration of ANKRA and fragments. C1 and C11 correspond to fragments encoded by two clones isolated from the screens. *C*, liquid β-galactosidase activity assays. Expression plasmids for C1 and C11 were transformed into Y187, which was then mated with AH109 harboring the bait expression plasmid or the empty vector expressing the Gal4 DNA-binding domain (*DBD*) as indicated. As an additional control, the expression plasmid pACT2 for the Gal4 activation domain (*AD*) was co-transformed with the bait expression plasmid. Average values of β-galactosidase activities in yeast extracts were determined and are shown with standard deviations. *RLU*, relative light units. *D–F*, *in vitro* pull-down assays. FLAG-HDAC4 was expressed in SF9 cells, immobilized on M2 agarose, and incubated with [^{35}S]methionine-labeled ANKRA proteins as indicated. Bound proteins were eluted for SDS-PAGE and autoradiography. *Input lanes* represent 20% of [^{35}S]methionine-labeled proteins used for binding assays. Plain SF9 extracts were used as mock controls.

overlap with each other (Fig. 1*B*). As shown in Fig. 1*C*, both specifically activated β-galactosidase expression, indicating that the interaction is specific. To substantiate this, we performed *in vitro* binding assays. FLAG-tagged HDAC4 was expressed in SF9 cells and immobilized on M2 agarose for binding to C1 and C11 produced using an *in vitro* transcription and translation system in the presence of L-[^{35}S]methionine. Bound proteins were resolved by SDS-PAGE and detected by autoradiography. As shown in Fig. 1, *D* and *E*, HDAC4 interacted with both fragments, supporting that HDAC4 binds directly to ANKRA.

As expected, HDAC4 also specifically interacted with full-length ANKRA (Fig. 1*F*, *lanes 1–3*). By contrast, its N-terminal 121 residues failed to associate with HDAC4 (*lanes 4–6*), indicating that the ankyrin repeat domain is required for the binding. To further characterize this association, we expressed ANKRA as an MBP fusion protein and tested for binding to FLAG-HDAC4 immobilized on M2 agarose. Bound proteins were eluted and separated by SDS-PAGE for staining with Coomassie Blue. As shown in Fig. 2*A* (*lanes 1*, *2*, *4*, *5*, and *7* and *8*), HDAC4 retained MBP-ANKRA but not MBP. Importantly, the association was almost stoichiometric, suggesting that the binding to ANKRA is strong.

HDAC4 Interaction with RFXANK—ANKRA was originally identified in yeast two-hybrid screens where the cytoplasmic tail of megalin was used as bait (28), but its function is barely known. Ankyrin repeats are protein-protein interaction motifs implicated in a wide range of biological processes (46). The ankyrin repeat domain of ANKRA shows significant homology to RFXANK (sequence identity, ~60%; Fig. 2*B*) (28, 47). Because the ankyrin repeats of ANKRA appeared to mediate the interaction with HDAC4 (Fig. 1), we tested whether this deacetylase also binds to RFXANK. For this, RFXANK was expressed as an MBP fusion protein in *E. coli* and tested for binding to FLAG-HDAC4 expressed in SF9 cells and immobilized on M2 agarose. Bound proteins were eluted, and after separation by SDS-PAGE, they were detected by Coomassie staining. As shown in Fig. 2*A* (*lanes 3*, *6*, and *9*), HDAC4 specifically interacted with RFXANK albeit less strongly than with ANKRA.

We next analyzed the interaction using immobilized RFXANK. For this, MBP-RFXANK was immobilized on amylose-agarose and used for binding to HDAC4, synthesized, and labeled *in vitro* with L-[^{35}S]methionine. As shown in Fig. 2*C* (*lanes 1*, *2*, and *8*), MBP-RFXANK but not MBP retained HDAC4, providing further support for RFXANK interaction with HDAC4. To map the region mediating this interaction, we engineered truncation mutants of RFXANK by gradually removing its ankyrin repeats from the C-terminal end (Fig. 2*B*). These mutants were expressed in *E. coli* as MBP fusion proteins, immobilized on amylose-agarose, and tested for binding to labeled HDAC4. As shown in Fig. 2*C* (*bottom*), amounts of the fusion proteins retained on the resin were similar. Truncation to residue 248, 213, or 180 had modest effects on HDAC4 binding (Fig. 2*C*, *top*, *lanes 5–7*), whereas further removal of ankyrin repeat 2 exerted a more dramatic effect (*lane 4*). Trun-

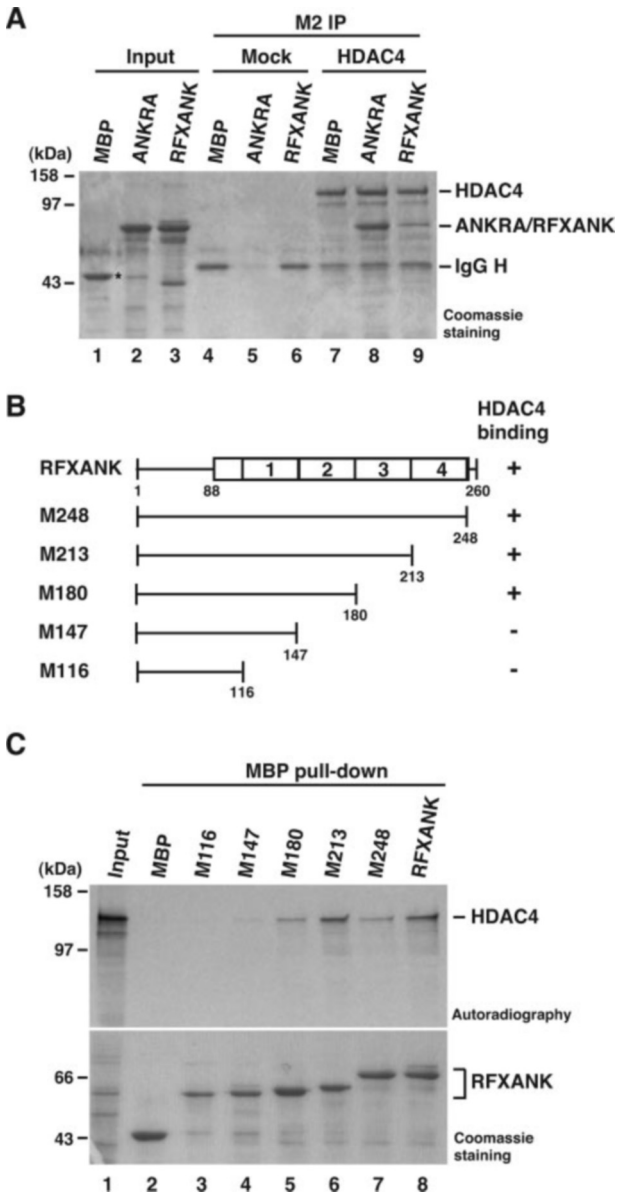

FIG. 2. **Direct interaction of HDAC4 with ANKRA and RFXANK.** *A*, FLAG-HDAC4 was expressed in SF9 cells, immobilized on M2 agarose, and incubated with MBP, MBP-ANKRA, or MBP-RFXANK as specified. Bound proteins were eluted for SDS-PAGE and Coomassie staining. Inputs (*lanes 1–3*) represent 20% of MBP or MBP fusion proteins used for binding assays. Bands corresponding to IgG heavy chain (*IgG H*) that randomly came off from M2 agarose are indicated at *right*. The MBP band on *lane 1* is marked with an *asterisk*. *B*, schematic illustration of RFXANK and truncation mutants. The ankyrin repeat region (*rectangles*, with ankyrin repeats *numbered*) is homologous to ANKRA (sequence identity, ~60%). The HDAC4 binding ability of RFXANK and truncation mutants is shown at *right* with *plus* and *minus* representing positive and negative interactions, respectively. *C*, *in vitro* MBP pull-down assays. MBP (*lane 2*) and MBP-RFXANK proteins (*lanes 3–8*) were expressed in *E. coli*, immobilized on amylose-agarose, and incubated with [^{35}S]methionine-labeled HDAC4. Bound proteins were eluted for SDS-PAGE, Coomassie staining (*bottom*), and autoradiography (*top*). *Input* (*lane 1*) represents 20% of labeled HDAC4 used for each binding assay.

cation to residue 116 completely abolished the binding (*lane 3*). Therefore, ankyrin repeats 1 and 2 are required for HDAC4 binding (Fig. 2, *B* and *C*).

Mapping of the RFXANK-binding Sites on HDAC4—To delineate the region important for RFXANK binding, we utilized a series of HDAC4 deletion mutants that has been described previously (Fig. 3*A*) (20, 34, 37, 48). They were synthesized, labeled *in vitro* with L-[^{35}S]methionine, and used for binding to MBP-RFXANK immobilized on amylose-agarose. Consistent with the fact that the N-terminal fragment of HDAC4 was used as bait in the two-hybrid screens (Fig. 1*A*), mutant 1–666 but not 621–1084 interacted with RFXANK (Fig. 3*B*). As expected, MBP-RFXANK retained full-length HDAC4 (Fig. 3*C*, *lanes 1–3*). Neither 208–1084 nor 315–1084 (*lanes 4–9*) was retained, indicating that the N-terminal 207 residues are required for the binding. To further define the binding site, deletion mutants 1–326, 1–279, 1–266, and 1–208 were analyzed (Fig. 3*A*). Although mutants 1–326 and 1–279 interacted strongly with RFXANK, the binding to mutant 1–266 was weaker, and 1–208 showed no detectable binding (Fig. 3*D*), suggesting that residues 209–278 are also essential for the interaction. Immobilized MBP-RFXANK efficiently retained mutant 118–326 but not 315–666 (Fig. 3, *E* and *F*). Together, these results indicate that a direct RFXANK-binding site resides within residues 118–279 of HDAC4.

To examine the binding *in vivo*, we performed co-immunoprecipitation. Expression plasmids for FLAG-HDAC4 and HA-RFXANK were transfected into HEK293 cells, and extracts were prepared for immunoprecipitation on M2 agarose. Precipitated proteins were eluted and analyzed by Western blotting with anti-FLAG and anti-HA antibodies. As shown in Fig. 3*G* (*lanes 1* and *7*), RFXANK co-precipitated with HDAC4. In the same assays, we also analyzed mutants 206–1084 and 315–1084, which showed no RFXANK interaction *in vitro* (Fig. 3*C*). Unexpectedly, both mutants co-precipitated RFXANK much more efficiently than wild-type HDAC4 (Fig. 3*G*, *lanes 1–3* and *7–9*), suggesting that RFXANK binds indirectly to these two mutants and the N-terminal 207 residues of HDAC4 inhibit this binding. To delineate the inhibitory domain, we tested mutants with smaller truncations. As shown in Fig. 3*G* (*lanes 4*, *5*, *10* and *11*), mutant 146–1084 co-precipitated RFXANK slightly more efficiently than wild-type HDAC4, whereas 189–1084 was almost as efficient as 206–1084 and 315–1084. In the same assays, mutant 621–1084 did not retain RFXANK, so the co-precipitation was specific. These results thus indicate that residues 147–188 are critical for the inhibition and that an indirect RFXANK-binding site resides within residues 315–620.

RFXANK associated with the N-terminal 279 residues of HDAC4 *in vitro* (Fig. 3, *C–F*), so the indirect binding site did not appear to overlap with the direct one. To substantiate this, we analyzed mutants 1–666, 1–326, and 315–666. As shown in Fig. 3*H* (*lanes 1–3* and *5–7*), 1–666 and 315–666 but not 1–326 co-precipitated RFXANK. Importantly, 315–666 was more efficient than 1–666. Moreover, mutant 488–666 was almost as efficient as 315–666, indicating that the indirect binding site is located within residues 488–666. These results also provide further support for the conclusion that the N-terminal region of HDAC4 dramatically inhibits the indirect binding. Because mutant 1–326 interacted with RFXANK *in vitro* (Fig. 3*D*) but not *in vivo* (Fig. 3*H*), RFXANK binding to residues 118–279 may be inhibited in cells. These results indicate that HDAC4 possesses two RFXANK-binding sites and further suggest that the binding is regulated *in vivo*.

Interaction of Different Class IIa HDACs with ANKRA and RFXANK—Because the two RFXANK-binding sites on HDAC4 are conserved among HDAC5, -7, and -9, we asked whether these three HDACs interact with RFXANK and ANKRA. To address this, *in vitro* binding assays were performed with HDAC5 and HDAC7 labeled *in vitro* with L-[^{35}S]methionine. They were incubated with MBP-ANKRA and -RFXANK immobilized on amylose-agarose. Bound proteins were eluted for SDS-PAGE, Coomassie staining, and autoradiography. For

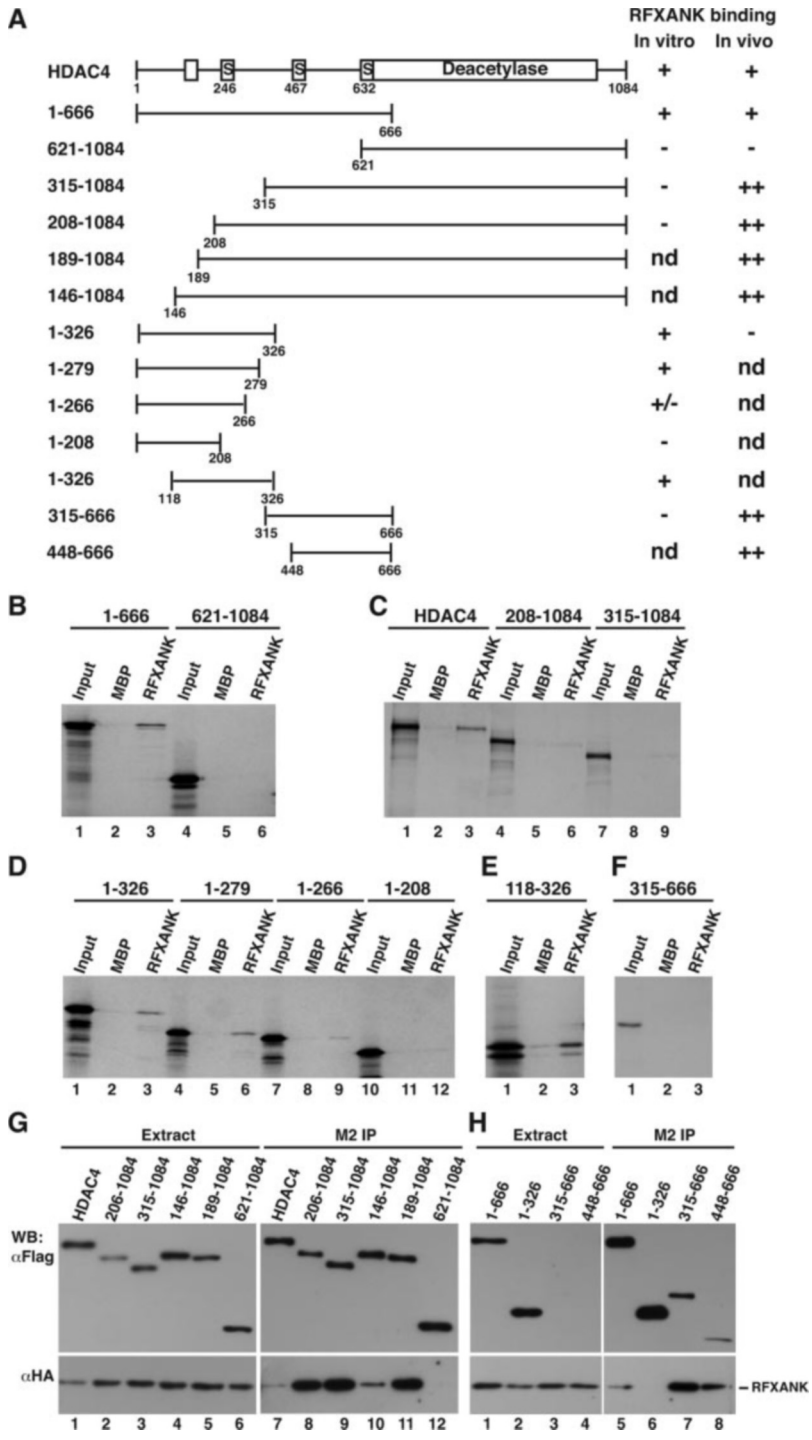

FIG. 3. **Mapping the RFXANK-binding sites on HDAC4.** *A*, schematic representation of HDAC4 and deletion mutants. The domain organization of HDAC4 is illustrated as in Fig. 1*A*. The RFXANK binding ability of HDAC4 and deletion mutants *in vitro* and *in vivo* is indicated at *right*: +, low affinity; ++, high affinity; −, no detectable binding; *nd*, not determined. *B–F*, *in vitro* MBP pull-down assays. MBP and MBP-RFXANK were expressed in *E. coli*, immobilized on amylose-agarose, and incubated with [^{35}S]methionine-labeled HDAC4 and mutants as specified. Bound proteins were eluted for SDS-PAGE and autoradiography. *Input lanes* represent 20% of labeled proteins used for each binding assay. *G* and *H*, co-immunoprecipitation (*IP*) assays. The expression plasmid for HA-tagged RFXANK was co-transfected into HEK293 cells with those for HDAC4 and deletion mutants (FLAG-tagged) as indicated. Extracts were prepared for immunoprecipitation on M2 agarose, and bound proteins were eluted with FLAG peptide for Western blotting (*WB*) with anti-FLAG and anti-HA antibodies. Expression of mutants 315–666 and 448–666 was barely detectable in extracts.

comparison, labeled HDAC4 was also assessed in the same set of experiments. As shown in Fig. 4*A*, like HDAC4, HDAC5 specifically interacted with ANKRA and less strongly with RFXANK (*lanes 1–8*). HDAC7 was found to bind RFXANK and ANKRA almost equally (*lanes 9–12*). We then compared RFXANK binding affinity of class IIa HDACs by co-immunoprecipitation. As shown in Fig. 4*B*, they co-precipitated RFXANK rather similarly. Therefore, RFXANK and ANKRA are able to interact with class IIa HDACs *in vitro* and *in vivo*.

Co-localization of RFXANK and ANKRA with Class IIa HDACs—To examine the subcellular localization of these ankyrin repeat proteins, we performed fluorescence microscopy. NIH3T3 cells were transfected with expression plasmids for GFP-RFXANK and -ANKRA, and transfected cells were examined for green fluorescence. Although GFP itself was pancellular, GFP-ANKRA and -RFXANK were found both in the nucleus and the cytoplasm with some nuclear enrichment (Fig. 5*A*). Consistent with previous reports (20, 37), HDAC4 was predominantly cytoplasmic, HDAC5 was nuclear, and HDAC7 was pancellular or slightly enriched in the nucleus (Fig. 5*B*). We then investigated whether RFXANK and ANKRA co-localize with these three HDACs. For this, an expression plasmid for GFP-RFXANK or -ANKRA was co-transfected into NIH3T3 cells with HA-tagged HDACs. As shown in Fig. 5, *C* and *D*, expression of the HDACs caused some enrichment of RFXANK and ANKRA in the nucleus. Moreover, the HDACs displayed some co-localization with these ankyrin repeat proteins. Intriguingly, HDAC4, but not HDAC5 or HDAC7, led to reloca-

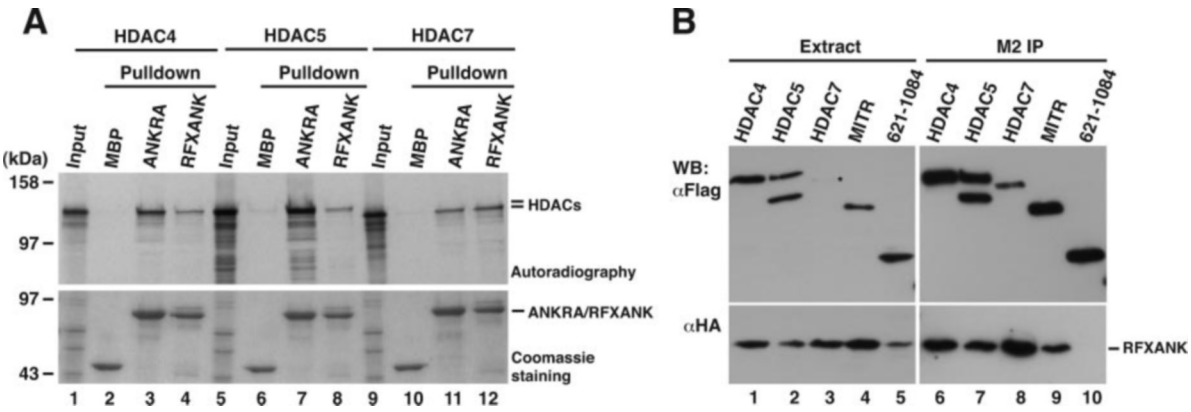

FIG. 4. **Interaction of ANKRA and RFXANK with class IIa HDACs.** *A*, *in vitro* MBP pull-down assays. MBP, MBP-ANKRA, and MBP-RFXANK were expressed in *E. coli*, immobilized on amylose-agarose, and incubated with [^{35}S]methionine-labeled HDAC4, -5, and -7 as indicated. Bound proteins were eluted for SDS-PAGE and autoradiography. *Input lanes* represent 20% of labeled proteins used for binding assays. *B*, co-immunoprecipitation (*IP*). The expression plasmid for HA-RFXANK was co-transfected into NIH3T3 cells with that for FLAG-tagged HDAC4, -5, -7, or MEF2-interacting transcription repressor (MITR). The HDAC4 mutant 621–1084 was used as the negative control. Extracts were prepared for immunoprecipitation on M2 agarose, and bound proteins were eluted with FLAG peptide for Western blotting (*WB*) with anti-FLAG and anti-HA antibodies as indicated. Expression of HDAC7 (*asterisk*, *lane 3*) was barely detectable in extracts. In HEK293 cells, RFXANK also co-precipitated with these HDACs (data not shown).

tion of GFP-RFXANK to nuclear dots in about 5–10% of transfected cells (Fig. 5*C*). This effect was more dramatic with ANKRA: in ~40% cells, it formed dots in the nucleus (Fig. 5*D*). Moreover, in some cells, HDAC4 co-localized with RFXANK or ANKRA in the dots (Fig. 5, *C* and *D*). Although the nature of the nuclear dots awaits further investigation, these results provide further support for the interaction of RFXANK and ANKRA with these HDACs *in vivo*.

Repression of MHCII Gene Expression by HDAC4—We next assessed whether RFXANK recruits HDAC4 to repress transcription. For this, RFXANK was expressed as a protein fused to the Gal4 DNA-binding domain, and the ability to regulate the transcriptional activity of the luciferase reporter Gal4-tk-Luc in which the luciferase gene is under the control of a thymidine kinase (tk) core promoter and six copies of the Gal4-binding site (34) was analyzed. As shown in Fig. 6*A*, Gal4-RFXANK activated the reporter activity (~10-fold), whereas HDAC4 inhibited the activity in a manner dependent on the Gal4 fusion protein, suggesting that RFXANK enlists HDAC4 to repress transcription.

RFXANK is required for assembling the RFX complex, which recognizes the X1 box of MHCII promoters and recruits the coactivator CIITA to activate transcription (30, 31), so we investigated whether class IIa HDACs repress CIITA-mediated activation of MHCII genes. This was first evaluated by transient transfection of NIH3T3 cells with a luciferase reporter under the control of an HLA-DRA promoter fragment. This reporter has been used to analyze the transcriptional regulation by RFX and CIITA (43). As shown in Fig. 6*B*, HDAC4 repressed the reporter activity in a dose-dependent manner.

CIITA is not expressed in fibroblasts like NIH3T3. As shown in Fig. 6*C*, expression of CIITA stimulated the DRA-Luc reporter activity (~35-fold) in these cells. Exogenous RFXANK and RFX5 had minimal effects on CIITA-mediated transactivation (data not shown), suggesting that levels of RFX5 and RFXANK are not limiting in NIH3T3. CIITA-dependent activation was inhibited by HDAC4 in a dose-dependent manner (Fig. 6*C*). HDAC5 also exerted inhibitory effects (Fig. 6*C*). Therefore, these HDACs are able to repress MHCII expression.

To investigate the physiological relevance, we assessed how HDAC4 regulates the expression of endogenous MHCII. For this, HeLa cells were transfected with the CIITA expression plasmid in the presence or absence of the HDAC4 expression plasmid. RNA was isolated for cDNA synthesis and real time PCR to quantify MHCII mRNA. As shown in Fig. 7*A*, CIITA stimulated the expression, whereas HDAC4 inhibited this stimulation. To determine which region of HDAC4 was involved, we analyzed two deletion mutants. Although mutant 621–1084 had minimal effects, mutant 1–666 blocked the activation of MHCII expression by CIITA (Fig. 7*A*). Interestingly, mutant 1–666 was slightly more potent than full-length HDAC4 (Fig. 7*A*). This mutant harbors both RFXANK-binding sites (Fig. 3). Moreover, it is exclusively nuclear and possesses a potent repression domain (34, 37).

We next analyzed how HDAC4 affects IFNγ-inducible gene expression. For this, HeLa cells were treated with IFNγ, and MHCII mRNA was quantified as above. As reported previously (43), IFNγ induced MHCII expression (Fig. 7*B*). HDAC4 and mutant 1–666, but not 621–1084, inhibited the induction (Fig. 7*B*). IFNγ is known to activate MHCII transcription by inducing CIITA expression (30, 32), so we analyzed effects of HDAC4 and mutants on CIITA mRNA levels. As shown in Fig. 7*C*, HDAC4 and the mutants had minimal effects on CIITA expression, indicating that the inhibitory effects of HDAC4 and mutant 1–666 on MHCII expression (Fig. 7*B*) are not due to reduction of CIITA expression. Therefore, HDAC4 is able to inhibit IFNγ-inducible expression of endogenous MHCII.

Interaction of HDAC4 and HDAC5 with CIITA—Because class IIa HDACs interact with RFXANK and repress RFX-dependent transcription, we wondered whether they also target RFX5, RFXAP, and CIITA. Among these, we found that HDAC4 and HDAC5 interacted with CIITA (Fig. 8*A*). Moreover, HDAC5 co-localized with CIITA (Fig. 8*B*). RFXANK interacts with RFX5 and CIITA (49), so we investigated whether HDAC4 interferes with the interactions. For this, co-immunoprecipitation was performed. As expected, RFX5 co-precipitated RFXANK (Fig. 8, *C–E*, *lanes 1*). Exogenous HDAC4 slightly affected the co-precipitation (Fig. 8*D*, compare *lanes 1* and *2*). Under the conditions used, RFXAP did not co-precipitate RFXANK (Fig. 8, *C–E*, *lanes 3* and *4*). Like RFX5, CIITA co-precipitated RFXANK (Fig. 8, *C–E*, *lanes 5*). Expression of HDAC4 affected the co-precipitation (compare *lanes 5* and *6*). As described above (Fig. 8*A*), CIITA also co-precipitated HDAC4 (Fig. 8, *C–E*, *lanes 6*). Importantly the binding affinity of HDAC4 to CIITA was quite comparable to that of RFXANK to CIITA (*lanes 5* and *6*). The biological significance for the interaction between RFXANK and CIITA has been well established (31, 32). These results thus

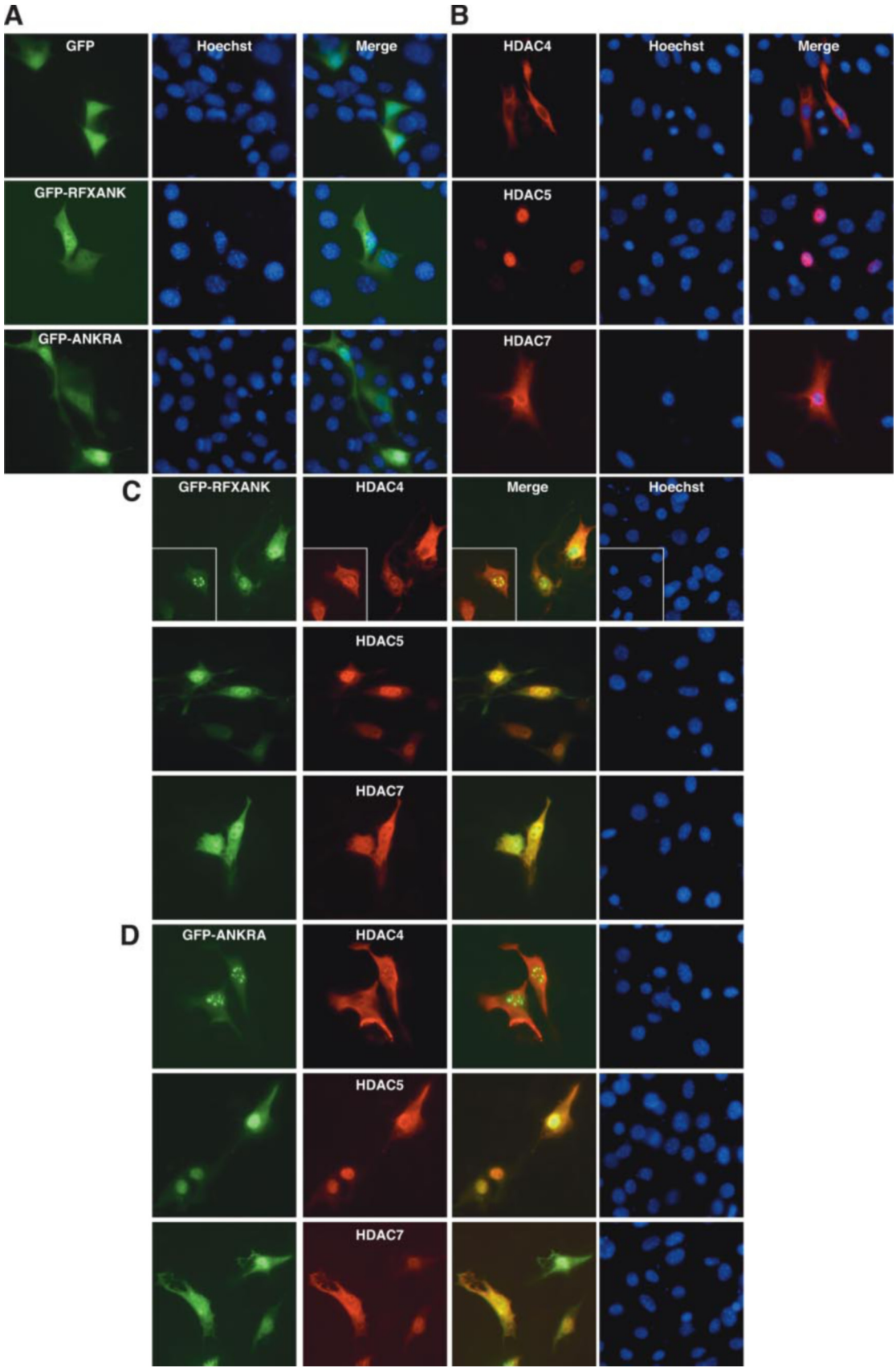

FIG. 5. **Subcellular localization of RFXANK, ANKRA, and class IIa HDACs.** *A*, expression plasmids for GFP-ANKRA and GFP-RFXANK were transfected into NIH3T3 cells. 24 h after transfection, cells were fixed and examined for green fluorescence. *B*, an expression plasmid for HA-tagged HDAC4, -5 or -7 was transfected into NIH3T3 cells. 24 h after transfection, the cells were fixed and stained with anti-HA antibody for indirect immunofluorescence microscopy. *C* and *D*, an expression plasmid for HA-tagged HDAC4, -5, or -7 was transfected into NIH3T3 cells along with an expression plasmid for GFP-ANKRA or -RFXANK as indicated. 24 h after transfection, cells were fixed and stained with anti-HA antibody to detect HDAC4, -5, or -7 by indirect immunofluorescence microscopy. *Green* fluorescence was used to locate GFP fusion proteins. Merged images are also shown with *yellow* signals indicating co-localization. GFP itself showed no co-localization with the HDACs (data not shown).

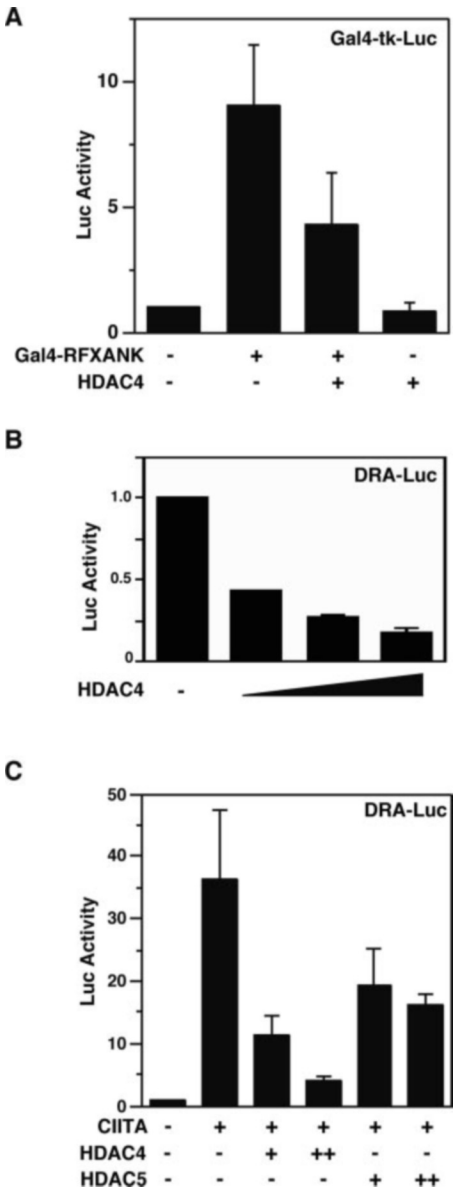

FIG. 6. **Repression of CIITA-dependent transcription by HDAC4 and HDAC5.** *A*, the luciferase reporter Gal4-tk-Luc was transfected into HEK293 cells along with a β-galactosidase expression plasmid and a plasmid expressing RFXANK as a protein fused to the Gal4 DNA-binding domain (residues 1–147). An HDAC4 expression plasmid was also co-transfected as indicated. The normalized luciferase activity from the transfection without any effector plasmids was arbitrarily set to 1.0. Average values of at least three independent experiments are shown with standard deviations. *B*, the luciferase reporter DRA-Luc (0.4 μg) was transfected into NIH3T3 cells with increasing amounts of the HDAC4 expression plasmid (0.05–0.2 μg). A cytomegalovirus β-galactosidase reporter (0.05 μg) was included for normalization of transfection efficiency. *C*, assays were performed as in *B* except that expression plasmids for CIITA and HDAC5 were also included as indicated.

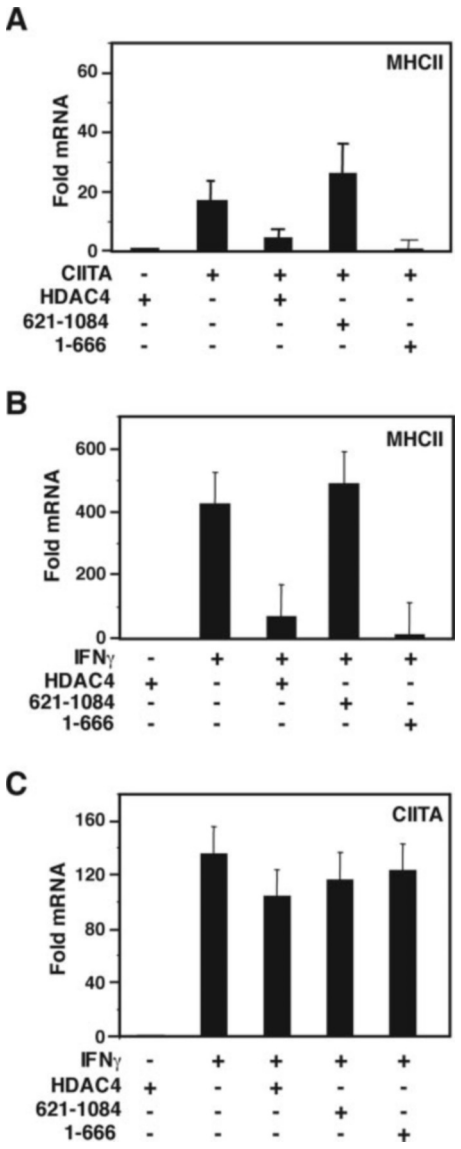

FIG. 7. **Repression of MHCII expression by HDAC4.** *A*, HDAC4 inhibits CIITA-dependent activation of MHCII expression. Real time PCR analysis was performed to quantify MHCII mRNA. CIITA and HDAC4 expression plasmids were transfected into HeLa cells as indicated, and RNA was isolated 24 h post-transfection. Samples were normalized to 18 S rRNA. *B* and *C*, HDAC4 represses IFNγ-inducible expression of MHCII. Real time PCR analysis was performed to determine levels of MHCII (*B*) and CIITA (*C*) mRNA. Expression plasmids for HDAC4 and mutants were transfected into HeLa cells followed by 24-h treatment with IFNγ (500 units/ml). For CIITA (*C*), mRNA transcribed from promoter IV was measured (68).

suggest that HDAC4 also targets CIITA and affects the assembly of the RFX-CIITA enhanceosome.

DISCUSSION

ANKRA and RFXANK as Novel Partners of Class IIa HDACs—Yeast two-hybrid screens with HDAC4 as bait (Fig. 1*A*) yielded two known interaction partners (MEF2C and 14-3-3ζ) and four novel ones (ANKRA, ATRX, REV3L, and microtubule-associated protein 1S), suggesting that HDAC4 and its homologs may also be involved in regulating cell processes other than transcriptional regulation. The high sequence similarity to RFXANK (28, 47) suggests a potential role of ANKRA in transcriptional regulation. Consistent with this notion, ANKRA interacted and displayed partial co-localization with HDAC4 and other class IIa HDACs (Figs. 1, 2*A*, 4, and 5*D*) and was able to enter the nucleus (Fig. 5, *A* and *D*). ANKRA has been shown to interact with membrane-associated receptors (28, 50), so an interesting possibility is that it acts as a signaling adaptor to facilitate the communication between the plasma membrane and the nucleus. This possibility needs to be investigated further.

The ankyrin repeat domain of ANKRA appeared to be necessary for HDAC4 interaction (Fig. 1, *B–F*). Similarly, the ankyrin repeat domain of RFXANK interacted with HDAC4 (Fig. 2). RFXANK was originally isolated from MHCII-deficient patients as one subunit of the trimeric transcription factor RFX (51, 52) and contains four ankyrin repeats in its C-terminal

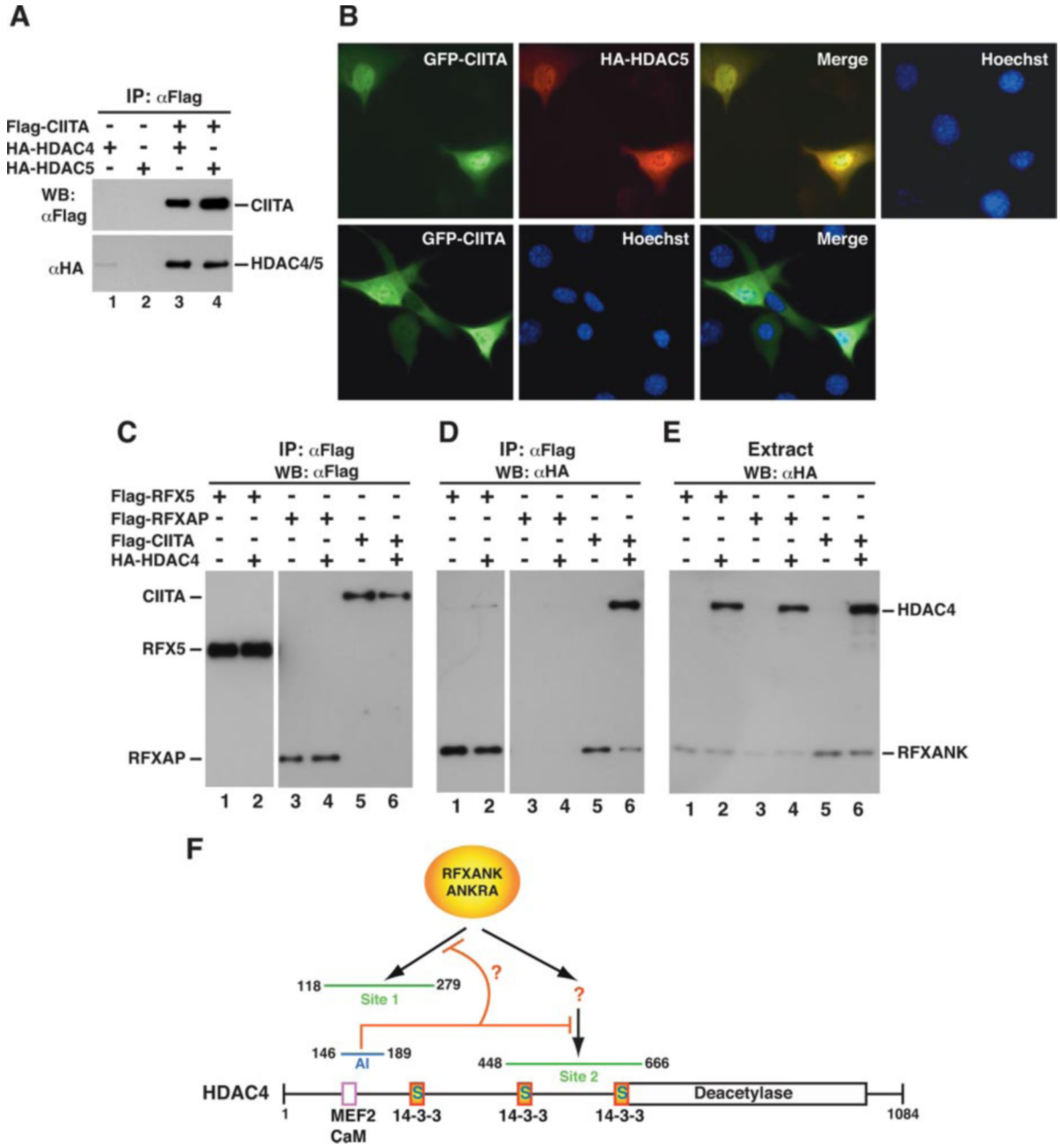

FIG. 8. **Interaction of HDAC4 and HDAC5 with CIITA.** *A*, expression plasmids for HA-HDAC4 and -HDAC5 were transfected into HEK293 cells with an expression plasmid for FLAG-CIITA as specified. Extracts were prepared in buffer B for immunoprecipitation (*IP*) on M2 agarose, and bound proteins were eluted with FLAG peptide for Western blotting (*WB*) analysis with anti-FLAG (*upper*) or anti-HA (*lower*) antibody. *B*, an expression plasmid for GFP-CIITA was co-transfected into NIH3T3 cells with (*upper*) or without (*lower*) an expression plasmid for HA-HDAC5. 24 h post-transfection, cells were fixed and stained with anti-HA antibody to detect HDAC5 by indirect immunofluorescence microscopy. Hoechst 33258 was used to stain DNA for nuclei visualization. *C–E*, the expression plasmid for HA-RFXANK was transfected into HEK293 cells along with an expression plasmid for FLAG-RFX5, -RFXAP, or -CIITA. The HA-HDAC4 expression plasmid was also co-transfected as indicated. Extracts were prepared in buffer K for immunoprecipitation on M2 agarose, and bound proteins were eluted for Western blotting with anti-FLAG (*C*) or anti-HA (*D*) antibody. Western blotting analysis of the extracts with anti-HA antibody is also shown (*E*). *F*, schematic about the interaction of HDAC4 with RFXANK or ANKRA. The domain organization of HDAC4 is depicted as in Fig. 1*A*. The calmodulin (*CaM*)-binding motif overlaps with that for MEF2. The two RFXANK-binding sites (Fig. 3) are indicated with *green lines*. RFXANK and perhaps also ANKRA bind directly to residues 118–279 (*Site 1*) but only indirectly to residues 448–666 (*Site 2*). Although it is unclear how the interaction at Site 1 is inhibited *in vivo*, residues 146–189 (*blue line*) act as the autoinhibitory (*AI*) domain to negatively regulate the interaction of RFXANK at Site 2. It remains to be determined what mediates this interaction *in vivo*. The model also applies to the other three class IIa HDACs.

region (Fig. 2*B*). Ankyrin repeats are common sequence motifs, and each repeat comprises 33 residues (46). Such motifs have been found in cell cycle regulators, signaling modulators, transcription factors, cytoskeletal organizers, and toxins. One major function of these motifs is to mediate protein-protein interaction. Ankyrin repeats of RFXANK provide an interaction platform to assemble the RFX complex (53, 54). Repeats 2–4 mediate the binding of RFXANK to CIITA, and repeat 2 is essential for the binding (49). Our results indicate that the ankyrin repeat domains of RFXANK and ANKRA are required for HDAC4 association (Figs. 1–2). Further mapping revealed that repeats 1 and 2 of RFXANK are important for the binding (Fig. 2, *B* and *C*), suggesting that the binding site for HDAC4 overlaps with that for CIITA. In agreement with this, HDAC4 competed with CIITA in binding to RFXANK (Fig. 8, *C–E*) and inhibited MHCII expression (Figs. 6 and 7).

We also mapped the RFXANK-binding sites on HDAC4. *In vitro* binding assays located one binding site to residues 118–279 of HDAC4 (Fig. 3, *A–F*). Co-immunoprecipitation revealed that this site is not functional *in vivo*, and an indirect binding site resides within residues 448–666 (Fig. 3, *G* and *H*). It is presently unclear how the indirect interaction is mediated *in vivo* (Fig. 8*F*). One possibility is that a third protein mediates the binding. Alternatively, a modification event controls the binding. Intriguingly, an N-terminal region containing residues 146–189 of HDAC4 negatively regulated the binding (Fig. 3, *G* and *H*). In addition, effects of HDAC4 on DRA-Luc reporter activity were cell line-dependent (Fig. 6 and data not shown). These results strongly suggest that the interaction of RFXANK with HDAC4 and its homolog is regulated *in vivo* (Fig. 8*F*). The two RFXANK-binding sites overlap with motifs required for interaction with MEF2, calmodulin, and 14-3-3 proteins (Fig. 8*F*), so an interesting possibility awaiting further investigation is whether these known partners are involved in the regulation.

Roles of Class IIa HDACs in Regulating MHCII Expression—Consistent with their physical interaction with RFXANK and CIITA, class IIa HDACs repressed CIITA-dependent transcription in reporter gene assays (Fig. 6). HDAC4 also inhibited IFNγ-inducible gene expression (Fig. 7). These results reinforce the notion that class IIa HDACs are transcriptional corepressors (6, 8).

How about the underlying repression mechanisms? There are at least four possibilities. First, mutant 1–666 of HDAC4 was able to inhibit MHCII expression (Fig. 7*B*), so this deacetylase may act through its N-terminal repression domain (8, 55). Second, HDAC4 and other class IIa members may mediate histone deacetylation, which is known to be important for MHCII expression. Both CIITA and nuclear factor Y interact with p300/CBP and p300/CBP-associated factor (56, 57). The role of HDACs has been implicated in the control of MHCII gene expression. The HDAC inhibitor trichostatin A rescues MHCII expression in tumor cells and mature dendritic cells (58, 59). HDAC1 has been shown to be recruited by transcription factor YY1 to a cis-acting element located in the first exon of the HLA-DRA promoter (60). Moreover, this recruitment leads to repression of inducible HLA-DRA activation (60). IFNγ regulates MHCII expression through CIITA induction. Chromatin immunoprecipitation revealed that IFNγ treatment increases CIITA binding to the HLA-DRA promoter and stimulates histone acetylation (61). Conversely, removal of IFNγ decreases CIITA association with the promoter and reduces histone acetylation (61). The decrease of histone acetylation may be due to active deacetylation. Therefore, association of HDAC4 with RFXANK may deacetylate histones to inhibit MHCII expression.

Third, class IIa HDACs may deacetylate transcription factors involved. Related to this, the transcriptional activity of CIITA is known to be up-regulated by acetylation (62, 63). CIITA interacts with HDAC1 (43) and class IIa HDACs (Fig. 8*A*), so these enzymes may cooperate with each other to deacetylate CIITA and inhibit transcription. These deacetylases have recently been shown to regulate the phosphorylation and sumoylation of MEF2 (48). By analogy, they may also modulate the phosphorylation and sumoylation of CIITA and/or RFX subunits. Finally, the finding that HDAC4 modestly interfered with the interaction of RFXANK with RFX5 and CIITA (Fig. 8, *C–E*) suggests that this deacetylase and its homologs may hinder enhanceosome assembly. In agreement with this, trichostatin A treatment enhances the binding of CIITA and RFX5 to MHCII promoters (43). Additional analyses are needed to distinguish between different possibilities raised here.

Targeting of CIITA by Class IIa HDACs—The finding that HDAC4 and HDAC5 interacted with a transcriptional coactivator like CIITA (Fig. 8) is unexpected. One precedent is myocardin, a potent coactivator that was recently shown to interact with HDAC5 (64). Theoretically, interaction of class IIa HDACs with coactivators needs to be properly regulated *in vivo*. Related to this, these HDACs are subject to regulation by dynamic nucleocytoplasmic shuttling, and protein kinases, such as calcium/calmodulin-dependent kinase and protein kinase D (also known as protein kinase C μ), phosphorylate class IIa HDACs at the 14-3-3 binding sites to promote the cytoplasmic localization (19–22, 24–27). Our findings thus raise the possibility that protein kinases act through class IIa HDACs to modulate IFNγ signaling. Of note, CIITA has also been shown to mediate transcriptional repression (33, 65), so it may recruit these HDACs to inhibit transcription. CIITA is a member of the nucleotide-binding oligomorization domain/caterpiller family (66, 67), so it will be interesting to determine whether class IIa HDACs also target other members of this important family.

Conclusions—The results presented herein demonstrate that class IIa HDACs interact with both ANKRA and RFXANK through their ankyrin repeats. Importantly, the interaction is regulated *in vivo* (Fig. 8*F*). Another group has independently identified RFXANK as a binding partner of HDAC5 in yeast two-hybrid screens.[2] Our results also show that by targeting RFXANK and CIITA, HDAC4 inhibits MHCII expression. Sequence homology to RFXANK and association with class IIa HDACs suggest that ANKRA may play a role in transcriptional control. Therefore, ANKRA, RFXANK, and CIITA are novel targets of class IIa HDACs.

Acknowledgments—We thank E. Seto, S. Khochbin, and X. Zhou for kindly providing HDAC expression plasmids.

REFERENCES

1. Kouzarides, T. (2000) *EMBO J.* **19,** 1176–1179
2. Sterner, D. E., and Berger, S. L. (2000) *Microbiol. Mol. Biol. Rev.* **64,** 435–459
3. Yang, X. J. (2004) *BioEssays* **26,** 1076–1087
4. Cohen, T., and Yao, T. P. (2004) *Sci. STKE* **2004,** pe42
5. Cress, W. D., and Seto, E. (2000) *J. Cell. Physiol.* **184,** 1–16
6. Khochbin, S., Verdel, A., Lemercier, C., and Seigneurin-Berny, D. (2001) *Curr. Opin. Genet. Dev.* **11,** 162–166
7. Grozinger, C. M., and Schreiber, S. L. (2002) *Chem. Biol.* **9,** 3–16
8. Verdin, E., Dequiedt, F., and Kasler, H. G. (2003) *Trends Genet.* **19,** 286–293
9. Hubbert, C., Guardiola, A., Shao, R., Kawaguchi, Y., Ito, A., Nixon, A., Yoshida, M., Wang, X. F., and Yao, T. P. (2002) *Nature* **417,** 455–458
10. Palazzo, A., Ackerman, B., and Gundersen, G. G. (2002) *Nature* **421,** 230
11. Matsuyama, A., Shimazu, T., Sumida, Y., Saito, A., Yoshimatsu, Y., Seigneurin-Berny, D., Osada, H., Komatsu, Y., Nishino, N., Khochbin, S., Horinouchi, S., and Yoshida, M. (2002) *EMBO J.* **21,** 6820–6831
12. Zhang, Y., Li, N., Caron, C., Matthias, G., Hess, D., Khochbin, S., and Matthias, P. (2003) *EMBO J.* **22,** 1168–1179
13. Haggarty, S. J., Koeller, K. M., Wong, J. C., Grozinger, C. M., and Schreiber, S. L. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 4389–4394
14. North, B. J., Marshall, B. L., Borra, M. T., Denu, J. M., and Verdin, E. (2003) *Mol. Cell* **11,** 437–444
15. McKinsey, T. A., Zhang, C. L., and Olson, E. N. (2002) *Trends Biochem. Sci.* **27,** 40–47
16. Lemercier, C., Brocard, M. P., Puvion-Dutilleul, F., Kao, H. Y., Albagli, O., and Khochbin, S. (2002) *J. Biol. Chem.* **277,** 22045–22052
17. Jin, Y. H., Jeon, E. J., Li, Q. L., Lee, Y. H., Choi, J. K., Kim, W. J., Lee, K. Y., and Bae, S. C. (2004) *J. Biol. Chem.* **279,** 29409–29417
18. Vega, R. B., Matsuda, K., Oh, J., Barbosa, A. C., Yang, X., Meadows, E., McAnally, J., Pomajzl, C., Shelton, J. M., Richardson, J. A., Karsenty, G., and Olson, E. N. (2004) *Cell* **119,** 555–566
19. Grozinger, C. M., and Schreiber, S. L. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 7835–7840
20. Wang, A. H., Kruhlak, M. J., Wu, J., Bertos, N. R., Vezmar, M., Posner, B. I., Bazett-Jones, D. P., and Yang, X. J. (2000) *Mol. Cell. Biol.* **20,** 6904–6912
21. McKinsey, T. A., Zhang, C. L., Lu, J., and Olson, E. N. (2000) *Nature* **408,** 106–111
22. Kao, H. Y., Verdel, A., Tsai, C. C., Simon, C., Juguilon, H., and Khochbin, S. (2001) *J. Biol. Chem.* **276,** 47496–47507
23. Dequiedt, F., Kasler, H., Fischle, W., Kiermer, V., Weinstein, M., Herndier, B. G., and Verdin, E. (2003) *Immunity* **18,** 687–698
24. Vega, R. B., Harrison, B. C., Meadows, E., Roberts, C. R., Papst, P. J., Olson, E. N., and McKinsey, T. A. (2004) *Mol. Cell. Biol.* **24,** 8374–8385
25. Parra, M., Kasler, H., McKinsey, T. A., Olson, E. N., and Verdin, E. (2005) *J. Biol. Chem.* **280,** 13762–13770

[2] E. N. Olson, personal communication.

26. Dequiedt, F., Van Lint, J., Lecomte, E., Van Duppen, V., Seufferlein, T., Vandenheede, J. R., Wattiez, R., and Kettmann, R. (2005) *J. Exp. Med.* **201,** 793–804
27. Chang, S., Bezprozvannaya, S., Li, S., and Olson, E. N. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102,** 8120–8125
28. Rader, K., Orlando, R. A., Lou, X., and Farquhar, M. G. (2000) *J. Am. Soc. Nephrol.* **11,** 2167–2178
29. Boss, J. M. (1997) *Curr. Opin. Immunol.* **9,** 107–113
30. Reith, W., and Mach, B. (2001) *Annu. Rev. Immunol.* **19,** 331–373
31. Ting, J. P., and Trowsdale, J. (2002) *Cell* **109,** (suppl.) S21–S33
32. Boss, J. M., and Jensen, P. E. (2003) *Curr. Opin. Immunol.* **15,** 105–111
33. Xu, Y., Wang, L., Buttice, G., Sengupta, P. K., and Smith, B. D. (2004) *J. Biol. Chem.* **279,** 41319–41332
34. Wang, A. H., Bertos, N. R., Vezmar, M., Pelletier, N., Crosato, M., Heng, H. H., Th'ng, J., Han, J., and Yang, X. J. (1999) *Mol. Cell. Biol.* **19,** 7816–7827
35. Champagne, N., Bertos, N. R., Pelletier, N., Wang, A. H., Vezmar, M., Yang, Y., Heng, H. H., and Yang, X. J. (1999) *J. Biol. Chem.* **274,** 28528–28536
36. Wang, B., Yang, H., Liu, Y. C., Jelinek, T., Zhang, L., Ruoslahti, E., and Fu, H. (1999) *Biochemistry* **38,** 12499–12504
37. Wang, A. H., and Yang, X. J. (2001) *Mol. Cell. Biol.* **21,** 5992–6005
38. Verdel, A., and Khochbin, S. (1999) *J. Biol. Chem.* **274,** 2440–2445
39. Kao, H. Y., Downes, M., Ordentlich, P., and Evans, R. M. (2000) *Genes Dev.* **14,** 55–66
40. Fischle, W., Dequiedt, F., Fillion, M., Hendzel, M. J., Voelter, W., and Verdin, E. (2001) *J. Biol. Chem.* **276,** 35826–35835
41. Zhou, X., Richon, V. M., Rifkind, R. A., and Marks, P. A. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 1056–1061
42. Wright, K. L., Chin, K. C., Linhoff, M., Skinner, C., Brown, J. A., Boss, J. M., Stark, G. R., and Ting, J. P. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 6267–6272
43. Zika, E., Greer, S. F., Zhu, X. S., and Ting, J. P. (2003) *Mol. Cell. Biol.* **23,** 3091–3102
44. Bertos, N. R., Gilquin, B., Chan, M., Yen, T. J., Khochbin, S., and Yang, X. J. (2004) *J. Biol. Chem.* **279,** 48246–48254
45. Wong, A. W., Ghosh, N., McKinnon, K. P., Reed, W., Piskurich, J. F., Wright, K. L., and Ting, J. P. (2002) *J. Immunol.* **169,** 3112–3119
46. Sedgwick, S. G., and Smerdon, S. J. (1999) *Trends Biochem. Sci.* **24,** 311–316
47. Long, A. B., and Boss, J. M. (2005) *Immunogenetics* **56,** 788–797
48. Grégoire, S., and Yang, X. J. (2005) *Mol. Cell. Biol.* **25,** 2273–2282
49. Nekrep, N., Geyer, M., Jabrane-Ferrat, N., and Peterlin, B. M. (2001) *Mol. Cell. Biol.* **21,** 5566–5576
50. Lim, H. H., and Park, C. S. (2005) *Mol. Biol. Cell* **16,** 1013–1025
51. Masternak, K., Barras, E., Zufferey, M., Conrad, B., Corthals, G., Aebersold, R., Sanchez, J. C., Hochstrasser, D. F., Mach, B., and Reith, W. (1998) *Nat. Genet.* **20,** 273–277
52. Nagarajan, U. M., Louis-Plence, P., DeSandro, A., Nilsen, R., Bushey, A., and Boss, J. M. (1999) *Immunity* **10,** 153–162
53. Nekrep, N., Jabrane-Ferrat, N., and Peterlin, B. M. (2000) *Mol. Cell. Biol.* **20,** 4455–4461
54. DeSandro, A. M., Nagarajan, U. M., and Boss, J. M. (2000) *Mol. Cell. Biol.* **20,** 6587–6599
55. Yang, X. J., and Grégoire, S. (2005) *Mol. Cell. Biol.* **25,** 2873–2884
56. Fontes, J. D., Kanazawa, S., Jean, D., and Peterlin, B. M. (1999) *Mol. Cell. Biol.* **19,** 941–947
57. Kretsovali, A., Agalioti, T., Spilianakis, C., Tzortzakaki, E., Merika, M., and Papamatheakis, J. (1998) *Mol. Cell. Biol.* **18,** 6777–6783
58. Landmann, S., Muhlethaler-Mottet, A., Bernasconi, L., Suter, T., Waldburger, J. M., Masternak, K., Arrighi, J. F., Hauser, C., Fontana, A., and Reith, W. (2001) *J. Exp. Med.* **194,** 379–391
59. Magner, W. J., Kazim, A. L., Stewart, C., Romano, M. A., Catalano, G., Grande, C., Keiser, N., Santaniello, F., and Tomasi, T. B. (2000) *J. Immunol.* **165,** 7017–7024
60. Osborne, A., Zhang, H., Yang, W. M., Seto, E., and Blanck, G. (2001) *Mol. Cell. Biol.* **21,** 6495–6506
61. Beresford, G. W., and Boss, J. M. (2001) *Nat. Immunol.* **2,** 652–657
62. Spilianakis, C., Papamatheakis, J., and Kretsovali, A. (2000) *Mol. Cell. Biol.* **20,** 8489–8498
63. Raval, A., Howcroft, K., Weissman, J. D., Kirshner, S., Zhu, X. S., Yokoyama, K., Ting, J., and Singer, D. (2001) *Mol. Cell* **7,** 105–115
64. Cao, D., Wang, Z., Zhang, C. L., Oh, J., Xing, W., Li, S., Richardson, J. A., Wang, D. Z., and Olson, E. N. (2005) *Mol. Cell. Biol.* **25,** 364–376
65. Nozell, S., Ma, Z., Wilson, C., Shah, R., and Benveniste, E. N. (2004) *J. Biol. Chem.* **279,** 38577–38589
66. Inohara, N., and Nunez, G. (2003) *Nat. Rev. Immunol.* **3,** 371–382
67. Ting, J. P., and Davis, B. K. (2005) *Annu. Rev. Immunol.* **23,** 387–414
68. Dong, Y., Rohn, W. M., and Benveniste, E. N. (1999) *J. Immunol.* **162,** 4731–4739