MOLECULAR AND CELLULAR BIOLOGY, May 2008, p. 3437–3445 Vol. 28, No. 10
0270-7306/08/$08.00+0 doi:10.1128/MCB.01611-07

Histone Deacetylase 5 Acquires Calcium/Calmodulin-Dependent Kinase II Responsiveness by Oligomerization with Histone Deacetylase 4▽

Johannes Backs,[1,2] Thea Backs,[1,2] Svetlana Bezprozvannaya,[1] Timothy A. McKinsey,[3] and Eric N. Olson[1]*

Department of Molecular Biology, University of Texas Southwestern Medical Center, Dallas, Texas[1]; Department of Cardiology, University of Heidelberg, Heidelberg, Germany[2]; and Gilead Colorado, Inc., 7575 West 103rd Avenue, Westminster, Colorado 80021[3]

Received 31 August 2007/Returned for modification 26 September 2007/Accepted 1 March 2008

Calcium/calmodulin-dependent protein kinase II (CaMKII) phosphorylates histone deacetylase 4 (HDAC4), a class IIa HDAC, resulting in the cytosolic accumulation of HDAC4 and the derepression of the transcription factor myocyte enhancer factor 2. Phosphorylation by CaMKII requires docking of the kinase to a specific domain of HDAC4 not present in other HDACs. Paradoxically, however, CaMKII signaling can also promote the nuclear export of other class IIa HDACs, such as HDAC5. Here, we show that HDAC4 and HDAC5 form homo- and hetero-oligomers via a conserved coiled-coil domain near their amino termini. Whereas HDAC5 alone is unresponsive to CaMKII, it becomes responsive to CaMKII in the presence of HDAC4. The acquisition of CaMKII responsiveness by HDAC5 is mediated by HDAC5's direct association with HDAC4 and can occur by phosphorylation of HDAC4 or by transphosphorylation by CaMKII bound to HDAC4. Thus, HDAC4 integrates upstream Ca^{2+}-dependent signals via its association with CaMKII and transmits these signals to HDAC5 by protein-protein interactions. We conclude that HDAC4 represents a point of convergence for CaMKII signaling to downstream HDAC-regulated genes, and we suggest that modulation of the interaction of CaMKII and HDAC4 represents a means of regulating CaMKII-dependent gene programs.

Histone deacetylases (HDACs) repress transcription by deacetylating nucleosomal histones and other components of the transcriptional machinery, thereby promoting chromatin compaction and perturbing the protein-protein interactions essential for gene activation. The four class IIa HDACs (HDAC4, -5, -7, and -9) have become the focus of intense interest because of their ability to respond to extracellular signals by regulated phosphorylation, which provides a mechanism for linking stimuli at the cell membrane with the genome (1, 13, 24, 26). These HDACs have been implicated in a variety of developmental and disease-related processes based on the phenotypes of HDAC knockout mice (6, 7, 37, 40). HDAC4 acts as a repressor of chondrocyte hypertrophy and endochondral bone development (37). HDAC5 and MEF2-interacting transcription repressor (MITR; a splice variant of HDAC9 that lacks the C-terminal half) play redundant roles in regulating stress-dependent cardiac growth and skeletal-muscle gene expression (6, 29, 40), and HDAC7 is required for T-cell survival (31) and vascular integrity as a consequence of its ability to repress the expression of matrix metalloproteinase 10 (7).

Class IIa HDACs contain a C-terminal deacetylase-like domain and an N-terminal extension that mediates interactions with transcriptional activators, such as myocyte enhancer factor 2 (MEF2) and calmodulin-binding transcription activators (CAMTAs) (13, 23, 30, 34). The interaction of MEF2 or CAMTA with the N-terminal extension of class IIa HDACs silences the expression of their target genes. The N-terminal regulatory domain also mediates interactions with class I HDACs and other corepressors, such as the C-terminal binding protein (CtBP), SMRT, and N-CoR (1, 41).

Signaling by G protein-coupled receptors leads to the phosphorylation of class IIa HDACs at three conserved serine residues in the N-terminal regulatory domain. Numerous calcium-dependent protein kinases, including protein kinase D (PKD), calcium/calmodulin-dependent protein kinases (CaMKs), and microtubule-associated regulatory kinase (Mark), phosphorylate these sites, depending on the stimulus and the cell type (5, 18, 20, 25, 26, 36). Phosphorylation creates docking sites for the 14-3-3 chaperone protein, which binds phospho-HDACs and escorts them from the nucleus to the cytoplasm, relieving downstream transcription factors, such as MEF2, from their repressive influence (15, 27, 28).

We recently showed that CaMKII specifically regulates the phosphorylation and nuclear export of HDAC4, but not HDAC5 or MITR, by virtue of a unique CaMKII docking site on HDAC4 (2, 44). These findings were puzzling in light of other studies showing that CaMKII also promotes nuclear export of HDAC5 (8, 21, 39). In the present study, we investigated the molecular basis for the CaMKII responsiveness of class IIa HDACs. We show that HDAC4 and HDAC5 oligomerize through a conserved amino-terminal alpha-helical domain and that HDAC4 thereby confers CaMKII responsiveness to HDAC5, which does not directly interact with CaMKII. These findings identify HDAC4 as a nodal regulator of CaMKII signaling via its recruitment of CaMKII and association with HDAC5. These findings have implications for understanding the mechanism of action of CaMKII in a variety of cell types and the role of class IIa HDACs as mediators of diverse cellular processes.

* Corresponding author. Mailing address: Department of Molecular Biology, UT Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75390-9148. Phone: (214) 648-1187. Fax: (214) 648-1196. E-mail: eric.olson@utsouthwestern.edu.

▽ Published ahead of print on 10 March 2008.

MATERIALS AND METHODS

Plasmids and adenoviruses. Expression constructs for constitutively active CaMKII (Myc-tagged CaMKIIδB T287D), HDAC4, HDAC5, HDAC7, and MITR were described previously (2). Point mutations were introduced with the QuikChange kit (Stratagene). Deletion mutants of HDAC4 and HDAC5 were generated by PCR.

For adenovirus production, Myc-tagged CaMKIIδB T287D (2) was subcloned into pShuttle-CMV (QBiogen), and adenovirus was generated in HEK293 cells according to the manufacturer's recommendations. Clonal populations of virus were obtained using the agar overlay method, and titers were determined with the Adeno-X rapid titer kit (Clontech).

Cell culture and transfection assays. COS cells and C2C12 myoblasts were maintained in Dulbecco modified Eagle medium with fetal bovine serum (10%), L-glutamine (2 mM), and penicillin-streptomycin. Transfection of COS cells was performed with FuGene 6 (Roche Molecular Biochemicals) according to the manufacturer's instructions. HDAC4 knockdown using HDAC4 short interfering RNA (siRNA; On-Targetplus SMARTpool HDAC4 mouse; Dharmacon) was performed with C2C12 myoblasts according to the manufacturer's instructions. The knockdown efficiency was determined by HDAC4 immunoblot analyses 48 h after siRNA transfection.

Indirect immunofluorescence. COS cells were grown on glass coverslips, fixed in paraformaldehyde (4%), permeabilized in 0.1% Triton X-100, and blocked in phosphate-buffered saline containing goat serum (5%). Primary antibodies against FLAG (monoclonal or rabbit; Sigma) were used at a dilution of 1:200. Secondary antibodies conjugated to either fluorescein or Texas Red (Vector Laboratories) were also used at a dilution of 1:200. All images were captured at a magnification of ×40.

Coimmunoprecipitation and immunoblotting. COS cells and C2C12 myoblasts were harvested 1 day after transfection in Tris (50 mM, pH 7.4), NaCl (150 mM), EDTA (1 mM), Triton X-100 (1%) supplemented with protease inhibitors (Complete; Roche), and phenylmethylsulfonyl fluoride (1 mM). The cells were further disrupted by passage through a 25-gauge needle, and cell debris was removed by centrifugation. FLAG-tagged proteins were immunoprecipitated with an M2-agarose conjugate (Sigma) and thoroughly washed with lysis buffer. Bound proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes, and immunoblotted as indicated in the figures with either a polyclonal anti-Myc antibody (rabbit; Santa Cruz) or a monoclonal anti-FLAG antibody (M2; Sigma). The proteins were visualized with a chemiluminescence system (Santa Cruz). In the C2C12 myoblasts, HDAC4 was immunoprecipitated with rabbit polyclonal anti-HDAC4 (Biomol) and adenoviral expressed Myc-CaMKII was immunoprecipitated with rabbit polyclonal anti-Myc antibody (Santa Cruz), followed by incubation with protein G beads (Roche). Bound HDAC5 was determined by immunoblotting using goat polyclonal anti-HDAC5 (Santa Cruz).

Statistical methods. In the immunofluorescence experiments, the nucleocytoplasmic distribution of class IIa HDACs was analyzed in three different randomly chosen areas with at least 30 cells per area (in total, >100 cells were counted per experiment). The means ± standard errors of the mean (SEMs) of the results for the three areas are shown in the figures. The immunoblots (HDAC4 protein/GAPDH [glyceraldehyde-3-phosphate dehydrogenase] protein ratio) were analyzed using ImageJ software. The values shown represent the means ± SEMs of the results for three independent knockdown experiments.

RESULTS

HDAC4 coexports HDAC5 and MITR in response to CaMKII. The four class IIa HDACs, HDAC4, -5, -7, and -9, contain homologous phosphorylation sites in their N-terminal regulatory regions that mediate nuclear export in response to PKD, CaMKI and -IV, and Mark (5, 26, 36). However, as shown in Fig. 1A, constitutively active CaMKII promotes the nuclear export of HDAC4 and, to a lesser degree, HDAC7, but not HDAC5 or MITR (a splice variant of HDAC9 that lacks the C-terminal half). Intriguingly, when HDAC4 was coexpressed with HDAC5 or MITR in transfected COS cells, HDAC5 and MITR were translocated with HDAC4 from the nucleus to the cytoplasm and became colocalized to the same subcellular compartment (Fig. 1B). In contrast, HDAC4 did not enhance the nuclear-to-cytoplasmic translocation of HDAC7, nor did HDAC4 colocalize with HDAC7 in the cytoplasm in response to CaMKII. The ability of HDAC4 to regulate the nuclear-to-cytoplasmic translocation of HDAC5 and, to a lesser degree, of MITR was HDAC4 dosage dependent (Fig. 1B), suggesting a stoichiometric mechanism.

The translocation of MITR to the cytoplasm in response to CaMKII signaling was especially intriguing because MITR lacks a nuclear export signal and remains localized exclusively to the nucleus even when phosphorylated by other kinases (e.g., PKD, CaMKI, or CaMKIV) that promote the translocation of other class IIa HDACs to the cytoplasm (26, 36). The observation that MITR colocalized with HDAC4 to the cytosol in response to CaMKII thus suggested a coshuttling mechanism.

Interaction between HDAC4 and HDAC5 is mediated by an N-terminal coiled-coil domain. To determine whether the colocalization and coshuttling of HDAC4 with HDAC5 and MITR were mediated by a physical interaction between these proteins, we performed coimmunoprecipitation assays with Myc- and FLAG-tagged proteins. As shown in Fig. 2A, HDAC4 could form homo-oligomers and hetero-oligomers with HDAC5 and, to a lesser degree, with MITR but showed almost no detectable interaction with HDAC7. We also found that HDAC5 associated strongly with itself and, to a lesser extent, with HDAC4, HDAC7, and MITR. HDAC7 also interacted more strongly with itself than with other HDACs. Thus, the extent to which the different class IIa HDACs were coexported from the nucleus with HDAC4 in response to CaMKII signaling correlated with the strength of their interaction with HDAC4, although the coimmunoprecipitation assay was slightly more sensitive than the nuclear export assay.

To identify the domain of HDAC4 that associated with HDAC5, we expressed truncated HDAC4 proteins together with HDAC5. Coimmunoprecipitation revealed that amino acids 66 to 208 of HDAC4, a region that also encompasses the MEF2 binding domain, are necessary to bind HDAC5 (Fig. 2B).

A sequence comparison of the N termini of class IIa HDACs revealed that HDAC4, HDAC5, and MITR share a homologous glutamine-rich coiled-coil domain (amino acids 68 to 155 of HDAC4) within the region that mediates dimerization. HDAC7 lacks this region of homology. This domain has been predicted to mediate protein-protein interactions (http://ca.expasy.org/prosite/). Consistent with this notion, a mutant HDAC4 protein lacking this region (HDAC4 Δcc) was impaired in its ability to associate with HDAC4 and HDAC5. However, it should be noted that two truncated HDAC4 proteins containing this domain (HDAC4 1-161 and HDAC4 1-190) were also unable to hetero-oligomerize with full-length HDAC5, suggesting that this interaction requires an additional domain (amino acids 161 to 208) or an extended secondary structure to appropriately orient or expose the coiled-coil domain. The MEF2 binding domain is not required for this interaction because an HDAC4 mutant lacking amino acids 163 to 184, which are critical for MEF2 binding (Fig. 2D), was still associated with HDAC5 (Fig. 2B and D). We also ruled out the possibility that HDAC4 and HDAC5 interact via CtBP, a known corepressor of class IIa HDACs (41) that binds to a P-X-D-L-R motif (amino acids 48 to 52 of HDAC4), because (i) HDAC4 66-1084 was able to bind to HDAC5 (Fig. 2B), and

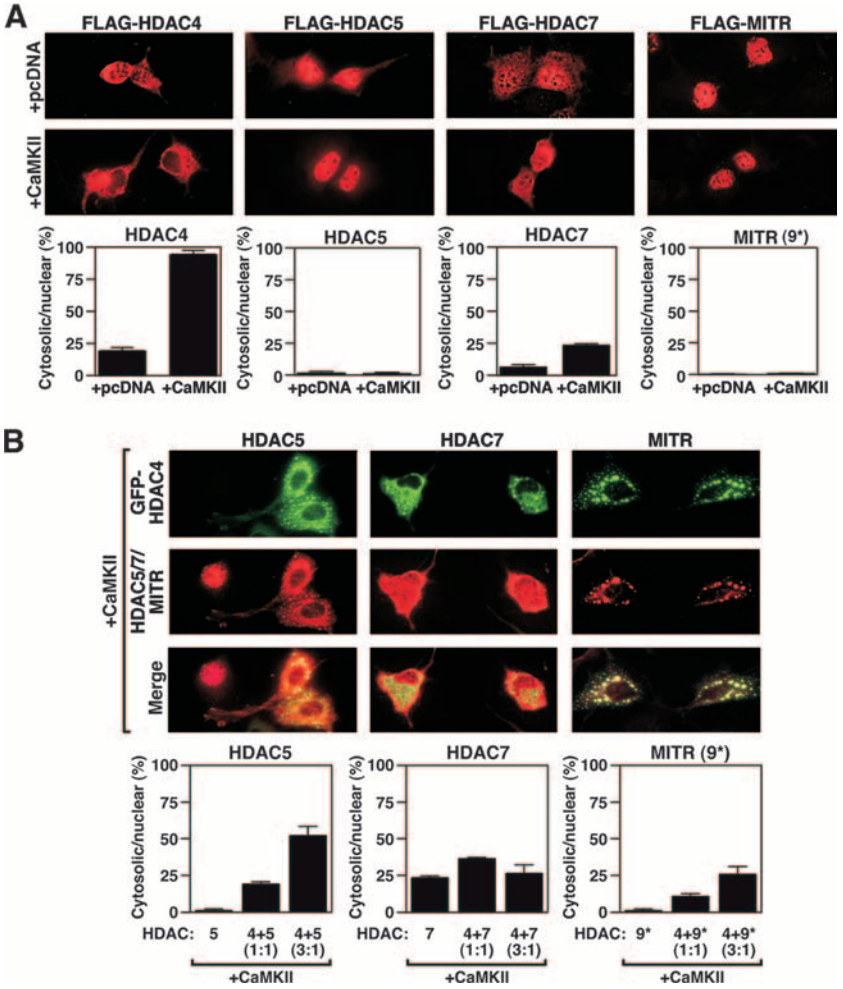

FIG. 1. Coshuttling of HDAC4 and HDAC5. (A) COS cells were transfected with expression plasmids encoding FLAG-HDAC4, -HDAC5, -HDAC7, and -MITR (referred to as 9*) in the absence or presence of expression plasmids encoding constitutively active CaMKII, or they were transfected with pCDNA1 as a control. The subcellular localization of these HDACs was determined 1 day after transfection by immunostaining for the FLAG epitope tag. CaMKII induced complete cytosolic accumulation of HDAC4 and partial nuclear export of HDAC7. (B) Green fluorescent protein (GFP)-HDAC4 was coexpressed with FLAG-HDAC5, -HDAC7, and -MITR in COS cells. HDAC4 colocalized with HDAC5 and MITR. CaMKII induced the nuclear export of HDAC5 and, to a lesser degree, of MITR in the presence of HDAC4 but not in its absence. The percentage of cells showing the localization of the indicated HDAC in the cytoplasm relative to that in the nucleus when cotransfected with pcDNA or activated CaMKII is shown beneath each set of panels. At least 100 HDAC-expressing cells were analyzed under each condition. The data are expressed as the means ± SEMs of the results for three randomly chosen areas per slide. In panel B, HDAC4 was coexpressed with HDAC5, HDAC7, or MITR at 1:1 and 3:1 ratios, as indicated.

(ii) HDAC4 Δcc, which could not bind to HDAC5, still bound to the CtBP (Fig. 2D). Figure 2E summarizes the mapping of the HDAC4 association domain.

Coshuttling requires interaction between class IIa HDACs and CaMKII docking to HDAC4. The HDAC4 Δcc mutant, which cannot oligomerize, contains the CaMKII docking site (surrounding R601) and CaMKII phosphorylation sites (S467 and S632) and thus responded to CaMKII signaling by shuttling to the cytosol (Fig. 3A). However, HDAC4 Δcc failed to induce the redistribution of HDAC5 to the cytosol in the presence of CaMKII, confirming that the coiled-coil region of HDAC4 is critical for coshuttling. Another HDAC4 mutant (HDAC4 R601F), which is unable to bind or respond to CaMKII (2), still colocalized with HDAC5 in the nucleus but lacked the ability to confer CaMKII responsiveness to HDAC5 (Fig. 3B).

"Piggyback" phosphorylation versus transphosphorylation. We imagined two potential explanations for the ability of HDAC4 to confer CaMKII responsiveness to HDAC5: (i) a "piggyback" mechanism in which CaMKII could specifically phosphorylate only HDAC4, which might then act as a chaperone to export HDAC5 from the nucleus to the cytoplasm without HDAC5 itself being phosphorylated, and (ii) a mechanism whereby HDAC4 could serve as a docking site for CaMKII, bringing the kinase into close proximity to HDAC5 and allowing transphosphorylation of HDAC5. To distinguish between these possibilities, we tested whether an HDAC5 mutant lacking the signal-responsive phosphorylation sites (S259/

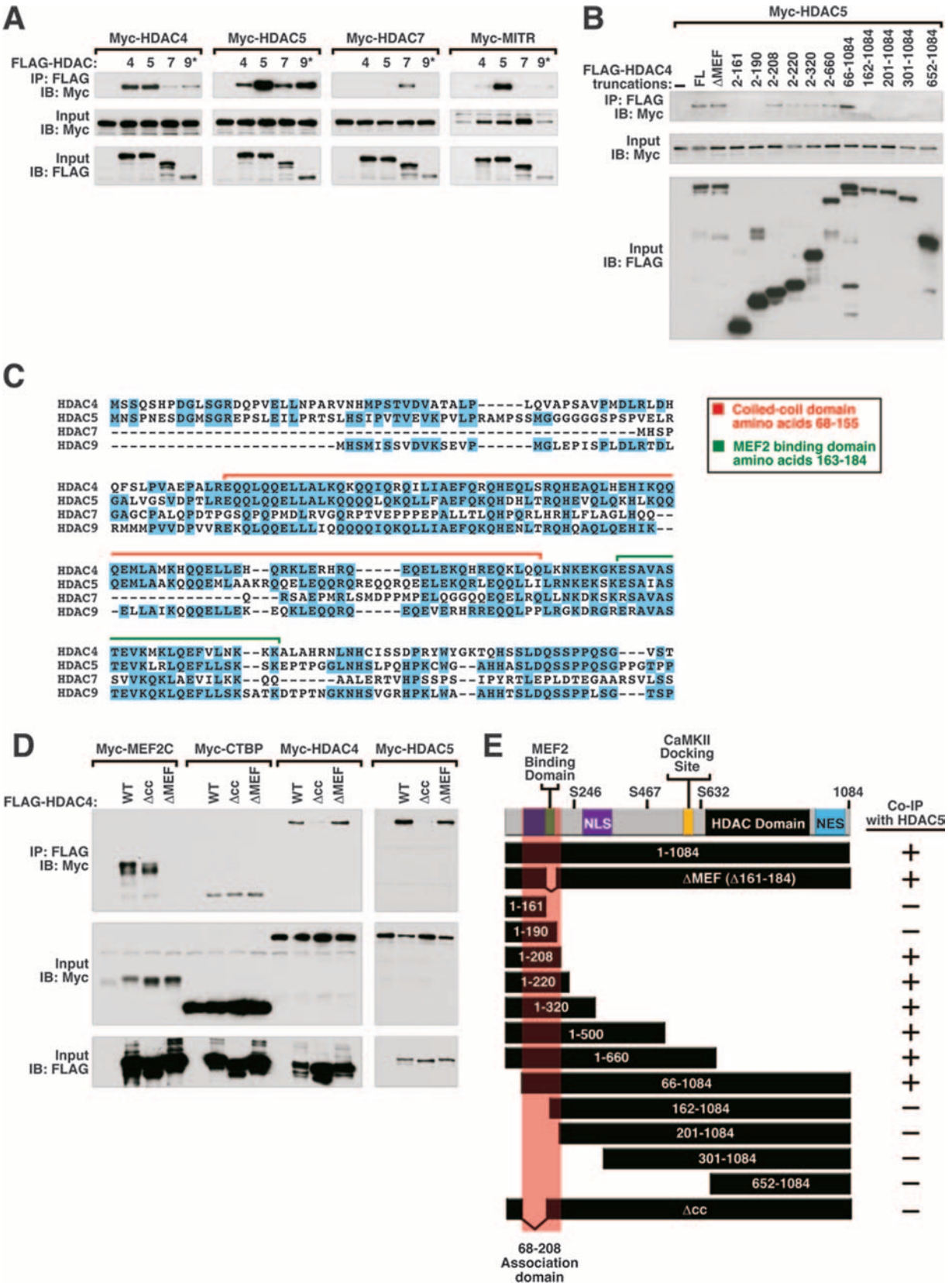

FIG. 2. HDAC4 hetero-oligomerizes with HDAC5. (A) COS cells were transfected with expression plasmids encoding epitope-tagged HDACs, as indicated. Immunoprecipitation (IP) with anti-FLAG followed by immunoblotting (IB) with anti-Myc revealed interactions between HDAC4, HDAC5, and MITR (referred to as 9*). The middle and lower panels show input proteins as detected by IB. (B) COS cells were transfected

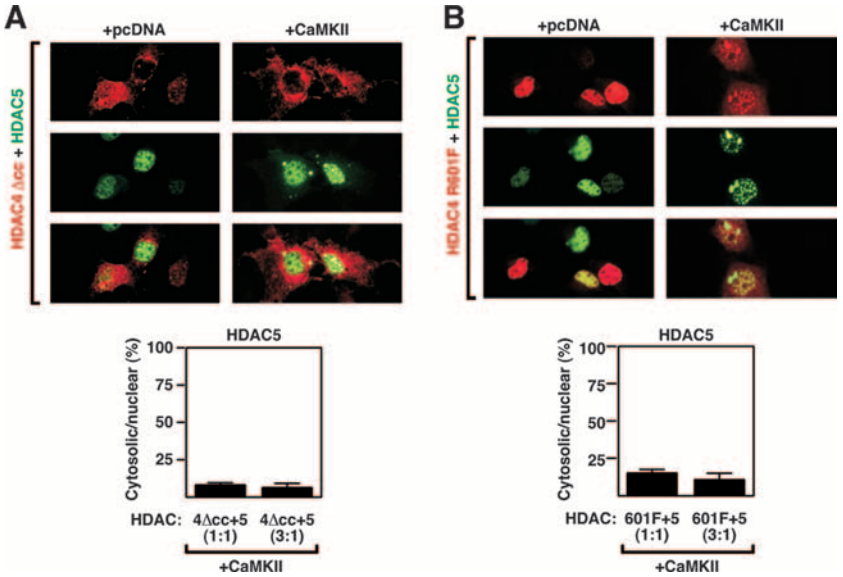

FIG. 3. Hetero-oligomerization and CaMKII docking are required for coshuttling. FLAG-HDAC4 lacking the coiled-coil domain (Δcc) (A) or encoding a disrupted CaMKII docking site (R601F) (B) was expressed in COS cells together with GFP-HDAC5. The coiled-coil domain deletion abolished the colocalization of HDAC4 with HDAC5 and prevented the coshuttling of HDAC5 to the cytosol in response to constitutively active CaMKII. Mutating the CaMKII docking site did not affect colocalization with HDAC5 in the nucleus but did prevent CaMKII responsiveness of the HDAC4-HDAC5 complex. The percentage of cells showing HDAC5 in the cytoplasm when cotransfected with HDAC4 Δcc or HDAC4 R601F and activated CaMKII is shown beneath each set of panels. At least 100 HDAC-expressing cells were analyzed under each condition. The data are expressed as the means ± SEMs of the results for three randomly chosen areas per slide. The HDAC4 mutants were coexpressed with HDAC5 at 1:1 and 3:1 ratios, as indicated.

498A [S/A]) could translocate from the nucleus to the cytoplasm in the presence of HDAC4. Conversely, we tested whether a signal-resistant HDAC4 mutant lacking serines 246, 467, and 632 (HDAC4-S/A) could translocate to the cytoplasm with wild-type HDAC5. Remarkably, HDAC4 coshuttled the signal-resistant HDAC5-S/A mutant to the cytosol in response to CaMKII, suggesting a "piggyback" mechanism (Fig. 4A). On the other hand, HDAC4-S/A was also exported to the cytoplasm in the presence of wild-type HDAC5, albeit to a lesser degree than wild-type HDAC4, consistent with a transphosphorylation mechanism (Fig. 4B).

In the experiment shown in Fig. 4A, HDAC4 provides the phosphorylation sites; thus, increasing amounts of HDAC4 enhance the coshuttling of HDAC5. In Fig. 4B, HDAC5 provides the phosphorylation sites; thus, the HDAC4-S/A–HDAC5 complex shuttles to the cytosol only when sufficient amounts of HDAC5 are present. Consistent with this interpretation, increasing the ratio of HDAC4-S/A to HDAC5 does not enhance nuclear export, as was seen with the wild-type HDAC4 protein. Conversely, when we changed the ratio of HDAC4-S/A to HDAC5 from 1:1 to 1:3, 40% of HDAC5 accumulated in the cytosol in response to CaMKII. In a control experiment (Fig. 4C), HDAC4-S/A was coexpressed with HDAC5-S/A, resulting in no response to CaMKII. We conclude that HDAC4 provides a docking site for CaMKII within an HDAC hetero-oligomer, and once docked, CaMKII can phosphorylate either HDAC4 or its dimerization partner, in this case HDAC5, with the consequent nuclear export of the multiprotein HDAC-CaMKII complex.

Endogenous HDAC4 interacts with endogenous HDAC5 in C2C12 myoblasts. We asked whether hetero-oligomerization between endogenous HDAC4 and HDAC5 indeed occurs under physiological conditions in C2C12 myoblasts. When endogenous HDAC4 was immunoprecipitated using an HDAC4 antibody, a significant portion of endogenous HDAC5 was bound to HDAC4 (Fig. 5B, compare lanes 3 and 5). The same experiment was performed after knocking down HDAC4 with an siRNA. The exposure of the C2C12 myoblasts to HDAC4 siRNA resulted in an approximately 50% decrease in HDAC4 protein (Fig. 5A). Consistently, under these conditions, 50% less HDAC5 coimmunoprecipitated with HDAC4, confirming the specificity of this experiment (Fig. 5B, compare lanes 5 and 6).

We showed previously that HDAC4 interacts strongly and directly with CaMKII (2). Given the interaction between endogenous HDAC4 and HDAC5 and the coshuttling of the two

with expression plasmids encoding Myc-HDAC5 and FLAG-HDAC4 and various deletion mutants of FLAG-HDAC4. IP followed by IB revealed that HDAC5 binds to HDAC4 through its N-terminal extension. (C) Sequence alignment of the N-terminal regions of HDAC4, -5, -7, and -9. (D) COS cells were transfected with expression plasmids encoding Myc-MEF2C, -CtBP, -HDAC4, -HDAC5, FLAG-HDAC4 wild type (WT), and FLAG-HDAC4 mutants lacking the coiled-coil domain (Δcc) and the MEF2 binding domain (ΔMEF2). Protein-protein interactions were detected by IP followed by IB. (E) Schematic diagram of HDAC4 and deletion mutants and summary of interactions with HDAC5 as detected by coimmunoprecipitation. NLS, nuclear localization sequence; NES, nuclear export sequence.

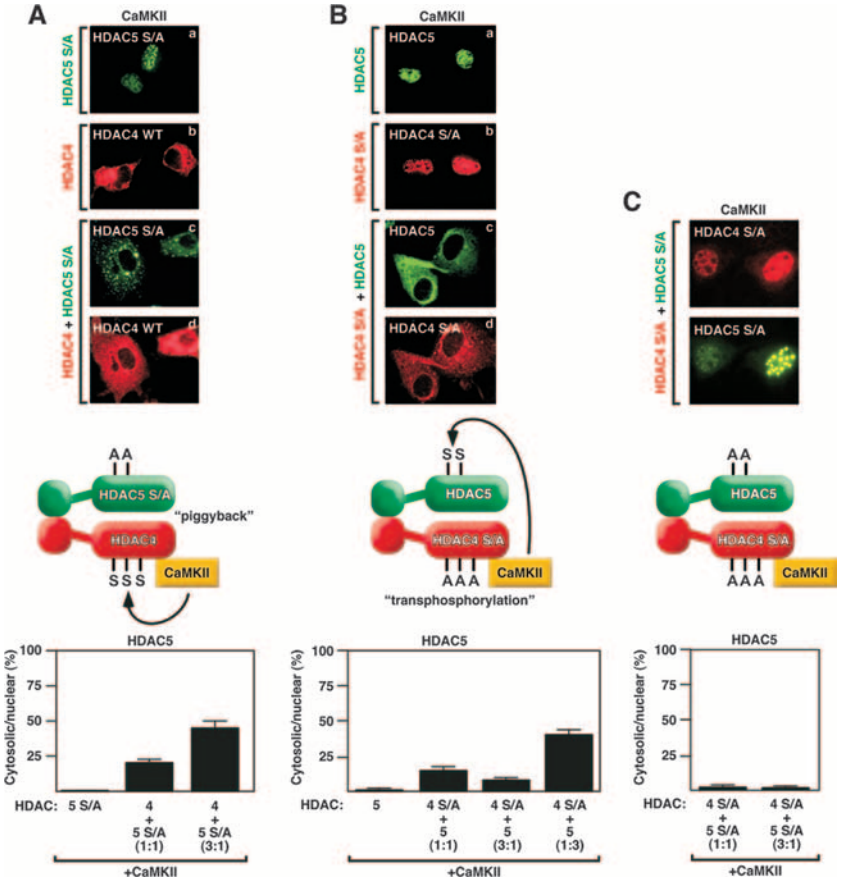

FIG. 4. "Piggyback" phosphorylation versus transphosphorylation by CaMKII. (A) Green fluorescent protein (GFP)-HDAC5 lacking the phosphorylation sites Ser-259 and -498 (HDAC5-S/A; green) and FLAG-HDAC4 (red) were expressed separately (a and b) or together (c and d) in COS cells with constitutively active CaMKII. HDAC5-S/A did not respond to CaMKII alone but was exported from the nucleus to the cytoplasm when expressed together with FLAG-HDAC4. (B) GFP-HDAC5 (green) and FLAG–HDAC4-S/A (in which serines 246, 467, and 632 were replaced with alanine) (red) were expressed separately (a and b) or together (c and d) in COS cells with constitutively active CaMKII. Neither of the HDACs alone can respond to CaMKII, but when expressed together, both were exported from the nucleus to the cytoplasm in response to CaMKII. The percentage of cells showing HDAC5 in the cytoplasm when cotransfected with HDAC4 and activated CaMKII is shown beneath each set of panels. At least 100 HDAC-expressing cells were analyzed under each condition. The data are expressed as the means ± SEMs of the results for three randomly chosen areas per slide. HDAC4 was coexpressed with HDAC5 at 1:1, 3:1, and 1:3 ratios, as indicated. (C) When GFP–HDAC5-S/A and FLAG–HDAC4-S/A were coexpressed in COS cells, neither protein was exported from the nucleus to the cytoplasm in response to CaMKII. Schematic diagrams of the interactions of HDAC4 and HDAC5 are shown in each panel.

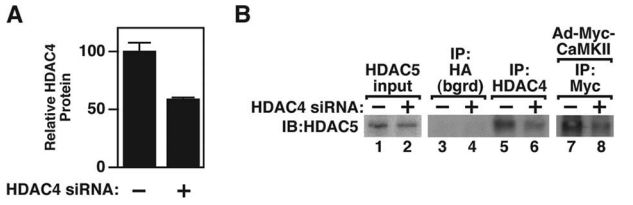

FIG. 5. Interaction of endogenous HDAC4 and HDAC5 in C2C12 myoblasts. (A) The exposure of C2C12 myoblasts to HDAC4 siRNA achieved ~50% downregulation of endogenous HDAC4 protein, as determined by immunoblot (IB) analysis and quantification by ImageJ software. (B) C2C12 myoblasts were treated with HDAC4 siRNA, as indicated. "Input" shows the level of endogenous HDAC5 (lanes 1 and 2). IP:HA designates a control IP using antihemagglutinin antibody to show background (bgrd) (lanes 3 and 4). Endogenous HDAC4 was immunoprecipitated (IP), and bound HDAC5 was determined by IB under control conditions and after HDAC4 knockdown (lanes 5 and 6). C2C12 cells were also infected with adenovirus (Ad) encoding Myc-CaMKII. Following IP with anti-Myc, the presence of HDAC5 in the CaMKII complex was determined by IB for endogenous HDAC5 (lanes 7 and 8).

proteins in the presence of CaMKII, we hypothesized that endogenous HDAC5 would be contained within a CaMKII complex with HDAC4. Indeed, we found that HDAC5 coimmunoprecipitated with CaMKII and that the association of CaMKII with HDAC5 was diminished by HDAC4 siRNA (Fig. 5B, compare lanes 7 and 8). Since CaMKII does not interact directly with HDAC5 (2), these findings are consistent with the conclusion that HDAC4 recruits CaMKII to an HDAC4-HDAC5 multiprotein complex.

DISCUSSION

The results of this study show that HDAC4 can interact with HDAC5 and can thereby confer CaMKII responsiveness to HDAC5, which does not bind CaMKII directly (2). These findings provide a potential explanation for the observations that CaMKII can promote the nuclear export of HDAC5 and that CaMKII inhibitors block the nuclear export of HDAC5 (8,

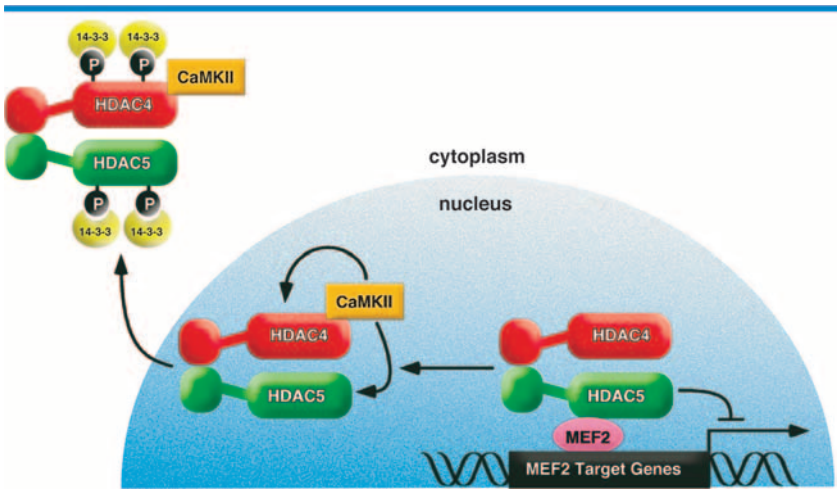

FIG. 6. Model of CaMKII- and HDAC4-dependent nuclear export of HDAC5. CaMKII interacts with a specific docking site on HDAC4, which results in the phosphorylation of HDAC4 and 14-3-3 protein-mediated nuclear export in response to signals that activate CaMKII. While CaMKII does not bind directly to HDAC5, hetero-oligomerization of HDAC5 with HDAC4 enables CaMKII to transphosphorylate HDAC5. 14-3-3 protein binding to either HDAC4 or HDAC5 is sufficient to induce nuclear export of the HDAC4-HDAC5 complex with consequent activation of transcription factors, such as MEF2, which are repressed by class IIa HDACs.

21, 39). The oligomerization of HDAC4 with HDAC5 is mediated by an N-terminal alpha-helical coiled-coil domain, which allows for the formation of a multiprotein HDAC complex in which CaMKII bound to HDAC4 can phosphorylate HDAC5 and derepress target genes that have been repressed by HDAC5 (Fig. 6). We conclude that HDAC4 functions as an integrator of CaMKII signaling and as a regulator of HDAC5 through its association with CaMKII.

Oligomerization of HDAC4. We demonstrate that HDAC4 confers CaMKII responsiveness mainly to HDAC5 and, to a lesser degree, to MITR but not to HDAC7. Accordingly, the glutamine-rich coiled-coil domain near the N terminus of HDAC4, which mediates HDAC4 oligomerization, is highly conserved between HDAC4 and HDAC5. MITR contains a similar, but less conserved, coiled-coil domain close to its N terminus, potentially explaining the partial response of MITR to CaMKII/HDAC4 signaling. In contrast, HDAC7 lacks this domain and does not undergo nuclear export with HDAC4 in response to CaMKII signaling. Recently, the same glutamine-rich region of HDAC4 was shown to adopt an alpha-helical structure that assembles as a homotetramer (17). In contrast to most coiled-coil proteins, the HDAC4 tetramer lacks regularly arranged apolar residues and an extended hydrophobic core. Instead, the protein interfaces consist of multiple hydrophobic patches interspersed with polar interaction networks, wherein clusters of glutamines engage in extensive intra- and interhelical interactions. We conclude that the degree of sequence homology of the N-terminal coiled-coil domain determines the degree of oligomerization and coshuttling between class IIa HDACs.

HDAC4 as a nodal regulator of CaMKII signaling. The requirement of HDAC4 for the recruitment of CaMKII and the phosphorylation of HDAC5 in a multiprotein complex implies that the stoichiometry between HDAC4 and HDAC5 influences transcriptional responsiveness to CaMKII signaling. Indeed, our results show that the extent of nuclear export of HDAC5, and thereby the extent of transcriptional derepression by CaMKII, depends on the ratio of HDAC4 to HDAC5, in that higher relative amounts of HDAC4 result in more efficient nuclear export of HDAC5 in response to CaMKII signaling. Conversely, reduced levels of HDAC4 diminish the responsiveness of HDAC5 to CaMKII. It is interesting, in this regard, that two studies reporting that CaMKII regulates the nuclear export of HDAC5 used hippocampal and cerebellar granule neurons (8, 21), which express high levels of endogenous HDAC4 (our unpublished data).

In addition to CaMKII, several other kinases, including CaMKI, CaMKIV, PKD, Mark, and Sik, can phosphorylate class IIa HDACs with little or no apparent preference for one HDAC isoform over another (3, 5, 18, 20, 26, 36). Thus, it is curious that phosphorylation of HDAC4 by CaMKII requires the direct docking of the kinase on the N-terminal regulatory domain but that regulation of HDAC5 requires a physical association with the HDAC4-CaMKII complex. Presumably, there are some cell types and situations in which these other kinases act on all available class IIa HDACs, and there are others in which CaMKII acts more specifically on HDAC4. In this regard, CaMKII is a multifunctional kinase that can phosphorylate multiple target proteins, many of which are involved in excitation-contraction coupling or apoptosis in addition to transcription (16). It is interesting to speculate that pathophysiological circumstances associated with high HDAC4 levels may render cells preferentially responsive to CaMKII signaling.

Implications. CaMKII regulates a variety of calcium-dependent processes that require transcriptional activation, such as neuronal and skeletal-muscle differentiation, cardiomyocyte hypertrophy, and neural-crest migration (10–12, 14, 19, 42). Signaling by CaMKII to class II HDACs has been implicated in each of these processes (2, 8, 21, 22, 33, 39). We showed recently that HDAC4 and HDAC5 are enriched in fast-fiber-dominant skeletal muscles, thereby repressing the slow-fiber

phenotype (32). In the current study, we provide direct evidence that HDAC4 and HDAC5 interact in C2C12 myoblasts, thereby rendering HDAC5 CaMKII responsive. Likewise, Ca^{2+}/calmodulin signaling is known to drive the expression of slow-skeletal-muscle genes (38), implying that HDAC4 may act as a nodal point for CaMKII-induced fiber-type switching, connecting this kinase to HDAC5 as well. It should also be noted that during skeletal-muscle atrophy, HDAC4 levels are dramatically upregulated (4), implying that CaMKII, under such conditions, may signal to HDAC5 to an even greater extent. Whether such regulation is causative or compensatory for skeletal-muscle atrophy needs to be elucidated. An additional level of HDAC4 regulation involves its translational inhibition by microRNAs. In this regard, HDAC4 was reported as a target of miR-1 in skeletal muscle and as a target of miR-140 in chondrocytes (9, 35). We speculate that these microRNAs may diminish the responsiveness of certain downstream genes to CaMKII signaling.

While we focused in the present study on the observation that HDAC4 confers CaMKII responsiveness to downstream target genes, it is also possible that other as-yet-unidentified kinases may interact specifically with other class IIa HDACs and thereby act through the type of cross-phosphorylation reaction we have described between HDAC4 and HDAC5. One can also imagine that hetero-oligomerization between class IIa HDACs has consequences for the sensitivity of downstream transcription factors that interact specifically with individual class IIa HDACs. For example, a transcription factor that interacts specifically with HDAC5 could be repressed by HDAC4 as a consequence of HDAC4's oligomerization with HDAC5 without the transcription factor interacting directly with HDAC4. Such combinatorial interactions may contribute to the diversity of transcriptional responses to specific stress stimuli and explain why different cell types with similar signaling cascades respond in different ways to the same signal.

Summary. The hetero-oligomerization of HDAC4 with HDAC5 represents a novel mechanism for coupling specific upstream signaling molecules with downstream transcriptional targets. These findings also suggest that therapeutic strategies to disrupt the interaction between HDAC4 and HDAC5 could serve to maintain HDAC5 in the nucleus, thereby sustaining the repressive state of target genes of MEF2 or of other transcription factors, such as CAMTAs. Given the involvement of HDAC4 and HDAC5 in pathological processes such as skeletal-muscle atrophy or cardiac hypertrophy (4, 12, 19, 42, 43), such an approach could potentially bypass more-global effects of CaMKII inhibition.

ACKNOWLEDGMENTS

We thank Kunhua Song, David Patrick, and Rhonda Bassel-Duby for helpful discussions; Philip J. Papst for adenovirus production; and Claudia Heft for excellent technical assistance.

This study was supported by grants from the NIH, the Donald W. Reynolds Center for Clinical Cardiovascular Research, the Muscular Dystrophy Association, and the Robert A. Welch Foundation to E.N.O. and from the Deutsche Forschungsgemeinschaft (BA 2258/2-1; Emmy Noether-Programm) to J.B.

REFERENCES

1. **Backs, J., and E. N. Olson.** 2006. Control of cardiac growth by histone acetylation/deacetylation. Circ. Res. **98:**15–24.
2. **Backs, J., K. Song, S. Bezprozvannaya, S. Chang, and E. N. Olson.** 2006. CaM kinase II selectively signals to histone deacetylase 4 during cardiomyocyte hypertrophy. J. Clin. Invest. **116:**1853–1864.
3. **Berdeaux, R., N. Goebel, L. Banaszynski, H. Takemori, T. Wandless, G. D. Shelton, and M. Montminy.** 2007. SIK1 is a class II HDAC kinase that promotes survival of skeletal myocytes. Nat. Med. **13:**597–603.
4. **Bodine, S. C., E. Latres, S. Baumhueter, V. K. Lai, L. Nunez, B. A. Clarke, W. T. Poueymirou, F. J. Panaro, E. Na, K. Dharmarajan, Z. Q. Pan, D. M. Valenzuela, T. M. DeChiara, T. N. Stitt, G. D. Yancopoulos, and D. J. Glass.** 2001. Identification of ubiquitin ligases required for skeletal muscle atrophy. Science **294:**1704–1708.
5. **Chang, S., S. Bezprozvannaya, S. Li, and E. N. Olson.** 2005. An expression screen reveals modulators of class II histone deacetylase phosphorylation. Proc. Natl. Acad. Sci. USA **102:**8120–8125.
6. **Chang, S., T. A. McKinsey, C. L. Zhang, J. A. Richardson, J. A. Hill, and E. N. Olson.** 2004. Histone deacetylases 5 and 9 govern responsiveness of the heart to a subset of stress signals and play redundant roles in heart development. Mol. Cell. Biol. **24:**8467–8476.
7. **Chang, S., B. D. Young, S. Li, X. Qi, J. A. Richardson, and E. N. Olson.** 2006. Histone deacetylase 7 maintains vascular integrity by repressing matrix metalloproteinase 10. Cell **126:**321–334.
8. **Chawla, S., P. Vanhoutte, F. J. Arnold, C. L. Huang, and H. Bading.** 2003. Neuronal activity-dependent nucleocytoplasmic shuttling of HDAC4 and HDAC5. J. Neurochem. **85:**151–159.
9. **Chen, J. F., E. M. Mandel, J. M. Thomson, Q. Wu, T. E. Callis, S. M. Hammond, F. L. Conlon, and D. Z. Wang.** 2006. The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. Nat. Genet. **38:**228–233.
10. **Chin, E. R.** 2005. Role of Ca2+/calmodulin-dependent kinases in skeletal muscle plasticity. J. Appl. Physiol. **99:**414–423.
11. **Colbran, R. J., and A. M. Brown.** 2004. Calcium/calmodulin-dependent protein kinase II and synaptic plasticity. Curr. Opin. Neurobiol. **14:**318–327.
12. **Colomer, J. M., L. Mao, H. A. Rockman, and A. R. Means.** 2003. Pressure overload selectively up-regulates Ca2+/calmodulin-dependent protein kinase II in vivo. Mol. Endocrinol. **17:**183–192.
13. **Fischle, W., V. Kiermer, F. Dequiedt, and E. Verdin.** 2001. The emerging role of class II histone deacetylases. Biochem. Cell Biol. **79:**337–348.
14. **Garriock, R. J., and P. A. Krieg.** 2007. Wnt11-R signaling regulates a calcium sensitive EMT event essential for dorsal fin development of Xenopus. Dev. Biol. **304:**127–140.
15. **Grozinger, C. M., and S. L. Schreiber.** 2000. Regulation of histone deacetylase 4 and 5 and transcriptional activity by 14-3-3-dependent cellular localization. Proc. Natl. Acad. Sci. USA **97:**7835–7840.
16. **Grueter, C. E., R. J. Colbran, and M. E. Anderson.** 2007. CaMKII, an emerging molecular driver for calcium homeostasis, arrhythmias, and cardiac dysfunction. J. Mol. Med. **85:**5–14.
17. **Guo, L., A. Han, D. L. Bates, J. Cao, and L. Chen.** 2007. Crystal structure of a conserved N-terminal domain of histone deacetylase 4 reveals functional insights into glutamine-rich domains. Proc. Natl. Acad. Sci. USA **104:**4297–4302.
18. **Harrison, B. C., M. S. Kim, E. van Rooij, C. F. Plato, P. J. Papst, R. B. Vega, J. A. McAnally, J. A. Richardson, R. Bassel-Duby, E. N. Olson, and T. A. McKinsey.** 2006. Regulation of cardiac stress signaling by protein kinase d1. Mol. Cell. Biol. **26:**3875–3888.
19. **Hoch, B., R. Meyer, R. Hetzer, E. G. Krause, and P. Karczewski.** 1999. Identification and expression of delta-isoforms of the multifunctional Ca2+/calmodulin-dependent protein kinase in failing and nonfailing human myocardium. Circ. Res. **84:**713–721.
20. **Li, X., S. Song, Y. Liu, S. H. Ko, and H. Y. Kao.** 2004. Phosphorylation of the histone deacetylase 7 modulates its stability and association with 14-3-3 proteins. J. Biol. Chem. **279:**34201–34208.
21. **Linseman, D. A., C. M. Bartley, S. S. Le, T. A. Laessig, R. J. Bouchard, M. K. Meintzer, M. Li, and K. A. Heidenreich.** 2003. Inactivation of the myocyte enhancer factor-2 repressor histone deacetylase-5 by endogenous Ca(2+) /calmodulin-dependent kinase II promotes depolarization-mediated cerebellar granule neuron survival. J. Biol. Chem. **278:**41472–41481.
22. **Liu, Y., W. R. Randall, and M. F. Schneider.** 2005. Activity-dependent and -independent nuclear fluxes of HDAC4 mediated by different kinases in adult skeletal muscle. J. Cell Biol. **168:**887–897.
23. **Lu, J., T. A. McKinsey, R. L. Nicol, and E. N. Olson.** 2000. Signal-dependent activation of the MEF2 transcription factor by dissociation from histone deacetylases. Proc. Natl. Acad. Sci. USA **97:**4070–4075.
24. **Martin, M., R. Kettmann, and F. Dequiedt.** 2007. Class IIa histone deacetylases: regulating the regulators. Oncogene **26:**5450–5467.
25. **McKinsey, T. A.** 2007. Derepression of pathological cardiac genes by members of the CaM kinase superfamily. Cardiovasc. Res. **73:**667–677.
26. **McKinsey, T. A., C. L. Zhang, J. Lu, and E. N. Olson.** 2000. Signal-dependent nuclear export of a histone deacetylase regulates muscle differentiation. Nature **408:**106–111.
27. **McKinsey, T. A., C. L. Zhang, and E. N. Olson.** 2000. Activation of the myocyte enhancer factor-2 transcription factor by calcium/calmodulin-dependent protein kinase-stimulated binding of 14-3-3 to histone deacetylase 5. Proc. Natl. Acad. Sci. USA **97:**14400–14405.

28. **McKinsey, T. A., C. L. Zhang, and E. N. Olson.** 2001. Identification of a signal-responsive nuclear export sequence in class II histone deacetylases. Mol. Cell. Biol. **21:**6312–6321.
29. **Méjat, A., F. Ramond, R. Bassel-Duby, S. Khochbin, E. N. Olson, and L. Schaeffer.** 2005. Histone deacetylase 9 couples neuronal activity to muscle chromatin acetylation and gene expression. Nat. Neurosci. **8:**313–321.
30. **Miska, E. A., C. Karlsson, E. Langley, S. J. Nielsen, J. Pines, and T. Kouzarides.** 1999. HDAC4 deacetylase associates with and represses the MEF2 transcription factor. EMBO J. **18:**5099–5107.
31. **Parra, M., H. Kasler, T. A. McKinsey, E. N. Olson, and E. Verdin.** 2005. Protein kinase D1 phosphorylates HDAC7 and induces its nuclear export after T-cell receptor activation. J. Biol. Chem. **280:**13762–13770.
32. **Potthoff, M. J., H. Wu, M. A. Arnold, J. M. Shelton, J. Backs, J. McAnally, J. A. Richardson, R. Bassel-Duby, and E. N. Olson.** 2007. Histone deacetylase degradation and MEF2 activation promote the formation of slow-twitch myofibers. J. Clin. Invest. **117:**2459–2467.
33. **Shen, T., Y. Liu, W. R. Randall, and M. F. Schneider.** 2006. Parallel mechanisms for resting nucleo-cytoplasmic shuttling and activity dependent translocation provide dual control of transcriptional regulators HDAC and NFAT in skeletal muscle fiber type plasticity. J. Muscle Res. Cell. Motil. **27:**405–411.
34. **Song, K., J. Backs, J. McAnally, X. Qi, R. D. Gerard, J. A. Richardson, J. A. Hill, R. Bassel-Duby, and E. N. Olson.** 2006. The transcriptional coactivator CAMTA2 stimulates cardiac growth by opposing class II histone deacetylases. Cell **125:**453–466.
35. **Tuddenham, L., G. Wheeler, S. Ntounia-Fousara, J. Waters, M. K. Hajihosseini, I. Clark, and T. Dalmay.** 2006. The cartilage specific microRNA-140 targets histone deacetylase 4 in mouse cells. FEBS Lett. **580:**4214–4217.
36. **Vega, R. B., B. C. Harrison, E. Meadows, C. R. Roberts, P. J. Papst, E. N. Olson, and T. A. McKinsey.** 2004. Protein kinases C and D mediate agonist-dependent cardiac hypertrophy through nuclear export of histone deacetylase 5. Mol. Cell. Biol. **24:**8374–8385.
37. **Vega, R. B., K. Matsuda, J. Oh, A. C. Barbosa, X. Yang, E. Meadows, J. McAnally, C. Pomajzl, J. M. Shelton, J. A. Richardson, G. Karsenty, and E. N. Olson.** 2004. Histone deacetylase 4 controls chondrocyte hypertrophy during skeletogenesis. Cell **119:**555–566.
38. **Wu, H., S. B. Kanatous, F. A. Thurmond, T. Gallardo, E. Isotani, R. Bassel-Duby, and R. S. Williams.** 2002. Regulation of mitochondrial biogenesis in skeletal muscle by CaMK. Science **296:**349–352.
39. **Wu, X., T. Zhang, J. Bossuyt, X. Li, T. A. McKinsey, J. R. Dedman, E. N. Olson, J. Chen, J. H. Brown, and D. M. Bers.** 2006. Local InsP3-dependent perinuclear Ca2+ signaling in cardiac myocyte excitation-transcription coupling. J. Clin. Invest. **116:**675–682.
40. **Zhang, C. L., T. A. McKinsey, S. Chang, C. L. Antos, J. A. Hill, and E. N. Olson.** 2002. Class II histone deacetylases act as signal-responsive repressors of cardiac hypertrophy. Cell **110:**479–488.
41. **Zhang, C. L., T. A. McKinsey, J. R. Lu, and E. N. Olson.** 2001. Association of COOH-terminal-binding protein (CtBP) and MEF2-interacting transcription repressor (MITR) contributes to transcriptional repression of the MEF2 transcription factor. J. Biol. Chem. **276:**35–39.
42. **Zhang, R., M. S. Khoo, Y. Wu, Y. Yang, C. E. Grueter, G. Ni, E. E. Price, W. Thiel, S. Guatimosim, L. S. Song, E. C. Madu, A. N. Shah, T. A. Vishnivetskaya, J. B. Atkinson, V. V. Gurevich, G. Salama, W. J. Lederer, R. J. Colbran, and M. E. Anderson.** 2005. Calmodulin kinase II inhibition protects against structural heart disease. Nat. Med. **11:**409–417.
43. **Zhang, T., and J. H. Brown.** 2004. Role of Ca2+/calmodulin-dependent protein kinase II in cardiac hypertrophy and heart failure. Cardiovasc. Res. **63:**476–486.
44. **Zhang, T., M. Kohlhaas, J. Backs, S. Mishra, W. Phillips, N. Dybkova, S. Chang, H. Ling, D. M. Bers, L. S. Maier, E. N. Olson, and J. H. Brown.** 2007. CaMKIIdelta isoforms differentially affect calcium handling but similarly regulate HDAC/MEF2 transcriptional responses. J. Biol. Chem. **282:**35078–35087.