3610–3617 *Nucleic Acids Research, 2016, Vol. 44, No. 8* *Published online 23 December 2015*
doi: 10.1093/nar/gkv1496

Evidence for a non-canonical role of HDAC5 in regulation of the cardiac Ncx1 and Bnp genes

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Received April 8, 2015; Revised November 13, 2015; Accepted December 10, 2015

ABSTRACT

Class IIa histone deacetylases (HDACs) are very important for tissue specific gene regulation in development and pathology. Because class IIa HDAC catalytic activity is low, their exact molecular roles have not been fully elucidated. Studies have suggested that class IIa HDACs may serve as a scaffold to recruit the catalytically active class I HDAC complexes to their substrate. Here we directly address whether the class IIa HDAC, HDAC5 may function as a scaffold to recruit co-repressor complexes to promoters. We examined two well-characterized cardiac promoters, the sodium calcium exchanger (Ncx1) and the brain natriuretic peptide (Bnp) whose hypertrophic upregulation is mediated by both class I and IIa HDACs. Selective inhibition of class IIa HDACs did not prevent adrenergic stimulated Ncx1 upregulation, however HDAC5 knockout prevented pressure overload induced Ncx1 upregulation. Using the HDAC5$^{(-/-)}$ mouse we show that HDAC5 is required for the interaction of the HDAC1/2/Sin3a co-repressor complexes with the Nkx2.5 and YY1 transcription factors and critical for recruitment of the HDAC1/Sin3a co-repressor complex to either the Ncx1 or Bnp promoter. Our novel findings support a non-canonical role of class IIa HDACs in the scaffolding of transcriptional regulatory complexes, which may be relevant for therapeutic intervention for pathologies.

INTRODUCTION

Humans express 18 types of histone deacetylases (HDACs) that differ in structure, subcellular location and mechanism(s) of regulating other molecules, which is primarily through the removal of acetyl groups. Zinc dependent HDACs are grouped into three classes, I, II and IV. Class I HDACs (HDAC1–3, HDAC8) have N-terminal catalytic deacetylase domains, a nuclear localization signal (HDAC1, HDAC3 and HDAC8) or lack a nuclear export motif (HDAC1 and HDAC2). The majority of class I HDACs are localized in the nucleus. But with both a nuclear localization signal and two different nuclear export sequences, HDAC3 has been found in both the cytoplasm and the nucleus (1–3). In addition, HDAC1 has been detected in the cytosol of damaged axons in brains of human patients with multiple sclerosis (4,5). HDACs 1–3 have high catalytic activity for deacetylation of Nϵ-acetyllysine residues. Class I HDACs do not function autonomously but are the catalytic core of large co-repressor complexes. HDAC1 and HDAC2 are found in the CoREST, Sin3 and NuRD complexes (6). HDAC3 is a part of a distinct complex that contains either nuclear receptor co-repressor (NCOR) or its homolog silencing mediator of retinoic and thyroid receptors (SMRT) (7). Class II HDACs are divided into class IIa (HDAC4, -5, -7, -9) and class IIb (HDAC6, -10). The class IIb HDACs are found in the cytosol and have two catalytic domains. Both domains show high catalytic activity in HDAC6 while only one domain is active in HDAC10. Contrarily, class IIa HDACs are known for having multiple conserved domains in their N-terminal half that facilitate the direct interaction of HDAC4, -5 and -9 with several transcription factors including MEF2, SRF and RFXANK (8,9). Class IIa HDACs, are directly involved in tissue-specific gene regulation and play a role in development and pathology. Inhibition of class IIa HDACs has been proposed for the treatment of cardiac hypertrophy, myocardial infarction, colitis and Huntington's disease (9–17). The subcellular location of class IIa HDACs is regulated by multiple kinases. Phosphorylation of class IIa HDACs initiates association with 14–3–3 proteins, which shuttle and retain the HDACs in the cytosol. Thus, phosphorylation can regulate class IIa HDACs ability to access their nuclear transcription binding partners. The mechanisms by which class IIa HDACs regulate gene expression are poorly understood. The catalytic

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activity of class IIa HDACs is much lower than class I and IIb HDACs due to the replacement of a conserved catalytic Tyr residue with a His in all vertebrate phylum and no natural substrate has been conclusively identified (18). Several possibilities have been raised to account for the poor activity. Class IIa HDACs could hydrolyze a still undiscovered substrate, require allosteric stimulation by covalent modification or interact with another factor(s) that would enhance its activity. Alternatively, because class IIa HDACs are associated with many cardiotrophic gene promoters others and we hypothesize that they may serve as a scaffold to recruit the catalytically active class I HDAC complexes to their substrate.

In this study we present direct evidence for the non-catalytic role of HDAC5. We demonstrate that in the absence of HDAC5, Ncx1 is not upregulated in cardiac hypertrophy, whereas pharmacological inhibition of HDAC5 deacetylase activity has no effect on expression. Furthermore, the amount of recruitment of HDAC1/Sin3a repressor complex to the Ncx1 and Bnp promoters is HDAC5-dependent.

MATERIALS AND METHODS

Adult cardiac myocyte cell culture

Adult feline cardiac myocytes were isolated via a hanging heart preparation using enzymatic digestion and cultured by the protocols approved by the Institutional Animal Care and Use Committee as described previously (IACUC) (19). Summarized here, freshly isolated cardiac myocytes were plated on laminin coated tissue culture plates at an initial plating density of 7.5 × 104 cells/ml. The cardiac myocytes were infected with pAd-Track adenovirus containing a full-length −1831 base pair wild-type Ncx1 promoter luciferase reporter gene construct (1831Ncx1) and cytomegalovirus driven green fluorescent protein (GFP) (20,21) at an approximate multiplicity of infection of 1.5 resulting in the infection and gene transfer to be greater than 85% of the plated cells based on GFP. Media was changed 12 h after infection. Cells were pretreated with either broad HDAC inhibitor, suberoyl anilide hydroxamic acid (SAHA), commercially known as Vorinostat, Class I HDAC inhibitor, MS-275 (commercially known as Entinostat), (1 μM) or Class IIa HDAC inhibitor (diphenyl acetic hydroxamic acid) (dPAHA) (10 μM) for 30 min. Cells were then treated with or without β1- and β2-adrenergic agonist 0.1 μM isoproterenol (Iso), or the α-adrenergic agonist phenylephrine (PE) (10 μM). Cells were lysed in reporter buffer 24 h after treatment and luciferase activity was determined relative to GFP levels.

Luciferase reporter assay

Promoter activation was determined using luciferase bioluminescence assay as previously described (21,22). Briefly, isolated adult cardiomyocytes were incubated 50 μl of luciferin mixture (Promega, Madison, WI). Light emission was measured using an Auto Lumat LB 953 luminometer. To measure GFP fluorescence, 50 μl of crude lysate was added to 100 μl of PBS and the sample read in a fluorometer with excitation of 488 nm and emission of 510 nm. Luciferase readings were then normalized by GFP expression and noted as Relative Light Units.

CBL5 wild-type and HDAC5 knockout mice

The HDAC5$^{-/-}$ mouse was re-derived in a C57BL/6 background from Dr. Eric Olson's original HDAC5$^{-/-}$ C57BL/6 mouse (23), which was a generous gift from Dr. Olson. Subsequent genotyping was performed by PCR with the following primers:

HDAC5 sense, 5′-CAAGGCCTTGTGCATGCTGGGC TGG-3′;
HDAC5 antisense,5′-CTGCTCCCGTAGCGCAGGGT CCATG-3′;
and LacZ, antisense 5′-GCCCGTTTGAGGGGACGA CGACAGTATCG-3′.

All procedures performed were approved by the Institution Animal Care and Use Committee of the Medical University of South Carolina in accordance with National Institutes of Health guidelines.

Transverse aortic constriction

Acute left ventricular (LV) pressure overload was created by microsurgical transverse aortic constriction (TAC), as described previously (24). Male C57 mice were between 10 and 12 weeks of age at the time of TAC. Mice were injected (intraperitoneal) with Entinostat (MS-275, 10 mg/kg/day), a class I selective inhibitor with a binding preference for HDACs 1, 2 and 3 or vehicle immediately following surgery. Hearts were removed from deeply anesthetized animals 72 h post-TAC and tissue processed for Western analysis. Each experimental cohort included n = 4; experiments were repeated thrice. All procedures performed were approved by the IACUC of the Medical University of South Carolina in accordance with National Institutes of Health guidelines.

Western blot analysis

25 mg of excised left ventricle from each experimental group was homogenized, lysed using Nonidet-P-40 or Radio Immuno Precipitation Assay Buffer that were supplemented with protease and phosphatase inhibitors on ice for 2 h. Protein concentrations of the lysates were determined by the Bio-Rad protein assay. Protein from the cell lysates was resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and then transferred to a Polyvinylidene fluoride membrane. The membrane was then incubated overnight with antibodies against NCX1(P-13) or GAPDH(FL-334), Santa Cruz Biotechnology overnight at 4°C. Membranes were washed and then incubated with appropriate horse-radish peroxidase conjugated secondary antibodies for 1–2 h. The proteins were visualized by Enhanced Chemiluminescence and imaged on a GE Image Quant LAS4000 imager or HyBlot CL film, Metuchen, NJ. Western blot analyses were repeated a minimum of three times to assess reproducibility and consistency.

Co-immunoprecipitation

Interactions between specific molecules were determined via co-immunoprecipitation (Co-IP) assays as previously described (21,25). Briefly, 250 μg of protein from freshly harvested wild-type or HDAC5$^{-/-}$ was precleared for 30 min and incubated overnight with 5–10 μg of HDAC1(C-19 or H-51), HDAC2(H-54), HDAC5(H-74 or G-18), Nkx2.5(H-114), YY1(C-20), mSin3a(AK-11), N-CoR(C-20), CoREST (H-65 or C-20) or normal Rabbit or Goat IgG(sc-2028) -isotype control immuno-precipitating antibody, Santa Cruz Biotechnology, with constant end-to end rotation. A/G agarose coupled beads were added to each sample and incubated for 1 h, again with end-to end rotation. Beads were harvested and sequentially washed to remove non-specific proteins and residual salts. Protein–protein complexes were eluted with SDS and resolved using SDS-PAGE. Western blot analysis using the antibodies against proteins of interest from Santa Cruz biotechnology as described above was used to assess protein abundance. Co-IP assays were done in duplicate and repeated three times.

Sequential double Co-IP

In order to assess putative protein–protein interactions in complexes containing more than two molecules, we performed two sequential Co-IP analyses from the same pool of lysate as described above in the Co-IP method above with the following modifications: In the primary Co-IP (1°), 500 μg of lysates were incubated with 5 μg of Nkx2.5 antibody overnight at 4°C. Then A/G agarose beads were added to immuno-precipitates for 1 h, after which beads were washed and protein complexes eluted. The eluted proteins were then incubated a second time (2°) with either HDAC1(C-19)) or HDAC5(G-18). After the 2° antibody incubation, protein–protein complexes were immuno-precipitated using agarose A/G beads, washed and eluted. Western blot analyses proceeded as described above from this point.

Chromatin immunoprecipitation

Wild-type, male mice at 10–12 weeks of age were either subjected to a sham operation (control) or TAC to induce pressure overload hypertrophy. After 72 h, mice were euthanized and hearts were harvested, homogenized. 25 mg of homogenates from each group were supplemented with paraformaldehyde in order to crosslink protein-DNA complexes. Samples were gently swirled every 5 min during this incubation. Cross-linking activity was quenched with the addition of 1.25 M glycine for 5 min. The homogenate was then suspended in lysis buffer, and incubated on ice for 1 h with gentle vortexing every 10 min. The cell lysate was sonicated for 8 cycles (10 s 'on' 10 s 'off'), and the cell debris was spun down via centrifugation. The supernatant was diluted and equal amounts of lysate were then precleared with A/G coupled agarose beads for 20 min. After which, lysates were then incubated with 5 μg of immunoprecipitation antibody HDAC1(H-51X chip-grade), HDAC2(H-54), HDAC3(H-99 chip-grade), HDAC4(H-92X chip-grade) HDAC5(H-74), HDAC9(H-45), Nkx2.5(H-114), YY1(C-20), mSin3a(AK-11X chip-grade) or Normal Rabbit IgG(sc-2027) from Santa Cruz, Biotechnology overnight 4°C with constant end-to-end rotation. Complexes were immuno-precipitated with agarose A/G coupled beads, sequentially washed and eluted from beads. Samples were reverse cross-linked and chromatin was then isolated from each input sample; this sample was quantified for normalization of the amount of template used for polymerase chain reaction (PCR). The mouse *Ncx1* proximal promoter was PCR amplified from the immunoprecipitated and non-immunoprecipitated (input control) chromatin.

Ncx1 (proximal)
Sense: 5′-CGACTGCCAATCTAAAGCATATTT-3′
Anti: 5′-AGCCTGTCCTTAGTGTCTCT-3′
NCBI Reference Sequence: NM_001112798.1 >gi\163914366\ref\NM_001112798.1
Bop (proximal)
Sense: 5′-CCCCACACTCGCCACAGGCTG- 3′
Anti: 5′-GATTTATCAGCTGCATTCCTGCCGGCTG-3′
NCBI Reference Sequence:NC_000070.6 >gi\372099106:147985789–147987205

Primers that amplified regions distal to the proximal promoter were also used as negative PCR controls. Experiments were repeated twice and performed on an n = 4 mice per experimental group.

Statistical analysis

Analysis of Variance (ANOVA) or Student's T-test was used to determine statistical significance. We used Graphpad Prism®, Image J, or Excel, to generate graphs. *P*-values < 0.05 were considered significant. Values for all measurements were expressed as the mean ± standard deviation.

RESULTS

Class I HDAC but not class IIa HDAC deacetylase activity regulates Ncx1 expression

To test the hypothesis that class IIa HDACs serve as scaffolds, we examined two well-characterized cardiac promoters, the sodium calcium exchanger (Ncx1) and the brain natriuretic peptide (Bnp) whose hypertrophic upregulation is inhibited by HDAC inhibitors (25,26). Importantly, key transcription factors regulating Ncx1 and Bnp expression, Nkx2.5 and YY1, respectively, interact with HDAC5 and these interactions are implicated to play a critical role in the regulation of these genes during both normal and pathological conditions. In addition to HDAC5, class I HDACs HDAC1 and HDAC2 are also recruited to the Ncx1 proximal promoter through interactions with the Nkx2.5 transcription factor. Acetylation of Nkx2.5 appears to be required for the recruitment of the HDAC1/2/5 complex to the Ncx1 promoter (25,27). In addition, the transcription factor YY1 also interacts with class I HDACs and is regulated by acetylation (28).

Since both class I and class IIa HDACs are recruited to the proximal promoter of Ncx1, we assessed whether the catalytic activity of both class I and IIa HDACs is rele-

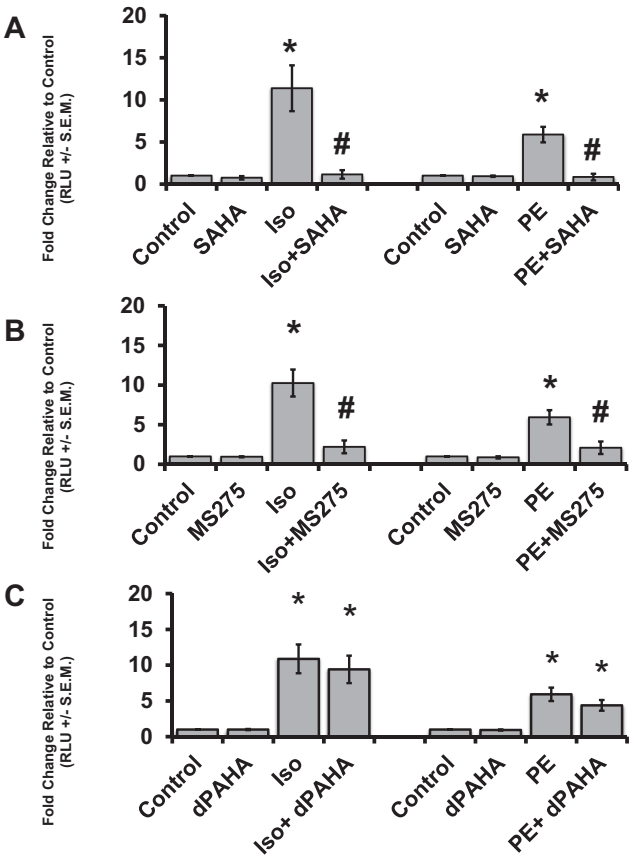

Figure 1. Inhibition of class I HDAC activity represses adrenergic stimulated Ncx1 promoter activation. Isolated adult cardiomyocytes were infected with adenoviruses containing 1831Ncx1 promoter luciferase reporter construct (multiplicity of infection (MOI) 1.5) and treated with either 1 μM isoproterenol (Iso) or 10 μM phenylephrine (PE) following pretreatment with either broad HDAC inhibitor, (**A**) SAHA Class I selective HDAC inhibitor or (**B**) MS275 (1 μM). (**C**) Isolated adult cardiomyocytes were infected with adenoviruses containing 1831Ncx1 promoter luciferase reporter construct (MOI 1.5) and treated with either 1 μM isoproterenol (Iso) or 10 μM phenylephrine (PE) following pretreatment with Class IIa HDAC inhibitor, dPAHA (10 μM). Cells for (**A**, **B** and **C**) were lysed in reporter buffer 24 h after infection and luciferase activity was determined relative to GFP levels. MS275 significantly inhibited Ncx1 promoter upregulation. PAHA had no effect on Ncx1 promoter activity. $^{*}P < 0.05$ respective to control, $\#P < 0.05$ respective to ± PE or ISO treatment (Student's *t*-test; $n = 4$).

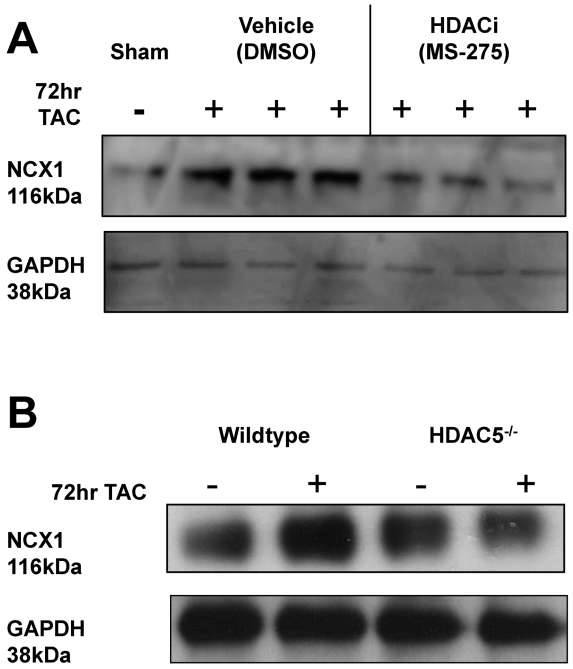

Figure 2. Pressure overload induced NCX1 upregulation is prevented by Class I HDAC inhibition and Class IIa HDAC5 deletion. (**A**) Age and sex-matched wild-type mice were subjected to TAC induced pressure overloaded for 72 h. At the time of surgery mice were injected (intraperitoneal, IP) with the Class I selective HDAC inhibitor, MS275 (10 mg/kg/day) and IP injected daily every 24 h. After 72 h, hearts were harvested, homogenized and lysed. Protein in lysates was quantified and 30 μg of each experimental group were then resolved on SDS-PAGE gel and transferred to a membrane for overnight immuno-blotting with anti-NCX1 antibody and GAPDH (loading control). (**B**) Age and sex-matched wild-type and HDAC5$^{-/-}$ mice were subjected to TAC induced pressure overload for 72 h. After 72 h, hearts were harvested and whole heart tissue homogenates were subjected to Western blotting as above with anti-NCX1 antibody and GAPDH (loading control). Western blots shown represent assays that were done in duplicate and repeated three times.

vant and/or required in hypertrophic-stimulated transcriptional regulation. We found that the class I and IIb inhibitor SAHA and the class I HDAC inhibitor MS-275 functionally abrogates α/β agonist mediated Ncx1 upregulation in a dose dependent manner (Figure 1A and B). The class IIa selective drug dPAHA (29) inhibits recombinant human HDAC5 activity with an IC50 of 280 nM (Supplementary Figure S1). But treatment of adult cardiomyocytes with dPAHA, fails to suppress α and β agonist stimulated Ncx1 transcriptional activation (Figure 1C).

This observation suggests that although both class I and class IIa HDACs are recruited to the proximal promoter of Ncx1, only the class I HDACs (HDAC1 and HDAC2) and not class IIa (HDAC5) are acting enzymatically to promote upregulation. MS-275 treatment of mice subjected to pressure overload by TAC confirms that class I HDACs impact Ncx1 upregulation in hypertrophy (Figure 2A). Therefore, we assessed if the loss of HDAC5 affects Ncx1 expression during hypertrophy. Notably, there is no pressure-overload induced upregulation of Ncx1 expression in the HDAC5 knockout mice (Figure 2B). These data suggest the possibility of a non-canonical and/or non-catalytic role for HDAC5 in the regulation of cardiotrophic genes.

The transcription factor, Nkx2.5 interacts with the HDAC1/2/Sin3a-corepressor complex

In order to characterize the possible non-catalytic role of HDAC5 in the regulation of Ncx1, we determined if HDAC interacted with class I HDACs and co-repressor complexes. Class IIa HDACs have been shown to interact with HDAC3 and with the HDAC3 associated co-repressor complex N-CoR/SMRT (30), but there are no reports of the class IIa HDACs existing in complex with HDAC1 or HDAC2 or their co-repressor complexes. Figure 3A shows that HDAC5 is co-immunoprecipitated with HDAC1, HDAC2 and the Sin3a co-repressor complex. Figure 3B shows that in addition to Sin3a, HDAC5 is co-immunoprecipitated with the

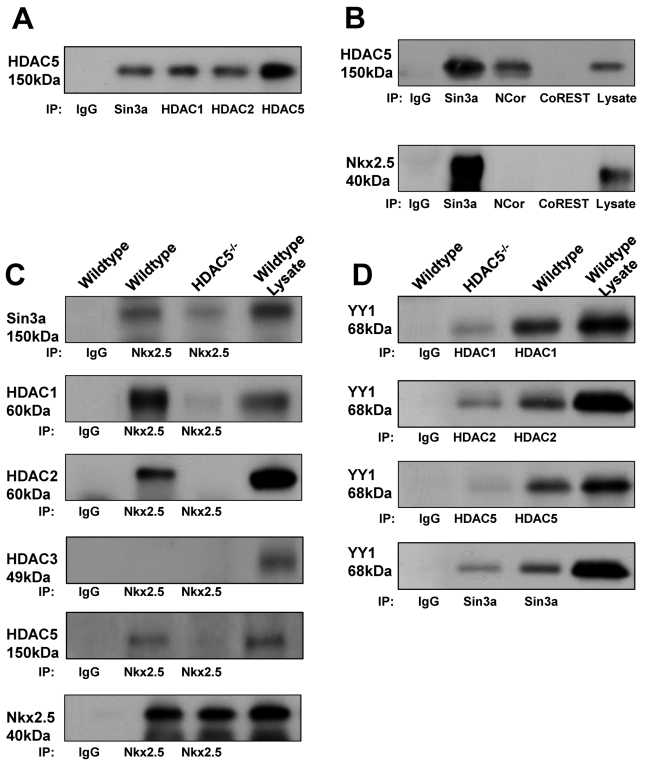

Figure 3. Nkx2.5 fails to co-immunoprecipitate HDAC2 and Sin3a in HDAC5-KO. 250 μg of protein from freshly harvested tissue from wild-type or HDAC5-knockout hearts was precleared and incubated overnight with 5–10 μg of HDAC5 (**A**, **C**), HDAC1, 2 (**A**, **D**), Nkx2.5 (**C**), YYI (**C**), Sin3a (**B**, **D**) NCor, CoREST (**B**) or IgG (**A-D**)-isotype control Ab, Santa Cruz Biotechnology, with constant end-to end rotation. A/G agarose beads were added to each sample and incubated for 1 h, again with end-to end rotation. Beads were harvested and sequentially washed to remove non-specific proteins. Protein complexes were eluted with SDS and resolved on SDS-page gel for western blot analysis using the antibodies against proteins of interest. Western blots shown represent co-immunoprecipitation assays that were done in duplicate and repeated three times.

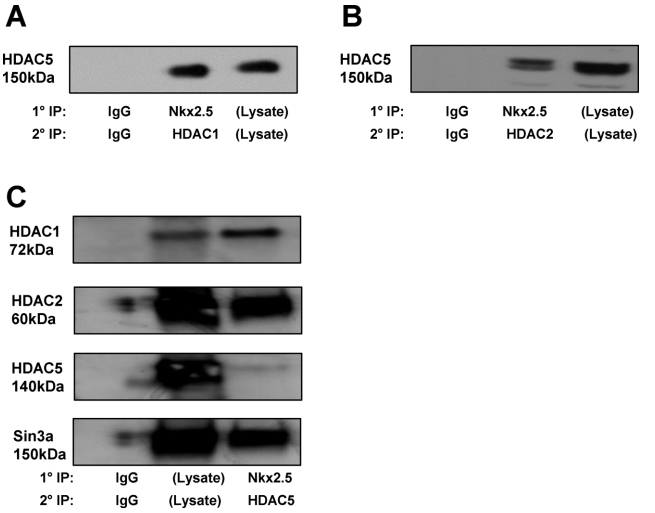

Figure 4. Sequential co-immunoprecipitation shows HDAC1/2-Sin3a co-repressor interactions with Nkx2.5. Interactions between specific molecules were determined via co-immunoprecipitation assays with 250 μg of precleared lysate from 10 to 12 weeks old male mice whole hearts. In the primary co-immunoprecipitation (1°), lysates were incubated with 5 μg of Nkx2.5 antibody overnight. Then A/G agarose beads were added to immuno-precipitates for 1 h, after which beads were washed and protein complexes eluted. The eluted proteins were then incubated a second time (2°) with either HDAC1 (**A**, **B**) or HDAC5 (**C**) antibody overnight. The following day, A/G agarose beads were added to immuno-precipitates for 1 h. Beads were harvested and sequentially washed and protein complexes were eluted with SDS then resolved on SDS-page gel for western blot analysis using the antibodies against proteins of interest. Western blots shown represent co-immunoprecipitation assays that were done in duplicate and repeated three times.

N-CoR but not the CoREST co-repressor complex. Nkx2.5 is associated with Sin3a but not N-CoR; therefore, we focused on HDAC5's interaction with the Sin3a complex.

Loss of HDAC5 reduces Sin3a-HDAC1/2 interactions

We asked whether HDAC5 might have a non-catalytic and non-canonical role as a scaffolding molecule, by examining how the interactions of Nkx2.5 to the HDAC1/2/5/Sin3a complex are impacted by the absence of HDAC5 (HDAC5$^{-/-}$). Co-immunoprecipitation shows that Nkx2.5 interacted with the HDAC1/2/5/Sin3a complex but not HDAC3 in wild-type mouse hearts. There was a profound reduction of HDAC1/2 and Sin3a interaction with Nkx2.5 in the HDAC5$^{-/-}$ mouse heart (Figure 3C). Previous work reported that HDAC5 and the transcription factor YY1 mediated BNP suppression in cardiac hypertrophy (31), therefore we examined whether HDAC5 might also serve a similar role. HDAC1, HDAC2, HDAC5 and Sin3a were all present in the YY1 co-immunoprecipitation (Figure 3D). Importantly, we observed a reduction of HDAC1/2/Sin3a interactions with YY1 when co-immunoprecipitation was performed using HDAC5$^{-/-}$ ventricle tissue lysates. These data suggest the association of HDAC1, HDAC2 and Sin3a with Nkx2.5 is compromised and functionally impacted when the class IIa HDAC5, is absent.

The co-immunoprecipitation experiments carried out in Figure 3C demonstrates that Nkx2.5 associates with HDAC1, HDAC2 and Sin3a but does not prove that these factors are all in the same complex. In order to test whether these factors were associated in the same complex we carried out sequential co-immunoprecipitation. Tissue lysates from wild-type control hearts were precipitated using anti-Nkx2.5 followed by a second immunoprecipitation with anti-HDAC1. Western analysis showed that HDAC5 was present in the second co-immunoprecipitation (Figure 4A). Next lysates were immunoprecipitated with anti-Nkx2.5 followed by a second immunoprecipitation with HDAC2 followed by Western analysis for HDAC5 (Figure 4B). Last, lysates were immunoprecipitated with anti-Nkx2.5 a second immunoprecipitation with anti-HDAC5 followed by Western analysis for HDAC1, HDAC2 HDAC5 and Sin3a. Together, these sequential co-immunoprecipitations confirmed Nkx2.5/HDAC5/HDAC1, Nkx2.5/HDAC5/HDAC2 and Nkx2.5/HDAC5/Sin3a are in complex.

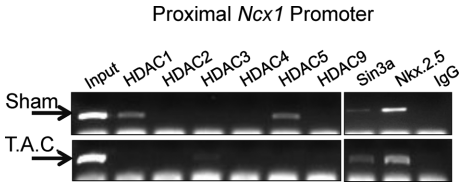

Figure 5. Pressure overload alters the recruitment of co-repressor complex molecules on the proximal promoter of *Ncx1*. Wild-type, male mice at 10–12 weeks of age were either subjected to a sham operation (control) or TAC to induce pressure overload hypertrophy. After 72 h, mice were euthanized and hearts were harvested, homogenized. Homogenates of each group were supplemented with paraformaldehyde in order to crosslink protein-DNA complexes. Samples were gently swirled every 5 min during this incubation. Cross-linking activity was quenched with the addition of 1.25 M glycine for 5 min. The homogenate was then suspended in lysis buffer, and incubated on ice for 1 h with gentle vortexing every 10 min. The cell lysate was sonicated for 8 cycles (10 s 'on' 10 s 'off'), and the cell debris was spun down. The supernatant was diluted and used equal amounts of lysate was then precleared with A/G agarose beads and supplemented with 5 μg of immunoprecipitation antibody [HDAC1, 2, 3, 4, 5, Sin3a or Nkx2.5] overnight 4°C with constant end-to-end rotation. Complexes were immune-precipitated with agarose A/G beads then sequentially washed and eluted from beads. Samples were reverse cross-linked and chromatin isolated from each input sample was quantified for normalization of the amount of template used for PCR. The mouse *Ncx1* proximal promoter was PCR amplified from the immunoprecipitated and non-immunoprecipitated (input control) chromatin. Primers that amplified regions distal to the proximal promoter were also used as negative PCR controls. Representative image of experiment that was done in duplicate and repeated three times, each time using different mice.

HDAC1 and HDAC5 are not associated with the Ncx1 promoter with pressure overload

Our previous work showed that HDAC1, HDAC2 and HDAC5 were associated with the Ncx1 proximal promoter in control adult feline cardiomyocytes and heart (20). Here we examined if the recruitment of these HDACs is changed in ventricles subject to pressure overload. ChIP analysis revealed HDAC1, HDAC5, Sin3a and the transcription factor Nkx2.5 are clearly associated with the proximal promoter of the Ncx1 gene in surgical sham mouse ventricles (Figure 5). As expected, HDAC1 and HDAC5 are no longer present on the Ncx1 proximal promoter 72 h post-TAC. Nkx2.5 is present in both the sham and pressure overloaded ventricle. Interestingly, Sin3a and HDAC3 are associated with the Ncx1 promoter with pressure overload.

Loss of HDAC5 reduces Sin3a-HDAC1 recruitment to the Ncx1 and Bnp promoters

ChIP analysis shows that HDAC5, HDAC1, HDAC2 and Sin3a are recruited to the proximal region of both the Ncx1 and Bnp promoters containing the Nkx2.5 and YY1 binding sites, respectively, in wild-type control mouse ventricles (Figure 6A and B). Although Nkx2.5 and YY1 are still present, HDAC1 is not recruited to the Ncx1 and Bnp promoters and Sin3a recruitment is greatly diminished in the HDAC5 knockout mouse ventricle. HDAC2 recruitment does not decrease on the Ncx1 promoter and is only slightly diminished on the Bnp promoter in the HDAC5 knockout.

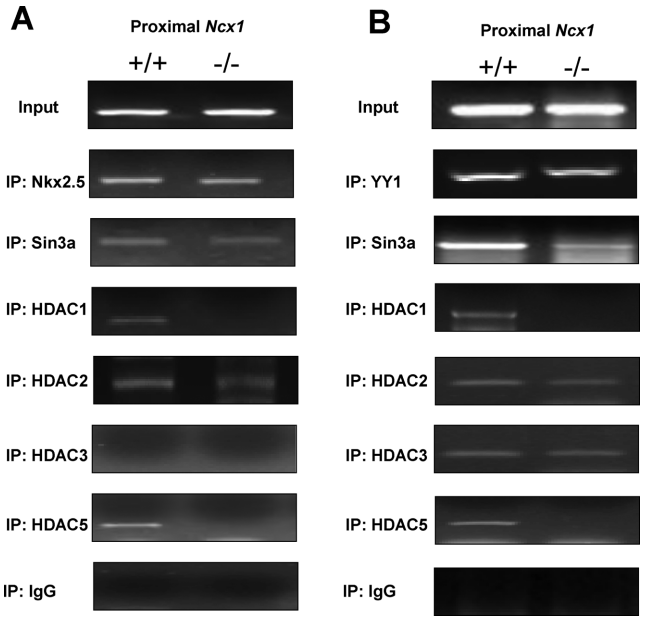

Figure 6. HDAC5 knockout abrogates recruitment of *Ncx1* and *Bnp* co-repressor complex molecules. 25 mg of fresh heart tissue from 10 to 12 weeks old wild-type and HDAC5$^{-/-}$ mice were minced, homogenized then immediately cross-linked with formaldehyde at a final concentration of 1% for 10 min at room temperature. Homogenates of each group were supplemented with paraformaldehyde in order to crosslink protein-DNA complexes. Samples were gently swirled every 5 min during this incubation. Cross-linking activity was quenched with the addition of 1.25 M glycine for 5 min. The homogenate was then suspended in lysis buffer, and incubated on ice for 1 h with gentle vortexing every 10 min. The cell lysate was sonicated for 8 cycles (10 s 'on' 10 s 'off'), and the cell debris was spun down. The supernatant was diluted and used equal amounts of lysate was then precleared with A/G agarose beads and supplemented with 5 μg of immunoprecipitation antibody [HDAC1, 2, 3 or 5, Sin3a (**A**), YY1 (**B**), or IgG (isotype control, A and B] overnight 4°C with constant end-to-end rotation. Complexes were immune-precipitated with agarose A/G beads then sequentially washed and eluted from beads. Samples were reverse cross-linked and chromatin isolated from each input sample was quantified for normalization of the amount of template used for PCR. The mouse *Ncx1* and *Bnp* proximal promoters was PCR amplified from the immunoprecipitated and non-immunoprecipitated (input control) chromatin. Primers that amplified regions distal to the proximal promoter were also used as negative PCR controls. Representative image of experiment that was done in duplicated and repeated three times, each time previously described. Image is representative of experiment that was done in duplicate and repeated three times.

DISCUSSION

We report here that the class IIa HDAC5 serves a key non-enzymatic role in the regulation of gene expression in cardiac hypertrophy. Our data show that the consequence of knocking out HDAC5 from a biological system is functionally different from inhibiting its catalytic activity. Essentially, removing HDAC5 results in the reduced recruitment of the HDAC1/Sin3a co-repressor complex to the proximal promoters of Ncx1 and Bnp. We observe that the Nkx2.5 and YY1 transcription factors interact with co-repressor complexes containing HDAC5, HDAC1, HDAC2 and Sin3a and that HDAC5 is required for this interaction. This was validated by sequential co-immunoprecipitation that revealed combinatory interactions between Nkx2.5/HDAC5/HDAC1,

Nkx2.5/HDAC5/HDAC2 and Nkx2.5/HDAC5/Sin3a. Furthermore, ChIP analysis shows that recruitment of an HDAC1/Sin3a co-repressor complex to the Ncx1 and Bnp promoters is HDAC5-dependent. Therefore our studies provide support for a role of class IIa HDAC5 as a scaffold or perhaps linker molecule for recruiting HDAC1/2-Sin3a co-repressor complexes.

Early studies demonstrated that class IIa HDACs are associated with HDAC3 and interact with the SMRT/N-CoR co-repressor complex (30). Eric Verdin's group demonstrated that when class IIa HDACs associate with the HDAC3/SMRT/N-CoR co-repressor complex they do not contribute to its enzymatic activity (32). Although these studies show that class IIa HDACs could interact with the HDAC3/SMRT/N-CoR co-repressor complex further studies were necessary to directly determine whether class IIa HDACs might still be playing a functionally relevant role in regulating gene expression while within these complexes. A study by Mihaylova *et al.* shows that the class IIa HDACs, HDAC4 and HDAC5 recruit HDAC3 to regulate the activation of FOXO via deacetylation (33). However, Mihaylova *et al*. did not show whether recruitment of HDAC3 also included NCOR or SMRT complex members.

Several previous studies support a non-enzymatic role for class IIa HDACs, yet have failed to provide direct evidence to support this postulation. Because of their extremely low activity against acetyl-lysine moieties, class IIa HDACs have been referred to as pseudo-enzymes (18). One example of this pseudo and somewhat enigmatic phenomenon can be seen in class IIa HDAC9. The HDAC9 splice variant (MITR) that encodes only the N-terminal domain with no catalytic domain is still able to inhibit MEF2 dependent transcription in the cardiac myocyte (34,35). Impairment of MEF2 dependent transcription is mediated via the recruitment of co-repressors such as CtBP and HP1 to binding domains on the N-terminus of HDAC9 (35,36). Another study demonstrated that the deacetylase domain of HDAC9 was not required for its negative regulatory effect of adipogenic differentiation (37).

Additional studies have determined that overexpression of class IIa HDACs can regulate cellular functions independently of acetylation. The physical presence of class IIa HDACs can indirectly regulate transcription by possible recruitment of sumoyl-conjugating enzymes to promoters and inhibiting transcription by the sumoylation of MEF2 (38,39). HDAC4 inhibits androgen receptor activity by facilitating androgen receptor sumoylation (40). Overexpression of HDAC7 in cultured neurons blocks low potassium-induced cell death. Interestingly, deletion of the HDAC7 catalytic domain has no impact on HDAC7s neuroprotective effect (41). Again giving support to non-canonical/non-enzymatic roles for class IIa HDACs.

Another intriguing idea is that class IIa HDACs could possibly serve as readers to bring the co-repressor complexes to their substrates. Data from a chemical phylogenetic study by Bradner's group demonstrated that class IIa deacetylase domains function as high affinity acetyl-lysine binding domains rather than functional deacetylases (42). They showed that the binding affinities for class IIa HDACs for acetyl-lysine substrates are within the range of binding affinities reported for bromodomains. Since class IIa HDACs have a high affinity for acetyl-lysine substrates it is interesting that previous work has shown that the acetylation of Nkx2.5 appears to be required for the recruitment of HDAC5/1/Sin3a co-repressor complexes (25).

In conclusion, we present data for the first time directly supporting the role of HDAC5 as a scaffold recruiting a chromatin modifying co-repressor complex to specific transcription factors on unique gene promoters. These data for HDAC5 may reflect one of the functions of class IIa HDACs in transcriptional regulation distinct from its catalytic activity.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENT

We would like to thank Joe Hill, Craig Crosson and Besim Ogretman for their critiques of our manuscript.

FUNDING

Funding for open access charge: National Institutes of Health [R01HL095696, R01HL094545, in part]; Merit Award from the Veterans' Affairs Health Administration [BX002327]; South Carolina Clinical & Translational Research (SCTR) Institute, with an academic home at the Medical University of South Carolina NIH/NCATS UL1 TR000062 [to D.R.M.]; NIH Postdoctoral Fellowship [T32HL07260 to L.G.H., S.H.W.].
Conflict of interest statement. None declared.

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