Modulation of chromatin position and gene expression by HDAC4 interaction with nucleoporins

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Class IIa histone deacetylases (HDACs) can modulate chromatin architecture and transcriptional activity, thereby participating in the regulation of cellular responses such as cardiomyocyte hypertrophy. However, the target genes of class IIa HDACs that control inducible cardiac growth and the broader mechanisms whereby these deacetylases modulate locus-specific gene expression within chromatin remain a mystery. Here, we used genome-wide promoter occupancy analysis, expression profiling, and primary cell validation to identify direct class IIa HDAC4 targets in cardiomyocytes. Simultaneously, we identified nucleoporin155 (Nup155) as an HDAC4-interacting protein. Mechanistically, we show that HDAC4 modulated the association of identified target genes with nucleoporins through interaction with Nup155. Moreover, a truncated mutant of Nup155 that cannot bind HDAC4 suppressed HDAC4-induced gene expression patterns and chromatin–nucleoporin association, suggesting that Nup155-mediated localization was required for HDAC4’s effect on gene expression. We thus propose a novel mechanism of action for HDAC4, suggesting it can function to dynamically regulate gene expression through changes in chromatin–nucleoporin association.

Introduction

In response to various disease-causing stimuli the adult myocardium undergoes hypertrophic growth (Dorn et al., 2003). Class IIa histone deacetylases (HDACs) are signal-regulated effectors of gene expression that modulate chromatin structure and can suppress the hypertrophic growth of cardiomyocytes (McKinsey et al., 2000; Zhang et al., 2002; Backs et al., 2006; Ago et al., 2008). Class IIa HDACs—HDAC4, HDAC5, HDAC7, and HDAC9—are unique among the four groups of HDACs and contain a large N-terminal regulatory domain that is subject to phosphorylation as a means of inducibly controlling their nuclear egress to permit gene activation (Verdin et al., 2003; Ago et al., 2008; Haberland et al., 2009). Although class IIa HDACs can bind myocyte enhancer factor-2 (MEF2) in mediating transcriptional repression of select genes (Zhang et al., 2002), their target loci in vivo and the mechanisms whereby they integrate their actions over these endogenous loci in cardiomyocyte nuclei remain unknown.

The nuclear envelope is a highly specialized membrane that surrounds the eukaryotic cell nucleus and provides attachment sites for the lamina, nuclear pore complex (NPC), and chromatin (Hetzer et al., 2005). The NPC is a large protein assembly composed of multiple copies of roughly 30 distinct nucleoporins (NUPs) that regulates the trafficking of macromolecules between the nucleoplasm and cytosol but also provides anchoring sites for chromatin (Lim et al., 2008). Inactive heterochromatin is often partitioned against the inner face of the nuclear membrane in association with the nuclear lamina, while active gene regions and euchromatin may be associated with NUPs at the periphery, or more centrally in the nucleus (Akhtar and Gasser, 2007). Although mechanisms regulating chromatin occupancy within these domains are poorly defined, NUP–chromatin association can be modified by acetylation/deacetylation. Indeed, general class I and class II HDAC blockade by trichostatin A (TSA) induced the association of differentially expressed genes with NUPs at the NPC (Brown et al., 2008).
It was also recently discovered that the association of chromatin with NUPs is important for gene expression (Vaquezias et al., 2010). However, the regulation of these processes, direct binding partners, and the acetyltransferases or deacetylases involved are unknown. Here, we show a novel paradigm whereby select gene loci are subject to inducible regulation through class Ila HDAC binding to nucleoporin 155 (Nup155), which can modulate the hypertrophic growth program in cardiomyocytes.

Results and discussion

To investigate the mechanisms whereby class Ila HDACs regulate the growth of cardiac myocytes we first identified HDAC4 target genes. HDAC4 was mildly overexpressed in primary cultures of neonatal rat ventricular cardiomyocytes (NRVMs) using recombinant adenoviruses expressing HDAC4 or control β-galactosidase, after which RNA was collected for transcript profiling. The array expression data showed that 1,130 genes were changed with HDAC4 overexpression, of which 815 genes were downregulated and 315 up-regulated (Fig. S1). Gene-grouping analysis using DAVID (Dennis et al., 2003; Huang et al., 2009) showed that HDAC4-repressed genes were enriched for cardiac transcripts, such as myofilament genes (12.3-fold enrichment, benjamini P < 2.2E-14), and heart contraction genes (11.4-fold enrichment, benjamini P < 1.8E-7). Interestingly, we also observed a significant enrichment for Ca2+ ion homeostasis genes (5.8-fold enrichment, benjamini P < 3.3E-6), a cardiac gene partner, and the acetyltransferases or deacetylases involved are unknown. Here, we show a novel paradigm whereby select functional gene groups in the heart. We also performed a search for evolutionary conserved motifs in the promoters (∼2,000 to +500 bp) of the HDAC4 target genes. However, multiple analyses did not reveal a conserved DNA sequence motif, suggesting that HDAC4 can function more broadly and is not restricted to conserved transcription factor binding sites.

We next experimentally validated the DamID and expression analysis results for the selected gene groups. Quantitative RT-PCR across 11 selected genes showed that the HDAC4 targets were either significantly repressed or activated, consistent with the expression arrays following expression of HDAC4 (Fig. 1 A). We also analyzed several of the direct HDAC4 target genes by chromatin immunoprecipitation (ChIP) for their promoter regions, which validated the DamID screen and showed occupancy of HDAC4 within 10 selected gene regions corresponding to their promoters (Fig. 1 B, band sizes given in Materials and methods). Changes in gene expression through HDAC4 also correlated with changes in protein levels of key Ca2+-handling genes (Fig. 1 C). We observed a reduction in expression of the Na+/Ca2+ exchanger (NCX1), phospholamban (PLN), and the sarcoplasmic reticulum Ca2+ ATPase (Serca2), a profile that should alter Ca2+ handling in myocytes. Indeed, we directly measured intracellular Ca2+ concentration and handling in stimulated cardiomyocytes using a fluorescent Ca2+ indicator (Fig. 1, D and E). Expression of HDAC4 resulted in a significant increase in baseline Ca2+ levels and a significant decrease in the transient relaxation time constant. Thus, HDAC4 acts in an orchestrated manner to alter the expression of several Ca2+-handling genes, validating the expression and promoter occupancy screen with a functional correlate.

To investigate the transcriptional mechanisms whereby HDAC4 might selectively control gene expression in the heart we performed a modified yeast two-hybrid screen, termed the Ras recruitment system (RRS; Aronheim, 2004). We used the N-terminal regulatory domain of HDAC4 (aa 3–666) as the bait with a heart cDNA library to identify prey plasmids that specifically interacted. This screen identified the C terminus of the Nup155 (aa 886–1391) as an HDAC4 binding partner. Examination in yeast showed that an HDAC4 N-terminal sequence (aa 3–281) was sufficient to bind Nup155 C terminus (Fig. 2 A). Coimmunoprecipitation (colP) in mammalian cells using HA-tagged full-length Nup155 and His-tagged HDAC4 fragments showed that peptides containing amino acid 3–220 and 3–185 of HDAC4 were sufficient for the interaction, whereas a 3–165 fragment was not, suggesting that the interacting domain in HDAC4 is between amino acids 165 and 185 (Fig. 2 B). This domain in HDAC4 also mediates an interaction with MEF2 and HDAC1 (Chan et al., 2003). Finally, recombinant GST was fused to the C terminus of Nup155 (aa 886–1391) and used to assess...
interaction with the recombinant HDAC4 N terminus (aa 3–628) fused to the maltose-binding protein (MBP). This analysis showed that GST-Nup155 was unable to bind MBP alone, although it did bind MBP-HDAC4 recombinant protein (Fig. 2 C, asterisk). GST alone did not bind MBP-HDAC4.

Coimmunoprecipitation experiments showed that endogenous HDAC4 interacted with endogenous Nup155 in extracts from primary cultures of NRVMs (Fig. 2 D). We used the well-validated mAb414 monoclonal antibody raised against the entire rat nuclear pore, which recognizes several NUPs but not Nup155 (Davis and Blobel, 1986), to show that other NUPs (bands at 62 and 107 kD) may be part of this complex (Fig. 2 D, and unpublished data). Remarkably, immunofluorescent staining of cardiomyocytes showed that endogenous HDAC4 is enriched at the nuclear periphery, and partially colocalized with the mAb414 antibody (Fig. 2 E). Interestingly, HDAC5 was not able to bind Nup155 (unpublished data), suggesting greater specificity for HDAC4. Collectively, these data suggest that Nup155 and HDAC4 can interact in conjunction with other NUPs, especially at the nuclear periphery where the NPC is located.

Global HDAC inhibition was previously shown to alter chromatin association with the NUPs for several gene loci (Brown et al., 2008), and a study in Drosophila cells showed that mAb414-positive NUPs were found to associate with sites of active transcription (Capelson et al., 2010). Therefore, we hypothesized that HDAC4 could modify the association of select genomic loci with the NUPs, and that the interaction between HDAC4 and Nup155 was required for this change in association. To test this hypothesis we initially performed ChIP assays using the mAb414 antibody in NRVMs, with or without expression of
Next we wanted to verify that the association with nucleoporins is required for HDAC4 action on gene expression. Unfortunately, knockdown of Nup155 resulted in cardiomyocyte death (unpublished data). To circumvent this problem we constructed a truncated Nup155 mutant, referred to as Nup155\(-\text{C}\) (aa 1–886), which lacks the HDAC4-interacting C-terminal domain. Analysis in cardiomyocytes showed that this truncation mutant is able to localize in a similar manner to full-length Nup155 (Fig. 3 B, arrowheads). Importantly, overexpression of Nup155\(-\text{C}\) did not seem to result in overt nuclear pore transport defects or otherwise compromise the health of cultured cells (Fig. S2). Overexpression of Nup155\(-\text{C}\) by adenoviral-mediated gene transfer in NRVMs also did not result in major alterations in other NUPs as assessed with m414 or Nup153 antibody (unpublished data).

Remarkably, adenoviral overexpression of Nup155\(-\text{C}\) replaced endogenous full-length Nup155 and now no longer permitted endogenous HDAC4 to associate with nucleoporins.

HDAC4 and TSA treatment. Many of the identified HDAC4 target genes indeed displayed some degree of association with the NUPs. Remarkably, as we hypothesized, overexpression of HDAC4 decreased the association of many target loci with the NUPs, and the addition of TSA restored this association (Fig. 3 A). Interestingly, several HDAC4 targets showed a reversed association pattern. Loci like Pln, Fos, Tnfrsf12a, and Csrp3 displayed low association with NUPs at baseline but overexpression of HDAC4 enhanced the association, which was reversed with TSA (Fig. 3 A). Although this differential behavior of specific loci has been described in yeast (Casolari et al., 2004) and mammalian cells (Brown et al., 2008), the reasons for it are unknown. The reversal of the dissociation by TSA shows that the process is acetylation/deacetylation dependent, although the enzymes involved, such as association with class I HDACs, and the recipients of the acetyl group, are unknown. Thus, our results identify a mechanism whereby HDAC4 can modify chromatin association of select gene loci with NUPs.

Figure 2. HDAC4 partners with Nup155 and associates with the NUPs. (A) Yeast two-hybrid growth assay with baits containing either the N terminus (aa 3–281 or 3–666) or C terminus (aa 632–1084) of HDAC4 (HD4) cotransfected with the library plasmid, which identified the Nup155 C terminus. Inducible promoters on library and bait plasmids allows differential activation of expression [−/+], showing growth and therefore interaction between Nup155 and both N-terminal constructs of HDAC4, but not with the C-terminal construct. (B) Western blots for the indicated proteins from mammalian cells transfected with the indicated constructs. For the bottom two blots, HA-Nup155 was immunoprecipitated (IP) followed by Western blotting for His-HDAC4 fragments or HA-Nup155. The asterisks show the fragments that interact. Input represents 10% of protein before IP. (C) Coomassie gel showing interaction between the C terminus of Nup155 as a GST fusion protein and the N terminus of HDAC4 as an MBP fusion protein. The asterisk shows the pull-down of MBP-HDAC4 with GST-Nup155 from the GST binding column. Input protein is shown on the left. (D) Western blots from NRVMs after endogenous HDAC4 immunoprecipitation (IP) or control IgG to investigate Nup155 and nucleoporin (mAb414) interaction. (E) Immunofluorescent staining of NRVMs for endogenous HDAC4 (green, arrowheads), the NUPs (mAb414, red), and DNA (ToPro3, blue) showing partial colocalization of HDAC4 with NUPs.
In contrast, the Pln locus demonstrates a reverse pattern. The Nlrx1 locus was used as a control, which showed no significant changes in intranuclear location (not depicted). These results corroborate the ChIP experiments and support the dynamic movement of specific loci to the nuclear periphery, the ability of HDAC4 to modify this balance, and the requirement of HDAC4–Nup155 interaction for this mechanism.

Consistent with the changes described above, detailed mRNA expression analysis using qRT-PCR showed that overexpression of Nup155ΔC reversed the expression pattern of HDAC4 for many of its target genes (Fig. 5 A). Functionally, these changes in gene expression correlated with alterations in cardiomyocyte hypertrophy. Indeed, although overexpression of HDAC4 reduced sarcomeric organization at baseline in quiescent NRVMs, expression of the Nup155ΔC mutant induced or- ganization of sarcomeres and induced an increase in cell size, likely by relieving HDAC-dependent repression of select genes involved in myocyte growth (Fig. 5, B–D).

To further verify dynamic association between NUPs and HDAC4 on select target genes in NRVMs we performed fluorescent in-situ hybridization (FISH) combined with confocal microscopy and quantified the results in both a blinded and unblinded manner, producing similar results (Fig. 4, A and B). In contrast, the Pln locus demonstrates a reverse pattern. The Nlrx1 locus was used as a control, which showed no significant changes in intranuclear location (not depicted). These results corroborate the ChIP experiments and support the dynamic movement of specific loci to the nuclear periphery, the ability of HDAC4 to modify this balance, and the requirement of HDAC4–Nup155 interaction for this mechanism.

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Collectively, we identified clusters of sarcomeric genes and Ca2+-handling genes that are directly repressed by HDAC4 and showed that HDAC4 exists in a complex with Nup155 and mAb414-positive NUPs, and that HDAC4 modified the
Materials and methods

Cell culture

Neonatal cardiomyocytes were isolated from 1–3-d-old Sprague Dawley rat pups using the Neonatal Cardiomyocyte Isolation System (Worthington) according to the manufacturer’s instructions. In brief, hearts were rinsed with ice-cold HBSS and trypsinized for 16 h at 4°C. Hearts were incubated with trypsin inhibitor at 37°C, and then incubated for 60 min with a collagenase solution at 37°C with gentle mixing. The cell pellet was triturated and resuspended in M199 media (Invitrogen) containing 10% fetal calf serum. Cells were then preplated for 1 h to deplete culture of fibroblasts, and nonadherent cells were plated on gelatin-coated culture dishes. After plating, cells were washed twice with serum-free or serum-containing culture media, and grown for 24–72 h.

Adenoviral and lentiviral vector production

The Gateway system (Invitrogen) was used to generate pEntry clones for adenoviral and lentiviral vector production. Adenoviral vectors were generated as described previously (Xu et al., 2006). NRVMs were infected for 2 h at 10 multiples of infection; cells were washed twice and incubated for 24–48 h. Lentiviral vectors were produced by the Viral Vector Core at the Translational Core Laboratories, Cincinnati Children’s Hospital Research Foundation (Cincinnati, OH). Lentiviral infection was performed overnight with addition of hexadimethrine bromide; cells were washed twice and incubated for 24–48 h.

Figure 4. HDAC4 regulates spatial chromatin organization. [A] Confocal FISH of selected loci (green dots, arrows) with nuclear Topro3 counterstain (blue) in NRVMs showing predominant peripheral nuclear localization of the Nppb, Acta1, Cacna1c during control conditions, a shift to a more central nuclear position under HDAC4 (HD4) overexpression, and reversal of the HDAC4 effect by expression of Nup155ΔC. In contrast, the Pln locus demonstrates a reverse pattern. (B) Quantitative summary of the FISH data showing the percentage of peripheral nuclear FISH signals (white bar) versus central nuclear signals (black bar). *, P < 0.001; significance of HDAC4-expressing vs. control and HDAC4+ Nup155ΔC, n = 250.
Ras recruitment system (RRS) yeast two-hybrid

The RRS screen was performed as described previously (Aronheim, 2004). In brief, the bait was a hybrid protein with the mammalian activated Ras protein lacking its farnesylation CAAX box fused to the human HDAC4 N terminus (aa 3–666). The bait plasmid was cotransfected into Cdc25-2 yeast cells with a myristoylated heart cDNA library (CryoTrap; Agilent Technologies). The expression of the cDNA library is designed under the control of the Gal1-inducible promoter, while the expression of the bait is controlled by a Met-off inducible promoter. Plates were incubated for 7 d at the permissive temperature of 24°C and were subsequently replica plated onto inductive medium and incubated at the restrictive temperature of 36°C. Colonies that exhibit efficient growth were selected and grown on appropriate glucose plates for 2 d. Subsequently, galactose and methionine dependency was assayed by replica plating at the restrictive temperature of 36°C. Colonies that exhibited efficient cell growth and full dependency were further analyzed. Plasmid DNA was extracted and the identified library plasmids were sequenced. The bait plasmid was cotransfected into Cdc25-2 cells with either the specific (HDAC4 aa 3–666) library plasmid identified by sequencing. Identified library plasmids were transfected in Cdc25-2 cells with either the specific (HDAC4 aa 3–666) or nonspecific baits (HDAC4 aa 632-end) and the interaction was reexamined using the galactose and methionine dependency test at the restrictive temperature of 36°C.

Commmunoprecipitation

Cultured cells were washed with ice-cold phosphate-buffered sodium (PBS) buffer and lyzed in buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, and 1% NP-40. 400 µg of protein was immunoprecipitated with anti-HA, HDAC4, or IgG antibodies and protein A/G agarose beads (Santa Cruz Biotechnology, Inc.). Beads were washed three times with buffer and suspended in 30 µl of Laemmli buffer. Proteins were separated on an SDS gel, blotted, and detected with primary anti-His antibody (Cell Signaling Technology), HA antibody (Cell Signaling Technology), Nup155 antibody (custom made from YenZym Antibodies, LLC.), nucleoporin mAb414 antibody (Covance), and HDAC4 antibody (Abcam). Secondary alkaline phosphatase–conjugated antibodies were also used (Santa Cruz Biotechnology, Inc.).
Chromatin immunoprecipitation
NRVMs were infected with adenoviral vectors encoding β-galactosidase, flag-HDAC4, and Nup155ΔC. Chromatin immunoprecipitation was performed using the EZ-Chip kit (Millipore) according to the manufacturer’s instructions. Antibodies for immunoprecipitation were anti-FLAG (Sigma-Aldrich) or anti-nucleoporin mAb414 (Covance). PCR was performed using primers specified as follows (site in base pairs, the forward band is shown in parenthesis): Acta1 (218) 5’-GGGTGGGAGTGTGTGTTCCGAGCTCTTCGTTGGA-3’; Cacna1c, 5’-CTCCAATTGCTTCTGGCTCT-3’; Fos (170) 5’-GCCAGCTGGTCCGATTTGAC-3’; Fosl1 5’-ACAATCCACGATGCAGAAGCT-3’. qRT-PCR
NRVMs were infected with adenoviral vectors encoding β-galactosidase or flag-HDAC4 and incubated in serum-free or 10% fetal bovine serum containing growth medium for 48 h. RNA was extracted using Trizol (Invitrogen). Reverse transcription (RT) reaction was performed using the SuperScript III kit (Invitrogen) according to the manufacturer’s instructions. Quantitative real-time PCR was performed with sybr-green and taq polymerase master mix (Applied Biosystems). Data were normalized to the expression of GAPDH. Primers used were: Acta1 5’-TGGCCGACCTTACTGACCTTGGTA-3’, Nup155 5’-CATTCCTCCAGTTTTGGAC-3’. Data were visualized using a confocal microscope.

Analysis of evolutionary conserved DNA motifs
Promoters of HDAC4 target genes (~2,000 to ~500 relative to the transcriptional start site) were extracted and cross-checked with genomic information of Homo sapiens, Rhesus macaca, Mus musculus, Bos taurus, and Canis familiaris to limit the analysis to evolutionary conserved sequences using GALAXY (Taylor et al., 2007). A search using MEME (Bailey and Elkan, 1994) and TOMTOM (Gupta et al., 2007) was used to identify conserved transcription factor–binding sites. In addition, a search for conserved transcription factor-binding sites was performed using GATHER (Chang and Nevins, 2006) and TRANSFAC databases.

Analysis of Ca2+ transients in cardiomyocytes
NRVMs plated on laminin-coated coverslips were loaded with 5 µmol/L Indo-1. Individual cells were excited at 355 nm and the fluorescence emission ratio (405/485) was collected every 20 ms using a 40x oil objective and a photomultiplier detection system (Photon Technology International). Cells were electrically stimulated (80 V, 5 ms, 0.5 Hz) using platinum electrodes. Ca2+ transient amplitudes were calculated by measuring the change in 405/485 fluorescence with each electrical pulse from 10 to 30 cells for each treatment. These Ca2+ measurements were conducted in normal or K+ Ringer’s solution (in mM: 14.5 NaCl, 5 KCl, 2 CaCl2, 1 MgCl2, and 10 Heps, pH 7.4).

Fluorescent in situ hybridization (FISH)
NRVMs were plated on glass slides, infected with adenoviral vectors encoding β-galactosidase, Flag-HDAC4, or Nup155ΔC, and grown for 24–48 h. Cells were pretreated with 0.8% sodium citrate and fixed in ice-cold 3:1 methanol/acetic acid. Slides were treated with protease solution (FISH specimen pretreatment reagent kit; Abbott Molecular) for 10 min at 37°C. Denaturation was performed with 70% formamide/2x SSC solution for 5 min at 70°C. Probes were generated using a nick translation reagent kit (Abbott Molecular) with Green-dUTP (Enzo Life Sciences) according to the manufacturer’s instructions, with bacterial artificial chromosome (BAC) clones as templates. Probes were suspended in buffer (C-DNAhyb; Instutus Biotechnologies) and denatured in the slides for 5 min. Slides were incubated overnight at 37°C. Washing was performed in 0.4x SSC/0.3% NP-40 for 2 min at 73°C and in 2x SSC/0.1% NP-40 for 1 min at room temperature. Nuclear were counterstained with TO-Pr3 (Invitrogen). Specimens were visualized using a confocal microscope.

Transcriptome analysis
NRVMs were infected with adenoviral vectors encoding β-galactosidase or Flag-HDAC4, and incubated in serum-free or 10% fetal bovine serum containing growth medium for 48 h. RNA was extracted using the RNeasy Fibrous Tissue kit (QIAGEN) according to the manufacturer’s instructions. Expression pattern was analyzed using GeneChip Rat Gene 1.0 ST Arrays (Affymetrix) according to the manufacturer’s instructions. The Affymetrix Whole Transcript Sense Target Labeling Assay was used to create biotin-labeled sense-strand cDNA targets for hybridization to the Gene 1.0 ST Arrays. Hybridization cocktail for a single probe array contained 2.5 µg of fragmented cRNA, 50 pM Control Oligonucleotide B2 (Affymetrix), 20x Eukaryotic Hybridization Controls (1.5 pM bioC, 5 pM bioB, 25 pM bioD, 100 pM cre, Affymetrix), 7% DMSO, and 1x hybridization buffer. GeneChip Rat Gene 1.0 ST Array was hybridized at 45°C for 18 h in the GeneChip Hybridization Oven 640 (Affymetrix). Expression data has been submitted to GEO Datasets under accession no. GSE19771.
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