Nuclear receptor co-repressors are required for the histone-deacetylase activity of HDAC3 in vivo

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Histone deacetylase 3 (HDAC3) is an epigenome-modifying enzyme that is required for normal mouse development and tissue-specific functions. In vitro, HDAC3 protein itself has minimal enzyme activity but gains its histone-deacetylation function from stable association with the conserved deacetylase-activating domain (DAD) contained in nuclear receptor co-repressors NCOR1 and SMRT. Here we show that HDAC3 enzyme activity is undetectable in mice bearing point mutations in the DAD of both NCOR1 and SMRT (NS-DADM), despite having normal levels of HDAC3 protein. Local histone acetylation is increased, and genomic HDAC3 recruitment is reduced though not abrogated. Notably, NS-DADM mice are born and live to adulthood, whereas genetic deletion of HDAC3 is embryonic lethal. These findings demonstrate that nuclear receptor co-repressors are required for HDAC3 enzyme activity in vivo and suggest that a deacetylase-independent function of HDAC3 may be required for life.

RESULTS

NS-DADM mice are born and live to adulthood
To determine whether NCOR1 and SMRT are required for HDAC3 enzyme activity in vivo, we generated C57BL/6 mice bearing mutations in the DAD of both alleles of Ncor1 and Ncor2. In each case, the mutant protein contains a single tyrosine-to-alanine

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substitution in the DAD (Y478A in NCOR1, Y470A in SMRT) that prevents interaction with and activation of HDAC3 (ref. 34). The N-DADm mice have been described previously35, and the S-DADm mice were generated in the C57BL/6 strain background by using a similar strategy (Supplementary Fig. 1). Mutants heterozygous for N-DADm and S-DADm were mated to generate mice that are heterozygous for both mutant alleles, and those mice were mated with each other to obtain male and female double-homozygous-mutant NS-DADm mice. Because this breeding strategy produces only one NS-DADm and one wild-type mouse of the same gender for every 32 pups, larger numbers of each genotype were generated by crossing wild-type and NS-DADm mice with each other. Notably, whereas loss of Ncor1, Ncor2 or Hdac3 is embryonically lethal26,28,39,40, the NS-DADm mice exhibited no detectable embryonic lethality and lived to adulthood (Table 1).

HDAC3 is enzymatically inactive in NS-DADm mice

We next studied the expression and function of HDAC3 in the tissues of mice bearing the DAD mutations in NCOR1 and SMRT. Gene expression of Hdac3 is normal in the livers of NS-DADm mice (Fig. 1a). Similarly, levels of hepatic HDAC3 protein were indistinguishable from those of wild-type mice (Fig. 1b). Because expression of HDAC3, NCOR1 and SMRT was not significantly altered by the presence of the DAD mutations (Supplementary Fig. 2a,b), we were able to test the hypothesis that endogenous HDAC3 requires NCOR1 or SMRT for its activity in vivo. Notably, whereas HDAC3 activity was readily measured in immunoprecipitates from wild-type liver, it was undetectable in liver from the NS-DADm mice (Fig. 2a), and this was not due to inefficient immunoprecipitation relative to wild type (Fig. 2b). Similar loss of HDAC3 enzyme activity was observed in heart (Fig. 2c) and skeletal-muscle (Fig. 2d) tissues. Moreover, no HDAC3 enzyme activity was detectable in embryos harvested on day 12.5 (Fig. 2e), thus demonstrating the importance of the NCOR1 and SMRT DADs in all tissues and that no other factor substitutes prenatally as an HDAC3 activator. Because of the background of the HDAC3 enzyme assay, it is possible that a small amount of residual activity exists. However, these data prove that NCOR1 and SMRT are required for the vast majority of the HDAC3 enzymatic activity in the tissues examined. Thus, the nuclear receptor co-repressors are required for HDAC3 enzyme activity in vivo.

HDAC3 genome binding is reduced in the NS-DADm mice

Because HDAC3 is thought to be recruited to the genome by NCOR1 and SMRT, we hypothesized that recruitment would be reduced in the NS-DADm mice. To test this, we located and quantified the recruitment of HDAC3 to mouse liver by using chromatin immunoprecipitation with HDAC3-specific antibody, followed by massively parallel DNA sequencing (ChIP-seq). At 5 p.m., when circadian genomic HDAC3 recruitment is maximal in mouse liver16, we detected HDAC3 at 5,799 sites in wild-type mice, the majority of which were distant from transcription start sites or were present in introns (Supplementary Fig. 3), consistent with prior findings41. By contrast, under the same stringent peak-calling criteria, only 600 HDAC3-binding regions were detected in the NS-DADm liver, the majority of which overlapped with wild-type binding (Supplementary Fig. 4). It should be noted that HDAC3 binding remained detectable at most sites. The strength of binding in the NS-DADm liver decreased ~62.4% on average (Fig. 3a), and individual HDAC3-binding sites reflect this decrease (Fig. 3b). The reduction of HDAC3 recruitment in the NS-DADm liver was validated at ten sites by ChIP followed by quantitative PCR (ChIP-qPCR) (Fig. 3c). The partial genomic interaction of HDAC3 is likely due to another region of NCOR1 or SMRT29,34 or to direct interaction between HDAC3 and other transcription factors, neither of which would activate the HDAC3 enzyme. Nevertheless, these data demonstrate that the NCOR1 and SMRT DADs are critical for normal genomic recruitment of HDAC3 in vivo.

Local histone acetylation is increased in NS-DADm liver

Deletion of HDAC3 has been shown to result in increased local histone acetylation in liver16 and macrophages18, which is consistent with its in vitro histone deacetylase activity. We next tested whether the loss of HDAC3 activity alters local histone acetylation in vivo. Indeed, histone
H3 Lys9 acetylation (H3K9ac), an activating mark\(^42\), was locally increased at the ten sites where loss of HDAC3 recruitment was validated by ChIP-qPCR but not at control sites in the Rplp0 (Arbp) and Ins1 (Ins) genes where HDAC3 is not bound (Fig. 4a). Similar results were obtained after analysis of acetylated histone H3 Lys27 (H3K27ac), another activating mark (Fig. 4b). Of note, the degree of elevated acetylation is comparable to that in the HDAC3 knockout liver (Supplementary Fig. 5a). Loss of HDAC3-DAD interaction did not alter the genomic recruitment of NCOA1 (Fig. 4c) or SMRT (Supplementary Fig. 5b), nor did the complete absence of HDAC3 affect the genomic recruitment of NCOA1 or SMRT (Supplementary Fig. 5c,d). Thus, the loss of HDAC3-DAD interaction leads to increased local nucleosomal histone acetylation in NS-DADm mice, despite normal binding of NCOA1 and SMRT at these genomic locations.

**NS-DADm and HDAC3-null phenotypes are distinct**

The viability of the NS-DADm mice, which carry the mutant alleles in every cell, was quite different from the embryonic lethality of mice with germ-line deletion of HDAC3 (refs. 39,40), despite the fact that HDAC3 activity was undetectable in embryos from NS-DADm mice (Fig. 2e). Because a major difference between these two genetic models is that HDAC3 protein is absent in the knockout but fully present in the NS-DADm mice, this result suggested that embryonic lethality from loss of HDAC3 is due to a deacetylation activity-independent function.

We also compared the livers of NS-DADm mice with those lacking HDAC3 in the liver. Deletion of hepatic HDAC3 (liver HDAC3 knockout) was accomplished by injecting 10-week-old male C57BL/6 mice bearing floxed Hdac3 alleles (Hdac3\(^\text{f/f}\)) with an adenovirus with either Cre recombinase or GFP as control. As previously described, the loss of HDAC3 in liver dramatically increased hepatic triglyceride levels\(^{10,16,17}\). Both mouse models exhibit undetectable hepatic HDAC3 activity (Fig. 5a) and increased local histone acetylation at the lost binding sites\(^{16}\) (Figs. 3 and 4), as well as increased liver triglycerides. However, the approximately two-fold increase of triglyceride activity in the NS-DADm livers was considerably more modest than the dramatic five- to ten-fold increase observed in the livers lacking HDAC3 protein in the liver (Fig. 5b,c). This suggests that the continued presence of the inactive HDAC3 protein in the NS-DADm mice serves a function that normally contributes to the overall biological activity of HDAC3. Indeed, whereas hepatic cholesterol levels increased in the liver HDAC3 knockout, no significant change in cholesterol accumulation was observed in the NS-DADm mice (Fig. 5d,e), which suggests that the cholesterol accumulation in the absence of HDAC3, which has been also observed by others\(^10\), involves a deacetylation-independent function of HDAC3.
Figure 5 The liver phenotype of NS-DADm mice was more modest than that for mice lacking hepatic HDAC3. (a) HDAC activity measured after immunoprecipitation with HDAC3-specific antibody or IgG in wild type, NS-DADm, Hdac3+f/f AAV-GFP and Hdac3f/f AAV-Cre liver. (b–e) Hepatic triglyceride (b,c) and liver cholesterol (d,e) in wild-type, NS-DADm, Hdac3f/f AAV-GFP and Hdac3f/f AAV-Cre mice as indicated. n = 4–5; error bars, s.e.m. *P < 0.05 and **P < 0.01 by Student’s two-tailed t test.

Consistent with the physiological data, comparisons of transcriptomes revealed that the loss of HDAC3 in liver had more dramatic effects on gene expression than did loss of HDAC3-DAD interaction in NS-DADm mice (Fig. 6a). In keeping with previous results17, pathway analysis highlighted the upregulation of many genes associated with lipid metabolism in livers lacking HDAC3 protein (Fig. 6b), many of which could be confirmed by reverse-transcription qPCR (RT-qPCR) (Fig. 6c). By contrast, abrogation of HDAC3 enzyme activity but not the HDAC3 protein in NS-DADm liver caused upregulation of fewer genes involved in lipid metabolism (Fig. 6b,d). These results reinforce the conclusion that although the NCOR1 and SMRT DADs control HDAC3 activity and local histone deacetylation, these effects contribute relatively modestly to the total effects of HDAC3 protein on hepatic gene expression and the physiology of liver lipid metabolism.

DISCUSSION

We have introduced point mutations into both alleles of NCOR1 and SMRT that specifically abolish their ability to activate HDAC3. Using this mouse model, we show that the DADs of nuclear receptor co-repressors NCOR1 and SMRT are required for HDAC3 enzyme activity in vivo. This finding is notable, as HDAC3 has been shown to have pivotal roles in transcriptional regulation1,6,7, cell cycle progression11–13, inflammation18, developmental events10 and metabolism16,17. Our data clearly show that NCOR1 or SMRT is required for nearly all HDAC3 enzyme activity in vivo.

HDAC3 clearly functions as an epigenomic modifier in the liver16, and indeed histone acetylation was increased in the NS-DADm liver at genomic locations where HDAC3 normally binds. This is probably explained by the loss of HDAC3 catalytic activity. In addition, HDAC3 occupancy on the genome was significantly reduced in the NS-DADm, thus demonstrating the importance of the NCOR1 and SMRT DADs in recruiting HDAC3 to the genome and providing a second mechanism for increased histone acetylation at sites of endogenous HDAC3 recruitment in wild-type mice. Histone acetylation and deacetylation alter chromosome accessibility and affect functions of transcription factors acting on the genome1. Many inhibitors of the enzyme activity of class I HDACs are being developed to treat diseases including several types of cancer14,42. Moreover, approximately 10% of currently prescribed drugs directly target transcription factors46, including tamoxifen for breast cancer and bicalutamide for prostate cancer, which target nuclear receptors by regulating their interaction with HDAC3 (refs. 47,48). Therefore, our findings toward understanding basic HDAC biology have important therapeutic implications.

The NS-DADm mice exhibited mild hepatic steatosis, and molecular analysis revealed reduced or absent HDAC3 binding and increased local histone acetylation at upregulated lipid-metabolic genes. Therefore, these effects are likely due to the absence of HDAC3-dependent histone deacetylation. Nevertheless, mice lacking HDAC3 in liver manifest much more severe hepatic steatosis10,16,17 along with disrupted cholesterol homeostasis10,17, whereas NS-DADm mice exhibited no detectable alteration in hepatic cholesterol. Furthermore, although absence of cardiac HDAC3 causes lethality or diet-induced heart failure depending on when the HDAC3 is deleted17,40, NS-DADm mice have normal hearts and are able to tolerate a high-fat diet (data not shown). Thus, although our studies show that the nuclear receptor co-repressors are required for the deacetylase activity of HDAC3 in vivo, they also suggest a DAD-independent or deacetylase-independent

Figure 6 Gene expression changes in NS-DADm livers were mild compared to those in HDAC3 protein depletion. (a) Venn diagram showing overlap of upregulated genes of NS-DADm versus HDAC3 liver-specific knockout (Hdac3f/f AAV-Cre) from liver microarray. (b) Biochemical pathways showing genes that are upregulated in both NS-DADm and H dac3f/f AAV-Cre livers (red) and upregulated only in HDac3f/f AAV-Cre liver (blue). VLDL, very-low-density lipoprotein. (c,d) RT-qPCR validation of results summarized in b. n = 4–5; error bars, s.e.m. *P < 0.05, **P < 0.01 and ***P < 0.001 by Student’s two-tailed t test.
role of HDAC3. In addition to their roles in adult tissues, germ-line deletion of NCoR1 (ref. 26), SMRT59 or HDAC3 (refs. 10,40) all cause embryonic lethality. The viability of the N-DADm mice demonstrated for the first time, to our knowledge, that corepressors NCOR1 and SMRT that interact with HDAC3 but do not activate the enzyme34. Nonetheless, the present study in NS-DADm mice demonstrates for the first time, to our knowledge, that corepressors NCOR1 and SMRT that interact with HDAC3 but do not activate the enzyme34.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Complete list of significantly changed genes is available at Gene Expression Omnibus, with accession code GSE42541.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

S.-H.Y. and M.A.L. conceived of the hypothesis and designed the experiments. S.-H.Y., Z.S. and M.B. performed the experiments. H.-W.L and K.-J.W. analyzed bioinformatics data. S.-H.Y., Z.S. and M.A.L. analyzed and interpreted the data. S.-H.Y. and M.A.L. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice. NS-DADm mice were generated from crossing N-DADm and S-DADm mice, and the generation of mice lacking Hdac3 in liver by injection of AAV-TBG-Cre into Hdac3 mice has been previously described16,17. Mice were housed under a 12-h light and 12-h dark cycle (lights on at 7 a.m. and lights off at 7 p.m.). We used adult male mice at the age of 3–7 months in all experiments, except where otherwise indicated. We harvested tissues at 5 p.m. without previously restricting food or water for the mice, unless otherwise indicated. All the animal care and use procedures followed the guidelines of the Institutional Animal Care and Use Committee of the University of Pennsylvania in accordance with the guidelines of the US National Institutes of Health.

Antibodies and reagents. HDAC3 antibodies for ChIP-seq, immunoprecipitation and western blot were purchased from Abcam (ab7030) and Millipore (05-813, clone 3G6). Acetylated H3K9 and H3K27 antibodies were purchased from Millipore (07-352) and Abcam (ab4729), respectively. IgG was purchased from Sigma (I8140).

Immunoprecipitation and western blotting. Liver tissue was homogenized in modified radioimmunoprecipitation assay (RIPA) buffer supplemented with protease inhibitors. Lysates were incubated on ice for 1 h and clarified by centrifugation. Supernatants were precleared and incubated with anti-HDAC3 antibody at 4 °C overnight followed by 1-h incubation with Protein A–agarose beads at 4 °C. Immunoprecipitates were washed five times with modified RIPA buffer, eluted by boiling in SDS loading buffer and subjected to immunoblot analysis.

For the western blot of the total lysates, tissues were lysed in modified RIPA buffer supplemented with protease inhibitors, and the samples were resolved by Tris-glycine SDS-PAGE, then transferred to polyvinylidene fluoride (PVDF) membranes and blotted with the indicated antibodies. All antibodies were used at 1:1,000 to 1:5,000 dilutions.

HDAC activity assay. Immunoprecipitation with HDAC3 antibody and control IgG were performed as described above. Immunoprecipitates were washed two times with modified RIPA buffer and two times with 1× PBS, followed by HDAC assay according to the manufacturer’s instructions (Active Motif, 56200).

ChIP-seq. ChIP was performed independently on liver samples from four or five different mice. Detailed procedures were previously described16,18. High-throughput sequencing was done by the Functional Genomics Core (J. Schug and K. Kaestner) of the Penn Diabetes Research Center using the Illumina Genome Analyzer IIx, and sequence reads were mapped to the mm8 mouse genome by using ELAND pipeline. In each ChIP-seq sample, all the duplicate reads were removed except for one for each genomic position.

Computational analysis for HDAC3 peaks. Peak calling was carried out by using HOMER59 with a default option (FDR = 0.001) on Hdac3 wild type and NS-DADm samples against the matching input sample, and then 1 RPM cutoff was applied. In the case of wild-type samples, two replicates were pooled into one before the peak calling, so the maximum tag counts per position were set to be two (-tbp 2). HDAC3 binding sites were annotated with the following priorities: (1) pTSS (proximal promoter, from −1 kb to 100 b around TSS), (2) TTS (from −100 b to 1 kb around TTS), (3) exon, (4) intron, (5) dtSS (distal promoter, from −10 kb to +1 kb around TSS) and (6) intergenic (everything else). The heat maps of normalized HDAC3 binding profiles surrounding peak centers were generated by using the heatmap2 function in R package.

Quantitative PCR. Quantitative PCR was performed with Power SYBR Green PCR Master Mix and the PRISM 7500 instrument (Applied Biosystems), and analyses were performed by the standard curve method. Primer sequences are provided in Supplementary Table 1.

Tissue triglyceride and cholesterol. Liver samples were homogenized in tissue lysis buffer (140 mM NaCl, 50 mM Tris, 1% Triton-X-100, pH 8.0). Triglyceride and cholesterol concentrations in the lysates were then quantified by using LiquiColor Triglyceride Procedure No. 2100 (Stanbio) and Cholesterol LiquiColor Test (Stanbio), respectively.

Microarray. Total RNA was extracted from liver by using the RNasy tissue Mini kit (Qiagen) according the manufacturer’s instructions. Preparation of RNA for hybridization to Affymetrix MoGene 1.0 ST and scanning of the arrays were performed by the University of Pennsylvania Microarray Facility (https://somapps.med.upenn.edu/brb/portal/microarr/) according to the manufacturer’s instructions. Robust multiarray averaging (RMA) signal extraction, normalization and filtering were performed by the Microarray Facility’s bioinformatics group (http://www.bioinformatics.upenn.edu/index.html) using Partek Genomics Suite.

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