Class I and Ila Histone Deacetylases Have Opposite Effects on Sclerostin Gene Regulation

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Background: Gene regulation of the bone repressor sclerostin (SOST) is only poorly understood.

Results: SOST gene suppression by parathyroid hormone is partially mediated by HDAC5 inhibiting MEF2, and SOST gene expression requires class I HDAC activity.

Conclusion: SOST gene expression is negatively regulated by HDAC5 and positively by class I HDACs.

Significance: Class I HDAC inhibitors represent a novel approach for bone forming osteoporosis therapies.

Adult bone mass is controlled by the bone formation repressor sclerostin (SOST). Previously, we have shown that intermittent parathyroid hormone (PTH) bone anabolic therapy involves SOST expression reduction by inhibiting myocyte enhancer factor 2 (MEF2), which activates a distant bone enhancer. Here, we extended our SOST gene regulation studies by analyzing a role of class I and Ila histone deacetylases (HDACs), which are known regulators of MEF2s. Expression analysis using quantitative PCR (qPCR) showed high expression of HDACs 1 and 2, lower amounts of HDACs 3, 5, and 7, and no expression of HDACs 8 and 9 in constitutively SOST-expressing UMR106 osteocytic cells. PTH-induced Sost suppression was associated with specific rapid nuclear accumulation of HDAC5 and co-localization with MEF2s in nuclear speckles requiring serine residues 259 and 498, whose phosphorylations control nucleocytoplasmic shuttling. Increasing nuclear levels of HDAC5 in UMR106 by blocking nuclear export with leptomycin B (LepB) or overexpression in transient transfection assays inhibited endogenous Sost transcription and reporter gene expression, respectively. This repressor effect of HDAC5 did not require catalytic activity using specific HDAC inhibitors. In contrast, inhibition of class I HDAC activities and expression using RNA interference suppressed constitutive Sost expression in UMR106 cells. An unbiased comprehensive search for involved HDAC targets using an acetylome analysis revealed several non-histone proteins as candidates. These findings suggest that PTH-mediated Sost repression involves nuclear accumulation of HDAC inhibiting the MEF2-dependent Sost bone enhancer, and class I HDACs are required for constitutive Sost expression in osteocytes.

Sclerostin (SOST), encoded by the SOST gene, is a crucial inhibitor of bone formation that is exclusively secreted by osteocytes in adult bone (1). SOST deficiency leads to drastic high bone mass disorders as illustrated in the human loss-of-function hereditary disorders van Buchem disease and sclerosteosis. Similarly, Sost knock-out mouse models display enormously elevated bone mass and strength due to increased bone formation in the entire skeleton and throughout adult growth (2, 3). Conversely, transgenic mice overexpressing SOST have low bone mass and strength due to a decrease in bone forming osteoblasts (4, 5). SOST inhibits bone formation by antagonizing canonical Wnt signaling, which is required for normal osteoblastogenesis and control of osteoclastogenesis (6–9). It does so by binding to the Wnt co-receptors Lrp5 and -6 preventing their interaction with the Wnt-Frizzled receptor complex, which triggers Wnt signaling in target cells. We and others have shown that suppression of SOST expression is a key mechanism as to how intermittent parathyroid hormone (PTH) treatment leads to bone mass elevation (3, 10, 11). Furthermore, we and others have shown that SOST is a direct target gene of PTH (12, 11) and that PTH exerts its repressive effect by inhibiting myocyte enhancer factor 2 (MEF2) transcription factors, which are binding to a distant downstream SOST gene enhancer that is required for SOST expression in adult bone (4, 13). In agreement with an important role of MEF2s in SOST gene control and, thus, adult bone metabolism MEF2C was recently identified as one of 20 loci affecting bone mineral density in a meta-analysis of five genome-wide association studies of femoral neck and lumbar spine bone mineral density (14).

MEF2s are widely expressed regulators of cell differentiation and organogenesis, and are well known for their role in the development of skeletal muscle, heart, vasculature, neurons, and T-cells (15). Their role in the control of adult bone metabolism is only poorly understood so far. In vertebrates, there are four genes MEF2A, -B, -C, and -D, which are highly homologous in their MADS and MEF2 domains mediating DNA binding, dimerization, and co-factor recruitment, but are highly divergent in their C-terminal transcriptional activation domains. Our previous quantitative real-time RNA expression analyses indicated that Mef2a, -c, and -d, but not b are quantitatively PCR; SILAC, stable-isotope labeling with amino acids in cell culture; TSA, trichostatin A; TRITC, tetramethylrhodamine isothiocyanate.

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2 The abbreviations used are: SOST, sclerostin; EGFP, enhanced green fluorescent protein; HCA, high content analysis; HDAC, histone deacetylase; HDI, HDAC inhibitor; LepB, leptomycin B; MEF2, myocyte enhancer factor 2; PMA, phorbol 12-myristate 13-acetate; PTH, parathyroid hormone; qPCR, quantitative PCR; SILAC, stable-isotope labeling with amino acids in cell culture; TSA, trichostatin A; TRITC, tetramethylrhodamine isothiocyanate.
endochondral ossification via activation of Collagen 10

expressed in adult bone (13). Mef2c plays a crucial role in bone development by controlling chondrocyte hypertrophy during endochondral ossification via activation of Collagen 10 and Runx2 genes (16). The activity of MEF2s is controlled by a variety of signaling pathways including acetylation by p300, phosphorylation by mitogen-activated protein kinases, association with calcineurin-dephosphorylated NFAT, sumoylation by SUMO2 and -3, and interaction with class IIa histone deacetylases (HDACs) whose nuclear to cytoplasmic distribution is controlled by calcium-regulated protein kinases (15, 17–19). The first three mechanisms stimulate MEF2 activity, whereas the latter two are inhibitory. Inhibition of MEF2 activity by class IIa HDACs is well known in regulating cardiomyocyte hypertrophy (20). The inhibitory effect of class IIa HDACs on MEF2s does not involve their catalytic activity, but is due to the formation of a transcriptional repressor protein-protein complex involving the recruitment of class I HDACs such as HDAC3, which deacetylate MEF2s (21). The transcription inhibition activity of class IIa HDACs is regulated by nucleocytoplasmic shuttling (22). Upon stimulation by a variety of physiological signals, three conserved serine residues are phosphorylated leading to binding of the 14-3-3 chaperone protein, which induces nuclear export of HDACs and thereby derepression of target genes such as MEF2s.

The role of HDACs in bone metabolism is not well understood. There are 11 mammalian HDACs, which are grouped into four classes based on protein domain arrangement similarities (23). Class I comprises HDAC1, -2, -3, and -8, class IIa HDAC4, -5, -7, and -9, class IIb HDAC6 and -10, and class IV HDAC11. In vitro experiments using HDAC inhibitors (HDIs) have shown inhibition of osteoclasts differentiation (24–26) and stimulation of osteoclast apoptosis (27) suggesting that HDACs promote bone resorption. However, suppression of class I HDAC3 and class IIa HDAC7 in bone marrow stromal cells showed opposite effects on osteoclastogenesis indicating a possible functional difference between class I and class IIa HDACs in osteoclast differentiation (28). Moreover, HDIs were shown to stimulate osteoblast differentiation inducing Type I collagen, osteopontin, bone sialoprotein, and osteocalcin gene expression (29–31). A role of HDACs in osteocytes, the most abundant bone cell, has not been described so far. Overall, in vitro experiments suggest that HDACs inhibit bone formation and stimulate bone resorption although there may be opposing effects of HDAC isoforms. Complex effects of HDACs have also been described in bone metabolism in vivo. Rodent studies using the two clinically approved HDIs valproate and suberoylanilide hydroxamic acid showed negative effects on bone mass with reductions in bone mineral content, trabecular bone volume, bone formation, and osteoblast number (31). However, local increases in mineral apposition rates were observed in suberoylanilide hydroxamic acid-treated mice confirming in vitro studies that HDIs stimulate osteoblasts activity, but decrease osteoblast number and/or formation. Clinical findings with the two HDIs show a similar picture as seen in animals. Valproate, which is used a long time for the treatment of epilepsy and bipolar disease, has been associated with bone mineral density decrease in several studies (31). However, it is currently unclear how HDIs affect human bone remodeling leading to bone mass reductions due to inconsistent results of serum and urinary biomarkers for bone resorption and formation.

Here, we show roles of HDACs in regulating the expression of the key bone formation inhibitor SOST using the validated osteocytic UMR106 cell model. PTH-mediated Sost inhibition was accompanied by specific nuclear import of class IIa HDAC5 and association with MEF2 transcription factors. HDAC5 inhibited the SOST bone enhancer in a MEF2-dependent but catalytically independent manner. Finally, we report that class I HDACs 1, 2, and 3 are required for Sost expression suggesting opposing roles and a complex interplay of class IIa and class I HDACs in regulating Sost expression.

**EXPERIMENTAL PROCEDURES**

**Reagents and Cloning**—Human parathyroid hormone (hPTH) (1–34) was synthesized at Novartis. Leptomycin B, phorbol 12-myristate 13-acetate (PMA), trichostatin A (TSA), and apicidin were purchased from Sigma. Forskolin was from Biomol. The class II HDAC inhibitor compound 2 (32) was re-synthesized and its specificity validated at Novartis (Table 1). Human HDAC expression plasmids (pcDNA3.1 HDAC1, -2, -3, -4, -5, -6, and -10) were a generous gift from R. Vega (Novartis). All C-terminal green fluorescent protein (GFP)-tagged HDACs were in pEGFP-N1 (Clontech) except HDAC7, which was in pEGFP-N2 (Clontech). Luciferase reporter plasmids with the human SOST promoter alone or in combination with the SOST bone enhancer element have been described (13). siRNAs against rat Hdac2 and -3 and non-targeting negative control siRNAs were purchased from Dharmacon and siRNA against Hdac1 was bought from Qiagen. Cloning of the serine 259 and serine 498 to alanine HDACS double mutant was done by GenScript from our wild-type (wt) human HDAC5 plasmids. All other mutants were cloned according to standard procedures. V5-tagged HDAC5 expression plasmid (pHDC5-V5) was generated at Solvias by replacing the EGFP coding region of pHDAC5-EGFP with a V5-encoding oligonucleotide primer pair using XhoI and XbaI restriction sites and the following oligonucleotide pair: TCGAGGTTAAGCCTATCCCTAACCCTCTCCTCCTCGGATTCTTACTGTAAGAGCCCGCAGCGCGCGCGACT’ (forward) and CTAGAGTCGCGGCCC-GCCCGCTTTAGTGATACTCAGACCCGAGAGGAGGT-TAGGGATTAGCTTTACCC (reverse).

**Cell Culture and Gene Expression Analyses**—The UMR106 cell line was bought from ATCC and maintained in DMEM/F-12 (Invitrogen) containing 10% FCS (AMIMED), 1% non-essential amino acids (Invitrogen), 1% Glutamax (Invitrogen), and antibiotics. Total RNA was isolated using RNeasy (Qiagen) and reverse-transcribed into cDNA using the high-capacity cDNA archive kit (Applied Biosystems) according to the manufacturer’s recommendations. Quantitative real-time reverse transcription-PCR (qPCR) expression analysis was performed on an ABI Prism 7900 HT sequence detection system using TaqMan assays and the Universal PCR Master mixture all from Applied Biosystems. For Sost gene expression analysis following compound treatments UMR106 cells were seeded in 96-well plates (Nunc) at a density of 10,000 cells per well. The next day the culture medium was replaced with fresh culture.
medium containing the desired compounds and the cells were incubated for 4 h. Subsequently, Sost mRNA gene expression determinations were done using the FastLane One-Step QuantiTect Multiplex RT-PCR kit (Qiagen). The following rat TaqMan® assays were used: β2m (Rn00560865_m1) for normalization, Sost (Rn00577971_m1), Hdac1 (Rn01519307_g1), Hdac2 (Rn0193634_g1), Hdac3 (Rn01405468_m1), Hdac4 (Rn01427053_m1), Hdac5 (Rn01464251_g1), Hdac7 (Rn01533232_m1), Hdac8 (Rn01419050_m1), and Hdac9 (Rn01769547_m1 and Rn01769541_m1).

High Content Analysis (HCA)—UMR106 cells were seeded in 96-well plates (PerkinElmer Life Sciences) at a density of 5,000 cells per well. The next day, cells were transfected with 40 ng of GFP-HDAC or FLAG-tagged HDAC expression plasmids using FuGENE6 (Roche Applied Science) and OptiMEM (Invitrogen). 24 h later, cells were treated with compounds (n = 3–4) before fixation with 3.7% formalin (Sigma). Nuclei were stained with DAPI (Sigma). Intracellular HDAC distribution was measured using a Cellomics Array Scan II system with a ×20 objective and the cytoplasm to nucleus translocation BioApplication. The individual ratios of the nuclear to cytoplasmic signal (CircRingAvgIntrnRatioCh2) were determined for transfected cells (n > 100) and averages were calculated. FLAG-tagged HDAC immunostaining was performed using mouse anti-FLAG M2 monoclonal antibody (Sigma) at 1:1,000 dilution and Alexa Fluor® 488 goat anti-mouse IgG (H+L) (Invitrogen) at 1:500 dilution. HCA of FLAG-tagged HDACs was performed as with GFP-tagged samples.

Confocal Microscopy—UMR106 cells were seeded in 8-well μ-slide (ibidi GmbH) at a density of 15,000 cells per well. Transfection and treatments was done for HCA. Cells were permeabilized with phosphate-buffered saline (PBS) containing 0.2% Triton X-100 (Fluka) before blocking with the same solution containing 5% goat serum (Jackson ImmunoResearch). The cells were then first incubated with rabbit polyclonal anti-MEF2 antibody (Santa Cruz sc-10794 at 0.2 μg/μl) at 1:100 in blocking solution and then with TRITC-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch) at 1:200 in blocking solution containing DAPI (Sigma). Confocal microscopy was done with a LSM710 Axio observer inverted confocal microscope using ZEN 2009 Light Edition software (Zeiss).

Chromatin Immunoprecipitation—UMR106 cells were grown in 15-cm cell culture dishes until 80–90% confluence. After medium change, cells were transfected overnight with HDAC5-V5 expression plasmid using Lipofectamine LTX Plus (Invitrogen) followed by stimulation of one dish with 100 nM hPTH or solvent control for 1 h in fresh growth medium. ChiP was basically done using the SimpleChiP Enzymatic Chromatin IP kit with magnetic beads (Cell Signaling Technology). Chromatin shearing was performed using an S-220 ultrasonicator (Covaris). Samples were immunoprecipitated with a polyclonal rabbit anti-V5 tag antibody (Abcam) or control rabbit IgG (Cell Signaling Technology) and bound Sost enhancer DNA was quantified by quantitative real-time PCR using the following primers: CTCTGATGTTCCCCAAACC (forward) and AGAAACTGGGCCAGTGTGC (reverse).

Reporter Gene Assays—UMR106 cells were seeded in 48-well plates (Nunc) at a density of 20,000 cells per well. The next day, cells were transfected with 40 ng of reporter plasmid, 2 ng of HDAC expression plasmid, and 50 ng of CMV β-Gal plasmid using FuGENE6 (Roche Applied Science) and OptiMEM (Invitrogen). After 24 h the cells were lysed with passive lysis buffer (Promega) and luciferase activity was measured using a Mithras CB 940 multimode reader (Berthold) and luciferase assay reagent (Promega).

HDAC Inhibition Assays—Compound dilutions in DMSO were incubated with human HDACs1, -3, -4, -5, and -6 catalytic domains (final protein concentration 1.65 ng/ml) in assay buffer (25 mM Tris/Cl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl2, 0.1 mg/ml of BSA, 10 μM Ac-Gly-Ala-Lys(TFA)-AMC substrate solution and incubated for 1 h at 37 °C. The reaction was stopped by adding a solution of 10 μM TSA and trypsin (0.02 mg/ml). The concentration of the cleaved AMC substrate was measured by fluorescence readings at 360 nm excitation and 460 nm emission.

siRNA Transfections—siRNA stock solutions of 12.5 ng/μl were prepared in siRNA dilution buffer (Qiagen). 10,000 cells per well in 96-well plates (Nunc) were transfected directly upon seeding with 18.75 ng of siRNA using HiPerFect transfection reagent (Qiagen) diluted in OptiMEM (Invitrogen). After 24 h, medium was exchanged and 48 h later gene expressions were determined using the FastLane One-Step QuantiTect Multiplex RT-PCR kit (Qiagen).

HDAC Assays—Total activity of HDAC class I and II enzymes was measured using the HDAC-Glo I/II Assay and Screening System (Promega). UMR106 cells were seeded at a density of 10,000 cells per well in 96-well plates. The next day, cells were incubated with 1 μM PTH or 10 μM apicidin for 60 min before luciferase activity was measured according to the manufacturer’s instructions.

Whole Cell Acetylome Analysis—Global acetylation changes in response to the HDI apicidin were determined by stable-isotope labeling with amino acids in cell culture (SILAC) using the SILAC™ Protein ID and Quantitation Media Kit (Invitrogen) according to the manufacturer’s instructions. UMR106 cells were either grown in medium with unlabeled Lys and Arg (light sample) or in the presence of [13C6]Lys and [13C6,15N4]Arg (heavy sample). Cells grown in heavy medium served as control, whereas cells grown in light medium were treated with either 1 μM apicidin or 100 nM PTH for 4 h before lysis. Enrichment of acetyllysine peptides using a pan-acetyllysine antibody was performed as published (33) with minor modifications. Briefly, cells were lysed by sonication in a lysis buffer containing 20 mM Heps, pH 8, and 8 mM urea. After centrifugation, the lysate was diluted to 2 M with 20 mM Heps and trypsinized overnight, using a trypsin to protein ratio of 1:50. Digestion was stopped by the addition of 1% trifluoroacetic acid, after checking by SDS-PAGE. After desalting on SepPak cartridges (Waters), acetyllysine-containing peptides were enriched by immunoaffinity chromatography as described (33). The enriched fraction was desalted on SepPak and lyophilized before further fractionation by hydrophilic interaction chromatography. Peptide mixtures were resuspended in 80% acetonitrile/H2O and separated on a TSK-Amide column (250 × 4 mm), using a linear gradient from 80% acetonitrile/H2O to 60/40 acetonitrile/H2O at a flow rate of 0.5 ml/min. Fractions of
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FIGURE 1. Expression of class I and IIa Hdacs in UMR106 cells. RNA expressions of class I and IIa Hdacs were determined by qPCR. Relative expression levels are expressed as the mean ± S.E. of two independent experiments. Expression of Hdac3 was arbitrarily taken as 100%.

RESULTS

Expression of Class I and IIa HDACs in UMR106 Cells—Class IIa Hdacs and Hdac3 expressions were determined in UMR106 cells because these HDACs are known repressors of MEF2s (15, 17, 21), and, thus, we hypothesized that they could be involved in the inhibition of MEF2s by PTH for controlling Sost expression in osteocytes. UMR106 cells are a valid cell model to study Sost gene suppression involving nuclear accumulation of these HDACs. UMR106 cells were transfected with expression plasmids for GFP-HDAC fusion proteins and the effect of PTH on their subcellular distributions was determined using fluorescence microscopy and HCA. Microscopic analysis of non-treated cells showed considerable cell-to-cell variation, but in general HDAC3 and -5 were predominantly nuclear, HDAC4 mostly in the cytoplasm and HDAC7 in both compartments (Fig. 2A). Quantitative analysis using HCA confirmed these differences as shown in Fig. 2B. Upon PTH treatment, only HDCA5 showed a clear change in subcellular distribution by further increasing nuclear localization to become exclusively nuclear (Fig. 2, A and B). Time course analysis using HCA showed that PTH rapidly induced nuclear accumulation of HDCA5 reaching a plateau after about 20 min (Fig. 2D). In contrast, nucleocytoplasmic distributions of HDAC3, -4, and -7 were only marginally affected by PTH in agreement with non-quantitative microscopic analysis. HDAC4 and -7 showed a continuous small nuclear increase, whereas HDAC3 slowly increased in the cytoplasm. Intracellular distribution was also analyzed for HDAC1, -2, -6, and -10. HDAC1 was predominantly nuclear, whereas HDACs 2, 6, and 10 were predominantly cytoplasmic (Fig. 2C). Neither showed marked changes in subcellular distribution upon PTH treatment, which is consistent with the fact that these HDACs have not been reported to shuttle between the cytoplasm and nucleus. HDACs are well known as transcription regulators in the nucleus. However, functional roles have also been described in the cytoplasm such as transcription regulators in the nucleus. However, functional roles have also been described in the cytoplasm.
as regulation of translation (38). In addition to inducing nuclear localization of HDAC5, PTH led to the appearance of many distinct nuclear spots suggesting further compartmentalization of HDAC5 within the nucleus (Fig. 2A). In summary, PTH leads to a specific rapid and strong nuclear localization of HDAC5 and the formation of distinct subnuclear speckles suggesting a role in repressing MEF2-mediated SOST gene expression.

**LepB Induces Nuclear Accumulation of HDAC5 and Inhibits SOST Expression**—Having shown that PTH-mediated Sost expression inhibition in UMR106 cells is associated with specific nuclear accumulation of the MEF2 inhibitor HDAC5, we analyzed whether there is a causal relationship. To this end, we induced nuclear accumulation of HDAC5 by inhibiting its nuclear export and analyzed the effect on endogenous Sost expression. HDAC5 is known to be exported by the specific export receptor CRM1 (39), which can be inhibited by the specific antagonist leptomycin B (40). Leptomycin B (LepB) treatment led to strong nuclear accretion of HDAC5 (Fig. 3A) surpassing PTH-induced nuclear accumulation. Addition of PTH to LepB showed a minimal further nuclear accumulation. Next, we analyzed the effect of blocking nuclear import on HDAC5 subcellular distribution. PMA blocks nuclear import by repressing the nuclear localization signal activity (41). Consistently, it led to strong nuclear depletion of HDAC5 (Fig. 3, A and B). Interestingly, PTH was not able to override the action of PMA demonstrating that import inhibition by PMA is dominant over nuclear accumulation by PTH. We also investigated whether a PTH-mediated nuclear increase of HDAC5 involved the cAMP pathway as previously shown to be involved in Sost repression (12). The cAMP stimulator forskolin fully mimicked
strong nuclear accretion of HDAC5 as observed with PTH (Fig. 3A). Next, we analyzed the effect of LepB-induced HDAC5 nuclear accumulation on endogenous Sost expression in UMR106 cells. LepB inhibited Sost expression in a time- and dose-dependent manner (Fig. 3, C and D). LepB inhibited Sost expression with an IC$_{50}$ of 1.2 nM corresponding to inhibition of CRM1 export receptor (42, 43). Maximal repression of about 60% was reached after 4 h. In summary, these data suggest a causal role of HDAC5 nuclear accumulation in PTH-mediated Sost repression.

**Mutational Analysis of HDAC5 Shuttling**—Serine residues 259 and 498 are critically involved in the regulation of HDAC5 nuclear export (44). Thus, we have analyzed their role in the regulation of HDAC5 subcellular distribution by PTH in UMR106 cells. Mutation of serine 498 to alanine did not significantly change basal and PTH-induced nuclear export compared with wt HDAC5 (Fig. 4A). In contrast, mutation of serine 259 to alanine resulted in an increased basal nuclear localization, which was increased by PTH to a similar level as seen with wt HDAC5. When both serine residues were mutated to alanines (S2A) full nuclear accumulation was observed of this mutant HDAC5 independent of PTH. Next, we analyzed whether MEF2 binding interferes with nuclear export of HDAC5. A MEF2-binding deficient HDAC5 mutant (dMEF2) was created by mutating lysine 184 and arginine 186 to alanines as reported (45). In addition, we generated the double mutant S2A-dMEF2. As shown in Fig. 4A, basal and PTH-induced subcellular distributions of the dMEF2 mutant were not significantly different from those of wt HDAC5, whereas distributions of S2A-dMEF2 were very similar to those of the S2A mutant suggesting that MEF2 binding is not involved in the control of nuclear export of HDAC5. Mutants were also tested for their behavior in blocking HDAC5 nuclear import by PMA, which we showed to be dominant over sup-

![FIGURE 3. The nuclear export inhibitor LepB inhibits Sost expression. A, regulation of HDAC5-cytoplasmic shuttling by LepB, PMA, and forskolin. UMR106 cells were preincubated for 30 min with 40 nM LepB, 100 nM PMA, 10 μM forskolin or solvent control (−) and then stimulated with 100 nM PTH or solvent control for 1 h followed by HCA of HDAC5 nucleocytoplasmic distribution. Shown are mean ± S.E. of three independent experiments normalized to control. B, representative image of PMA-induced HDAC5-GFP nuclear export. Time (C)- and dose (D)-dependent inhibition of Sost expression by LepB. UMR106 cells were incubated with LepB as indicated in the figure and subsequently Sost expression was analyzed by qPCR. Sost expression levels were normalized to β2-microglobulin expression. Shown are relative Sost expression level mean ± S.E. of two independent experiments.]

![FIGURE 4. Effect of PTH and PMA on HDAC5 shuttling mutants. A, intracellular localizations of wt HDAC5-GFP and the mutants serine 498 to alanine (S498A), serine 259 to alanine (S259A), both serine to alanine (S2A), lysine 184 and arginine 186 to alanine (dMEF2), and all four residues to alanines (S2A-dMEF2) were analyzed after transfection of the corresponding expression plasmids into UMR106 cells using HCA. Transfected cells were incubated with solvent control, 100 nM PTH (A), or 100 nM PMA (B) for 1 h. Shown are the relative mean ± S.E. of at least four experiments normalized to untreated wt HDAC5.]

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pressing export by PTH (Fig. 3A). Both single serine mutants (S498A and S259A) were equally well prevented from nuclear import as wt HDAC5 (Fig. 4B). In contrast, the double mutant S2A was accumulated in the nucleus and this could not be inhibited by PMA. In summary, HDAC5 mutational analysis suggests that PTH-mediated export inhibition mainly involves serine 259, but in addition requires serine 498 for full effect. In contrast, one of the two serine residues is sufficient to confer suppression of nuclear import by PMA.

Nuclear Accumulation of HDAC5 Leads to Speckle Formation and Co-localization with MEF2—Confocal microscopy was used to analyze the subnuclear localization of HDAC5 and association with MEF2. Endogenous MEF2 transcription factors and transfected HDAC5-GFP showed a diffuse nuclear distribution (Fig. 5A). HDAC5-GFP, but not MEF2, was also weakly detected in the cytoplasm. PTH treatment led to complete nuclear localization of HDAC5 and a drastic change in subnuclear localization in the form of speckles (Fig. 5B), which was already hinted at with conventional microscopy (Fig. 2A). Interestingly, PTH also induced speckle formation of MEF2 that co-localized with HDAC5 speckles. Nuclear accumulation of HDCA5 by LepB or using the S2A mutant produced the same nuclear speckle formation and co-association with MEF2 (Fig. 5, C and D). In contrast, the MEF2 binding-deficient HDAC5 mutant (dMEF2) formed PTH-induced nuclear speckles as wt HDAC5, but did not induce co-localization of MEF2 (Fig. 5E) suggesting that HDAC5 recruits MEF2 to nuclear speckle domains. Finally, we used ChIP to directly analyze the effect of PTH on Sost enhancer chromatin binding by HDAC5. UMR106 cells were transfected with V5-tagged HDAC5, followed by treatment with PTH or solvent for 1 h. Subsequently, we immunoprecipitated with anti-V5 or control IgG and used qPCR to measure HDAC5-V5 association with the Sost bone enhancer. PTH massively induced HDAC5-V5 chromatin binding (Fig. 5F). These results further support the conclusions that PTH induces nuclear accumulation of HDAC5 and binding to the Sost bone enhancer in association with MEF2.

HDAC5 Inhibits SOST Bone Enhancer Activity through MEF2—Previously, we have shown that MEF2 activates SOST bone enhancer activity that is required for SOST expression in adult bone (13). Thus, we have analyzed the effect of varying levels of nuclear HDAC5 on SOST enhancer activity using reporter gene assays. Overexpression of wt HDAC5 had little effect on activity of the SOST promoter, but decreased SOST enhancer activity by 39% (Fig. 6). The nuclear-enriched S2A HDAC5 mutant inhibited SOST enhancer activity much stronger (64%) and again affected SOST promoter activity only marginally. In contrast, HDAC5 mutants deficient of Mef2 binding (HDAC5-dMef2 and HDAC5-S2A_dMef2) only marginally inhibited SOST bone enhancer activity. These data suggest that nuclear HDAC5 inhibits SOST bone enhancer activity by masking MEF2.

Pharmacological Inhibition of HDACs—To investigate whether HDAC5 catalytic activity is involved in Sost gene regulation, UMR106 cells were treated with a series of HDIs analyzing their effect on Sost expression. First, we tested the specific and potent class II HDI compound 2 (32) inhibiting HDAC4 and -5 catalytic activities with IC_{50} values in the lower nanomolar range. Compound 2 was re-synthesized and its specificity confirmed (Table 1). It only weakly inhibited Sost expression at 10 μM (Fig. 7A) and did not relieve PTH-induced SOST repression (data not shown) indicating that HDAC5 catalytic activity is not required for its regulation of Sost expression. In contrast, the pan-HDI TSA (46, 47) very potently inhibited endogenous Sost expression >95% with an IC_{50} of 2.4 nm suggesting that Sost expression may depend on class I HDAC catalytic activity. To corroborate and further analyze the requirement of class I HDAC isoform activities for Sost expression we tested the specific class I HDAC inhibitor apicidin (46 – 48). Apicidin efficiently inhibited Sost expression >95% with an IC_{50} of 24 nm. Because controversial data have been reported on inhibition of HDAC1 by apicidin (46, 48), we further used HDI MS-275, which preferentially inhibits HDAC1 (46, 47, 49, 50). In contrast to TSA and apicidin, MS-275 did not inhibit SOST expression, but showed a bell-shaped about 2-fold stimulation with a maximum at 1 μM. A similar response was obtained with MGCD0103 (51, 52), another potent HDAC1 inhibitor (data not shown). As the class I HDAC8 is not expressed in UMR106 cells (Fig. 1), these data suggest that Sost expression requires HDAC2 and -3 catalytic activities. Overexpression of HDACs2 and -3 did not significantly affect SOST promoter and enhancer activities indicating that their activities are not limiting in UMR106 cells (data not shown). In summary, these data suggest that the catalytic activity of HDCA5 is not required for its role in PTH-mediated Sost repression. In contrast, catalytic activities of HDACs 2 and 3 appear to be required for Sost expression in UMR106 cells.

siRNA-mediated Knock-down of Class I HDACs—Having shown that Sost expression requires class I HDAC catalytic activities we analyzed the role of class I HDAC isoforms in Sost expression by siRNA-mediated knock-down (Fig. 7B). UMR106 cells were transfected with siRNAs against class I Hdac1, 2, and 3, which are expressed in these cells (Fig. 1). Specific down-regulation of the corresponding genes was observed achieving reductions of 85% for Hdac1, 71% for Hdac2, and 54% for Hdac3 expressions. Hdac1, -2, and -3 knock-down led to SOST expression inhibitions of 39, 21, and 20%, respectively. A combination of Hdac2 and -3 siRNAs resulted in an additive Sost expression inhibition of about 40%, and a synergistic inhibition of 79% was observed when all three siRNAs were combined. These results suggest all three Hdac1, -2, and -3 isoforms are required for full Sost expression in UMR106 cells.

Acetylome Analysis—Having shown that HDAC2 and -3 catalytic activities are required for Sost expression in UMR106 cells we performed an acetylome analysis to elucidate potential target proteins using the specific HDI apicidin. It inhibited global cellular HDAC activity >97% (Fig. 8). In contrast, PTH did not significantly change overall cellular HDAC activity. To identify potential HDAC targets we preformed large scale mapping of lysine acetylation sites and measured acetylation changes in response to the HDAC inhibitor apicidin using immunofinity enrichment of acetylated peptides and SILAC-based quantitative mass spectrometry (33). As expected, apicidin treatment resulted in significantly increased acetylation levels (Fig. 9B), whereas there was little effect on overall protein levels judging from the distribution of all peptides, acetyllysine and non-
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A

MEF2
HDAC5-GFP
Overlay

B

C

D

E

F

% Input

0.5%

0.4%

0.3%

0.2%

0.1%

0.0%

anti-V5

IgG control

- PTH
- Solvent
acetylysine-containing peptides, which co-purified (Fig. 9A). Because of the asymmetric distribution of the acetylysine-peptides, the ratio distribution of all peptides was used to determine the cutoffs for significance (Fig. 9A). Based on these cutoffs, apicidin treatment triggered increased acetylation of 40 different peptides (Table 2). As expected and confirming inhibition of HDAC activities, many hyper-acetylated proteins were histones and histone-related proteins. These proteins are unlikely to be specifically involved in Sost gene regulation. The non-histone proteins with increased acetylation upon apicidin treatment are listed in Table 3. These 12 proteins are potential targets of HDAC2 and -3 in UMR106 cells that might play a role in Sost gene regulation. In summary, we identified a number of non-histone proteins as potential targets for HDAC2 and -3-mediated Sost gene expression stimulation.

**DISCUSSION**

Osteocyte-secreted sclerostin is a key repressor of bone formation. Its absence leads to massive bone overgrowth of the whole skeleton as observed in sclerosteosis and Van Buchem disease patients. On this basis, novel bone anabolic osteoporosis therapies are being developed, for example, by suppressing the activity of sclerostin with antibodies (53). With the exception of daily PTH injections there are currently no treatments available for the restoration of lost bone in severe osteoporosis patients. Interestingly, stimulation of bone formation by intermittent PTH involves suppression of Sost expression as an important mechanism further portending to the sclerostin pathway as a central control mechanism in adult bone metabolism (10, 12). Despite its significance in bone mass control regulation of SOST expression is only poorly understood. Previously, we have shown that PTH down-regulation of Sost expression is mediated by interfering with a crucial MEF2 binding site in the distant bone enhancer of the SOST gene (13). Our present results further elucidate the molecular mechanisms of SOST gene control by uncovering a PTH-induced repressive role of class Ila HDAC5 acting on MEF2 transcription factors and an unanticipated stimulatory role of class I HDACs catalytic activity for which we identified potential targets using a comprehensive acetylamine analysis. Our study is the first report suggesting a role of HDACs in osteocytes to regulate adult bone metabolism via controlling SOST gene expression. In addition, we demonstrate opposite effects of class I and Ila HDACs on Sost gene regulation and consequently on adult bone mass control via the same pathway. Expression analysis by qPCR showed expression of several members of class I and Ila HDACs in osteocytic UMR106 cells, but only HDAC5 showed nuclear accumulation upon PTH treatment. In addition to nuclear buildup, PTH induced localization of HDAC5 to a multitude of small subnuclear bodies and recruitment of MEF2s to the same compartments. Similar structures called nuclear speckles have been described for the recruitment of HDAC4 and -5 to chromatin-bound MEF2C and nuclear receptors by the nuclear corepressor SMRTe (54). Whereas SMRTe similarly recruited both HDAC4 and -5 into the nucleus for repression of target transcription factors such as MEF2s, we observed selective employment of HDAC5 by PTH for Sost gene repression. HDAC4 was expressed at much lower levels in UMR106 cells, was mainly located in the cytoplasm, and was not imported into the nucleus by PTH. Furthermore, increasing nuclear levels of HDAC5 by overexpression or by blocking its nuclear export corepressor CRM1 with LepB inhibited expression in SOST reporter gene assays and the endogenous Sost gene, respectively. Finally, we observed massive recruitment of HDAC5 to the MEF2 response element-containing Sost bone enhancer region using ChIP. These results strongly suggest a critical co-repressor role of HDAC5 in SOST gene regulation and, thus, bone mass control. This is also supported by the identification of HDAC5 as one of 20 bone mineral density loci in a meta-analysis of five genome-wide association studies of femoral neck and lumbar spine bone mineral density in almost 20,000 subjects (14) and by the discovery that microRNA-2861-mediated HDAC5 repression regulates osteoblast differentiation and bone mass in humans (55). Interestingly, HDAC4, but not
Opposite SOST Gene Regulation by Class I and IIa HDACs

other class IIa HDACs, are critically involved in limiting chondrocyte hypertrophy during bone development demonstrating a functional specification of class IIa HDACs in controlling bone development and mature bone homeostasis (56). The transcriptional activity of class IIa HDACs is regulated by dynamic nucleocytoplasmic shuttling (44). Our demonstration of increased nuclear HDAC5 levels by blocking its export receptor CRM1 with LepB and decreased nuclear HDAC5 levels by inhibiting nuclear import with PMA support such an active equilibrium of cytoplasmic and nuclear HDAC5 in UMR106 cells. This is further supported by the rapid disappearance of PTH-induced nuclear accretion of HDAC5 within 10 min when PTH was washed out (data not shown). Consistent with our earlier report that Sost gene repression by PTH involves the cAMP pathway, PTH-induced HDAC5 nuclear accumulation was fully mimicked by cAMP induction with forskolin suggesting an important role for nuclear HDAC5 in PTH-induced Sost gene repression. However, despite inducing higher nuclear accumulation of HDAC5 than with PTH LepB maximally suppressed SOST expression only by 60–70% (Fig. 3C) compared with 90% by PTH (12). Thus, there may be further mechanisms of PTH-mediated SOST gene repression independent of HDAC5 nuclear accumulation, which remain to be uncovered.

Two conserved serine residues in the N-terminal half of HDAC5 provide phosphorylation sites for protein kinase D and calcium/calmodulin-dependent protein kinase-mediated regulation of nucleocytoplasmic shuttling (17, 44). HDACs phosphorylated at these sites are bound by the chaperone protein 14-3-3, which frees HDAC-bound MEF2s and induces nuclear expulsion by uncovering a nuclear export sequence that is recognized by the export receptor CRM1. We have observed that the two serine residues are also critically involved in nuclear-cytoplasmic localization of HDAC5 in UMR106 cells. When both were mutated to alanines HDAC5 was fully accumulated in the nucleus as seen after PTH treatment. However, in contrast to the reported results obtained in COS cells (44) Ser-498 alone had little effect on shuttling, whereas Ser-259 alone was already responsible for about half of the effect suggesting that it

FIGURE 7. The class I HDACs 1, 2, and 3 are required for Sost expression. A, dose-response curves of HDIs compound 2, TSA, apicidin, and MS-275. UMR106 cells were incubated with increasing concentrations of HDIs for 4 h followed by Sost expression analysis using qPCR. Shown are mean ± S.E. of two independent experiments. B, siRNA-mediated knock-down of Hdadcs 1, 2, and 3 and effect on Sost expression. UMR106 cells were transfected upon seeding with individual HDAC siRNAs or combinations thereof as indicated in the figure. After 72 h, gene expression levels of Hdad1, -2, -3, and Sost were determined using qPCR. Expressions were normalized to β2-microglobulin expression and non-targeting control siRNA, which was set at 100%. Shown are mean ± S.E. of at least 2 independent experiments.

FIGURE 8. Global cellular HDAC activity is suppressed by apicidin but not by PTH. Overall relative HDAC activities were determined in UMR106 cells. Cells were treated with solvent control (○), 1 μM PTH, or 10 μM apicidin for 1 h before measuring total HDAC activities using a luciferase reporter kit. Shown are mean ± S.E. of a representative experiment.
plays a dominant role in PTH-mediated control of HDAC5 subcellular trafficking in osteocytes. A number of kinase pathways have been reported to be involved in the phosphorylation of class IIa HDACs in different tissues and in response to a variety of stimuli (22). In addition, myosin phosphatase and protein phosphatase 2A have been described to dephosphorylate class IIa HDACs. Despite this plethora of pathways there are examples of distinct pathways regulating specific HDACs suggesting high specificity for particular physiological signals in particular tissues. For example, protein kinase D and microtubule affinity-regulating kinases are specifically involved in HDAC-mediated regulation of cardiac growth and remodeling genes in pathological myocyte hypertrophy (20). Furthermore, CaMKII specifically phosphorylates HDAC4, but not HDAC5, -7, and -9, in this process. The signaling pathways involved in PTH-mediated regulation of HDAC5 nucleocytoplasmic shuttling in osteocytes remain to be elucidated. A likely pathway candidate is the one involved in PTH-related peptide-mediated inhibition of chondrocyte hypertrophy (57). It involves activation of protein phosphatase 2A, which dephosphorylates HDAC4 leading to repression of MEF2C-mediated collagen X expression. Activation of protein phosphatase 2A was proposed to occur by cAMP-stimulated PKA. However, we have not observed an inhibition of Sost repression by PTH using the specific PKA inhibitor H89 or the protein phosphatase 2A inhibitor calyculin A (data not shown). Thus, comprehensive genomics and proteomics approaches will be required to uncover the pathway that signals from PTH-induced cAMP to nuclear accumulation of HDAC5 in osteocytes. Class IIa HDACs nucleocytoplasmic shuttling is also regulated at nuclear import. PMA was shown to inhibit HDAC4 nuclear import by repressing nuclear localization signals also involving the chaperone protein 14-3-3 (41). Our results showing cytoplasmic accumulation of HDAC5 by PMA are consistent with this observation. Along the same line, elimination of the two crucial phosphoserine binding sites for 14-3-3 prohibited PMA action. In addition, we have investigated the individual contribution of the two serine residues and demonstrate that either of them is enough to confer full cytoplasmic retention by PMA. These data suggest that the binding requirements of 14-3-3 to HDACs are diverging between its roles in nuclear export and import. Moreover, we observed that HDAC5 nuclear import inhibition by PMA was dominant over PTH-induced nuclear accumulation. Obviously, inhibition of HDAC5 nuclear export by PTH cannot lead to nuclear accumulation if nuclear import of cytoplasm-produced HDAC5 is prevented in the first place. However, this result also suggests that if PTH stimulated nuclear import of HDAC5 it would have to act more upstream than PMA. The signaling pathways regulating class IIa HDACs nuclear import are only poorly understood. The action of PMA appears to be mediated by PKCδ and PKCe, but not PKCα, however, not acting directly on HDACs (41).

Our results using the specific class II HDI compound 2 (32) suggest that HDAC5 catalytic activity is not required for PTH-mediated inhibition of Sost expression. This is consistent with a large body of evidence showing that class IIa HDACs repress target transcription factors by direct protein-protein binding and recruitment of co-repressors such as SMRT/N-CoR independently of their inefficient catalytic activity (17, 22). Surprisingly, the pan-specific HDI TSA fully suppressed Sost expres-
### TABLE 2

Peptides with increased acetylation based on log2 ratio significance cutoffs — 0.44 and 1.04 from all peptides

| IPI accession | Gene Names | Protein Description | Acetyl (K) Probabilities | Number of Acetyl (K) | Log2Ratio acpidelin/ control |
|---------------|------------|---------------------|--------------------------|---------------------|-----------------------------|
| IP00036308.1  | H2a/hist1h2b | 15 kDa protein, histone cluster 1, H2a/histone H2B, histone cluster 1, H2b;15 kDa protein, histone cluster 3, H2b;15 kDa protein, histone cluster 1, H2b;15 kDa protein, histone cluster 1, H2b | K(0.096)GSK(0.996)K(1)AVTK | 2 | 2.09 |
| IP00038309.2 | Hist1h2bbl | 15 kDa protein, histone H2B, various isoforms | GLGK(1)GAGK(1)R | 2 | 1.83 |
| IP00054053.1 | Hist1h2bbl | 15 kDa protein, histone H2B, various isoforms | GLGK(1)GAGK(1)R | 2 | 1.83 |
| IP00054054.1 | Hist1h2bbl | 15 kDa protein, histone H2B, various isoforms | GLGK(1)GAGK(1)R | 2 | 2.32 |
| IP00076428.1 | H3T3b/H3b.3b | similar to H3 histone, family 2 isoform 2;Histone H3.3;Histone H3.1;15 kDa protein;similar to CG31613-PA;histone cluster 2, H3c2;19 kDa protein;15 kDa protein | K(1)QATK(1)AAR | 2 | 2.64 |
| IP00054061.1 | Hist1h2bbl | histone H2B, various isoforms | K(0.096)GSK(0.996)K(1)AVTK | 2 | 2.67 |
| IP00056081.8 | Hist1h2bbl | 15 kDa protein, similar to Histone H2B 291B | PETA(1)SAPAPK | 2 | 2.77 |
| IP00036188.7 | Hist1h2bbl | 15 kDa protein, histone H2B, various isoforms | PETA(1)SAPAPK | 2 | 3.03 |
| IP00054041.1 | Hist1h2bbl | histone H2B, various isoforms | PETA(1)SAPAPK | 2 | 3.03 |
| IP00036188.7 | Hist1h2bbl | histone H2B, various isoforms | PETA(1)SAPAPK | 2 | 3.03 |
| IP00056041.1 | Hist1h2bbl | histone H2B, various isoforms | PETA(1)SAPAPK | 2 | 3.03 |
| IP00056041.1 | Hist1h2bbl | histone H2B, various isoforms | PETA(1)SAPAPK | 2 | 3.03 |

Acetyl (K) probabilities display the sequence with site assignment probabilities following each Lys residue (between 0 and 1). The sum of the probabilities equals the total number of acetyllysines in the peptide, i.e. the value in the column. Number of acetyl (Lys) shows the number of acetylated lysines in the peptide.
tion in UMR106 cells like PTH indicating a positive effect of class I HDACs in Sost expression. HDAC I activities do not appear to be limiting in UMR106 cells as overexpression of HDACs 1–3 did not increase Sost expression. Consistently, we found high expression levels of HDAC1 and -2. Further analysis using class I subtype-specific HDIs combined with our HDAC expression analysis suggest that the catalytic activity of HDAC2 and -3, but not 1 and 8 are required. Gene expression knockdown experiments using RNA interference confirmed the stimulatory role of HDAC2 and -3, but showed an additional role for HDAC1. These data suggest that all three class I HDACs expressed in UMR106 cells are required for Sost expression, but only the catalytic activity of HDAC2 and -3. Direct deacetylation of MF2D by HDAC3 has been reported (21). However, this resulted in a repression of MF2D-dependent transcription and inhibition of myogenesis. Thus, it remains to be analyzed how HDAC1, -2, and -3 activate Sost transcription and whether it involves direct actions on MEF2s. To identify putative targets for HDAC1, -2, and -3 regulating Sost expression in osteocytes we did a comprehensive acetylome analysis. The specific HDAC2, -3, and -8 inhibitor apicidin almost completely suppressed HDAC activity in UMR106 cells. Because we could not detect HDAC8 expression in these cells this suggests that HDAC2 and -3 are required for Sost expression. PTH had no effect on overall HDAC activity. This is consistent with our findings that PTH-induced Sost gene repression by HDAC5 did not require catalytic activity of HDAC5. Furthermore, we did not observe differences in protein acetylation by PTH treatment. Analysis of acetylated proteins after apicidin treatment revealed a number of non-histone proteins as promising candidates for regulating Sost expression by HDAC2 and -3. Most of them are associated with transcription regulation. Brpf1, Brpf3, Ep300, Ing4, and Meaf6 are components of chromatin remodeling and transcription co-activator multiprotein complexes with histone acetyltransferase activities. Hmga1 and Nudt21 are involved in RNA processing, and Mll3 possesses histone methyltransferase activity.

A specific role of these candidates in Sost gene regulation remains to be investigated. Interestingly, our acetylation analysis did not detect MEF2s as putative targets indicating that they are not involved in Sost gene regulation by HDAC2 and -3 actions. Thus, it will be interesting to elucidate the upstream signaling pathways and downstream transcription co-factors that regulate Sost gene expression by class I HDACs. Last but not least, class I HDIs and particularly, specific HDIs of HDAC2 and -3 represent a novel approach for the development of novel bone forming osteoporosis therapies.

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