Bone is a highly metabolic organ that undergoes continuous remodeling to maintain its structural integrity. During development, bones, in particular osteoblasts, rely on glucose uptake. However, the role of glucose metabolism in osteoblasts is unknown. Osteocytes are terminally differentiated osteoblasts orchestrating bone modeling and remodeling. In these cells, parathyroid hormone (PTH) suppresses Sost/sclerostin expression (a potent inhibitor of bone formation) by promoting nuclear translocation of class IIa histone deacetylase (HDAC) 4 and 5 and the repression of myocyte enhancer factor 2 (MEF2) type C. Recently, Scriptaid, an HDAC complex co-repressor inhibitor, has been shown to induce MEF2 activation and exercise-like adaptation in mice. In muscles, Scriptaid disrupts the HDAC4/5 co-repressor complex, increases MEF2C function, and promotes cell respiration. We hypothesized that Scriptaid, by affecting HDAC4/5 localization and MEF2C activation, might affect osteocyte functions. Treatment of the osteocytic Ocy454-12H cells with Scriptaid increased metabolic gene expression, cell respiration, and glucose uptake. Similar effects were also seen upon treatment with PTH, suggesting that both Scriptaid and PTH can promote osteocyte metabolism. Similar to PTH, Scriptaid potently suppressed Sost expression. Silencing of HDAC5 in Ocy454-12H cells abolished Sost suppression but not glucose transporter type 4 (Glut4) up-regulation induced by Scriptaid. These results demonstrate that Scriptaid increases osteocyte respiration and glucose uptake by mechanisms independent of HDAC complex inhibition. In osteocytes, Scriptaid, similar to PTH, increases binding of HDAC5 to Mef2c with suppression of Sost but only partially increases receptor activator of NF-κB ligand (Rankl) expression, suggesting a potential bone anabolic effect.

Bone undergoes continuous modeling and remodeling to maintain its structural integrity. Glucose metabolism is a critical component of skeletal metabolism, and bone cells, mostly osteoblasts, take up one-fifth of the glucose consumed by muscles. They sense and use glucose through a glucose transporter-1 (GLUT1)/AMP-activated protein kinase (AMPK)3–dependent pathway independent of insulin signaling (1). Glucose uptake in cells of the osteoblast lineage is necessary for osteoblast differentiation, bone formation, and glucose homeostasis (1). It has been previously shown that PTH promotes bone anabolism, in part, by stimulating anaerobic glycolysis in osteoblasts while suppressing glucose oxidation through the TCA cycle (2). This suggests that hormones like PTH can control bone homeostasis by regulating cell respiration and energy consumption. Whereas the role of glucose in osteoblasts is well-established, its effect on osteocytes is still unknown. Osteocytes are the most abundant bone cells, deeply buried in the mineralized matrix. They reside in lacunae, and they connect with adjacent osteocytes and other bone cells, including osteoblasts, osteoclasts, and bone marrow cells via their dendritic processes that lie within the canaliculi. This extensive cellular network is needed to control and maintain skeletal homeostasis (3–5). Osteocytes regulate bone modeling and remodeling, hematopoiesis, and adipocyte metabolism through secreted factors (5–8). Among them, one important factor is sclerostin, a glycoprotein encoded by the Sost gene that potently inhibits osteoblast differentiation and bone formation (6, 9, 10). Recent studies demonstrated that osteocytes also control adipose tissue and metabolism, in part through secreted sclerostin (7, 8, 11–13).

This work was supported in part by National Institutes of Health Grants RO1-AR06221-05 (to P. D. P.) and AR067285 and DK116716 (to M. N. W.). The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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3 The abbreviations used are: AMPK, AMP-activated protein kinase; C/EBPα, CCAAT/enhancer−binding protein; H3K9ac, histone 3 lysine 9; HDAC, histone deacetylase; MEF2, myocyte enhancer factor 2; IRE, insulin-responsive element; NCoR, nuclear receptor co-repressor; OCR, oxygen consumption rate; O/E&NF1, Olf-1/EBF&nuclear factor 1; OEBE, osteocyte-enriched calcivarial bone explant; PTH, parathyroid hormone; sh, small hairpin; SMRT, silencing mediator of retinoic acid and thyroid hormone receptor; SREBP1, sterol regulatory element-binding protein 1; TF, transcription factor; TCA, tricarboxylic acid; TSA, trichostatin A; ANOVA, analysis of variance; qPCR, quantitative PCR; IP, immunoprecipitation; αMEM, α-minimum essential medium.
Scriptaid, an HDAC co-repressor inhibitor, induces MEF2 activation and exercise-like adaptation in skeletal muscle (14). In these cells, Scriptaid disrupts the HDAC co-repressor complex composed of HDAC4/5, HDAC3, silencing mediator of retinoic acid and thyroid hormone receptor (SMRT), and nuclear receptor co-repressor (NCoR). Disruption of the complex induces the nuclear export of HDAC4/5, activation of MEF2, and subsequently expression of metabolic genes. In osteocytes, PTH suppresses Sost expression by inducing HDAC4/5 nuclear translocation and binding to MEF2C (15) suggesting that Scriptaid might regulate Sost expression in osteocytes. To test this hypothesis, the clonal osteocytic cell line Ocy454-12H (16), calvarial bone explants, and primary osteocytes were treated with Scriptaid to determine the effects of this compound in bone cells. In these cells, Scriptaid potently suppressed Sost, while it significantly increased metabolic genes such as Glut4, pyruvate dehydrogenase lipoamide kinase isozyme 4 (Pdk4), citrate synthase (Cs), and ATP synthase subunit 5 delta (Atp5d). Moreover, both Scriptaid and PTH stimulated mitochondrial respiration, but only Scriptaid significantly increased glucose uptake in osteocytes. Scriptaid induced nuclear translocation of HDAC5, although it had no effect on HDAC4. Next, we used small hairpin (sh) RNA to silence HDAC5 in Ocy454-12H cells. In these cells, Scriptaid-induced Sost suppression was abolished. Importantly, shHDAC5 cells showed preserved Glut4 up-regulation indicating an HDAC5-independent mechanism. Deletion of additional putative transcription factor—binding sites in the Glut4 promoter partially inhibited its up-regulation by Scriptaid, demonstrating that they are involved in controlling osteocyte metabolism and glucose uptake. Similarly, bone explants and primary osteocytes treated with Scriptaid showed a significant increase in Glut4, Pdk4, Cs, and Atp5d expression and suppression in Sost. Taken together, these data demonstrate that in osteocytes Scriptaid reduces Sost expression through an HDAC5-dependent mechanism, although it promotes metabolism and glucose uptake through OLF-1/EBF&nuclear factor 1 (O/E&NF1) and specificity protein 1 (SP1) and sterol regulatory element—binding protein 1 (SREBP1) and CCAAT/enhancer—binding protein (C/EBPα)-dependent mechanisms independent of HDAC5. Scriptaid and its derivative can therefore be used not only to induce exercise-like adaptation in skeletal muscle but also to promote bone anabolism through Sost suppression and Glut4 stimulation.

Results
Scriptaid and PTH-regulated expression of metabolic genes in an osteocytic cell line (Ocy454-12H)

Because previous studies demonstrated that Scriptaid induces muscle-adaptive responses by increasing metabolic genes’ expression (14, 17), lipid oxidation, and glucose utilization, we sought to examine whether Scriptaid stimulates metabolism in osteocytes. Ocy454-12H cells, a clonal osteocytic cell line, were selected for their high sclerostin expression compared with the original Ocy454 cells. As expected, Scriptaid induced histone 3 lysine 9 (H3K9ac) and global histone 3 acetylation (Fig. 1, A – C), confirming its activity in these cells. The drug also significantly increased the expression of genes involved in glucose uptake (Glut4) (Fig. 1D), lipid metabolism (Pdk4) (Fig. 1E), and mitochondrial activity (Atp5d) (Fig. 1G). Treatment with Scriptaid had no significant effect on genes related to TCA cycle (Cs) (Fig. 1F), glucose utilization (HKII) (Fig. 1H), and glucose transporter 1 (Glut1) (Fig. 1I). Taken together, these results suggest that Scriptaid regulates cell respiration and metabolism in osteocytes similarly to its effect in muscle and adipose cells. Dose-response analysis demonstrated that 500 nm was sufficient to significantly up-regulate Glut4 and Pdk4 expression (Fig. 1, N and O).

PTH is secreted by the parathyroid gland and regulates calcium and phosphate homeostasis and bone remodeling by binding to and activating the PTH1 receptor. It has been shown that PTH exerts its anabolic effect in bone, in part by inducing glucose utilization and metabolism in osteoblasts (2). Thus, we sought to explore the effects of PTH on osteocytes’ metabolism. As expected, treatment with PTH did not induce H3K9 and global histone 3 acetylation in Ocy454-12H cells (Fig. 1, A – C); however, it significantly increased the expression of Glut4, Pdk4, and Atp5d (Fig. 1, J, K, and M) but had no effect on Cs (Fig. 1L).

Similar results were obtained when cells were treated with trichostatin A (TSA), a highly-selective class I HDAC inhibitor (Fig. 1, P – S), whereas MC1568, a class II-a–specific inhibitor, had no effect (Fig. 1, P – S). Taken together, these data demonstrate that Scriptaid, PTH, and TSA regulate the expression of genes linked to glucose metabolism in osteocytes.

Scriptaid and PTH increased glucose utilization and cell respiration in Ocy454-12H cells

Treatment of Ocy454-12H cells with Scriptaid (Fig. 2, A and B) or PTH (Fig. 2, C and D) significantly increased GLUT4 protein expression, as shown by Western blot analysis. To further analyze osteocyte respiration and oxidative capability, Ocy454-12H cells were treated with Scriptaid or PTH for 4 h before their metabolic analysis. Both Scriptaid and PTH significantly increased osteocytes basal, maximal, and nonmitochondrial respiration and ATP production (Fig. 2, E–J), but they had no significant effect on spare respiration capability (Fig. 2L). In addition, Scriptaid increased the osteocytes’ proton leak, whereas PTH had no effect (Fig. 2K). Glucose uptake was also significantly increased in Ocy454-12H cells upon Scriptaid administration, whereas PTH stimulation only marginally increased glucose uptake (p = 0.07) (Fig. 2L).

Scriptaid and PTH suppressed Sost expression and regulated bone-remodeling genes

In muscle cells, Scriptaid blocks the formation of the HDAC co-repressor complex containing HDAC4/5, SMRT, NCoR, and HDAC3 and releases the transcriptional activity of MEF2. MEF2, in turn, promotes the transcription of several genes, including Glut4. In osteocytes, PTH promotes HDAC4/5 dephosphorylation, nuclear translocation, and MEF2C-mediated suppression of Sost expression. We hypothesized that Scriptaid might reduce HDAC4/5–mediated suppression of MEF2C and increase Sost expression in osteocytes. Ocy454-12H cells were treated with Scriptaid or PTH for 4 h prior to RNA isolation and
gene analysis. Scriptaid significantly suppressed Sost (Fig. 3A), matrix extracellular phosphoglycoprotein (Mepe) (Fig. 3C), osteoprotegerin (Opg) (Fig. 3D), and Mef2c (Fig. 3H) and increased Rankl (Fig. 3B) expression and the Rankl/Opg ratio (Fig. 3I). The compound had no effect on dentin matrix protein 1 (Dmp1) (Fig. 3E), Hdac4 (Fig. 3F), Hdac5 (Fig. 3G), Hdac1 (Fig. S1A), or Hdac2 (Fig. S1B) and only minimally up-regulated Hdac3 (Fig. S1C) expression. Dose-response analysis showed that Scriptaid significantly down-regulated Sost and Mepe at doses as low as 10 nM (Fig. 3P and Q) and significantly up-regulated Rankl at the dose of 1 μM (Fig. 3Q). As expected, and as reported previously (18), PTH significantly suppressed Sost (Fig. 3J) and Mepe (Fig. 3L) but had no effect on Opg (Fig. 3M) and Mef2c (Fig. 3N) expression. The hormone significantly up-regulated Rankl (Fig. 3K), as reported previously, and significantly increased the Rankl/Opg ratio (Fig. 3O). Interestingly, although the degrees of Sost and Mepe inhibition were similar between Scriptaid and PTH, the hormone increased Rankl expression by ~60-fold (Fig. 3K), whereas Scriptaid only induced a 3-fold increase (Fig. 3B). Additionally, TSA showed effects similar to Scriptaid, whereas the selective class IIa HDAC inhibitor MC1568 had no significant effect (Fig. 3, S and T). In summary, these data demonstrate that Scriptaid, similarly to PTH, promotes bone remodeling by suppressing Sost and increasing Rankl expression.

Scriptaid increased the expression of both metabolic and skeletal genes in bone explants and primary osteocytes

To further investigate the effects of Scriptaid and PTH on bone metabolism, we isolated osteocyte-enriched calvarial bone explants (OEBE) from 4-week-old WT animals (19) and treated them with Scriptaid or PTH for 6 h prior to RNA isolation. Similar to its effects in Ocy454-12H cells, Scriptaid significantly increased the expression of Glut4, Pdk4, Cs, Atp5d, HkII, and Glut1 in cells treated with Scriptaid (1 μM) (D–I) or PTH (1 nM) (J–M) for 4 h. N and O, Glut4 and Pdk4 dose response in cells treated with Scriptaid for 4 h. P–S, Glut4, Pdk4, Atp5d, and HkII in cells treated with TSA (1 μM) or MC1568 (1 μM) for 4 h, relative to β-actin. One-way ANOVA was performed for B–C and N–S with vehicle as comparison groups. Unpaired t tests were performed for D–M. For all graphs, n = 3, and *, p < 0.05; **, p < 0.01; and ***, p < 0.001; data are expressed as means ± S.D.

Finally, Scriptaid significantly increased Glut4 and suppressed Sost (Fig. 4, Q and R) in primary osteocytes isolated from the long bones in mice. Rankl expression was only marginally affected by the treatment (Fig. S2G, p = 0.08).
Scriptaid promoted HDAC5 nuclear translocation and its binding to MEF2C

Next, we sought to elucidate the molecular mechanisms by which Scriptaid regulates gene expression in osteocytes. We have previously demonstrated that, in these cells, PTH suppression of Sost is mediated by HDAC4/5 nuclear translocation and binding to MEF2C (15, 20); therefore, we investigated whether similar mechanisms were also elicited upon Scriptaid treatment. As shown in Fig. 5, treatment with Scriptaid did not induce HDAC4 nuclear translocation (Fig. 5, A and B) but significantly promoted accumulation in the nucleus of HDAC5 (Fig. 5, C and D), suggesting that Scriptaid-mediated suppression of Sost is HDAC5-dependent cytoplasmic translocation.

To further investigate whether Scriptaid controls Sost expression by promoting HDAC5 nuclear translocation and binding to MEF2C, we performed immunoprecipitation. As expected, Scriptaid significantly increased HDAC5 binding to MEF2C (Fig. 5, E and G–I) but had no effect on HDAC4 (Fig. 5, E and F). These results indicated that in osteocytes, Scriptaid, similar to PTH, inhibits Sost expression by promoting HDAC5 translocation to the nucleus and its binding to MEF2C.

Silencing of HDAC5 blocked Scriptaid suppression of Sost

To investigate whether HDAC4 and HDAC5 are required for Scriptaid-mediated gene regulation, we generated Ocy454-12H in cells lacking either HDAC4 or HDAC5 by using CRISPR/Cas9 and shRNA techniques. Immunoblotting analysis demonstrated efficient depletion of these proteins (Fig. 6A and Fig. S3A). As expected, deletion of HDAC4 did not impair Scriptaid-mediated regulation of Sost and Glut4 expression (Fig. S3, E and F) nor affect basal gene expression (Fig. S3, B–D) demonstrating that mechanisms other than HDAC4 must be involved.

On the contrary, the silencing of HDAC5 significantly up-regulated Sost (Fig. 6B) (as reported previously) (20) and impaired its suppression in response to Scriptaid (Fig. 6K). As shown in Fig. 6, F and L, expression or up-regulation of Glut4 upon Scriptaid treatment was still present in Ocy454–shHDAC5 cells. In addition, HDAC5 silencing moderately decreased Dmp1 (Fig. 6E) and showed no effects on Rankl, Mepe, Pdk4, Cs, Atp5d, and HkII gene expressions (Fig. 6, C, D, and G–J).

Ocy454-12H cells in which MEF2C expression was reduced using shRNA were used to further validate this model. Sost expression was significantly lower in shMEF2C cells (Fig. S3, G and H) whereas, as expected, Glut4 expression was unaffected, and cells were fully responsive to Scriptaid treatment (Fig. 6M).

Taken together, these results demonstrate that Sost regulation is mediated by HDAC5 nuclear translocation, whereas Glut4 up-regulation is independent of HDAC4/5.

Figure 2. Regulation of cell respiration and glucose utilization by Scriptaid and PTH. Ocy454-12H cells were treated with Scriptaid or PTH prior to protein isolation or functional assays. Western blot analysis for GLUT4 in cells treated with Scriptaid (1 and 10 μM) or PTH (10 nM) for 6 h. Loading was relative to tubulin. B and D, quantification or GLUT4, relative to tubulin; MW = molecular weight; Veh, vehicle. E, OCR in Ocy454-12H cells treated with Scriptaid (1 μM) or PTH (10 nM). F–K, quantification for OCR in basal respiration, maximal respiration, nonmitochondrial respiration, ATP production, spare respiratory capability, and proton leak, normalized to vehicle. L, glucose uptake in Ocy454-12H cells treated with Scriptaid (10 μM) or PTH (50 nM) for 4 h, normalized to vehicle. One-way ANOVA were performed for B and F–L with vehicle as comparison groups. Unpaired t test was performed for D. For all graphs, n = 3, and *, p < 0.05 and **, p < 0.01; data are expressed as means ± S.D.
**CRT2C nuclear translocation was induced by Scriptaid but did not mediate Glut4 up-regulation**

Because Glut4 up-regulation by Scriptaid is HDAC4/5-independent, we sought to explore other potential factors. It has been reported previously that salt-inducible kinase 2 (SIK2) and its downstream target protein cAMP-regulated transcription co-activator 2 (CRT2) play critical roles in regulating its downstream target protein cAMP-regulated transcription factor 2 (CRTC2) nuclear translocation in osteocytes. We hypothesize that Scriptaid might regulate Glut4 expression through the SIK2/CRTC2 signaling pathway. Western blot analysis of subcellular localization of CRTC2 revealed CRTC2 nuclear translocation in osteocytes.

To investigate whether the SIK2/CRTC2 signaling pathway mediates Glut4 up-regulation, SIK2 (shSIK) and CRT2 (shCRT2) were silenced using shRNAs (Fig. S4, C and D). Interestingly, shSIK2 and shCRT2 cells significantly reduced their basal expression of Sost (Fig. S4E). However, silencing of SIK2 and CRT2 did not significantly affect Rankl, Mepe, and metabolic gene expression, including Pdk4, Atp5d, Cs, and HkiII (Fig. S4, F–K). Both low (100 nM) and high (500 nM) doses of Scriptaid significantly increased Glut4 expression in shSIK2 and shCRT2 cells (Fig. S4L), suggesting a SIK2/CRTC2-independent mechanism. Taken together, these results suggest that Scriptaid and PTH regulate gene expression via distinct intracellular mechanisms.

**IRE-containing O/E&NF1 (IRE (O/E&NF1)) and three TF (SP1, SREBP1c, and C/EBPα)-binding sites mediated Scriptaid-induced osteocyte Glut4 up-regulation**

To explore an alternative mechanism by which Scriptaid regulates Glut4 expression, we searched for additional putative TF-binding sites in the mouse Glut4 gene promoter region. The majority of the known TF-binding sites are located within 1200 bp upstream of the Glut4 transcription start site. Three fragments of the Glut4 promoter (sequence spanning GLUT4 1.3 kb: −1239 to +34; GLUT4 0.8 kb: −756 to +34; and GLUT4 0.5 kb: −463 bp to +34) were cloned into pGL3-basic reporter vector, and luciferase activity was analyzed (Fig. 7A). Scriptaid significantly increased luciferase activity in the GLUT4 1.3-kb region (−250%) compared with the truncated 0.8-kb (∼100%) and the 0.5-kb (∼100%) regions, suggesting that the sequence between −1239 and −756 bp and between −463 and −0 bp mediate Scriptaid effects on Glut4 (Fig. 7B).

The −1239 to −463-bp region contains both IRE (O/E&NF1) and MEF2-binding sites; therefore, we individually mutated or deleted these regions (Fig. 7A). Mutation of the IRE region significantly reduced the Scriptaid-mediated Glut4 reg-

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**Figure 3. Regulation by Scriptaid and PTH of bone-remodeling genes.** Ocy454-12H cells were treated with Scriptaid, PTH, TSA, or MC1568 prior to RNA isolation. Real-time qPCRs for Sost, Rankl, Mepe, Dmp1, Hdac4, Hdac5, Mef2c, and Rankl/Opg ratio in cells treated with Scriptaid (1 μM) (A–I) and Sost, Rankl, Mepe, Opg, Mef2c, and Rankl/Opg ratios in cells treated with PTH (1 nM) (J–O) for 4 h are shown. Sost, Rankl, and Mepe dose response in cells treated with Scriptaid for 4 h (P–R) is shown. Sost, Rankl, and Dmp1 in cells treated with TSA (1 μM) or MC1568 (1 μM) for 4 h (S and T), relative to β-actin, are shown. Unpaired t test was performed for A–O. One-way ANOVA were performed for P–T with vehicle as comparison groups. For all graphs, n = 3, and *, p < 0.05; **, p < 0.01; and ***, p < 0.001; data are expressed as means ± S.D.
ulation, whereas mutation of the MEF2 region had no effect (Fig. 7C). This suggested that IRE, but not MEF2, is important for Scriptaid-induced \textit{Glut4} expression. Next, we generated GLUT4 1.3-kb vectors in which the O/E&NF1-binding site (O/N Del) or the whole IRE region (IRE Del) was deleted (Fig. 7D), indicating that the IRE (O/E&NF1) region is required for \textit{Glut4} regulation by Scriptaid. Finally, the GLUT4 1.3-kb luciferase plasmid with deletion of three transcription factor–binding sites, including SP1, SREBP1c, and C/EBP\(_{\alpha}\) (3TF Del), was generated for validation (Fig. 7A). A 3TF deletion also impaired Scriptaid up-regulation (Fig. 7E). Administration of Vorinostat, a Food and Drug Administration–approved drug for cutaneous T cell lymphoma structurally related to Scriptaid, also significantly increased luciferase activity in the GLUT4 1.3-kb promoter plasmid (Fig. 7F).

To further validate this finding, we generated Ocy454-12H cells in which C/EBP\(_{\alpha}\) was silenced by shRNA (Fig. 7G). In these cells, \textit{Glut4} up-regulation by Scriptaid was significantly reduced (~7- to ~2-fold) (Fig. 7H), indicating that both O/E&NF1 and three transcription factor–binding sites play important roles in the Scriptaid up-regulation of \textit{Glut4} expression.

**Discussion**

The adult skeleton maintains its structural integrity by continuous remodeling, i.e. the highly-orchestrated coupling of bone formation and bone resorption. Remodeling is regulated by environmental factors (load), hormones, and nutritional cues, and it requires energy in the form of glucose. Indeed, glucose metabolism is a critical component of skeletal metabolism, and bone cells, mostly osteoblasts, consume close to one-fifth of the glucose consumed by muscles. These cells sense and use glucose through a GLUT1 and AMPK-dependent pathway independent of insulin signaling. Osteoblasts require glucose for their differentiation and collagen synthesis. In turn, these cells act as an integral component of the whole-body glucose homeostasis by secreting growth factors and hormones, like osteocalcin, which, in its under-carboxylated form, regulates pancreatic insulin secretion.

Osteocytes, deeply embedded in the mineralized matrix of the bone, are the most abundant cells in the adult skeleton, and they have emerged as main orchestrators of both osteoblast and osteoclast functions through factors such as sclerostin, receptor activator of nuclear factor \(\kappa\)B ligand (RANKL), and osteoprotegerin. Despite their key role in skeletal homeostasis, their energy consumption and glucose metabolism are still unknown.
Figure 5. HDAC4/5 subcellular translocation and binding to MEF2C in response to Scriptaid. Ocy454-12H cells were treated with Scriptaid prior to protein isolation. Western blot analysis for HDAC4 (A) and HDAC5 (C) in cells treated with Scriptaid (10 μM) for 1 h. Nuclear (N) proteins were normalized to SP1, and cytoplasmic (C) proteins were normalized to tubulin. Quantification for nuclear/cytoplasmic ratio of HDAC4 (B) and HDAC5 (D), normalized to vehicle; MW = molecular weight; Veh, vehicle. Co-immunoprecipitation of MEF2C IP to HDAC4/5 (E) and HDAC5 IP to MEF2C (H) is shown. Quantification for co-immunoprecipitation of HDAC4 (F) and HDAC5 (G) binding to MEF2C, normalized to MEF2C is shown; MEF2C (I) binding to HDAC5, normalized to HDAC5, is shown. For all graphs, unpaired t tests were performed; n = 3–4, and *, p < 0.05 and ***, p < 0.001; data are expressed as means ± S.D.

Figure 6. Metabolic and bone-remodeling gene regulation by Scriptaid in Ocy454-12H-shHDAC5 cells. A. Western blot analysis for HDAC4 and HDAC5 in shHDAC5 cells for characterization; MW = molecular weight; Veh, vehicle. B–J, real-time qPCR for basal Sost, Rankl, Mepe, Dmp1, Glut4, Pdk4, Cs, Atp5d, and HklII in control and shHDAC5 cells. K and L, real-time qPCR for Sost and Glut4 in control and shHDAC5 cells treated with Scriptaid (100 and 500 nM) for 4 h, relative to β-actin. M, real-time qPCR for Glut4 in control and shMEF2C cells treated with Scriptaid (500 nM) for 4 h. Unpaired t tests were performed for B–J. One-way ANOVA multiple comparison tests within sample cell line were performed for K–M with vehicle of treatment of same cell as comparison groups. For all graphs, n = 3, and *, p < 0.05; **, p < 0.01; and ***, p < 0.001; data are expressed as means ± S.D.
Several hormones, including PTH, promote bone anabolism, by stimulating in osteoblasts anaerobic glycolysis while suppressing glucose oxidation through the TCA cycle. In osteocytes, PTH exerts its bone-forming effect by suppressing Sost through dephosphorylation and nuclear translocation of class Ia HDAC4 and HDAC5 and inhibition of MEF2C.

Recent studies have identified a class II HDAC repressor, Scriptaid, capable of promoting, in muscles, MEF2 expression and exercise-like adaptation. In skeletal muscles, administration is associated with the expression of genes involved in glucose (Glut4 and HkII), lipid (Pdk4), and mitochondrial (Cs and Atp5d) metabolism (14, 17).

Here, we report that both Scriptaid and PTH stimulate osteocyte respiration and glucose utilization through an HDAC4/5-independent mechanism. Moreover, both Scriptaid and PTH potently suppress Sost mRNA by mechanisms dependent on HDAC5, as reported previously.

HDACs are a family of enzymes that facilitate acetyl group removal from lysine residues on histones. HDACs are divided into four classes (class I, II, III, and IV) with HDAC4 and HDAC5 belonging to class II (22). HDAC4 and HDAC5 lack strong catalytic capability, and they form complexes with class I HDACs, such as HDAC3, to inhibit transcription factors. In bone, several studies have indicated that HDACs play an important role in skeletal homeostasis (23). HDAC3 binds to RUNX2 and reduces osteocalcin expression (24), whereas conditional deletion of HDAC3 in osteoblasts and osteocytes accelerates bone loss with aging (25). HDAC4 inhibits RUNX2 and regulates endochondral bone formation (26). In osteocytes, PTH (or cAMP)-dependent inhibition of SIK2 induces HDAC4/5 dephosphorylation and their translocation into the nucleus where they bind to and suppress MEF2C with subsequent Sost suppression (15, 20). Similarly, HDAC5 null mice have increased sclerostin expression, and reduced trabecular bone (20) and deletion of HDAC5 in Ocy454 cells significantly increase Sost (as shown in Fig. 6B).

Scriptaid has a weak affinity to class I HDACs but works mainly as an HDAC co-repressor complex inhibitor (14, 27). However, in osteocytes, Scriptaid induces HDAC5 nuclear translocation and suppresses MEF2C and Sost expression suggesting that, in different cells, HDACs (or additional complex factors) form different complexes with opposite effects. In osteocytes, Scriptaid (and TSA) promotes HDAC5 nuclear translocation and MEF2C inhibition, whereas in skeletal muscle it promotes MEF2 transcriptional activity (20). We can speculate that these opposite effects might be due to different co-factors or co-repressors differentially expressed in bone or muscle. One possibility is that part of the inhibitory effect of Scriptaid and TSA is mediated by class I HDACs, such as HDAC3. However, preliminary data (not shown) demonstrated that Scriptaid treatment does not induce HDAC3 nuclear
translocation in osteocytes, suggesting that other co-factors might be involved.

In osteocytes, both PTH and Scriptaid increase energy metabolism and glucose utilization through an HDAC4/5-independent mechanism, as shown by a full metabolic response in cells where HDAC4 and HDAC5 were deleted or silenced. In muscle cells, Glut4 expression is MEF2-dependent (14), and in adipocytes Glut4 is suppressed via HDAC4- and CTRC2-dependent mechanisms (21). However, in our study either HDAC4/5 or CTRC2 deletions did not impair Scriptaid-induced Glut4 up-regulation, suggesting different regulation machinery.

Ex vivo OEBEs closely recapitulate in vivo bone elements, and they were used, in this study, to study the effects of Scriptaid and PTH. As expected, both drugs suppressed Sost and Mepe mRNAs, suggesting an anabolic effect. Interestingly, Rankl was significantly reduced by Scriptaid in OEBEs and was only marginally (although not significantly) increased in primary osteocytes, suggesting cell-specific effects of this compound. In contrast, PTH had similar effects both in vitro and ex vivo. It is important to note that the increase in Rankl expression differs in magnitude between Scriptaid and PTH, with the latter having a more potent effect (~3- and 60-fold, respectively). We can speculate that this blunted Rankl activation by Scriptaid might be beneficial to the bone. We do not have a clear explanation of the opposite effects of Scriptaid on Rankl expression, but we can speculate that in ex vivo bone explants other cells, such as endosteal or periosteal osteoblasts or endothelial cells, might contribute to the regulation of this transcript. It is important to note that in osteocytes, Rankl varies among these different experimental models with OEBEs having the highest expression of the transcript (~250-fold compared with Ocy454-12H and ~50-fold compared with primary osteocytes). We can speculate that cells with high Rankl expression are potentially less sensitive to its regulation by Scriptaid. In addition, other Rankl-expressing cells, besides osteocytes, might differentially regulate Rankl in response to Scriptaid treatment. PTH stimulation of Rankl was also blunted in OEBE, with only a 3-fold increase in expression compared with the 60-fold increase present in Ocy454-12H cells. Further studies will be needed to tease out these different cellular responses (7, 16).

In OEBEs, PTH did not significantly regulate the expression of metabolic genes, including Glut4, Pdk4, Atp5d, and Cs, as it did in osteocytic cell lines and only moderately regulated HkII expression. Several factors can account for the differences between the in vitro and the ex vivo models. PTH bioavailability might be different between in vitro and ex vivo conditions, and the dose and time used in this experiment might not be the optimal one to elicit PTH maximal effects. In addition, although OEBE is a well-accepted osteocyte-rich model, other cells might still be present (such as osteoblasts, marrow adipocytes, endothelial cells, fibroblast or other mesenchymal cells) that might not regulate metabolic gene expression in response to PTH treatment. Indeed, recent studies reported that PTH treatment suppresses Glut4 expression and insulin-stimulated glucose uptake in differentiated 3T3-L1 adipocytes (28). One limitation of this experiment is that only relative changes in gene expression were analyzed. Indeed, any real-time PCR experiment has an intrinsic variability that can be improved by including a panel of endogenous controls (or housekeeping genes) to correct for sample variation. In our experiments, all data were normalized to a single housekeeping gene, β-actin, based on initial studies demonstrating that this gene did not change in Ocy454-12H cells upon Scriptaid treatment. Nevertheless, we cannot exclude that some of the variability among our real-time data might not be due to changes in this reference gene.

In summary, we have reported here for the first time that Scriptaid, an HDAC complex disruptor, can significantly suppress Sost and Mepe expression and at the same time induce osteocyte respiration and glucose uptake. Similar effects were also induced by PTH, suggesting, in part, some common signaling mechanisms. In contrast, Scriptaid was unable to regulate additional PTH-responsive genes (Table S1) (15), demonstrating independent signaling pathways. We can speculate that Scriptaid induces exercise-like responses both in muscle and in bone, and it might be a good candidate for the treatment of disuse-induced osteopenia and muscle atrophy. Additional studies are needed to demonstrate the therapeutic efficacy of this compound in vivo. Currently, some Scriptaid derivatives like Vorinostat are approved for the treatment of hematologic diseases, suggesting that, in these conditions, the drug might also protect the skeleton from detrimental bone loss.

**Experimental procedures**

**Compounds and antibodies**

Scriptaid was purchased from Santa Cruz Biotechnology (Dallas, TX). Trichostatin A and MC1568 were purchased from Sigma. Synthetic human PTH(1–34) peptide was kindly provided by Dr. Ashok Khatri (Peptide/Protein Core Facility, Partners Research Core and Massachusetts General Hospital, Boston, MA).

For primary antibodies, antibodies against GLUT4 (catalog no. 2213), HDAC5 (catalog no. 20458), MEF2C (catalog no. 5030), H3K9ac (catalog no. 9649), and β-tubulin (catalog no. 5346) were purchased from Cell Signaling Technology (Danvers, MA). HDAC4 (catalog no. ab12172) antibody was purchased from Abcam (Cambridge, MA). SP1 (catalog no. 07-645) and CRTC2 (catalog no. ST1099) antibodies were from EMD Millipore (Burlington, MA).

For secondary antibodies, the antibody against rabbit (catalog no. 7074) was from Santa Cruz Biotechnology (Dallas, TX), and the antibody against mouse (catalog no. sc-516102) was from Cruz Biotechnology.

**Cells and in vitro culture**

Ocy454-12H cells were generated from single-cell cloning of high GFP-expressing Ocy454 cells and screened for high sclerostin expression as reported recently (8, 18). Cells were routinely maintained on noncollagen-coated cell culture flask (Corning) in α-minimum essential medium (αMEM) supplemented with 10% heat-inactivated fetal bovine serum and 1% antibiotic/antimycotic (αMEM complete medium) (Gibco, Gaithersburg, MD). For all cell culture experiments, cells were plated at 100,000 cells/ml in 6-well (2 ml/well), 12-well (1 ml/well), or 24-well (0.5 ml/well) plates or 10-cm tissue culture
dishes (10 ml/dish) and allowed to reach confluence at 33 °C for 3 or 4 days, prior to being moved to 37 °C and cultured for the indicated times to induce differentiation.

**Osteocyte-enriched calvarial bone explants**

OEBEs were isolated from 4-week-old WT C57BL female mice purchased from Charles River Laboratories (Wilmington, MA). In brief, after 1 day of acclimatization, mice were sacrificed, and calvarial bones were aseptically isolated, washed in PBS, and sequentially digested in αMEM containing collagenase (1 mg/ml collagenase type I/II ratio of 1:4; Worthington), 1 mM CaCl₂, 10 mM HEPES (Life Technologies, Inc.) to remove periosteal and endosteal osteoblasts. Calvarial explants were cultured overnight at 37 °C in 5% CO₂ in complete αMEM. 6 h before RNA extraction, calvarial bones were cut into two halves through sagittal suture and randomly distributed into four groups: 10 μM Scriptaid; 100 nm hPTH(1–34); DMSO (as vehicle for Scriptaid); and complete medium (as vehicle for PTH). After 6 h of treatment, OEBEs were homogenized, and RNA was extracted as described below.

**Primary osteocyte isolation**

Primary osteocytes were isolated, as described previously (29), from 4-week-old WT C57Bl6 male mice purchased from Charles River Laboratories (Wilmington, MA). In brief, mice were sacrificed, and humeri, femora, and tibias were aseptically dissected and cultured in αMEM with 10% penicillin and streptomycin. Epiphyses were cut off, and the bone marrow was flushed out. The bone diaphyses were then cut lengthwise and then cut into 1–2 mm lengths. The bone fragments were sequentially digested in αMEM containing collagenase (1 mg/ml collagenase type I/II ratio of 1:4; Worthington); 1 mM CaCl₂, 10 mM HEPES, pH 7.4 (Life Technologies, Inc.). Additional digestions were performed in 5 mM EDTA/Dulbecco’s PBS solution containing 1% BSA. Digestions were carried out at 37 °C with agitation (300 rpm). At the end of the digestions, bone fragments were washed in medium, collected, and cultured in complete αMEM at 37 °C in 5% CO₂. The primary osteocytes migrated from the bone fragments, and when they reached confluence, they were trypsinized, plated, and treated with Scriptaid as described above. All cells were used in the first passage.

**Quantitative real-time PCR**

For in vitro cultures, total RNA was isolated using RNeasy Plus mini kit (Qiagen, Germantown, MD) and quantified using NanoDrop 2000 (Thermo Fisher Scientific). First-strand cDNA was generated using qScript cDNA SuperMix (Quantabio, Beverly, MA) and 50–1000 ng of total RNA.

For ex vivo OEBEs, bone explants were homogenized in TRIzol (Thermo Fisher Scientific) using a tissue homogenizer (TissueLyser, Qiagen, Qiagen, Germantown, MD). Total RNA was isolated using PureLink RNA mini kit (Ambion, Life Technologies, Inc.). cDNA was synthesized with Takara PrimeScript RT reagent kit plus gDNA Eraser (Clontech) starting with 250–1000 ng of total RNA.

Real-time qPCR was performed using SYBR Green Master Mix (Thermo Fisher Scientific) in a StepOnePlus real-time PCR system (Applied Biosystems). Target genes were normalized to one housekeeping gene (β-actin) because its expression did not change upon Scriptaid treatment. Primer sequences are listed in Table S2.

**Western blotting, immunoprecipitation, and subcellular protein fractionation**

Whole-cell lysates were prepared in ice-cold lysis buffer (1% Triton X-100, 50 mM Tris-base, 1 mM EDTA, 1.5 mM MgCl₂, 150 mM NaCl, 10% glycerol) with 10% protease inhibitor mixture and 2% phosphatase inhibitor mixtures 2 and 3 (Sigma) according to the manufacturer’s recommendations. Protein concentrations were quantified with the protein assay from Bio-Rad. 20 μg of sample protein lysate was heated in Laemmli SDS reducing sample buffer (Boston Bio Products, Ashland, MA) at 95 °C for 5 min. The lysate was separated in 7–10% Tris-glycine denaturing gels (Life Technologies, Inc.) and transferred to polyvinylidene difluoride membranes using the Trans-Blot Turbo (Bio-Rad) system according to the manufacturer’s recommendations. The membranes were blocked with 5% BSA or 5% nonfat milk in TBS containing 0.1% Tween 20 (TBST) at room temperature for 1 h and then incubated with primary antibodies overnight at 4 °C according to the manufacturer’s recommended concentration (1:500–1:1000 dilution). After washing, the secondary antibody was incubated for 1 h at room temperature according to the manufacturer’s recommendations (1:500–1:2000 dilution), and the blot was developed using enhanced chemiluminescence (Thermo Fisher Scientific) or SuperSignal West Femto Chemiluminescent Substrate (Thermo Fisher Scientific).

For immunoprecipitation, whole-cell lysate was isolated from cells cultured in 10-cm dishes (Corning, Corning, NY) with TNT lysis buffer (200 mM NaCl, 20 mM Tris-HCl, pH 8, 0.5% Triton X-100) supplemented with protease and phosphatase inhibitors mixtures 2 and 3 (Sigma). Lysates were pre-cleared with protein A/G–agarose beads (Pierce, Thermo Fisher Scientific), and 3 mg of total protein in 800 μl of lysis buffer were incubated with 5–6 μg of antibody at 4 °C overnight. The next day, targeted protein complexes were precipitated with the beads and washed 3–4 times in cold TNT buffer. The proteins were eluted in 60 μl of Laemmli SDS reducing sample buffer (Boston Bio Products, Ashland, MA) at 95 °C for 5 min, and 20 μl of the sample were separated in 7–10% Tris-glycine denaturing gels and detected as described above.

The nuclear and cytoplasmic fractions of protein lysates were isolated using a commercial kit (NE-PER catalog no. 78833, Thermo Fisher Scientific). 20 μg of the sample was used for Western blot analysis as described above.

**Global histone 3 acetylation assay**

The global H3 acetylation assay was performed following the manufacturer’s protocol from Global Histone H3 acetylation assay kit (Epigentek, Farmingdale, NY). In brief, histone proteins were isolated and purified from Ocy454-12H cells after treatment with Scriptaid. After quantification, 2 μg of histone proteins were incubated with capture and detection antibodies. The ratio of acetylated histone 3 was quantified.
through horseradish peroxidase-conjugated secondary antibody using a microplate reader.

**Cell bioenergetics and mitochondrial stress test (Seahorse XF)**

Ocy454-12H cells were plated in 96-well Agilent Seahorse XF cell culture microplate at 20,000 cells/well (Agilent, Santa Clara, CA) and cultured in αMEM complete medium (as described above) at 33 °C in a humidified 5% CO₂ incubator for 4 days. On the experimental day, cells were treated with Scriptaid, PTH, or DMSO (vehicle), at the indicated concentration, for 4 h prior to analysis with Seahorse XFe 96 analyzers (Agilent, Santa Clara, CA) following the manufacturer’s recommendation (14). After sequentially injected oligomycin, p-trifluoromethoxyphenylhydrazone (FCCP), and antimycin, cells were restricted or stimulated to specific respiration status. Oxygen consumption rates (OCR) were automatically measured by the analyzer through each period, and respiration and energy production capabilities were calculated. CyQuant (Thermo Fisher Scientific) was used to normalize for cell number by measuring DNA concentration.

**Glucose uptake assay**

Glucose uptake cell-based assay kit (Cayman Chemical, Ann Arbor, MI) was used to measure glucose uptake, following the manufacturer’s recommendation. Briefly, cells were plated in 96-well black plates with the clear bottom at 20,000 cells/well in αMEM complete medium, at 33 °C overnight. The next day, cells were cultured in no-glucose Dulbecco’s modified Eagle’s medium (catalog no. 11966025, Gibco, Gaithersburg, MD) and treated for 4 h with Scriptaid, PTH, or DMSO (vehicle) at the indicated concentration. At the end of the treatment, fluorescent deoxyglucose derivative (2-NBDG) was added to each well to a final concentration of 100 μM/mL, and cells were incubated for an additional 1 h. Glucose uptake was measured at 485 nm excitation/535 nm emission using Tecan Infinite M1000 Microplate Reader (Tecan, Switzerland). Fluorescence intensity was normalized with the protein content in each well.

**shRNA gene silencing and CRISPR/Cas9 gene deletion**

The shRNA and CRISPR/Cas9 techniques were used to generate Ocy454 cells with genes silencing or deletion of targets described below (15, 20). The sequences for shRNA and CRISPR/Cas9 gRNAs are listed in Table S3. Briefly, for shRNA, targeting sequences were cloned into PLKO.1-puro backbone (gift from Bob Weinberg, Addgene catalog no. 8453, Cambridge, MA) (30) and plasmids were amplified in One-Shot Stbl3 chemically competent *Escherichia coli* cells (Invitrogen™, Thermo Fisher Scientific, Waltham, MA) following the manufacturer’s recommendation. HEK 293T cells were plated at 440,000 cells/well in 6-well plates and after 24 h transfected with shRNA plasmid along with pMD2.G (gift from Didier Trono, Addgene catalog no. 12259, Cambridge, MA) and psPAX2 (gift from Didier Trono, Addgene catalog no. 12260, Cambridge, MA) using FuGENE-HD (Promega, Madison, WI) according to manufacturer’s protocol. Lentivirus particles and Polybrene (5 mg/ml) overnight at 33 °C. The medium was replaced every day with fresh complete αMEM containing puromycin (4 μg/ml) to efficiently transfected select cells. Cells were maintained in complete αMEM with puromycin (4 μg/ml) at 33 °C.

For CRISPR/Cas9 sgRNAs targeting HDAC4 were cloned into pSpCas9(BB)-2A-GFP (PX458) (gift from Feng Zhang, Addgene catalog no. 48138, Cambridge, MA), which co-expresses Cas9, sgRNA, and enhanced GFP (15, 31). The transfection was described in the previous study (15). In brief, empty vectors were used to generate controls. Ocy454 cells were cultured in a 6-well plate transfected with 1 μg of plasmid/well at 33 °C. After 48 h of transfection, eGFP+ cells were sorted by FACs and single cells plated in a 96-well plate. The αMEM complete medium was changed every 3–4 days until the colony formed. Cells were trypsinized and transferred to cell culture flask (Corning) and maintained as the regular cell culture in vitro described above.

**Transient transfections and luciferase reporter assays**

Transient transfection of luciferase reporter plasmids was performed in the Ocy454-12H cell line using FuGENE-HD (Promega, Madison, WI) according to manufacturer’s protocol. In brief, the synthetic mouse DNA sequences containing different lengths of mouse Glut4 gene promoter sequence were cloned into pGL3-basic luciferase reporter vectors (Promega, Madison, WI). Full-length (−1294 + 34 bp) or truncated Glut4 promoters in the pGL3 firefly luciferase plasmids were co-transfected with Renilla luciferase plasmids driven by cytomegalovirus or thymidine kinase promoter (GLUT4-luciferase: *Renilla* = 25:1 to 50:1) at 500 ng of total DNA quantity. 24 h after transfection, the culture medium was replaced by fresh complete αMEM containing the indicated chemicals, and 4 h later the luciferase assays were performed and quantified using dual-luciferase reporter assay system (Promega, Madison, WI) according to the manufacturer’s protocol.

For promoter sequence mutation and deletion, primers were designed with NEBaseChanger™ tool, and the Q5 mutagenesis kit (New England Biolabs, Ipswich, MA) was utilized to perform mutated PCR according to manufacturer’s protocol. Mutant plasmids were transfected in Ocy454-12H cells and assayed for luciferase as described above.

**Statistical analysis**

Data are presented as means ± S.D. Statistically significant differences were analyzed by unpaired *t* test or ANOVA using Prism (GraphPad, San Diego). Values of *p* < 0.05 were considered significant. The experiments were conducted in duplicate or triplicate and repeated three times unless specified in the figure legends.

**Author contributions**—N. S. and P. D. P. conceptualization; N. S., E. A., C. S., M. N. W., and P. D. P. resources; N. S. and P. D. P. data curation; N. S. and P. D. P. formal analysis; N. S. and P. D. P. validation; N. S., Y. U., E. A., A. K., R. N. C., T. K., and P. D. P. investigation; N. S., Y. U., A. K., C. S., M. N. W., and P. D. P. methodology; N. S. and P. D. P. writing—original draft; N. S. and P. D. P. project administration; Y. U., M. N. W., and P. D. P. writing—review and editing; M. N. W. and P. D. P. funding acquisition; P. D. P. supervision.
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