Published in final edited form as:

Crit Rev Eukaryot Gene Expr. 2011 ; 21(2): 101–113.

Hdac-Mediated Control of Endochondral and Intramembranous Ossification

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Abstract

Histone deacetylases (Hdacs) remove acetyl groups (CH₃CO-) from ε-amino groups in lysine residues within histones and other proteins. This post-translational (de) modification alters protein stability, protein-protein interactions, and chromatin structure. Hdac activity plays important roles in the development of all organs and tissues, including the mineralized skeleton. Bone is a dynamic tissue that forms and regenerates by two processes: endochondral and intramembranous ossification. Chondrocytes and osteoblasts are responsible for producing the extracellular matrices of skeletal tissues. Several Hdacs contribute to the molecular pathways and chromatin changes that regulate tissue-specific gene expression during chondrocyte and osteoblast specification, maturation and terminal differentiation. In this review, we summarize the roles of class I and class II Hdacs in chondrocytes and osteoblasts. The effects of small molecule Hdac inhibitors on the skeleton are also discussed.

Keywords

Lysine deacetylase; chondrocytes; cartilage; osteoblasts; Runx2

INTRODUCTION

The vertebrate skeleton is an endocrine and mechanical organ that facilitates locomotion, protects vital organs, provides a niche for cells of the immune system, and regulates mineral homeostasis, metabolism, and male fertility.^{1–4} Disruptions in normal skeletal development and age-associated declines in skeletal maintenance have negative consequences on overall health and quality of life. Skeletal development and regeneration proceed by two general processes: endochondral bone formation and intramembranous ossification.^{5, 6} In each case, mesenchymal progenitors condense and begin developmental programs that include chondrogenesis (as is the case during endochondral bone formation) and/or osteoblastogenesis. In adults, the skeleton is constantly being remodeled in response to physiological needs and repaired to heal fractures. During remodeling and repair, the vasculature and perichondrium bring mesenchymal progenitors to skeletal sites. The differentiation of mesenchymal cells into chondrocytes or osteoblasts, and their ability to maintain the differentiated phenotypes that exert autocrine, paracrine, and/or endocrine control requires the expression of particular gene programs and the stable repression of genes that are not needed for skeletal functions. These cell type-specific gene expression programs must be maintained in daughter cells during proliferation phases to prevent trans- or de-differentiation of the cell phenotype. Epigenetic processes such as DNA methylation,

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histone acetylation and methylation, and microRNA programming contribute to the differentiation of mesenchymal cells to the chondrocyte or osteoblast lineage as well as the maintenance and functional activities of these cell types. In this review, we will focus on the roles that histone deacetylases (Hdacs) play in regulating gene expression in chondrocytes and osteoblasts during ossification.

Hdacs and Gene Expression

Gene expression requires that transcriptional units in DNA are made accessible to RNA polymerase II. Transcription factors bind to promoter and enhancer elements and facilitate the recruitment of RNA polymerase II. Although some transcription factors are required for the formation of specific tissues and are often referred to as master transcription factors (*e.g.*, Runx2 in osteoblasts and Sox9 in chondrocytes^{7–9}), it is the temporal, spatial, and combinatorial control of multiple transcription factors that provides tissue-specific regulation of gene expression.^{10, 11} Transcription factors bind to DNA and provide docking sites for transcriptional co-activators and repressors, including lysine deacetylases (Kdacs) and their enzymatic counterparts, lysine acetyl transferases (Kats).

Histone deacetylases (Hdacs) are Kdacs that regulate transcription in an epigenetic manner by affecting chromatin structure and transcription factor activity. Hdacs remove negatively charged acetyl groups (CH₃CO⁻) from ϵ -amino groups of lysine residues in histones, to restore the positively charged side chain. This alters the structural confirmation of histones, prevents associations with bromodomain-containing proteins, increases the affinity of the lysine side chain for negatively charged DNA, and thereby facilitates chromatin condensation and transcriptional repression. Hdacs can also deacetylate lysine residues on non-histone proteins, including Runx2 and other transcription factors,^{12–15} affecting their stability and/or cellular localization to influence gene expression programs.

The 18 Hdacs in the human and genomes are subdivided into four groups based on their structure and function (Figure 1). Class I Hdacs (Hdacs 1, 2, 3, and 8) are structurally similar to each other and to the *S. cerevisiae* protein Rpd3.¹⁶ They are broadly expressed in most tissues and are usually localized to cell nuclei.^{17, 18} Class II Hdacs (Hdacs 4, 5, 6, 7, 9, and 10) are similar to the *S. cerevisiae* protein Hda1¹⁶ and demonstrate a more tissue restricted expression pattern making them important contributors to cell-specific differentiation programs. Furthermore, class II Hdacs shuttle between the cytoplasm and nucleus in response to activation of specific kinase signaling pathways. Hdacs 6 and 10 are subgrouped as class IIb Hdacs, due to the presence of two catalytic domains. Unlike the Zn²⁺-dependent catalytic domains of class I and II Hdacs, class III Hdacs (the sirtuins (Sirts), homologs of the *S. cerevisiae* protein Sir2) require NAD⁺ for their enzymatic activity. Sirts 1, 6, and 7 are predominantly localized to cell nuclei, whereas Sirt2 is cytoplasmic and Sirts 3–5 are present in mitochondria.¹⁹ Hdac11, which is the sole member of class IV, shares similarities with both class I and class II Hdacs. Here the roles of class I and II Hdacs in endochondral and intramembranous ossification are reviewed.

Hdac Control of Endochondral Ossification

Endochondral ossification is a highly organized and tightly controlled developmental process and Hdacs play crucial roles in its orchestration (Figure 2). Endochondral ossification gives rise to the majority of bones in the skeleton, including the long bones and vertebrae, and evolves via successive steps of mesenchymal condensation, chondrogenesis, chondrocyte maturation and hypertrophy, and finally vasculogenesis and osteoblast recruitment.

The initial steps of endochondral ossification involve the condensation of mesenchymal cells. The roles of the various Hdacs in these first steps are difficult to ascertain because many Hdac knockout mice die during early embryogenesis. This is the case for Hdac1, 3 and 7 as germline deletion of these genes causes lethality prior to embryonic day 10.5 (E10.5).^{20–22} In contrast, Hdac2, 4, 5, 6, 8 and 9 do not appear to regulate mesenchymal condensation because individual deletion of these enzymes allows early endochondral bone formation.^{23–25} It is unclear if this is due to the fact that these Hdacs are not expressed in the mesenchymal progenitors or if another protein compensates for their loss.

Several Hdacs contribute to the next phase of endochondral ossification, which is the commitment of mesenchymal precursors to the chondrocyte lineage.^{7, 26} Hdac1 associates with Nkx3.2, an essential transcriptional repressor governing the formation of the cartilage anlagen during endochondral ossification.^{27, 28} Furthermore, Hdac1 represses Smad-dependent signaling to control responses to Bmp2, a critical chondrogenic factor.²⁹ Hdac4 and Hdac5 also regulate Smad signaling downstream of Tgf- β and thus may participate in controlling the process of chondrogenesis.³⁰

Chondrocytes within the growth plate promote bone lengthening through proliferation, deposition of an extracellular matrix (ECM), and hypertrophy. Hdac1 regulates expression of several cartilage ECM genes including aggrecan, collagen type 2, collagen type 9, and cartilage oligomeric protein (Comp).^{31–33} Hdac2 also regulates expression of chondrocyte specific matrix genes, including collagen type 2, aggrecan and collagen type 11.³⁴ *Hdac1* KO embryos die prior to chondrogenesis, but *Hdac2* deletion causes only partially penetrant embryonic lethality, with significantly decreased survival of post-natal null animals.²⁴ Of interest, a runted phenotype was observed in the surviving *Hdac2* null animals, suggesting impairment of growth plate development. Double mutant *Hdac5:Hdac9* mice are also smaller in size despite the fact that loss of either *Hdac5* or *Hdac9* has no effect.²⁵ These data suggest that Hdacs contribute to chondrocyte proliferation and/or hypertrophy, but some redundancy exists amongst these Hdacs in regulating the endochondral ossification process.

Genetic deletion of *Hdac3* and *Hdac4* revealed the roles of these proteins in chondrocyte maturation.^{23, 25, 35} Hdac3 and Hdac4 control chondrocyte hypertrophy through their interaction with key transcription factors Runx2 and Mef2, as well as with the co-repressor Zfp521.^{23, 35, 36} Although global loss of *Hdac3* results in embryonic lethality, conditional loss of *Hdac3* function within osterix-expressing cells decreased body size.³⁵ This smaller overall body size was accompanied by a narrower growth plate and a relative expansion of the hypertrophic zone.³⁵ Additional studies are needed with more cartilage specific drivers of Cre (e.g., Col2-Cre) to fully characterize the role of Hdac3 in chondrocytes. In contrast to Hdac3, mice with germline deletion of *Hdac4* function are viable, but exhibit premature endochondral ossification.²³ *Hdac4* ablation accelerated chondrocyte hypertrophy, as evidenced by increased expression of Ihh, Runx2 and collagen type X within the developing growth plate.²³ The effect mimicked Runx2 overexpression in chondrocytes.²³ It was thus concluded that Hdac4 binds to Runx2 and represses Runx2-induced hypertrophy.^{23, 37} Zfp521, a zinc-finger protein and transcription co-repressor that is crucial for maintaining PTH-inducible chondrocyte proliferation, is a component of the Runx2-Hdac4 repressor complex.³⁶ Hdac4 also inhibits Mef2C transcriptional activity to control chondrocyte hypertrophy.^{23, 37}

During the late stages of endochondral ossification, hypertrophic chondrocytes down-regulate expression of type 2a collagen, begin to secrete type X collagen, and calcify the extracellular matrix.³⁸ With further maturation, hypertrophic chondrocytes produce vascular endothelial growth factor (Vegf), which promotes vascularization of the growth plate. Hypertrophic chondrocytes also produce matrix metalloproteinases (Mmps), which aid in

calcified cartilage matrix turnover occurring in primary spongiosa formation. Hdacs regulate Vegf and Mmp13 expression in multiple cell types, thus they are likely to contribute growth plate biology as well.^{39–42}

Ultimately chondrocytes undergo apoptosis, leaving behind the calcified cartilage matrix as a scaffold for bone formation. Vascularization facilitates the recruitment of osteoblast progenitor cells required for the replacement of the collagen type II-dominated matrix with a collagen type I-dominated bone matrix and the formation of the primary spongiosa. These newly recruited precursors contribute to formation of osteoblasts responsible for trabecular (or cancellous) bone deposition; meanwhile, perichondral cells adjacent to the zone of hypertrophy become osteoblasts that form the bony collar. Osteoblast differentiation requires the master transcription factor Runx2.^{8, 9} Several Hdacs (Hdac1, 3, 4, 5, 6 and 7) are expressed in osteoblasts and bind Runx2.^{14, 43–45} Conditional loss of *Hdac3* within osteo-chondroprogenitor cells decreases bone density and reduces osteoblast numbers, but increases marrow adiposity.⁴⁶ These data point to a potential role for *Hdac3* in regulating lineage commitment of mesenchymal cells. Interactions between Runx2 and Hdac3 are enhanced in both premature and mature osteoblasts by the co-repressor Zfp521 to control not only osteoblast differentiation, but also osteoblast-dependent osteoclastogenesis.⁴⁷

A role for Hdac1 during bone formation was supported by the differential expression of Hdac1 during osteoblast differentiation. Hdac1 expression and presence on key osteoblast differentiation gene promoters (such as osterix and osteocalcin) declines during osteoblast maturation.⁴⁸ siRNA-mediated suppression of Hdac1 induces osteoblast differentiation.⁴⁸ Mechanistically, Hdac1 regulates osteoblast differentiation through a physical association with Runx2 to decrease transcriptional activity and repress the stimulatory effects of p300.⁴⁸

Several class II Hdacs are also involved in osteoblast maturation. Hdac4 regulates osteoblast maturation by controlling expression of Mmp13 through a Runx2-dependent and PTH-sensitive mechanism.⁴⁹ Hdac4 and Hdac5 also regulate Tgf- β -dependent signaling and downstream gene repression in osteoblasts through an interaction with a Runx2/Smad3 complex.³⁰ Hdac6 is expressed in differentiated osteoblasts and binds Runx2 to repress gene expression, but its time in the nucleus is very short as it is rapidly exported from the nucleus by unknown signals.^{43, 50} *Hdac6* deficiency results in a minor increase in trabecular bone density.⁵¹ Hdac7 also inhibits the activity of Runx2 and slows osteoblast maturation.⁵² Bmp2 disrupts the interaction between Runx2 and Hdac7 by stimulating protein kinase D, which phosphorylates Hdac7, promotes its association with 14-3-3 proteins and facilitates nuclear export.⁵⁰ Bmp2 also induces nuclear export of Hdac4 in committed osteoblasts, but it did not effect the localization of Hdac5, which remained in the nucleus, or Hdac6, which was firmly cytoplasmic.⁵⁰ Thus, one mechanism of action for anabolic agents is to alter class II Hdac compartmentalization within the cell.

Hdacs Regulate Intramembranous Ossification and Craniofacial Development

In addition to the important role Hdacs play in endochondral ossification, intramembranous ossification is likewise regulated by Hdac functions (Figure 3). Since Runx2 plays an important role in mediating osteoblast differentiation in both types of bone formation, it is not surprising that many Hdac mutants also display craniofacial abnormalities reminiscent of defects in intramembranous ossification. For instance, *Hdac3* deficiency in osterix-positive osteo-chondroprogenitor cells decreased skull bone density as manifested by decreased calvarial thickness and increased porosity.³⁵ In addition to Hdac3, Hdac8 is essential for proper skull development as evidenced by the effect of germline *Hdac8* deletion. Conditional Hdac8 deletion using Wnt1-Cre decreased expression of the homeobox transcription factors Otx2 and Lhx1 and hindered intramembranous ossification.⁵³ This phenotype was not observed with conditional deletion of *Hdac8* in osteoblasts or in

mesenchymal precursor cells with either Col1-Cre or Twist-1-Cre respectively.⁵³ Thus, Hdac8 plays a crucial role in the specification of neural crest progenitor cells to the osteoblast lineage.

HDACS in Human Skeletal Disease

Given the important role of Hdac activity in bone cell function and murine skeletal development, it is not surprising that aberrant class II Hdac activity is associated with skeletal diseases in humans, including osteoarthritis and osteoporosis.

HDAC4 and HDAC5—Recently, *HDAC5* was identified as a locus affecting BMD in a genome-wide association study (GWAS),⁵⁴ and HDAC5 levels were elevated in two juveniles with primary osteoporosis.⁵⁵ Consistent with these findings, increasing Hdac5 (via antagonizing a natural Hdac5 repressor, miR-2861) decreased bone formation and caused bone loss in animal models.⁵⁵ Inactivating mutations in *HDAC4* are associated with brachydactyly,⁵⁶ but otherwise HDAC4 has yet to be conclusively linked to skeletal disease in humans. However, both HDAC4 and HDAC5 interact with *MEF2C*, another BMD-affecting locus identified in the GWAS described above and later confirmed in a second population cohort.⁵⁷ HDAC4 and HDAC5 repress MEF2C's transcriptional activity.⁵⁴ This interaction is relevant because MEF2 transcription factors are implicated in the regulation of adult bone mass,⁵⁸ likely in relation to Wnt signaling, as MEF2 transcription factors (including MEF2C) are essential for the transcriptional activation of *SOST*, the gene that produces the Wnt pathway inhibitor sclerostin.⁵⁸

HDAC7—Cartilage from osteoarthritis patients demonstrated elevated levels of HDAC7 mRNA as compared to cartilage obtained from healthy controls.⁵⁹ Furthermore, HDAC7-positive immunohistochemical staining was more prevalent in the middle- and deep zones of the articular cartilage of OA patients as compared to healthy controls. HDAC7 represses expression of MMP13,⁵⁹ a matrix metalloproteinase linked to cartilage destruction, and thus HDAC7 increases may contribute to the progression and severity of cartilage breakdown.

Hdac Inhibitors and Bone Health

Pharmaceutical Hdac inhibitors are being rapidly developed and tested in clinical trials against a wide spectrum of diseases and clinical conditions including cancer, arthritis, traumatic brain injury, and cystic fibrosis.^{60–63} Since class I and class II Hdac activity is already known to be associated with several human skeletal diseases (as described above), the potential for inhibitor therapies that target the enzymatic action of these Hdacs appears enticing. Early studies, predominantly conducted using in vitro models, supported the idea that Hdac inhibitors could be promising skeletal therapies as they inhibited osteoclasts and stimulated osteoblasts.^{64–68}

These in vitro studies, however, are sharply contrasted by clinical reports of bone loss in patients on long-term valproate therapy. In several patient cohorts, prolonged exposure to valproate decreased bone mineral density in both axial and appendicular sites in children and adults,^{69–71} leading to increased fracture risk.⁷² These studies have many confounding factors, including general decreased physical activity of epileptic patients, but more mechanistic studies utilizing rodent models have confirmed that Hdac inhibition has negative consequences on bone. Mice and rats both demonstrate reduced bone mineral content and compromised skeletal architecture following valproate administration.^{73, 74} Suberoylanilide hydroxamic acid (SAHA, also known as vorinostat or ZolinzaTM), another FDA-approved Hdac inhibitor, also appears to have negative consequences on the skeleton. A preclinical animal model designed to test the effects of SAHA on tumors growing within the bone microenvironment confirmed that SAHA reduced tumor burden in long bones of

immunocompromised mice, but unexpectedly found that bone mass of the contralateral limbs was simultaneously reduced.⁷⁵ An independent investigation conducted with C57BL/6 mice concluded that SAHA administration led to a reduction in trabecular bone mass and architecture.⁷⁶ Understanding the mechanisms behind Hdac inhibitor-induced bone loss requires a more in-depth consideration of the effects of these treatments on the cells responsible for bone resorption (osteoclasts) and bone formation (osteoblasts), as well as on the progenitor pools from which these cells arise.

Hdac Inhibition and Bone Resorption

Osteoclasts are bone-specific macrophages that are essential for bone formation, elongation, and remodeling as they stimulate osteoblast maturation, remove damaged bone, and influence bone shape. Several studies demonstrated that Hdac inhibition decreases osteoclast survival and activity *in vitro*. The Hdac inhibitor trichostatin A (TSA) promoted apoptosis in mature osteoclasts derived from bone marrow cells.⁶⁶ Similarly, the short chain fatty acid sodium butyrate suppressed osteoclast differentiation from hematopoietic precursors *in vitro*.^{67, 77} Depsipeptide, in the cyclic peptide class of Hdac inhibitors, inhibited osteoclastogenesis by increasing interferon- β production, and decreasing expression of pro-osteoclastogenesis factors c-Fos and Socs-3.⁶⁷ Studies with human osteoclasts generated from peripheral blood mononuclear cells indicate that simultaneous inhibition of both class I and II Hdacs may be necessary for osteoclastic suppression by Hdac inhibitors. Thus, pan Hdac inhibitors (*e.g.*, 1179.4b⁷⁸ and SAHA) suppressed osteoclast activity *in vitro*, whereas drugs that preferentially targeted class I (MS-275) or class II (2664.12⁷⁹) Hdacs were less effective until utilized as a combined treatment.⁸⁰ However, molecular studies suggest that targeting certain class II Hdacs may not prevent osteoclastogenesis at all, and instead may actually support osteoclast differentiation. Hdac3 suppression via shRNA inhibited osteoclast differentiation, whereas Hdac7 suppression increased osteoclast differentiation compared to controls, likely because of loss of its suppressive effect on *Mitf*-dependent transcriptional activity.⁸¹

Hdac6 is another important class II Hdac in osteoclasts. Hdac6 is a tubulin deacetylase that regulates tubulin polymerization and cellular attachment.^{82, 83} Hdac6-regulated tubulin deacetylation controls podosome formation and osteoclast migration along the bone surface.^{84, 85} C-Cbl isoforms displace Hdac6 from tubulin and prevent osteoclast apoptosis while stabilizing microtubules and podosomes. Taken together, these studies indicate that osteoclasts are generally intolerant of class I Hdac inhibition *in vitro*, but further research is needed to understand the role of specific Hdacs in osteoclast development and survival.

Reconciling the effects of Hdac inhibitors on osteoclasts *in vitro* with their effects on bone resorption *in vivo* is difficult. Clinical reports offer conflicting evidence; serum and urinary markers of bone resorption were reported to increase, decrease, or remain unchanged in patients on valproate therapy.^{86–88} Animal models, as present, have also failed to demonstrate a consistent *in vivo* effect of Hdac inhibition on bone resorption. Valproate-treated C3H/HeJ mice experience compromised trabecular architecture with no histomorphometric changes in osteoclast number. In immunocompromised mice, SAHA induced bone loss was associated with an increase in serum and histological markers of bone resorption,⁸⁹ but in normal C57BL/6 mice, SAHA administration caused bone loss without inducing changes in circulating or histomorphometric indices of bone resorption.⁷⁶ Thus, understanding the effects of Hdac inhibition on bone resorption *in vivo* requires further study.

Hdac inhibition and bone formation

A large body of literature demonstrates that Hdac inhibitors promote osteoblast differentiation *in vitro* or *ex vivo*.^{64, 90} Valproate, TSA, sodium butyrate, and MS-275 all have stimulatory effects on several osteoblast cell lines, primary calvarial osteoblasts, and in calvarial organ cultures.^{64, 68, 91} In particular, TSA increased expression levels of osteoblast marker genes including collagen type I and osteopontin, and all four of these Hdac inhibitors increased Runx2-dependent transcriptional activity without negatively affecting cell viability.⁶⁴ Hdac inhibitors have been consistently reported to increase osteoblast gene expression, mineralized matrix deposition, alkaline phosphatase production, and Runx2 transcriptional activity *in vitro* by numerous independent researchers.^{64, 90, 92–94}

Similar to what happens with osteoclasts, these effects of Hdac inhibitors on osteoblasts *in vitro* do not translate to a similar response *in vivo*. Although SAHA-treated C57BL/6 mice had increased indices of local osteoblast activity including mineral apposition rate (MAR) and bone formation rate (paralleling increases in osteoblastic gene expression and mineralized matrix production seen in osteoblasts following *in vitro* exposure to Hdac inhibitors^{64, 93, 94}), levels of the circulating bone formation marker P1NP were reduced, and histological indices of osteoblast numbers were decreased. These confounding data highlight a limitation of dynamic histomorphometry, particularly MAR measurements, which only measure the activity of mature osteoblasts where bone is present. Biochemical markers are generally more reflective measures of overall bone formation activity. However, circulating osteocalcin (a marker of bone formation) was reported to be both higher^{86, 88, 95} and lower^{87, 96} in patients receiving long-term valproate therapy compared to controls. Recent studies indicate that human cells treated with valproate reduce production of type I collagen and osteonectin (a collagen binding glycoprotein necessary for maintenance of bone mass), supporting a direct effect of valproate that may contribute to bone loss.⁹⁷

Why, then, do the *in vitro* stimulatory effects of Hdac inhibitors on osteoblasts not translate to increased bone formation *in vivo*? The deleterious effects of Hdac inhibitors on osteoblast progenitors may explain this. Hdac inhibitors promote apoptosis by activating both extrinsic (death ligands/receptors) and intrinsic (mitochondrial proteins) pro-apoptotic pathways.^{98, 99} This is one reason that Hdac inhibitors are such effective anti-cancer therapeutics, as they preferentially target rapidly proliferating cells and induce apoptosis. However, this mechanism raises concern regarding the effects of Hdac inhibitors in regenerative organs, like bone, especially when considering their consequences on self-renewing mesenchymal progenitor cell populations. Mouse embryos from valproate or TSA dams show increased somatic apoptosis, likely contributing to axial skeletal malformations,¹⁰⁰ which may explain why children born to mothers treated with valproate have an increased chance of developing craniofacial bone defects.¹⁰¹ More importantly, adult bone marrow stromal cells, from which osteoblasts arise, appear susceptible to Hdac inhibition. Both mouse and human bone marrow stromal cells undergo DNA damage, cell cycle arrest, and apoptosis following culture with Hdac inhibitors.^{76, 93, 94, 102} Interestingly, once these cells are sufficiently differentiated into mature, matrix producing osteoblasts, Hdac inhibitors fail to cause cell death and instead begin to have stimulatory effect.^{76, 92} Thus, although Hdac inhibitors may stimulate mature, functional osteoblasts, this positive effect is outweighed by their simultaneous depletion of the immature osteoblast progenitor pool, leading to a net decrease in osteoblasts and overall bone formation.

Conclusion / Future Directions

Class I and II Hdacs contribute greatly to skeletal development by binding to transcription factors that control gene expression during specific stages of chondrocyte and osteoblast maturation. It is clear that broad inhibition of Hdacs with pharmaceuticals has adverse

effects on mesenchymal precursors and bone formation. Conditional deletion of some individual Hdacs has revealed stage-specific roles, but systematic deletion of every Hdac in skeletal tissues during relevant stages of differentiation is necessary to fully understand their roles during development. This may ultimately lead to better designs for Hdac inhibitors that effectively treat cancer and other diseases without harming the skeleton. Studies are also needed to determine how Hdacs contribute to orthopedic problems such as fracture repair and if existing Hdac inhibitors are effective at treating heterotopic ossification.

Tissue-restricted expression and intracellular mobility between the cytoplasm and nucleus are well-described characteristics of class II Hdacs. Surprisingly, very little is known about how class II Hdacs are regulated at the transcriptional and translation levels. Moreover, the signaling pathways and posttranslational modifications that influence cytoplasmic-nuclear shuttling are incompletely understood. This knowledge could provide new mechanistic insights for regulating the expression and/or localization of specific enzymes.

There are 18 genes that encode Hdacs in the human and mouse genomes, meanwhile there are greater than 1800 transcription factor genes. It is clear that transcriptional control of gene expression in chondrocytes, osteoblasts, and all other cell types is specified by the combinatorial association of the transcription factors with DNA sequences, each other, and cofactors like Hdacs. New genome-wide sequencing technologies will provide a plethora of data in the next few years that will increase our understanding of the genetic and epigenetic events regulating gene expression. It will also be important to know how interactions between transcription factors and co-repressors are regulated in each tissue. As was discussed, Runx2 binds multiple Hdacs but it is not understood if these are high-affinity interactions or if signaling pathways influence their pairing. Phosphorylation of specific serine residues in Runx1 releases or prevents interactions between Runx1 and Hdac1/Hdac3 in myeloid cells.¹⁰³ Similar events may influence the activity of Runx2 and other transcription factors involved in endochondral and intramembranous bone formation. Thus, while much has been accomplished in the last decade, more work is needed to understand the mechanisms of action of Hdacs in the skeleton.

Acknowledgments

The National Institutes of Health supported this work through grants AR48147, AR56950, AR60140, and DE20194.

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Figure 1.
Mammalian class I, II and IV histone deacetylases.

A. Endochondral bone formation

B.

Figure 2. Hdacs regulate endochondral ossification

A. Summary of endochondral ossification process. **B.** Hdacs play distinct roles in growth plate maturation zones. Hdacs control chondrocyte maturation by regulating expression of matrix genes.

Figure 3. Hdacs regulate intramembranous bone formation

A. Summary of intramembranous bone formation. **B.** Hdac-mediated control of during osteoblast differentiation.