Involvement of Histone Deacetylase 4 (HDAC4) in Osteoclast Function

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Dedication

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Abstract

Bone modeling and remodeling during development and bone integrity throughout life are normally regulated via complex coupled actions of bone-resorbing osteoclasts and bone-forming osteoblasts. Loss of coupling between bone formation and bone resorption leads to pathogenesis of bone metabolic diseases including osteoporosis or osteopetrosis. Defects in osteoclast activity, whether increased activity or deficiency is responsible for bone destruction in many bone diseases such as osteoporosis, osteopetrosis and rheumatoid arthritis rather than impaired osteoblastic bone formation. Despite our growing knowledge in the mechanisms involved in the regulation of osteoclast differentiation and function, there is still a lot unknown. For this reason, it is important to understand the molecular mechanisms underlying how the activity of these bone-resorbing cells are regulated in order to develop effective therapies for bone disorders. Histone deacetylation is one such potential mechanism. It has been reported that class IIa histone deacetylase (HDAC), which include HDAC4, 5, 7 and 9 are regulators of osteoclastogenesis. Evidence from our lab and other labs using in vitro cell culture and in vivo mouse model systems indicated that HDAC7 and 9 are negative regulators of osteoclast differentiation and activity. However, whether the other class IIa members are functionally significant in osteoclasts is largely unknown. The aim of this research was to use in vitro osteoclast cell culture assays in conjunction with an in vivo mouse model to investigate the role(s) of HDAC4 in osteoclasts. HDAC4 conditional knockout (4cKO) mice exhibited increased bone mass phenotype (osteopetrosis) cause by decreased bone-resorbing activity of osteoclasts. HDAC4-deficient osteoclasts show reduced resorptive activity resulting from impaired signaling downstream of the M-CSF and αvβ3 integrin and diminished M-CSF mediated adhesion and migration. Moreover, I demonstrated that c-Src activation in osteoclasts is regulated by HDAC4. The results of this thesis have identified HDAC4 as an essential regulator of osteoclast bone resorption activity both in vivo and in vitro.
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List of Abbreviations

µCT: Microcomputed Tomography
4cKO: HDAC4 conditional knockout, HDAC4<sup>flox/flox</sup>;cfms-Cre
4WT: HDAC4 wild type, HDAC4<sup>flox/flox</sup>
Acp5: gene name for TRAP enzyme
AKT: Protein kinase B
BMM: Bone marrow macrophages
BMU: Basic multicellular unit
BV: Bone volume
BV/TV: Bone volume to total volume
BS: Bone surface
BMPs: Bone morphogenetic proteins
cFms: Receptor for M-CSF (Csf1r)
Csf1r: Gene name for colony stimulating factor
Ctsk: Cathepsin K
c-FOS: FBJ osteosarcoma oncogene
c-Src: Cellular sarcoma
CEBPα: CCAAT/enhancer binding protein-α
CTX: C-terminal telopeptide
De-stamp: Dendritic expressed seven transmembrane protein
ERK 1/2: Extracellular signal-reduced kinase 1/2
HAT: Histone acetyltransferase
HDAC: Histone deacetylase
HDRP: Histone deacetylase related protein (MITR)
HDI: HDACs inhibitor
IL: Interleukin
IP: Immunoprecipitation
JNK: c-Jun N-terminal kinase
M-CSF: Macrophage colony stimulating factor
MEF: Myocyte enhancer factor
MNC: Multinuclear cell
MITF: Microphthalmia transcription factor
MEK 1/2: Mitogen activated protein kinases kinase
MMP9: Matrix metalloproteinase 9
NFATc1: Nuclear factor of activated T cells
Oc.N/BS: Number of osteoclasts per bone surface
Oc.S/BS: Osteoclast surface per bone surface
PU.1: transcription factor PU.1
P1NP: Procollagen type I N-terminal propeptide
p38 MAPK/CSBP: p38 MAP Kinase, Cytokinin Specific Binding Protein
RANK: Receptor activator of NF-κB
RANKL: Receptor activator of NF-κB ligand
TGF-β: Transforming growth factor β
TNF-α: Tumor necrosis factor α
Tb.N: Trabecular number
Tb.Sp: Trabecular spacing
Tb.Th: Trabecular thickness
TRAP: Tartrate-resistant acid phosphatase
TRAcP5b: Tartrate -resistant acid phosphatase 5b
TV: Total volume
WT: Wild type
Wnt: Wingless integrated 1
Chapter 1. Introduction, Statement of Purpose and Specific Aims

1.1. Introduction: Normal bone remodeling

Bone is a highly complex specialized connective tissue that undergoes remodeling (replacement of old bone) throughout life so as to maintain skeletal integrity and bone mass. Human skeleton has two types of bones, cortical and trabecular, that gets remodeled. The trabecular bone is more actively remodeled than cortical bone because of the much larger surface area to volume ratio. Bone tissue is made up of organic and inorganic (mineralized) components (1). The organic component includes bone cells, type I collagen, proteoglycans and numerous noncollagenous proteins, while the inorganic component is comprise of calcium hydroxyapatite providing rigidity and strength (2). It is estimated that 10% of human skeleton is completely remodeled every year (3, 4).

During bone remodeling, a balance exists between bone formation by osteoblasts and resorption by osteoclasts. This balance between these two processes is essential for maintaining bone mass and mineral homeostasis in adult life (5). The overall process of bone remodeling is a tightly controlled and coordinated, and regulated by various local and systemic factors (1). Hormones such as calcitonin (CT), parathyroid hormone (PTH), estrogen, growth factors including bone morphogenetic proteins (BMPs), wingless integrated 1(Wnts) and transforming growth factor β (TGF-β) and cytokines such as macrophage colony-stimulating factor (M-CSF, also called CSF-1), receptor activator of nuclear factor κβ (RANKL), interleukins (IL) and tumor necrosis factor α (TNF-α), play a critical role in bone remodeling process by regulating bone cells, osteoblasts and osteoclasts.

In normal physiological bone remodeling, the tight coupling of bone formation by osteoblast and resorption by osteoclasts requires communication between different bone cells. Cells of the osteoblast lineage (osteoblasts, osteocyte and bone-lining cells) and bone-resorbing cells (osteoclasts) are arranged in a systematic unit called bone multicellular units (BMU) (6). Osteoblasts arise from mesenchymal stem cells and are responsible for bone matrix synthesis and its mineralization (7). Osteocytes play an important role in regulating bone remodeling process (8). Osteoclasts are multinucleated
giant cells that develop from hematopoietic stem cells of the monocyte/macrophage lineage, and are responsible for removing the organic and inorganic components of bone matrix using acid secretion and cysteine proteinases (9). The purpose of BMU is to promote normal bone resorption by supplying connective tissues and blood vessels.

The remodeling cycle consists of activation, resorption, reversal, formation and quiescence phases, as shown in figure 1 (3) (4). During the activation phase, pre-osteoclasts are attracted by activation signals to the remodeling site, where they fuse to form multinucleated osteoclasts. The resorption phase begins with mature osteoclasts digging out a cavity termed a resorption pit whereas the name implies resorption occurs. The bone resorption ends with apoptosis of osteoclasts. During the reversal phase, mesenchymal stem cells, pre-cursors to osteoblasts, appear along the resorption pit, where they proliferate and differentiate into pre-osteoblasts. At the formation phase, pre-osteoblasts mature into osteoblasts at the surface of the resorption pit, and release osteoid at the site, forming a new soft nonmineralized matrix. The new bone matrix is then mineralized with calcium and phosphorous (hydroxyapatite) (3, 4). During quiescence phase, the new bone surface is covered with lining bone cells and the cells remain dormant until a new remodeling cycle is initiated. Resorption phase is short (2 to 4 weeks), while formation phase can continue for 3 to 6 months (3). Imbalance between bone formation and resorption leads to severe bone diseases, and most clinical bone diseases are associated with osteoclast dysregulation in the remodeling process. Thus, understanding how osteoclasts are regulated is necessary.

![Figure 1. The process of bone remodeling in healthy bone.](https://basicmedicalkey.com/structure-and-function-of-the-musculoskeletal-system/)
1.1.2 Osteoclastic Bone Resorption

Osteoclasts are large multinuclear cells formed by fusion of mononuclear precursors. These cells are highly mobile and alternate between migratory and bone-resorbing stages (10, 11). The bone resorption process begins with osteoclasts attaching firmly to bone surface using integrins such as αvβ3 (12). Following attachment to bone surface, osteoclasts polarize to create a tight sealing zone in close proximity to the bone surface and a ruffled border, a highly convoluted membrane that faces the resorbing surface. The formation of sealing zone is important for osteoclast polarization since it forms a diffusion barrier and allows the directional secretion of hydrochloric acid and lysosomal enzymes into the ruffled border (10). The mineral component of bone is degraded through acidification by electrogenic vacuolar H+ ATPases proton pump (V-type ATPase), Cl⁻ channel, and a basolateral chloride-bicarbonate exchanger (13). The production of bicarbonate ions by carbonic anhydrase and release of H⁺ regulates osteoclast intracellular pH (14). The organic component of the bone matrix is degraded by secretion of acidic lysosomal proteinases such as cathepsin K, TRAP and MMP9. Cathepsin K is a major proteinase in the degradation of bone matrix in the resorption pit (15). The products of matrix degradation are endocytosed by osteoclasts and transported in vesicles to and released at cell’s extracellular environment (16). In order to arrange these highly polarized structures, osteoclast must migrate, adhere to the bone surface, detach and move to new resorption site. In this sense, crosstalk between signaling pathways in osteoclasts is an important step in regulating this process.
1.1.3 Osteoclasts

Osteoclasts are multinucleated giant cells originated from hemopoietic stem cells of the monocyte/macrophage lineage that are responsible for bone resorption under both normal conditions during development and remodeling, and pathological conditions (9). Osteoclast differentiation is a multi-step process that includes cell proliferation, commitment and fusion. Osteoclast differentiation is dependent on two important cytokines: macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear kappa B ligand (RANKL) (9, 10). These cytokines are produced by osteoblasts and stromal cells in the proximity of osteoclasts and are required for differentiation, survival and bone-resorption (18). The differentiation process begins with the hematopoietic stem cell becoming multipotent progenitor cell then committing to be a cell of monocyte/macrophage lineage. Under the presence of M-CSF and RANKL, the cells proliferate, exit the cell cycle to become committed osteoclast precursor and then differentiate into mononuclear prefusion osteoclasts. Eventually, mononuclear osteoclast will fuse into multinuclear osteoclasts, become polarized, activated and resorb bone (see Figure 3).
1.1.4 RANK Signaling

In osteoclast precursors and mature osteoclasts, binding of RANKL to its receptor RANK supply the critical signal to drive osteoclast differentiation from hematopoietic stem cells as well as activate mature osteoclasts (19). The essential role of RANKL and RANK in osteoclasts was identified by genetic experiments which demonstrated that mice lacking either Rank or Rankl had severe osteopetrosis due to lack of osteoclasts (20, 21). RANKL is membrane bound protein that may also be released by osteoblasts or stromal cells to activate osteoclast differentiation. RANK is a member of tumor necrosis factor receptor (TNFR) superfamily, which lacks intrinsic enzymatic activity and is required to activate downstream signaling cascades. Therefore, RANK transduces intracellular signals by recruiting adaptor molecules such as TNF receptor-associated factor 6 (TRAF6), which then activates NF-κB, PI3K/Akt, and MAP kinases (ERK, p38, and JNK) pathways to regulate osteoclastogenesis (22). This interaction also triggers signaling cascades that activate transcription factors NF-κB and NFATc1. These transcription factors activate genes such as c-Fos, dc-stamp, and cathepsin K that have been associated with osteoclast differentiation, survival, and activity (19).

1.1.5 M-CSF Signaling

M-CSF is critical for both osteoclast development and function. The role of M-CSF in osteoclast formation was first observed in op/op mice, which demonstrated that deletion of M-CSF results in osteopetrotic mice due to lack of osteoclasts (23). Additionally, it was reported that deletion of M-CSF receptor, c-Fms, also results in osteopetrotic mice (24). c-Fms, a platelet-derived growth factor (PDGF) receptor family member, is a sole cell surface
receptor of M-CSF (25, 26). Binding of M-CSF to its cell surface receptor c-Fms results in receptor dimerization, and hence activation of its intrinsic receptor tyrosine kinase activity, leading to autophosphorylation of c-Fms domains. Each phosphorylated residue then acts as a docking site for phosphotyrosine-binding (PTB) or Src homology region 2 (SH2) containing proteins, which amplify and activate signaling that promotes osteoclast differentiation, survival and activity. Activation of PI3K/Akt and ERK1/2 pathways regulate proliferation of osteoclast precursors and osteoclast survival (27). c-Src activation is important for osteoclast proliferation as well as cytoskeletal reorganization for osteoclast bone resorption (28). It has also been reported that crosstalk between αvβ3 integrin and M-CSF regulates cytoskeleton rearrangement, as both signals regulate osteoclast bone resorption (29, 30).

1.1.6 Histone Deacetylases (HDACs)

Acetylation of core histones, as well as non-histone proteins, is becoming more evident as a widespread reversible posttranslational modification in regulating gene expression and various cellular processes. The opposing activities of two type of enzymes, histone acetyltransferases (HATs) and histone deacetylases (HDACs), control the reversible acetylation process (31, 32). HATs promote transcription activation, whereas HDACs favors transcriptional repression as shown in Figure 4. Association of HATs and HDACs with sequence specific DNA-binding proteins permit gene-specific activation and repression, respectively (31).

![Figure 4. Histone acetyltransferases (HATs) and Histone deacetylases (HDACs). Cartoon illustrating the roles of HATs and HDACs in acetylation status of histones.](image-url)
1.1.6.1 Mammalian HDACs

Based on their similarities to yeast deacetylase domain, 18 mammalian HDACs have been identified and classified into four classes based on their catalytic mechanisms as shown in Figure 5 (31). Class I, II and IV are zinc-dependent enzymes, whereas class III enzymatic activity is dependent on NAD+. Class I HDACs consist of HDAC1, 2, 3 and 8, which are ubiquitously expressed and located in nuclei, with the exception of HDAC3, which is both nuclear and cytoplasmic. Class II HDAC4, 5, 6, 7, 9 and 10, exhibit tissue-specific expression patterns, with highest expression in brain and skeletal muscle, and can shuttle between nucleus and cytoplasm in response to intracellular signaling. Class III HDACs consists of sirtuins (SIRT 1-7), which require NAD+ for activity (33). Class IV HDAC 11, is the sole member of this class and has homology to both class I and II (34). HDACs have been shown to regulate many biological processes such as cell cycle progression, differentiation and development. Several HDACs inhibitors have been developed to help with treatments of human diseases; however, these inhibitors were reported to not be specific, as they target both class I and II HDACs (35). Therefore, the effects of individual HDACs, such as class IIa HDACs on osteoclasts and bone in health and disease state is pivotal.

Figure 5. The four classes of HDACs in mammals - class I, class IIa, class IIb, class II, class III and class IV. Image obtained from (36).
1.1.6.2 Class II HDACs

Based on their domain organization, class II HDACs are further divided into class IIa (HDAC4, 5, 7 and 9) and class IIb (HDAC6 and 10). In recent years, class IIa HDACs have been accepted as critical regulators of a multitude of biological processes (37), with aberrant expression or activity being linked with wide range of human diseases such as cancer (38). It has been reported that HDAC4, 5, and 7 regulate chondrocyte and osteoblast differentiation and activity, suggesting that these HDACs are critical regulators of endochondral ossification (36). Recently, HDAC7 and 9 have been reported both in vivo and in vitro as negative regulators of osteoclast differentiation and function (39-41). HDAC4 was shown to regulate osteoclast differentiation in vitro (42), however HDAC4 in vivo role in osteoclastogenesis has not been examined.

1.1.6.3 Histone deacetylase 4 (HDAC4)

HDAC4, a key member of the class IIa HDACs, is expressed in various tissues and plays a central role in the formation of the skeleton (43, 44) (45). HDAC4 has been shown to regulate chondrocyte hypertrophy in part by regulating the activity of transcription factor RUNX2, a master gene of skeletogenesis, in proliferating chondrocytes (43). Given that RUNX2 expression in osteoblasts regulates endochondral bone formation, this suggest that HDAC4 also regulates bone formation (7). Mice lacking HDAC4 die perinatally stage due to ectopic ossification of endochondral cartilage (43). In osteoclasts, HDAC4 is expressed during early stages of osteoclastogenesis (42). The Mansky lab has previously demonstrated that knockdown of HDAC4 using shRNA causes an increase in osteoclast number, size and resorption (42). However, the in vivo significance of HDAC4 expression in osteoclast differentiation and function has not been studied.

1.2 Statement of Purpose

1.2.1 Significance of Research

To develop novel pharmacologic agents that arrest the progression of skeletal disorders such as osteoporosis, we need to know how osteoclasts differentiation and function is regulated. Incomplete understanding of the molecular mechanisms which regulate osteoclast function limit this process. A novel approach for the treatment of skeletal
disorders is via HDAC regulation of bone resorption. Given the core biological functions of HDACs, it is not surprising that they are involved in many pathological processes, and their inhibition could provide clinical benefits. Recently, HDAC inhibitors (HDIs) vorinostat and romidepsin, were approved by the US FDA for the treatment of T cell lymphoma and ovarian/cervical cancers (35). The first generation of HDIs promoted the acetylation of histone and non-histone proteins to increase or decrease expression of multiple genes. Moreover, long-term use to these drugs leads to detrimental side effects such as loss of bone density.

The next generation of HDIs is predicted to target specific HDACs. However, there is a compelling need to first understand the role of individual HDACs in regulating osteoclasts and more generally in bone in physiological and pathological disorders. Conditional knockout animals can provide valuable information on suitable targets, potential therapeutic benefits and define the role of specific HDACs on osteoclasts and bone.

The *in vivo* role of HDAC4 in osteoclast function, and the mechanism(s) by which HDAC4 regulates osteoclast function have not been examined. These gaps in our knowledge limit the development of HDAC member-specific HDIs that maintain therapeutic benefits but minimize detrimental side effects. The data outlined in this thesis will address these important questions and are vital towards the development of new clinical therapeutics based on each HDACs in osteoclasts.

1.2.2 Hypothesis

I hypothesize that HDAC4 regulates osteoclast function through its interactions with key osteoclast proteins.

1.3 Specific Aims

1.3.1 Specific Aim 1: Characterize the *in vivo* phenotype and cellular effects of conditional knockout of HDAC4 (4cKO) in osteoclasts progenitors.
To understand the role of HDAC4 in osteoclast, I have generated a conditional knockout mice model for HDAC4 expression in osteoclasts by mating $Hdac4^{fl/fl}$ mice to $c-Fms-Cre$ mice.

1.3.2 Specific Aim 2: Characterize the molecular mechanisms by which HDAC4 regulates osteoclast differentiation and function

Change in gene expression is required for sufficient osteoclast differentiation. To determine global changes in gene expression in HDAC4 conditional knockout (4cKO) osteoclasts, I performed RNA-SEQ on HDAC4 WT (4WT) and 4cKO osteoclasts.

The results of this thesis will address the role of HDAC4 on osteoclasts. It will further our knowledge in the effects of individual HDACs in osteoclasts and bone. This knowledge is vital towards the development of new clinical therapeutics for the treatment of bone diseases based on each HDACs in osteoclasts.
Chapter 2. Regulation of Osteoclast Differentiation and Skeletal Maintenance by Histone Deacetylases

Chapter 2 incorporates review paper: Regulation of Osteoclast Differentiation and Skeletal Maintenance by Histone Deacetylases. Faulkner B, Astleford K, and Mansky KC. Molecules 2019; 24(7): 1355

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Chapter Summary

The roles of HDACs in osteoclasts differentiation and function have not been widely studied as they have been in osteoblast, nevertheless this is a developing area of scientific interest. The aim of this review was therefore to summarize the general properties of HDACs and the research that have revealed their important functions in osteoclasts. Understanding the biological significance of HDACs will not only provide new insights into the mechanisms of HDACs involved in mediating biological response, but also form a platform to develop pharmacologic agents to achieve clinical implications.

2.1 Introduction

A common misconception about the skeleton is that it is static, however, bone is an ever-changing organ that is remodeled through tight coupling of bone resorption followed by formation of new bone (as reviewed (46-48)). These processes are performed by bone resorbing osteoclasts and bone forming osteoblasts. Skeletal homeostasis depends on strict control over the number of active osteoclasts at any site (19). Since osteoclasts are a perpetrator of many skeletal diseases, understanding the mechanisms that regulate their activity during the bone remodeling process is necessary (as reviewed in (49-51)).

The dynamic and responsive nature of bone during the remodeling process requires temporal changes in gene expression within the osteoclast lineage (as reviewed in (52)). Combination of transcription factors and co-factors binding to DNA sequences plays an important role in chromatin remodeling and cellular signaling events in regulating osteoclasts, thus impacting bone remodeling. Additionally, regulation of gene expression is controlled, in part, by histone deacetylases (HDACs) (31), which are intracellular
enzymes that directly affect chromatin structure, transcription factor activity (53), signaling events, and thus affect the activities required for bone remodeling. Understanding how these molecular and cellular switches work has become an important mechanism(s) to consider in regards to developing targeted bone therapies. HDACs are clinically relevant because many molecules that inhibit their activities are used alone or in combination with other drugs to treat diseases such as osteoporosis and cancer (54).

To develop therapies that halt progression of skeletal diseases, we need to understand how osteoclasts operate including the mechanisms that regulate their generation, regulation and bone resorbing activity. Therefore, transcription factors, co-factors, transcriptional regulators and co-regulators must be studied to determine their effects on osteoclastogenesis. Recently, a great deal of progress has been made in investigating these mechanisms, and the bone degradation process using experimental mouse models and patients with abnormal bone phenotypes (9, 49, 55, 56).

In this review, we focus on regulation of osteoclasts by transcriptional factors and introduce important findings on how osteoclasts are regulated by HDACs to exert bone-resorbing activity. We have organized this review according to the different categories of regulation. We will begin by discussing osteoclasts and their transcriptional factors followed by the influence of HDACs on osteoclast differentiation and activity.

2.2 Osteoclast Biology

Bone is comprised of multiple cell types, including osteoblasts, which are mesenchymal-derived cells responsible for synthesizing new bone, osteocytes which are terminally differentiated osteoblasts and bone degrading osteoclasts, which are hematopoietic in origin (10). Bone homeostasis and normal function result from the tightly regulated interactions between osteoblasts, osteocytes and osteoclasts. To maintain this balance in the adult skeleton, osteoblasts and/or osteocytes produce two necessary cytokines, macrophage colony stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL) to promote osteoclast differentiation (57).

Osteoclasts are giant multinucleated cells of hematopoietic origin that degrade the bone matrix. They are formed by fusion of mononuclear precursors of the monocyte/macrophage lineage, and are the primary resorptive cells of the skeleton (19, 58). One of the most remarkable features of the osteoclast is its ruffled membrane, which is the resorptive
organelle of the cell. When this membrane comes into contact with bone, skeletal matrix degradation follows (13). Most skeletal disorders manifest when osteoclast activity is dysfunctional (50, 51). Increased bone mass phenotype (osteopetrosis) is associated with low osteoclast activity while decrease bone mass phenotype (osteoporosis) is associated with increased osteoclast activity.

Progression from multipotent progenitors into specialized, terminally differentiated cells involves carefully regulated patterns of gene expression to control lineage specification and emergence of the cellular phenotype. This process requires coordinated action of transcription factors with co-activators and co-repressors to bring about proper activation and inhibition of gene expression. In the following section, we will describe what is known about transcription factors that regulate osteoclast differentiation (Figure 6).

2.3 Transcriptional Regulators of Osteoclast Gene Expression

2.3.1 PU.1

PU.1 is a member of the ETS-domain transcription factor family (59). It has been shown that mice lacking PU.1 have a deficiency in both macrophage and osteoclast differentiation, and this established PU.1 as one of the earliest markers of the osteoclast lineage (60). During osteoclast differentiation, the expression level of PU.1 is unchanged or modestly increased (60, 61). In the early stages of commitment to the monocyte/macrophage lineage, PU.1 upregulates expression of Csf1r the receptor for M-CSF one of the essential ligands for osteoclast differentiation (62). Along with other osteoclast transcription factors, PU.1 regulates expression of Tnfrsf11a the gene for RANK, the receptor for RANKL, the other essential cytokine for osteoclast differentiation (63). Lastly PU.1 along with other osteoclast transcription factors, c-FOS, MITF and NFATc1, regulates multiple genes necessary for osteoclast differentiation including cathepsin K (Ctsk), Acp5, the gene for TRAP, dendritic cell seven transmembrane protein (Dc-stamp), and Oscar (61, 64, 65).

2.3.2 MITF

Microphthalmia transcription factor (MITF) is a basic helix-loop-helix-leucine zipper transcription factor (66-68). It has been established that MITF plays an essential role in
regulating gene expression during osteoclast differentiation. This observation was based on a bone marrow transplantation experiment which showed that osteopetrotic condition in mi/mi mutant mouse could be cured using bone marrow from a wild type littermate (69). Additionally, mononuclear osteoclasts from these mi/mi mice were unable to fuse, form a ruffled border and resorb bone (70). These results not only confirmed osteoclasts hematopoietic origin but also established MITF as a necessary transcription factor during osteoclast differentiation. MITF along with PU.1 was shown to upregulate the expression of Tnfrsf11a (71). Studies on MITF in osteoclasts have also revealed that it can transcriptionally regulate osteoclast genes (Acp5, Oscar and Ctsk) along with PU.1 (64, 72, 73).

2.3.3 CEBPα

The CCAAT/enhancer binding protein-α (C/EBPα) is a member of C/EBPα family of transcription factors which act as master regulators of various cellular processes (74). C/EBPα has been shown to be important for differentiation of myeloid progenitors (75). Recently, C/EBPα was shown to be highly expressed in osteoclasts (76). Overexpression studies revealed that PU.1 upregulates expression of C/EBPα while C/EBPα upregulates expression of c-FOS and NFATc1 (76, 77). Forced expression studies revealed that C/EBPα can specify mouse bone marrow cells to become osteoclasts, suggesting that C/EBPα can induce osteoclast lineage priming and plays a role in regulating gene expression during the early stages of osteoclast differentiation (76). In addition, C/EBPα deficient mice were shown to have severe osteopetrosis due to impaired osteoclast development (78, 79). In a subsequent study, overexpression of C/EBPα was shown to promote osteoclast differentiation and induce expression of osteoclast genes Nfatc1, Ctsk, and Acp5 (80). Besides its role in regulating osteoclast differentiation, functional experiments revealed that C/EBPα plays a role in bone resorption (80). Together all these studies suggest that C/EBPα is essential for osteoclast differentiation and activity.

2.3.4 MEF2

The myocyte enhancer factor 2 (MEF2) family of transcription factors play an important role in many cellular processes including cell differentiation and apoptosis (81-83). These proteins can affect the expression of many genes by binding with other
transcription factors (81-83). One of these binding partners is HDACs. Each individual HDAC contains a MEF2-interacting transcriptional repressor homology domain suggesting that they have the ability to regulate the MEF2 family (81-83). MEF2 family consists of 4 members, MEF2A, B, C and D (81-83). Osteoclasts primarily express MEF2A and D (Mansky, unpublished observation). Osteoclasts null for MEF2A and D express reduced levels of c-FOS and NFATc1 suggesting that MEF2 may regulate expression of these transcription factors in osteoclasts (Mansky, unpublished observation).

2.3.5 c-FOS

c-FOS is a member of activator protein -1 (AP-1) family of transcription factors, and its expression is induced early during osteoclast differentiation (84, 85). c-FOS acts as an important switch between osteoclast and macrophages differentiation, and in its absence osteoclasts do not form (84). This observation was made in c-Fos knockout mice which develop osteopetrosis due to the inability of cells to commit to osteoclast lineage (84, 86). c-FOS and NF-κB both target and upregulate Nfatc1 expression in osteoclasts and at least in part explain why c-Fos knockout mice do not develop osteoclasts (85).

2.3.6 NF-κB

Nuclear factor kappa-light-chain-enhancer of activated B cells or NF-κB is a pleiotropic transcription factor part of the Rel subfamily of proteins (87). In osteoclasts, NF-κB regulates formation, function and survival (87-89). NF-κB is activated downstream of RANK signaling that ultimately results in the activation of NFATc1 to induce osteoclastogenesis (87, 90). The inhibitory protein IκB localizes NF-κB in the cytoplasm until it is activated by dimerizing with Rel family proteins promoting nuclear translocation to activate transcription (87-89). Loss of the p50 and p52 subunits of NF-κB results in an osteoporotic bone phenotype in mice most likely due to the inability to activate Nfatc1 expression (90).

2.3.7 NFATc1

NFAT is a family of transcription factors that regulate the expression of cytokines and other immunoregulatory genes (91). NFATc1 mediates RANKL-induced osteoclast formation, and its overexpression in c-Fos deficient cells rescues osteoclastogenesis (85,
At early stages of osteoclast differentiation, RANKL increases the stability of NFATc1 protein by stimulating calcineurin-mediated dephosphorylation of NFATc1 in the cytosol, causing it to translocate to the nucleus (93). However, during late stage osteoclastogenesis, M-CSF downregulates NFATc1 protein levels, and eventually NFATc1 is degraded through ubiquitin-proteosome pathway in the cytoplasm (94). This degradation is mediated by Cbl-b and c-Cbl ubiquitin ligases in a Src-dependent manner (94). While NFATc1 is termed the “master regulator” and is sufficient for osteoclast differentiation, ChIP experiments demonstrated that NFATc1 regulates osteoclast gene expression in a complex with PU.1, MITF and c-FOS (95). There are no studies yet to demonstrate how or if MEF2 and C/EBPα interact with this complex to regulate osteoclast gene expression.

**Figure 6. Expression of transcription factors and HDACs during osteoclast differentiation.** Cartoon illustrates expression of transcription factors and HDACs during osteoclast differentiation based on studies presented in this review. HDACs in red inhibit osteoclast differentiation, HDACs in green promote osteoclast differentiation and the function of HDACs in orange are not known due to lack of animal studies (96).

### 2.4 Histone Deacetylases (HDACs)
Histones are major regulators of gene expression and transcription, and their state of activeness and inactiveness controls this process (44). Acetylation is an important modification to histone tails that allow for genes to be transcribed by releasing DNA so it can unwind and become accessible for transcription (44). There are two enzymes that play an important role in this process; histone acetyltransferases (HATs) and histone deacetylases (HDACs) (44). HATs are enzymes that will acetylate histone tails resulting in the relaxation of the chromatin and allowing for transcription factors to bind to their promoters (44). For transcription to be repressed, HDACs are recruited to remove acetyl groups on the tails, allowing chromatin to rewind around histones (44). Additionally, some HDAC members have the ability to directly interact with transcription factors to repress transcription (31). Understanding transcriptional regulation by HATs and HDACs in bone is vital in the study of differentiation and disease in which these two enzymes play a role.

2.4.1 HDAC Classes

There are 18 HDACs within the human genome, each of which are distributed into 4 different classes: class I HDACs (HDACs 1,2,3,8), class II HDACs (HDACs 4,5,6,7,9,10), class III HDACs (Sirtuins 1-7) and class IV HDACs (HDAC11) (31). They are divided based on their enzymatic activity, location within the cell and their sequence homology to yeast (31). The structure and function of HDACs are similar to one another and are also closely related to that of budding yeast, however; each class has their own unique catalytic domain (31). Class I HDACs are homologs to yeast RPD3 and are primarily localized in the nucleus (31). They contain a catalytic domain unique to the class I HDACs and predominantly target histone substrates (31). Class II HDACs are homologs to yeast HDA1 (97, 98). These HDACs are separated into two subclasses; class IIA and class IIB (44). Class IIA HDACs are transcriptional repressors that are shuttled between the nucleus and the cytoplasm by the chaperone protein 14-3-3 (44, 98). Class IIB HDACs differ from IIA, in that they contain two catalytic domains and they are primarily found in the cytoplasm (97). Class III HDACs, or sirtuins, are homologs to yeast Sir1 (99). These HDACs are dissimilar to the class I and class II HDACs in that they are NAD⁺ dependent and contain an NAD⁺ binding site (99). Class III HDACs are known to deacetylate histones and regulate transcription factors (99). They can regulate transcription factors by altering their binding to DNA, modifying their location within the cell or changing their interaction with
other proteins (99). HDAC11 is the sole member of the class IV HDACs (100). It shares a similar structure to that of the class I HDACs, however little is known about the mechanism by which HDAC11 regulates transcription (100).

2.4.2 HDACs in bone development

Bone development occurs throughout life. The three bone cells that are involved in development, growth and remodeling are osteoblasts, osteocytes and osteoclasts. The two main types of bone formation/ossification are intramembranous and endochondral. Endochondral ossification is the process by which long bones and vertebrae form while intramembranous ossification results in formation of flat bones of skull, mandible and clavicle. It has been reported that some HDACs have roles in the ossification process. Intramembranous bone defects in mouse models associated with altered class I HDAC expression have been described (36, 101, 102). HDACs have also been shown to contribute to different steps of endochondral bone ossification. Germline deletion of HDAC1, 3 and 7 are embryonic lethal and die before endochondral ossification begins (103-105). Deletion of HDAC2, 4, 5, 6, 8 and 9 are not embryonic lethal and do not appear to disrupt early endochondral ossification (43, 106, 107). HDAC3 and HDAC4 have been shown to play a role in chondrocyte maturation (43, 104, 108). There are currently no published studies demonstrating that HDACs play a role in regulating osteoclast activity during bone development. For a more complete review of HDAC mediated control of endochondral and intramembranous ossification see (109).

2.4.3 HDACs and skeletal maintenance

Skeletal maintenance requires a tightly coordinated activity of bone cells osteoclast, osteoblasts and osteocytes (48). Some of the 18 human HDACs have been shown to play a part in skeletal bone maintenance. Multiple HDACs (1, 3, 4, 5, 6 and 7) are expressed in osteoblasts and have been shown to regulate osteoblast differentiation and gene expression (110-116) as well as osteocyte activity (117, 118). More complete reviews on regulation of osteoblasts and osteocyte activity by HDACs are (6, 36, 109, 119). In section 5 of this review, we will discuss what is known concerning the role of regulating osteoclasts during maintenance or remodeling phase of the skeleton.
2.4.4 HDACs and skeletal diseases

Currently in humans, mutations in HDAC2, 4,5,6 and 8 have been shown to affect the skeleton (120-128). These HDACs mutations results in either loss-of-function, deletion or gain of function in patients. Some of the defects in bone caused by these mutations are low bone mineral density, brachydactyly, and skeletal abnormalities in the craniofacial region, the spine and the growth plates (as reviewed in (36)). Further investigations are needed in order to understand the altered HDACs functions. It has also been reported that many HDACs are expressed in human articular cartilage and expression of some of the HDACs was even higher in joints from osteoarthritic patients (129-133). The causative role for the altered HDACs function needs to be investigated. Usually, the amount of bone resorbed by osteoclasts is balanced by the amount of bone formed by osteoblasts. Nevertheless, in increased bone resorption conditions such as osteoporosis and Paget’s disease, bone resorption surpass bone deposition which results in bone loss. The roles of HDACs in regulating osteoclasts in these bone resorption conditions is not understood and is an emerging area of scientific interest.

2.5 Role of HDACs in Osteoclasts

2.5.1 Class I HDACs

Class I HDACs are thought to be expressed in most cell types (31). HDAC1 and 2 expression is almost exclusively nuclear as both proteins lack a nuclear export signal (31). However, HDAC3 has both a nuclear import and export signal but is almost always found in the nucleus perhaps due to the fact that it recruited to the nucleus via its interactions with HDAC4, 5 and 7 when they are bound to DNA (134-136). There are currently no published animal model studies analyzing the role of HDAC1, 2, 3 or 8 in osteoclasts using conditional mouse models. These studies are critical to understanding the role of class I HDACs in osteoclast differentiation as well as skeletal maintenance. Chemical inhibitors and siRNA studies are informative as they can be used to distinguish between effects of HDACs regulating osteoclast differentiation versus activity but they can have off target effects making the results difficult to interpret. Animal models are critical to understanding the role of class I HDACs in regulating osteoclasts and skeleton maintenance.

2.5.1.1 HDAC1
HDAC1 is a transcriptional repressor that is present early during osteoclast differentiation (41). It is expressed in osteoclast precursors and then drops significantly after RANKL stimulation (41). The main role of HDAC1 in osteoclasts is to act as a co-repressor (137). It does this by being recruited to the promoters of osteoclast genes such as Nfatc1 and Oscar to prevent their expression (137). By ChIP analysis, MITF and PU.1 have been shown to be recruited to Ctsk and Acp5 promoters with M-CSF stimulation along with co-repressors CtBP, Sin3A and HDAC1 (138).

2.5.1.2 HDAC2

In contrast to HDAC1, HDAC2 expression increases during osteoclast differentiation (139). Studies have shown that knock down of HDAC2 in osteoclasts not only inhibits osteoclast differentiation, but also hinders actin ring formation, fusion, and osteoclast activity (139). HDAC2 was shown to activate Akt, which removed an inhibitor, FoxO1, and demonstrated that HDAC2 is not an inhibitor of osteoclast differentiation.

2.5.1.3 HDAC3

HDAC3 is expressed in osteoclast precursors, and its expression remains low during osteoclast differentiation (41). Knock down of HDAC3 results in inhibition of osteoclastogenesis resulting from the down regulation of osteoclast genes Nfatc1, Ctsk and Dc-stamp (140). Loss of HDAC3 expression is similar to the phenotype seen with osteoclasts treated with broad spectrum HDAC inhibitors (HDIs) such as trichostatin A (TSA) and sodium butyrate (NaB) (141, 142).

2.5.1.4 HDAC8

HDAC8 expression is low during early osteoclast differentiation, and its expression increase during late osteoclast differentiation (41). Not much is known about HDAC8 in osteoclasts, and as stated above further animal model studies will need to be done to understand its role in regulating differentiation and activity.

2.5.2 Class II HDACs

Based on cellular signals, class II HDACs are able to shuttle between the cytoplasm and the nucleus. At least in muscle cells HDAC4, 5 and 7 have a very regulated process of shuttling between the nucleus and cytoplasm (31, 143, 144); however, the subcellular
localization of class II HDACs has not been well studied in osteoclasts. HDAC4, 5, 7 and 9 all have a binding site in their amino terminus for C-terminal binding protein (CtBP), MEF2 and 14-3-3 (31, 135, 145). One interesting question that has emerged pertaining to HDACs 4, 5, 7 and 9 given their similarity is why are they not functionally redundant in osteoclasts. To answer this question, studies need to be performed to identify targets and partners of class II HDACs so as to understand the mechanisms by which class II HDACs regulate osteoclast differentiation and activity.

2.5.2.1 HDAC4

HDAC4 is expressed in osteoclast precursors, and its expression begins to decrease as osteoclasts become mature (41, 42). Knock down of HDAC4 by shRNA increases osteoclasts in size and number (42). With the increase in osteoclast differentiation there was also an up regulation of osteoclast genes (c-fos, Nfatc1, Dc-stamp, and Ctsk) and increases in osteoclast activity (42). To further study the role of HDAC4 in regulating osteoclast differentiation, conditional deletion of Hdac4 using Cfms-Cre was created and demonstrated to have no gross phenotypic changes in bone structure. More focused analysis surprisingly revealed an increase in bone volume in trabecular bone at 3 months of age suggesting an increase in bone mass phenotype (Mansky, unpublished observation). Moreover, bone resorption was reduced in HDAC4cKO mice (Mansky, unpublished observation). The mechanism(s) by which HDAC4 regulates osteoclast differentiation i.e. targets and partners is currently unknown and is being evaluated using an osteoclast specific HDAC4 mouse model (Mansky, unpublished observation).

2.5.2.2 HDAC5

HDAC5 is expressed later in osteoclast differentiation, having the highest expression around the time of osteoclast fusion (41, 42). Similar to HDAC4, HDAC5 knock down by shRNA results in an increase in osteoclast differentiation, an upregulation of osteoclast genes and an increase in resorption (42). Global deletion of HDAC5 in mice demonstrated no gross defect at birth, and mice are of normal size (107). In two other separate studies, HDAC5 knockout mice were reported to have reduced trabecular bone density at 2-3 month of age (114, 117). One study suggested loss of HDAC5 expression leads to an increase in RANKL expression by osteoblasts, which stimulated greater bone resorption by the
osteoclasts (114). However, since multiple types of bone cells are being affected in the global HDAC5 null mouse, and the mechanism(s) for bone loss is not clear more focused tissue specific studies need to be conducted. These mechanisms will be explored using an osteoclast specific HDAC5 mouse model (Mansky, unpublished observation). Mechanistically HDAC5 has been shown to deacetylate NFATc1 leading to NFATc1’s instability and a potential mechanism by which HDAC5 inhibits osteoclast differentiation (146).

2.5.2.3 HDAC6

During osteoclast differentiation, HDAC6, a class IIb HDAC protein, expression was shown to peak around osteoclast fusion and to be expressed predominately in cytoplasm (41, 42). By using shRNA, knockdown of HDAC6 expression in mouse bone marrow macrophages (BMMs) did not display any phenotype nor did it seem to affect expression of major osteoclast genes (42). HDAC6 plays an important role in destabilizing the osteoclast cytoskeleton and inhibiting osteoclast migration and podosome formation (147, 148). This observation revealed a molecular mechanism in which RhoA-mDia2-HDAC6 forms a complex and regulate podosome patterning (147). It was demonstrated using microinjection experiments that injecting either activated RhoA or mDia2 caused microtubule deacetylation together with podosome belt disruptions (147).

2.5.2.4 HDAC7

HDAC7 is expressed early in osteoclast differentiation and continues at a low level of expression throughout differentiation (41, 42). HDAC7 has been shown to have a role in regulating osteoclast differentiation (39, 40, 140). Osteoclasts with reduced HDAC7 expression resulted in accelerated osteoclast differentiation and increased size of TRAP positive multinucleated osteoclasts (39, 40, 140). This discovery was ascribed to the capacity of HDAC7 to suppress MITF transcriptional activity (39). It was reported that the N-terminus tail of HDAC7 was sufficient to bind and suppress MITF activity, suggesting that the deacetylase domain of HDAC7 is not required for its repression function (39). Conditional knockdown of HDAC7 using LysM-Cre, that targets monocytes and myeloid lineage cells which includes osteoclasts, was found to enhance osteoclast differentiation and resulted in an osteopenic skeletal phenotype (39, 40). Besides MITF, HDAC7 was
shown to inhibit β-catenin activity and cyclin D1 expression in the presence of RANKL (40). These studies suggest that HDAC7 is a negative regulator of osteoclastogenesis.

2.5.2.5 HDAC9

HDAC9 is expressed immediately after RANKL stimulation and then expression reduces to undetectable levels (41, 42). Osteoclast differentiation and bone resorption were highly elevated in HDAC9 KO mice (41). Bone marrow transplantation experiments revealed that the osteoclasts defects in HDAC9 KO are intrinsic primarily to the hematopoietic cell lineage, since when HDAC9 KO bone marrow was transplanted to wild-type mice, it resulted in osteopenia, and when wild-type bone marrow was transplanted to HDAC9 KO mice, a rescue occurred (41). HDAC9 was found to take part in a negative regulatory circuit with PPARγ and RANKL signaling. Both PPARγ and RANKL can inhibit Hdac9 mRNA expression levels while HDAC9 forms a complex with NCoR and SMART to inhibit PPARγ activity (41). In conclusion, these studies demonstrate that HDAC9 expression inhibits osteoclastogenesis. Similar to HDAC5, creating a conditional mouse model of HDAC9 would allow for studies to be performed that analyze the role of HDAC9 in osteoclasts without the confusion of HDAC9’s role in regulating the other cells of the skeleton.

2.5.2.6 HDAC10

Like HDAC6, HDAC10 is a part of the class IIb HDAC family (149). The biological role of HDAC10 in osteoclasts is largely unknown however, what is known is that HDAC10 expression increases during differentiation and peaks around fusion (41, 42). Additionally, suppression of HDAC10 by shRNAs result in increased osteoclastogenesis, expression of osteoclast genes (c-fos, Nfatc1, Dc-stamp, and Ctsk), and osteoclast activity (42). This suggests that HDAC10 acts as an inhibitor of osteoclast differentiation; however, studies should be done in an animal model to confirm cell culture studies as well as identify osteoclast targets and partners of HDAC10.

2.5.3 Class III HDACs

Class III HDACs are a family of NAD+ dependent deacetylases known as sirtuins (150). These proteins are involved with many physiological processes such as cellular
metabolism, DNA repair, cell growth and autophagy (150). There are seven known sirtuins; however, only three have been studied in osteoclasts. There is not much known about sirtuins 2, 4, 5 and 7 in osteoclasts, and further studies will need to be performed to characterize their roles in regulating osteoclast differentiation and activity.

2.5.3.1 Sirtuin 1

Sirtuin 1 (SIRT1) is a repressor of osteoclast differentiation and activity by inhibiting RANKL signaling (151). SIRT1 is able to do this by deacetylating and activating a group of inhibitors of osteoclastogenesis known as forkhead box proteins (FOX) (151). Loss of SIRT1 expression results in an increase in osteoclast formation and activity due to the increase of acetylated FOX proteins (151).

2.5.3.2 Sirtuin 3

In osteoclasts, Sirtuin 3 (SIRT3) expression is induced by RANKL stimulation (152). Loss of SIRT3 in osteoclasts results in osteopenia in mice due to an increase in osteoclast number (152). This indicates that SIRT3 is a negative regulator of osteoclast differentiation (152). Additionally, the loss of SIRT3 results in an increase in osteoclast specific genes such as Oscar, Nfatc1 and Atp6v0d2 (152). Interestingly, SIRT3 does not regulate osteoclast activity meaning its ability to resorb mineral is unaffected by loss of the gene (152). Lastly, it is suggested that SIRT3 can negatively regulate osteoclast differentiation by controlling AMPK activity (152).

2.5.3.3 Sirtuin 6

Sirtuin 6 (SIRT6) is expressed in osteoclasts once monocytes are stimulated with M-CSF and RANKL (153). SIRT6 acts as a transcriptional repressor through inhibiting NF-κB transcription (153). This inhibition activity results from the deacetylation of H3K9 on the promoters of target genes for NF-κB (153). Therefore, overexpression of SIRT6 results in inhibition of osteoclastogenesis and enhanced osteoclast differentiation is measured when SIRT6 is lost (153).

2.5.4 Class IV HDACs

HDAC11, the sole member of class IV HDAC, is expressed late in osteoclast differentiation where it is most highly expressed after three days of RANKL stimulation
HDAC11 appears to be most closely related to HDAC3 and 8; however, its classification has not been determined since its sequence identity with other HDACs is limited (100, 154). Loss of HDAC11 expression in osteoclasts results in an increase in osteoclast differentiation as well as osteoclast activity. Interestingly, HDAC11 does not affect c-fos or Nfatc1, but increases the expression of both Dc-stamp and Ctsk, suggesting that it plays a role on osteoclast fusion as well as activity but independent of regulating Nfatc1 expression or activity (42). These results suggest HDAC11 acts as a repressor, however, further animal model studies will need to be performed to characterize its role in regulating osteoclast differentiation and activity.

2.6 Effects of HDAC Inhibitors (HDIs) on the Skeleton

To date the majority of studies have analyzed the effects of HDACs and HDIs on the effect of individual bone cells (as reviewed in (6, 155). It has become apparent that maintaining a healthy skeleton requires continued crosstalk between osteoclasts, osteoblasts and osteocytes (Figure 7). Studies assessing the crosstalk between the various cells will need to be done to assess the efficacy of using broad or specific HDIs in treating skeletal diseases.

Figure 7. Coupling of bone formation and bone resorption. Cartoon illustrates that osteoblasts express M-CSF and RANKL to stimulate osteoclast differentiation and bone resorption. Osteoclasts also express and release factors that regulate osteoblast differentiation and activity (96).
Since the 1960s, valproate as an HDI has been used as treatment for epilepsy, bipolar and other mood disorders. In several patient groups, prolonged exposure to valproate leads to a decrease in bone mineral density and an increase in fracture risk (156-159). In the valproate studies the mechanism(s) resulting in the bone loss are not clear as changes in bone biomarkers was not conclusive (160-164). Additionally, valproate has other activities besides inhibiting HDACs (165, 166). In animal studies of periodontitis, a broad acting HDI resulted in bone loss and reduced the number of osteoclasts in the gingiva and alveolar bone (167). In animal models of rheumatoid arthritis, topical treatment with either broad spectrum HDI, phenyl butyrate or TSA, suppressed joint swelling, levels of TNF-α with no evidence of joint destruction (168). Class and isozyme specific HDI, NW-21 (targets HDACs1 and 2), MS-275 (targets HDAC1) and BML-275 (targets HDAC6) have been shown to have anti-arthritis activities in rodent models (169, 170). A number of broad acting and a HDAC6 specific HDI are currently being used in the clinic to treat multiple myeloma (171-174). While HDIs may help patients that suffer from skeletal diseases such as osteopetrosis, they may have off-target effects that cause bone loss in other patients. Additionally, these HDIs are broad acting and do not target specific HDACs. Producing new HDIs in the future that target specific HDACs may be more beneficial since HDACs have differing role in osteoclasts.

2.6.1 HDAC Inhibitors (HDIs) and Fracture healing

After initial trauma, bone heals by direct intramembranous or indirect fracture healing involving intramembranous and endochondral bone formation (as reviewed in (175)). Bone healing process involves an acute inflammatory response and the recruitment of mesenchymal stem cells so as to initiate formation of primary cartilaginous callus. The callus is then subjected to revascularization and calcification and eventually remodeled by osteoblasts and osteoclasts (175). It has been reported that HDIs can increase bone healing process. Lee et al reported that they saw greater bone formation in rabbit calvarial bone fracture model when cyclic depsipeptide largazole was added to a macroporous biphasic calcium phosphate scaffold compared to scaffold control. The activity of the largazole was attributed to increase expression of Runx2 and BMPs (176). This finding requires further investigation because as reported in the previous section, HDI administration in humans has been shown to have negative effects on bone mass.
2.7 Conclusions

Over the years we have learned a great deal about the mechanisms by which the osteoclast is formed and resorbs bone. Most of this progress has come about as a result of rapid advances in cell and molecular biology studies. We now know more about the enzymes, signaling pathways, and cytokines which are important for osteoclast formation. While treatments such as HDIs may be beneficial for some skeletal diseases, this new level of understanding will result in the generation of new therapies that specifically target osteoclast generation and/or activity. Despite these advances, several key questions remained unanswered, one which relates to the detailed mechanism of osteoclast regulation by HDACs a process that seems to affect its function. More importantly, however, is the issue of roles of different HDACs at molecular and cellular levels in bone biology since, much remains unknown. A better understanding of the roles of HDACs in regulating osteoclasts, especially during bone resorption, will also provide insights into the cellular and molecular mechanisms that regulate skeletal development and homeostasis.

Chapter 3. Class II and IV HDACs function as inhibitors of osteoclast differentiation


* Authors contributed equally to this work

Author contributions

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Chapter summary
Previous research studies using *in vitro* and *in vivo* models have demonstrated the roles for several HDACs in regulating chondrocyte and osteoblast differentiation and activity. By comparison, there is very little data on the roles of individual HDACs in osteoclast formation and function. For this reason, the aim of this study was therefore to investigate the gene expression patterns and the effects of suppressing individual class II (*Hdac*4, 5, 6, 9, and 10) and class IV (*Hdac11*) HDACs during osteoclast differentiation *in vitro*. It was hypothesized that that loss of any one HDAC would be compensated for by the remaining proteins *in vitro*.

### 3.1 Introduction

Bone is a dynamic tissue that is constantly remodeled through degradation by osteoclasts and renewal by osteoblasts (19). Proper coordination between these cell types is essential for maintaining structural integrity of the skeleton throughout development. Osteoclasts are large, multinucleated cells derived from hematopoietic stem cells/monocyte precursors (19). Osteoclast differentiation is governed mainly by two important cytokines: macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor kappa B ligand (RANKL) (9, 10, 177, 178). RANKL stimulates the expression of key transcription factors such as NF-κB, c-Fos, MITF, and NFATc1 (68, 178-180) that are necessary for osteoclast differentiation and maturation. Osteoclasts are needed for normal bone functions such as bone remodeling and fracture repair. However, uncontrolled osteoclast activity can lead to skeletal disorders such as osteoporosis. Therefore, it is important to determine mechanisms that regulate transcription of osteoclast genes. This knowledge may reveal key modulators of bone resorption that can be considered as therapeutic targets.

The existence of tissue-specific transcription factors alone is inadequate to control temporal gene expression; co-factors are needed for chromatin remodeling and recruitment of RNA polymerase II. Histone acetyltransferases (HATs) and histone deacetylases (HDACs) control gene expression markings by being drafted to target genes through interactions with specific transcription factors (181). Gene activation is linked to the recruitment of HATs by transcription factors. Conversely, HDACs bind to the same transcription factors and promote transcriptional repression. Thus, HATs and HDACs act
as molecular switches for the functions of transcription factors. These processes have become essential mechanisms to examine regarding our understanding of bone physiology and diseases.

HDACs are negative regulators of transcription, and have been shown to induce specific changes to gene expression in various biological processes by deacetylating both histone and non-histone proteins (44, 182, 183). Rooted in structural and functional similarities to yeast deacetylases, the 18 HDACs in the human genome are categorized into four classes: class I HDACs (HDAC1, 2, 3 and 8), class II HDACs (HDAC4, 5, 6, 7, 9 and 10), class III HDACs (Sirtuins1-7) and class IV HDACs (HDAC11) (6, 31, 184-188). HDACs are emerging as important regulators of skeletal homeostasis (6). Various in vitro studies have reported that HDAC inhibitors can repress osteoclast differentiation (141, 189, 190). These inhibitors are broad-spectrum compounds that target multiple HDACs. However, the specific effects of individual HDACs on osteoclasts are largely unknown.

Previous work from our lab demonstrated that suppression of Hdac7 and Hdac3 have opposite effects on osteoclast differentiation in vitro (140). Hdac7 suppression enhances differentiation, whereas suppression of Hdac3 inhibits osteoclast differentiation. Our lab (39) as well as Jin et al (40) demonstrated that HDAC7 inhibits osteoclastogenesis and bone resorption in vivo. We found that HDAC7 represses osteoclast differentiation through interacting with the transcription factor MITF (39), while Jin et al. showed that HDAC7 regulates NFATc1 activity (40). Additionally, Jin et al (41) demonstrated that HDAC9 suppresses osteoclastogenesis through negatively regulating PPARγ. These findings suggest that each HDAC member can induce suppression of osteoclast differentiation through distinct and possibly multiple mechanisms. However, it is still unclear whether other HDAC members play separate and pivotal roles in osteoclastogenesis.

The goal of this study was to investigate the functions of class II and IV HDACs during osteoclastogenesis and to determine whether any redundant roles exist for the class II and IV HDACs. Our results revealed that suppression of individual HDACs enhance osteoclast differentiation, potentially through repression of different proteins within the same cellular pathways. We anticipate that characterization of changes in gene expression patterns due to specific suppression of each HDAC in osteoclasts will further our understanding of how
class II and IV HDACs regulate osteoclastogenesis. This knowledge will lead to potential new therapies in treating resorption-mediated bone diseases.

3.2 Experimental Procedures

3.2.1 Ethics. The use and care of the mice was reviewed and approved by the University of Minnesota Institutional Animal Care and Use Committee, IACUC protocol number 150732820A. Euthanasia was performed by CO₂ inhalation. Isolation and culture of osteoclasts from mouse bone marrow cells, as well as virus generation and viral transduction of osteoclasts were performed under approval of the University of Minnesota Institutional Biosafety Committee, permit number 1506-32712H.

3.2.2 Primary Osteoclast Cell Culture. Primary bone marrow macrophages (BMMs) were isolated from the femora and tibiae of C57BL/6 mice. The femora and tibiae were dissected out and adherent tissue was removed. The epiphyses of these long bones were removed, and the bone marrow was flushed from the diaphysis. Red blood cells were lysed from the flushed marrow using red blood cell lysis buffer (150 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA, pH 7.4), and the resulting cells were plated and cultured overnight in 100 mm tissue culture dishes (MidSci) in osteoclast media (phenol red-free alpha-MEM [Gibco] with 5% fetal bovine serum [Hyclone], 25 units/mL penicillin/streptomycin [Invitrogen], 400 mM L-Glutamine [Invitrogen], and supplemented with 1% CMG 14-12 culture supernatant containing M-CSF). CMG 14-12 cell line was obtained from Dr. Sunao Takeshita (Nagoya City University, Nagoya, Japan)(191). The non-adherent cell population was then re-plated in 12-well plates (MidSci) at 2 x 10⁵ cells/well in osteoclast media supplemented with 1% CMG 14-12 culture supernatant for 48 hours. Cells were then fed every two days with osteoclast media containing 1% CMG culture supernatant plus 10 ng/mL RANKL (R&D Systems) to stimulate osteoclastogenesis.

3.2.3 Tartrate resistant Acid Phosphatase (TRAP) Staining. After five days of culture with 1% CMG 14-12 culture supernatant and 10 ng/ml RANKL, primary osteoclasts were rinsed in PBS and fixed with 4% paraformaldehyde for 20 minutes. Cells expressing TRAP
were stained with Naphthol AS-MX phosphate and Fast Violet LB salt protocol as previously described (39). The stained cells were then imaged and photographed with light microscopy and analyzed using NIH ImageJ to measure the number and size of TRAP-positive multinuclear cells.

3.2.4 Demineralization Assay. BMMs were plated on calcium phosphate-coated plates (Corning) and cultured as above. After five days of stimulation with 1% CMG 14-12 culture supernatant and 10 ng/ml RANKL, plates were processed according to the manufacturer’s instructions, the demineralized area was photographed by dark field microscopy and analyzed using NIH ImageJ.

3.2.5 Lentiviral Infection of Osteoclasts. Lentiviral vectors (Open Biosystems) encoding shRNAs against Hdac4 (TRCN0000039249 and TRCN0000039252), Hdac5 (V2LMM 72835 and V3LMM 432047), Hdac6 (V2LMM 61798 and V2LMM 79557), Hdac9 (V3LMM 481592 and V3LMM 481594), Hdac10 (V3LMM 425386 and V3LMM 425387), Hdac11 (V2LMM 24029 and TRCN0000039224), or a control shRNA were used to produce replication-defective lentivirus according to the manufacturer’s protocols. Viral stocks were titrated by infection in HeLa cells. BMMs were isolated and cultured as described above. 48 hours after seeding the non-adherent population, lentiviruses were added at 10 MOI and incubated for 18 hours at 37°C in the presence of 1% CMG 14-12 culture supernatant. The following day cultures were stimulated with 1% CMG 14-12 culture supernatant and 10 ng/ml RANKL. Cells were used for either RNA extraction after 48 hours with 1% CMG 14-12 culture supernatant and RANKL treatment, or TRAP staining or demineralization assays after 5 days with 1% CMG 14-12 culture supernatant and RANKL.

3.2.6 RNA Isolation and Real-time PCR. RNA was harvested from cells plated in triplicate using TRIZOL Reagent (Ambion, Life Technologies) and quantified using UV spectroscopy. cDNA was then prepared from 1 µg of purified RNA using iScript cDNA Synthesis Kit (Bio-Rad) per the manufacture’s protocol. Quantitative real-time PCR (qRT-PCR) was performed in duplicate using CFX Connect Real-Time PCR system (Bio-Rad).
Each 20 µl reaction mixture contained 1 µl cDNA, 10 µL iTaq Universal Sybr Green Supermix, and 500 nM forward and reverse primers. The PCR conditions were as follows: 95°C for 3 minute, and 40 cycles of 94°C for 15 seconds, 56°C for 30 seconds, and 72°C for 30 seconds, followed by melt curve analysis (95°C for 5 seconds, 65°C for 5 seconds, and then 65°C to 95°C with 0.5°C increase for 5 seconds). Experimental genes were normalized to *Hprt*. Primers amplified with equal efficiencies. Their sequences used are listed in Table 1. All measurements were analyzed using the ΔΔCT method.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers (Forward and Reverse)</th>
<th>Sequence 5’→3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cathepsin K (Ctsk)</td>
<td>Forward</td>
<td>AGGGAAGCAAGC ACTGGATA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GCTGGCTGGAATC ACATCTTT</td>
</tr>
<tr>
<td>C-Fos</td>
<td>Forward</td>
<td>CCAAGCGGAACAGATCAA ACTT</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TCCAGTTTTTCCTTTCCTTTCAGCA</td>
</tr>
<tr>
<td>DC-STAMP</td>
<td>Forward</td>
<td>CAGACTCCCAAATGCTGGAT</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CTTGTGGAGGAACCTAAGCG</td>
</tr>
<tr>
<td>NFATC-1</td>
<td>Forward</td>
<td>TCATCTGTCCAACACG AA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TCACCCCTGTTCTTCTCCT</td>
</tr>
<tr>
<td>HDAC4</td>
<td>Forward</td>
<td>CAT GGG TAC TGC TGT AGG GG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>ATG AGC TCC CAA AGC CAT C</td>
</tr>
<tr>
<td>HDAC5</td>
<td>Forward</td>
<td>CGTTCCTCGTCCGTCTG CT</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>ATGCCATCTGCGACTCGT</td>
</tr>
<tr>
<td>HDAC6</td>
<td>Forward</td>
<td>GGA GAC AAC CCA GTA CAT GAA TGA A</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CGG AGG ACA GAG CCT GTA G</td>
</tr>
<tr>
<td>HDAC9</td>
<td>Forward</td>
<td>CCAAGTCGTGGGCATCTTT</td>
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<td></td>
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<td>TGTTCCTCTCCTCCAGGT TTT</td>
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<tr>
<td>HDAC10</td>
<td>Forward</td>
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<tr>
<td>HPRT</td>
<td>Forward</td>
<td>GAGGAGTCTGT GATGTTGCCAG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GGCTGGCTATAGGCTC ATAGTGC</td>
</tr>
</tbody>
</table>

**Table 1. Sequence of primers used for qRT-PCR**

### 3.2.7 Immunoblot Analysis

Protein cell lysates were harvested from primary osteoclasts in modified RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% IGEPAL, 0.25% sodium
deoxycholate, 1 mM EDTA) supplemented with Halt Protease & Phosphatase Inhibitor Cocktail (Thermo Scientific). Lysates were cleared by centrifugation. Proteins were resolved by SDS-PAGE, transferred to PVDF membrane (Millipore), blocked, and blotted in primary antibody overnight at 4°C. The next day, blots were incubated with horseradish peroxidase conjugated secondary antibody (G.E. Healthcare) for 1 hour at room temperature. Antibody binding was detected using western blotting detection kit (Western Bright Quantum, Advansta). The following primary antibodies were all used at 1:1000 dilution: polyclonal HDAC4 produced using peptide corresponding to amino acids 14-28 of human HDAC4 – Sigma Aldrich (H9536, Antibody ID# AB477079); monoclonal HDAC5 produced using peptide corresponding to amino acids 371-443 of human HDAC5 – Santa Cruz Biotechnology (SC-133225, Antibody ID# 2116791); polyclonal HDAC9 produced using peptide corresponding to amino acids 112-129 of HDAC9 – Thermo Scientific (PA5-23346, Antibody ID# AB2540870); polyclonal MITR produced from peptide against human HDAC9 – Sigma Aldrich (SAB4503694, Antibody ID# AB10751345); polyclonal HDAC10 produced using peptide corresponding to amino acids 2-16 of human HDAC10 – Sigma Aldrich (H3413, Antibody ID# AB261940); polyclonal HDAC11 produced using peptide corresponding to amino acids 2-16 of human HDAC11 – Sigma Aldrich (H4539, Antibody ID# AB532246) and polyclonal ACTIN – Santa Cruz Biotechnology produced using carboxy terminus of human actin (SC-1616, Antibody ID# AB630836). The secondary antibodies used were all used at 1:10,000 dilution: anti-rabbit (NA-934, Antibody ID# AB772206) and anti-goat (SC-2020, Antibody ID# AB631728). All densitometry data was generated using the Image Lab Software (Bio Rad) following the manufacturer’s instructions for normalizing data to a housekeeping protein. The calculated volume intensity for HDAC expression was normalized to its corresponding actin from the same membrane.

3.2.8 Statistical Analysis: All experiments were run in triplicate, performed three times, and results are expressed as mean ± standard deviation. Student’s t-test or one-way ANOVA analysis followed by a Tukey’s multiple comparison test were used to compare data using Graph-Pad Prism version 7.
3.3 Results

3.3.1 Expression of class II and IV HDACs during Osteoclastogenesis

To investigate the roles of class II and IV HDACs during osteoclastogenesis we first examined their expression through the process of osteoclast differentiation. Non-adherent cells from C57BL/6 (wild-type) mouse bone marrow cells were cultured with 1% CMG for two days to promote bone marrow macrophages (BMMs) proliferation, and then treated with M-CSF plus RANKL for four more days to stimulate osteoclast differentiation. All Hdac members in class IIa, IIb and IV were expressed in both proliferating BMMs (day zero with M-CSF treatment) and differentiating osteoclasts (day one-day four with M-CSF plus RANKL treatment) (Figure 8A). Among these HDACs, the expression of Hdac4, Hdac7, Hdac9 and Histone deacetylase related protein (Hdrp/Mitr) were significantly down-regulated upon RANKL stimulation (day zero vs day two) and expression remained low during osteoclast differentiation (day one-day four). Conversely, Hdac5, Hdac6, Hdac10 and Hdac11 expression increased with RANKL expression (Figure 8A, day zero vs day two). Jin et al (41) reported similar trends in HDAC mRNA expression. Hdac11, Hdac5 and Hdac7 were the most highly expressed Hdac RNAs during osteoclast differentiation. As expected, the expression of the osteoclast marker gene Cathepsin K (Ctsk) was significantly increased upon RANKL stimulation and served as a positive control for osteoclast differentiation (Figure 8A, bottom right panel). HDAC protein expression as measured by western blot demonstrated similar expression patterns to the mRNAs (Figure 8B). These results show that HDAC class II and IV members are differentially expressed during osteoclast differentiation.
Figure 8. Class II and IV Hdac expression during osteoclast differentiation. BMM cells from C57BL/6 mice were cultured in M-CSF only (day zero) or in M-CSF plus RANKL (day two and day four) to stimulate osteoclast differentiation. Class II and IV Hdac RNA expression (A) on day zero, day two and day four of osteoclast differentiation. qRT-PCR data shown are the mean of three independent experiments. * p < 0.05 comparing vs. day zero, ns = not significant. Representative protein expression (B) of class II and IV HDACs on each day of osteoclast differentiation. Displayed relative expression values are HDAC expression relative to the normalized expression on day zero as calculated by Image Lab Software (BioRad). In the case of HDAC9 no band was identified for day zero so data is expressed relative to day one. The same lysates were analyzed for HDAC9 and MITR expression and therefore, have the same actin loading control.
3.3.2 Effect of shRNA Suppression of Hdacs on Osteoclast Differentiation

Since we detected expression of all the examined Hdacs in osteoclast cultures, we next asked how suppression of each individual Hdac would affect osteoclast formation or function. To this end, we used lentiviral vectors encoding shRNAs against these Hdacs. Prior to beginning RANKL stimulation, BMMs from wild-type mice were infected with one of two lentiviral vectors encoding an shRNA against an Hdac or a control shRNA. For each Hdac, we obtained similar results with both shRNAs indicating that the outcomes were not due to off-target effects. Following infection with shRNAs, we confirmed target gene knockdown by qRT-PCR and examined changes in the expression of c-Fos, Nfatc1, De-stamp and Ctsk genes important for osteoclast formation or function. Because our experiments produced similar results in our analysis of osteoclast differentiation and activity assays using the two different Hdac shRNA, we grouped our PCR data together from the Hdac shRNA experiments for qRT-PCR. We employed TRAP staining and demineralization assays to measure osteoclast differentiation, fusion and activity.

3.3.2.1 Suppression of Hdac4 Increases Osteoclastogenesis

First, we investigated the effects of silencing Hdac4 on osteoclastogenesis (Figure 9). Cells infected with Hdac4 shRNA #1 or #2 showed enhanced osteoclast differentiation compared to control shRNA (Figure 10A). The observed TRAP-positive multinuclear cells (MNCs) from both Hdac4-shRNA cultures were more numerous, and their size significantly increased (3-fold) relative to the control (Figure 10B-C). Demineralization assays on calcium phosphate-coated plates demonstrated cells infected with Hdac4 shRNA #2 produced significantly increased total number of pits, average pit size, and total percent demineralized area compared to control shRNA infected cells. Hdac4 shRNA #1 infected cells showed similar trends (Figure 10D-E). Moreover, BMMs infected with either Hdac4 shRNA showed Hdac4 RNA expression levels reduced by approximately 50% (Figure 9), and Hdac4 protein expression levels reduced between 30-65% (Fig 10F). Hdac4 shRNA increased expression of c-Fos, Nfatc1, De-stamp and Ctsk compared to control shRNA (Figure 10G-J). Taken together these results reveal that Hdac4 suppression increased
osteoclast differentiation by producing larger and more numerous osteoclasts, suggesting HDAC4 inhibits osteoclastogenesis.

Figure 9. mRNA expression of $Hdac4$ shRNA. qPCR of control and $Hdac4$ expressing cells. *** $p < 0.001$
Figure 10. Accelerated osteoclast differentiation in Hdac4-suppressed osteoclasts. Representative images of TRAP staining (A) of osteoclast cultures infected with control or Hdac4 shRNA-expressing lentivirus. Hdac4#1 represents Hdac4 shRNA #1 and Hdac4#2 represents Hdac4 shRNA #2. Number (B) and size (C) of TRAP-positive MNCs. Representative images (D) and quantification (E) of demineralization activity of control and Hdac4 shRNA-expressing osteoclast cultures grown on calcium phosphate-coated plates. Scale bar represents 200 μm. Western blot (F) of control and Hdac4 shRNA-expressing cells with relative expression of shRNA expressing cells relative to control expressing cells indicated under the blots. Expression profile (G-J) of osteoclast genes c-Fos, Nfatc1, De-stamp and Cisk. Data presented are the mean of three independent
experiments. * p < 0.05; ** p < 0.01; *** p < 0.001; ns = not significant compared to control infected cells.

3.3.2.2 *Hdac5* is a Negative Regulator of Osteoclastogenesis

We investigated the effects of suppressing expression of the Class IIa deacetylase *Hdac5* (Figure 11). Osteoclast differentiation in either *Hdac5*-shRNA culture was enhanced compared to control shRNA (Figure 12A). The average size of TRAP-positive MNCs in *Hdac5*-shRNA cells was significantly increased (Figure 12B). However, the number of TRAP-positive MNCs per well in *Hdac5*-shRNA cells did not significantly change compared to control shRNA (Figure 12C). When osteoclast activity was determined, cells infected with each *Hdac5*-shRNA had significantly increased total number of pits, average pit size, and total percent demineralized area (Figure 12D-E). The cultured osteoclasts also showed reduced *Hdac5* mRNA expression levels (Figure 11), and approximately 70-80% reduction in HDAC5 expression as measured by western blot (Figure 12F). *Hdac5*-suppressed osteoclasts showed an upward but not significant change in expression of *c-Fos* (Figure 12G); while expression of genes important for osteoclast formation or function including *Nfatc1*, *Dc-stamp*, and *Ctsk* (Figure 12H-J) were significantly increased. These findings indicate that *Hdac5* suppression enhances osteoclast differentiation through increased expression of genes such as *Nfatc1*, *Dc-stamp*, and *Ctsk* but independent of changes to *c-Fos*.
Figure 11. mRNA expression of *Hdac5* shRNA. qPCR of control and *Hdac5* expressing cells. *** p <0.001

Figure 12. Suppression of *Hdac5* enhances osteoclast differentiation. Representative images of TRAP staining (A) of osteoclast cultures. *Hdac5*#1 represents *Hdac5* shRNA #1 and *Hdac5*#2 represents *Hdac5* shRNA #2. Quantification of the number (B) and
average size of TRAP-stained multinucleated osteoclasts (C). Representative photographs (D) and quantification (E) of demineralization activity of control and Hdac5 shRNA-expressing osteoclast cultures grown on calcium phosphate-coated plates. Scale bar represents 200 μm. Western blot (F) of control and Hdac5 shRNA-expressing cells with relative expression of shRNA expressing cells relative to control expressing cells indicated under the blots. Expression profile (G-J) of osteoclast genes c-Fos, Nfatc1, Dc-stamp and Ctsk. Data presented are the mean of three independent experiments. * p <0.05; ** p <0.01; *** p < 0.001; ns = not significant compared to control infected cells.

3.3.2.3 Hdac6 Suppression Does Not Affect Osteoclast Differentiation

HDAC6, a member of class IIB HDAC family and tubulin deacetylase, has been shown to regulate tubulin dynamics and stability of the podosome belt in mature osteoclasts (192, 193). However, the role of HDAC6 in early stages of osteoclast differentiation is unknown. There was no significant change in the number or size of TRAP-positive MNCs with Hdac6 suppression (Figure 13A-C). Moreover, Hdac6-shRNA #1 showed no significant changes in the demineralization assay (Figure 13D-E). Commercially verifiable antibodies to HDAC6 are not available; therefore, we verified both Hdac6 shRNAs significantly repressed Hdac6 expression by qPCR (Figure 13F). However, c-Fos, Nfatc1, Dc-stamp, and Ctsk were not significantly changed by Hdac6 suppression (Figure 13 G-J). These data show that knockdown of HDAC6 does not have any impact on osteoclast differentiation, at least within the parameters and genes analyzed.
Figure 13. **Hdac6 suppression does not affect osteoclast differentiation.** Representative images of TRAP staining (A) of osteoclast cultures. *Hdac6*#1 represents *Hdac6* shRNA #1 and *Hdac6*#2 represents *Hdac6* shRNA #2. Quantification of (B) number and (C) size of TRAP-positive MNCs. Representative photographs (D) and quantification (E) of demineralization activity of control and *Hdac6* shRNA-expressing osteoclast cultures grown on calcium phosphate-coated plates. Scale bar represents 200 μm. qRT-PCR (F) of control and *Hdac6* shRNA-expressing cells. Expression profile (G-J) of c-Fos, *Nfatc1*, *De-stamp*, and *Ctsk*. Data presented are the mean of three independent experiments. ***p < 0.001; ns = not significant compared to control infected cells.
3.3.2.4 Suppression of *Hdac9* Inhibits Osteoclastogenesis

We next used shRNAs to characterize the role of *Hdac9* and *Mitr* in osteoclasts. Although our gene expression data (Figure 8) indicate that MITR is the predominant form of HDAC9 in osteoclasts, we were unable to identify shRNAs that distinguish between *Mitr* and full-length *Hdac9*. Consequently, both shRNAs used are predicted to target *Hdac9* and *Mitr* (Figure 14). Suppression of *Hdac9* increased the size of TRAP-positive MNCs, but had little effect on the number of osteoclasts formed (Figure 15A-C). Demineralization assays showed that in *Hdac9*-shRNA infected cells, average pit size and demineralized area were significantly increased (Figure 15D). As expected, in *Hdac9*-suppressed cells there was a reduction in *Hdac9* protein expression (Figure 15F) as well as *Hdac9* RNA (Figure 14). *Hdac9*-suppressed cells revealed a slight but not significant reduction in *c-Fos* expression (Figure 15G), while expression of *Nfatc1, De-stamp,* and *Ctsk* was increased compared with control (Figure 15H-J). These observations indicate that HDAC9/MITR inhibits osteoclastogenesis.

![Figure 14](image_url)

**Figure 14. mRNA expression of *Hdac9***. qPCR of control and *Hdac9* expressing cells. *p < 0.05
Figure 15. Suppression of *Hdac9* Inhibits Osteoclast Differentiation. Representative images of TRAP staining (A) of osteoclast cultures infected with control or *Hdac9* shRNA-expressing lentiviruses. *Hdac9*#1 represents *Hdac9* shRNA #1 and *Hdac9*#2 represents *Hdac9* shRNA #2. Number (B) and size (C) of TRAP-positive MNCs. Representative photographs (D) and quantification (E) of demineralization activity of control and *Hdac9* shRNA-expressing osteoclast cultures grown on calcium phosphate-coated plates. Scale bar represents 200 μm. Western blot (F) of control and *Hdac9* shRNA-expressing cells with relative expression of shRNA expressing cells relative to control expressing cells.
indicated under the blots. Expression profile (G-J) of c-Fos, Nfatc1, Dc-stamp and Ctsk. Data presented are the mean of three independent experiments. * p < 0.05; ** p < 0.01; ns = not significant compared to control infected cells.

3.3.2.5 HDAC10 Activity Inhibits Osteoclastogenesis

HDAC6 and HDAC10 are members of class IIb HDAC family. HDAC6 functions as a tubulin deacetylase, and its role as a disrupter of the actin belt in mature osteoclasts has been well established (147, 194), whereas the biological function(s) of HDAC10 remain largely unknown (195, 196). To explore HDAC10’s role in osteoclast differentiation, we examined the effects of suppressing its expression (Figure 16A), finding that in Hdac10-suppressed cells showed a decrease in the mean number of TRAP-positive MNCs per well (Figure 16B). The average size of TRAP-positive MNCs in Hdac10-suppressed cells was significantly increased (Figure 16C). Demineralization assays show total number of pits, average pit size and percent demineralized area was increased with knockdown of Hdac10 (Figure 16D-E). As expected, Hdac10-suppressed osteoclasts showed a reduction in Hdac10 mRNA expression compared to control shRNA (Figure 16F). We were unable to obtain a reliable western blot verifying HDAC10 protein reduction in shRNA expressing cells due to technical issues. Expression of c-Fos, Nfatc1, Dc-stamp, and Ctsk was significantly increased (Figure 16G-J). These data indicate that reduction of Hdac10 expression enhanced osteoclast formation, suggesting that HDAC10, unlike HDAC6, may negatively regulate osteoclast differentiation.
Figure 16. *Hdac10* suppression accelerates osteoclast differentiation. Representative images of TRAP staining (A) of osteoclast cultures. *Hdac10* #1 represents *Hdac10* shRNA #1 and *Hdac10* #2 represents *Hdac10* shRNA #2. Quantification of number (B) and size (C) of TRAP-positive MNCs. Representative images (D) and quantification (E) of demineralization activity of control and *Hdac10* shRNA-expressing osteoclast cultures grown on calcium phosphate-coated plates. Scale bar represents 200 μm. qRT-PCR (F) of control and *Hdac10* shRNA-expressing cells. Expression profile (G, H, I and J) of *c-Fos*, *Nfatc1*, *Dc-stamp*, and *Ctsk*. Data presented are the mean of three independent experiments. * p < 0.05; *** p < 0.001; ns = not significant compared to control infected cells.

3.3.2.6 HDAC11 Inhibits Osteoclastogenesis
We examined the effects of suppressing Hdac11 on osteoclast differentiation (Figure 17). Cells infected with either Hdac11 shRNA had enhanced osteoclast differentiation (Figure 18A). Quantitative analysis of these cultures indicated that there was no significant difference in the average number of TRAP-positive MNCs formed in either Hdac11 shRNA cultures compared with control shRNA (Figure 18B). However, the average size of TRAP-positive MNCs was increased in both Hdac11 shRNA cultures (Figure 18C). Hdac11 shRNA #2 in demineralization assays increased the total number of pits, average pit size and total percent demineralized area significantly (Figure 18D-E). Hdac11 shRNA #1 infected cells did not show a significant difference in average pit size but did show a difference in the total number of pits and total percent demineralized area (Figure 18D-E). The Hdac11 shRNAs moderately reduced Hdac11 RNA (Figure 17) and protein expression (Figure 18F), which led to increased De-stamp and Ctsk expression (Figure 18I-J) but no change in c-Fos (Figure 18G) and Nfatc1 expression (Figure 18H). These results reveal that Hdac11 suppression enhanced osteoclast formation, thus suggesting Hdac11 acts as an inhibitor of osteoclast differentiation.

Figure 17. mRNA expression of Hdac11. qPCR of control and Hdac5 expressing cells. *** p <0.001
Figure 18. Suppression of *Hdac11* inhibits osteoclast differentiation. Representative images of TRAP staining (A) of osteoclast cultures. *Hdac11*#1 represents *Hdac11* shRNA #1 and *Hdac11*#2 represents *Hdac11* shRNA #2. Histomorphometric analysis of TRAP-stained osteoclasts (B-C). Representative photographs (D) and quantification (E) of demineralization activity of control and *Hdac11* shRNA-expressing osteoclast cultures grown on calcium phosphate-coated plates. Scale bar represents 200 μm. Western blot (F) of control and *Hdac11* shRNA-expressing cells with relative expression of shRNA expressing cells relative to control expressing cells indicated under the blots. Expression
profile (G, H, I and J) of c-Fos, Nfatc1, Dc-stamp, and Ctsk. Data presented are the mean of three independent experiments. * p < 0.05; ** p < 0.01; *** p < 0.001; ns = not significant compared to control infected cells.

4. Discussion

To better understand how osteoclast differentiation and activity are regulated, it is necessary to investigate classes of transcriptional activators and repressors that function in osteoclasts. HDACs are a well-known class of transcriptional repressors that have been shown to be active in osteoclasts. Due to their high sequence homology and interactions with the same proteins in other cell types (184, 197), some HDACs may perform redundant roles in regulating osteoclast differentiation. However, recent studies demonstrated two class II HDACs, HDAC7 and HDAC9, repressed osteoclast differentiation using unique mechanisms. Deletion of either Hdac7 or Hdac9 in BMMs resulted in enhanced osteoclast differentiation in vitro, and increased bone resorption and osteopenia in mice in vivo (39, 41). Our lab concluded HDAC7 interacted with and repressed the activity of MITF (39), another group demonstrated via reporter assays that HDAC7 might repress NFATc1 activity (40), and HDAC9 repressed PPAR-γ (41). Though these mechanisms are different, it was still possible that the remaining Class II HDACs performed redundant roles in osteoclasts, in which case we hypothesized that loss of any one HDAC would be compensated for by the remaining proteins. To address this issue, we analyzed mRNA and protein expression, and the effects of shRNA-mediated knockdown of most class II (HDAC4, 5, 6, 9, and 10) and class IV (HDAC11) HDACs during osteoclast differentiation. To help identify how each HDAC was affecting osteoclasts, we used TRAP staining, demineralization assays, and qRT-PCR to evaluate changes in differentiation, activity, or gene expression due to Hdac knockdown. Individual knockdown of Hdac4, 5, 9, 10, and 11 produced similar phenotypes, which was observed as increased size of multinuclear TRAP-positive MNCs and percent demineralization of a calcium phosphate matrix, but little to no change in multinuclear TRAP-positive osteoclast number (Figures 9, 10, and 12-14). Additionally, temporal expression patterns of these HDACs were also different from each other, which is in agreement with a previous study (41). The presence of similar osteoclast phenotypes when different HDACs are knocked down and their
distinct expression profiles suggests that most HDACs have at least some unique targets rather than fully redundant roles.

**Inhibiting differentiation in early stages of osteoclast differentiation.** *Hdac9* and the related *Mitr* are highly expressed in BMMs but drastically decrease in expression after RANKL stimulation. *Hdac4* follows a similar trend but maintains a low level of expression throughout differentiation (Figure 8A and 8B). The initially high levels of HDAC9 and HDAC4 in BMMs which begin decreasing with the onset of RANKL stimulation could indicate that the presence of these HDACs is associated with maintaining proliferation rather than differentiation. Knockdown of *Hdac4* showed a small but significant increase in osteoclast number (Figure 10B) and a trend towards increased c-Fos expression (Figure 10G). Supporting this, osteoclasts from *Hdac4*-KO mice showed increased c-Fos expression (data not shown, manuscript in preparation). Knockdown of *Hdac9* demonstrated increased differentiation marker expression and osteoclast size but not increased osteoclast number or c-Fos expression, indicating HDAC9 may not be involved in proliferation through regulating c-Fos expression (Figure 15, (41)). Previous research on HDAC9 in osteoclasts showed both increased cell number and size of osteoclasts from global *Hdac9*-KO mice (41). These contrasting results could be explained by the global deletion of *Hdac9* affecting the BMM population at a stage before our transient shRNA studies began. Considering this, both previous and our work show upregulated expression of genes important for differentiation, implying knockdown of *Hdac9* promotes a switch from proliferation to differentiation earlier than normal (Figure 15).

MITR is a truncated splice variant of HDAC9 that displays co-repressor activity despite lacking the C-terminal deacetylase catalytic domain. Specific suppression of *Mitr* but not of *Hdac9* expression in neurons leads to cell death, demonstrating a necessary role for MITR separate from HDAC9 (198, 199). It remains unknown whether MITR plays a distinct role in osteoclasts. The commercially available *Hdac9* shRNAs we used are predicted to target both the full-length *Hdac9* and *Mitr* transcripts (BLAST alignment and data not shown), and the global *Hdac9*-KO mice have reduced HDAC9 and MITR protein levels (200). Future work should examine potential roles that are specific to MITR but separate from those of HDAC9.
Repressors of differentiation, fusion, and activity: Contrary to Hdac4, Hdac9, and Mitr’s early expression, Hdac5, Hdac10, and Hdac11 have expression levels that increase after RANKL treatment (Figure 8). Knockdown of any of these three HDACs results in increased osteoclast size and percent demineralization with little to no change in the number of osteoclasts (Figures 12, 16, and 18). Supporting this, knockdown of each HDAC increased De-stamp expression, which would lead to more fusion and, consequently, larger cells. However, knockdown did not affect c-Fos expression or cell number, indicating these HDACs mostly affect genes regulating differentiation, fusion, and possibly activity instead of proliferation (Figures 12, 16, and 18). Knockdown of either Hdac5 or Hdac10 showed a trend of increased Nfatc1 expression (Figure 12H and 16H). NFATc1 regulates expression of genes important for cell fusion (De-stamp) and resorption activity (Ctsk), as well as its own autoamplification after it is stably induced during differentiation (94, 201, 202). Therefore, HDAC-mediated inhibition of Nfatc1 expression could reduce NFATc1 activity, concomitantly decreasing expression of genes that promote differentiation, fusion, and activity of osteoclasts. Indeed, Hdac5 shRNA treatment showed trends of increased Nfatc1 and De-stamp expression with significantly increased Ctsk expression (Figure 12). Supporting this, HDAC5 activity promotes deacetylation and, consequently, destabilization of NFATc1 (146). Generally, class IIa HDACs rely on recruitment of class I HDACs, typically HDAC3, for deacetylation of targets (185). Interestingly, HDAC10 interacts with all class I and IIa HDACs and could potentially act as an HDAC recruiter for the repression of targets (31). Future HDAC5 and HDAC10 studies should concentrate on a potential role for their repression of NFATc1 either cooperatively or separately.

Suppression of Hdac11 resulted in a milder phenotype than either Hdac5- or Hdac10-shRNA treatment (Figure 18). This is a surprising result to us considering HDAC11 is more closely related to class I than II HDACs (31), and we have previously shown that loss of expression of Hdac3, a class I HDAC, inhibits osteoclast differentiation (140). Distinct from Hdac5 and Hdac10, Hdac11 knockdown only increased De-stamp expression (Figure 18I). This potentially means HDAC11 specifically targets the De-stamp promoter or fusion genes without affecting the transcription of proteins such as NFATc1, which regulate both differentiation and fusion of osteoclasts. HDAC11 may interact with NFATc1 in a protein-
protein interaction to regulate NFATc1’s ability to activate targets such as Dc-stamp. The mechanism(s) by which HDAC11 inhibits osteoclast differentiation will be explored in future work. HDAC11 has previously been shown to associate with and deacetylate the IL-10 promoter in bone marrow-derived antigen-presenting cells (203). Surprisingly, HDAC6 and HDAC11 physically interact and oppose each other in regarding to Il-10 expression; both HDAC6 and HDAC11 associate with the Il-10 promoter, but HDAC6 promotes expression while HDAC11 represses expression (204). With the varied expression of different HDACs throughout differentiation, a similar mechanism may exist in osteoclasts. However, as explained in detail below, HDAC6 most likely is not exerting an opposing action on HDAC11 activity in osteoclasts.

**The role of HDAC6:** HDAC6 can be recruited to gene promoters to modulate gene expression (204, 205). Though we successfully reduced Hdac6 expression, no observable effects on gene expression were seen (Figure 13). Since our four examined genes are far from an exhaustive characterization of altered gene expression by HDAC suppression, it is possible that HDAC6 does have some role in regulating osteoclast-specific gene expression that is outside of our examination or it does so in a redundant manner with another HDAC. Classically, HDAC6 is known to deacetylate α-tubulin, leading to changes in microtubule stabilization and dynamics (206, 207). It may be that HDAC6’s role in regulating osteoclast differentiation is limited to effecting changes to microtubules but not gene expression. Further characterizing potential gene targets of HDAC6 in osteoclasts is beyond the scope of this work and may be further examined in the future. *In vivo* knockdown of HDAC6 in embryonic stem cells displayed hyperacetylated tubulin (208). Supporting this, studies in osteoclasts demonstrated that inhibition of HDAC6 activity with chemical inhibitors led to hyperacetylated tubulin and stabilized podosomes (147, 209). Microtubules are important in helping form and stabilize the podosome belt, which facilitates cell adhesion and matrix resorption by osteoclasts (210). Knowing this, we predicted knockdown of Hdac6 to produce a significant effect in the demineralization assay; however, we did not observe any phenotype in terms of osteoclast size, number, or activity after Hdac6 depletion by shRNA (Figure 13). Though these results are surprising, they are not completely unexpected considering the conflicting results between HDAC6 knockdown and inhibition of activity in other cell types. Importantly, chemical inhibition
of HDAC6 led to hyperacetylated tubulin and reduced microtubule dynamics, while siRNA-mediated knockdown of *Hdac6* also showed hyperacetylated tubulin but did not affect microtubule dynamics (148, 211). In this way, we would not expect *Hdac6* knockdown to impact osteoclast size, number, or activity. Future HDAC6 research should concentrate on additional transcriptional regulatory roles or potential redundancy with Sirtuin 2, a class III HDAC known to also deacetylate α-tubulin (212).

Overall, we have demonstrated for the first time the results of specifically knocking down *Hdac4, 5, 6, 10,* and *11* in osteoclasts. We found that HDACs differ in their temporal expression pattern during osteoclast differentiation (Figure 19). Except for HDAC6, our studies indicate that class II and IV HDACs have non-redundant roles during osteoclast differentiation. This study serves as an important starting block from which future work examining individual HDACs in osteoclasts should benefit.

![Figure 19. Expression pattern of class II and IV HDACs during osteoclast differentiation.](image)

Illustration of the stages of osteoclast differentiation with HDAC expression as determined by the western blots presented in Figure 8.
Chapter 4. Histone Deacetylase 4 (HDAC4) Regulates Osteoclast Bone Resorption and Bone Mass

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(Manuscript in preparation)

Short Title: HDAC4 Regulates Osteoclast Bone Resorption

Chapter Summary
The results of chapter 3 suggested that in vitro, members of class II and IV HDACs are differentially expressed during osteoclast differentiation and suppress osteoclast differentiation and function. Suggesting that HDACs are functionally significant in osteoclasts. Previous studies have shown that class IIa HDACs members, HDAC7 and HDAC9 can regulate osteoclast differentiation and bone resorption both in vivo and in vitro. No studies have assessed the in vivo role of the other class IIa HDAC members during osteoclastogenesis. HDAC4 is a class IIa HDAC whose importance during osteoclast development is not understood. In vitro, suppression of HDAC4 enhances osteoclast differentiation and function is unknown. For this reason, the aim of this study was to determine the role of HDAC4 in osteoclast formation and function by using in vitro osteoclast culture and in vivo HDAC4 conditional knockout mice model. It was hypothesized that mice null for HDAC4 expression in osteoclasts will be osteopenic due to enhanced osteoclast formation.

4.1 Introduction
Normally bone remodeling during development and bone integrity throughout life are regulated by bone-forming osteoblasts and bone resorbing-osteoclasts (213, 214). The balance between bone formation and resorption is what maintains bone mass. When an imbalance occurs, bone metabolic diseases appear such as osteoporosis (213, 214). Osteoclasts are giant multinucleated cells that are responsible for bone resorption (9, 10). They differentiate from monocyte/macrophage lineage in response to two critical cytokines receptor activator of NF-κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) (179, 215, 216). Jointly, RANKL and M-CSF promote proliferation and survival of osteoclast precursors and their differentiation into mature bone-resorbing osteoclasts (179). While bone resorption is tightly regulated in the skeleton, the molecular
mechanisms underlying it are not fully understood. Hence, it is important to understand how osteoclast activity is being regulated in order to develop targeted therapies for bone disorders.

Acetylation of lysine residues in histones, and/or transcription factors, is emerging as a widespread posttranslational mechanism involved in the regulation of several cellular functions (182, 217, 218). Histone deacetylases (HDACs) are enzymes that catalyze the removal of acetyl groups from histone and non-histone proteins, and in general act to repress transcription (31, 32, 44, 183). Eighteen mammalian HDACs have been identified thus far, and they are divided into four classes based on their homology to yeast deacetylases domain. They are class I HDACs (HDAC1, -2, -3, and -8), class II (HDAC4, -5, -6, -7, -9 and -10), class III (Sirtuins 1–7) and class IV (HDAC11) (6, 31, 42, 96). The class II HDACs can be further subdivided into class IIa (HDAC4, -5, -7, and -9) and class IIb (HDAC6 and -10) based on the presence in class IIa members of an extended N-terminal domain, and a C-terminal deacetylase domain, that are important in regulating their functions (188). HDACs have not only emerged as critical regulators of biological processes such as cell growth, differentiation, survival, proliferation and apoptotic programs, but also are a key regulator of skeletal homeostasis (6, 31, 44) (219). Several in vitro studies have demonstrated that osteoclast differentiation can be suppressed by using broad-spectrum HDAC inhibitors (141, 189, 220) that target different HDAC family members. However, there is a gap in the knowledge concerning the specific roles individual HDACs play in bone, particularly in osteoclasts. This is a critical question to answer since several HDACs inhibitors currently in clinical trials for the treatment of diseases such as cancer, diabetes, cognitive disorders and neurodegeneration are class specific (221, 222).

Recently, data demonstrating that class IIa HDACs act as regulators of osteoclast differentiation and activity has started to emerge. Overexpression of HDAC7 in mouse bone marrow macrophage (BMMs) inhibits fusion of osteoclast precursors through regulating MITF transcriptional activity (140). In a subsequent study, overexpression of HDAC7 in BMMs inhibited both number and size of TRAP positive multinuclear cells (39). Conversely, it has been shown that loss of HDAC7 expression enhances osteoclast differentiation. In this study, HDAC7 was found to reduce NFATc1 suppression of β-catenin in the presence of RANKL (40). Lastly, osteoclast differentiation and bone
resorption was significantly elevated in HDAC9-null mice suggesting that HDAC9 similar to HDAC7 act as an inhibitor of osteoclast differentiation (41).

HDAC4 is a class IIa HDAC that has been associated with muscle and bone development (43, 145, 223-226). In vitro studies have shown that HDAC4 can mediate these effects by repressing MEF2C and RUNX2 using its N-terminal binding domain (43, 145, 223, 225, 226). In-vivo, HDAC4 together with RUNX2 have been shown to be important for bone development. HDAC4 knockout mice displayed premature ossification of endochondral bone because of early onset of chondrocyte hypertrophy resulting in postnatal lethality; however, the role of HDAC4 in regulating osteoclast differentiation has not been well studied (43). An in vitro study using shRNA against HDAC4 in osteoclasts suggested that HDAC4 similar to HDAC7 and HDAC9 acts as an inhibitor of osteoclastogenesis (42). However, whether HDAC4 is a physiological relevant regulator of osteoclast differentiation and bone resorption is still an unanswered question, and the mechanisms underlying potential HDAC4 regulation of osteoclasts is unclear.

In this current study, we investigated the consequences of HDAC4 disruption during osteoclast differentiation, migration and bone resorptive activity in vivo and in vitro. We demonstrated that HDAC4 positively regulates the bone-resorbing activity of osteoclasts and that mice with HDAC4 null osteoclasts exhibit increase bone mass. Bone marrow macrophages (BMMs) derived from HDAC4 conditional knockout (4cKO) mice differentiated normally into multinuclear TRAP positive cells but had decreased bone resorption activity both in vivo and in vitro. We found that ERK1/2 and AKT signaling was reduced in 4cKO osteoclasts. Lastly, we demonstrate that 4cKO osteoclasts have impaired signaling downstream of M-CSF and αvβ3 integrin. Thus, our study suggests that HDAC4 regulates osteoclast function by impairing activation of c-Src and ERK1/2 through a mechanism involving αvβ3-integrin and M-CSF mediated signaling.

4.2 Experimental Procedures

4.2.1 Mice

Hdac4 floxed mice (43) were obtained from Dr. Eric Olson (UT Southwestern Medical Center) in a C57BL/6 background. c-fms-Cre mice, which expresses Cre recombinase in the cells of myeloid lineage that includes osteoclasts and their monocyte/macrophage
progenitors were purchased from the Jackson Laboratory. To obtain mice with Hdac4-deficient osteoclasts (4cKO), Hdac4<sup>flox/flox</sup> mice were bred with c-fms-Cre mice. The mice were of same age and same gender (male). In all experiments, Hdac4<sup>flox/flox</sup> Cre<sup>−</sup> mice (4WT) served as control. The presence of the floxed Hdac4 gene was determined using the primer 5′-ATCTGCCCACCAGAGTATGTG-3′ (forward) and primer 5′-CTTGTTGAGAACA-AACTCCTGCAGCT-3′ (reverse).

4.2.2 Ethics
All mice were housed in the animal care unit of the University of Minnesota and maintained according to the guidelines set by the Institutional Animal Care (IACUC) and the Committee of the Office for the Protection of Research Subjects. All experimental protocols were approved by IACUC (protocol 180636053A) at the University of Minnesota. Euthanasia was performed by CO2 inhalation.

4.2.3 Primary Osteoclast culture
Cultures of murine osteoclast differentiation was performed as previously described (42). Bone marrow cells were flushed from the femora and tibiae of 4WT or 4cKO 4-week-old male mice. Harvested cells were incubated in culture dishes overnight in phenol red-free alpha-MEM (Gibco) containing 5% fetal bone serum (Atlanta Biologicals), 25 units/mL penicillin/streptomycin (Invitrogen), 400 mM L-Glutamine (Invitrogen) in the presence of 1% CMG 14-12 culture supernatant containing M-CSF. CMG14-12 cells were obtained from Dr. Sunao Takeshita (Nagoya City University, Nagoya, Japan). Non-adherent cells were counted, plated in tissue culture plates, and cultured for 2 days with 1% CMG culture supernatant containing M-CSF. After 2 days of culture, the cells are considered to be bone marrow-derived macrophages (BMMs). The BMMs were cultured for additional 4-5 days in phenol red-free alpha-MEM containing 5% fetal bone serum, 25 units/mL penicillin/streptomycin, 1% CMG 14-12 culture supernatant containing M-CSF and 10 ng/mL RANKL (R&D Systems) to obtain osteoclasts.

4.2.4 Enzyme-linked immunosorbent assays (ELISA)
The in vivo bone resorption and formation levels were measured from serum of 3 and 6-month-old male mice following ELISA kits manufacturer’s instructions. Blood samples were collected from 3 and 6-month-old male mice by cardiac puncture, and separated into
three phases by centrifugation for 10 minutes at 13000 rpm at 4°C. After centrifugation, serum was collected from the top of the microfuge tube and transferred into clean microfuge tube, leaving behind red blood cells. Serum CTX was measured using RatLaps ELISA kit; serum TRAP was measured using Mouse TRAP (TRAcP 5b) ELISA kit; and serum PINP was measured using Rat/Mouse PINP EIA ELISA kit. All the ELISA kits were purchased from Immunodiagnostic Systems (IDS) Inc (Gaithersburg, MD, USA).

4.2.5 Real-time PCR

Total RNA was harvested using TRIZOL Reagent (Ambion, Life Technologies) and quantified using UV spectroscopy. Single stranded cDNA was prepared from 1 µg of purified RNA using iScript cDNA Synthesis Kit (BIO-RAD) as per manufacturer’s protocol. Quantitative real-time PCR was performed on CFX Connect Real-Time PCR system (Bio-Rad) using iTaq Universal Sybr Green Supermix (BIO-RAD) according to manufacturer’s instructions. All reactions were performed in triplicate. Experimental genes were normalized to Hprt. Primer sequences were as follows: Hprt (Forward) 5’-GAGGAGTCCTGTAGTATGGCCAG- 3’;(Reverse) 5’-GGCTGGCCATATAGGCTACGTGC -3’; De-stamp (Forward) 5’-CAGACTCCAAATGCTGGAT -3’; (Reverse) 5’ CTTGTGGGAGGAACCTAAGCG -3’; c-Fos (Forward) 5’- CCAAGCGGAGACGATCACTT- 3’; (Reverse) 5’-TCCAGTTTTTCCTTCTTTTCAAGCAGA -3’; Nfatc-1 (Forward) 5’-TCATCCTGTCCAAACACAAAA -3’; (Reverse) 5’- TCACCCCTGTGCTTCTCCTC- 3’; Ctsk (Forward) 5’- AGGGAGAAGCAGACTGGATA- 3’; (Reverse) 5’-GCTGGCTGGAATCACATCTT-3’; Mmp9 (Forward) 5’-GTTTTTGATGCTATTGCTAGATCCA -3’; (Reverse) 5’-CCCACATTGGCAGTCCAGAGAAGA-3’. β3 integrin (Forward) 5’-TTACCCCGTGGACATCCTA-3’ ;(Reverse) 5’-AGTCTTCCATCCAGGGAATA -3’; αv integrin (Forward) 5’- CCTCAGAGGGAGATGTCACAC –3’; (Reverse) 5’-AACTGCCAAGATGATGACTGGATA- 3’; Rank receptor (Forward) 5’-CCAGGACAGGGCTGATGAGA- 3’; (Reverse) 5’-TGGCTGACATCACCAGGATGACA- 3’; c-Fms (CSF1R) 5’-TCGGAGAAAGGTTGAGATGCTG-3’; (Reverse) 5’-TGCTAAAGGCGTCATCCT-3’.
4.2.6 Western blot analysis and antibodies

Cell protein lysates were harvested from 4WT or 4cKO primary osteoclasts using modified RIPA buffer (50 mM Tris ph 7.4, 150 mM NaCl, 1% IGEPAL, 0.25% sodium deoxycholate, 1 mM EDTA) containing Halt Protease & Phosphatase Inhibitor Cocktail (Thermo Scientific). Lysates were cleared by centrifugation at 12,000 x g at 4°C. Proteins were resolved by SDS-PAGE, transferred to PVDF membrane (Millipore), blocked, and blotted in primary antibody overnight at 4°C. Next day, blots were incubated with horseradish peroxidase conjugated secondary antibody (G.E. Healthcare) for 1 hour at room temperature. Antibody binding was detected using western blotting detection kit (WesternBright™ Quantum, Advansta). Images were acquired using Chemitouch (BioRad) and cropped in Adobe Photoshop. The sources of the antibodies are follows: rabbit anti-HDAC4 (H9536) was from Sigma Aldrich, rabbit anti-Src (36D10) and phospho-Src (Y416) antibodies; rabbit anti-MEK1/2 (D1A5) and phospho-MEK1/2 (S217/221) 41G9 antibodies; rabbit anti-p44/42 MAPK (ERK 1/2) and phospho-p44/42 MAPK (T202/Y204) antibodies; rabbit anti-p38 and phospho-p38 (T180/182) antibodies; rabbit anti-Akt pan (C67E7) and phospho-Akt (S473) (193H12); rabbit anti-Pyk2 and phospho-Pyk2 (Y402) antibodies were obtained from Cell signaling (Beverly, MA). All densitometry data was generated using the Image Lab Software (Bio Rad) following the manufacturer’s instructions for normalizing data to a housekeeping protein.

4.2.7 Tartrate Resistant Acid Phosphatase (TRAP) staining

For TRAP staining of 4WT or 4cKO osteoclast cultures, cells were washed with 1X PBS, fixed with 4% paraformaldehyde for 20 minutes and then incubated with pre-warmed TRAP staining solution (0.1M Acetate buffer, 0.3M Sodium Tartrate, 10mg/ml Naphthol AS-MX phosphate, Triton X-100, water, and Fast Red Violet LB salt) (BD Biosciences Technical Bulletin #445) at 37°C for 30 minutes. Color development was stopped by washing three times with 1X PBS. Stained cells were then imaged and photographed with light microscopy and analyzed using NIH Image J to measure the number and size of TRAP-positive osteoclasts.

4.2.8 Demineralization assays
For demineralization assay, BMMs from 4WT or 4cKO 4-week-old male mice were plated at a density of 1x10^5 cells/well on calcium phosphate-coated plates (Osteo Assay Surface plates, Corning) and cultured with 1% CMG 14-12 culture supernatant containing 1.2 µg/mL M-CSF and 10 ng/mL RANKL for 5 days. Medium was then removed from wells, 10% bleach solution was added to wells and incubated for 20 minutes at room temperature. After incubation period, bleach solution was aspirated and the wells were washed two times with water. The wells were then allowed to air dry completely at room temperature for 5 hours. Demineralized area was visualized using light (darkfield) microscopy, and images were taken for quantification. Visual enumeration of demineralization areas was done using NIH Image J software.

4.2.9 Cell adhesion assay
Untreated tissue culture 12-well plate were coated with 5ug/ml of vitronectin and incubated overnight at 4°C. Prior to use the plate was pre-warmed at room temperature for 1 hour. Day 2 pre-osteoclasts that were plated on a 12-well plate at a density of 2 x 10^5 cells/well were scraped and transferred one well to one well of the vitronectin coated plate. The plates were then incubated for 15 or 30 minutes at 37 ºC in 5% CO_2. After incubation time periods, cells were washed twice with 1xPBS, fixed with paraformaldehyde (PFA), and TRAP stained. Cells were then observed and captured with light microscopy and the measurements were analyzed using NIH Image J software.

4.2.10 αvβ3 integrin-mediated signaling and western blot for p-Src and p-ERK 1/2 (p-44/42) MAPK
Untreated tissue culture plate was coated with 5ug/ml of vitronectin and incubated overnight at 4°C. Plate was then placed at room temperature for 1 hour to warm up. 4WT and 4cKO day 2 pre-osteoclasts were serum and cytokine starved for 4 hours. The pre-osteoclasts were then lifted and re-plated onto wells coated with 5ug/ml of vitronectin (A) or left in suspension (S) for 30 minutes, followed by western blot for phospho-ERK1/2 and phospho-Src.

4.2.11 M-CSF signaling and western blot for p-ERK 1/2
BMMs from of 4-week-old 4WT or 4cKO were cultured with 1% CMG 14-12 culture supernatant containing M-CSF until day 1 of osteoclast differentiation. Cells were then
serum and cytokine starved for 4 hours. The adherent osteoclasts were then stimulated with M-CSF for 0, 5, 15, 30 or 60 minutes prior to cell lysis. Western blot analysis was performed using anti-44/42 MAPK (ERK 1/2) and anti-phospho-p44/p42 MAPK (ERK1/2) antibodies.

4.2.12 Cell migration assays
Pre-osteoclast migration assays used transwell migration kit (Corning, NY, USA). 4WT or 4cKO pre-osteoclasts were plated at a density of 2 x 10^5 cells/well on a 12-well plate and allowed to grow until day 2 of osteoclast differentiation. Cells were then scraped, combined and counted. 1 x 10^5 cells/well in 1% CMG 14-12 culture supernatant containing M-CSF in osteoclast media were plated in upper chamber. In the lower chamber, 700ul of osteoclast media was added with no cytokine. The transwell plate was incubated at 37°C for 2 hours. After 2 hours, 1% CMG 14-12 culture supernatant containing M-CSF was added to the lower chamber, and cells were allowed to migrate overnight. The cells in the lower chamber were fixed using 4% paraformaldehyde for 20 minutes. The fixed cells were then stained with TRAP. Light microscopy was used to visualized and take images of TRAP cells for quantification. Image analysis was done using NIH Image J software.

4.2.13 Microcomputed Tomography (µCT) Analysis
Mouse femora were dissected out, defleshed, and frozen in PBS. Before scanning, frozen femora were equilibrated to room temperature and scanned in PBS with a 1 mm aluminum filter using the XT H 225 micro-computed tomography machine (Nikon Metrology Inc., Brighton, MI, USA) at an isotropic voxel size of 7.4 µm. Samples were scanned at 120 kV, 61 µA, 720 projections, 2 frames per projection, and an integration time of 708 milliseconds. CT Pro 3D (Nikon metrology, Inc., Brighton, MI, USA) was used to make 3D reconstruction volumes for each scan. VGStudio MAX 3.2 (Volume Graphics GmbH, Heidelberg, Germany) was used to convert 3D reconstruction volumes to bitmap datasets. Morphometric analyses were performed using SkyScan CT-Analyser (CTAn)(Bruker micro-CT, Belgium) following Bruker’s instructions and reported guidelines for the field3. The region of interest for trabecular bone analysis in the distal metaphysis started 1 mm proximal to the growth plate and extended 2 mm proximally towards the diaphysis. The region for cortical bone analysis was a 0.5 mm region at the
mid-diaphysis. Automated contouring was used to determine the region of interest boundaries for both trabecular and cortical bone with manual editing as needed. Global thresholding was used to segment bone from surrounding tissue for both 3D trabecular and 2D cortical analyses. One threshold value was used for all cortical analyses, and a different threshold value was used for all trabecular analyses. CT-Volume (Bruker micro-CT, Belgium) was used to create all 3D models from bitmaps corresponding to cortical and trabecular regions analyzed; however, only a 1 mm region of the most distal trabecular region was used to create a model.

4.2.14 In vivo TRAP staining
3-month-old male mice were sacrificed by CO₂ inhalation, and their femurs were isolated and fixed in an aqueous buffered zinc formalin (Z-FIX) (Anatech ltd, Battle Creek, MI) overnight, followed by decalcification in 10% EDTA, pH7.4, for 14 days, paraffin embedded, sectioned and stained for TRAP. Osteoclast parameters were measured and expressed in units following guidelines established by the America Society for Bone and Mineral Research histomorphometry nomenclature committee and analyzed using NIH Image J.

4.2.15 Subcellular protein fraction Assay
Cell membrane and cytosolic fractions were prepared using Mem-PER Plus Membrane Protein Extraction Kit from Pierce (89842). Briefly, we used 4WT and 4cKO osteoclasts were plated at a density of 5 x 10⁶ cells/dish on two 10cm dishes and allowed to grow until day 2 of osteoclast differentiation. Media was then removed, and 1X PBS was added and cells were then scraped off the surface. The harvested cell suspensions were centrifuged at 300 xg for 5 minutes. The cell pellets were then washed with 3 mL a Cell Wash Solution and centrifuged at 300 xg for 5 minutes. Supernatant was removed and the cell pellet was resuspended in 1.5mL of Cell Wash Solution, transferred to a clear tube and centrifuged at 300 xg for 5 minutes. After centrifugation, supernatant was discarded. To the cell pellet, 0.75mL of Permeabilization Buffer containing protease and phosphatase inhibitors was added, vortex briefly and incubated at 10 minutes at 4°C with constant mixing. After incubation, the permeabilized cells were centrifuged at 16,000 xg for 15 minutes at 4°C. The supernatant which contains cytosolic proteins was carefully removed and transferred
into a new tube. To the remaining cell pellet, 0.5 mL of Solubilization Buffer containing protease and phosphatase inhibitors was added and the cell pellet was resuspended by pipetting up and down. The tube was then incubated at 4°C for 30 minutes with constant mixing. The tube was then centrifuged at 16,000 xg for 15 minutes at 4°C. The supernatant containing solubilized membrane and membrane-associated proteins was transferred to a new tube. Western blotting analysis was then performed.

4.2.1.6 Statistical analysis

All experiments were completed in triplicate and performed independently three times. The quantitative data are represented as mean ± standard deviation (S.D.). Statistical significances were determined using Student’s t-test with p values ≤0.05 considered significant as determined by Prism.

4.3 Results

4.3.1 Hdac4 conditional knockout (4cKO) male mice exhibit increase bone mass at 3-month of age

To investigate the role of Hdac4 in regulating osteoclast differentiation and activity, we generated osteoclast-specific Hdac4 cKO (4cKO) mice by mating Hdac4fl/fl (4WT) mice with cFms-Cre transgenic mice, in which the Cre recombinase gene is inserted into c-Fms locus and expressed in osteoclasts (227). 4cKO mice were born alive and did not exhibit any abnormal phenotype. Immunoblot confirmed cFms-Cre-mediated deletion of HDAC4 throughout osteoclastogenesis (Figure 20A). Microcomputed tomography (micro-CT) analysis of the distal femur revealed an increase in bone mass phenotype in the 4cKO male mice at 3-month of age compared to 4WT. Bone volume/tissue volume (BV/TV) and trabecular number were significantly increased. Trabecular separation (Tb.Sp) and trabecular thickness (Tb.Th) were not significant different between 4WT and 4cKO; however, trabecular separation did trend towards a decrease (Figure 20 B-F). There was no significant difference in 4cKO female BV/TV as compared to 4WT littermates at 3-month of age (data not shown). However, when the 4cKO male mice were aged to 6-month, micro-CT analysis revealed that the increased bone mass phenotype that was seen at 3-month of age had resolved itself. There was no significant difference in BV/TV, Tb.N , Tb.Sp , and Tb.Th between 4cKO and 4WTmice (Figure 21). In additional, there were no changes and
differences in cortical parameters of 4cKO and 4WT mice at 3- and 6-months of age (data not shown). These data suggest that HDAC4 plays an important role in regulating bone homeostasis in an age-dependent manner.

**Figure 20. HDAC4 conditional knockout (4cKO) male mice exhibit increase bone mass at 3-months of age.** (A) Expression of HDAC4 in osteoclasts from 4WT mice and 4cKO mice was analyzed by Western blotting. β-Actin was used as a loading control. (B) Representative micro-CT images of the distal femur in a 4WT mouse (left) and 4cKO mouse (right) at 3-month of age. (C–F) Graphs show quantitative data from the micro-CT analyses. BV/TV, bone volume per total volume; Tb.N, trabecular number; Tb.Th, trabecular thickness; Tb.Sp, trabecular separation. Data are represented as the mean ± SD (*P < 0.05; n = 10 male mice in each group). P-values are based on Student’s t-test. P < 0.05, is considered significant. NS, not significant.
Figure 21. Micro-CT analysis revealed that 4cKO male mice resolves the increase bone mass phenotype (osteopetrotic) at 6-month of age. Graphs show quantitative data.

4.3.2 Deletion of HDAC4 in osteoclast does not affect in vitro osteoclast differentiation

To assess the role of HDAC4 in the osteoclast differentiation process, we cultured 4WT and 4cKO BMMs with M-CSF and RANKL. RANKL-induced osteoclast differentiation was evaluated by tartrate resistant acid phosphatase (TRAP) staining at day 2, 3 and 4, and mRNA expression levels of osteoclast differentiation markers genes (c-Fos, Nfatc1 and Dc-stamp). After 2-day exposure to cytokines, 4WT and 4cKO BMMs cultures had many TRAP-expressing mononuclear osteoclasts (Figure 22A). By day 3 and 4, both BMMs cultures had differentiated into TRAP positive multinuclear osteoclasts. There was no significant difference in the number of TRAP+ multinuclear osteoclasts in 4WT and 4cKO BMMs cultures (Figure 22B); however, the 4cKO osteoclasts, had a trend towards
increased size at day 3 compared to their 4WT counterparts. The mRNA expression of osteoclast differentiation marker genes (c-Fos, Nfatc1 and Dc-stamp) did not differ between 4cKO and 4WT osteoclasts; even though, the mRNA expression levels of marker genes in 4cKO osteoclasts showed an increase (Figure 23). The serum levels of TRAP5b, an in-vivo marker of TRAP activity, was not significantly different between 4cKO and 4WT mice at 3-month (Figure 24A) or 6-month of age (Figure 24 B). TRAP stained bone sections of 3-month old 4cKO and 4WT male mice revealed that 4cKO male mice exhibited a significant increase in the osteoclast surface/bone surface (Oc.S/BS) but not in osteoclast number/bone surface (Oc.N/BS, not shown) (Figure 24C). This data suggests that osteoclast differentiation is enhanced in-vivo in the 4cKO mice compared to their 4WT littermates.
Figure 22. Enhanced in vivo differentiation of 4cKO osteoclasts. (A) BMMs from 4WT and 4cKO mice were cultured in the presence of M-CSF and RANKL for 2, 3 or 4 days, followed by staining for TRAP$^+$ cells. (B) Graphs show quantitative data for number and size of TRAP$^+$ multinucleated cells generated from 4WT and 4cKO BMMs. Only cells containing more than 3 nuclei were counted as osteoclasts and quantified. Data are
represented as the mean ± SD. Data shown are representative of three independent experiments. (C) TRAP expression of decalcified bone sections of the femur from 3-month old 4WT male mice (left) or 4cKO male mice (right) was assessed by TRAP staining and the following parameter, Oc.N/BS, number of osteoclasts per bone surface and Oc.S/BS, osteoclast surface per bone surface was measured. Representative TRAP-stained images are shown. Black boxes indicate the region of magnification. Data are represented as the mean ± SD (***p < 0.001, n = 4 male mice in each group). P-values are based on Student’s t-test. p < 0.05, is considered significant.

Figure 23. Genetic deletion of Hdac4 results in no change in the expression of osteoclast differentiation markers in vitro. Analysis of the osteoclastogenic marker genes c-Fos, Nfatc1 and Dc-stamp, was performed at day 0–4 from 4WT or 4cKO osteoclasts by qPCR. Data shown are representative of three independent experiments. p < 0.05, is considered significant.
Figure 24. *Hdac4* deletion does not affect TRAP activity *in vivo*. Blood plasma serum of 3-month (A) and 6-month (B) old 4WT and 4cKO male mice were analyzed for TRAP form 5b (TRACP 5b), a marker for TRAP activity. N = 10 male mice in each group. Data analyzed by Student’s t-test. p< 0.05, is considered significant. NS, not significant.

4.3.3 Bone resorption is decreased in 4cKO male mice

Since the increased bone mass phenotype of 4cKO male mice could not be explained by the increased *in vivo* osteoclast differentiation we next analyzed the activity of the osteoclasts from the 4cKO mice. We first generated TRAP-expressing multinuclear osteoclasts on calcium phosphate plates. After 5 days in M-CSF and RANKL, the demineralized area was visualized using light microscope (Figure 25A). The 4cKO male mice exhibited significant decrease in percent demineralized area and average number of demineralized areas, but not in average size of demineralized areas (Figure 25B). The serum levels of C-terminal cross-linking telopeptide of type 1 collagen (CTX), a bone resorption *in-vivo* marker, were significantly decreased in 4cKO mice compared with 4WT mice (Figure 25C) at 3-month of age. There was no significant change in genes necessary for osteoclast activity (*Acp5, Ctsk* and *Mmp9*) (Figure 26). We found that resorptive capacity of 4cKO osteoclasts was not significantly different from 4WT at 6-month of age as measured by serum CTX but there was a trend towards decrease in the 4cKO mice (Figure 27). The serum levels of PINP, an *in-vivo* marker for bone formation, were not significantly different between 4cKO and 4WT mice at 3-month and 6-month of age, despite a trend towards increased PINP levels in 4cKO mice (Figure 28 A/B). These findings suggest that the increase bone mass phenotype of 4cKO mice at 3-month of age is a result of a decrease in bone resorbing activity of osteoclasts.
Figure 25. HDAC4 aids the bone-resorbing activity of osteoclasts. (A) Osteoclasts from 4WT and 4cKO mice were cultured on calcium phosphatase surface for 5 days, and the demineralized areas were visualized by light microscope and quantified using NIH Image J software. Representative images of demineralized area are shown. (B) Graphs shows percent demineralized area, average size and average number quantification. Data are represented as the mean ± SD (****p < 0.0001; *p < 0.05). Data shown are representative of three independent experiments. (C) Serum concentration of CTX, an in-vivo marker of bone resorption, in 4WT mice and 4cKO mice was assessed by ELISA. Data are represented as the mean ± SD (*p < 0.05, n = 10 male mice in each group). P-values are based on Student’s t-test. p < 0.05, is considered significant. NS, not significant.
Figure 26. Hdac4 deletion in osteoclasts does not alter expression of osteoclast activity marker genes. Analysis of the osteoclast activity marker genes Acp5, Ctsk and Mmp9 was performed at day 0–4 from 4WT or 4cKO osteoclast by qPCR. Data shown are representative of three independent experiments. p < 0.05, is considered significant.

Figure 27. Bone resorption was trending toward decrease in 4cKO male mice at 6-month of age. Blood plasma serum of 6-month old 4WT and 4cKO mice was analyzed.
for CTX, a marker for global bone resorption. N = 10 male mice in each group. Data analyzed by Student’s t-test. p < 0.05, is considered significant.

![Graph showing PINP levels in 4WT and 4cKO mice](image)

**Figure 28. Deletion of Hdac4 mice cause a trend towards increase bone formation in vivo.** Blood plasma serum of 3-month (A) and 6-month (B) old 4WT and 4cKO male mice was analyzed for PINP, a marker for bone formation. N = 10 male mice in each group. Data analyzed by Student’s t-test. p < 0.05, is considered significant. NS, not significant.

### 4.3.4 Reduced ERK and AKT activation in 4cKO osteoclasts

Since we did not measure any changes in gene expression in the 4cKO osteoclasts, we next analyzed intracellular signaling pathways to determine if signaling pathways were altered to account for the change in osteoclast activity. As assessed by immunoblotting, the phosphorylation of MAPKs, in particular extracellular signal-related kinase 1/2 (ERK1/2), was significantly decreased at day 0 and 1 in 4cKO osteoclasts as compared with 4WT osteoclasts, while the phosphorylation of p38 was only significantly increased at day 1. In contrast, the phosphorylation of JNK was unchanged by Hdac4 deletion (Figure 29A, B). These results suggest that expression of HDAC4 is necessary for activation of ERK1/2 MAPK signaling in osteoclast precursors.

Since, PI3-K/AKT pathway has been shown to regulate osteoclast development (228), we also examined the phosphorylation of AKT in the cultured osteoclasts. As shown in Figure 29A and B, we found that the levels of AKT phosphorylation were also significantly reduced in 4cKO osteoclasts at day 0 and 1 compared to 4WT osteoclasts, suggesting that the functional abnormality observed in 4cKO osteoclasts are also AKT dependent.
Together, these results show that HDAC4 expression is required for activation of ERK and AKT signaling.

We next asked whether M-CSF or RANKL activation was impaired in its ability to activate ERK1/2 signaling since both cytokines have been demonstrated to activate MAPK signaling in osteoclasts (229). We generated osteoclasts from 4cKO and 4WT mice by culturing BMMs in M-CSF till day 1. The cells were then cytokine starved, stimulated with M-CSF for 0, 5, 15, and 30 minutes, lysed and extracted protein for western blot. We assessed total and phosphorylated levels of ERK1/2. As seen in Figure 29C, following M-CSF stimulation, the magnitude and duration of ERK1/2 phosphorylation was dampened in 4cKO osteoclasts compared to 4WT osteoclasts. This reduced M-CSF induced activation of ERK1/2 suggests a requirement for HDAC4 in the regulation of ERK1/2 by M-CSF. There was no difference observed in the ability of RANKL to activate ERK1/2 in the 4WT or 4cKO osteoclasts.
Figure 29. HDAC4 positively regulates the activity of ERK1/2 and AKT. (A) 4WT and 4cKO BMMs were cultured in M-CSF and RANKL for indicated time. The whole-cell lysates from 4WT and 4cKO osteoclasts were then subjected to Western blot using antibodies against phospho-ERK 1/2, phospho-JNK, phospho-p38, and phospho-AKT. Total-ERK 1/2, total-JNK, total-p38 and total-AKT served as a loading control. (B) Graphs shows quantifications of the Western blots. Data are represented as the mean ± SD (*P < 0.05). Data quantified are representative of three independent experiments. P-values are based on Student’s t-test. p < 0.05, is considered significant. (C) Pre-osteoclasts were stimulated with M-CSF for 0, 5, 15, 30 minutes, followed by western blot for phospho-ERK 1/2. β-actin served as a loading control.

In canonical MAPK signaling, ERK1/2 activation is dependent on MEK1/2 (230). Because 4cKO osteoclasts have reduced ERK1/2 activation, we asked whether upstream regulator of ERK1/2 signaling, MEK1/2 is also affected. We cultured BMMs cells
isolated from 4cKO and 4WT mice in M-CSF and RANKL. Generated osteoclasts were then collected, lysed and protein extracted for western blot. As shown in Fig. 5A, the phosphorylation of MEK1/2 was unchanged by Hdac4 deletion, suggesting that the reduced ERK1/2 phosphorylation seen in 4cKO osteoclasts was not due to significant change in MEK1/2 activity. Thus, changes in MEK 1/2 activation was not responsible for ERK1/2 changes we observed in 4cKO osteoclasts.

Figure 30. Hdac4 deletion in osteoclasts does not alter MEK1/2 activation. 4WT and 4cKO BMMs were cultured with M-CSF and RANKL up to 4 days. MEK1/2 phosphorylation was determined by immunoblot. Total-MEK 1/2 served as a loading control. Data quantified are representative of three independent experiments. P < 0.05, is considered significant.

4.3.5 Impaired αvβ3 Integrin Signaling Activation in 4cKO Osteoclasts

Resorption by osteoclasts requires migration across the bone surface and the ability to bind to the bone surface through αvβ3 engagement with RGD containing proteins in the bone matrix (11, 12, 231). Mice that are null for ERK1/2 expression in osteoclasts have reduced migration and adhesion (232). Since the 4cKO osteoclasts had reduced ERK1/2 activation, we asked whether deletion of Hdac4 affects αvβ3 integrin-mediated adhesion and signaling. We differentiated 4WT and 4cKO BMMs cells till day 2, then re-plated the preosteoclasts on vitronectin (VTN), the αvβ3 ligand, and incubated the cells for 15 and 30-minute period. As seen in Figure 31A, the number of adherent 4cKO osteoclasts was decreased significantly after 15 and 30 minutes compared to 4WT osteoclasts. This data suggests that HDAC4 is necessary for αvβ3 integrin-mediated adhesion. We next asked whether HDAC4 is also required for activations of upstream regulators of integrin-mediated signaling. We cultured 4WTand 4cKO BMMs in M-CSF and RANKL. After 2 days, we cytokine-starved and preosteoclasts were lifted and re-plated on the αvβ3 ligand, vitronectin (VTN, (A), or maintained in suspension (S) for 30-minutes. After 30-minute incubation, cells were lysed, and Src and ERK1/2 activation, as manifested by
phosphorylation, was assessed by immunoblot. As shown in Figure 32B, Src and ERK1/2 activation are decreased in 4cKO, supporting our hypothesis that HDAC4 is required for integrin-mediated signaling.

Because adhesion was affected in 4cKO osteoclasts, we next examined whether deletion of Hdac4 affected migration of preosteoclasts using transwell migration assay in response to M-CSF. As shown by Figure 31C, 4cKO pre-osteoclasts migrated at lower numbers compared to 4WT pre-osteoclasts in response to M-CSF. Defective adhesion and migration of 4cKO pre-osteoclasts suggests a requirement for HDAC4 in the activation of αvβ3 integrin, which may underlie the reduced bone resorption activity detected in 4cKO osteoclast in vivo and in vitro.

**Figure 31.** Defective αvβ3-dependent signaling in 4cKO osteoclasts. (A) M-CSF mediated pre-osteoclast adhesion to αvβ3. 4WT or 4cKO BMMs were cultured with M-CSF and RANKL for 2 days to generate pre-osteoclasts. The cells were lifted and re-plated onto vitronectin-coated wells for 15 or 30 minutes, followed by TRAP staining. Data are represented as the mean ± SD (*p < 0.05). Data shown are representative of three independent experiments. (B) 4WT or 4cKO pre-osteoclasts were cytokine/serum starved, re-stimulated, lifted and re-plated onto vitronectin-coated wells for 30 minutes (A) or left
in suspension (S), followed by western blot for phospho-Src and phospho-ERK1/2. Total-Src and total-ERK 1/2 served as a loading control. (C) Transwell migration of day 2 pre-osteoclasts in response to M-CSF. Data are represented as the mean ± SD (**p < 0.01). Data shown are representative of three independent experiments. P-values are based on Student’s t-test.

4.3.6 Pyk2 phosphorylation is reduced in 4cKO osteoclasts

It has been previously shown that activation of αvβ3 integrin causes phosphorylation of Pyk2Y402, a calcium-dependent tyrosine kinase that is a FAK family member which then activates c-Src by binding to its SH2 domain in osteoclasts (233). Additionally, we have shown in this Chapter that Src phosphorylation is decreased in 4cKO osteoclasts after adhesion (Figure 31B), suggesting that HDAC4 is required for integrin-mediated signaling in osteoclasts. Because activation of Src by αvβ3 requires phosphorylation of Pyk2 (233), we evaluated whether HDAC4 is required for activation of Pyk2 in osteoclasts. To examine this, we analyzed phosphorylation of Pyk2 in 4WT and 4cKO differentiating osteoclasts. We found that Pyk2 phosphorylation was decreased in cells derived from 4cKO osteoclasts (Figure 33), indicating that HDAC4 is required for activation of Pyk2.
**Figure 32. Pyk2 phosphorylation is decreased in 4cKO osteoclasts.** Expression levels of Pyk2 protein during osteoclastogenesis using 4WT and 4cKO BMMs in culture with M-CSF plus RANKL from 0 to 4 days. Graph shows quantitative data of western blots. Data are represented as the mean ± SD (*p < 0.05). Data shown are representative of three independent experiments.

### 4.3.7 Src phosphorylation is decreased in plasma membrane fraction of 4cKO osteoclasts

The Src family of protein kinases are nonreceptor tyrosine kinases which associates with cell membrane to regulates different cellular events such as differentiation, adhesion and migration (234). It has been reported that Src-membrane association-dissociation is regulated by CREB binding protein (CBP)-mediated acetylation (235). Since we observed reduced adhesion, migration and Src phosphorylation in 4cKO osteoclasts, we asked whether HDAC4 regulates Src-membrane association-dissociation. To delineate whether Src-cell membrane association changes in osteoclasts, we fractionated Src from two cell compartments: plasma membrane and cytoplasm using 4WT and 4cKO osteoclasts. Src phosphorylation was detected in the cytoplasmic fractions of both 4WT and 4cKO osteoclasts. While Src from plasma membrane fraction of 4WT osteoclasts had detectable phosphorylation, Src from plasma membrane fraction of 4cKO osteoclasts had undetectable phosphorylation (Figure 33). Our data suggests that HDAC4 regulates Src phosphorylation in the membrane of osteoclasts. This data agrees with previous data from Figure 31 suggesting that integrin activation of Src which would take place at the plasma membrane is impaired in HDAC4 null osteoclasts. Lastly this data suggests that loss of HDAC4 expression does not appear to affect total Src expression as there was no difference in total Src expression between 4WT and 4cKO osteoclasts (middle panel, Fig 33).
Figure 33. Src phosphorylation is decreased in plasma membrane fraction of 4cKO osteoclasts. Src recovered from plasma membrane (M) and cytoplasmic (C) fractions from 4WT and 4cKO osteoclast were analyzed for phosphorylation. Representative result is shown.

4.4 Discussion

Osteoclast dysfunction has been implicated in several bone disorders. Osteoclast hyperactivity is associated with diseases such as osteoporosis and cancer metastasis to bone; whereas osteoclast deficiency leads to diseases such as osteopetrosis (19, 236). We have begun to understand the mechanisms that regulate osteoclast differentiation and function; however, there is still a lot that is unknown. Recently, class IIa HDACs which include HDAC4, 5, 7 and 9 have been reported to be regulators of osteoclastogenesis. Our lab reported that HDAC7 regulates osteoclast differentiation by inhibiting MITF’s activation of osteoclast genes (39, 140), Jin et al. reported that HDAC7 negatively regulates osteoclastogenesis through suppressing Nfatc1 expression and preventing down-regulation of β-catenin and cyclin D1 (40). HDAC9 inhibits osteoclastogenesis through suppression of PPARγ/RANKL signaling (41). Our present study surprisingly demonstrates that HDAC4 positively regulates the bone resorption activity of osteoclasts in vitro and in vivo by impairing activation of c-Src and ERK1/2 through a mechanism involving αvβ3-integrin and M-CSF mediated signaling. Together these studies suggest
that class IIa HDACs are not redundant in their regulation of osteoclastogenesis. Consequently, deletion of HDAC4 in osteoclasts leads to an increase bone mass phenotype due to dysfunctional osteoclasts.

Male mice null for HDAC4 in osteoclasts exhibited significantly decreased bone resorption and increased bone mass at 3-months of age. This finding suggests that unlike HDAC7 and -9 which negatively regulate osteoclast differentiation and activity (39-41), HDAC4 positively regulates osteoclast activity. HDAC4’s role in regulating osteoclast differentiation are not as clear. In our previous study, osteoclasts infected with an shRNAs against HDAC4 showed larger osteoclasts compared to control infected cells. However, in our current study we did not see a significant difference in size or number of TRAP positive cells in vitro but we did measure an increase in size in TRAP positive cells in vivo. It is not clear whether our results with the Hdac4 shRNAs were due to off target effects against other class IIa HDACs during osteoclast differentiation. An alternative explanation for the difference results could be to the timing of loss of HDAC4 expression during osteoclast differentiation.

Based on the skeletal phenotype of $Hdac4^{fl/fl}$;C-fms Cre mice HDAC4’s main role in osteoclasts involves regulating activity, as opposed to differentiation. Given the in vivo osteoclast defects in 4cKO mice, we examined the capacity of osteoclast precursors to differentiate in response to the osteoclastogenic cytokines RANKL and M-CSF, as well as the resorptive capacity of the osteoclasts, in vitro. Expression of early osteoclast differentiation marker genes such as c-Fos, Nfatc-1 and Dc-stamp and late marker genes such as Acp5, Ctsk and Mmp9 were not significantly different in 4cKO osteoclasts compared to 4WT osteoclasts. This data suggests that HDAC4 does not regulate osteoclasts through regulation of these genes. Despite the ability of the mutant cells to fuse and become TRAP positive multinucleated cells, 4cKO osteoclasts had markedly diminished capacity to resorb bone. The finding of suppressed osteoclast function in the face of elevated osteoclast number in vivo is reminiscent of β3 integrin-deficient mice, whose bone mass is enhanced (12, 29).

In an attempt to elucidate the mechanisms by which HDAC4 enhances the bone-resorbing activity of osteoclasts, we demonstrated that the activity of ERK1/2 was significantly decreased in 4cKO osteoclasts. Previous studies have established the role of
ERK1/2 in osteoclasts. He et al. reported that ERK1/2 positively regulates osteoclast function (232), providing grounds for our proposal that the decreased bone-resorbing activity of 4cKO osteoclasts, at least in part, is caused by the decreased in activity of ERK1/2. Although multiple studies have reported in the antiapoptotic effects of ERK1/2 in osteoclasts (237, 238), we did not find a decrease or increase in survival of 4cKO osteoclasts (data not shown). This difference may be explained by the duration of ERK1/2 phosphorylation and its subcellular localization. Chen et al. reported that both duration of ERK1/2 activity and localization of ERK1/2 are important for the proper function of ERK1/2 (239). Further studies are needed to determine the mechanisms by which HDAC4 regulates ERK1/2 activation.

In osteoclasts, differentiation and function involve activations of many signaling pathways such as ERK1/2 by M-CSF, through its cell surface receptor c-FMS and the αvβ3 integrin (12, 24, 231, 240, 241). Given the in vitro reduction of ERK1/2 phosphorylation in 4cKO osteoclasts, we asked whether ERK1/2 activation in response to M-CSF is impaired. We find that ERK 1/2 activation after M-CSF stimulation is HDAC4 dependent. The importance of ERK 1/2 signaling pathway in regulating osteoclast differentiation and function has been shown with Erk1−/− and Erk2−/− mice, which reported that ERK1 positively regulates osteoclast function (232). The fact that 4cKO osteoclasts have defective M-CSF induced activation of ERK 1/2 suggests a requirement for HDAC4 in the regulation of ERK1/2, which may partially underlie the defects in bone resorptive activity in 4cKO osteoclasts in vitro.

Bone resorption by osteoclasts involve the binding of osteoclasts to bone via αvβ3 integrin. The αvβ3 integrin is highly expressed in osteoclasts and mediates the ability of the cell to polarize, spread, migrate and degrade bone (11, 12). β3 integrin-deficient mice are osteopetrotic as a result of failure of their osteoclasts to spread (12). To determine if HDAC4 is required for osteoclast attachment, we analyzed αvβ3 – and M-CSF-mediated adhesion in 4WT and 4cKO preosteoclasts. The finding that fewer 4cKO preosteoclasts adhered to vitronectin coated plates during stimulation with M-CSF indicates a defect in the ability of osteoclasts to bind through interactions between αvβ3 integrin and its ligand vitronectin to initiate bone resorption. To confirm the role of HDAC4 during osteoclast attachment, we analyzed αvβ3-dependent activation of two kinases, namely c-Src and
ERK1/2, in 4WT and 4cKO preosteoclasts. We find that both c-Src and ERK 1/2 signals were reduced in 4cKO pre-osteoclasts, suggesting that HDAC4 is necessary for αvβ3-integrin-mediated signaling in osteoclasts during attachment. Because, 4cKO preosteoclasts do not adhere very well and activate αvβ3-dependent kinases (c-Src and ERK 1/2), we asked whether migratory capacity of the preosteoclasts is affected, using transwell migration in response to M-CSF. We find that 4cKO preosteoclasts migrated at a lower number in response to M-CSF. Thus, our results suggest preosteoclast migration across the bone might be blocked in cells lacking HDAC4, suggesting that HDAC4 is required for osteoclast migration in vitro. The finding of suppressed osteoclast function, in the face of impaired adhesion and migration is reminiscent of Erk1 knockout mice, who have an enhanced bone mass (232).

Acetylation as a regulatory mechanism of enzymatic activity has been highly conserved in evolution. HDACs have been shown to modify histone and non-histone protein to regulate protein function (31, 183). A recent study using cancer cells reported that CREB binding protein (CBP), an acetyltransferase, regulates c-Src-cell membrane association and dissociation, hence its function (235). Our data suggest that HDAC4 may be deacetylating c-Src since we found that 4cKO osteoclasts have impaired c-Src activation. Sunjay et al. reported that when αvβ3 integrin is activated it results in phosphorylation of Pyk2, which then activates c-Src (233). Given the reduced c-Src activation we observed in 4cKO osteoclasts (Chapter 4, Figure 31B), we examined whether HDAC4 is required for activation of Pyk2 in osteoclasts. We observed diminished Pyk2 phosphorylation in 4cKO osteoclast, suggesting Pyk2 activation in osteoclasts may require HDAC4. Therefore, the reduced bone resorption activity in 4cKO osteoclasts may be a result of αvβ3-Pyk2-c-Src being unable to be activated.

Our studies do not rule of the possibility that Src activity may affect Pyk2 phosphorylation directly through mechanism involving Src phosphorylation rather than acetylation. Previously, it has been shown that Src-Pyk2 interaction requires Src SH2 domain, and that the domain is important in Src phosphorylation of Pyk2 (242). Furthermore, it has been reported that Src retains a basal activity in cells via an auto-inhibitory mechanism, whereby a tyrosine phosphate Y526 or Y527 in the carboxyl terminal tail is phosphorylated allowing for interaction with Src homology 2 (SH2) domain.
preventing catalytic activity (235). Src activation is initiated when the carboxyl terminal tyrosine phosphate is removed, causing a conformation change and subsequently, autophosphorylation (243). The fact that both Src and Pyk2 phosphorylation are abrogated in 4cKO osteoclast could suggest that Src is catalytically inactive, which in turn prevents interaction with Pyk2. This abolished interaction between Src and Pyk2 might impact Pyk2 activation during osteoclastogenesis. This would seem to suggest that Src activation occurs separately from HDAC4 complex assembly and can exert its effects on Pyk2 directly. This provides yet an additional explanation of the importance of Src activation. Future studies are needed to precisely determine how Src-Pyk2 mechanism works in 4cKO osteoclasts.

Among the various properties known to be important to Src, dynamic association with plasma membrane is one of them (235, 244). Given we observed reduced adhesion, migration and Src phosphorylation in 4cKO osteoclasts (Figure 31), we asked whether Src membrane association was affected using cytoplasmic and plasma membrane fractions. We found that Src phosphorylation was detected in both cytoplasmic fractions of 4WT and 4cKO osteoclasts, but it was undetectable in the plasma fraction of 4cKO osteoclasts (Figure 33), suggesting that HDAC4 regulates Src phosphorylation in the membrane of osteoclasts. Furthermore, our data suggest that loss of HDAC4 expression in osteoclasts does not alter total Src expression as we did not detect changes in total Src between 4WT and 4cKO osteoclasts. In summary this data agrees with our previous data from Figure 31 suggesting that integrin activation of Src which would take place at the plasma membrane is impaired in HDAC4 null osteoclasts.

A limitation of this study is that deacetylation of Src was assessed only in cell lysates of osteoclasts. Further studies are needed to determine if c-Src acetylation status dictates cellular location. In addition, we did not elucidate which sites in c-Src unique domain and catalytic domain are the potential HDAC4 deacetylation sites in osteoclasts.

In conclusion, our study identified HDAC4 as the significant regulator of osteoclast bone resorption both in vivo and in vitro. The characterization of 4cKO mice, molecular and cellular assays lends further support for the importance of the HDAC4 in osteoclasts. Collectively my data suggests that expression of HDAC4 regulates Pyk2 and Src phosphorylation in osteoclasts. We hypothesize that the changes in Pyk2 and Src results in changes in adhesion and migration and ultimately resorption. Several questions that
remain to be answered are 1) similar to cancer cells is deacetylation of Src necessary for activation of Src at the membrane, 2) is HDAC4 deacetylating Src which allows Src to be phosphorylated at the membrane, 3) can only deacetylated Src associate with integrins to allow for phosphorylation and 4) is ERK1/2 phosphorylation regulated by Src, M-CSF and/or RANKL signaling? (Fig 34)

**Figure 34. Mechanism of HDAC4 regulation of osteoclast function.** Illustration of a pathway of HDAC4 regulation of osteoclast function.
Chapter 5. RNA-Sequence based detection of differentially expressed genes in 4cKO osteoclasts

Chapter summary

The results of chapter 4 demonstrated that mice model lacking HDAC4 in osteoclasts had increased bone mass phenotype (osteopetrosis) resulting in decreased osteoclast-bone resorption with no effect on osteoclast differentiation. Moreover, the expression of the osteoclast differentiation markers genes from 4cKO osteoclasts did not differ from 4WT osteoclasts. It has been reported that in muscle cells, class IIa family members, HDAC4, 5 and 7 are evolutionary conserved and can compensate for each other to regulate gene expression. Given this, the aim of this study was to investigate global changes in gene expression in 4cKO osteoclasts by performing RNA-Sequence on 4WT and 4cKO osteoclasts.

5.1 Introduction

Of the class IIa HDACs, HDAC 4, 5 and 7 are more evolutionarily conserved and share approximately 57% similarity (31). During various stages of muscle differentiation, it has been shown that HDAC 4, 5 and 7 complement each other to regulate gene expression in response to specific signals (31, 226). However, it is not known whether the same process occurs in osteoclast. Since suppression of a single HDAC, either HDAC7 or 9 will enhance osteoclast formation, this data suggested that the class IIa HDACs are not functionally redundant (42, 140). Also, these results suggest that each HDAC provides a carefully regulated set of changes in gene expression to regulate osteoclast differentiation and activity.

5.2 Experimental Procedure

5.2.1 Primary Osteoclast Culture

Cultures of murine osteoclast differentiation was performed as previously described (42). Briefly, bone marrow cells were flushed from the femora and tibiae of 4WT or 4cKO 4-week-old male mice. Harvested cells were incubated in culture dishes overnight in phenol red-free alpha-MEM (Gibco) containing 5% fetal bone serum (Atlanta Biologicals),
25 units/mL penicillin/streptomycin (Invitrogen), 400 mM L-Glutamine (Invitrogen) in the presence of 1% CMG 14-12 culture supernatant containing M-CSF. CMG14-12 cells were obtained from Dr. Sunao Takeshita (Nagoya City University, Nagoya, Japan). Non-adherent cells were counted, plated in tissue culture plates, and cultured for 2 days with 1% CMG culture supernatant containing M-CSF. After 2 days of culture, the cells are considered to be bone marrow-derived macrophages (BMMs). The BMMs were cultured for additional 2 days in phenol red-free alpha-MEM containing 5% fetal bone serum, 25 units/mL penicillin/streptomycin, 1% CMG 14-12 culture supernatant containing M-CSF and 10 ng/mL RANKL (R&D Systems) to obtain osteoclasts.

5.2.2 RNA-Sequence.

4WT and 4cKO BMMs were differentiated into osteoclasts and harvested on day 2 using RNeasy Plus Mini Kit (QIAGEN, Germantown, MD, USA). 50bp FastQ paired-end reads were trimmed via Trimmomatic (v 0.33). For each sample, quality control on raw data sequence was performed using FastQC. Read mapping was performed using Hisat2 (v2.0.2) and mouse genome was used a reference. Gene quantification was achieved using Cuffquant for FPKM values and Feature Counts for raw read counts. Pathway analysis was performed using DAVID.

5.3 Results

To determine global changes in gene expression in 4cKO osteoclasts, I performed RNA-Sequence experiment using 4WT and 4cKO osteoclasts on day 2 of osteoclast differentiation. A total of 745 mRNA genes were differentially expressed when considering exclusively a significance threshold of $P$-value $\leq 0.05$. When we performed a more stringent analysis ($P$-value $\leq 0.01$), 715 genes were differentially expressed (Table 2 showing representative genes).

We used DAVID analysis to identify pathways to which differentially expressed genes belong to as well as to explore the existence of signaling networks connecting differentially expressed genes. This information should be interpreted with caution because, in general, pathways were represented by a small number of genes and statistical significance was not
very high. As shown in Table 3, we found 14 regulatory networks related to variety of functions.

<table>
<thead>
<tr>
<th>Feature ID</th>
<th>EDGE test: cfms vs HDAC4, tagwise dispersions - FDR p-value correction</th>
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</thead>
<tbody>
<tr>
<td>Dok2</td>
<td>0</td>
</tr>
<tr>
<td>Igf2bp3</td>
<td>0</td>
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<tr>
<td>Arl11</td>
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<td>Lox</td>
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<td>Tgfbr3</td>
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<td>Map2k3os</td>
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<tr>
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Table 2: Representative list of differentially expressed genes in HDAC4 osteoclasts. P value < 0.01.

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<tr>
<th>Pathway</th>
<th>Genes Within Pathway</th>
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<tr>
<td>Rap1 signaling pathway</td>
<td>Adcy6, Angpt1, Cdh1, Csfl, Epha2, Fgf12, Fpr1, Hgf, Igf1, Itga2b, Itgb2l, Kdr, Kit, Pdgfc, Pdgfrb, Rapgef3, Rasgrp2, Thbs1, Vegfa</td>
</tr>
<tr>
<td>Oocyte Meiosis</td>
<td>Adcy6, Bub1, Cdc20, Cdk1, Cpeb1, Igf1, Mad2l1, Pgr</td>
</tr>
<tr>
<td>p53</td>
<td>Ccnb1, Ccnb2, Cnd2, Cdk1, Chek1, Gadd45a, Igf1, Serpine1, Sfn, Thbs1</td>
</tr>
<tr>
<td>Cell Adhesion</td>
<td>Cdc22, Cdc274, Cdc4, Cdh1, H2-Aa, H2-Db1, H2-Ab, H2-T24, Itgb2l, Nrcam, Pecam1, Sdc2, Sdc3, Sell, Selp, Siglec1, Vcam1, Vcan</td>
</tr>
<tr>
<td>Cell Cycle</td>
<td>Bub1, Ccnb1, Ccnb2, Cnd2, Cdc20, Cdc25c, Cdk1, Chek1, Gadd45a, Mad2l1, Sfn, Tgfb2, Tgfb3</td>
</tr>
<tr>
<td>PI3K/Akt</td>
<td>Angpt1, Ccnd2, Colla2, Colla1, Colla2, Ccnd2, Col5a2, Col6a1, Col6a2, Col6a3, Csf1, Csf3r Epha2, Fgf12, Fn1, Hgf, Igf1, Il7r, Itga2b, Kdr, Kit, Lamb1, Myb, Pdgfc, Pdgfrb, Sgk1, Thbs1, Thbs2, Tnc</td>
</tr>
<tr>
<td>Hippo</td>
<td>Afp, Birc5, Ccnd2, Cdh1, Ctgf, Fzd6, Itgb2l, Serpine1, Tgfb2, Tgfb3, Wnt6</td>
</tr>
<tr>
<td>HIF1</td>
<td>Angpt1, Eno1b, Eno2, Igf1, Nos2, Serpine1, Timp1, Trf, Vegfa</td>
</tr>
<tr>
<td>Hemopoietic</td>
<td>Anpep, Cd22, Cd33, Cd36, Cd4, Cd7, Csf1, Csf3r, Fcgr1, Flt3, Gp1ba, Gp1bb, Gp5, Gp9, H2-Db1, Il1b, Il1r1, Il7r, Itga2b, Kit, Siglec1</td>
</tr>
<tr>
<td>FoxO</td>
<td>Ccnb1, Ccnb2, Ccnd2, Gadd45a Igf1, Il7r, Irs2, Klf2, Plk2 Sgk1, Tgfb2, Tgfb3, Tnfsf10</td>
</tr>
<tr>
<td>Focal Adhesion</td>
<td>Cav1, Ccnd2, Colla2, Colla1, Colla2, Colla2, Coll3a1, Coll5a2, Coll6a1, Coll6a2, Coll6a3, Fn1, Hgf, Igf1, Itga2b, Kdr, Lamb1, Myl9, Mylk, Parva, Pdgfc, Pdgfrb, Thbs1, Thbs2, Tnc, Vegfa</td>
</tr>
<tr>
<td>CMP PGK</td>
<td>Adcy6, Atp1a3, Ednrb, Gucy1a3, Gucy1b3, Irs2, Mrvi1, Myl9, Mylk, Pde3a, Prkg1, Trpc6</td>
</tr>
<tr>
<td>Cytokine cytokine interaction</td>
<td>Cd3, Ccl4, Ccl5, Ccr2, Ccr5, Ccr7, Ccr9, Csf1, Csf3r, Cxcl9, Cxcr2, Flt3, Hgf, Il1b, Il1r1, Il7r, Kdr, Kit, Mpl, Pdgfc, Pdgfrb, Pppb Tgfb2, Tgfb3, Tnfrsf18, Tnfsf10</td>
</tr>
</tbody>
</table>

Table 3: List of Regulatory pathways identified in HDAC4 osteoclasts using David analysis.

5.4 Discussion

Hdac4 is highly expressed during early stages of osteoclastogenesis (chapter 3). Mice lacking HDAC4 in osteoclasts have increase bone mass phenotype and decrease bone resorption with no effect on osteoclast differentiation (chapter 4). Moreover, analysis of gene expression of osteoclast differentiation markers showed that gene marker expression did not differ from 4WT and 4cKO osteoclasts (chapter 4). Hence, this study aimed to investigate global gene changes in 4cKO osteoclasts in vitro using RNA-sequence. In this study, we confirmed that deletion of HDAC4 in osteoclasts does not affect the expression of osteoclasts marker genes since they did not appear in our list of differentially regulated genes. We do show that deletion of HDAC4 in osteoclasts affected genes that participate in many regulatory pathways in osteoclasts which suggests that HDAC4 may be involved in the regulation of gene expression important in other phases of osteoclast life cycle. However, it should be noted that our RNA-sequence data focused only on day 2 of differentiation, maybe if we looked at different day of differentiation we might have identified more differentially expressed genes or even osteoclast marker genes that HDAC4 targets, hence, have a more global picture of HDAC4 biological role(s) and activities. We need to critically consider these non-histone targets of HDAC4, which contains many important regulatory proteins. Future studies of these targets will expand our knowledge of biological role(s) of HDAC4 in osteoclasts.
Chapter 6: Conclusions and Future Directions

6.1 Conclusions

Since their development, HDAC inhibitors (HDIs) have been accepted as promising drugs for the treatment of several diseases such as cancers (221, 245) and epilepsy (246). The majority of research into these drugs have involved broad spectrum acting HDIs which are not only non-HDAC class specific but also are non-member specific. It should be noted that broad spectrum HDIs are more effective in hematologic cancers than in solid cancers (247), suggesting that these HDIs may also be effective in other hematopoietic cell types such as osteoclasts to affect bone resorption and bone mass.

It has been reported in several human studies that long term use of broad HDI such as valproate causes decrease in bone mineral density (BMD) (157, 159). In vitro studies using broad spectrum HDIs such as trichostatin A (TSA) and sodium butyrate (NaB) have shown to promote osteoblast differentiation while suppressing osteoclast differentiation, suggesting bone protective effect (141, 248). The reason for this observation is that individual class members have been shown to play opposite roles during bone remodeling. For example, it has been observed that osteoclastogenesis is inhibited by HDAC7 (40, 140), while enhanced by HDAC3 (140). Of note, to date most of the studies of HDAC’s regulation of bone have been conducted using in vitro cultures and broad-spectrum HDIs. These approaches may distort the specific HDACs functions as a result of multitarget inhibition, pharmacologic effects, and thus making it impossible to determine the specific physiological role of each HDACs in skeletal maintenance.

The particular focus of this research involved identify the in vivo role of HDAC4 in osteoclast differentiation and function. The hypothesis that defined this body of work is Hdac4 regulates osteoclast differentiation and function through its interactions with key osteoclast proteins. This hypothesis was confirmed by the results of the study.

The skeleton is a metabolically active and regenerative organ. Dynamic and response nature of bone during bone remodeling process requires temporal changes in gene expression within the cells (osteoblasts, osteoclasts and osteocytes) required for maintenance of skeletal mass (as reviewed in (6)). Regulation of gene expression is in part accomplished by histone deacetylases which are intracellular enzymes that induce chromatin condensation and transcriptional repression. Key roles of HDACs in
chondrocytes and osteoblast differentiation and functions have been shown through in vivo and in vitro models, but in comparison little is known about the roles of HDACs in osteoclasts. Recent research observations suggest that clinically usage of broad-spectrum HDI to treat diseases such as epilepsy and cancer, may affect the bone cell population responsible for bone mass. The aim of Chapter 2 (96) was to highlight the progress that has been made in understanding how individual HDACs and their targets contribute to osteoclast biology and skeletal maintenance. Even though some aspects of the functions of individual HDACs in osteoclasts have been elucidated, a more comprehensive in vivo data are required to deduce potential therapeutic benefits. Conditionally knockout mouse models can provide knowledge about specific targets and help define the roles of each HDACs in osteoclasts.

In order to understand the roles of individual HDACs in osteoclastogenesis, the gene expression patterns and the effects of suppressing individual class II (HDAC4, 5, 6, 9 and 10) and class IV (HDAC11) throughout osteoclast formation was assessed in vitro (Chapter 3 (42)). mRNA and protein expression of HDACs in both classes were found to be differentially expressed during osteoclast differentiation. Furthermore, suppression of class II and IV HDACs resulted in accelerated osteoclast differentiation. Recent in vivo and in vitro studies have also revealed that both HDAC7 and HDAC9 suppress osteoclast differentiation (39-41, 140). These findings and our results suggest that several HDACs regulate osteoclastogenesis and further studies are needed to determine the specific roles each HDAC plays in this process. Studies using conditional knockout mouse models will be important in elucidating which HDACs are physiologically important regulators of osteoclastogenesis and bone resorption. These attempts will eventually facilitate the development of class/member-specific HDI which not only will give therapeutic benefits but also minimize bone loss side effects. In designing these HDI, it is important to keep in mind the species of the cells studied as there may be differences in human and mice models.

Overall the results obtained using a shRNA assay demonstrates the importance of HDACs in osteoclast regulation. The in vivo role of many HDACs, their intercellular mechanism (s), and targets involved in osteoclast differentiation and function are still unknown.
A key finding of the *in vitro* study described in Chapter 3 (42) was that suppression of HDAC4 accelerated osteoclast differentiation. Importantly, mRNA expression of key osteoclast genes including *c-Fos*, *Nfatc1*, *Dc-stamp* and *Cathepsin K*, which are important for differentiation were found to be upregulated. This suggested that HDC4 has a role in the regulation of osteoclast formation and activity. In regards to other aspects of bone development, HDAC4 has been shown to be critical in chondrocyte and osteoblast differentiation by interacting with RUNX2, a master transcription factor of osteoblasts and inhibiting its function (43). *In vivo*, HDAC4 null mice have been shown to have severe bone malformations due to chondrocyte hypertropy and do not survive weaning age (43). To date, there had been no reports on the *in vivo* role of HDAC4 in osteoclast formation and activity.

Based on the promising *in vitro* effects of HDC4 suppression (Chapter 3), studies were carried out to assess the *in vivo* role of HDC4 in osteoclasts (Chapter 4). It was hypothesized that mice null for HDAC4 expression in osteoclasts would have enhanced osteoclast formation, resulting in an osteopenic phenotype. Interestingly, micro CT (µCT) analysis revealed that 4cKO mice exhibited an increase bone mass phenotype (osteopetrosis). This increase bone mass was due to increased bone volume/tissue volume ratio (BV/TV) and trabecular number (Tb.N). This observation was surprisingly since it has been shown that other class IIa HDACs, HDAC7 and HDAC9 have osteopenic phenotype. This finding suggested that there is no redundancy of individual HDACs required for osteoclast differentiation and function. Additionally, 4cKO osteoclasts had decreased bone resorption both *in vivo* and *in vitro* with no effect on osteoclast differentiation. These results were not consistent with the *in vitro* shRNA findings (Chapter 3) which showed that suppression of HDAC4 increased demineralization activity (bone resorption) and suppressed osteoclast differentiation. The discrepancy in the results could be due to the fact that the HDAC4 inhibition is dependent upon the stage of development and the timing of shRNA treatment, although further investigations are needed.

In an attempt to elucidate the mechanism by which HDAC4 increases the bone-resorbing activity of osteoclasts, we demonstrated that the activity of MAPK, in particular ERK 1/2, was decreased in 4cKO osteoclasts. Many previous studies have reported the role of ERK 1/2 in osteoclasts. A recent study reported that ERK1 positively regulates
osteoclast function (232), suggesting that the decreased bone-resorbing activity of 4cKO osteoclasts is, at least in part, caused by the decreased activity of ERK 1/2. We speculated that the decreased in bone-resorbing activity observed in 4cKO osteoclasts was caused by the defects in osteoclasts adhesion and migration. As we anticipate, 4cKO pre-osteoclasts migrated at a lower number in response to M-CSF and fewer of them adhered to vitronectin during stimulation with M-CSF. This suggested that the decreased bone resorption activity in 4cKO osteoclasts may be caused by the reduction of both adhesion and migration. Prompted by this observation, we assess the role of HDAC4 during osteoclast attachment by analyzing αvβ3-dependent activation of two kinases, namely c-Src and ERK 1/2, in 4cKO osteoclasts. The finding that both of these signals were reduced in 4cKO osteoclasts suggested that HDAC4 may regulate αvβ3 integrin activity, further studies are needed to delineate this.

The study described in Chapter 4 is the first to demonstrate the in vivo role of HDAC4 in osteoclastic bone resorption. The results from this mouse model suggest that HDAC4 plays a separate and pivotal role in osteoclast function, and has the potential to be used for the development of member-specific HDAC inhibitor to directly target bone mass increase disorders which activities of osteoclasts are decreased.

Given the close similarity in shared sequence between class IIa HDACs, HDAC4, 5, and 7, global changes in gene expression in 4cKO osteoclasts was examined using RNA-sequence in Chapter 5. RNA-sequence data showed 4cKO osteoclasts had 745 differentially expressed genes which participated in various regulatory pathways including cell adhesion in osteoclasts. This was consistent with the observation that HDAC4 is involved in osteoclast adhesion in vitro (Chapter 4). Overall RNA-sequence results suggest that HDAC4 biological activities in osteoclasts is vast. In-depth investigation of the RNA-sequence data will reveal novel mechanism(s) by which HDAC4 is involved and this will give further understanding of the overall effects on osteoclasts.

In summary, this thesis confirms that HDAC4 positively regulates the bone-resorbing function of osteoclasts through its interactions with key osteoclast proteins. Using both in vitro and in vivo strategies, I have identified HDC4 as an important and physiological relevant positive regulator of osteoclastic bone resorption. The results of the studies
described in this thesis shed light onto the mechanism of HDAC4 in regulating osteoclastic bone resorption as summarized in Figure 33. ERK1/2 and c-Src were identified as key osteoclasts proteins that are affected with loss of HDAC4 expression. Further studies of targets of HDC4 are required to fully understand the mechanisms of transcriptional regulation of osteoclast function. Knowledge from the targets of HDAC4 regulation will improve our understanding of HDAC4 biological activity. Future investigations may also give insight for development of highly selective pharmacologic drugs which are HDAC4 based for treatment of diseases. This is the first in vivo study that reports critical function of HDAC4 in osteoclast bone resorption.

6.2. Future Directions

Overall the studies described in this thesis show that HDAC4 play a key role in osteoclast function. An interesting finding was that deletion of Hdac4 in osteoclasts results in an increase bone mass phenotype (osteopetrosis) in mice. This result highlighted that class IIa HDACs regulate targets in a non-redundant manner since two other class IIa members HDAC7 and HDAC9 have opposite phenotypes. Studies of the HDACs regulation of osteoclasts are complicated by the lack of more comprehensive in vivo data. This data can help with understanding of specific role(s) of each HDACs members in bone remodeling. For example, some important observations can be made from the studies described in this thesis. Both in vivo and in vitro, deletion of Hdac4 in osteoclasts affected osteoclast bone resorption. Deletion of Hdac4 did not affect osteoclast size and number in vitro but did measure larger osteoclasts in vivo.

A number of key future directions can be identified by the work presented in this thesis:

- More studies are necessary to assess HDAC4 other important substrates such as non-histone targets during osteoclastogenesis to determine if they play a role in HDAC4 biological activity and function in osteoclasts.
- Appropriate acetylation assays need to be developed/utilized to determine which c-Src protein domain is a potential HDAC4 acetylation site
- It would be interesting to determine if the deacetylase activity is dispensable to HDAC4 phenotype in osteoclasts
• Studies are needed to investigate the role of HDAC4 in adult skeleton and aging to determine if deletion of Hdac4 affect young and adult skeleton differently
• It would be interesting to determine whether RANKL signaling regulates phosphorylation of HDAC4 in osteoclasts
• It is important to determine the effects of deleting Hdac4 in osteoclasts on other bone remodeling cells such as osteoblasts and osteocytes.
• There is a need to determine HDAC4’s unique as well as redundant role(s) in bone development

The results of this thesis have demonstrated the significant function of HDAC4 in regulating osteoclast bone resorption. Even though our knowledge of HDACs roles in osteoclastogenesis and bone resorption is limited, the data presented here, and work of others, demonstrates that regulation of osteoclasts by HDACs is important for bone remodeling, hence skeletal maintenance. This level of understanding will result in the development of therapy that are class/member specific HDI. This may also open an avenue to generate therapy that are pathology-associated HDAC.
Bibliography:


71. Ishii J, Kitazawa R, Mori K, McHugh KP, Morii E, Kondo T, Kitazawa S. Lipopolysaccharide suppresses RANK gene expression in macrophages by down-


103.


129. Hong S, Derfoul A, Pereira-Mouries L, Hall DJ. A novel domain in histone deacetylase 1 and 2 mediates repression of cartilage-specific genes in human


208. Zhang Y, Li N, Caron C, Matthias G, Hess D, Khochbin S, Matthias P. HDAC-6 interacts with and deacetylates tubulin and microtubules in vivo. EMBO J.


