Mechanisms Underlying the Regulation and Functions of HDAC7 in Osteoclast Differentiation

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DEDICATIONS

To my family: my grandmother, my parents and my parents-in-law for their love and support.

To my husband, Jihoon, for everything

ABSTRACT

The purpose of this research is to characterize the regulation and functions of Histone Deacetylase 7 (HDAC7) in osteoclast differentiation. Histone Deacetylases (HDACs) are negative regulators of transcription.[1] Endochondral bone formation including maturation of chondrocytes and osteoblasts is regulated by HDACs.[2] It has been shown that the suppression of HDAC7 enhanced osteoclast formation while the overexpression of myc-HDAC7 inhibits osteoclast formation.[3] Hence, it has been suggested that the stimulation of HDAC7 might be a unique therapeutic strategy to reduce osteoclastic bone loss.[3] However, the mechanism and the molecular pathways regulating how HDAC7 inhibits osteoclast formations have not been examined. In this study, we hypothesized that the deacetylase catalytic activity of HDAC7 is necessary for suppression of osteoclast formation. It is also hypothesized that the subcellular localization of HDAC7 in nucleus facilitated by the phosphorylation of serine residues at N-terminal with Receptor activator of nuclear factor-kappaB ligand (RANKL) stimulation is the one of the mechanism that HDAC7 controls osteoclast differentiation. However, from this study, it is found that HDAC7 deacetylase activity is dispensable for HDAC7-mediated inhibition of osteoclastogenesis. It is also concluded that the presence of HDAC7, not necessarily the localization of HDAC7 in the nucleus, is necessary to repress the osteoclastogenesis.

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List of Abbreviations

ANOVA	One-way analysis of variance
Atp6v0d2	d2 isoform of vacuolar ATPase Vo domain
Bcl6	B cell lymphoma 6
c-Fms	colony-stimulating factor-1 receptor
CFU-GM	Granulocyte-macrophage colony-forming unit
ChIP	Chromatin immunoprecipitation
Ct	Threshold cycle
CTR	Calcitonin receptor
CTSK	Cathepsin K
CTX-1	Type I collagen fragments
DC-STAMP	Dendritic cell-specific transmembrane protein
dsDNA	Double strand DNA
Gapdh	Glyceraldehyde 3-phosphate dehydrogenase
GEFs	Guanine nucleotide exchange factors
HATs	Histone acetyltransferases
HDAC	Histone deacetylase
HDAC7	Histone deacetylase 7
IP	Immunoprecipitation
IRF-8	Interferon regulatory factor-8
M-CSF	Macrophage colony stimulating factor
MafB	v-mar musculoaponeurotic fibrosarcoma oncogene family member protein B
MITF	Microphthalmia transcription factor
MMPs	Matrix metalloproteinases
NaB	Sodium butyrate
NES	Nuclear export sequence
NFAT-c1	Nuclear factor of activated T-cells
NLS	Nuclear localization sequence
OPG	Osteoprotegerin
OSCAR	Osteoclast-associated receptor
PTH	Parathyroid hormone
RANK	Receptor activator of nuclear factor-kappaB
RNAKL	RANK ligand
SAHA	Suberoylanilide hydroxamic acid
TGFβ	Transforming growth factor-beta
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TRAIL	Tumor necrosis factor-Related Apoptosis-Inducing Ligand
TRAP	Tartrate-Resistant Acid Phosphatase
TSA	Trichostatin A

1. INTRODUCTION

1.1 Background and Significance

Bone is a dynamic organ that undergoes remodeling through resorption by osteoclasts and synthesis by osteoblasts. Pathologic bone loss, such as osteoporosis, periodontal disease, idiopathic condylar resorption of the temporomandibular joint, rheumatoid arthritis and Paget's disease, are resulted from the excessive bone degradation by osteoclasts relative to the formation by osteoblasts. A better understanding of molecular mechanisms that control osteoclast differentiation and activation will help us with the development of improved therapies to prevent pathologic bone loss.

Osteoclast differentiation requires stringent control of gene activation and suppression in response to physiological cues. Histone acetylations and deacetylation play essential roles in modifying chromatin structures and regulate gene expression. Histone deacetylation induces a closed-chromatin configuration and transcriptional repression.[4,5] In addition, histone deacetylases (HDACs) also influence the activity of a broad array of proteins by removing post-translational acetylation modifications.[4,5] In recent years, a number of HDAC inhibitors have emerged as potential treatments for cancers, asthma, HIV and central nervous system disorders.[6-9] Non-specific inhibition of histone deacetylase functions in osteoclast has been reported to inhibit receptor activator of nuclear factor-kappa B Ligand (RANKL)-mediated osteoclast differentiation by stimulating their apoptosis.[10] However, it has been shown that the suppression of HDAC7 unexpectedly enhanced *in vitro* differentiation of osteoclasts using bone marrow cultures, while the overexpression of HDAC7 inhibited their formation.[3] Hence, HDAC7 has been identified as a novel regulator of osteoclast formation that has potentially significant implications for the development of improved therapeutic interventions for the prevention or treatment of excessive bone-loss disease.

Even though it is suggested that the stimulation of HDAC7 might be a unique therapeutic strategy to reduce osteoclastic bone loss, the mechanism and the molecular pathways regulating how HDAC7 inhibits osteoclast formations have not been examined. The study outlined in this paper will address questions about the role of HDAC7 in osteoclastogenesis and its regulation. By doing this study, we hope to provide new light into understanding of molecular pathways regulating osteoclastogenesis and to potentially lead to new therapies with ultimate goal of improving clinical outcomes for a number of significant bone disease.

2. Review of the Literature

2.1 Bone

Bone is a living tissue that is involved in the vital functions in human. It is a rigid structure necessary to support the body and to protect organs beneath them. It also serves as attachment sites for muscles, tendons and ligaments for movement and also serves as a storage site for the two most abundant minerals in the body, calcium and phosphate ions, in order to regulate mineral balance. Finally, bone is the site of hematopoiesis producing blood cells. Bone is a rigid yet dynamic organ that undergoes a remodeling. It involves resorption and synthesis keeping the bone healthy and repairing damages. The inorganic phase of the bone is composed of hydroxyapatite crystals and the organic part of the bone is composed of 90% type I collagen.[11]

2.2 Bone Cells

Osteoclasts, osteoblasts and osteocytes are the main cells involved in bone remodeling. Osteoclasts are derivatives of the myeloid stem cell lineage. Single cells of the monocyte-macrophage lineage fuse to become multinucleated osteoclasts containing four to twenty nuclei once fully differentiated.[12] Their main function is to resorb bone and they are found in bone resorption sites.

Osteoblasts and osteocytes are derivatives of the mesenchymal stem cell lineage. Osteoblasts' main function is to form bone. They are found on bone forming surfaces and secrete bone matrix proteins. Both osteoblasts and osteocytes regulate osteoclastic activity.

Physiologic bone resorption involves the coupling of both osteoblasts/osteocytes and osteoclasts. Pathologic bone loss due to an increase in the rate of bone degradation by osteoclasts relative to formation by osteoblasts can result in gross perturbations in skeletal structure and function as observed in osteoporosis or cancer-associated bone disease.[13]

2.3 Life Cycle of Osteoclasts

Osteoclasts are tissue-specific macrophage polykaryons. They are hematopoietic in origin and derived from granulocyte-macrophage colony-forming unit (CFU-GM). This granulocyte-macrophage progenitor cells are induced by macrophage-colony stimulating factor (M-CSF) and RANKL to form immature osteoclasts. These immature osteoclasts are in turn fused to become multinuclear osteoclasts that are found on the surface of bone resorption sites. They are then activated to become bone-resorbing osteoclasts. Osteoclasts resorb bone by a process of osteoclast attachment, polarization, formation of sealing zone at the ruffled border and resorption.[14] Factors such as transforming growth factor-beta (TGFβ) or bisphosphonates then induce osteoclast apoptosis (***Figure 1***).[15]

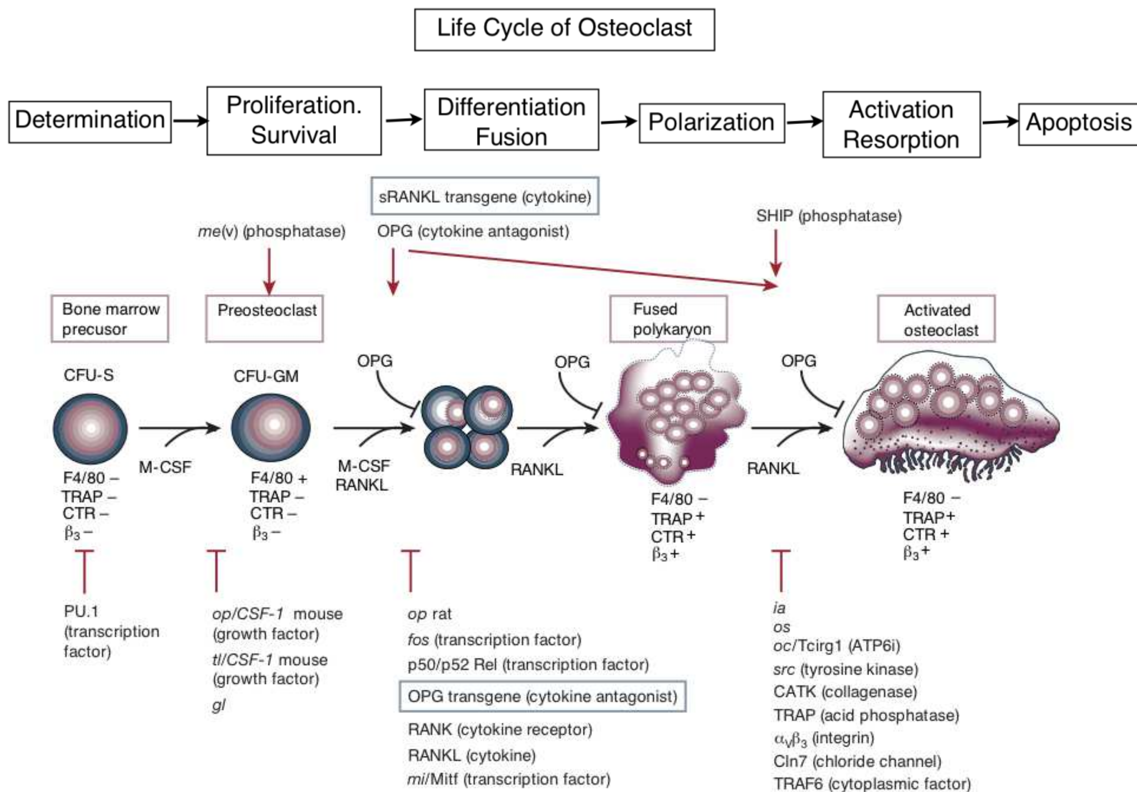


Figure 1. Transcription and signaling factors involved in osteoclastogenesis (Boyle et al., 2003)

2.4 Factors Regulating Osteoclast Differentiation

2.4.1 Bone Microenvironment and Macrophage Colony-Stimulating Factor (M-CSF)

The bone microenvironment plays an important role in osteoclast formation. It has been shown that M-CSF binds to its receptor, colony-stimulating factor-1 receptor (c-Fms), which allows osteoclast precursors to survive and proliferate. Stromal cells or osteoblasts produce M-CSF, which induces osteoclast formation from progenitor cells. On the other hand, marrow stromal cells or osteoblasts from op/op mice that lack M-CSF do not support osteoclast differentiation.[16] When purified recombinant human M-CSF was exogenously re-introduced to these op/op mice, 50-fold larger number of macrophages was detected.[16] These results suggest that both contact with stromal cells and M-CSF are requisite for osteoclast differentiation under physiological conditions.[16]

2.4.2 Receptor Activator of Nuclear Factor-kappaB ligand (RANKL) and Osteoprotegerin (OPG)

Current understanding of osteoclast differentiation and activation has come from the analysis of a family of biologically related tumor necrosis factor receptor (TNFR) called Receptor activator of nuclear factor-kappa B (RANK) and TNF-like proteins called RANK ligand (RANKL) and osteoprotegerin (OPG).

RANKL is a transmembrane protein that belongs to the tumor necrosis factor (TNF) superfamily. Certain stimulus to marrow stromal cells induces increased levels of RANKL on their cell surface. RANKL then binds the RANK receptor on osteoclast precursors. RANKL activates signals through NF-KappaB and other kinases, which in turn promotes osteoclast differentiation and activation. RANK signaling pathway hence induces bone resorption and skeletal remodeling.[17]

On the other hand, OPG, also produced by marrow stromal cells, acts as a decoy receptor preventing RANKL to bind RANK receptor on osteoclasts and prevents osteoclast precursor cells from surviving and maturing. It blocks the fusion/differentiation stage of osteoclast differentiation, rather than the proliferative phase. OPG also binds to Tumor necrosis factor-Related Apoptosis-Inducing Ligand (TRAIL).[18]

Thus, RANKL and OPG are important regulators produced by the marrow microenvironment, and the ratio of RANKL to OPG regulates osteoclast formation and osteoclast activity (***Figure 2***).[19]

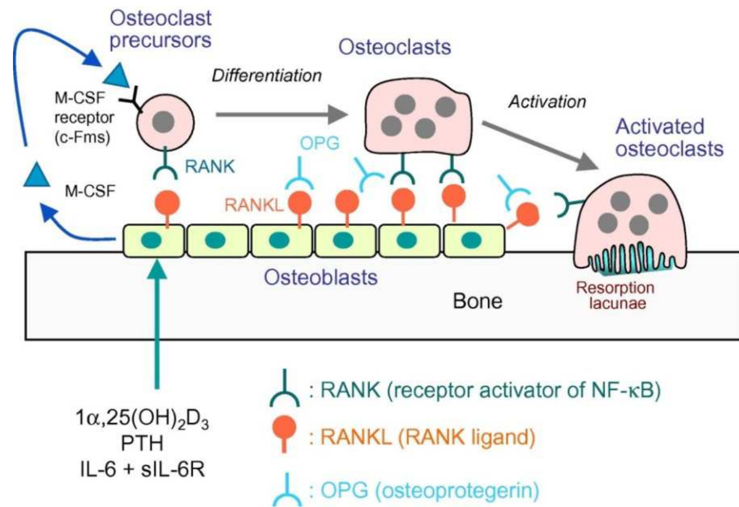


Figure 2. Interaction between RANKL/RANK/OPG (Suda et al., 2012)
RANKL and OPG are expressed by osteoblasts and bone marrow stromal cells. RANKL binds to the RANK receptor cells of the osteoclast lineage to induce differentiation and activation of osteoclasts. OPG is the decoy receptor that prevents RANKL from binding to its RANK receptor.

2.4.3 Systemic hormones and other local factors for bone remodeling

Systemic hormones and other local factors determine the level of RANKL and OPG presented by osteoblasts. The ratio between RANKL and OPG is indirectly controlled by systemic hormones, such as parathyroid hormone (PTH), Vitamin D (1,25-Dihydroxyvitamin D) and calcitonin. [20] PTH has been shown to stimulate osteoclastic bone resorption by increasing acid production by osteoclasts, whereas calcitonin decreases acid secretion.[20]

2.5 Mechanisms of Osteoclastic Bone Resorption

Osteoclasts attach to the bone, polarize, form a sealing zone at the ruffled border, resorb, and eventually undergo apoptosis. The ruffled border is a "villous organelle" found in osteoclasts.[14] When the sealing zone is formed, podosomes localizes to form an actin ring, which separates the extracellular space from the acidic resorptive microenvironment.[15] Osteoclasts resorb bone by secreting proteases, such as cathepsin K (a cysteine proteinase) and matrix metalloproteinases (MMPs) (***Figure 3***).[15] Osteoclasts also secrete protons and acid that activates tartrate-resistant acid phosphatase (TRAP) and cathepsin K which degrades bone mineral and collagen matrices. An acidic environment as low as pH 3 to 4, produced by hydrochloric acid secretion at the ruffled border, causes the hydroxyapatite in bone to be resorbed extracellularly.[11]

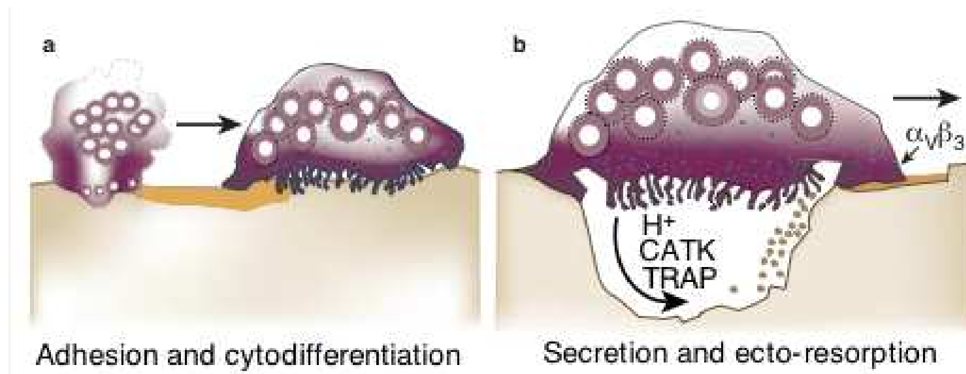


Figure 3. Activation of bone resorption (Boyle et al., 2003)

a. *Multinulceated polykaryons are recruited by the action of CSF-1 and RANKL, which then adhere to bone and undergo cytodifferentiation into a mature osteoclast*

b. *RANKL stimulates osteoclast activation by inducing secretion of protons and lytic enzymes into a sealed resorption vacuole formed between the basal surface of the osteoclast and the bone surface. Acidification of this compartment by secretion of protons leads to the activation of TRAP and CATK, which are the two main enzymes responsible for the degradation of bone mineral and collagen matrices.*

2.6 Genes Involved in Osteoclast Differentiation and Fusion

At least 24 genes or loci have been shown to positively and negatively regulate osteoclastogenesis and osteoclast activation (***Figure 1***).[15]

2.6.1 *c-Fos*

c-Fos is a transcription factor that act as a key mediator of the lineage commitment between osteoclasts and dendritic cells, which are also derived from monocyte/macrophage precursor cells. Mice deficient in *c-Fos* display osteopetrotic phenotype due to an osteoclast differentiation defect, while the number of the macrophages increases.[21]

2.6.2 *Nuclear Factor of Activated T Cells Cytoplasmic 1 (NFAT-c1)*

Nuclear Factor of Activated T cells Cytoplasmic 1 (NFAT-c1) is a transcription factor that acts as the master regulator of osteoclast differentiation and activation.[17] *NFAT-c1*$^{-/-}$ stem cells fail to develop into osteoclasts *in vitro*, and ectopic NFATc1 expression shows precursor cell differentiation in the absence of RANKL.[14] As a master regulator, NFAT-c1 regulates various genes, such as *osteoclast-associated receptor* (OSCAR), *calcitonin receptor* (CTR), *Acp5* (TRAP), and *cathepsin K* (CTSK).[17] NFATc1 also regulates other transcription factors, such as AP-1, PU.1, and MITF in cooperation with a number of osteoclast specific genes.[17] Recent studies have shown that *dendritic cell-specific transmembrane protein* (DC-STAMP) and the *d2 isoform of vacuolar ATPase Vo domain* (Atp6v0d2) are upregulated under NFATc1 activity.[22]

RANKL specifically and potently induces NFAT-c1. The induction of NFATc1 is dependent on both the TRAF6-NF-kB and c-Fos pathways. The activation of NFATc1 is also mediated by a specific phosphatase, calcineurin, which is activated by calcium-calmodulin signaling. The *Nfatc1* promoter contains NFAT binding sites. Hence, NFAT-c1 autoregulates its own promoter during osteoclastogenesis and this results in the robust induction of NFAT-c1.[23]

NFAT-c1 activity is negatively regulated by other group of transcription factors such as interferon regulatory factor-8 (IRF-8), B cell lymphoma 6 (Bcl6) and the v-mar musculoaponeurotic fibrosarcoma oncogene family member protein B (MafB).[24-26] Thus, NFAT-c1 controls the cell fate in the osteoclast lineage by inducing the repression of negative regulators, as well as through its effect on positive regulators.

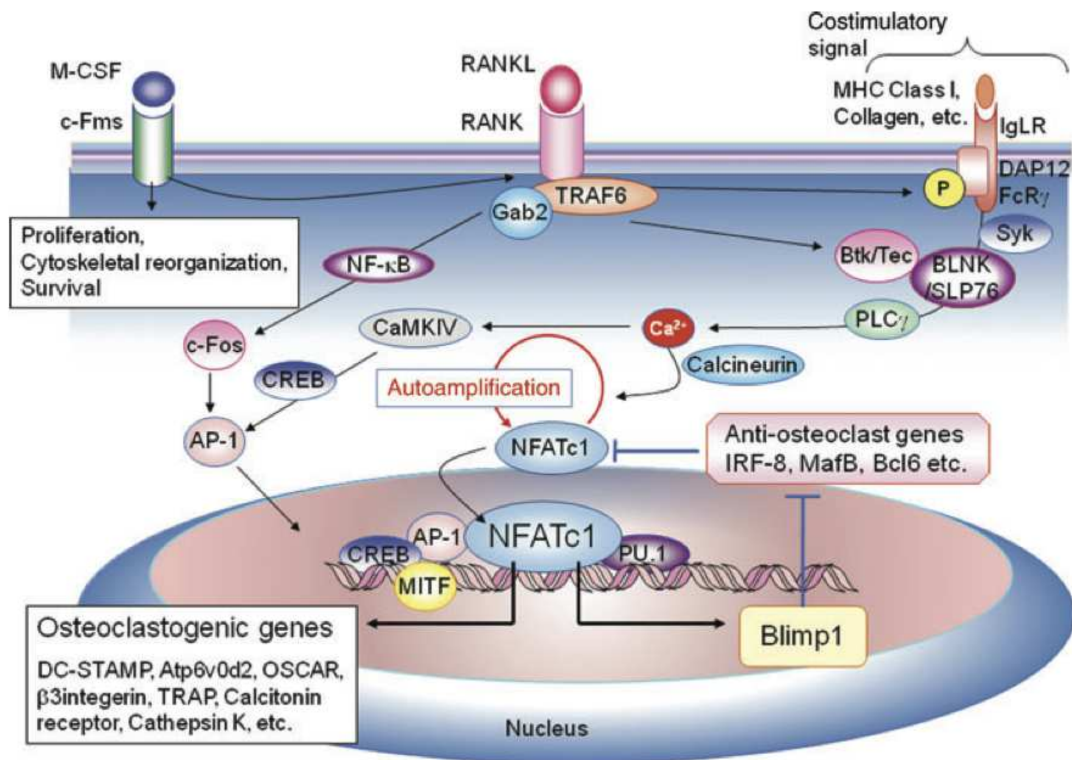


Figure 4. Signal transduction in osteoclast differentiation. (Takayanagi et al., 2012)
RANKL binding to its receptor RANK results in the recruitment of TRAF6, which activates NF-kB and JNK-c-Jun. RANKL also stimulates the induction of a component of AP-1, c-Fos. AP-1 contributes to the robust induction of NFATc1, which is based on an autoamplifying mechanism that is effected through persistent calcium signal-mediated activation of NFATc1 (NFATc1 binds to NFAT-binding sites on its own promoter, constituting a positive feedback loop). In the nucleus, NFATc1 cooperates with other transcription factors, such as AP-1, PU.1, CREB and MITF to induce various osteoclast-specific genes. On the other hand, NFATc1 activity is negatively regulated by other transcription factors, such as IRF-8, Bcl6 and MafB during osteoclastogenesis. Thus, NFATc1 choreographs the determination of cell fate in the osteoclast lineage by inducing the repression of negative regulators as well as through its effect on positive regulators.

2.6.3 *Dendritic Cell-specific Transmembrane Protein (DC-STAMP)*

Dendritic cell-specific transmembrane protein (DC-STAMP) is a gene that aids in the fusion of mononuclear osteoclast precursors into multinuclear osteoclasts. DC-STAMP contains seven transmembrane regions expressed on the surface of osteoclast cells.[27-29] RANKL induces activation of osteoclastic precursor cells through NFAT-c1, and this activates fusion genes including *DC-STAMP* and *Atp6v0d2*.[27-29] DC-STAMP-expressing osteoclast becomes the master-fusing cell, which takes the lead in "cellocytosing" another cell, and fuses with a DC-STAMP-negative follower cell. The ligand for DC-STAMP may be membrane bound or soluble.[14] Mice that are *DC-STAMP*$^{-/-}$ express an osteopetrotic phenotype and contain no multinuclear osteoclasts.[14,28,29] Resorption occurs in *DC-STAMP*$^{-/-}$ osteoclasts, but it is less efficient, suggesting that unfused mononuclear osteoclasts are not as functional as multinuclear fused osteoclasts.[30] (***Figure 5***)

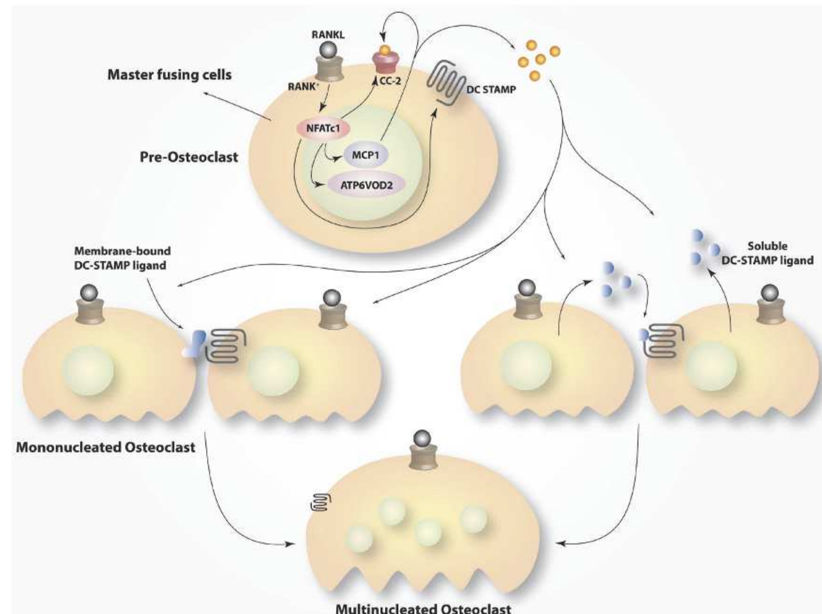


Figure 5. The fusion of mononuclear pre-osteoclasts. (Yavropoulou et al., 2008)
RANKL induces the expression of DC-STAMP and Atp6vod2. The DC-STAMP-expressing osteoclast becomes the master-fusing cell, which can fuse with a DC-STAMP-negative follower cell. The ligand for DC-STAMP may be membrane-bound or soluble.

2.6.4 *Beta 3 integrin (β3 integrin)*

A ring-like actin structure of osteoclasts forms the sealing zone, which isolates the resorptive microenvironment from the general extracellular space. Then, a villous organelle unique to the resorbing osteoclasts, known as the ruffled membrane, is formed within this sealing zone. Failure of polarization and organization of cytoskeletal structure within osteoclasts results in osteoclast dysfunction and varying degrees of osteopetrosis.[14] Alpha-v-beta-3 (αvβ3 integrin) is the principal osteoclast integrin, which mediates recognition of mineralized matrix.[14] Upon occupation of αvβ3 integrin, c-SRC kinase binds directly to the terminal three amino acids of the β3 integrin subunit.[14] Activation of the receptor leads to phosphorylation of the tyrosins within the ITAM, SRC-family kinases, which in turn recruits and activates Syk kinase.[14] Activated ITAM-bound Syk targets the Vav family of guanine nucleotide exchange factors (GEFs), mainly Vav3 which leads to induction of Rho GTPase, Rac. This cascade leads to organization in the osteoclast cytoskeleton.[14] (***Figure 6***)

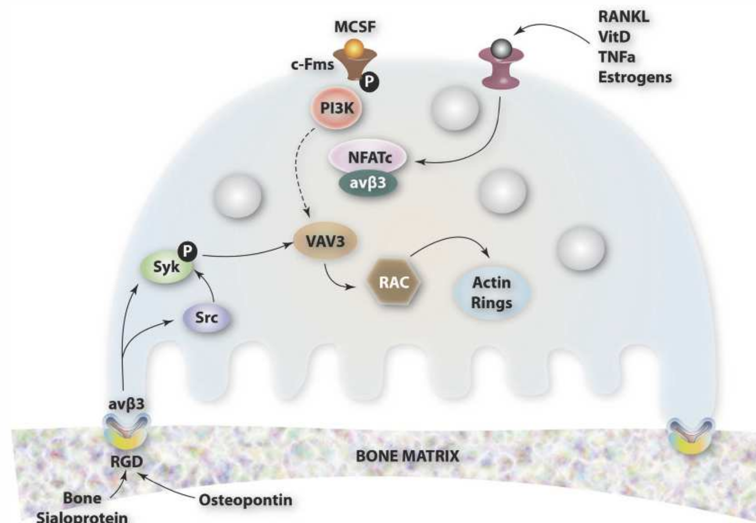


Figure 6. Activation of multinuclear immature osteoclast. (Yavropoulou et al., 2008)
Upon occupation of the αvβ3 integrin, Syk and c-Src are recruited independently to its cytoplasmic domain. C-Src phosphorylates and activates Syk which in turn induces VaV3. Both occupancy of M-CSF and αvβ3 integrin collaborate to phosphorylate VaV3 which then activates Rac leading to cytoskeletal organization. NFAT binds to the promoter of αvβ3 integrin, and increases its expression.

2.6.5 *Cathepsin K (CTSK)*

Cathepsin K (CTSK) is a proteinase that degrades and removes Type I Collagen. CTSK is expressed in fully differentiated osteoclasts. Normally, collagen is broken down to type I collagen fragments (CTX-1); however, in *cathepsin K* knockout mice, these collagen fragments are not present in cell cultures of osteoclastic cells. In addition, a defective CTSK in human results in a pycnodysostotic phenotype, which is characterized by a lack of normal collagen breakdown and sclerotic bone that is prone to fracture.[11]

2.6.6 *Tartrate-Resistant Acid Phosphatase (TRAP)*

Tartrate-Resistant Acid Phosphatase (TRAP) degrades bone mineral and collagen matrices. It is widely used as an enzymatic marker of mature osteoclasts and is expressed in multiple tissues, most notably in bone, liver, spleen, thymus, and colon. *Acp5* is the gene encoding TRAP protein and *Acp5* knockout mice exhibit an osteopetrotic phenotype, with fatter, shorter limbs and axial skeletons as well as altered epiphyseal growth plates.[31] These mice also demonstrate inefficient immune and inflammatory responses as TRAP proteins are prevalent in immune cells.[31]

2.7 Histone Deacetylase (HDAC)

Histone acetylations and deacetylations play essential roles in modifying chromatin structure and regulate gene expression. Histone deacetylases (HDACs) are a group of enzymes responsible for broadly promoting transcriptional repression by removing acetyl groups from histone core proteins at target gene promoters.[32] Histone deacetylation induces a closed-chromatin configuration and transcriptional repression (***Figure 7***).[33] HDACs also influence the activity of a broad array of proteins by removing post-translational acetylation modifications.[4,5]

Eighteen distinct human HDACs are identified and grouped into three classes based on their primary homology to three *saccharomyces cerevisiae* HDACs. Class I HDACs (HDAC1, -2, -3, -8 and -11) are predominately nuclear proteins expressed in most tissues and cell lines. Class II HDACs are further subdivided into two subclasses, IIa (HDAC4, -5, -7 and -9) and IIb (HDAC6 and HDAC10), based on sequence homology and domain organization. Class III HDACs show no homology to class I and II proteins.[1]

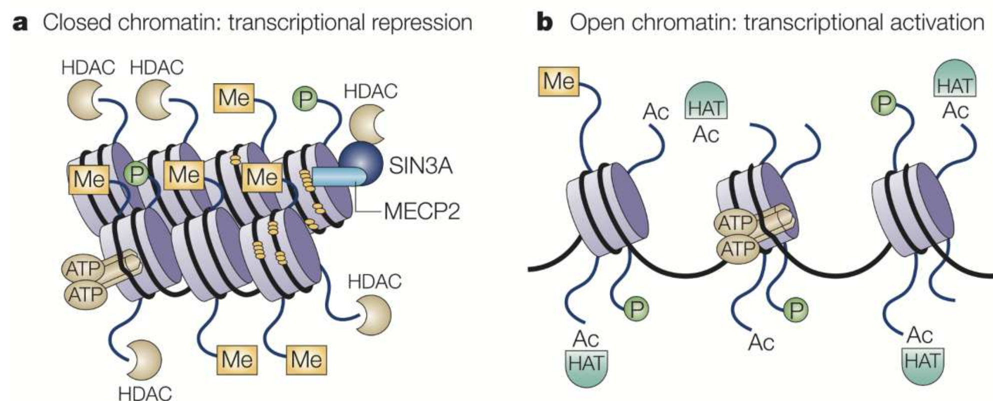


Figure 7. Histone deacetylases and histone acetyltransferases **(Johnston et al., 2002)**
Nucleosomes consist of DNA (black line) wrapped around histone octomers (purple). Nucleosome structure can be regulated by the opposing actions of histone acetyltransferases (HATs) and histone deacetylases (HDACs). (a) DNA methylation and histone deacetylation induce a closed-chromatin configuration and transcriptional repression. (b) Histone acetylation and demethylation of DNA relaxes chromatin, and allows transcriptional activation.

2.7.1 Class IIa HDACs

Class IIa HDACs contain similar overall domain structures, possessing an amino-terminal domain of roughly 450–500 amino acids including a nuclear localization sequence (NLS) and sequence that mediates protein–protein interactions.[1] A similarly sized carboxy-terminal domain comprises of the deacetylase catalytic domain and a nuclear export sequence (NES) (***Figure 8***).[1] Class IIa HDACs has three unique properties as listed below.

2.7.1.a *Tissue-specific expression*

Unlike class I HDACs, which are ubiquitously expressed, the class IIa HDACs exhibit tissue restricted expression patterns. This restricted expression pattern suggests that Class II HDACs are involved in a cellular differentiation and developmental process.[34] Three of the class IIA HDACs, HDAC4, -5 and -9, show highest expression in heart, skeletal muscle and brain.[1] It has also been found that HDAC7 is most highly expressed in CD4/CD8 double-positive thymocytes.[1] Dr. Mansky's lab identified the presence of HDAC7 in osteoclasts.

2.7.1.b Recruitment of co-repressor proteins

Class IIa HDACs contain functional deacetylase catalytic domains. However, they have not been shown to function by directly deacetylating histones. Instead, Class IIa HDACs recruit repressor complexes that contain Class I HDACs and co-repressor proteins (***Figure 8***).[1] The amino termini of class IIa HDACs have also been shown to possess deacetylation-independent repression activities including recruitment of CtBP and HP1 co-repressor proteins, directly inhibiting the ability of transcription factors to bind DNA or sequestering transcription factors into inactive subnuclear bodies.[1]

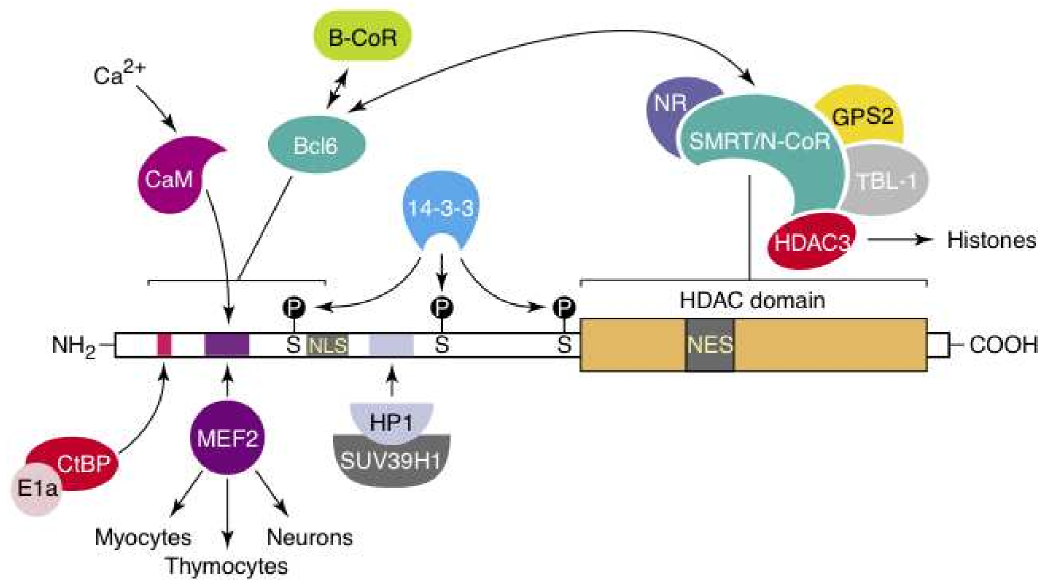


Figure 8. Interaction partners of class IIa histone deacetylases (HDACs). (Verdin et al., 2003)

Class IIa HDACs interact with several partners through distinct domains. The N-terminal domain is used primarily as a targeting domain for distinct promoters by the MEF2 transcription factors. Interaction of class IIa HDACs with HP1, CtBP and the SMRT/N-CoR and B-CoR co-repressor complexes mediates the transcriptional repressive activities of class II HDACs.

2.7.1.c Nucleus-cytoplasm subcellular localization

Another distinct feature of class IIa HDACs is that they shuttle between nucleus and cytoplasm by binding to 14-3-3 proteins.[1] Phosphorylation-dependent binding of 14-3-3 proteins to the N-termini of class IIa HDACs masks the nuclear localization signal and prevents nuclear import.[1] This binding is dependent on the phosphorylation of two or three conserved N-terminal serine residues in class IIa HDACs and mediates their cytoplasmic sequestration (***Figure 9***).[1] Mutation of these sites prevents the export of class IIa HDACs from the nucleus to the cytoplasm.[35]

It is thought that 14-3-3 proteins modify the subcellular localization of targets by interfering with nuclear import and export signals.[1] A nuclear localization signal contains an arginine/lysine-rich motif.[1] This nuclear localization signal has been mapped in HDAC4 and -5, and appears to be conserved in HDAC7 and MITR/HDAC9.[1] Binding of 14-3-3 proteins to the

N-termini of class II HDACs, which is triggered by phosphorylation, masks the nuclear localization signal and prevents nuclear import.[1] It was suggested that phosphorylation of the N-terminus of class IIa HDACs and/or recruitment of 14-3-3 proteins induces a long distance conformational change that unmasks a latent nuclear export signal in their C-terminus.[1]

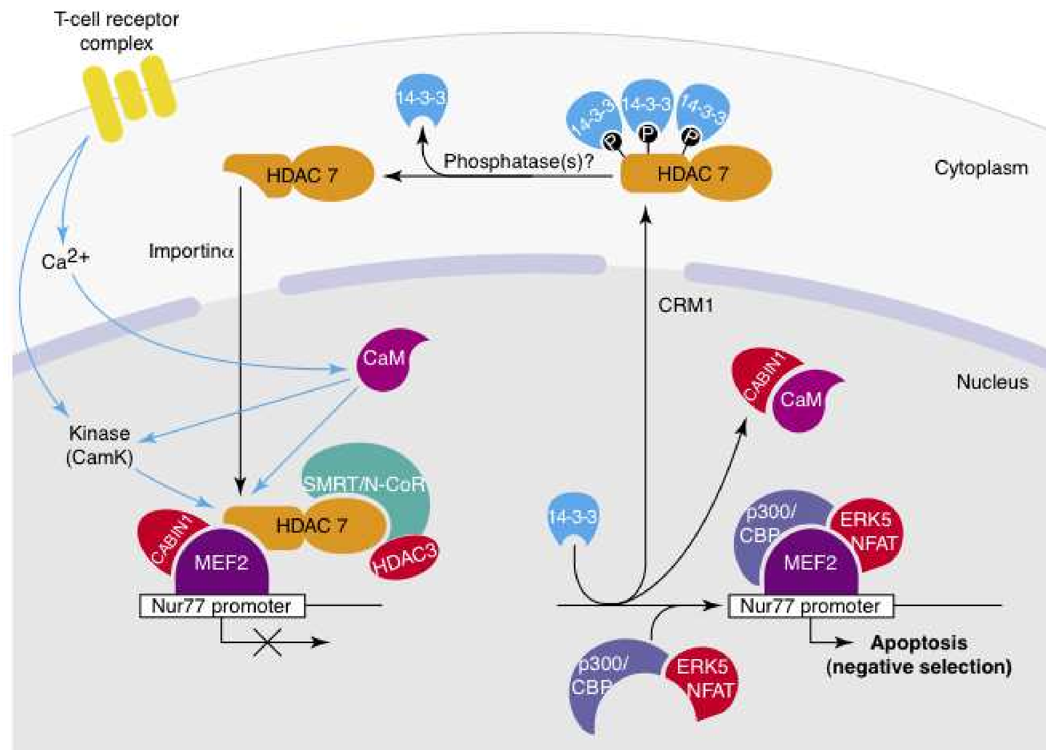


Figure 9. Regulation of Nur77 promoter activity in developing thymic T cells by class II histone deacetylases (HDACs). (Verdin et al., 2003)
Class II HDACs, primarily HDAC7 in the thymus, repress the latent activating potential of MEF2 transcription factors, both by preventing the binding of coactivators and by direct enzymatic activities associated with class IIa HDACs. Phosphorylated class IIa HDACs and mediate CRM1-dependent nuclear export, allowing the sustained expression of MEF2 target genes. For activation of transcription, coactivators (p300/CBP) and activators (NFAT, ERK5) are recruited to the promoter. Unknown cytoplasmic phosphatases deposphorylate class II HDACs, leading to their release from 14-3-3 proteins.

2.7.2 HDAC Inhibitors and osteoclasts

In recent years, a number of HDAC inhibitors have emerged as potential treatments for cancers, asthma, HIV and central nervous system disorders.[6-9] Non-specific inhibition of histone deacetylase function in osteoclasts has been reported to inhibit RANKL-mediated osteoclasts

differentiation.[10] These findings indicate that HDACs' activity is required for osteoclastogenesis. General HDAC inhibitors such as trichostatin A (TSA) and sodium butyrate (NaB) have been shown to inhibit osteoclast differentiation by affecting NF-κB and p38 MAP kinase signaling.[10] Another general HDAC inhibitor suberoylanilide hydroxamic acid (SAHA), currently under clinical trials for its ability to suppress tumor cell proliferation, was shown to inhibit osteoclast differentiation by enhancing apoptosis of osteoclasts.[36] However, it has been shown that suppression of HDAC7 unexpectedly enhances *in vitro* differentiation of osteoclasts using bone marrow cultures, while overexpression of HDAC7 inhibited their formation.[3] Hence, HDAC7 has been identified as a novel regulator of osteoclast formation, opposite to the function of other HDACs. HDAC7 has potentially significant implications for the development of therapeutic interventions for the prevention or treatment of excessive bone-loss disease.

2.8 Microphthalmia-Associated Transcription Factor (MITF)

Microphthalmia transcription factor (MITF) is a basic helix-loop-helix transcription factor that is necessary for osteoclast differentiation.[37] It has been shown that MITF acts synergistically with PU.1 and NFAT-c1 to activate *Apc5*, *Cathepsin K* and *OSCAR* promoters during osteoclast differentiation.[38,39] Mice that are homozygous for the $MITF^{mi}$ mutation show an osteopetrotic phenotype with lack of osteoclast differentiation.[37]

M-CSF signaling alone regulates MITF nuclear localization and recruitment of MITF to target promoters.[39] However, even with the presence of MITF in nucleus with stimulation with M-CSF alone, it does not activate gene expression. Rather, co-stimulation with M-CSF and RANKL is required to induce expression of osteoclast differentiation gene.[38] Dr. Mansky's lab has hypothesized that there are cellular inhibitors that regulate MITF activation of osteoclast gene expression during M-CSF signaling which may be removed with RANKL stimulation (***Figure 10 C***).[12] It has also shown that HDAC7 and MITF interact in RAW 264.7 cells and primary

osteoclasts during M-CSF signaling, and this inhibits its transcriptional activity.[3] Dr. Mansky's lab also found that the interaction between HDAC7 and MITF was reduced by stimulation with RANKL (***Figure 10 A and B***).[12] Hence, it was hypothesized that HDAC7 may be a novel MITF repressor which is localized between nucleus and cytoplasm by RANKL stimulation (***Figure 10 C***).

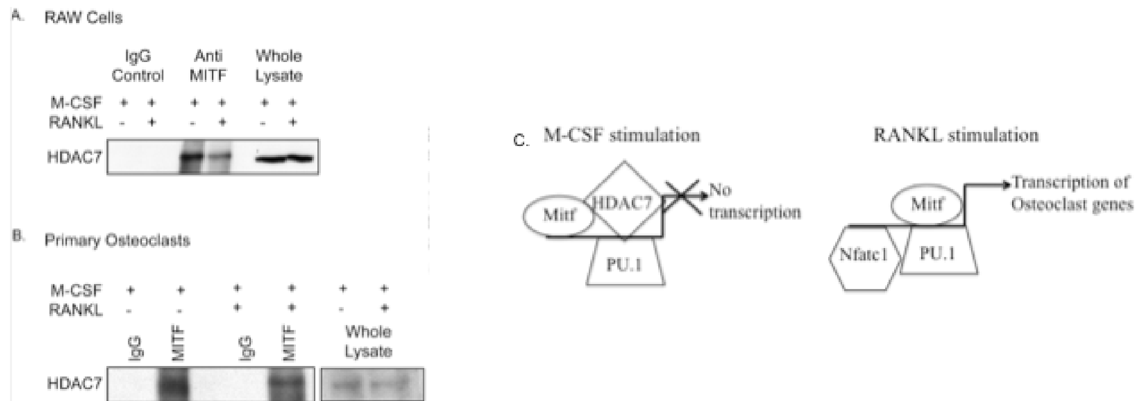


Figure 10. Interaction between HDAC7 and MITF (Jensen et al., 2011)
(A) Western blots of MITF immunoprecipitates (IP) from RAW 264.7 c4 cells and (B) primary mouse osteoclasts immunoblotted against HDAC7. (C) Possible model of interaction between HDAC7 and MITF. With M-CSF stimulation of osteoclasts, MITF and HDAC7 interact on osteoclast promoters and prevent MITF from activating genes necessary for osteoclast differentiation. With RANKL stimulation of osteoclast precursors, MITF and HDAC7 can no longer interact and MITF in complex with PU.1 and Nfatc1 activate genes necessary for osteoclast differentiation.

3. HYPOTHESIS

This research will address following hypothesis

1. The deacetylase activity of HDAC7 is necessary to inhibit osteoclast differentiation
2. HDAC7 cytoplasmic localization is necessary to promote osteoclast differentiation
3. HDAC7 nuclear localization is necessary to repress osteoclast differentiation

4. SPECIFIC AIMS

1. To determine the effect of a deactivating point mutant in the deacetylase domain in HDAC7 in osteoclastogenesis.
2. To determine the effect of deletion of nuclear localizing signal sequence in HDAC7 on osteoclastogenesis
3. To determine the effect of mutation of four serine residues to alanine in amino-terminus of HDAC7 on osteoclastogenesis.

5. EXPERIMENTAL DESIGN/MATERIAL AND METHODS

5.1 <u>Generation of Knockout Mice</u>

Since a global knockout of HDAC7 is embryonic lethal with cardiovascular defects,[40] a mouse model that is null for HDAC7 expression specifically in osteoclasts were made by using CRE-mediated recombination to specifically delete HDAC7 in osteoclasts. HDAC7$^{flox/flox}$ mice were originally created by Dr. Eric Olson (University of Texas Southwestern Medical Center) as described in Chang et al.[40] Mice were crossed with the LysM-Cre expressing mouse (Jackson Labs). Expression of the CRE recombinase mediates deletion of exons 2 through 10 including the entire amino terminus of the HDAC7 gene, resulting in null expression of HDAC7.[40] Mice were genotyped for LysM-Cre and flox-HDAC7 using qPCR. Mice that have the genotype

$HDAC7^{+/+}$;LysM-Cre will be refer to as WT and mice with genotype $HDAC7^{flox/flox}$;LysM-Cre will be refer to as KO in this paper. Use and care of the mice in this study was approved by the University of Minnesota Institutional Animal Care and Use Committee.

5.2 Primary Osteoclast Cell Culture and Lentiviral Infection of Osteoclasts

Four week old HDAC7 $^{+/+}$;LysMCre (WT) and $HDAC7^{fl/fl}$;LysMCre (KO) mice were euthanized and osteoclast progenitor cells were isolated by flushing bone marrow with serum free media from femurs of WT and KO mice. The cells were cultured in alpha-MEM media for three days in the presence of 50 ng/ml M-CSF on non-tissue culture dishes. M-CSF selects for osteoclasts by promoting survival and proliferation of osteoclast precursors. Cells were counted and plated on either 12 well plate for TRAP staining or 6 well plates for RNA extraction for RT-PCR. The adherent cell population, containing the primary osteoclasts, was infected with lentiviral vectors (Open Biosystems) that expressing EGFP (Control), wild type HDAC7 (HDAC7), deletion of NLS sequence ($HDAC7^{\Delta NLS}$), mutation of four serine residue (Ser-155, 181, 321 and 449) to alanine ($HDAC7^{4A}$)[41] and deletion of function of deacetylase catalytic domain by point mutation in carboxy-terminal domain ($HDAC7^{\Delta DA}$).[42] Following the infection, primary osteoclast cultures were stimulated with M-CSF (30ng/ml) and RANKL (60ng/ml) for three days at 37’C.

5.3 TRAP staining

Cells were rinsed in PBS, fixed in 4% paraformaldehyde for 20 min, and stained using acid phosphoric reagents with tartrate [5 mg Napthol AS-MX phosphate, 0.5 ml N,N-dimethyl formamide, 50 ml acetic acid buffer (1 ml acetic acid, 6.8 g sodium acetate trihydrate, 11.5 g sodium tartrate in 1 L water), and 25 mg Fast Violet LB salt]. Triplicate samples were used.

5.4 Histomorphometric Analysis of Osteoclasts

Osteoclasts that had been stained with TRAP were photographed at 4X magnification. Cells were analyzed using NIH Image J to measure the number and size of TRAP-positive osteoclasts.

5.5 Quantification of Gene Expression

Total RNA from the cultured osteoclasts was extracted using TriZol Reagent (Invitrogen) and quantified by UV spectroscopy. cDNA was generated using 2ug of RNA and reverse transcriptase polymerase chain reaction using iScript cDNA synthesis kit (Bio-Rad).

PCR reactions, data quantification, and analysis were performed using MyIQ Single-Color Real-Time PCR Detection System (Bio-Rad) via the iCycler thermal cycler machine. RT-PCR is a tool that allows relatively quick and precise quantification of mRNA levels where genes of interest are amplified and measured in real time. Double stranded DNA (dsDNA) induces dyes to fluoresce once bound; the thermal cycler machine can by utilized to detect dsDNA via the fluorescent dye, SYBR green (Bio-Rad IQ SYBRGreen Supermix). RT-PCR using cDNA from each osteoclast treatment group was run in minimum of duplicates for each of the five genes of interest, including *cFos*, *Nfat-c1*, *DC-STAMP*, *β3 integrin*, *Cathepsin K*, and the internal standard housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (*Gapdh)* gene.

Values were normalized to Gapdh mRNA. *Gapdh* acts as a housekeeping gene, or an internal standard, in which comparisons amongst other genes may be made. This signal from the internal control was used to normalize sample data to account for tube-to-tube differences caused by variable RNA quality or RT efficiency, inaccurate quantitation or pipetting.

Threshold cycle (Ct) values were gathered after each RT-PCR run from the MyIQ software and a series of calculations comparing each target gene to the reference gene, *Gapdh*,

were computed using Microsoft Excel. These calculations led to the average expression and fold change values that are entered into GraphPad for statistical analysis.

Reaction sample mixtures were comprised of nucleotides, forward and reverse primers specific for the target gene, osteoclast cDNA, and SYBRGreen (Bio-Rad IQ SYBR Green Supermix). Primer sequences used were *Gapdh* (Forward) 5'-TGC ACC ACC AAC TGC TTA G-3'; (Reverse) 5'-GAT GCA GGG ATG ATG TTC-3'; *cFos* (Forward) 5'-CTG TCA CCG TGG GGA TTA AG-3'; (Reverse) 5'-CCT ACT ACC ATT CCC CAG CC-3'; *Nfatc1*(Forward) 5'-TCA TCC TGT CCA ACA CCA AA-3'; (Reverse) 5'-TCA CCC TGG TGT TCT TCC TC-3'; *DC-STAMP* (Forward) 5'-GGG GAC CAG CAG TAT TTT CCT GA-3'; (Reverse) 5'-TGG CAG GAT CCA GTA AAA GG-3'; *β3 integrin* (Forward) 5'-CTG GTA AAA CGC GTG AAT-3'; (Reverse) CGG TCA TGA ATG GTG ATG AG-3'; *Cathepsin K* (Forward) 5'-AGG GAA GCA AGC ACT GGA TA-3'; (Reverse) 5'-GCT GGC TGG AAT CAC ATC TT-3'. All measurements were performed in duplicates and analyzed using the 2-(ΔΔ) Ct method.

5.6 Statistical Analysis

All experiments were run in minimum of duplicates and results are expressed as mean ± SD. Statistical analysis included one-way analysis of variance (ANOVA) measuring significance between each treatment group. Further statistical analysis using Tukey's Multiple Comparison Test was done to identify which of the treatment group comparisons from the ANOVA were statistically significant. Significance is indicated by $p \leq 0.05$. All statistical analysis is performed using Prism 5 (Graphpad Software, Inc. San Diego, CA).

6. Results

6.1 Deacetylase activity of HDAC7 is not necessary to inhibit osteoclast differentiation

It has been shown by others as well as the Mansky lab that the deacetylase activity of HDAC7 is not necessary for its repressive activity.[1,3] To determine if HDAC7's deacetylase activity is necessary to inhibit osteoclastogenesis, HDAC7 with a point mutation which disrupts the deacetylase activity (HDAC7$^{\Delta DA}$) was overexpressed in WT and KO osteoclast progenitor cells using lentiviral vector. Following the infection, primary osteoclast cultures were stimulated with M-CSF and RANKL for three days. Osteoclast differentiation was studied by measuring size and number of the cells with TRAP staining and by measuring changes in gene expression by real time RT-PCR.

While control culture readily formed TRAP-positive osteoclasts upon stimulation with RANKL, osteoclast formation was strongly reduced in both cultures infected with HDAC7 and HDAC7$^{\Delta DA}$ in both WT and KO cells (***Figure 11A and C***). Quantitative analysis of these cultures indicated that the size of TRAP-positive cells were significantly reduced in both HDAC7 (4 fold reduction, $p \leq 0.001$) and HDAC7$^{\Delta DA}$ (4 fold reduction, $p \leq 0.001$) in WT cells (***Figure 11B***). In KO cells, the size of TRAP-positive cells were also significantly reduced in both HDAC7 (4.2 fold reduction, $p \leq 0.001$) and HDAC7$^{\Delta DA}$ (4.8 fold reduction, $p \leq 0.001$) (***Figure 11D***). The average size difference between TRAP-positive multinucleated osteoclasts infected with lentiviral vectors encoding HDAC7 and HDAC7$^{\Delta DA}$ were not statistically significant in both WT and KO.

Same pattern observed in cell numbers. The number of TRAP-positive cells were reduced in both HDAC7 (2.3 fold reduction, $p \leq 0.001$) and HDAC7$^{\Delta DA}$ (2.1 fold reduction, $p \leq 0.001$) in WT cells (***Figure 11B***). In KO cells, the number of TRAP-positive cells were also reduced in both HDAC7 (2.6 fold reduction, $p \leq 0.001$) and HDAC7$^{\Delta DA}$ (2.8 fold reduction, $p \leq 0.001$)

(***Figure 11D***). The difference between average number of TRAP-positive osteoclasts with HDAC7 and HDAC7$^{\Delta DA}$ constructs were not statistically significant in both WT and KO.

The quantitative real time RT-PCR analysis demonstrated that the gene expression for *c-Fos* were not significantly different among control, HDAC7 and HDAC7$^{\Delta DA}$ constructs both in WT and KO as these genes are expressed in early in osteoclast differentiation. This indicates that these cells are committed to osteoclast lineage.

As expected, the expression of osteoclast marker gene *NFAT-c1* (1.9 fold reduction in WT, no significance; 2.3 fold reduction in KO, $P \leq 0.001$), *DC-STAMP* (3.6 fold reduction WT, $P \leq 0.05$; 2.2 fold reduction in KO, no significance), *β3 integrin* (1.4 fold reduction in WT, no significance; 2.7 fold reduction in KO, no significance) and *CTSK* (2.3 fold reduction in WT, $P \leq 0.01$; 4.1 fold reduction in KO, $P \leq 0.05$) were reduced in WT and KO cells infected with HDAC7.

In addition, the quantitative real time RT-PCR analysis of WT and KO osteoclast progenitor cells infected with HDAC7$^{\Delta DA}$ demonstrated that the expression of osteoclast marker gene *NFAT-c1* (2.2 fold reduction in WT, no significance; 1.7 fold reduction in KO, $P \leq 0.5$), *DC-STAMP* (3.1 fold reduction in WT, $P \leq 0.05$; 2.1 fold reduction in KO, no significance), *β3 integrin* (5.2 fold reduction in WT, no significance; 3.45 fold reduction in KO, no significance) and *CTSK* (1.7 fold reduction in WT, $P \leq 0.05$; 2.8 fold reduction in KO, no significance) were also reduced.

The gene expression of *NFAT-c1*, *DC-STAMP*, *β3 integrin* and *CTSK* in WT and KO osteoclast cells between HDAC7 and HDAC7$^{\Delta DA}$ were not significantly different. These data indicate that HDAC7 without deacetylase catalytic activity is sufficient to repress osteoclastogenesis. Hence, deacetylase activity of HDAC7 is not necessary to inhibit osteoclast differentiation.

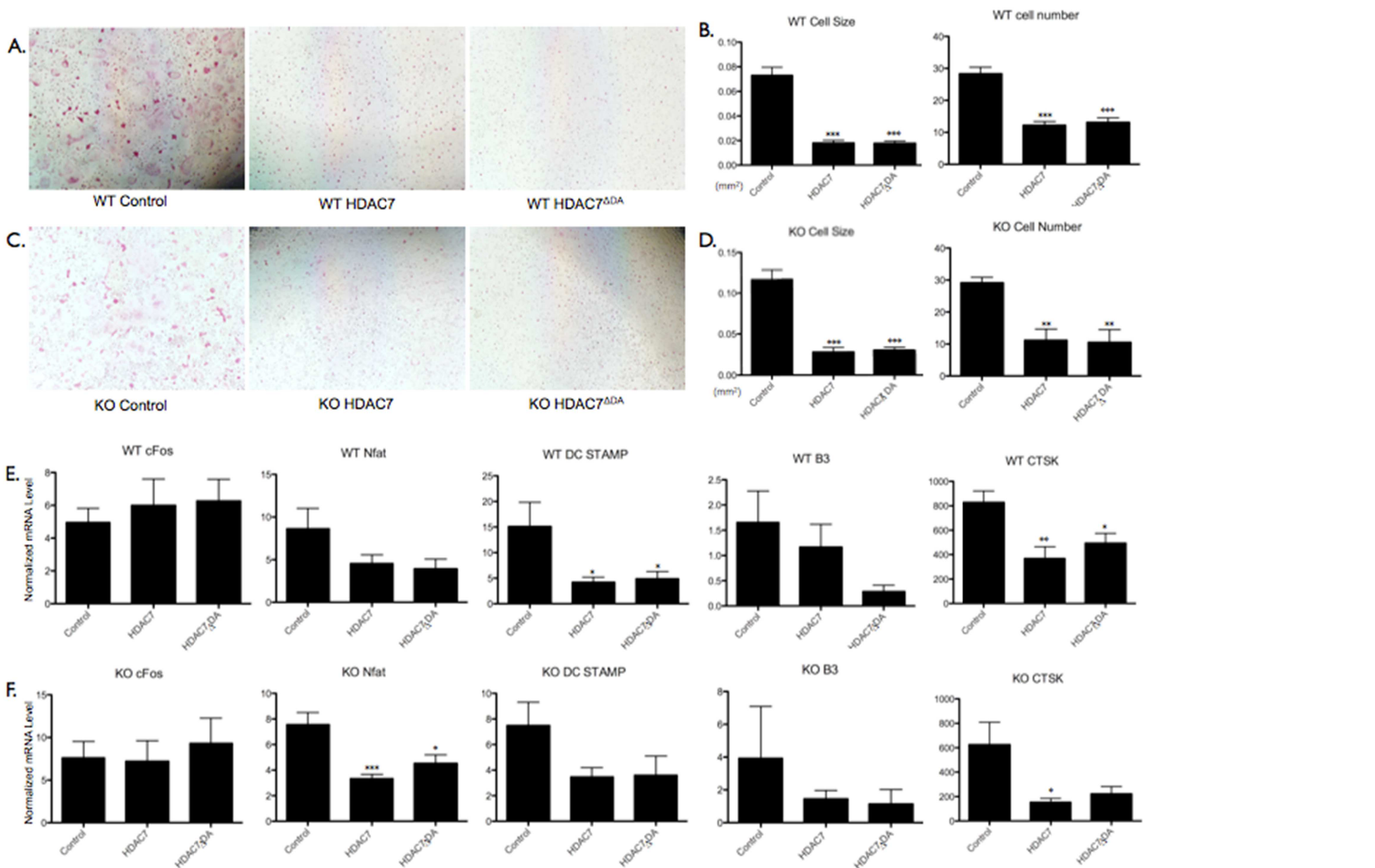


Figure 11. Deacetylase activity of HDAC7 is not necessary to inhibit osteoclast differentiation

*(A) TRAP staining of osteoclast cultures infected with control, HDAC7 or HDAC7$^{\Delta DA}$ lentiviral vector in WT cells (B)Histomorphometric analysis of TRAP-stained WT osteoclasts (C) TRAP staining of osteoclast cultures infected with control, HDAC7 or HDAC7$^{\Delta DA}$ lentiviral vector in KO cells (D)Histomorphometric analysis of TRAP-stained KO osteoclasts (E) Expression profile of c-Fos, Nfat-c1, DC-STAMP, β3 integrin, and CTSK in WT cells. * $p \leq 0.05$, ** $p \leq 0.02$ and *** $p \leq 0.001$*

6.2 HDAC7 cytoplasmic localization is sufficient to suppress osteoclast differentiation

In order to determine whether cytoplasmic localization of HDAC7 is necessary to promote osteoclast differentiation, a constitutively cytoplasmic mutant HDAC7 was constructed with deletion of nuclear localization signal (HDAC7$^{\Delta NLS}$) and introduced to cells by using lentiviral vector. Nuclear localization signal is located in N-termini of HDAC7 and deletion of such sequence prevents nuclear import of the HDAC7 resulting in HDAC7 localization in cytoplasm. We hypothesized that cytoplasmic localization of HDAC7 will promote osteoclast differentiation.

While control culture readily formed TRAP-positive osteoclasts upon stimulation with RANKL, unexpectedly, osteoclast formation was significantly reduced in both cultures infected with HDAC7 and HDAC7$^{\Delta NLS}$ in both WT and KO cells (***Figure 12 A and C***). Quantitative analysis of these cultures indicated that the size of TRAP-positive cells were significantly reduced in both HDAC7 (4 fold reduction, $p \leq 0.001$) and HDAC7$^{\Delta NLS}$ (5 fold reduction, $p \leq 0.001$) in WT cells (***Figure 12 B***). In KO cells, the size of TRAP-positive cells were also significantly reduced in both HDAC7 (4.2 fold reduction, $p \leq 0.001$) and HDAC7$^{\Delta NLS}$ (3 fold reduction, $p \leq 0.001$) (***Figure 12D***). The average size difference between TRAP-positive osteoclasts infected with lentiviral vectors encoding HDAC7 and HDAC7$^{\Delta NLS}$ were not statistically significant in both WT and KO.

Same pattern observed in cell numbers. The number of TRAP-positive cells were significantly reduced in both HDAC7 (2.3 fold reduction, $p \leq 0.001$) and HDAC7$^{\Delta NLS}$ (2.4 fold reduction, $p \leq 0.001$) in WT cells (***Figure 12B***). In KO cells, the number of TRAP-positive cells were significantly reduced in both HDAC7 (2.6 fold reduction, $p \leq 0.001$) and HDAC7$^{\Delta NLS}$ (2.3 fold reduction, $p \leq 0.001$) (***Figure 12D***). The difference between average number of TRAP-positive osteoclasts infected with HDAC7 and HDAC7$^{\Delta NLS}$ constructs were not statistically significant in both WT and KO.

The gene expression for c-Fos were not significantly different between control, HDAC7 and HDAC7$^{\Delta NLS}$ constructs both in WT and KO as these genes are expressed in early in differentiation. This indicates these cells are committed to osteoclast lineage.

The expression of osteoclast marker gene *NFAT-c1*, *DC-STAMP*, *β3 integrin* and *CTSK* were reduced in WT and KO cells infected with HDAC7 as described in result 6.1 section.

Unexpectedly, the expression of osteoclast marker gene *NFAT-c1* (2.9 fold reduction in WT, $P \leq 0.05$; 1.6 fold reduction in KO, $P \leq 0.5$), *DC-STAMP* (4.5 fold reduction in WT, $P \leq 0.05$; 1.6 fold reduction in KO, no significance), *β3 integrin* (3.5 fold reduction in WT, no significance; 1.65 fold reduction in KO, no significance) and *CTSK* (2.6 fold reduction WT, $P \leq 0.01$; 2 fold reduction in KO, $P \leq 0.01$) were also reduced in WT and KO cells with HDAC7$^{\Delta NLS}$ construct.

The gene expression of *NFAT-c1*, *DC-STAMP*, *β3 integrin* and *CTSK* of WT and KO osteoclast cells between HDAC7 and HDAC7$^{\Delta NLS}$ were not significantly different. These data indicate that even when HDAC7 is localized in cytoplasm, osteoclastogenesis is suppressed by HDAC7. Hence, it is shown that HDAC7 cytoplasmic localization is sufficient to suppress osteoclast differentiation.

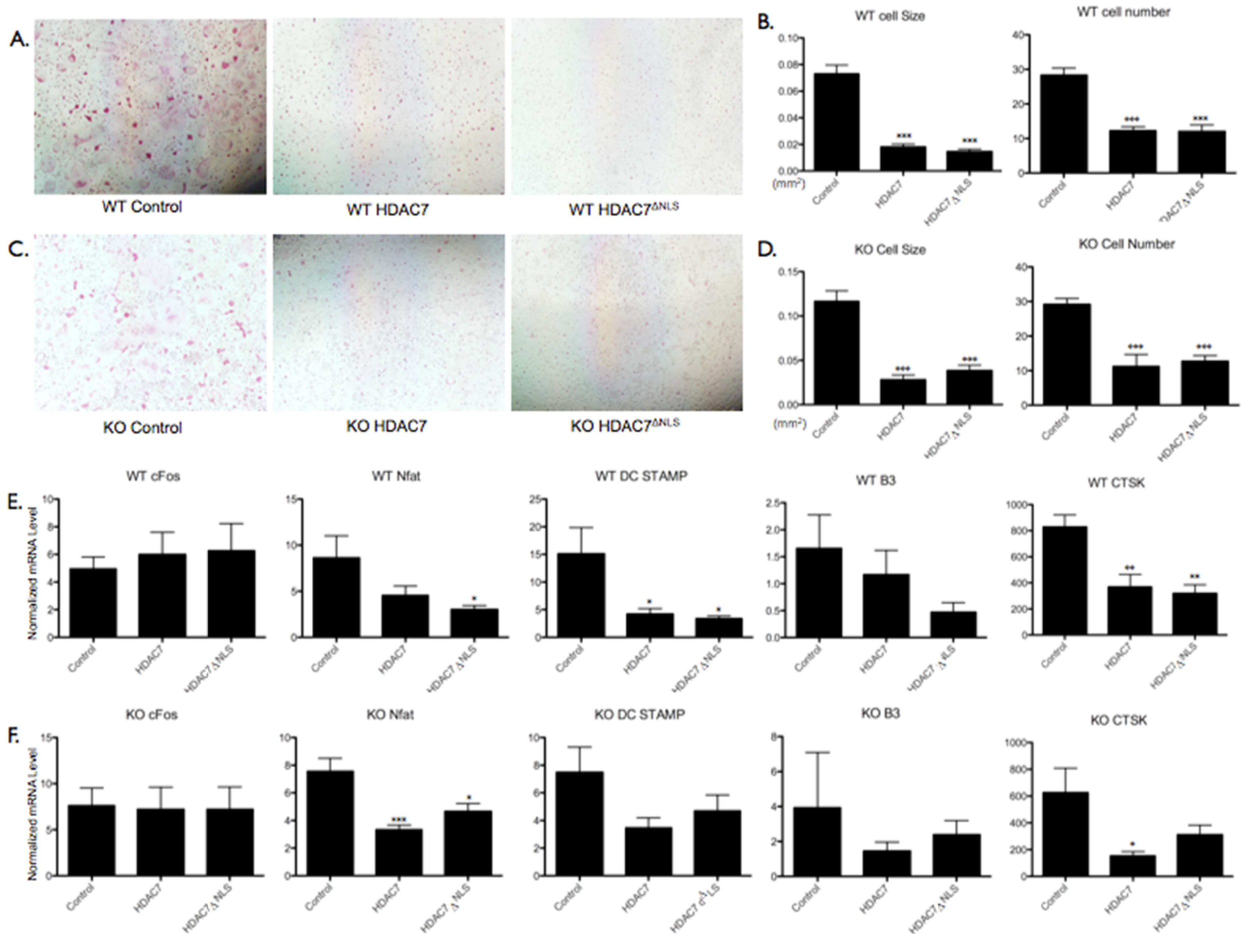


Figure 12. HDAC7 cytoplasmic localization is sufficient to suppress osteoclast differentiation

*(A) TRAP staining of osteoclast cultures infected with control, HDAC7 or HDAC7$^{\Delta NLS}$ lentiviral vector in WT cells (B)Histomorphometric analysis of TRAP-stained WT osteoclasts (C) TRAP staining of osteoclast cultures infected with control, HDAC7 or HDAC7$^{\Delta NLS}$ lentiviral vector in KO cells (D)Histomorphometric analysis of TRAP-stained KO osteoclasts (E) Expression profile of c-Fos, Nfat-c1, DC-STAMP, β3 integrin, and CTSK in WT cells. * $p \leq 0.05$, ** $p \leq 0.02$ and *** $p \leq 0.001$*

6.3 HDAC7 nuclear localization does not further repress of osteoclast differentiation

In order to determine whether nuclear localization is necessary to repress osteoclast differentiation, a constitutively nuclear HDAC7 was constructed by fabricating HDAC7 with four serine residues mutated to alanine (HDAC7^{4A}) and introduced to cells using lentiviral vector. As a Class IIa HDACs, HDAC7 has been known to be transported out from nucleus to cytoplasm by phosphorylation-dependent binding of 14-3-3 proteins to the N-terminal.[1] This binding is dependent on the phosphorylation of N-terminal serine residues and mutation of these sites prevents the export of class IIa HDACs from the nucleus to the cytoplasm.[35]

While control culture readily formed TRAP-positive osteoclasts upon stimulation with RANKL, osteoclast formation was reduced in both cultures infected with HDAC7 and HDAC7^{4A} in WT and KO cells (***Figure 13A***). Quantitative analysis of these cultures indicated that the size of TRAP-positive cells were significantly reduced in both HDAC7 (4 fold reduction, $p \leq 0.001$) and HDAC7^{4A} (4.3 fold reduction, $p \leq 0.001$) in WT cells (***Figure 11B***). However, in KO cells, the size of TRAP-positive cells were significantly reduced in HDAC7 (4.2 fold reduction, $p \leq 0.001$) but reduced with less degree in HDAC7^{4A} (2.1 fold reduction, $p \leq 0.001$) (***Figure 11D***). The average size difference between TRAP-positive osteoclasts with HDAC7 and HDAC7^{4A} constructs were not statistically significant in both WT and KO.

The number of TRAP-positive cells were reduced in both HDAC7 (2.3 fold reduction, $p \leq 0.001$) and HDAC7^{4A} (2.8 fold reduction, $p \leq 0.001$) in WT cells (***Figure 11B***). However, in KO cells, the number of TRAP-positive cells were reduced only in HDAC7 (2.6 fold reduction, $p \leq 0.001$) but not in HDAC7^{4A} (***Figure 11D***). The difference between average number of TRAP-positive osteoclasts with HDAC7 and HDAC7^{4A} were not statistically significant in WT but in KO, the difference was significant between HDAC7 and HDAC7^{4A} (2.3 fold increase from HDAC7 to HDAC7^{4A}, $p \leq 0.01$).

The gene expression for c-Fos were not significantly different between control, HDAC7

and HDAC7^{4A} constructs both in WT and KO as these genes are expressed in early in differentiation indicating that these cells are committed to osteoclast lineage.

The expression of osteoclast marker gene *NFAT-c1*, *DC-STAMP*, *β3 integrin* and *CTSK* were reduced in WT and KO cells infected with HDAC7 as described in result 6.1 section.

The expression of osteoclast marker gene *CTSK* (3.3 fold reduction in WT, $P \leq 0.01$; 3.2 fold reduction in KO, $P \leq 0.05$) was the only pair with reduced gene expression both in WT and KO cells with HDAC7^{4A} mutation. The gene expression of *CTSK* between HDAC7 and HDAC7^{4A} were not significantly different in both WT and KO osteoclast cells.

Even though it is not statistically significant, the gene expression of *NFAT-c1* (2.5 fold increase from HDAC7 to HDAC7^{4A}, no significance) and *DC-STAMP* (2.5 fold increase from HDAC7 to HDAC7^{4A}, no significance) in WT cells with the HDAC7^{4A} construct were increased compared to those with HDAC7. In addition, the gene expression of *β3 integrin* in WT cells with HDAC7^{4A} were reduced compared to cells with HDAC7, while that in KO cells with HDAC7^{4A} increased compared to cells with HDAC7 construct.

Thus, there is no indication that any osteoclast marker genes in WT or KO osteoclast cells with HDAC7^{4A} construct further repress the osteoclastogenesis significantly. These data along with previous results with HDAC7$^{\Delta NLS}$ construct indicates that localization of HDAC7 may not be the sole mechanism regulating HDAC7 activity.

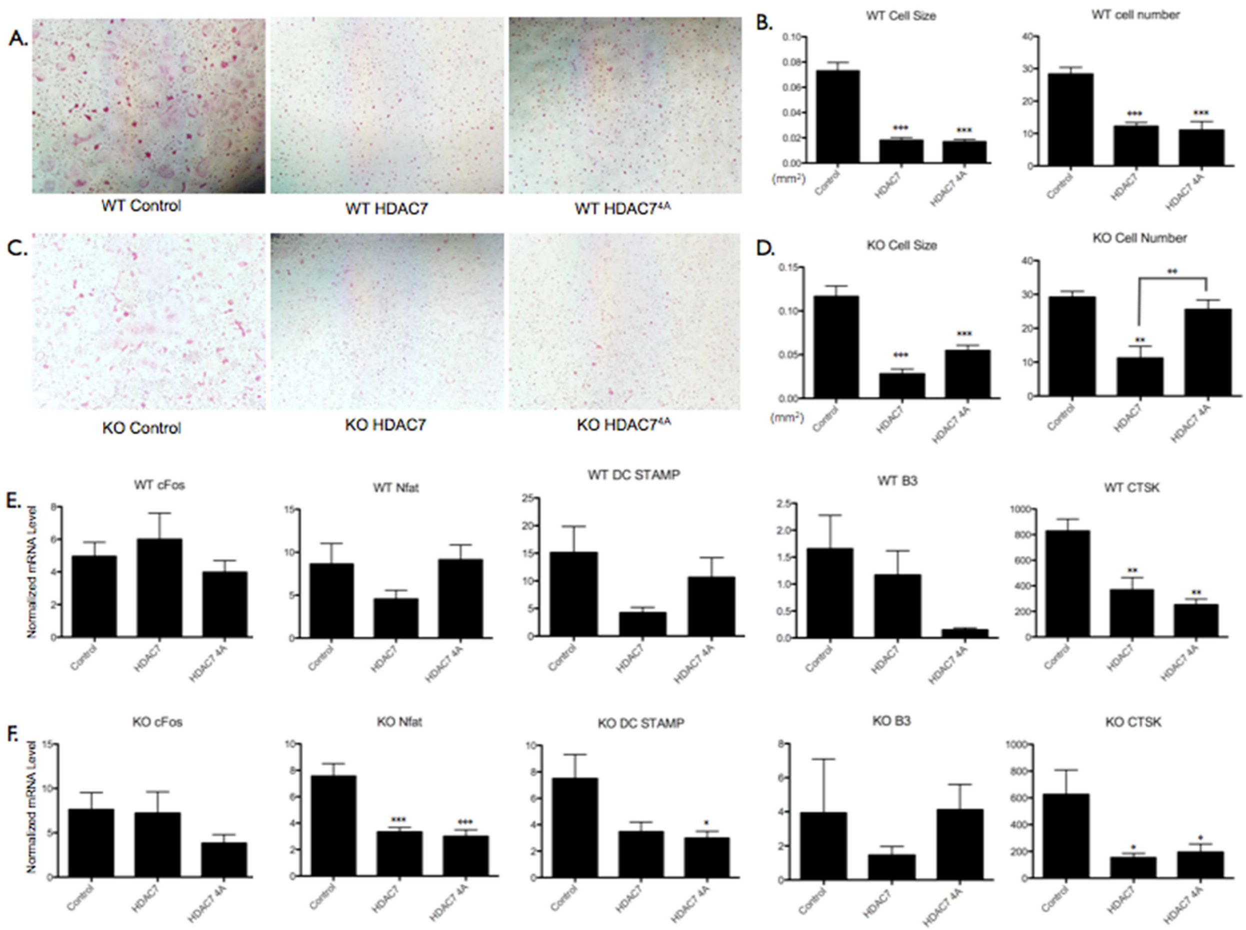


Figure 13. HDAC7 nuclear localization does not further repress of osteoclast differentiation

*(A) TRAP staining of osteoclast cultures infected with control, HDAC7 or HDAC7^{4A} lentiviral vector in WT cells (B)Histomorphometric analysis of TRAP-stained WT osteoclasts (C) TRAP staining of osteoclast cultures infected with control, HDAC7 or HDAC7^{4A} lentiviral vector in KO cells (D)Histomorphometric analysis of TRAP-stained KO osteoclasts (E) Expression profile of c-Fos, Nfat-c1, DC-STAMP, β3 integrin, and CTSK in WT cells. * $p \leq 0.05$, ** $p \leq 0.02$ and *** $p \leq 0.001$*

7. Discussion

To understand how osteoclasts maintain their fidelity of skeletal gene expression and to understand the spectrum of regulatory signals that govern their differentiation, it is essential to characterize the promoter regulatory elements as well as protein/DNA and protein/protein interactions that determine the level of each gene's transcription.

In this study, it has been hypothesized that HDAC7 is acting as a repressor of osteoclastogenesis with its deacetylase activity by inducing a closed-chromatin configuration. However, this study revealed that repression of osteoclastogenesis was observed even without deacetylase catalytic function in HDAC7. This result supports the fact that HDAC7 is a class IIa HDAC which represses gene expression by recruitment of co-repressor proteins and repressor complexes containing class I HDACs.[1] The amino termini of HDAC7 without functional deacetylase catalytic domain are sufficient to repress osteoclast gene expression. This would be possible by HDAC7 recruiting co-repressors, inhibiting the binding of transcriptions factors to DNA or sequestering transcription factors into inactive subnuclear bodies.

Based on previous findings in Dr. Mansky's lab, it has been hypothesized that HDAC7 may act as a co-repressor recruited to the promoter of essential osteoclast genes by associating with MITF and potentially other transcription factors and suppressed their gene expression. It is also hypothesized that RANKL stimulation will disrupt these repressive interactions and enabling osteoclast gene expression and subsequent differentiation (***Figure 10***).[3] Class IIa HDACs are known to shuttle between nucleus and cytoplasm by phosphorylation-dependent binding to 14-3-3 proteins.[1] To test if regulation of HDAC7 in osteoclasts is also done by nuclear-cytoplasm shuttling, lentiviral constructs with empty vector (Control) as a positive control, that with wild type HDAC7 (HDAC7) as a negative control, that with a constitutively nuclear HDAC7 mutant

(HDAC7^{4A}) and that with a constitutively cytoplasmic HDAC7 mutant (HDAC7$^{\Delta NLS}$) were transduced in WT and KO osteoclast cells.

The preliminary data from Dr. Mansky's lab showed that similar levels of HDAC7 expression throughout the osteoclast differentiations and the majority of HDAC7 is in the cytoplasm with smaller amount of HDAC7 present in the nucleus at all time through differentiation (***Figure 14***).[12] Based on these preliminary results, locations of HADC7 in each cell line with mutant constructs was anticipated as shown in ***Figure 15***.

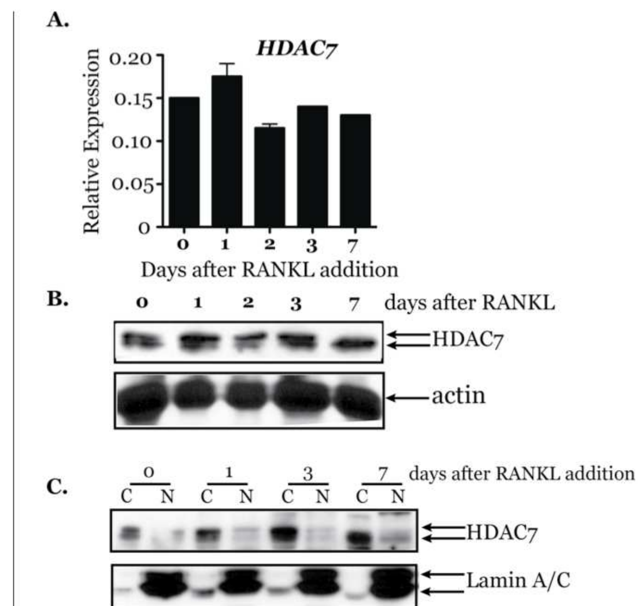
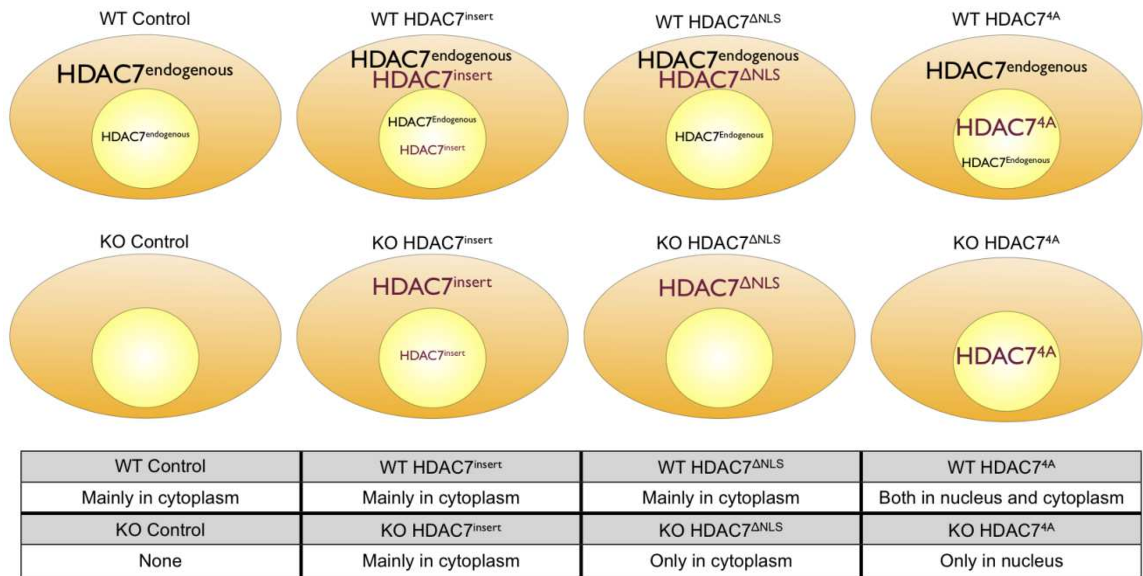


Figure 14. HDAC7 expression during osteoclast differentiation. (A) HDAC7 RNA expression during osteoclast differentiation. (B) Whole cell lysates were analyzed by SDS-PAGE and Western blotting against HDAC7 (Abcam) and actin (Sigma). (C) BMMs were treated with various days with RANKL and M-CSF and subcellular fractions were prepared. Cytoplasmic (C) and nuclear (N) fractions were analyzed by SDS-PAGE and by Western blotting with an antibody against HDAC7 (Abcam) and lamin A/C (Cell Signaling).



WT Control	WT HDAC7^{insert}	WT HDAC7$^{\Delta NLS}$	WT HDAC7^{4A}
Mainly in cytoplasm	Mainly in cytoplasm	Mainly in cytoplasm	Both in nucleus and cytoplasm
KO Control	KO HDAC7^{insert}	KO HDAC7$^{\Delta NLS}$	KO HDAC7^{4A}
None	Mainly in cytoplasm	Only in cytoplasm	Only in nucleus

Figure 15. Localization of HDAC7 in each osteoclast cell culture

When constitutively cytoplasmic HDAC7$^{\Delta NLS}$ lentiviral constructs were introduced in WT osteoclast cells with endogenous HDAC7 mainly in cytoplasm, the osteoclast gene expression, cell size and cell count were reduced to the comparable level with those in WT osteoclast cell transduced with wild type HDAC7. In addition, when same lentiviral constructs were introduced in KO osteoclast cells without endogenous HDAC7, the osteoclast gene expression, cell size and cell count were reduced slightly less but to the comparable level with KO osteoclast cells transduced with wild type HDAC7. This indicates that the presence of HDAC7 mainly in cytoplasm or only in cytoplasm still represses the osteoclast differentiation.

When constitutively nucleus HDAC7^{4A} lentiviral constructs were introduced in WT osteoclast cells with endogenous HDAC7 mainly in cytoplasm, osteoclast cell size and cell count were reduced to the comparable level with those in WT osteoclast cell transduced with wild type HDAC7 but osteoclast gene expression was not reduced significantly. On the other hand, when same lentiviral constructs were introduced in KO osteoclast cells without endogenous HDAC7, the osteoclast gene expression (except *c-Fos* and *β3 integrin*) and cell size were reduced to the

comparable level with KO osteoclast cell transduced with wild type HDAC7 but cell number was not reduced significantly. This inconsistent result indicates that the presence of HDAC7 in nucleus does not further suppress the osteoclast differentiation.

Both study designs indicate that the presence of HDAC7, not necessarily the localization of HDAC7 in the nucleus, is necessary to repress the osteoclastogenesis. This result supplements with Dr. Mansky's preliminary data showing that HDAC7 may not undergo significant nuclear-cytoplasmic redistribution during osteoclastogenesis. Hence it is indicated that nuclear-cytoplasmic shuttling may not be the primary means of regulating HDAC7's repressive activity in osteoclasts. These unexpected results prompt us to think of alternative models to explain the mechanism by which HDAC7 suppresses osteoclast differentiation. One alternative hypothesis is that HDAC7 is sequestering transcription factors, such as MITF and NFAT-c1 that are known to be shuttled between cytoplasm and nucleus. By associating these transcription factors within the cytoplasm, HDAC7 may prevent these transcription factors from reaching into the nucleus (***Figure 16***). In these alternative models, MITF-HDAC7 complex is not directly regulating at the osteoclast gene promoters. Chromatin immunoprecipitation (ChIP) assays may be helpful to determine if a MITF-HDAC7 complex directly regulates these genes.

To add credibility to this study, supplemental experiments are recommended to identify location of HDAC7 with each constructed cell lines. Immunofluorescence microscopy or western blotting of nuclear and cytoplasmic fractions can be used to identify nuclear/cytoplasmic distribution of HDAC7 within each cell line. Such method will enable us to quantify subcellular distribution of HDAC7 with single-cell resolution.

One of the limitations of this study is that we cannot control the amount of lentiviral vector introduced in the cell line. One can titrate the amount of adenovirus vector introduced in cell lines. Another study using adenoviral vectors with HDAC7 mutations used in this study may complement this study.

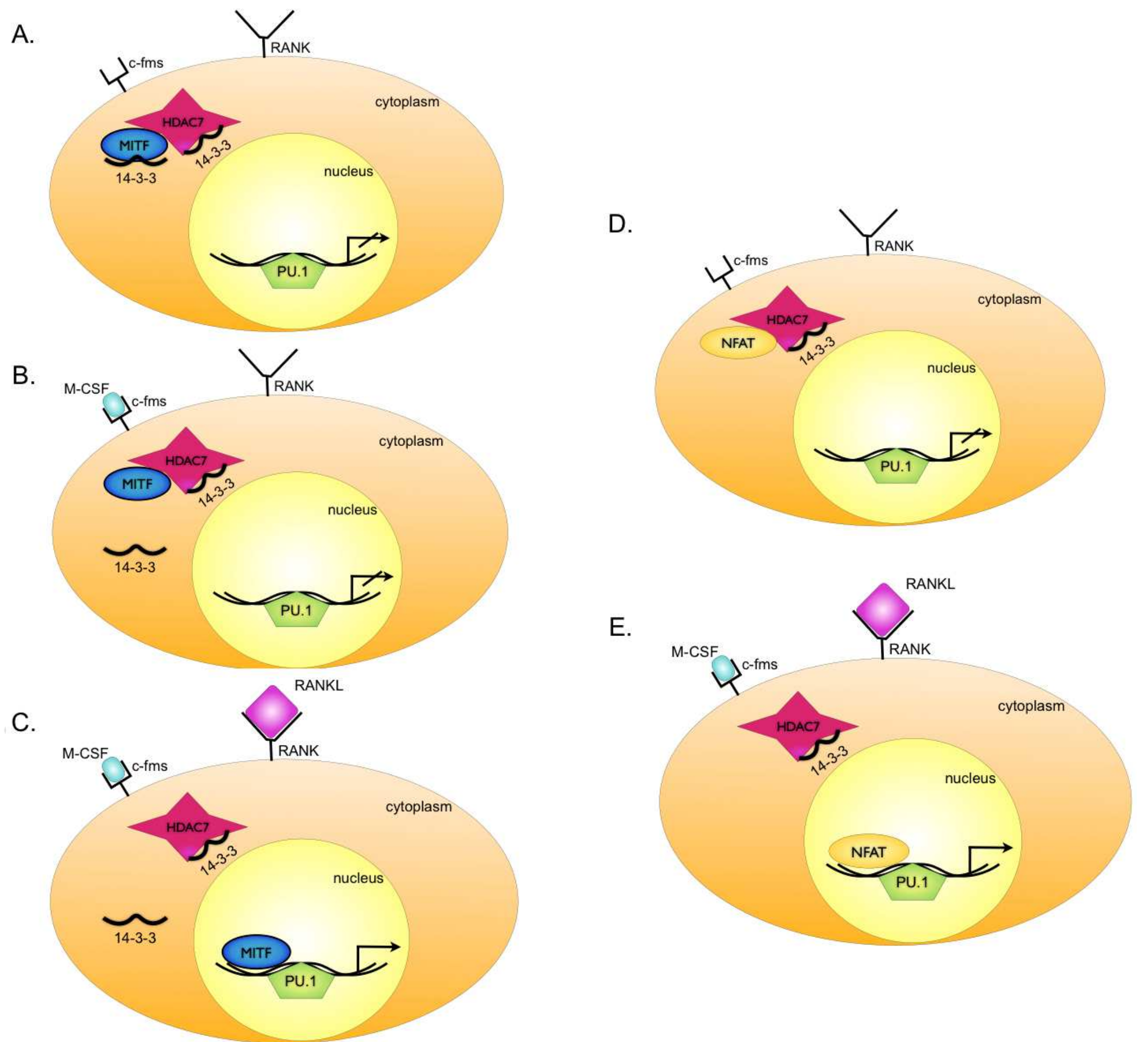


Figure 16. Possible models of mechanism by which HDAC7 regulates osteoclast transcription factors.

Model 1.(A) MITF and HDAC7 are associated together in cytoplasm preventing MITF to reach into the nucleus. (B) With M-CSF stimulation of osteoclasts, 14-3-3 protein is released from MITF but HDAC7 is still bound to MITF preventing from transporting into the nucleus. (C) With RANKL stimulation of osteoclast precursors, HDAC7 can no longer interact with MITF and MITF moves in to nucleus and form a complex with PU.1 activate genes necessary for osteoclast differentiation.
Model 2.(D) NFAT and HDAC7 are associated together in cytoplasm preventing NFAT to shuttle into the nucleus. (E)With M-CSF and RANKL stimulation, NFAT separates from HDAC7 and transported into nucleus and activates genes necessary for osteoclast differentiation.

This study opens up new opportunities to explore other possible mechanisms of regulation of HDAC7's suppressive activity in osteoclasts. Understanding the mechanisms by which HDAC7 represses MITF function in the context of the larger transcription complex of c-

Fos/MITF/PU.1/NFAT-c1 at osteoclast promoters will increase our understanding of skeletal development, maintenance and pathologic states. Such studies to identify the regulatory inputs that coordinate osteoclast activity will be a significant area for future studies as it will also help us understand the mechanism of bone remodeling and unwanted root resorption with orthodontic tooth movement and pathologic condylar resorption of temporomandibular joint.

Orthodontists are heavily relying on biological response of bone for tooth movement and its surrounding system including temporomandibular joint. There has been a study where they found the expression of RANKL in the synovial tissue from patient with internal derangement in temporomandibular joint.[43] Another study also found that there is an increase in RANKL to OPG ratio in synovia of patients with temporomandibular joint disorders.[44] These studies indicate that there is a room for developing a novel therapy to treat patients with temporomandibular disorder with HADC7 gene therapy.

Often times orthodontists face relapse with their patients after orthodontic treatment. There has been an animal study that showed local administration of OPG decreased post-orthodontic tooth relapse.[45] Another animal study also found that local OPG gene transfer to periodontal tissues inhibited relapse after orthodontic tooth movement without systematic effect.[46] These studies also indicate possible use of HDAC7 gene therapy to enhance post-orthodontic stability.

Orthodontists sometimes face unwanted side effect of root resorption during orthodontic treatment. There has been a study showing that RANKL to OPG ratio increase on pressure side and decrease in the ratio in the tensile side and this change was paralleled to the number of odontoclasts and tooth resorption.[47] This study showing

crucial involvement of RANKL and OPG in root resorption indicates that there is a room for use of HDAC7 gene therapy for prevention of root resorption during orthodontic treatment.

There is also a recent study that suggested using the RANKL to OPG ratio in saliva as functional biomarkers that could assist in the screening of the orthodontic treatment in clinical practice as they found time-related changes in salivary levels of the RANKL to OPG ratio through orthodontic treatment.[48] Orthodontists are also very concerned with efficiency of tooth movement without unwanted side effects. There has been an animal study that showed local RANKL gene transfer to periodontal tissue accelerated orthodontic tooth movement.[49] In addition, some other researchers compared this accelerated tooth movement with RANKL gene transfer treatment to tooth movement with corticotomy surgery and they found higher effectiveness of tooth movement with RANKL gene transfer than surgical method.[50] Hence, there may be another area where suppression of HDAC7 function or HDAC7 inhibitor can be used for accelerated tooth movement.

As illustrated above, there are many areas where understanding of molecular mechanism of regulation and functions of HDAC7 in osteoclastogenesis can help us develop therapies applied in the field of orthodontics and this will be a significant area for future studies.

8. Conclusion

1. HDAC7 deacetylase activity is dispensable for HDAC7-mediated inhibition of osteoclastogenesis.

2. Presence of HDAC7, not necessarily the localization of HDAC7 in the nucleus, is necessary to repress the osteoclastogenesis.

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