TROPOMYOSIN 4, MYOSIN IIA, AND MYOSIN X ENHANCE OSTEOCLAST FUNCTION THROUGH REGULATION OF CELLULAR ATTACHMENT STRUCTURES

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy
in the Graduate School of The Ohio State University

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2008

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ABSTRACT

Osteoclasts perform bone resorption vital to the maintaining of healthy bone and blood calcium levels. Osteoclasts function by forming unique actin attachment structures for migration (podosomes) and bone resorption (actin rings). For these studies, the role of two actin binding protein classes, tropomyosins and myosins, are studied to reveal their function in osteoclast differentiation and podosome/actin ring dynamics.

Our research initially demonstrated the presence of nine tropomyosin isoforms in distinct locations in osteoclasts. Tm4 co-localized with the interior faces of actin rings and podosomes. RNAi-mediated suppression led to reductions in actin ring thickness, bone resorption, and motility. Overexpression of exogenous Tm4 resulted in abnormal, thicker podosomes that were unusually distributed along with reduced cell motility and abolished bone resorption. We hypothesize that the main role of Tm4 is regulation of the adhesion structures of osteoclasts by stabilizing filamentous actin in podosomes and actin rings and thus affecting migration and bone resorption.

Myosins are a class of molecular motors that provide the ATP-dependent force to generate movement such as vesicle transport, cell migration, and cell division. Our previous studies showed MyoIIA to be distributed within podosomes and the actin ring of polarized osteoclasts. During differentiation, MyoIIA protein levels temporarily diminished corresponding to fusion initiation. Cathepsin B regulated the temporary
protein decrease. RNAi of MyoIIA was used during the latter stages of osteoclastogenesis. This suppression generated very large, less motile osteoclasts that were a result of increased fusion (elevated numbers of nuclei per cell) and cell spreading (perimeter per nuclear number). While the large cells, which formed large actin rings, were capable of resorbing bone, their diminished motility resulted in minimal changes in resorption. These data suggest MyoIIA plays an inhibitory role during osteoclastogenesis to limit the extent of precursor fusion and to promote osteoclast motility.

In mature osteoclasts, podosomes are arranged in belt-like formations at the cell periphery from a microtubule-dependent process. The multifaceted Myo10 has been shown to bind multiple podosome regulatory proteins including microtubules. Initial analysis showed Myo10 localized immediately adjacent to podosome belts, suggesting a potential role in podosome positioning. RNAi suppression of Myo10 resulted in diminished spreading of osteoclasts and a microtubule-based podosome patterning defect. Dominant negative overexpression of the MyTH4 (microtubule binding) tail domain similarly inhibited podosome positioning. Overexpression of full-length Myo10 led to increased podosome belt formation in both osteoclasts and macrophages. Analysis of osteoclasts during podosome positioning shows Myo10 localized between podosomes and the surrounding microtubule system. These results suggest Myo10 links the microtubule network to podosomes through its MyTH4 tail domain.

Together Tm4, MyoIIA, and Myo10 seem to be playing various roles in regulating osteoclast attachment and function.
ACKNOWLEDGMENTS

I wish to thank my mentor Dr. Beth Lee for all her support and guidance. She has continuously listened to and answered every question no matter how small or larger they are. Without her help and guidance I would not have accomplished such a diverse thesis. Additionally I would like to thank my thesis committee, Dr. Tony Brown, Dr. Harold Fisk, and Dr. Arthur Strauch for always patiently directing me to do better research and grow as a student. I would also like to thank the present and past members of the Lee lab for all their guidance and continuous support especially Preeyal Kotadiya who has worked along side me these past few years on the osteoclast projects.

I would also like to thank my family, especially my husband Brian who continuously listens to me jabber about science and has had to deal with me through the stressful and not so stressful times of being a graduate student. His continued support is always appreciated. A special thanks to my parents who have supported me through all my adventures in science and life.

I would like to thank Dr. Mike Ostrowski and Dr. Kim Mansky for giving me my first research job at OSU to training me in all the basics for molecular biology which made entering a graduate program possible.

I am grateful for all the continued help from the Campus Microscopy Facility for help with obtaining the beautiful confocal imagines. This research was supported by grants from the National Institute of Health to Dr. Beth Lee.
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TABLE OF CONTENTS

Abstract.......................................................................................................................... ii
Acknowledgements........................................................................................................ iv
Vita..................................................................................................................................... v
List of Figures.................................................................................................................. x
List of Abbreviations........................................................................................................ xii

Chapters:

1. Introduction.................................................................................................................. 1
   1.1 The Skeleton.......................................................................................................... 1
      1.1.1 Bone............................................................................................................... 1
      1.1.2 Bone Cells..................................................................................................... 3
      1.1.3 Diseases of Bone............................................................................................ 5
   1.2 The Osteoclast........................................................................................................ 7
   1.3 Osteoclast Differentiation.................................................................................... 7
      1.3.1 Environmental Factors.................................................................................. 10
      1.3.2 Signaling Events............................................................................................ 11
   1.4 Osteoclast Function.............................................................................................. 13
      1.4.1 Osteoclast Migration.................................................................................... 14
      1.4.2 Podosome Function...................................................................................... 15
      1.4.3 The Actin Ring............................................................................................... 16
      1.4.4 Resorption Enzymes.................................................................................... 17
   1.5 Cytoskeleton......................................................................................................... 19
   1.6 Actin-myosin Network.......................................................................................... 20
   Chapter 1 figures....................................................................................................... 23

2. Tropomyosins Regulate Osteoclast Function............................................................ 30
   2.1 Introduction.......................................................................................................... 30
   2.2 Tropomyosin Genes and Products......................................................................... 32
   2.3 Tropomyosin in Osteoclasts................................................................................ 32
      2.3.1 Analysis of Presence of Tms....................................................................... 33
      2.3.2 Localization of Tms..................................................................................... 33
      2.3.3 Discussion of Tms in Osteoclasts................................................................. 35
2.4 Tm4 Regulates Osteoclast Function through Actin Adhesion Complexes…..38

2.4.1 Tropomyosin 4 Localizes to the Actin Adhesion
Structures in Osteoclasts……………………………………………38

2.4.2 siRNA Knock-down of Tm4 RNA and Protein…………………..39

2.4.2.1 Tm4 Suppression Results in Decreased
Migration and Extra Actin Structures…………………..…….40

2.4.2.2 The Decrease in Tm4 Leads to Thinner Actin
Rings and Decreased Bone Resorption…………………..…….40

2.4.2.3 Reduced Tm4 Levels Leads to Altered V-ATPase and α-actinin Expression…………………..…….42

2.4.3 Overexpression of Tm4……………………………………………..43

2.4.3.1 Overexpression of Tm4 Leads to Altered
Podosome Formation………………………………………..…….44

2.4.3.2 Increased Tm4 Leads to Abnormal Actin Ring
Formation in Resorbing Osteoclasts……………………………..…….45

2.4.4 Tm4 Interacts Directly with Only TRIP6 in Osteoclasts……….….46

2.4.5 Discussion of Tm4…………………………………………………47

Chapter 2 Figures……………………………………………………….………..52

3. Myosin IIA Regulates Osteoclast Fusion and Function…………………..66

3.1 Conventional Myosins………………………………………………………66

3.2 Non-muscle MyoIIA ………………………………………………………..68

3.2.1 Myosin IIA Diseases …………………………………………..…….69

3.3 Myosin IIA Localization in Osteoclasts……………………………..…….69

3.4 Natural Expression of MyoIIA During Osteoclastogenesis………..…….71

3.4.1 Decreased Half Life of MyoIIA Protein Leads to The
Temporary Decrease ……………………………………………………..72

3.4.2 IIA Degradation is Cathepsin B Mediated……………………………..…….73

3.5 RNAi Mediated Decrease of MyoIIA………………………………………..74

3.5.1 Increased Cell Size Due to Fusion…………………………………….75

3.5.2 MyoIIA Suppression Leads to Larger Actin Rings…………………..…….80

3.5.3 Suppression Leads to Decrease Motility without
Altering Resorption…………………………………………………..…….81

3.6 Discussion of MyoIIA…………………………………………………..…….82

Chapter 3 Figures………………………………………………………………..88
4. Myosin X Regulates Podosomes and Actin Rings in Osteoclasts
   4.1 Introduction.............................................................................98
   4.1.1 Structure of Myo10.................................................................99
   4.1.2 Functions of Myo10...............................................................100
   4.2 Down-regulation of Myo10 Leads to a Podosomal Patterning
      Defect in Osteclasts.................................................................101
      4.2.1 Myo10 is Enriched Adjacent to Adhesion Structures........101
      4.2.2 SiRNA Treatment of Myo10.................................................102
      4.2.3 Suppression Leads to Decrease in Cell Spreading............103
      4.2.4 RNAi-mediated Myo10 Suppression Results in
         Mislocalized Microtubules.....................................................104
      4.2.5 RNAi-mediated Myo10 Suppression Inhibits Podosome
         Belt Formation.................................................................105
      4.2.6 Overexpression of MyTH4-containing Tail Truncates
         Inhibits Podosome Belt Formation......................................106
      4.2.7 Overexpression of Full-length Myo10 Leads to Increased
         Podosome Belt Formation..................................................108
      4.2.8 Myo10 Localizes between Podosomes and Microtubules
         During Osteoclast Podosome Patterning..............................109
      4.2.9 Discussion of Myo10 Involvement in Osteoclast
         Podosome Patterning........................................................110
   4.3 Myo10 Plays Multiple Roles beyond Podosome Patterning........113
      4.3.1 Suppression of Myo10 Leads to Decrease in Actin Ring Size....113
      4.3.2 Overexpression of Each Domain Causes Defective Podosome....115
      4.3.3 Overexpression of PH Domains Link Myo10 Podosome
         Defects to PI3K Signalling.................................................116
   4.4 Discussion of Myo10 in Osteoclasts......................................117
   Chapter 4 figures........................................................................121

5. Discussion..................................................................................134
   5.1 Importance of Osteoclasts in Human Health..........................134
   5.2 Importance of Actin Binding Proteins in Cancer......................135
   5.2.1 Invadopodia and Podosomes: Invadosomes.........................135
   5.3 Tm4, MyoIIA, and Myo10 in Osteoclast Function......................137
   Chapter 5 Figures........................................................................143
6. Methods ................................................................................................. 145

6.1 Reagents ............................................................................................. 145
6.2 Cell Culture ......................................................................................... 146
6.3 Western Analysis .................................................................................. 146
6.4 Immunocytochemistry and Microscopy ............................................. 147
6.5 Competitive RT-PCR of mRNA ....................................................... 148
6.6 Immunoprecipitation ......................................................................... 149
6.7 Knockdowns of Genes Studied ............................................................ 150
6.8 Osteoclast Resorption and Motility Assays ....................................... 152
6.9 Overexpression of Proteins ................................................................. 153
6.10 Microtubule Depolymerization ......................................................... 153
6.11 Statistical Analysis ............................................................................ 153

References ............................................................................................... 154
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Bone formation and remodeling</td>
<td>23</td>
</tr>
<tr>
<td>1.2</td>
<td>Osteoclast formation</td>
<td>24</td>
</tr>
<tr>
<td>1.3</td>
<td>Osteoclast Environmental effectors</td>
<td>25</td>
</tr>
<tr>
<td>1.4</td>
<td>RANKL signaling pathways which lead to survival, resorption, and differentiation of osteoclasts</td>
<td>26</td>
</tr>
<tr>
<td>1.5</td>
<td>Podosome composition and formation</td>
<td>27</td>
</tr>
<tr>
<td>1.6</td>
<td>Osteoclast resorption</td>
<td>28</td>
</tr>
<tr>
<td>1.7</td>
<td>Actin binding protein modulating actin</td>
<td>29</td>
</tr>
<tr>
<td>2.1</td>
<td>Tropomyosin genes</td>
<td>52</td>
</tr>
<tr>
<td>2.2</td>
<td>Possible Tm non-muscle gene products</td>
<td>53</td>
</tr>
<tr>
<td>2.3</td>
<td>Tm Antibodies</td>
<td>54</td>
</tr>
<tr>
<td>2.4</td>
<td>Expression of Tm in osteoclasts</td>
<td>55</td>
</tr>
<tr>
<td>2.5</td>
<td>Tm distribution migration pattern</td>
<td>56</td>
</tr>
<tr>
<td>2.6</td>
<td>Tm distribution in osteoclasts during resorption</td>
<td>57</td>
</tr>
<tr>
<td>2.7</td>
<td>Colocalization of Tms</td>
<td>58</td>
</tr>
<tr>
<td>2.8</td>
<td>Tm4 localizes to the interior edge of the actin structures in osteoclasts</td>
<td>59</td>
</tr>
<tr>
<td>2.9</td>
<td>RNAi of Tm4</td>
<td>60</td>
</tr>
<tr>
<td>2.10</td>
<td>Tm4 reduction causes change in internal actin structures</td>
<td>61</td>
</tr>
<tr>
<td>2.11</td>
<td>Decreased Tm4 lead to thinner actin rings and decreased resorption</td>
<td>62</td>
</tr>
<tr>
<td>2.12</td>
<td>Tm4 decrease affects addition protein localization</td>
<td>63</td>
</tr>
<tr>
<td>2.13</td>
<td>Overexpression increased podosome height while decreasing migration</td>
<td>64</td>
</tr>
<tr>
<td>2.14</td>
<td>Over-expression of Tm4 causes disruption of bone resorption</td>
<td>65</td>
</tr>
<tr>
<td>2.15</td>
<td>Tm4 interacts directly with one protein in osteoclasts</td>
<td>66</td>
</tr>
<tr>
<td>3.1</td>
<td>MyoIIA overlaps with actin ring and podosomes</td>
<td>88</td>
</tr>
</tbody>
</table>
3.2 MyoIIA protein during osteoclast differentiation.................................89
3.3 Myosin IIA levels decrease by cathepsin B mediated degradation.............90
3.4 Myosin IIA siRNA mediated knock-down...........................................92
3.5 Decrease in MyoIIA leads to larger cells............................................93
3.6 MyoIIA decrease leads to a decrease in microtubule acetylation..............94
3.7 MyoIIA decrease leads to increase in cell fusion..................................95
3.8 MyoIIA decrease leads to larger actin rings.....................................96
3.9 Functional assays of MyoIIA knock-downs........................................97
4.1 Schematic representation of Myo10.....................................................121
4.2 Distribution of Myo10 in mature osteoclasts...................................122
4.3 Suppression of Myo10 in osteoclasts..................................................123
4.4 Suppression of Myo10 leads to a decrease in cell spreading....................124
4.5 Suppression of Myo10 leads to mislocalization of microtubules at the cell periphery.................................................................125
4.6 Suppression of Myo10 leads to an inability to regenerate podosome belts after microtubule depolymerization........................................126
4.7 Overexpression of the MyTH4 domain suppresses podosome belt formation, while overexpression of full-length Myo10 promotes belt and ring formation...127
4.8 Myo10 is positioned between microtubules and podosomal rings and belts......129
4.9 Myo10 localization on bone is absent in actin ring...............................130
4.10 Myo10 siRNA decrease causes a decrease in ring size and bone resorption....131
4.11 Overexpression of tail domains causes abnormal podosome formation........132
4.12 Overexpression of PH domain causes PI3 Kinase related defect in podosome patterning and formation.......................................................133
5.1 Invadosomes.....................................................................................143
5.2 Localization of Tm4, MyoIIA, and Myo10.............................................144
LIST OF ABBREVIATIONS

a.a.- amino acid
ATP- Adenosine 5'-triphosphate
CaII- carbonic anhydrase II
CatB- cathepsin B
CatK- cathepsin K
CLC7- chloride channel 7
DNA- Deoxyribonucleic acid
ECM- extra cellular matrix
FBP11- mammalian homologue of yeast splicing factor Prp40
FERM- band 4.1, ezrin, radixin, and moesin domain
FRAP- Fluorescence recovery after photobleaching
GAPDH- Glyceraldehyde 3-phosphate dehydrogenase
HMM- heavy meromyosin
HMW- high molecular weight
IL-1- Interleukin 1
IP- immunoprecipitation
I.S.- internal standard
LMW- low molecular weight
MBM- mouse bone marrow
M-CSF- macrophage colony stimulation factor
MITF- Microphthalmia transcription factor
MMP- Matrix metalloproteinase
Myh- myosin heavy chain
MyoIIA- non-muscle myosin IIA
MyTH4- myosin tail homogy domain 4
NFAT- Nuclear factor of activated T-cells
NFκB- nuclear factor-kappa B
OPG- Osteoprotegerin-RANK
OPGL- osteoprotegerin ligand- RANKL
PCR- polymerase chain reaction
PEST- proline-, glutamic acid-, serine-, and threonine-rich domain
PH- pleckstrin homology domain
PIP- Phosphatidylinositol trisphosphate
PI3K- Phosphoinositide Kinase-3
PLCγ- polyphosphoinositides by phospholipase C
PTH- parathyroid hormone
PU.1- SFFV Proviral Integration 1
RANKL- Receptor Activator for Nuclear Factor κ B Ligand
RNA- Ribonucleic acid
RT-PCR- Reverse transcriptase-PCR
s.d.- standard deviation
siRNA- small interfering RNA
TGF-β- Transforming growth factor beta
Tm- tropomyosin
Tm4- tropomyosin 4
TNF- Tumor necrosis factors
TNF-α- Tumor necrosis factor alpha
TRAF (2,5,6)- TNF Receptor Associated Factor
TRAP- tartrate-resistant acidic phosphatase
TRIP6- Thyroid receptor interacting protein 6
TRPC3- "canonical" transient receptor potential 3
VASP- vasodilator-stimulated phosphoprotein
V-ATPase- vacuolar type ATPase
W-ASP- Wiskott–Aldrich syndrome protein
1.1 The Skeleton

The main role of an organism’s skeleton, exoskeleton or endoskeleton, is to support and protect its internal organs. The endoskeleton of mammals not only protects vital organs such as the brain, spinal chord, lungs, and heart, but it also supports muscle attachment for increased mobility, incubates developing immune system cells such as red and white blood cells, and serves as a reservoir of calcium and phosphate for the body. There are over 200 bones in the human body, and unlike most organs, they are not confined to one location or structural shape within the body. In adult humans the skeleton is composed of two tissues made by specific cells, primarily bone and to a lesser extent cartilage.

1.1.1 Bone

Bone is a rigid organ that, contrary to its appearance, is not uniformly solid. The layers of cortical and trabecular bone are composed of the same material but are organized differently, resulting in a difference in density. Cortical bone forms the hard outer layer of compact bone tissue that is denser than the spongy, porous trabecular tissue inside each bone.
Around 30% of bone is composed of organic bone matrix and 70% is non-organic compounds. Approximately 90% of organic bone matrix is composed of type I collagen. During bone formation the organic osteoid (collagen) is produced first, then coated by the mineralization of inorganic calcium phosphate particles (hydroxyapatite, \( \text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2 \)). Though predominantly composed of collagen and hydroxyapatite the bone matrix also contains osteonectin and osteocalcin, which assist in the binding of hydroxyapatite to the collagen. Bone sialoprotein I & II, fibronectin, and thrombospondin are cell attachment factors present in the bone matrix while biglycan and decorin help regulate the collagen structure.

Bone formation is a carefully controlled process and any imbalance can lead to major defects in skeletal health. In endochondral bone formation, first, a cartilage model for the future bone is formed by chondrocytes. Next, osteoblasts produce the collagen network that is later calcified by hydroxyapatite on the cartilage backbone. This mineralization occurs when there is a high concentration of calcium and phosphate ions present which precipitate as hydroxyapatite. During mammal development the growth plate is where chondrocytes continue to make cartilage that is later ossified. Through a human life cycle, the peak bone mass is acquired at adolescent and growth plates diminish as adolescence is passed. Figure 1.1A demonstrates how bone forms utilizing endochondral formation. Intramembranous ossification which does not use a cartilage model for ossification is responsible for flat bone formation such as the skull and scapula (Figure 1.1B). During intramembranous ossification mesenchymal tissue is transformed
into bone via osteoblast producing the collagen and hydroxyapatite network in the correct location (Gilbert 2000).

Though bone has a solid static reputation, it is actually continuously remodeled throughout an organism’s life time. In adult humans, 10% of the total bone mass is replaced each year, which calculates to the entire human skeleton being replaced once every decade of adult life (Alliston, 2002). Bone remodeling occurs through distinct steps (Figure 1.1A): First osteoclasts are recruited and activated to resorb a specific area of bone. After the osteoclasts have resorbed the correct amount of bone, they apoptose and mononuclear pre-osteoblasts fill in the area that had just been degraded. Next, the osteoblasts mature and begin the process of laying down new bone matrix. Once the correct levels of bone density are reached, the osteoblasts rest until they need to begin the cycle again. Though resorption is followed by formation continuously throughout an organism’s life, bone formation and degradation occur at different rates. For example, if bone formation in a specific area takes three months to build, the same amount of bone is degraded in two to three weeks. Constant remodeling is necessary for proper bone health and a balance of formation and resorption must occur for bone growth and healing, to facilitate tooth eruption, and to produce correct levels of blood calcium for other bodily functions.

1.1.2 Bone Cells

Chondrocytes are the first cell type that is activated in endochondral ossification, where a cartilaginous template must be first organized. Chondrocytes produce cartilaginous matrix and proteoglycans that are the backbone for future bone formation.
Chondrocytes differentiate from the mesenchymal stem cells by various known factors including parathyroid hormone related peptide and bone morphogenetic proteins. The large, round, mononuclear chondrocytes are the only cell type present in cartilage as they organize into columns at growth plates (Figure 1.1A).

Osteoblasts are the cells responsible for the synthesis and distribution of collagen and minerals needed for endochondral and intramembranous ossification making them necessary for longitudinal growth, bone mineralization, and bone remodeling. Like the chondrocytes, mononuclear osteoblasts differentiate from mesenchymal stem cells but can be distinguished from chondrocytes by their large amount of rough endoplasmic reticulum. Osteoblasts do not function alone but in groups of cells normally adjacent to newly synthesized bone and producing many growth factors to stimulate the cells around them. During bone formation, some osteoblasts are encased within the bone matrix that they produce and become osteocytes.

Osteocytes are terminally differentiated, smaller osteoblasts that have become trapped within the bone matrix and function to help regulate bone remodeling. Though the cells are completely encased in bone, they have long extensions through the bone matrix which allow them to contact other osteocytes. They are thought to be able to sense mechanical stress and signal to adjacent cells to start the bone remodeling process. The osteocytes, encased by bone and distinguished by their long processes, are resorbed along with bone during bone resorption.
Bone lining cells are a group of thin elongated cells that cover the surface of bone when it is not being remodeled. They are thought to be inactive or pre-osteoblastic cells that must move to expose bone before osteoclasts can resorb bone.

The last cell type found in bone is the osteoclast (Figure 1.2A) which is the only cell type capable of bone resorption. These giant, multinucleated cells (20-100 µm) utilize acidification and proteolysis to degrade the hydroxyapatite and collagen laid by the osteoblasts. Unlike chondrocytes and osteoblasts, osteoclasts are derived from macrophage/monocyte precursors. Activation of osteoclast differentiation occurs by secreted factors in the bone microenvironment supplied mainly by osteoblasts along with lesser contributions by fibroblasts, T-cells, and lymphocytes. Osteoclasts are distinguished by multinucleation, expression of the protein TRAP (tartrate-resistant acidic phosphatase), and the ability to resorb bone.

1.1.3 Diseases of Bone

Several diseases arise when the equilibrium of bone formation to resorption is disrupted. On the extreme ends, osteoporosis develops when there is too little formation compared to resorption and osteopetrosis occurs when there is too much formation compared to resorption. Another common disease, osteosclerosis, is a localized area of increased bone mass that can be caused by osteopetrosis or a variety of other diseases.

Osteoporosis is the most common bone degenerative disease with the estimate at 1 in 3 women and 1 in 12 men over the age of 50 worldwide developing osteoporosis. It is characterized by reduced bone mass with a predisposition to fracture. Osteoporotic bone tends to be unstable and brittle due to excessive resorption compared to bone.
formation. Osteoporosis occurs when osteoblasts do not form enough bone or osteoclasts resorb too much bone. Most osteoporosis drugs and therapies focus on inhibiting osteoclastic function as the latter has been found to be the main cause of osteoporosis.

Osteopetrosis, which is phenotypically the opposite of osteoporosis, is a group of heritable diseases characterized by the occurrence of too much bone mass. It is rarer than osteoporosis and estimated to occur only 1 in 100,000-500,000 births. The deficiency of bone resorption and thus remodeling leads to thicker, but not stronger bones. Normal remodeling keeps bone healthy. With a composition of elastic collagen and dense apatite, a lack of remodeling and replacing existing collagen leads to brittle bones. Osteopetrosis is a rare disease that in severe cases is fatal if left untreated. Distinguished by brittle bones, lack of bone marrow cavities, lack of tooth eruption, and stunted growth, there are several naturally occurring mouse models available to study this disease, such as both the grey lethal mouse and microphthalmic mouse models. Osteopetrosis is mainly caused by the lack of functional osteoclasts or by a decrease in osteoclast formation. Though osteopetrosis is rare in humans, it has given great insight into gene responsible for osteoclast function. Along with naturally occurring mouse models, several more models have been made by knocking out genes that are specific for osteoclast function such as the a3 subunit of the V-ATPase and the proto oncogene c-Src.

The second most common bone disease, Paget’s disease, occurs primarily in older adults. Paget’s disease is not a result of an imbalance in the bone equilibrium but rather an increase of bone remodeling by both osteoclasts and osteoblasts. Paget’s disease leads to bone pain, deformities, and fractures due to the high increase in turnover of bone mass.
So far researchers believe it may be triggered by viral infection in individuals with a genetic predisposition (Roodman 2005, Reddy 2001). More rare, diseases such as osteogenesis imperfecta, a disease of collagen formation leading to brittle bones, or osteosarcoma, bone cancer resulting in bone tumors often of uncontrolled bone growth, can occur in humans.

1.2 The Osteoclast

The osteoclast was first described by Albert Koelliker in Wuertsburg in 1873 (Lerner, 2000). The osteoclast is a giant multinucleated cell of up to 100 μm in diameter with an average 4-20 nuclei per cell (Figure 1.2A). It is responsible for daily control of calcium and phosphate homeostasis. Osteoclasts live their short life cycle within the bone environment and are activated to form and degrade bone by changes in the environment. The osteoclast is the focus of many bone diseases including both osteoporosis and osteopetrosis drug therapies.

1.3 Osteoclast Differentiation

Naturally occurring mouse models that lack normal osteoclast activity tend to have defects in osteoclast differentiation. These models have been a great benefit to scientists in elucidating the mechanisms involved in differentiation of osteoclasts from monocyte precursors to multinucleated resorbing cells. The osteoclast is a multinucleated cell formed by precursor cell fusion. Osteoclast fusion/differentiation occurs through several well defined steps, as illustrated by Figure 1.2B. First, stem cells are stimulated to commit to the pre-osteoclast lineage of cells, which proliferate until stimulated to fuse. Once fused, the osteoclasts tightly attach to the bone matrix and polarize to generate a
resorptive phenotype. Shortly after resorbing bone, osteoclasts proceed through pre-programmed cell death as they are only activated for a short time to control the amount of resorption that takes place.

Osteoclasts were originally thought to be of the same mesenchymal lineage as other bone cells such as osteoblasts and chondrocytes, but early studies proved that osteoclasts originate from hematopoietic precursors. Walker (1975a/b) performed two experiments that proved osteoclasts were from the hematopoietic lineage. First, spleen cells from healthy mice were infused into osteopetrotic mice, which resulted in cured osteopetrosis. Second, spleen cells from osteopetrotic mice were transformed into normal irradiated mice causing the recipient animals to develop osteopetrosis. These two experiments showed that the osteoclast precursors were in the spleen cells thus inferred by the transference of osteopetrosis to be of hematopoietic origin. Later, it was shown that osteoclast precursors are from macrophage origin and lack Fc and C3 receptors of macrophages but display their own unique combination of receptors for TRAP, vitronectin, and calcitonin (Baron 1986, Athanasou 1988, Udagawa 1990).

Initial osteoclast studies relied on utilizing either mature osteoclasts that were harvested from the surface of bone or osteoclast precursors that were co-cultured with osteoblasts or stromal cells to induce differentiation of the precursors into osteoclasts. These methods, which were useful at the time, are not extensively still in use. The first method of harvesting mature osteoclasts from bone created problems with cell viability since removing mature osteoclasts from bone is a harsh process. Further, both processes caused a mixture of cells to be present which made biochemical analysis difficult to
interpret. Using spleen or bone marrow precursor cells, researchers found the proportion of osteoclasts in the culture could be enhanced by treatment with 1, 25-dihydroxyvitamin D3. However this treatment also enhanced granulocytes and macrophages in the mixture as they are derived from the same precursors as osteoclasts (Takahashi 1988, Kurihara 1990).

The key to osteoclast differentiation was discovered through a study looking for new tumor necrosis factor related receptor (TNF-R) molecules in rat intestines for therapeutic uses (Simonet 1997). Simonet et al. classified OPG (osteoprotegerin) as a truncated form of TNF-R like protein with no transmembrane domain. Overexpression of OPG in mice caused severe osteopetrosis, suggesting a function in bone resorption. The osteopetrosis was caused by a lack of osteoclasts indicating the role of OPG in osteoclastogenesis. Simonet et al. hypothesized OPG to be a secreted molecule with an unknown receptor. A year later the same lab identified the ligand for OPG which they termed OPGL but it is now known as RANKL (Receptor Activator for Nuclear Factor κ B Ligand) (Lacey 1998). RANKL alone was demonstrated to cause osteoclast precursors to form mature osteoclasts. RANKL has since been found to exist as both membrane bound and secreted forms and is secreted by osteoblast and stromal cells. At the same time a second lab characterized RANKL, also finding that it caused osteoclast differentiation (Yasuda 1998). Mice lacking RANKL show severe osteopetrosis with a complete lack of osteoclasts (Kong 1999). It is now common practice to use M-CSF (macrophage colony stimulation factor) to enhance proliferation and survival of macrophage/osteoclast precursors, along with soluble RANKL, to induce
osteoclastogenesis in osteoclasts from bone marrow, spleen cells, and RAW264.7 macrophage cell line. Osteoclast precursors express the receptor RANK on their surface to interact with RANKL, which in turn activates many genes including NF-κβ (nuclear factor-κβ) and NFATc1 (nuclear factor of activated t cells, cytoplasmic, calcineurin-dependent 1).

1.3.1 Environmental Factors

Osteoclast activity and differentiation is controlled by a variety of factors excreted from other cells types within the bone environment. The main environmental factors that affect osteoclast differentiation are RANKL and OPG, the decoy which can bind and block RANKL action on osteoclasts. Both of these are proteins secreted by osteoblasts and stromal cells to allow for positive and negative control of bone degradation (Figure 1.3). Regulation of osteoclasts can be divided into those proteins that enhance osteoclasts by increasing proliferation, survival, and fusion and those that inhibit osteoclasts. The list of proteins affecting osteoclasts grows each day.

Osteoclast activity is enhanced by vitamin D3, PTH (parathyroid hormone), TNF-α (Tumor necrosis factor alpha), and IL-1 (Interleukin 1). Vitamin D3 causes an increase in RANKL expression by osteoblasts and thus osteoclast fusion (Kitazawa 1999). PTH increases RANKL also along with decreasing OPG by stimulating osteoblasts (Horwood 1998, Lee 1999). TNF-α and IL-1 both stimulate the production of M-CSF (Hofbauer 1999a). M-CSF is a protein involved mainly in macrophage differentiation but promotes osteoclasts by increasing the pool of precursor cells and promoting cell survival.
Osteoclasts activity is inhibited by bisphosphonates, estrogen, calcitonin, and TGF-β (Transforming growth factor beta). Bisphosphonates are pharmaceutical compounds that currently are the major treatment for osteoporosis. They do not affect osteoclast formation but rather cause osteoclast apoptosis (Rogers 2003). Osteoclasts lack estrogen receptors, but osteoblasts, which have estrogen receptors, are stimulated to increase production of decoy OPG (Collier 1998, Hofbauer 1999b). Calcitonin decreases bone resorption by inhibiting osteoclast motility (Marzia 2006). TGF-β also causes an increase in decoy OPG (Takai 1998). So far, the main enhancing and inhibitory molecules for osteoclast differentiation regulate osteoclasts through RANKL/OPG and M-CSF signaling.

1.3.2 Signaling Events

M-CSF/RANKL stimulation of osteoclast precursors causes the cells to terminally differentiate into multinucleated fused cells via a long cascade of signaling events. Using a variety of mouse models and microarray techniques, scientists have found many genes that are regulated in both a M-CSF and RANKL dependent manner. Within osteoclasts RANKL, M-CSF, integrin, and functional genes from signaling cascades regulate differentiation and resorption by osteoclasts. The initial osteoclast signaling events are started by M-CSF stimulation.

M-CSF (macrophage colony stimulating factor) is a secreted protein that causes the differentiation from monocyte precursors to macrophages. M-CSF expression is stimulated by the transcription factor PU.1 (SFFV Proviral Integration 1). Mice devoid of the transcription factor PU.1 are osteopetrotic and lack both macrophages and
osteoclasts (Tondravi 1997). M-CSF was found to be important for osteoclast differentiation by a natural occurring osteopetrotic mouse model op which later was found to be the result of a frame shift in the M-CSF gene (Yoshida 1990). M-CSF is responsible for regulating several genes including Bcl2, MITF (microphthalmia transcription factor), and cbl during osteoclast differentiation and function (McGill 2000, Nakamura 2003, and Weilbaecher 2001). M-CSF promotes cell survival and proliferation by increasing the pool of macrophages that can differentiate into osteoclasts and thus is necessary for osteoclast differentiation.

The main focus of study in the field of osteoclast signaling over the past ten years since it was discovered has been RANKL. RANKL is required for osteoclast differentiation and activates several different signaling cascades once precursors are stimulated (Figure 1.4). TRAF6 (TNF Receptor Associated Factor) is initially activated by RANKL and alone or together with TRAF2/TRAF5, it can activate genes responsible for multiple functions including survival, resorption, differentiation, actin ring formation, and transcriptional activation (Review Reddy 2004). TRAF6 starts a signaling cascade that ends with NFAT (Nuclear factor of activated T-cells), c-Fos, c-Jun, MITF, and NFkB entering the nuclei and activating transcription for genes necessary for osteoclast differentiation and function. Both c-Fos and TRAF6 are necessary for osteoclast differentiation as knockout mice develop osteopetrosis (Lomega 1999, Wagner 2003). MITF is required for terminal differentiation as MITF knockout mice also exhibit osteopetrosis due to a loss of multinucleated cells. This loss may be due in part to MITF being a target of RANKL signaling necessary for further gene activation (Mansky 2002).
RANKL signaling can also regulate resorption genes through stimulation of c-Src kinase to regulate Pyk2 and PI3K (Phosphoinositide Kinase-3), both important in osteoclast resorption.

Along with RANKL signaling which accounts for the majority of the gene regulation during differentiation, mature osteoclasts also respond to integrin mediated signaling, primarily through αvβ3. Ligation of αvβ3 in the presence of RANKL induces c-Src phosphorylation of Pyk2 (Lakkakorpi 2003), which in turn initiates a signaling cascade that mediates osteoclast resorption. αvβ3 interacts with RGD sequences, which are found in the bone protein osteopontin. The RGD sequence has been shown to directly interact with αvβ3 integrin and thus increase PI3K activity (Hruska 1995). Phosphorylation of p130cas and PI3K are also dependent on αvβ3 integrin signaling in osteoclasts (Nakamura 2003, Golden 2004).

1.4 Osteoclast Function

Osteoclasts function by secreting acid to dissolve the hydroxyapatite bone complex and proteases and collagenases to degrade the organic bone substrates. Osteoclasts migrate to sites of exposed bone where bone lining cells have moved to reveal the bone surface. Once in the correct location, the cell attaches to the bone tissue via αvβ3 integrins, and polarizes to form the actin ring/sealing zone. Within the actin ring is a specialized apical secretory domain termed the ruffled border (Figure 1.2A). Enzymatic proteins and protons are secreted from the ruffled border to cause bone degradation. Following transcytosis of bone degradation products out the basolateral membrane, the osteoclast either moves to a new location or undergoes apoptosis.
1.4.1 Osteoclast Migration

To migrate, osteoclasts use a novel cell adhesion complex termed the podosome. Podosomes were first discovered in macrophages as unique actin based foci that appeared to be much closer to the substratum than the rest of the cell and therefore were hypothesized to be migratory machinery (Trotter 1981). Via TEM they were further characterized in cells of mesenchymal and monocytic origin or virally-transformed cells as an actin core with surrounding proteins that create short protrusions on the ventral surface of the cells (Marchisio 1984). These clusters of F-actin perpendicular to the substrate were termed podosomes as they had a foot-like appearance and were assumed to be used in cell migration and attachment (Tarone 1985). Osteoclasts, which are of monocytic origin, do not express focal adhesions or stress fibers but only podosomes. Podosomes are composed of actin binding proteins, signaling kinases, and extra cellular matrix (ECM) degrading proteins and share some proteins with focal adhesions, yet are unique adhesion structures. Podosomes contain focal adhesion proteins, integrins, tensin, vinculin, p130cas, paxillin, and talin, but they also contain MMP’s (matrix metalloproteinases), α-actinin, WASp (Wiskott–Aldrich syndrome protein), gelsolin, and Arp2/3, which focal adhesions do not (Figure 1.5A). The major distinction between focal adhesions and podosomes is that podosomes contain an actin core where focal adhesions do not (Figure 1.5A/B).

Podosome composition has been extensively studied by various labs and is gaining interest as a new actin based attachment structure with potential ECM degrading
abilities. Podosomes are distinguished by their unique tightly bundled F-actin core orientated perpendicular to the substrate, which is surrounded by a light cloud of G- and F-actin. Podosome accessory proteins are divided into those that localize in the core and those that localize in the surrounding cloud (complete list Linder 2003). The actin core has been measured to be 0.3-0.5 μm in diameter with a 0.5-1.0 μm height and was found by FRAP analysis to continuously turn over (Destaiing 2003, Evans 2006).

1.4.2 Podosome Function

Due to their close proximity to the substrate, podosomes have been hypothesized to be used by osteoclasts to migrate and adhere to various substrates in place of stress fibers and focal adhesions. Podosomes only form on the substrate side of the osteoclast, and the cell continuously generates new podosomes at the leading edge of the cell. It also has been hypothesized that podosomes participate in ECM degradation and cell invasion as they contain MMP’s, known for matrix degradation (Linder 2003). Podosomes are formed in cells at regularly spaced intervals and can be stable for hours while having a median time span of two minutes (Destaiing 2003). Podosomes form de novo within the cell and can either fuse to adjacent podosomes to form one or split from larger podosomes to form two (Evans 2006). In mature osteoclasts, podosomes are arranged in belt-like formations at the cell periphery. Through live cell imaging of GFP-actin labeled osteoclasts, it was determined that this pattern of positioning results from a stepwise process as the cell matures (Destaiing 2003). Initially, individual podosomes are gathered in clusters internal to the cell periphery which then enlarge to form small rings within the interior of the cell. From this point, the podosome rings expand to form a belt at the cell
periphery, with this belt composed of one or two rows of individual podosomes (Figure 1.5C). The latter step in osteoclast podosome patterning was found to be a microtubule dependent event, as nocodazole inhibits the transition from small rings to peripheral belts, while having no effect on podosome cluster and ring formation. By EM analysis Evans et al. demonstrated each macrophage podosome is in close proximity to a microtubule, occasionally overlapping (Evans 2006). Using microtubule and actin inhibitors researchers found podosome formation and function depends on the microtubule system but the microtubules do not depend on the actin podosomes (Linder 2000, Destaing 2003, Evans 2006). This procession from podosome cluster to peripheral belt is reiterated upon recovery if microtubules are temporarily depolymerized through cold or nocodazole treatments. Through various studies overall podosome formation in osteoclasts depends on microtubules, tyrosine kinases, actin formation, and Rho GTPases.

1.4.3 The Actin Ring

The actin ring, also known as the sealing zone, is the actin based structure that osteoclasts form when they are on bone. It consists of a 4 µm x 4 µm tightly packed ring of actin filaments orientated perpendicular to the substrate (Saltel 2004). Actin ring/sealing zones do not form on glass or plastic, but require bone or synthetic bone hydroxyapatite coated substrates. Similar to podosomes, the actin ring is a zone of continuous actin turnover (Saltel 2004). Figure 1.6A demonstrates the phenotypes seen as resting osteoclasts change to be able to resorb bone. The actin ring tightly attaches via integrins during osteoclast activation to create a polarized morphology. Figure 1.6B shows F-actin in a single confocal section or a three dimensional side view from a
confocal z-scan. The actin ring contains the same proteins as podosomes with actin in the core, but not necessarily in the same arrangement. Thus it is thought that the actin ring and podosomes are two separate actin structures, although several groups continue to support the initial thought in osteoclast cell biology that the actin ring is derived from podosomes. Currently there is no concrete evidence to support either theory. Similar to podosomes, the actin ring is also microtubule dependent in formation (Saltel 2004). The actin ring is necessary for Resorption, and the polarization which occurs during actin ring formation is necessary for creation of the ruffled border, the osteoclast resorptive organelle.

1.4.4 Resorption Enzymes

The actin ring forms a tight attachment to the bone substrate for the cell to create an acidic microenvironment between itself and the bone for degradation. Within the ring exists the specialized osteoclast resorptive structure, the ruffled membrane, an invaginated cell membrane that contains ion transporters and secrets specific enzymes essential for resorption. Human genetic forms of osteopetrosis tend to have a defect in one of the following functional genes –several of which localize to the ruffled membrane- needed for proper bone resorption: carbonic anhydrase II (CaII), Vacuolar type-ATPase (V-ATPase), Cathepsin K (CatK), MMP, and chloride channel 7 (CLC7).

CaII and MMP’s do not localize to the ruffled border but assist the other proteins that localize there. CaII is distributed near the apical membrane and catalyzes the reaction that forms protons to be used by the V-ATPases, proton transporting complexes inserted into the ruffled border membrane (Gay 1974, Vaanannen 1984). V-ATPases are
responsible for generating the proton efflux that dissolves hydroxyapatite. MMP’s are endopeptidases that can be secreted or membrane associated and aid in digestion of ECM proteins along with participating in cell proliferation, migration, and adhesion. The exact role of MMP’s in osteoclasts is not yet defined but MMP’s 1, 2, 3, 9, 12, 13, and 14 have been found in osteoclasts and affect osteoclast function to various degrees.

During osteoclast polarization, V-ATPases, CatK, and CLC7 localize to the ruffled border. Early in osteoclast biology it was discovered the region between the cell and the bone was highly acidified compared to the surrounding environment which led to investigating the mechanism behind the localization of the acidification (Baron 1985). V-ATPases reside in endosomes, lysosomes, and secretory vesicles as a proton pump. Blair et al. found the vacuolar ATPase was utilized in osteoclasts to create the acidic microenvironment between the cell and the bone (Blair 1989). This high acidity dissolves the hydroxyapatite and facilitates activity of proteases to dissolve the collagen backbone of the bone substrate. The a3 subunit of the V-ATPase was discovered to be specifically up-regulated during osteoclastogenesis and the mouse knock-out model mimicked the human cases of mutations in a3 when it caused severe osteopetrosis (Li 1999, Kornak 2000). The chloride channel CLC7 acts in parallel with the V-ATPase to dissipate the electrical gradient generated by the efflux of protons. Together CLC7 and V-ATPase create hydrochloric acid (HCl) in the space between the cells and the bone. CatK is a cysteine protease mainly expressed in osteoclasts and secreted to aid in digestion of the bone matrix. Cathepsins are normally found within lysosomes for protein degradation, and they have optimal activity in acidic conditions. Osteoclasts
express the cathepsins B, C, D, E, G, K, and L, though only CatK has been highly studied as it is excreted from the ruffled border into the acidic microenvironment, and its absence in osteoclasts causes osteopetrosis.

1.5 Cytoskeleton

The cytoskeleton is responsible for a wide variety cell functions including maintenance of cell shape, cell movement, organelle trafficking, and other various processes. Cytoskeletal filaments are classified into three types: microfilaments, intermediate filaments, and microtubules. Microfilaments are composed of actin monomers that form long polar helical actin fibrils that associate with various actin binding proteins including the motor protein, myosin (Figure 1.7). As discussed previously, actin plays an important role in osteoclast function by forming the structures used for migration and resorption. Along with podosomes and the actin ring, actin is responsible for trafficking organelles within the cell that are essential for osteoclast function. In one example, the V-ATPase complex is transported from internal vesicles to the ruffled border upon osteoclast activation via microfilaments (Lee 1999). Osteoclasts also express the intermediate filament vimentin which has been less studied due to osteoclast resorption being dependent on the actin based structures. Intermediate filaments are composed of non-polarized filaments and are typically used by the cell for structure and shape. There have been no detailed analysis of intermediate filaments in osteoclasts as of yet. The last and thickest filaments found in osteoclasts are the cylindrical hollow microtubules. Microtubules are composed of alpha-and beta-tubulin heterodimers that bind their own unique set of accessory proteins along with their
associated motor proteins, kinesin and dynein. Microtubules have been found in osteoclasts radiating from the perinuclear region of the cells as mouse osteoclasts are terminally differentiated and do not form microtubule organization centers (Mulari 2003). As discussed previously, microtubule disrupting drugs affect the formation of podosome belts and actin rings (Destain 2003, Saltel 2004).

1.6 Actin-myosin Network

The actin network is involved in a variety of cellular processes that require movement of the cell or particles within the cell. Cell motility and osteoclastic bone resorption depend on the actin cytoskeleton, membranes, and adhesion systems working in coordination. Myosins are a large group of actin motor proteins that provide the motor force needed for migration, changes in cell shape, phagocytosis, cell extensions, and organelle trafficking. Myosins bind actin filaments and hydrolyze ATP to produce the movement of actin and various cargos. Myosins are composed of a motor domain which binds actin and hydrolyzes ATP, an IQ region which binds myosin light chains or calmodulin-like proteins to regulate the myosin’s function, and a tail which anchors the myosin or is used as a cargo carrier. Myosins, classified by their motor domains, tend to be the most similar in the motor region and vary the most in the tail region. Utilizing measurements of actin binding potentials and duty ratios, myosins can be classified in terms of processivity and cargo binding to indicate the different function of the tail region of the various myosins. The myosin proteins are divided into up to 37 classes, based on similarities in the motor and tail regions (Odronitz 2007). In humans there have been 40 myosin genes discovered from 12 classes of myosins (Berg 2001).
Tropomyosins, alpha helical coiled-coil dimers, bind along the grooves of filamentous actin to regulate the access of actin binding proteins including myosins. Tropomyosins were first discovered in 1948 in muscle cells and thus have been highly studied in muscle cells (Bailey 1948). Tropomyosin in its relaxed position covers myosin binding sites in muscle cells. Stimulation with calcium acts upon the regulatory troponin complex in muscle cells, and the tropomyosin moves to expose the myosin sites to allow binding of myosin to actin (Gordonn 2000). In 1972 tropomyosins were found in non-muscle cells running either along the length of the actin or only in specific regions of the microfilaments (Cohen 1972). There are four tropomyosin genes that with alternative promoters and alternative exons lead to over forty isoforms, including cell specific variants. Muscle cells tend to express only one or two high molecular weight (HMW) tropomyosins while non-muscle cells tend to express six to eight high and low molecular weight (LMW) isoforms. Non-muscle tropomyosins have been implicated in an assortment of functions including cytokinesis, vesicular transport, mitosis, and embryonic development (Hook 2004). With the variety of four genes, multiple splice forms, homo- and heterodimerization capabilities, along with tissue specific gene products, the tropomyosin gene family forms numerous proteins with the capabilities for a wide range of functions within one cell.

Tropomyosins and myosins form two large classes of proteins that both work to regulate actin microfilaments and each other in cells. Due to the importance of the actin structures in osteoclast function (podosomes and actin ring), our lab has chosen to focus on the actin-myosin network, specifically tropomyosins and myosins, to try to better
understand how osteoclasts function in migration and resorption. The following studies focus on tropomyosins and myosin isoforms MyoIIA and Myo10 in osteoclasts.
Figure 1.1: Bone formation and remodeling. (A) Endochondral ossification: The diagram illustrates bone formation as chondrocytes lay down the cartilaginous back bone for the osteoblasts to build bone upon. Right side describes the normal bone remodeling process of osteoclast differentiation, resorption by osteoclasts, and then new bone formation by osteoblasts (Baron 2006). (B) Intramebraneous ossification: The diagram illustrates bone formation as mesenchymal cells differentiate and begin to for ossification centers which are expanded to form flat bones (Gilbert 2000).
Figure 1.2: Osteoclast formation. (A) Representation of a polarized osteoclast with multiple nuclei and multiple membrane domains (left) and cross section of an osteoclast (Alberts 2002). (B) Osteoclast differentiation through various stages along with the genes involved.
Figure 1.3: Osteoclast environmental effectors. (Khosla 2001) RANKL and OPG regulate osteoclast differentiation and function through osteoblast/stromal cell signaling of soluble or membrane bound RANKL.
Figure 1.4: RANKL signaling pathways which lead to survival, resorption, and differentiation of osteoclasts. RANKL binding of RANK activates TRAF6 which in turn activates survival pathways, cytoskeletal changes for resorption, and transcription factors required for osteoclast differentiation. OPG is a decoy that can stop these processes by inhibiting the binding of RANKL to RANK. (Modified Reddy 2004)
Figure 1.5: **Podosome composition and formation.** (A) Podosome structure including a sample of actin binding proteins that regulate podosomes. (B) SEM scans of podosomes (Luxenberg 2007). (C) The three steps of podosome formation: clusters, rings, and belts along with confocal image representations of each step (Destaing 2003).
**Figure 1.6: Osteoclast resorption.** (A) Diagram shows the steps of actin (green) changes during osteoclast resorption with the formation of an actin ring to allow for polarization. Resting cells exhibit only a meshwork of internal actin which is arranged into an actin ring for the other three steps. (B) Confocal images of a cell actin ring (red) through the cell (top) and z-scan side view of the same cell (bottom)
Figure 1.7: Actin binding protein modulating actin. Various actin binding proteins regulate actin differently as shown in both lamellipodium and filopodium. (Borisy 2000)
TROPOMYOSINS REGULATE OSTEOCLAST FUNCTION

2.1 Introduction

Tropomyosins function by binding and stiffening actin filaments along with mediating access of other actin binding proteins within cells (Kojima 1994). Each tropomyosin binds six-seven actin monomers and if at saturation (which typically does not happen in vivo) each filament of actin greater than the length of seven monomers would have two tropomyosins bound, one in each groove. Tropomyosins have been shown to stabilize actin and lessen its susceptibility to actin binding proteins such as coflin and Arp2/3 (DesMarias 2002). It was shown that tropomyosins can dissociate gelsolin from gelsolin-actin complexes and transforms the actin into long fibrils (Nyakern-Meazza 2002).

The genetic regulation of the tropomyosin genes has led to cellular specificity of tropomyosin isoform expression. Tropomyosins are divided into high molecular weight HMW (mainly α and β gene products) and low molecular weight forms LMW (mainly γ and δ gene products) due to alternative promoters. Along with the alternative promoters,
tropomyosins differ at the exons 6 (6a and 6b) and 9 (9a, 9b, 9c, and 9d) (Figure 2.1). The alternative splicing allows homo- or hetero-dimers to form, although non-muscle Tropomyosins tend to only naturally form homodimers. $\alpha / \beta$ tropomyosin heterodimers lead to more thermodynamically stable tropomyosins, but both $\alpha$ and $\beta$ tropomyosin are HMW tropomyosins mainly expressed in muscle cells (Gimona 1995).

With the wide diversity of tropomyosin gene products from the four genes that vary by as little as one exon, there has been speculation that the four tropomyosin genes formed from duplications during evolution leading to functional redundancy. Recent studies have shown functional redundancy to not be the case since various tropomyosins fail to compensate for the loss of other tropomyosins. Braverman et al. showed that Tm1, but not Tm2, which differ only by the expression of exon 6, can rescue the phenotype of loss of stress fibers due to transformation even though both Tm1 and Tm2 localize to the stress fibers (Braverman 1996). Lin et al. suggested in 1988, using the antibodies available, that tropomyosin isoforms localized in distinct regions of the cells to accomplish different actin based functions. Distribution of tropomyosins in LLC-PK1 renal epithelial cells showed high molecular weight tropomyosins in stress fibers and low molecular weight tropomyosins in adhesion belts (Temm-Grove 1998). As antibodies have become available that can distinguish better between isoforms, it has been seen that the various tropomyosins localize to specific regions and perform different functions. Tm5NM1 (6a exon) has been localized to stress fibers where Tm5NM2 (6b exon) was found associated with the Golgi in NIH 3T3 fibroblasts (Percival 2004). While in the
neuronal cell line B35, Tm5NM1 recruits non-muscle myosin II to stress fibers, brain specific TmBr3 stops myosin II from being recruited (Bryce 2003).

Another new area of interest in tropomyosin comes from the expression changes of various tropomyosins in cancer cells. Cancer cells up-regulate LMW tropomyosin isoforms while down-regulating HMW tropomyosin isoforms during transformation associated with cell morphology and motility changes (Stehn 2006). LMW tropomyosins can be considered an indication of increased motile phenotypes associated with cancer.

2.2 Tropomyosin Genes and Products

Each tropomyosin gene consists of up to nine exons and vary in their expression of exons 1, 2, 6ab, 9a-d. Eighteen isoforms have been identified from these four genes in various non-muscle/non-brain tissues (Figure 2.2). In non-muscle cells, it is common to have several isoforms present in distinct distributions. Most antibodies available recognize several subsets of tropomyosin gene products. We utilized six antibodies that in combination could recognize all eighteen isoforms (Figure 2.3). Cloning via RT-PCR with specific primers was done to determine more specifically which tropomyosin each antibody recognized in osteoclasts. Antibody specificity has been shown by the various over- and under-expression studies in our lab and in this thesis.

2.3 Tropomyosin in Osteoclasts

Osteoclasts exhibit two fairly unique actin arrangements that had no previously known content of tropomyosin. As these structures contain high amounts of F-actin, especially the 4 µm thick actin ring, we expected to see tropomyosins colocalizing to these structures.
2.3.1 Analysis of Presence of Tropomyosins

To determine which tropomyosins were present in osteoclasts, I first utilized Western analysis of NIH 3T3 cell lysates, RAW264.7 macrophage cell lysates, and RAW264.7 derived osteoclast cell lysates to be able to see which sets of tropomyosin are present and the relative expression of each tropomyosin set. NIH 3T3 cell tropomyosin expression has been well characterized and served as a control for the correct band size seen by the various antibodies (Percival 2000). I found by Western analysis that each antibody recognized at least one tropomyosin in the cells to varying degrees of expression. RT-PCR of each tropomyosin set followed by subcloning and sequencing allowed us to determine which tropomyosin mRNAs were expressed in osteoclast and to what ratios (Figure 2.3). The cloning along with Western analysis showed that tropomyosins 2, 3, 5a, 5b, 4, 5NM1, 5NM11, 5NM4, and 5NM7 were expressed in osteoclasts (Figures 2.3 & 2.4). Western analysis of both undifferentiated and differentiated RAW264.7 cells allowed identification of those tropomyosins up-regulated or down-regulated in mature osteoclasts. I found both Tm2/3 and Tm5a/b (products of the alpha tropomyosin gene) were up-regulated in osteoclasts (Figure 2.4A and B) while Tm5NM11 was down-regulated (Figure 2.4E). The remaining three tropomyosins are expressed at the same level in macrophages and osteoclasts.

2.3.2 Localization of Tropomyosins

After we found which tropomyosins were present, I utilized immunocytochemical staining to visualize where each tropomyosin localized. Confocal imagining was used to view the base of each osteoclast showing tropomyosin in relation to the podosomes and
actin rings residing at the substrate/base side of the cell. Using phalloidin to stain the F-actin structures, we compared each tropomyosin or tropomyosin pairs to the main actin structures. The lower abundance tropomyosins 2/3, 5NM1, 5NM11, and 5NM4/7 when plated on glass gave a mainly disperse pattern with little to no specific association with the podosome cloud or core (Figure 2.5). Tropomyosins 5NM1, 5NM11, and 5NM4/7 all were mostly absent from the podosome region. The two higher abundance tropomyosins, 4 and 5ab, specifically associated with the podosomes (Figure 2.5). Tm5a/b co-localized with the cloud of actin around the podosomes while Tm4 associated with the actin core.

On bone, Tm2/3 and 5NM1 were slightly concentrated within the actin ring (Figure 2.6). 3D-analysis through confocal z-stack images shows that Tm2/3 was loosely intercalated into the actin ring (McMichael 2006). Tm5NM11 showed no preferential staining to the actin ring while Tm 5NM4/7 was completely absent and concentrated just outside the actin ring (Figure 2.6). In contrast tropomyosins 4 and 5a/b both strongly overlapped with the actin ring (Figure 2.6). Z-stack analysis showed Tm5a/b to localize to the interior and outside edge of the ring while Tm4 localized to the interior, outside, and inside edge of the actin ring (5a/b data McMichael 2006, 4 data Figure 2.8). I performed dual staining of Tm5a/b and Tm2, both products of the alpha gene, to see that Tm5a/b antibody only showed little recognition of Tm2/3. The merge indicates that there is little overlap between the two antibodies though they can potentially recognize the same tropomyosin gene products (Figure 2.7A). Dual staining of the other tropomyosins in various combinations showed little to no overlap also (Figure 2.7B). Figure 2.7C compares all the localizations of the tropomyosins on glass and bone.
These results indicated that the tropomyosins present in osteoclasts are segregated into separate areas of the cell with the potential to regulate specific F-actin structures and functions. While nine tropomyosin isoforms are present, only two, Tm4 and Tm5a/b localized to the podosomes. Tm2/3, 5NM1, 5NM11, and 5NM4/7 all were distributed more uniformly through the cell and potentially regulate unknown cellular structures and functions.

2.3.3 Discussion of Tropomyosins in Osteoclasts

Our initial findings of multiple tropomyosins in osteoclasts are consistent with what has been shown for macrophages and other non-muscle cells. All osteoclast tropomyosins were also present in macrophages precursors except for Tm2/3. Macrophages do not express Tm2/3 or any other high molecular weight tropomyosins (Nakumura 1995). High molecular weight tropomyosins are expressed in muscle cells and in the stress fibers of most non-muscle cells. A loss of HMW tropomyosins is found in cancer and the transformation of cells to a more motile phenotype (Stehn 2006). Osteoclasts and macrophages are both highly motile cells, yet only osteoclasts express the HMW Tm2/3. Because on both bone and glass, Tm2/3 was distributed throughout the cell with no specific overlap with the adhesion structures, it is possible it plays some role in maintaining cell shape and/or size in the much larger osteoclasts. Recently, cell staining in our lab has shown both β-actin and γ-actin are present in osteoclasts. Though the main osteoclast F-actin structures stain for β-actin, γ-actin is found dispersed throughout both the podosomes and actin rings similar to Tm2/3 staining (unpublished...
data). It is possible that specific tropomyosins in osteoclasts regulate beta and gamma actin separately.

Our initial studies have shown Tm2/3 and Tm5a/b to be specifically up-regulated during osteoclast differentiation. Tm2/3 and Tm5a/b are all gene products from the alpha tropomyosin gene and possibly are regulated similarly, though, they both differ in the exon 1 (1a and 1b) suggesting alternative promoters that might have different regulatory mechanisms. Up-regulation of tropomyosins in response to external stimuli has been seen previously as Tm4 is up-regulated when stimulated with follicle stimulating hormone in granulosa cell differentiation and during dedifferentiation of smooth muscle cells (Grieshaber 2003, Abouhamed 2003). Further studies are being conducted in the lab to see how Tm2/3 and Tm5a/b expression are regulated during osteoclast differentiation.

Our findings indicate that Tm4 and Tm5a/b strongly associate with podosomes in osteoclasts. This is the first study where tropomyosins were studied in a cell type that naturally produces podosomes. Tanaka et al. previously found tropomyosins distributed around the podosomes in transformed cells (cells which naturally do not exhibit podosomes but are induced after transformation), similar to Tm5a/b staining in osteoclasts, but no tropomyosin in the actin core (Tanaka 1993). With podosomes becoming extensively studied in the past few years, the debate has begun that transformed cell lines exhibit invadapodia and not true podosomes (Linder 2003). Though they are very similar in content, the invadapodia actin core is longer than the podosome core and thought to be used for migration and extra cellular degradation. The
initial tropomyosin study of podosomes also used antibodies that did not recognize Tm4-which is the only tropomyosin we found in the podosome core (Tanaka 1993). More specific antibodies have become available to distinguish between the various tropomyosins since these first studies; therefore the divergence of our results from published data could be consistent.

The gamma gene products, tropomyosins 5NM1, 5NM4/7, and 5NM11 are more unclear in their function as none of these associate with the actin adhesion structures for osteoclast motility and resorption. In neurons, 5NM1 resides in stress fibers to recruit nonmuscle myosin IIA, but osteoclasts do not have stress fibers (Bryce 2003). On the contrary, nonmuscle myosin IIA in osteoclasts surrounds the podosome core and is in the actin ring where Tm5NM1 is not highly expressed (Krits 2002). Tm5NM2 was associated with the Golgi while we found in osteoclasts Tm5NM11 gives a Golgi like distribution pattern (Percival 2004). The final gamma gene products Tm5NM4/7 also were unique by being the only tropomyosins completely absent from both the podosome and actin ring regions. Tm5NM4/7 were found to overlap with the unconventional myosin X (unpublished data). The direct overlap of Tm5NM4/7 and myosin X leaves one to wonder whether particular tropomyosins may regulate specific myosin classes in osteoclasts.

Previous tropomyosin data indicates that tropomyosins are absent from dynamic actin at the leading edge of lamellipodia. In osteoclasts Tm4 and Tm5a/b are present in the dynamic actin structures but they are not localized to the fast growing end of the filaments. The podosome actin core extends from inside the cell towards the substrate
which the cell sits upon. Osteoclast actin adhesion structures have Arp2/3 at the membrane surface of each structure where the tropomyosins are not present (Hurst 2004). Though they are not localized to the fast growing end of the F-actin in osteoclasts, it is likely that these two tropomyosin proteins will greatly affect osteoclast migration and resorption.

2.4 Tm4 Regulates Osteoclast Function through Actin Adhesion Complexes

After initially finding nine tropomyosins present in osteoclasts, we chose to first examine Tm4 in greater detail due to its strong localization to the actin core of the podosome and in the actin ring. Further, Tm4 is the only product of the delta tropomyosin gene which makes it the easiest of the osteoclast tropomyosin isoforms to genetically manipulate with knock-down and over-expression studies. Additionally Tm4 in mice and humans are highly similar as they differ only by 4 amino acids (Forry-Schaudies 1990). Tm4, along with other various LMW tropomyosins, is up-regulated in a variety of cancer and transformed cell lines, such as breast cancer and invasive bladder cancer tumors. Tm4 is expressed in a variety of tissues including kidney, lung, liver, spleen, stomach, fibroblasts, brain, and heart (Schevzov 2005).

2.4.1 Tropomyosin 4 Localizes to the Actin Adhesion Structures in Osteoclasts

Initial observations showed Tm4 localized to the actin core of podosomes and overlapped with the actin ring in resorbing osteoclasts. I utilized confocal Z-stack imaging to view the localization of Tm4 further. Figure 2.8A demonstrates that Tm4 forms a cap on the actin ring, localizing to the exterior, top, and interior sides of the actin ring denoted by arrows and the yellow merge. Figure 2.8B shows confocal z-sections
through the podosomes when starting at the base of the cell (top picture) the podosomes are stained red indicating that it is actin alone. As the pictures progress further into the cells (steps of +.51um) the podosomes become yellow, demonstrating that Tm4 localizes to the interior ends of the F-actin in the actin ring and the podosomes. Tropomyosins have been found to prevent depolymerization of filamentous actin from the pointed (slow growing end) of actin and are essential to regulate filament length by regulating depolymerization (Broschat 1990). Due to the localization of Tm4 and the role tropomyosins are known to play on the pointed end of F-actin where Tm4 localizes, we propose that Tm4 is binding the pointed end of podosomes and actin rings to protect the actin from severing proteins and to regulate the length of these structures.

2.4.2 siRNA Knock-down of Tm4 RNA and Protein

To investigate the role of this protein further, Tm4 expression was knocked down via RNA interference in both the RAW264.7 cell line and primary bone marrow derived osteoclasts. Small interfering RNA’s (siRNA) that specifically targeted Tm4 or a non-targeting control were transfected into osteoclasts and the expression levels (both mRNA and protein) were analyzed over the following three days post transfection. Using an internal standard with competitive RT-PCR I found Tm4 mRNA was suppressed one day following the transfection and remained suppressed through the three days analyzed (Figure 2.9 top panels of A, RAW264.7 and B, MBM). Protein levels were also assessed and found to decrease by about 50% one day after transfection and remained decreased over the same time period while the levels of GAPDH or β-actin did not change (Figure 2.9 bottom panels A and B). Quantification showed a 50% decrease for both marrow and
RAW264.7 osteoclasts protein and mRNA over a three day time course (Figure 2.9A/B). Though only a 50% decrease in expression occurred, the transfection efficiency was over 95% and immunocytochemical analysis indicated that it was a uniform decrease in Tm4 in all the cells in the culture (Figure 2.9E).

2.4.2.1 Tm4 Suppression Results in Decreased Migration and Extra Actin Structures

Osteoclasts plated on glass were transfected with control oligo or siRNA, then labeled with phalloidin and a Tm4 antibody. As expected, there was still Tm4 present in the cells as the protein decrease from Figure 2.9 showed only a 50% decrease. While there were no significant changes in the podosome structure, there were changes to the actin within the osteoclast (Figure 2.10A). Tm4 siRNA-treated cells formed unusually long actin fibrils not seen in the control transfected cells within the cells where there was a significant lack of Tm4 (Figure 2.10A, B). Osteoclasts do not form stress fibers or focal adhesions naturally because they utilize their own unique F-actin attachment structures. Using transwell migration assays, the Tm4 siRNA-treated cells showed a 40% decrease in migration through 8.0 µm pores when stimulated by osteopotin to migrate from the top to the bottom of the chamber (Figure 2.10C). This decrease in migration could be partially caused by the additional unusual actin structures.

2.4.2.2 The Decrease in Tm4 Leads to Thinner Actin Rings and Decreased Bone Resorption

To assess the F-actin ring structures of osteoclasts with suppressed Tm4 levels, I collected confocal Z-stack images of cells plated on ivory labeled with fluorescent
phalloidin. The actin ring thickness of untransfected, control siRNA transfected, and Tm4 siRNA transfected cells was measured at its thickest point. Figure 2.11 demonstrates the decrease in actin ring height (from substrate to top of F-actin ring) in Tm4 suppressed cells from 9.3 µm to 4.9 µm in RAW264.7 osteoclasts and 9.5 µm to 5.4 µm in marrow osteoclasts (quantification Figure 2.11B). Since ivory can give an uneven plane for the cells to sit on, the results were confirmed on more uniform synthetic bone substrate and found to almost identically match in the decrease of the actin ring height (Figure 2.11B). Given that the F-actin within the actin ring is perpendicular to the substrate, these results indicate a decrease in the length of the filamentous actin within the actin ring. These data demonstrated that the 50% decrease in Tm4 protein led to a 50% decrease in the thickness of the actin rings, indicating that Tm4 plays a role in stabilizing osteoclast actin rings.

Since there were significant changes in the resorptive organ of the Tm4 siRNA-treated cells, I examined the cells resorption capacity. To evaluate osteoclast resorption, RAW264.7 osteoclasts were plated on osteological synthetic bone substrate and allowed to resorb the hydroxyapatite for three days. I found the decrease in Tm4 leads to a 40% decrease in the number of clearings formed. It is possible that the thinnest actin rings measured do not have resorptive capabilities since there needs to be a correct actin ring attachment for the ruffled border to form within and for resorption to occur. The decrease in the number of clearings along with a decrease in area per clearing led to an 80% decrease in resorption (Figure 2.11C). Additionally, Tm4 siRNA-treated cells generated clearings with an altered shape. When a normal osteoclast resorbs bone, the
area beneath the ring is resorbed and typically tends to be a rounded clearing. However, the Tm4 siRNA-treated cells generated incompletely resorbed clearings on both synthetic and ivory bone substrates (Figure 2.11D). These incomplete pits could be due to altered cell motility also which we saw previously with a decrease in Tm4 total migration.

2.4.2.3 Reduced Tm4 Levels Leads to Altered V-ATPase and α-actinin Expression

In order for osteoclasts to resorb bone, they must create a tight adhesion via the actin ring and then localize enzymes such as V-ATPases within the actin ring- in the ruffled border. Since there was a difference in the ring thickness, we chose to assess whether the V-ATPases were correctly localized to the ruffled border to find if these thinner rings were functional. Immunolocalization was performed using antibodies that recognize specific subunits the V-ATPases in cells plated on ivory. In typical cells and the control cells, the V-ATPases were enriched within the actin ring 100% of the time (Figure 2.12A). In about 20% of the Tm4 siRNA-treated cells, the V-ATPases failed to localize within the actin ring, indicating that not all of the thin rings formed functional ruffled borders for resorption, which would account partially for the decrease in the number of pits in siRNA-treated discs on Figure 2.11 (Figure 2.12A). Additionally, protein levels were measured by Western analysis and showed that there were no significant differences in the protein levels of the V-ATPases compared to the control. As well, V-ATPases from metabolically S35 labeled cells were immunoprecipitated to show that the complex still properly formed in the siRNA-treated cells. Therefore, the difference in V-ATPase localization between control and Tm4 siRNA-treated cells seems to be from improper V-ATPase trafficking and not expression or complex formation.
Along with the V-ATPases, several actin binding proteins were examined to see if the decrease in Tm4 altered the location of other various proteins found to be expressed in podosomes and actin rings. I examined the proteins paxillin, cofilin, p130cas, vinculin, integrin beta1, integrin beta3, gelsolin, and α-actinin and found only α-actinin changed in its localization when Tm4 decreased. In podosomes, the cross linking/actin bundling protein α-actinin localizes to the core at the top of podosomes and the cloud surrounding the bottom of the podosomes. Additional podosome analysis recently has shown that the podosome cloud that surrounds podosomes radiates from the podosome core creating an F-actin structure similar to an upside down umbrella (Luxenburg 2006). At the base of the cell, α-actinin is found surrounding the podosomes as seen in the control cells (Figure 2.12B top panels). In Tm4 siRNA-treated cells at the base of the podosomes, α-actinin was found in the core (Figure 2.12B bottom panels). This could possibly be explained if the podosomes also decreased in height as the actin ring did, but the difference of a structure only 1-2 microns cannot be accurately measured with the resolution of the confocal microscope used for these experiments. On bone, resorbing osteoclasts also had a change in α-actinin when Tm4 decreased. In normal cells, alpha-actinin is present through the top half of the actin ring same as Tm4 distribution, but when Tm4 decreases α-actinin localizes to the middle of the thinner rings (Figure 2.12C).

2.4.3 Overexpression of Tm4

Because Tm4 suppression led to a decrease in the actin ring thickness and altered actin structures, I overexpressed Tm4 to see if it would also alter the actin structures in osteoclasts. Stable cells lines were created with the cDNA for Tm4 or an empty vector as
a control in RAW264.7 macrophages and individual clones were picked that overexpressed Tm4 to varying degrees (Figure 2.13A). These cells were differentiated into osteoclasts for the following experiments. By Western analysis the best clones expressed only a two-fold increase of Tm4, yet all the clones tested generated altered actin structures displayed in both Figures 2.13 and 2.14. The promoter for the overexpression vector was human elongation factor 1 alpha. When Tm4 was overexpressed with a stronger CMV promoter, the cells died- indicating that the cells could not tolerate a high overexpression of Tm4. Overexpression also did not occur if the 3’UTR was attached to the Tm4 coding region, therefore my clone contains only the coding region of Tm4. The stably expressing Tm4 RAW264.7 cells were able to form osteoclasts at the same rate as the control cells.

2.4.3.1 Overexpression of Tm4 Leads to Altered Podosome Formation

Initially the overexpressing osteoclasts were assessed on glass in their migrating phenotype. Osteoclasts were grown on glass coverslips and stained with phalloidin to view the F-actin structures within the cells. Overexpressing cells had brighter labeled podosomes that were unusually distributed throughout the cell rather than near the cell edge as shown in the control (Figure 2.13B). Because the overexpressing cells had such bright podosomes, I used confocal Z-stack imaging to measure the height of the podosomes. Compared to the control, the height of the podosomes increased from 2 μm to 3.5 μm (Figure 2.13C). When α-actinin and paxillin were assessed to reaffirm the structures seen are podosomes, the majority were similarly localized in the core and around the core in both the control and overexpressing lines (Figure 2.13D). Along with
the phenotypic analysis, I used transwell migration assays to determine if the abnormal podosomes were affecting migration. Migration decreased 65% in overexpressing cells (Figure 2.13E). In few instances, less than 10% of the cells, Tm4 over-expressing cells formed what phenotypically appeared as actin rings when plated on glass. To assess if these were functional actin rings, V-ATPase distribution was examined. In the rare control cells that exhibited brighter densely packed podosomes similar to actin rings (affect possibly due to dense podosome rings that are in the process of expanding), the V-ATPase was distributed throughout the cell (Figure 2.13F, upper panels). In the Tm4-RT clones, the V-ATPase expression is concentrated within the actin ring-like structure, suggesting functional actin ring formation on non-bone substrates (Figure 2.13F, bottom panels).

2.4.3.2 Increased Tm4 Leads to Abnormal Actin Ring Formation in Resorbing Osteoclasts

To assess the resorbing phenotype of the overexpressing cells, osteoclasts of both control and overexpressing tropomyosin clones were plated on ivory. Control cells were able to polarize and form proper actin rings, while overexpressing clones were unable to form actin rings. Overexpressing cells formed patches of actin where the actin ring should have been at the base of the cell (Figure 2.14A). Together with the abnormal actin at the base of the cell, the patches of actin were also formed around the cell membrane of the cells. When the cells were costained with Tm4, Tm4 localized to the actin patches at the base and membrane of the cell (Figure 2.14B). The overexpression cells lacked concentration of V-ATPase, visualized using antibodies to the a3 subunit, within the
abnormal actin structures as the control cells properly localized the V-ATPases within the actin rings of cells plated on bone (Figure 2.14C). There were no changes in expression of the V-ATPases by Western analysis. As expected without proper actin ring formation or V-ATPase localization, under normal conditions where the cells were plated on the synthetic bone substrate for three days, the overexpressing cells were unable to resorb bone (Figure 2.14D). Normal bone resorption data consists of cell plated on the synthetic bone substrate for three days. Since these cells did not resorb any of the substrate, while the control resorbed normal amounts, the experiment was repeated and the cells were left on the synthetic bone substrate for eight days. The overexpressing clones resorbed less than 1% of the total disc which equated to approximately 9% of control levels after this unusually longer time period.

2.4.4 Tm4 Interacts Directly with Only TRIP6 in Osteoclasts

Current publications have shown that Tm4 is only known to bind two proteins: FBP11 (mammalian homologue of yeast splicing factor Prp40), an elongation splicing protein, and TRIP6 (Thyroid receptor interacting protein 6), a focal adhesion nuclear shuttling protein (Yi 2002, Lin 2004). To try to understand the phenotypes seen in the knock-down and over-expression studies, a Tm4 antibody was used to immunoprecipitate (IP) metabolically radioactively labeled proteins from osteoclasts. The resulting IP was split in half and so that half remained untreated and half was treated with an actin depolymerization G-buffer (Figure 2.15A). When Tm4 was immunoprecipitated with intact F-actin, it was found Tm4 and the resulting actin was bound by five proteins, but after actin depolymerization, Tm4 bound only one protein. IP’s using Tm4 and TRIP6
without G-buffered followed by Western analysis showed the five proteins to be myosin IIa, TRIP6, Actin, Tm4, and Tm5a/b with the 100kDa protein remaining unknown as it could be any protein that interacts with actin or the remaining binding proteins listed above (Figure 2.15B). The only protein that directly interacted was the 50kDa TRIP6. Immunolocalization was performed to see if TRIP6 localized to areas known to have Tm4 content (the core of podosomes and top half of the actin ring). The nuclear shuttling protein TRIP6, contrary to other published cells types, localizes mainly in the nuclei with a slight localization in the podosomes (Figure 2.15C). On bone, TRIP6 still resides mostly in the nuclei and was very slightly intercalated into the actin ring (Figure 2.15D). With very little overlap between TRIP6 and Tm4, this potential interaction has many aspects left to be explored.

2.4.5 Discussion of Tm4

These studies indicate that Tm4 plays a role in osteoclast function by regulating the actin structures used for migration and resorption. Tm4 is unique from the other osteoclast tropomyosins by genetics and localization. First, Tm4 is the only gene product of the delta gene which made it easier than the other tropomyosins to genetically manipulate with RNA interference. The other tropomyosin genes expressed in osteoclasts have several isoforms present, possibly differing only by one exon. Most of these similar isoforms cannot be individually separated from their closest isoform via Western analysis. The gamma gene has four isoforms alone expressed in osteoclasts. Second Tm4 is unique by being the only tropomyosin to localize in the core of podosomes. Further analysis of the podosomes and actin rings shows that Tm4 localizes
to the interior end of each structure, suggesting a common function in osteoclast regulation of the actin attachment structures.

These studies indicate that Tm4 plays multiple roles in the osteoclasts along with podosome/actin ring regulation. Our results found Tm4 to be expressed in NIH 3T3 fibroblast cells which do not exhibit podosomes or actin rings but have stress fibers and focal adhesions, both structures which osteoclasts lack. As mentioned previously, Tm4 which is expressed at various levels in many tissues is found in many tissues which do not have podosomes either. Tm4 has a common role in motile events since it is present in the motile extension of the neuron growth cone (Had 1994), in cells changing to a motile phenotype during dedifferentiation of smooth muscle cells (Abouhamed 2003), and in cancer cells where it is up-regulated when the cells metastasize. In osteoclasts, Tm4 seems to play not only a role in migration but also resorption.

For our studies, we found the main role of Tm4 is to regulate the migration and resorption F-actin structures. We propose Tm4 is regulating both podosomes and actin rings by preventing access to proteins such as those that may increase actin turnover to the interior end of each structure. My knockdown and over-expression studies are consistent with this in that when Tm4 decreased, the actin ring decreased in thickness, while over-expression Tm4 caused the podosomes to increase in height. When there is less Tm4, other proteins such as gelsolin or cofilin can bind and change the actin structures in osteoclasts. Tropomyosins have already been shown to regulate the access of gelsolin and cofilin to actin (Ishikawa 1989, Bernstein 1982). Additionally, since endogenous Tm4 forms a cap on the actin ring, it is possible that Tm4 may be holding
specific actin ring regulatory proteins in the correct conformation needed for proper actin ring formation. Studies have found the Arp2/3 complex localizes specifically to the bottom half of the actin ring while I found Tm4 localizes to the top half of the actin ring (Hurst 2004). I found specifically α-actinin is mislocalized when Tm4 decreases in both podosomes and the actin ring. While Tm4 suppression could lead to a decrease in podosome height, due to the restrictions of the optical resolution of the confocal, I was unable to generate accurate measurements. Either way, over- or under-expressing Tm4 decreased resorption and motility. The osteoclasts were unable to tolerate any large change either way in expression levels as I was only able to knock-down expression by 50% and increase expression 2-fold. Attempts to over-express using a strong viral promoter resulted in cell death. Also attempts at utilizing the full length Tm4 with the 3’UTR, was unsuccessful in up-regulating Tm4 expression.

Less clear are the multiple additional roles Tm4 plays in osteoclasts. When Tm4 decreased in cells on glass in the migratory phenotype, there was an abundance of internal fibril actin accumulated within the cells. I found Tm5a/b and non-muscle myosin IIA (MyoIIA) were bound to these structures possibly facilitating their formation, but it is unknown why they occurred at the absence of Tm4 since Tm5a/b and myosin IIA are naturally distributed in osteoclasts when these structures are not present. It is possible the normal distribution of Tm5a/b and MyoIIA are disrupted and they bind actin usually not available when Tm4 decreases, since there are all three bind the same pieces of F-actin through the Tm4 IP’s. Second, the decrease of Tm4 in resorbing osteoclasts led to a mislocalization of α-actinin and V-ATPases. Tm4 does not directly bind α-actinin
though it resides in the same cellular location in actin rings and podosomes. It is possible α-actinin is one of the actin binding proteins that Tm4 regulates indirectly by stabilizing the F-actin in the correct conformation for α-actinin to bind. V-ATPases are transported from membranes to the ruffled border for proper bone resorption to occur. It has been previously reported that V-ATPase transport depends on actin since multiple V-ATPase subunits bind directly and indirectly to F-actin (Lee 1999). At the same time, it was demonstrated that the actin that binds the V-ATPase binds non-muscle myosin IIA. While Tm4 only binds one protein directly in osteoclasts, the filamentous actin Tm4 regulates also includes myosin IIA. Again, Tm4 may be indirectly regulating V-ATPase localization by partially regulating the binding of myosin IIA to specific actin used for V-ATPase transport.

When Tm4 was over-expressed on bone, we would expect the cells to form thicker actin rings due to the thinning of the actin ring during siRNA treatment and thickening of podosome during overexpression, but the osteoclasts were unable to form actin rings at all. While on non-bone substrate of glass, pseudo-actin ring formation occurred in Tm4 over-expression cells, but on bone these cells lacked actin rings and exhibited large patches of actin around the secretory domain of the osteoclast cell membrane. These patches could be inhibiting the transcytosis needed for proper bone resorption. Transcytosis is the method used by osteoclasts to remove the by-products of osteoclast resorption. Large amounts of inorganic and organic material that is resorbed is released by passing through the cell secretory domain without the cell detaching from the resorptive site (Salo 1997). If the overexpressing cells have a deficiency in transcytosis,
then forming actin rings could possibly be detrimental to the cell when plated on bone, not glass. Forming an actin ring on glass would result in no resorption products passing through the cell. Therefore due to the membrane actin patches formed by Tm4 overexpression, Tm4 may be inhibiting resorption and actin ring formation of cells on bone only.

In summary, Tm4 appears to regulate the F-actin within the podosomes and actin rings by stabilizing these structures. Minimal changes in Tm4 by increasing or decreasing protein expression causes the height of both the podosomes and actin rings to change and significant alterations to appear within the cells. Because Tms are known to regulate actin by preventing or giving access of other actin regulatory proteins to specific actin, Tm4 is consistent in playing a role in the dynamics of actin networks.
Figure 2.1: Tropomyosin genes. Exon maps of the four tropomyosin mammalian genes with alternative promoters to regulate expression of exons 1 (green) along with alternative exons expressed for exon 6 and exon 9 (yellow) allowing for multiple isoforms.
Figure 2.2: Possible tropomyosin non-muscle gene products. Exon maps of the 18 known available tropomyosin isoforms from the four genes in non-muscle, non-brain tissues.
<table>
<thead>
<tr>
<th>Antibody</th>
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<th>Possible Tm</th>
<th>Osteoclast Tm</th>
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<td></td>
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<td>γ (9d)</td>
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<td>γ (9c)</td>
<td>5NM 4, 7, 8, 9, 10</td>
<td>5NM 7(95%)</td>
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</tbody>
</table>

**Figure 2.3: Tm Antibodies.** Each antibody commercially provided is known to recognize a set of tropomyosin genes (2nd column) and isoforms (3rd column). Cloning with exon specific primers along with PCR sequencing indicated which tropomyosin each antibody was recognizing in osteoclasts (4th column). The percent of each isoform occurrence is listed behind each tropomyosin recognized in osteoclasts. Antibody specificity has been confirmed through the use of each antibody in over-expression and under-expression of individual tropomyosins.
Figure 2.4: Expression of tropomyosin in osteoclasts. Western analysis was performed with NIH 3T3, macrophage, and osteoclast whole cell lysates with the various tropomyosin antibodies to detect the expressed tropomyosin in osteoclasts. Ten micrograms of total protein were loaded per lane. Antibodies used were (A) Tm311, (B) AB5441, (C) AB5449, (D) AB5447, (E) AB5443, and (F) AB5445.
**Figure 2.5: Tropomyosin distribution migration pattern.** RAW264.7 derived or MBM derived osteoclasts were plated on glass to cause the formation of podosomes. These cells were coimmunostained with each tropomyosin antibody and phalloidin to visualize the F-actin structures. Single confocal sections were taken to view the localization of each tropomyosin compared to the podosomes at the cell periphery. The tropomyosin proteins were labeled in green (far left) while the actin was labeled red (middle). The first two panels of Tm5ab and Tm4 are a close-up of the section boxed in the merge to show the colocalization of these two proteins with the podosomes.
**Figure 2.6: Tropomyosin distribution in osteoclasts during resorption.** RAW264.7 derived or MBM derived osteoclasts were plated on thinly sliced ivory to cause the formation of the actin ring. These cells were coimmunostained with each tropomyosin antibody and phalloidin to visualize the F-actin structures. Single confocal sections were taken to view the localization of each tropomyosin compared to the actin ring. The tropomyosin proteins were labeled in green (far left) while the actin was labeled red (middle).
**Figure 2.7: Colocalization of tropomyosins.** Osteoclasts were plated on glass and stained for two different tropomyosins along with F-actin then viewed by confocal microscopy. Actin was visualized with fluorescent phalloidin and then using the confocal computer program the color removed to view dual green/red staining. (A) Tm5ab (green, far left) did not colocalize with Tm2/3 (red, left) as seen in the merge with very little costaining. (B) Tm5NM1 (top green, far left) and Tm2/3 (top red, left) though both found disperse throughout the cell, did not show significant colocalization. Tm5ab (bottom green, far left) along with Tm4 (bottom red, left) show distinct podosome staining (actin- white, far right) with very little overlap in the podosome region. (C) Table to define where each tropomyosin localized in cells on glass and ivory.
Figure 2.8: Tm4 localizes to the interior edge of the actin structures in osteoclasts. Multiple confocal sections of Tm4 (green) stained osteoclasts on ivory (A) and glass (B) along with F-actin attachment structures (red). (A) Side views of an osteoclast on ivory shows Tm4 colocalizes to the interior section of the F-actin in the actin ring indicated by arrow heads. The distribution shows Tm4 forms a cap on the actin ring along with localizing to a lesser extent at the cell interior at the plasma membrane. (B) A close up view of the podosomes starting at the base of the cell and progressing up into the cell 0.5 µm in each successive picture. Tm4 colocalizes to the interior end of the podosomes also indicated by the yellow co-staining in the most interior picture at 1.02 µm.
Figure 2.9: RNAi of Tm4. Tm4 expression was suppressed via small interfering RNA’s. (A) The RNA levels decreased over a three day time course for RAW264.7 osteoclasts (top panel) when competitive RT-PCR compared to an internal standard. Over the same time course the protein also decreased 50% compared via Western blotting along with the controls of GAPDH and beta actin. Graphical representation of the decrease of protein and RNA for RAW264.7 cells average. (B) The RNA decrease was also seen in MBM osteoclasts (top panel) and protein decrease also (lower panels). Graphical representation of the decrease of protein and RNA for MBM average. (C) Control and Tm4siRNA treated cells plated on glass and stained with Tm4 and actin to show the overall uniform decrease of protein expression. A second siRNA to Tm4 produced similar results for all siRNA experiments shown with Tm4.
Figure 2.10: Tm4 reduction causes change in internal actin structures. RAW264.7 derived osteoclasts were plated on glass to analyze the podosomes after the siRNA decrease of Tm4. (A) siRNA treated cells exhibit extra actin fibrils (red middle) within the cells where there is no Tm4 present (green top) while no distinguishable difference in the podosome structures. Control cells did not exhibit these extra structures. Scale bars indicate 10 μm. (B) A closer magnification of the siRNA-treated osteoclasts in A to show the extra actin fibrils seen in the siRNA treated cells. Scale bars indicate 10 μm. (C) Migration was measure via transwell migration assays and the siRNA treatment caused a 40% decrease in migration of cells lacking Tm4 (mean +/- s.d.).
Figure 2.11: Decreased Tm4 lead to thinner actin rings and decreased resorption.
Osteoclasts were plated on ivory and the actin ring was measured using confocal z-stack function. (A) A single representation of both the base actin ring structure and the side view of the cell which show the decrease of the actin ring thickness (white brackets) (B) The thickest point of several actin rings of untransfected, control transfected, and siRNA transfected cells shows the 50% decrease in the ring thickness in both RAW264.7 and MBM derived osteoclasts. (C) RAW264.7 osteoclasts were also plated on BD osteological discs to measure the decrease in resorption in number of pits, percent per pit, and total resorption. (D) Control vs. siRNA resorption that show the change in the shape of the resorptive areas with the arrows indicating the incompleteness of the siRNA treated resorptive areas in both synthetic and ivory substrates.
Figure 2.12: Tm4 decrease affects additional protein localization. (A) Osteoclasts plated on ivory showed reduced localization of the V-ATPase within the actin ring in 20% of the Tm4 siRNA-treated osteoclasts. Control panels show the V-ATPase enhanced within the actin ring while the Tm4 siRNA treated cell lacks the specific localization of the V-ATPase. (B) Control osteoclasts on glass show α-actinin (green/left) localizes around the podosomes (red/middle) at the base of the podosomes but the Tm4 siRNA-treated cells show a distribution of α-actinin to the core of the podosomes at the podosome base. (C) Osteoclasts on bone show that α-actinin (green) localizes to the top third of the actin ring but when the Tm4 is reduced in the cell, the α-actinin (green) is reduced to just the middle of the thinner ring.
Figure 2.13: Overexpression increased podosome height while decreasing migration. Tm4 without the 3'UTR was cloned into a mammalian expression vector and overexpressed in RAW264.7 cells. (A) Western analysis of individual clones that were selected giving up to a two fold increase of Tm4 expression (top panel). GAPDH and beta actin were also blotted as loading controls (lower panels). (B) Confocal pictures of the control and Tm4 cells lines stained for F-actin. The Tm4 clones gave a disperse patterning of podosomes demonstrating the mislocalized, thickened podosomes not seen in the control. Scale bars=10µm. (C) Podosome height was measured by Z-stack analysis and the average height shows the increase in the podosome height of the Tm4 clones (mean +/- s.d.). (D) Overexpressing cells were stained with α-actinin (left panels-red) and paxillin (right panels- red) along with actin (green) to reaffirm the extra actin structures to be podosomes. Scale bars=10µm. (E) Transwell migration was used to measure the migration ability of the clones and the average migration decreased 60% (mean +/- s.d.). (F) Psuedo-actin rings formed by RAW264.7 cell control and Rl clone costained with actin (left) and V-ATPase (right). Scale bars=10µm.
Figure 2.14: Over-expression of Tm4 causes disruption of bone resorption. Control and Tm4 expressing osteoclasts were plated on thinly sliced ivory. (A) Control and Tm4 clones were stained with phalloidin to visualize F-actin. Confocal Z-stack images were taken and the base of the cell is shown where the actin ring should be as it is in the control but not in Tm4 clone 2.2. Along with disrupting the base actin structures, there are abnormal actin patches at the cell membrane. Arrows indicate the abnormal F-actin patches in membrane. (B) Rt 1.2 clone stained to show colocalization of Tm4 (right) with extra membrane actin patches (left) in osteoclasts. (C) V-ATPase expression was imaged with antibodies recognizing α3subunit (right) and pictured to show no specific V-ATPase localization (left) in Tm4 clones (D) Resorption was measured on BD osteological discs and under normal conditions of three days (left bars) show the Tm4 clone produced no resorption. When the mature cells were left on the discs for eight days there was little resorption totaling 9% of the control (right bars) (mean+/−s.d.).
Figure 2.15: Tm4 interacts directly with one protein in osteoclasts. (A) Immunoprecipitation using S35 labeled cells shows when the actin is not depolymerized (left ln) Tm4 binds 5 proteins but after G-buffer actin depolymerizer (right) Tm4 binds only TRIP6. (B) Western analysis of IP samples using mouse antibodies to Tm4, TRIP6, and MOPC then probed with rabbit antibodies show that Tm4 interacts with myoIIA, TRIP6, Actin, Tm4 and Tm5ab. (C) On glass osteoclasts were stained for TRIP6 (red, middle) to show the distribution in relation to F-actin (green, left). (D) On bone osteoclasts were stained for TRIP6 (red, middle) to show its distribution in relation to F-actin (green, left). Confocal z-scan shows the distribution of TRIP6 in the actin ring along with a zoom of the actin ring to show TRIP6 only loosely intercalated into the ring.
CHAPTER 3

MYOSIN IIA REGULATES OSTEOCLAST FUSION AND FUNCTION

3.1 Conventional Myosins

Conventional, or class II, myosins were the first described and largest class of myosin proteins in vertebrates. In muscle cells, class II myosins are highly studied for their function in muscle contraction and consist of at least 38 known isoforms in animals (Sellers 2000, Berg 2001). All class II myosin dimers consist of two heavy chains, and four regulatory light chains. The class II myosin consists of an N-term globular head with an ATP based actin binding domain and a C-terminal tail rod domain. Class II myosins self associate by dimerizing the two heavy chains to form a coil-coiled between the tail end of the myosin. The coiled-coils form bipolar myofilaments which bind actin at the head domain and additional myosin II proteins at the tail domain, and can move actin in an ATP dependent manner. Class II myosins can be proteolytically cleaved between the head and tail to form the S1 domain consisting of the actin binding head and light chains. Another cleavage site resides 40kDa into the tail coiled-coil tail and

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2 The results of this chapter are in preparation for manuscript submission.
produces the S2 domain, consisting of the actin-binding head and part of the coiled-coil, also known as heavy meromyosin (HMM) (Sellers 2000).

While all class II molecules are composed of two heavy chains, two essential light chains, and two regulatory chains, their unique activities are a function of their particular heavy chain isoforms. Although the nonmuscle heavy chain isoforms share extensive structural homology, they have been shown to demonstrate distinct patterns of expression (Maupin 1994, Conrad 1995, Rochlin 1995, Kolega 1998), enzyme kinetics and activation (Kovacs 2003, Rosenfeld 2003, Wang 2003, Golomb 2004), and cellular function (Cai 2006, Sandquist 2006, Swailes 2006). There are three known non-muscle forms of class II myosins from three separate genes: MyoIIA (myh9), MyoIIB (myh10), and MyoIIC (myh14) (Berg 2001). These isoforms are 64-90% similar in amino acid sequence (Golomb 2004, Leal 2003). Studies in various non-muscle cell types have shown that MyoIIA and MyoIIB localize to stress fibers and lamellipodia (Maupin 1994). Due to their biochemical differences, MyoIIA localizes first to these structures and disappears first in lamellipodia leading MyoIIB to be present more often in the trailing edge while MyoIIA is distributed in the leading edge (Kolega 1998). In osteoclasts, we previously demonstrated that myosins IIA and IIB localize to distinct subcellular regions within osteoclasts, with MyoIIA strongly segregating to both podosomes and the actin ring of the sealing zone (Krits 2002).

3.2 Non-muscle MyoIIA

Non-muscle myosin IIA (MyoIIA) has been implicated in many cellular functions. It had been highly studied in non-muscle cells where it assists in cell
migration by holding the tension needed within the cell during the migration process (Clark 2007). MyoIIA is the only isoform present in platelets but is expressed with both myoIIB and myoIIC in many cell types. Several labs have tried unsuccessfully to make myosin IIA knock-out mice for further studies. Conti et al. found the homozygous knockouts were embryonic lethal on day E7.5 due to a defect in cell-cell adhesion. Embryoid bodies removed for study from knock-out mice constantly shed cells and failed to adhere to each other (Conti 2004). MyoIB knock-out mice are also non-viable as they die from heart and brain defects before or shortly after birth (Tullio 1997).

In vitro studies of MyoIIA have found MyoIIA to be involved in a wide variety of cellular functions, including cytokinesis, cell contractility, and adhesion and motility. When MyoIIA was decreased via RNAi in breast cancer cells, migration and cell spreading decreased (Betapudi 2006). When MyoIIA light chains were inhibited, motility was also inhibited (Gillespie 1999). In fibroblasts, where MyoIIA extensively co-localizes with stress fibers, a decrease in MyoIIA led to increased migration and defective stress fibers (Even-Ram 2007).

Myosin IIA has also been demonstrated as important in cell division. Knecht et al. utilized antisense RNA to decrease myoII levels in dictyostelium and the cells became multinucleated due to failed cell division (Knecht 1987). Further studies showed that the dictyostelium elongated as they proceeded through the mitosis steps but failed to form a cleavage furrow properly which led to their multinucleation (Zang 1997). In muscle cells, MyoIIA is only temporarily expressed immediately before cell division, and then degraded once the cell has divided (Grainger 1991). Immunolabeling of MyoIIA in
dividing cells demonstrated its localization in the cleavage furrow during mitosis and meiosis (Simerly 1998).

3.2.1 Myosin IIA Diseases

Myosin IIA is the only non-muscle myosin that is mutated in several human diseases, which share similar phenotypes. Recently, these diseases were reclassified together as myh9 associated diseases that include May-Hegglin, Fletcher syndrome, Sebastian syndrome, and Epstein syndrome (Heath 2001). Symptoms of these diseases include giant platelets, thrombocytopenia, and granulocyte inclusions along with a range of additional symptoms specific to each disease. Documented mutations ranging from a.a.371- a.a.1933 include the motor domain and the coiled-coil tail and either stabilize or destabilize the protein and its interactions. The two prevalent myh9 symptoms are in the cell types that only express MyoIIA and not any other class II myosin isoforms: granulocytes and megakaryocytes. In granulocytes the mutations cause a dominant negative effect while in megakaryocytes it is a haploinsufficient effect (Pecci 2005). Granulocytes and megakaryocytes both differentiate from hematopoietic precursors, as do osteoclasts. As mentioned previously, MyoIIA knock-out in mice lead to embryonic lethality, so researchers have yet to find a mouse model to study myh9 diseases to further classify potential phenotypes.

3.3 Myosin IIA Localization in Osteoclasts

Previously in the lab, MyoIIA and MyoIIB were found to localize to distinct F-actin pools in osteoclasts. While MyoIIA localized to the osteoclast attachment structures, MyoIIB was completely absent in these regions. To examine further, I utilized

70
confocal imaging to determine the precise location of MyoIIA in osteoclast attachment structures. Using isoform specific antibodies and phalloidin F-actin staining, I found that MyoIIA distinctly localizes to the cloud of actin surrounding the podosomes (Figure 3.1A). Confocal z-sectioning shows that MyoIIA is distributed around and over the top of the podosomes but is completely absent from the podosome core (Figure 3.1A, right panel). On bone, MyoIIA is distributed over the interior face of the actin ring (Figure 3.1B). Because of this distribution into osteoclast adhesion structures and findings in other cells showing MyoIIA to be associated with dynamic Rho-kinase dependent functions such as adhesion and locomotion, we hypothesized that MyoIIA may play a vital role in cell motility and the bone resorption function. In this study we have examined cellular expression of MyoIIA during osteoclastogenesis, and along with RNAi-mediated suppression of the protein, have confirmed its role in cell spreading, motility, and sealing zone formation. However, this study also unexpectedly revealed a role for MyoIIA in regulating pre-osteoclast fusion during osteoclastogenesis.

3.4 Expression of MyoIIA during Osteoclastogenesis

As a first step in examining the role of MyoIIA in osteoclast function, RAW264.7 macrophages or mouse bone marrow osteoclast precursors were differentiated in vitro over a seven-day culture period. Western analysis demonstrated that differentiating osteoclasts temporarily decreased their cellular levels of the MyoIIA heavy chain during the middle of this differentiation period, corresponding to the time when precursor cells cease mitosis and begin to fuse (Tanaka 1993b, Takahashi 1994). Figure 3.2A shows representative Western blots of this process in RAW264.7 cells, while the graph in Figure
3.2B shows quantitation of three such experiments each for RAW264.7 cells and mouse marrow precursors. In both cases, MyoIIA levels decreased by about half by day 4 of culture, and returned to baseline levels by day 7. In contrast, levels of β-actin and the housekeeping protein GAPDH remained constant (Fig. 3.2A). Additionally, expression levels of the MyoIIB isoform were unchanged over the same period. To determine the mechanism behind this transient decrease, MyoIIA heavy chain mRNA levels were assayed by competitive RT-PCR. As shown in Figure 3.2C, these remained steady during the differentiation period, indicating that the temporary loss of MyoIIA resulted from a translational or post-translational mechanism. To further define this mechanism, pulse-chase analysis was performed on differentiating RAW 264.7 cells at days 0, 3, and 7 of culture to determine MyoIIA heavy chain half-life. Figure 3.2D shows that while day 0 and day 7 cells exhibited only a 10-20% loss of MyoIIA after nine hours of chase (following a two hour pulse), the day 3 cells lost ~70% of their labeled MyoIIA over the same time period. These results demonstrate that the temporary decrease in MyoIIA expression during mid-osteoclastogenesis is due to increased degradation of the protein.

3.4.1 MyoIIA Degradation is Cathepsin B Mediated

Differentiating osteoclasts were tested with a panel of specific protease inhibitors to identify the class of enzyme responsible for the transient decrease in MyoIIA expression. RAW264.7 cells on day 3 of culture were treated with a panel of these compounds for 6 hours. The proteasomal inhibitors MG132 and ZLLF did not suppress MyoIIA degradation, nor did the cysteine protease inhibitor E64 (Figure 3.3A), or the specific calpain inhibitor calpeptin. A single non-specific inhibitor of cysteine proteases,
ALLM, produced a slight suppression of degradation. However, because ALLM is established as having a degree of reactivity with cathepsins B and L, cathepsin inhibitor I (which inhibits B, and to a lesser extent, L) and cathepsin L inhibitor I also were tested on differentiating osteoclasts. Cells treated with cathepsin inhibitor I expressed MyoIIA at levels greater than that of control cells, while cathepsin L inhibitor I did not (Figure 3.3A). These data indicate a role for cathepsin B (catB) in mediating MyoIIA degradation. Previous studies (Blair 2000) have demonstrated increasing levels of both catB protein and activity in lysates from differentiating human osteoclast precursors. I confirmed and extended these studies by taking advantage of a commercially available cell-permeable cathepsin B substrate that fluoresces upon cleavage, allowing visual identification of active catB within live cells. Examination of osteoclasts at varying stages of maturation demonstrated a distinct rise in active catB levels when osteoclast precursors shift from a mononucleated to multinucleated stage. Figure 3.3B shows an example of day 3 osteoclast cultures containing a mixture of mononuclear (open arrowheads) and multinuclear (closed arrowhead) cells. Additionally, it illustrates the intense catB activity present in mature, 7-day osteoclast cultures. Quantification of staining intensity in day 3 cultures revealed a clear rise in active cathepsin B levels as pre-osteoclasts undergo multinucleation (Figure 3.3B, right). This rise in activity temporally correlates with the loss of MyoIIA expression demonstrated in Figure 3.2A. These results suggest a role for cathepsin B in mediating the regulated proteolysis of MyoIIA during mid-osteoclastogenesis. These results were confirmed by a time course experiment in which cathepsin inhibitor I was added to cultures during days 1-4 of
osteoclast differentiation. Addition of the inhibitor on a daily basis maintained MyoIIA protein at baseline levels (Figure 3.3C).

To determine whether inhibition of cathepsin B activity had any effect on osteoclast formation, cathepsin inhibitor I was added to day 3 pre-osteoclasts and the resulting cells were examined the next day. Figure 3.3D illustrates that inhibitor-treated cells showed a significant decrease in multinucleation, relative to vehicle-only controls. Further, to determine the effects of catB gain-of-function, a murine cDNA cloned was obtained by RT-PCR and overexpressed in RAW264.7 macrophages (Figure 3.3E, left). Upon differentiation into mature osteoclasts, catB overexpressing cells demonstrated an 84% increase in nuclear number, with a similar accompanying increase in cell perimeter (Figure 3.3E, right). These results suggest that cathepsin B activity has an effect on osteoclast fusion, potentially by regulating proteolysis of MyoIIA.

3.5 RNAi Mediated Decrease of MyoIIA

To determine whether cellular modulation of MyoIIA levels might affect osteoclast differentiation, RNA interference was used to knock down MyoIIA expression in the latter half (day 4 onward) of the osteoclast differentiation process, thus preventing recovery of its expression levels after the normal transient decrease. Two siRNAs specific to the MyoIIA heavy chain were designed and tested for efficacy in both RAW264.7 and mouse marrow cells, as were three control double-stranded oligonucleotides. Control C0 was a commercially purchased non-targeting dsRNA oligonucleotide, while controls C1 and C2 were mutated versions of siRNA1 and siRNA2, respectively. Cells were transfected on day 4 of culture and assayed for
MyoIIA mRNA and protein levels two and three days later, respectively. Figure 3.4A (top panel) demonstrates that both targeting siRNAs diminished levels of MyoIIA mRNA, while the three control dsRNA oligonucleotides had no effect. The lower panels of Figure 3.4A confirm a significant loss of MyoIIA protein after siRNA treatment, while cellular GAPDH and β-actin levels remained constant. The remaining panels of Figure 3.4 illustrate the time course of diminished MyoIIA expression achieved with siRNA1. Notable decreases in MyoIIA mRNA were evident as early as one day post-transfection (Figure 3.4B), with suppressed protein levels evident one day later (Figure 3.4C). GAPDH and β-actin levels were unaffected over the length of the time course.

Quantitation of multiple experiments with siRNA1 showed MyoIIA protein to drop to ~60% of control levels by two days post-transfection in both RAW264.7 and marrow cells, and to diminish further to about 30% of controls by five days post-transfection (Figure 3.4D). In a single time course experiment, similar results were obtained with siRNA2 in RAW264.7 cells. These results demonstrate that MyoIIA levels can be efficiently and specifically suppressed in the latter half of the osteoclast differentiation process.

### 3.5.1 Increased Cell Perimeter Due to Fusion and Cell Spreading

An immediately obvious consequence of MyoIIA suppression was the generation of osteoclasts with larger surface areas than normal osteoclasts or control-transfected cells. Generation of podosomal actin cores appeared unaffected. Figure 3.5A shows photomicrographs of phalloidin-labeled control- and siRNA-treated cells, demonstrating the large size of cells subjected to RNAi. The left and center panels illustrate typical
fields of control- and siRNA-treated cells, while the right panel shows an example of the enormous size to which the siRNA-treated cells could develop (all photos are at the same scale). These differences were quantified in Figure 3.5B, which shows that for both siRNA1- and siRNA2-treated cells, average cell perimeter roughly doubled when compared to controls. Although this figure illustrates only results using the control C0 oligonucleotides, further experiments showed that transfection with control oligonucleotides C1 and C2 produced osteoclasts of the same size as C0-transfected cells.

To determine whether the large siRNA-treated cells were the product of increased pre-osteoclast fusion, control- or siRNA-treated cells (at 4 days following transfection) were labeled with bisbenzimide, and their nuclei were enumerated. Figure 3.5C illustrates that nuclear number approximately doubled in either RAW264.7 or marrow cultures when cells were treated either with siRNA1 or siRNA2. While the increased nuclear number is indicative of enhanced cell fusion in the siRNA-treated cells, it has been demonstrated that inhibition or suppression of myosin II results in failure of cytokinesis (Straight 2003; Straight 2005), suggesting the formal possibility that the increased nuclear number in siRNA-transfected cells may be a result of nuclear replication without accompanying cell division. We considered this possibility to be unlikely because our differentiating osteoclasts were not transfected with siRNAs or controls until day 4 of osteoclastogenesis, when cell fusion had begun and the cells presumably were already post-mitotic. However, to formally assess this possibility, control- and siRNA-transfected cells were treated with the DNA replication inhibitor hydroxyurea for 3 days (starting at the time of transfection, i.e. day 4 of culture) to inhibit any ongoing mitosis.
Hydroxyurea had no effect on resulting cell size, indicating that the increased nuclear number seen in siRNA-treated cells was a result of precursor fusion, not aborted cytokinesis. Additionally, to determine whether siRNA-treated cells might undergo greater fusion than controls due to enhanced cell survival, nuclear number and apoptosis (via TUNEL assay) were measured in control and siRNA-treated samples. No significant differences were seen.

Although these data demonstrate a role for MyoIIA in mediating osteoclast fusion, numerous studies in other cell types have suggested a role for this protein in cell spreading (Wylie 2003, Betapudi 2006, Cai 2006, Takizawa 2007). Therefore, I sought to determine whether the larger surface area of siRNA-treated cells was a result solely of increased pre-osteoclast fusion or whether cell spreading also was affected. I first investigated whether I could quantify a relationship between nuclear number and cell perimeter in normal osteoclasts generated from RAW264.7 cells. A survey of ~300 cells at different stages of maturation did indeed indicate that cell perimeter increases proportionately to nuclear number (Figure 3.5D, WT cells). In further experiments, control- and siRNA-treated cells also were examined for the relationship between nuclear number and cell perimeter. These cells were transfected on day 4 of differentiation and assayed 4 days later, using the same protocol as that described in Figure 3.4. As shown in Figure 3.5C, control-treated cells, which possessed up to 16 nuclei, had a slightly greater perimeter than wild-type cells of the same nuclear number. This increased spreading may be attributable to the lipid-based transfection methods used in this study. In contrast, siRNA-treated cells, which possessed up to 28 nuclei, demonstrated
perimeters that were similar to control cells at low nuclear number, but increased rapidly as nuclear number increased. These results, which were very similar for both siRNA1 and siRNA2, show that loss of MyoIIA increases not only cell fusion, but also cell spreading in highly multinucleated cells, resulting in formation of extremely large osteoclasts such as that shown in Figure 3.5A. Nearly identical results were obtained from marrow-derived osteoclasts.

To further characterize the increase in cell spreading due to suppression of MyoIIA, I assessed the microtubule acetylation. Acetylation is an indicator, though not a cause of, microtubule stability. In osteoclasts, microtubules play a role in podosome patterning, and as the cells increase in perimeter and form podosome belts during osteoclast maturation, levels of microtubule acetylation increase (Destaing 2005). Recently it was discovered that microtubules and myosin IIA affect each other in that as MyoIIA levels decrease, microtubule acetylation increases in a stress fiber based cell type (Even-Ram 2007). I assessed the siRNA-treated osteoclasts for changes in microtubule acetylation. Control cells exhibited a high amount of acetylation at the base of the cell within the podosome belt, as expected in mature 7-8 day old osteoclasts (Figure 3.6A, top panel). MyoIIA siRNA-treated cells, on the other hand, have slightly less acetylation when the same average perimeter osteoclast was viewed, but when the extremely large cells were viewed the absence of acetylation was even more striking (Figure 3.6A, lower panels). Western analysis confirmed that over the four day post transfection time course in control cells, microtubule acetylation increased (as it would as the cell matured and grew in size) (Figure 3.6B, left). On the other hand, over the same 4 day post
transfection period, the siRNA-treated cells increased dramatically in perimeter, but microtubule acetylation levels stayed even or possibly slightly decreased (Figure 3.6B, right).

To further support the role of MyoIIA in cell fusion, in separate experiments undifferentiated RAW264.7 cells were transfected with the control or siRNA1 on day 0 of differentiation. One set of experiments left the cells for seven days without RANKL treatment and in the second set of experiments, the RAW264.7 cells were immediately differentiated by the addition of RANKL for two days before viewing. For both conditions, the resulting cells were stained with phalloidin for F-actin and bisbenzamide to visualize the nuclei along with TRAP staining to indicate the formation of osteoclasts. Without RANKL stimulation, MyoIIA siRNA-treated cells formed multinucleated cells which were not TRAP positive (Figure 3.7A). MyoIIA is known to play a role in cell division, so the average nuclear number was counted to see if the increase was due to bi-nuclear cells from failed cell division. Nuclear number indicated that there was cell fusion occurring at the suppression of MyoIIA; the average number of nuclei for MyoIIA siRNA-treated cells was 3.7 nuclei per cell versus the expected two resulting from a failed cell division (Figure 3.7A, graph). These multinucleated cells were not TRAP positive indicating they are either osteoclast precursors or giant cells of fused macrophages. Second, early suppression of MyoIIA with the addition of RANKL leads to an increase in the average nuclear number after only two days of differentiation. The control cultures had only 10 multinucleated TRAP positive cells (osteoclasts) after two days of RANKL treatment with an average 2.3 +/- 0.3 (mean +/- sem) which would be
expected from the short time of differentiation (Figure 3.7B). The cells, which were first treated with siRNA1 to decrease the MyoIIA prematurely during differentiation, showed 26 multinucleated TRAP positive cells (osteoclasts) with an average 4.5+/-0.4 (Figure 3.7B). The siRNA treatment prior to differentiation produced an increase in the nuclear number of osteoclasts beyond normal.

### 3.5.2 MyoIIA Suppression Leads to Larger Actin Rings

Because of MyoIIA’s presence in the actin ring (sealing zone) of polarized osteoclasts, I sought to determine whether actin ring formation was affected by suppression of its expression. When plated on bone, siRNA-treated cells demonstrated obviously larger actin rings than control cells (Figure 3.8A). The increased size of actin rings is demonstrated graphically in Figure 3.8B. To determine whether the enlarged actin rings were a consequence of the enhanced overall size of the cells or a direct effect of MyoIIA suppression on ring formation, I first examined the relationship between cell size and actin ring circumference in normal polarized RAW264.7-derived osteoclasts on bone. As demonstrated in Figure 3.8D (WT cells), it was determined that there exists a relationship between nuclear number and ring circumference; that is, larger cells tend to generate larger rings. When control- and siRNA-transfected cells were assayed for nuclear number and actin ring size, the control cells behaved similarly to wild-type. In contrast, siRNA-treated cells generated actin rings approximately 25% larger than expected, indicating a modest but significant effect of myosin IIA suppression on actin ring size. Both siRNAs produced similar results; however, only siRNA1 is shown for figure clarity. Additionally, siRNA1 was tested on marrow-derived osteoclasts. This
treatment produced results similar to those in RAW264.7 cells; the actin rings generated by the siRNA were ~38% greater in perimeter than control transfectants. Along with larger actin rings, rarely I found the osteoclasts would exhibit multiple rings (Figure 3.8C). This could be a unique actin ring phenotype or a time sensitive method where as the multiple normal sized rings at a later point develop into the large actin rings measured in Figure 3.8A/B.

3.5.3 Suppression Leads to Decrease Motility without Altering Resorption

Because myosin IIA plays roles both in cell adhesion and formation of the sealing zone, I assessed the effects of its suppression on cell motility and bone resorption. Most motility assays are dependent on cell size, relying either on the ability of cells to migrate through filters of a particular pore size, or of their ability to migrate and generate a path over a given surface area. Because normal MyoIIA-suppressed cells are much larger than their control counterparts, I first needed to produce culture conditions in which the siRNA-treated cells produced were of similar size as the controls. To achieve this, differentiating RAW264.7 osteoclast cultures were washed extensively just prior to transfection with either anti-MyoIIA or control- siRNAs. This treatment resulted in removal of mononuclear cells (which adhere to culture plates less firmly than osteoclasts), and inhibited further cell fusion. The resulting cultures produced cells very similar in nuclear number and perimeter (control: 4.4 +/- 2.8 nuclei, 501.4 +/- 206.0 microns, n = 47; siRNA1: 4.8 +/- 2.5 nuclei, 508.1 +/- 216 microns, n = 53). When tested in Transwell migration assays (Figure 3.9A), the siRNA-treated cells showed
significantly diminished motility (inhibition of ~35-50%), indicating a role for MyoIIA in cell osteoclast migration.

Bone resorption in MyoIIA-suppressed cells was tested in two types of assays. The resorptive capacity of osteoclasts was assayed on synthetic bone substrate (Figure 3.9B) or on ivory slices (Figure 3.9C). In neither case was resorption affected by the siRNA1-mediated loss of MyoIIA expression, in spite of the diminished motility of these cells. We expect that maintenance of resorptive capacity may be a function of the siRNA-treated cells possessing more or larger sealing zones, which could counteract the effects of lowered motility.

3.6 Discussion of MyoIIA

The roles of myosin IIA in cell function have been studied extensively in model systems from lower eukaryotes to mammalian cells. However, this study is the first detailed examination of MyoIIA activity in cells that use podosome-based structures for adhesion to a substrate. Although modulation of osteoclast spreading and motility was an expected effect of MyoIIA suppression, we also found that this myosin plays a crucial role in mediating cell fusion.

Alteration of the actin cytoskeleton is a critical process in cell-cell fusion. For example, myoblast fusion is dependent on Rac-mediated regulation of actin polymerization via a WASP (Wiskott-Aldrich syndrome protein) family member (Kim 2007, Massarwa 2007). Fusion-related cytoskeletal rearrangements have been implicated in events ranging from migration to cell adhesion to the fusion process itself (rev. in (Chen 2007). Myosin II activity appears to be necessary for mediating cadherin-based
contact in both Drosophila embryos (Bertet 2004) and cultured mammalian epithelial
cells (Krendel 1999). Further, embryoid bodies isolated from mouse embryos deficient in
MyoIIA showed decreased cell-cell adhesion with reduced E-cadherin staining at the
contacts (Conti 2004). More recently, MyoIIA was shown to be required for changes in
cell shape and adhesion essential for myoblast fusion (Shewan 2005). However, in these
models myosin II appeared to play a positive role in cell-cell contact and fusion, while
our study demonstrates a negative effect of MyoIIA on osteoclast fusion. These
apparently contradictory findings are understandable, however, when one compares the
morphologies of osteoclasts and epithelial- or fibroblast-like cells. Osteoclasts, unlike
most other cell types, do not express stress fibers; nor do they possess an intense
meshwork of cortical actin. Their adhesion structures turn over rapidly, and they possess
the ability to rapidly polarize and depolarize. Further, cadherins have not been
implicated in mediating cell-cell contact required for osteoclastogenesis. Therefore, it is
not surprising that MyoIIA may play an entirely different role in osteoclast function than
in the cells that have been studied to date. Although the structural components regulating
pre-osteoclast fusion are not well understood, in recent years a number of candidate cell
surface molecules have been implicated, including receptors CD44 (Sterling 1998, Cui
2006), CD47 and its ligand macrophage fusion receptor (MFR, also known as signal
regulatory protein alpha, SIRP) (Saginario 1998, Han 2000, Lundberg 2007), the
purinergic receptor P2X7 (Steinberg 2007), and the disintegrin and metalloproteinase
ADAM8 (Choi 2001). A recently identified seven-transmembrane-spanning receptor, the
dendritic cell-specific transmembrane protein, DC-STAMP, is essential for osteoclast
fusion both in vitro and in vivo (Kukita 2004, Yagi 2005). More recently, the d2 subunit of proton-translocating V-ATPases, a membrane subunit isoform expressed predominantly in osteoclasts, similarly was demonstrated to be required for fusion in vitro and in vivo (Lee 2006). The mechanism by which myosin IIA suppresses cell fusion is unclear. One possibility is that MyoIIA, which is distributed fairly homogenously in monocytic/macrophage osteoclast precursors, may provide intracellular tension or even a physical barrier that prevents fusion from occurring. Alternately, MyoIIA may play a role in limiting cell-cell contact in these precursors through interaction with cell surface proteins. For example, we previously showed that osteoclast V-ATPases, which contain the d2 subunit required for fusion in vitro and in vivo, directly bind a complex of F-actin and MyoIIA ((Lee 1999) and unpublished data). It is possible that myosin IIA may play a role in fusion by interacting with membrane proteins such as d2, DC-STAMP, CD44, and ADAM8. In another study, MyoIIA antisense treatment also led to defects in membrane resealing in cells (Togo 2004). It is also possible that MyoIIA is responsible for the mechanism of sealing and resealing the membranes during cell fusion as it is correctly orientated at the cell periphery in normal osteoclasts.

The role of cathepsin B in regulating MyoIIA expression is at first examination somewhat surprising, given that this protease exists in cells primarily in lysosomes or on the cell surface, while MyoIIA is a cytosolic protein. However, this study is not the first demonstration of a role for cathepsin B as a key regulator in multinucleated cell formation. Multiple reports have implicated cathepsin B in promoting myoblast fusion. First, cathepsin B activity increases during fusion of postmitotic myoblasts into myotubes...
Further, diminution of cathepsin B levels by either retroviral gene trapping or cell-permeable inhibitors negatively affects myoblast fusion (Jane 1994, Jane 2002a). These findings have obvious parallels to those described in this study. Most recently, cathepsin B has been shown to localize to the cytoplasmic face of the plasma membrane of differentiating myoblasts at caveolae, suggesting a role in mediating cytoskeletal rearrangements required for cell fusion (Jane et al., 2006). Although we have not yet determined whether cathepsin B is capable of directly degrading nonmuscle myosin IIA, it is well established that the related skeletal muscle myosin II heavy chain is exceedingly susceptible to the effects of this protease (Schwartz 1977), suggesting a potential direct effect. Moreover, a panel of protease inhibitors very similar to ours was used to examine the enzymatic basis for proteolytic breakdown of actin-rich dendritic spines in N-methyl-D-aspartate-stimulated neurons. Only inhibitors of cathepsin B were capable of preventing this degradation, and the cytosolic protein MARCKS (myristoylated alanine-rich C kinase substrate) was shown to be a target of the enzyme (Graber 2004). Thus, cathepsin B-mediated degradation of cytoskeletal proteins may be a universal mechanism by which extensive changes in cell shape are mediated.

In addition to our findings showing a role for MyoIIA in osteoclast precursor fusion, I also showed it to inhibit osteoclast spreading. These results are consistent with those obtained from epithelia-derived carcinoma cells, in which siRNA-mediated knockdown of MyoIIA resulted in more extensive cell spreading and dynamic protrusions than controls (Sandquist 2006). Moreover, siRNA-mediated knockdown of MyoIIA in either embryonic stem cells or human foreskin fibroblasts produced cells that
were flattened and possessed long protrusions (Even-Ram 2007). However, the precise mechanisms by which epithelia-derived cells and fibroblasts utilize MyoIIA for maintaining cell shape necessarily differ from those of osteoclasts. In the former cell types, MyoIIA is present in stress fibers that terminate in focal adhesions, and is required to mediate cell tension and contractility (Chrzanowska-Wodnicka 1996, Zamir 2000). The role of MyoIIA in osteoclasts is less clear. Osteoclasts form neither stress fibers nor focal adhesions, but do generate a meshwork of MyoIIA-containing actin fibers that appear to connect networks of podosomes (Krits 2002). I also demonstrated that siRNA-treated osteoclasts are capable of generating sealing zones. However, our current study demonstrates that suppression of MyoIIA causes osteoclasts to form sealing zones 25-40% larger than expected, based on cell size. These findings are consistent with the notion that MyoIIA regulates contractility, and thus, spreading of the actin ring, as well as spreading of the entire cell.

In Transwell migration assays, MyoIIA-suppressed cells demonstrated lower motility than control cells. However, my examination of cell motility was somewhat hindered by the increased size of the siRNA-treated cells relative to controls. Although we attempted to correct for size issues with my culture conditions, we were limited to examining only small osteoclasts (~4 nuclei) under these conditions. A more detailed examination of cell migration will be possible using time-lapse recordings. Indeed, using these types of measurements, it was recently possible to demonstrate that MyoIIA-suppressed human foreskin fibroblasts showed enhanced random migration, but decreased directional migration (Even-Ram 2007). This latter finding is consistent with
our results, in which directional mobility across a membrane was assessed. It would be of interest to determine how overall migration velocity may be affected in siRNA-treated osteoclasts.

In summary, this study revealed a not-unexpected role for myosin IIA in mediating osteoclast spreading, motility, and sealing zone formation, but also indicated a somewhat surprising function in regulating cell fusion during late osteoclastogenesis. These data also suggest a role for cathepsin B as a regulator of multinucleation in osteoclasts. Future studies to elucidate the mechanisms behind these events should generate new insights into the process of regulated cell fusion.
Figure 3.1: Myosin II A is strongly localized to adhesion structures of osteoclasts. Osteoclasts were cultured on glass coverslips (top panels) or thinly sliced ivory (bottom panels). (A) Myosin II A (green/left) colocalizes around the phallloidin stained podosomes. Z-Stack analysis shows that MyoII A localizes around and the top of the podosome (far right). (B) During resorption MyoII A is concentrated in the actin ring and Z-stack images demonstrate that MyoII A is present at the inner face of the sealing zone.
Figure 3.2: Myosin IIA is transiently and post-translationally downregulated during osteoclastogenesis. (A) Osteoclasts were cultured to maturity over a seven-day period. Cells lysates probed on immunoblots for MyoIIA, GAPDH, and β-actin show a transient decrease in MyoIIA during mid-osteoclastogenesis. (B) Multiple experiments like that in panel A, for both RAW264.7- and marrow-derived cells, were quantified. Results shown are the mean of three experiments + std dev. (C) MyoIIA mRNA levels during osteoclastogenesis were examined by competitive RT-PCR. The upper band represents the signal from MyoIIA mRNA, while the lower band represents an internal standard. (D) Pulse-chase analysis was performed to determine the half-life of MyoIIA protein on days 0, 3, or 7 of RAW264.7 culture. Results shown are the average of three experiments +/− std dev.
A

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D

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CatB  
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per cent of control

Nuclei  
Perimeter
**Figure 3.3: Inhibition of cathepsin B during osteoclastogenesis prevents loss of myosin IIA and inhibits multinucleation.** (A) Inhibitors of calpain (ALLM), protease (E64D), proteosome (ZLLF, MG132), Cathepsin (CathI), or DMSO (vehicle) were added to day 3 osteoclast cultures for 6hrs. Western analysis shows that the inhibitor CathI caused an increase in the levels of myosin IIA protein compared to the vehicle control with beta actin to show equal loading between samples. (B) Magic Red cathepsin B substrate was use to localize active cathepsin B in live day 3 and day 7 osteoclast cultures. Cathepsin B is stained in red and the nuclei in blue. Open arrows indicate the mononuclear cells in the culture which exhibit much less active cathepsin versus the closed arrows pointing to the multinucleated cells. The middle panel shows a closer view of the cathepsin B substrate in a multinucleated cell. The right panel shows day 7 osteoclasts which exhibit a larger amount of active cathepsin B. The intensity of cathepsin B was measured on day 3 and cells with more than one nucleus had almost three times the amount of active cathepsin B. (mean +/- s.d.) (C) A timecourse of MyoIIA expression demonstrates that continual addition of cathepsin inhibitor I to differentiating cultures inhibits suppression of MyoIIA levels. (D) Addition of cathepsin inhibitor I to cultures on days 3-4 results in suppression of osteoclast multinucleation.(mean +/- s.d.) (E) Western analysis shows catB expression in clonal RAW264.7 macrophages stably transfected with either empty vector (C) or catB-expressing plasmid (1-3). Only clones 2 and 3 demonstrated overexpression of catB and were chosen for analysis. The graph on the right demonstrates average relative nuclear number and cell perimeter for mature osteoclasts derived from clone 2 or the empty vector control. Clone 3 showed similar result. (mean +/- s.d.)
Figure 3.4: Myosin IIA siRNA mediated knock-down. (A) Two days post transfection siRNA1 and siRNA2 decrease the RNA expression measured by competitive RT-PCR with internal standard (I.S.) compared to controls C1 for siRNA1, C2 for siRNA2 and C0 is a non-specific control (top panel). Western analysis for the same controls and siRNA along with GAPDH and beta actin loading controls show the decrease in protein expression three days post transfection (bottom panels). (B) The RNA decrease occurs one day post transfection and remains decreased over four days post transfection measured by RT-PCR with internal standard (top panel). Protein was measured over the same time course via Western blot. (C) The protein decrease was measured by Western analysis two to five days post transfection to show that the protein remained decreased over the same time course (mean +/- s.d.).
Figure 3.5: Suppression of myosin IIA during late osteoclastogenesis results in increase fusion and spreading. (A) Control- or siRNA1-transfected cultures were labeled with fluorescent phalloidin to show the peripheral podosome belt. Scale bar = 200 µm. (B) RAW264.7- or marrow-derived osteoclasts were treated with control oligonucleotides, siRNA1, or siRNA2. The perimeters of siRNA-treated marrow cells were statistically different from controls, as were the perimeters of siRNA-treated RAW264.7 cells. (C) Nuclei were enumerated in RAW264.7- or marrow-derived osteoclasts treated with control or siRNA oligonucleotides. SiRNA-treated RAW264.7 cells demonstrated greater nuclear number than controls, as did marrow cells. (D) Nuclear number versus cell perimeter was plotted for wild type (WT) RAW264.7 osteoclasts, as well as for control- or siRNA-transfected cells. For the perimeter outcome, the slopes of the log-transformed data were not significantly different (P = 0.253) and thus a common slope was used in the final model. Pairwise comparisons between each siRNA and control cells showed significant differences at the intercept and at ten nuclei.
Figure 3.6: MyoIIA decrease leads to a decrease in microtubule acetylation. (A) Microtubule acetylation was examined by costaining osteoclasts for F-actin (green/left) and acetylated microtubules (red/middle). Both smaller and larger osteoclasts were examined in the MyoIIA siRNA treated cells to show the greater decrease in acetylation in larger osteoclasts. (B) Western analysis from Figure 3.4B shows only a slight decrease in microtubule acetylation overall when the whole culture-cell lysates is assayed.
Figure 3.7: MyoIIA decrease leads to increase in cell fusion. (A) MyoIIA siRNA or control siRNA were transfected and the cells were left for seven days (normal osteoclast time course). Control cells remained mononuclear but siRNA IIA cells became multinucleated (top left panels actin green, nuclei blue). TRAP staining indicates they are not osteoclasts but the blue nuclear stain indicated multinucleation (top right panels). Quantification shows the increase from 1 to 3.7 nuclei per cell (mean +/-s.d.). (B) MyoIIA was decreased via siRNA treatment and RANKL was added immediately following transfection for two days. Control cells show as expected the small increase in multinucleation that occurs as cells differentiate (top left panels red actin, blue nuclei) and the increase in multinucleated TRAP positive cells (top right panels) while the increase in multinucleation was greater in MyoIIA siRNA treated cells. Quantification shows the increase from 2 to 4.4 nuclei per cell after MyoIIA decreased (mean +/-s.d.).
Figure 3.8: Suppression of myosin IIA during late osteoclastogenesis results in increased sealing zone perimeter. Osteoclasts were plated on ivory after siRNA transfected to view the resorption phenotype via phalloidin staining combined with confocal microscopy. (A) Examples of sealing zones from control- or siRNA-treated cells on ivory are shown. Cells were labeled with fluorescent phalloidin and photographed at the same scale. (nuclear number for both = 7 nuclei). Scale bars = 20µm. (B) The sealing zone perimeters of siRNA-treated marrow cells were statistically different from controls, as were the perimeters of siRNA-treated RAW264.7 cells. (C) The second phenotype that was rarely seen was the multiple average sized ring in one siRNA cell compared to control cells that normally exhibited one ring per cell. Scale bars = 20µm. (D) Nuclear number versus actin ring perimeter was plotted for wild type (WT) RAW264.7 osteoclasts, as well as for control- or siRNA-transfected cells. As in the cell perimeter data above, the slopes of the log-transformed data were not significantly different (P = 0.925), leading to the use of a common slope model. Comparison of wild-type and control cells showed no significant difference, while the actin rings of siRNA1 cells were significantly different from controls at 10 nuclei and at the intercept.
Figure 3.9: Suppression of myosin IIA results in diminished motility, but preserved bone resorptive capacity in osteoclasts. (A) siRNA-treated RAW264.7 osteoclasts showed diminished motility in Transwell migration assays relative to controls. (B) Control- or siRNA1-treated RAW264.7-derived osteoclasts were assayed for resorptive capacity on synthetic bone substrate; no significant differences were noted. (C) Control- or siRNA1-treated marrow-derived osteoclasts were assayed for resorptive capacity on ivory slices; as with the RAW cells, no differences were noted.
CHAPTER 4

MYOSIN X REGULATES PODOSOMES AND ACTIN RINGS IN OSTEOCLASTS

4.1 Introduction

Myosin X (Myo10), a low abundance (consisting of only hundreds to a few thousand copies per cell), widely distributed, vertebrate myosin was initially characterized as residing in dynamic regions such as the lamellipodia, membrane ruffles, and filopodia after initial discovery in 1994 during a PCR screen of the inner ear of bullfrogs (Solc 1994, Berg 2000). Myo10, the founding member of class X myosins and currently the only known isoform, contains a motor domain which can bind actin in an ATP dependent manner, and a unique tail containing a coiled-coil for dimerization, a PEST region which can be cleaved by calpain, three pleckstrin homology (PH) domains, one myosin tail homology domain 4 (MyTH4) and a band 4.1, ezrin, radixin, and moesin domain (FERM) domain (Figure 4.1) (Berg 2000, Yonezawa 2000). Myo10’s motor possesses a weak affinity for actin in the presence of ATP, classifying the motor as

3 The results of this chapter are in preparation for manuscript submission.
similar to non-processive, conventional myosin IIA, yet it has a high duty ratio indicating a cargo carrying function for this new myosin class (Homma 2001, Homma 2005).

4.1.1 Structure of Myo10

PH domains, the 11th most common domains in human proteins, have been shown to be involved in protein-protein interactions along with mediating signaling transduction through binding of phosphoinositides (Lemmon 2004). PH domains, which have low sequence homology, are classified by their 3D structure containing a seven stranded $\beta$-sandwich with a C-terminal $\alpha$-helix (Lemmon 2000). Through yeast screening, researchers found most PH domains do not recognize specific phosphoinositides, but some can bind phosphoinositides strongly to mediate PI3K signaling and target proteins to the cellular membrane (Yu 2004). Utilizing its second PH domain, Myo10 binds PIP2/PIP3 in vivo (Isakoff 1998, Yonezawa 2003). Additionally using the combined three Myo10 PH domains coupled to a GFP tag, researchers utilized single molecule imaging to demonstrate that Myo10 PH domains in live cells are bound to the plasma membrane and released. These data suggest that Myo10 PH domains are used for either recruitment or tethering to the plasma membrane for signaling events (Mashanov 2004).

The less-studied myosin tail homology domain 4 (MyTH4) domains are found in four myosin classes (VII, X, XII, XV) of which only four including Myo10 are present in vertebrate tissues. With less than 50% homology, MyTH4 domain function has been minimally studied (Chen 2001). Recent focus has shown MyTH4 domains in plant kinesin like protein to be involved in microtubule binding independent of ATP activation.
Myo10 MyTH4 domain directly binds microtubules linking the spindle assembly and positioning during meiosis of Xenopus oocytes (Weber 2004).

FERM domains link cell membrane proteins such as integrins to the cytoskeleton (Chishti 1998). FERM domains range in homology from 71-15%; that of Myo10 is 28% similar to the FERM domain of talin (Chishti 1998). Through studies of multiple FERM domains, they have been further classified into three subdomains: a domain similar to PH domains in protein folding, an Ena-VASP homology domain (EVH1) and phosphotyrosine binding domain (PTB) (Hamada 2000). Multiple potential binding partners have been investigated, but so far it has been demonstrated only that Myo10 FERM domains bind the NPXY domain of β integrins (Zhang 2004). It is unknown if Myo10 FERM domain can also activate integrin signaling as the talin FERM domain can. Thus, PH, MyTH4, and FERM domains allow Myo10 to bind and transport multiple proteins, possibly even simultaneously.

4.1.2 Functions of Myo10

With wide distribution throughout vertebrate tissues, Myo10 has been implicated in a variety of cell functions. Through live cell imaging of GFP Myo10, it was determined that Myo10 participates in forward and rearward intra-filopodial motility (Berg 2002). Also, Myo10 contributes in cell-cell adhesion transportation of VASP to filapodia tips, and Fc-mediated phagocytosis (Cox 2002, Tokuo 2004, Yonezawa 2003). Through overexpression and coimmunoprecipitation studies along with GFP live cell imaging of whole Myo10 or tail fragments, Myo10 has been implicated in providing a motor link between cytoskeleton and integrin signaling, filapadia formation, anchoring
and spindle assembly during meiosis (Zhang 2004, Bohil 2006, Weber 2004, Toyoshima 2007). In this chapter, we have examined the role of Myo10 in osteoclasts, which exhibit dynamic F-actin attachment structures different from the cells in which Myo10 was previously studied.

4.2 Down-regulation of Myo10 Leads to a Podosomal Patterning Defect in Osteoclasts

We considered Myo10 as a potentially important contributor to osteoclast attachment and migration due to its capacity to bind integrins and as an effector of PI3 kinase, a signaling molecule known to play a crucial role in osteoclast spreading and motility (Lakkakorpi 1997, Pilkington 1998). Here we demonstrate that Myo10 plays a role in podosome positioning and subsequent cell spreading and migration by linking podosomes to the microtubule network. This work is the first to define a role for Myo10 in podosome-based adhesion, as well as demonstrating its role as a linker between the two cytoskeletal systems.

4.2.1 Myo10 is Enriched Adjacent to Adhesion Structures

Myo10 distribution was visualized by immunocytochemistry in mature RAW264.7- and mouse bone marrow-derived osteoclasts plated on glass coverslips. In RAW264.7 osteoclasts, Myo10 demonstrated a generally diffuse distribution throughout the cytoplasm, but was mostly absent from the peripheral podosome belt and indeed was somewhat enriched immediately adjacent to this belt (Figure 4.2A, arrows). A similar distribution was found in mouse bone marrow-derived osteoclasts (Figure 4.2B, arrow). The enrichment of Myo10 near the podosome belt was variable in that it could occur at
the inner or outer rim of the belt, or on both sides, as in Figure 4.2A. The general exclusion of Myo10 from podosomes suggests that it is not a component of osteoclast adhesion structures. However, given its ability to bind integrins as well as to mediate effects of PI3 kinase (a signaling molecule with known effects on osteoclast spreading and migration), Myo10’s distribution was suggestive of a potential role in podosome formation or positioning. Thus, its role in osteoclast attachment was explored further.

4.2.2 SiRNA Treatment of Myo10

In an effort to examine the function of Myo10 in osteoclasts, RNA interference was used to suppress Myo10 expression in both RAW264.7- and mouse bone marrow-derived osteoclasts. Targeting and negative control siRNAs were transfected on day 4 of osteoclast differentiation and assayed for Myo10 mRNA levels two days post-transfection. Three negative control double-stranded oligonucleotides failed to decrease the levels of Myo10 mRNA in RAW264.7 osteoclasts while both siRNA 73578 and 73762 decreased the mRNA expression by approximately 90% (n = 3) when measured by competitive RT-PCR (Figure 4.3A, top panel). Four days post-transfection in RAW264.7 osteoclasts, Myo10 protein levels were reduced to 62.0 +/- 4.5% (siRNA 73578) or 44.2 +/- 5.6% (siRNA 73782) of control levels (n = 3; Figure 4.3A, middle panel). Similar efficiencies of knockdown were achieved in mouse marrow-derived osteoclasts (Figure 4.3B). At 2 days post-transfection, Myo10 mRNA was decreased to 20 – 30% of controls. At 4 days post-transfection, Myo10 protein was reduced to 33.8 +/- 3.3% (siRNA 73762) and 46.9 +/- 5.7% (siRNA 73578) of controls (n = 3). To further explore the timing of Myo10 suppression, control- or siRNA 73578-treated cells
were examined over a four-day period. As shown in Figures 4.3C, modest levels of knockdown were achieved early post-transfection, but were substantial at day 4. This lag in kinetics of suppression was most likely due to the substantial stability of Myo10 protein. Myosins tend to be stable proteins with lengthy half-lives; for example, nonmuscle myosin IIA has a half life of 5 days (Grainger 1991). For all subsequent experiments with siRNA-treated osteoclasts, cells were examined on day 4 post-transfection when Myo10 protein levels were at their lowest. Later time points were not considered because the osteoclasts were, by 4 days post-transfection (8 days total in culture), approaching the limits of their vigor in culture.

4.2.3 Suppression Leads to Decrease in Cell Spreading

SiRNA-treated osteoclasts initially were labeled with fluorescent phalloidin to determine podosome structure and cell spreading. While the siRNA-treated cells did not exhibit any immediately apparent changes in the podosomes at the cell periphery, they exhibited a readily noticeable difference in perimeter when compared to controls (Figure 4.4A). As shown in Figure 4.4B, siRNA-treated cells possessed a cell perimeter similar to that of control cells over the first 3 days post-transfection, but were noticeably diminished in perimeter, to about 60% of controls, on day 4 when Myo10 protein expression was at its lowest (refer to Figure 4.3D). These results show a direct correlation between levels of Myo10 protein expression and osteoclast perimeter. Figure 4.4C shows the changes in perimeter for both RAW264.7- and marrow-derived osteoclasts treated with siRNA 73578 for 4 days. Although only one control is shown in this figure, subsequent experiments were performed comparing all three siRNA controls,
which gave identical results (data not shown). Because osteoclasts in these culture systems continue to fuse and become more multinucleated during their time in culture, the nuclei in siRNA- and control-treated cells were enumerated to determine whether Myo10 suppression might have an effect on cell fusion. Figure 4.4C illustrates that no differences in nuclear number were caused by Myo10 knockdown, indicating the changes in cell perimeter to be solely a function of osteoclast spreading. In addition to defects in spreading, the motility of siRNA-treated cells was examined in Transwell migration assays. When stimulated to undergo directional migration by addition of osteopontin to the underside of Transwell filters, siRNA-treated osteoclasts (either RAW264.7- or marrow-derived) demonstrated levels of migration only 40% of that of controls (Figure 4.4D).

4.2.4 RNAi-mediated Myo10 Suppression Results in Mislocalized Microtubules

I next examined the distribution of known binding partners of Myo10 to determine whether the decrease in Myo10 led to their mislocalization. Myo10 siRNA-treated cells, along with control-treated cells, were doubly labeled for tubulin and F-actin. Initial observations at low magnification revealed apparently normal microtubule networks in siRNA-treated cells (Figure 4.5A). However, closer examination demonstrated that microtubules in control cells extended to the podosome belt, while the microtubules of siRNA-treated cells did not (Figure 4.5B, large panels). Confocal Z-stack imaging was used at the cell periphery to better understand the changes in the microtubule/podosome association in Myo10-suppressed cells. Control cells were thin at the cell periphery, with the microtubule network extending to podosomes in a mostly
level plane at the base of the cell. However, siRNA-treated cells were thickened, consistent with diminished spreading, and the microtubule ends generally were not present in the same plane as podosomes (Figure 4.5B, side views). These results show that although the microtubule network is intact and well-spread in both control- and siRNA-treated cells, the latter show aberrant association of microtubules with the podosome belt.

4.2.5 RNAi-mediated Myo10 Suppression Inhibits Podosome Belt Formation

Podosome positioning at the cell periphery in macrophages and osteoclasts is dependent on intact microtubules (Destaing 2003, Evans 2003, Linder 2000). Because Myo10 siRNA-treated cells show aberrant interaction between these structures, I transiently depolymerized microtubules in these cells and assessed how Myo10 suppression affected podosome repositioning. Control- and siRNA-treated cells were placed at 4°C for three hours, which caused complete disruption of the microtubule network and a collapse of the podosome belt. Cold treatment was used to depolymerize microtubules, rather than the more specific microtubule inhibitor nocodazole, because nocodazole caused cell death prior to collapse of the podosome belts. The initial three hour cold treatment caused all the cells to be devoid of a microtubule network or a podosome belt in both the control- and siRNA- treated cells (Figure 4.6A, top panels). After a 30 minute recovery at 37°C, microtubules were regenerated in both control- or siRNA-treated cells. However, although many control cells were able to recover their podosome belts at the periphery, siRNA-treated cells were unable to do so (Figure 4.6A, middle panels). Instead, podosomes were diffusely spread or in clusters. Even after 24
hours of recovery, the siRNA-treated cells still were unable to regenerate peripheral podosome belts, but instead organized their podosomes in internal clusters or small rings (Figure 4.6A, bottom panels). Figure 4.6B shows a low magnification view of both control- and siRNA-treated cells after 24 hours recovery, along with individual osteoclasts, to depict the phenotypes seen in each condition. Almost 100% of the control cells recovered their peripheral podosome belts after 24 hours, while less than 3% of siRNA-treated cells did so (Figure 4.6C). Instead, Myo10 siRNA-treated cells mostly showed podosome patterning in clusters and rings. Thus, the defect in Myo10-suppressed cells appears to be in podosome patterning and not formation, as Myo10 siRNA-treated cells form similar numbers of podosomes as control cells, but do not localize them correctly.

4.2.6 Overexpression of MyTH4-containing Tail Truncates Inhibits Podosome Belt Formation

To delineate regions of Myo10 involved in podosome patterning, and to support the emerging hypothesis that Myo10 links podosomes to the microtubules during osteoclast podosome positioning, I created plasmid constructs that contained various Myo10 tail domains alone or in combination in the pEF6/V5-His expression vector (Figure 4.7A). These constructs, in addition to an empty vector for control, were transfected in RAW264.7 cells to generate stably transfected clones that overexpressed each domain. The cells were then differentiated into osteoclasts and stained with phalloidin to visualize the F-actin podosome structures and general cell morphology. Because MyTH4 is the microtubule binding domain of Myo10 and the siRNA-treated
cells exhibited a microtubule-dependent defect in podosome patterning, I examined the podosome phenotypes of control and MyTH4 overexpression cells on glass. Additionally, because it has been reported that the combination of MyTH4-FERM is more efficient at binding microtubules than the MyTH4 domain alone in proteins that contain both domains in combination (Narasimhulu 1998, Weber 2004), the effects of overexpressing the MyTH4-FERM domains were assessed. Overexpression of truncated Myo10 tail domains results in dominant negative inhibition of Myo10 function (Bohil 2006, Cox 2002, Weber 2004). At least four clones for each construct were examined; all demonstrated similar results. MyTH4 and MyTH4-FERM overexpressing osteoclasts were unable to form podosome belts, but instead formed internal rings and clusters (Figure 4.7B). To be sure this was not a differentiation-dependent patterning effect, microtubules in mature osteoclasts were depolymerized by cold treatment, and cells were allowed to recover overnight. MyTH4-expressing osteoclasts, like siRNA-treated cells, could not recover podosome belts and were inhibited from transitioning out of the internal ring stage (Figure 4.7C). The MyTH4-FERM expressing cells demonstrate a more extreme phenotype and could recover only very small rings after depolymerization, potentially due to the effects of FERM domain binding on its partners. Additionally, overexpression of the MyTH4-FERM domains caused osteoclast perimeter to decrease by ~20% when normalized for nuclear number (data not shown). These results demonstrate that the MyTH4 domain plays a crucial role in positioning podosomes within osteoclasts.
4.2.7 Overexpression of Full-length Myo10 Leads to Increased Podosome Belt Formation

To reaffirm the role of Myo10 in podosome positioning, full length Myo10 was cloned into the pEF6/V5-His expression vector and overexpressed in RAW264.7 cells. The empty vector was used as a control for these studies. I initially noted that RAW264.7 cells could not readily tolerate long-term overexpression of the full-length myosin. Of three experiments where 12 individual clones were chosen for expansion each time, only two clones (FLX1 and FLX2) survived to produce enough cells for further study. Western analysis demonstrated that FLX1 and FLX2 expressed Myo10 at 140 ± 1.3% and 199 ± 3.5% of control cells, respectively (data not shown). The pEF-V5 vector uses the human elongation factor-1α promoter to keep overexpression levels low compared to more commonly used viral promoters, but even under these conditions, RAW264.7 cells apparently tolerate long-term overexpression of Myo10 poorly.

The resulting clones were plated on glass and the F-actin phenotype was examined by labeling with fluorescent phalloidin. Initially, undifferentiated RAW264.7 macrophages were examined (Figure 4.7D, top panels). While control transfectants exhibited normal F-actin distribution, clones FLX1 and FLX2 generated noticeable podosome belts, a phenotype normally present only in osteoclasts. When the clones were differentiated into osteoclasts, control cells generated typical podosome belts, but the podosomes of FLX1 and FLX2 were arranged in unusual conformations (Figure 4.7D, bottom panels). Myo10-overexpressing cells produced podosomes in multiple ring and belt structures. Higher magnification of one of these cells demonstrates the appearance
of intensely labeled belts with multiple internal rows of podosomes (Figure 4.7E). Thus, while suppression of Myo10 function by either RNA interference or dominant negative inhibition by the MyTH4 domain inhibited podosome belt formation, overexpression of the full-length Myo10 forced an increase in peripheral and internal podosome patterning. These results support a role for Myo10 in osteoclast podosome patterning through interactions between the MyTH4 tail domain and microtubules.

4.2.8 Myo10 Localizes Between Podosomes and Microtubules during Osteoclast Podosome Patterning

Because the podosome defect in Myo10-suppressed cells occurs during the transition from the podosome ring to peripheral belt- a microtubule dependent event- and dominant negative overexpression of the MyTH4 domain caused a podosome patterning defect, I reexamined the distribution of endogenous Myo10 in relationship to microtubules and F-actin. Figure 4.8A illustrates a cell in the transition stage between an internal ring and a podosome belt. The individual and merged images demonstrate a thin band of Myo10 surrounding the podosome rings, with microtubules positioned outside but terminating near the Myo10 (arrows). These photomicrographs visually suggest an arrangement in which Myo10 acts as an intermediary between podosomes and microtubules, consistent with our previously described findings. Additionally, Figures 4.8B shows high magnification images of a podosome belt in a mature osteoclast. These panels demonstrate enrichment of Myo10 adjacent to the podosome belt (arrow), with microtubules terminating near the Myo10 band. Figure 4.8C demonstrates how Myo10 colocalizes with both microtubules and actin. These findings, coupled with our studies
demonstrating effects of Myo10 over- and under-expression, lead us to propose that Myo10 acts as a direct linker between microtubules and podosomes through binding of both the MyTH4 domain and F-actin. A greater magnification of an additional cell shows the colocalization of Myo10 with both microtubules and internal-non-podosome associated F-actin (Figure 4.8C).

4.2.9 Discussion of Myo10’s Involvement in Osteoclast Podosome Patterning

Podosomes are actin-based structures that interact with, and are dependent on, intact microtubules for formation and function. While a few proteins have been proposed to link the podosome and microtubule networks, Myo10 is the first potential linker demonstrated to directly bind both actin and microtubules. Linder et al. (2000) suggested a role for the actin binding protein WASP in mediating podosome to microtubule interaction through a second intermediate, CIP4 (CDC42 interacting protein 4). The WASP polyproline domain that binds CIP4, a microtubule binding protein, was found to block podosome regeneration without affecting the microtubule network when injected into macrophages (Linder 2000). Perhaps relevant to these findings, Myo10 has been proposed to interact with and transport VASP, a WASP related protein that shares similar structural motifs such as the polyproline domain, to the tip of filopodia (Tokuo 2004). A second protein, kinesin KIF1C, also has been proposed as the microtubule-podosome link (Kopp 2006). Again, it was suggested that KIF1C binds microtubules and a second intermediate binds actin. Immunoprecipitation of KIF1C from HUVECs resulted in co-precipitation of nonmuscle myosin IIA, which was purported to directly interact with KIF1C, although conditions by which these experiments were performed may have
resulted in precipitation of indirectly bound actin-associated proteins. Although Kopp et al. found non-specific inhibition of nonmuscle myosin IIA in macrophages via blebbistatin (which also inhibits macrophage myosin IIB) decreases podosome formation, our own studies in osteoclasts demonstrated that specific inhibition of MyoIIA via RNA interference did not affect podosome formation (chapter 3). Nonetheless, multiple linker proteins may be responsible for microtubule regulation of podosome function, and our results here indicate that Myo10 may mediate this connection via its microtubule-binding MyTH4 tail domain.

MyTH4 domains are present in four myosin classes (VII, X, XII, XV) and in a plant kinesin (Sousa 2005). Of these, vertebrate tissues express only myosins VII, X, and XV, of which only Myo7a is expressed in osteoclasts (our unpublished data). The Myo10 MyTH4 domain is less than 50% similar to other vertebrate MyTH4 domains (Chen 2001). First classified in the Arabidopsis kinesin like calmodulin binding protein (KCBP), the MyTH4 domain binds microtubules independently of ATP hydrolysis (Narasimhulu 1998, Reddy 1996). Subsequently, Myo10 was shown to directly bind microtubules during cell division for nuclear spindle anchoring through its MyTH4 domain (Weber 2004). Because Myo10 is known to bind microtubules and actin, we hypothesized that Myo10 might link microtubules to podosomes in osteoclasts. Our data support our hypothesis in that Myo10 inhibition, either by RNA interference or overexpression of truncates caused a microtubule-dependent podosome defect. By overexpressing individual tail domains, I found the defect was caused by the MyTH4 domain and not the PH or FERM domains (to be discussed further in this chapter).
Conversely, overexpression of full-length Myo10 resulted in excessive podosome belt formation.

Myo10 inhibition in osteoclasts also led to a corresponding decrease in cell spreading and motility. Modulation of Myo10 levels in other cell types has produced varying effects on spreading. RAW264.7 macrophages, the precursors of one of our osteoclast models, similarly demonstrated reduced spreading upon Myo10 inhibition by expression of dominant negative tail domains (Cox 2002). Similar results were obtained in COS-1 cells (Yonezawa 2003). In contrast, suppression of Myo10 by RNA interference or overexpressing of tail truncates in HeLa cells led to loss of filopodia and an increase in cell spreading (Bohil 2006). The decrease in cell spreading of the osteoclasts did not coincide with a change in the number or distribution of podosomes and therefore might be attributed to Myo10 functioning in an additional role in podosome/attachment dynamics in osteoclasts.

In summary, the results presented suggest that Myo10 plays a role in mediating podosome patterning in osteoclasts. Myo10-suppressed cells exhibited a microtubule-dependent defect in latter stages of podosome belt formation, accompanied by decreased cell spreading and motility. Conversely, overexpression of full length Myo10 led to abnormal, increased numbers of podosome belts in macrophages and osteoclasts. Immunolocalization studies were consistent with a role for Myo10 as a microtubule-podosome linker, due to its enrichment between these structures in podosome rings and belts. Together these results suggest one role of Myo10 in osteoclasts is to link the actin-based podosomes to microtubules during podosome positioning in osteoclasts.
4.3 Myo10 Plays Multiple Roles beyond Podosome Patterning

Along with the role in osteoclast podosome patterning, the expression and function of Myo10 on bone and the additional tail domains has been preliminarily examined. Myo10 may be playing additional roles in osteoclasts beyond podosome patterning through its PH and FERM domains. Although recent evidence suggests PH domains are utilized more for protein-protein interaction, the Myo10 PH domains have been shown to bind PI3 kinase substrates (Isakoff 1998). PI3 kinase signaling is essential for proper osteoclast function as the irreversible PI3K inhibitor wortmannin disrupts podosome formation along with actin ring and ruffled border formation (Lakkakorpi 1997, Golden 2004). Myo10 also has been shown to bind beta integrins via the FERM domain (Zhang 2004). Both PI3 kinase signal transduction and integrin mediated signaling and attachment play roles in proper osteoclast function (Duong 2000, Faccio 2003, Hruska 1995). The following studies are to better examine potential relationships between these activities and Myo10 function. Additionally, the role of Myo10 in relation to the sealing zone of polarized osteoclasts has been initially studied since it has been shown that osteoclasts from Pyk2-/- mice exhibiting a podosome patterning defect similar to that caused by Myo10 suppression in osteoclasts also have slight bone resorption deficiencies (Gil-Henn 2007).

4.3.1 Suppression of Myo10 Leads to a Decrease in Actin Ring Size

Myo10 was found to only associate strongly with podosomes during the transition between podosome rings to belts. Osteoclast actin ring patterning occurs at a much quicker rate and a stepwise positioning is not normally examined in fixed cells on bone.
As with podosome belts on glass, Myo10 does not associate with the actin ring of polarized cells. Figure 4.9A shows the distribution of Myo10 in RAW264.7 osteoclasts with the actin ring in one confocal section at the base of the cell and confocal z-sectioning of Myo10 throughout the cell. In mature MBM osteoclasts, Myo10 shows the same distribution throughout the cell, but no overlap with the actin ring (Figure 4.9B).

RAW264.7 and MBM derived osteoclasts were control oligonucleotide or siRNA-treated, replated on ivory, and labeled with fluorescent phalloidin to determine changes in actin ring structure and spreading. Initial observations showed a decrease in the perimeter of the actin ring, as demonstrated in Figure 4.10A by osteoclasts containing five nuclei each. Figure 4.10B shows the changes in average (+/− s.d.) perimeter for both RAW264.7- and MBM-derived osteoclasts treated with siRNA 73578 for 4 days. Although only one control is shown in the top figure for both marrow and RAW264.7 derived osteoclasts, subsequent experiments were performed comparing each siRNA to their individual mutated controls in RAW264.7 osteoclasts, which gave identical results. The nuclei in siRNA- and control-treated cells were counted to determine the fusion of osteoclasts. Figure 4.10B illustrates that no differences in nuclear number were caused by Myo10 knockdown, indicating the changes in cell perimeter to be solely a function of osteoclast actin ring spreading. These results were similarly seen on cells on glass (Figure 4.4C). In addition to defects in actin ring perimeter, the resorption of Myo10 siRNA-treated cells was examined by utilizing synthetic substrate assays. When plated two days post transfection for three additional days on synthetic bone substrate, Myo10 siRNA-treated RAW264.7 osteoclasts demonstrated decreases in number of clearings,
area per clearing, and total resorption (Figure 4.10C). Similar to Pyk2-/- osteoclasts, which also demonstrated a podosome ring to belt transition defect, suppression of Myo10 also led to decreased resorption due to small actin rings (Gil-Henn 2007).

### 4.3.2 Overexpression of Each Myo10 Tail Domain Causes Defective Podosomes

Overexpression of Myo10 tail domains other than MyTH4 were performed by creating plasmid constructs that contained various Myo10 tail domains alone or in combination in the pEF6/V5-His expression vector (Figure 4.11/top). These constructs, in addition to an empty vector for control, were transfected in RAW264.7 cells to generate stably transfected clones that overexpressed each domain. The cells were then differentiated into osteoclasts and stained with phalloidin to visualize the F-actin podosome structures and general cell morphology. Overexpression of truncated Myo10 tail domains results in dominant negative inhibition of Myo10 function and therefore allowed us to determine whether the domains other than MyTH4 affect podosome formation and patterning through their own mechanisms (Bohil 2006, Cox 2002, Weber 2004). At least four clones for each construct were examined; all demonstrated similar results. As previously demonstrated, MyTH4 and MyTH4-FERM created podosome patterning defects at the microtubule dependent transition between podosome ring to podosome belt, similar to those resulting from Myo10 suppression (Figure 4.7, Figure 4.11). PH domains or PH-MyTH4 domain overexpression did not generate microtubule podosome patterning defects, but instead caused almost complete loss of podosomes and generation of osteoclasts with a “spiky” perimeter (Figure 4.11). Finally, overexpression of the FERM domain alone caused a podosome patterning defect of cloud to ring...
transition. These results demonstrate that each domain plays a different role in podosome formation and positioning, supporting the hypothesis that Myo10 plays multiple functions in osteoclasts.

4.3.3 Overexpression of PH Domains Link Myo10 Podosome Defects to PI3K Signaling

To further examine the connection of Myo10’s PH domains with PI3 kinase signaling, I created overexpression plasmids containing either the full three PH domains, PH domain 1A-2, PH domain 1A-1B, PH domain 3, along with an empty vector control (Figure 4.12A). Myo10 has an unusual PH domain arrangement in that the second PH domain splits the first domain into two halves (Figure 4.1). In the three Myo10 PH domains, the 1B section contains the PIP2/PIP3 binding site, so the constructs were created to examine the difference between the PI3K substrate site and the additional PH domains of Myo10 (Yonezawa 2003). The stable cells lines were then differentiated into osteoclasts and examined on glass by fluorescent phalloidin staining to view podosome formation and positioning. Control cells exhibited normal rows of podosomes at the cell periphery, but the full PH domains caused a decrease in the number of podosomes, and the cell periphery changed to a spiky shape as in seen in Figure 4.11 and Figure 4.12. Overexpression of the PH1A-2 domain caused no change from the control cells as the podosomes were similar in number and position (Figure 4.12B). Overexpression of PH1A-1B caused a decrease in the number and placement of podosomes at the cell periphery, but not the spiky appearance caused by the full three domains (Figure 4.12B). Overexpression of the PH3 domain caused a slight decrease in the amount of podosomes,
yet they were still positioned mainly around the cell periphery as seen in the control (Figure 4.12B). These data suggest the PI3K substrate binding activity of the PH1B domain is the cause of the decrease in podosome formation by overexpression of Myo10 PH domains.

To support the role of Myo10 PI3K substrate binding activity, osteoclasts were treated with either vehicle as a control or wortmannin for 60 minutes and the cells examined by phalloidin staining and confocal imaging. Similar to the combined three PH domain phenotype of Figure 4.12B, wortmannin treated osteoclasts exhibited a decrease in podosome number and formation of spiky cell membrane at the cell periphery (Figure 4.12C). This further suggests the phenotype seen in the overexpression of Myo10 PH domains is related to PI3 kinase signaling in osteoclasts.

4.4 Discussion of Myo10 in Osteoclasts

The relatively recently described class X myosin was first characterized as the founding member of a group of myosins containing multiple pleckstrin homology domains (Berg 2000). Myo10 function has been studied in a variety of tissues including neurons, oocytes, hepatocytes, macrophages, and multiple cell lines; yet this is the first study to examine the role of Myo10 in osteoclasts. In other cell types, Myo10 has been shown to be involved in producing protrusions at the cell membrane as it plays a role in filopodia extensions and is localized in lamellipodia and phagocytic cups. Thus, Myo10 associates with regions of dynamic actin. This study extends this general finding to osteoclasts, which utilize podosome-based attachment structures both for migration and for formation of a tight sealing zone critical for osteoclast bone resorptive capacity.
Initial results demonstrate the main role of Myo10 in osteoclasts to be involved in podosome patterning in the microtubule dependent step of transitioning from podosome ring to belt as the phenotype seen in siRNA mediated knock-down of Myo10 resulted in cells blocked at the podosome ring stage. Similarly, Pyk2-/- mice, which exhibit mild osteopetrosis, have a podosome transition defect in their inability to make podosome belts at the cell periphery (Gil-Henn 2007). Pyk2-/- osteoclasts were able to only form small actin rings in cells on bone, similar to the results seen in the Myo10 siRNA-treated osteoclasts, which both led to a decrease in bone resorption. Like the transition from podosome ring to belt, formation of the actin ring/sealing zone is a microtubule-dependent event (Jurdic 2006). It is possible that Myo10 is involved in enlargement of the actin ring similar to the role it plays in expanding podosome belts on glass. Because I examined only mature actin rings, any transient involvement in Myo10 in the ring would have been undetected. Actin ring formation is difficult to examine because of its rapidity and because of problems inherent in visualizing fine detail in cells cultured on bone slices.

Overexpression of the various tail domains alone and in combination indicates Myo10 plays multiple roles in podosome formation and positioning in osteoclasts. Myo10 is involved in PI3 kinase signaling as it has been shown by several labs the PH domains bind PIP2/PIP3 and alone cause plasma membrane localization. To further support this, I also found the PH domains on Myo10 to be important in podosome formation (an event dependent on PI3 kinase signaling), with the PIP2/PIP3 binding PH domain causing the most severe effects on podosome formation when overexpressed.
Interestingly, through the study of other PH domains and specifically split PH domains, it has been suggested that there exists the possibility of split PH domain complementation. Researchers found that the C-terminal half of the phospholipase Cγ (PLCγ) PH domain directly lines up with the N-terminal half of the plasma membrane channel TRPC3 PH domain, and by utilizing the halves from each, they were able to pull down each other (Lemmon 2002). Is it possible the split PH domain of Myo10 interacts with an additional split PH domain in another protein? Since both halves of the Myo10 PH domain align, it is unlikely, but it would add to the multiple functions of Myo10 if it indeed functions in that manner.

Taken together, these results demonstrate that Myo10 is necessary for multiple roles in osteoclast podosome and actin ring formation and stability of these essential osteoclast actin structures. When individual tail domains were overexpressed to give a dominant negative effect, the multiple roles of Myo10 were evident at different stages of podosome formation and patterning. Initial formation of podosomes is affected by the PH domains and PI3 kinase signaling potential of Myo10, while the transition from podosome cloud to podosome ring is affected by overexpression of the FERM domain. Not only does wortmannin and inhibition of PI3K in osteoclasts cause the loss of podosomes, it also affects the redistribution of αvβ3 integrins which are essential for osteoclast function (Faccio 2002). Myo10 potentially could link PI3 kinase signaling by PH domains to β-integrin signaling through the FERM domain. Finally, Myo10 uses the MyTH4 domain to transition podosomes from the podosome ring to belt by binding
microtubules. Throughout the process of osteoclast podosome formation and patterning, we can see that the tail domains of Myo10 play multiple roles.
Figure 4.1: Schematic representation of Myo10. Myo10 representation showing the multiple domains of Myo10 and known binding partners for each of the tail domains (Sousa 2005).
Figure 4.2: Distribution of Myo10 in mature osteoclasts. (A) RAW264.7 osteoclasts were plated on glass and examined by confocal microscopy for the localization of Myo10 (left-green) and F-actin (middle-red). Higher magnification of the same cell demonstrates Myo10 is generally excluded from the podosome belt (lower panels). Scale bars = 10 µm. (B) MBM derived osteoclasts were plated on glass and examined by confocal microscopy for the localization of Myo10 (left-green) along with phalloidin F-actin (middle-red). The distribution of Myo10 in both RAW264.7- and marrow-derived osteoclasts was similar. Scale bars = 10 µm.
Figure 4.3: Suppression of Myo10 in osteoclasts. (A) Competitive RT-PCR and Western analysis of RAW264.7-derived osteoclasts. SiRNA’s 73762 and 73578 decreased Myo10 mRNA expression while three negative controls (a generic non-targeting double-stranded oligonucleotide and mutant versions of 73762 and 73578) did not affect Myo10 expression. mRNA was measured 2 days post transfection. Protein levels measured by Western analysis 4 days post transfection also demonstrate siRNA-specific loss of Myo10 while exhibiting no change in any of the controls. β-actin was used as a control for expression. (B) Competitive RT-PCR and Western analysis of marrow-derived osteoclasts. SiRNA’s 73762 and 73578 elicited specific knockdown of Myo10, similar to that seen in RAW264.7-derived cells. (C) Western analysis shows time course of Myo10 protein expression following transfection of siRNA 73578. Multiple experiments were quantified and graphed to show relative to control transfectants (mean+/-s.d.).
Figure 4.4: Suppression of Myo10 leads to a decrease in cell spreading. (A) Four days post transfection, osteoclasts treated with a control oligonucleotide or siRNA 73578 were fixed and stained with phalloidin. Further experimentation with siRNA 73762 and additional negative controls verified the results (data not shown). Confocal images demonstrate the decrease in cell perimeter resulting from Myo10 suppression. Scale bar = 50 µm. (B) Osteoclast perimeter was quantified for control- and siRNA 73578-treated cells 1-4 days post-transfection (mean +/- s.d.). (C) SiRNA treatment of RAW264.7 and mouse bone marrow (MBM) derived osteoclasts resulted in decreased perimeter 4 days post-transfection without affecting nuclear number (mean +/- s.d.). (D) Motility was measured in both RAW264.7 and mouse bone marrow (MBM) osteoclasts via Transwell migration assays. Myo10 suppression resulted in loss of motility in both cell types (mean +/- s.d.).
Figure 4.5: Suppression of Myo10 leads to mislocalization of microtubules at the cell periphery. Osteoclasts were plated on glass and examined for microtubule (red-middle) and actin (green-left) distribution in control- and siRNA 73578-transfected cells. (A) Low magnification views show that microtubule networks and podosome belts are intact in control- and siRNA-treated cells. Scale bars = 20 µm. (B) Confocal images demonstrate that microtubules in siRNA-treated cells do not terminate in the same plane as podosomes. Arrowheads in the large merged panels point to microtubule ends in control and siRNA-treated cells. The lack of association between microtubules and podosomes in siRNA-treated cells is further demonstrated by XZ images (right panels) showing microtubule ends generally absent from the plane in which podosomes reside in siRNA-treated cells. Scale bars = 10 µm.
Figure 4.6 Suppression of Myo10 leads to an inability to regenerate podosome belts after microtubule depolymerization. Osteoclasts at 4 days post-transfection with control or targeting siRNAs were cold-treated to depolymerize microtubules, allowed to recover, fixed and stained for microtubules and actin. (A) Osteoclasts cold-treated were allowed to recover for 0, 0.5, or 24 hours at 37°C. Cells plated on glass were viewed after fixation and labeling for microtubules (red-middle) and actin (green-left). Control cells recovered peripheral podosome belts following microtubule disruption and recovery, but siRNA73578-treated cells did not. Scale bars = 20 µm. (B) Low magnification views of cells recovered for 24 hours demonstrate the differences in podosome distribution between control- and siRNA73578-treated cells. Bottom pictures illustrate the phenotypes that occurred most often in each culture. Scale bars = 30 µm. (C) Graphical representation of the proportion of cells exhibiting a podosomal belt versus podosome clusters/rings (mean+/-.s.d.).
Figure 4.7: Overexpression of the MyTH4 domain suppresses podosome belt formation, while overexpression of full-length Myo10 promotes belt and ring formation. (A) Diagram of Myo10 tail domain clones. Each was overexpressed in multiple RAW264.7 cell clones and differentiated into osteoclasts. (B) Osteoclasts overexpressing MyTH4 and MyTH4-FERM were labeled with fluorescent phalloidin and revealed that dominant negative overexpression of the microtubule binding domain caused the cells to be unable to form podosome belts. Scale bars = 20 µm. (C) Microtubules were depolymerized and allowed to recover 24 hr; the resulting phenotype was viewed by fluorescent phalloidin labeling. Again, MyTH4 and MyTH4-FERM overexpressing cells were unable to form podosome belts. Scale bars = 20 µm. (D) Overexpression of full-length Myo10 promotes podosome belt formation in macrophages (top panels). In mature osteoclasts, overexpression of Myo10 induces excessive formation of podosome belts and rings. Scale bars = 50 µm. (E) A high magnification view of a mature osteoclast overexpressing Myo10 illustrates excessive podosome belt and ring formation (top panel). The right photomicrograph, taken 2.6 µm above the coverslip surface, demonstrates that the figure shows a single cell. Scale bar = 20 µm.
Figure 4.8: Myo10 is positioned between microtubules and podosomal rings and belts. (A) An osteoclast with an internal ring was labeled for Myo10 (green-top left), F-actin (red-top right), and microtubules (blue-bottom left). Myo10 localizes between F-actin and microtubules. Scale bars = 10 µm. (B) An osteoclast containing a podosome belt was labeled for Myo10 (green-top left), F-actin (red-top right), and microtubules (blue-bottom left) to show Myo10 localizes between both microtubules and podosomes. Scale bar = 10 µm. (C) An osteoclast containing a transitioning podosome belt was labeled for Myo10 (green-left), F-actin (red-top middle), and microtubules (blue-bottom middle) to show Myo10 localizes with both microtubules and podosomes. Scale bar = 10 µm.
Figure 4.9: Myo10 localization on bone is absent in actin ring. Osteoclast Myo10 distribution viewed by confocal microscopy along with confocal xyz sectioning. RAW264.7 and MBM osteoclasts viewed by confocal microscopy Myo10 (green left) and F-actin phalloidin (red, middle) and merge in z-scan. (A) RAW264.7 osteoclasts show the distribution of Myo10 compared to the actin ring along with confocal side view showing Myo10 does not overlap with the actin ring. Myo10 is absent in the ring and localizes completely internal to the ring structure. (B) MBM osteoclast also stained with Myo10 to show the internal localization and separation of Myo10 from the actin ring.
Figure 4.10: Myo10 siRNA decrease causes a decrease in actin ring perimeter size and bone resorption. Osteoclasts, both RAW264.7 and MBM derived, were plated on ivory and stained with fluorescent phalloidin to view the actin ring. (A) Phalloidin stained siRNA73578-treated cells were visualized using confocal microscope to show the decrease in the perimeter of the actin ring with si73578 compared to the control (nuclear number for both 5 nuclei). Scale bar = 30µm. (B) Perimeter was measured and compared along with nuclear number to show the decrease in actin ring perimeter is due to spreading. Top graph shows ring and nuclear number for RAW264.7 and MBM osteoclasts using siRNA73578 and the bottom graph shows ring and nuclear number using siRNA73762 and siRNA73578 along with their specific controls (mean +/- s.d.). (C) Resorption was measured using synthetic substrate and found that the siRNA Myo10 smaller rings resorbed less in number of pits, area per pit, and total area (mean +/- s.d.).
Figure 4.11: Overexpression of tail domains causes abnormal podosome formation.

Diagram of the various tail pieces that were overexpressed in RAW264.7 cells and differentiated into osteoclasts. MyTH4 contain tail pieces demonstrate podosome ring to belt transition defect while FERM domain demonstrates cloud to ring defect. PH containing domains have less podosomes total and completely disorganized podosome formation. Scale bars = 20µm.
Figure 4.12: Overexpression of PH domain causes PI3 Kinase related defect in podosome patterning and formation. (A) Diagram demonstrating the three Myo10 PH domain constructs which were overexpressed in RAW264.7 cells. (B) Stable cell clones were differentiated, and phalloidin F-actin visualization demonstrates in the control vector only osteoclasts form a band of podosomes at the cell periphery. Overexpression of the full PH domain causes the podosomes to decrease in number and the cell to lose the normal peripheral shape. Each PH domain was overexpressed and cells were stained with phalloidin to view podosome distribution. Only overexpression of the PH1A-1B (PIP2/3 interacts with Myo10 PH1B domain) still had aberrant podosomes and shape. (C) Wortmannin, an inhibitor of PI3 Kinase caused the cells to exhibit a similar decrease of podosomes and change in cell peripheral shape.
5.1 Importance of Osteoclasts in Human Health

Osteoclasts function to regulate resorption of organic and inorganic bone substrates to provide proper bone mass and constant skeletal balance. Osteoclasts are required to produce bones strong enough to support daily activities through proper bone remodeling without over- or under-resorption. Additionally, osteoclasts play a crucial role in regulation of overall body calcium and phosphate levels. Bone stores almost 100% of the body’s calcium and roughly 85% of the body’s phosphate. Osteoclastic bone resorption releases stored calcium into the blood when necessary to maintain proper calcium levels when not enough calcium is ingested or there is a net loss of calcium through urine, skin, hair, and nails.

Calcium is responsible for many cellular processes. Calcium regulates muscle contraction by mediating myosin/actin interaction via troponin binding. Therefore, muscle contraction is dependent on correct levels of calcium circulating throughout the body. Calcium regulates cell signaling by activating or deactivating many proteins involved in cell signaling. Calcium ions help regulate phospholipase C which hydrolyzes PIP2 in PI3 kinase signaling cascades (Kang 2007). Calcium also plays a role in cell
survival. In gingival fibroblasts increased intracellular calcium leads to increased apoptosis (Hattori 2008). In general, calcium plays a role in multiple signaling pathways within cells because it is stored in various compartments, and changes in the influx of calcium through calcium channels can trigger signaling events. Osteoclasts must therefore play a duel role of not only regulating skeletal health, but overall body health through the release of calcium into the blood.

5.2 Importance of Actin Binding Proteins in Cancer

Cancer cells metastasize by detaching from their normal location, moving through surrounding cells, and increasing or changing to a motile/invasive phenotype. For each step, there must be changes in the internal cell architecture. Cancerous cells must change from a stationary, sometimes non-dividing quiescent state, to a more motile phenotype. In recent years, research has begun to focus on the unique attachments of podosomes and invadopodia, collectively termed invadosomes, as unique attachment/extra cellular matrix (ECM) degrading protrusions. While osteoclasts and their hematopoietic precursors naturally utilize podosome based attachment, cancer cells exploit the chemotaxis increased mobility of these structures.

5.2.1 Invadopodia and Podosomes: Invadosomes

Invadosomes are unique attachment structures as they contain ECM degrading abilities not seen in focal adhesions. These ventral protrusions, which can be triggered to form in many cell types by over activation of Src kinase, attach to and degrade the ECM via matrix metalloproteinases (MMP). Podosomes and invadopodia share the same overall structure but differ in the length of their actin core. Podosomes are normally 1-2
μm in height (actin core length) while invadopodia are typically 4-6 μm (Figure 5.1). Invadopodia also typically form only clusters without the usual podosome cloud/core composition of adhesive binding proteins such as integrins. Further, invadopodia are stable for hours, while podosomes are typically stable for only minutes. Invadopodia have been shown to be a sign of increased cancer metastasis. Injection of breast cancer cells with and without invadopodia into nude mice results in increased metastasis when the cells have invadopodia (Thompson 1992, Coopman 1998). Further, unpublished data from several labs have suggested that there exists a possible progression from podosomes to invadopodia. These groups suggest that podosomes are the preliminary form of invadopodia as cells search for a degradable matrix, and once it is found, invadopodia form. Recently, it was demonstrated that smooth muscle cells, which can be induced to form podosomes on glass, spontaneously form complete functional invadopodia when cultured on thin fibrous ECM (Gimona 2008). Since invadopodia and podosomes share the same architecture and are never present simultaneously in the same cell, it is possible that podosomes are immature invadopodia. Our results show overexpression of Tm4 led to an increase of the height of the podosomes (or possibly short invadopodia) suggesting that Tm4 could play a role in stabilizing the transition from podosome to invadopodia. Additionally the formation of pseudo-actin rings on glass suggests Tm4 plays a role in stabilizing the actin within the podosomes to allow conformational changes. Live cell imaging is needed to determine whether the overexpression of Tm4 causes changes in the characteristics of the invadosomes formed. Overexpression of Tm4 allowed the cell to ignore the substrate and form actin rings and possibly invadopodia on glass.
Recently researchers found an invadosome model that patterns the placement of invadosomes similar to osteoclasts. RSV-transformed BHK cells (which are both classified as forming invadopodia and podosomes depending on the literature) show self organization of invadosomes into invadosome rings that transition to belts at the cell periphery (Badowski 2007). Using the RSV-transformed BHK cells and osteoclasts, they further demonstrated ring expansion from invadosome ring to belt is necessary for ECM degradation and transmigration through a cell monolayer (Badowski 2007). Our studies with Myo10 suggest Myo10 plays a role in transitioning osteoclast podosome rings to belts, and its wide distribution makes it possible to function similarly in other cell types such as RSV-transformed BHK cells. The defect of Myo10 suppression made osteoclasts unable to recover podosome belts, and also affected osteoclast resorption via actin ring formation. So the question remains, is an osteoclast actin ring a specialized invadosome for which podosomes are the immature model? Invadopodia-containing cells transport large vesicles filled with ECM degradation products through the cells during cancer progression, as seen by electron microscopy, similar to the trancytosis of degraded bone matrix through osteoclasts (Bowden 2001). As this area of research expands, it may give more answers to the podosome/actin ring relationship in osteoclasts. Future studies of Tm4 and Myo10 function may help shed light on this new area of research.

5.3 Tm4, MyoIIA, and Myo10 in Osteoclast Function

In an attempt to further define osteoclast function, our studies have focused on the relation of three actin binding proteins, Tm4, MyoIIA, and Myo10, in osteoclast migration and resorption. Each protein localizes to different areas around the actin
attachment structures in osteoclasts, yet together they regulate the same structures for proper osteoclast function. Tm4 localizes to the podosome core while MyoIIA is in the podosome cloud and Myo10 is adjacent to podosomes. On bone, Tm4 is highly concentrated in the actin ring while MyoIIA is partially concentrated in the ring, and Myo10 completely absent (Figure 5.2). The difference in distribution leads to many possible varied functions of these three proteins in osteoclasts.

MyoIIA was the only member of the three actin binding proteins studied found to participate in cell fusion, as it is naturally down-regulated at the start of fusion and up-regulated at the end of fusion. MyoIIA is known in other cell types to play a role in maintaining membrane tension homeostasis to allow for cell migration and function (Clark 2008). MyoIIA has also been suggested to facilitate membrane resealing by lowering membrane tension to making membrane break resealing easier for the cell (Togo 2003, Togo 2004). Cells have been shown to compensate for disruption in the plasma membrane by decreasing membrane tension. Thus, it is possible that osteoclast fusion is facilitated by decreasing membrane tension as two cells must break their plasma membranes and reseal with each other to fuse. An alternative hypothesis involves MyoIIA’s role in cytokinesis. Early dictyostelium studies demonstrated that if MyoIIA was down-regulated, incomplete cell division occurred resulting in a cell with two nuclei. In our studies, decreasing MyoIIA in the absence of RANKL led to multinucleation in RAW264.7 cells, but instead of two nuclei per cell as seen in the dictyostelium studies, they averaged 3.7 nuclei per cell. It is unknown if the natural decrease of MyoIIA occurs in osteoclasts before or after the cells become post-mitotic as these events occur around
the same time during the seven day differentiation period. It is possible that MyoIIA is
down regulated before osteoclast precursors become post mitotic, resulting in an initial
multinucleation that enhances cell fusion. Interestingly, the primary defect in Paget’s
disease (chapter 1) is an osteoclast phenotype in which cells differentiate more quickly
and form extremely large osteoclasts of up to 100 nuclei per cell (Reddy 2001). It is
possible that the disease has a connection to myosin IIA regulation in osteoclasts as
decreasing MyoIIA prematurely causes the cells to differentiate faster. Additionally, if
MyoIIA is not up-regulated correctly at the end of differentiation, the cells continue to
fuse to form large cells due to increased multinucleation.

The two myosin motors studied played opposing roles in cell spreading. While
suppression of MyoIIA caused an increase in cell spreading, suppression of Myo10
caused a decrease in cell spreading. Both MyoIIA and Myo10 utilize the microtubule
system to regulate cell spreading. MyoIIA regulates microtubule acetylation and thus the
stability of the podosome belt, while Myo10 utilizes the intact microtubule system to
transition the podosomes from podosome ring to belt. As mentioned previously, MyoIIA
has been shown to participate in creating cellular membrane tension by mediating stress
fiber formation to retain cell shape and allow for proper migration (Clark 2007).
Osteoclasts are a stress fiber free system, yet MyoIIA plays a role in cell tension by
affecting microtubule acetylation. Loss of acetylation in the MyoIIA knock-down
indicates a loss of stable peripheral belt podosomes. To better observe these cells, live
cell imaging of GFP-actin labeled cells could show if there are changes in the stability of
the podosomes at the periphery. This would also be useful to observe the phenotype of
loss of podosome ring to belt transition seen in the less spread Myo10 knock-down osteoclasts.

Suppression of all three proteins studied and increased levels of Tm4 caused a decrease in directional migration of 40-60%. This decrease could be attributed to different changes in the podosomes for each protein. Overexpressing Tm4 increased the height of podosomes, possibly enhancing the stability of the longer podosomes and thus affecting migration. Decreasing Tm4 caused mislocalization of bundling protein α-actinin and possibly the height of the podosomes, suggesting an increase in podosome turnover which would potentially lead to increased migration. Further, the unusual extra fibril actin within cells with lowered Tm4 levels could cause tension changes and thus decreased migration. The stability of under- and over-expression of Tm4 podosomes should be examined further to better understand how Tm4 is affecting migration. Live-cell imaging utilizing GFP actin and FRAP analysis, could indicate whether there are changes in the podosome turnover, which I suspect changes due to the amount of Tm4 bound to the podosome core. Decreasing MyoIIA led to increased spreading suggesting changes in tension though no significant changes were seen in the actual podosome structure of fixed cells. Decreased and increased Myo10 led to podosome patterning defects, thus affecting migration. Though they all caused different effects to the podosomes, the results for each were decreased migration by under- and over-expression studies. Live-cell imaging would allow better understanding of migration and podosome function in these studies as actin turn over of the podosome core can be measured using
FRAP, and time-lapse live cell imaging can demonstrate an average podosome life span and positioning.

Suppression of both Tm4, which overlaps the actin ring, and Myo10, which is absent in the actin ring, causes a decrease in resorption due to altered actin ring formation. Lowered Tm4 levels caused the actin ring to diminish in height while suppression of Myo10 caused the rings to decrease in perimeter, each leading to resorptive defects. Myo10 actin ring formation needs to be defined further with live-cell imagining. Step by step formation of actin rings has yet to be defined as distinctly as podosome dynamics. Myo10 leaves questions unanswered: Are the smaller actin rings formed and then utilized, or does the cell try continuously to form larger rings from the smaller rings, both resulting in decreased resorption? MyoIIA knock-downs, which had larger than normal actin rings, showed no changes in resorption due to being able to compensate for the increased actin ring perimeter with decreased migration.

From these studies, we propose Tm4 regulates the adhesion structures of osteoclasts by stabilizing actin in the podosome core and actin rings, and thus affecting osteoclast migration and resorption. We hypothesize MyoIIA is playing a negative regulatory role in cell fusion during osteoclast differentiation, decreasing to allow initial fusion and increasing to prevent extremely large, less motile cells to form. Although suppression of MyoIIA leads to osteoclast cell perimeters and actin ring perimeters twice as large as controls, there are no significant changes in resorption due to the decrease in motility; thus, decreasing MyoIIA does not affect resorption. From our studies, we propose the primary role of Myo10 is in transitioning the podosome ring to podosome
belt in osteoclasts via its ability to bind both microtubules and actin. Together, our results have started the studies in attempting to elucidate common and osteoclast specific functions of tropomyosins and myosins through osteoclast biological studies.
Figure 5.1 Invadosomes. Diagram illustrating the few differences between podosomes and invadopodia. Invadopodia exhibit longer actin cores along with increased ECM degradation. Mature invadopodia also have smaller attachment at the contact end of the actin core (Gimona 2008).
Figure 5.2 Localization of Tm4, MyoIIA, and Myo10. Diagram demonstrating the unique localization of the three proteins studied in this thesis with Tm4 in podosome core and actin ring, MyoIIA in podosome cloud and in actin ring, and Myo10 around podosome area and absent from the actin ring.
CHAPTER 6

MATERIALS AND METHODS

6.1 Reagents

For detection of tropomyosins, AB5441, AB5449, AB5447, AB5443, and AB5445 were purchased from Chemicon International. Rabbit polyclonal antibodies against the MyoIIA heavy chain, Tm311, and α-actinin antibodies were purchased from Sigma. An anti-paxillin antibody and a mouse anti-TRIP6 antibody were purchased from BD transductions laboratories. An anti-alpha tubulin antibody was purchased from Molecular probes (Invitrogen corp). Anti-mouse Tm4 was purchased from Abnova. Loading control antibodies to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin, both mouse monoclonal antibodies, along with rabbit TRIP6 were purchased from Abcam. Antibodies against V-ATPase subunits E1 and a3 were generated in this laboratory and were described previously (Jeyaraj 2005). Anti-cathepsin B antibody was purchased from Calbiochem. Anti-Myo10 antibody was provided to us from Richard Cheney (Berg 2000).

Protease inhibitors were purchased from EMD Biosciences and used at various concentrations: ALLM (50 µM), E64d (50 µM), ZLLF (25 µM), MG132 (2 mM),
Calpeptin (5 µM), Calpastatin peptide (1 µM), Cathepsin Inhibitor I (50 µM), and Cathepsin L Inhibitor I (50 µM).

Active CatB was visualized using Magic Red CatB detection kit following the manufacturer’s directions (Immunochemistry Technologies, LLC.)

6.2 Cell Culture

Osteoclasts were generated either from RAW264.7 macrophages (ATCC) or murine bone marrow as previously described (Krits 2002). Briefly, RAW264.7 cells were passaged in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% heat inactivated fetal bovine serum, and penicillin/streptomycin. For generation of osteoclasts, the macrophage precursors were plated at 20,000 cells/cm² in the same medium with the addition of 100 ng/ml of a GST-RANKL fusion protein, replacing the medium every 2-3 days. For bone marrow derived osteoclasts, marrow and spleen cells from male Swiss-Webster mice (Harlan), 4-8 weeks in age, were incubated overnight in αMEM containing 10% heat inactivated fetal bovine serum and 20 ng/ml M-CSF (R&D Systems). On the following day, non-adherent cells were collected and incubated in the same medium with the addition of 100 ng/ml GST-RANKL, replacing the medium every 2-3 days. Mature osteoclasts TRAP+ (both RAW264.7- and marrow-derived) were present by day 6-7 of culture.

6.3 Western Analysis

For Western analysis, osteoclast lysates were harvested with M-PER reagent (Pierce) and were run in pre-cast PAGE gels (Bio-Rad Corp.), and transferred to Hybond membrane (GE HealthCare). Primary antibodies were allowed to bind to the membranes
using standard methodology and were detected using horseradish peroxidase-labeled secondary antibodies coupled with Pierce SuperSignal West Pico Chemiluminescent reagents.

6.4 Immunocytochemistry and Microscopy

Osteoclasts were cultured either on glass coverslips or thinly-cut ivory slices. Cells briefly were fixed in a solution of 1% formaldehyde in pH 6.5 stabilization buffer (127 mM NaCl, 5 mM KCl, 1.1 mM NaH$_2$PO$_4$, 0.4 mM KH$_2$PO$_4$, 2 mM MgCl$_2$, 5.5 mM glucose, 1 mM EGTA, 20 mM Pipes), and were subsequently more extensively fixed and permeabilized 1hr in a solution of 2% formaldehyde, 0.2% Triton X-100, and 0.5% deoxycholate in the same stabilization buffer (Zeng 2000). When staining microtubules, 0.05-0.1% glutaraldehyde was added to both the initial and second fixatives. Primary antibodies were added in a standard PEG blocking buffer and were detected using Alexa-conjugated secondary antibodies (Invitrogen Corp.). F-actin was labeled using Alexa-conjugated phalloidin, also from Invitrogen. Cells were visualized using a Zeiss 510 META laser scanning confocal microscope (Campus Microscopy and Imaging Facility, The Ohio State University). Actin ring, podosome, and pit thickness was determined by generating multiple Z-stack images and measuring these structures at their thickest points.

Cell perimeter and ring perimeter were measured using SigmaScan Pro 5.0 software (SPSS Science, Chicago, IL). Nuclei were visualized with bisbenzimide staining for 10 minutes following the secondary staining. Nuclei were counted and each sample size contained similar total nuclei per coverslip.
6.5 Competitive RT-PCR of mRNA

To determine mRNA expression levels of Tm4, MyoIIA, and Myo10, RT-PCR primers were created that corresponded to murine sequences. For an internal standard, a cDNA was created that corresponded to the expected PCR product using the primers above, but contained an internal deletion, a T7 promoter element, and a tail of 15 adenosines, as previously described (Jeyaraj 2005, McMichael 2007). This product was transcribed in vitro using the MAXIscript system (Ambion), and the resulting RNA (the internal standard) was added to 1 µg of osteoclast total cellular RNA prior to reverse transcription and PCR. These reactions were performed using the Superscript First-strand synthesis System and TaqDNA Polymerase, both from Invitrogen. The resulting RT-PCR products were run in a 2% gel and then stained with ethidium bromide to visualize the relative intensities of the bands which were measured using Quantity One software (Bio-Rad Corp.). Primers and deletions created for each gene:

Tm4 sense 5’-AGCCCCACACTTTGAAGCAC-3’
Tm4 antisense 5’-CCTGGAATAAGACGCTTGCTCC-3’
(17% deletion, 10 pg standard to 1 µg sample)

MyoIIA sense 5’-AGACCGCTGATGCTATGAACCG-3’
MyoIIA antisense 5’-ATGGTCAGAGCAGTCTCAAGGC-3’. (33% deletion, 10 pg standard to 1 µg sample)

Myo10 sense 5’-AACAATGGACAGCTTCTTTCCCG
Myo10 antisense 3’-GCATAGCATTCTTGGCAATGG
(21% deletion, 3 pg standard to 1.5 µg sample)
6.6 Immunoprecipitation

Performed as previously described (Lee 1999), briefly, for radioactive labeling cells were labeled overnight in 90% methionine and cystine free media supplemented with 50 μCi/ml Tran\(^{35}\)S-label (MP Biomedicals, Irvine, CA). For western analysis, cells were not radioactively labeled. The cells were solubilized in a Triton X-100 buffer (1% Triton X-100, 20 mM Tris-Cl, pH 7.4, 1 mM EDTA, 1 mM DTT, 0.1% SDS, 10% glycerol, 5 mM sodium azide, and protease inhibitors) and centrifuged to remove insoluble material. Following a preclearing incubation with protein A-Sepharose, the lysates were incubated with Tm4, TRIP6, or MOPC antibody for 2-4 hours at 4°C and then with 50% protein A-Sepharose for 30 minutes at 4°C. The immune complexes were washed several times in NET-GEL buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 5 mM sodium azide, 0.1% NP-40, and 0.25% gelatin) and then half the radioactive samples were incubated overnight in Protein G-buffer (20 mM Tris-Cl, 0.5 mM CaCl\(_2\), 0.2 mM DTT, 100 mM NaCl, 5 mM MgCl\(_2\), 0.5 mM ATP, 10 μM DNase I). After several washes with NET-GEL buffer the samples were run in SDS-PAGE. For radioactive samples the gel was treated with Fluoro-Hance (RPI Corp., Mt. Prospect, IL), dried, and exposed to film. For Western analysis, the proteins were transferred to a membrane and probed using standard methodologies.

6.6 Pulse-chase Analysis for MyoIIA

Osteoclasts on day 0, day 3, and day 7 of differentiation were labeled two hours in methionine and cysteine free media supplemented with 45 μCi/ml Tran\(^{35}\)S-label (MP Biomedicals). The cells were then incubated in complete media for the various time
points before immunoprecipitation. Immunoprecipitation was performed as previously described (Lee 1999). The cells were solubilized in M-PER supplemented with protease inhibitors and centrifuged to remove insoluble material. The precleared lysates were then incubated with MyoIIA antibody for 12-16 hours at 4°C and then with 30 μl protein A-Sepharose for 30 minutes at 4°C. The complexes were then washed once with NET-GEL buffer and incubated with G-buffer for two hours. The precipitates were run in a SDS-PAGE gel, treated with Fluoro-Hance (RPI), and exposed to film. Quantity One software was used to quantify the decrease in intensity of the myosin IIA immunoprecipitate.

6.7 Knockdowns of Genes Studied

To knock down murine expression, siRNA’s were designed and synthesized by Ambion. One siRNA of each was used for all of the experiments and a second siRNA was used to confirm the results seen by the first siRNA. For all experiments, a non-targeting dsRNA from Ambion was used as a negative control, although siRNA’s homologous to each siRNA but containing three point mutations also were used for confirmation in the MyoIIA and Myo10 experiments. RAW264.7 cells were plated and stimulated with GST-RANKL to form osteoclasts. On day 5 of differentiation for Tms or day 4 for myosins, siRNA or an equal concentration of a control siRNA was added to Lipofectamine 2000 (Invitrogen Corp.) in plain DMEM and added to the cells. The medium was replaced after five hours. For immunocytochemical analysis, the cells were scraped and replated on ivory slices or glass coverslips immediately following the transfection. For RNA analysis, total cellular RNA was harvested after the transfection with RNA-Bee (Tel-test, Inc.). For protein analysis, whole cell lysates were harvested
post transfection with M-PER (Pierce Biotechnology). Time course of days post
transfection for both RNA and protein were done and the optimal day was used for the
experiments. Concentration was also optimized to find the lowest concentration to
decrease protein levels.

Bone marrow-derived osteoclasts were transfected via electroporation. On day
four of differentiation in culture, after osteoclasts had formed, cells were scraped,
pelleted, and resuspended in siPORT buffer (Ambion). The cells were electroporated at
250V/ 50μF with the siRNA solution or an equal concentration of a control siRNA
(Ambion), then plated in standard differentiation medium on glass or ivory for
immunocytochemistry, or plastic for RNA and protein analysis. Control transfections
utilizing a fluorescent oligo demonstrated that the transfection efficiencies for both
RAW264.7 and MBM osteoclasts was greater than 95%.

Following are the sequences or ambion siRNA# and the concentration used:

Tm4 siRNA: ID 293590  75 µM
MyoIIA: siRNA1  5’-GGCUGAUUUCUGCAUUAUCtt-3’ (sense)

5’-GAUAAUGCAGAAAUCAGCCtt-3’ (antisense)  50 µM
MyoIIA: siRNA2  5’-GGUGAACAAGGACGACAUCtt-3’ (sense)

5’-GAUGUCGUCCUUUGUUCACCtt-3’ (antisense).  25 µM
Myo10 siRNA: ID 73762  50 µm

ID 73578  75 µm
6.8 Osteoclast Resorption and Motility Assays

Four/five days after initial RANKL stimulation, osteoclasts were transfected and immediately plated on BD BioCoat Osteologic Discs (BD Biosciences) or thinly cut ivory slices. Control and siRNA-treated cells were kept on the discs/bone for 3 days. The cells were removed by the addition of bleach for 5 minutes and several washes with water or by scraping. Resorption clearings were assayed by photographing the resulting discs under low magnification, and quantifying resorbed areas with SigmaScan Pro 5.0 software (SPSS Science). Equal numbers of images were compared among test groups. On ivory the cells were removed and the pits stained with acid hematoxylin for ten minutes followed by excessive washing with water. The cells were viewed by confocal microscopy and the pit depth and perimeter was measured using the confocal software.

Motility was measured by the use of 8.0 μm pore Transwell migration chambers (Corning Life Sciences). The bottom side of the membrane was coated with collagen (3 mg/ml diluted 1:2 with 100% ethanol) and dried overnight. Immediately following the transfection, the cells were scraped and replated at a low density on the upper side of the chamber. After 72 hours, the cells were stimulated overnight to migrate by the addition of 40 μg/ml osteopontin peptide to the bottom of the well. Cells on the upper side of the membrane were removed with a cotton swab, and the remaining cells were fixed and stained for tartrate resistant acid phosphatase using a Leukocyte Acid Phosphatase kit (Sigma). For myosin IIA, on day 4 before transfection mononuclear cells were removed from the cultures and the remaining osteoclasts were transfected with the siRNAs to control for excessive cell fusion and a difference in cell size between the control and
siRNA-treated cells. For Myo10, the cells were replated one day post transfection and stimulated three days post transfection.

6.9 Overexpression of Proteins

For generation of expression plasmids, fragments containing primarily coding regions and little untranslated region were TOPO-cloned into the eukaryotic expression vector pEF6/V5-His (Invitrogen Corp.). Myo10 domains: FLX a.a.1-2065, PH a.a. 1167-1462, MyTH4 a.a. 1541-1712, FERM a.a.1752-2065, PH1A-1B a.a.1168-1313, PH1A-1B a.a. 1168-1381, and PH3 a.a. 1383-1463. Each construct was stably transfected into RAW264.7 macrophages; the empty pEF6/V5-His vector was stably transfected into cells as a control via Lipofectamine and Plus reagent transfections (Invitrogen Corp., Carlsbad, CA). Cells were maintained in 3 μg/ml blasticidin for selection and individual clones were picked for the studies.

6.10 Microtubule Depolymerization

To depolymerize microtubules, osteoclasts were placed on ice at 4°C for three hours. 37°C complete media containing RANKL was added immediately following cold treatment and the cells recovered for the times indicated in the figures.

6.11 Statistical Analysis

Pairwise comparison of samples were performed using Student’s t-test. Linear regression was used to model the relationship between cell or actin ring perimeter and the number of nuclei, allowing for different slope and intercept terms for each cell condition and was performed by the Center for Biostatistics at The Ohio State University College of Medicine. Statistical comparison was deemed significant at P < 0.05.
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