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The Role of Autophagy and IL-17 in Bone Resorption

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The Role of Autophagy and IL-17 in Bone Resorption

by

Carl J. DeSelm

A dissertation presented to the Graduate School of Arts and Sciences of Washington University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

May 2012

Saint Louis, Missouri
ABSTRACT OF THE DISSERTATION

The Role of Autophagy and IL-17 in Bone Resorption

by

Carl J. DeSelm

Doctor of Philosophy in Biology and Biomedical Sciences

Molecular Cell Biology

Washington University in St. Louis, 2012

Professor Steven Teitelbaum, Chairperson

Osteoclasts are essential for skeletal homeostasis (Teitelbaum, 2000). These macrophage-lineage cells function by generating a polarized microenvironment between themselves and bone wherein skeletal matrix is degraded. This resorptive compartment is isolated from the general extracellular space by an actin ring which encompasses the ruffled border, a convoluted plasma membrane structure formed by its fusion with lysosome-related vesicles containing an electrogenic H+ATPase, a chloride channel, LAMP-1, and cathepsin K. In consequence, resorption of bone reflects secretion of HCl to mobilize mineral, and cathepsin K to degrade the collagen-rich organic matrix, into the resorptive space (Stenbeck, 2002; Zhao et al., 2008). We determined that the two ubiquitin-like conjugation systems required for macroautophagy are necessary for generation of the osteoclast ruffled border. These autophagy proteins regulate the cell’s capacity to efficiently secrete lysosomal proteins into the resorptive microenvironment both in vitro and in vivo and thus its ability to degrade bone. These findings provide a novel role for autophagy proteins and a possible mechanism to explain human genome
wide association data suggesting a link between the autophagy pathway and predisposition to osteoporosis (Zhang et al.).

Further studies in this thesis implicate IL-17 signaling in osteoporosis. Although not traditionally thought of as an inflammatory disease, estrogen deficiency osteoporosis is partially mediated by T cells, at least in mice (Weitzmann and Pacifici, 2007). Th17 cells, which secrete IL-17, mediate a number of inflammatory and autoimmune conditions, including arthritis-mediated bone destruction. We find IL-17 critical in the pathogenesis of bone loss due to estrogen deficiency. IL-17 receptor deficient mice are protected from bone loss after ovariectomy, as are mice treated with a neutralizing IL-17 antibody. Mice treated with a small molecule inhibitor of IL-17 production are similarly protected. The effect of IL-17 on osteoclasts is indirect, via osteoblastic upregulation of RANKL. The SEFIR domain of IL-17R is critical for osteoblast-mediated osteoclast formation and RANKL production. The adaptor protein Act1, specifically its SEFIR domain, is required for osteoblast-mediated osteoclast formation in response to IL-17, and Act1-/- mice are also protected from bone loss induced by estrogen deficiency.
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Most importantly, I would like to thank my mentor, Dr. Steven Teitelbaum, for encouraging my ideas, appreciating my accomplishments, even when they were small, guiding me when I needed help, always solving my dilemmas, and always pushing me to strive for excellence. Before I joined the lab I knew Dr. Teitelbaum was a great scientist, but upon leaving I can now say he is a great mentor, as well as role model.

Outside of the lab, I would like to thank my mother, for always believing, always supporting, and always finding ways to help me succeed; I likely would not be at this point without her help. Thank you dad, for always pushing me to identify and stay on the noble path, and to always consider the greater good beyond myself. Thank you to the rest of my family – Katie, Ollie, and John – for being there for me throughout the years.

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Preface

This thesis addresses several aspects of bone biology, focusing on novel mechanisms in both the bone resorbing osteoclasts and bone forming osteoblasts. The projects described in this manuscript progressed enough to reach public presentation and/or publication, but they are the minority. My favorite in the pool of unsuccessful projects was an attempt to derive human osteoblasts and osteoclasts from skin fibroblasts, which would allow one to grow and study an individual’s bone-forming and bone-resorbing cells to characterize their skeletal disease using only a skin biopsy. It would also provide the opportunity to expand and/or modify the cells and re-introduce them, if the methods became safe, into the same person to provide new or more functional bone forming or resorbing cells without worry of rejection. The plan was ambitious, since osteoclasts had never been differentiated from human stem cells, and since methods for inducing pluripotency in fibroblasts had just been discovered. Nonetheless, we were able to create pluripotent stem cells from fibroblasts, and were able to differentiate mouse stem cells to osteoblasts and osteoclasts, but never succeeded in pushing human induced stem cells into osteoblast or osteoclast lineages.

In the middle of this stem cell project, near the time of my qualifying exam, I attended a talk on a new and interesting topic of autophagy, which I learned from Pubmed was completely unstudied in the bone. The Virgin Lab was kind to share some mouse bones from their autophagy deficient animals, so I started a side project to test whether these autophagy proteins had a role in osteoclasts. It eventually became apparent the cells had a defect in secretion, which was a hypothesized but unestablished role for autophagy, thus spiking interest in the Virgin Lab and providing rationale for my mentor
to support pursuit of the project. I am very fortunate for the patience and freedom allowed by my mentor, Steve Teitelbaum, who with great foresight gave me the promising project of studying the role of IL-17 on bone disease in the very beginning, and likely had points of annoyance along the way as I sacrificed time on this project to pursue others as they jumped to mind. I am also lucky that even when I worked on IL-17, Steve allowed me to pursue higher risk experiments that were not part of the defined path, which often did not work. In the end I matured greatly, and learned how to better recognize good and bad projects and identify the types of experiments that will quickly disprove an idea or maximize its human relevance.

Since all projects in this thesis relate to the skeleton and to the osteoclast, the general introduction provides background to importance of this cell and how it functions. Within each chapter is more specific background to each project.
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Atg Proteins Drive Lysosomal Secretion by Osteoclasts
Chapter 1: Introduction

**Bone**

Bones survive hundreds of years outside the body, but inside roughly 10% of each bone is broken down per year in the adult, and 30-100% per year in the child (Nanci, 2008). Bone is composed of an organic component, which is 90% type I collagen and 10% other proteins such as osteocalcin and osteopontin, and an inorganic phase, which is mainly calcium phosphate in the form of calcium hydroxyapatite. The osteoclast, the lone bone-resorbing cell, secretes acid to dissolve the inorganic calcium phosphate component, and proteases to break down the organic matrix. Each phase of bone degradation is usually followed by a phase of bone formation by the osteoblast, resulting in healthy bone remodeling and only gradual changes in bone mass. In pathological states, such as osteoporosis, osteoclast activity is increased relative to that of the osteoblast, causing net bone loss. Thus, most drug treatments for osteoporosis target the osteoclast. On the contrary, when osteoclast activity is genetically impaired, bone density is increased leading to osteopetrosis.

**Osteoclast Development and Signaling**

Osteoclasts are large, multinucleated cells that form by multiple fusions of monocyte/macrophage precursors (Figure 1.1). Osteoclast differentiation and activation is driven by macrophage-colony stimulating factor (M-CSF), and receptor activator of nuclear factor-kappaB ligand (RANKL). RANKL is primarily produced by the osteoblast, and is required for osteoclastogenesis (Theill, Boyle, & Penninger, 2002).
Figure 1.1: The bone resorbing osteoclast

Electron micrograph of an osteclast *in vivo*. Osteoclasts are multinucleated cells that secrete hydrogen ions and lysosomal enzymes over the bone surface. Convoluted, finger-like projections of plasma membrane form at areas of active secretion.
RANKL mainly exerts its differentiating effect by activating nuclear factor kappa B (NFκB) signaling downstream of its receptor, RANK. A TNF-receptor family member, RANK lacks intrinsic enzymatic activity, but recruits TRAF6, which then leads to not only NFκB activation, but also that of mitogen-activated kinases (MAPKs), including Jun N-terminal kinase (JNK) and p38 (Kobayashi et al., 2001; Lomaga et al., 1999; Naito et al., 1999; Wong et al., 1998).

TRAF proteins are important in nearly all NFκB signaling pathways (Hacker & Karin, 2006; Scheidereit, 2006). Of the seven TRAF proteins, TRAFs 2-7 have an N-terminal RING domain common to many E3 ubiquitin ligases. Ubiquitin ligase activity has been most clearly established for TRAF 2 and 6. Unlike most ubiquitin ligases, which target proteins for degradation, TRAF6-mediated ubiquitination primarily activates downstream kinase cascades via K63 polyubiquitination (Deng et al., 2000; Wang et al., 2001). TRAF6 E3 ligase activity is important in the activation of the IKK complex leading to both canonical and noncanonical NF-κB pathways (Chen et al., 2006), and a point mutation in a conserved region of the RING domain abolishes ubiquitination and NFkB signaling (Deng et al., 2000). Auto-ubiquitination of TRAF6 via a Lys63-linked polyubiquitin chain also mediates the recruitment of other mediators, resulting in phosphorylation of IKK (Kanayama et al., 2004). Although TRAF6 knockout (KO) mice exhibit severe osteopetrosis, it is unclear whether the defect is primarily due to osteoclastogenesis or function. TRAF6 KO mice created by Naito et al showed significantly fewer osteoclasts and impaired differentiation in vitro, while the knockout mice created by Lomaga et al showed normal numbers of osteoclasts in vivo but impaired ability of the cells to resorb bone (Kobayashi et al., 2001; Lomaga et al., 1999; Naito et
NF-κB signaling is essential for osteoclast development. Mammals express five NF-κB proteins that belong to two classes. Proteins of the first class do not require processing to become activated, and consist of RelA (p65), c-Rel, and RelB. Proteins complexes containing RelA and c-Rel are kept inactive in the cytoplasm by binding to IkB inhibitor proteins. Upon activation of the pathway, IkB kinase (IKK) phosphorylates IkB, leading to its degradation. IKK exists in a complex consisting of NEMO, IKKα, and IKKβ. The second class of proteins are synthesized as large precursors, p105 and p100, and are proteolytically processed into mature p50 and p52 NF-κB proteins, respectively. In the canonical pathway, p50 dimerizes with RelA or c-Rel. In the alternative pathway, p52 dimerizes with RelB (Figure 1.2) (Hayden & Ghosh, 2008; Karin & Lin, 2002). Canonical pathway activation occurs in response to all NF-κB-activating stimuli, while the alternative pathway is only activated by the TNF family cytokines RANKL, CD40L, BAFF, and lymphotixin-β. Both pathways are operant in the osteoclast (Vaira et al., 2008), and p50-/-p52-/- mice develop osteopetrosis due to defective osteoclastognesis (Franzoso et al., 1997; Iotsova et al., 1997).

p62, one Traf6 binding partner, has been implicated osteoclastogenesis and NFκB signaling. Null mutation of p62 in osteoclast precursor cells causes severe impairment of osteoclast formation in a culture system. However, p62-deficient mice exhibit no defects unless they are challenged by osteoclastogenic stimuli such as PTH-related peptide (PTHrP) (Duran et al., 2004). On the other hand, p62 mutations in humans are associated with Paget's disease of bone (PDB), a genetic disorder characterized by large osteoclasts and focal, disorganized increase of bone turnover (Laurin, Brown, Morissette, &
Raymond, 2002). In these PDB patients, mutant p62 proteins with reduced ability to bind ubiquitin and ubiquitin-conjugated proteins are expressed (Cavey et al., 2005). p62 is degraded by autophagy (Bjorkoy et al., 2009), and we observe p62 accumulation in Atg5-, Atg7-, and Atg16L-deficient osteoclasts, all of which have impaired autophagy.

**Figure 1.2: The NFκB Pathway**

Following receptor ligation, signaling to IKK proceeds through TRAF/RIP complexes, generally in conjunction with TAK1, leading to canonical NF-κB signaling, or through TRAFs and NIK leading to the noncanonical NFκB pathway. IKK activation results in IκB phosphorylation and degradation in the canonical pathway or p100 processing to p52 in the noncanonical pathway. Phosphorylated NFκB dimers bind to κB DNA elements and induce transcription of target genes. Adapted from Matthew S. Hayden and Sankar Ghosh, Shared Principles in NF-κB Signaling.
**Osteopetrosis and Disorders of High Bone Density**

Since this thesis aims to characterize novel molecular pathways that, when absent, contribute to high bone density, it is useful to consider other proteins and pathways responsible for diseases of high bone mass. Much has been learned about the critical molecules in osteoclast biology by genetic disorder causing high bone density.

Osteopetrosis refers to disorders arising from reduced bone resorption, while disorders of increased bone formation are simply called high bone mass syndromes (de Vernejoul & Kornak). Osteosclerosis refers to more radio-opaque, and thus denser, bones of normal size – in the long bones this results from trabecular thickening, and can be either osteoblast or osteoclast related. Hyperostosis refers to cortical thickening of long bones or enlargement of affected skull (de Vernejoul & Kornak).

Osteopetrosis is divided an autosomal dominant (ADO) adult type that is less severe (Albers-Schonberg disease) and an autosomal recessive (ARO) type that is typically fatal during infancy or early childhood if untreated. It is estimated around 60% of patients with ARO have a mutation in the TCIRG1 gene encoding the $\alpha_3$ subunit of the proton pump that brings $H^+$ into the resorptive space, while around 13% have a mutation in the CLCN7 chloride channel. The chloride channel is necessary to maintain electroneutrality in the bone pit (Jentsch, Friedrich, Schriever, & Yamada, 1999). Although the proton pump subunit was initially thought to be specific for the osteoclast, it has since been found in gastric parietal cells where it is involved in gastric acid secretion and calcium absorption, which may be responsible for a rickets-like phenotype in children with the mutation (Schinke et al., 2009). In a smaller number of patients, the $OSTM1$ gene is mutated (Chalhoub et al., 2003). These mutations lead to normal or
increased numbers of osteoclasts, but defective function. More recently, mutations are described in RANKL or RANK that lead to lower numbers of osteoclasts (Guerrini et al., 2008; Sobacchi et al., 2007).

ADO prevalence is estimated at 5 per 100,000, and 60-80% of individuals with radiographic signs of ADO experience clinical problems. Fractures occur in 80%, most commonly the femur; some patients have more than 10 fractures (Benichou, Laredo, & de Vernejoul, 2000; Waguespack, Hui, Dimeglio, & Econs, 2007). These patients often have mutations in CLCN7, which are less severe than those CLCN7 mutations causing ARO (Cleiren et al., 2001).

Cargonic anhydrase II (CAII) is present in many tissues, including brain, kidney, erythrocytes, cartilage, lung, and stomach. In osteoclasts, it is necessary for intracellular production of H+ ions that are transported by the proton pump into the resorptive space (Gay, 1996). Mutation of this enzyme leads to osteopetrosis with renal tubular acidosis (Sly, Hewett-Emmett, Whyte, Yu, & Tashian, 1983).

A specific high bone density disorder attributed to Cathepsin K deficiency is termed Pycnodysostosis. The first suspected case may be have been the French impressionist painter Henri de Toulouse-Lautrec (Maroteaux & Lamy, 1965), but now over 100 cases are described. It is autosomal recessive, leading to short stature with a relatively large cranium. Cathepsin K is specific for the osteoclast, and is a protease secreted above the bone to degrade its organic component (Gelb, Shi, Chapman, & Desnick, 1996).

Disorders of high bone density due to osteoblast activity are also described. Progressive Diaphyseal Dysplasia (PDD), also known as Camurati-Engelmann disease, is due to mutation in \( TGF\beta 1 \) that causes premature activation of the protein (Janssens et al.,
2000; Janssens, ten Dijke, Ralston, Bergmann, & Van Hul, 2003). Though the protein is ubiquitously expressed, the disease is largely limited to the skeleton. Osteopoikilosis, or spotted bone disease, is due to *LEMD3* mutation, which encodes a nuclear membrane protein that inhibits Smad proteins, which mediate TGF-β signaling (Hellemans et al., 2004).

The Wnt pathway is mutated in several osteoblastic bone disorders, including Worth syndrome (*LRP5* mutation) (Van Wesenbeeck et al., 2003), osteopathia striata (*WTX* mutation) (Jenkins et al., 2009; Viot et al., 2002), Van Buchem disease, and sclerosteosis (sclerostin mutation) (Balemans et al., 2002; Hamersma, Gardner, & Beighton, 2003).

Mutation of *HPGD*, the main enzyme responsible for degrading prostaglandins, causes Pachydermomperiostosis. The disorder leads to gradual enlargement of the hands and feet, arthralgia, skin thickening, and clubbing (Seifert et al., 2009; Uppal et al., 2008). Prostaglandins have multiple effects on osteoblasts and osteoclasts (Blackwell, Raisz, & Pilbeam).

It is interesting to note that disorders from decreased osteoclast activity, such as osteopetrosis or pycnodysostosis, result in frequent fractures, while disorders with increased bone formation from either increased activity of the Wnt pathway or activating mutation of TGF-β are not associated with fracturing. This discrepancy may be due to decreased elasticity of the skeleton or defective repair of micro-damage resulting from low bone remodeling. Although therapeutics that inhibit osteoclasts generally reduce fracture rate (Iwamoto, Sato, Takeda, & Matsumoto, 2008), it is still debated whether these drugs may also lead to pathologic fractures in some circumstances (Black et al.).
Therapeutic inhibition of osteoclasts may still allow for cyclic periods of bone resorption between doses, which may reduce the negative side effects resulting from complete blockade of bone turnover.

Human genome wide association analysis (GWAS) studies are implicating an increasing number of genes and pathways in bone homeostasis. It will be important to determine the effect of the identified genes in the osteoclast or osteoblast, as characterization of these novel pathways in the bone may lead to new types of drugs, exemplified by current successful clinical trials using sclerostin and RANKL antibodies. The following chapters implicate two new pathways in bone homeostasis, from the molecular level to the living animal. Ultimately, knowledge of these pathways may better our understanding of bone disease, or lead to better treatments.
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(1998). The TRAF family of signal transducers mediates NF-kappaB activation
Chapter 2: Autophagy Proteins Mediate Lysosome Secretion and Bone Resorption

Abstract

Osteoclasts are essential for skeletal homeostasis (Teitelbaum, 2000). These macrophage-lineage cells function by generating a polarized space between themselves and bone wherein skeletal matrix is degraded. This resorptive compartment is isolated from the general extracellular space by an actin ring which encompasses the ruffled border, a convoluted plasma membrane structure formed by its fusion with lysosome-related vesicles containing an electrogenic H+ATPase, a chloride channel, LAMP-1, and cathepsin K. Secreted HCl then mobilizes bone mineral while cathepsin K degrades the collagen-rich organic bone matrix (Stenbeck, 2002; H. Zhao et al., 2008). We determined that the two ubiquitin-like conjugation systems required for macroautophagy are necessary for generation of the osteoclast ruffled border and the cell’s capacity to efficiently secrete lysosomal proteins into the resorptive microenvironment both in vitro and in vivo. These findings provide a novel role for autophagy proteins and a possible mechanism to explain human genome wide association data suggesting a link between the autophagy pathway and predisposition to osteoporosis (Zhang et al.).
**Introduction**

**Autophagy**

Autophagy (more specifically macroautophagy, referred to here as autophagy) is a pathway whereby cytoplasmic material and organelles are removed from the cell by self-digestion. This process of self-degradation is traditionally thought of as a mechanism by which cells may survive periods of starvation, and also serves the beneficial function of clearing aberrant or long-lived proteins and organelles. Although basal levels of autophagy exist in most cells, autophagy is also induced by a variety of cellular stressors, such as starvation (Mizushima, Yamamoto, Matsui, Yoshimori, & Ohsumi, 2004), growth factor deprivation (Lum et al., 2005), ER stress (Yorimitsu, Nair, Yang, & Klionsky, 2006), microbial infection (Nakagawa et al., 2004), and hypoxia (Papandreou, Lim, Laderoute, & Denko, 2008). Autophagy work was pioneered in yeast, where over 30 autophagy related genes (Atg’s) have been discovered. Many mammalian homologues of these yeast proteins are now known, and the functions of autophagy have expanded beyond the simple role of cell survival during starvation.

**Autophagosome Formation and Maturation**

The process of autophagy first involves enclosure of cytoplasmic material, most often non-specifically, into double-membraned vesicles known as autophagosomes (Figure 1). Autophagosomes receive proton pumps and lysosomal enzymes (in that order, as suggested by (Dunn, 1990)) from late endosomes or multivesicular bodies, thus slowly becoming more acidic (Berg, Fengsrud, Stromhaug, Berg, & Seglen, 1998; Dunn, 1990; Gordon, Hoyvik, & Seglen, 1992; Lawrence & Brown, 1992; Liou, Geuze, Geelen, & Slot, 1997; Punnonen, Autio, Kaija, & Reunanen, 1993; Tooze et al., 1990). The outer...
membrane of the autophagosome eventually fuses with the lysosome, delivering its contents still contained within the inner membrane. Thus, the inner membrane as well as its contents are degraded by lysosomal enzymes. Lysosomal transporters then export amino acids and other degradation products back to the cytoplasm for re-use (Mizushima, 2007).

While lysosomes and endoplasmic reticulum (ER) contain numerous integral membrane proteins, only two have been identified on the autophagosome: Atg9 and vacuolar membrane protein 1 (VMP1). (Fengsrud, Erichsen, Berg, Raiborg, & Seglen, 2000; Punnonen, Pihakaski, Mattila, Lounatmaa, & Hirsimaki, 1989; Rez & Meldolesi, 1980). Atg9 has six transmembrane domains and cycles between the TGN and late endosomes in a ULK1 (yeast Atg1 homologue) dependent manner. Knocking down Atg9 impairs autophagy (Young et al., 2006), and Atg9 is proposed to deliver lipids to the forming autophagosome (Kim, Huang, Stromhaug, & Klionsky, 2002). VMP1 localizes predominantly to the ER, and colocalizes with LC3 and Beclin1 on autophagosome membranes (Ropolo et al., 2007). Overexpression of VMP1 induces autophagosomes, even under nutrient-rich conditions, and depletion using siRNA inhibits autophagosome formation after starvation.

The source of the membrane that forms autophagosomes in mammalian cells remains a mystery. In yeast, a unique compartment known as the pre-autophagosomal structure forms the autophagosome (Kim et al., 2002), but such a structure has not been identified in mammals. Conventional SNARE membrane fusion machinery, including N-ethylmaleimide-sensitive factor (NSF), soluble NSF attachment protein (SNAP), and SNAP receptors, are not needed for autophagosome formation in yeast (Ishihara et al.,
Additionally, mutants defective in Sec12 or the Sec23–Sec31 complex, which are needed for COPII vesicle formation and transport of vesicles from the ER to the Golgi, have no autophagy defects (Hamasaki, Noda, & Ohsumi, 2003; Ishihara et al., 2001). Thus, unconventional vesicle trafficking and membrane formation mechanisms may be used during autophagosome formation.

Once autophagosomes are formed, yeast studies have shown they fail to fuse with the vacuole (the yeast lysosome) in the absence of Ypt7 (the yeast homolog of Rab7) (Kirisako et al., 1999), Vam3 (a syntaxin homolog) (Darsow, Rieder, & Emr, 1997), Sec18 (yeast homolog of NSF), or Vti1, a SNARE receptor (Ishihara et al., 2001). Vti1 (Atlashkin et al., 2003) and Rab7 (Gutierrez, Munafo, Beron, & Colombo, 2004; Jager et al., 2004) are confirmed regulators of mammalian autophagosome-lysosome fusion.

Protein Conjugating Systems

Two novel protein conjugating systems, similar to the ubiquitin conjugating system, are utilized during autophagosome formation. These systems involve Atg12–Atg5 and LC3–phosphatidylethanolamine (PE) conjugation, and require other proteins that act in a similar manner to E1 and E2 enzymes in ubiquitin conjugation. The first step in ubiquitination is formation of a high-energy bond between the C-terminus of ubiquitin and the E1 enzyme, which uses ATP. Next, activated ubiquitin is transferred to a cysteine on one of the E2 enzymes. Finally, E3 enzymes mediate the ligation of ubiquitin to a lysine on a target protein (Wooten & Geetha, 2006).

In developing autophagosomes, Atg5 localizes mainly to the outer side of the developing membrane, but is not present after sealing. In a manner similar to ubiquitination, a lysine on Atg5 (K130) is covalently attached to a C-terminal glycine on
Atg12 (Mizushima, Sugita, Yoshimori, & Ohsumi, 1998). In this conjugation, Atg7 acts similar to an E1 enzyme, and Atg10 mirrors an E2 enzyme by transferring Atg12 to Atg5 (Mizushima, Yoshimori, & Ohsumi, 2003). Several Atg12–Atg5 conjugates are then linked in a polymer by Atg16L (Mizushima, Kuma et al., 2003; Mizushima, Yoshimori et al., 2003). This polymer is proposed to coat the developing membrane and assist in its curvature.

Yeast Atg8 was the first autophagosome marker to be identified (Kabeya et al., 2000), and is still used as a marker for autophagy activation. Atg8 has at least eight mammalian homologues, which can be divided into two groups by amino acid homology: the microtubule associated protein 1 light chain 3 (LC3) subfamily and the GABA-receptor-associated-protein (GABARAP) subfamily. In humans, three different genes encode three highly homologous LC3 proteins, known as LC3α, LC3β, and LC3γ. Mice have homologous LC3A and LC3B. The GABARAP family includes GABARAP, golgi-associated ATPase enhancer of 16 kDa (GATE-16) (Sagiv, Legesse-Miller, Porat, & Elazar, 2000), GABARAPL1, and GABARAP-L3 (He et al., 2003; Xin et al., 2001). While past autophagy research primarily focused on LC3, recent siRNA experiments show both the LC3 and the GABARAP families are required for autophagy, but at slightly different steps in autophagosome maturation (Weidberg et al.).

Upon autophagy induction, levels of LC3B-II correlate with elevated levels of autophagic vesicles either by electron microscopy or immunofluorescence, whereas LC3A-II levels do not (Klionsky et al., 2008). Here, LC3B will be referred to as LC3. LC3 associates with both the inner and outer membranes of the autophagosome (Kabeya et al., 2000), but dissociates from the outer membrane during maturation (Jager et al.,
2004; Kabeya et al., 2000). The LC3 conjugation process is more involved than that of Atg5. First, LC3 must be cleaved by Atg4 to expose a glycine residue necessary for conjugation. LC3 is then activated by Atg7 (E1 enzyme) and transferred to Atg3 (E2 enzyme), and finally linked to phosphatidylethanolamine (PE) (Ichimura et al., 2000; Kabeya et al., 2004; Sou, Tanida, Komatsu, Ueno, & Kominami, 2006). In yeast, the Atg12-Atg5 conjugate acts like an E3 enzyme by enhancing the conjugation of PE to Atg8 (yeast homologue of LC3) (Hanada et al., 2007), suggesting the Atg12-Atg5 conjugate promotes protein-lipid conjugation. Linkage of LC3 to PE results in movement of LC3 from its cytosolic residence to the autophagosome membrane.

Membrane localized, PE-conjugated LC3 is termed LC3-II, while the cytosolic, non-conjugated LC3 is termed LC3-I, and they can be differentiated by Western (Kabeya et al., 2000). The ratio of LC3-II/LC3-I reflects the relative amount of autophagy activity (Kabeya et al., 2000). For normal progression of autophagy, an additional step of deconjugation of LC3 from PE is necessary, catalyzed by Atg4 (Mizushima, Yoshimori et al., 2003). Atg4 also processes the other LC3 mammalian homologues, including the GABARAP family (Hemelaar, Lelyveld, Kessler, & Ploegh, 2003). The purpose of PE-lipidation of LC3 is suggested to aid in membrane docking and fusion, and can cause hemifusion of membranes in vitro (Nakatogawa, Ichimura, & Ohsumi, 2007). While LC3-II is usually found on the autophagosome membrane, it can also be recruited to phagosomes in response to Toll-like receptor signaling, in an Atg5- and Atg7-dependent manner. Recruitment of LC3-II to the phagosome results in phagosome fusion with late-endosomes and lysosomes (Sanjuan et al., 2007).

LC3B has been knocked out in mice, and unlike mice lacking other autophagy
proteins (such as Atg5, Atg7, Atg3, or Beclin1), the LC3B-/ mouse survives normally and does not exhibit defective autophagy (Cann et al., 2008), suggesting other LC3 homologues compensate.

Upstream of these conjugation pathways, autophagosome formation is commonly initiated by the inactivation of mammalian target of Rapamycin (mTor), which leads to activation of the class III phosphatidylinositol 3-kinase (PI3K), Vps34 (Yan & Backer, 2007). Vps34 activation is necessary for nucleation of the isolation membrane in the first steps of autophagosome formation (Petiot, Ogier-Denis, Blommaart, Meijer, & Codogno, 2000).

**Autophagy and Secretion**

The autophagy pathway is implicated in secretion in a small number of cell types. Mice hypomorphic for the autophagy protein Atg16L have a defect in paneth cells resembling that of human subjects with an Atg16L polymorphism making them more susceptible to Chron’s disease. These cells appear constipated with lysozyme, have abnormal distribution of the enzyme within the cytoplasm, and are relatively absent of the enzyme in the intestinal lumen, suggesting impaired secretion of lysozyme in these cells (Cadwell et al., 2008). In *Saccharomyces cerevisiae* and *Pichia pastoris* yeast, secretion of the acyl-coA binding protein Acb1 does not involve conventional secretion through the ER, but rather is enclosed in an autophagosome which fuses with recycling endosomes to form a multivesicular body, which then fuses with the plasma membrane in a SNARE protein dependent manner (Duran, Anjard, Stefan, Loomis, & Malhotra, ; Manjithaya, Anjard, Loomis, & Subramani). Other secretory cells, including melanocytes and β-cells of the pancreas, are functionally affected by deletion of autophagy genes, although
direct involvement of autophagy proteins in the secretory process is not shown in these cells (Ebato et al., 2008; Ganesan et al., 2008; Jung et al., 2008).

The Osteoclast Ruffled Border and Cytoskeleton

Two of the most striking and characteristic morphologic features of the osteoclast are the actin ring and ruffled border. The actin ring, literally a ring of actin filaments that forms over a tight, circular attachment to the bone, is necessary for bone resorption (Lakkakorpi, Tuukkanen, Hentunen, Jarvelin, & Vaananen, 1989). The membrane beneath the actin ring becomes convoluted due to a large number of vesicles fusing with the plasma membrane in this region, forming a “ruffled border.” Degraded bone matrix is thought to transcytose from the ruffled border to the opposite end of the osteoclast, allowing for its removal (Nesbitt & Horton, 1997; Salo, Lehenkari, Mulari, Metsikko, & Vaananen, 1997).

Neither the actin ring nor ruffled border form in osteoclasts grown on plastic or glass surfaces, suggesting matrix interactions are critical for osteoclast activation. Cell/matrix interactions are commonly mediated by integrins, and the major integrin in the mature osteoclast is αvβ3 (Inoue, Namba, Chappel, Teitelbaum, & Ross, 1998), which binds collagen, vitronectin, and other bone matrix proteins. Osteoclasts cultured from mice lacking αvβ3 form actin rings but have abnormal ruffled borders and shallow resorption pits (McHugh et al., 2000). αvβ3 leads to cytoskeletal rearrangement in the osteoclast by activating RhoA and Rac GTPases from their GDP- to GTP-bound forms (Faccio, Takeshita, Zallone, Ross, & Teitelbaum, 2003). Additionally, the tyrosine kinase c-src associates with the integrin and becomes activated upon integrin ligation. C-src then phosphorylates syk kinase, which is recruited to the activated integrin (Miyazaki
et al., 2004; Schwartzberg et al., 1997). Syk ultimately activates the Rac GTPase through Vav3, a Rac-specific guanine nucleotide exchange factor (GEF) in the osteoclast (Faccio et al., 2005).

**Lysosome Secretion**

Lysosomes are specialized organelles for intracellular protein degradation, but a small number of cells secrete their lysosomes for extracellular purposes. Some have suggested secretory lysosomes are specialized and different from those mediating protein degradation. This idea is challenged by the observation that all lysosomes can fuse with the plasma membrane in response to membrane damage (Andrews, 2000; Rodriguez, Webster, Ortego, & Andrews, 1997). This fusion is mediated by synaptotagmin VIIa and may repair plasma membrane damage and dispose of surplus lysosomal membrane (Martinez et al., 2000). In contrast, some cells with secretory lysosomes possess specific proteins that regulate their secretion, suggesting certain markers may exist that distinguish secretory from degradative lysosomes. For example, in melanocytes, the lysosome-like melanosome is bound to Rab27a, which localizes the melanosome to the plasma membrane in a myosin-V (an actin molecular motor) dependent manner (Wu et al., 2002). In the osteoclast, secretory lysosomes contribute to generating the ruffled membrane, but specific markers of secretory lysosomes are not known. Rab7, which in other cells is important in late endosome/lysosome trafficking, may be the most likely candidate since it is localized at (Palokangas, Mulari, & Vaananen, 1997), and likely important for trafficking material to, the ruffled border (Zhao, Laitala-Leinonen, Parikka, & Vaananen, 2001).

The resorption lacuna, enclosed by the ruffled border and bone surface, is
reminiscent of an “external lysosome.” LAMP1 and LAMP2, two integral membrane proteins of the lysosome, are enriched at the ruffled border (Palokangas et al., 1997). Cathepsins are lysosomal proteinases, and Cathepsin K is found exclusively in the osteoclast, where it is necessary for bone matrix degradation (Drake et al., 1996). Cathepsin K is found concentrated at the ruffled border and resorption lacuna (Yamaza et al., 1998). Deficiency of Cathepsin K in humans causes pycnodysostosis, a disease characterized by short long bones, craniofacial malformations, and malocclusion of the teeth, all due to osteoclast dysfunction (de Vernejoul & Benichou, 2001). The large area of the ruffled border as well as the speed at which a resorption pit is formed in vitro suggest the ruffled border is derived by exocytosis of lysosomes, endosomes, or other storage granule-like structures. Thus, much attention has been given to the role of lysosomes, especially “secretory lysosomes,” in osteoclast function.

Importantly, autophagy loss is associated to lysosome dysfunction in intestinal cells deficient of Atg5, Atg16L, or Atg7 (Cadwell, Patel, Komatsu, Virgin, & Stappenbeck, 2009). Human polymorphisms in Atg16L that reduce its function are associated with similar lysosome defects in intestinal Paneth cells, and this dysfunction is associated with Crohn’s disease. In patients with this polymorphism, and in mice lacking these genes, lysozyme mislocalizes throughout the Paneth cell and the cell is dysfunctional. The rationale for the mislocalization is unclear, as this is a novel, but clinically important role for autophagy proteins. We find the osteoclast parallels the Paneth cell in several ways; a lysosomal enzyme (CatK) is mislocalized within the cell, the cells are dysfunctional, and in mice this leads to a disease phenotype (in this case, protection from osteoporosis). However, unlike the Paneth cell, osteoclasts can be cultured in large quantities and
manipulated in vitro. Thus, the osteoclast may provide a system to study the mechanisms behind this novel role for autophagy proteins. Furthermore, our results suggest an osteoclast-specific autophagy inhibitor may be an effective treatment for osteoporosis.

*Atg5* and *Atg7* knockout mice die within the first day of life (Komatsu et al., 2005; Kuma et al., 2004). Thus, after visualizing LC3 within the cell, we examined the bones and osteoclasts of mice containing loxP-flanked *Atg5* or *Atg7* genes (Hara et al., 2006; Komatsu et al., 2005) bred to mice expressing the Cre recombinase under control of Lysozyme-M, a monocyte/granulocyte specific promoter (Clausen, Burkhardt, Reith, Renkawitz, & Forster, 1999).
Results

Lipidated LC3 Localizes to the Osteoclast Ruffled Border in an Atg5 Dependent Process

To determine LC3 localization, we cultured bone marrow macrophages of GFP-LC3 transgenic mice (Mizushima et al., 2004) on bone fragments to generate osteoclasts. By confocal microscopy, GFP-LC3 was found in concentrated in the actin ring in a number of osteoclasts (Figure 2.1). Cathepsin K co-localized within the actin ring confirming that GFP-LC3 is within the resorptive microenvironment (Yamaza et al., 1998) (Figure 2.1). We did not observe GFP-LC3/cathepsin K co-localization external to the confines of the actin ring, suggesting that LC3 is not on the same vesicles as those containing Cathepsin K. To test whether Thus, in addition to its known residence on autophagosomes and aggregates of cytoplasmic proteins (Kuma, Matsui, & Mizushima, 2007), LC3 transits to the region of the ruffled border in bone-residing osteoclasts.

LC3 Associates Directly with the Ruffled Border

To better characterize the LC3-containing compartment of the osteoclast, we performed iodixanol density gradient fractionation (Gille & Nohl, 2000; Graham, Ford, & Rickwood, 1994) of osteoclasts grown on bone powder. Previously this method was used to demonstrate ruffled-border components resolved at the densest level of the gradient (H. Zhao et al., 2008). In both $Atg^{5\text{flox/}\text{fl}}$-LyzM-Cre+ and $Atg^{5\text{flox/}\text{fl}}$ samples, the densest fractions contained ruffled border and plasma membrane proteins flotillin and β3s integrin. In $Atg^{5\text{flox/}\text{fl}}$ samples, LC3 was also found exclusively at this location, while $Atg^{5\text{flox/}\text{fl}}$-LyzM-Cre+ osteoclasts contained a significant amount of LC3 in a less
dense, perhaps cytoplasmic fraction (Figure 2.2). These results are consistent with IF data suggesting LC3 is present in the ruffled border compartment in $\text{Atg}^{{5}\text{flax/flox}}$ but not $\text{Atg}^{{5}\text{flax/flox}}$-LyzM-Cre+ osteoclasts, but it does not identify the specific membranes containing the LC3.
We considered the possibility that LC3 localization at the ruffled border might reflect the accumulation of autophagosomes in this area. ImmunoEM on bone-residing GFP-LC3 transgenic osteoclasts revealed that LC3 polarized toward the bone-apposed membrane in osteoclasts that had not yet initiated bone resorption or ruffled border formation (Figure 2.3). Importantly, in osteoclasts with developed ruffle borders, LC3 was found directly associated with the ruffled border domain of the plasma membrane in the absence of autophagosomes (Figure 2.4).

During autophagy, LC3 is localized to autophagosomes after LC3I is conjugated to PE on glycine 120 generating LC3II. To determine the role of PE conjugation in LC3 localization to the resorptive microenvironment, we retrovirally transduced osteoclasts with either wild-type GFP-LC3 or the non-conjugatable mutant GFP-LC3G120A. While WT GFP-LC3 was constrained by actin rings in 30% of osteoclasts, this occurred in less than 10% of cells transduced with GFP-LC3G120A (Figure 2.5). Thus lipidation of LC3 on glycine 120 is required for efficient transport of LC3 to the resorptive microenvironment.
**Figure 2.3: LC3 Polarizes to the Bone-Apposed Membrane**

GFP-LC3 transgenic osteoclasts grown on bone slices were immunostained with gold labeled anti-GFP (top) or isotype control (bottom) antibody.
Figure 2.4: LC3 Directly Associates with the Ruffled Border

A) High magnification of the ruffled border of a GFP-LC3 transgenic osteoclast grown on bone, immunostained with gold labeled anti-GFP, demonstrates LC3 associates directly with the ruffled border membrane, independent of autophagosomes or other organelles. B) GFP-LC3 is also seen on autophagosomes scattered throughout the osteoclast cytoplasm.
LC3II is targeted to specific locations by a complex of proteins including Atg5 covalently conjugated to Atg12 (Checroun, Wehrly, Fischer, Hayes, & Celli, 2006). Atg5 is required for the lipidation and binding of LC3 to autophagosomes and phagosomes (Mizushima et al., 2001; Sanjuan et al., 2007). To determine the role of Atg5 in LC3II localization in osteoclasts we bred Atg5$^{flax/flax}$ mice to those expressing the Cre recombinase under control of the lysozyme M promoter (LyzM-Cre) which is constitutively active in myeloid lineage cells, including osteoclasts (Clausen et al., 1999; Z. Zhao et al., 2008). Western blot of Atg5$^{flax/flax}$-LyzM-Cre+ osteoclasts revealed efficient excision of Atg5 and inhibition of autophagy as manifest by accumulation of p62 and decreased LC3II (Figure 2.6). Atg5$^{flax/flax}$-LyzM-Cre+ bone marrow cells exhibited a slight delay in osteoclast formation, but by day five formed normal numbers

![Figure 2.5: GFP-LC3, but not GFP-LC3(G120A), Localizes to the Actin Ring](image)

Quantification of percentage of osteoclasts with GFP concentrated within the actin ring. Osteoclasts were transduced with GFP, GFP-LC3 or GFP-LC3G120A and grown on bone for 6 days; osteoclasts with a complete actin ring were analyzed. Data from 2 experiments (n=40 cells).
**Figure 2.6: Atg5 is Efficiently Deleted in LyzM-Cre+ Osteoclasts**

Western blot of Atg5, p62, LC3, and actin in Atg5flox/flox and Atg5flox/flox-LyzM-Cre+ osteoclasts generated on plastic for 6 days, n=6 experiments.
of bone resorptive polykaryons (Figure 2.7). The delay in osteoclast formation may be due to a slight defect of proliferation of precursors (Figure 2.8). Apoptosis levels, measured by anti-histone ELISA death detection kit, were equivalent (Figure 2.9). We observed no difference between $Atg5^{flox/flox}$-LyzM-Cre$^+$ and $Atg5^{flox/flox}$ cells in the
expression of osteoclast differentiation markers (Figure 2.10), the packaging of cathepsin K (Figure 2.10), the average number of nuclei per osteoclast (Figure 2.11), or their ability to form actin rings on bone (Figure 2.12). Therefore Atg5 is not required for osteoclast formation or polarization of the cell’s fibrillar actin.

Figure 2.8: Atg5-Deficient pre-Osteoclasts are Less Proliferative

BrdU incorporation over 18 hours, beginning two days after RANKL and M-CSF were added to macrophages to induce osteoclastogenesis, showed Atg5flox/flox have slightly higher proliferation rates compared with Atg5flox/flox-LyzM-Cre+ cells.

Figure 2.9: WT and Atg5-Deficient pre-Osteoclasts have Equal Levels of Apoptosis

Death detection assay using anti-Histone antibody based ELISA performed on pre-osteoclasts (bone marrow macrophages exposed to RANKL and M-CSF for two days) showed no difference between Atg5flox/flox or Atg5flox/flox-LyzM-Cre+ cells.
Figure 2.10: Atg5-Deficient Osteoclasts Differentiate Normally

A) Immunoblot of markers of osteoclast differentiation in Atg5flox/flox and Atg5flox/flox-LyzM-Cre+ bone marrow macrophages treated with M-CSF and RANK ligand. B) Ultrathin cryosections of Atg5flox/flox (upper panel) and Atg5flox/flox-LyzM-Cre+ (lower panel) osteoclasts immunogold labeled for cathepsin K (10 nm gold particles). Depletion of Atg5 does not result in an altered subcellular localization of cathepsin K, representative images from two experiments. E, endosome; PM, plasma membrane; SL, secretory lysosome.
We next transduced \textit{Atg}^{5\text{flox/flox}}-\text{LyzM-Cre}^+ or \textit{Atg}^{5\text{flox/flox}} cells with GFP-LC3, which localized to the ruffled border in the control but not \textit{Atg}5-deficient osteoclasts (Figure 2.13, Figure 2.14). Hence, \textit{Atg}5-dependent lipid conjugation is required for LC3 transport to the resorptive microenvironment.
Atg5 and Atg7 are Necessary for Efficient Bone Resorption

Since these data suggested that autophagy-associated ubiquitin-like conjugation systems might participate in osteoclast secretory function, we assessed the bone resorptive capacity of Atg5-deficient cells. We cultured $Atg5^{flox/flox}$-LyzM-Cre+ or $Atg5^{flox/flox}$ osteoclasts on bone for six days and measured resorption pits by confocal microscopy (Salo et al., 1997). Atg5 was critical for efficient bone resorption as pits
formed by $Atg5^{\text{flox/flox}}$ osteoclasts were approximately twofold deeper than those

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![GFP-LC3 transduced $Atg5^{\text{flox/flox}}$](image4)  ![GFP-LC3 transduced $Atg5^{\text{flox/flox}}$](image5)  ![GFP-LC3 transduced $Atg5^{\text{flox/flox}}$](image6)

**Figure 2.13: GFP-LC3 Localization to the Ruffled Border is $Atg5$-Dependent**

$Atg5^{\text{flox/flox}}$ and $Atg5^{\text{flox/flox}}$-LyzM-Cre+ osteoclasts transduced with GFP-LC3 were generated on bone. Actin and GFP were immunostained and visualized by confocal microscopy. Representative images at the level of the actin ring, with cell perimeter outlined in white.
Figure 2.14: GFP-LC3 Localization is Atg5-Dependent

Quantification of percentage of Atg5flox/flox and Atg5flox/flox-LyzM-Cre+ transduced osteoclasts with GFP-LC3 concentrated within the actin ring. Data from 2 experiments (n=40 cells).

Figure 2.15: Atg5-Deficient Osteoclasts Exhibit Defective Bone Pit Formation

Atg5flox/flox and Atg5flox/flox-LyzM-Cre+ bone-residing osteoclasts were immunostained for actin (red) and exposed to wheat germ agglutinin conjugated to FITC, which stains cells and bone pits (green). Bone was visualized in z plane by confocal microscopy before (top) and following removal of cells (bottom). Representative images from 2 experiments, cell perimeters outlined in white.
excavated by Atg5flox/flox or Atg5flox/flox-LyzM-Cre+ osteoclasts. Bone pit volume was similarly reduced (Figure 2.17).

To determine if other essential autophagy proteins are important for bone

Figure 2.16: Quantification of Bone Pit Formation in Atg5-Deficient Osteoclasts

Quantification of bone pit depth generated by Atg5flox/flox or Atg5flox/flox-LyzM-Cre+ osteoclasts. To pool 4 experiments data from each was normalized to the average depth generated by Atg5flox/flox cells as 100%, analyzed using paired t-test.

Figure 2.17: Atg5-Deficient Osteoclasts Resorb Less Bone Volume

Three dimensional images of wheat germ agglutinin-FITC-stained bone pits excavated by Atg5flox/flox and Atg5flox/flox-LyzM-Cre+ osteoclasts were obtained by confocal microscopy, and pit volume determined using LSM software (1 of 2 experiments shown). Excavated by Atg5flox/flox-LyzM-Cre+ cells (Figure 2.15, Figure 2.16). Bone pit volume was similarly reduced (Figure 2.17).
resorption, we deleted Atg7, which is required for the conjugation of Atg5 to Atg12, from osteoclasts (Komatsu et al., 2005; Z. Zhao et al., 2008; Zhao et al., 2007). Immunoblotting of lysates of \( \text{Atg7}^{\text{flox/flox}} - \text{LyzM-Cre}^{+} \) osteoclasts revealed a substantial reduction in Atg7 and inhibition of LC3-I to LC3-II conversion (Figure 2.18, Figure 2.19). Similar to those lacking Atg5, the depth of bone pits generated by Atg7-deficient osteoclasts was decreased (Figure 2.20). The magnitude of bone pit depth reduction consequent to Atg5 or Atg7 deficiency was similar to that observed with deletion of cathepsin K or the \( \alpha \nu \beta \) integrin, which are required for efficient osteoclast resorptive function (McHugh et al., 2000; Saftig et al., 1998).
Atg5 and Atg7 are Required for Lysosomal Secretion at the Ruffled Border

Bone resorption requires secretion of matrix-degrading molecules at the ruffled border suggesting that the shallow pits generated by Atg5-deficient osteoclasts may reflect defective focal secretion. We therefore examined this resorptive organelle in more detail by assessing the localization of lysosomal proteins. We selected LAMP1 as an
integral membrane protein in the lysosome-like vesicles whose contents are secreted by osteoclasts and cathepsin K as a lysosomal protease delivered by these vesicles to the resorptive microenvironment (Akamine et al., 1993; Maeda, Akasaki, Yoshimine, Akamine, & Yamamoto, 1999; Palokangas et al., 1997; Yamaza et al., 1998). Cathepsin K and LAMP1 localized principally within the actin ring in 50-60% of $\text{Atg5}^{\text{flox/flox}}$. 

Figure 2.21: Cathepsin K Localization to the Actin Ring is $\text{Atg5}$-Dependent

Representative confocal images of $\text{Atg5}^{\text{flox/flox}}$ or $\text{Atg5}^{\text{flox/flox-LyzM-Cre+}}$ bone-residing osteoclasts immunostained for actin (green) and CatK (red), cell perimeters outlined in white.
osteoclasts (Figure 2.21, Figure 2.22) but only 20-30% of $\text{Atg5}^{\text{flox/flox}}$-$\text{LyzM-Cre}^+$ osteoclasts (Figure 2.22). Transport of Cathepsin K to the resorptive microenvironment was also defective in Atg7-deficient osteoclasts (Figure 2.23).

**Figure 2.22: Cathepsin K and Lamp-1 Localization are Impaired in Atg5-Deficient Osteoclasts**

Quantification of percentage of Atg5$flox/flox$ or Atg5$flox/flox$-$\text{LyzM-Cre}^+$ osteoclasts on bone with CatK or LAMP1 concentrated within the actin ring, data pooled from 3 independent experiments.

**Figure 2.23: Cathepsin K and Lamp-1 Localization to the Actin Ring are Defective in Atg7-Deficient Osteoclasts**

Quantification of percentage of Atg7$flox/flox$ and Atg7$flox/flox$-$\text{LyzM-Cre}^+$ osteoclasts grown on bone with CatK or LAMP1 localized in the actin ring, 2 pooled experiments.
We considered possible explanations for the role of autophagy proteins in the secretory function of osteoclasts other than a direct role in secretion from the ruffled border. The quantities of pro-cathepsin K and its mature active form were normal in plastic-grown Atg5-deficient osteoclasts indicating proper synthesis and activation of this lysosomal enzyme (Rieman et al., 2001) (Figure 2.24). Further, constitutive and receptor-mediated endocytosis which occurs in the resorptive environment, as analyzed by fluorescent dextran (Figure 2.25) or transferrin uptake (Figure 2.26) (Palokangas et al; Stenbeck and Horton, Endocytic trafficking in actively resorbing osteoclasts), were equivalent in Atg5\textsuperscript{flox/flox}-LyzM-Cre\textsuperscript{+} and Atg5\textsuperscript{flox/flox} osteoclasts on bone. Autophagy gene deficiency disrupts intracellular membranes and mitochondria in hepatocytes and Paneth cells (Cadwell et al., 2008; Komatsu et al., 2005). However, analysis of osteoclasts by electron microscopy revealed no morphological abnormalities of mitochondria or intracellular membranes in Atg5-deficient osteoclasts (not shown). These data are consistent with a selective role for autophagy proteins in the secretory process that occurs at the ruffled border.

![Figure 2.24: Atg5\textsuperscript{flox/flox}-LyzM-Cre\textsuperscript{+} Osteoclasts Normally Express and Process Cathepsin K](image)

Western blot of actin and the pro- and active forms of Cathepsin K in lysates of Atg5\textsuperscript{flox/flox} and Atg5\textsuperscript{flox/flox}-LyzM-Cre\textsuperscript{+} osteoclasts, representative of 4 experiments.
**Figure 2.25: Constitutive Endocytosis Occurs in WT and Atg5-Deficient Osteoclasts**

Atg5<sup>flox</sup>/flox and Atg5<sup>flox</sup>/flox-LyzM-Cre<sup>+</sup> osteoclasts were grown on bone and incubated with FITC-Dextran (green) for 20 minutes, then washed, fixed and stained for a membrane marker (red) and visualized by confocal microscopy (z-plane is shown).

**Figure 2.26: Receptor-Mediated Endocytosis Occurs in WT and Atg5-Deficient Osteoclasts**

Atg5<sup>flox</sup>/flox and Atg5<sup>flox</sup>/flox-LyzM-Cre<sup>+</sup> osteoclasts were grown on bone and incubated with transferrin (blue) for 20 minutes, then washed, fixed and stained for actin (red) and visualized by confocal microscopy (z-plane is shown).
Given that the ruffled border is derived from fusion of lysosome-like vesicles, suggests Atg5 deficiency would result in defective formation of this resorptive organelle.

**Figure 2.27: Examples of Absent, Immature, and Mature Ruffled Borders**

Representative electron micrographs of tibia-residing osteoclasts demonstrating absent (top), immature (middle), and mature (bottom) ruffled borders adjacent to sealing zones (asterisk).

(Helfrich & Gerritsen, 2001; Josephsen et al., 2009; Kornak et al., 2001; McHugh et al.,
2000; Reinholt et al., 1999). We therefore ultrastructurally examined the ruffled borders of osteoclasts in tibiae of $\text{Atg}^5\text{flox/flox}$ and $\text{Atg}^5\text{flox/flox}$-LyzM-Cre+ mice, grading them independently by two genotype-blinded observers as absent, immature, or mature (Figure 2.27). $\text{Atg}^5\text{flox/flox}$-LyzM-Cre+ osteoclasts were more than ten-fold predisposed to lack sealing-zone (i.e. actin ring)-adjacent ruffled borders than those of $\text{Atg}^5\text{flox/flox}$ mice (Figure 2.28). When present, convoluted membranes were less likely to be mature in $\text{Atg}^5\text{flox/flox}$-LyzM-Cre+ osteoclasts. These in vivo observations suggest that Atg5 participates in generating the ruffled border via fusion of lysosome-like vesicles with the bone-apposed plasma membrane.

![Graph showing the percentage of Atg5flox/flox and Atg5flox/flox-LyzM-Cre+ cells graded as absent, immature, or mature.](image)

**Figure 2.28: Atg5 is Important for Formation of the Ruffled Border**

Percentage of Atg5flox/flox-LyzM-Cre+ or Atg5flox/flox osteoclasts exhibiting absent, immature, or mature ruffled borders. Chi-square test for trend was performed, n=30 cells from one mouse of each genotype, a second mouse exhibited similar findings.

Atg5-Atg12 Conjugation and Atg4b are Necessary for Efficient Lysosome Localization and Bone Resorption

To determine if the role of Atg5 in osteoclast secretory function requires
conjugation to Atg12, as is true for its role in macroautophagy, we expressed wild-type Atg5 or Atg5 with its Atg12-conjugating lysine mutated to arginine (Hamacher-Brady et al., 2007; Mizushima et al., 2001) in $\text{Atg5}^{\text{floxflo}}$-LyzM-Cre+ or $\text{Atg5}^{\text{floxflo}}$ osteoclasts.

Atg5, but not Atg5$^{\text{K130R}}$, complexed with Atg12. Moreover, only wild-type Atg5 normalized LC3II generation (Figure 2.29), cathepsin K localization (Figure 2.30), and bone pit formation (Figure 2.31) in $\text{Atg5}^{\text{floxflo}}$-LyzM-Cre+ cells.

Hence the Atg5-Atg12 conjugate regulates osteoclast secretory function. Interestingly, expression of Atg5$^{\text{K130R}}$ in $\text{Atg5}^{\text{floxflo}}$ osteoclasts decreased bone pit depth and prompted abnormal cathepsin K localization, consistent with a dominant-negative function noted in other cells (Hamacher-Brady et al., 2007; Pyo et al., 2005).

The Atg5-Atg12 complex is important for LC3 conjugation, but along with Atg7, these proteins may have other activities (Jounai et al., 2007; Virgin & Levine, 2009).
Figure 2.30: Atg5, but not Atg5-K130R Mutant, Rescues Cathepsin K Localization to the Actin Ring

Percentage of cells with CatK concentrated within the actin ring in Atg5flox/flox or Atg5flox/flox-LyzM-Cre+ osteoclasts retrovirally transduced with mCherry-Atg5 or mCherry-Atg5K130R, pooled from 3 independent experiments.

Figure 2.31: Atg5, but not Atg5-K130R Mutant, Rescues the Osteoclast’s Ability to Resorb Bone

Osteoclasts of the indicated genotype were infected with control, Atg5, or Atg5-K130R, and grown on bone. Average bone pit depth was determined by confocal, and normalized to Atg5-flox/flox control. Pooled from 3 experiments (analyzed by paired t-test).
Atg7-independent autophagy is implicated in mitochondrial clearance from reticulocytes (Zhang et al., 2009), and Ulk-1 (a mammalian homologue of Atg1) is required for both reticulocyte clearance of mitochondria (Kundu et al., 2008) and, along with Beclin-1, for Atg5/Atg7-independent autophagy (Nishida et al., 2009). Therefore, we retrovirally transduced osteoclasts with a mutant form of Atg4B (Atg4B<sup>C74A</sup>) which binds LC3 and blocks its conjugation to PE (Figure 2.32). Importantly, Atg4B<sup>C74A</sup> inhibits autophagy without disrupting formation of the Atg5-Atg12 conjugate (Fujita, Hayashi-Nishino et al., 2008). Atg4B<sup>C74A</sup> expression reduced bone pit formation (Figure 2.33) and caused mislocalization of cathepsin K (Figure 2.34). Expression of this construct in GFP-LC3 osteoclasts also inhibited LC3 localization to the ruffled border (Figure 2.35, Figure 2.36 A). These data indicate that LC3 lipilation and the Atg5-Atg12 complex are both required for efficient osteoclast secretion.
Figure 2.33: Dominant Negative Atg4b Reduces Osteoclast-Mediated Bone Pit Formation

Depth of bone pits excavated by wild-type osteoclasts retrovirally transduced with empty vector (control) or Atg4b-C74A, pooled from 3 independent experiments normalized to vector control (analyzed by paired t-test). All Atg4b-C74A constructs contain mStrawberry.

Figure 2.34: Dominant Negative Atg4b Impairs Cathepsin K Localization to the Resorptive Membrane

Quantification of CatK localization in actin rings in wild-type osteoclasts transduced with control or Atg4b-C74A, 1 of 3 experiments shown.
Figure 2.35: Dominant Negative Atg4b Impairs Localization of LC3 to the Ruffled Border

Control or dominant negative Atg4B infected osteoclasts, viewed from side by confocal microscopy. Green = GFP-LC3, Blue = CatK, Red = Actin.
Figure 2.36: A) Dominant Negative Atg4b Impairs LC3 Localization to the Actin Ring. B, C) Atg5 is necessary for Rab7 to localize to the ruffled border

Quantification of percentage of cells with GFP-LC3 localized in the actin ring of control or Atg4b-C74A transduced GFP-LC3 osteoclasts, 1 of 2 experiments shown. B, C) Confocal images of bone-residing Atg5flox/flox and Atg5flox/flox-LyzM-Cre+ osteoclasts. Immunostaining of actin (red) and Rab7 (green) shows localization of Rab7 within the actin ring in Atg5flox/flox that is diminished in Atg5flox/flox-LyzM-Cre+ osteoclasts, representative of four experiments, cell perimeters outlined in white.

Rab7 generates the ruffled border, modulates bone resorption and promotes autophagosome maturation (Zhao et al., 2001; Gutierrez et al., 2004; Harrison et al., 2003; Jager et al., 2004; Yamaguchi et al., 2009; Tabata et al., 2010). Indicating that
ruffled border formation by this small GTPase is Atg5-dependent, Rab7 appeared at the ruffled border in 60% of control osteoclasts, but only 16% of those lacking Atg5 (Figure 2.36 B). In comparison, Rab7 localization to the area surrounding the actin ring does not require Atg5 (Figure 2.36 B, C).

**Atg5 Loss in the Osteoclast Protects from Estrogen-deficiency Osteoporosis in vivo**

To mirror the most common human cause of osteoporosis, namely estrogen deficiency, we tested the effect of osteoclast Atg5 deletion on skeletal mass after ovariectomy. Analysis of trabecular bone volume by micro-CT established a small but significant increase in naïve $Atg5^{flax/flax}$-LyzM-Cre+ versus $Atg5^{flax/flax}$ mice after eight weeks (Figure 2.37), and a slightly larger increase after 9 months (Figure 2.38). Importantly, absence of Atg5 in osteoclasts reduced ovariectomy-induced bone loss by approximately 50% (Figure 2.39). Confirming global suppression of bone resorption, serum levels of c-telopeptide of type I collagen (CTx) was reduced two weeks after ovariectomy (Figure 2.40). Thus, Atg5 and LC3B regulate osteoclast function, *in vitro* and *in vivo*. 
Figure 2.37: Mice with Atg5-Deficient Osteoclasts Have Slightly Higher bone Volume at Eight Weeks

Percentage of marrow space occupied by trabecular bone (BV/TV) of 8-week old female Atg5floxFlox or Atg5floxFlox-LyzM-Cre+ measured by micro-computed tomography, pooled from 3 experiments, n=12 total mice per genotype. BV/TV = Bone Volume/Total Volume.
Figure 2.38: Mice with Atg5-Deficient Osteoclasts have Higher Bone Volume at Nine Months

Percentage of marrow space occupied by trabecular bone (BV/TV) of 9-month old female Atg5floxflox or Atg5floxflox-LyzM-Cre+ measured by micro-computed tomography, n=6 total mice per genotype. BV/TV = Bone Volume/Total Volume.
**Figure 2.39: Mice Lacking Atg5 in the Osteoclast are Protected from Ovariectomy-Induced Osteoporosis**

Quantification of percentage of ovariectomy-induced bone loss one day following basal determination of BV/TV. Ovariectomy (ovx) or sham operation was performed on Atg5floxflox (8 ovx, 5 sham) or Atg5floxflox-LyzM-Cre+ (7 ovx, 4 sham) mice. BV/TV was reanalyzed after 4 weeks. The average bone loss of sham-operated Atg5floxflox animals was subtracted from all measurements, pooled from 2 (sham) or 3 (ovx) experiments.

**Figure 2.40: Serum CTx Levels are Reduced in Mice Lacking Atg5 in the Osteoclast**

Serum was collected from six hour starved mice, two weeks post-ovariectomy, and CTx levels were determined by ELISA (n=6 from each genotype).
**Discussion**

Our data establish an unexpected role of the ubiquitin-like conjugation systems required for autophagy in the bone resorptive process. Atg5, Atg7, and LC3 (based on data from expression of a dominant negative form of Atg4B), participate in generating the most important functional organelle of the osteoclast, its ruffled membrane. This organelle is formed by fusion of lysosome-like vesicles to the bone-apposed plasma membrane, mirroring that of autophagosomes and phagosomes, containing Toll-like receptor ligands (Sanjuan et al., 2007), to lysosomes. Each process involves the ubiquitin-like conjugation apparatus essential for macroautophagy (Figure 2.41). Taken together with human genetic data (Pan et al., ; Zhang et al.), an immediate practical implication of our findings is the candidacy of autophagy proteins as anti-resorptive targets for treatment of pathological bone loss. Importantly our findings also indicate that therapeutic modulation of these genes, or the autophagy pathway, for other purposes, may impact the skeleton and thus should be accompanied by evaluation of bone density.

Molecularly, our data suggest autophagy machinery may enjoy a general role in targeting lysosomes to specific intracellular membranes.

Traditionally, autophagy has been described as a pathway involving two ubiquitin-like conjugation pathways that leads to autophagosome formation and protein degradation. A major implication of our findings is that the cascade of “autophagy” proteins may mediate effects independent of the autophagosome. Unfortunately, no proteins have been identified that explicitly mediate autophagosome formation or function without affecting the rest of the pathway. Atg9 is an integral membrane protein thought to deliver membrane to the autophagosome, but unfortunately even Atg9 is
important for LC3 conjugation (Matsunaga et al., 2009; Yamada et al., 2005; Zhong et al., 2009).

**Figure 2.41: Model of Autophagy Machinery in the Osteoclast**

LC3 localizes to the bone-apposed plasma membrane upon lipidation to PE. Proper localization requires the autophagy proteins Atg5 and Atg7, as well as Atg5-Atg12 conjugation. Rab7 also localizes to the ruffled border in an Atg5-dependent manner. At the plasma membrane, LC3 promotes trafficking, tethering or fusion of lysosomal vesicles, promoting ruffled border formation, secretion of Cathepsin K, and bone resorption.

Although LC3 becomes lipitated and associated with the bone apposed resorptive membrane, its exact function here is unclear. ImmunoEM showing lack of LC3 on
lysosomal membranes or on autophagosomes near the ruffled border help exclude the possibility that LC3 associates with either lysosomes or autophagosomes that fuse with the plasma membrane. Direct fusion of autophagosomes with the membrane would also result in lipids or cytosolic components in the resorptive space, which we do not observe. LC3 at the resorptive membrane may directly mediate fusion of incoming lysosomes, as it does with autophagosome and with membranes in vitro. Alternatively, it may tether secretory vesicles near the membrane, or may serve as an adaptor for other proteins, such as Synaptotagmin VII (H. Zhao et al., 2008), that must localize here to mediate fusion. To distinguish between adaptor protein or membrane fusion/tethering functions, it would be interesting to make corresponding mutations described in Atg8 (the yeast LC3 homologue) that abolish its membrane hemifusion function without affecting its PE lipidation (Nakatogawa et al., 2007). These proteins, in addition to WT, could be over-expressed to see if they restore CatK localization and bone pit resorption.

It is still not known why LC3 associates specifically with the autophagosome and not other organelles, and an unanswered question here is what targets LC3 to the ruffled border rather than surrounding membrane. One possibility is that the distinct lipid composition of the bone-apposed plasma membrane attracts appropriate Atg proteins, which then recruit LC3. Forced localization of Atg16L to the plasma membrane using a prenylation motif promoted recruitment of LC3-II to this site (Fujita, Itoh et al., 2008). The prenylated Atg16L promoted GFP-LC3 conjugation to PE independent of starvation. Lipidation was also insensitive to wortmannin, which inhibits PI3K and traditional autophagy, suggesting that the main function of PI3P formation by the type III PI3K is to localize the Atg16L complex to the PI3P-rich isolation membrane of the autophagosome.
Although PI3K is already known to be important for osteoclast activation, it would be interesting to see if the ruffled border is indeed concentrated in PI3P.

In any given culture of wild type osteoclasts on bone, my experience is that 50% or more of the cells will exhibit CatK and Lamp1 localized within the actin ring. However, we observed LC3 localized in less than 30% of osteoclasts. Nearly every osteoclast that had LC3 within the actin ring also had CatK. There are three possible explanations for the relative abundance of CatK at this area relative to LC3. One is a sensitivity issue; perhaps LC3, even when GFP tagged and transgenically or retrovirally expressed, is less easily visualized than CatK. The second is a kinetics issue; perhaps LC3 only initiates or enhances the fusion process, or perhaps it dissipates more quickly than the CatK. Finally, vesicles that carry CatK or Lamp1 may have parallel pathways for reaching and fusing with the ruffled border that do not rely on LC3. One way to address the kinetics issue would be live cell imaging on bone. GFP-LC3 transgenic macrophages could be transduced with RFP-actin, differentiated to osteoclasts, then given a lysotracker dye and imaged on bone substrate over time to visualize the order of events. I would expect to see actin ring formation first, followed GFP-LC3 localization within the actin ring, and finally lysosome accumulation within the area, leading to lysosome fusion with the membrane and bone resorption. One caveat of this experiment is that even transduced GFP-LC3 is difficult to visualize; we had to immunostain GFP-LC3 transgenic cells with FITC conjugated anti-GFP antibody to see the fluorescence.

All of our studies, like many in the past, rely entirely on GFP-LC3 rather than endogenous LC3. There are three caveats to this approach. One is that over-expressed GFP–LC3 can be incorporated into protein aggregates independent of autophagy.
Fortunately, although we observe LC3 enrichment at the ruffled border, it does not appear in clumps or aggregates. A second is that transfection procedures used to introduce GFP-LC3 can activate autophagy (Klionsky et al., 2008). We also observe transduction procedures affect osteoclast activity, which is why all experiments had control vector transductions. Finally, GFP-LC3 simply may not behave the same as endogenous LC3. GFP-LC3 has been used rather than endogenous LC3 in the majority of previous autophagy studies for convenience, and besides non-specific aggregation the GFP tag has not been reported to affect the protein’s function, but we still cannot rule out it has an artificial effect.

One implication of our findings is that drugs or proteins that affect autophagy may also affect the bone. In many cases, autophagy modifiers are already known to have skeletal effects. Consistent with autophagy proteins mediating osteoclast secretory activity, Rab7 is necessary for both autophagosome formation and fusion of autophagosomes with lysosomes (Gutierrez et al., 2004; Jager et al., 2004), as well as for targeting the hydrogen pump to the ruffled border for bone resorption (Zhao et al., 2001). Hypoxia activates autophagy (Semenza, ; Tracy et al., 2007). Rab7 is also associated with osteoporosis and osteoclast activation in vitro (Knowles & Athanasou, 2009; Miller et al., 2006). Wortmannin, an inhibitor of PI3K and autophagy (Blommaart, Krause, Schellens, Vreeling-Sindelarova, & Meijer, 1997), blocks bone resorption (Nakamura et al., 1995). The same may be true of other PI3-kinase inhibitors used to inhibit autophagy, such as 3-methyladenine and wortmannin (Seglen & Gordon, 1982), but these drugs likely also affect osteoclast activity independently (Chen et al., 2004). Hydroxychloroquine, another autophagy inhibitor, increases bone density (Stapley,
2001), and is a common treatment for arthritis (Bingham & Miner, 2007). However, since this drug increase lysosomal pH leading to less protease activity, one would expect it to inhibit osteoclast bone resorption by direct lysosomal action. One would expect the same of the autophagy inhibitor Bafilomycin A1, which inhibits fusion of autophagosomes with both endosomes and lysosomes (Mousavi et al., 2001; Yamamoto et al., 1998), but also inhibits the Na+H+ pump on the lysosome. Thus, molecules and proteins known to activate or inhibit autophagy have consistent effects on bone resorption, but all of these mediators are non-specific.

One of the initial reasons I was drawn to study autophagy in the osteoclast was the connection between autophagy, the adaptor protein p62, and the osteoclastic disorder known as Paget’s disease of bone. Paget’s is a painful bone disease characterized by focal lytic areas of increased bone turnover, where the osteoclasts are oversized and overactive. Osteoblasts react by increasing their activity, leading to high levels of alkaline phosphatase, and formation of disorganized, weak bone prone to fracture (Laurin, Brown, Morissette, & Raymond, 2002). It is one of the more common bone diseases among Caucasians, with 3% affected over the age of 50 (Helfrich & Hocking, 2008). The most common genetic mutation found in Paget’s disease patients is in p62. p62 interacts with ubiquitinated proteins to mediate aggregate formation, but also binds LC3, and is thus thought to target ubiquitinated aggregates to the autophagosome (Komatsu & Ichimura, ; Komatsu et al., 2007). In the osteoclast and other cells, p62 also mediates Traf6 and NFkB activation as a molecular scaffolding protein (Chamoux et al., 2009; Duran et al., 2004; Yip, Feng, Pavlos, Zheng, & Xu, 2006). Null mutation of p62 in osteoclast precursor cells causes severe impairment of osteoclast formation in a culture
system. However, p62-deficient mice exhibit no defects unless they are challenged by osteoclastogenic stimuli such as PTH-related peptide (PTHrP) (Duran et al., 2004). It is possible the secretion phenotype we observe in autophagy deficient osteoclasts is related to buildup of protein aggregates that impair cellular function. To test this hypothesis, it would be interesting to see if loss of p62 rescues the phenotype, by breeding p62-/− mice with \( \text{Atg}^{\text{flax/flax}} \)-LyzM-\text{Cre}+ mice, or using p62 siRNA. This method has worked in hepatocytes, where loss of p62 reduces the number of protein aggregates in autophagy deficient cells and restores cellular function (Komatsu et al., 2007). Alternatively, it is possible LC3 functions to bind p62 and localize it to the ruffled border where it has a separate function, but in my p62 immunofluorescence studies, I did not find p62 localized within the actin ring in WT cells.

In the course of this project, before either of the two papers were published using GWAS to show the autophagy pathway is significantly associated with bone phenotypes in humans, we analyzed a similar databank of polymorphisms and tested for their link to bone mineral density. Although individually none of the autophagy proteins were significantly associated, we noted that most of them were close, which is probably why the two other groups used a pathway-based analysis to achieve significance. In the study of height variability (Pan et al.), genes most significantly associated were Atg5, Atg12, Atg4, Atg3, Beclin1, GABARAPL1, and GABARAPL2. In young individuals, height is influenced by chondrocytes, osteoblasts, and osteoclasts as bone modeling occurs. Additionally, it is estimated that patients lose an average of 4.5 cm between 40 and 70 years old (Briot, Legrand, Pouchain, Monnier, & Roux), and height loss is predictive of fragility fractures and osteoporosis (Gunnes, Lehmann, Mellstrom, & Johnell, 1996;
Siminoski et al., 2005). Since the average age of patients in the GWAS study for height was over 70, both bone growth during youth and bone loss during age are relevant. Although both depend on osteoclast activity, effects of autophagy on osteoblast and chondrocytes should also be considered in future studies (Srinivas & Shapiro, 2006).

**Methods**

**Mice**  The generation of \( \text{Atg}^5\text{flox/flox}, \text{Atg}^7\text{flox/flox}, \text{Atg}^5\text{flox/flox-LyzM-Cre+}, \) and GFP-LC3 mice has been previously reported (Hara et al., 2006; Komatsu et al., 2005; Mizushima et al., 2004; Z. Zhao et al., 2008). To generate \( \text{Atg}^7\text{flox/flox-LyzM-Cre+} \) mice, we bred \( \text{Atg}^7\text{flox/flox} \) mice to mice expressing the Cre recombinase from the Lysozyme M locus (strain #004781, Jackson Laboratory, Bar Harbor, ME). \( \text{Atg}^5\text{flox/flox}, \text{Atg}^7\text{flox/flox}, \text{Atg}^5\text{flox/flox-LyzM-Cre+} \) mice were genotyped as described (Stephenson et al., 2009). GFP-LC3 mice were genotyped with primers GFP-1: 5′-TCCTGCTGGAGTTCGTGACC-3′ and LC3: 5′-TTGCGAATTCTCAGCCGTCTTCA TCTCTCTCGC-3′ using PCR [94°C(4 min); 35 cycles of 94°C (30 sec), 57°C (30 sec), 72°C (1 min); 72°C (5 min)]. Mice were maintained at Washington University School of Medicine in accordance with institutional policies for animal care and usage.

**Cell culture**  Osteoclasts were grown in alpha10 media, containing alpha-MEM (Sigma Aldrich, St. Louis, MO), 10% fetal calf serum (Hyclone, Waltham, MA), 100U/mL penicillin and 100ug/mL streptomycin. Plat-E retrovirus packaging cells were purchased from Cell Biolabs, Inc. (San Diego, CA) and maintained in DMEM media (Cellgro, Manassas, VA) containing 10% fetal calf serum (Hyclone, Waltham, MA), and 2mM L-glutamine (Gibco, Carlsbad, CA). Osteoclasts were differentiated from bone.
marrow as described (H. Zhao et al., 2008). Briefly, bone marrow was extracted from mice and cultured in the presence of 10% CMG 14-12 supernatant (Takeshita, Kaji, & Kudo, 2000), an M-CSF-containing cell supernatant, on day -4. After 4 days (day 0), cells were lifted and replated on plastic or bovine bone fragments in alpha10 media supplemented with 2% CMG and 100ng/mL recombinant RANKL. Bone-grown cells were fixed in 4% paraformaldehyde 6 or 7 days after plating for immunofluorescence and bone pit measurements. Plastic-grown cells were fixed on days 3, 4, and 5 in 4% paraformaldehyde/PBS for osteoclastogenesis analysis or lysed on various days for immunoblot analysis.

**Retroviral DNA constructs** mCherry-ATG5WT and mCherry-ATG5K130R were generated in the laboratory of Dr. A. B. Gustafsson (Hamacher-Brady et al., 2007). mStrawberry-ATG4BC74A was kindly provided by Dr. T. Yoshimori (Fujita, Hayashi-Nishino et al., 2008). The eGFP construct was purchased from Clontech (Mountain View, CA). All constructs were cloned into the pMXs-IRES-Puro retroviral vector (Cell Biolabs, Inc., San Diego, CA). eGFP-IRES-Puro retrovirus and vector only retrovirus were used as controls.

**Proliferation and cell death ELISA assay** BrdU proliferation assay was performed by incubating cells for 18 hours in BrdU, followed by ELISA detection using the cell proliferation Biotrak ELISA system (Amersham). Cell death was analyzed in quadruplicate using cell death detection ELISAPLUS kit (Roche), which detects cytoplasmic histone-associated DNA fragmentation.
Retroviral transduction  Retroviral transduction of bone marrow macrophages was done as previously described, with slight modifications (H. Zhao et al., 2008). Briefly, 8ug of retroviral DNA was transfected into Plat-E cells using the Fugene HD transfection reagent (Roche, Basel, Switzerland) the same day as bone marrow isolation. Plat-E media was replaced 24 hours later and virus harvested 48 and 72 hours after for transduction of plated bone marrow cells. To transduce the bone marrow cells, culture media was replaced at 48 and 72 hours with alpha10 containing 25% Plat-E viral media, 10% CMG 14-12 supernatant, and 4ug/mL polybrene. At 96 hours the transduction media was replaced with alpha10 with 10% CMG 14-12 supernatant and 2ug/mL puromycin. Cells were allowed to grow for 3 days before being harvested and plated to form osteoclasts. Control, untransduced cells were killed by the puromycin.

Immunofluorescence  Osteoclasts grown on bone fragments were fixed in 4% paraformaldehyde for 10 minutes and washed in PBS. Cells were permeabilized in 0.1% Triton-X for 10 minutes at room temperature and blocked for 1 hour in PBS containing 0.2% BSA, 10% normal goat serum, and 10% normal rabbit serum. The following antibodies and detection reagents were used: anti-cathepsin K (Millipore, Billerica, MA) (1:500 dilution), mouse IgG1 isotype control (Southern Biotech, Birmingham, AL), anti-GFP conjugated to Alexa 488 (Molecular Probes, Carlsbad, CA), polyclonal rabbit antibody conjugated to Alexa 488 isotype control (anti-fluorescein, Molecular Probes, Carlsbad, CA), anti-CD107a (eBioscience, San Diego, CA), rat anti-mouse IgG2a isotype control (anti-mouse CD8a, BD Biosciences, San Jose, CA), phalloidin-Alexa 555 and phalloidin-Alexa 488 (Molecular Probes, Carlsbad, CA), goat anti-rat-Alexa 488 (Molecular Probes, Carlsbad, CA), goat anti-mouse Cy5 (Jackson Immunoresearch, West
Grove, PA), and donkey anti-mouse-Alexa 555 (Jackson Immunoresearch, West Grove, PA). All antibodies were diluted in blocking buffer. Primary antibodies were applied overnight. After washing in PBS, secondary antibodies were applied for 1 hour at room temperature. Bones were then mounted in 30% glycerol or VECTASHIELD Mounting Media with DAPI (Vector labs, Burlingame, CA) and imaged on a Nikon Eclipse epifluorescent microscope (Nikon, Melville, NY), Olympus BX51 epifluorescent microscope (Olympus, Center Valley, PA), or Axiovert 100M confocal microscope (Zeiss, Thornwood, NY). Confocal images were analyzed using LSM 510 software (Zeiss, Thornwood, NY). For localization of GFP-LC3, cathepsin K, and LAMP1, confocal microscopy was used to identify cells with complete actin rings. If the area outlined by the actin ring was completely occupied by CatK in any z-plane containing the actin ring, and CatK was not visualized outside the ring in those z-planes, the cell was said to have positive CatK localization. The same procedure was followed for LAMP1 and GFP-LC3 localization. All data was collected by a blinded observer.

**Immuno-electron microscopy.** For immunolocalization at the electron microscopy level, osteoclasts on bone chips were fixed in 4% paraformaldehyde/0.05% glutaraldehyde (Polysciences Inc., Warrington, PA) in 100mM PIPES/0.5mM MgCl₂, pH 7.2 for 1 hr at 4°C. Samples were infiltrated overnight in the cryoprotectant 2.3M sucrose/20% polyvinyl pyrrolidone in PIPES/MgCl₂ at 4°C. To permeabilize cells for antibody labeling samples were plunge frozen in liquid nitrogen and subsequently thawed in PBS at room temperature. This technique was confirmed to permeabilize the host cell membrane and intracellular organelle membranes. Samples were probed with the rabbit anti-GFP (Abcam 6556, 1:500) followed by FluoroNanogold anti-rabbit Fab (1:250)
(Nanoprobes, Yaphank, NY) and silver enhancement (Nanoprobes HQ silver enhancement kit). Samples were washed in phosphate buffer and postfix in 1% osmium tetroxide (Polysciences Inc., Warrington, PA) for 1 hr. Samples were then rinsed extensively in dH₂O prior to en bloc staining with 1% aqueous uranyl acetate (Ted Pella Inc., Redding, CA) for 1 hr. Following several rinses in dH₂O, samples were dehydrated in a graded series of ethanol and embedded in Eponate 12 resin (Ted Pella Inc.). Sections of 95 nm were cut with a Leica Ultracut UCT ultramicrotome (Leica Microsystems Inc., Bannockburn, IL), stained with uranyl acetate and lead citrate, and viewed on a JEOL 1200 EX transmission electron microscope (JEOL USA Inc., Peabody, MA). All prelabeling experiments were conducted in parallel with isotype controls or omission of the primary antibody. These controls were consistently negative at the concentration of Nanoprobes conjugated secondary antibodies used in these studies.

**Bone pit depth measurements** Osteoclast-generated bone pits were stained by removing the cells from bone fragments with a soft brush and incubating with 20μg/mL FITC conjugated wheat germ agglutinin (Sigma, St. Louis, MO) for 30 minutes at room temperature. After washing the bones, pits were imaged by confocal microscopy. The pit depth was measured from the surface of the bone down to the deepest point in the pit. All data was collected by a blinded observer.

**Nuclei Counts** Osteoclasts were grown on bone for 6 days and fixed and permeabilized as described above. Cells were stained with Alexa 555-conjugated phalloidin and mounted with VECTASHIELD Mounting Media with DAPI to visualize
actin rings and nuclei. Nuclei per cell were quantified counting any cell with at least three nuclei.

**Osteoclastogenesis** Osteoclasts were grown on plastic in 48-well plates for 3-5 days and fixed with 4% PFA, as described above. TRAP staining on fixed cells was performed using a commercially available kit according to the manufacturer's instructions (Sigma, St. Louis, MO). All TRAP+ cells with at least three nuclei were counted, with 2-3 wells counted per genotype.

**ImmunobLOTS** Cells were lysed in RIPA buffer + protease inhibitors for 10 minutes on ice, the lysates were clarified, 5x Laemmlli sample buffer added, and the samples boiled. Immunoblotting was performed using antibodies against ATG5 (Nanotools, Teningen Germany) (1:200 dilution), LC3 (Cosmo Bio Co, LTD, Tokyo, Japan) (1:500 dilution), ATG7 (Sigma-Aldrich, St. Louis MO) (1:2,000 dilution), β-actin (Sigma-Aldrich, St. Louis MO) (1:40,000 dilution), p62 (Progen Biotechnik, Heidelberg, Germany) (1:1,000 dilution), Atg4B (MBL, Woburn, MA) (1:1,000 dilution), c-Src (monoclonal antibody directed against c-Src was a gift from A. Shaw) (final concentration: 2ug/mL), β3 integrin (Cell Signaling Technology) and cathepsin K (Millipore, Billerica, MA) (1:500 dilution). Quantification of immunoblot results was performed using a Storm 840 phosphorimager (Molecular Dynamics, Piscataway, NJ) and ImageQuant software (GE, Piscataway, NJ).

**Microcomputed tomography and ovariectomy** Trabecular volume in the distal femoral metaphysis (right leg) was measured using in vivo microcomputed tomography (vivaCT 40, Scanco Medical, Brüttsellen, Switzerland) while the mice were anesthetized.
with isofluorane. A threshold linear attenuation coefficient of $1.2 \text{ cm}^{-1}$ was used to differentiate bone from non-bone. A threshold of 120 was used for evaluation of all scans. 30 slices were analyzed, starting with the first slice in which condyles and primary spongiosa were no longer visible. Trabecular volume was measured one day before ovariectomy or sham operation (basal bone volume) and 28 days after surgery (post-ovx). For ovariectomies, mice were anesthetized with ketamine/xylene delivered by intraperitoneal injection, and ovaries were removed through two small dorsal incisions. Sham operated mice were anesthetized and opened equivalently, but ovaries were not removed.

**Electron microscopy**  Proximal tibiae of 2-month-old mice were fixed with 4% PFA overnight and decalcified in 14% EDTA for 7 days. The samples were then postfixed in 2% OsO4/3% K-ferrocyanide for 2 hr, dehydrated in ethanol, and embedded in Epon LX 112. Ultrathin sections were stained briefly with lead citrate and examined using a JEOL 100SX transmission electron microscope.

**Transmission electron microscopy.** For immunolocalization at the electron microscopy level, osteoclasts on bone chips were fixed in 4% paraformaldehyde/0.05% glutaraldehyde (Polysciences Inc., Warrington, PA) in 100mM PIPES/0.5mM MgCl$_2$, pH 7.2 for 1 hr at 4°C. Samples were infiltrated overnight in the cryoprotectant 2.3M sucrose/20% polyvinyl pyrrolidone in PIPES/MgCl$_2$ at 4°C. To permeabilize cells for antibody labeling samples were plunge frozen in liquid nitrogen and subsequently thawed in PBS at room temperature. This technique was confirmed to permeabilize the host cell membrane and intracellular organelle membranes. Samples were probed with the rabbit
anti-GFP (Abcam 6556, 1:500) followed by FluoroNanogold anti-rabbit Fab (1:250) (Nanoprobes, Yaphank, NY) and silver enhancement (Nanoprobes HQ silver enhancement kit). Samples were washed in phosphate buffer and postfixed in 1% osmium tetroxide (Polysciences Inc., Warrington, PA) for 1 hr. Samples were then rinsed extensively in dH2O prior to en bloc staining with 1% aqueous uranyl acetate (Ted Pella Inc., Redding, CA) for 1 hr. Following several rinses in dH2O, samples were dehydrated in a graded series of ethanol and embedded in Eponate 12 resin (Ted Pella Inc.). Sections of 95 nm were cut with a Leica Ultracut UCT ultramicrotome (Leica Microsystems Inc., Bannockburn, IL), stained with uranyl acetate and lead citrate, and viewed on a JEOL 1200 EX transmission electron microscope (JEOL USA Inc., Peabody, MA). All prelabeling experiments were conducted in parallel with isotype controls or omission of the primary antibody. These controls were consistently negative at the concentration of Nanoprobes conjugated secondary antibodies used in these studies.

Transmission electron microscopy (Cathepsin K) For the immunolocalization of cathepsin K, osteoclasts grown on a plastic substrate were fixed in 4% w/v paraformaldehyde in 0.1 M phosphate buffer (pH=7.4) (Slot and Geuze, 2007). Preparation of the samples, cryosectioning and immunogold labeling was performed as described (Slot and Geuze, 2007). Cathepsin K was detected with a mouse monoclonal antibody from Millipore (Billerica, MA) (Van Meel et al., 2011). Subsequently, a rabbit polyclonal antibody against mouse immunoglobulins was used as a bridging step between the cathepsin K antibody and protein A-gold (Dako, Heverlee, Belgium).
**Serum Ctx measurements**  Blood was collected by cheek puncture after 6 hours starvation. Plasma was obtained using plasma separator tubes with lithium heparin (Becton Dickinson). Serum CTx-I, a specific marker of osteoclastic bone resorption, was measured using a RatLaps ELISA kit from Nordic Bioscience Diagnostics A/S.

**Actin ring formation**  Osteoclasts on bone were incubated in cold alpha10 media for 30 minutes to disrupt actin rings, followed by warm alpha10 with 10% FBS, RANKL, and M-CSF. Cells were fixed at the indicated times, and actin rings were stained and counted.

**Statistics**  All data was analyzed with Prism software (Graphpad, San Diego, CA), using two-tailed unpaired Student’s t tests, unless otherwise indicated. Error bars represent standard deviation.

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Abstract

Although not traditionally thought of as an inflammatory disease, estrogen deficiency osteoporosis is partially mediated by T cells, at least in mice (Weitzmann & Pacifici, 2007). Th17 cells, which secrete IL-17, are implicated in a number of inflammatory and autoimmune conditions, including arthritis-mediated bone destruction. We find IL-17 critical in the pathogenesis of bone loss due to estrogen deficiency. IL-17 receptor deficient mice are protected from bone loss after ovariectomy, as are mice treated with a neutralizing IL-17 antibody. The effect of IL-17 on osteoclasts is indirect, via osteoblastic upregulation of RANKL. The SEFIR domain of IL-17R is critical for osteoblast-mediated osteoclast formation and RANKL production. The adaptor protein Act1 is required for osteoblast-mediated osteoclast formation in response to IL-17, and Act1 deficient mice are also protected from bone loss induced by estrogen deficiency.
Introduction

Autoimmune and inflammatory conditions have long been known to cause bone loss, suggesting a connection between the T-cell and the bone-resorbing osteoclast. Osteoclast formation requires two cytokines: Receptor Activator of NFκB Ligand (RANKL) and Macrophage Colony Stimulating Factor (M-CSF), produced primarily by osteoblasts and bone marrow stromal cells (Lacey et al., 1998; Yasuda et al., 1998). Though RANKL and M-CSF are essential, additional cytokines can modulate osteoclast formation. The best studied are TNFα and IL-1, which upregulate RANKL production on osteoblasts (Cenci et al., 2000; Hofbauer et al., 1999; Lam et al., 2000; Sherman, Weber, Datta, & Kufe, 1990; Wei, Kitaura, Zhou, Ross, & Teitelbaum, 2005).

CD4+ T helper cells are heterogeneous and can be characterized by the cytokine profiles they produce. CD4+ T helper type 1 (Th1) cells produce interferon (IFN)-γ, and regulate influx of macrophages. CD4+ Th2 cells produce mainly IL-4 and induce immunoglobulin (Ig)E production, recruit eosinophils to inflammatory sites, and help clear parasites. Treg cells develop to inhibit over-activation of the T-cell response, to save normal tissue from unnecessary destruction. More recently, a population of CD4+ T cells was identified that preferentially produces IL-17A and IL-17F, as well as IL-22, termed Th17 cells (Bettelli et al., 2006; Harrington et al., 2005; Mangan et al., 2006; Park et al., 2005). Th17 cells are thought to arise when naïve CD4+ are stimulated with TGF-β, which induces both RORγt and Foxp3 transcription factors. These cells now have dual potential to differentiate into either the Th17 or Treg cells depending on the cytokine environment. In the presence of proinflammatory cytokines such as IL-6, IL-21, or IL-23, and low concentrations of TGF-β, RORγt expression is further upregulated, whereas
Foxp3 is inhibited, and Th17 cells develop. In contrast, in the absence of proinflammatory cytokines, high concentrations of TGF-β along with retinoic acid and IL-2 are optimal for Foxp3 expression and promote Treg cell differentiation (Zhou et al., 2008). RORγt is required for Th17 cell differentiation and is both necessary and sufficient for induction of IL-17 and other genes characteristic of Th17 cells, but RORα also plays a role (Ivanov et al., 2006; Yang et al., 2008; Zhang, Meng, & Strober, 2008). Other transcription factors are necessary for Th17 development and mainly work by modifying RORγt expression or function, including interferon regulatory factor 4 (IRF4), the aryl hydrocarbon receptor (AHR), runt-related transcription factor 1 (RUNX1), Stat3, and basic leucine zipper transcription factor, ATF-like (BATF) (Brustle et al., 2007; Chen et al., 2006; Quintana et al., 2008; Schraml et al., 2009; Veldhoen et al., 2008; Zhang et al., 2008). Th17 cells can be induced from naïve T-cells using only IL-6 and TGF-β (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen, Hocking, Atkins, Locksley, & Stockinger, 2006), while TNF-α, IL-23, and IL-1β amplify their development (Sutton, Brereton, Keogh, Mills, & Lavelle, 2006), and IFNγ and IL-4 are inhibitory (Harrington et al., 2005).

IL-17A is the founding member of the IL-17 family, but five other members have been described. Th17 cells produce IL-17A and IL-17F, which are most similar in homology and function (Kolls & Lindén, 2004). IL-17 forms a dimer, with A/A being the most potent, followed by A/F and F/F (Liang et al., 2007). Here we will refer to IL-17A simply as IL-17. IL-17 is an important mediator of inflammation, recruiting neutrophils and inducing TNF-α, IL-1β, IL-6, IL-8, and other inflammatory cytokines by macrophages, fibroblasts, and other cells. IL-17 activates a number of kinase cascades in
many cell types, including canonical NFkB, Akt, JNK, ERK 1/2, and p38 (Kim et al., 2007; Liu et al., 2009; Shalom-Barak, Quach, & Lotz, 1998).

The IL-17 receptor (IL-17R, or IL-17AR) binds both IL-17 and IL-17F (Shen & Gaffen, 2008), and contains a cytoplasmic SEFIR domain (similar expression to fibroblast growth factor genes, IL-17 receptors and Toll–IL-1R) (Novatchkova, Leibbrandt, Werzowa, Neubuser, & Eisenhaber, 2003). This domain is required for the activation of Mitogen-Activated Protein Kinases (MAPKs) and the transcription factors NF-κB, C/EBPβ, and C/EBPδ (Maitra et al., 2007). The SEFIR domain is similar to the Toll-interleukin 1 receptor (TIR) domain. Both SEFIR and TIR are implicated in homotypic dimerization (Novatchkova et al., 2003), and both are required for activation of NFkB in fibroblasts. A single point mutation within the SEFIR (V553H) is sufficient to eliminate IL-17-dependent NFkB activation (Maitra et al.). Deletion of the SEFIR motif also prevents ERK activation.

Activated IL-17R interacts with the adaptor protein Act1 (transcription factor NF-kB activator 1). Act1 was identified as an activator of NFkB, p38, and JNK (Leonardi, Chariot, Claudio, Cunningham, & Siebenlist, 2000; Li et al., 2000). Fibroblasts lacking Act1 fail to activate NFkB when IL-17 stimulated, but ERK phosphorylation after 24 hours of stimulation is unaffected (Qian et al., 2007). Act1 contains two TRAF binding sites, as well as a helix-loop-helix and a coiled-coil domain (Li et al., 2000). Like the IL-17R, Act1 has a SEFIR domain, which mediates its interaction with IL-17R upon activation. After binding IL-17R, Act1 recruits transforming growth factor β–activated kinase 1 (TAK1) and TRAF6 to mediate downstream signaling (Qian et al., 2007; Schwandner, Yamaguchi, & Cao, 2000). Act1 contains a Ubox-like domain, which can
act as an E3 ubiquitin ligase to ubiquitinate TRAF6. Ubiquitination is K63-dependent, and is critical for IL-17 mediated NFκB, JNK, and ERK activation (Liu et al., 2009). Although previous data suggests TRAF6 ubiquitinates itself, TRAF6 is not ubiquitinated in IL-17-stimulated Act1 KO MEFs or Act1 KO MEFs reconstituted with Ubox-mutant Act1, suggesting Act1 may be the ubiquitin ligase necessary for TRAF6 ubiquitination (Liu et al., 2009; Petroski et al., 2007). TRAF6 KO cells reconstituted with RING domain mutant TRAF6 also have impaired activation of NFκB in response to IL-17, suggesting Act1 E3 ligase may be important for TRAF6 ubiquitination, but TRAF6 E3 ligase activity is still necessary for downstream signaling (Liu et al., 2009).

The IL-17R is not the only pathway that utilizes Act1. Act1 also regulates signaling from the TNF receptor family members BAFFR, which is required for B-cell maturation, and CD40, which mediates T-cell dependent B-cell activation (Kanamori, Kai, Hayashizaki, & Suzuki, 2002; Qian, Zhao, Jiang, & Li, 2002). Act1 KO mice have more peripheral B cells, leading to lymphadenopathy, splenomegaly, hypergammaglobulinemia, and the production of auto-antibodies (Qian et al., 2004). Stimulation of splenic B cells from Act1 KO mice with CD40 and BAFF (B cell–activating factor belonging to the TNF family) results in enhanced cellular differentiation and survival. These effects are associated with enhanced IκB phosphorylation, processing of NF-κB2, and activation of JNK, ERK, and p38, suggesting Act1 negatively regulates these signaling events in response to BAFF ligand and CD40 antibody, in contrast to its activating role in response to IL-17 (Qian et al., 2004). However, cellular specificity may exist, since epithelial cells utilize Act1 as a positive mediator of NFκB activation in response to CD40L (Qian et al., 2002).
While NFkB signaling in osteoclasts is well studied, only recently was the role of NFkB in the osteoblast established. Genetic NFkB inhibition in mature osteoblasts causes increased bone formation in vivo and in vitro, resulting in osteopetrosis (Chang et al., 2009). The mechanism is thought to involve prolonged JNK phosphorylation leading to Fra-1 (encoded by Fosl-1, an AP-1 type transcription factor) expression. Knocking down Fra-1 inhibited bone mineralization and reduced levels of the bone matrix genes Ocn, Ibsp and Col1a2 (Chang et al., 2009). Previously it was found that osteoblast-specific expression of transgenic Fra-1 leads to osteosclerosis due to enhanced osteoblastogenesis and bone formation (Jochum et al., 2000), while mice with osteoblast-specific deletion of Fra-1 are osteopenic with a defect in osteoblast differentiation, but no effect on the number of osteoblasts (Eferl et al., 2004). Although the primary ligand was not identified for the osteoblast, these data suggest NFkB signaling in mature osteoblasts inhibits bone formation, while in osteoclasts the same signaling causes increased bone resorption.

Rheumatoid arthritis (RA) is a classic example of inflammatory disease causing bone and joint destruction. It was originally thought RA was predominantly Th1 mediated (Kotake et al., 2001; Yamada et al., 2008). However, the primary Th1 cytokine, IFNγ, was found to suppress osteoclastogenesis by inducing TRAF-6 degradation (Huang, O'Keefe, & Schwarz, 2003; Pang, Martinez, Jacobs, Balkan, & Troen, 2005; Takayanagi et al., 2000), and other Th1 cytokines including GM-CSF, IL-12, and IL-18 were shown to inhibit bone resorption in vitro (Amcheslavsky & Bar-Shavit, 2006; Nakashima & Takayanagi, 2008; Tunyogi-Csapo et al., 2008). In vivo, IFNγ is protective against arthritis, and IFNγ deficient mice are more susceptible to
autoimmune arthritis manifestations (Chu, Song, Mayton, Wu, & Wooley, 2003; Irmler, Gajda, & Brauer, 2007). The primary culprit is now thought to be the Th17 cell, for its ability to produce or induce the bone destructive cytokines TNFα, IL-1, IL-6 and IL-17. Th17 cells enhance osteoclastogenesis in coculture much more efficiently than Th1 or Th2 cells (Sato et al., 2006). The pro-osteoclastic effect of IL-17 is thought to be indirect, by stimulation of osteoblasts to express RANKL (Page & Miossec, 2005; Sato et al., 2006), but IL-17 was also found to directly enhance osteoclastogenesis in a monoculture of human macrophages (Yago et al., 2009). In another study, IL-17 actually inhibited osteoclastogenesis from RAW264.7 macrophages (Kitami et al.). TNFα, produced by both Th17 and Th1 cells, has a synergistic effect with IL-17 on many targets (Miossec, 2003).

Osteoporosis in humans most commonly occurs post-menopause, during which estrogen levels decrease and osteoclast numbers increase. The mechanism by which low estrogen leads to bone loss is complex, but is thought to occur through osteoblast-mediated osteoclast formation and activation. Although osteoporosis is not associated with inflammation, T-cells and TNFα are proposed to play a critical role. Nude mice lacking T-cells are protected from bone loss induced by ovariectomy, while nude mice reconstituted with T-cells are not (Cenci et al., 2000; Roggia et al., 2001); (Felix, Cecchini, & Fleisch, 1990; Kong et al., 1999; Roggia et al., 2001). TNF-/- mice are protected from ovariectomy-induced osteoporosis (Roggia et al., 2001), as are transgenic mice overexpressing a soluble TNF-receptor (Ammann et al., 1997), and mice treated with inhibitory TNF-binding protein (Kimble, Bain, & Pacifici, 1997). The source of the TNF in this circumstance is T-cells, not BMMs. The type of T-cell involved has not been
studied, but similar to the case of RA-mediated bone loss, Th1 cells are unlikely culprits because of the bone-protective function of IFNγ. Recently, IL-17R-/- mice were reported to have more fat and more leptin, as well as more whole-body bone loss after ovariectomy as measured by Dual energy X-ray absorptiometry (DEXA) (Goswami, Hernandez-Santos, Zuniga, & Gaffen, 2009). However, fat distribution independently affects bone density readings by DEXA (Formica, Loro, Gilsanz, & Seeman, 1995; Hangartner & Johnston, 1990; Tothill, Hannan, Cowen, & Freeman, 1997; Tothill, Laskey, Orphanidou, & van Wijk, 1999; Wren, Kim, Janicka, Sanchez, & Gilsanz, 2007), making the technique less appropriate than micro-CT in many circumstances. Given the role of IL-17 in inflammatory bone loss, the importance of TNFα in osteoporosis, and the synergistic effects of TNFα and IL-17 on target tissues (Miossec, 2003), we studied the effect of IL-17 signaling on osteoclastogenesis and osteoporosis.
Results

IL-17R Deficiency or Treatment with IL-17 Blocking Antibody Protects Mice From Bone Loss Induced by Estrogen Deficiency

To test whether IL-17 signaling is important in the pathogenesis of osteoporosis, we mimicked the most common human form of osteoporosis, that of estrogen deficiency, by removing ovaries (ovx) in mice. Micro-CT, a sensitive and specific measure of bone volume, was used to assess trabecular bone. While WT mice exhibited significant bone loss after ovx, minor, non-significant changes were observed in IL-17R KO animals (Figure 3.1). To further establish the role of IL-17 in this process, we selected an IL-17 blocking antibody for its ability to inhibit osteoclastogenesis in a coculture of BMMs

![Figure 3.1: IL-17R KO Mice are Protected From Post-ovx Bone Loss](image)

Wild-type and IL-17R knockout mice were subjected to ovariectomy or sham procedure. Four weeks later, trabecular Bone Volume per Total Volume was measured in the distal femora using micro-CT.
with osteoblasts and IL-17 (Figure 3.2). Both two weeks (Figure 3.3) and four weeks
(Figure 3.4) post-ovx, injection of the blocking antibody protected animals from bone
loss, while injection of an isotype control antibody had no effect. To test another
experimental system, we ovariectomized mice lacking BATF, which is reported to be
essential for Th17 cell differentiation (Schraml et al., 2009). To our surprise, BATF KO
mice were not protected from bone loss (Figure 3.5). Thus, Th17-independent sources of
IL-17, perhaps derived from bone marrow resident cells, mediate bone loss induced by
ovariectomy. These data suggest IL-17 is important in the pathogenesis of osteoporosis,
and suggest pharmacologic inhibition with either antibody against IL-17 may ameliorate
osteoporosis induced by estrogen loss.

Figure 3.3: IL-17 Blocking Antibody Begins to Protect Mice from Ovx-Induced Bone Loss at Two Weeks

Ovariectomized mice were treated with IL-17 blocking antibody or isotype control for two weeks and distal femora were analyzed by micro-CT. n=5-7 mice per group. All significant p-values are shown.
Figure 3.4: IL-17 Blocking Antibody Protects from Ovx-Induced Bone Loss at Four Weeks

Ovariectomized mice were treated with IL-17 blocking antibody or isotype control daily for four weeks and distal femora were analyzed by micro-CT. n=5-7 mice per group.
Since different groups report IL-17 having both a direct and indirect effect on osteoclast development, we first cultured BMMs with varying concentrations of M-CSF, RANKL, and IL-17, and observed no differences in osteoclast formation (Figure 3.6). To test the function of IL-17 signaling in the osteoblast, we first determined the IL-17 receptor is expressed in osteoblasts, and its expression is unaffected by TNFα or IL-17 stimulation (Figure 3.7). Next we cultured BMMs in combination with primary osteoblasts and varying IL-17 concentrations. IL-17 potently induced osteoclast formation, even at low levels (Figure 3.8). RANKL mRNA expression in the osteoblast is induced by IL-17 (Figure 3.9), explaining the cytokine’s pro-osteoclastogenic effect. IL-17 does not affect osteoblast proliferation (Figure 3.10). This suggests IL-17 is unable to...
directly induce osteoclastogenesis, but instead acts through the osteoblast.

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**Figure 3.6: IL-17 Does not Directly Affect Osteoclast Formation**

Bone marrow macrophages were grown in the presence of RANKL (100 ng/ml), M-CSF (20 ng/ml), and varying concentrations of IL-17 for 3, 4, or 4 days, then fixed and trap stained.
The IL-17R SEFIR Domain is Necessary for Osteoblast-induced Osteoclast Formation

To determine the domains of IL-17R necessary for osteoclast formation, we retrovirally transduced primary IL-17R KO osteoblasts with WT or mutant IL-17R. Coculture with WT BMMs and low or high levels of IL-17 demonstrated efficient osteoclastogenesis in cultures containing IL-71R KO osteoblasts reconstituted with WT IL-17R, a mutant lacking the distal cytoplasmic domain (d665), or a point mutant in a hypothetical disulfide bond-forming residue (R549A) (Figure 3.11, Figure 3.12). However, osteoblasts reconstituted with IL-17 receptor lacking the SEFIR domain (dSEFIR) were unable to induce osteoclastogenesis in response to IL-17 (Figure 3.11). Osteoblasts containing WT, d665, or R549A mutant IL-17 receptor displayed efficient RANKL mRNA induction in response to IL-17, while dSEFIR reconstituted osteoblasts exhibited poor IL-17-induced RANKL expression (Figure 3.13, Figure 3.14). These results suggest the SEFIR domain is critical for osteoclast induction by IL-17.
Figure 3.8: IL-17 Potently Enduces Osteoclastogenesis in Coculture

Bone marrow macrophages and primary osteoblasts were incubated with vitamin D and varying concentrations of IL-17. Osteoblasts were lifted, then osteoclasts were fixed and TRAP stained.
Act1 is Critical for IL-17 Dependent Osteoclast Formation

Act1 is downstream of not only IL-17, but also the TNFR superfamily members CD40 and BAFFR (Li, 2008), and can act as an E3 ubiquitin ligase for TRAF6 (Liu et al., 2009). RANK, the TNFR family member in the osteoclast, requires TRAF6 for signaling (Lomaga et al., 1999; Naito et al., 1999), so we tested whether Act1 deficiency affects osteoclast differentiation in response to RANKL. Osteoclast differentiation is...
equivalent in Act1 WT and KO osteoclasts, suggesting Act1 is not a downstream mediator of RANK signaling (Figure 3.15). Additionally, Act1 deficiency does not
affect osteoblast differentiation (Figure 3.16) or proliferation (Figure 3.17). Coculture of BMMs with WT or KO Act1 osteoblasts and IL-17 revealed enhanced osteoclast
formation only in the presence of Act1 (Figure 3.18).

**Figure 3.14: qPCR of RANKL Expression of IL-17R Mutants Stimulated with IL-17**

Wild-type and IL-17R knockout osteoblasts were transduced with empty vector (EV) or various IL-17R mutants and stimulated with 10 ng/ml IL-17. RANKL expression was measured by qPCR.
Figure 3.15: Act1 does not Mediate Osteoclast Differentiation

Act1 WT and KO bone marrow macrophages were grown with RANKL and MCSF for the indicated number of days, then fixed and trap stained.
Figure 3.16: Osteoblasts Differentiate Normally in the Absence of Act1

Primary osteoblasts were isolated from Act1 WT and KO calvaria, differentiated for 20 days, and stained for alkaline phosphatase.

Figure 3.17: Act1 Deficiency Does not Affect Osteoblast Proliferation

Act1 WT and KO osteoblasts were cultured with BrdU for 16 hours, after which BrdU incorporation was determined by absorptiometry.
**Figure 3.18: Act1 is Necessary for IL-17-Induced Osteoclast Formation in Coculture**

Act1 KO osteoblasts were transduced with empty vector or WT Act1, and Act1 KO osteoblasts were transduced with empty vector. These osteoblasts were cocultured with bone marrow macrophages in the presence of Vitamin D and the indicated amount of IL-17. Osteoblasts were lifted and osteoclasts were trap stained.

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**Act1 Expression, but not IL-17RA, is Regulated by Estrogen *in vitro* and *in vivo***

These data raise the possibility that estrogen exerts its bone-sparing effects, at least in part, by suppressed expression of Il-17 effectors, such as Act1. To determine if such is the case, we first established that estrogen supplementation does not significantly affect expression of IL-17RA (Figure 3.19) and Act1 (Figure 3.20) mRNA by WT osteoblasts.
We then exposed osteoblasts to estrogen for 5 days followed by 5 days in the absence of the steroid. As control, the osteoblasts were maintained in the presence of the steroid for the entire 10 days of culture. Estrogen withdrawal does not affect IL-17RA mRNA (Figure 3.21) expression but increases that of Act1 (Figure 3.22). To confirm the in vivo significance, mice were subjected to Ovx or sham procedures, and two weeks later femora were removed, pulverized (bone marrow was first removed by centrifugation), and extracted of mRNA. Act1 levels significantly increase post-ovx compared to sham, while IL-17R do not significantly change (Figure 3.23, Figure 3.24).

![Figure 3.19: Estrogen Does not Significantly Affect IL-17R Expression](image)

Wt osteoblasts were exposed to estrogen (10nm) with time. IL-17RA mRNA was measured by qPCR.
Figure 3.20: Estrogen Does not Significantly Affect Act1 Expression

Wt osteoblasts were exposed to estrogen (10nm) with time. Act1 mRNA was measured by qPCR.

Figure 3.21: Estrogen Deprivation Does not Significantly Affect IL-17R Expression

Wt osteoblasts were exposed to estrogen (10nm) for five day followed by five days of continued estrogen (E2) or estrogen deprivation (E2 deprivation). IL-17R expression was measured by qPCR.
Figure 3.22: Estrogen Deprivation Significantly Increases Act1 Expression

Wt osteoblasts were exposed to estrogen (10nm) for five day followed by five days of continued estrogen (E2) or estrogen deprivation (E2 deprivation). Act1 expression was measured by qPCR.

Figure 3.23: Act1 Expression Increases in the Bone following Ovariectomy

Wt mice were subjected to OVX or sham procedures, and bone devoid of marrow was harvest for mRNA. Act1 expression was measured by qPCR.
Figure 3.24: IL-17R Expression Does Not Significantly Change in the Bone following Ovariectomy

WT mice were subjected to OVX or sham procedures, and bone devoid of marrow was harvest for mRNA. IL-17R expression was measured by qPCR.

Act1 Deficiency Protects Mice from Estrogen Loss Osteoporosis

Micro-CT analysis of trabecular bone established that baseline bone volume is unaffected by global loss of Act1 (Figure 3.25). However, after ovariectomy, Act1 KO mice maintain higher bone mass (Figure 3.25), which is consistent with an essential downstream role of Act1 in IL-17-induced bone pathology.
Figure 3.25: Act1-deficient Mice are Protected from OVX-induced Bone Loss

Eight-week old mice were subjected to ovariectomy or sham procedure, and bone volume/total volume (BV/TV) of distal femora was measured by micro-CT after four weeks. n = 6 mice per group.

**Figure 3.25: Act1-deficient Mice are Protected from OVX-induced Bone Loss**

Eight-week old mice were subjected to ovariectomy or sham procedure, and bone volume/total volume (BV/TV) of distal femora was measured by micro-CT after four weeks. n = 6 mice per group.
Discussion

IL-17 is an inflammatory cytokine associated with a variety of disorders, including rheumatoid arthritis, where it promotes osteoclast formation and bone resorption. Osteoporosis resulting from estrogen deficiency is dependent on T cells, specifically the TNF produced by T cells. We find bone loss due to estrogen deficiency is also dependent on IL-17. IL-17R KO mice exhibit minimal changes in trabecular bone after ovariectomy, as do WT mice treated with IL-17 blocking antibody. The downstream IL-17R signaling protein Act1 is critical in this process, as Act1 KO mice are protected from the bone loss of ovariectomy. IL-17 promotes bone loss by stimulating RANKL production in the osteoblast, a process that requires the SEFIR domain of the IL-17 receptor. Similarly, Act1 is essential in the osteoblast, but not osteoclast, for IL-17-mediated bone resorption. Unexpectedly, a previous report found IL-17R deficient mice actually lose more whole-body bone density after ovariectomy than do WT controls (Goswami et al., 2009). As mentioned earlier, these differences may be due age differences in the mice, or to the use of DEXA versus micro-CT, since DEXA measurements of bone density are influenced by fat distribution (Formica et al., 1995; Hangartner & Johnston, 1990; Tothill et al., 1997; Tothill et al., 1999; Wren et al., 2007), which is potentially affected by IL-17 (Goswami et al., 2009; Shin, Shin, & Noh, 2009; Sumarac-Dumanovic et al., 2009).

The necessity of both IL-17 and TNF in estrogen deficiency bone loss may be explained by the effect of IL-17 and TNF on target tissues alone or in combination. IL-17 and TNFα have a synergistic effect on most cells, including the osteoblast (Chabaud et al., 2000; Chabaud, Lubberts, Joosten, van Den Berg, & Miossec, 2001; Chabaud, Page,
& Miossec, 2001; Hata et al., 2002; Jones & Chan, 2002; Katz, Nadiv, & Beer, 2001; Koshy et al., 2002; Laan et al., 1999; LeGrand et al., 2001; Martel-Pelletier, Mineau, Jovanovic, Di Battista, & Pelletier, 1999; Miossec, 2003). Thus, similar to reports that blockade of IL-17 can prevent TNFα-induced arthritis (Lubberts, Koenders, & van den Berg, 2005), inhibiting the IL-17 pathway may effectively diminish the effect of TNFα in estrogen-loss osteoporosis.

It is interesting that mice lacking the IL17R are protected from osteoporosis, but mice lacking BATF, which is required for Th17 cell development, are not. It is possible cells besides Th17 cells are producing IL-17 in the bone microenvironment or elsewhere, providing sufficient levels of the cytokine for bone loss. IL-17 is known to be produced by mast cells, γδ T cells (CD4– and CD8– T cells), a memory subset of cytotoxic T cells, invariant natural killer T cells, macrophages, and neutrophils (Ferretti, Bonneau, Dubois, Jones, & Trifilieff, 2003; Happel et al., 2003; Hueber et al., 2003; Ivanov et al., 2007; Kolls & Linden, 2004; Lockhart, Green, & Flynn, 2006; Maek, Buranapraditkun, Klaewsongkram, & Ruxrunghtham, 2007; Michel et al., 2007; Shin, Benberou, Esnault, & Guenounou, 1999; Song et al., 2008). One study found the major source of IL-17 in the arthritic joint is the mast cell (Hueber et al.). It is also possible that BATF deficiency does not completely abolish the Th17 population in our experimental osteoporosis model.

IL-1 and lipopolysaccharide (LPS) are osteoclastogenic and their receptors share a common Toll/IL-1R (TIR) sequence. IL-17 receptors contain an intracellular SEFIR domain, homologous to the TIR motif, a docking site for adaptor proteins (Maitra et al., 2007). A distal component of the SEFIR domain, known as TILL, is unique to IL-17RA and in other circumstances, its mutation disables the receptor’s activity. The TILL-
residing amino acid, R549, generates a key salt bridge in the homologous TIR domain. Recently, Onishi et al established that signals emanating from IL-17RA require a large extension distal to the TILL component of the SEFIR domain (Onishi et al). While deletion of the distal SEFIR and its entire TILL sequence, in IL-17RA, forestalls IL-17-stimulated osteoclastogenesis and RANKL expression by osteoblasts, the process occurs independently of R549 and its putative salt bridge formation. This observation is in keeping with the fact that IL-17RA^{R549A} does not impair IL-17/TNF- induced signaling in transformed cells (Maitra et al., 2007). Thus, while the LPS, IL-1 and IL-17 are each osteoclastogenic, homologous components of their receptors may function differently.
Methods

Mice All animals were housed in the animal care unit of the Washington University School of Medicine and were maintained according to guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care. All animal experimentation was approved by the Animal Studies Committee of the Washington University School of Medicine.

Histological analysis Tibias were harvested, cleaned of soft tissue, and fixed in 10% buffered formalin overnight. For non-decalcified sections, tibias were dehydrated by incubation in an ethanol gradient (20%, 30%, 50%, 70% ETOH, for 30 minutes each). Bones stored in 70% ETOH were then embedded in methylmethacrylate and cut into 5µm longitudinal sections. Non-decalcified sections were left unstained. For decalcified sections, following fixation, tibias were rinsed in water then incubated in 14% EDTA (pH 7.2) for 7 days with solution changes every 3.5 days. Following decalcification, tibias were rinsed in water then incubated in the ETOH gradient described above. Bones stored in 70% ETOH were then embedded in paraffin, cut into 4µm longitudinal sections, and stained with TRAP or hematoxylin and eosin for visualization of osteoclasts or osteoblasts, and measurements were made using BioQuant software (Bioquant; Nashville, TN).

Bone formation assay (calcein double labeling) Mice were injected with 7.5mg/kg calcein (Sigma-Aldrich; St. Louis, MO). Five days later mice were injected again, and tibias were harvested after an additional 2 days for histologic analysis. Dynamic assessment of trabecular bone formation was determined on non-decalcified sections.
using BioQuant software (Bioquant; Nashville, TN). This software provided measures of bone surface, percent single- (sLS/BS) and double-labeled (dLS/BS) bone surface, mineralizing surface (MS/BS), mineral apposition rate (MAR), and bone formation rate (BFR/BS). Trabecular bone measurements were taken from a region encompassing a 500µm-long field, across the width of the bone (located 100µm below the growth plate). For each animal, 2 serial sections were analyzed and the measurements averaged. Pictures were obtained using an Olympus 1X51 fluorescent microscope fitted with Olympus DP70 camera (Olympus; Center Valley, PA).

**Cell culture** Osteoclasts were grown in alpha10 media, containing alpha-MEM (Sigma Aldrich, St. Louis, MO), 10% fetal calf serum (Hyclone, Waltham, MA), 100U/mL penicillin and 100ug/mL streptomycin. Plat-E retrovirus packaging cells were purchased from Cell Biolabs, Inc. (San Diego, CA) and maintained in DMEM media (Cellgro, Manassas, VA) containing 10% fetal calf serum (Hyclone, Waltham, MA), and 2mM L-glutamine (Gibco, Carlsbad, CA). Osteoclasts were differentiated from bone marrow as described (Zhao et al., 2008). Briefly, bone marrow was extracted from mice and cultured in the presence of 10% CMG 14-12 supernatant (Takeshita, Kaji, & Kudo, 2000), a M-CSF-containing cell supernatant. After 4 days, cells were lifted and replated on plastic or bovine bone fragments in alpha10 media supplemented with 2% CMG and 100ng/mL recombinant RANKL. Bone-grown cells were fixed in 4% paraformaldehyde 6 or 7 days after plating. Plastic-grown cells were fixed on days 3, 4, and 5 in 4% paraformaldehyde/PBS for osteoclastogenesis analysis or lysed on various days for immunoblot analysis.
**RT-PCR** RNA was isolated using RNeasy kits (Qiagen); RLT lysis buffer was supplemented with β-mercaptoethanol (1%). Purified RNA was treated with DNase I (Invitrogen; Carlsbad, CA) prior to reverse transcription (RT). RT was performed using SuperScript III (Invitrogen). A negative control using RNA not subjected to reverse transcription was included in each assay. Quantitative PCR (qPCR) was performed using Applied Biosystems’s (Foster City, CA) Power SYBR green master mix and gene specific primers. The qPCR reaction was run on Applied Biosystem’s ABI Prism 7000. Transcript levels were normalized to cyclophilin.

**Quantitative RT-PCR** For ex-vivo RNA analysis, bone marrow was removed from femora by centrifugation. The remaining bone was frozen in liquid nitrogen and pulverized using a Braun Mikrodismembrator (Sartorius BBI Systems Inc., Bethlehem, PA) in an RNAase-free environment. RNA was extracted using Trizol (Invitrogen) following manufacturer’s protocol. Quantitative PCR was performed using TaqMan probes to IL-17RA (Invitrogen, Mm00434214) Act1 (Invitrogen, Mm00506094), RANKL (Invitrogen, Cat #4331182) and as a control, Cyclophillin (Invitrogen) or GAPDH as described.

**Tumor and bone metastasis models** For intracardiac injections, the operator was blinded to genotype. Mice were anesthetized and injected via the left ventricular chamber with $1 \times 10^5$ B16-FL cells in 50 µL PBS (Hirbe et al., 2007). Bioluminescence imaging was performed on days 7, 10, and 12 after B16-FL cell injection. For in-vivo imaging, mice were injected i.p. with 150 mg/kg D-luciferin (Biosynthesis) in PBS 10 min before imaging. Imaging was performed using a charge-coupled device camera (IVIS 100;
exposure time of 1 or 5 min, binning of 8, field of view of 15 cm, f/stop of 1, and no filter) in collaboration with the Molecular Imaging Center Reporter Core at Washington University. Mice were anesthetized by isoflurane (2% vaporized in O₂), and shaved to minimize attenuation of light by pigmented hair. For analysis, total photon flux (photons per second) was measured from a fixed region of interest in the tibia/femur or the mandible using Living Image 2.50 and IgorPro software (Wavemetrics).

**Flow Cytometry** Spleen cells were prepared by gently crushing the tissue and filtering through a 40-µm cell strainer (BD Falcon). Cells were harvested, centrifuged, and incubated in red blood cell (RBC) lysis buffer. In stimulation experiments, cells were incubated in 50 ng/ml PMA and 500 ng/ml ionomycin (Sigma-Aldrich) for 4 h at 37°C. Brefeldin A (Sigma-Aldrich) was added during the last 2 h of culture at 10 µg/ml. Cells were stained with LIVE/DEAD Fixable Dead Cell Stain Kits (Molecular Probes, Invitrogen) followed by the appropriate surface antibody (anti-CD4) for 20 min at 4°C. Stained cells were fixed in 2% paraformaldehyde for 20 min at RT before permeabilization with 0.05% saponin. Intracellular staining for the cells was conducted at 4°C for 30 min using Alexa Fluor 488 anti-mouse IL-17A (1:1000 dilution) (eBioscience). Samples were gated on live cells using the above LIVE/DEAD cell stain. All samples were analyzed on a FACSCalibur or FACSCanto (BD) and data were analyzed with FlowJo software (TreeStar). A total of 30,000 events were collected per sample.

**Signaling experiments** For BMM signaling, macrophages were grown in α-10 medium and 1/10 CMG14-12 supernatant for 3 days. Cells were then starved of serum and M-
CSF for 24 hours before being stimulated with either RANKL, M-CSF, IL-17, or TNFα. For pre-osteoclast signaling, BMMs were grown for 48 hours in CMG and RANKL before 4 hours of serum starvation, followed by cytokine stimulation for the appropriate time periods. Total cell lysates were collected and Western blot analysis was performed.

**Immunoprecipitation** Cells were washed in cold PBS and lysed on ice in buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 0.2% NP-40, 1 mM EDTA, and protease inhibitors). Lysates were passed through a 25-gauge needle 10 times, incubated on ice for 30 minutes, and then clarified by centrifugation at 13,000 g for 15 minutes. One milligram of protein was incubated with 5–10 µg of antibodies overnight with rotation. Protein A/G agarose was then added and incubated with rotation for 3 hours at 4°C. Immunoprecipitates were washed 4 times in lysis buffer, and solubilized proteins were separated by SDS-polyacrylamide gels.

**Proliferation and cell death ELISA assay** BrdU proliferation assay was performed by incubating cells for 18 hours in BrdU, followed by ELISA detection using the cell proliferation Biotrak ELISA system (Amersham). Cell death was analyzed in quadruplicate using cell death detection ELISAPLUS kit (Roche), which detects cytoplasmic histone-associated DNA fragmentation.

**Retroviral DNA constructs** All constructs were cloned into the pMXs-IRES-Puro retroviral vector (Cell Biolabs, Inc., San Diego, CA). Vector only retrovirus were used as controls.
Retroviral transduction  Retroviral transduction of bone marrow macrophages was done as previously described (Zhao et al., 2008). 10 ug of retroviral DNA was transfected into Plat-E cells using the Fugene HD transfection reagent (Roche, Basel, Switzerland) the same day as bone marrow isolation. Plat-E media was replaced 24 hours later and virus harvested 48 and 72 hours after for transduction of plated bone marrow cells. To transduce the bone marrow cells, culture media was replaced at 48 and 72 hours with alpha10 containing 50% Plat-E viral media, 10% CMG 14-12 supernatant, and 4ug/mL polybrene. At 96 hours the transduction media was replaced with alpha10 with 10% CMG 14-12 supernatant and 2 ug/mL puromycin. Cells were selected for 3 days before being harvested and plated to form osteoclasts, at which time non-transduced control cells were killed by the puromycin.

Osteoclastogenesis  Osteoclasts were grown on plastic in 48-well plates for 3-5 days and fixed with 4% PFA, as described above. TRAP staining on fixed cells was performed using a commercially available kit according to the manufacturer's instructions (Sigma, St. Louis, MO). All TRAP+ cells with at least three nuclei were counted, with 2-3 wells counted per genotype.

Calvaria osteoblast isolation and culture  Whole calvaria were extracted from 3-4 day old pups and cleaned of soft and periostial tissue. Osteoblasts were liberated by 3x 20 minute collagenase treatment at 37 degrees, shaking. Osteoblasts were transduced with appropriate virally expressed proteins when 50% confluent, selected with the appropriate antibiotic for 4 days, then plated for experiments. For cocultures, BMMs were cultured for 4 days prior to being lifted and then cultured with osteoblasts in α-MEM media
containing pen/strep, 10% FBS and 10nM VitD3, plus IL-17 or other cytokines where indicated.

**Immunoblots**  Cells were lysed in RIPA buffer + protease inhibitors for 10 minutes on ice, the lysates were clarified, 5x Laemmlli sample buffer added, and the samples boiled. Immunoblotting was performed using antibodies against β-actin (Sigma-Aldrich, St. Louis MO) (1:40,000 dilution), c-Src (monoclonal antibody directed against c-Src was a gift from A. Shaw) (final concentration: 2ug/mL), β3 integrin, JNK, p-JNK, p38, p-p38, Akt, p-Akt, ERK, p-ERK, IκB, p-IκB (Cell Signaling Technology); monoclonal anti-HA (Covance); cathepsin K (Millipore, Billerica, MA) (1:500 dilution), and Alexa Fluor 488–conjugated phalloidin (Invitrogen). Quantification of immunoblot results was performed using a Storm 840 phosphorimager (Molecular Dynamics, Piscataway, NJ) and ImageQuant software (GE, Piscataway, NJ).

**Microcomputed tomography and ovariectomy**  Trabecular volume in the distal femoral metaphysis (right leg) was measured using *in vivo* microcomputed tomography (vivaCT 40, Scanco Medical, Brüttisellen, Switzerland) while the mice were anesthetized with isofluorane. A threshold linear attenuation coefficient of 1.2 cm$^{-1}$ was used to differentiate bone from non-bone. A threshold of 220 was used for evaluation of all scans. 30 slices were analyzed, starting with the first slice in which condyles and primary spongiosa were no longer visible. Measurements were made 28 days after sham operation or ovariectomy (ovx), unless otherwise indicated. Measurements included bone volume/total volume (BV/TV), connectivity density (Conn. Dens), structure model index (SMI), trabecular separation (Tb.Sp), trabecular thickness (Tb.Th), and trabecular
number (Tb.N). For ovariectomies, all mice were aged 8 weeks at time of ovx. Mice were anesthetized with ketamine/xylene delivered by intraperitoneal injection, and ovaries were removed through two small dorsal incisions. Sham operated mice were anesthetized and opened equivalently, but ovaries were not removed.

**Electron microscopy**  Proximal tibiae of 2-month-old mice were fixed with 4% PFA overnight and decalcified in 14% EDTA for 7 days. The samples were then postfixed in 2% OsO4/3% K-ferrocyanide for 2 hr, dehydrated in ethanol, and embedded in Epon LX 112. Ultrathin sections were stained briefly with lead citrate and examined using a JEOL 100SX transmission electron microscope.

**Serum Ctx measurements**  Blood was collected by cheek puncture after 6 hours starvation. Plasma was obtained using plasma separator tubes with lithium heparin (Becton Dickinson). Serum CTx-I, a specific marker of osteoclastic bone resorption, was measured using a RatLaps ELISA kit from Nordic Bioscience Diagnostics A/S.

**Statistics**  All data was analyzed with Prism software (Graphpad, San Diego, CA), using two-tailed unpaired Student’s t tests, unless otherwise indicated. Error bars represent standard deviation.
References


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Chapter 4: Halofuginone Prevents Osteoporosis in Mice

Abstract

Osteoporosis is characterized by low bone mass, leading to fracture in an estimated one of two women, and one of five men over 50 (Cooper, Gass & Dawson-Hughes, 2006). Bone loss develops when activity of the bone-resorbing osteoclast outweighs that of the bone-forming osteoblast. Although the osteoblast creates new bone, it also produces RANKL, the most potent osteoclast activator. RANKL production is stimulated by IL-17, leading to bone loss in inflammatory arthritis (Miossec, 2003), and contributing to bone loss in estrogen deficiency (other paper). IL-17 is primarily produced by Th17 T cells, which can be effectively inhibited by the small molecule Halofuginone (Sundrud et al., 2009). We find Halofuginone treatment protects mice from estrogen-deficiency bone loss, a model of the most prevalent form of osteoporosis in humans. Halofuginone does not directly inhibit osteoclast formation, but rather reduces the number of IL-17 producing T cells. Less osteoblastic IL-17 stimulation results in less RANKL production, and lower serum markers of bone resorption. Thus, Halofuginone is a novel potential therapeutic for bone loss in the estrogen-deficient state.
Introduction

For centuries the roots of *Dichroa febrifuga* have been used in ancient Chinese herbal medicine to treat malarial fevers. The active ingredients were identified as febrifugine and its stereoisomere, isofebrifugine. Halofuginone (7-bromo-6-chloro-3-[3-(3-hydroxy-2-piperidinyl)-2-oxopropyl]-4(3H)-quinazolinone) is a febrifugine analogue used in commercial poultry production for coccidiosis infection. Its utility in treating fibrotic conditions, such as scleroderma, in humans has since been established. In 2009, it was reported in *Science* to also inhibit production of IL-17 (Sundrud et al., 2009).

The anti-sclerosis properties of Halofuginone are thought to arise from its ability to inhibit production of type I collagen by fibroblasts, which is the main constituent of fibrous tissue. Halofuginone inhibits collagen α1(I) mRNA and protein levels in a variety of cells, including mouse skin fibroblasts, avian growth plate chondrocytes, a transformed rat embryo cell line, vascular smooth muscle cells, bovine aortic endothelial cells, and rat liver stellate cells (Bruck et al., 2001; Choi, Callow, Sehgal, Brown, & Ryan, 1995; Granot, Halevy, Hurwitz, & Pines, 1993). Collagen type II or III were not inhibited in these studies (Choi et al., 1995; Granot et al., 1993). Halofuginone is shown to inhibit fibrosis accumulation in rat urethral stricture formation (Nagler et al., 2000), thioacetamide- and dimethylnitrosamine-induced rat cirrhosis (Bruck et al., 2001; Pines, Knopov, Genina, Lavelin, & Nagler, 1997), rat pulmonary fibrosis after bleomycin treatment (Nagler et al., 1996), and tight skin (Tsk)+ and cGvHD-afflicted mice (Levi-Schaffer, Nagler, Slavin, Knopov, & Pines, 1996; McGaha, Phelps, Spiera, & Bona, 2002; Pines et al., 2001). The drug was effective whether given orally, locally, or intraperitoneally. The mechanism by which Halofuginone decreases collagen type I is
unclear, but seems to require new protein synthesis, since cycloheximide or actinomycin D blocks the suppressive effect of halofuginone on collagen α1(I) mRNA expression (Halevy, Nagler, Levi-Schaffer, Genina, & Pines, 1996). Its antifibrotic effects may be due to inhibition of TGFβ1 signaling (McGaha et al., 2002), but at concentrations that inhibit IL-17 production, no TGFβ inhibition is seen (Sundrud et al., 2009). Rather, Halofuginone induces the amino acid starvation response, which through unknown mechanisms prevents IL-17 production and Th17 development, a process that cannot be rescued by forced RORγt expression (Sundrud et al., 2009). In most animal models of fibrosis, regardless of the tissue, halofuginone had a minimal effect on collagen content in the control, nonfibrotic animals, whereas it exhibited a profound inhibitory effect in the fibrotic organs. In culture, halofuginone was effective in reducing collagen synthesis by fibroblasts after they had been stimulated with a profibrotic agents, but had a very small effect on collagen synthesis in control cells (McGaha et al., 2002). Even in animal models of pre-existing fibrosis, Halofuginone treatment can reduce fibrotic levels to normal levels (Nagler et al., 1998).

Given the promise of Halofuginone in treatment of systemic sclerotic conditions, Phase I-III studies have been performed using the drug topically, as well as a Phase I study for oral administration. The oral study was double-blind and involved 26 healthy, male volunteers receiving between 0.07 to 2.5 mg/d with food. Single, oral doses of 0.07 and 0.5 mg Halofuginone were found to be safe and well tolerated, with no clinically significant adverse events. At 1.5 to 2.5 mg, Halofuginone was moderately tolerated, with incidence of nausea and vomiting associated with dose escalation. A daily dose of 1.5 mg Halofuginone was designated as the maximal tolerated dose. A later study found
that dividing the dose into several daily portions allowed greater intake without increasing gastrointestinal adverse events (Pines, Snyder, Yarkoni, & Nagler, 2003).

Since earlier studies in this thesis implicate IL-17 in the pathogenesis of osteoporosis, we tested whether Halofuginone administration post-ovariectomy has an effect on IL-17 production and bone mass, and find Halofuginone to be a novel potential therapeutic for treatment of osteoporosis.
**Results**

**Halofuginone Protects from Osteoporosis**

To mirror the most common form of osteoporosis in humans, we produced an estrogen-deprived state by ovariectomizing (ovx) mice. Beginning the day of ovx, mice were treated with vehicle or Halofuginone at a dose reported to inhibit IL-17 production (Sundrud et al., 2009). After two weeks, bone volume (BV) was significantly reduced in vehicle treated ovx mice relative to sham, but not in ovx mice treated with Halofuginone (Figure 4.1). Bone volume per total volume (BV/TV) was significantly lower in vehicle treated ovx mice compared with either sham or Halofuginone treated ovx mice. Flow cytometry analysis of spleen cells showed the percentage of CD4+ T-cells producing IL-17 was significantly reduced in Halofuginone treated mice after two weeks (Figure 4.2). Carboxy-terminal collagen crosslinks (CTx) are released from the bone upon resorption, and serum levels are used to measure levels of systemic bone resorption (Rosen et al., 2000). Two weeks after ovx, vehicle treated mice have significantly higher levels of serum CTx, while Halofuginone treated mice have a blunted, non-statistically significant, increase in the serum marker of bone resorption (Figure 4.3). By four weeks, BV/TV, BV, connectivity density, trabecular thickness, and trabecular number were all significantly reduced in ovx mice compared with sham, but not in Halofuginone treated ovx mice relative to sham (Figure 4.4). These data indicate Halofuginone treatment after ovx protects mice from bone loss associated with the estrogen-deprived state.

**Halofuginone does not directly affect osteoclast formation or bone formation**

To exclude the possibility that Halofuginone directly inhibits osteoclasts, we culutered bone marrow macrophages in RANKL and M-CSF plus varying concentrations
Figure 4.1: Halofuginine Protects from Ovx-Induced Bone Loss at Two Weeks

Ovariectomized mice were treated with vehicle or halofuginone for two weeks and distal femora were analyzed by micro-CT. BV = bone volume; TV = total volume; BV/TV = bone volume/total volume; SMI = Structure Model Index; Tb.N = trabecular number; Conn.Dens = connectivity density; Tb.Sp = trabecular space; Tb.Th = trabecular thickness.
Figure 4.2: Halofuginone Treatment Reduces Th17 Cells After Two Weeks

Mice were treated with Halofuginone (HL) or vehicle control daily for two weeks, beginning the day of ovariectomy. Splenic cells were harvested, stimulated with ionomycin, and analyzed by flow cytometry, gating on live cells.

Figure 4.3: Halofuginine Treatment Reduces Serum CTx

Ovariectomized mice were treated with vehicle or HL daily and after two weeks serum was collected and analyzed by ELISA for CTx. Ovx vehicle treated animals had significantly increased levels of CTx, while Ovx HL treated animals had moderately elevated, non-statistically significant changes relative to sham operated mice. n = 5 mice per group.
Figure 4.4: Halofuginine Protects from Ovx-Induced Bone Loss at Four Weeks

Ovariectomized mice were treated with vehicle or HL daily for four weeks and distal femora were analyzed by micro-CT. Two independent experiments were performed (one experiment shown), 5-7 mice in each group for each experiment.
of Halofuginone. While 10 ug/ml was toxic to macrophages in the culture, other concentrations had no effect on osteoclast formation (Figure 4.5).

Since Halofuginone inhibits type I collagen synthesis by fibroblasts and other cells, we tested whether bone formation rates are reduced in mice treated with the chemical. Calcein double labeling of mice treated for two weeks with Halofuginone or PBS control showed equivalent bone formation rates (Figure 4.6), indicating Halofuginone does not affect bone formation.

**Halofuginone treated mice have less body fat**

IL-17A inhibits adipocyte differentiation from human mesenchymal stem cells (Shin, Shin, & Noh, 2009). IL17R-/− mice are heavier and have higher leptin levels, which is a marker of adipocyte mass (Galic, Oakhill, & Steinberg, ; Goswami, Hernandez-Santos, Zuniga, & Gaffen, 2009). Leptin can stimulate IL-17 production (Liu
et al., 2008), and blood IL-17 levels are increased in obese women (Sumarac-Dumanovic et al., 2009). These data implicate the Th17 axis in adiposity, so we used magnetic resonance imaging (MRI) as a sensitive measure of body composition after 12 weeks of treatment with Halofuginone or vehicle control. We expected inhibition of IL-17 may lead to increased adiposity, but instead Halofuginone treated mice had significantly less total fat mass, in addition to reduced percent body fat (Figure 4.7). Additionally, Halofuginone treatment resulted in a small but statistically significant increase in percent lean mass (Figure 4.7).
Figure 4.6: Halofuginone does not Affect Bone Formation

Mice were treated daily with HL or vehicle control for 14 days. Calcein was injected on day 7 and 12, and Mineral Apposition Rates (MAR) and Bone Formation Rates (BFR) were determined.
Figure 4.7: Body Composition of Halofuginone-Treated Mice

Eight week old mice were treated with Halofuginone or PBS for 12 weeks, after which body content was measured using MRI. n=6 mice per group.
**Discussion**

Halofuginone is reported to inhibit IL-17 production *in vivo*, and since IL-17 is implicated in bone destruction in arthritis (Lubberts) and osteoporosis (previous chapter), we tested the effect of Halofuginone on bone loss in the mouse model of osteoporosis. We find treatment with Halofuginone prevents the majority of the bone loss induced by ovariectomy. Since Halofuginone inhibits collagen type I synthesis, we expected bone formation rates might be reduced in Halofuginone treated animals. However, no differences were observed in bone formation. One explanation for this is that Halofuginone inhibits collagen synthesis more in conditions where fibrosis is extravagant, and not at baseline (McGaha et al., 2002). Another explanation is that at the concentrations used in these experiments, IL-17 production is affected, but TGFβ signaling is not (Sundrud et al., 2009), and it is the effects on TGFβ that are thought to interfere with collagen synthesis (McGaha et al., 2002). Since IL17R-/- mice are protected from osteoporosis, Halofuginone likely works on bone by reducing IL-17 production, leading to less IL-17 signaling in osteoblasts, less RANKL production, and less osteoclastic bone resorption (as shown in the previous chapter). Additionally, Th17 cells express high levels of RANKL and may directly mediate bone destruction (Sato et al., 2006), suggesting that reduction of CD4+ IL-17+ T-cells using Halofuginone may have beneficial effects on the bone independent of the osteoblast.

It is interesting to note that BATF KO mice, which are reported to lack Th17 cells (Schraml et al., 2009), are not protected from ovx-induced bone loss, in contrast to mice treated with Halofuginone or IL-17 blocking antibody (previous chapter). This suggests Halofuginone does more than inhibit Th17 cell development, and may inhibit production
of IL-17 by other cell types as well. If it is not Th17 cells, it will be interesting to
determine the IL-17 producing cell type responsible for bone loss induced by estrogen
deficiency.
Methods

Mice  All animals were housed in the animal care unit of the Washington University School of Medicine and were maintained according to guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care. All animal experimentation was approved by the Animal Studies Committee of the Washington University School of Medicine.

Reagents  Halofuginone was purchased from Mingdou Chemical Company Ltd. (Shanghai, China).

Histological analysis  Tibias were harvested, cleaned of soft tissue, and fixed in 10% buffered formalin overnight. For non-decalcified sections, tibias were dehydrated by incubation in an ethanol gradient (20%, 30%, 50%, 70% ETOH, for 30 minutes each). Bones stored in 70% ETOH were then embedded in methylmethacrylate and cut into 5µm longitudinal sections. Non-decalcified sections were left unstained. For decalcified sections, following fixation, tibias were rinsed in water then incubated in 14% EDTA (pH 7.2) for 7 days with solution changes every 3.5 days. Following decalcification, tibias were rinsed in water then incubated in the ETOH gradient described above. Bones stored in 70% ETOH were then embedded in paraffin, cut into 4µm longitudinal sections, and stained with TRAP or hematoxylin and eosin for visualization of osteoclasts or osteoblasts, and measurements were made using BioQuant software (Bioquant; Nashville, TN).

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tibias were harvested after an additional 2 days for histologic analysis. Dynamic assessment of trabecular bone formation was determined on non-decalcified sections using BioQuant software (Bioquant; Nashville, TN). This software provided measures of bone surface, percent single- (sLS/BS) and double-labeled (dLS/BS) bone surface, mineralizing surface (MS/BS), mineral apposition rate (MAR), and bone formation rate (BFR/BS). Trabecular bone measurements were taken from a region encompassing a 500µm-long field, across the width of the bone (located 100µm below the growth plate). For each animal, 2 serial sections were analyzed and the measurements averaged. Pictures were obtained using an Olympus 1X51 fluorescent microscope fitted with Olympus DP70 camera (Olympus; Center Valley, PA).

Cell culture Osteoclasts were grown in alpha10 media, containing alpha-MEM (Sigma Aldrich, St. Louis, MO), 10% fetal calf serum (Hyclone, Waltham, MA), 100U/mL penicillin and 100µg/mL streptomycin. Plat-E retrovirus packaging cells were purchased from Cell Biolabs, Inc. (San Diego, CA) and maintained in DMEM media (Cellgro, Manassas, VA) containing 10% fetal calf serum (Hyclone, Waltham, MA), and 2mM L-glutamine (Gibco, Carlsbad, CA). Osteoclasts were differentiated from bone marrow as described (Zhao et al., 2008). Briefly, bone marrow was extracted from mice and cultured in the presence of 10% CMG 14-12 supernatant (Takeshita, Kaji, & Kudo, 2000), a M-CSF-containing cell supernatant. After 4 days, cells were lifted and replated on plastic or bovine bone fragments in alpha10 media supplemented with 2% CMG and 100ng/mL recombinant RANKL. Bone-grown cells were fixed in 4% paraformaldehyde 6 or 7 days after plating. Plastic-grown cells were fixed on days 3, 4, and 5 in 4%
paraformaldehyde/PBS for osteoclastogenesis analysis or lysed on various days for immunoblot analysis.

**RT-PCR** RNA was isolated using RNeasy kits (Qiagen); RLT lysis buffer was supplemented with β-mercaptoethanol (1%). Purified RNA was treated with DNase I (Invitrogen; Carlsbad, CA) prior to reverse transcription (RT). RT was performed using SuperScript III (Invitrogen). A negative control using RNA not subjected to reverse transcription was included in each assay. Quantitative PCR (qPCR) was performed using Applied Biosystems’s (Foster City, CA) Power SYBR green master mix and gene specific primers. The qPCR reaction was run on Applied Biosystem’s ABI Prism 7000. Transcript levels were normalized to cyclophilin.

**Flow Cytometry** Spleen cells were prepared by gently crushing the tissue and filtering through a 40-µm cell strainer (BD Falcon). Cells were harvested, centrifuged, and incubated in red blood cell (RBC) lysis buffer. In stimulation experiments, cells were incubated in 50 ng/ml PMA and 500 ng/ml ionomycin (Sigma-Aldrich) for 4 h at 37°C. Brefeldin A (Sigma-Aldrich) was added during the last 2 h of culture at 10 µg/ml. Cells were stained with LIVE/DEAD Fixable Dead Cell Stain Kits (Molecular Probes, Invitrogen) followed by the appropriate surface antibody (anti-CD4) for 20 min at 4°C. Stained cells were fixed in 2% paraformaldehyde for 20 min at RT before permeabilization with 0.05% saponin. Intracellular staining for the cells was conducted at 4°C for 30 min using Alexa Fluor 488 anti-mouse IL-17A (1:1000 dilution) (eBioscience). To determine background levels of cytokine staining, a set of cells was not stimulated, but was treated with brefeldin A and stained for intracellular cytokines, as
was done for experimental cells. Samples were gated on live cells using the above LIVE/DEAD cell stain. All samples were analyzed on a FACSCalibur or FACSCanto (BD) and data were analyzed with FlowJo software (TreeStar). A total of 30,000 events were collected per sample.

**Osteoclastogenesis** Osteoclasts were grown on plastic in 48-well plates for 3-5 days and fixed with 4% PFA, as described above. TRAP staining on fixed cells was performed using a commercially available kit according to the manufacturer's instructions (Sigma, St. Louis, MO). All TRAP+ cells with at least three nuclei were counted, with 2-3 wells counted per genotype.

**Calvaria osteoblast isolation and culture** Whole calvaria were extracted from 3-4 day old pups and cleaned of soft and periostial tissue. Osteoblasts were liberated by 3x 20 minute collagenase treatment at 37 degrees, shaking. Osteoblasts were transduced with appropriate virally expressed proteins when 50% confluent, selected with the appropriate antibiotic for 4 days, then plated for experiments. For cocultures, BMMs were cultured for 4 days prior to being lifted and then cultured with osteoblasts in α-MEM media containing pen/strep, 10% FBS and 10nM VitD3, plus IL-17 or other cytokines where indicated.

**Microcomputed tomography and ovariectomy** Trabecular volume in the distal femoral metaphysis (right leg) was measured using *in vivo* microcomputed tomography (vivaCT 40, Scanco Medical, Brüttisellen, Switzerland) while the mice were anesthetized with isofluorane. A threshold linear attenuation coefficient of 1.2 cm$^{-1}$ was used to differentiate bone from non-bone. A threshold of 220 was used for evaluation of all
scans. 30 slices were analyzed, starting with the first slice in which condyles and primary spongiosa were no longer visible. Measurements were made 28 days after sham operation or ovariectomy (ovx), unless otherwise indicated. Measurements included bone volume/total volume (BV/TV), connectivity density (Conn. Dens), structure model index (SMI), trabecular separation (Tb.Sp), trabecular thickness (Tb.Th), and trabecular number (Tb.N). For ovariectomies, all mice were aged 8 weeks at time of ovx. Mice were anesthetized with ketamine/xylene delivered by intraperitoneal injection, and ovaries were removed through two small dorsal incisions. Sham operated mice were anesthetized and opened equivalently, but ovaries were not removed.

**MRI** Measurements of body composition were performed using the magnetic resonance imaging facility located at the Animal Model Research Core at Washington University (CNRU grant NIH P30 DK56341).

**Serum Ctx measurements** Blood was collected by cheek puncture after 6 hours starvation. Plasma was obtained using plasma separator tubes with lithium heparin (Becton Dickinson). Serum CTx-I, a specific marker of osteoclastic bone resorption, was measured using a RatLaps ELISA kit from Nordic Bioscience Diagnostics A/S.

**Statistics** All data was analyzed with Prism software (Graphpad, San Diego, CA), using two-tailed unpaired Student’s t tests, unless otherwise indicated. Error bars represent standard deviation.
References


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Chapter 5: Conclusion

The first chapter in this thesis establishes autophagy proteins as novel regulators of lysosome secretion in osteoclasts. We show that the autophagy protein LC3 localizes to the ruffled border in wild type bone grown osteoclasts, but not those lacking the autophagy protein ATG5. ATG5 deficient osteoclasts form more shallow bone pits, and also are defective in localizing Cathepsin K and Lamp1 to the ruffled border, suggesting a defect in secretion. Osteoclasts lacking Atg7 display a similar phenotype. Dominant negative Atg4b also prevents LC3 localization to the ruffled border, resulting in mislocalized Cathepsin K and Lamp1, and impaired bone resorption. The lysine on position 130 of Atg5 is critical for its function of both LC3 and CatK localization, as wild type Atg5 but not K130R-Atg5 successfully restores the osteoclast phenotype.

Consistent with a role in secretion, ruffled border formation is attenuated in osteoclasts lacking Atg5. Although the precise role of LC3 at the ruffled border is unclear, it is likely not localized here by autophagosomes, as immunoEM against GFP-LC3 reveals the protein is concentrated near the bone-apposed plasma membrane, not on autophagosomes, and is directly associated with ruffled border plasma membrane in actively resorbing osteoclasts. These data may help explain GWAS data implicating the autophagy cascade in risk for osteoporosis and height variability.

Again focusing on mechanisms of bone loss, Chapter 3 implicates a novel pathway in the pathogenesis of osteoporosis. Although osteoporosis is not traditionally thought of as an inflammatory disease, a critical role for T cells has been established by Dr. Pacifici’s group. The specific type of T cell involved has not been investigated, but
Th17 cells are an obvious candidates given their strong effects on bone resorption during arthritis, in contrast to the anti-osteoclastic effects of the Th1 cytokine IFNγ. Indeed, we find IL17R−/− mice are protected from ovariectomy-induced bone loss. Furthermore, IL-17 neutralizing antibody injected post ovariectomy protects them from bone loss, as does a drug recently reported to inhibit IL-17 production (Halofuginone).

IL-17 does not directly stimulate osteoclasts or their precursors, but rather acts on the osteoblast to increase RANKL production. Neutralizing IL-17 antibody abolishes the osteoclastogenic effect of IL-17 in coculture. Structure/function analysis of the IL-17 receptor indicates the SEFIR domain is critical for RANKL upregulation. This domain likely functions by interacting with the scaffolding protein Act1, and Act1−/− mice are also protected from bone loss due to ovariectomy. Although Act1 is a ubiquitin ligase for TRAF6, we find Act1 does not have a direct role in osteoclast formation, but rather is critical in the osteoblast for IL-17 induced RANKL expression.

These experiments provide evidence for a non-canonical role of the autophagy cascade in lysosome secretion. Additionally, this thesis identifies autophagy proteins and the IL-17 pathway as critical regulators of osteoclast function and bone resorption. Finally, a novel therapeutic for osteoporosis is described, which we have submitted for patent.
Chapter 6: Future Directions

Autophagy in the Osteoblast

Background

The autophagy pathway is linked to bone density and height variation in human GWAS analysis (Pan et al.,; Zhang et al.), and this thesis demonstrates the importance of autophagy on osteoclast secretion. However, autophagy is most critical in long-lived cells, such as neurons, which must not only survive varying nutritional states but must also dispose of unwanted proteins and damaged organelles efficiently, as cell death and regeneration is not an option. Osteoclasts are relatively short lived cells, which may be why autophagy is not used for osteoclast survival. Osteoblasts, however, survive for long periods, then become osteocytes which live for decades in the bone. Therefore, autophagy’s role in survival of osteoblasts and osteocytes may be critical. Furthermore, osteoblasts have a similar secretory apparatus as osteoclasts (Zhao et al., 2008). They synthesize and secrete type I collagen, non-collagen proteins, and growth factors, creating the bone matrix (osteoid) (Harada & Rodan, 2003). Another round of secretion occurs as osteoblasts secrete matrix vesicles, which are membrane-bound extracellular structures that mediate mineralization of the osteoid (Anderson, 1995). Therefore, we hypothesize autophagy is important for osteoblast survival or function.

Experimental Design

We will breed the collagen I-cre (Coll-cre) mouse (from Fanxin Long), which expresses cre recombinase in osteoblasts, to Atg5-flox/flox and Atg7-flox/flox mice (from Skip Virgin). The bone phenotype will be assessed by in vivo uCT at ages of 8
weeks, and older if a phenotype is observed. Tetracycline double label will be performed to assess bone formation rates in vivo. Osteoid and osteoblast number will be determined histologically. Cre+ and cre- osteoblasts will be isolated from calvariae, cultured, and assayed for proliferation rate (BrdU incorporation), bone nodule formation, apoptosis, and differentiation. Differentiation will be assessed by staining cells for alkaline phosphotase (ALP), and by analyzing RNA levels of ALP and osteocalcin at various days of differentiation (0, 3, 7, 14, 21). Bone nodules will be assessed by alizarin red staining. Results of these experiments will dictate further direction, such as pursuing the effect of autophagy on the osteocyte.
**TI-Vamp and Secretion in the Osteoclast**

**Background**

Osteoclasts secrete lysosomes and endosome-derived organelles to resorb bone. Thus far, all that is known of vesicle exocytosis in osteoclasts is that Synaptotagmin-VII is necessary (Zhao et al., 2008), acting as a SNARE-regulating calcium sensor. The direct involvement of specific SNARE proteins has not been shown. TI-VAMP (VAMP7) is a v-SNARE involved in lysosome-late endosome fusion (Pryor et al., 2004), lysosome-plasma membrane fusion (Logan et al., 2006; Mollinedo et al., 2006), lysosome-autophagosome fusion (Fader, Sanchez, Mestre, & Colombo, 2009), and granule exocytosis (Logan et al., 2006; Mollinedo et al., 2006) in various cell types, and is thus a prime candidate for mediating lysosome exocytosis in the osteoclast.

**Experimental Design**

No TI-Vamp knockout or floxed mice currently exist. Thus, siRNA knockdown or overexpression of the protein are the best available techniques. If siRNA data demonstrates a strong phenotype, it may be grounds to create a knockout and/or floxed TI-VAMP mouse. Important siRNA-based assays will be CTx of media surrounding bone grown osteoclasts, bone pit formation (morphology and depth), visualization of CatK by confocal microscopy to observe for mislocalization and accumulation, and perhaps electron microscopy of bone grown osteoclasts to assess ruffled border formation. Significant defects in these assays with least two different siRNA constructs should be demonstrated before a knockout of floxed mouse is made.
Plekhm1 in Autophagy and Bone Resorption

Background

In the search for novel regulators of autophagy and bone resorption, Plekhm1 (pleckstrin homology domain containing, family member 1) is an important candidate. Plekhm1 is a homologue of Rubicon, a newly discovered negative regulator of autophagy. Rubicon localizes to the endosome/lysosome, and binds Vps34 and UVRAG (Zhong et al., 2009). Knockdown of Rubicon promotes autophagy (Matsunaga et al., 2009), while forced expression of Rubicon caused aberrant late endosomal/lysosomal structures and impaired autophagosome maturation (Zhong et al., 2009).

Plekhm1 was identified as a spontaneous mouse mutation that caused osteopetrosis. Mutant osteoclasts lack ruffled borders and lack extracellular TRAP, but accumulate the protein intracellularly, suggesting a defect in secretion (Reinholt et al., 1999). Like Rubicon, Plekhm1 localizes to lysosomes and late endosomes (Van Wesenbeeck et al., 2007). Plekhm1 mutation is associated with osteopetrosis in humans (Van Wesenbeeck et al., 2007).

Although thus far unpublished, Rubicon and Plekhm1 are fairly homologous. A BLAST search for Plekhm1 returns Rubicon, and a Clustal W multiple sequence alignment reveals similarity between the two proteins (Figure 6.1). While Plekhm1 is highly expressed in mouse osteoclasts (Figure 6.2), Rubicon is not (Figure 6.3). It is possible Plekhm1 and Rubicon have similar functions, with Plekhm1 being dominantly expressed in osteoclasts, and Rubicon in other cell types.

If Plekhm1 and Rubicon have similar functions, one would expect Plekhm1 to negatively regulate autophagosome maturation. It is already known Plekhm1 positively
Key:
Red letters = small and hydrophobic, Blue = acidic, Magenta = basic, Green = basic with a hydroxyl and amine. Below the alignment:
*** means the residues or nucleotides in that column are identical in all sequences in the alignment.
.: means conserved substitutions have been observed.
. means semi-conserved substitutions are observed.

Figure 6.1: Sequence alignment of Plekhm1 and Rubicon

Human Plekhm1 (sequence obtained from Pubmed: NM_014798.2) and Human Rubicon (sequence obtained from Pubmed: KIAA0226) mRNA sequences were aligned using Clustal W2 alignment tool (available at http://www.ebi.ac.uk/Tools/clustalw2/index.html).
Figure 6.2: Plekhm1 mRNA is expressed at high levels in macrophage lineage cells, including osteoclasts.

The first vertical line represents the median gene expression level across all tissues. Bone cells are represented in light green. Osteoclasts express high levels of Plekhm1 compared to osteoblasts and other non-macrophage cells.

Source: biogps.gnf.org
Figure 6.3: Rubicon mRNA is expressed at high levels in macrophage lineage cells, including osteoclasts

Bone cells are represented in light green. Osteoclasts express low levels of Rubicon. Source: biogps.gnf.org/, search term: KIAA0226
regulates secretion in the osteoclast. Thus, my hypothesis is that 1) Plekhm1 negatively regulates traditional autophagy, and 2) Plekhm1 acts as the molecular switch that channels autophagy related proteins to perform secretory duties rather than autophagosome formation.

**Experimental Design**

The first task will be to determine whether Plekhm1 regulates traditional autophagy. Based on personal observation, autophagosomes are relatively lacking in osteoclasts, so one approach would be to knockdown Plekhm1 in the osteoclast using retroviral expression of shRNA and monitor autophagosome formation. LC3-positive puncta could be measured by immunofluorescence, but the best method would be to identify and count autophagosomes by electron microscopy (the cells could be grown on plastic rather than bone, making the process easier). Other cell types could also be used, and in cells that have higher basal autophagosome formation, overexpression of Plekhm1 may inhibit autophagosome formation. LC3 conjugation should not be used in these studies, because if Plekhm1 diverts autophagy machinery for secretory function, LC3 conjugation will likely be intact.

In Plekhm1 knockdown cells, it would be interesting to see if less LC3 localizes to the ruffled border of bone grown osteoclasts, and vice versa in Plekhm1 overexpression. One would have to infect GFP-LC3 transgenic osteoclasts with the shRNA or expression vector, then stain for GFP and actin, as described in Chapter 2.

Since Plekhm1 has been knocked out by the International Knockout Mouse Consortium, it would be interesting to characterize the phenotype, specifically looking at
autophagy. One would expect higher autophagy flux in cells of this mouse, although cell
to cell variability may be observed. Based on expression data, the best cells to
characterize may be macrophages and osteoclasts. An osteoclast phenotype is already
known, but given that Plekhm1 is even more highly expressed in macrophages, and the
autophagy pathway seems to be important for many functions in these cells, it would be
particularly interesting to study macrophages in these knockout mice.

If Plekhm1 is identified as a regulator of autophagy, it would be the first molecular
switch to date that determines whether autophagy machinery are used for autophagosome
formation or for other uses. Thus, it would be necessary to determine how the protein
itself is regulated, and perform a structure/function analysis.

**Conclusion**

Although this thesis describes autophagy proteins as regulators of osteoclast
function, they may have additional roles in the osteoblast. The autophagy cascade may
have a similar function in both cell types, as in the case of Synaptotagmin VII, or may
simply function in osteoblast survival. Given GWAS data implicating the autophagy
cascade in bone density, it would be informative to characterize these proteins in both of
the cell types that affect bone turnover (the osteoclast and the osteoblast).

TI-VAMP is a SNARE protein important for autophagosome-lysosome fusion, as
well as secretion of lysosomes in a variety of cell types. From the autophagy viewpoint,
it is the best candidate for regulating secretion of lysosomes via autophagy-cascade
proteins; from the general lysosome secretion viewpoint, it is the best candidate SNARE
protein for mediating lysosome-plasma membrane fusion in the osteoclast. Thus, pursuit
of the role of this protein in osteoclast function seems high-yield.
Finally, Plekhm1, being homologous to a negative regulator of autophagosome formation (Rubicon) and being critical for osteoclastic secretion, is positioned as a potential novel regulator of autophagy, especially in cells like the osteoclast that lack high levels of Rubicon. Plekhm1 may serve as a molecular switch, directing autophagy machinery from traditional functions, including autophagosome formation, toward non-canonical roles, such as secretion.
References


