Osteoclastogenesis: Roles of Filamin A and SBDS, and their Regulation of Rho GTPases during Pre-Osteoclast Migration

by

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Abstract

Osteoclasts are multinucleated, bone resorbing cells that carry out their function using specialized actin-based structures called actin rings and podosomes. Rho GTPases function as molecular switches that regulate the actin cytoskeleton in osteoclasts and many other cell types. Filamin A (FLNa) and SBDS are two proteins that have the potential to interact with both F-actin and Rho GTPases, and thus regulate osteoclast formation, differentiation, or function. We found that in FLNa-null pre-osteoclasts, activation of RhoA, Rac1, and Cdc42 was perturbed, leading to defective pre-osteoclast migration prior to fusion. Ablation of SBDS resulted in the blockage of osteoclast differentiation downstream of RANK and defective RANKL-mediated upregulation of Rac2 that is required for pre-osteoclast migration. Therefore, both FLNa and SBDS are required to coordinate Rho GTPase activation during osteoclastogenesis, in addition to a role for SBDS in osteoclast differentiation downstream of RANK.
Acknowledgements

I would like to take this opportunity to thank my family and friends for supporting me through this long journey. I am indebted to my supervisor and my lab colleagues for guiding me in my research.
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<td>α-MEM</td>
<td>α-minimal essential medium</td>
</tr>
<tr>
<td>ABD</td>
<td>Actin binding domain</td>
</tr>
<tr>
<td>ADAM</td>
<td>A disintegrin and metalloprotease</td>
</tr>
<tr>
<td>Akt</td>
<td>Thymoma viral proto-oncogene</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein-1</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>Bcl</td>
<td>B-cell leukemia/lymphoma</td>
</tr>
<tr>
<td>BMC</td>
<td>Bone mineral content</td>
</tr>
<tr>
<td>BMD</td>
<td>Bone mineral density</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>CA</td>
<td>Constitutively-active</td>
</tr>
<tr>
<td>CaM kinase II</td>
<td>Ca²⁺/calmodulin-dependent protein kinase II</td>
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<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DAP12</td>
<td>DNAX-activating protein 12</td>
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<tr>
<td>DAPI</td>
<td>4',6'-diamidino-2-phenylinodole</td>
</tr>
<tr>
<td>DC-STAMP</td>
<td>Dendritic cell-specific transmembrane protein</td>
</tr>
<tr>
<td>DN</td>
<td>Dominant-negative</td>
</tr>
<tr>
<td>DRF</td>
<td>Diaphanous-related formin</td>
</tr>
<tr>
<td>DXA</td>
<td>Dual-energy x-ray absorptiometry</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>F-actin</td>
<td>Filamentous actin</td>
</tr>
<tr>
<td>FBE</td>
<td>Free-barbed-end</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FcRγ</td>
<td>Fc receptor common γ subunit</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FLN</td>
<td>Filamin</td>
</tr>
<tr>
<td>fMLP</td>
<td>Formyl-methionyl-leucyl-phenylalanine</td>
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<tr>
<td>FOV</td>
<td>Field of view</td>
</tr>
<tr>
<td>FPP</td>
<td>Farnesyl diphosphate</td>
</tr>
<tr>
<td>FSD</td>
<td>Functional secretory domain</td>
</tr>
<tr>
<td>G-actin</td>
<td>Globular actin</td>
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<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
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<tr>
<td>GDI</td>
<td>Guanine nucleotide dissociation inhibitor</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
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<tr>
<td>GGPP</td>
<td>Geranylgeranyl diphosphate</td>
</tr>
<tr>
<td>GGTase</td>
<td>Geranylgeranyl transferase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony stimulating factor</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse radish peroxidase</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IP₃</td>
<td>Inositol-1,4,5-trisphosphate</td>
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<td>IPTG</td>
<td>Isopropyl-beta-D-1-thiogalactopyranoside</td>
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<tr>
<td>ITAM</td>
<td>Immunoreceptor tyrosine-based activation motif</td>
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<td>c-jun N-terminal kinase</td>
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<tr>
<td>LIMK</td>
<td>LIM kinase</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage colony stimulating factor</td>
</tr>
<tr>
<td>MFR</td>
<td>Macrophage fusion receptor</td>
</tr>
<tr>
<td>MITF</td>
<td>Micro-ophthalmia-associated transcription factor</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>N-BP</td>
<td>Nitrogen-containing bisphosphonate</td>
</tr>
<tr>
<td>NFATc1</td>
<td>Nuclear factor of activated T cell, cytoplasmic 1</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear Factor kappa B</td>
</tr>
<tr>
<td>OCG</td>
<td>Osteoclastogenesis</td>
</tr>
<tr>
<td>ODF</td>
<td>Osteoclast differentiation factor</td>
</tr>
<tr>
<td>OG</td>
<td>Octyl-glucoside</td>
</tr>
<tr>
<td>OPG</td>
<td>Osteoprotegerin</td>
</tr>
<tr>
<td>OPGL</td>
<td>Osteoprotegerin ligand</td>
</tr>
<tr>
<td>OSCAR</td>
<td>Osteoclast-associated receptor</td>
</tr>
<tr>
<td>PAK</td>
<td>p21-activated kinase</td>
</tr>
<tr>
<td>PAMPs</td>
<td>Pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
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<tr>
<td>PI(3,4,5)P₃</td>
<td>Phosphatidylinositol 3,4,5 trisphosphate</td>
</tr>
<tr>
<td>PI(4,5)P₂</td>
<td>Phosphatidylinositol 4,5 bisphosphate</td>
</tr>
<tr>
<td>PI3-K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PI4P-5K</td>
<td>Phosphatidylinositol 4-phosphate 5-kinase</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>PTH</td>
<td>Parathyroid hormone</td>
</tr>
<tr>
<td>PVNH</td>
<td>Periventricular nodular heterotopia</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative real-time polymerase chain reaction</td>
</tr>
<tr>
<td>RANK</td>
<td>Receptor Activator of Nuclear Factor kappa B</td>
</tr>
<tr>
<td>RANKL</td>
<td>Receptor Activator of Nuclear Factor kappa B ligand</td>
</tr>
<tr>
<td>RGD</td>
<td>Arg-Gly-Asp</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho kinase</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SBDS</td>
<td>Shwachman-Bodian-Diamond syndrome</td>
</tr>
<tr>
<td>SDF-1</td>
<td>Stromal cell-derived factor-1</td>
</tr>
<tr>
<td>SDS</td>
<td>Shwachman-Diamond syndrome</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TRACP</td>
<td>Tartrate-resistant acid phosphatase</td>
</tr>
<tr>
<td>TRANCE</td>
<td>Tumor necrosis factor-related activation-induced cytokine</td>
</tr>
<tr>
<td>WASP</td>
<td>Wiskott-Aldrich syndrome protein</td>
</tr>
<tr>
<td>WIP</td>
<td>WASP interacting protein</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
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</table>
Chapter 1: Literature Review
I. Osteoclasts and normal bone physiology

Osteoclasts are multinucleated, terminally differentiated cells of hematopoietic origin. Their primary role is to resorb bone; this function is required for physiological bone turnover. Together with bone-forming osteoblasts, and osteocytes embedded in bone, these three cell types regulate bone homeostasis, ensuring that “old” bone is remodeled and replaced by mechanically-sound new bone. Osteoclasts function secondarily to control calcium flux into and from the extracellular fluids, as bone is the major reservoir of calcium in the body. The mammalian osteoclast contains on average 8 nuclei, and *in vitro* diameters can reach ~300 µm. To resorb bone, a mature osteoclast enters into a resorption cycle. In this sequence of events, osteoclasts initially migrate to the resorption site. Once the osteoclast attaches to the bone surface, a new membrane domain is formed which results in the development of a sealed-off, membrane-bound compartment that contacts the bone matrix. This compartment permits the secretion of enzymes and hydrochloric acid into the enclosed space, resulting in the dissolution of hydroxyapatite, and degradation of the organic matrix of the bone. A number of diseases, including postmenopausal osteoporosis, rheumatoid arthritis, periodontitis, Paget’s disease, and bone tumors are associated with excessive osteoclast-mediated bone resorption, resulting in pathological loss of bone mass.

A large-scale global effort is currently devoted to the development of osteoclast inhibitors in order to prevent excessive osteoclast resorption activity.

a. Origins

Early experiments by Walker demonstrated that normal bone resorption in mice with inherited osteopetrosis could be resumed by an intravenous bone marrow transplant from normal littermates, showing that osteoclast precursors come from a hematopoietic origin. The same concept of bone marrow-sourced osteoclast progenitors was soon demonstrated in humans when
an infant with otherwise fatal osteopetrosis was cured after a bone marrow transplant from her HLA-MLC-matched brother.\textsuperscript{6} The specific hematopoietic stem cell population responsible for the generation of osteoclast precursors was determined by Udagawa \textit{et al.} to be a member of the monocyte/macrophage family, using a co-culture system of mouse marrow-derived stromal cell line (ST2) and bone marrow mononuclear cells.\textsuperscript{7}

\textbf{b. Osteoclastogenesis}

Osteoclasts are derived from the fusion of progenitors from the monocyte-macrophage lineage.\textsuperscript{2} \textit{In vivo}, osteoclastogenesis (OCG) is supported by direct cell-to-cell contact between Receptor Activator of Nuclear Factor kappa B ligand (RANKL) expressed on the surface of osteoblasts and bone marrow stromal cells, and RANK, its receptor on the surface of osteoclast precursors.\textsuperscript{2} The other critical ligand for OCG is macrophage colony stimulating factor (M-CSF). M-CSF is also produced by bone marrow stromal cells, and has additional roles in proliferation of precursor cells and survival for mature osteoclasts.\textsuperscript{8} Both RANKL and M-CSF are essential for OCG: mice with ablated genes for RANKL or its receptor, RANK, are completely devoid of osteoclasts resulting in severe osteopetrosis.\textsuperscript{9} Mice homozygous for the recessive mutation osteopetrosis (op/op) exhibit severe deficiencies in mature macrophages and osteoclasts which are attributed to a mutation in the M-CSF gene.\textsuperscript{10} Further, the addition of RANKL and M-CSF to cells of the mononuclear phagocyte lineage \textit{in vitro} is sufficient for the generation of mature functional osteoclasts.\textsuperscript{11} An additional molecule that negatively regulates OCG is osteoprotegerin (OPG), which is produced by osteoblasts and secreted in a soluble form which acts as a decoy receptor for RANKL, thereby moderating net RANKL activity. This was first shown by Simonet \textit{et al.} who showed that transgenic mice over-expressing OPG exhibited non-lethal osteopetrosis.
and concurrent defects in osteoclast differentiation. Thus, modulation of osteoclastogenesis in vivo is determined by the ratio of RANKL and OPG.

c. *In vitro* generation of osteoclasts

Osteoclasts can be differentiated in vitro using two methods: 1) stimulating enriched hematopoietic cells (e.g. bone marrow cells, spleen cells, M-CSF-stimulated bone marrow-derived macrophages) with a combination of RANKL and M-CSF, or 2) co-culturing these same cells with cells that produce these stimulating factors, i.e. osteoblasts or activated T-cells. Osteoclast formation occurs in 2 overall stages: during the first three days in culture, there is M-CSF-dependent proliferation of monocyte/macrophage cells, with expression of tartrate-resistant acid phosphatase (TRACP) toward the end of this period; from day 3 onward, M-CSF-dependent proliferation slows down, and TRACP-positive mononuclear cells begin to fuse into polykaryons, with the expression of more specific osteoclast markers, e.g. cathepsin K and calcitonin receptors.

d. M-CSF and RANKL

M-CSF is a homodimeric cytokine of the colony-stimulating factor family, which stimulates proliferation and subsequent differentiation of cells of the macrophage/osteoclast lineage, and migration of mature osteoclasts. Expression of the receptor for M-CSF (c-Fms), a member of the receptor tyrosine kinase family, is a required feature of osteoclast precursors. RANKL, also named osteoclast differentiation factor (ODF), osteoprotegerin ligand (OPGL), or TRANCE, is a member of the tumor necrosis factor (TNF) receptor-ligand family and is a long-sought after factor that stimulates osteoclast development. RANKL is a homotrimer, however unlike other TNF family members it has four unique surface loops as distinguishing features.
The addition of M-CSF to osteoclast precursors induces the expression of RANK, which together with cell adherence allows differentiation to proceed.² Some of the features of intracellular signaling by c-Fms and RANK have been elucidated:²¹ upon M-CSF occupation c-Fms oligomerizes and autoprophosphorylates at seven tyrosine residues, thereby activating c-Src, extracellular signal-regulated kinase (ERK1/2) and thymoma viral proto-oncogene 1 (called Akt) via phosphatidylinositol 3-kinase (PI3-K). Tyr⁵⁹⁹ is an important residue which is responsible for binding c-Src, while Tyr⁷²¹ binds PI3-K.¹⁹ PI3-K and c-Src are recruited to c-Fms via SH2 interactions, and c-Src in turn activates PI3-K via SH3 interactions, which leads to phosphatidylinositol 3,4,5 trisphosphate (PIP₃) binding on Vav3, a guanine nucleotide exchange factor (GEF) which activates Rho small GTPases.²⁰ Vav3 and Rac are downstream of c-Fms, and upon M-CSF binding, induce cytoskeletal remodeling and spreading in osteoclasts that is prevented by the PI3-K inhibitor Wortmannin.²⁰ More recently, the c-Fms-Tyr⁵⁹⁹/c-Src complex has also been shown to be indispensable for signaling via the immunoreceptor tyrosine-based activation motif (ITAM)-containing DAP12, which becomes phosphorylated, and subsequently recruits the protein tyrosine kinase Syk via its SH2 domain and activates it via autophosphorylation, leading to downstream effects in cytoskeletal reorganization.²¹ The downstream effects include the proliferation of osteoclast precursors, generating survival signals, and activation of Rac which begins the reorganization of the actin cytoskeleton.²⁰ Survival signals downstream of c-Fms are mediated by the transcription factor micro-ophthalmia-associated transcription factor (MITF), which ultimately regulates the transcription of the anti-apoptotic factor B-cell leukemia/lymphoma 2 (Bcl-2).²²

RANKL-RANK interaction recruits TRAF6, a plasma membrane adaptor protein that coordinates the downstream activation of the transcription factors Nuclear Factor kappa B
(NFκB), and activator protein-1 (AP-1) complex via one of its components c-Fos, and the MAP kinases, including c-jun N-terminal kinase (JNK) and p38.\textsuperscript{18} c-Fos and NFκB are critical transcription factors for osteoclastogenesis as mice with mutations in c-Fos or NFκB (p50/p52) are osteopetrotic as a direct result of defective osteoclast differentiation that can be reversed by marrow transplantation.\textsuperscript{23,24} AP-1 is a dimeric complex composed of different Fos, Jun, and ATF family members, and it has been shown that an AP-1 dimer with c-Fos and any Jun protein induces osteoclastogenesis,\textsuperscript{25} illustrating redundancies in the Jun family members.

Another signaling cascade downstream of RANK is the calcium/calcineurin pathway which activates a key osteoclastogenic transcription factor, nuclear factor of activated T cell, cytoplasmic 1 (NFATc1), which has been shown to be the transcription factor most highly upregulated by RANKL.\textsuperscript{26} Mice with NFATc1 mutation are osteopetrotic and exhibit reduced numbers and sizes of osteoclasts.\textsuperscript{27} Cathepsin K, the calcitonin receptor, the β3 integrin subunit, dendritic cell-specific transmembrane protein (DC-STAMP), and Atp6v0d2 (a subunit of the vacuolar ATPase), all of which are important to osteoclast formation or function, are under the control of NFATc1.\textsuperscript{28-31}

e. Other important transcription factors

The transcription factor PU.1, found exclusively on myeloid and B-cells, is required for early osteoclast and macrophage differentiation, as PU.1\textsuperscript{-/-} mice exhibit osteopetrosis and a complete lack of osteoclasts and this phenotype is fully reversed by bone marrow transplantation.\textsuperscript{32} Mutations in MITF results in osteopetrosis in the Mitf\textsuperscript{mi/mi} mouse as a result of defects in the gene encoding the helix-loop-helix-leucine zipper protein family of transcription factors,\textsuperscript{33} which is responsible for the transcription of genes required for osteoclast function, e.g. TRACP and carbonic anhydrase II.\textsuperscript{34}
f. Osteoimmunology

A number of cytokines, receptors, signaling pathways, and transcription factors play overlapping roles in both bone metabolism and the immune system. The crosstalk and interaction between these two systems have prompted the coining of the term “osteoimmunology”. Although under physiologic conditions RANKL is only produced by osteoblasts and bone marrow stromal cells, it can also be produced by activated T lymphocytes under inflammatory conditions, resulting in local bone resorption. Furthermore, under chronic systemic activation of T cells, e.g. in autoimmune diseases, viral infections, local inflammation within bone as a result of metastasis, infections, and fractures, or joint inflammation in arthritis, production of proinflammatory cytokines such as TNF-α and IL-1β further upregulates RANKL expression in osteoblasts and stromal cells. To counterbalance this, activated T cells also secrete interferon-γ (IFN-γ), which blocks RANKL-induced osteoclast differentiation by ubiquitination of TRAF6, and this mechanism is proposed to block uncontrolled bone destruction under inflammatory T-cell responses. Other inflammatory cytokines that exhibit synergism with RANKL in inducing osteoclastogenesis include IL-6, IL-11, and IL-15, while IL-4, IL-10, IL-12, IL-13, IL-18, GM-CSF, and the above-mentioned IFN-γ have inhibitory effects on resorption. Furthermore, B-cells can exert an effect on osteoclasts as B-cell deficient mice exhibit osteoporosis, owing to deficient OPG, as B cells are thought to be the major source of OPG in the bone marrow.

In addition to the adaptive immune system, osteoclasts also interact with the innate immune system. Toll-like receptors (TLRs) are pattern recognition receptors that form an ancient defense mechanism against invading pathogens by recognizing specific pathogen-associated molecular patterns (PAMPs). Activation of TLRs induces the synthesis and release of proinflammatory cytokines, thus influencing bone remodeling. Additionally, TLRs are found on
both osteoblasts and osteoclasts, potentially allowing pathogens to interact directly with these bone remodeling cells.  

**g. NFATc1 – the master regulator of osteoclastogenesis**

RANKL stimulation of osteoclast precursors results in the activation of NFκB and AP-1 in the early phase. During the later stage of osteoclastogenesis, the gene most highly induced by RANKL is NFATc1,\(^{26}\) which is now known to be the master regulator of osteoclast differentiation. There are five members in the NFAT family, however all but one is regulated by the serine/threonine phosphatase calcineurin that is activated by intracellular Ca\(^{2+}\).\(^{18}\) Calcineurin dephosphorylates the serine residues in NFATs which liberates their nuclear-localization signal that allows for their translocation into the nucleus.\(^{18}\) Immunosuppressants such as FK506 and cyclosporine A, which are inhibitors of calcineurin, effectively suppress osteoclastogenesis.\(^{18}\) Under RANKL stimulation, sustained Ca\(^{2+}\) oscillations are observed in osteoclast precursors, and when Ca\(^{2+}\) signals are suppressed by chelation, RANKL-mediated osteoclastogenesis is suppressed as a result of failure of NFATc1 activation.\(^{26}\) Although NFATc1-deficient mice are embryonically lethal as a result of defects in cardiac valve morphogenesis,\(^{40}\) NFATc1-deficient embryonic stem cells have been shown to be unable to differentiate into osteoclasts in response to RANKL and M-CSF,\(^{26}\) illustrating its crucial role in osteoclastogenesis. Since then, this has been demonstrated *in vivo* using a chimeric mouse approach to circumvent the NFATc1-deficient embryonic lethality.\(^{41}\)

**h. Intracellular Ca\(^{2+}\) signalling**

Intracellular Ca\(^{2+}\) signals are mediated via ITAMs present on Fc receptor common \(\gamma\) subunit (FcR\(\gamma\)) as well as DNAX-activating protein 12 (DAP12) adaptor proteins.\(^{18,42}\) FcR\(\gamma^{-/-}\) DAP12\(^{+/+}\) double-knockout mice exhibit severe osteopetrosis as a result of defective osteoclast
In osteoclast precursors, FcR\(\gamma\) and DAP12 associate with multiple immunoreceptors that supply a costimulatory signal for osteoclastogenesis and activate the Ca\(^{2+}\) signaling cascade via phospholipase C\(\gamma\) (PLC\(\gamma\)).\(^{43}\) FcR\(\gamma\)/DAP12\(^{-/-}\) cells exhibit defective Ca\(^{2+}\) oscillations and NFATc1 activation, suggesting that the ITAM signals provided by FcR\(\gamma\) and DAP12 are required for NFATc1 activation via Ca\(^{2+}\) signals. Upon stimulation, ITAMs are phosphorylated, which recruits Syk via SH2 domain,\(^{18,44}\) after which Syk becomes phosphorylated (possibly by non-receptor tyrosine kinases Btk and Tec).\(^{18}\) Phosphorylation of Syk is crucial downstream of ITAM during osteoclastogenesis as FcR\(\gamma\)/DAP12\(^{-/-}\) bone marrow cells fail to differentiate into osteoclasts due to a defect in Syk phosphorylation.\(^{44}\) Phosphorylated Syk activates PLC\(\gamma\) by phosphorylation,\(^{43}\) and subsequent cleavage of membrane phospholipid phosphatidylinositol-4,5-bisphosphate (PIP\(_2\)) to inositol-1,4,5-trisphosphate (IP\(_3\)) and diacylglycerol (DAG). IP\(_3\) induces Ca\(^{2+}\) release from endoplasmic reticulum stores followed by store-operated calcium entry of extracellular Ca\(^{2+}\). It is likely that only the PLC\(\gamma\)2 isoform mediates ITAM-stimulated Ca\(^{2+}\) signaling, as targeted deletion of PLC\(\gamma\)2 in mice produces an osteopetrotic phenotype as a result of the failure to upregulate NFATc1 downstream of FcR\(\gamma\)/DAP12 leading to defect in osteoclastogenesis.\(^{45}\)

i. Genes Regulated by NFATc1

Activation of NFATc1 is followed by its recruitment to the NFATc1 promoter and this association persists during the later stages of osteoclast differentiation, illustrating an autoamplification mechanism.\(^{18}\) An additional level of regulation to ensure specificity comes from the epigenetic activation of only the NFATc1 promoter via histone acetylation and methylation of histone H3 lysine 4, characteristic of a transcriptionally active locus.\(^{18}\) Transcriptional targets of NFATc1 include TRACP, calcitonin receptor, cathepsin K, \(\beta\)3 integrin subunit, osteoclast-
associated receptor (OSCAR, an immunoglobulin-like receptor involved in the cell-cell interaction between osteoblasts and osteoclasts), and DC-STAMP.\textsuperscript{18} NFATc1 has also been shown to form a complex with AP-1 which is important for inducing TRACP, calcitonin receptor, and in its own autoamplification.\textsuperscript{18,26} Furthermore, NFATc1 may complex with PU.1 and MITF to regulate cathepsin K and the OSCAR promoters.\textsuperscript{46} Thus, it appears that NFATc1 regulates the expression of various osteoclast genes at different times either on its own or in a complex with AP-1, PU.1, or MITF.

j. Pre-osteoclast fusion

The mechanisms involved in the fusion of monocytes and macrophages to become osteoclasts and giant cells, respectively, have only become clearer in recent years. Several receptors have been identified early on to play a role in cell fusion. Macrophage fusion receptor (MFR, also known as SIRPα) was identified as a cell surface protein that is strongly induced transiently at the onset of macrophage fusion\textsuperscript{47} and it binds to its ligand CD47\textsuperscript{48} on its fusion partner to mediate membrane fusion. There are some data suggesting that MFR-CD47 interaction may be important during OCG.\textsuperscript{49} Another cell surface protein with some homology to MFR is CD44, which is also transiently up regulated during macrophage fusion;\textsuperscript{50} however its ligand has not been identified.

i. DC-STAMP

A protein that has been garnering much recent attention is dendritic cell-specific transmembrane protein (DC-STAMP). It was originally identified in dendritic cells and consists of 470 amino acids with seven putative transmembrane regions.\textsuperscript{51} DC-STAMP expression is highly upregulated by RANKL,\textsuperscript{52} and it has been shown to be essential for the fusion of osteoclast precursors and macrophages.\textsuperscript{30,52,53} DC-STAMP-deficient mice lack multinuclear
osteoclasts and exhibit an osteopetrotic phenotype, not due to defects in cell differentiation but solely due to a fusion defect. Yagi et al. also showed that not all fusing cells require the expression of DC-STAMP; fusion of mononuclear osteoclasts can occur with one “founder” cell expressing DC-STAMP with a “follower” cell that does not in a process of cellocytosis. Cellocytosis has been described as the internalization of one cell by another after receptor binding, causing the internalized cell to be surrounded by two plasma membranes and the subsequent recycling of the plasma membranes and integration of the internalized cytoplasm and organelles. Kukita et al. showed that multinucleation was blocked by siRNA and an antibody specific to DC-STAMP, while overexpression of DC-STAMP enhanced osteoclastogenesis and TRACP activity. Using RAW264.7 cells to study fusion after stimulation by RANKL, lipopolysaccharide, TNF-α, or peptidoglycan, signalling post-cell fusion is thought to be mediated via PI3-K, Src, ERK and JNK, as their respective inhibitors abolished cell fusion.

ii. Other candidate receptors and potentiators of cell fusion

There are numerous other potentiating molecules that enhance pre-osteoclast fusion. Recently, the matrix metalloproteinase MT1-MMP has garnered some attention for its role in pre-osteoclast fusion. It was shown to regulate lamellipodia formation, migration and cell morphology prior to fusion, and these processes are mediated via Rac1 and its adaptor p130Cas. The regulation of fusion does not happen via its protease activity, but rather through its cytosolic tail (Tyr 573) as an adaptor for p130Cas binding and subsequent Rac1 activation. A disintegrin and metalloprotease 8 (ADAM8) has been shown to enhance pre-osteoclast fusion, as mice overexpressing ADAM8 exhibit osteopenia, decreased trabecular bone volume, and hypermultinucleated osteoclasts with increased bone resorptive activity. ADAMs are transmembrane proteins that maintain cell-cell or cell-matrix adhesions, and can cleave
extracellular parts of transmembrane proteins. The increased fusion events in ADAM8-overexpressing pre-osteoclasts are attributed to inducing increased DC-STAMP expression.\textsuperscript{56} The P2X7 receptor, an ATP-gated ion channel and a non-selective plasma membrane pore expressed in cells of hematopoietic origin, is an important mediator of pre-osteoclast fusion. Macrophages in culture expressing high levels of P2X7 exhibit much higher rates of multinucleation, and this is further supported by the spontaneous fusion of HEK293 cells after transfection with P2X7.\textsuperscript{57} M-CSF and RANKL-mediated fusion of human monocytes/osteoclast precursors is abolished by P2X7 blocking antibodies and pharmacological antagonists.\textsuperscript{58,59} Extracellular adenosine, which is generated at the expense of ATP that is released from P2X7, enhances fusion via binding to the A2A adenosine receptor.\textsuperscript{58} These results show that P2X7 and the purinergic pathway function together in osteoclast fusion. However, an earlier study showed that P2X7 was not required for murine pre-osteoclast fusion,\textsuperscript{60} which may indicate a species-specific role for P2X7.

k. Bone resorption

Once the mature osteoclast is fully differentiated, it is able to begin the process of bone resorption. Osteoclasts that are adherent on a mineralized substrate alternate between two phases: resorption and migration.\textsuperscript{61} During the resorptive phase, osteoclasts are polarized, where the cell spreading decreases, cell thickness is increased, and the actin ring is formed which surrounds the resorption lacunae. During the migration phase, this polarization is lost, and the actin cytoskeleton has a non-specific organization. The exact mechanism by which an osteoclast is targeted to the resorption site is unknown, but potential chemoattractants include peptides derived from the bone matrix, type 1 collagen peptides, \(\alpha_2\)HS glycoprotein, osteocalcin, stromal cell-derived factor-1 (SDF-1), and dying osteocytes.\textsuperscript{3} Firm adhesion is generated through the
osteoclast’s main integrin for matrix recognition, $\alpha_v\beta_3$, which recognizes Arg-Gly-Asp (RGD) sequences in bone matrix proteins such as osteopontin, vitronectin, and bone sialoprotein.\(^8\) Binding induces conformational changes which is followed by outside-in signaling to begin the organization of the actin cytoskeleton. A resorption lacuna is formed at the osteoclast/bone interface, bounded by the villous-like ruffled membrane and the circumferential actin ring or sealing zone structure. Acidified vesicles carrying the H$^+$-ATPases are transported toward the osteoclast/bone interface via microtubules, and the fusion of these vesicles with the cell membrane releases protons into the lacuna space, localizes the H$^+$-ATPases, and greatly increases the membrane surface area. The lacuna is further acidified by the proton pumps which pump H$^+$ into the microenvironment, resulting in a pH of $\sim$4.5.\(^3\) The protons are generated by carbonic anhydrase II, a highly-expressed enzyme in osteoclasts, which converts H$_2$O and CO$_2$ into H$^+$ and HCO$_3^-$\(^-\).\(^3\) To prevent alkalinisation of the cytosol, HCO$_3^-$ is exchanged for extracellular Cl$^-$ via a chloride channel, which subsequently enters the lacuna via channels charge-coupled to the H$^+$-ATPase. The hydroxyapatite is demineralized by HCl according to the following reaction: $[\text{Ca}_3(\text{PO}_4)_2]_3\text{Ca(OH)}_2 \rightarrow \text{Ca}^{2+} + \text{HPO}_4^{2-} + \text{H}_2\text{O}$, while the organic matrix is degraded by collagenolytic enzymes such as cathepsin K and matrix metalloproteinases (MMPs).\(^3\) Although there are different types of cathepsins, cathepsin K is regarded as the main enzyme for bone matrix degradation in osteoclasts. A mutation in cathepsin K in humans (resulting in a condition called pycnodysostosis) and in mice results in osteopetrosis.\(^{34}\) Meanwhile, the various types of MMPs have overlapping degradation functions in osteoclasts as individual mutations produce only mild phenotypes.\(^{34}\) The resorption products are removed from the lacuna by endocytosis, and these endocytic vesicles are targeted to the functional secretory domain (FSD) located on the plasma membrane opposite to the ruffled border, in a
process called transcytosis and these products are released into the extracellular fluid. When resorption is complete, the osteoclast detaches from the bone surface, and either migrates to a new site for resorption or undergoes apoptosis.

1. Integrin αvβ3

Integrins are heterodimeric, transmembrane glycoprotein receptors that mediate cell-cell or cell-matrix interactions. Integrin αvβ3, which binds RGD sequence-containing molecules in the bone matrix, is the most abundantly-expressed integrin on the osteoclast, and it initiates initial osteoclast adhesion. It is absolutely required for proper osteoclast function, but not for osteoclastogenesis. Mice with β3 deletion develop osteopetrosis, even though they have a 3.5-fold increase in osteoclast numbers in vivo vs. heterozygous littermates. This discrepancy is attributed to the dysfunction of the cell in their abnormal actin cytoskeleton, and an inability to resorb dentin, to form actin rings or a ruffled membrane, and to migrate.

Being an integrin, αvβ3 is also regulated by inside-out signaling, such as when c-Fms is bound to M-CSF that activates αvβ3 by changing it into its high-affinity binding state. Occupied c-Fms forms a stable interaction with αvβ3. They also work collaboratively during osteoclastogenesis as they share common downstream signaling components such as extracellular signal-regulated kinases (ERKs) and c-Fos. Faccio et al. demonstrated that osteoclastogenesis in β3−/− mice can be rescued by increased stimulation by M-CSF (but not RANKL) via the rescued activation of ERK and c-Fos. M-CSF and αvβ3 both induce cytoskeletal reorganization via activation of Rho GTPases. Specifically, Vav3, a GEF for Rac, is required for actin cytoskeleton organization, polarization, spreading and resorptive activity of the activated osteoclast downstream of αvβ3 and the M-CSF receptor, without which the Vav3-deficient mouse exhibits osteopetrosis. Vav3’s upstream regulator is Syk tyrosine kinase. αvβ3 also associates
with c-Src, a tyrosine kinase, that is activated upon $\alpha_v\beta_3$ occupation and subsequently recruits and phosphorylates Syk, which phosphorylates Vav3. Targeted disruption of c-Src results in non-functional osteoclasts and osteopetrosis, which is attributed to a failure to organize the osteoclast cytoskeleton, and the lack of a ruffled border, and its function is mediated by both its role as an adaptor molecule and its kinase activity. Lastly, a third transmembrane adaptor protein, the above-mentioned DAP12, is required for providing a co-stimulatory signal for integrin $\alpha_v\beta_3$ in addition to its role in $\text{Ca}^{2+}$ signaling. DAP12$^{-/-}$ osteoclasts have impairment in M-CSF and $\alpha_v\beta_3$ signalling since its 2 tyrosine residues located within its ITAM domain are phosphorylated by c-Src, allowing subsequent binding by Syk and its phosphorylation by c-Src, and ultimately cytoskeletal remodelling. Thus the complex of $\alpha_v\beta_3$, c-Src, DAP12, Syk, Vav3, and Rac regulates the osteoclast cytoskeleton and bone resorption.

m. Actin cytoskeleton and osteoclasts

The actin cytoskeleton is a dynamic and constantly-remodelling structure that provides a scaffold for the cell’s spatial organization and movement. In non-muscle cells, the actin cytoskeleton consists of a pool of globular monomeric actin (G-actin), which can reversibly polymerize into filamentous actin (F-actin), thereby changing the mechanical properties of the cell. It is well known that actin plays an important role in mature osteoclast biology as osteoclasts are dynamic, adherent cells that undergo migration, polarization during resorption, and transmigration through cell layers. Actin is especially crucial for osteoclast’s primary adhesive structures known as podosomes, which make up the most prominent component of the actin cytoskeleton in monocyte-derived cells. Actin polymerization and depolymerization within podosomes regulate osteoclast adhesion to the substratum.
i. Podosomes

Instead of developing stress fibers and focal adhesions, cells from the myeloid lineage such as osteoclasts, macrophages, and dendritic cells develop structures called podosomes that are important for motility and cell adhesion. Podosomes are highly-dynamic dot-like structures, composed primarily of F-actin, with individual podosomes having a lifespan of 2-12 min. It is postulated that osteoclasts use the speed of podosome assembly/disassembly to migrate quickly during bone resorption. The podosome core contains F-actin with a high concentration of actin-regulating proteins including cortactin, Wiskott-Aldrich syndrome protein (WASP), WASP interacting protein (WIP), Arp2/3, gelsolin, and CD44, and the core is surrounded by scaffolding proteins (e.g. vinculin, paxillin, talin), kinases (c-Src, Pyk2), integrins (e.g. αvβ3), and Rho GTPases Rho, Rac, and Cdc42. In addition, a loose network of radial F-actin cables, termed actin cloud, surrounds the core. The signaling mechanisms regulating podosome core formation are distinct from those regulating the surrounding actin cloud subdomain. c-Src−/− osteoclasts, which lack actin cloud, have podosome defects and ultimately a failure in bone resorption. Thus, c-Src was postulated to regulate the formation, structure, lifespan, and rate of actin polymerization in podosomes and in the actin cloud. The actin cloud domain is linked to the extracellular matrix (ECM) via αvβ3 and its regulation also involves Pyk2 and c-cbl. Meanwhile, the non-integrin proteoglycan CD44, a receptor for hyaluronic acid, has the important function as a nucleator of podosome cores via its binding with WASP. Both actin core and cloud subdomains are involved in, and have additive effects on cell adhesion.

ii. Actin ring

The formation of a sealed-off microenvironment within which bone resorption occurs is dependent on cell polarization, and the formation of a sealing zone, alternatively termed actin
ring, which surrounds it that also mediates strong anchorage to the bone. The actin ring measures 4 µm in width and height, and has an inner and outer lining of vinculin.\textsuperscript{69} The actin ring is generated by the dense interconnection of adjacent podosomes rich in F-actin, and this ring is further stabilized by acetylated microtubules.\textsuperscript{61} Interestingly, this actin ring structure is only formed when osteoclasts are attached to bone or a mineralized matrix (e.g. dentin and hydroxyapatite-embedded collagen). When osteoclasts are grown on glass \textit{in vitro}, much smaller podosome clusters form during the initial stages of osteoclastogenesis, which rapidly and spontaneously expand into dynamic, short-lived podosome rings, with a lifespan of under 30 min.\textsuperscript{69} In mature osteoclasts, stable podosome belts are formed at the cell periphery, with a lifespan of 1 to 12 hr.\textsuperscript{69,73} The progression from a podosome ring to the podosome belt is made by new podosome additions to the periphery of the ring and not to the center, thus circumferentially expanding the ring into the belt by “oriented treadmilling”.\textsuperscript{69}

\textbf{iii. Regulation of podosomes and actin ring by phosphoinositide signaling}

A further layer of complexity in podosome regulation comes from integrin $\alpha_v\beta_3$ and Rho GTPase-mediated phosphoinositide signaling.\textsuperscript{70} Rho GTPases Rac1, Cdc42, and RhoA activate phosphatidylinositol 4-phosphate 5-kinase (PI4P-5K) and PI3-K downstream of $\alpha_v\beta_3$, producing phosphatidylinositol 4,5 bisphosphate (PI(4,5)P$_2$) and PI(3,4,5)P$_3$, respectively. PI(4,5)P$_2$ interacts with gelsolin, which results in a decrease in gelsolin/actin complexes, actin uncapping and increase in barbed end actin polymerization,\textsuperscript{74} ultimately regulating podosome assembly/disassembly.\textsuperscript{70} Secondly, PI(4,5)P$_2$ interacts with WASP to regulate actin ring formation.\textsuperscript{70} WASP-null murine osteoclasts lack podosomes, actin rings, and suffer resorptive defects \textit{in vivo} and \textit{in vitro}.\textsuperscript{75} PI(3,4,5)P$_3$, on the other hand, serves as a scaffold for gelsolin and interacts with a gelsolin signaling complex required for regulation of podosome dynamics.\textsuperscript{70}
Differential regulation of actin ring and podosomes are demonstrated by gelsolin-ablated osteoclasts that are unable to form podosomes or to migrate, but these osteoclasts express actin rings containing WASP which functionally resorb bone.70,76

II. Rho GTPases

Rho GTPases make up a distinct family within the superfamily of Ras-related small GTPases and they act as intracellular second messengers to transmit extracellular stimuli which are crucial for regulating cell shape, motility, and adhesion through the reorganization of the actin cytoskeleton. They are present in all eukaryotic cells, and there are 22 mammalian genes identified thus far encoding for as many Rho GTPases.77 These “molecular switches” cycle between an active GTP-bound state, and an inactive GDP-bound state. The activation/inactivation of Rho GTPases are mediated via their regulatory proteins: guanine nucleotide exchange factors (GEFs) that catalyze the exchange of GDP for GTP; GTPase activating proteins (GAPs) that stimulate their intrinsic GTPase activity converting GTP to GDP, thereby inactivating the GTPase; and guanine nucleotide dissociation inhibitors (GDIs) that sequester the GTPases to prevent spontaneous activation.77 GTP-bound Rho GTPases interact with downstream effectors to ultimately affect cellular function.

a. Rac, Cdc42, and Rho GTPases

In mammalian cells, the most extensively-studied of the Rho GTPases are Rac, Cdc42, and Rho. Rac has 3 isoforms, 1, 2, and 3; however only Rac1 and Rac2 are present in osteoclasts and their precursors, with Rac1 expressed at a higher level.15 Rac, Cdc42, and Rho are the main GTPases that regulate the cellular actin cytoskeleton.77 In broad terms, the activation of Rac1 induces lamellapodia formation, which are broad sheets of cytoplasm extending from the leading edge in the direction of cell movement. Cdc42, meanwhile, induces the extension of
filopodia, which are thin, finger-like projections at the front of the moving cell, postulated to sense the extracellular environment.\textsuperscript{78,79} Finally, Rho is required for cell retraction by activating the actin:myosin contractile machinery, and has been shown to localize to the rear (uropod) of a migrating cell. In certain cells, Rho is also involved in the formation of stress fibers, actin bundles that promote cell attachment through focal adhesions.\textsuperscript{80} Activation of these GTPases is regulated spatially and temporally in order to coordinate cell shape, adhesion, motility, and in some cells, phagocytosis.

b. Actin filament polymerization and cellular migration

During migration, cells respond to an extracellular stimulus (e.g. a chemoattractant) and the actin cytoskeleton polarizes, where actin polymerization at the front of the cells and actin:myosin contraction at the rear provides the driving force for movement. The formation of both lamellipodia and filopodia at the leading edge require actin polymerization which pushes the leading edge membrane forward. Both Rac and Cdc42 induce actin polymerization via the Arp2/3 complex which is found in all eukaryotic cells, and functions in nucleating new actin filament formation.\textsuperscript{77} Activation of Arp2/3 by Rac is mediated via the WAVE family proteins,\textsuperscript{77} and in neutrophils this is augmented by a positive feedback loop via PI3-K and its lipid product PI(3,4,5)P₃.\textsuperscript{81} The selective accumulation of PI(3,4,5)P₃ at the front is further enhanced through the lipid phosphatase PTEN to the sides and rear of the migrating cell.\textsuperscript{82} Activation of Arp2/3 by Cdc42 is mediated via Wiskott-Aldrich syndrome protein (WASP).\textsuperscript{77} Extension of filopodia induced by Cdc42 activation is postulated to function more in terms of directional sensing. When macrophages migrate in a gradient of M-CSF, both Rac and Rho are required for migration towards M-CSF, but when a dominant-negative Cdc42 is introduced, macrophages move randomly and directionality is lost.\textsuperscript{83} This supports the thought that filopodia formation
induced by Cdc42 is responsible for sensing the extracellular environment, e.g. for chemoattractants.

Rho, on the other hand, can also induce actin polymerization via the formin family of proteins, e.g. diaphanous-related formin (DRF) and mDia1. Rho-GTP binding to mDia1 relieves its autoinhibition, exposing its FH1 and FH2 domains. FH2 allows its binding to the barbed ends of actin filaments, and the FH1 domain interacts with a profilin/actin complex to deliver it to the filament for elongation. However, Rho is thought to mainly function in the rear of the migrating cell to regulate cellular retraction. Rho-GTP activates its downstream effector serine/threonine Rho kinase (ROCK), which phosphorylates and inactivates myosin light chain phosphatase. This results in the accumulation of phosphorylated myosin light chain, thus activating the actin:myosin contractile machinery.

c. Spatio-temporal regulation of Rac/Cdc42/Rho activation

The actin filament-severing protein cofilin provides increased numbers of actin free barbed ends to act as new sites for actin filament elongation. However, at sites of membrane protrusion, active Rac and Cdc42 also activate LIM kinases (LIMKs) via p21-activated kinases (PAKs), which phosphorylates and inactivates cofilin. LIMK can also be activated via Rho and its downstream effector ROCK leading to inactivation of cofilin. The seemingly contradictory processes at sites of membrane protrusion between Arp2/3-mediated filament elongation and LIMK-mediated cofilin inhibition to prevent free barbed end formation may be explained by each event occurring within spatially distinct compartments within the leading edge of the cell.

In addition to operating at the front of the migrating cell, Rac-GTP has been shown to also be present at the rear of migrating neutrophils where Rho is thought to function. Since no
protrusions are found at the rear of a migrating cell, Rac-GTP must have an alternative function at the rear. Rac1 (and not Rac2) has been shown to be required globally to activate Rho and myosin at the uropod,\textsuperscript{88} thus not only is Rac1 required for leading edge protrusion, it also regulates Rho activation.

Recent studies have focused on the crosstalk and antagonism between Rac and Rho during chemotaxis, as both GTPases appear to be present at the leading and trailing edges of a migrating cell. Rac1 was shown to locally inhibit Rho backness program at the leading edge of a migrating cell.\textsuperscript{88} Conversely, a newly identified GAP specific for Rac, FilGAP, is a downstream effector of ROCK which mediates the inactivation of Rac by Rho.\textsuperscript{89} FilGAP binds filamin A (see below), and this binding targets FilGAP to sites of membrane protrusion to mediate the antagonism of Rac by Rho to suppress leading edge protrusion. To add another level of complexity, using biosensors to study the localization and activities of Rho GTPases in embryonic fibroblasts, RhoA, Rac1, and Cdc42 are all active at the leading edge.\textsuperscript{90} Importantly, RhoA activation is synchronous with edge advancement, whereas Cdc42 and Rac1 are activated 2 µm behind the edge with a delay of 40 s, showing a spatio-temporal regulation of their antagonistic activities.

\textbf{d. Rho GTPases and the osteoclast}

The current paradigm for drug discovery requires the identification of a target involved in the disease process (e.g. enzyme or receptor) and the development of an appropriate interfering ligand (activator, inhibitor or selective modulator). However, bisphosphonates have been used for over 20 years in diseases of rapid bone destruction (e.g. post-menopausal osteoporosis, Paget's disease, bone metastases), without fully understanding their molecular mechanism of action. Over the last few years, there has been significant progress in elucidating the mechanism
The main goal is to decrease osteoclast-mediated bone loss by inhibiting osteoclast function. While they serve their intended purpose in decreasing osteoclastic bone resorption and increasing bone mass, they come with unexpected side effects and adverse outcomes, including in rare instances osteonecrosis of the jaw with N-BPs. These observations prompted scientists to study the mechanisms by which bisphosphonates alter osteoclast function in hopes of developing improved pharmacological agents to inhibit pathological bone resorption.

**e. Post-translational prenylation**

N-BPs affect a crucial post-translational modification of Rho GTPases in osteoclasts. Rho GTPases are localized to membrane compartments, and this is dependent on post-translational prenylation whereby a hydrophobic isoprenoid lipid group is added to the C-terminal prenylation motifs. The 20-carbon isoprenoid geranylgeranyl group is covalently linked to Rac, Cdc42, and Rho, and this modification is mediated by the protein:prenyl transferase enzyme geranylgeranyl transferase (GGTase) I. N-BPs such as risedronate and alendronate prevent prenylation of GTPases in osteoclasts, by inhibiting farnesyl diphosphate (FPP) synthase, resulting in depletion of FPP and geranylgeranyl diphosphate (GGPP) which are required as precursors for prenylation. Bone resorption by prenylation-defective osteoclasts is inhibited in vivo. Furthermore, inhibition of GGTase I, thus preventing prenylation, has negative effects on the osteoclast cytoskeleton, osteoclast formation, activity, and survival, strengthening the evidence for Rho GTPase function in osteoclast biochemistry.

**f. Actin rings, podosomes and Rho GTPases**

It has been well established that Rho GTPases regulate the actin cytoskeleton and F-actin-based structures. Since podosomes comprise primarily of F-actin, it is not surprising that
Rho GTPases also regulate podosomes. Inhibition of Rho, Rac, and Cdc42 with *C. difficile* toxin B causes actin ring disruption and inhibition of osteoclast activity.\(^{92}\) When osteoclasts are attached to bone, actin ring formation and osteoclast polarization are associated with high basal RhoA activity.\(^{73}\) Inhibition of RhoA activity by exoenzyme C3 reverts the actin ring structure into the podosome belt, a structure rarely seen in osteoclasts grown on bone, and resorption activity is inhibited *in vitro*.\(^{96,97}\) Similarly when osteoclasts are grown on glass, podosome belts are also observed when RhoA is inhibited; however, active RhoA in osteoclasts grown on glass induces the formation of podosome clusters instead.\(^{73}\) Thus, RhoA appears to induce the formation of actin ring/sealing zone only when accompanied by signals from a mineralized extracellular matrix (ECM). Additional evidence for the requirement for signals from the ECM come from early experiments where Rho and Cdc42 enhances PIP\(_2\)-mediated activation of WASP, formation of WASP-Arp2/3 complex, and actin ring formation in osteoclasts in conjunction with stimulation by osteopontin.\(^{98}\) Less is known about how Rac1 and Cdc42 regulate podosomes in osteoclasts, however alteration in Rac1 activity has been shown to disrupt podosomes in multinucleated macrophages, and active Cdc42 disrupts podosome belts in osteoclasts.\(^{98}\) All three GTPases also function to regulate podosome and actin ring dynamics via the phosphatidylinositide pathway (Section I.m.iii). Further support for a role of Rho GTPases in podosome regulation is the presence of Rho regulators such as p190rhoGAP at these sites.\(^{61}\)

**g. Regulation of osteoclast physiology by Rho GTPases**

In addition to regulating the osteoclast actin cytoskeleton, increasing evidence suggests Rho GTPases to also be important in osteoclast formation, function, and survival. Using adenovirus infection methods to introduce constitutively active or dominant negative Rac1 into murine osteoclasts *in vitro*, Rac1 was shown to be involved in mediating osteoclast survival
downstream of the M-CSF receptor and this was modulated via PI3K/Akt pathways. Rac1 was also shown to play a role in the resorptive activity and in osteoclast cell spreading and membrane ruffling. Using mice with conditional Rac1, Rac2, and double null mutations, both Rac1 and Rac2 were found to be required for normal osteoclast formation and differentiation, although Rac1 was deemed the key isoform since the effects of Rac2 deletion were milder. Rac1 was essential for pre-osteoclast chemotaxis towards M-CSF, generation of actin free barbed ends for actin filament elongation after M-CSF stimulation, RANKL-induced reactive oxygen species (ROS) generation, and cell spreading. Others have shown an important role for Rac2 in osteoclast function and in the skeleton: Rac2−/− mice were found to have a slight increase in trabecular bone mass, although there were increased numbers of osteoclasts in vivo. Rac2−/− osteoclasts were found to be defective in chemotaxis, resorptive activity, cell spreading, and had abnormal accumulation of actin and disruption of the actin ring. However, the requirement for Rac2 in osteoclast biology is not universally recognized, as Lee et al. found that only Rac1 and not Rac2 was activated downstream of RANK leading to activation of NFκB.

Using mice with loss- or gain-of-function mutations in Cdc42, it was demonstrated that Cdc42 loss-of-function mutation resulted in mice with an osteopetrotic phenotype, and osteoclasts isolated from these mice possessed reduced resorptive activity, whereas Cdc42 gain-of-function mutation resulted in mice with an osteoporotic phenotype with osteoclasts having increased resorptive activity.

Recent evidence suggests regulators of Rho GTPases also have significant effects on osteoclast function. Dock5, a GEF for Rac1, is required for osteoclast activity in vitro and in vivo, by regulating Rac1 activity that affects actin ring formation and cellular adhesion. Dock5−/− mice exhibit increased trabecular bone mass although these mice have normal osteoclast
numbers, suggesting Dock5 is essential in osteoclast resorptive function. Further indirect evidence regarding the importance of Dock5 to osteoclast biology comes from the fact that it is one of a few RhoGEFs that become highly upregulated by RANKL during murine osteoclast differentiation.

h. Reactive oxygen species and Rho GTPases

A new player in the regulation of osteoclastogenesis is reactive oxygen species (ROS), and it was shown that stimulation with RANKL activates a TRAF6-Rac1-NADPH oxidase (Nox) complex, producing a transient increase in ROS, which is required for osteoclast differentiation. Interference with TRAF6, Rac1, or Nox1 activity chemically, or by introducing dominant negative mutants or by RNA interference resulted in impaired ROS production and a termination in osteoclast differentiation. The mechanism by which ROS mediates osteoclast differentiation may be a result of turning on a key gene in osteoclast differentiation. Recently, Kim et al. showed that RANKL activates Rac1 and stimulates the production of ROS, which activates PLCγ1 and the subsequent increase in intracellular calcium resulting in a sustained Ca^{2+} oscillation. Calcineurin is activated which goes on to activate NFATc1, the master gene in osteoclast differentiation.

III. Filamin A

a. Discovery

Filamin (FLN) was first discovered accidentally as a by-product in an attempt to isolate a Ca^{2+}-sensitive myosin from rabbit macrophages. It is an actin crosslinking protein, amongst others being spectrin, fimbrin, and α-actinin. Only one molecule of this “actin binding protein” is required per filament of actin to induce gelation, and it was termed an “actin-saving protein” for its ability, on reduce quantities of actin, to form an actin network of similar properties as one
polymerized at high concentrations. This actin binding protein was eventually termed “filamin” because antibodies that recognized it decorated actin-rich stress fibers in chicken fibroblasts.

b. Filamin structure and actin crosslinking

There are three human FLN isoforms, filamins A, B, and C, sharing 70% sequence homology, with FLNa being the most abundant and widely-expressed. FLNa has been shown to be the most potent actin-filament-crosslinking protein. The main role of FLNa is to crosslink F-actin into high-angle orthogonal networks. It is this high-angle actin filament branching induced by FLNa which separates its superior crosslinking efficiency over that of other actin crosslinking proteins, which are thought to form only parallel actin bundles. FLNa can be found at T, X, and Y-junctions where actin filaments branch or overlap, and FLN has been shown to be present uniformly throughout the actin cytoskeletal network right up to the plasma membrane. Under electron microscopy, human FLN appears to have a V-shaped structure.

Structurally, human FLNa exists as a homodimer (280 kDa), and each monomer is composed of 24 β-pleated sheet immunoglobulin (Ig) repeats, each with ~96 amino acid residues folded into antiparallel overlapping domains to generate rod structures. The N-terminal actin-binding domain (ABD), consisting of a stretch of 275 amino acids, is composed of two calponin homology domains found in many actin-filament binding proteins such as β-spectrin, dystrophin, α-actinin, nesprin, plectin, and fimbrin. Two hinge regions between repeats 15-16 and 23-24 contain calpain cleavage sites and provide the FLNa homodimer with flexibility. The sequential rigid rod structures separated by flexible hinges allow FLNa to act like a “molecular leaf spring” with a mix of flexibility and stiffness required to crosslink actin filaments at
perpendicular angles. The C-terminal repeat 24 comprises the dimerization domain of FLNa, and this domain is absolutely required for high avidity F-actin binding and crosslinking. Using an extensive library of FLNa mutants, Nakamura et al. showed that in addition to the ABDs, Ig repeats 9-15 also contain a supplementary actin-binding domain that augments high avidity F-actin binding, although repeats 9-15 on their own cannot cause actin gelation.

c. Functions of Filamin A

i. Actin cytoskeleton stabilization

FLNa has been shown to be important in different cell types for regulating the actin cytoskeleton, thus affecting cell shape, locomotion, cell-cell adhesion and other actin-based cellular processes. The stability of the cortical actin cytoskeleton has been shown to be regulated by FLNa, as cells lacking this actin binding protein exhibit circumferential blebbing due to cortical instability and an inability to withstand internal hydrostatic pressures. One interesting function is its role in mechanoprotection in cells subjected to shear stress, by transducing extracellular signal from the β1 integrin to stiffen the actin cytoskeleton to resist strain. It also binds migfilin, an important adaptor protein at sites of cell-cell and cell-ECM contact, to connect the plasma membrane at sites of adhesion to the actin cytoskeleton.

ii. Cellular migration

FLNa is critically required for normal cortical neuron migration from their native neural crest location to the cerebral cortex during brain development. A null-mutation in the FLNa gene results in the X-linked syndrome periventricular nodular heterotopia (PVNH) in which heterotopic neurons accumulate in the lateral ventricles due to a failure to undergo radial migration from the subventricular zone to the neocortex, thus creating foci which are sources of seizure activity in females; null mutations in FLNa are usually embryonic lethal in males. The
FLNa-deficient M2 cell line derived from human malignant melanoma exhibits defective migratory ability that is restored upon FLNa transgene rescue.\textsuperscript{114} Depletion of FLN in motile Dictyostelium amoebae results in a defect in pseudopod extension leading to impaired locomotion and chemotaxis.\textsuperscript{118} Observations from the PVNH patients, M2 cell line, and Dictyostelium amoebae suggests that FLNa plays a major role in cellular migration and chemotaxis.

**iii. Syndromes resulting from FLNa mutations**

In addition to neuronal defects present in PVNH, the importance of FLNa \textit{in vivo} is reflected by the myriad of other syndromes as a result of missense mutations in the FLNa gene, including otopalatodigital syndrome, frontometaphyseal dysplasia, and Melnick Needle syndrome which are characterized by a combination of skeletal dysplasia, and anomalies in the craniofacial, cardiovascular, genitourinary, and intestinal structures.\textsuperscript{119} The mechanisms by which FLNa mutations affect these structures are obscure, partly due to a scarcity of animal models recapitulating these defects. Using a murine model, Feng \textit{et al.}\textsuperscript{120} showed cell motility-independent roles for Flna in that Flna is important for cell-cell contacts and adherens junctions during the development of many organs, including in developing blood vessels, heart, and brain, providing some insights into the syndromes caused by human FLNa mutations.

**iv. Filamin A binds actin network regulators**

FLNa has more than 20 identified binding partners, ranging from transmembrane proteins (including integrins), membrane channels, signaling intermediates, and transcription factors, and most of these interactions occur near the C-terminus.\textsuperscript{113} Most of the FLN-interacting proteins bind to FLN between repeats 16 and 24.\textsuperscript{108,112} The second-last rod domain has been shown to bind to important signaling intermediates for actin cytoskeleton remodeling, namely Rho
GTPases, and some of their regulatory cofactors and effectors. Specifically, the 23rd repeat of the FLNa homodimer has been identified as a binding site for the Rho GTPases Rac, Cdc42, RhoA (constitutive binding sites),121,122 the Rac guanine nucleotide exchange factors (GEF) Trio, and the Rho GTPase effectors Pak1, ROCK and FilGAP,89,113,123 all of which play regulatory roles for actin cytoskeleton remodeling. It has been shown that some of these effectors function only when bound to FLNa.89 The binding of these important actin regulatory molecules suggests a secondary role for FLNa in mediating signal transduction in addition to its main role in actin crosslinking. The localization of Rho GTPases within close proximity of their regulatory proteins and effectors to actin filaments via FLNa likely permits the tight regulation of local actin cytoskeleton remodeling.108,113

d. Regulation of Filamin

Although there is increasing recent data on how FLN regulates multiple signaling cascades, how itself is regulated remains largely unexplored. FLNs are phosphorylated by multiple serine/threonine protein kinases: protein kinase A (PKA), protein kinase C, Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaM kinase II), and p90 ribosomal S6 kinase.113 In platelets, phosphorylation of FLNa by PKA increases its resistance to cleavage by calpain;124 and phosphorylation of purified chicken gizzard FLN by CaM kinase II decreases its actin-binding affinity.125 The importance of FLN regulation to the actin cytoskeleton as a whole remains obscure.

IV. Shwachman-Diamond syndrome

Shwachman-Diamond syndrome (SDS) (OMIM 260400) is an autosomal recessive, multi-system disorder first described in the 1960’s, with hallmark features of bone marrow failure, exocrine pancreatic insufficiency, and skeletal abnormalities.126-128 It is the third most common
inherited bone marrow failure disorder, following Fanconi anemia and Diamond-Blackfan anemia, and it is the second-most common cause of pancreatic insufficiency in children, following only cystic fibrosis. SDS is usually diagnosed during infancy due to a failure to thrive; mean birth weight is at the 25th percentile, and over half of patients are below the third percentile for height after year 1. SDS patients are very susceptible to recurrent viral, bacterial, and fungal infections. Neutropenia is the most common clinical manifestation of bone marrow failure that is present in 88%-100% of patients, and fluctuations in neutrophil levels can be cyclical in nature. Neutrophils of affected individuals have been shown to exhibit impaired chemotaxis and deficiency in migration. Other manifestations of bone marrow failure include pancytopenia, anemia, and thrombocytopenia, and patients are at increased risk of developing aplastic anemia and acute myeloid leukemia. The pancreas exhibits fatty tissue infiltration of the acini, although the pancreatic ducts and islets are spared, resulting in impaired enzyme output and low serum amylase and trypsinogen levels. Manifestations of pancreatic insufficiency include malabsorption, steatorrhea, malnutrition, and fat-soluble vitamin deficiencies; pancreatic dysfunction may improve spontaneously with age in 50% of SDS patients.

a. Genetic features

SDS results from mutations in the ubiquitously expressed, conserved Shwachman-Bodian-Diamond syndrome (SBDS) gene, located on chromosome 7q11. About 90% of SDS patients harbor mutations in SBDS, and usually results from a conversion of the gene into an adjacent pseudogene (SBDSP) with 97% nucleotide sequence identity resulting in early truncation of the gene product. Genome analysis of affected individuals show that there are 2 predominant mutations that account for more than 95% of mutant alleles, the most common being a splice donor mutation 258 +2T → C in intron 2, found in both homozygous and heterozygous individuals, causing premature
truncation by frameshift, and 183-184TA → CT in exon 2 causing premature truncation by introducing an in-frame stop codon, which accompanies other mutations and is never homozygous. *SBDS* has a 1.6kb transcript, and is predicted to encode a protein of 250 amino acids.\textsuperscript{134} *SBDS* protein family is present widely in nature, and its homologues have been identified in 159 species, including from all sequenced archaeal and eukaryotic genomes and all eukaryotic kingdoms.\textsuperscript{135} The protein is expressed in many tissues other than in the pancreas and bone marrow.\textsuperscript{134} An early study on the phylogeny of *SBDS* suggests it belongs to a family of proteins that are enriched for RNA metabolism and/or ribosome-associated functions.\textsuperscript{135}

**b. SBDS function**

**i. RNA metabolism and ribosome biogenesis**

The exact function of the *SBDS* protein remains unclear. In recent years through studies of orthologs in yeast and Archaea\textsuperscript{136,137} and patient bone marrow cells,\textsuperscript{138-140} it has been postulated to function in RNA metabolism and ribosome biogenesis. Crystal structure analysis of the *SBDS* homologue in *Archaeoglobus fulgidus* showed an N-terminus where the majority of disease-conferring mutations are located, and a C-terminal domain with structural homology with known RNA-binding domains.\textsuperscript{136} Proteomic and genetic analysis of *SBDS* homologues in *Saccharomyces cerevisiae* showed an association with more than 20 proteins involved in ribosome biosynthesis and interactions with proteins involved in RNA and rRNA processing.\textsuperscript{136} Study of another yeast ortholog Sdo1 showed that it is required for late 60S ribosomal subunit maturation and translational activation of ribosomes by allowing 80S ribosome assembly.\textsuperscript{137} Human cell lines and primary cells obtained from SDS patients support a role for *SBDS* in ribosome biogenesis. SDS cells are hypersensitive to an inhibitor of rRNA transcription that is restored by wild-type *SBDS* complementation,\textsuperscript{140} and also exhibit a down-regulation of ribosomal protein genes.\textsuperscript{138} The authors also found that *SBDS* migrates
with the 60S subunit in sucrose gradients, co-precipitates with 28S rRNA, and furthermore rejects a function for SBDS in general protein translation.\textsuperscript{140} In HEK293 cells, SBDS depletion results in accumulation of a small RNA, suggesting a role in rRNA biosynthesis.\textsuperscript{141} In addition, alterations (up- and down-regulation) of genes involved in brain development and function, bone morphogenesis, blood cell proliferation and differentiation, cell adhesion, DNA damage repair, and RNA modifications were observed.\textsuperscript{141}

**ii. Cell proliferation and viability**

In addition to ribosome/RNA-related functions, Sbds is required for early murine embryogenesis and cell proliferation,\textsuperscript{142} and it has also been implicated in cell division/cell proliferation, potentially by its microtubule co-localization and stabilization of the mitotic spindle,\textsuperscript{143-145} and cellular stress responses to various types of DNA damage and ER stressors.\textsuperscript{146} Multiple studies have shown SBDS-depleted cells to be apoptotic, are more sensitive to apoptotic stimuli, or exhibit suppressed expression of genes relating to inhibition of apoptosis,\textsuperscript{138,145,147,148} and that it co-immunoprecipitates with proteins important for DNA repair,\textsuperscript{146} which may explain why SDS patients experience increased risk of developing hematological malignancies.

**iii. Actin dynamics and chemotaxis**

SBDS is proposed to mediate normal F-actin polymerization/depolymerization properties required for neutrophil chemotaxis, and it was found to colocalize with F-actin and Rac2, a regulator of actin dynamics.\textsuperscript{131} SDS neutrophils also exhibit an inability to orient correctly in spatial gradients of chemoattractant.\textsuperscript{132} Indirect evidence supporting a function for SBDS in cellular chemotaxis and migration comes from an amoeba model used to study neutrophils, in which SBDS is enriched in the anterior pseudopods of chemotaxing amoebae in a spatial gradient of chemoattractant.\textsuperscript{149} Thus,
SBDS appears to have extra-ribosomal functions, which is further evidenced by its expression in both the nucleolus and the cytosol.

c. Skeletal phenotype

Skeletal defects are a prominent feature of SDS, and they have been reported in at least a large majority of SDS patients. There is no phenotype-genotype correlation, as patients with the same mutations could present with very different skeletal phenotypes and with different severities. In early cross-sectional studies, metaphyseal dysostosis was observed in 40-80% of SDS patients, and rib and/or thoracic cage abnormalities in 30-50% of SDS patients.150-152 More recently in a longitudinal study on 15 SDS child patients, Mäkitie et al. tracked age-related changes in their skeletal manifestations.133 They found that all of the SDS patients studied displayed varying skeletal abnormalities including delayed appearance of secondary ossification centers, variable widening and irregularity of the metaphyses in early childhood followed by progressive thickening and irregularity of the growth plates, and thin cortices with generalized osteopenia of the long bones.133 The longitudinal study also showed that the abnormalities in the epiphyses tended towards normalization with age while those in the growth plates and metaphyses worsened. Radiographic analysis of skeletal radiographs showed various degrees of skeletal defects in the thoracic cage, wrist/hand, pelvis/hips, knees, spine, and skull that varied with time. In addition, a subset of patients showed early signs of osteoporotic vertebral deformities and disturbances in bone homeostasis manifesting in low-turnover osteoporosis.133,153 These patients had markedly reduced bone mineral density in the lumbar spine, proximal femur, and whole body, reduced bone mineral content, and on occasion vertebral compression fractures. Transiliac bone biopsies showed reduced trabecular bone volume, reduced numbers of osteoclasts and osteoblasts, and reduced amount of osteoid, suggestive of a primary defect in bone metabolism in SDS.
d. Oral manifestations of SDS

Oral and dental diseases and conditions including periodontitis, delayed eruption of the permanent dentition, increased caries risk in primary and permanent dentitions, and increased soft tissue pathoses, have been reported.$^{154}$
V. Objectives and hypotheses

Rho GTPases have been known as the main regulators of the actin cytoskeleton in many different cell types. They are absolutely required for membrane protrusion and retraction during migration and for the polarization of the cell. Not only are they important in regulating the multiple actin-based structures in the osteoclast such as podosomes and actin ring, they have also been shown to function as second messengers in osteoclast differentiation and function. Many studies have confirmed roles for Rac1, Rac2, Cdc42, and RhoA in different aspects of osteoclast differentiation and/or function, using experimental approaches including transgenic mice, pharmacological manipulation, gene silencing, and the addition of constitutively active or dominant negative mutants.

Mutations in both FLNa and SBDS genes in humans result in an altered bone phenotype. The skeletal phenotype in SDS is better-characterized compared to that in the various syndromes caused by FLNa mutations. The latest study of SDS patients show that 100% have skeletal abnormalities: histology of bone biopsies demonstrate reduced levels of osteoclasts, osteoblasts, and osteoid, and bone scans reveal reduced bone mineral densities, all indicative of low-turnover osteoporosis. These results suggest a primary defect in bone homeostasis involving osteoclasts and/or osteoblasts.

We have unpublished evidence which shows that FLNa is required for neutrophil chemotaxis by regulating RhoA activation and uropod retraction, in addition to migratory role in cortical neurons and M2 cells. Both neutrophils and osteoclast precursors are derived from the same myelogramulocytic progenitor cells, and it is possible that the same neutrophil defects associated with FLNa mutation are exhibited by osteoclast precursors as well. Since FLNa binds actin filaments, Rho GTPases, and their regulators, thereby localizing them in close proximity, a
possible role exists for FLNa in osteoclast biochemistry due to its regulation of the primary structure actin filaments or through its integration of signals that regulate the actin cytoskeleton. It is interesting that like FLNa, SBDS has also been shown to regulate migration and chemotaxis in neutrophils and other cells by interacting with actin and Rac. Thus, the well-characterized neutrophil defects observed in SDS could also be translated into defects in osteoclasts, manifesting in a dysregulation of bone homeostasis and the observed skeletal phenotype.

The main objectives of this thesis are to study the roles of filamin A and SBDS in regulating Rho GTPases during the different stages of osteoclast differentiation, thereby offering some insight into their respective skeletal phenotypes in human mutations.

Hypotheses:

1. Filamin A coordinates Rho GTPase activation in osteoclasts and thus regulates subsequent actin-based processes such as cell migration.

2. Similar to functional defects seen in SDS neutrophils, SBDS ablation in osteoclasts results in functional defects as a result of deficiencies in the regulation of the actin cytoskeleton by Rac.
Chapter 2

Filamin A regulates monocyte migration through Rho small GTPases during osteoclastogenesis
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Contributions: RL designed and performed research, collected, analyzed, and interpreted data, performed statistical analysis, and wrote and revised the manuscript; YW designed research and analyzed data; KC performed research, collected and analyzed data, and performed statistical analysis; CS designed research and analyzed data; JM performed research and analyzed data; M Grynpas analyzed data; and M Glogauer designed research, analyzed data, and wrote the manuscript.
Abstract

Osteoclastogenesis (OCG) results from the fusion of monocytes after stimulation with macrophage-colony stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL). Migration of monocytes in to close proximity precedes critical fusion events that are required for osteoclast formation. Cellular migration requires leading edge actin cytoskeleton assembly which drives cellular locomotion. Filamin A (FLNa) cross-links F-actin filaments in the leading edge of migrating cells and has also been shown to regulate signal transduction during cell migration. However, little is known about the possible role of FLNa in osteoclastogenesis. **Objective:** To investigate the role of FLNa in osteoclastogenesis. **Methods:** Bone marrow monocytes isolated from the tibias and femurs of wild type (WT) and Flna-null mice were cultured for 6 days with M-CSF and RANKL, and osteoclasts were identified by TRACP-staining. The Flna-null mouse skeletal phenotype was characterized using DEXA to analyze the skeleton, as well as tests on blood chemistry. Osteoclast levels in vivo were quantified by counting of TRACP-stained histological sections of distal femurs. To elucidate the mechanisms by which FLNa regulates osteoclastogenesis, migration, actin polymerization, and activation of Rho GTPases Rac1, Cdc42 and RhoA were assessed in monocytes during in vitro OCG. Deficiencies in migration were rescued using constitutively-active Rac1 and Cdc42 TAT fusion proteins. The RANKL signaling pathway was evaluated for activation by monitoring nuclear translocation of NFκB and c-jun, and expression of key osteoclast genes using qRT-PCR. **Results:** Flna-null monocytes formed fewer osteoclasts in vitro, and those that were formed were smaller with fewer nuclei. Decreased OCG was reflected in vivo in TRACP-stained histological bone sections. Flna-null monocytes experienced impaired migratory ability. When OCG was performed at increasing starting cellular plating densities in order to decrease
intercellular distances, there was progressive rescue of Flna-null osteoclast formation comparable to WT levels, confirming Flna regulates monocyte migration pre-fusion. Activation of the actin cytoskeleton regulators Rac1, Cdc42, and RhoA, and actin free-barbed end generation were partially or completely abrogated in Flna monocytes; however monocyte migration was restored upon rescuing with constitutively-active Rac1 and Cdc42 TAT fusion proteins. **Conclusion:** Filamin A is required for osteoclastogenesis by regulating actin dynamics via Rho GTPases which control monocyte migration.

**Key Words:** osteoclast, filamin A, Rho GTPases, cell migration, actin dynamics
Introduction

Osteoclasts are bone-resorbing cells that are important in bone homeostasis and remodeling. They are also involved in chronic osteolytic diseases such as periodontitis, osteoporosis and rheumatoid arthritis where they pathologically resorb bone. Osteoclasts are large multinucleated cells formed by the fusion of mononuclear monocytes derived from hematopoietic progenitors in the bone marrow after stimulation by the cytokines RANKL and M-CSF. A major cellular event required for osteoclastogenesis (OCG) is cell migration which brings cells in close proximity prior to fusion. Murine and human monocytes have been shown to chemotax toward a variety of bone matrix peptides. Osteoclasts also exhibit migratory ability toward M-CSF and TGF-β. Cellular migration and membrane fusion require dynamic actin cytoskeleton reorganization, and it has been firmly established that the Rho family small GTPases Rac1, Cdc42, and RhoA regulate the actin cytoskeleton. These GTPases act as molecular switches to control actin-dependent structures and processes in OCG and in mature osteoclast function, including chemotaxis, actin filament elongation, actin ring and sealing zone formation, and cell polarity.

Filamins are actin-binding proteins which crosslink F-actin into high-angle orthogonal networks, and its homodimeric structure and multiple spring-like hinge regions impart flexibility and elasticity to the crosslinked actin cytoskeleton. Filamin molecules can be found at T-, X-, and Y- junctions where actin filaments branch or overlap. There are three human filamin isoforms, filamins A, B, and C, which share 70% sequence homology, with filamin A (FLNa) being the most abundant and widely-expressed. FLNa has been shown to be important in different cell types during cellular migration. FLNa is critical for normal cortical neuron migration from their native neural crest location to the cerebral cortex during brain development,
and its importance is shown by the development of periventricular nodular heterotopia (PVNH) in which a null-mutation in the *FLNa* gene causes neurons to accumulate in the lateral ventricle due to their defective migration.\textsuperscript{117} The FLNa-deficient M2 cell line, derived from human malignant melanoma, also exhibits defective migratory ability that is restored upon FLNa transgene rescue.\textsuperscript{114}

The ability of Rho GTPases to regulate the cortical actin cytoskeleton during migration would suggest their presence of at the cell periphery. Rac1 and Cdc42, and to a lesser extent RhoA have been observed to translocate from the cytosol to the cell membrane in rat osteoclasts stimulated with M-CSF.\textsuperscript{20} Using constitutively active mutants, Sakai \textit{et al.}\textsuperscript{20} showed that Rac1 induced lamellipodia formation, Cdc42 induced some filopodia and to a lesser extent lamellipodia formation, and RhoA induced cell retraction. Since FLNa is present at both the leading and trailing edges of polarized cells,\textsuperscript{113,160} it has been proposed that FLNa functions to properly localize Rho-family GTPases at both poles of migrating cells,\textsuperscript{89,108,113} allowing for Rac1 and Cdc42 activation at the leading edge and RhoA activation at the trailing edge. The 23\textsuperscript{rd} of 24 beta-sheet repeats of the FLNa homodimer have been identified as a binding site for Rac, Cdc42, Rho, the Rac guanine nucleotide exchange factor (GEF) Trio, the RhoGEF Lbc, and the RhoGTPase effectors Pak1, ROCK and FilGAP,\textsuperscript{89,113,123} making FLNa an ideal scaffolding protein to integrate the Rho GTPase signaling cascade.

The mechanisms and signaling elements involved in osteoclastogenesis are only starting to emerge. Particularly lacking is information regarding the regulation of monocyte migration pre-fusion, and the role of FLNa in monocyte migration and ultimately osteoclastogenesis. Since FLNa is required in the signaling cascade in regulating the actin cytoskeleton during cell migration and actin remodeling,\textsuperscript{114,117} FLNa potentially plays an important role in the many
actin-dependent processes of osteoclastogenesis. The objective of this study is to investigate the role of FLNa in regulating monocyte migration and osteoclastogenesis, and the involvement of Rho family GTPases that transduce their signal via FLNa. Utilizing mice with a conditional Flna gene knock-out only in cells from the granulocyte lineage (monocytes and neutrophils) and a previously-described in vitro OCG model, we characterized the role of FLNa in osteoclastogenesis. We show here that FLNa is required for monocyte migration during osteoclastogenesis via its role in regulating Rho GTPase-mediated actin remodeling.

Materials and Methods

Animals

All procedures described were performed in accordance with the Guide for the Humane Use and Care of Laboratory Animals and were approved by the University of Toronto Animal Care Committee. Mice containing the conditional knock-out of the X-linked Flna gene (Flna-null) were generated as described previously. Briefly, a conditional knockout strategy was used with loxP sites inserted into introns 2 and 7 of the mouse Flna gene (Flna or male Flna mice). Deletion of the Flna gene in granulocytes (neutrophils and monocytes) was accomplished by breeding these mice with mice expressing Cre-recombinase under control of the granulocyte-specific lysozyme M promoter which is active during early embryogenesis. Cre-mediated recombination deletes exons 3-7, producing a non-sense mutation with early Flna truncation at amino acid 121. To confirm deletion of the Flna gene, tail snips were used to prepare DNA for PCR analysis as described previously. Littermates with unsuccessful Cre-mediated recombination were used as wild-type control (WT).
Monocyte isolation

Tibia and femur bones from 8 to 12-week-old WT and Flna-null mice were dissected aseptically under a laminar air flow hood. The ends of the bones were carefully cut, and bone marrow was flushed out using a needle and syringe containing α-MEM (Life Technologies). Cell aggregates were broken by repeated aspiration using the same syringe and a 20 G needle. Cells were washed once, and resuspended in α-MEM supplemented with 10% fetal bovine serum (FBS) and antibiotics (164 IU/ml penicillin G, 50 µg/ml gentamicin, and 0.25 µg/ml fungizone). To remove stromal cells, bone marrow cells were cultured overnight (in a humidified incubator at 37°C with 5% CO₂) in T-75 tissue culture flasks after which cells in the supernatant were pelleted and resuspended in 4 ml of α-MEM. The cell suspension was gently layered over 4 ml of Ficoll-Paque PLUS (Amersham Biosciences) and centrifuged at 350 g for 30 min at 4°C. The cell layer at the solution interface enriched in monocytes was removed with a sterile Pasteur pipette and washed in excess α-MEM. Cells were counted using a Z1 Coulter counter (Coulter Electronics) and resuspended in α-MEM growth medium at 0.5x10⁶ cells/ml.

Validation Experiments

To ensure that Flna was deleted at the protein level, cell lysates from freshly-isolated WT and Flna-null monocytes were subjected to SDS-PAGE and Western blotting (see below) using these antibodies: rabbit polyclonal anti-mouse filamin A (A301-135A, Bethyl Laboratories, Inc.; 1:2000) followed by HRP-conjugated donkey anti-rabbit IgG (NA934V, GE Healthcare; 1:2000). Total c-FMS (M-CSF receptor) and RANK protein expression was evaluated by Western blotting on monocyte lysates using primary antibodies rabbit anti-c-Fms/CSF-1R (C-20, sc-692, Santa Cruz Biotechnology; 1:600) and goat anti-RANK (N-20, Santa Cruz Biotechnology; 1:600), respectively, followed by HRP-conjugated donkey anti-rabbit IgG
and HRP-conjugated rabbit anti-goat IgG (30220, Alpha Diagnostic Intl, Inc.; 1:2500), respectively. Primary and secondary antibodies used for Western blotting were diluted in TBS-T with 5% milk. The number of osteoclast progenitors and mesenchymal stem cells were evaluated using colony-forming unit assays as previously described. The levels of cell surface c-Fms and RANK were measured by incubating 10^6 monocytes with either 2 µg rat anti-cFms (0.N.179; Santa Cruz Biotechnology) followed by 2 µg FITC goat anti-rat IgG (CLCC40101; Cedarlane) or with 2 µg goat anti-RANK (N-20) followed by 2 µg FITC swine anti-goat IgG (CLCC50001; Cedarlane). FITC fluorescence was evaluated by flow cytometry.

**Osteoclastogenesis**

Osteoclastogenesis (OCG) was initiated by plating 5 x10^4 cells/well in a 6-well tissue culture plate with supplementation by 20 ng/ml M-CSF (M9170, Sigma) and 100 ng/ml recombinant sRANKL purified as previously described. Cells were cultured for 6 days, with a change of cell culture media and cytokine supplementation every other day. On day 6, cells were washed twice with PBS, fixed with 4% paraformaldehyde (PFA), and stained for TRACP (see below). The number of TRACP-positive osteoclasts and the number of nuclei within these osteoclasts were counted in 10 random fields of view (FOV). In some experiments, monocytes were added to wells containing a calcium phosphate-coated BioCoat Osteologic disc (BD Biosciences) or a OsteoSite dentine disc (Immunodiagnostic Systems Ltd.), and OCG was performed as above. Resorption of dentin was evaluated by staining dentin discs with 1% toluidine blue in a 0.5% sodium tetraborate solution for 3 min, washed, and viewed by light microscopy. Subsequent experiments to determine the effect of monocyte plating density on OCG were performed by plating 1.25x10^5, 2.5x10^5, and 5x10^5 cells/well in 6-well plates and culturing them over 6 days as
above. For these experiments, all the osteoclasts in the culture wells with >20 nuclei/osteoclast were counted.

**TRACP Staining**

Staining for tartrate-resistant acid phosphatase (TRACP) was done as previously described. Briefly, cultured osteoclasts were washed once with pre-warmed PBS and fixed with 4% PFA for 15 min at room temperature. Fixed cells were washed with PBS, then incubated in a solution of naphthol AS-BI phosphate and fast red TR salt (Sigma) in 0.2 M acetate buffer (pH 5.2) containing 100 mM sodium tartrate (Sigma) for 10 min at 37°C. TRACP-stained cells were washed twice with water, cells were viewed with a Leitz Wetzlar microscope at 200x magnification, and images were taken with a PixeLink camera.

**Transwell (Boyden chamber) Migration Assay**

Monocyte migration was evaluated in an assay using Transwell permeable supports with 5 µm membrane pores in a 24-well plate (Corning Life Sciences), as previously described. These inserts were first incubated in α-MEM growth medium for 30 min at 37°C, after which 0.5x10^6 cells (in 200 µl) were added to the Transwell insert and incubated for 2 hr at 37°C to allow for cell attachment to the membrane. Non-adherent cells were removed, and the inserts were placed in 600 µl growth medium containing either RANKL (100 or 500 ng/ml) or M-CSF (20, 50, or 100 ng/ml) and further incubated for 2 hours at 37°C to allow for migration. The inserts were then fixed in 4% PFA, stained with 0.165 μM 4’6,-diamidino-2-phenylindole (DAPI; Sigma), and washed thoroughly with PBS. Cells that have adhered to the top of the membrane but have not migrated through the membrane were removed by gentle wiping with a cotton swab and rinsing with PBS, and the membranes were cut out from the inserts and mounted onto glass.
slides. Cell nuclei in 10 random FOVs per sample were counted using a Nikon Eclipse E400 fluorescent microscope at 200x magnification.

**Actin Free-Barbed End (FBE) Assay**

An actin FBE assay as described by Glogauer et al.\(^{164}\) was used to measure actin FBE generation in WT and Flna-null monocytes. Monocytes (0.8x10\(^6\) cells) were stimulated with M-CSF (20 ng/ml) or PBS vehicle for 40 min in culture media. Resting and activated monocytes were resuspended in 90 µl HANKs and permeabilized for 10 s with 0.2% octyl glucoside (OG) (PHEM buffer containing 10 µM phallacidin, 42 nM leupeptin, 10 mM benzamidine, and 0.123 mM aprotinin). Permeabilization was stopped with addition of 3 vol of buffer B (1 mM Tris, 1 mM EGTA, 2 mM MgCl\(_2\), 10 mM KCl, 5 mM β-mercaptoethanol, and 5 mM ATP; pH 7.4). This cell mixture was transferred to a 96-well microplate, after which pyrene-labelled rabbit skeletal muscle actin (Cytoskeleton) was added (1 µM final conc.). The ability of these cells to accelerate actin assembly was measured by the increase in pyrene fluorescence using a microplate reader (FLUOstar optima; BMG Labtech) with fluorescence excitation and emission wavelengths of 355 and 405 nm, respectively. The rate of fluorescence increase as pyrene-actin is polymerized, is proportional to the number of actin FBEs.\(^{164}\) The increase in actin polymerization rate was expressed as fold increase above unactivated levels within each genotype. To confirm that actin FBEs were measured and not pointed ends, 2 µM cytochalasin D was added prior to addition of rabbit actin to block barbed ends,\(^{165}\) which effectively eliminated the fluorescence increase.

**Immunoprecipitation, SDS-PAGE and Western Blotting**

To evaluate activation of Rho GTPases, monocytes freshly isolated after Ficoll centrifugation were plated on 10 cm petri dishes and cultured for 2 days. On average, four mice from each
genotype were sacrificed to yield adequate cells for each experiment (totaling about $4 \times 10^7$ cells plated). Cells that remained in suspension after 2 days were aspirated, and adherent monocytes were washed with α-MEM once and subjected to activation by M-CSF (20 ng/ml) or treated with PBS vehicle for 4 hr at 37°C. Monocytes were subsequently lysed with 200 µl ice-cold RIPA buffer (Sigma) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1x protease inhibitor (BD pharmpingen) for 5 min and collected by cell scraping. Cell lysates were centrifuged for 1 min at maximum speed to pellet cellular debris. To assess total Rho GTPase content, 20 µl of cell lysates were added to 5 µl of 5x Laemmli sample buffer, boiled for 10 min and reserved for subsequent SDS-PAGE and Western blotting. To assess activated (GTP-bound) Rho GTPase content, the remainder of the cell lysates were added to either 20 µg of PAK-GST glutathione sepharose beads (for Rac1 and Cdc42) or 60 µg Rhotekin-RBD beads (for RhoA) (both from Cytoskeleton, Inc.) and rotated for 1 hr at 4°C to allow for binding. Beads were subsequently washed twice with a cold wash buffer (20 mM Hepes pH 7.4, 142.5 mM NaCl, 4 mM EGTA, 4 mM EDTA, 1% NP-40, 10% glycerol) and boiled for 10 min with the addition of 20 µl 2x Laemmlie sample buffer to release bound proteins. Immunoprecipitated and total protein samples were separated by SDS-PAGE on a 12% polyacrylamide gel and transferred to a nitrocellulose membrane (Amersham Biosciences). Non-specific protein binding sites were blocked with Tris-buffered saline plus 0.05% v/v Tween 20 (TBS-T) with 5% nonfat milk powder for 1 hr. Membranes were incubated with a primary antibody for 1 hr (see below), washed 3 times with TBS-T, incubated with a corresponding secondary antibody for 1 hr, and washed 3 times with TBS-T. Membranes were developed using Amersham ECL Plus (GE Healthcare) and the resulting chemiluminescence was exposed to film (Clonex Corp). Films were scanned (Epson scanner) and band intensities were quantified by densitometry using Image
J 1.41 software. Results from the immunoprecipitation were normalized against corresponding total GTPase levels, and the degree of Rho GTPase activation was expressed as folds over unactivated controls.

Primary antibodies used were as follows: mouse monoclonal anti-Rac1 (23A8, Upstate Biotechnologies; 1:2000 in TBS-T/5% milk); rabbit monoclonal anti-RhoA (67B9, Cell Signaling Technology; 1:1000 in TBS-T/5% BSA); mouse monoclonal anti-Cdc42 (B-8, Santa Cruz Biotechnology, Inc; 1:100 in TBS-T/5% BSA). Secondary antibodies used were as follows (all diluted in TBS-T/5% milk): HRP-conjugated sheep anti-mouse IgG (610-603-002, Rockland Inc; 1:4000 for Rac1 and 1:1000 for Cdc42); HRP-conjugated donkey anti-rabbit IgG (NA934V, GE Healthcare; 1:2000).

**TAT Protein Constructs and Migration Rescue**

Constructs of TAT fusion proteins for protein transduction were kindly provided by Dr. Gary Bokoch (Scripps Research Institute, La Jolla, CA). Constructs for constitutively active (CA) Rac1 and Cdc42 (G12V), dominant negative (DN) Rac1 and Cdc42 (T17N), and empty vector pTAT-HA were transformed into BL21(DE3)pLysS competent cells. Transformed cells were grown in 250 ml of Luria broth for 6 hrs at 37°C with shaking, after which IPTG (1 mM final conc.) was added to induce protein expression and grown for 2 hrs. Bacteria were pelleted at 4°C, and TAT fusion proteins were purified using Ni-NTA columns (#30600, Qiagen) following manufacturer’s instructions. The resulting proteins were dialyzed in Slide-A-Lyzer dialysis cassettes (#66203, Thermo Scientific) in PBS overnight at 4°C. Protein concentrations were measured using the BCA protein assay kit (#23225, Pierce). Entry of TAT fusion proteins into purified monocytes was tested by incubating 0.5x10⁶ cells with a range of concentrations of proteins for varying lengths of time, washing the cells twice, and performing western blots for
the HA tag. We determined that entry reached a maximal level after 10 min, and the optimal protein concentration was 500 nM, which is in a similar range of concentrations used by others.\textsuperscript{166-168} To observe the effects of TAT fusion proteins on migration, cells were pre-incubated with 500 nM TAT proteins alone or in combination for 10 min at 37\textdegree C prior to being added to Transwell supports. Migration assay was performed as described above.

\textit{NF\kappa B and c-jun Activation}

To evaluate whether the RANKL-RANK signaling axis is intact in Flna-null cells, activation of the transcription factors NF\kappa B and \textit{c-jun} was measured. Definitive activation was defined by their translocation into the nucleus. Osteoclasts were cultured as described above for 6 days in 8-well chamber slides, and NF\kappa B and \textit{c-jun} localization was visualized by immunostaining using a NF\kappa B and \textit{c-jun} activation kit (8400301, Thermo Scientific) and fluorescence microscopy at 400x magnification (Nikon Eclipse E1000).

\textit{Quantitative Real-Time PCR}

On days 1-5 of OCG, RNA was extracted from WT and Flna-null cultures (Qiagen RNeasy mini kit). Total RNA (500 ng) was reverse transcribed into cDNA using Superscript II (Invitrogen Life Technologies; Carlsbad, CA) and Oligo-dT\textsubscript{18}VN primer (ACGT Corp; Toronto, Canada). Primers were designed from the GenBank accession numbers BC029644 for TRACP (F5'-ACGGCTACTTTGCGGTTTCA-3'; R5'-TCCTTGGGAGGCTGGTCTT-3'), NM\_007802 for cathepsin K (F5'-GAAGAAGACTCACCAGAAGCAG-3'; R5'-TCCAGGTTATGGGCAGAGATT-3'), AB109560 for DC-STAMP (F5'-TACGTGGAGAGAAGCAAGGAA-3'; R5'-ACACTGAGACGTGGTTTAGGAAT-3'), and M32599 for GAPDH (F5'-CCTTCCGTGGTCTACCCC-3'; R5'-GCCCAAGATGCCCTTCAGT-3'). Using a dilution series, we determined that a 1:10 cDNA
dilution was optimal to yield PCR efficiency of between 95-105%, thus a 1:10 cDNA dilution was used for all reactions. Quantitative real-time PCR was performed in 20 µl reactions containing 5 µl diluted cDNA and 10 µl SsoFast EvaGreen Supermix (Bio-Rad; Hercules, CA) using the BioRad CFX96 Real Time System. Each reaction was done in triplicate. PCR conditions were as follows: initial denaturation at 95°C for 30 sec, annealing temperature at 60°C for 1 min, extension at 72°C for 1 min, repeated for 35 cycles. A final extension at 72°C for 10 min concluded the reaction. Data were normalized with internal GAPDH control as previously described using the \( 2^{-\Delta \Delta C_T} \) method.\(^{169}\) Gene expression in Flna-null cells was expressed relative to the WT gene expression profile.

**Dual Energy X-Ray Absorptiometry (DEXA)**

Sex and age-matched 3 month-old mice were sacrificed by CO₂ asphyxiation and kept at -20°C until analysis. DEXA was performed using an animal PIXImus densitometer (Lunar; GE Corp) by a single investigator. Bone mineral density (BMD) and bone mineral content (BMC) were analyzed for the whole body, right femur, and lumbar vertebra, and fat content was measured for the whole body. Heads were excluded by masking.

**Histology**

Femurs from 3 month-old mice were isolated intact, dissected free of soft tissue, and fixed in 10% formalin (pH 7.4) for 1 week. The bones were demineralized in 10% EDTA (pH 7.4) for 10 days, washed three times in PBS, and incubated in distilled water overnight. Samples were dehydrated, embedded in paraffin, and sectioned (5 µm), and just before staining they were deparaffinized by xylene (3 x 5 min) and rehydrated through decreasing concentrations of alcohols. TRACP staining was done as described above and counter-stained with hematoxylin. The number of osteoclasts within the whole femoral head in three histological sections per
mouse was counted and results were expressed as mean osteoclast number per femoral head section.

**Blood Chemistry and Bone Marker Analysis**

Blood was collected from anesthetized mice by cardiac puncture. Measurements of circulating osteocalcin levels were performed on serum samples using a mouse osteocalcin EIA kit (BT-470, Biomedical Technologies Inc.) according to manufacturer’s instructions. To measure alkaline phosphatase activity of osteogenic bone marrow precursor cells, total bone marrow cells were cultured in 6 well plates (2 x 10^5 cells/well) for 10 days in the presence of ascorbic acid (50 µg/ml) and the resultant alkaline phosphatase activity was measured using an alkaline phosphatase kit (85L2-KT, Sigma) as described previously. For measurements of blood ionized calcium and parathyroid hormone (PTH) levels, serum samples were processed using standard hematological methods by Idexx Laboratories (Toronto).

**Statistical Analysis**

For experiments in which there were multiple observations per sample, numerical results were expressed as mean ± SEM. For experiments in which there was only a single observation per sample, results were expressed as mean ± SD. Each experiment had a sample size of n≥3. Statistical analysis was performed using student’s t-test unless otherwise specified. For DEXA analysis, two-way ANOVA with post-hoc multiple comparisons LSD test was used to measure statistical significance. p<0.05 was considered statistically significant.

**Results**

**Characterization of Flna-null monocytes**

Western blotting confirmed that Flna was only expressed in WT cells but not in the Flna-null monocytes (Fig. 1A). Expression levels of c-Fms and RANK in total lysates (Fig. 1A) and
on the cell surface (not shown), were normal in Flna-null monocytes. To ensure that the number of osteoclast progenitors was not altered as a result of the Flna gene deletion, bone marrow stem cells were cultured in a colony-forming assay as described by Wang et al.\textsuperscript{100} We found no significant difference in the number of osteoclast progenitors between WT and Flna-null mice (Fig. 1B). A fibroblast colony forming unit assay to evaluate the number of mesenchymal stem cells as described by Castro et al.\textsuperscript{162} also showed no significant difference between the WT and Flna genotypes (Fig. 1C), nor was monocyte adhesion to tissue culture plastic altered (not shown). These results confirmed that the Flna deletion did not alter important monocyte characteristics which could otherwise affect OCG, thus allowing us to utilize this mouse model to test the effects of Flna deletion on osteoclastogenesis.

\textit{In vitro osteoclastogenesis}

To determine the effects of the targeted Flna gene knock-out in monocytes on osteoclast formation, an \textit{in vitro} osteoclastogenesis assay was performed, using monocytes isolated from WT and Flna-null mice. The number of TRACP-positive osteoclasts and the number of nuclei per osteoclast were quantified. Flna-null monocytes had a profound defect in their ability to form osteoclasts (Fig. 2A). There was a significant four-fold decrease in Flna-null osteoclasts with 3-7 nuclei compared to WT osteoclasts (Fig. 2B). Larger WT osteoclasts with 8 or more nuclei were also observed, whereas large Flna-null osteoclasts were entirely absent. However, the formation of TRACP-positive Flna-null osteoclasts with 1 to 2 nuclei was similar to that of WT. In addition to culturing osteoclasts on plastic petri dishes, they were also cultured on a calcium phosphate Osteologic disc. Under this condition, Flna-null monocytes also formed fewer and smaller osteoclasts compared to WT monocytes. In each FOV, the number of nuclei within TRACP-positive, multinucleated osteoclasts and the total number of nuclei in all cells were counted as an
indicator of fusion efficiency. A significant two-fold decrease in fusion efficiency was observed in Flna-null cells compared to WT (Fig. 2C), illustrating decreased fusion events in Flna-null cultures.

**Monocyte Migration**

In the early phase of osteoclastogenesis monocytes migrate toward each other via chemotaxis, followed by their subsequent fusion. Since decreased osteoclastogenesis in Flna-null monocytes could be due to either defective migration or fusion, or both, we evaluated both parameters. Migration was assessed in a Boyden chamber, using M-CSF and RANKL as chemoattractants. WT monocytes migrated toward M-CSF at all concentrations used compared to Flna-null monocytes, with migration reaching a maximal level at 20 ng/ml M-CSF (Fig. 3A). Flna-null monocytes did not migrate even at the highest M-CSF concentration used. When RANKL was used as the chemoattractant, significantly more WT monocytes migrated in a dose-dependent manner, while Flna-null monocytes did not migrate appreciably (Fig. 3B).

**Rescuing defective osteoclastogenesis in Flna-null monocytes**

To assess the importance of migration during osteoclastogenesis, and to determine whether osteoclastogenesis in migration-defective Flna-null monocytes could be rescued, monocytes were cultured at increasing cell densities, with the rationale that Flna-null cells grown in close proximity could overcome their defect in migration in order to fuse (Fig. 4A,B). A decrease in OCG for Flna-null monocytes was already observed when cultured at a density of 5 x 10^4 cells/well, thus a minimal plating density of 1.25 x 10^5 cells/well was used as a starting point. For these experiments, all large osteoclasts in the culture dish with more than 20 nuclei per cell were counted to quantify any recovery in OCG in migration-defective Flna-null monocytes. At 1.25 x 10^5 cells/well, Flna-null monocytes continued to exhibit a significant defect in the
formation of large osteoclasts with >20 nuclei compared to WT monocytes. However, as the density increased four-fold to 5 x 10^5 cells/well, there was complete recovery in the formation of these large osteoclasts to WT levels, illustrating that migration-defective Flna-null monocytes could fuse to the same degree as WT monocytes if intercellular distances decreased. The Flna-null osteoclasts formed at the highest density were also equally functional as their WT counterparts, as evidenced by the same degree of mineral resorption when they were grown on dentin discs (Fig. 4A).

*Actin dynamics, Rho family GTPase activation and rescuing defective migration using TAT fusion proteins*

The ability of cells to undergo directed locomotion depends on the availability of high-affinity actin free barbed ends (FBEs) needed for actin polymerization at the leading edge of migrating cells. To explore the mechanisms by which Flna regulates migration, the ability of activated WT and Flna-null monocytes to facilitate the assembly of exogenously-added pyrene-actin was measured, with the rate of actin polymerization being proportional to the number of actin free high affinity filament ends (FBE). After activation with M-CSF, the rate of actin polymerization was two-fold higher for WT monocytes than for Flna-null monocytes (Fig. 5A). These results suggest that Flna regulates osteoclast formation during the pre-fusion phase by facilitating actin FBE generation and the actin polymerization necessary for cellular migration.

As mentioned above, the Rho family of small GTPases Rac1, Cdc42, and RhoA, all mediate different aspects of actin dynamics during cell migration. The activation of these small GTPases was investigated to elucidate the signaling mechanisms through which filamin A regulates actin remodeling. Levels of activated, GTP-bound isoforms of the GTPases were compared in resting and M-CSF-stimulated monocytes from WT and Flna-null mice. Since Flna
binds these GTPases, we hypothesized that Flna-null monocytes have a defect in the activation of these GTPases, resulting in defective migration. After stimulation by M-CSF, normal activation of Rac1 and Cdc42 seen in WT monocytes was absent in Flna-null monocytes (Fig. 5B,C). Flna-null monocytes also exhibited a complete defect in RhoA activation, compared to significant RhoA activation for WT monocytes (Fig. 5B,C). There was no significant difference in the total Rac1, Cdc42, or RhoA expression between WT and Flna-null monocytes.

To confirm that defective Rho GTPase activation downstream of Flna led to the impaired migration, we attempted to rescue Rac1 and Cdc42 in the Flna-null monocytes by introducing constitutively active Rac1 (Rac1-CA) and Cdc42 (Cdc-CA) TAT fusion proteins into the monocytes undergoing M-CSF mediated chemotaxis (Fig. 5D). Rac1-CA and Cdc-CA, when added in combination, fully rescued Flna-null monocyte migration to M-CSF, while adding them individually resulted in partial rescue. As negative controls, dominant negative (DN) forms of Rac1 and Cdc-42 dampened the level of migration toward M-CSF when used alone, and completely eliminated all migration when used in combination in WT cells. Addition of pTAT-HA alone did not alter migration characteristics when compared with vehicle-treated controls in WT or Flna-null monocytes, showing that the observed effects were specific to the GTPase fusion proteins. These results confirmed that active Rac1 and Cdc42 in combination rescued migration-defective Flna-null cells, thus directly linking monocyte migration to the activation of these Rho GTPases downstream of Flna.

_Activation of RANKL-RANK signaling pathway and expression of key osteoclast genes_

We examined whether RANKL-RANK signaling was intact in Flna-null monocytes/osteoclasts by evaluating NFkB and _c-jun_ activation. Both transcription factors were activated and translocated to the nucleus of both WT and Flna-null multinucleated osteoclasts.
(Fig.6A), illustrating intact RANKL-RANK signaling. In a small subpopulation of mononuclear WT and Flna-null osteoclasts, both \textit{c-jun} and NFκB also translocated to the nucleus, while only \textit{c-jun} was activated in the majority of mononuclear osteoclasts. Key osteoclast genes TRACP, cathepsin K, and the fusion receptor DC-STAMP were measured using quantitative real-time PCR during the first five days of OCG (Fig. 6B). We found an overall reduction in TRACP and cathepsin K expression in Flna-null osteoclast cultures vs. WT during the 5 days; however, DC-STAMP expression was statistically unchanged between the two genotypes.

\textit{Characterization of Flna-null mouse phenotype}

To investigate whether decreased \textit{in vitro} osteoclastogenesis was associated with \textit{in vivo} skeletal effects, the mouse skeleton was analyzed. Surprisingly DEXA results showed that Flna-null mice were osteoporotic. When comparing differences in genotype alone (thus disregarding the gender variable), bone mineral content (BMC) in the whole body, right femur, and lumbar vertebrae were all significantly decreased in Flna-null mice compared to WT ($p<0.05$), while decreases in bone mineral density (BMD) were significant in the whole body and lumbar vertebrae ($p<0.05$). When comparing bone parameters within gender, DEXA showed that Flna-null mice had significantly decreased BMC in the whole body in males, and in the lumbar vertebrae in males and females (Fig. 7A). Male Flna-null mice also had significantly decreased BMD in the whole body, right femur, and lumbar vertebrae (Fig. 7B). However, histologic analysis of femoral head sections showed decreased TRACP staining in Flna-null bone sections vs. WT sections (Fig. 7C). The number of small osteoclasts with only two nuclei was not significantly different between WT and Flna-null mice, however WT mice had significantly more osteoclasts with three or more nuclei than Flna-null mice, mirroring the results seen \textit{in vitro}. 
(Fig. 7D). Incidental findings of the Flna knock-out phenotype included increased weight and significantly increased percentage body fat (Fig. 7E).

To reconcile how Flna-null mice could be mildly osteoporotic while concurrently having reduced histological bone osteoclast levels, we measured the bone formation markers alkaline phosphatase\(^{170}\) and serum osteocalcin\(^{171}\) to assess the level of bone formation (Fig. 7F). As suspected, Flna-null total bone marrow cells cultured under mineralizing conditions resulted in significantly reduced alkaline phosphatase activity vs. WT bone marrow cells. This was corroborated by significantly reduced serum osteocalcin levels as measured by an ELISA. Steady-state serum ionized calcium was measured as an indicator of bone turnover; we found that calcium levels were mildly but significantly reduced in Flna-null mice (Fig. 7G). However, no difference in serum parathyroid hormone levels was detected in the two mouse genotypes (Fig. 7G).

**Discussion**

Filamin A is a ubiquitous, cytoplasmic actin-binding protein that plays regulatory roles in cell migration, actin cytoskeleton reorganization, and cell signaling.\(^{89,111,123}\) Its role in the actin cytoskeleton has been firmly establish as a cross-linker of F-actin into high angle orthogonal networks, thus stabilizing the actin cytoskeleton.\(^{108}\) It not only binds actin, but has also been shown to bind to transmembrane receptors, signaling intermediates and transcription factors,\(^{108,113}\) suggesting that it may be a key signaling intermediate in a number of actin-based cellular processes. In addition to neuronal defects present in PVNH, the physiological importance of FLNa \textit{in vivo} is reflected by the myriad of other syndromes as a result of missense mutations in the \textit{FLNa} gene, including otopalatodigital syndrome, frontometaphyseal dysplasia,
and Melnick Needles syndrome which are characterized by a combination of skeletal dysplasia, and anomalies in the craniofacial, cardiac, genitourinary, and intestinal structures.\textsuperscript{119,120,172}

\textit{Characterization of the filamin A-null phenotype and osteoclastogenesis}

In this study, we showed that Flna is required for osteoclast formation, as Flna-null monocytes had deficiencies in osteoclastogenesis both \textit{in vivo} and \textit{in vitro}. As shown by our validation experiments, the Flna knock-out in granulocytes did not alter the number of mesenchymal stem cells or osteoclast progenitor cells, nor did it decrease monocyte or pre-osteoclast cell adhesion which could account for the observed defect in osteoclastogenesis. This is consistent with others who have also shown that Flna-deficient melanoma cells display normal adhesive properties.\textsuperscript{114} Under normal \textit{in vitro} plating densities, Flna-null osteoclasts were smaller, less numerous, and contained fewer nuclei per osteoclast. Quantification of osteoclasts \textit{in vivo} in the distal femoral head revealed similar results, illustrating the \textit{in vivo} physiological relevance of Flna in osteoclastogenesis. Although decreased \textit{in vivo} osteoclast numbers would suggest an osteopetrotic phenotype as a result of decreased bone remodelling, DEXA surprisingly showed that Flna-null mice had a mild osteoporotic phenotype. We investigated whether Flna-null mice had a deficiency in osteogenic potential. Two indicators showed this to be valid: bone marrow cells cultured from Flna-null mice had significantly decreased alkaline phosphatase activity suggestive of decreased osteoblast precursor numbers and/or activity; and serum osteocalcin levels, a serum bone formation marker,\textsuperscript{171} were also decreased vs. WT. Thus, Flna-null mice had a phenotype consistent with low-turnover osteoporosis, where decreases in BMD, BMC, and osteoclast and osteoblast numbers are observed.\textsuperscript{153} Decreased \textit{in vivo} osteoclast numbers manifested in a mild but significant decrease in serum ionized calcium, but changes in PTH levels could not be detected in our animal cohort, likely as a result of the decrease in serum
calcium not reaching a threshold to stimulate increased PTH production. A full-scale histomorphometric analysis of Flna-null mice is currently underway to further characterize the Flna-null skeleton.

The cause for the decrease in osteogenesis in Flna-null mice is unknown, but it is likely due to a parallel decrease in osteoclast numbers, since osteoblastic and osteoclastic activities are coupled during bone remodelling to maintain homeostasis. We attribute the incidental finding of the large increase in body fat and weight in Flna-null mice to a shift in mesenchymal stem cell differentiation from osteogenesis to adipogenesis. Osteoblasts and adipocytes are derived from common multipotential mesenchymal stem cell progenitors, with Runx2 driving osteoblasts and PPARγ driving adipocytes. It has been shown that the number of osteoblasts and adipocytes have an inverse relationship. When a dominant-negative N-cadherin mutant was expressed transgenically in osteoblast progenitors thus reducing its osteogenic potential, mice had increased adipogenesis. Conversely, PPARγ-deficient mice were shown to exhibit decreased adipogenesis and increased osteogenesis.

Filamin A regulates monocyte migration

As Flna is an important regulator of the actin cytoskeleton, we hypothesized that its absence likely disrupted normal cellular processes in osteoclastogenesis which require rapid actin reorganization, such as during monocyte migration. Using M-CSF and RANKL as chemoattractants, we showed that Flna is crucial to monocyte migration. Previous studies in other cell types have shown that Flna is important for cell migration. Flna has been shown to be required for the stabilizing of the orthogonal actin network in the leading edge lamellae of migrating cells, while migration-defective Flna-deficient M2 cells show cortical instability resulting in surface blebbing and abnormally long, thin actin filaments. Flna is also needed
for the actin reorganization responsible for filipodia formation leading to cell migration. Human FLNa loss-of-function mutations result in periventricular nodular heterotopia due to migratory defects in cortical neurons. We recently found that Flna-null neutrophils exhibit defective uropod retraction during neutrophil chemotaxis, which was responsible for their reduced rate of migration toward fMLP (unpublished data). To rescue osteoclastogenesis in migration-defective Flna-null monocytes, we cultured monocytes at increasing densities to determine if Flna-null monocytes could overcome their locomotive defect and fuse if intercellular distances decreased. We confirmed that when cell densities quadrupled, in effect decreasing intercellular distances that monocytes must travel before fusing, there was significant recovery of the formation of large, fully functional Flna-null osteoclasts approaching WT levels, indicating that Flna-null monocytes were competent in fusing if the element of migration was eliminated. This experiment also showed the importance of monocyte migration in osteoclastogenesis, a topic that has not been adequately addressed in the literature.

**Actin polymerization dynamics**

Cellular locomotion requires rapid actin cytoskeleton reorganization. Actin monomers polymerize at the leading edge of migrating cells, forming flat, broad cellular protrusions called lamellipodia, and thin finger-like protrusions called filipodia. This polymerization is dependent on the availability of high-affinity actin free barbed ends (FBEs) from which new actin filaments are produced to drive migration, due to the barbed end being more thermodynamically favoured for elongation by new actin monomers. To investigate whether decreased migration in Flna-null monocytes could be a result of a defect in actin nucleation, we measured the ability of activated WT and Flna-null monocytes to generate actin FBEs. Activated, Flna-null monocytes showed half the actin polymerization rate compared to activated WT monocytes,
reflecting a two-fold reduction in available actin FBEs. Using monocytes, we previously showed that decreased actin FBE generation accompanies decreased migration. This confirms that Flna is required for normal actin polymerization dynamics during cell migration.

**Cell signaling and osteoclast gene expression**

Osteoclastogenesis requires the differentiation cytokine RANKL binding its receptor RANK, leading to intracellular RANK-TRAF-6 interaction and subsequent activation of transcription factors NFκB and AP-1 (consisting of c-\textit{fos} and c-\textit{jun}). Although Flna has a signaling role in mediating actin remodeling, major RANKL signaling pathways in osteoclasts do not involve Flna. We showed that the RANKL-RANK signaling axis remained intact in both mononucleated and multinucleated Flna-null osteoclasts, as seen in the nuclear localization of NFκB which is the definitive indicator of active NFκB. RANKL, while required as a differentiation factor in osteoclastogenesis, has a secondary role as an anti-apoptotic factor whose action is mediated by the JNK/c-\textit{jun} pathway. Moreover, c-\textit{jun} complements AP-1 activation upstream of NFATc-1. We show here that Flna-null osteoclasts also expressed active c-\textit{jun} localized within the nuclei. Thus, Flna does not mediate osteoclast differentiation or survival downstream of RANK, but it is required in the early stages where dynamic actin remodeling is crucial for pre-osteoclast migration.

RANKL-inducible DC-STAMP expression, required for pre-osteoclast fusion, remained unaltered in Flna-null cells at the transcript level, while both cathepsin K and TRACP were decreased compared to WT cells. We attribute these results to decreased numbers of multinucleated Flna-null osteoclasts contributing to lower cathepsin K and TRACP expression, while DC-STAMP would be expected to be expressed at WT levels since Flna-null pre-
osteoclasts have the potential to fuse if grown at high cellular densities, indicating normal levels of fusion receptor expression.

**Rho family GTPases directly regulate monocyte migration**

It is well established that the actin cytoskeleton is regulated via Rho family GTPases. For example Rac1 and Cdc42 are important in the leading edge lamellae where the actin polymerization rate is the highest.\textsuperscript{179-181} Previous studies have shown that M-CSF stimulation of osteoclasts leads to activation of Rac, Cdc42, and RhoA.\textsuperscript{20,159} We investigated whether decreased actin polymerization and the resultant migratory defect in Flna-null monocytes accompany decreased Rho GTPase activation. While total levels of Rac1, Cdc42, and RhoA were similar in WT and Flna-null monocytes, activation of all three GTPases was impaired in Flna-null monocytes, confirming Flna is necessary in the signal transduction cascade leading to Rac1, Cdc42, and RhoA activation downstream of the M-CSF receptor. These results are in line with multiple studies investigating the role of Rho GTPases in osteoclast migration and osteoclastogenesis.\textsuperscript{73,96,159} A recent study from our laboratory showed that osteoclast progenitors lacking Rac1 suffered significant defects in osteoclast formation with concomitant defects in migration, FBE generation, and reactive oxygen species generation.\textsuperscript{100}

Using TAT fusion proteins, we showed that Rac1 and Cdc42 directly regulate migration in monocytes. TAT fusion proteins, composed of an N-terminal, 11 amino acid sequence derived from HIV, are widely acknowledged to easily enter difficult-to-transfect cells, such as primary monocytes and osteoclasts.\textsuperscript{167,168,182} Many have shown that TAT proteins enter almost 100% of the cell population within minutes and remain in the cytosol for up to several days in culture. Constitutively active isoforms of Rac1 and Cdc42, when added in combination to Flna-null monocytes, fully rescued their impaired migration to WT levels, while the presence of each one
individually only partially rescued migration, implicating the requirement for at least both Rac1 and Cdc42 for optimal migration. These results corroborate with the signaling role of Flna in coordinating the activation of Rho GTPases, in that Flna-null monocytes will migrate as long as Rho GTPases are activated. Our group has previously shown that RhoA activation is dependent on the presence of active Rac1, and we believe that the addition of Rac1-CA stimulates the activation of native RhoA, thus making the need for adding RhoA-TAT redundant. Thus, we offer direct evidence that filamin A regulates monocyte migration via Rho GTPase activation.

**Conclusion**

This is the first study to demonstrate a role of Flna in osteoclast biology. This novel study provides the first evidence that filamin A regulates osteoclastogenesis by controlling actin dynamics via Rho family GTPase activation, enabling pre-fusion monocyte migration. Although Flna is not a member of the classical RANKL-RANK signaling pathway in osteoclasts, its importance in the early stages of osteoclastogenesis should not be ignored.

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A) Western blot analysis showing Flna protein expression only in WT monocytes. β-actin was used for loading control. RANK and c-FMS protein expression in WT and Flna-null monocytes were equal as well. B) Osteoclast progenitor numbers in WT and Flna-null mice were not significantly different as determined by a colony-forming unit (CFU) assay ($p>0.2$, $n=4$). Bone marrow stem cells were isolated and cultured for 6 days in 1% MethoCult medium supplemented with 60 ng/ml M-CSF and 40 ng/ml RANKL. The number of large colonies containing $>10$ cells was determined, each corresponding to one osteoclast progenitor. C) A colony-forming unit assay for fibroblasts was performed, which is a representation of the level of mesenchymal cells in each genotype, revealing no significant difference between WT and Flna-null mice ($p>0.2$, $n=4$).
Flna-null monocytes had a marked defect in osteoclastogenesis (OCG) after 6 days in culture. A) Representative photomicrographs of OCG, showing more large osteoclasts in WT than Flna-null cultures. B) WT monocytes were able to form significantly more small osteoclasts with 3 to 7 nuclei as well as larger osteoclasts with 8 or more nuclei vs. Flna-null monocytes (*p<0.01, n=3). There was no difference in the level of small, TRACP-positive osteoclasts with 1 or 2 nuclei. C) Monocytes were cultured for 6 days on calcium phosphate Osteologic discs to induce osteoclast formation and subsequently TRACP-stained. The number of nuclei within TRACP-positive osteoclasts (OCs) and total nuclei per field of view (FOV) were quantified and this was expressed as percent nuclei in OCs/FOV as an index for fusion efficiency. Significantly higher percentage of total nuclei was found within WT osteoclasts than Flna-null osteoclasts, illustrating decreased fusion events in Flna-null cultures (**p<0.05, n=3).
Monocyte migratory ability through a porous membrane was measured using M-CSF and RANKL as chemoattractants in a Boyden chamber set-up. The level of migration was expressed as the average number of DAPI-labelled nuclei counted per FOV. A) WT monocytes showed significantly increased migration toward M-CSF vs. Flna-null monocytes at all concentrations used (*p<0.05, n=3). There was no appreciable migration of Flna-null monocytes toward M-CSF even at 100 ng/ml. B) WT monocytes exhibited a dose-dependent increase in migration toward RANKL that was significantly higher than Flna-null monocytes (*p<0.05, n=3). There was a small but insignificant increase in Flna-null monocyte migration when comparing 0 and 500 ng/mL RANKL (p>0.4).
Effects of monocyte density on osteoclastogenesis. A) Representative photomicrographs of osteoclasts formed by WT and Flna-null monocytes cultured at three densities in 6-well plates after 6 days. Flna-null monocytes formed fewer and smaller osteoclasts vs. WT at 1.25x10^5 cells/well, but formed similar numbers and sizes of osteoclasts as WT at 5x10^5 cells/well. Resorption of dentin disks by WT and Flna-null osteoclasts grown at the highest density showed equally resorptive osteoclasts, as shown by the resorption pits after staining in a 1% toluidine blue solution (right panels). B) Quantitative data of results in (A) illustrating the progressive recovery of osteoclastogenesis for Flna-null monocytes at increasing cell densities. In this experiment, all large osteoclasts (those with \( \geq 20 \) nuclei per cell) in each culture well were counted. Osteoclasts (OC) were categorized into those with 20-29 nuclei, 30-39 nuclei, 40-49 nuclei, and \( > 50 \) nuclei. At the two lower monocyte densities, there were significantly more WT than Flna-null osteoclasts in most of the \# nuclei/OC categories (*p<0.05, n=3). At 5x10^5 cells/well, there was no difference between the number of large osteoclasts formed by WT and Flna-null monocytes. Statistical analysis was performed by comparing WT vs. Flna-null cell counts at each cell density and for each nuclei/OC category.
Fig. 5

A

![Bar chart showing fold increase in actin polymerization rate](image)

B

<table>
<thead>
<tr>
<th>M-CSF</th>
<th>WT (--)</th>
<th>Flna-null (+)</th>
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<tr>
<td>Cdc42</td>
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C

![Bar chart showing fold change in active GTPase level](image)
A) The rate of actin polymerization was evaluated in resting and activated WT and Flna-null monocytes, and results were expressed as fold increase in actin polymerization rate over resting values. Activation was performed by stimulating monocytes with M-CSF (20 ng/ml) for 40 min. WT monocytes exhibited a two-fold higher actin polymerization rate after activation than Flna-null monocytes (*p<0.05, n=5). Actin polymerization was completely abrogated with the pre-incubation of monocytes with cytochalasin D (2 µM) which blocks free barbed ends (FBEs, not shown), demonstrating that this assay measures actin free barbed ends and not pointed ends. B) Immunoprecipitation (IP) of active GTP-bound forms of RhoA, Rac1, and Cdc42 in resting and M-CSF-stimulated WT and Flna-null monocytes. Representative Western blots from a minimum of three experiments, showing the increase in RhoA, Rac1, and Cdc42 activation for WT monocytes after M-CSF stimulation. There was no appreciable activation of these Rho GTPases for Flna-null monocytes, and no difference in total levels of these GTPases in each genotype. C) Quantification of band intensities using Image J software. IP results were normalized against corresponding total GTPase levels, and the degree of Rho GTPase activation was expressed as fold change in active GTPase level over resting values (*p<0.05; n=3 for Rac1 and Cdc42, n=4 for RhoA). D) Migration of Flna-null monocytes toward 100 ng/ml M-CSF was rescued by pre-incubating cells with constitutively-active (CA) Rac1 and Cdc42 TAT fusion proteins prior to starting the migration assay. The Rac1 and Cdc-CA TAT proteins did not further increase the migration of WT monocytes; however, dominant-negative (DN) forms of the proteins had an additive effect on inhibiting WT monocyte migration. Conversely, Rac1-CA and Cdc-CA individually increased Flna-null monocyte migration, and when added together, fully rescued their migration to WT levels. Statistical significance was measured by comparing the number of monocytes that migrated in the presence of M-CSF to resting values within each treatment group (*p<0.05, n=3).
A) Integrity of the RANKL-RANK signalling pathway in Flna-null cells was compared to WT cells by immunostaining for nuclear localization of NFκB and c-jun after 6 days in culture with M-CSF and with or without RANKL. Both multinucleated WT and Flna-null osteoclasts showed clear nuclear localization of NFκB and c-jun in the presence of RANKL, as well as TRACP-positive staining. A few mononuclear osteoclasts also showed nuclear staining of NFκB (inset), but NFκB staining was cytoplasmic in most mononuclear osteoclasts. When no RANKL was added, neither NFκB nor c-jun was activated as shown by their cytoplasmic staining, and TRACP-staining was negative. B) Expression of cathepsin K, TRACP, and DC-STAMP were measured over a 5-day course of OCG by qRT-PCR using GAPDH as an internal control. Gene expression in Flna-null cells are expressed relative to WT cells. Both cathepsin K and TRACP expression were reduced in Flna-null cells, reaching statistical significance on 3 to 4 days out of 5 (*p<0.05, n=3). However, DC-STAMP expression in Flna-null cells was unchanged from WT levels.
Fig. 7

A

BMC

M F M F M F

Whole Body R Femur L Vertebra

0.4 0.3 0.2 0.1 0 0.05

B

BMD

M F M F M F

Whole Body R Femur L Vertebra

0.08 0.06 0.04 0.02

C

WT Fima-null

D

mean OC/section

2 3 4 5 6 >7

nuclei/osteoclast

WT Fima-null
DEXA analysis was performed on age-matched male (M) and female (F) WT and Flna-null mice to characterize the mouse skeletons. When gender was not factored into the analysis, Flna-null mice had significantly decreased BMC compared to WT in the whole body, right femur, and lumbar vertebrae, and significantly decreased BMD in the whole body and lumbar vertebrae (both $p<0.05$, not shown) A) When gender was factored into the analyses, BMC measurements showed that Flna-null mice had reduced bone mineral content compared to WT mice, reaching statistical significance for male mice in the whole body, and male and female mice in the lumbar vertebrae (*$p<0.05$, n=4). B) BMD measurements showed that Flna-null mice had a mild decrease in bone density, reaching statistical significance for male mice in the whole body, right femur, and lumbar vertebrae (*$p<0.05$, n=4). C) In vivo osteoclast numbers were quantified in histological sections of WT and Flna-null femoral heads after TRACP staining. Representative photomicrographs taken at 100x magnification of WT and Flna-null femoral head sections illustrating more TRACP staining of large multinucleated cells in WT sections. Osteoclasts are indicated by the arrows. D) The number of osteoclasts (OC) and the number of nuclei within each were quantified in three separate histological sections per mouse and expressed as mean OC per femoral head section. No significant difference was observed in the number of osteoclasts with 2 nuclei between WT and Flna-null sections, but WT sections had significantly more osteoclasts with 3 or more nuclei vs. Flna-null sections (*$p<0.05$, n=3). E) Increases in % body
fat and weight were observed in Flna-null mice (*p<0.05, n=4). F) Two indicators of bone formation, serum osteocalcin level and alkaline phosphatase activity in cultured BM cells, were both significantly decreased in Flna-null mice (*p<0.05, both n=4). G) A small but significant decrease was observed in serum ionized calcium levels in Flna-null mice, but parathyroid hormone (PTH) levels were not changed (*p<0.05, n=6).
Chapter 3

Sbds is required for Rac2-mediated monocyte migration and signaling downstream of RANK during osteoclastogenesis
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Contributions: RL designed and performed research, collected, analyzed, and interpreted data, performed statistical analysis, and wrote and revised the manuscript; KC performed research, collected and analyzed data, and performed statistical analysis; YW designed research and analyzed data; JR contributed vital tools (bred transgenic mice), analyzed data, and wrote the manuscript; and MG designed research, analyzed data, and wrote the manuscript.
Abstract

Shwachman-Diamond syndrome (SDS) results from mutations in the SBDS gene, characterized by exocrine pancreatic insufficiency, and hematological and skeletal abnormalities. Neutropenia and neutrophil dysfunction are hallmark features of SDS; however, causes for the bone defects have not been well studied. Dysfunction of bone-resorbing osteoclasts, formed by the fusion of monocytic progenitors derived from the same granulocytic precursors as neutrophils, could be responsible. We report that SBDS is required for in vitro and in vivo osteoclastogenesis (OCG). Sbds-null murine monocytes formed osteoclasts of reduced number and size due to impaired migration and fusion required for OCG. Phenotypically, Sbds-null mice exhibited low-turnover osteoporosis consistent with findings in SDS patients. Western blotting of Rho GTPases that control actin dynamics and migration showed a 5-fold decrease in Rac2, while Rac1, Cdc42, and RhoA were unchanged or only mildly reduced. Although migration was rescued upon Rac2 supplementation, OCG was not. This was attributed to impaired signaling downstream of RANK and reduced expression of the RANKL-dependent fusion receptor DC-STAMP. We conclude that SBDS is required for OCG by regulating monocyte migration via Rac2 and osteoclast differentiation signaling downstream of RANK. Impaired osteoclast formation could disrupt bone homeostasis resulting in skeletal abnormalities seen in SDS patients.
Introduction

Shwachman-Diamond syndrome (SDS) is an autosomal recessive disorder with hallmark features of bone marrow failure, exocrine pancreatic insufficiency, and skeletal abnormalities. Neutropenia is the most common clinical manifestation of bone marrow failure, but patients may also experience pancytopenia, anemia, and thrombocytopenia, and be at increased risk of developing aplastic anemia and acute myeloid leukemia. In early cross-sectional studies, metaphyseal dysostosis was observed in 40-80% of SDS patients, and rib and/or thoracic cage abnormalities in 30-50% of SDS patients. More recently in a longitudinal study, all of the SDS patients studied displayed varying skeletal abnormalities including delayed appearance of secondary ossification centers, variable widening and irregularity of the metaphyses in early childhood followed by progressive thickening and irregularity of the growth plates, and generalized osteopenia. In addition, a subset of patients showed early signs of osteoporotic vertebral deformities and disturbances in bone homeostasis manifesting in low-turnover osteoporosis. Oral and dental diseases and conditions including periodontitis, delayed eruption of the permanent dentition, increased caries risk in primary and permanent dentitions, and increased soft tissue pathoses, have also been reported.

SDS results from mutations in the ubiquitously expressed, conserved Shwachman-Bodian-Diamond syndrome (SBDS) gene. The exact function of the SBDS protein remains unclear; however, in recent years through studies of orthologs in yeast and Archaea and patient bone marrow cells, it has been postulated to function in RNA metabolism and ribosome biogenesis. SBDS is an essential gene in embryogenesis, and SBDS has also been implicated in cell division and cellular stress responses.
In addition to concerns of neutropenia and recurrent infections, neutrophils of affected individuals have been shown to exhibit impaired chemotaxis and deficiency in migration.\textsuperscript{131,132} A less-characterized abnormality with SDS patients is their defect in bone homeostasis, a parameter that is normally maintained via the balanced activities of bone-producing osteoblasts, and bone-resorbing osteoclasts.\textsuperscript{173} A unique feature of osteoclast formation, or osteoclastogenesis (OCG), involves multiple fusions of pre-osteoclasts derived from cells from the monocyte-macrophage lineage, a process requiring a dynamic actin cytoskeleton that is regulated by small GTPases.\textsuperscript{2,155} Two critical ligands, receptor activator of nuclear factor kappa B ligand (RANKL) and macrophage-colony stimulating factor (M-CSF) are essential for OCG: mice with ablated genes for RANKL or its receptor, RANK, are completely devoid of osteoclasts.\textsuperscript{9} Further, the addition of RANKL and M-CSF to cells of the mononuclear phagocyte lineage \textit{in vitro} is sufficient for the generation of mature functional osteoclasts.\textsuperscript{2} Since both neutrophils and monocytes are derived from the same myeloid granulocyte progenitors, and neutrophil numbers and function are impaired in SDS, osteoclasts may also be negatively affected resulting in loss of bone homeostasis.

The objective of this study was to investigate whether SBDS is required for \textit{in vitro} and \textit{in vivo} osteoclastogenesis, and if so, the possible mechanisms involved, using mice with conditional Sbds-ablation only in granulocytes, and an \textit{in vitro} OCG model we previously established.\textsuperscript{100,183}

\textbf{Materials and Methods}

\textit{Animals and isolation of bone marrow monocytes}

All procedures were performed in accordance with the Guide for the Humane Use and Care of Laboratory Animals and were approved by the University of Toronto Animal Care
Committee. Mice containing conditional (S⁺) and null Sbds alleles (S⁻) were generated as described. Ablation of Sbds in mature myeloid lineages were obtained by breeding mice heterozygous for the Sbds alleles (S⁺⁻ and S⁻⁻) to those with Cre recombinase under control of the LysM promoter (LCre/Cre), to obtain S⁺⁻ LCre/Cre mutant (Sbds-null) and S⁺⁻ LCre/Cre control (WT) genotypes. Mice were maintained on a mixed BL6/SV129 background. DNA was extracted from tail specimens to establish genotypes. PCR genotyping was performed by amplification with primers located in intron 1 of Sbds (5’AAACTGAGGCAGGAGGATTG and 5’CCCTGGAAAATGACCATAATGT) to detect S⁺ (wild-type) and S⁻ (conditional) alleles with fragments of 484 and 399 bp, respectively. The S⁻ and LCre alleles were identified as described.

To isolate bone marrow osteoclast precursors (monocytes), tibia and femora from 8 to 12-week-old mice were dissected aseptically under a laminar air flow hood, and cells were processed and isolated as previously described. Monocytes were resuspended in α-MEM supplemented with 10% fetal bovine serum and antibiotics (164 IU/ml penicillin G, 50 µg/ml gentamicin, and 0.25 µg/ml fungizone) at 0.5x10⁶ cells/ml.

**Osteoclastogenesis**

Monocytes were induced to form osteoclasts by plating 5x10⁴ cells/well in a 6-well tissue culture plate supplemented with 20 ng/ml M-CSF (M9170, Sigma) and 60 ng/ml recombinant murine sRANKL (Peprotech). Cells were cultured for 6 days, with a change of cell culture media and cytokine supplementation every second day. To evaluate the effect of increased initial cell density on osteoclastogenesis, WT and Sbds-null monocytes were also plated at 2.5x10⁵ cells/well and grown for 6 days. On day 6, cells were washed twice with PBS, fixed with 4% paraformaldehyde (PFA), and stained for tartrate-resistant acid phosphatase (TRACP). Fixed cells
were incubated in a solution of naphthol AS-BI phosphate and fast red TR salt (Sigma) in 0.2 M acetate buffer (pH 5.2) containing 100 mM sodium tartrate (Sigma) for 10 min at 37°C and subsequently washed twice with water. Red-stained cells were viewed with a Leitz Wetzlar microscope at 200X magnification, and images were taken with a PixeLink camera. The number of TRACP-positive osteoclasts and the number of nuclei within these osteoclasts were counted in 10 random fields of view (FOV).

**Histology and Dual Energy X-Ray Absorptiometry (DXA) analysis of the mouse skeleton**

DXA was performed on 12-week-old female mice after CO₂ asphyxiation, using an animal PIXIImus densitometer (Lunar; GE Corp). Data for bone mineral density (BMD) and bone mineral content (BMC) were collected for the lumbar vertebrae, left femur, and the entire skeleton after masking of the heads, as well as percentage body fat, by a single operator. Femurs for histology and subsequent TRACP-staining were processed as previously described using standard histological methods. Osteoclasts were quantified in 3 histological sections per mouse and expressed as mean osteoclast number per bone section.

**Transwell (Boyden chamber) migration assay**

Monocyte migration was evaluated using Transwell permeable supports with 5 μm membrane pores in a 24-well plate (Corning Life Sciences), as previously described. These inserts were first incubated in α-MEM growth medium for 30 min at 37°C, after which 0.5x10⁶ cells (in 200 μl) were added to the Transwell insert and incubated for 2 hr at 37°C to allow for cell attachment to the membrane. Non-adherent cells were removed, and the inserts were placed in 600 μl growth medium alone or supplemented with M-CSF (20 or 100 ng/ml) and further incubated for 2 hours at 37°C to allow for migration. The inserts were then fixed in 4% PFA, stained with 0.165 μM 4’,6-diamidino-2-phenylindole (DAPI; Sigma), and washed thoroughly with PBS. Cells adhered to the
top of the membrane but had not migrated through the membrane were removed by gentle wiping with a cotton swab and rinsing with PBS, and the membranes were cut out from the inserts and mounted onto glass slides for counting of cell nuclei. Cell nuclei in 10 random FOVs per sample were counted manually using a Nikon Eclipse E400 fluorescent microscope at 200X magnification.

*Western blotting*

At the indicated time points, monocytes/osteoclasts in culture were washed twice with PBS, and lysed with 100 µl ice-cold RIPA buffer (Sigma) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1x protease inhibitor (BD Pharmingen) for 5 min and collected by cell scraping. Cell lysates were centrifuged for 1 min at 15,000g to pellet cellular debris. 5x Laemmli sample buffer was added to the lysates and boiled for 10 minutes. Samples were subjected to SDS-PAGE using a 12% polyacrylamide gel, transferred onto nitrocellulose membrane (Amersham Biosciences), and immunoblotting was performed as previously described. Immuno-reactive protein was detected using chemiluminescence with Amersham ECL Plus (GE Healthcare) upon exposure to Bioflex MSI film (Clonex Corp). Band intensities on scanned films were quantified by densitometry using Image J 1.41 software and normalized against β-actin levels used as internal loading controls. Proteins in Sbds-null cells were presented as proportions of WT. Primary antibodies used were as follows: goat polyclonal anti-Sbds (S-15, Santa Cruz Biotechnology, Inc. (SCBT); 1:100); rabbit polyclonal anti-c-Fms/CSF-1R (C-20, SCBT; 1:600); goat polyclonal anti-RANK (M-20, SCBT; 1:600); mouse monoclonal anti-Rac1 (23A8, Upstate Biotechnologies; 1:2000); rabbit polyclonal anti-Rac2 (Upstate Biotechnologies; 1:5000); mouse monoclonal anti-RhoA (26C4, SCBT; 1:200); mouse monoclonal anti-Cdc42 (B-8, SCBT; 1:100). Secondary antibodies used were as follows: HRP-conjugated sheep anti-mouse IgG (610-603-002, Rockland Inc; 1:4000 for Rac1, 1:1000 for Cdc42, 1:2000 for RhoA); HRP-conjugated donkey anti-rabbit IgG

TAT protein constructs and rescue of migration and osteoclastogenesis

Constructs of TAT fusion proteins for protein transduction were kindly provided by the late Dr. Gary Bokoch (Scripps Research Institute, La Jolla, CA). TAT fusion proteins for wild-type (WT) and constitutively active (CA) Rac2 (G12V), Rac1-CA (G12V), and empty vector pTAT-HA were produced and purified as described. We previously determined optimal TAT fusion protein entry into monocytes with a maximal level after 10 min, and a protein concentration of 500 nM, which is comparable to conditions used by others. To observe the effects of TAT fusion proteins on migration, cells were pre-incubated for 10 min prior to being added to Transwell supports, and migration assays was performed as described above. When used in OCG rescue experiments, fresh culture media containing TAT proteins (500 nM), RANKL and M-CSF was changed daily for 6 days.

Evaluation of the RANK signaling pathway

To investigate whether signaling downstream of RANK was intact in Sbds-null cells, activation of Nuclear Factor kappa B (NFκB) and nuclear factor of activated T cells c1 (NFATc1), two transcription factors crucial for osteoclastogenesis, was measured and compared to activation of c-Jun, which is not essential. Activation of the transcription factors NFκB and c-Jun was measured firstly by their translocation into the nucleus. Osteoclasts were cultured for 6 days in 8-well chamber slides at 1x10^4 cells/well, and NFκB and c-Jun localization was evaluated by immunostaining using a NFκB and c-Jun activation kit (8400301, Thermo Scientific) following manufacturer’s directions and visualized by fluorescence microscopy at 400X magnification (Nikon Eclipse E1000). Alternatively, RANK signaling was evaluated by
measuring NFκB and NFATc1 content in nuclear extracts isolated from WT and Sbds-null osteoclasts cultured after 4 days with M-CSF and in the absence or presence of RANKL. Nuclear extracts were obtained using a nuclear extract kit (Active Motif) following manufacturer’s directions and protein concentrations were measured using the BCA method. Activation of NFATc1 was evaluated in 5 µg of nuclear extract by ELISA using the TransAM NFATc1 transcription factor assay kit (Active Motif) which binds NFATc1 using a consensus binding sequence. Similarly, activation of NFκB was measured in 0.5 µg of nuclear extract using the TransAM NFκB p65 Chemi kit (Active Motif). Specificity of NFATc1 and NFκB binding was assessed using the provided WT consensus and mutant oligonucleotide probes as competitors in the ELISA.

**Quantitative Real-Time PCR**

On days 2, 4, and 6 of OCG, RNA was extracted from WT and Sbds-null cultures (Qiagen RNeasy mini kit). Total RNA (500 ng) was reverse transcribed into cDNA using Superscript II (Invitrogen Life Technologies; Carlsbad, CA) and Oligo-dT18VN primer (ACGT Corp; Toronto, Canada). Primers were designed from the GenBank accession numbers NM_009007 for Rac1 (F5’-GAGACGGAGCTGTTGGTAAAA-3’; R5’-ATAGGCCCAGATTCACTGGTT-3’), NM_009008 for Rac2 (F5’-GACAGTAAGCCGGTGAACCTG-3’; R5’-CTGACTAGCGAGAAGCAGATG-3’), NM_007779 for c-Fms (F5’-TGTCATCGAGCCTAGTGGC-3’; R5’-CGGGAGATTCAGGGTCCAAG-3’), NM_007779 for c-Fms (F5’-TGTCATCGAGCCTAGTGGC-3’; R5’-CGGGAGATTCAGGGTCCAAG-3’), AF019046 for RANK (F5’-CTAATCCAGGGAAGCAAT-3’; R5’-GACACGGGCATAGAGTCAGTTC-3’), AB109560 for DC-STAMP (F5’-TACGTGGAGAGCAAGCAGATG-3’), AB109560 for DC-STAMP (F5’-TACGTGGAGAGCAAGCAGATG-3’), NM_009424 for TRAF6 (F5’-ACACTGAGACGTGTTTAGGAAT-3’), NM_009424 for TRAF6 (F5’-ACACTGAGACGTGTTTAGGAAT-3’).
CCTTTCAGCGACCCACAATC-3’; R5’-ACTTCGTGGCTGGAAACCCT-3’), and M32599 for GAPDH (F5’-CCTTCCGTGTTCCTACCCC-3’; R5’-GCCCAAGATGCCCCTTCAGT-3’). A 1:10 cDNA dilution was used for all primer pairs to yield optimal PCR efficiency. Quantitative real-time PCR (qRT-PCR) was performed in 20 µl reactions containing 5 µl diluted cDNA and 10 µl SsoFast EvaGreen Supermix (Bio-Rad; Hercules, CA) using the BioRad CFX96 Real Time System. Each reaction was performed in triplicate. PCR conditions were as follows: initial denaturation at 95°C for 30 sec, annealing temperature at 60°C for 1 min, extension at 72°C for 1 min, repeated for 35 cycles. A final extension at 72°C for 10 min concluded the reaction. Data were normalized with internal GAPDH control as previously described.183

Statistical analysis

For experiments in which there were multiple observations per sample, numerical results were expressed as mean ± SEM. For experiments in which there was only a single observation per sample, results were expressed as mean ± SD. Each experiment had a sample size of n≥3. Statistical analysis was performed using student’s t-test. p<0.05 was considered statistically significant.

Results

Osteoclastogenesis is impaired in Sbds-ablated osteoclast progenitors

Sbds-null monocytes had a severe defect in forming osteoclasts in vitro. TRACP staining was clearly and significantly reduced in Sbds-null vs. WT cultures (Fig. 1A-C). Significantly-reduced numbers of small (3-4 nuclei/cell), medium (5-7 nuclei/cell), and large osteoclasts (≥8 nuclei/cell) were generated from Sbds-null monocytes after 6 days in culture. Prolonging the number of days in culture to a total of 9 days did not increase the size or number of Sbds-null osteoclasts (not shown).
To ensure that the conditional Sbds knock-out did not affect important cellular characteristics limiting osteoclastogenesis, we measured the levels of osteoclast progenitors in a stem cell colony-forming assay as previously described, and the receptors to the two essential cytokines RANKL and M-CSF, RANK and c-Fms, respectively (Fig. 1D-E). Sbds-null osteoclast progenitor levels were unchanged from WT levels indicating unaffected cell proliferative activity, and RANK and c-Fms protein levels in early pre-osteoclasts after 2 days in culture were also unaltered. Thus, WT and Sbds-null pre-osteoclasts during early cell culture were quantitatively and qualitatively similar.

*In vivo* osteoclastogenesis was evaluated by TRACP-staining of femoral histological sections. Distal femur bone sections showed distinctly greater TRACP-staining in WT sections, and significantly-higher numbers of multinucleated osteoclasts than in Sbds-null sections (Fig. 1F). Phenotypically, WT and Sbds-null mice had similar body mass (22.0±1.8g and 20.5±1.3g respectively; n=6, p>0.05), indicating no significant difference in growth and development. However, DXA analysis showed that both bone mineral density and content were significantly reduced in Sbds-null mice as quantified in the whole skeleton and locally in the lumbar vertebra and left femur (Fig. 1G). Additionally, DXA showed that Sbds-null mice had significantly-higher percentage body fat compared to WT littermates (28.8±2.4% and 23.5±1.7%, respectively; n=6, p<0.005).

*Sbds regulates both migration and fusion of monocytes*

Impaired osteoclast formation could have resulted from defective monocyte migration or fusion mechanisms, or both. We showed previously that migration of monocytes into close proximity of neighboring cells is an important step in osteoclastogenesis prior to fusion, and this process is controlled by multiple Rho GTPases. Sbds-null monocytes migrated significantly less than WT monocytes at both 20 and 100 ng/mL M-CSF (Fig. 2). To measure if Sbds-null monocytes
also had a fusion defect, we cultured cells at both low and high initial plating densities, the rationale being if fusion mechanisms were unaffected in Sbds-null cells, osteoclast formation could be rescued if Sbds-null monocytes were grown close together (i.e. at high initial plating density). Significantly-more large, multinucleated osteoclasts were observed in WT cultures at the high concentration than at the low concentration (Fig. 3). However, this was not observed in Sbds-null cells, and the number of multinucleated osteoclasts was similar when grown at either concentration. Thus, Sbds-null monocytes have both migration and fusion defects.

*Sbds affects expression of genes crucial to osteoclastogenesis and actin dynamics*

Expression of genes critical to osteoclastogenesis was examined by qRT-PCR on days 2, 4 and 6 of culture (Fig. 4A). These included the pre-osteoclast fusion receptor DC-STAMP, the cytokine receptors RANK and c-Fms, TRAF6 (the downstream binding adaptor protein of RANK), and the Rho GTPases Rac1 and Rac2 (both important in actin remodeling and previously shown to be required for distinct aspects of osteoclastogenesis). During early osteoclastogenesis (day 2), transcripts of membrane proteins involved in cell signaling, i.e. RANK, c-Fms and TRAF6, were similar between Sbds-null and WT cells. However, on days 4 and 6, expression of all three genes increased two- to three-fold in WT osteoclasts, while their expression in Sbds-null osteoclasts remained unchanged from day 2 levels, resulting in only 40 to 60% of WT levels by day 6. These results suggest impairment of osteoclast differentiation signaling. DC-STAMP transcripts doubled in WT cells between days 2 and 6; however that of Sbds-null cells was consistently lower and remained at less than 20% of WT levels on day 6. Reduced DC-STAMP expression could explain the fusion defect observed in Sbds-null cells. Rac1 transcript levels were similar between WT and Sbds-null cells on days 2 and 4, but decreased slightly to 80% of WT levels by day 6 in Sbds-null cells.
Meanwhile, Rac2 levels were notably decreased in Sbds-null osteoclasts throughout the course of osteoclast formation, with only 40% on day 2 and <20% of WT levels by day 6.

We suspected deficient RANK signaling in Sbds-null osteoclasts due to decreased RANK and TRAF6 transcripts, and the failure to up-regulate DC-STAMP. Since there was a mild decrease in Rac1 and a severe decrease in Rac2, we investigated whether their gene expression is mediated by RANKL stimulation as an explanation to their decreased expression. Cells were cultured for 6 days with M-CSF and in the presence and absence of RANKL, and gene expression was compared with that of DC-STAMP which is known to be up-regulated by RANKL (Fig. 4B). Both DC-STAMP, and surprisingly Rac2, were significantly up-regulated by RANKL in WT cells by 21-fold and 3.2-fold, respectively, while in Sbds-null cells DC-STAMP only increased 7-fold and Rac2 remained unchanged. Conversely, Rac1 gene expression was not increased significantly with RANKL co-stimulation vs. M-CSF alone in either cell type (~1.2-fold for both, \( p > 0.05 \)). These results show that Sbds-null cells exhibit an impaired up-regulation of RANKL-dependent DC-STAMP and Rac2, whereas Rac1 gene expression is RANKL independent.

To verify decreased Rac during normal osteoclastogenesis, we measured protein levels of Rac1 and Rac2 on day 6 of osteoclast cultures (Fig. 5). Sbds-null osteoclasts expressed mildly-reduced Rac1 and severely-reduced Rac2 protein levels (85% and <20% of WT, respectively), confirming the decreased transcript levels observed. Evaluation of two other members of the Rho GTPase family not directly involved with osteoclast differentiation, Cdc42 and RhoA, showed unchanged levels between WT and Sbds-null osteoclasts.

Rescuing impaired migration and osteoclastogenesis by Rac1/Rac2 supplementation

A major role for Rho GTPases is to regulate the actin cytoskeleton during cellular migration. We have already shown that Rac2 is the major Rho GTPase that was down-regulated in
Sbds-null osteoclasts. Previously, we successfully employed TAT fusion proteins tethered to Rho GTPases to rescue impaired migration.\textsuperscript{183} To determine if addition of exogenous Rac2 could rescue migration in Sbds-null monocytes, we added TAT proteins fused to either wild-type Rac2 (Rac2-WT) or constitutively-active Rac2 (Rac2-CA) (Fig. 6A). Only Rac2-CA increased basal WT monocyte migration in the absence of M-CSF, and neither Rac2-CA nor Rac2-WT further increased WT monocyte migration in the presence of M-CSF. In Sbds-null monocytes, Rac2-CA increased basal migration slightly, but in the presence of M-CSF both Rac2-CA and Rac2-WT significantly rescued migration to nearly WT levels. Further supplementation of Sbds-null monocytes with Rac1-CA in conjunction with Rac2-CA did not further improve their migratory ability.

Although Sbds-null monocyte migration was nearly fully-rescued by supplementation of Rac2, osteoclastogenesis was not. Addition of the same concentrations of Rac2 TAT fusion proteins that rescued migration did not increase pre-osteoclast fusion (Fig. 6B).

\textit{Sbds is required for activation of osteoclast transcription factors downstream of RANK}

Since expression of RANK and TRAF6 was reduced in Sbds-null osteoclasts on days 4 and 6 of osteoclastogenesis, and there was a partial to total failure in the up-regulation of RANKL-dependent DC-STAMP and Rac2, we evaluated whether signaling downstream of RANK was impaired in Sbds-null osteoclasts after 6 days in culture. Nuclear localization of NFκB, a marker of RANK activation, was analyzed using fluorescence microscopy (Fig. 7A). Both large and small WT multinucleated osteoclasts showed clear nuclear localization of NFκB indicative of its active status and intact RANK signaling. However, NFκB staining was cytoplasmic in Sbds-null cells. Similar results were observed on day 4 (not shown). Staining for c-Jun, a transcription factor that mediates the anti-apoptotic effect of RANKL in osteoclasts\textsuperscript{178} but not directly downstream of RANK or
absolutely required for OCG,\textsuperscript{18} was nuclear in both WT and Sbds-null cells, illustrating that cell signaling in an unrelated pathway was not affected.

As further evidence of disturbed RANK signaling, activation of NFκB and NFATc1, another RANKL-dependent transcription factor crucial for osteoclastogenesis, was assessed in nuclear extracts purified from day 4 osteoclasts by ELISA (Fig. 7B, D). Under RANKL co-stimulation, WT nuclear extracts had a 5.8-fold increase in NFκB binding and 2.5-fold increase in NFATc1 binding compared to WT cells cultured with M-CSF only. There was no appreciable increase in NFκB or NFATc1 content in nuclear extracts of Sbds-null osteoclasts with RANKL co-stimulation. Specificity of NFκB and NFATc1 binding was confirmed by the ablated signal only in the presence of WT and not the mutant consensus oligonucleotide competitor (Fig. 7C, E). Thus, impaired activation of NFκB and NFATc1 transcription factors reflect an uncoupling of two divergent arms of signaling downstream of RANK.

**Discussion**

The spectrum of abnormalities including the hematological issues present in Shwachman-Diamond syndrome have been of interest for some time, however, mechanisms underlying the more recent observations of skeletal dysplasias have not been well-studied. Although SBDS is ubiquitously-expressed, SDS defects are relatively organ specific, i.e. pancreas, liver, bone marrow, and bone. Osteoclasts, cells responsible for bone remodeling through resorption, could potentially contribute to disruptions in bone homeostasis resulting in abnormalities observed in bones of SDS patients. Since global deletion of Sbds in mice results in early embryonic lethality,\textsuperscript{142} we used a Cre-Lox conditional knock-out strategy in which Sbds was deleted from cells of the myeloid lineages
only (monocyte/macrophage and neutrophils) to generate viable mice that enabled us to study osteoclastogenesis both *in vitro* and *in vivo*.

*Sbds is required for osteoclastogenesis*

We show that Sbds was required for the formation of large, multi-nucleated osteoclasts not only *in vitro*, but also *in vivo*. An earlier report showed a requirement for SBDS in maintaining viability of granulocyte precursors as SBDS knocked-down neutrophilic cells experienced increased apoptosis. However, others reported that SBDS was required for differentiation but not proliferation of primary mouse hematopoietic progenitors. We found that osteoclast precursor proliferation is independent of Sbds, and early Sbds-null pre-osteoclasts were qualitatively similar to WT pre-osteoclasts, as evidenced by similar expression of RANK and c-Fms on day 2. We therefore investigated potential mechanisms that could cause impaired osteoclast differentiation in Sbds-null osteoclast precursors.

*Migration is impaired in Rac2-deficient Sbds-ablated cells*

In addition to experiencing neutropenia, SDS neutrophils have chemotatic defects characterized by inabilities to orient toward a spatial chemoattractant gradient and to polarize. The SBDS homolog in *Dictyostelium discoideum*, an amoeba model to study neutrophils, is enriched in pseudopods of migrating cells, further implicating a role for SBDS in chemotaxis. Migration of monocytes/pre-osteoclasts into close proximity prior to fusion is an important step in osteoclastogenesis, and we have shown previously that defective migration alone could contribute to decreased osteoclast formation. Ablation of Sbds in monocytes resulted in impaired migration toward even the highest concentration of M-CSF used. We have shown that defective activation of multiple Rho GTPases, namely Rac1, Cdc42 and RhoA, was responsible for impaired monocyte migration. Moreover, previous studies have demonstrated that Rac2-deficient neutrophils exhibit
defective chemotaxis. As normal actin dynamics that is regulated by Rho GTPases is required for cellular migration/chemotaxis, we investigated whether members of the Rho GTPase family were deficient in quantity or defective in their activation. RhoA and Cdc42 protein levels in day 6 Sbds-null cultures were unaffected, while Rac1 was only mildly reduced and Rac2 was severely reduced. Unexpectedly, we found that in WT cells Rac2 mRNA was up-regulated by RANKL co-stimulation, while no appreciable increase was observed with Rac1. While both the closely-related Rac1 and Rac2 have been shown to be required for osteoclastogenesis, it is interesting that only Rac2 is downstream of RANK. We have unpublished data that in RAW264.7 cells, Rac2 expression was up-regulated by RANKL stimulation at the mRNA and protein level, whereas Rac1 expression remained constant. Others have also shown the RANKL-dependent up-regulation of Rac2 at the transcript level in bone marrow-derived osteoclasts and RAW cells. Since RANK signaling is impaired in Sbds-null cells (see below), it is likely that Rac2 expression does not reach adequate levels to regulate migration. The fact that Rac1 is not downstream of RANK also explains its relatively normal expression level despite RANK signaling impairment.

To clarify the role of Rac2 in monocyte migration, we rescued migration-defective, Rac2-deficient Sbds-null cells with TAT-Rac2 fusion proteins. TAT is an 11 amino acid peptide derived from HIV that we and others have used to transduce difficult-to-transfect primary cells including monocytes and osteoclasts. We observed that migration was nearly fully-rescued by either Rac2-WT or Rac2-CA, demonstrating the requirement for Rac2 in association with Sbds in monocyte migration and that the migration defect was due to decreased Rac2 expression in Sbds-null cells, not in its activation. Our results are supported by others who showed that in neutrophilic PLB-985 cells, F-actin, SBDS, and Rac2 colocalized in cellular protrusions after stimulation, and SDS neutrophils exhibited disturbed F-actin polymerization and delayed polarization, suggesting
SBDS and Rac2 may play an interdependent regulatory role in controlling actin dynamics required for directed migration. Moreover, Rac2 had been shown to regulate cofilin dephosphorylation and ARP2/3 de novo nucleation important in actin free-barbed end generation, required for leading edge protrusion in cells undergoing chemotaxis. Thus, multiple lines of evidence point to a role for Rac2 in conjunction with SBDS in regulating monocyte migration. Rac1 co-supplementation did not result in further augmentation of migration in Sbds-null monocytes, likely due to adequate native Rac1 levels to meet the minimum threshold required for migration. In recent studies, SBDS has been shown to be localized to, and stabilize mitotic spindles in human myeloid progenitors and bone marrow stromal cells, and bound to microtubules in in vitro binding studies, suggesting a role for SBDS in cell division. As microtubules are intricately linked with the actin cytoskeleton and actin-rich podosomes in osteoclasts, SBDS may additionally mediate actin dynamics via interactions with microtubules.

*Sbds regulates genes important in pre-osteoclast differentiation and fusion*

We investigated impairments in cell signaling, differentiation, and expression of the required cytokine/fusion receptors and adaptor intermediates as potential causes for impaired migration and fusion. A major signaling axis for osteoclastogenesis is the RANK-TRAF6-NFκB axis which requires the major differentiation cytokine RANKL for activation. RANKL-RANK binding is followed by the recruitment of adaptor molecule TRAF6 to the cytoplasmic tail of RANK and subsequent downstream activation of transcription factors NFκB and c-Fos, the latter within the activator protein-1 (AP-1) complex. Reduced RANK and TRAF6 expression in Sbds-null cells on days 4 and 6 likely contributed to reduced signal transduction along this axis required for osteoclast differentiation, fusion, and maturation. Moreover, in addition to being the main proliferation factor, M-CSF functions secondarily as a differentiation factor for osteoclasts; therefore although c-Fms
signaling is not downstream of RANK, decreased c-Fms expression could have had additive deleterious effects on osteoclast differentiation. Impaired RANK signaling was confirmed by greatly-reduced NFκB activation in Sbds-null osteoclasts. A second major transcription factor crucial for osteoclastogenesis is NFATc1. Activation of NFATc1, a RANKL-dependent transcription factor that is up-regulated in later-stage osteoclast differentiation via Ca\textsuperscript{2+}/calcineurin,\textsuperscript{18} was also impaired in Sbds-null cells, further evidence that RANK signaling was disturbed. Finally, Rac2 expression, which we show to be regulated by RANK, was severely low in Sbds-null cells, which ultimately prevented effective migration.

Without adequate activation of NFκB and NFATc1, crucial genes for osteoclastogenesis are not turned on, of which DC-STAMP is one example. Expression of DC-STAMP, the crucial fusion receptor absolutely required for osteoclast multinucleation\textsuperscript{30} and normally up-regulated by RANKL,\textsuperscript{52} was low in Sbds-null cells on day 2 and was reduced by more than 5-fold vs. WT on day 6. Down-regulation of DC-STAMP in Sbds-null cells likely resulted from impaired RANK signaling; this alone could explain the inability to rescue pre-osteoclast fusion by increasing cell plating density, as well as by supplementation with TAT proteins as this would not be expected to correct the deficiency in DC-STAMP expression. Hesling \textit{et al.}\textsuperscript{141} showed that in HEK293 cells, SBDS knock-down resulted in both up- and down-regulation of genes required for brain, skeletal, and blood cell development. Importantly, \textit{FOS} – one of two major components of the AP-1 complex that becomes activated during osteoclastogenesis, saw a 5.3-fold decrease in transcript level. Thus, it appears that Sbds has pleiotropic effects on the regulation of certain genes, without which subsequent osteoclast differentiation and fusion become blocked. We observed that activation of c-Jun, an anti-apoptotic mediator of RANKL in osteoclasts and a non-essential transcription factor in OCG, was not impaired in Sbds-null cells, indicating only select signaling pathways were affected.
Interestingly, HeLa cells depleted of SBDS exhibited a 3- to 6-fold increase in mRNA and secreted protein levels of osteoprotegerin (OPG), a decoy RANKL receptor. Whether Sbds-null pre-osteoclasts intrinsically produce OPG to sequester RANKL, thus preventing RANK activation leading to decreased OCG, awaits further investigation.

**Linking Sbds with bone homeostasis in SDS**

The activities of bone-producing osteoblasts must be kept in balance with that of bone-resorbing osteoclasts to maintain homeostasis. Osteoblast activity in excess of balanced osteoclast activity results in osteopetrosis, while the opposite leads to osteoporosis. A recent study of 11 SDS patients showed they exhibit low-turnover osteoporosis characterized by low bone mass (decreased BMD and BMC), reduced bone turnover, reduced numbers of osteoclasts, osteoblasts and osteoid, and vertebral fragility. These results corroborate with earlier findings of generalized osteopenia and early osteoporotic vertebral deformities in SDS patients. We show that the Sbds-null mouse phenotype accurately recapitulates those findings of SDS patients, i.e. decreased BMD, BMC and *in vivo* osteoclastogenesis. It may be counterintuitive that decreased *in vivo* osteoclastogenesis produces an osteoporotic phenotype, as the opposite would be expected. Moreover, a number of murine models in which osteoclastogenesis is either severely inhibited or altogether absent, e.g. gene mutations in M-CSF, PU.1, or RANKL, result in overt osteopetrosis. This can be explained by the complete uncoupling of bone remodeling homeostasis as a result of the non-physiological, severe impairment in bone resorption, resulting in net bone deposition. In our study, numbers of multinucleated, TRACP-positive osteoclasts, although decreased, were still present *in vivo*, thus we hypothesize that there was an over-compensation in the decrease of osteoblast activity, resulting in a net loss of BMC/BMD, and osteoporosis. Interestingly, osteoblasts and adipocytes are derived from the same mesenchymal stem cells, with Runx2 and
PPARγ driving the osteoblast and adipocyte lineages, respectively,\textsuperscript{174,175} and the numbers of each cell type share an inverse relationship.\textsuperscript{162,174} Thus the incidental finding of increased adipogenesis in Sbds-null mice is indirect evidence of decreased osteoblastogenesis and a resultant push toward the adipocyte lineage during stem cell differentiation. This is supported by previous work from our lab where a filamin A targeted knock-out to granulocytes resulted in decreased \textit{in vitro} and \textit{in vivo} osteoclastogenesis and a mild osteoporotic phenotype due to decreased bone formation, as measured by decreases in bone formation markers such as serum osteocalcin and alkaline phosphatase activity of osteoblast progenitor cells.\textsuperscript{183} The dysregulation in the crosstalk between osteoclasts and osteoblasts in Sbds-null mice await planned future osteogenic and histomorphometric studies. It follows that decreased osteoclastogenesis in SDS patients could potentially contribute to an imbalance in bone homeostasis resulting in an associated compensatory decrease in osteoblastogenesis and the array of skeletal abnormalities observed. This study was the first to evaluate osteoclast formation in Shwachman-Diamond syndrome, and validates an animal model to study osteoclast dysfunction in SDS. The evidence presented on Sbds ablation illustrates impaired monocyte migration due to a failure in RANKL-mediated up-regulation of Rac2 and impaired signaling downstream of RANK, resulting in decreased pre-osteoclast differentiation and osteoclastogenesis.

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Figure 1.
Figure 1. Sbds is required for *in vitro* and *in vivo* osteoclastogenesis.

(A) Photomicrographs of TRACP-stained osteoclasts derived from WT and Sbds-null mice after 6 days in culture with RANKL and M-CSF (both taken at 200X magnification). Significantly more large multinucleated osteoclasts were observed in WT cultures. (B) The marked difference in osteoclastogenesis between WT and SBDS-null cultures is visible to the eye. Shown are cultures derived from 3 separate WT (left) and Sbds-null (right) mice. (C) The number of osteoclasts and the number of nuclei per osteoclast were quantified. Sbds-null osteoclast cultures exhibited decreased osteoclastogenesis compared to WT cultures, as shown by significantly lower numbers of small (3-4 nuclei/osteoclast), medium (5-7 nuclei/osteoclast) and large (≥ 8 nuclei/osteoclast) osteoclasts. (*p<0.01, n=8) (D) Western blots showing that Sbds was deleted from Sbds-null monocytes. Levels of RANK and c-Fms between WT and Sbds-null cells were similar after two days of culture with RANKL and M-CSF. β–actin was used as loading control. (E) There was no difference in osteoclast progenitor levels between WT and Sbds-null mice as measured by a colony-forming assay after 10 days in culture with M-CSF and RANKL. (*p>0.05, n=3) (F) Photomicrographs of TRACP-stained distal femoral bone sections at low (100X) magnification, and the boxed areas at high (400X) magnification, representative of more intense staining in WT sections. Osteoclasts were quantified in 3 bone sections per mouse and 3 mice per genotype (right); significantly more small, medium and large osteoclasts were counted in WT sections. (*p<0.001, **p<0.005, n=3) (G) DXA analysis of age-matched female animals showed that WT mice had significantly higher bone mineral density (BMD, top panel) and bone mineral content (BMC, lower panel) in the whole body, lumbar vertebrae, and left femur than Sbds-null mice. (*p<0.01, **p<0.005, n=6)
Figure 2. Sbds ablation results in decreased monocyte migration. Migration of monocytes was evaluated in a Boyden chamber assay and two concentrations of M-CSF as a chemoattractant. Sbds-null monocytes migrated significantly less vs. WT monocytes at both 20 and 100 ng/mL M-CSF. (*p <0.01, n=5)
Figure 3. Sbds-null monocytes lack the ability to fuse even at high cell densities. Monocytes were cultured at two initial plating densities in 6-well plates, low (5x10^4 cells/well) and high (2.5x10^5 cells/well), to observe the effects of cell density on osteoclastogenesis. Representative photomicrographs taken at 200X magnification of WT (top two panels) and Sbds-null (lower two panels) cultures are shown on days 2, 4, and 6 of osteoclastogenesis, and representative TRACP–stained images of day 6 WT and Sbds-null cultures are shown on the right. At the five-fold-higher initial plating density, WT cells formed clearly more large multinucleated osteoclasts than at the lower density. However, this increase in osteoclastogenesis was not observed for Sbds-null cells, suggestive of a fusion defect.
Figure 4. Sbds ablation results in the down-regulation of multiple genes important in osteoclastogenesis. (A) Quantitative real-time PCR was used to quantify gene expression on days 2, 4, and 6 of osteoclast cultures. Results are expressed as fold vs. GAPDH expression used as internal control. Sbds-null cells expressed significantly lower transcript levels of DC-STAMP and Rac2 on day 2 compared to WT cells, while expression of c-Fms, TRAF6, and Rac1 were unchanged. RANK expression was mildly increased in Sbds-null cells on day 2. All 6 genes were down-regulated by day 6 in Sbds-null cells; however Rac1 was least affected at about 80% of WT levels. (*p<0.01, n=3) (B) To examine whether Rac1 and Rac2 gene expression is RANKL-dependent, cells were cultured for 6 days in M-CSF alone or in combination with RANKL, and qRT-PCR was performed. Results are expressed as fold activation in the presence of RANKL vs. M-CSF alone, and compared to DC-STAMP expression which is known to be RANKL-dependent. As expected, DC-STAMP was highly up-regulated by RANKL in WT cells, but this up-regulation was much less pronounced in Sbds-null cells. Rac1 gene expression was RANKL-independent; however Rac2 was up-regulated 3.2-fold in WT cells but not in Sbds-null cells. Thus, Sbds-null cells exhibit defective RANK-mediated up-regulation of Rac2 and DC-STAMP. (*p<0.05, n=3)
Figure 5. Quantification of Rho GTPase levels important in regulating actin dynamics and migration. Rho GTPase protein levels were analyzed on day 6 by Western blotting. (A) Sbds-null cells expressed slightly lower Rac1, and greatly-reduced Rac2 levels vs. WT cells. Levels of Cdc42 and RhoA were unchanged between WT and Sbds-null cells. Band intensities were normalized to β-actin used as loading control. (B) Quantification of band intensities by densitometry illustrating slightly-reduced Rac1 and greatly-reduced Rac2 levels. (*p<0.01, n=4)
Figure 6. Supplementation of Sbds-null monocytes with Rac2 TAT fusion proteins rescues migration but not osteoclastogenesis. (A) Migration of Sbds-null monocytes toward 100 ng/ml M-CSF was rescued by pre-incubating cells with wild-type (WT) or constitutively-active (CA) Rac2 TAT fusion proteins prior to the migration assay. Addition of the empty vector (pTAT-HA) had no effect on migration of WT or Sbds-null monocytes. Only Rac2-CA increased basal WT monocyte migration in the absence of M-CSF, and neither Rac2-CA nor Rac2-WT appreciably further increased WT monocyte migration in the presence of M-CSF. Rac2-CA mildly increased basal SBDS-null monocyte migration, and in the presence of M-CSF both Rac2-CA and Rac2-WT increased Sbds-null monocyte migration to nearly WT levels. Co-supplementation of cells with equimolar Rac1-CA and Rac2-CA (Rac1/2-CA) did not further increase migration of WT or Sbds-null monocytes in the presence or absence of M-CSF over and above levels achieved by Rac2-CA alone. Statistical significance was measured by comparing the number of monocytes that migrated in the presence of M-CSF to resting values within each treatment group. (*p<0.005, **p<0.01, n≥3) (B) Osteoclastogenesis was not rescued in Sbds-null cultures treated with Rac2-CA or Rac1/2-CA at concentrations that increased migration. Small (3-4 nuclei/OC), medium (5-7 nuclei/OC), and large (≥8 nuclei/OC) osteoclasts were quantified after TRACP staining as described in methods. In both genotypes, there was no difference in the number of osteoclasts in each size category after treatments by pTAT-HA, Rac2-CA, or Rac1/2-CA vs. vehicle alone. (p>0.05, n=4)
Figure 7. Sbds ablation is associated with impaired RANK signaling. (A) Signaling downstream of RANK was evaluated by immunostaining for NFκB on day 6 of osteoclastogenesis. NFκB was found to be activated only in WT osteoclasts as shown by its exclusively nuclear distribution (arrows); Sbds-null osteoclasts did not exhibit active NFκB as shown by its cytoplasmic distribution. Both Sbds-null and WT osteoclasts demonstrated activated, nuclear c-Jun, a transcription factor not directly involved in RANK signaling in osteoclasts. The staining pattern was identical in day 4 osteoclasts. (B) Alternatively, activation of NFκB was measured by performing an ELISA on NFκB content in nuclear extracts of day 4 osteoclasts. Stimulation of WT cells by M-CSF and RANKL resulted in a significant 5.8-fold increase in nuclear NFκB signal vs. stimulation by M-CSF alone. (*p<0.005, n=4) There was an
insignificant increase in nuclear NFκB signal after stimulation of Sbds-null cells with M-CSF and RANKL. \((p=0.065)\) (C) Specificity of the NFκB ELISA was verified with the addition of WT or mutant oligonucleotide competitors. WT oligo, representing the native consensus binding sequence of NFκB (5’-GGGACCTTCC-3’), significantly inhibited NFκB binding in the positive control (Jurkat cells stimulated with PMA and A23187), WT and Sbds-null nuclear extracts, whereas the mutant consensus oligonucleotide had no effect. Specificity of 1 signifies 100% specificity. \((*p<0.01, n=4)\) (D) Translocation of NFATc1 into the nucleus was evaluated as a second marker of the RANK signaling pathway by performing an ELISA on NFATc1 content in day 4 nuclear extracts. Stimulation of WT cells by M-CSF and RANKL resulted in a significant 2.5-fold increase in nuclear NFATc1 signal vs. stimulation by M-CSF alone. There was no increase in nuclear NFATc1 signal after stimulation of Sbds-null cells with M-CSF and RANKL. \((*p<0.005, n=4)\) (E) NFATc1 ELISA showed good specificity as the WT oligo, representing the native consensus binding sequence of NFATc1 (5’-AGGAAA-3’), significantly inhibited NFATc1 binding in the positive control (leucoagglutinin-stimulated Jurkat cells), WT and Sbds-null nuclear extracts, whereas the mutant consensus oligonucleotide had no effect. \((*p<0.005, **p<0.01, n=4)\)
Chapter 4

Rho GTPase techniques in Osteoclastogenesis
Roland Leung and Michael Glogauer

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Abstract

Historically, in vitro culturing of primary osteoclasts involved co-culturing of mononuclear monocytes with bone marrow stromal cells, thereby providing the cytokines required for osteoclast formation and multinucleation. Since the identification and cloning of Receptor Activator of Nuclear Factor kappa B ligand (RANKL), culturing primary osteoclasts in vitro has become much simplified. It has become apparent that the actin cytoskeleton is extremely important for the osteoclast, not only in terms of structural support, but also for adhesion, polarization, and migration. Rho family GTPases are the primary regulators of the actin cytoskeleton. In this chapter, we describe simple techniques for culturing primary osteoclasts from murine bone marrow cells, evaluating the activation states of Rho GTPases in osteoclasts, measuring the migratory abilities of monocytes, and introducing proteins of interest into osteoclasts using the TAT construct.

Key words: osteoclast, monocyte, Rho-GTPases, TAT fusion protein, migration
Osteoclasts are multinucleated, bone-resorbing cells that play important roles in bone homeostasis, a parameter that is normally maintained via the balanced activities of osteoclasts and bone-producing osteoblasts. Osteoclast activity in excess of that of osteoblasts results in a net loss of bone mass, whereas the opposite results in osteopetrosis. Pathologically, osteolytic diseases such as periodontitis, osteoporosis and rheumatoid arthritis exhibit excessive osteoclast activities. A unique feature of osteoclast formation, or osteoclastogenesis, involves multiple fusions of mononuclear monocytes/pre-osteoclasts differentiated from granulocytic progenitors in the bone marrow. Fusion is preceded by cell migration which brings cells in close proximity. In vivo, osteoclastogenesis is supported by cell-to-cell contact between the Receptor Activator of Nuclear Factor kappa B ligand (RANKL) on the surface of osteoblasts and bone marrow stromal cells, and its receptor RANK on the surface of osteoclast precursors. RANKL and Macrophage-Colony Stimulating Factor (M-CSF) are two cytokines essential for osteoclastogenesis, since mice with ablated genes for RANKL, its receptor RANK, and M-CSF are completely devoid of osteoclasts and exhibit osteopetrosis. Furthermore, the addition of RANKL and M-CSF to cells of the mononuclear phagocyte lineage in vitro is sufficient for the generation of mature functional osteoclasts.

It is well known that the actin cytoskeleton plays an important role in mature osteoclast biology as osteoclasts are dynamic, adherent cells that undergo migration, polarization during resorption, and transmigration through cell layers. Actin is especially crucial for osteoclast’s primary adhesive structures known as podosomes, which make up the most prominent component of the actin cytoskeleton in monocyte-derived cells. Actin polymerization and depolymerization within podosomes regulate osteoclast adhesion to the substratum. Cellular migration and
membrane fusion during osteoclastogenesis also require dynamic actin cytoskeleton reorganization, and it has been firmly established that the Rho family small GTPases Rac1, Rac2, Cdc42, and RhoA regulate the actin cytoskeleton. These GTPases control actin structures and actin-based cell processes both during osteoclast formation and in mature osteoclast function, including actin filament elongation during chemotaxis, formation of actin ring and sealing zone, cell spreading, and mature osteoclast polarization to form the resorption lacunae. Following stimulation, GTPases release GDP and bind to GTP, a reaction mediated by guanine nucleotide exchange factors (GEFs). In their active GTP-bound state, Rho GTPases interact with effector proteins to promote cellular responses. The active state of GTPases is transient because of their intrinsic GTPase activity, which is stimulated further by GTPase activating proteins (GAPs).

Many studies have shown the importance of Rho GTPases to osteoclast formation and/or function. The role of Rho GTPases in podosome regulation is supported by the presence of p190rhoGAP at these sites. The signaling role of Rho GTPases in osteoclast migration toward M-CSF is illustrated by the activation of RhoA and Rac downstream of the β3 integrin, a main osteoclast adhesive and signaling molecule. We showed that osteoclast progenitors lacking Rac1 suffered significant defects in osteoclast formation with concomitant defects in migration, defective actin elongation, and reactive oxygen species generation. Others have shown the importance of Rac1 in osteoclast function and survival. In osteoclast cell-spreading and cytoskeletal remodeling studies, Sakai et al. showed that Rac1 induced lamellipodia formation and Cdc42 induced moderate filopodia/lamellipodia formation, whereas RhoA had the opposite effect of inducing cell retraction. A very recent paper described the role of Cdc42 in osteoclast resorption activity, activation and differentiation signaling induced by M-CSF and RANKL, and polarization.
In this chapter, we describe a method to differentiate monocytes/pre-osteoclasts isolated from the murine bone marrow into mature, functional osteoclasts. To study the activation of Rac, Cdc42, and RhoA, we describe a pull-down assay that assess the active, GTP-bound forms of the GTPases, using M-CSF as the agonist which has been shown to activate these GTPases.\textsuperscript{20,99,159} Secondly, since it can be difficult to introduce new transgenes into primary cells, including osteoclasts, we also describe a simple method for protein transduction into primary pre-osteoclasts/osteoclasts using a TAT-construct coupled to a protein of interest. This method could be used to introduce Rho GTPases into pre-osteoclasts/osteoclasts.

2. \textit{Materials}

2.1 \textbf{Mouse Dissection and Pre-Osteoclast Isolation}

1. Dissection tools: 1 pair of surgical scissors and tissue forceps (sterilized in an autoclave)
2. 70\% ethanol
3. Sterile surgical gloves (optional)
4. 10 mL syringes
5. Sterile needles: 21 gauge and 30 gauge
6. Alpha minimal essential medium (\(\alpha\)-MEM, Life Technologies)
7. Sterile 60mm petri dishes
8. 15 mL conical tubes
9. Ficoll-Paque PLUS (Amersham Biosciences)
10. Cell counter: Z1 Coulter counter (Coulter Electronics) or a hemocytometer

2.2 \textit{In vitro Osteoclast Culture}

1. \(\alpha\)-MEM growth medium, containing 10\% fetal bovine serum and antibiotics (final concentrations 164 IU/ml penicillin G, 50 \(\mu\)g/ml gentamicin, and 0.25 \(\mu\)g/ml fungizone)
2. M-CSF (10 µg, Sigma). Dilute in 100 µl sterile water and 900 µl PBS to yield a stock concentration of 10 ng/µl, and stored in single-use aliquots at -20°C

3. RANKL (10 µg, Peprotech). Dilute in 500 µl PBS with dissolved 0.05% bovine serum albumin (Sigma)

4. 6-well tissue culture plates

5. 4% paraformadehyde (PFA) fixative solution

6. naphthol AS-BI phosphate and fast red TR salt (Sigma) in 0.2 M acetate buffer (pH 5.2) containing 100 mM sodium tartrate (Sigma)

2.3 Rho-GTPase Activation Assay

1. Phosphate-buffered saline (PBS) with calcium chloride and magnesium chloride (Sigma)

2. RIPA buffer (Sigma, ready-to-use solution containing 150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0)

3. PAK-GST glutathione sepharose beads (Cytoskeleton, Inc.) reconstituted in 500 µl water to yield a stock concentration of 1 mg/mL, and stored in single-use aliquots at -80°C

4. Rhotekin-RBD GST glutathione sepharose beads (Cytoskeleton, Inc.) reconstituted in 600 µl water to yield a stock concentration of 3.3 mg/mL, and stored in single-use aliquots at -80°C

5. 50x protease inhibitor (BD Pharmingen)

6. 1 mM phenylmethylsulfonyl fluoride (PMSF) (dissolved in ethanol)

7. Tube rotator

8. PBD/RBD wash buffer (20 mM Heps pH 7.4, 142.5 mM NaCl, 4 mM EGTA, 4 mM EDTA, 1% NP-40, 10% glycerol; store at 4°C)

9. Investigator’s choice of SDS-PAGE and immunoblot equipment
10. 12% polyacrylamide gels

11. 2x Laemmli buffer

12. Tris-buffered saline (137 mM sodium chloride, 20 mM Tris, pH 7.6) with v/v 0.05% Tween-20 (TBS-T)

13. Chemiluminescence reagent (Amersham ECL Plus, GE Healthcare)

14. X-ray film

**Antibodies required for immunoblot**

1. Primary antibodies
   a. mouse monoclonal anti-Rac1 (23A8, Upstate Biotechnologies; 1:2000 in TBS-T/5% milk)
   b. rabbit polyclonal anti-Rac2 (Upstate Biotechnologies; 1:5000 in TBS-T/5% milk)
   c. mouse monoclonal anti-RhoA (26C4, Santa Cruz Biotechnology, Inc; 1:200 in TBS-T/5% BSA)
   d. mouse monoclonal anti-Cdc42 (B-8, Santa Cruz Biotechnology, Inc; 1:100 in TBS-T/5% BSA).

2. Secondary antibodies (all diluted in TBS-T/5% milk):
   a. HRP-conjugated sheep anti-mouse IgG (610-603-002, Rockland Inc; 1:4000 for Rac1, 1:1000 for Cdc42, 1:2000 for RhoA)
   b. HRP-conjugated donkey anti-rabbit IgG (NA934V, GE Healthcare; 1:2000)

2.4 TAT Protein Constructs and Migration Rescue

1. 6His-pTAT-HA expression vector

2. BL21(DE3)pLysS competent E. coli

3. LB agar plates with the appropriate antibiotic to select for ligated expression vector
4. Luria broth

5. Isopropyl β-D-1-thiogalactopyranoside (IPTG, Sigma)

6. QIAexpress Ni-NTA Fast Start Kit (#30600, Qiagen) For the beginner, this kit offers all of the reagents necessary to easily and quickly purify 6His-tag recombinant proteins.

7. Dialysis equipment (Slide-A-Lyzer cassettes from Thermo Scientific)

8. Phosphate-buffered saline

9. 5mL sterile syringe and 21 gauge needle

10. BCA protein assay kit (#23225, Pierce)

11. Transwell permeable supports with 5µm membrane pores in 24-well plates (Corning Life Sciences)

12. 0.165 µM 4’6,-diamidino-2-phenylindole (DAPI, Sigma)

13. Cotton swabs

14. Scapel and forceps

15. Microscope slides

16. Fluorescent mounting medium

3. Methods

3.1 Mouse Dissection, Pre-Osteoclast Isolation and In vitro Osteoclast Culture

1. Euthanize animals in accordance with ethical guidelines employed at the researcher’s institution. Two commonly-used methods for euthanasia are cervical dislocation or carbon dioxide asphyxiation.

2. Euthanized mice should be immersed in, or sprayed well with 70% ethanol to decrease the chance of contamination of cell cultures. All procedures involving mice dissection and tissue culture should be done aseptically in a laminar air flow hood.
3. Using a pair of sterile forceps, lift the skin on the ventral surface of the mouse at the midpoint between the front and hind legs. Ensure that you do not lift the deeper peritoneal lining with the skin.

4. With sterile scissors, make a small 5mm cut in the skin where it has been lifted.

5. Using sterile surgical gloves, or gloves that have been well-sprayed with 70% ethanol, grab the skin on either side of the cut with your thumb and index finger and pull firmly in opposite directions, until the hind legs pull through the skin. This exposes the muscle of the hindlegs.

6. While holding the hindfoot with your non-dominant hand, de-flesh the femur and tibia using scissors as much as possible. The fibula can be discarded at this stage. (Note 1)

7. Separate the hind legs from the body at the femur-ilio joint. By holding the leg with forceps, cut off the hindfoot.

8. Separate the tibia and femora at the joint using scissors. Continue to de-flesh ensuring that the bones are clean and free of soft tissue. Place the cleaned bones in cold α-MEM in a petri dish and proceed to isolate the bones from the other leg. (Note 2)

9. Prepare a 10 mL syringe with α-MEM and attach a 30-gauge needle.

10. Cut both ends of the tibia/femora to expose the bone marrow. Hold the bone securely at mid-shaft with forceps, and insert the tip of the needle into one end of the bone and flush the bone marrow over a 60 mm sterile petri dish. Repeat this process by flushing the bone marrow from the opposite cut-end. The bone should appear chalky-white once the bone marrow has been flushed out. Repeat for the other bones.
11. Once all the bone marrow has been flushed into the petri dish, attach a 21-gauge needle to the same syringe and aspirate the cell suspension several times to break up cell aggregates.

12. Pipette cell suspension into a 15 mL conical tube avoiding tissue debris and bone spicules.

13. Pellet bone marrow cells by spinning at 1500 g for 5 min at room temperature.

14. Aspirate the supernatant from the tube, being careful not to disturb the cell pellet. Wash the pellet once with fresh α-MEM, and resuspend the pellet in 10 mL α-MEM with 10% FBS and antibiotics. Cuture cells overnight in a 100 mm petri dish in a humidified incubator at 37°C with 5% CO2. This removes stromal cells, while most osteoclast precursors will remain in suspension.

15. Collect the supernatant into a 15mL conical tube. Pellet the cells at 1500 g for 5 min.

16. Resuspend the pellet containing osteoclast precursors with 4 mL α-MEM. Using an automatic pipette on its slowest setting, or using sterile Pasteur pipettes attached to a bulb, gently layer the cell suspension over 4 ml of Ficoll-Paque PLUS in a 15 mL tube, being careful not to disturb the solution interface. The α-MEM should form a distinct layer above the Ficoll. (Note 3)

17. Centrifuge at 350g for 30 min at 4°C. The cell layer at the solution interface is enriched in osteoclast precursor cells. Using a sterile Pasteur pipette attached to a bulb, transfer the cells at the interface into a 15 mL conical tube.

18. Wash the cells 3 times with α-MEM and resuspend with 5 mL α-MEM growth medium. Count the cells either using an electronic cell counter (e.g. Coulter Z1) or a hemocytometer. Adjust the cell concentration to 0.25 x 10^6 cells/mL.
19. Add 2 mL of cells per well to a 6-well plate, resulting in $0.5 \times 10^6$ cells/well. Supplement with 20 ng/mL M-CSF and 100 ng/mL RANKL. Culture for 6 days, with a change of media and cytokine supplementation every other day. Increase initial plating density if larger or more osteoclasts are desired. (Note 4)

20. Under light microscopy, search for large, multinucleated osteoclasts. Alternatively for easier identification, osteoclasts can be stained for tartrate-resistant acid phosphatase (TRACP). Wash culture wells once with pre-warmed PBS and fix cells with 4% PFA for 15 min at room temperature. Wash fixed cells with PBS, stain with TRACP staining solution for 10-15 min at 37°C, then wash twice with water. TRACP-positive osteoclasts will appear pink-red.

### 3.2 Rho-GTPase Activation Assay

1. Plate osteoclast precursors isolated from Ficoll centrifugation (see Section 3.1. step 18) in two 100 mm petri dishes and culture for 2 days in $\alpha$-MEM growth medium without cytokine supplementation. On average, bone marrow from four mice should be isolated to yield adequate cells for each experiment (totaling about $2 \times 10^7$ cells plated per dish). (Note 5)

2. After 2 days, aspirate non-adherent cells, and gently wash adherent cells three times with PBS. (Note 6)

3. Immediately add $\alpha$-MEM growth medium to one petri dish (control cells) and to the other $\alpha$-MEM growth medium supplemented with M-CSF (20 ng/ml) and incubate for 4 hr at 37°C. M-CSF concentration and length of activation should be optimized for each investigator. (Note 7)
4. Aspirate culture media, immediately lyse cells with 200 µl ice-cold RIPA buffer supplemented with 1 mM PMSF and protease inhibitor, and collect by cell scraping using a rubber policeman. Transfer control and activated cell lysates to pre-chilled 1.5 mL microfuge tubes.

5. Centrifuge lysates for 1 min at 13000 g and at 4°C to pellet cellular debris.

6. To assess total Rho GTPase content in control and activated cells, add 20 µl of cell lysates to 5 µl of 5x Laemmli sample buffer, boil the mixture for 5 min and reserve for subsequent SDS-PAGE and immunoblotting.

7. To assess activated (GTP-bound) Rho GTPase content, add the remainder of the cell lysates to either 20 µg of PAK-GST glutathione sepharose beads (for Rac1, Rac2 and Cdc42) or 60 µg Rhotekin-RBD GST glutathione sepharose bead (for RhoA). Secure tubes to a rotator and incubate for 1 hr at 4°C and 40 rpm to allow for binding.

8. Pellet the beads for 1 min at 13000 g and at 4°C. Wash beads three times with ice-cold wash buffer and aspirate without disturbing the bead pellet.

9. Add 20 µl 2x Laemmli sample buffer and boil for 5 min to release bound proteins. These proteins are GTP-bound forms of the Rho GTPases. Spin down and pellet the beads.

10. Fractionate the total GTPase and activated GTPase samples by SDS-PAGE on a 12% polyacrylamide gel. Load the total protein and “pulled-down” protein samples in neighbouring wells, being careful to exclude the beads.

11. Transfer the fractionated proteins onto a nitrocellulose membrane using your desired apparatus.
12. Perform immunoblotting for the desired Rho GTPase: Rac1, Rac2, or cdc42 if PAK-GST beads are used, and Rho A if Rhotekin-RBD beads are used. Antibodies and their recommended starting dilutions are listed in Section 2.3.

13. Immuno-reactive proteins are observed by chemiluminescence and exposure to film.

14. Develop the film and quantify the band intensities by densitometry.

15. For both the control and activated lysates, normalize the band intensity of the pulled-down, activated protein against the corresponding total protein level. Express the degree of Rho GTPase activation by dividing the normalized value of the activated sample by the normalized value of the control sample (Figure 1).

3.3 TAT Protein Constructs and Migration Rescue

This method can be used to introduce Rho-GTPases at all stages of osteoclastogenesis. The value of protein transduction is the ability to modulate the global activation states of these GTPases by introducing constitutively-active and dominant-negative mutants into osteoclasts, and monitoring the associated changes in the cells.

1. Clone the cDNA of the protein of interest and its mutants into the multiple cloning site of the 6His-pTAT-HA expression vector. The 6His tag allows for affinity purification of the recombinant protein.

2. Transform the ligated TAT plasmids into BL21(DE3)pLysS competent cells.

3. Streak a LB agar plate containing the appropriate antibiotic with the ligated plasmid, and incubate at 37°C overnight.

4. Pick a colony and inoculate 10 mL of LB with antibiotic. Grow overnight at 37°C in a shaking incubator.
5. Add 1 mL of overnight culture to 100 mL of LB with antibiotic. Incubate at 37°C with shaking until OD$_{600}$ reaches 0.6.

6. Add IPTG (1 mM final conc.) to induce protein expression and grow for 3-4 hrs.

7. Pellet bacteria at 4000 g for 15 min at 4°C.

8. Purify TAT fusion proteins using Ni-NTA columns (QIAexpress Ni-NTA fast Start Kit) under denaturing conditions.

9. Dialyze eluted proteins in Slide-A-Lyzer dialysis cassettes in a large volume of PBS overnight at 4°C. Repeat dialysis after a change of PBS for several more hours. (Note 8)

10. Precipitates are commonly observed after dialysis; remove the protein sample with a syringe and needle and spin down the precipitate.

11. Measure protein concentration in the supernatant using the BCA protein assay kit.

3.3.1 Optimizing TAT fusion protein entry into cells

1. Prepare several 1.5 mL microfuge tubes each with 0.5x10^6 monocytes in 1 mL of α-MEM growth media.

2. Add increasing concentrations of purified TAT proteins into each tube, from 50 nM to 1 µM, and incubate at 37°C for 5 to 30 min.

3. Pellet cells by centrifugation at maximal speed, and wash 3 times with pre-warmed PBS.

4. Add 2x Laemlli sample buffer and boil for 5 min.

5. Perform immunoblotting on the HA tag to identify the optimal concentration and incubation time in order for maximal TAT protein entry. We find that entry reaches a maximum level after 10 min, and the optimal protein concentration to elicit function is 500 nM, which is in a similar range of concentrations used by others.\textsuperscript{166-168}

3.3.2 Studying the effects of TAT fusion proteins on migration
1. Incubate Transwell inserts in α-MEM growth medium for 30 min at 37°C.

2. Add 0.5x10^6 cells (in 200 µl) to Transwell inserts and incubate for 2 hr at 37°C to allow for cell attachment to the membrane.

3. Remove non-adherent cells by turning over the inserts.

4. Place inserts in 600 µl growth medium containing 100 ng/mL M-CSF and further incubate for 2 hours at 37°C to allow for migration.

5. Fix the cells on the membrane with 4% PFA.

6. Stain cell nuclei with DAPI solution for 10 min in the dark, then wash thoroughly with water.

7. With gentle wiping using a cotton swab and frequent rinsing with PBS, remove cells that have adhered to the top of the membrane but have not migrated through the membrane.

8. Cut out membrane from the insert and mount onto a glass slide using fluorescent mounting medium.

9. Using a fluorescent microscope at 200x magnification, count the number of cell nuclei in 10 random fields of view in order to quantify the mean number of cells that have migrated through the membrane.

10. To study the effects of the TAT fusion proteins on migration, pre-incubate monocytes with 500 nM TAT proteins for 10 min at 37°C prior to being added to Transwell supports. Evaluate the level of migration in control cells and in the presence of TAT proteins (Figure 2).
Notes:

1. Removing soft tissue while the legs are still attached to the body makes it much easier, since you can pull the leg straight while the weight of the body holds the mouse in place.

2. Separate the femur and tibia by feeling for a depression in the joint with scissors. To remove the remaining soft tissue, place the cutting edge of the scissors at mid-shaft and scrape towards the ends.

3. To ensure that the solution interface is not disturbed, place the tip of the pipette against the side of the tube when layering cells.

4. Osteoclastogenesis performed using this method routinely results in close to 100% of cells staining for TRACP by day 6.

5. Rho GTPase activity can also be assessed during later-stage osteoclast differentiation.
   Culture osteoclasts with M-CSF and RANKL supplementation for the desired number of days. Wash cells with PBS, and incubate cells with α-MEM growth medium alone to return Rho GTPases to their inactive state. The investigator should optimize the length of time that cells are starved of cytokines, as prolonged periods of time without cytokine supplementation will result in cell death. Proceed with step 3.

6. Non-adherent cells contain lymphocytes and do not adhere to tissue culture plastic.

7. The amount of GTPase activation can be variable depending on experimental conditions. Investigators should plan control experiments with increasing concentrations of M-CSF and varying activation times in order to identify optimal activation conditions.

8. Different pore sizes are available; choose a pore size that is smaller than the predicted size of the TAT-protein to prevent sample loss due to dialysis. Because the protein sample is injected into the cassette through a syringe port using a small needle and
syringe, one must be careful not to pierce the membrane during injection to prevent accidental loss of the sample.
Immunoprecipitation (IP) of active GTP-bound Rac1 in resting and M-CSF-stimulated monocytes. After activation by M-CSF (20 ng/mL for 4 hr), significantly more Rac1-GTP is pulled down by the RBD beads.

The ability of select Rho-GTPases and their mutants to affect the migration of monocytes toward a 100 ng/mL M-CSF chemoattractant gradient is examined by TAT protein transduction. The TAT fusion proteins are pTAT-HA (empty vector), and constitutively-active (CA) and dominant negative (DN) forms of Rac1 and Cdc42. When there is no chemoattractant, Rac1-CA and Cdc-CA increases cell migration slightly when added individually and in combination. In the presence of M-CSF, neither Rac1-CA nor Cdc-CA further increases migration even when added in combination. Rac1-DN and Cdc-DN significantly reduces migration toward M-CSF when added individually, and completely inhibit all migration when added in combination. (*p<0.05 comparing M-CSF vs. no M-CSF for each treatment group)
Chapter 5

Summary and Conclusions
Phenotypes of Flna-null and Sbds-null mice

The two animal models that we used to study the role of filamin A and SBDS in regulating Rho GTPases during osteoclastogenesis have revealed a similar phenotype that has not been commonly reported in the literature. In both animals, bone density scans showed that the mice had decreased bone mineral density and bone mineral content, and histological analysis of femurs showed a decreased level of osteoclasts *in vivo* which was confirmed by decreased osteoclastogenesis *in vitro*. These were perplexing findings that appeared at first contradictory, as it has been commonly reported in the literature that animals harbouring mutations that affected osteoclast differentiation exhibit an osteopetrotic phenotype, generally with increased trabecular thickness, decreased marrow spaces and increased bone densities. For example, murine models in which loss-of function or null mutations in genes including, but not limited to RANK/RANKL, M-CSF, c-Src, NFATc1, NFκB, and β3 integrin give rise to mice that exhibit osteopetrosis.

Since the regulation of bone remodelling to maintain a state of homeostasis is determined by the balanced activities of bone resorbing osteoclasts and bone producing osteoblasts, a decrease in concurrent osteoblastic activity with that observed in osteoclast activity may explain the phenotypes of the Flna-null and Sbds-null mice. This was exactly what was shown in the Flna-null mice, in that osteoblastic activity was significantly decreased as determined by bone formation markers such as serum osteocalcin and alkaline phosphatase activity of whole bone marrow cells in a mineralizing medium. Indirect evidence for the inhibition of osteoblast formation and/or function comes from the increased adipogenesis in these mice. Differentiation from mesenchymal progenitors into adipocytes is regulated by peroxisome proliferator-activated receptor (PPAR) γ and C/EBPs, while differentiation into osteoblasts is regulated by Runx2.
and Osterix. Since adipocytes and osteoblasts are produced by the same mesenchymal progenitor cells, inhibition in the signals that direct osteoblast differentiation can push the equilibrium toward adipocyte differentiation. Although no attempt was made to identify the source of the inhibitory signal from osteoclasts to downregulate osteoblast activity in these mice, it is possible that molecules liberated from bone during resorption are responsible. Bone is the most abundant source of transforming growth factor β (TGF-β), where it is deposited in the matrix in a latent form during bone formation, and it is released and becomes activated by the acidic environment created by osteoclasts when matrix is resorbed. TGF-β has been shown to have a biphasic effect on osteoblastogenesis: it promotes early recruitment and proliferation of osteoblast precursors and expression of matrix proteins, but it inhibits late osteoblast differentiation and mineralization and bone morphogenetic protein (BMP)-induced bone formation. Recently, a novel mechanism was discovered by which TGF-β promotes osteoblast differentiation by inhibiting the BMP-induced expression of the BMP antagonist noggin (thereby dampening the negative feedback loop), and prolonging BMP activity. The decrease in osteoclastogenesis in vivo may result in reduced TGF-β that becomes liberated from the bone reservoir, thereby decreasing early osteoblast differentiation in vivo. We saw that the Sbds-null mice had a similar phenotype as the Flna-null mice, in that they exhibited defective in vivo osteoclastogenesis, accompanied by decreased bone mineral density and content which are signs of osteoporosis, and increased adipogenesis. Although in the Sbds study we did not explore whether there was a defect in osteoblast differentiation, we made an educated inference that osteoblast differentiation was perturbed, based on the inverse increase in adipogenesis combined with the decreased osteoblast activity observed in the Flna-null mice. It is obvious that the crosstalk between osteoclasts and osteoblasts in vivo is crucial in regulating bone turnover, as
they co-exist in close proximity in space and time, thus changes in the activity of one cell type are expected to have modulating effects on the other.

Both transgenic mice had mutations only in cells from the granulocyte lineage, namely neutrophils and monocyte/macrophages, as a result of expressing the mutant gene downstream of the granulocyte-specific lysozyme M promoter. However, compensatory changes were observed in osteoblasts as well, demonstrating communication between the two cell types within the bone microenvironment. These findings are supported by others in the literature. In a recently published murine model in which PPARγ is over-expressed specifically in osteoblasts under the control of the procollagen type 1 promoter, male mice exhibited an osteoporosis phenotype, and female mice exhibited accelerated osteoporosis after ovariectomy. Bone mineral densities in males were 8-10% lower compared to WT, with highly decreased bone volume, trabecular thickness and number, and increased marrow spaces; dynamic bone formation studies showed that these mice had decreased bone formation and mineral apposition rates vs. WT. Osteoblastogenesis was inhibited \textit{in vivo}, as it was \textit{in vitro} as shown by decreased alkaline phosphatase activity and decreased transcripts of Runx2, Osterix, type 1 collagen, ALP, and osteocalcin. Osteoclast numbers and surface were decreased 40% and 58%, respectively \textit{in vivo}, and \textit{in vitro} osteoclastogenesis was inhibited by 35%, owing to an increase in OPG production by osteoblasts/stromal cells and a decrease in RANKL/OPG ratio in total bone marrow cultures. Interestingly, osteoclastogenesis induced by M-CSF and RANKL on isolated bone marrow monocytes did not exhibit a decrease in osteoclastogenesis, suggesting that there is no inherent defect in osteoclasts themselves, rather the osteoclastogenesis defect was due to inhibitory signals originating from osteoblasts. Taken together, these results unequivocally show that
overexpressing PPARγ in osteoblasts inhibited both osteoblast and osteoclast differentiation, demonstrating an intimate crosstalk between the two cell types.

In another animal model, an osteoblast-specific mutation also resulted in changes in osteoclast activity. To study the role of NFATc1 in osteoblasts, a constitutively active, nuclear-localizing NFATc1 mutation was introduced in osteoblasts (NFATc1<sup>nuc</sup> mice).<sup>27</sup> These mice exhibited profound increases in bone formation, osteoblast numbers <em>in vivo</em>, and increased serum bone formation markers. Meanwhile, the NFATc1<sup>nuc</sup> mutation was not expressed in monocytes isolated from the NFATc1<sup>nuc</sup> mice or in <em>in vitro</em> differentiated osteoclasts; however these mice exhibited a 4-fold increase in osteoclast numbers and 5-fold increase in urine deoxypyridinoline crosslinks, a marker for osteoclast activity.<sup>200</sup> The mechanism in osteoblast-induced increase in osteoclastogenesis involves an increase in monocyte chemoattractants CCL8, CCL6, and CCL12 present in bone, of which CCL8 expression by osteoblasts was directly upregulated by NFATc1. Increased <em>in vivo</em> osteoclastogenesis was not attributed to changes in RANKL, M-CSF or OPG expressed by osteoblasts which were comparable to WT mice.

These studies, along with ours, demonstrate that loss- or gain-of-function mutations in either osteoclasts or osteoblasts can have effects on the regulation of the other cell type, as their activities are normally coupled in order to maintain bone homeostasis. Uncoupling of their activities results in excessive bone production or bone resorption.

**Roles of FLNa and SBDS in regulating Rho GTPases**

The main goals of this thesis were to study the roles of two proteins, FLNa and SBDS, in osteoclastogenesis, topics that have not been addressed in the literature. The focus was on the
regulatory functions of these proteins on Rho GTPases, since they have been shown to regulate the activation of, and/or to localize with one or more members Rac1, Rac2, Cdc42, or RhoA.

Filamin A is well-recognized as a powerful actin-crosslinking protein, and in many cell types it is required for controlling cell processes involving the actin cytoskeleton. Structural, sequence, and binding studies have shown that not only does it bind actin filaments, it is a promiscuous binding partner for over 20 identified proteins, including Rac, Cdc42, RhoA, and their regulatory GEFs and GAPs. Using a mouse model in which Flna was deleted specifically in granulocytes, we show that Flna is required as a scaffold protein that coordinates the activation of Rac1, Cdc42, and RhoA, without which actin polymerization and pre-osteoclast migration are perturbed. Flna is not downstream of major osteoclast differentiation pathways, and activation of the two major transcription factors for osteoclast differentiation, NFκB and NFATc1, are not affected.

Sbds is required for osteoclast differentiation downstream of RANK. Sbds-null pre-osteoclasts have defects in RANKL-mediated upregulation of Rac2, which is required for their migration and is rescued upon Rac2 complementation. However, rescued migration was inadequate in rescuing osteoclastogenesis, as there was a general impairment in the RANKL-RANK-TRAF6 signaling pathway, resulting in the defective activation of NFκB and NFATc1. It is important to recognize that this mouse model accurately recapitulates the phenotype of SDS patients, and it should be of great benefit in future studies to investigate how Sbds regulates RANK signalling, and ultimately osteoclastogenesis.
Future Directions

Although we have shown that Sbds regulates osteoclastogenesis downstream of RANK, its exact role is still undefined. It will be of great interest to further clarify its role in regulating RANK signalling. Does Sbds play a role as a transcriptional regulator of osteoclast-specific genes that become up/downregulated? Could Sbds deletion promote the synthesis of proteins that inhibit osteoclast differentiation like OPG, as observed in HeLa cells?145 Does it function as a cytoskeletal adaptor protein during cellular migration where it localizes with F-actin and Rac2?

The phenotypes of the Flna-null and Sbds-null mice can be further characterized by histomorphometric studies, and dynamic bone formation studies evaluating osteoblast activity in vivo would complement the results from the osteoclast studies.

Finally, investigations into the crosstalk between osteoblasts and osteoclasts will shed light on how FLNa and SBDS mutations in osteoclast precursors signal to osteoblasts to regulate their function. Could it be a molecule that becomes released during bone resorption that signals to osteoblasts, like TGF-β, or an unidentified protein released from osteoclasts that functions as a paracrine signal to osteoblasts? Successful elucidation of these pathways may offer insight into the skeletal defects in patients with Shwachman Diamond syndrome and those with filamin A mutations.
Chapter 6

REFERENCES


