

Rac1 Inhibition Via *Srgap2* Restrains Inflammatory Osteoclastogenesis and Limits the Clastokine, SLIT3

Bongjin Shin,¹ Justine Kupferman,^{2†} Ewoud Schmidt,² Franck Polleux,² Anne M Delany,^{3*} and Sun-Kyeong Lee^{1*} 

¹Center on Aging, UConn Health, Farmington, CT, USA

²Department of Neuroscience, Columbia University, New York, NY, USA

³Center for Molecular Oncology, UConn Health, Farmington, CT, USA

ABSTRACT

The Rac1-specific guanosine triphosphatase (GTPase)-activating protein Slit-Robo GAP2 (*Srgap2*) is dramatically upregulated during RANKL-induced osteoclastogenesis. *Srgap2* interacts with the cell membrane to locally inhibit activity of Rac1. In this study, we determined the role of *Srgap2* in the myeloid lineage on bone homeostasis and the osteoclastic response to TNF α treatment. The bone phenotype of mice specifically lacking *Srgap2* in the myeloid lineage (*Srgap2*^{fl/fl}; *LysM-Cre*; *Srgap2* conditional knockout [cKO]) was investigated using histomorphometric analysis, in vitro cultures and Western blot analysis. Similar methods were used to determine the impact of TNF α challenge on osteoclast formation in *Srgap2* cKO mice. Bone parameters in male *Srgap2* cKO mice were unaffected. However, female cKO mice displayed higher trabecular bone volume due to increased osteoblast surface and bone formation rate, whereas osteoclastic parameters were unaltered. In vitro, cells from *Srgap2* cKO had strongly enhanced Rac1 activation, but RANKL-induced osteoclast formation was unaffected. In contrast, conditioned medium from *Srgap2* cKO osteoclasts promoted osteoblast differentiation and had increased levels of the bone anabolic clastokine SLIT3, providing a possible mechanism for increased bone formation in vivo. Rac1 is rapidly activated by the inflammatory cytokine TNF α . Supracalvarial injection of TNF α caused an augmented osteoclastic response in *Srgap2* cKO mice. In vitro, cells from *Srgap2* cKO mice displayed increased osteoclast formation in response to TNF α . We conclude that *Srgap2* plays a prominent role in limiting osteoclastogenesis during inflammation through Rac1, and restricts expression of the paracrine clastokine SLIT3, a positive regulator of bone formation. © 2019 American Society for Bone and Mineral Research.

KEY WORDS: INFLAMMATORY OSTEOLYSIS; Rac1; TUMOR NECROSIS FACTOR ALPHA; CLASTOKINE; SLIT3

Introduction

Osteoclasts are specialized multinucleated cells that resorb mineralized bone matrix. Their activity is essential for calcium homeostasis, for maintaining bone quality, and for returning repaired bone to its normal shape postfracture. However, excess osteoclast activity contributes to the development of prevalent bone diseases such as osteoporosis and to the pathology associated with osteolytic bone metastases and multiple myeloma.^(1–3)

Osteoclasts arise by fusion of macrophage/monocyte progenitors, which is induced by macrophage colony-stimulating factor (M-CSF) and receptor activator of NF- κ B ligand (RANKL), both of which can be synthesized by bone-forming osteoblasts.^(4,5) Moreover, the potent pro-inflammatory cytokine TNF α is known to stimulate osteoclastogenesis with or without involvement of RANKL.^(6–8) In vivo, TNF α increases osteoclast precursors,^(9–12)

promotes RANK expression, and stimulates the synthesis of M-CSF and RANKL by mesenchymal cells.^(13–15) This contributes to both localized bone loss at sites of inflammation as well as a generalized increase in osteoclast formation due to increased circulating TNF α .

In addition to resorbing bone, mature osteoclasts regulate the differentiation of bone-forming osteoblasts. The term clastokine was coined for osteoclast-derived factors that positively or negatively regulate osteoblast differentiation. Recently identified clastokines include bone morphogenetic protein 6 (BMP6), EphrinB2 (EFNB2), sphingosine 1-phosphate (S1P), collagen triple helix repeat containing 1 (CTHRC1), and SLIT guidance ligand 3 (SLIT3).^(16–19) The interplay of these paracrine factors between osteoclasts and osteoblasts contributes to the tight regulation of bone homeostasis.

Multiple signaling pathways regulate the differentiation and function of osteoclasts. Of these, the Rho family of guanosine

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Address correspondence to: Sun-Kyeong Lee, PhD, Center on Aging, UCONN Health, 263 Farmington Ave, Farmington, CT 06030, USA. E-mail: slee@uchc.edu
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*Sun-Kyeong Lee and Anne Delany should be considered co-corresponding authors.

†Current address: Justine Kupferman, Kallyope Inc., New York, NY 10016, USA.

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triphosphatases (GTPases), which includes RhoA, Cdc42, and Rac1, are responsible for cytoskeletal reorganization critical for cell motility and for the development of the specialized structures needed to facilitate bone resorption. During osteoclast maturation, actin polymerizes and forms podosomal rings at sealing zones on bone-resorbing surfaces. At the ruffled border within the sealing zone, protons and lysosomal enzymes are released into resorption lacuna to dissolve bone mineral and digest organic matrix.⁽²⁰⁾ Although all Rho GTPases have an impact on bone mass regulation and osteoclast function, their individual biological properties are subtle and distinct.^(21–23)

In osteoclasts, Rac1 regulates survival, migration, and actin organization, promoting resorption capacity, but not necessarily differentiation.^(23–26) Although the conformational change of Rac1 between inactive GDP-bound and active GTP-bound forms is facilitated by multiple guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs), respectively, only a small subset of these factors are established to function in osteoclasts. Rac1 deficiency or the deficiency of Rac1-specific guanine exchange factors (GEFs) (eg, Vav3, Dock5), or the failure to control Rac1 activity, causes bone disorders due to abnormal osteoclast activity.^(26–29) However, the impact of Rac1-specific GAPs on osteoclasts and bone is unknown.

In non-biased screen, we found SLIT-ROBO GTPase activating protein (*Srgap2*) RNA is dramatically upregulated in myeloid cells undergoing M-CSF plus RANKL-induced osteoclastogenesis, compared with cells treated with M-CSF alone. The expression of this Rac1-specific GAP in the osteoclast lineage has not been previously reported. In this study, we show that *Srgap2* strongly limits Rac1 activation and attenuates osteoclast-mediated bone resorption in vitro. However, mice lacking *Srgap2* specifically in the monocyte lineage (*Srgap2^{fl/fl};LysM-Cre*; *Srgap2* conditional knockout [cKO] mice) displayed increased bone formation and enhanced production of the bone-anabolic clastokine SLIT3. In contrast, *Srgap2* cKO mice exhibited augmented osteoclastogenic response to the pro-inflammatory cytokine TNF α in vivo and in vitro, suggesting that *Srgap2* plays a more prominent role in regulating osteoclastogenesis during inflammation.

Materials and Methods

Animals

Floxed *Srgap2* mice were generated using homologous recombination in C57BL/6J embryonic stem (ES) cells (see Fig. 1B for design). They were then crossed with *LysM^{Cre}* mice [*Lyz2^{tm1(Cre)}/f^o*; The Jackson Laboratory, Bar Harbor, ME, USA]. Wild-type (WT) and floxed *Srgap2* alleles were differentiated using PCR of genomic DNA (Fig. 1D). *LysM^{Cre+/-}; Srgap2^{fl/fl}* mice were crossed with *Srgap2^{fl/fl}* to generate littermate controls (*LysM^{Cre-/-}; Srgap2^{fl/fl}*) and mice with monocyte lineage-restricted deletion of *Srgap2* (*LysM^{Cre+/-}; Srgap2^{fl/fl}*; *Srgap2*-cKO). All controls and *Srgap2*-cKO mice used for experiments were 7 to 9 weeks old.

Mice were maintained and handled in accordance with the guidelines of the Institutional Animal Care and Use Committee at UConn Health. Littermates were housed in a temperature and humidity controlled specific pathogen-free facility in micro-isolator cages on a 12-hour light/dark schedule. Mice had access to standard mouse chow and water *ad libitum*, and were provided with species-appropriate enrichment. Recombinant mouse TNF α (50 μ g/kg) was injected supracalvarially daily for 4 days; 24 hours later, calvarial tissues were removed for

histology. Mice of each genotype were randomly assigned to vehicle or TNF α treatment groups.

Micro-CT and histomorphometric analyses

Right femurs from *Srgap2*-cKO and littermate control mice were fixed in 70% ethanol. Metaphyseal region of the distal femur was analyzed at 8 μ m voxel size resolution using micro-CT (μ CT) (μ CT40; Scanco Medical AG, Bassersdorf, Switzerland) to quantify trabecular and cortical morphometry.⁽³⁰⁾ The sample sizes used are sufficient to detect a 20% to 25% difference in bone parameters with 80% power, $p < .05$ with 14% coefficient of variation. Left femurs were fixed in 4% paraformaldehyde, decalcified in 14% EDTA, embedded in paraffin blocks, sectioned at 7 μ m thickness, and subjected to tartrate-resistant acid phosphatase (TRAP) and hematoxylin staining.

For dynamic bone histomorphometry, 10 mg/kg calcein (Sigma-Aldrich, St. Louis, MO, USA) and 30 mg/kg alizarin-3-methyliminodiacetic acid (Sigma-Aldrich) were injected into mice 7 and 2 days prior to euthanasia. Femurs were fixed in 4% paraformaldehyde for 6 days, incubated in 30% sucrose overnight and embedded in OCT compound (Thermo Fischer Scientific, Waltham, MA, USA). Femurs were sectioned at 7 μ m thickness using a cryostat (Leica, Wetzlar, Germany) and tape transfer system (Section-lab, Hiroshima, Japan; <http://section-lab.jp/>). Sections were analyzed using Osteomeasure software (OsteoMetrics, Decatur, GA, USA) and quantified 0.2 mm from growth plates and cortices, as recommended by the Nomenclature Committee of the American Society for Bone and Mineral Research.⁽³¹⁾

ELISA

Serum was harvested at time of euthanasia from animals at 8 weeks of age and subjected to ELISA as per the manufacturer's protocol. Bone resorption was quantitated with CTX (Mouse CTX ELISA; MyBioSource, San Diego, CA, USA), and bone formation was quantitated with P1NP (Mouse P1NP ELISA; MyBioSource) ELISAs. SLIT3 ELISA (Biomatik, Cambridge, ON, Canada) was performed using serum from control and *Srgap2*-cKO mice, as well as conditioned media from bone marrow monocyte (BMM) cultures that were treated with M-CSF and RANKL for 5 days.

RANKL-induced and TNF-induced osteoclast formation assay

Bone marrow cells were flushed from tibias and femurs with α MEM and cultured overnight on tissue culture plastic to remove stromal cells. Nonadherent cells were subjected to Ficol-Hypaque (GE Healthcare, Piscataway, NJ, USA) density gradient to prepare BMMs from the buffy coat layer. BMMs (2×10^4 cells/well in 96-well plate) were cultured with recombinant human M-CSF (30 ng/mL) and human RANKL (30 ng/mL) or mouse TNF α (30 ng/mL) for up to 5 days. Culture medium and cytokines were refreshed at day 3. Multinucleated osteoclasts were stained for TRAP and cells with three or more nuclei were counted.

Recombinant human M-CSF and human RANKL were expressed in our laboratory using the constructs kindly provided by Dr. D. Fremont from Washington University and Dr. Y. Choi from University of Pennsylvania, respectively. Recombinant murine TNF was prepared in our laboratory as follows: a murine TNF- α cDNA fragment encoding amino acid residues 83 to 235 was cloned by PCR using primers

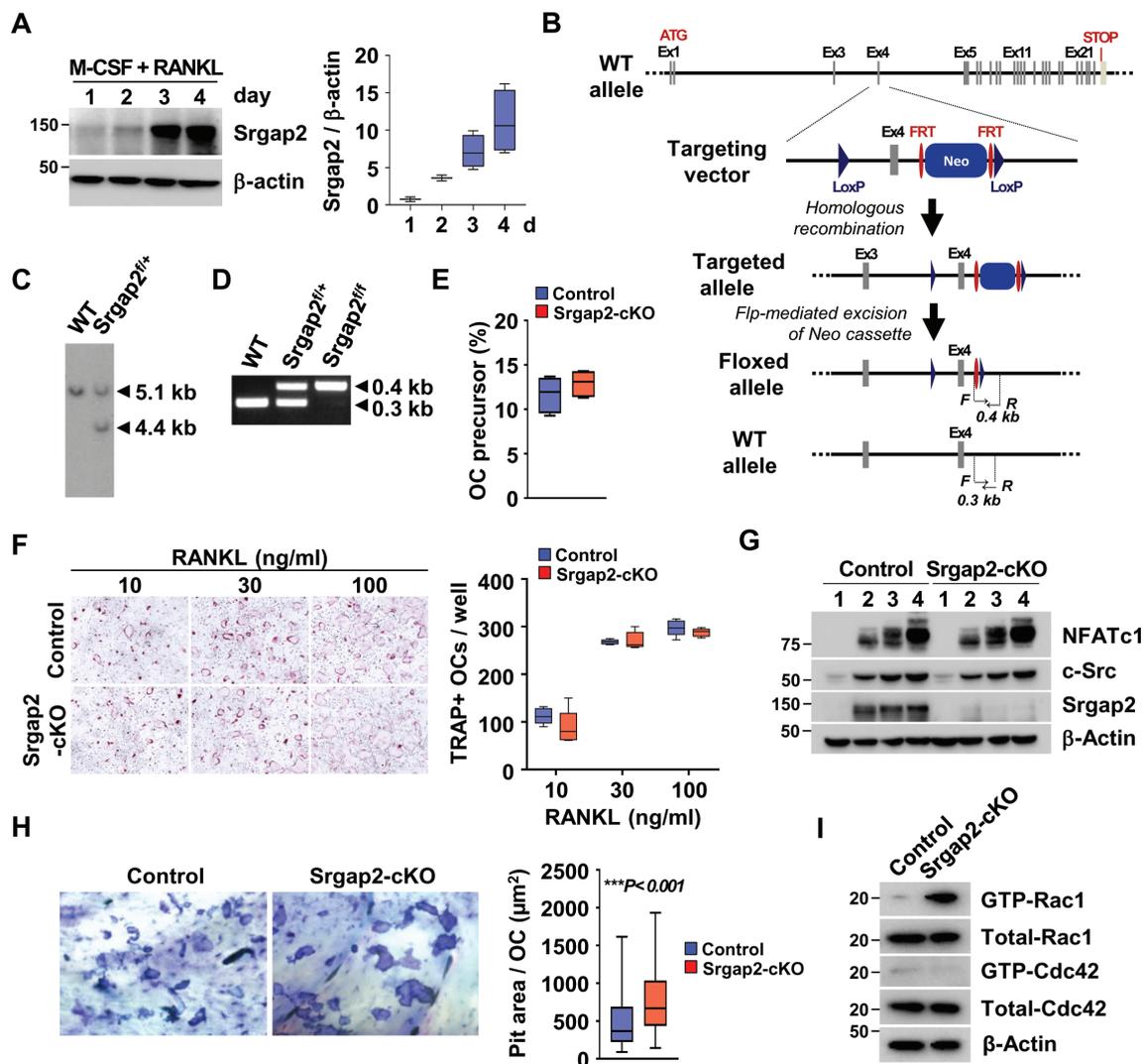


Fig. 1. Deficiency of *Srgap2* affects function of osteoclasts. (A) *Srgap2* expression during osteoclast differentiation with M-CSF and RANKL for up to 4 days. (B–D) Schematic representation of *Srgap2* genomic locus and targeting vector used to induce homologous recombination in mouse ES cells. Flp-mediated recombination of the FRT sites deleted the neomycin cassette. The LoxP sites flanking Exon 4 will allow Cre-recombinase mediated excision of Exon 4 and efficient interruption of open reading frame. (C) Southern blot analysis of *Afl*II-digested genomic DNA using a probe distinguishing the targeted allele (4.4 kb) from the WT allele (5.1 kb). (D) The WT and *Srgap2*^{fl/fl} alleles can be genotyped by PCR of genomic DNA using primers indicated in bottom two panels of B. (E) Flow cytometric analysis of a CD11b^{low}CD45R⁺CD3[–]CD115⁺ population for osteoclast precursors was performed. (F) Osteoclast formation assay with 30 ng/mL M-CSF and increasing RANKL concentrations using BMMs from *Srgap2*-cKO and littermate control mice. (G) Western blot analysis of the osteoclastogenic factors NFATc1 and c-Src during days 1 to 4 of RANKL-induced osteoclastogenesis. Deletion of *Srgap2* was confirmed by *Srgap2* Western blotting; β-actin was used as control. (H) Bone resorption assay. Equal numbers of osteoclasts differentiated on collagen type I-coated plates were cultured on bone slices for 24 hours and pit area per osteoclast was measured. A representative of the pit formation stained by 1% toluidine blue is shown. (I) BMMs were cultured with M-CSF and RANKL for 3 days and starved serum for 2 hours. Active forms of Rac1 and Cdc42 were detected by pull-down assays. Total Rac1 and Cdc42 were detected in whole cell lysate and β-actin was used as an internal control. ****p* < .001. Data represent mean ± SE. Cells were isolated from female mice. ES = embryonic stem.

5'-CCCCATATGCTCAGATCATCTTCTCAA-3' and 5'-CCCCTCGAGT-CACAGAGCAATGACTCC-3'. The PCR product was digested with NdeI and XhoI, and cloned into a pET28a expression vector (EMD Biosciences, Billerica, MA, USA) to generate a histidine (HIS)-fusion protein. HIS-TNFα was expressed in *Escherichia coli* BL21 cells (Stratagene, La Jolla, CA, USA).

Resorption pit formation assay

BMMs were cultured on collagen type I-coated plates with hM-CSF (30 ng/mL) and hRANKL (30 ng/mL) for 5 days. Multinucleated osteoclasts were collected by digestion using 0.1% collagenase P (Roche Diagnostics, Mannheim, Germany), and equal numbers of osteoclasts were cultured on bovine cortical bone

slices with hM-CSF (30 ng/mL) and hRANKL (30 ng/mL) for an additional 24 hours. Cells were removed by sonication, and bone slices were stained with 1% toluidine blue. The resorption pits were viewed via light microscopy (Olympus BX53; Olympus, Waltham, MA, USA) and measured using Olympus CellSens imaging software.

Flow cytometry

BMMs were incubated in ACK lysing buffer (Thermo Fisher Scientific) for 5 min and washed with Hanks' balanced salt solution containing 10mM HEPES (pH 7.4) and 2% FBS. Cells were incubated with fluorochrome-conjugated antibodies: anti-CD3, anti-CD45R (B220), anti-CD11b (Mac-1), anti-CD117 (c-kit), and anti-CD115 (c-Fms) for 30 min on ice. All antibodies were purchased from eBioscience (Santa Clara, CA, USA). After washing, cells were analyzed using a LSR flow cytometer and FlowJo software (FlowJo, LLC, Ashland, OR, USA).

Osteoblast differentiation assay

To prepare primary osteoblasts, cells were liberated from neonatal C57BL/6 mouse calvaria by five sequential 15-min incubations with 0.1% bacterial collagenase (Collagenase P; Roche Diagnostics), and 0.1% trypsin. Cells were collected by centrifugation after each digestion and washed with DMEM and 10% heat-inactivated fetal calf serum (HIFCS; Hyclone Laboratories, Logan, UT, USA). Cells obtained from digestions four to five were pooled and used as primary osteoblasts.⁽³²⁾

Osteoclast conditioned medium was collected from cells on day 5 of culture and concentrated 100-fold using 100-kDa pore size Amicon Ultra-4 Centrifugal Filter Unit (Millipore, Billerica, MA, USA), to eliminate low molecular weight proteins. Concentrated osteoclast conditioned medium was added at 1:50 ratio onto osteoblasts cultured with 8 mM β -glycerophosphate and 50 μ g/mL ascorbic acid. Recombinant SLIT3 0.5 μ g/mL (R&D Systems, Minneapolis, MN, USA) was used as a positive control. Osteoblast differentiation was induced for up to 12 days. Culture medium was replaced every 3 days. In some experiments, neutralizing antibody to SLIT3 (5 μ g/mL; R&D Systems) was added to cultures along with the osteoclast conditioned medium.

Osteoblastic cells were stained for alkaline phosphatase activity using Leukocyte Alkaline Phosphatase Kit (Sigma-Aldrich). Alkaline phosphatase activity per microgram of protein was evaluated using the substrate 2 mg/mL p-nitrophenyl phosphate as described,⁽³³⁾ and absorbance was measured at 415 nm. For calcium deposition assay, cells were fixed in 4% paraformaldehyde for 5 min and stained with 1% Alizarin red S (ARS, pH 5.5). The ARS stains were eluted in 10% acetic acid and absorbance was measured at 415 nm.

RNA extraction and RT-PCR

TRI reagent (Molecular Research Center, Cincinnati, OH, USA) was used to extract total RNA from either control or *Srgap2*-cKO BMM cultures that had been treated with M-CSF and RANKL for 4 days. Total RNA was converted to cDNA by reverse transcriptase (High Capacity cDNA Reverse Transcription Kit; Applied Biosystems, Carlsbad, CA, USA) using random hexamer primers, and aliquots of reverse transcription (RT) mixtures were used for PCR amplification. PCR amplification was done using gene-specific real-time PCR primers (Applied Biosystems) and expressed as fold differences compared to WT controls.

Western blot antibodies

Rabbit polyclonal anti-*Srgap2* antibody generated in house by Dr. Franck Polleux was previously described.⁽³⁴⁾ The following commercial antibodies were used for Western blotting: mouse anti-NFATc1 (BD Biosciences, San Jose, CA, USA); rabbit anti-c-Src, rabbit anti-p-IkB, rabbit anti-p-ERK, rabbit anti-ERK, rabbit anti-p-JNK, rabbit anti-JNK, rabbit anti-p-p38, rabbit anti-p38, and rabbit anti- β -actin antibodies (Cell Signaling Technology, Beverly, MA, USA); mouse anti-Myc (Millipore); anti-SLIT3 antibody (Abnova, Taipei, Taiwan).

Rho GTPase activity assay

BMMs were cultured with hM-CSF and hRANKL (both at 30 ng/mL) or hM-CSF and mTNF α for 3 days and serum starved for 2 hours. Whole-cell extracts were incubated with GST-fused PAK1 (p21 activated kinase 1 protein) PBD (p21 binding domain) overnight. GTP-bound Rac1 or Cdc42 were pulled down by GST pull-down assay (Cell BioLabs, Inc., San Diego, CA, USA).

Retroviral transduction

Myc-tagged constitutively active form of Rac1V12 and Cdc42V12 in pMX-puro vectors were transfected into the retrovirus packaging cell line, Plat-E (Cell Biolabs, Inc.) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Retroviral vectors and Plat-E cells were kindly provided by H. Kim (University of Pennsylvania, Philadelphia, PA, USA).⁽²⁵⁾ After 48 hours, retroviral supernatants plus 8 μ g/mL polybrene were used to transduce BMMs expanded for 2 days in medium containing hM-CSF (150 ng/mL). Transduced BMMs were detached with Trypsin/EDTA and re-plated with hM-CSF (30 ng/mL) and puromycin (2 μ g/mL) for an additional 2 days. Puromycin-resistant cells were used and expression of the constructs was confirmed by Western blotting using anti-Myc antibody.

Statistical analysis

All data are expressed as mean \pm SE. In vitro data were obtained from at least three independent experiments. Statistical analysis was evaluated by paired Student's *t* test to compare two groups. Results were considered significant when $p < .05$.

Results

Srgap2 deficiency increases Rac1 activity and resorption activity

As reported,⁽³⁵⁾ a CD11b^{-/low}CD45R⁻CD3⁻CD115⁺ population from mouse bone marrow contains a highly efficient population of osteoclast precursors. Microarray analysis performed on this FACS-sorted population cultured with M-CSF or M-CSF and RANKL showed a 22-fold increase in *Srgap2* expression in cells cultured with RANKL (Supporting Fig. 1A). We therefore determined *Srgap2* protein levels during osteoclastogenesis by culturing BMMs with M-CSF and RANKL. *Srgap2* protein was progressively induced and highly expressed in preosteoclasts and multinucleated osteoclasts on day 3 and 4 of differentiation, imply that *Srgap2* may play a role in osteoclast differentiation and/or function (Fig. 1A). For comparison, we also determined whether cultured neonatal mouse calvarial osteoblasts express *Srgap2* (Supporting Fig. Fig. 1B). We found that these primary osteoblasts expressed similar levels of *Srgap2* protein when

cultured in standard growth medium and when cultured in osteogenic medium, suggesting constitutive expression.

To determine the role of *Srgap2* in osteoclast differentiation and function, we generated *Srgap2* cKO mice carrying *LoxP* sites flanking Exon 4 (*Srgap2^{fl/fl}*; Fig. 1B–D), and induced monocyte/macrophage lineage-restricted deletion by crossing floxed mice with mice expressing Cre under the control of the lysozyme 2 promoter (*LysM^{Cre}*) (B6N.129P2(B6)-*Ly2z^{tm1(cre)/lfo}/J*; The Jackson Laboratory). To determine whether deletion of *Srgap2* in *LysM*-expressing cells impacted the abundance of osteoclast precursors in bone marrow, we performed flow cytometric analysis (Fig. 1E). Both control (*Srgap2^{fl/fl}*) and *Srgap2*-cKO (*Srgap2^{fl/fl}; LysM-Cre*) mice had a similar number of the CD11b^{-low}CD45R⁻CD3⁻CD115⁺ osteoclast precursor population. The ability of *Srgap2* to regulate osteoclast formation was evaluated using BMMs from *Srgap2*-cKO and control mice cultured with M-CSF and RANKL at various concentrations. *Srgap2*-deficient BMMs formed multinucleated osteoclasts similar to control cells, and normally expressed osteoclastogenic marker proteins NFATc1 and c-Src (Fig. 1F,G). However, resorption pit assays performed using equal numbers of purified mature osteoclasts cultured on bone slices showed that *Srgap2*-cKO cells generated increased pit area compared to controls, although osteoclasts from control and *Srgap2*-cKO mice express similar levels of cathepsin K (Fig. 1H; Supporting Fig. 5A).

Because it is known that the GAP protein domain of *Srgap2* specifically inhibits Rac1 activity in neuronal cells,⁽³⁶⁾ we assessed Rac1 activity in osteoclast precursor cells. BMMs were cultured with M-CSF and RANKL for 3 days, then serum and cytokines were depleted to determine basal level of Rac1 activity. The active GTP-bound form of Rac1 was greater in *Srgap2*-cKO cells compared to control, whereas there was no difference in Cdc42 activation, suggesting specificity for Rac1 in the osteoclast lineage as well as in neuronal cells (Fig. 1I). These results indicate that *Srgap2* modulates Rac1 activity and osteoclast function without altering RANKL-induced osteoclast differentiation.

Deletion of *Srgap2* in myeloid cells increases bone mass due to enhanced bone-forming activity

To determine the physiological role of *Srgap2* in myeloid lineage cells in the skeleton, we performed μ CT analysis of femurs from 8-week-old control and *Srgap2*-cKO mice. In males, significant differences in cortical bone parameters were not observed between genotypes (Supporting Fig. 2A). In contrast, female *Srgap2*-cKO mice displayed significantly reduced femur length, cortical area and marrow area compared to littermate controls, while cortical thickness was similar to control mice (Fig. 2A). Although osteoclasts from *Srgap2*-cKO mice showed increased bone resorption activity in vitro, trabecular bone volume (BV/TV) was normal in *Srgap2*-cKO male mice and significantly increased (29%) in *Srgap2*-cKO female mice compared to controls (Supporting Fig. 2B; Fig. 2B). In the females, this increased trabecular bone volume was driven by an increase in trabecular number and decreased trabecular spacing, without alterations in trabecular thickness. Due to the sex-specific impact of *Srgap2* deletion in the monocyte lineage, we focused our subsequent analyses on bones and cells from females.

Static and dynamic histomorphometric analysis showed a significant increase in osteoblast surface (Ob.S/BS, 22%), mineralizing surface (MS/BS, 30%), and bone formation rate (BFR/BS, 68%) in *Srgap2*-cKO female mice, without alterations in eroded surface (ES/BS) and osteoclast surface (Oc.S/BS) (Fig. 3A,B). Evaluation of serum markers for bone formation and resorption corroborated these histomorphometry data. Specifically, P1NP levels were significantly higher in serum from *Srgap2*-cKO mice when compared to control mice, while no significant differences were observed in the CTX levels between control and *Srgap2*-cKO mice (Fig. 3C). Together, these results suggest that *Srgap2* deficiency in the myeloid lineage promotes osteoblast activity, resulting in increased bone formation and BV/TV.

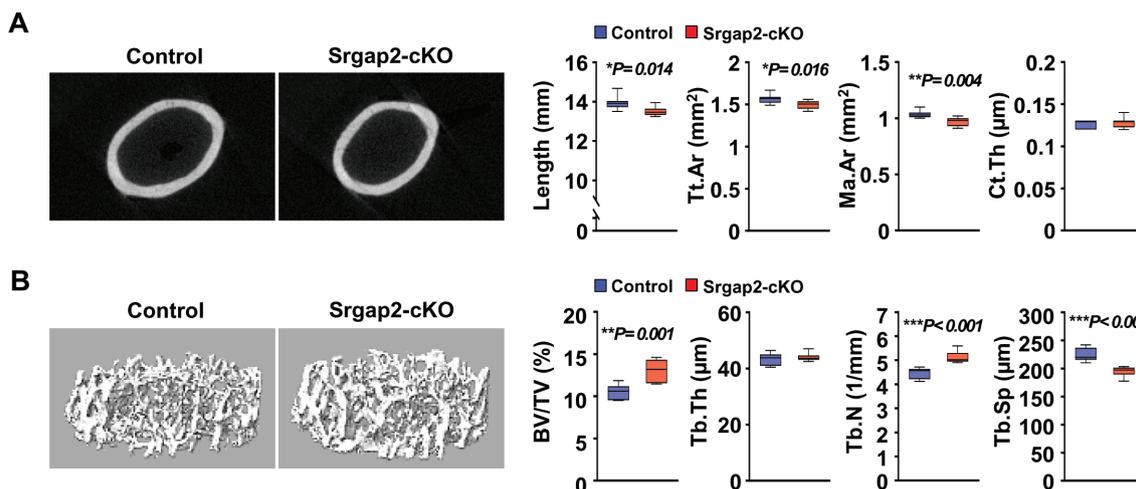


Fig. 2. μ CT analysis of femurs from *Srgap2*-cKO female mice. (A) μ CT analysis of cortical bone of femurs from 8-week-old *Srgap2*-cKO and littermate controls: Tt.Ar, Ma.Ar, and Ct.Th. (B) μ CT analysis of trabecular bone of femurs from 8-week-old *Srgap2*-cKO and littermate control mice: BV/TV, Tb.Th, Tb.N, and Tb.Sp. $n = 7-8$. * $p < .05$, ** $p < .01$, *** $p < .001$. Data represent mean \pm SE. Tt.Ar = total cortical area; Ma.Ar = marrow area; Ct.Th = cortical thickness; BV/TV = bone volume/total volume; Tb.Th = trabecular thickness; Tb.N = trabecular number; Tb.Sp = trabecular spacing.

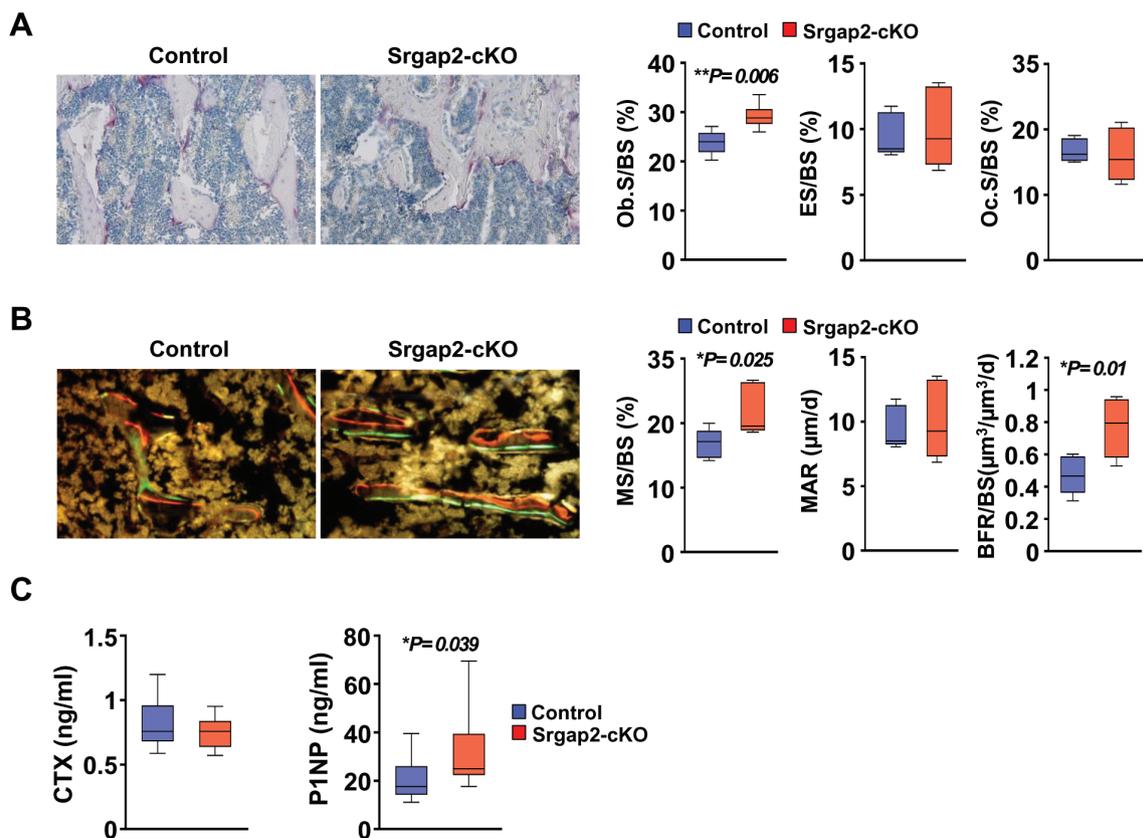


Fig. 3. Histomorphometric analysis of femurs from *Srgap2*-cKO female mice. (A) Static histomorphometric analysis of femurs from 8-week-old female *Srgap2*-cKO and littermate control mice. Sections were stained for TRAP activity and counterstained with hematoxylin: Ob.S/BS, ES/BS, and Oc.S/BS. (B) Dynamic histomorphometric analysis of femurs double-labeled with calcein and alizarin complexone from 8-week-old *Srgap2*-cKO and littermate control mice: MS/BS, MAR, and BFR/BS. $n = 5-6$. (C) Serum CTX and P1NP were measured from control and *Srgap2*-cKO mice. $n = 12-13$. * $p < .05$, ** $p < .01$. Data represent mean \pm SE. Ob.S/BS = osteoblast/bone surface; ES/BS = eroded surface/bone surface; Oc.S/BS = osteoclast surface/bone surface; MS/BS = mineralized surface/bone surface; MAR = mineral apposition rate; BFR/BS = bone formation rate/bone surface.

Rac1 activation by *Srgap2* deficiency in myeloid cells enhances SLIT3

Given that deletion of *Srgap2* in the myeloid lineage caused an increase in bone formation, we considered potential clastokines that may mediate such a response. Recently, SLIT3 was identified by two different groups as an osteoclast-derived and osteoblast-derived bone anabolic factor that acts in a paracrine fashion.^(19,37) Western blot analysis confirmed that SLIT3 protein was induced in BMMs cultured in the presence of M-CSF and RANKL; moreover, expression of SLIT3 was greater in differentiated osteoclasts from *Srgap2*-cKO mice compared to control (Fig. 4A). ELISA confirmed the increased levels of SLIT3 secreted into conditioned medium from *Srgap2*-cKO osteoclasts (Fig. 4B).

Srgap2-cKO cells display increased Rac1 activation (Fig. 1); to establish if upregulation of SLIT3 could be mediated by Rac1 activation, we cultured BMMs expressing constitutively active Rac1 or Cdc42 with M-CSF and RANKL and examined SLIT3 levels. Western blot analysis revealed that SLIT3 was greater in constitutively active Rac1-expressing BMMs compared to empty vector or BMMs expressing constitutively active Cdc42 (Fig. 4C). To further confirm the activation of Rac1 caused enhanced SLIT3 expression, we used NSC23766, a specific inhibitor of Rac1

activation.⁽³⁸⁾ As shown in Fig. 4D, SLIT3 expression was reduced in cultures that were treated with NSC23766 in both control and *Srgap2*-cKO cells. Because it is possible to detect bone-derived peptides in serum, we measured SLIT3 in serum from WT and cKO mice (Fig. 4E). However, significant differences in serum SLIT3 levels between WT and *Srgap2*-cKO mice were not detected. This was not wholly unexpected because Slit3 can be expressed in other tissues such as neuronal cells, heart, and kidney. Therefore, the increased levels SLIT3 derived from *Srgap2*-deficient monocyte lineage cells is not likely to have a systemic effect in vivo. Overall, these results indicate that SLIT3 is highly expressed in *Srgap2*-deficient osteoclastic cells, an effect that can be driven by Rac1 activation.

Conditioned medium from *Srgap2*-deficient osteoclastic cells promotes osteoblast differentiation

Although SLIT3 is a bone anabolic clastokine, other osteoclast-derived coupling factors have been published (eg, BMP6, EphrinB2, CTHR1, Wnt10b, and SPHK1). To determine whether the expression of these might be altered in *Srgap2*-cKO cells, we examined their mRNA expression in BMM cultures by real-time PCR. As shown in Supporting Fig. 3, none of these factors

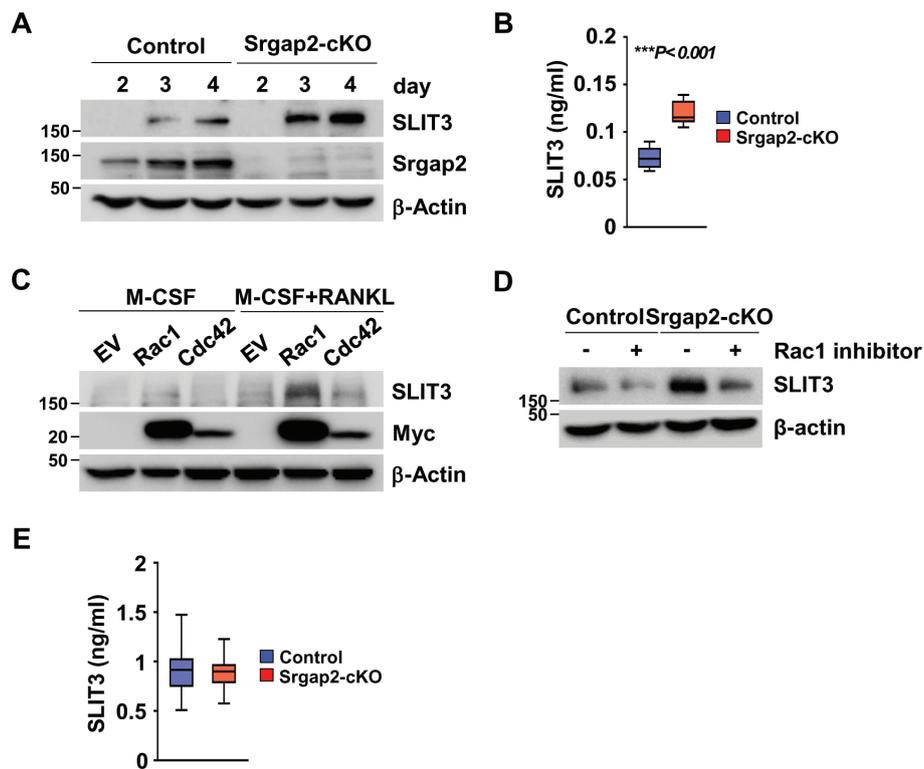


Fig. 4. Protein levels of SLIT3 in Srgap2-deficient and Rac1-activated osteoclasts. (A) SLIT3 was detected by Western blotting of lysates from Srgap2-cKO and control BMMs cultured with M-CSF and RANKL for up to 4 days. Srgap2 was detected to confirm the genotype and β -actin was used as an internal control. (B) SLIT3 levels were measured in the conditioned medium from control and Srgap2-cKO cells that were treated with M-CSF and RANKL for 5 days. (C) SLIT3 proteins were detected by Western blotting in WT BMMs transduced with EV, Myc-tagged constitutively active Rac1, or Myc-tagged constitutively active Cdc42. Transduced BMMs were cultured with M-CSF and RANKL for 4 days. Transduced genes were confirmed by Western blotting with anti-Myc antibody and β -actin was used as a loading control. (D) BMM cells from control and Srgap2-cKO mice were cultured with or without NSC23766, a specific Rac1 inhibitor, for 4 days in the presence of M-CSF and RANKL. (E) SLIT3 levels in the serum from control and Srgap2-cKO mice. $n = 12-13$. *** $p < .001$. EV = empty vector.

were significantly altered in Srgap2-cKO osteoclasts. To confirm that SLIT3 secreted from Srgap2-deficient myeloid cells enhances osteoblast differentiation, we collected conditioned medium from Srgap2-cKO and control osteoclast cultures and examined its impact on osteoblast differentiation. Most osteoclast-derived coupling factors published thus far are 27-kDa to 56-kDa proteins, whereas SLIT3 is a much larger ~168-kDa protein. Therefore, we used a 100K normal molecular weight limit (NMWL) filter to first exclude molecules smaller than 100 kDa and to concentrate osteoclast culture supernatants. Conditioned medium concentrates were used to treat primary calvarial osteoblast cells cultured in the presence of β -glycerol phosphate and ascorbic acid (osteogenic medium). Alkaline phosphatase (ALP) activity is critical for collagen crosslinking and is an early osteogenic marker. Whereas recombinant SLIT3 increased both ALP staining and activity assays, culture supernatant from Srgap2-cKO osteoclasts further enhanced osteoblast differentiation compared to culture supernatant from controls (Fig. 5A,B). Deposition of mineralized matrix is a hallmark of differentiated osteoblasts. ARS staining and quantification revealed increased calcium deposition in the cell layer of cultures treated with supernatant from Srgap2-cKO osteoclasts (Fig. 5A,B). Likewise, mRNA expression of osteoblastic marker genes such as

ALP, bone sialoprotein (BSP), and osteocalcin (OCN) were also significantly increased in cultures that received supernatant from Srgap2-cKO osteoclasts (Fig. 5C). To confirm this enhancement in osteoblast differentiation in Srgap2-cKO conditioned medium-treated cultures was due to increased SLIT3 expression, we used neutralizing antibody specific to SLIT3 in similar conditioned medium transfer experiments. As shown in Fig. 5D, conditioned medium from Srgap2-cKO cultures enhanced both ALP activity and ARS; this effect was ablated in cultures co-treated with SLIT3-specific neutralizing antibody. These data indicate that increased SLIT3 secretion by Srgap2 osteoclasts promotes osteoblastic differentiation.

Deficiency of Srgap2 in myeloid cells enhanced TNF α -induced osteoclastogenesis

Because Rac1 is rapidly activated by TNF α and required for TNF α -induced MAP kinase signaling,⁽³⁹⁾ we investigated if Srgap2 deficiency in the myeloid lineage affects TNF α -induced osteoclastogenesis in vivo and in vitro. Recombinant TNF α was injected into mice over the calvaria once a day for 4 days, and TRAP+ osteoclast surface area was evaluated. TRAP-stained calvarial sections showed that TNF α challenge led to a significant

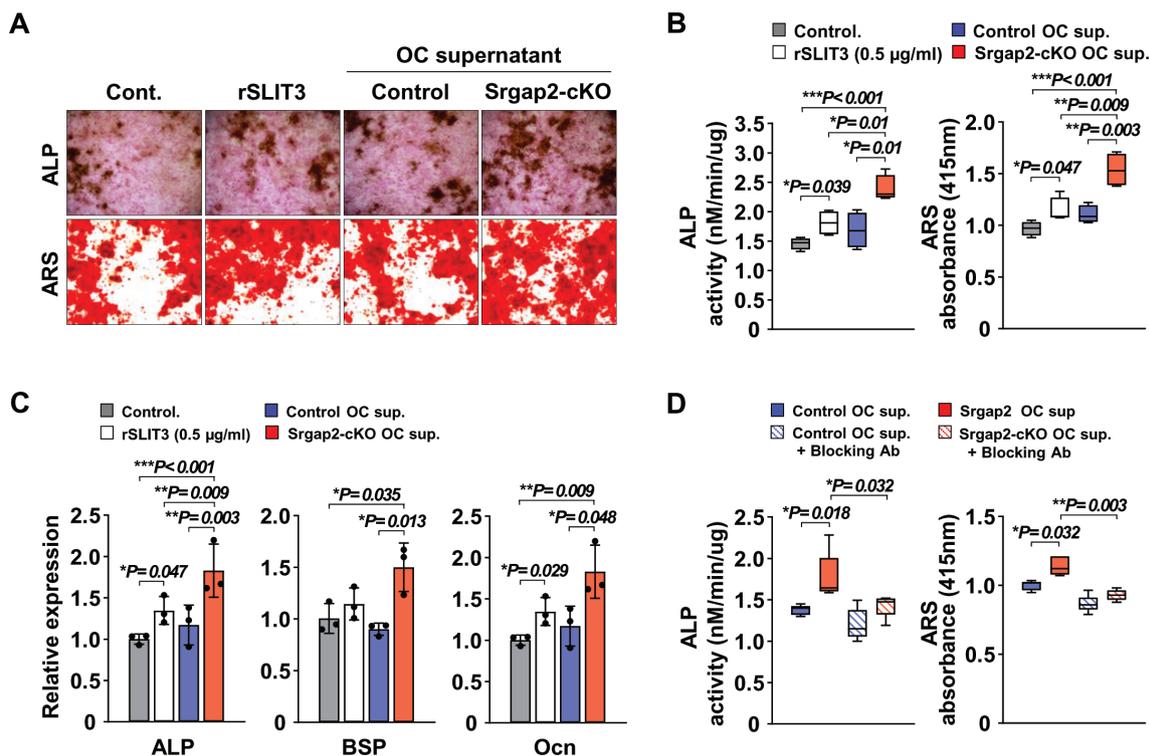


Fig. 5. Conditioned medium from Srgap2 deficient osteoclastic cells promotes osteoblastic differentiation in vitro. rSLIT3, conditioned medium of osteoclast cultures from Srgap2-cKO or control mice were added to osteoblasts cultured with β -glycerophosphate and ascorbic acid. (A) Representative ALP and ARS staining of osteoblastic cultures. (B) ALP activity assay and ARS eluted from stained wells was measured at 415 nm absorbance. (C) Osteoblastic gene expression levels, ALP, BSP, Ocn relative to Gapdh were analyzed by quantitative real-time PCR. (D) ALP activity and ARS measurement after treating the cells with control or Srgap2-cKO osteoclast conditioned medium in the presence or absence of SLIT3 neutralizing antibody, to block SLIT3 activity. * $p < .05$, ** $p < .01$, *** $p < .001$. Data represent mean \pm SE. rSLIT3 = recombinant SLIT3; ALP = alkaline phosphatase; ARS = Alizarin red S; BSP = bone sialoprotein; Ocn = osteocalcin.

increase in TRAP⁺ osteoclast surface area in Srgap2-cKO mice compared to TNF α -treated littermate controls (Fig. 6A). In vitro studies confirmed that Srgap2-cKO BMMs were more responsive to TNF α -induced osteoclast formation in vitro (Fig. 6B). This is in contrast to RANKL-induced osteoclast formation, which was comparable between Srgap2-cKO and control (Fig. 1F). Further, TNF α treatment more dramatically increased NFATc1 protein levels in Srgap2-cKO cells (Fig. 6D).

To examine the impact of TNF α on basal Rac1 activation, BMMs were cultured with M-CSF in the presence or absence of TNF α for 3 days, then serum and cytokines were depleted. The active GTP-bound form of Rac1 level was greater in Srgap2-cKO cells compared to control, both in the presence or absence of TNF α (Fig. 6C). Although TNF-induced osteoclast formation was enhanced in Srgap2 cultures and these cells displayed increased basal level of Rac1 activation, TNF-induced osteoclastic differentiation was not accompanied by an increase in SLIT3 expression (Supporting Fig. 4). This is in contrast to RANKL-induced osteoclastogenesis, which causes a dramatic increase in SLIT3 expression (Fig. 1A).

To investigate the mechanism that leads to increased osteoclast formation in Srgap2-cKO in response to TNF α treatment, we examined whether Srgap2 deficiency affected TNF α -induced NF- κ B and MAP kinase activation. I κ B phosphorylation, an indicator of NF- κ B activation, was highly induced by TNF α stimulation

in Srgap2-cKO BMMs (Fig. 6E,F). In contrast, TNF α activation of MAP kinase pathway components (JNK, ERK, and p38) was similar in control and Srgap2-cKO cells. To confirm that upregulation of NF- κ B by TNF α can be mediated by Rac1 activation, we treated BMMs expressing constitutively active Rac1 with TNF α . We found that I κ B phosphorylation was increased by TNF α in constitutively active Rac1-expressing BMMs (Fig. 6G). Further, treatment with the Rac1 inhibitor NSC23766 dramatically downregulated I κ B phosphorylation in both control and Srgap2-cKO cells cultured in TNF α (Fig. 6H). Taken together, these results demonstrate that Rac1 is highly activated in TNF α -treated Srgap2-cKO cells, with a corresponding activation of NF- κ B and enhanced osteoclastogenesis, suggesting Srgap2 control of Rac1 activity is an important anti-inflammatory mechanism.

Discussion

This study connects Rac1 signaling activity in osteoclasts with clastokine-mediated bone anabolism and enhanced osteoclastogenesis in response to inflammatory cues. We showed that expression of the Rac1-specific GTPase-activating protein Srgap2 is progressively increased during the course of RANKL-induced osteoclastogenesis in vitro. Deletion of Srgap2 resulted in high levels of Rac1 activation. Although this modestly increased

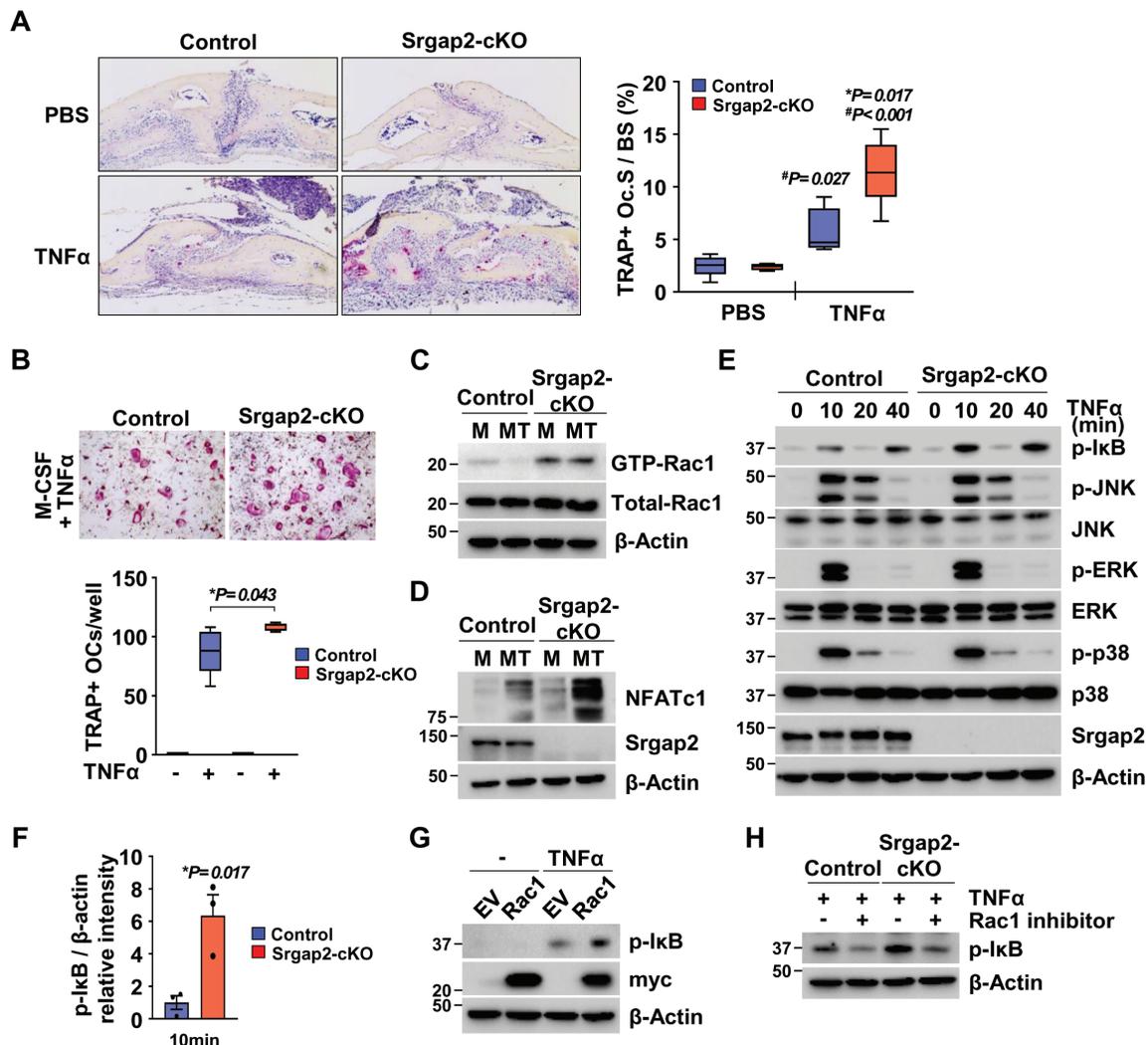


Fig. 6. Regulation of Rac1 activity by Srgap2 is critical for TNF α -induced osteoclastogenesis in vivo and in vitro. (A) Srgap2-cKO and littermate control mice were injected with PBS or TNF α daily for 4 days over the calvaria. Calvarial sections were stained for TRAP with hematoxylin counterstain. TRAP(+) osteoclast surface area was quantified. (B) BMMs from Srgap2-cKO and littermate control mice were cultured with M-CSF and TNF α for 5 days. A representative image of osteoclasts stained for TRAP is shown and TRAP(+) osteoclasts were counted. (C) BMMs were cultured with M-CSF with or without TNF α for 3 days and starved serum for 2 hours. Active form of Rac1 was detected by pull-down assays. Total Rac1 was detected from whole cell lysate and β -actin was used as an internal control. (D) Western blot analysis of NFATc1 in cultures of Srgap2-cKO and control BMMs cultured in M-CSF alone M or MT for 3 days. (E) BMMs from Srgap2-cKO and littermate mice cultured with M-CSF for 3 days were starved serum for 2 hours and then exposed to TNF α for up to 40 min. Effect of Srgap2 deficiency on TNF α signaling were assessed by Western blotting of phosphorylated of I κ B, JNK, ERK, and p38. Blots were reprobed for total JNK, ERK, and p38. Srgap2 was detected to confirm genotype and β -actin was used an internal control. (F) Fold induction of phospho-I κ B in Srgap2-cKO cells compared to control cells in response to TNF α for 10 min. (G) BMMs transduced EV or Myc-tagged constitutively active Rac1 were starved serum for 2 hours and then exposed to TNF α for 15 min. Phosphorylation of I κ B was assessed by Western blotting. Transduced genes were confirmed by Western blotting with anti-Myc antibody and β -actin was used an internal control. (H) BMMs from control and Srgap2-cKO mice were cultured with M-CSF for 3 days, serum starved, and pretreated with the Rac1 inhibitor (NSC23766) for 2 hours, then treated with TNF for 10 min. *Significant effect of Srgap2 deficiency, $p < .05$; significant effect of TNF α treatment, # $p < .05$. Data represent mean \pm SE. M = M-CSF alone; MT = M-CSF and TNF α ; EV = empty vector.

osteoclastic activity, it caused a dramatic increase in expression of the bone anabolic clastokine SLIT3. Thus, in vivo, Srgap2 deletion in the myeloid lineage promoted bone formation without altering osteoclastic parameters. Last, Srgap2-cKO mice displayed enhanced osteoclast formation in response to the inflammatory cytokine TNF α , an effect mediated by accentuated NF- κ B signaling. These data suggest that Srgap2 activity in the

osteoclast lineage restrains osteoclast-osteoblast coupling and tempers osteoclastogenesis in response to inflammatory mediators.

Osteoclasts express both Rac1 and Rac2, which appear to have overlapping and compensatory functions in this cell type. Previous studies exploring the role of Rac in osteoclasts have been primarily limited to "loss of function" studies performed

using mice deficient for Rac1 and/or Rac2 or the Rac-specific GTP exchange factors Vav3 and Dock5.^(23,28,29) These studies indicate that Rac activity is more important for RANKL-induced osteoclast function than formation; our *in vitro* studies support this concept (Fig. 1). Although Vav3 appears to be constitutively expressed during osteoclastogenesis *in vitro*, Dock5 expression is low in committed cells and increases during maturation.^(28,29) In this regard, the expression pattern of *Srgap2* is most similar to that of Dock5 (Fig. 1A). It is likely that based on their localization within the cell, these GAP/GEFs fine tune Rac1 activation in osteoclasts.

Our observation that *Srgap2* is expressed in the osteoclast lineage is novel; most of the previous work on *Srgap2* function was performed in neuronal cells. There, *Srgap2* decreases migration and promotes filopodia-like membrane protrusions needed for neurite outgrowth and branching, a process requiring *Srgap2* to recognize concave membrane curvatures.⁽⁴⁰⁾ Recently, *Srgap2* was shown to be critical for the polarization of neutrophils, stabilizing plasma membrane curvature and activating signaling cascades needed for polarization.⁽⁴¹⁾ Future studies could investigate localization of *Srgap2*, Rac1, and Rac-specific GEFs in osteoclasts cultured on bone slices, to examine distribution in basal versus apical surfaces.

Rac1 activation was strongly increased in *Srgap2*-cKO osteoclasts, and constitutive activation of Rac1 in osteoclastic cells promoted the expression of the cytokine SLIT3 (Figs. 1I and 4C). Other investigators demonstrated that, in BMMs treated with M-CSF and RANKL, the addition of exogenous SLIT3 inhibited Rac1 activation. This effect of SLIT3 on osteoclastic cells was mediated by the ROBO1 receptor, which interacts with the SH3 domain of *Srgap2*.^(19,42) These data suggest a feedback loop in which Rac activity stimulates the expression of SLIT3, which in turn acts in an autocrine manner to limit Rac activation in osteoclastic cells. However, our studies did not reveal decreased osteoclast parameters in *Srgap2*-cKO mice *in vivo*, nor was RANKL-induced osteoclastogenesis *in vitro* affected, suggesting perhaps other compensatory mechanisms.

In addition to autocrine activities, SLIT3 synthesized by osteoclasts has paracrine effects on osteoblasts. To our knowledge, SLIT3 is the only high molecular weight osteoclast-derived coupling factor described to date. SLIT3 synthesis is induced in *Srgap2*-cKO osteoclasts, which promoted osteoblastic differentiation *in vitro* (Fig. 5). Importantly, mice with myeloid lineage-restricted deletion of *Srgap2* displayed increased BV/TV and increased BFR, whereas osteoclast surface and eroded surface remained unchanged (Figs. 2 and 3). These results complement published studies demonstrating that deletion of *Slit3* in cathepsin K-expressing cells decreased bone formation rate and trabecular bone volume, although osteoclast number and eroded surface were also increased in this mouse model.⁽¹⁹⁾ Although the impact of osteoblast lineage-derived SLIT3 on the skeleton remains somewhat controversial,^(19,37) our data firmly support the concept that osteoclast-derived coupling factors, including SLIT3, play an important role in skeletal homeostasis.

BMMs from male and female mice express similar levels of SRGAP2, and *Srgap2*-cKO cells from both sexes display dramatically increased Rac1 activation (Supporting Fig. 5). However, at 8 weeks of age, we observed the increased trabecular bone phenotype only in female in *Srgap2*-cKO mice. Sex-restricted phenotypes in genetic mouse models are not uncommon.^(43–48) Skeletal sexual dimorphism depends not only on androgen action in males and estrogen action in females, but also on complex sex-specific and time-specific interactions between sex

hormones, the growth hormone/insulin-like growth factor-1 (GH/IGF-1) axis, and mechanical loading. It is worth noting that 8-week-old or 2-month-old C57BL/6 mice, particularly the males, are still growing.⁽⁴⁹⁾ Examination of more skeletally mature mice may reveal differences between control and *Srgap2*-cKO males, especially because C57BL/6 mice lose trabecular bone volume with maturity. The bone environment *in vivo* is complex, and studies are underway to determine the impact of *Srgap2* deletion in the monocyte lineage on bone quantity and quality in mature and aging mice of both sexes.

Although inactivation of *Srgap2* had no impact on RANKL-induced osteoclast differentiation, the absence of *Srgap2* had a profound stimulatory effect on TNF α -induced osteoclastogenesis, *in vivo* and *in vitro* (compare Figs. 1 and 6). In BMMs, Rac is rapidly activated by TNF α stimulation, through Src-mediated phosphorylation of the VAV family of Rho GEFs. Moreover, this Rac activation is thought to play a critical role in initiating the MAPK signaling cascade in TNF α -treated cells.⁽³⁹⁾ Although NF- κ B signaling was significantly augmented by TNF α in *Srgap2*-cKO BMMs, the p38, JNK, and ERK MAPK signaling pathways were not (Fig. 6). These data suggest that the increased osteoclastogenesis in TNF α -treated *Srgap2*-cKO mice was mediated primarily by a boost in NF- κ B signaling, and indicate that *Srgap2* activity plays a prominent role in limiting inflammatory osteolysis.

While little is known about the molecular mechanisms regulating *Srgap2* expression, intriguingly, only ~3.6 kb of intergenic DNA separates the *Srgap2* gene and its nearest neighbor *Fam72a*, and this genomic organization is conserved across species. *Srgap2* and *Fam72a* are transcribed in opposite directions and appear to share this short intergenic region as a promoter. In neuronal cells, this intergenic region can mediate co-transcription of *Srgap2* and *Fam72a* or their independent transcription, depending on cellular context.⁽⁵⁰⁾ However, transcription factors regulating the expression of *Srgap2* in osteoclasts or neuronal cells are not yet identified, and the expression of *Fam72a* in myeloid cells has not been reported. In contrast, we previously demonstrated that miR-29 family members target *Srgap2* mRNA. miR-29 positively regulates osteoclastogenesis and expression of the miR-29 family increases during osteoclast differentiation *in vitro*.⁽⁵¹⁾ It is likely that miR-29 tempers *Srgap2* expression in maturing osteoclasts. Although other miRNAs have been proposed to target *Srgap2*, these miRNA-target interactions have not yet been validated.⁽⁵²⁾

Although mice have only one *Srgap2* gene, humans have three paralogs because of partial human-specific gene duplication events. Human SRGAP2A is orthologous to murine *Srgap2*, whereas human SRGAP2C and SRGAP2B are truncated paralogs containing only a partial F-BAR domain.⁽⁵³⁾ SRGAP2C can dimerize with SRGAP2A and inhibit its function.^(34,54) The expression of SRGAP2 paralogs in human osteoclastic cells has yet to be determined. However, because deletion of *Srgap2* activity in mouse myeloid cells resulted in a bone-anabolic effect *in vivo*, it is tempting to speculate that interfering with *Srgap2* function using small molecules targeted to bone-resorbing surfaces or by increasing the expression of SRGAP2B/C may constitute a novel approach for treating osteoporosis.

In summary, upregulation of Rac1 activation due to *Srgap2* deficiency in osteoclasts revealed novel roles for Rac1 in bone homeostasis and in the inflammatory response. Our studies highlight the importance of osteoblast-osteoclast crosstalk in the maintenance of bone mass, and the potential role of SLIT3 in this process. Further, our work suggests that Rac1 activity plays a critical role in the control of inflammatory osteoclastogenesis.

Disclosures

The authors declare that they have no conflicts of interest with the contents of this article.

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Author's roles: FP, JK, and ES designed, created, and verified the *Srgap2* floxed mice and *Srgap2* antiserum. BS, AD, and SKL designed the skeletal research studies. BS and SKL performed the research and analyzed the data. BS, SKL, and AD wrote the manuscript; FP, JK, and ES edited the manuscript. BS, AD, and SKL take responsibility for data analysis. All authors approved manuscript submission.

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