Methods to investigate the role of Rho GTPases in osteoclast function

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Anne Morel[1,2], Anne Blangy[1,2,@] and Virginie Vives[1,2]

[1]CRBM CNRS UMR 5237, Montpellier, France

[2]Montpellier University, Montpellier, France

[@]Corresponding author:

Anne Blangy

Email: anne.blangy@crbm.cnrs.fr

Address:

CRBM CNRS UMR 5237

RhoGTPases in osteoclast biology

1919 route de Mende

34293 Montpellier Cedex 5

FRANCE

Tel: +33 43435 9508

Fax: +33 43435 9410

i. Summary/Abstract.

The actin cytoskeleton is essential for the biology of osteoclasts, in particular during bone resorption. As key regulators of actin dynamics, the small GTPases of the Rho family are very important in the control of osteoclast activity. The study of Rho GTPase signaling pathways is essential to uncover the mechanisms of bone resorption and can have interesting applications for the treatment of osteolytic diseases. In this chapter, we describe various technics to obtain primary osteoclasts from murine bone marrow cells, to measure RhoGTPase activation levels, to monitor bone resorption activity of osteoclasts and to introduce the expression of proteins of interest using a retroviral approach. We illustrate the different methods with experimental examples of the effect of Rac1 activation by the exchange factor Dock5 on bone resorption by osteoclasts.

ii. Key Words

Osteoclast, osteoporosis, GTPase, Rho, Rac, Cdc42, Dock5, actin, GEF, GAP

1. Introduction.

Osteoclasts are multinucleated myeloid cells specialized for bone resorption. In tight coordination with osteoblasts, the cells responsible for bone formation, they ensure the maintenance of bone homeostasis throughout life. Still, various situations can break this balance between bone resorption and bone formation in favor of bone resorption by osteoclasts, which leads to progressive bone loss and osteoporosis. It can be a consequence for instance of hormonal disorders such as menopause [1], of cancer, for instance multiple myeloma and bone metastases [2], and of inflammatory diseases including rheumatoid arthritis [3]. Controlling osteoclast activity to prevent pathological bone loss is an important challenge in osteolytic diseases; the identification of novel targets to develop anti-osteolytic treatments results an essential goal. Primary osteoclasts can be differentiated *ex vivo*, from mouse bone marrow or peripheral human blood for instance, using the cytokines macrophage-colony stimulating factor (M-CSF) and receptor activator of nuclear factor kappa B ligand (RANKL) [4]. These osteoclasts are functional to resorb the bone and they can be manipulated genetically or treated with chemical compounds to monitor the effect on their bone resorption activity.

To resorb the bone, osteoclasts assemble a specific adhesion structure called the sealing zone or actin ring, which seals the cell onto the surface of the bone. Within the actin ring, the osteoclasts secrete protons and proteases to dissolve and degrade, respectively, the mineral and the protein phases of the bone matrix. The sealing zone is based on a ring of densely packed podosomes, which are actin-based adhesion structures [5]. Thus, the regulation of actin dynamics by Rho GTPase signaling pathways plays an essential role during bone resorption [6]. In fact, actin polymerization and depolymerization mediates the adhesion of osteoclasts, their migration and the assembly/disassembly cycles of the sealing zone. The regulation of actin dynamics is also crucial during the differentiation of osteoclasts, which

involves in particular cell-cell fusions [7]. Not surprisingly, various Rho GTPases are expressed in osteoclasts [8] and they play important functions in many aspects of osteoclast biology, from differentiation and fusion of osteoclast precursors to osteoclast migration and bone resorption activity [9], including RhoA [10, 11], Rac1 and Rac2 [12–15], RhoU/Wrch-1[8, 16, 17], RhoE/Rnd3 [18] and Cdc42 [19].

Rho GTPases alternate between active GTP-bound and inactive GDP-bound states. As other small GTPases from the Ras superfamily, the activity cycle of Rho GTPases is regulated on one hand by guanine nucleotide exchange factors (GEFs), which activate the GTPases by catalyzing the release of GDP and allowing the binding of GTP [20, 21], and on the other hand GTPase-activating proteins (GAPs), which inactivate the GTPases by stimulating their GTP hydrolysis activity [22]. Once activated, Rho GTPases can bind numerous effector proteins to mediate their effect on actin dynamics. Various Rho GTPase GEFs [8] and GAPs are expressed in osteoclasts. Some were shown to control different processes in osteoclasts, namely GEFs for Rac: Dock5 [23], Vav3 [24] and FARP2 [25], for Cdc42: FGD6 [26] and for RhoA: Arhgef3 [27]; and GAPs for RhoA: Myo9b [28] and for Cdc42: Cdc42GAP [19]. Targeting RhoGTPase activity in osteoclasts with pharmaceutical compounds that affect these regulators constitutes a promising therapeutic approach to modulate the pathological excess of osteoclast activity [29].

The aim of this chapter is to describe a method to differentiate functional osteoclasts from monocytes obtained from mouse bone marrow. We then describe a method to assess the activation of the major Rho GTPases RhoA, Rac and Cdc42 in osteoclasts. We also present a technique to monitor the bone resorption activity of osteoclasts in culture. Finally we propose a retrovirus-mediated protocol to express proteins of interest in osteoclasts. We illustrate these protocols with examples of the effect of exchange factor Dock5 on the activation of Rac in osteoclasts and the consequences on their bone resorption activity.

2. Materials

2.1. Mouse dissection and bone marrow macrophages (BMMs) isolation

1. Phosphate buffered-saline (PBS, Lonza).

2. Fetal Bovine Serum (FBS), heat inactivated at 56°C for 30 minutes (BioWest).

3. 2% FBS diluted in PBS.

4. 15-mL conical tubes.

5. 6-well tissue culture plates, 10-mL syringes, gloves, sterile needles (23- and 26-gauge) and sterile gauze.

6. Bunsen burner.

7. 70% ethanol.

8. Dissection tools: 2 pairs of chirurgical scissors (14 cm, straight, sharp and 11.5 cm, straight, sharp), scalpel blades and forceps (14 cm, straight, serrated tips).

9. Sterile 70-µm cell strainers for 50 mL conical tubes (Sigma-Aldrich).

10. α-minimal essential medium (α-MEM) growth medium: (Lonza) containing 10% heat inactivated FBS, 2 mM glutamine (Lonza) and 100 U/mL penicillin and streptomycin (Lonza).

11. Malassez cell counting chamber.

12. 150-mm tissue culture dishes.

13. 100-mm sterile Petri dishes.

14. M-CSF (10 µg, Myltenyi). Dissolve 10 µg in 1 mL of sterile PBS containing 1 mg/mL ultra pure bovine serum albumin (BSA) (Sigma-Aldrich) to yield a stock concentration of 100 ng/mL. Store in 200 µL aliquots at -80°C.

15. Tissue culture room with regular equipment including a class II laminar air-flow hood, a humidity and CO_2 regulated-incubator, a low-speed Sorval Legend XI

centrifuge or equivalent, to spin down cells, and an inverted bright field microscope: Leica DM IL equipped with 10x, 20x and 40x HI PLAN objectives or equivalent, for live-cell observation.

15. Tissue culture facility: laminar flow cabinet, incubator, microscope, centrifuge.

2.2. In vitro osteoclast differentiation

1. PBS.

2. 0.25% Trypsin - 1 mM EDTA in PBS (Lonza).

3. α-MEM growth medium.

4. Malassez cell counting chamber.

5. 24-well tissue culture plates.

6. M-CSF.

7. RANKL (10 µg, Myltenyi). Dissolve 10 µg in 1 mL of sterile PBS containing 1 mg/mL ultra pure BSA (Sigma-Aldrich) to yield a stock concentration of 100 ng/mL. Store in 200 µL aliquots at -80°C.

2.3. Resorption assay

1. 24-well tissue culture plates.

2. α-MEM growth medium, M-CSF and RANKL

3. 15-mL conical tubes.

4. Accutase (Sigma-Aldrich).

5. Osteo Assay 96 stripwell plates, which are coated with inorganic crystalline calcium phosphate that mimics bone (Corning).

6. 96-well tissue culture plates.

7. PBS.

8. Formalin 3.6%: formalin 36% diluted 10 times in PBS.

9. Ethanol.

10. Acetone.

11. Sodium acetate/sodium tartrate solution: sodium acetate 0.1M, sodium tartrate 50 mM, pH 5.

12. Fast red violet LB salt: 30 mg Fast Red Violet LB salt (Sigma-Aldrich) in 10 mL of distilled water. Store 1 mL aliquots at -20°C.

13. NN dimethylformamide (DMF)-naphtol: 50 mg Naphtol AS – MX phosphate (Sigma-Aldrich) in 5 mL of DMF. Store 500 μL aliquots -20°C.

14. TRAP staining solution, to be prepared upon use: 50 mL of sodium acetate / sodium tartrate solution + 1 mL of fast red violet salt + 500 μL of NN dimethylformamide (DMF)-naphtol.

15. 1% Bleach in distilled water.

16. 5% Silver nitrate (AgNO$_3$) in distilled water.

17. 10% Formalin (Sigma-Aldrich) in distilled water.

18. 5% Carbonate sodium (Na$_2$CO$_3$) in 10% formalin.

19. ImageJ software, an open source image processing program (free download at https://imagej.net), with cell-counter plugin (https://imagej.nih.gov/ij/plugins/cell-counter.html).

20. Transmission Nikon SMZ1000 stereomicroscope equipped with a Achro 0,5x objective and a Nikon DXM 1200F CCD camera, or equivalent.

2.4. Rho-GTPases activation assay

1. 6-well tissue culture plates.

2. α-MEM growth medium, M-CSF and RANKL.

3. Dock5 inhibitor C21 CAS 54129-15-6 (Fisher Scientific), prepare a 20 mM stock in DMSO and store in aliquots at 4°C.

4. PBS.

5. FISH buffer: 10% glycerol, 50 mM Tris-HCl pH 7.4, 100 mM NaCl, 1% IGEPAL (Sigma-Aldrich) and 2 mM MgCl$_2$.

6. 1000× protease inhibitor cocktail (Sigma-Aldrich).

7. 1.5-mL microcentrifuge tubes.

8. Cell lifters.

9. 4× Laemmli buffer: 0.25 M Tris-HCl pH 6.8, 8% sodium dodecylsulfate (SDS), 40% glycerol, 8% β-mercaptoethanol and 0.02% bromophenol blue.

10. PAK-GST gluthatione sepharose beads (Cytoskeleton): reconstituted in 500 µL of sterile distilled water to yield a stock concentration of 1 mg/mL. Store 20 µL aliquots at -80°C.

11. 2× Laemmli buffer is obtained by dilution of 4× Laemmli in water.

12. Anti-Rac1 antibody (Millipore).

13. ImageJ software.

2.5. Rho-GTPases overexpression

1. 100-mm tissue culture plates.

2. Dulbecco's Modified Eagle Medium (DMEM) growth medium (Lonza) containing 10% fetal bovine serum, 2 mM glutamine (Lonza) and 100 U/mL penicillin and streptomycin (Lonza).

3. 1.5-mL microcentrifuge tubes.

4. 150 mM sodium chloride (NaCl) dissolved in sterile water.

5. Jet PEI DNA transfection reagent (Polyplus).

6. pMXS-Puro retroviral expression vector (Cell Biolabs).

7. Friend MLV-based Gag-Pol expression vector pC57GP [30], generous gift from Marc Sitbon, Institute of Molecular Genetics of Montpellier (IGMM), Montpellier, France.

8. VSV-G envelope glycoprotein expression vector pCSI-G [31], generous gift from Marc Sitbon, Institute of Molecular Genetics of Montpellier (IGMM), Montpellier, France.

9. Salmon sperm DNA (Sigma-Aldrich).

10. 0.45-μm disposable syringe filter units.

11. 20-mL syringes.

12. Sterile 100-mm Petri dishes.

13. α-MEM growth medium.

14. M-CSF.

15. Hexadimethrine bromide (Sigma-Aldrich), 8 μg/μL dissolved in sterile water. Store in aliquots at 4°C.

16. Puromycine dihydrochloride (Sigma-Aldrich), 10 μg/μL dissolved in sterile water. Store in aliquots at -20 °C.

3. Methods

3.1. Mouse dissection and bone marrow macrophages (BMMs) isolation

1. Pipet 5 mL of 2% FBS in PBS in each well of a 6-well tissue culture plate and place 20 mL of the same solution in a 50 mL conical tube. Keep both on ice during all the procedure.

2. Euthanize animals according to appropriate ethical guidelines. The commonly used method for euthanasia is cervical dislocation.

3. All procedures involving mouse dissection should be done aseptically next to a Bunsen burner and wearing gloves. Generously spread euthanized mice with 70% ethanol to minimize the chance of cell culture contamination.

4. With 14-cm scissors, make a small 5-mm cut on the ventral surface of the skin. Lift the skin to make sure the underlying peritoneum is not cut.

5. Grab the skin on either side of the cut and pull firmly in opposite directions along the longitudinal axis, until the skin is pulled off the legs. Completely separate the skin from the hind legs by pulling; be careful not to break the bones. Thereby the muscles of the hind legs are exposed.

6. Separate the legs from the body at the femur-ilium joint with 11.5-cm scissors, the femur head should remain with the leg. Place each leg in cold 2% FBS in PBS in a side well of the 6-well plate.

7. Cut off the foot and carefully remove most of the bones muscles using 11.5-cm scissors. Place each leg in cold 2% FBS in PBS in a middle well of the 6-well plate.

8. Complete flesh removal with gauze to ensure that the bones are clean and free from any soft tissue. Separate the femur and tibia at the joint using 11.5-cm scissors. Spray the 4 bones with 70% ethanol and distribute them in ice cold PBS 2% FBS in the 2 clean wells that are left in the 6-well plate.

9. Fill up a 10-mL syringe with 2% FBS in PBS from the 50-mL conical tube and attach a 23-gauge needle.

10. Cut the femurs in the middle using a scalpel blade to expose bone marrow. For each half, hold securely with forceps at mid-shaft, insert the needle into the cut-end side and flush the bone marrow into the 50-mL conical tube. Repeat until all the bone marrow is completely flushed out, the bone should whiten.

11. Attach a 26-gauge needle to the same syringe and repeat steps 9 and 10 to flush out the bone marrow of the tibias.

12. From this step, all procedures should be performed in a class II laminar air-flow hood.

13. Pipet up and down the bone marrow suspension several times in the 50-mL conical tube to break up the aggregates.

14. Filter the suspension through a 70-μm cell strainer adapted onto a new 50-mL conical tube, in order to eliminate bone particles and residual aggregates.

15. Pellet bone marrow cells by spinning at 5,000 xg for 5 minutes at room temperature.

16. Aspirate the supernatant, being careful not to disturb the cell pellet and resuspend the pellet in 10 mL of α-MEM growth medium.

17. Count cells with a Malassez counting cell chamber. Bone marrow extraction from one mouse produces 1 to 1.5×10^8 cells. Plate 3×10^7 cells per 150-mm tissue culture dish. Adjust medium to 15 mL per 150 mm tissue culture dish and mix well. Grow cells overnight in a humidified incubator at 37°C with 5.5% CO_2. Stromal cells will attach whereas osteoclast precursors will remain in suspension.

18. Collect the supernatant into a 50-mL conical tube and pellet the cells at 5,000 xg for 5 min **(see Note 1)**.

19. Resuspend the pellet containing osteoclast precursors with 10 mL of α-MEM growth medium and count cells with a Malassez counting cell chamber. One should be able to collect around 50% of the cells counted at step 17. Plate 5×10^6 cells per 100-mm Petri dish **(see Note 2)** in 10 mL of α-MEM growth medium supplemented with 100 ng/mL M-CSF. Culture for 5 days to obtain BMMs **(see Fig. 1A)**, change medium and cytokine every other day **(see Note 3)**.

3.2. In vitro osteoclast differentiation

1. Rinse 100-mm Petri dishes containing BMMs with PBS and remove supernatant.

2. Lift BMMs with 1 mL of trypsin-EDTA per dish, suspend in 10 mL of α-MEM growth medium and spin down 5 minutes at 5,000 xg.

3. Resuspend pellet with 10 mL of α-MEM growth medium and count cells using the Malassez counting cell chamber. On average, expect 2 to 5×10^5 BMMs per Petri dish.

4. Plate 3×10^4 BMMs per well in a 24-well tissue culture plate in 2 mL of α-MEM growth medium supplemented with 30 ng/mL M-CSF and 50 ng/mL RANKL.

5. Change medium every 2 days. Cells will start to fuse and form multinucleate cells 2 days later **(see Fig. 1B).** Osteoclasts will be obtained after another 1 to 2 days **(see Fig. 1C)**.

3.3. Resorption assay

1. Place 3×10^4 BMMs (from subheading 3.2, step 3) per well in a 24-well tissue culture plate in 2 mL of α-MEM growth medium with 30 ng/mL M-CSF and 50 ng/mL RANKL. Prepare 10 wells per condition to be tested.

2. After 2 days, lift the differentiating osteoclasts (pre-osteoclasts) with 100 µL of Accutase per well. Pool the cells from the 10 wells in a 15-mL conical tube and add 1-mL of α-MEM growth medium supplemented with 30 ng/mL M-CSF and 50 ng/mL RANKL. The volume should be 2.5 mL; if it is less, adjust with medium.

3. Dispense 225 µL of the suspension per well in 8 wells of an Osteo Assay stripwell and in 1 well of a 96-well tissue culture plate. The latter serves as a positive control to monitor osteoclast differentiation.

4. Change medium every other day.

5. Once osteoclasts appear in the control well, continue osteoclast culture in the Osteo Assay stripwell for 2 more days (**see Note 4**).

6. To count osteoclasts, stain 4 wells of the Osteo Assay stripwell for Tartrate Resistant Acid Phosphatase (TRAP) activity. TRAP enzyme is characteristic of osteoclasts, it converts p-nitrophenyl phosphate to p-nitrophenol under acidic conditions. For TRAP staining, fix cells for 10 minutes with 100 µL per well of 3.6% formalin, wash once with PBS, permeabilize with ethanol:acetone (1:1) for 1 minute, aspirate and add 200 µL of freshly thawed TRAP staining solution. In osteoclasts, the TRAP enzyme will convert the Naphtol AS – MX phosphate, the reaction product will combined with Fast Red Violet LB Salt Dye and result in a pink-red precipitate in the cell. Incubate for 20 minutes to 1 h at 37°C until the pink-red stain develops. Eliminate TRAP staining solution and air dry..

7. Image the entire wells with a Nikon SMZ1000 stereomicroscope or equivalent.

8. Quantify manually the total number of osteoclasts in each well with ImageJ software using the cell-counter plugin.

9. Stain the remaining 4 wells of the Osteo Assay stripwell with von Kossa, to reveal the mineralized substrate in black and measure the area resorbed by osteoclasts that will appear as white hole.., Aspirate the culture medium, add 100 µL of 1% bleach for 5 minutes and wash twice with distilled water. Drain upside down on a paper towel and add 100 µL of 5% AgNO$_3$ for 10 minutes. Wash with distilled water for 5 minutes, drain and add 100 µL of 5% Na$_2$CO$_3$ for 4 minutes. The mineral surface becomes black and the resorption areas will appear white. Drain solution and air-dry prior to imaging.

10. Imaging the entire wells with a Nikon SMZ1000 stereomicroscope or equivalent.

11. Quantify the total resorption area in each well with ImageJ software.

12. To obtain osteoclast specific activity, divide the average resorption area in the 4 wells stained with von Kossa by the average number of osteoclasts in the 4 wells stained with TRAP **(see Fig. 2)**.

3.4. Rac1 activation assay.

1. Plate 15×10^4 BMMs (from step 3 of subheading 3.2) in each well of a 6-well tissue culture plate in 5 mL of α-MEM growth medium supplemented with 30 ng/mL M-CSF and 50 ng/mL RANKL. Prepare a minimum of 3 wells per condition. Change medium every 2 days until osteoclasts appear.

2. Treat osteoclasts in at least three wells with 100 µM of C21 diluted in culture medium for 1 hour and treat the same number of control wells with 0.5% DMSO **(see Note 5)**.

3. From this step, proceed on ice in a cold room.

4. Wash osteoclasts with ice cold PBS and lyse cells for 5 minutes with 75 µL per well of FISH buffer supplemented with $1 \times$ protease inhibitor cocktail.

5. Scrape the cells using a cell lifter and pool lysates from the same condition into a 1.5-mL microfuge tube.

6. Centrifuge the lysates for 2 minutes at $17,000 \times g$ at 4°C to pellet cell debris.

7. To assess total Rho GTPase content in control and C21-treated cells, combine 20 µL of each cell lysate with 5 µL of $4 \times$ Laemmli buffer in a clean microfuge tube. Heat for 5 minutes at 95°C and store at -20°C.

8. To assess activated Rho GTPase add 4 µg of PAK-GST glutathione sepharose beads per tube of remaining lysates **(see Note 6)**.

9. Gently rotate the tubes on a wheel in the cold room for 45 minutes to 1 hour to allow the binding of the active GTPase to its effector PAK.

10. Briefly spin down the beads and eliminate supernatant.

11. Wash the beads 3 times with 1 mL of FISH buffer, centrifuge 1 minute at 10,000 xg between each wash.

12. Pellet the beads and resuspend in 20 µL of 2× Laemmli buffer, heat for 5 minutes at 95°C to release the bead-bound proteins and store at -20°C.

13. The samples (total and activated) are now ready to be run on a 12% SDS-PAGE gel and analyzed by western blot with an anti-Rac 1 antibody using ImageJ software **(see Fig. 3)**.

14. To establish the level of Rac1 activation in each sample, calculate the ratio between the amount of active Rac1 (bound to the PAK-GST glutathione sepharose beads) and the amount of Rac1 in the total cell lysate.

3.5. Rho-GTPase overexpression protocol

3.5.1. Retroviral supernatant production

1. Plate 2×10^6 HEK 293T cells in 100-mm tissue culture plates in 10 mL of DMEM growth medium. On the next day, the HEK 293T cells should be 60-70% confluent.

2. For retrovirus component transfection, prepare 2 mixes in 1.5 mL microcentrifuge tubes: (a) 500 µL of 150 mM NaCl and 14 µL of Jet PEI and (b) 500 µL of 150 mM NaCl and 7 µg of DNA mix: 3.5 µg pMXs-Puro based expression plasmid (in our case pMXs-Puro-GFP and pMXs-Puro-GFP-RacL61) + 1.22 µg pC57GP + 1 µg pCSI-G + 1.22 µg salmon sperm DNA **(see Note 7)**.

3. Mix (a) into (b) and incubate for 20 minutes at room temperature. Gently add the resulting mix dropwise with a 1000-µL micropipette into the medium of the HEK

293T cells and shake gently. Incubate for 4 to 5 hours and then replace with 10 mL of fresh DMEM growth medium.

4. After 3 days, carefully collect the culture medium with a 10-mL sterile plastic pipette (**see Note 8)** and pass through a 0.45-µm filter adapted to a 20-mL syringe (**see Note 9**). Keep the retroviral supernatant on ice until infection (**see Note 10**).

3.5.2. Infection of BMMs

1. Plate 5×10^6 freshly isolated osteoclast precursors (Subheading 3.1, step 19) in a 100-mm Petri dish with 10 mL of α-MEM growth medium supplemented with 100 ng/mL M-CSF (**see Note 11**).

2. Change for fresh medium after 2 days and leave for 3 more days.

3. Aspirate the culture medium and immediately infect the osteoclast precursors with 5 mL retroviral supernatant (see subheading 3.5.1, step 3) combined with 5 mL α-MEM growth medium, 4 µg/mL hexadimethrine bromide and 100 ng/mL M-CSF (**see Note 12**). Incubate for 24h.

4. Remove medium and add 10 mL α-MEM growth medium with 100ng/mL M-CSF and 3 µg/mL of puromycin to select infected cells. Use non-infected BMMs as control plate for selection efficiency.

5. After 2 days, replace medium by 10 mL of α-MEM growth medium with 100 ng/mL M-CSF.

6. Culture the infected BMMs from 1 to 4 days, depending on their confluence (**see Note 13**) before using them for osteoclast differentiation to observe the localization of the protein of interest for instance (**see Fig. 4)** and further experiments such as Rho GTPase pull down assays or resorption assays.

4. Notes

1. Optimize supernatant collection by tilting the plates; do not flush medium onto the cells attached at the bottom to collect more osteoclast precursors.

2. It is important to use Petri dishes and not tissue culture dishes otherwise osteoclast precursors adhere too firmly and it will be difficult to lift the cells without damaging, which is needed for splitting or replating at the appropriate density to perform the different assays described below.

3. Cells should never reach confluence, if necessary trypsinize and split 1:2 in new 100-mm Petri dishes.

4. During those 2 days, one can perform treatments with a pharmacological compound, for instance by adding 20 μM C21, an inhibitor of Dock5, which will hinder bone resorption.

5. Medium can be reduced to 2 mL for short-term treatments to save precious chemicals. To avoid acute DMSO toxicity, remove 2 mL of growth medium and add 100 μM C21. Remove the rest of medium and replace by the 2 mL of medium containing C21. Do the same for the DMSO-treated control. If using another solvent, test the toxicity on osteoclasts before doing the experiment.

6. Rac2 and Cdc42 activation can also be assessed with PAK-GST gluthatione sepharose beads whereas Rhotekin-RBD gluthatione sepharose beads have to be used for RhoA. These GTPases can be detected by western blot with the corresponding antibodies in subheading 3.4, step 13.

7. This protocol is appropriate to produce retroviruses that can infect osteoclast precursors from either mouse or human origin. If only working with mouse material, pMXs-derived expression plasmids alone can be transfected in Platinum-E (Plat-E) Retroviral Packaging Cell Line (Cell Biolabs). This produces ecotropic

retroviruses, which can only infect mouse or rat cells, that can be used to perform BMM infection as described in subheading 3.5.2.

8. The amount of virus produced depends on HEK 293T cell transfection efficiency. If using proteins with a fluorescent tag, it can be useful to check live by fluorescence microscopy.

9. The filtration allows the removal of the HEK 293T cells that can easily detach from the tissue culture plate upon supernatant collection.

10. Fresh retrovirus supernatants are more efficient for BMM infection, but they can also be frozen in aliquots, kept at -20°C for 24 hours and then at -80°C until further use.

11. When using fresh retroviral supernatants, the BMMs production procedure must be synchronized with viral supernatant collection (Subheading 3.5.1).

12. At this step, osteoclasts precursors must be around 70% confluent. If the confluence is higher, remove some cells with a short Accutase treatment.

13. The infection efficiency varies with the size and nature of the protein to be expressed.

Acknowledgements

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Figure Legends.

Figure 1: RANKL-induced *in vitro* osteoclast differentiation of BMMs. Phase contrast images, taken during the osteoclast differentiation procedure described in subheading 3.2, of (A) bipolar elongated BMMs (arrows), (B) undergoing fusion (arrows) after 2 days in the presence of RANKL and (C) forming differentiated osteoclasts after 4 days. Scale bar = 100 μm.

Figure 2: Resorption activity of osteoclasts expressing or not the Rac1 GEF Dock5. (A) Resorption areas (in white) were visualized after von Kossa staining of Osteo Assay stripwell plated with Dock5 wild-type (*Dock5$^{+/+}$*) or knockout (*Dock5$^{-/-}$*) osteoclasts [23] according to the method described in subheading 3.3. (B) Quantification of resorption area per osteoclast (OC) for each genotype, determined from the average and SEM of 4 wells per genotype as described in subheading 3.3, step 8.

**Figure 3: Effect of the Dock5 inhibitor C21 on the activity of Rac1 in osteoclasts.** Osteoclasts were treated for one hour with 0.5% DMSO containing (+) or not (-) 100 μM C21. (A) Total Rac1 and active GTP-bound Rac1 were analyzed by western blot according to the procedure described in Subheading 3.4. (B) For each condition the amount of Rac1 was determined using ImageJ and the percentage of active Rac1 normalized to total Rac1.

Figure 4: Expression of GFP and GFP-Rac1-Q61L in Dock5$^{-/-}$ osteoclasts. BMMs were prepared from Dock5$^{-/-}$ mice according to the method described in Subheading 3.2. Viral supernatants were produced from pMXs-Puro vectors expressing GFP or GFP-fused active Q61L mutant of Rac1 and used to infect Dock5-/- BMMs, according to the procedures described in Subheading 3.5. BMMs were differentiated into osteoclasts following the protocol in Subheading 3.4. Fixed osteoclasts were imaged for GFP (left panels) and Hoechst-

labeled nuclei (middle panels), with the two channels merged in the right panels to show the

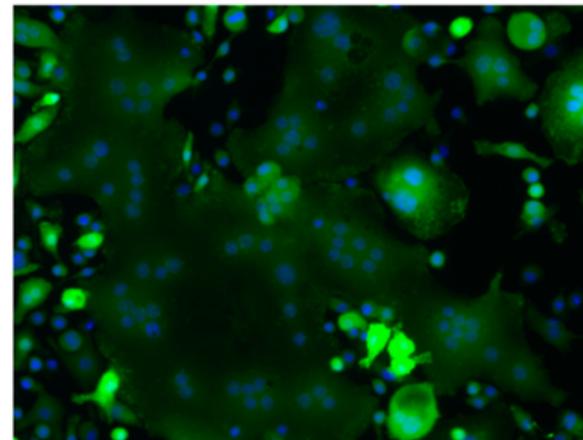multinucleated GFP-positive osteoclasts. Scale bar: 50 µm.

A

Dock5$^{+/+}$ | Dock5$^{-/-}$
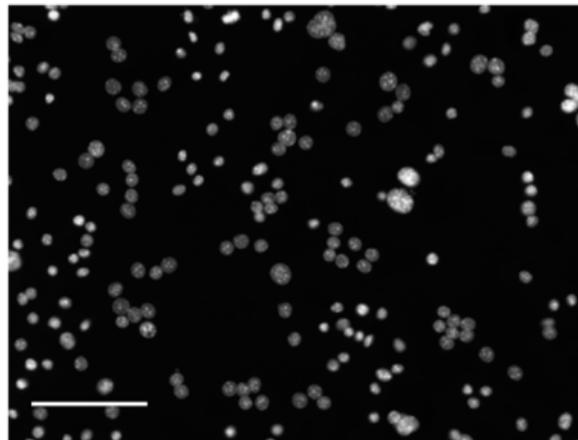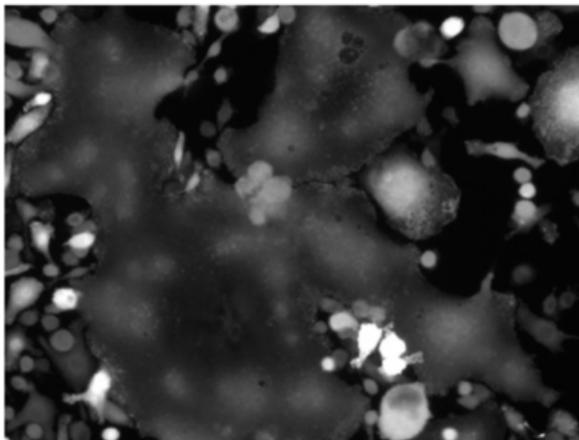
B

A

C21 (100 µM)

 −   +

active
Rac1

total
Rac1

B

Active Rac1 (%)

 −   +
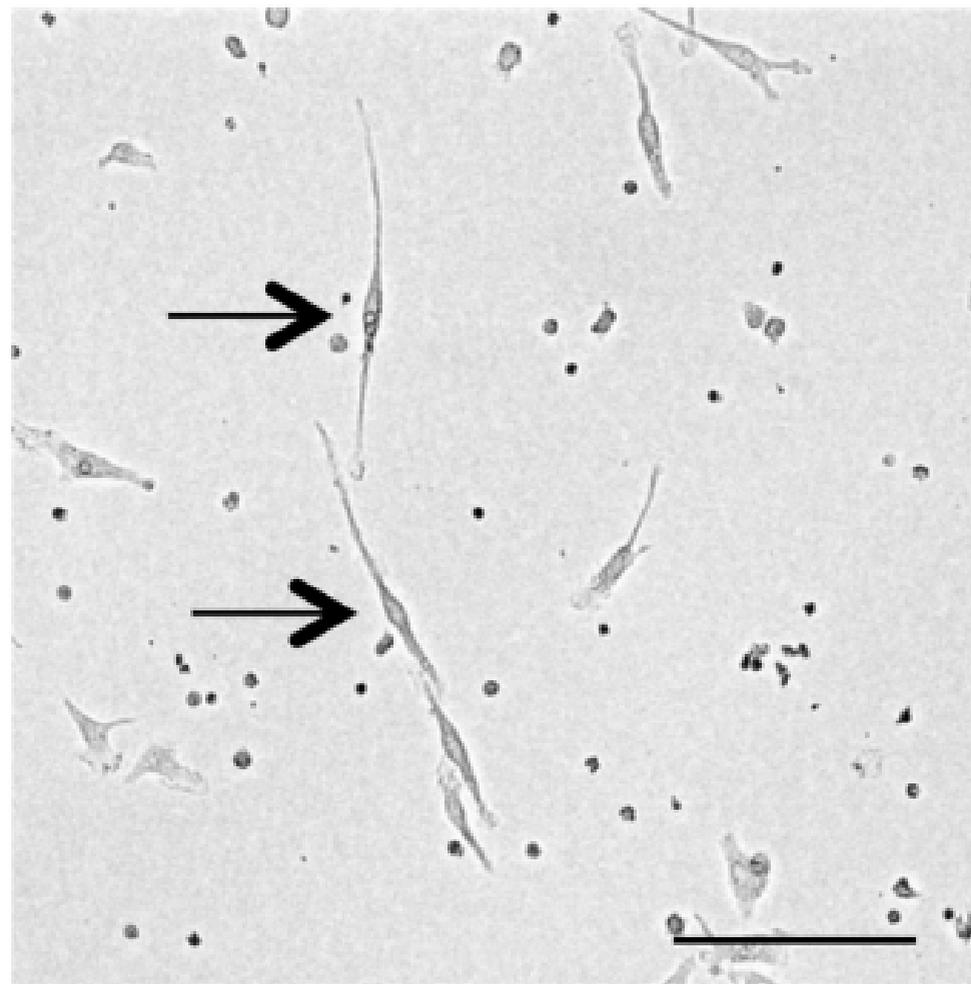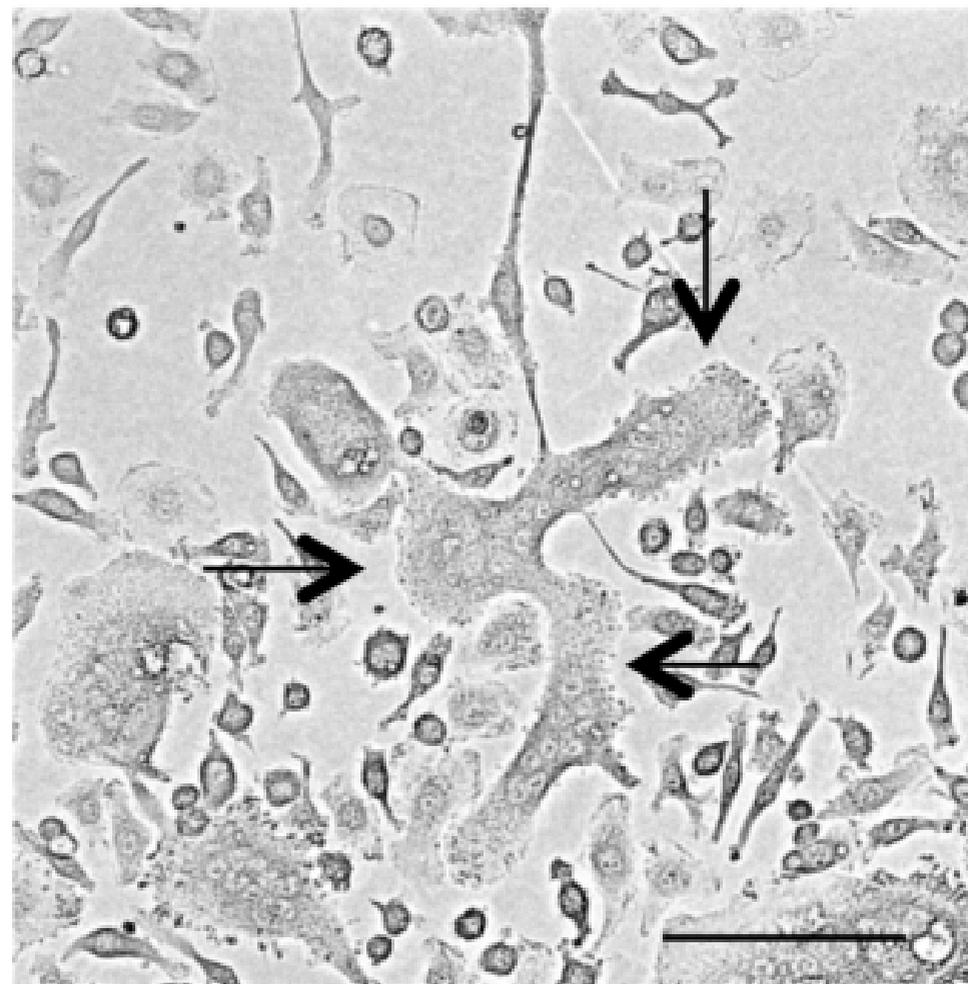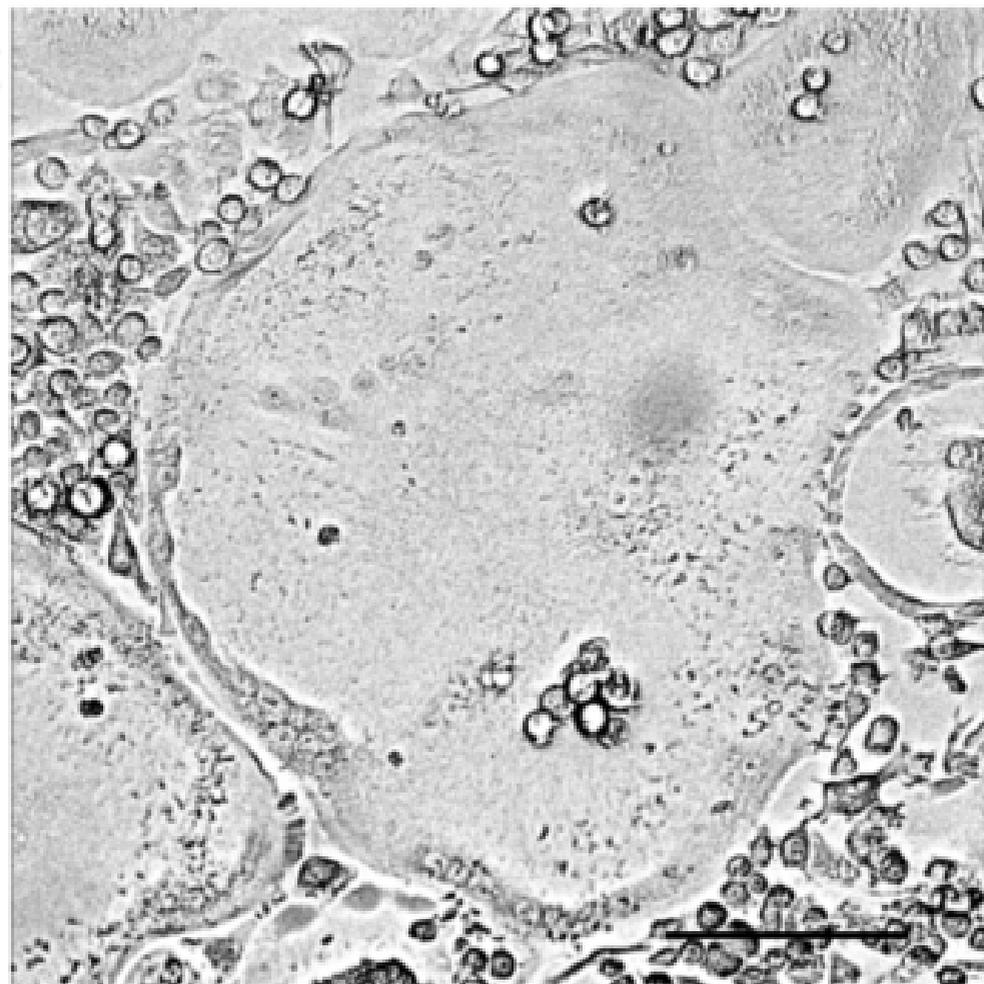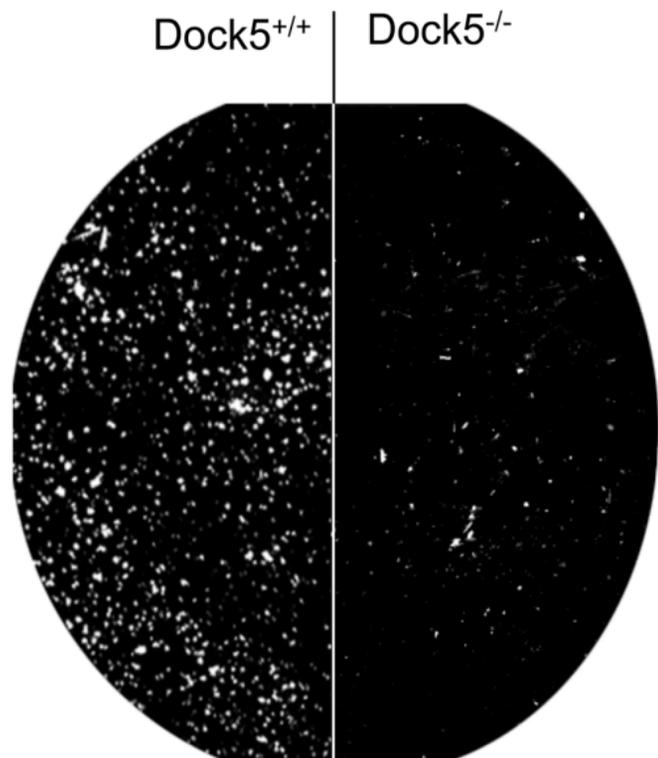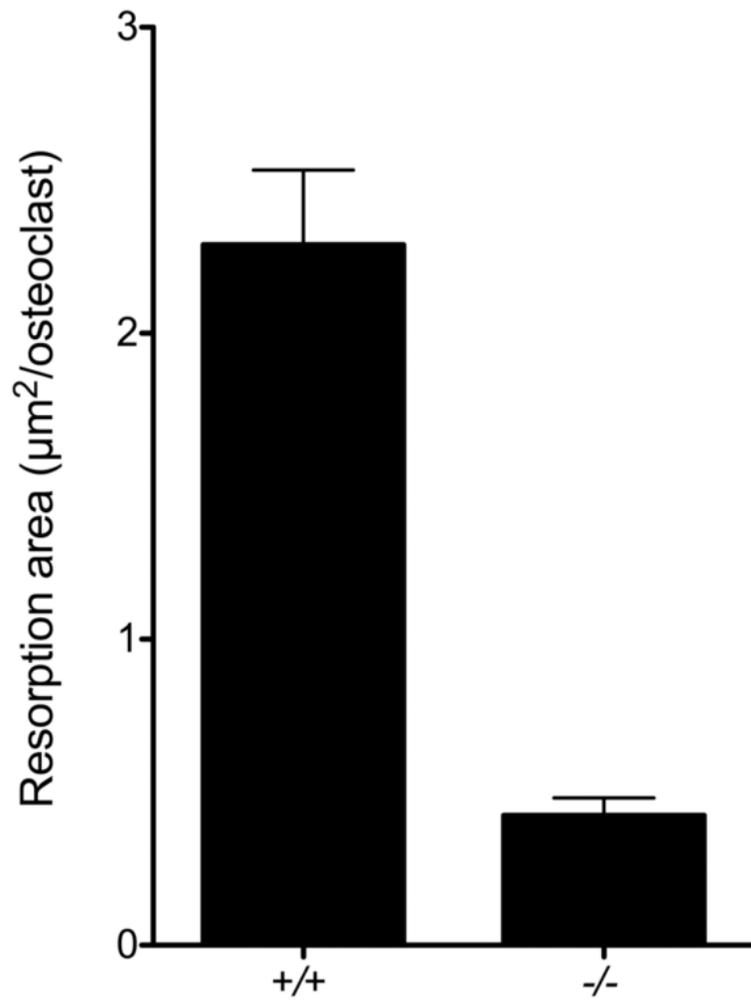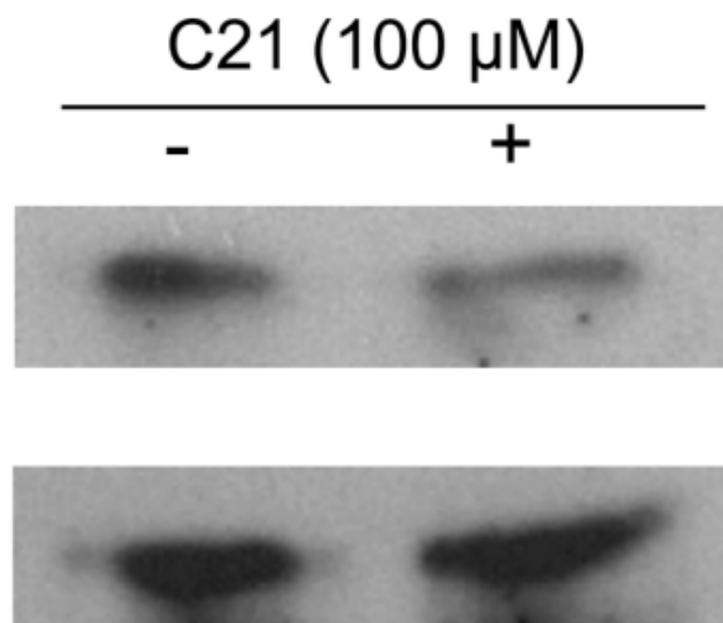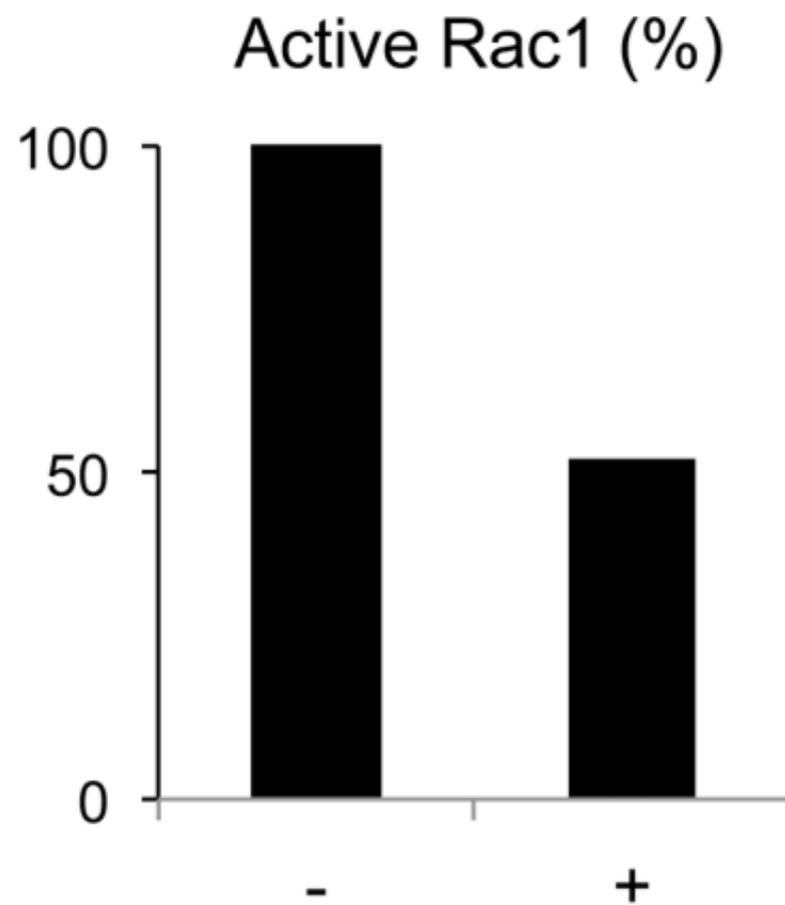
	GFP	Hoechst	Merge
Protein expressed: GFP			
GFP- Rac1- Q61L			