Cdc42 regulates bone modeling and remodeling in mice by modulating RANKL/M-CSF signaling and osteoclast polarization

Yuji Ito,1 Steven L. Teitelbaum,1 Wei Zou,1 Yi Zheng,2 James F. Johnson,2 Jean Chappel,1 F. Patrick Ross,1 and Haibo Zhao1

1Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, Missouri, USA.
2Division of Experimental Hematology, Children’s Hospital Medical Center, Molecular Developmental Biology Graduate Program, University of Cincinnati, Cincinnati, Ohio, USA.

The modeling and remodeling of bone requires activation and polarization of osteoclasts, achieved by reorganization of the cytoskeleton. Members of the Rho subfamily of small GTPases, including Cdc42, are known regulators of cytoskeletal components, but the role of these proteins in bone physiology and pathophysiology remains unclear. Here, we examined loss-of-function mice in which Cdc42 was selectively ablated in differentiated osteoclasts and gain-of-function animals wherein Cdc42Gap, a protein that inactivates the small GTPase, was deleted globally. Cdc42 loss-of-function mice were osteopetrotic and resistant to ovariectomy-induced bone loss, while gain-of-function animals were osteoporotic. Isolated Cdc42-deficient osteoclasts displayed suppressed bone resorption, while osteoclasts with increased Cdc42 activity had enhanced resorptive capacity. We further demonstrated that Cdc42 modulated M-CSF–stimulated cyclin D expression and phosphorylation of Rb and induced caspase 3 and Bim, thus contributing to osteoclast proliferation and apoptosis rates. Furthermore, Cdc42 was required for multiple M-CSF– and RANKL-induced osteoclastogenic signals including activation and expression of the differentiation factors MITF and NFATc1 and was a component of the Par3/Par6/atypical PKC polarization complex in osteoclasts. These data suggest that Cdc42 regulates osteoclast formation and function and may represent a promising therapeutic target for prevention of pathological bone loss.

Introduction

Generation and maintenance of skeletal mass and integrity requires continuous renewal of bone by a combination of modeling and remodeling that is mediated by osteoblasts and osteoclasts. In osteoporosis, resorption exceeds osteogenesis, while defective osteoclast formation and/or function results in bone accumulation (1).

Osteoclasts are multinucleated cells generated from mononuclear precursors of the monocyte/macrophage lineage. RANKL and M-CSF, the two principal osteoclastogenic cytokines, trigger signals that control precursor differentiation and activation of the mature resorptive cell (2, 3). Activated osteoclasts polarize by reorganizing their cytoskeleton. Upon matrix recognition, actin polymerizes into podosomal rings, adhesion structures at the sealing zone where the plasma membrane juxtaposes bone. This sealing zone surrounds a specialized domain, the ruffled border, through which protons and lysosomal enzymes are secreted into the resorption lacuna to dissolve bone mineral and digest organic matrix, respectively (4).

Rho, Rac, and Cdc42, the most studied Rho subfamily small GTPases, are key regulators of the actin cytoskeleton. Acting as molecular switches, by cycling between active, GTP-bound and inactive, GDP-bound forms, they play multiple roles in cell regulation (5). These inter-conversions are controlled by guanine nucleotide–exchange factors (GEFs) and GTPase-activating proteins (GAPs) (6). While the role of Rho, Rac, and Cdc42 in actin organization has been examined in a range of cell types, studies in osteoclasts have invariably involved in vitro experiments using chemical inhibitors or dominant-negative and/or overexpression of constitutively active mutants of the small GTPases (7, 8). Unfortunately, these approaches have yielded contradictory conclusions. Thus, two groups have suggested that Rho-A is critical for osteoclast podosome organization, motility, and bone resorption (9, 10). In contrast, Destaing et al. proposed that inhibited Rho-A yields podosome patterning via Rho-mDia2-HDAC6 microtubule acetylation (11). Similarly, ligation of the integrin αvβ3 activates Rac, which is required for actin ring formation (12), whereas dominant-negative Rac does not affect the osteoclast cytoskeleton, but rather regulates osteoclast apoptosis (8). The Wiscott-Aldrich syndrome protein (WASP) and actin-related protein 2/3 (Arp2/3) complex participate in actin ring formation and bone resorption (13, 14). Because they are Cdc42 effectors, this GTPase may regulate the resorptive polykaryon.

Here we used mice in which Cdc42 is absent only in mature osteoclasts (loss of function) or present as a basally active molecule (gain of function) in committed precursors to characterize the role of the GTPase, in vivo and in vitro. We find that the molecule, which is activated independently by both M-CSF and RANKL, regulates properties of the resorptive cell. Specifically, Cdc42 is essential for skeletal homeostasis during modeling and remodeling, and its deletion provides protection from oophorectomy-induced bone loss. Cdc42 exerts its skeletal effects by regulating proliferation of osteoclast precursors via GSK-3β/cyclin D/Rb and apoptosis of mature cells through Bim/caspase-9. It is also a component of the Par-3/Par-6/atypical PKC (Par-3/Par-6/aPKC) polarization complex, which governs the rate of actin ring formation. Cdc42 is therefore an essential component of RANKL and M-CSF signal-
ing, regulating osteoclast recruitment physiologically and in the context of postmenopausal osteoporosis.

**Results**

*Generation of osteoclast-specific Cdc42−/− mice and Cdc42Gap−/− radiation chimeras.* M-CSF and RANKL are the 2 major regulators of osteoclast differentiation. Kim et al. have shown the latter activates Cdc42 (15), and we found the same is true regarding M-CSF. As seen in Figure 1, within 5–15 minutes each cytokine activated the small GTPase.

These experiments suggest Cdc42 may regulate the osteoclast. To determine whether this is the case, we generated osteoclasts in which activity of the GTPase is suppressed or enhanced. Since conventional gene targeting of Cdc42 prompts early embryonic lethality,
we used loxP/Cre technology to produce a loss-of-function model. Thus, we crossed floxed Cdc42 and cathepsin K–Cre knockin mice (CtsKCre/+) thereby targeting the Cdc42 gene only in cells committed to the osteoclast phenotype (16, 17). The progeny of Cdc42+/CtsKCre/+ and Cdc42fl/flCtsKCre/+ breeding pairs were designated Cdc42 WT and Cdc42 osteoclast conditional knockout (Cdc42ΔOC/ΔOC) osteoclasts. Apoptotic osteoclasts are indicated with asterisks. Original magnification, ×200. (F and G) ELISA-determined osteoclast apoptosis. (H and I) Immunoblots of cleaved caspase-3, -9, and -8 and Bim in mature WT, Cdc42ΔOC/ΔOC, and Cdc42Gap−/− osteoclasts. 5′ represents mature osteoclasts deprived of cytokines and serum for 3 hours prior to assay. β-Actin served as a loading control. Data are presented as mean ± SD (**P < 0.01; ***P < 0.001).

Figure 3
Cdc42 regulates proliferation and survival of osteoclast lineage cells. (A) BrdU incorporation of WT, Cdc42−/−, and Cdc42−/− rescued with WT Cdc42 BMs exposed to M-CSF (50 ng/ml) for 12 hours. (B) BrdU incorporation by WT, Cdc42Gap−/−, Cdc42Gap−/− transduced with WT, or GAP-deficient mutant Cdc42GAPR305A BMs exposed to M-CSF (50 ng/ml) for 12 hours. (C and D) Immunoblots of D-type cyclins and phosphorylation of retinoblastoma protein (Rb) in response to M-CSF. β-Actin served as a loading control. (E) TRAP staining of WT and Cdc42ΔOC/ΔOC osteoclasts. Apoptotic osteoclasts are indicated with asterisks. Original magnification, ×200. (F and G) ELISA-determined osteoclast apoptosis. (H and I) Immunoblots of cleaved caspase-3, -9, and -8 and Bim in mature WT, Cdc42ΔOC/ΔOC, and Cdc42Gap−/− osteoclasts. 5′ represents mature osteoclasts deprived of cytokines and serum for 3 hours prior to assay. β-Actin served as a loading control. Data are presented as mean ± SD (**P < 0.01; ***P < 0.001).
Figure 4
Cdc42 regulates osteoclast differentiation in vitro. (A) TRAP staining of osteoclasts generated from WT and Cdc42−/− BMMs produced by transducing Cdc42fl/fl BMMs with retrovirus expressing Cre recombinase, and Cdc42−/− BMMs rescued with WT Cdc42. Original magnification, ×200. (B) Quantification of the number of TRAP+ multinucleated osteoclasts (MNCs) shown in A. (C) TRAP staining of osteoclasts generated from WT or Cdc42Gap−/− BMMs or Cdc42Gap−/− BMMs transduced with either WT or the GAP-deficient mutant, Cdc42GapR305A. Original magnification, ×40. (D) Quantification of results from C. (E) Osteoclast (OC) differentiation markers assessed by RT-PCR. Numbers represent densitometrically determined ratios relative to GAPDH. (F) Osteoclast differentiation markers assessed by immunoblot. Numbers represent densitometrically determined ratios relative to β-Actin. (G) Induction of NFATc1 protein during osteoclast differentiation of WT and Cdc42−/− BMMs. β-Actin served as loading control. (H) MITF phosphorylation with time of exposure to RANKL. β-Actin served as loading control. Data shown in B and D are presented as mean ± SD (**P < 0.01; ***P < 0.001).
The Journal of Clinical Investigation

Cdc42 promotes bone resorption in vitro. (A, C, and D) Bone resorption pits labeled by lectin (brown reaction product). Original magnification, ×200. (B and E) Culture medium CTx-1 determined by ELISA. Data are presented as mean ± SD (*P < 0.05).
In contrast to proliferation, apoptosis of WT, \( \text{Cdc42}^{\Delta OC/\Delta OC} \), and \( \text{Cdc42Gap}^{-/-} \) BMMs and pre-osteoclasts was indistinguishable in vitro (data not shown). However, mature osteoclasts lacking Cdc42 died more rapidly than WT upon withdrawal of M-CSF and RANKL (Figure 3, E and F). Consistent with these observations, survival of \( \text{Cdc42Gap}^{-/-} \) osteoclasts was prolonged (Figure 3G).

Apoptosis is induced via the intrinsic and/or extrinsic pathways. In the former, pro-apoptotic Bcl-2 family proteins promote cytochrome c release from mitochondria, and consequently, activation of caspase-9 and caspase-3. In the death receptor scheme, ligation of specialized receptors such as Fas activates initiator caspase-8 and ultimately, caspase-3 (22). To determine which pathway is involved in Cdc42-regulated apoptosis, we assessed the activity of caspases-8, -9, and -3 and the quantity of Bim, a pro-apoptotic Bcl-2 family member functional in osteoclasts (23). After 3 hours of cytokine and serum starvation, \( \text{Cdc42}^{2OC/2OC} \) osteoclasts, generated by 5 days in M-CSF and RANKL, contained more Bim and activated caspases-9 and -3 than WT cells (Figure 3H). In keeping with their resistance to apoptosis, \( \text{Cdc42Gap}^{-/-} \) osteoclast lineage cells produced a paucity of caspase-3 or activated caspase-9 (Figure 3I). In contrast to caspase-9, altered activation of caspase-8 was not detectably induced by either loss or gain of function of Cdc42. This in vitro role of Cdc42 in osteoclast apoptosis mirrors that occurring in vivo (see below).

The enhanced rate of apoptosis and inhibited precursor proliferation diminishes the abundance of mature polykaryons in \( \text{Cdc42}^{-/-} \) cultures, while re-expression of WT GTPase normalized the process (Figure 4, A and B). Alternatively, \( \text{Cdc42Gap}^{-/-} \) BMMs yielded increased numbers of osteoclasts, and the phenotype was reversed by re-expression of WT Cdc42Gap but not the GAP-deficient mutant (Figure 4, C and D). As confirmation that the small GTPase controls osteoclast number via its effects on maturation, expression of osteoclast mRNA (Figure 4E) and protein (Figure 4F) maturation markers was decreased in \( \text{Cdc42}^{-/-} \) precursors, while the same parameters were upregulated in \( \text{Cdc42Gap}^{-/-} \) cells. To explore further the role of Cdc42 in osteoclast differentiation, we examined NFATc1 and MITF, 2 transcription factors that regulate the process. While NFATc1 was reduced in the absence of Cdc42, RANKL-induced phosphorylation of MITF, which activates this molecule (24), was enhanced in \( \text{Cdc42Gap}^{-/-} \) cells (Figure 4, G and H). Thus, Cdc42 promotes osteoclast differentiation.
Cdc42 regulates osteoclast function in vitro. While our data indicate that Cdc42 controls osteoclast differentiation, these experiments provide no insights into the role of the small GTPase in the resorptive capacity of the cell. To address this issue, we generated osteoclasts on dentin slices using Cdc42Δ/ΔOC and Cdc42ΔGAP precursors as well as their retrovirally rescued counterparts. We visualized resorption lacunae by staining with wheat germ lectin conjugated to horseradish peroxidase and determined global bone degradation as a function of CTx-1 fragments released into culture medium. In keeping with their failure to generate mature osteoclasts, Cdc42Δ/ΔOC BMMs treated for 5 days with RANKL and M-CSF were unable to resorb bone, but the defect was rescued by retroviral reconstitution of the GTPase. Alternatively, Cdc42ΔMOC/AOC osteoclasts, which differentiate normally, formed

Figure 7
Par-3/Par-6/aPKCs complex mediates Cdc42-regulated osteoclast polarization. (A) RANKL stimulates threonine and tyrosine phosphorylation of aPKCs in mature osteoclasts. Mature osteoclasts, generated by 5 days’ exposure of WT BMMs to RANKL and M-CSF, were serum- and cytokine-starved and then treated with RANKL over time. Left: Lysates were immunoblotted for threonine-phosphorylated and total aPKC. Right: Lysates were immunoprecipitated for total phosphotyrosine (p-Y) using mAb 4G10 or aPKC. Immunoprecipitates were immunoblotted for aPKC. (B) Immunoprecipitation of endogenous aPKCs in osteoclasts transduced with empty vector (pMX), HA-tagged dominant-negative (Cdc42-17N), or HA-tagged constitutively active (Cdc42-12V) retroviruses. TCL: total cell lysates. (C) Co-immunoprecipitation of HA-tagged Par-6 with aPKCs and LGL in osteoclasts. (D) Co-immunoprecipitation of endogenous Par-3 in osteoclasts transduced with pMX, N-terminal–FLAG–tagged PKC-λ, (N-FLAG-aPKC), or C-terminal–FLAG–tagged PKC-λ, (C-FLAG-aPKC). (E) Co-immunoprecipitation of endogenous Par-3 in osteoclasts transduced with N-terminal HA-tagged par-6 (N-HA par-6) or C-terminal HA-tagged par-6 (C-HA par-6). (F) Selective inhibition of PKC isoforms in mature osteoclasts, cultured on bone slices, by 1 hour exposure to cell-permeable pseudo substrate (PS) peptide inhibitors of PKC-α, PKC-θ, PKC-ε, PKC-ζ, and Go 6976. (G) Osteoclasts were incubated with PKC-ζ inhibitor or carrier for 1 hour followed by washing and cultured for 1 or 3 hours. Cells were stained to visualize actin rings. Hoechst stain visualizes nuclei. Original magnification, ×200. (H) Quantification of the percentage of osteoclasts exhibiting actin rings illustrated in F (***P < 0.001).
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A  WT  Cdc42<sup>loco/loco</sup>

B  WT  Cdc42<sup>loco/loco</sup>

C  WT  Cdc42<sup>loco/loco</sup>

D  WT  Cdc42<sup>loco/loco</sup>

E

F

G

H

I

J

Cdc42 is essential for osteoclastic bone resorption in vivo and its ablation prevents ovariectomy-induced bone loss. Having established that Cdc42 regulates osteoclast function in vitro, we asked whether the same is true in vivo. Tooth eruption of Cdc42ΔOC/ΔOC mice was normal (data not shown), but the animals were smaller than WT mice (Figure 8A). Moreover, their femora were abnormally shaped, which indicated abnormal modeling (Figure 8B). Trabecular volume of the mutant bone was enhanced, as were trabecular number and thickness, and these changes were accompanied by decreased trabecular separation (Figure 8, D and E). Hence, Cdc42 is dispensable for ultimate formation of actin rings but regulates the rate at which these structures are generated.

Cdc42 regulates osteoclast polarization. Cdc42 regulation of actin ring formation is reminiscent of its role in generating and maintaining tight junctions in polarized epithelial cells. In this circumstance, the active GTPase stimulates assembly of the Cdc42/Par3/Par6/aPKC polarity complex (28, 29). Although bone-residing osteoclasts are polarized, the protein constituents that establish and sustain this polarity are unknown. Current models suggest the presence of 2 polarizing complexes: inactive Par-α/aPKC/LGL (lethal giant larvae) and active Cdc42/Par3/Par6/aPKC (30, 31). Binding of GTP-bound Cdc42 to Par6 activates aPKCs, which phosphorylate LGL, leading to its dissociation and replacement with Par3, the key event that triggers polarization. In the context of osteoclasts, RT-PCR and Western blots showed that the cells expressed Par-3 and Par-6α as well as the aPKCs PKC-ζ, and PKC-λ (Supplemental Figure 4). Of particular note, Par3 increases during osteoclast differentiation, suggesting, as in other cells, that it regulates polarity (32). Finally, RANKL activates aPKCs by phosphorylating threonine and tyrosine (Figure 7A) (33, 34). We therefore asked whether Cdc42 participates in the active polarizing complex in osteoclasts. To this end, we generated osteoclasts expressing dominant-negative (Cdc42-12V) or constitutively active (Cdc42-12V) Cdc42. aPKCs bind only the active form of this small GTPases (Figure 7B) despite similar expression of the mutant protein (Figure 7B). Both aPKC and LGL-1 form a complex with Par-6, which does not contain Par-3 (Figure 7C and data not shown). Furthermore, endogenous Par-3 co-immunoprecipitates with FLAG-tagged PKC-ζ and HA-tagged Par-6, but not with LGL-1 (Figure 7D, E and F, and data not shown). These observations suggest the existence of an active Cdc42/Par3/Par6/aPKC-λ complex in osteoclasts, formation of which is stimulated by RANKL.

The PKC family comprises 3 major subgroups: conventional, novel, and aPKCs. To discriminate between these family members in osteoclast polarization, we used inhibitors that block PKC-α, -β, -γ (Go6976, conventional), -θ, -ε (novel), or -λ, -ζ (atypical). Neither Go6976 nor peptides targeting novel PKCs altered the number of actin rings (Figure 7F) or osteoclast number (data not shown). In contrast, a peptide inhibitor of aPKCs (PS-aPKC) dose dependently blocked actin ring formation (Figure 7F, lower left panel) without altering osteoclast abundance (Figure 7F, lower right panel). This inhibitory effect of PS-aPKC was not due to toxicity, as osteoclast apoptosis, determined by DNA-fragmentation ELISA, was unaltered (data not shown) and withdrawal of the inhibitor for 3 hours normalized actin ring formation (Figure 7G and H).

Cdc42 is essential for osteoclastic bone resorption in vivo and its ablation prevents ovariectomy-induced bone loss. Having established that Cdc42 regulates osteoclast function in vitro, we asked whether the same is true in vivo. Tooth eruption of Cdc42ΔOC/ΔOC mice was normal (data not shown), but the animals were smaller than WT mice (Figure 8A). Moreover, their femora were abnormally shaped, which indicated abnormal modeling (Figure 8B). Trabecular volume of the mutant bone was enhanced, as were trabecular number and thickness, and these changes were accompanied by decreased trabecular separation (Figure 8,
C and E). These phenotypic changes indicated either arrested bone resorption or stimulated osteogenesis. Cdc42ΔOC/ΔOC mice, however, had normal circulating osteocalcin (WT 21.1 ± 14.7 versus Cdc42ΔOC/ΔOC 20.1 ± 6.7 ng/ml), which indicated that deletion of the GTPase in the osteoclast does not stimulate the osteoblast. Decreased bone resorption in Cdc42ΔOC/ΔOC mice in vivo was confirmed by serum CTx-1 (Figure 8F). Importantly, Cdc42ΔOC/ΔOC female mice were protected from ovariectomy-induced (OVX-induced) bone loss (Figure 8G).

Retarded bone resorption may reflect increased osteoclast apoptosis and/or diminished osteoclastogenesis. In fact, osteoclast abundance of Cdc42ΔOC/ΔOC mice was diminished, and the percentage of osteoclasts exhibiting features of apoptosis increased 4-fold (Figure 8, H and J).

These findings indicate that Cdc42 is critical for osteoclastic bone resorption. If this is so, Cdc42 gain-of-function mice should exhibit a reciprocal phenotype. In this regard, bone mineral density (BMD) of lumbar vertebrae of Cdc42Gap–/– marrow chimeras is decreased at 2 and 4 months of age (Figure 9A). Histomorphometric analysis of proximal tibiae of 4-month-old mice showed that Cdc42Gap–/– chimeras had a lower ratio of bone volume to tissue volume (BV/TV) and increased osteoclast surface (Figure 9, B and C). Enhanced osteoclast recruitment and activity in Cdc42Gap–/– mice were confirmed by increased serum TRAP5b and CTx-1, respectively (Figure 9D).

Discussion

Our findings, summarized in Figure 10, and based exclusively on the use of genetically manipulated mice and their derived primary cells, indicate what we believe is a novel and multifactorial role for Cdc42 in osteoclastogenesis and bone resorption in vitro and in vivo. Thus, Cdc42 is crucial for skeletal modeling, remodeling, and bone loss in the context of estrogen withdrawal. The small GTPase exerted its effects by regulating RANKL- and/or M-CSF–dependent precursor proliferation and differentiation, apoptosis of the mature osteoclasts, and their capacity to polarize.

Activated Cdc42, binding to specific effectors, modulates cytoskeleton organization, proliferation, apoptosis, matrix adhesion, polarization, membrane trafficking, and transcription (35). As each of these events is involved in osteoclast differentiation and function, we hypothesized that Cdc42 participates in the bone resorptive process. While the GTPase is known to be RANKL-activated in osteoclasts (15), most observations to date represent in vitro overexpression of dominant mutants and are contradictory (7, 8). Therefore, the significance of Cdc42 in osteoclast biology remains unresolved. By using genetic models of Cdc42 loss and gain of function, we established that the GTPase broadly regulates the osteoclast by previously uncharacterized means.

Cdc42, activated by M-CSF and RANKL, enhanced BMM proliferation and osteoclast differentiation and survival in vivo. Loss or gain of Cdc42 function selectively controlled RANKL stimulation of MAP kinases and Akt but not NF-κB. Similarly, upon c-Fms occupancy, the GTPase activates p38 and Akt but not ERKs. Consistent with our previous findings (21), downregulation of Akt/GSK-3β consequent to absent Cdc42 decreases D-type cyclins, phosphorylated Rb, and cell proliferation. Hence, the Akt/GSK-3β/D cyclin–Rb axis mediates Cdc42-regulated osteoclast precursor proliferation.

Figure 9

Decreased bone mass in Cdc42Gap–/– chimeric mice due to enhanced bone resorption. (A) BMD of lumbar vertebrae measured by DEXA in WT and Cdc42Gap–/– chimeric mice 2 months and 4 months after marrow transplantation. (B) TRAP-stained histological sections of proximal tibia of WT and Cdc42Gap–/– chimeric mice 4 months after transplantation (n = 10). Original magnification, ×40. (C) BV/TV and osteoclast perimeter per bone perimeter (OC Pm/B Pm) calculated from B. (D) Serum TRAP5b and CTx-1 in WT and Cdc42Gap–/– chimeric mice 4 months after transplantation, as measured by ELISA. Data are presented as mean ± SD (⁎P < 0.05; **P < 0.01; ***P < 0.001).
induction of MITF phosphorylation and activation is enhanced in Cdc42 gain-of-function cells, indicates that GTPase regulates osteoclast differentiation.

JNK activation by RANKL is a necessary event in the cytokine’s induction of NFATc1 (43). Similarly, RANKL or M-CSF phosphorylates p38, which stimulates MITF (41). The cytokines also activated Cdc42, which in turn induced phosphorylation of JNK and p38, indicating that GTPase mediated RANK and M-CSF stimulation of osteoclastogenic genes via selected MAPKs.

Cdc42 is central to cytoskeletal organization, which in the osteoclast can only be fully evaluated on mineralized substrate (44, 45). The major known effectors linking activated Cdc42 to actin include WASH, IQGAP, and PAKs. The WASH-Arp2/3 complex plays a critical role in actin ring formation and bone resorption (46–48). Thus, it is surprising that neither the appearance of filopodia (Supplemental Figure 5), an actin-enriched structure induced by Cdc42, nor the abundance of actin rings in osteoclasts were affected by absence of the GTPase, suggesting compensation by either Rac or related molecules (12). Indeed, Wrch1, a Rho GTPase that shares sequence similarities with Rac1 and Cdc42, regulates actin ring formation, osteoclast adhesion, and migration (49). On the other hand, CTX-1 in culture medium or in mouse blood was reduced in the absence of Cdc42, establishing its necessary, nonredundant role in the osteoclast in vitro and in vivo.

Despite the presence of actin rings in Cdc42-deficient osteoclasts, the formation rate of these cytoskeletal structures was prolonged. Modulation of the kinetics of actin ring generation by Cdc42 is likely essential for osteoclast function, as illustrated by substantial changes in medium CTX-1 in loss- and gain-of-function cultures. The fact that active but not dominant-negative Cdc42 associates with pAKCs in osteoclasts suggests that, as in other cells, this complex participates in polarization (48). Thus, Cdc42 governs an array of mechanisms by which RANKL and M-CSF promote osteoclast recruitment and function and presents as a candidate therapeutic target for postmenopausal osteoporosis.

Methods

Antibodies and reagents. Commercially available antibodies were purchased from the following resources: monoclonal anti–PKC-δ and Bim (BD Biosciences); JNK, p-JNK, p38, p-p38, Akt, p-Akt, ERK, p-ERK, IkB, p-IkB, GSK3β, p-GSK3β, p-Rb (807/811), p-ApKCs, cleaved caspase-3, and cleaved caspase-9 (Cell Signaling Technology); monoclonal anti-HA (Covance); rabbit anti–Par-3 and aPKCs (Invitrogen); monoclonal anti–cyclin D2, anti–cyclin D3, anti-NFATc1, anti–caspase-8, and anti–β-actin (Sigma-Aldrich). Rabbit anti-β-actin was kindly provided by Dr. Ulf Hume (University of Lund, Lund, Sweden). Rabbit anti–cyclin D1, anti–cyclin D2, anti–cyclin D3, anti–caspase-8, and anti–β-actin (Santa Cruz Biotechnology Inc.) and monoclonal anti-αT3 (Sigma-Aldrich) were purchased from Sigma-Aldrich. Rabbit anti-αT3 (Sigma-Aldrich) was kindly provided by Dr. Ulf Hume (University of Lund, Lund, Sweden). Rabbit anti–cyclin D1, anti–cyclin D2, anti–cyclin D3, anti–caspase-8, and anti–β-actin (Santa Cruz Biotechnology Inc.) and monoclonal anti-αT3 (Sigma-Aldrich) were purchased from Sigma-Aldrich. Rabbit anti–cyclin D1, anti–cyclin D2, anti–cyclin D3, anti–caspase-8, and anti–β-actin (Santa Cruz Biotechnology Inc.) and monoclonal anti-αT3 (Sigma-Aldrich) were purchased from Sigma-Aldrich. Rabbit anti–cyclin D1, anti–cyclin D2, anti–cyclin D3, anti–caspase-8, and anti–β-actin (Santa Cruz Biotechnology Inc.) and monoclonal anti-αT3 (Sigma-Aldrich) were purchased from Sigma-Aldrich. Rabbit anti–cyclin D1, anti–cyclin D2, anti–cyclin D3, anti–caspase-8, and anti–β-actin (Santa Cruz Biotechnology Inc.) and monoclonal anti-αT3 (Sigma-Aldrich) were purchased from Sigma-Aldrich. Rabbit anti–cyclin D1, anti–cyclin D2, anti–cyclin D3, anti–caspase-8, and anti–β-actin (Santa Cruz Biotechnology Inc.) and monoclonal anti-αT3 (Sigma-Aldrich) were purchased from Sigma-Aldrich.
aggregation was then added and incubated with rotation for 3 hours at 4°C. Immunoprecipitates were washed 4 times in lysis buffer, and solubilized proteins were separated by SDS-polyacrylamide gels.

\textbf{Immunofluorescence and actin ring formation assay.} Osteoclasts cultured on bone slices were fixed with 4% paraformaldehyde in PBS for 20 minutes. Free aldehyde groups were quenched with 50 mM NH₂Cl in PBS for 10 minutes, followed by washing and blocking in PBS/0.2% BSA/0.1% Saponin (PBSBS) for 30 minutes. Bone slices were incubated with primary and secondary antibodies in PBSBS for 45 minutes. F-actin was stained with Alexa Fluor 488–phalloidin. Samples were mounted with 90% glycerol/PBS and observed using a conventional microscope equipped with a charge-coupled device camera (Nikon USA) or a confocal laser scanning microscope (LSM Zeiss).

For actin ring re-formation assay, mature osteoclasts cultured on bone slices were washed twice with cold cytochrome-free medium followed by incubation at 37 °C for 30 minutes. RANKL (100 ng/ml) was then added for the indicated time. Bone slices were fixed with 4% paraformaldehyde in PBS for 20 minutes. F-actin and nuclei were stained with Alexa Fluor 488–phalloidin and Hoechst 33258, respectively. The number of total multinucleated osteoclasts and active osteoclasts with actin rings were counted under a conventional microscope equipped with a charge-coupled device camera (Nikon USA).

\textbf{Cdc42 activation assay.} Pre-osteoclasts stimulated with M-CSF or RANKL were lysed and Cdc42 activity was examined by an effector domain, GST-fusion pulldown protocol using EZ-detect Cdc42 activation kit (Pierce Biotechnology Inc.).

\textbf{Proliferation and cell death ELISA assay.} The BrdU ELISA was conducted using the cell proliferation Biotrak ELISA system (Amersham) as described previously (21). Cell death was analyzed in quadruplicate using cell death detection ELISAPLUS kit (Roche), which detects cytoplasmic histone-associated DNA fragmentation. All experiments were repeated two to three times.

\textbf{Medium CTx-1 assay.} Macrophages were cultured on bone with 100 ng/ml RANKL and 1/100 vol CMG14-12 supernatant for 5–6 days. Medium (α-10) was changed one day before harvesting. Medium CTx-1 concentration was determined using CrossLaps for Culture ELISA kit (Nordic Bioscience Diagnosis A/S).

\textbf{Statistics.} Data of 2-group comparisons were analyzed using a 2-tailed Student’s t test. Simultaneous comparison of more than 2 groups was performed using ANOVA. For all graphs and in the text, data are represented as mean ± SD.

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Address correspondence to: F. Patrick Ross or Haibo Zhao, Washington University School of Medicine, Department of Pathology and Immunology, Campus Box 8118, 660 South Euclid Avenue, 4940 Parkview Place, St. Louis, Missouri 63110, USA. Phone: 314.454.8463; Fax: 314.454.5505; E-mail: rossf@hss.edu (F.P. Ross); hzhao@uams.edu (H. Zhao).