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Loss of Cbl–PI3K Interaction Enhances Osteoclast Survival due to p21-Ras Mediated PI3K Activation Independent of Cbl-b

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ABSTRACT

Cbl family proteins, Cbl and Cbl-b, are E3 ubiquitin ligases and adaptor proteins, which play important roles in bone-resorbing osteoclasts. Loss of Cbl in mice decreases osteoclast migration, resulting in delayed bone development where as absence of Cbl-b decreases bone volume due to hyper-resorptive osteoclasts. A major structural difference between Cbl and Cbl-b is tyrosine 737 (in YEAM motif) only on Cbl, which upon phosphorylation interacts with the p85 subunit of phosphatidylinositol-3 Kinase (PI3K). In contrast to Cbl$^{-/-}$ and Cbl-b$^{-/-}$, mice lacking Cbl–PI3K interaction due to a Y737F (tyrosine to phenylalanine, YF) mutation showed enhanced osteoclast survival, but defective bone resorption. To investigate whether Cbl–PI3K interaction contributes to distinct roles of Cbl and Cbl-b in osteoclasts, mice bearing CblY737F mutation in the Cbl-b$^{-/-}$ background (YF/YF;Cbl-b$^{-/-}$) were generated. The differentiation and survival were augmented similarly in YF/YF and YF/YF;Cbl-b$^{-/-}$ osteoclasts, associated with enhanced PI3K signaling suggesting an exclusive role of Cbl–PI3K interaction, independent of Cbl-b. In addition to PI3K, the small GTPase Ras also regulates osteoclast survival. In the absence of Cbl–PI3K interaction, increased Ras GTPase activity and Ras–PI3K binding were observed and inhibition of Ras activation attenuated PI3K mediated osteoclast survival. In contrast to differentiation and survival, increased osteoclast activity observed in Cbl-b$^{-/-}$ mice persisted even after introduction of the resorption-defective YF mutation in YF/YF;Cbl-b$^{-/-}$ mice. Hence, Cbl and Cbl-b play mutually exclusive roles in osteoclasts. Whereas Cbl–PI3K interaction regulates differentiation and survival, bone resorption is predominantly regulated by Cbl-b in osteoclasts. J. Cell. Biochem. 115: 1277–1289, 2014.

KEY WORDS: Cbl-b; RANKL; AKT; SURVIVAL; GSK

Bone remodeling is a dynamic process, which involves resorption by osteoclasts and formation by osteoblasts. Perturbation of bone remodeling process leads to diseases, including osteoporosis and osteopetrosis [Teitelbaum, 2000]. The differentiation, survival and function of osteoclasts are tightly regulated by cell signaling events. Cbl family proteins, Cbl and Cbl-b, are E3 ubiquitin ligases with adaptor function that are abundantly expressed in osteoclasts and osteoblasts [Horne et al., 2005; Severe et al., 2013]. E3 ubiquitin ligase function of Cbl proteins facilitates negative regulation of cell signaling by targeting the phosphorylated receptor and non-receptor kinases and associated signaling molecules for proteasomal degradation by ubiquitylation system. The adaptor function of Cbl proteins facilitates signaling from several receptors including c-Fms, RANK and integrins, by forming molecular complexes via protein–protein interactions [Thien and Langdon, 2005; Swaminathan and Tsygankov, 2006].

In the skeletal system, Cbl and Cbl-b have been demonstrated to perform distinct functions in regulating bone formation and bone resorption [Horne et al., 2005; Severe et al., 2013]. In mice, the absence of both proteins resulted in embryonic lethality before day 10.5, suggesting overlapping functions during embryonic development [Naramura et al., 2002]. However, the absence of either protein resulted in distinct outcomes in skeletal tissue. Lack of Cbl protein resulted in decreased osteoclast migration and a developmental

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delay but adult mice had no overt skeletal phenotype [Chiusaroli et al., 2003]. In contrast, adult Cbl-b$^{-/-}$ mice had decreased bone volume because of hyper-resorptive osteoclasts [Nakajima et al., 2009].

The structural differences in their C-terminal half have been attributed to distinct functional roles of Cbl and Cbl-b, since the N-terminal half, which harbors the tyrosine kinase binding (TKB) domain and the RING finger domain, are highly conserved [Swaminathan and Tsygankov, 2006]. Both Cbl and Cbl-b undergo phosphorylation down stream of tyrosine kinase signaling pathways. However, the tyrosine phosphorylation site Y737 (Y731 in human) is unique to Cbl (not present on Cbl-b) and has a downstream sequence (EAM) that provides a docking site for the SH2 domain of the p85 regulatory subunit of phosphatidylinositol-3-kinase (PI3K) [Songyang and Cantley, 1995; Songyang and Cantley, 2004].

PI3Ks are lipid kinases that phosphorylate phosphoinositides at 3′ position. Members of the class I_A PI3K are made up of a heterodimeric p110 catalytic subunit and a p85 regulatory subunit. The p110 catalytic subunit binds to the p85 regulatory subunit with high affinity preventing the activation of PI3K. Upon engagement of the SH2 domain of the p85 subunit through binding to phosphorylated tyrosines of signaling proteins, the inhibitory effect of p85 on p110 is released resulting in increased lipid kinase activity [Foukas and Okkenhaug, 2003]. PI3K has been demonstrated to be important for osteoclast differentiation, survival and bone resorption due to its involvement downstream of RANK, c-Fms, and αvβ3 integrin signaling [Golden and Insogna, 2004]. p85$^{-/-}$ mice are osteopetrotic due to defective osteoclast function [Munugalavadla et al., 2008]. In addition, inhibition of PI3K activity decreased the bone resorbing activity of osteoclasts [Nakamura et al., 1995; Lakkakorpi et al., 1997]. Recent work by Shinohara et al. 2012 showed that osteoclast-specific deletion of the p85 genes results in an osteopetrotic phenotype caused by a defect in the bone-resorbing activity of osteoclasts [Shinohara et al., 2012].

Studies in a number of cell types have shown that stimulation through tyrosine kinase coupled receptors induces prominent association between Cbl and the p85 subunit of PI3K. In turn, a significant proportion of PI3K activity is attributable to Cbl–PI3K interaction following receptor stimulation [Ueno et al., 1998; Feshchenko et al., 1999; Hunter et al., 1999]. In contrast to this phosphorylation-dependent interaction of Cbl, the proline rich region of Cbl-b provides constitutive association with the p85 subunit of PI3K [Fang et al., 2001]. In osteoclasts, PI3K is activated and translocated to the actin-rich cytoskeletal fraction upon adhesion to matrix proteins [Lakkakorpi et al., 1997]. In macrophages, Src family kinase-mediated phosphorylation of Cbl is required for Cbl–PI3K association and for the transfer of this complex to the actin rich-cytoskeleton [Meng and Lowell, 1998]. Cbl–PI3K interaction is also critical for the spreading and motility of transformed fibroblasts [Feshchenko et al., 1999].

In osteoclasts, over-expression of CblY737F protein, which does not interact with PI3K, reduced in vitro bone resorbing activity of osteoclasts by 80% [Miyazaki et al., 2004]. In agreement with these observations, our recent work demonstrated that Cbl knock-in mice with a tyrosine to phenylalanine substitution at position 737, which prevents Cbl–PI3K interaction (YF mice), showed decreased osteoclast function and increased bone volume [Adapala et al., 2010a,b; Brennan et al., 2011]. Increased bone volume in the YF mice was in contrast to adult Cbl$^{-/-}$ mice, which do not show an overt bone phenotype, probably due to compensation by Cbl-b protein [Chiusaroli et al., 2003]. Characterization of YF osteoclasts also highlighted a negative role for the Cbl–PI3K interaction in osteoclastogenesis and survival. Loss of Cbl–PI3K interaction resulted in a two-fold increase in the number of osteoclasts in YF mice. Furthermore, the ex vivo differentiation of precursors into osteoclasts and the survival of mature osteoclasts was enhanced. At the molecular level, these effects were attributed to perturbed RANKL-mediated signaling [Adapala et al., 2010a].

In this report we evaluated: (1) whether Cbl-b influences the enhanced osteoclast survival due to increased PI3K activity observed in the absence of Cbl–PI3K interaction; (2) the molecular mechanism responsible for the increased PI3K activity in the absence of Cbl–PI3K interaction and (3) whether the YF mutation on Cbl has a dominant-negative role in osteoclast function for both Cbl and Cbl-b proteins. Our results demonstrate that irrespective of the presence of Cbl-b, (1) loss of Cbl–PI3K interaction resulted in up regulation of RANK signaling and increased PI3K activity in osteoclasts. (2) The survival of osteoclasts is enhanced due to increased Ras activity in the absence of Cbl–PI3K interaction; inhibition of Ras activation attenuated the enhanced PI3K signaling and abrogated enhanced survival resulted from the loss of Cbl–PI3K interaction. (3) The role of Cbl–PI3K interaction in bone resorption by osteoclasts is distinct from Cbl-b since loss of Cbl–PI3K interaction in Cbl-b$^{-/-}$ mice (YF/YF;Cbl-b$^{-/-}$) did not prevent osteopenia due to hyperactivity of osteoclasts observed in Cbl-b$^{-/-}$ mice. Thus, we provide direct evidence that Cbl and Cbl-b play distinct and mostly independent roles in osteoclast-mediated bone remodeling in which Cbl-b negatively regulates bone resorption, while Cbl–PI3K interaction regulates survival of osteoclasts mediated by RANK signaling.

MATERIALS AND METHODS

MICE

All mice used in the study were in a mixed C57BL/6JX129SvJ background. The generation of Cbl-b$^{-/-}$, Cbl$^{-/-}$, and YF/YF mice was described previously [Bachmaier et al., 2000; Chiang et al., 2000; Molero et al., 2006]. Mice harboring the CblY737 mutation in a Cbl-b$^{-/-}$ background were generated by mating Cbl$^{YF/YF}$ mice with Cbl-b$^{-/-}$ mice. Heterozygotes were backcrossed with Cbl-b$^{-/-}$ mice. Table I indicates the genotypes and the nomenclature used in the manuscript to describe WT, Cbl$^{-/-}$, YF/YF, Cbl-b$^{-/-}$, and YF/YF;Cbl-b$^{-/-}$ mice. The absence of Cbl-b and the substitution of tyrosine 737 by phenylalanine were verified by PCR analysis (Supplementary Fig. S1A) using the following primers: For YF mice,

TABLE I. Cbl Expression in the Mouse Genotypes Used in This Study

Genotype	Cbl	Cbl-b
WT	+/+	+/+
YF	YF/YF	+/+
YF/Cbl-b$^{-/-}$	YF/YF	−/−
Cbl-b$^{-/-}$	+/+	−/−
Cbl$^{-/-}$	−/−	+/+

common forward 5′-GAAGAGGACACAGAATATATGACTC-3′, WT reverse 5′-CTGGATGTTATACATCGCTTCAT-3′, YF reverse 5′-CTGGATGTTATACATCGCTTCGA-3′. For Cbl-b$^{-/-}$ mice common forward 5′-CCGACTGCATCCTGAATAGC-3′, WT reverse 5′-AATGG-TACAAGTGAGCCTG-3′, KO reverse 5′-TTCTGGATTCATCGACT GTG-3′.

Bone marrow-derived osteoclasts from YF/YF;Cbl-b$^{-/-}$ mice were tested for Y737 phosphorylation, Cbl–PI3K interaction and the expression of Cbl-b by Western blotting analysis (Supplementary Fig. S1B,C). All experiments were performed in compliance with the Institutional Animal Care and Use Committee at University of Connecticut Health Center.

REAGENTS

Minimum essential medium-α modification (α-MEM) and fetal bovine serum (FBS) were purchased from Sigma (St. Louis, MO). Collagen gel was obtained from Nitta Gelatin Co. (Osaka, Japan). Bacterial collagenase and dispase were purchased from Calbiochem (San Diego, CA). Antibodies against phospho-Cbl Tyr^{737}, phospho-AKT Thr^{308}, AKT, phospho-ERK, phospho-IKKα/β, phospho-GSKα/β, GSK3β, phospho-AKT substrates and GAPDH were purchased from Cell Signaling Technology (Danvers, MA). Anti-ERK1/2 and anti-p85 antibodies were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-Cbl, anti-Cbl-b and IKKα antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rhodamine, DAPI and Dead End Fluorometric TUNEL were obtained from Promega (Madison, WI). RANK ligand (RANKL) and MCSF were purchased from R&D Systems (Minneapolis, MN). 1,25-Dihydroxyvitamin D3, prostaglandin E2 and tartrate-resistant acid phosphatase (TRAP) staining kit were obtained from Sigma. FPT III was purchased from Calbiochem and LY294002 from Sigma. Ras activity was determined using a commercial kit purchased from Thermo Scientific (Rockford, IL).

ANALYSIS OF BONES

High-resolution images of long bones from 8-week-old male mice were acquired using a Skyscan 1172, 12 Mpix model, (Micro-photonics, Allentown, PA) as described previously [Brennan et al., 2011]. To measure the dynamic parameters of bone formation, Calcein (route i.p. 10 mg/kg body weight) was injected 9 and 2 days before harvesting the tibiae and femora of mice. Longitudinal sections (5 μM) were used to measure the fluorochrome label, and then deplasticized and stained with Masson Trichrome. Histomorphometry was performed on 3 tissue sections per limb using a Nikon 800 fluorescence microscope linked to a Bioquant Osteo II system (R&M Biometrics, Nashville, TN). Dynamic parameters of bone formation including mineralizing surface [MS/BS; %], mineral apposition rate [MAR = mM/day], bone formation rate [BFR/BS = MAR × (MS/BS)], osteoid volume [OV/BS] and osteoblast number [Ob.N./BS], were evaluated as previously described [Brennan et al., 2011].

CELL CULTURE

For generation of OCLs, bone marrow was isolated from long bones of 4- to 6-week-old mice. Following overnight incubation on tissue culture plastic, non-adherent cells were plated at 2.5×10^5/cm^2 in α-MEM, 10% FBS, penicillin/streptomycin containing 20 ng/ml M-CSF. Subsequently, cells were treated with M-CSF (20 ng/ml) and RANKL (50 ng/ml) for an additional 5–6 days. For some experiments, OCLs were also generated by the co-culture method as described previously [Miyazaki et al., 2004]. Briefly, primary osteoblastic cells were obtained from the calvaria of 1-day-old mice by enzymatic digestion, and bone marrow cells were obtained from long bones of 4- to 6-week-old mice. Bone marrow cells (10^5 cells/cm^2) were co-cultured with calvarial cells (2×10^4 cells/cm^2) on collagen gel-coated plates in the presence of 10 nM 1,25-dihydroxyvitamin D3 and 1 μM prostaglandin E2 (Sigma).

SURVIVAL ASSAY

Following differentiation with M-CSF and RANKL for 5 days, one set of 96-well plates was fixed with 10% formaldehyde (Sigma) in PBS (Mediatech, Manassas, VA). Other sets were either kept in α-MEM only or α-MEM containing RANKL (50 ng/ml) for 48 h. Cells were fixed and TRAP stained using a commercial kit (Sigma). The total number of TRAP+ multi-nucleated cells in the wells was determined. In other experiments, cells were cultured on coverslips in the presence of α-MEM only or were treated with RANKL (50 ng/ml) for 6 or 12 h. The cells were fixed with 10% formaldehyde in PBS and TUNEL assay was performed using the DeadEnd Fluorometric TUNEL kit (Promega) according to the manufacturer's protocol. TUNEL-positive cells were counted using a NIKON Eclipse E800 microscope (Melville, NY) at 20× magnification.

FLUORESCENCE MICROSCOPY

Cells were plated on sterile FBS-coated glass coverslips (Corning, Inc., Corning, NY). OCLs were fixed with 10% formaldehyde in PBS for 10 min and then permeabilized with ice-cold acetone (Sigma) for 5 min. Coverslips for actin labeling were incubated in a 1:40 dilution of rhodamine-phalloidin stock solution (Invitrogen) in PBS for 20 min followed by DAPI staining for nuclei. Cells were examined using a confocal imaging system (Leica TCS FP5 X, Wetzlar, Germany) and images were processed using Adobe photoshop CS4 Extended Edition.

PIT FORMATION ASSAY

OCLs were generated in co-culture as described above in collagen gel. After 6–7 days of co-culture, collagen was removed by gentle digestion with 0.1% collagenase, and then cells were seeded onto sterile dentine slices (ImmunoDiagnostic Systems Ltd., Boldon, UK) in 96-well plates. Forty eight hours later, dentine slices were immersed in 1 M ammonium hydroxide (Acros Organics, Fair Lawn, NJ) for 5 min, sonicated for 10 s, and then stained for 4 min with 1% toluidine blue (Sigma) in 1% sodium borate (Sigma) and briefly washed in water. Pit area was quantified with the measure tool in Adobe Photoshop CS4 Extended Edition and was normalized to the number of osteoclasts actually present in each sample, determined by counting OCLs present in a separate aliquot of OCL suspension.

EVALUATION OF BIOCHEMICAL ACTIVITIES

Cells were deprived of serum and growth factors as mentioned in the figure legends and then stimulated with RANKL (50 ng/ml). In some

experiments, cells were treated with either the Ras inhibitor FPTIII (100 μM) for 8 h or the PI3K inhibitor LY29400 (10 μM) for 1 h prior to the addition of the RANKL. Ras activity was determined using a pull down assay based on the Ras binding domain of Raf1 using a commercial kit (Thermo Scientific). For immunoprecipitation of p85, total cell lysate (500 μg) was incubated with anti-p85 antibodies (2 μg) and protein-A agarose slurry (50 μl) for 2 h at 4°C. Antigen-antibody complexes on the agarose beads were washed with cell lysis buffer and then subjected to Western blotting analysis as previously described. Phosphorylation of AKT, AKT substrates, ERK, and GSK was determined using phospho-specific antibodies as previously described [Adapala et al., 2010a]. The quantitation of bands in Western blots was performed by using Odyssey Infrared Imaging Systems software 2.1 (LICOR Biosciences).

STATISTICAL ANALYSIS

Experiments conducted in this study were repeated at least three times. The results obtained from a typical experiment were expressed as the mean ± SD. For two groups, significant differences were determined using student's *t* test, whereas for more than two groups, ANOVA with Tukey's multiple comparison test was performed using Prism 5 software. $P < 0.05$ versus control was considered statistically significant.

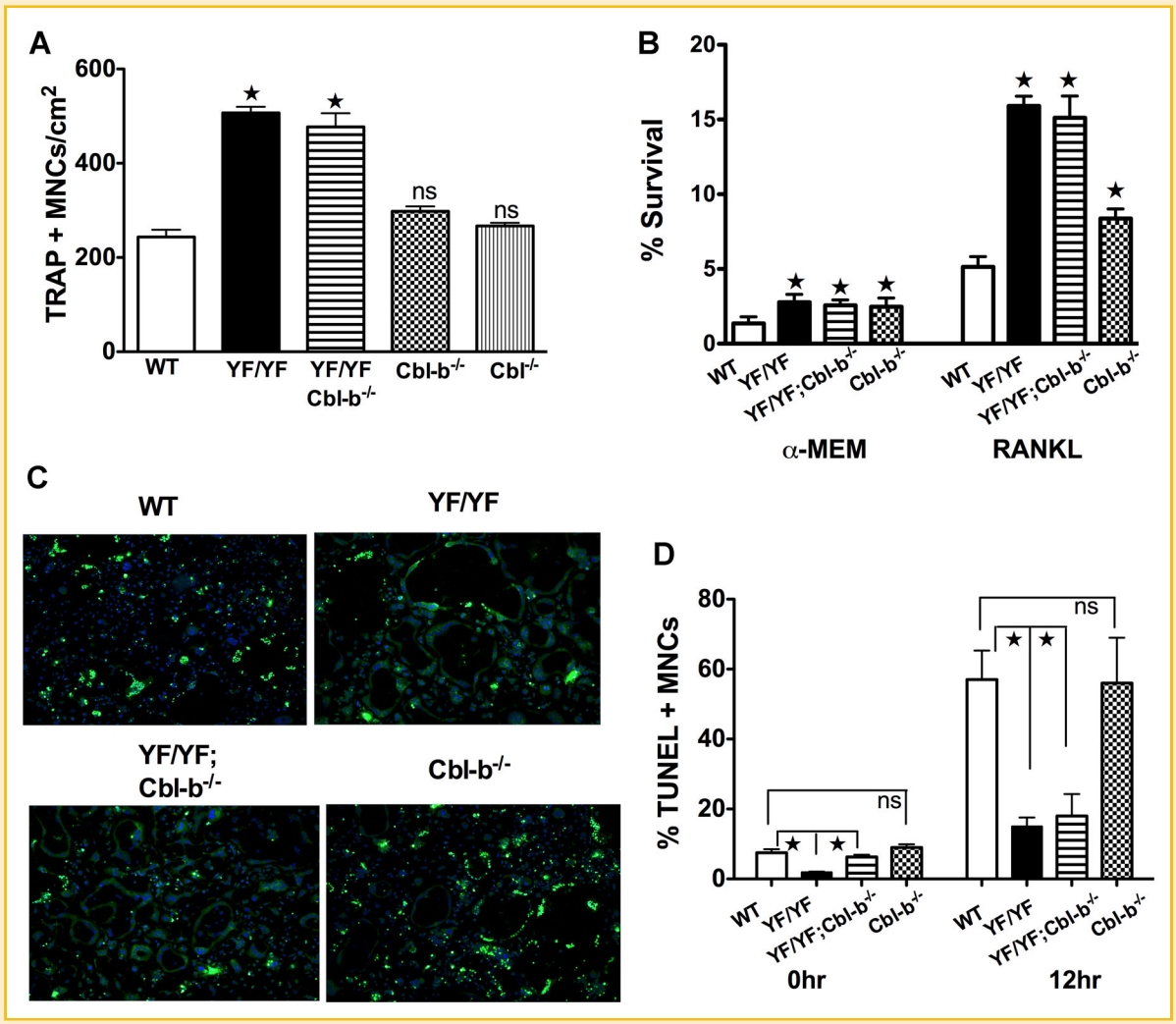

Fig. 1. Cbl–PI3K interaction regulates differentiation and survival independent of Cbl-b. Differentiation of bone marrow precursors into osteoclast like cells (OCLs) was performed by treating 5×10^5 cells/cm^2 bone marrow macrophages with MCSF (20 ng/ml) and RANKL (50 ng/ml) for 5 days, in a 96-well plate. A: TRAP stained cells with >3 nuclei were counted as OCLs. B: To perform survival assay, following the differentiation, one set of cultures were fixed to determine the starting number of cells. The culture medium in experimental plate was replaced with either basal medium (α-MEM) or basal medium containing only RANKL (50 ng/ml) for 48 h. After treatment, cells were fixed and TRAP stained. Survival data represent the percentage of the number of OCLs that remained in the culture after 48 h compared to starting number of cells at day 5. C,D: To determine onset of apoptosis, OCLs were cultured in basal medium containing RANKL (50 ng/ml) for 12 h. To visualize apoptotic OCLs at beginning of assay (0 h) and at 12 h, TUNEL staining was performed and nuclei were visualized with DAPI. C: Bright green TUNEL positive cells showing apoptotic nuclei are seen. D: Data are expressed as percentage of the number of TUNEL positive OCLs to the total number of OCLs in the well. (Data presented are representative of three independent experiments.) *$P < 0.05$ as compared with WT control was considered statistically significant. n.s., not significant compared to WT control.

RESULTS

ABSENCE OF CbL–PI3K INTERACTION RESULTED IN INCREASED NUMBERS OF OSTEOCLASTS

Osteoclast differentiation is dependent on RANK signaling, which involves signaling cascades in which Cbl and PI3K play important roles [Arron et al., 2001]. We have previously shown that addition of RANKL to osteoclast cultures resulted in increased phosphorylation of Y737 on Cbl and activation of PI3K signaling. In contrast, loss of Cbl–PI3K interaction due to the Y737F mutation resulted in increased PI3K activity in YF/YF OCLs compared to WT OCLs, which, in turn, correlated with enhanced differentiation [Adapala et al., 2010a]. To determine if Cbl-b plays a role in enhanced osteoclast differentiation in the absence of Cbl–PI3K interaction, osteoclastogenesis was tested in bone marrow macrophages derived from all the five genotypes (Table I). As shown in Figure 1A, in Cbl$^{-/-}$ and Cbl-b$^{-/-}$ cultures, the number of multi-nucleated TRAP+ cells (>3 nuclei/cell) was comparable to WT, whereas the number was increased in both YF/YF;Cbl-b$^{-/-}$ and YF/YF cultures. These results suggest that while the absence of Cbl-b had no effect on osteoclastogenesis, loss of Cbl–PI3K enhanced osteoclast differentiation.

OSTEOCLAST SURVIVAL IS REGULATED BY CbL–PI3K INTERACTION, INDEPENDENT OF CbL-B

One of the possibilities for the increased number of osteoclasts could be a prolonged persistence of cells in culture. Since the number of OCLs in WT and Cbl$^{-/-}$ cultures were comparable, in the next series of experiments only WT, YF/YF, Cbl-b$^{-/-}$, and YF/YF;Cbl-b−/− cultures were analyzed. After 48 h of RANKL treatment, in both YF/YF and YF/YF;Cbl-b$^{-/-}$ cultures, threefold more OCLs persisted in the culture (5.13% WT, 15.9% YF/YF; 15.1% YF/YF;Cbl-b$^{-/-}$) compared to WT. In contrast only 8.36% of Cbl-b$^{-/-}$ OCLs survived in response to RANKL (Cbl-b$^{-/-}$ 8.36%; $P < 0.05$) (Fig. 1B). Since the enhanced survival of OCLs may result from a delay in the onset of apoptosis [Adapala et al., 2010a], we also determined the percentage of apoptotic cells in the cultures by TUNEL staining and found that enhanced survival was associated with decreased apoptosis. In response to treatment with RANKL, 57% and 56% of OCLs stained TUNEL positive in WT and Cbl-b$^{-/-}$ cultures respectively. In contrast, only 15% of YF/YF and 18% of YF/YF;Cbl-b$^{-/-}$ OCLs stained TUNEL-positive upon treatment with RANKL (Fig. 1C,D). These results indicate that apoptosis developed more slowly in the absence of Cbl–PI3K interaction and Cbl-b did not influence osteoclast survival. Hence it appears that osteoclast survival is uniquely regulated by Cbl–PI3K interaction, independent of Cbl-b.

LOSS OF CBL–PI3K INTERACTION ENHANCES PI3K SIGNALING IN OCLS, INDEPENDENT OF CbL-B

Cbl proteins are phosphorylated downstream of RANK and are reported to modulate RANK expression and signaling [Arron et al., 2001]. We have reported that loss of Cbl–PI3K interaction in osteoclasts results in enhanced RANK signaling. Specifically, we showed that in YF/YF OCLs treatment with RANKL resulted in increased phosphorylation of ERK, IKK, and AKT [Adapala et al., 2010a]. Here, we examined the effect of the absence of Cbl-b and the loss of Cbl–PI3K interaction on PI3K activation in response

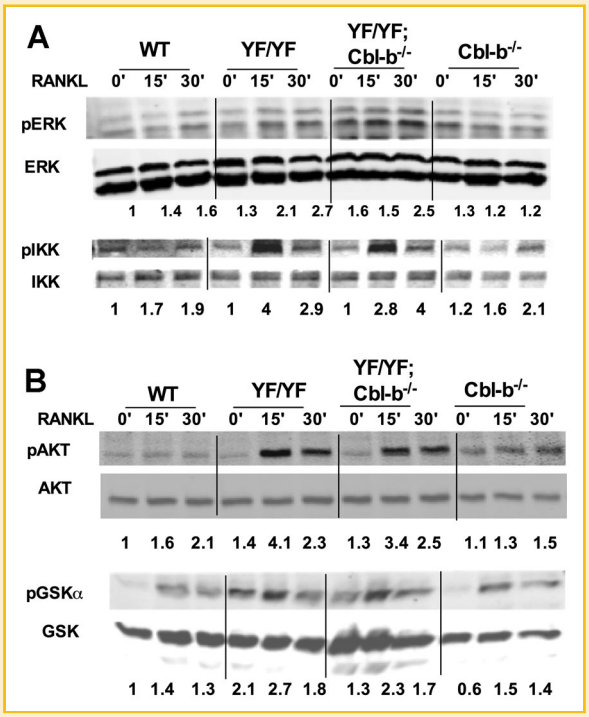

Fig. 2. Cbl but not Cbl-b modulates PI3K activity during RANK signaling in osteoclasts. OCLs were serum-starved for 90 min and then treated with RANKL (50 ng/ml) for the indicated times. A: Blots were probed with anti-phospho-ERK (Thr202/Tyr204) antibodies (top panel) or pIKKα/β (bottom panel) and then stripped and re-probed with total ERK or IKKα antibodies. B: OCLs were treated as described above and blots were probed with anti-phospho-AKT-Thr308 antibodies (top panel) or anti-phospho-GSKβ (bottom panel) then stripped and re-probed for total proteins. To correct for experimental variability, the amounts of total proteins in individual bands were quantified by using Odyssey Infrared Imaging Systems software 2.1 (LICOR Biosciences), and the ratio of phosphorylated protein to total protein is shown at the bottom of the panels. A representative experiment of four repetitions is shown.

to treatment with RANKL. Western blotting analysis demonstrated that treatment with RANKL resulted in increased phosphorylation of ERK (Fig. 2A, top panel) and IKK (Fig. 2A bottom panel) in both YF/YF and YF/YF;Cbl-b$^{-/-}$ OCLs compared to WT and Cbl-b$^{-/-}$ cells. Increased PI3K signaling in response to RANK activation was evidenced by increased phosphorylation of AKT (Fig. 2B, top panel) and GSK (Fig. 2B, bottom panel), a known substrate of AKT. These results suggest that the two major subsets of RANK signaling pathways show increased activation in the absence of Cbl–PI3K interaction, independent of Cbl-b.

INCREASED RANK SIGNALING DUE TO LOSS OF CbL–PI3K INTERACTION IS ASSOCIATED WITH INCREASED Ras ACTIVITY AND Ras–PI3K BINDING

We have previously proposed that the inability of Cbl to interact with PI3K increased the availability of PI3K, and enhanced the possibility to interact with other signaling proteins that regulate osteoclast differentiation and survival [Adapala et al., 2010a]. One of the important signaling molecules, which are known to regulate osteoclast survival is the small GTPase, p21-Ras. Bradley et al.

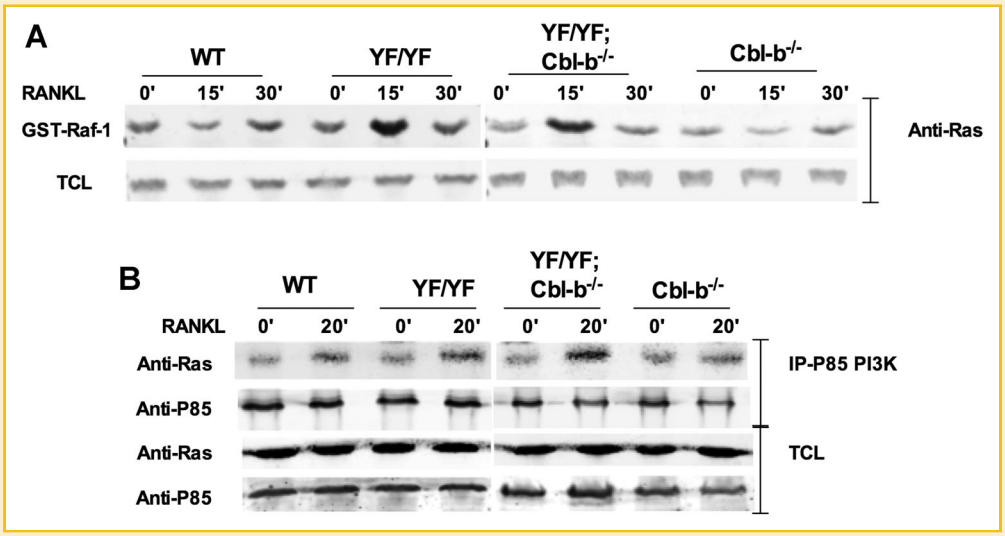

Fig. 3. In the presence or absence of Cbl-b, loss of Cbl–PI3K interaction is associated with increased Ras activity. A: Osteoclasts were serum-starved for 60 min and then treated with RANKL (50 ng/ml) for the indicated times. To determine Ras activity, cell lysates (500 μg) were incubated with GST-conjugated Raf-1 and then processed for Western blotting analysis. Blots were probed with anti-Ras antibody (top panel). Total cell lysate (50 μg) was processed for Western blotting and probed with anti-Ras antibody to determine the expression levels of Ras in cell lysates (bottom panel). B: To examine PI3K binding to Ras, osteoclasts were serum-starved for 60 min and then treated with RANKL (50 ng/ml). Total cell lysates (500 μg) were immunoprecipitated with anti-p85 PI3K antibody and then processed for Western blotting analysis. Blots were probed with anti-Ras antibody (top panel), and then stripped and re-probed with p85 antibody (bottom panel). Total cell lysate (TCL) were also examined for Ras and p85 expression.

have previously demonstrated the activation of PI3K mediated by Ras in osteoclasts [Bradley et al., 2008]. Upon stimulation, the GTP-bound form of Ras recruits the protein kinase Raf-1 to the cell membrane resulting in the activation of MEK1/2 and ERK1/2 pathway. Overexpression of a dominant negative from of Ras inhibited ERK phosphorylation, which compromised osteoclast survival [Miyazaki et al., 2000]. Upon treatment of OCLs with RANKL, increased phosphorylation of ERK in cells, which do not have Cbl–PI3K interaction, suggesting the involvement of Ras-Raf signaling pathway (Fig. 2A, top panel). As compared to WT and Cbl-b$^{-/-}$, stimulation with RANKL resulted in increased Ras activity in both YF/YF and YF/YF;Cbl-b$^{-/-}$ OCLs (Fig. 3A). Given that Ras is also known to interact with the p85 subunit of PI3K, we also determined Ras–PI3K association. Co-immunoprecipitation of Ras with p85 subunit of PI3K showed interaction between Ras and the p85 subunit of PI3K across all the genotypes (Fig. 3B). Cumulatively, these results suggest that in the absence of Cbl–PI3K interaction Ras activity and RANK signaling were increased and Cbl-b did not appear to play any significant role in this mechanism.

RAS ACTIVATES PI3K ACTIVITY IN THE ABSENCE OF CbL–PI3K INTERACTION RESULTING IN INCREASED AKT ACTIVITY AND OSTEOCLAST SURVIVAL

Since Cbl-b does not appear to play a significant role in regulation of PI3K activity downstream of RANK signaling (Fig. 3) and that both YF/YF and YF/YF;Cbl-b$^{-/-}$ OCLs demonstrate a similar increase in RANK mediated signaling events, we performed further analysis of Ras mediated PI3K activation downstream of RANK signaling comparing only WT and YF/YF;Cbl-b$^{-/-}$ OCLs.

First, we confirmed that in YF/YF;Cbl-b$^{-/-}$ OCLs increased AKT phosphorylation is a consequence of augmented PI3K activity. For these experiments, WT and YF/YF;Cbl-b$^{-/-}$ OCLs were treated with the PI3K inhibitor LY294002 and the effect on RANKL-mediated PI3K activity was examined by Western blotting. While treatment of OCLs with RANKL increased phosphorylation of AKT, GSK and AKT substrates, inhibition of PI3K activity significantly attenuated phosphorylation of AKT, GSK3β (Fig. 4A) and phospho-AKT substrates (Fig. 4B) in response to RANKL stimulation. In contrast, increased phosphorylation of AKT, GSK3β and AKT substrates was seen for both untreated and RANKL-treated YF/YF;Cbl-b$^{-/-}$ OCLs, while inhibition of PI3K activity did not diminish phosphorylation of AKT, GSK, and AKT substrates in YF/YF;Cbl-b$^{-/-}$ OCLs.

Next, we studied whether Ras mediates enhanced PI3K signaling in the absence of Cbl–PI3K interaction. For these experiments, Ras activity was modulated using FPT III, a known Ras inhibitor. Inhibition of Ras in WT or YF/YF;Cbl-b$^{-/-}$ OCLs resulted in decreased phosphorylation of ERK (Fig. 4C, upper panel), which is a known target of the Ras-Raf signaling pathway. However, Ras inhibition had no effect on the phosphorylation of AKT (Fig. 4C, middle panel), GSK (Fig. 4C, lower panel) or AKT substrates (Fig. 4D) in WT OCLs. In contrast, treatment with FPT III completely inhibited phosphorylation of AKT, AKT substrates, and GSKβ in YF/YF;Cbl-b$^{-/-}$ OCLs (Fig. 4C,D). These data suggest that in WT OCLs only ERK phosphorylation is dependent on Ras activity, while in the YF/YF;Cbl-b$^{-/-}$ OCLs, both ERK and AKT activation are Ras-dependent.

Finally, we examined the role of Ras activity on the onset of apoptosis in the presence or absence of Cbl–PI3K interaction, by TUNEL staining (Fig. 5A). In WT cultures 68.0% of OCLs were apoptotic when cultured in medium devoid of serum. Stimulation with RANKL decreased apoptosis of WT cells to 42.6%, and inhibition of Ras did not alter the survival of WT, since 41.1% cells exhibited apoptosis upon FPT III treatment. In contrast, 48.1% of

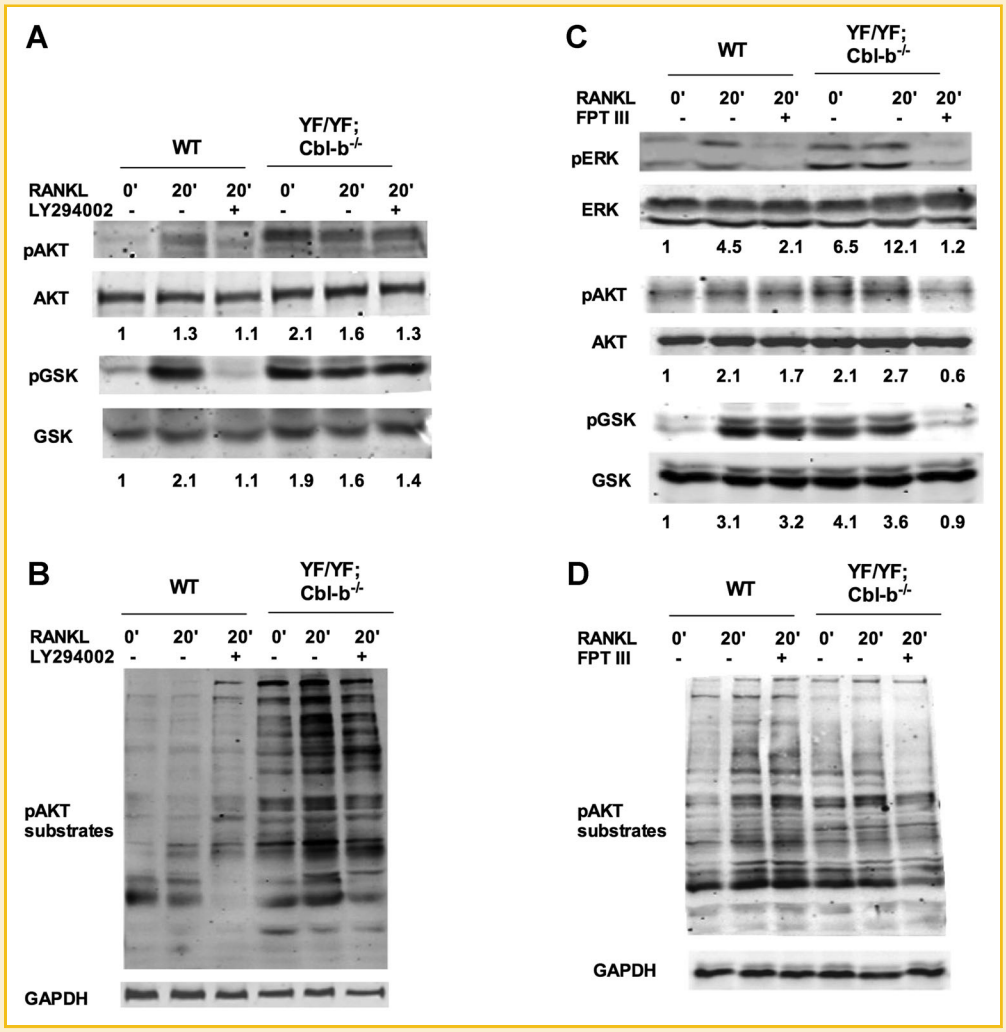

Fig. 4. Ras mediates increased PI3K activity due to loss of Cbl–PI3K interaction. OCLs were serum-starved for 60 min and then treated with RANKL (50 ng/ml) in the absence or presence of PI3K inhibitor LY294002 (10 μM) for 20 min. Total cell lysates were processed for Western blotting analysis. A: Blots were probed with anti-phospho AKT (Thr308) antibody (top panel), anti-phospho GSK3β antibody (bottom panel) or B: anti-phospho AKT substrates. After blotting with the indicated phospho-specific antibodies, blots were stripped and re-probed with antibodies to respective proteins or GAPDH to determine equal loading. The amounts of total proteins in individual bands were quantified as described above and the ratio of tyrosine phosphorylation to amount of total protein is shown at the bottom of the panels. To test the effect of Ras inhibition, OCLs were serum starved for 60 min then treated with RANKL (50 ng/ml) in the absence or presence of Ras inhibitor FPT III (100 μM) for 20 min. C: To confirm the efficiency of Ras inhibition, blots were probed with anti-phospho-ERK 42/44 (Thr202/Tyr204) (top panel), stripped, and re-probed with anti-ERK antibodies. To analyze PI3K signaling, blots were probed with anti-phospho AKT (Thr308) antibody (middle panel), anti-phospho GSK3β antibody (bottom panel) or anti-phospho AKT substrates antibody. D: The blots were stripped and re-probed for total proteins or GAPDH. The ratio of phosphorylated protein to total protein is shown below the panel.

YF/YF;Cbl-b$^{-/-}$ OCLs were apoptotic when cultured in medium devoid of serum and treatment with RANKL substantially decreased the numbers of apoptotic cells (to 24.2%). However, Ras inhibition removed protection from apoptosis in YF/YF;Cbl-b$^{-/-}$ OCLs and led to a significant increase in the numbers of apoptotic cells (YF/YF;Cbl-b$^{-/-}$ 76.2% vs. WT 41.1%; Fig. 5B). These results suggest that in WT OCLs RANK signaling promotes survival of OCLs through pathways involving Cbl–PI3K interaction and AKT activation, which are not affected by Ras inhibition. However, when Cbl–PI3K interaction is abrogated, Ras activity becomes essential for enhanced survival mediated by PI3K pathway.

DEFECTIVE OSTEOCLAST FUNCTION DUE TO THE LOSS OF CbL–PI3K INTERACTION DOES NOT RESCUE THE INCREASED BONE RESORPTION BY CbL-b$^{-/-}$ OSTEOCLASTS

Having studied the effect of Cbl–PI3K interaction and the absence of Cbl-b on osteoclast differentiation and survival, we next examined their effect on osteoclast function and bone remodeling. Mice, which lacked Cbl–PI3K interaction in a Cbl-b$^{-/-}$ background provided a model system to analyze the specific contributions of Cbl and Cbl-b proteins to skeletal remodeling. We first compared in vitro bone resorption in WT, YF/YF, YF/YF;Cbl-b$^{-/-}$, Cbl-b$^{-/-}$, and Cbl$^{-/-}$ osteoclasts. Pit resorbing activity of Cbl$^{-/-}$ OCLs was comparable to WT cells, while loss of Cbl–PI3K interaction in YF mice decreased pit

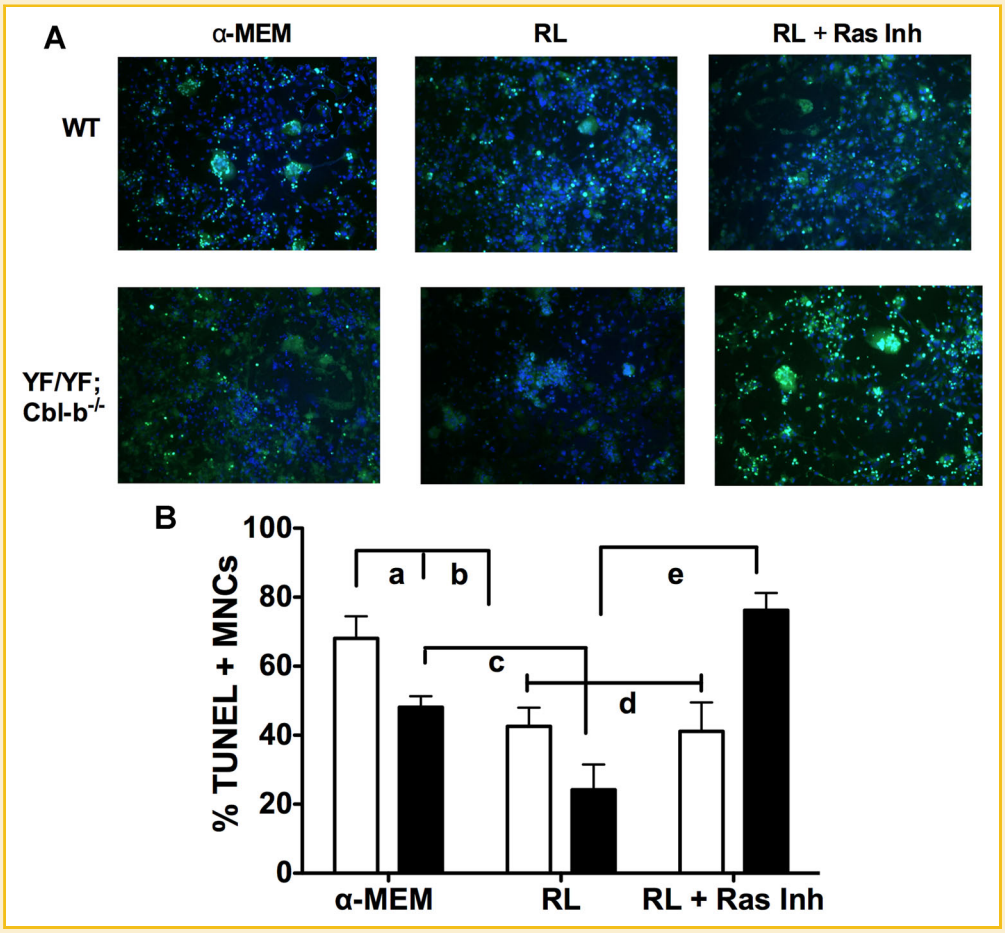

Fig. 5. Increase in PI3K activity mediated by Ras is required for enhanced survival in the absence of Cbl–PI3K interaction. OCLs were either untreated or were treated with RANKL (50 ng/ml) for 12 h in the absence or presence of Ras inhibitor, FPT III (100 μM), cells were fixed and TUNEL staining was performed. A: Photomicrographs of the TUNEL-stained OCL cultures are shown at 10× magnification. Bright green signal indicates DNA strand breaks during apoptosis. B: Histogram represents the percentage of TUNEL+ cells out of total number of multi-nucleated cells and data are expressed as percentage of TUNEL positive OCLs (open bars, WT; black bars, YF/YF;Cbl-b$^{-/-}$). a: $P < 0.05$ WT α-MEM control versus YF/YF;Cbl-b$^{-/-}$ α-MEM control. b: $P < 0.05$ WT α-MEM control versus WT RANKL treatment. c: $P < 0.05$ YF/YF;Cbl-b$^{-/-}$ α-MEM control versus YF/YF;Cbl-b$^{-/-}$ RANKL treatment. d: $P < 0.05$ WT without Ras inhibition versus WT with Ras inhibition e: $P < 0.05$ YF/YF;Cbl-b$^{-/-}$ without Ras inhibition versus YF/YF;Cbl-b$^{-/-}$ with Ras inhibition. RL, RANKL; Ras Inh, Ras inhibitor. A representative of three independent experiments is shown.

formation as previously reported (Adapala et al. 2010a and Fig. 6). Also, the absence of Cbl-b in osteoclasts resulted in 2.4-fold increase in pit formation ability as previously reported [Nakajima et al., 2009]. In spite of the loss of Cbl–PI3K interaction, YF/YF;Cbl-b$^{-/-}$ OCLs showed a twofold increase in pit formation (Fig. 6). Thus, the loss of Cbl–PI3K interaction resulted in decreased osteoclast activity. However, it did not confer protection from the hyperactive osteoclast function observed in the absence of Cbl-b. To study if the differences in bone resorption are a result of defects in actin ring formation, we analyzed cytoskeletal organization and did not find any major differences in actin cytoskeleton across all genotypes (Supplemental Fig. S2).

Following analysis of bone resorption, we studied the effect of Cbl-b and Cbl–PI3K interactions on bone remodeling in vivo comparing WT and YF/YF;Cbl-b$^{-/-}$ mice. Micro CT analysis of tibia and L2 vertebra from YF/YF;Cbl-b$^{-/-}$ mice showed that compared to WT mice there was a 56% and 59% decrease in bone volume in the tibia and L2 vertebra, respectively (Fig. 7A,B and Table II). The decrease in bone volume correlated with decreased trabecular number and thickness and increased trabecular separation, while no significant differences were observed in the cortical bone parameters (Table II and Supplementary Fig. S3). Histological analysis of tibiae from 8-week-old WT and YF/YF;Cbl-b$^{-/-}$ mice showed that the cancellous bone was decreased in YF/YF;Cbl-b$^{-/-}$ mice relative to age-matched WT mice (Fig. 7C). Bone formation rate and osteoblast numbers were comparable between WT and YF/YF;Cbl-b$^{-/-}$ mice (Table II). In agreement with the results in ex vivo cultures (Fig. 1), we also observed increased numbers of TRAP stained osteoclasts in the long bones of YF/YF;Cbl-b$^{-/-}$ (Fig. 7D and Table II). Additionally, serum levels of C-terminal collagen telopeptide levels (CTX), a marker of in vivo osteoclast activity, were significantly increased in YF/YF;Cbl-b$^{-/-}$ mice compared to WT mice (Fig. 7E). Collectively, the histomorphometric and histological analysis indicated two points: YF/YF;Cbl-b$^{-/-}$ mice had (1) decreased bone volume due to

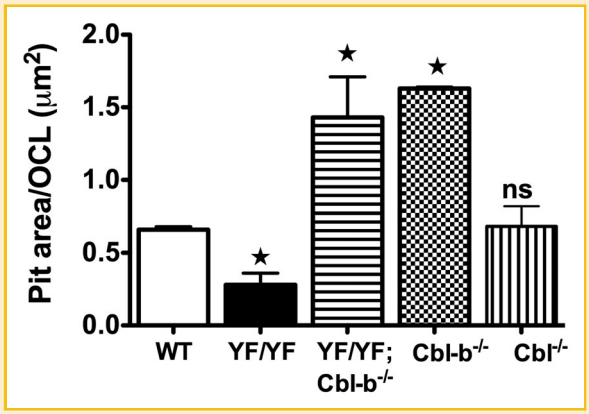

Fig. 6. Loss of Cbl–PI3K interaction distinctively decreases osteoclast function and does not interfere with hyperactivity observed in the absence of Cbl-b. Mature osteoclasts derived from bone marrow in co-culture with osteoblasts were plated on dentin discs for 48 h. OCLs were removed and the dentin discs were stained with toluidine blue. The area of pits on dentin discs was determined using Adobe photoshop CS4. The graph shows pit area normalized to numbers of osteoclasts and data are expressed as area of dentin resorbed per osteoclast. A representative experiment of three repetitions is shown. *$P < 0.05$ was considered statistically significant as compared to WT control. n.s., not significant, compared to WT.

increased osteoclast activity like Cbl-b$^{-/-}$ mice and (2) increased numbers of mature osteoclasts like YF mice. Hence, defective osteoclast function, due to the loss of Cbl–PI3K interaction did not negate the hyperactivity of osteoclasts in the absence of Cbl-b, suggesting that Cbl-b negatively regulates bone resorption and Cbl–PI3K interaction does not contribute to this process.

DISCUSSION

Using single knockout mice and over expression of Cbl proteins in osteoclasts, it has been postulated that Cbl and Cbl-b have both overlapping and distinct functions in bone remodeling [Chiusaroli et al., 2003; Nakajima et al., 2009]. In this report, we have formally demonstrated that Cbl and Cbl-b distinctly regulate osteoclast survival and function. Skeletal characterization of the YF/YF; Cbl-b$^{-/-}$ mice in which Cbl-b is absent and Cbl–PI3K interaction is lost, led to the following significant observations. First, Cbl–PI3K interaction is necessary for maintaining optimal PI3K signaling. Second, loss of Cbl–PI3K interaction resulted in increased Ras activation, which led to increased osteoclast survival. Third, the loss of Cbl–PI3K interaction did not have any impact on the hyperactivity of Cbl-b$^{-/-}$ osteoclasts.

Both Cbl and Cbl-b share structural organization and considerable homology in the protein–protein interacting domains. However, one functionally important difference between them is the presence of a unique inducible binding site for the p85 subunit of PI3K at Y737 in Cbl, which enables Cbl to recruit PI3K to cell membranes [Ueno et al., 1998; Hunter et al., 1999; Lazaar et al., 2001]. It has been suggested that both Cbl and Cbl-b promote PI3K activation downstream of RANK signaling [Arron et al., 2001]. However, increased PI3K activity in response to RANKL in both YF OCLs and YF/YF;Cbl-b$^{-/-}$ OCLs indicated that Cbl-b does not relay the signal from RANK-TRAF-6-Src to PI3K (Fig. 2). In addition to increased AKT phosphorylation, ERK phosphorylation was also increased in YF/YF;Cbl-b$^{-/-}$ OCLs in response to RANKL treatment (Fig. 2A). We have previously reported that protein levels of the p85 subunit of PI3K are not altered in YF OCLs [Adapala et al., 2010a] and the absence of Cbl-b did not affect PI3K activity in YF/YF;Cbl-b$^{-/-}$ osteoclasts. Given these data, we hypothesized that PI3K is available for activation by parallel pathways, when not bound to Cbl.

PI3K coordinately activates AKT and MEK/ERK pathways, which regulate cell survival. Cross talk between AKT and MEK/ERK pathways in muscle and breast cancer cells involved AKT-mediated inactivation of Raf-1 repressing MEK pathway [Zimmermann and Moelling, 1999; Moelling et al., 2002]. In vascular smooth muscle cells, PI3K mediated repression of Raf-MEK-ERK pathway resulted in PDG-F-mediated differentiation of VSMCs [Reusch et al., 2001]. Osteoclast survival in response to IL-1 and M-CSF is regulated by a cross talk between PI3K and Ras-Raf signaling pathways [Miyazaki et al., 2000; Bradley et al., 2008]. Hyperactivation of Ras and PI3K is also known to alter osteoclast function by modulating neurofrombin, a GTPase activating protein for Ras in both mice and humans [Yang et al., 2006]. Thus coordinated regulation of Ras and PI3K is important for bone homeostasis.

We propose that Cbl spatio-temporally regulates PI3K to control survival of osteoclasts by limiting the availability of PI3K among different signaling pathways (Fig. 8). In WT osteoclasts, upon RANKL stimulation, the Src-Cbl-PI3K pathway activates AKT, and this optimal activation is required for normal cell survival. In this situation inhibition of Ras, while decreasing Ras-Raf-1-MEK-ERK signaling, does not affect osteoclast survival, since Src-Cbl-PI3K-AKT pathway is intact. However, when Cbl is unable to negatively regulate signaling by limiting the availability of PI3K, optimal AKT activation is lost, resulting in increased osteoclast survival due to cooperation between Ras and PI3K signaling. In this situation, inhibition of Ras results not only in inhibition of ERK activation, but also in AKT inhibition and as a result the survival of osteoclasts with YF mutation is compromised. Other proteins including ITAM receptors, DAP12 and FcγR, may also activate PI3K [Peng et al., 2010]. However, the contribution of ITAM signaling towards PI3K activation in our model system remains to be tested.

We previously reported that expression of the YF mutant in WT osteoclasts strongly reduced in vitro bone resorption [Miyazaki et al., 2004], which is similar to the effect of overexpressing wild type Cbl-b [Nakajima et al., 2009]. These observations raised the possibility that the CblYF mutant may have a dominant negative role in bone resorption. However, detailed skeletal characterization of the YF/YF;Cbl-b$^{-/-}$ mice and evaluation of in vivo and in vitro bone resorption parameters clearly demonstrated that the presence of the YF mutation in the Cbl-b$^{-/-}$ background does not rescue the increased bone-resorbing activity of Cbl-b$^{-/-}$ osteoclasts (Fig. 7 and Table II).

The ubiquitylation function of Cbl and Cbl-b has also been reported to differ in how they down regulate signaling effectors, like Syk and PLCγ [Fang and Liu, 2001; Yasuda et al., 2002; Zhang et al., 2004]. In addition, although Cbl and Cbl-b seem to have equal capacity to act as ubiquitin ligases toward a range of

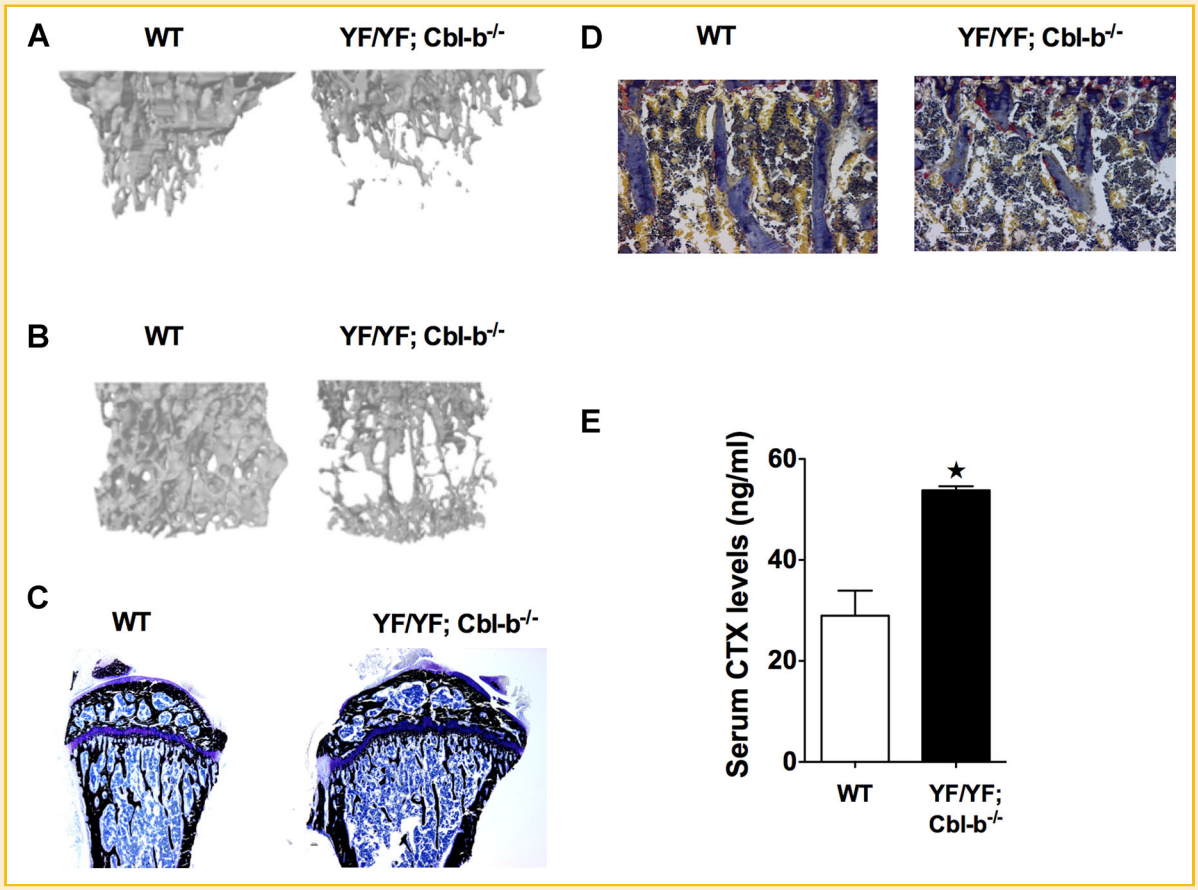

Fig. 7. Bone volume is decreased in mice lacking Cbl–PI3K interaction and Cbl-b. A,B: Three-dimensional images of trabecular region of tibia and L2 vertebral body of 8-week-old male mice demonstrating decreased bone volume in YF/YF;Cbl-b$^{-/-}$ mice. μCT analysis was performed at 59 kV source voltage, and 167 μA as source current using a SkyScan 1172 scanner and associated analysis software version 1.10 (SKYSSCAN, Aartselaar, Belgium), following JBMR guidelines. C: Photomicrographs (10× magnification shown) of von Kossa stained sections of tibiae from 8-week-old WT and YF/YF;Cbl-b$^{-/-}$ mice demonstrated decreased mineral content. Sections were counter-stained with toluidine blue. D: Representative histological sections of proximal tibiae showing TRAP activity reaction product (red) shown at 20× magnification. YF/YF;Cbl-b$^{-/-}$ sections had more numbers of TRAP+ cells. E: Measurement of serum levels of collagen telopeptide revealed increased osteoclast activity in YF/YF;Cbl-b$^{-/-}$ mice. n = 4–6 mice/genotype. Mean ± SD. *$P < 0.05$ as compared to control.

different proteins in in vitro ubiquitylation assays, the fates of their ubiquitylated substrates are sometimes different. For example, loss of Cbl-b in T cells does not alter the amount of p85 or Vav, which are known to be down-regulated by Cbl, but affects activation, localization, and association of these proteins with other signaling molecules [Bachmaier et al., 2000; Chiang et al., 2000; Fang and Liu, 2001]. In Cbl-b$^{-/-}$ osteoclasts, expression of a RING domain mutant, which abrogates E3 ligase activity, failed to rescue the increased pit formation [Nakajima et al., 2009]. These findings suggest that other features of Cbl-b contribute to its role in the negative regulation of osteoclast function.

In addition to osteoclast function, Cbl is also known to impact osteoblast differentiation and function in cell cultures downstream of integrin signaling and the FGF receptor [Kaabeche et al., 2004, 2005; Guenou et al., 2006; Severe et al., 2013]. However, the absence of Cbl or Cbl-b in mice did not affect osteoblast number or bone formation rate [Chiusaroli et al., 2003; Nakajima et al., 2009]. In contrast to these findings, we have previously reported that in YF mice both the numbers of osteoblasts and the bone formation rate were increased [Adapala et al., 2010a; Brennan et al., 2011]. Surprisingly, the bone formation parameters were unchanged in the YF/YF;Cbl-b$^{-/-}$ mice (Table II). Although speculative these data suggest that the absence of Cbl–PI3K interaction may affect osteoblast differentiation and function either directly or indirectly via secretion of clastokine(s).

In conclusion, we provide evidence that Cbl and Cbl-b perform distinct roles in osteoclast differentiation, survival and function due to the unique ability of Cbl to modulate PI3K availability, thereby controlling the degree of PI3K activity. Perturbation of Cbl binding to PI3K results in Ras-mediated hyper-activation of PI3K, affecting survival of osteoclasts. Additionally, although Cbl can negatively regulate bone resorption by influencing PI3K availability, it does not affect Cbl-b mediated bone resorption suggesting that these similar proteins have very distinct and diverse functions in osteoclasts.

TABLE II. Characterization of Tibia and L2 Vertebra of WT and YF/YF;Cbl-b$^{-/-}$ Male Mice

	BV/TV (%)	Tb.Th (mm)	TbN (1/mm)	Tb.Sp (mm)
Trabecular bone parameters				
Tibia				
WT	7.63 ± 0.87	0.05 ± 0.001	1.41 ± 0.29	0.41 ± 0.01
YF/YF;Cbl-b$^{-/-}$	4.30 ± 1.01*	0.04 ± 0.003*	0.85 ± 0.16*	0.48 ± 0.01*
L2 vertebra				
WT	11.29 ± 1.46	0.04 ± 0.002	2.06 ± 0.29	0.46 ± 0.01
YF/YF;Cbl-b$^{-/-}$	6.68 ± 1.34*	0.05 ± 0.003*	1.30 ± 0.23*	0.56 ± 0.01*

	Bone area (mm^2)	PPm (mm)	BM area (mm^2)	EPm (mm)	CTh 2-D (mm)
Cortical bone parameters					
WT	1.28 ± 0.06	6.11 ± 0.33	0.68 ± 0.06	4.63 ± 0.2	0.12 ± 0.01
YF/YF;Cbl-b$^{-/-}$	1.34 ± 0.10	5.59 ± 0.14	0.69 ± 0.04	4.73 ± 0.13	0.13 ± 0.01

	BFR/BS (μm^3/μm^2/day)	MAR (μm/day)	NOb/BPm (/mm)	NOc/BPm (/mm)
Static and dynamic histomorphometry				
WT	0.13 ± 0.04	1.2 ± 0.1	15.2 ± 4.5	3.7 ± 0.4
YF/YF;Cbl-b$^{-/-}$	0.12 ± 0.05	1.3 ± 0.2	12.5 ± 6.8	7.2 ± 0.6*

μCT analysis was performed on 8-week-old mice.
Values are mean ± SD; n = 4 WT, n = 4 YF/YF;Cbl-b$^{-/-}$.
TV, tissue volume; BV, bone volume; Tb.Th, trabecular thickness; Tb.N, trabecular number; Tb.Sp, trabecular separation; PPm, periosteal perimeter; BM area, bone marrow area; EPm, endosteal perimeter; CTh 2-D, cortical thickness-2 dimensional; NOb, number of osteoblasts; NOc, number of osteoclasts; BPm, bone perimeter; MS, mineralized surface; BS, bone surface; MAR, mineral apposition rate; BFR, bone formation rate.
*$P < 0.05$ compared to WT.

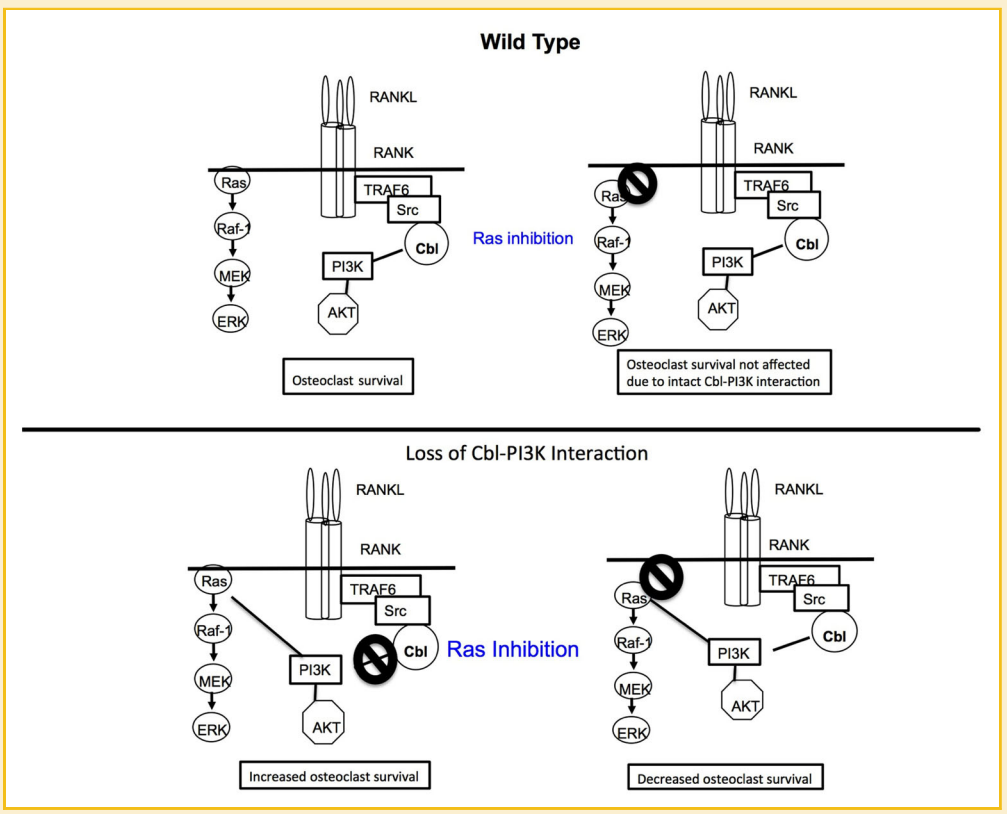

Fig. 8. Molecular regulation of osteoclast survival: In WT osteoclast, both RANK and Ras–Raf–1–MEK–ERK signaling contribute to cell survival, but survival is mainly regulated by Src–Cbl–PI3K pathway. When Cbl–PI3K interaction is lost due to the YF mutation, Ras mediates increased PI3K signaling. Inhibition of Ras activity decreases Ras–Raf–1–MEK–ERK signaling in both WT and YF osteoclasts, but survival is affected only in YF osteoclasts, since Src–Cbl–PI3K mediated AKT activation remains intact in WT osteoclasts in spite of Ras inhibition.

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