RBP-J imposes a requirement for ITAM-mediated costimulation of osteoclastogenesis

Susan Li, 1 Christine H. Miller, 1 Eugenia Giannopoulou, 1, 2 Xiaoyu Hu, 1, 3 Lionel B. Ivashkiv, 1, 3, 4 and Baohong Zhao 1, 3

Introduction

Osteoclasts, derived from monocyte/macrophage precursors, are the exclusive bone-resorptive cells that play an important role in physiological skeleton development and homeostatic bone remodeling in concert with bone-forming osteoblasts/osteocytes (1–3). Abnormal generation and function of osteoclasts lead to either osteopetrosis or excessive bone resorption, such as occurs in osteoporosis and in inflammatory arthritis (4–6). In addition to the cellular survival factor macrophage colony-stimulating factor (M-CSF), osteoclastogenesis requires 2 essential signals to induce osteoclast formation and bone resorption in DAPI2-deficient animals. Thus, RBP-J imposes a requirement for ITAM-mediated costimulation of RANKL or TNF-α-induced osteoclastogenesis. Mechanically, RBP-J suppressed induction of key osteoclastogenic factors NFATc1, BLIMP1, and c-FOS by inhibiting ITAM-mediated expression and function of PLCγ2 and activation of downstream calcium-CaMKK/PYK2 signaling. Moreover, RBP-J suppressed Plgc2 expression and downstream calcium oscillations indirectly by a TGF-β/PLCγ2/calcium axis. Together, our findings indicate that RBP-J suppresses ITAM-mediated costimulation, thereby limiting crosstalk between ITAM and RANK/TNFR signaling and allowing fine tuning of osteoclastogenesis during bone homeostasis and under inflammatory conditions. Furthermore, these data suggest that environmental cues that regulate RBP-J expression/function potentially modulate the requirement for costimulatory signaling for osteoclast differentiation and bone remodeling.

Osteoclastogenesis requires activation of RANK signaling as well as costimulatory signals from immunoreceptor tyrosine-based activation motif-containing (ITAM-containing) receptors/adaptors, predominantly tyrosine kinase–binding proteins DAP12 and FcγR, in osteoclast precursors. It is not well understood how costimulatory signals are regulated and integrated with RANK signaling. Here, we found that osteopetrotic bone phenotypes in mice lacking DAP12 or DAP12 and FcγR are mediated by the transcription factor RBP-J, as deletion of Rbpj in these mice substantially rescued the defects of bone remodeling. Using a TNF-α–induced model of inflammatory bone resorption, we determined that RBP-J deficiency enables TNF-α to induce osteoclast formation and bone resorption in DAPI2-deficient animals. Thus, RBP-J imposes a requirement for ITAM-mediated costimulation of RANKL or TNF-α-induced osteoclastogenesis. Mechanistically, RBP-J suppressed induction of key osteoclastogenic factors NFATc1, BLIMP1, and c-FOS by inhibiting ITAM-mediated expression and function of PLCγ2 and activation of downstream calcium-CaMKK/PYK2 signaling. Moreover, RBP-J suppressed Plgc2 expression and downstream calcium oscillations indirectly by a TGF-β/PLCγ2/calcium axis. Together, our findings indicate that RBP-J suppresses ITAM-mediated costimulation, thereby limiting crosstalk between ITAM and RANK/TNFR signaling and allowing fine tuning of osteoclastogenesis during bone homeostasis and under inflammatory conditions. Furthermore, these data suggest that environmental cues that regulate RBP-J expression/function potentially modulate the requirement for costimulatory signaling for osteoclast differentiation and bone remodeling.

The main ITAM-containing adaptors expressed by myeloid osteoclast precursors are DNAx-activating protein 12 (DAP12) and Fc receptor common γ subunit (FcγR). These adaptors associate with and mediate signaling by various receptors, including DAP12-associated triggering receptor expressed in myeloid cells 2 (TREM2) and signal-regulatory protein β 1 (SIRPβ1), FcγR-associated osteoclast-associated receptor (OSCAR), paired immunoglobulin-like receptor-A (PIR-A), and FcRs. The ligands for many of these receptors are not known but appear to be constitutively expressed and mediate basal signaling. Ligation of ITAM-coupled receptors in myeloid cells leads to the phosphorylation of ITAM tyrosine residues by Src family kinases. The tyrosine kinase Syk is then recruited and activates signaling molecules, such as BTK/TEC, the scaffolding adaptor B cell linker (BLNK)/SLP76, and phospholipase-Cγ2 (PLCγ2). Phosphorylation of PLCγ2 increases its catalytic function and results in elevated intracellular calcium levels that activate calcium-mediated signaling pathways. Calcium-activated signaling molecules calcium/calmod-
ulin-dependent protein kinase kinase (CaMKK), CaMK, protein tyrosine kinase 2 (PYK2; also known as PTK2B), and calcineurin further transmit calcium signaling to activate transcriptional factors, such as CREB, MEF2, and NFATc1, to induce various cellular activities (9, 12–17). Activation of PLCγ2 and downstream calcium-signaling pathways plays an essential role in NFATc1 induction during osteoclastogenesis (16, 18, 19).

Recent work has revealed an integration of RANK- and ITAM-signaling pathways by a multiprotein signaling complex comprising BTK/TEC/BLNK/SLP76/PLCγ2, which activates downstream calcium signaling and NFATc1 induction (19, 20). Deletion of PLCγ2 in mice results in an osteopetrotic phenotype due to a defect in osteoclastogenesis with impaired NFATc1 induction, demonstrating a key role for PLCγ2 in NFATc1 induction and osteoclast differentiation (19). Diminishment of ITAM-mediated costimulatory signaling in osteoclast precursors as occurs in Dap12−/−Fcrg−/− cells leads to attenuation of PLCγ2 activation followed by impaired NFATc1 induction and osteoclast differentiation even in the presence of RANKL (18, 19). Thus, ITAM-mediated costimulatory signals and PLCγ2 activity, which determine downstream calcium signaling, are required for osteoclast differentiation. RANK signaling induces a transient increase in PLCγ2 activity, but does not regulate its expression and is not required for basal PLCγ2 activity in osteoclast precursors. Instead, basal PLCγ2 activity, which has been observed in myeloid cells including osteoclast precursors (17, 21–24), appears to be maintained by tonic ITAM signaling mediated by engagement of ITAM-associated receptors by constitutively expressed ligands (13); consistently, addition of ligands/agonists for ITAM-associated receptors is not required for osteoclast differentiation in vitro. Importantly this basal level of costimulation and PLCγ2 activation together with RANKL is sufficient for NFATc1 induction and osteoclast differentiation (16, 18, 19). The ligands for FcRγ- and DAP12-associated receptors that mediate tonic signaling are suggested to be expressed by osteoblasts/stromal cells and osteoclast precursors, respectively (18, 24). Thus, cell-cell interaction could provide basal/tonic ITAM-mediated PLCγ2/calcium costimulatory signaling during osteoclastogenesis. There has been great interest in delineating the mechanisms by which ITAM signals crossregulate heterologous receptors, such as TLRs and cytokine receptors (13), but less is known about mechanisms/factors in the cells that fine tune ITAM-mediated PLCγ2/calcium signaling.

Recombinant recognition sequence binding protein at the J chain (RBP-J, also called RBP-Jκ, CSL, or CBF1) functions as a central transcription factor that receives input from several signaling pathways, such as the canonical NOTCH pathway, the WNT/β-catenin pathway, the NF-kB pathway, and the TLR- and TNF-signaling pathways (25–31), to mediate diverse cellular activities (25). We have recently identified RBP-J as a key inhibitor suppressing inflammatory osteoclastogenesis and bone resorption (29). To further dissect mechanisms by which RBP-J inhibits osteoclastogenesis, we tested whether RBP-J regulates ITAM-mediated costimulatory signaling during osteoclast differentiation and bone remodeling. We found that RBP-J deficiency substantially rescued impaired osteoclast formation and osteopetrotic bone phenotype in vivo in both Dap12−/− and Dap12−/−Fcrg−/− mice and enabled TNF-α to induce inflammatory bone resorption in Dap12−/− mice. Mechanistically, RBP-J inhibited induction of NFATc1, BLIMPI, and c-FOS by suppressing PLCγ2 expression and activity and downstream calcium-CaMKK-PYK2 signaling via the TGF-β pathway during osteoclastogenesis. Thus, RBP-J deficiency allows RANK or TNFR signaling to induce osteoclast differentiation independently of ITAM-mediated costimulation. These results indicate that RBP-J imposes a requirement for ITAM-mediated costimulation and limits the crosstalk between ITAM and RANK signaling, thereby restraining both pathways in osteoclastogenesis.

Results

RBP-J deficiency reverses the osteopetrotic bone phenotype of Dap12−/− and Dap12−/−Fcrg−/− mice. Loss of Dap12 or both Dap12 and FcRγ in mice leads to severe osteopetrosis due to impaired osteoclastogenesis and sorptive function (18, 24, 32, 33). To investigate the role of RBP-J in ITAM-mediated bone remodeling, we first deleted Rbpj in myeloid lineage osteoclast precursors in Dap12−/−, Fcrg−/−, and Dap12−/−Fcrg−/− mice by crossing these mice with Rbpjlox/ΔM (Rbpjlox/ΔM/LysM-Cre− mice as described in the literature; ref. 29) to generate Rbpjlox/ΔM/Dap12−/−, Rbpjlox/ΔM/Fcrg−/−, or Rbpjlox/ΔM/Dap12−/−Fcrg−/− (hereafter referred to as TKO) mice, respectively. Age- and sex-matched mice with a LysM-Cre− genotype (hereafter referred to as control) were used as control mice. Consistent with the literature (18, 24), there were no significant bone defects in Fcrg−/− mice, and RBP-J deficiency in Rbpjlox/ΔM/Fcrg−/− mice did not affect osteoclast differentiation and bone phenotypes of Fcrg−/− mice (data not shown). Thus, in the present study, we focused on the effect of RBP-J on the Dap12−/− and Dap12−/−Fcrg−/− mice, which exhibit a severe osteopetrotic phenotype. In order to avoid potential effects of global deficiency of DAPI2 or FcRγ, we generated bone marrow chimeric mice by transplanting the bone marrows of control, Rbpjlox/ΔM/Dap12−/−, Dap12−/−Fcrg−/−, Rbpjlox/ΔM/Dap12−/−Fcrg−/−, or TKO mice to age-matched male C57BL/6 recipient mice as described in Methods. Dap12−/− and Dap12−/−Fcrg−/− chimeric mice exhibited an osteopetrotic bone phenotype that was more severe in the double-knockout mice (Figure 1, A and B), which is consistent with previous literature (18, 24). In the presence of intact ITAMs, Rbpjlox/ΔM/Dap12−/− mice with 70% to 80% deletion did not exhibit obvious bone defects compared with control mice in homeostatic conditions (ref. 29, Figure 1, and Table 1). Strikingly, RBP-J deficiency in Rbpjlox/ΔM/Dap12−/− or TKO chimeric mice relieved the osteopetrotic phenotype caused by lack of DAPI2 or both DAPI2 and FcRγ, respectively. Rbpjlox/ΔM/Dap12−/− mice exhibited trabecular bone volume, number, thickness, and spacing similar to those of control mice (Figure 1, A and B, and Table 1, and Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI71882). Furthermore, the serum levels of tartrate-resistant acid phosphatase (TRAP), a biomarker reflecting the amount of osteoclasts and bone resorption in vivo, in Rbpjlox/ΔM/Dap12−/− or TKO chimeric mice were significantly enhanced relative to those of Dap12−/− or Dap12−/−Fcrg−/− chimeric mice, respectively (Figure 1C). Notably, serum TRAP levels in Rbpjlox/ΔM/Dap12−/− mice were comparable to those in control mice (Figure 1C and Table 1). These findings were further corroborated by markedly increased TRAP osteoclasts in Rbpjlox/ΔM/Dap12−/− or TKO chimeric mice compared with Dap12−/− or Dap12−/−Fcrg−/− chimeric mice, respectively (Figure 1,
Figure 1. RBP-J deficiency rescues the osteopetrotic bone phenotype in both Dap12−/− and Dap12−/−Fcrg−/− mice. (A) Microcomputed tomography (μCT) images of trabecular bones of the distal femurs isolated from the indicated bone marrow chimeric mice. Top of images is toward the growth plate. Scale bar: 1 mm. (B) Bone morphometric analysis of femurs isolated from the chimeric mice. BV/TV, bone volume per tissue volume; Tb.N, trabecular number; Tb.Th, trabecular bone thickness; Tb.Sp, trabecular bone spacing. n = at least 5 per group. *P < 0.05; **P < 0.01. (C) Concentration of basal serum TRAP obtained from the chimeric mice. n = at least 5 per group. *P < 0.05; **P < 0.01. (D) TRAP staining and (E) Histomorphometric analysis of the histological sections obtained from the metaphysis region of distal femurs of the chimeric mice. N. Oc./BS, number of osteoclasts per bone surface; Oc. S/BS, osteoclast surface per bone surface. n = 5 per group. *P < 0.05; **P < 0.01. Scale bar: 100 μm.
Dap12–/–Fcrg–/– enhanced the number and size of the TRAP + multinucleated cells enhanced in RANKL-treated TKO cells relative to (encoding cathepsin K), and Itgb3 Ctsk (encoding TRAP), osteoclast marker genes of TRAP+ small “crenated” osteoclasts (Figure 2A), while there described in previous literature (18, 24, 34), formed a low number of TRAP+ osteoclasts (Figure 2A), while there was no osteoclast formation in Dap12+/Fcg−/− cells (Figure 2A) in response to RANKL stimulation compared with control cell cultures. RBP-J deficiency in Dap12−/− BMMs, as described in previous literature (18, 24, 34), was also rescued in TKO cells (Figure 3B), suggesting that RBP-J constitutively plays an inhibitory role in this biological context.

RBP-J deficiency bypasses the requirement for ITAM-mediated costimulation of RANKL-induced osteoclastogenesis. We then examined in vitro osteoclast differentiation using bone marrow–derived macrophages (BMMs) as osteoclast precursors. Dap12−/− BMMs, as described in previous literature (18, 24, 34), formed a low number of TRAP+ small “crenated” osteoclasts (Figure 2A), while there was no osteoclast formation in Dap12−/−Fcg−/− cells (Figure 2A) in response to RANKL stimulation compared with control cell cultures. RBP-J deficiency in RbpjΔ/ΔDap12−/− cells significantly enhanced the number and size of the TRAP+ multinucleated cells (MNCs) to a level comparable to that seen in control cell cultures (Figure 2, A and B). Moreover, RBP-J deficiency in TKO cells substantially reversed the defects of osteoclastogenesis that resulted from Dap12 and FceγR deficiency and resulted in formation of a large number of TRAP+ MNCs (Figure 2, A and B). In parallel with the increased generation of TRAP+ polykaryons, the expression of osteoclast marker genes Acp5 (encoding TRAP), Ctsk (encoding cathepsin K), and Iftg3 (encoding β3 integrin) was dramatically enhanced in RANKL-treated TKO cells relative to Dap12−/−Fcg−/− cells, and the levels of Acp5 and CtsK were even comparable to the levels as induced by RANKL in control cell cultures (Figure 2C). Collectively, these results indicate that the defect in the osteoclast differentiation program in the absence of ITAM-mediated costimulation is almost completely reversed by RBP-J deficiency. This in vitro reversal of osteoclastogenesis defects by RBP-J deficiency further corroborates the reversal observed in vivo, supporting an important role of RBP-J in restraining ITAM-mediated osteoclast differentiation.

NFATc1 is well known as the “master regulator” of osteoclastogenesis. In addition to the regulation of Blimp1 by NFATc1 (35), genetic evidence obtained using Blimp1-KO cells or RNAi targeting of BLIMP1 shows that BLIMP1 conversely plays an important role in induction of NFATc1 (29, 36), indicating a critical reciprocal regulatory circuit between BLIMP1 and NFATc1 that coordinately controls the osteoclast differentiation program. Induction of NFATc1 required not only RANKL but also costimulatory signaling from ITAM-associated receptors (refs. 18, 19, and Figure 3A). In this study, we found that BLIMP1 induction also requires costimulatory signaling, as RANKL failed to induce BLIMP1 expression in Dap12−/−Fcg−/− cells (Figure 3A). Consistent with previous literature (29), deletion of RBP-J enhanced the expression of NFATc1, BLIMP1, and c-FOS (Figure 3, A and B). Strikingly, RBP-J deficiency in TKO cells completely rescued the impaired induction of NFATc1, BLIMP1, and c-FOS by RANKL in Dap12−/−Fcg−/− cells (Figure 3A). Although the kinetics of the induction of these transcription factors in TKO cells was slower than that in control cells, their expression levels were comparable to or even greater than control levels at late time points (Figure 3A). Furthermore, the diminished mRNA expression of Nfatc1, Blimp1, and Fos was also rescued in TKO cells (Figure 3B), suggesting that the regulation of the expression of Nfatc1, Prdm1 (which encodes Blimp1), and Fos (which encodes c-Fos) by RBP-J occurs at the transcriptional level. These results show that RBP-J deficiency bypassed the need for ITAM-mediated costimulation to induce NFATc1/BLIMP1/c-FOS expression and osteoclastogenesis. Thus, RBP-J imposes a requirement for costimulation of RANKL signaling.

RBP-J suppresses the expression and activity of basal PLCγ2 and downstream calcium-CaMKK-PYK2 signaling by regulating TGF-β signaling. We then asked how RBP-J imposes a requirement for ITAM-mediated costimulation. To address this question, we first performed gene-expression profiling using high-throughput sequencing of RNA (RNAseq) with control and RbpjΔ/Δ osteoclast precursors at baseline and after TNF-α stimulation to identify genes regulated by RBP-J during osteoclastogenesis. Gene-set enrichment analysis of the RBP-J-regulated genes revealed marked enrichment of calcium signaling pathway genes in RbpjΔ/Δ cells (P = 7.72 × 10−7). Consistent with regulation of calcium signaling by RBP-J, we observed that RBP-J deficiency significantly increased intracellular calcium levels (Figure 4A). Moreover, we measured calcium oscillations including oscillatory amplitude in single cells (Figure 4B) and oscillating/non-oscillating cell proportion in each cell culture (Figure 4C). We found that calcium oscillations in RBP-J-deficient cells were dramatically increased (Figure 4, B and C). We thus hypothesized
TEC (20). However, such induction of PLCγ2 activity over a low baseline by RANKL is not sufficient to induce osteoclastogenesis, as shown in Figure 2A and previous literature (18, 19), as there is almost no osteoclast differentiation in Dap12−/−Fcrg−/− cells. The essentially nondetectable basal PLCγ2 phosphorylation in Dap12−/−Fcrg−/− cells (Figure 4E) indicates that tonic ITAM signaling makes a predominant contribution to maintaining basal activity of PLCγ2. Although the importance of basal PLCγ2 activity that is calibrated by ITAM pathways in response to environmental cues was not previously fully appreciated, our observations suggest that such basal PLCγ2 function acts additively with RANKL-induced PLCγ2 activity to surpass a threshold required to induce NFATc1 and osteoclast differentiation. Consistent with this notion, we found that RBP-J deficiency in RbpjΔM/ΔM or TKO cells, which resulted in increased osteoclastogenesis (ref. 29 and Figure 2), concomitantly, significantly, and consistently that RBP-J deficiency bypasses the need for ITAM-containing adaptors by enhancing the expression or function of calcium signaling components downstream of these adaptors. As PLCγ2 is an important mediator of calcium signaling, we examined the effects of RBP-J deficiency on the expression and activity of PLCγ2. Interestingly, we found that RBP-J deficiency significantly increased PLCγ2 expression (Figure 4D). In contrast, PLCγ2 expression was strongly diminished in Dap12−/−Fcrg−/− cells, and this decrease was reversed in TKO cells (Figure 4D). These data indicate that RBP-J suppresses while ITAM signaling maintains basal PLCγ2 expression. Next, we examined whether PLCγ2 activity was affected. As expected, RANKL increased phosphorylation of PLCγ2 over baseline (Figure 4E); strikingly, basal PLCγ2 phosphorylation was essentially absent in Dap12−/−Fcrg−/− cells (Figure 4E, lane 11), although RANKL was able to induce a modest increase, presumably due to the activation of BTC/
enhanced the basal level of phospho-PLCγ2 compared with control or Dap12−/−Fcrg−/− cells (Figure 4E, lane 6 vs. lane 1 and lane 16 vs. lane 11), but did not always affect the early transient induction of phospho-PLCγ2 by RANKL (Figure 4E).

Previous data from our group and others show that NOTCH signaling inhibits osteoclastogenesis (29, 37). In the present study, we further found that inhibition of osteoclast differentiation by the NOTCH ligands delta-like1 and jagged1 is dependent on RBP-J (Supplemental Figure 3). Furthermore, activation of NOTCH signaling in osteoclast precursor cells suppressed phosphorylation of PLCγ2 and its total protein level (Supplemental Figure 4), indicating that NOTCH–RBP-J signaling negatively regulates PLCγ2 expression and further supporting the notion that the levels of PLCγ2 expression are important in determining its activity; the important adaptor functions of PLCγ2 (19) would also be compromised by its lower expression. We then investigated mechanisms that regulate PLCγ2 expression. We found that RBP-J deficiency increased Pleg2 mRNA amounts (Figure 4F). The elevation was modest, approximately a 30% increase compared with control cells, but was statistically significant (P < 0.01) and consistently similar to the elevation of PLCγ2 protein amounts in multiple experiments. RBP-J did not affect the mRNA stability of Pleg2 (Supplemental Figure 5), while the primary Pleg2 transcripts (unspliced transcripts of Pleg2) were significantly enhanced in the RBP-J–deficient cells (Figure 4G).

These data indicate that RBP-J regulates PLCγ2 expression at the transcriptional level, which was further corroborated by enhanced RNA polymerase II (Pol II) occupancy at the Pleg2 locus in RBP-J–deficient cells (Figure 4H). To address whether RBP-J directly regulates Pleg2, we obtained the set of known RBP-J target genes from published databases (38–40) and overlapped these targets with genes that were regulated by RBP-J in our RNAseq experiments (Supplemental Figure 6), but did not find Pleg2 included. ChIPseq data (38) did not show any significant chromosomal DNA-binding peaks of RBP-J in a range of at least 100-kb upstream or downstream of the transcriptional start site of Pleg2. These suggest that RBP-J negatively regulates Pleg2 transcription in an indirect manner.

Figure 3. RBP-J deficiency compensates for the defect of NFATc1, BLIMP1, and c-FOS induction in Dap12−/−Fcrg−/− cells by RANKL. (A) Immunoblot analysis of NFATc1, BLIMP1, and c-FOS expression in whole-cell lysates obtained from control, RbpjΔM/ΔM, Dap12−/−Fcrg−/−, and TKO BMMs at the indicated time points after stimulation with RANKL. GAPDH or β-tubulin was measured as loading control. Isoform A of NFATc1 is the major isoform induced during osteoclastogenesis. Data are representative of at least 3 independent experiments. Lanes separated by a black line indicate that they were run on the same gel but were noncontiguous. (B) Quantitative real-time PCR analysis of mRNA expression of Nfatc1 (encoding NFATc1), Prdm1 (encoding BLIMP1), and Fos (encoding c-FOS) induced by RANKL for 24 hours in osteoclastogenic cell cultures from the indicated BMMs. Data are representative of at least 3 independent experiments. **P < 0.01.
Insight into indirect mechanisms of regulation was provided by the striking finding that the direct RBP-J target gene *Tgfbr1* (encoding TGF-β receptor I) was expressed at a rate approximately 2.5 times higher in RBP-J–deficient osteoclast precursors than in control cells (Figure 5A). This suggested that RBP-J deficiency could enhance the responsiveness of osteoclast precursors to TGF-β that is produced by these cells and is also present in serum, and is an important factor for osteoclastogenesis (41, 42). Previous literature showing crosstalk between TGF-β signaling and the Notch pathway in other settings (43–46) supports the possibility of regu-
Figure 5. RBP-J suppresses PLC-γ2 expression and calcium oscillations via a TGF-β-dependent pathway. (A) Quantitative real-time PCR analysis of mRNA expression level of Tgfbr1 (encoding TGF-β receptor 1). Data are representative of at least 3 independent experiments. **P < 0.01. (B) Immunoblot analysis of phospho-SMAD2/3 in the cells pretreated with TGF-β receptor I inhibitor SB431542 (100 μM) or DMSO (vehicle) for 1 hour followed by stimulation with TGF-β (10 ng/ml) for the indicated times. β-Tubulin was measured as a loading control. (C) ChIP assay of SMAD2/3 occupancy at the upstream regulatory region of Plcg2 in BMMs stimulated without or with TGF-β (10 ng/ml) for 1 hour. Relative occupancy of SMAD2/3 in response to TGF-β was normalized to each basal control condition. Results are shown as average values from 3 independent experiments. *P < 0.01. (D) Immunoblot analysis of phospho-PLC-γ2 and total PLC-γ2 and (E) primary Plcg2 transcript expression in BMMs treated with SB431542 (100 μM) or DMSO for 6 hours. (F) Quantification of TRAP-positive MNCs in the culture of BMMs (≥3 nuclei per cell) treated with SB431542 (10 μM) or DMSO for 4 days in the presence of TNF-α. *P < 0.05; **P < 0.01. (G) Representative calcium oscillatory traces of cells stimulated without or with TGF-β (10 ng/ml) for 24 hours; oscillating cells were quantified. Controls for basal calcium oscillation data without TGF-β stimulation were the same as shown in Figure 4B. (H) Representative calcium oscillatory traces of cells treated with SB431542 (100 μM) or DMSO for 24 hours; oscillating cells were quantified. (H) *P < 0.05; **P < 0.01.

**RESEARCH ARTICLE**

The Journal of Clinical Investigation
loration of TGF-β responses by RBP-J. Furthermore, TGF-β increases intracellular calcium concentration and activates downstream calcium signaling and PKC (47–49), suggesting that TGF-β signaling may regulate PLCγ2. Indeed, we found that RBP-J deficiency enhanced the expression of TGF-β-responsive genes, such as Id1, Id3, Cdkn1a, Cdkn2b, and Smad7 (Supplemental Figure 7), indicating that RBP-J deficiency potentiates cell responses to TGF-β. Corroboratively, phosphorylation of the TGF-β-activated key signaling components SMAD2 and SMAD3 was also enhanced in RBP-J-deficient cells (Figure 5B and Supplemental Figure 8). Previous ChIPseq data (50) showed that TGF-β-induced SMAD2/3 binding peaks in the upstream regulatory region of Pleg2. In our system, we found that RBP-J deficiency modestly, but significantly and consistently, elevated the occupancy of SMAD2/3 in response to TGF-β at the same upstream regulatory region of Pleg2 locus as shown in the literature (50) in multiple experiments (Figure 5C). This further supports the notion that RBP-J regulates Pleg2 expression via a TGF-β-SMAD2/3 signaling pathway. As TGF-β signaling primes osteoclast precursors and facilitates osteoclastogenesis (41, 42), we next examined whether the enhanced TGF-β signaling in RBP-J-deficient cells contributes to the enhanced PLCγ2 expression and osteoclastogenesis. We blocked the kinase activity of TGF-β receptor I and downstream signaling using the inhibitor SB431542 (Figure 5B and Supplemental Figure 8) and found that the basal expression and phosphorylation of PLCγ2 (Figure 5, D and E) as well as osteoclastogenesis were significantly suppressed (Figure 5F). Similar results were obtained when specifically knocking down Tgfr1 (Supplemental Figure 9). These data suggest that repression of Tgfr1 expression by RBP-J results in decreased responsiveness of osteoclast precursors to TGF-β, with a concomitant decrease in TGF-β-mediated PLCγ2 expression. Consistent with a role for TGF-β in calcium signaling, we, for what we believe is the first time, observed that TGF-β significantly induced calcium oscillations in control BMMs (Figure 5, G and H). RBP-J deficiency markedly increased calcium oscillations in both basal and TGF-β stimulatory conditions (Figure 5, G and H). The TGF-β signaling inhibitor SB431542 dampened basal calcium oscillation in both cell types and decreased the proportion of oscillating cells in RBP-J-deficient cells to a level comparable to that of control cells (Figure 5, I and J). In addition, inhibition of TGF-β signaling markedly decreased TNF-α-induced calcium oscillations in the Rbpj Δ/Δ cells (Supplemental Figure 10). This indicates a significant contribution of endogenous TGF-β signaling to calcium oscillations in control BMM cells as well as the enhanced oscillatory state in RBP-J-deficient cells. Collectively, the results show that RBP-J deficiency elevates the basal PLCγ2/calcium pathway via TGF-β-Smad2/3 signaling.

To directly test whether increased PLCγ2-calcium signaling in RBP-J-deficient cells contributes to increased expression of downstream NFATc1, BLIMP1, and c-FOS, we blocked PLCγ2 activity before RANKL treatment using the well-known PLCγ2 inhibitor U-73122. Indeed, the increased induction of NFATc1, BLIMP1, and c-FOS by RANKL in Rbpj Δ/Δ or TKO cells was attenuated to a level comparable to that in control or Dap12 Δ/c-FcgRIγ double-deficient (Figure 6A and Supplemental Figure 11A). The inhibition of NFATc1 and BLIMP1 expression by U-73122 lasted for at least 2 days (Supplemental Figure 12). Correspondingly, inhibition of PLCγ2 activity significantly reduced the enhanced RANKL-induced osteoclastogenesis in RBP-J-deficient cells; the inhibitory effect was most pronounced in TKO cells (Figure 6B). Thus, enhanced osteoclastogenesis in RBP-J-deficient cells is dependent on PLCγ2 signaling. PLCγ2/calcium signals are transmitted by CaMKK and PYK2 in other systems (12), where CaMKK functions downstream of PLCγ2 but upstream of PYK2. Thus, we first used CaMKK inhibitor STO-609 to block its activity in the osteoclastogenic system and found that the induction of NFATc1, BLIMP1, and c-FOS was suppressed in control cells, while the enhanced induction of NFATc1, BLIMP1, and c-FOS by RBP-J deficiency in Rbpj Δ/Δ or TKO cells was attenuated to the level seen in control or in Dap12 Δ/c-FcgRIγ double-deficient cells, respectively (Figure 6C and Supplemental Figure 11B). Osteoclastogenesis was also inhibited by CaMKK blockade, and inhibition was most pronounced in TKO cells (Figure 6D). Furthermore, we observed that RANKL-induced phosphorylation of PYK2 (Figure 6E). RBP-J deficiency did not increase the RANKL-induced phosphorylation of PYK2, but significantly elevated its basal phosphorylation level and total amount in a CaMKK-dependent manner (Figure 6, E and F, and data not shown), which is likely due to increased upstream PLCγ2 signaling resulting from RBP-J deficiency. As such, the basal level of phosphorylation and total amounts of PYK2 were also higher in TKO cells than in Dap12 Δ/c-FcgRIγ double-deficient cells (Figure 6F). These results show that RBP-J regulates both PYK2 activity and total PYK2 amounts. Quantification data (Figure 6, E and F) showed that the active phospho-PYK2 relative to total PYK2 was still increased by RBP-J deficiency, indicating that the increased PYK2 activity by RBP-J is partially correlated with the increase in total amount of PYK2. Inhibition of PYK2 activity by its inhibitor AG 17 markedly suppressed the induction of NFATc1, BLIMP1, and c-FOS (Figure 6G) as well as osteoclastogenesis in control cells (Figure 6H), indicating that PYK2 plays an activating role in induction of NFATc1/BLIMP1/c-FOS and osteoclastogenesis. In RBP-J-deficient cells, inhibition of PYK2 activity by AG 17 dramatically decreased the enhanced expression level of NFATc1/BLIMP1/c-FOS (Figure 6G) and osteoclastogenesis, which was most pronounced in TKO cells (Figure 6H). The heightened suppression of osteoclastogenesis by PLCγ2, CaMKK, and Pyk2 inhibitors in TKO cells supports a critical role for augmented downstream PLCγ2 activity in compensating for missing upstream signals from DAP12 and FcγRI. Taken together, these findings indicate that RBP-J inhibits NFATc1/BLIMP1/c-FOS expression by keeping PLCγ2 in a basal suppressed state and thus downregulating downstream calcium-CaMKK-PYK2 signaling in osteoclastogenesis. These findings are consistent with a model whereby RBP-J deficiency rescues osteoclastogenesis by bypassing the need for upstream ITAM signaling by increasing TGF-β signaling to maintain downstream PLCγ2/calcium-CaMKK-PYK2 activity (Figure 6I).

RBP-J deficiency enables TNF-α to induce NFATc1 and BLIMP1 expression and osteoclastogenesis in the absence of ITAM-mediated costimulatory signaling. We reasoned that increased PLCγ2 activity in RBP-J-deficient cells would compensate for lesser induction of ITAM signaling by TNF-α than by RANKL (51) and thus contribute to increased TNF-α-mediated osteoclastogenesis. We tested this notion using a genetic approach to see whether TNF-α could induce osteoclastogenesis in RBP-J-deficient cells
Figure 6. RBP-J suppresses PLCγ2/calcium-CaMKK/Pyk2 signaling in osteoclast differentiation. (A) Immunoblot analysis of phospho-PLCγ2, NFATc1, Blimp1, and c-Fos expression at basal levels or stimulated with RANKL for 24 hours in the BMMs. The cells were pretreated for 30 minutes with PLCγ2 inhibitor U-73122 (10 μM) or its vehicle ethanol; inhibitor or vehicle was completely washed away before addition of RANKL. (B) Quantitation of TRAP-positive MNCs (≥ 10 nuclei per cell) that were pretreated for 30 minutes with U-73122 or ethanol and then cultured with RANKL for 6 days. (C) and (G) Immunoblot analysis of NFATc1, Blimp1, and c-Fos expression at basal levels or after being stimulated with RANKL for 24 hours with CaMKK inhibitor STO-609 (5 μg/ml) (C) or PYK2 inhibitor AG 17 (3 μM) (G) or vehicle DMSO in the BMMs. (D and H) Quantitation of TRAP-positive MNCs (≥ 10 nuclei per cell) that were stimulated with RANKL with STO-609 (D), AG 17 (H), or DMSO for 6 days. (E and F) Immunoblot analysis of phospho-PYK2 and total PYK2 expression in the indicated conditions (E) or at basal levels (F). The lanes separated by a black line were run on the same gel but were noncontiguous. Quantification of the immunoblot bands by densitometry were shown in the right panels of (E) and (F). β-Tubulin (A) or p38α (C and E–G) was measured as a loading control. (I) A model showing that RBP-J suppresses ITAM-mediated basal PLCγ2 expression/activity and downstream calcium-CaMKK-PYK2 signaling in induction of c-FOS, BLIMP1, and NFATc1 in a TGF-β dependent pathway.
even in the absence of the ITAM-containing adaptors DAP12 and FcRγ. In Dap12–/– and Dap12–/–Fcrg–/– cells, as in control cells, TNF-α alone did not significantly induce a osteoclast differentiation program, including expression of osteoclast marker genes and NFATc1/BLIMP1 induction (Figure 7, A–E). However, RBP-J deficiency resulted in formation of TRAP+ MNCs in response to TNF-α in the absence of DAP12 or DAP12/FcRγ (Figure 7, A and B). Despite a “crenated” morphology, these TRAP+ MNCs in RbpjΔM/ΔM/Dap12–/– and TKO cells expressed osteoclast marker genes Acp5, Ctsk, and Itgβ3 at levels similar to those induced in fully differentiated RbpjΔM/ΔM osteoclasts (Figure 7C and ref. 29). Furthermore, TNF-α induced expression levels of NFATc1 and BLIMP1 in RbpjΔM/ΔM/Dap12–/– and TKO cells comparable to that in RbpjΔM/ΔM cells (Figure 7, D and E, and data not shown). However, these crenated MNCs failed to fully spread on tissue culture plates and did not resorb dentin (data not shown). These results indicate that RBP-J deficiency that provides basal downstream PLCγ2-CaMKK-PYK2 signaling in the absence of upstream ITAMs enables TNF-α to fully induce the early osteoclast differentiation program in vitro in cultures of purified osteoclast precursors in the absence of stromal cells. However, ITAM-mediated costimulatory signaling is still required for osteoclast function, such as actin ring formation and resorptive activity, even in RBP-J-deficient cells (15, 32).

**Discussion**

Induction of osteoclastogenesis by RANKL requires calcium signaling to activate NFATc1, which is typically provided by costimulatory signals induced by ITAM-associated receptors to activate PLCγ2 and thus modulate intracellular calcium concentration. In this study, we identify RBP-J as a key transcription factor that controls calcium signaling downstream of ITAM-associated receptors by regulating PLCγ2 expression and function, thereby controlling NFATc1 and downstream osteoclastogenesis. Removal of RBP-J resulted in increased PLCγ2 expression and activity as well as basal calcium signaling that enabled NFATc1 activation independently of upstream signaling by the major ITAM-containing adaptors DAP12 and FcRγ. This newly described pathway was biologically important, as deletion of RBP-J resulted in ITAM-adaptor-independent RANKL-induced osteoclastogenesis in vitro and in vivo under homeostatic and inflammatory conditions. These results identify a mechanism that regulates NFATc1 activation in osteoclast differentiation and is important for regulation of bone resorption in vivo.

RBP-J expression decreases over time after RANKL stimulation during osteoclastogenesis (29), and thus the effect of genetic knockout of RBP-J mimics and reflects its role under physiological conditions. The decreased RBP-J expression observed after RANKL stimulation likely contributes to the sustained PLCγ2/calcium signaling that has been described after RANKL stimulation (18), thereby facilitating the ability of upstream ITAM-generated signals to promote osteoclast differentiation. As the expression and function of RBP-J are controlled by various inputs, such as NOTCH, WNT/β-catenin, NF-κB, TLR, and TNF signaling pathways (25–30), our findings suggest that these various pathways can fine tune osteoclastogenesis and bone resorption by determining the strength of upstream ITAM signals required to provide adequate costimulation for NFATc1 activation. This model helps explain why, under certain conditions, such as high concentrations of M-CSF (10), ovariectomy, or low-calcium diet, ITAM-containing adaptors are not required for osteoclast formation and bone remodeling at specific skeletal sites (11). Allelic variants of **RBP** have been recently linked with increased disease susceptibility of rheumatoid arthritis (RA) (53–55). In parallel with these findings, we found that RBP-J expression level was significantly lower in RA patients’ synovial fluid macrophages, which can function as osteoclast precursors, than in monocyte-derived macrophages from healthy donors (Supplemental Figure 14), which supports the pathological relevance of RBP-J to RA. Thus, we speculate that the expression/function of RBP-J can be altered in various environmental cues and diminished RBP-J expression and/or function may contribute to the decreased requirement for ITAM adaptors under certain stress conditions in vivo.
Figure 7. RBP-J deficiency allows TNF-α to induce osteoclast differentiation in Dap12<sup>−/−</sup> and Dap12<sup>−/−</sup>Fcrg<sup>−/−</sup> BMMs. BMMs derived from control, Dap12<sup>−/−</sup>, Rbpj<sup>ΔM/ΔM</sup>Dap12<sup>−/−</sup>, Dap12<sup>−/−</sup>Fcrg<sup>−/−</sup>, and TKO mice were stimulated with TNF-α for 5 days. TRAP staining was performed (A), and the number of TRAP-positive MNCs (≥ 3 nuclei per cell) per well was counted (B). TRAP-positive cells appear red in the photographs. Scale bar: 100 μm. Data are representative of at least 20 independent experiments. **P < 0.01. (C) Quantitative real-time PCR analysis of mRNA expression of Acp5 (encoding TRAP), Ctsk (encoding cathepsin K), and Itgb3 (encoding β3) in BMMs from control, Rbpj<sup>ΔM/ΔM</sup>, Dap12<sup>−/−</sup>Fcrg<sup>−/−</sup>, and TKO mice treated with TNF-α for the indicated times. Data are representative of at least 3 independent experiments. **P < 0.01. (D) Immunoblot analysis of NFATc1 and BLIMP1 expression in whole-cell lysates obtained from control, Rbpj<sup>ΔM/ΔM</sup>, Dap12<sup>−/−</sup>Fcrg<sup>−/−</sup>, and TKO BMMs after stimulation with TNF-α for 24 hours. p38α was measured as a loading control. Data are representative of at least 3 independent experiments. (E) Quantitative real-time PCR analysis of mRNA expression of Nfatc1 (encoding NFATc1) and Prdm1 (encoding Blimp1) induced by TNF-α in osteoclastogenic cell cultures from control, Rbpj<sup>ΔM/ΔM</sup>, Dap12<sup>−/−</sup>Fcrg<sup>−/−</sup>, and TKO BMMs. Data are representative of at least 3 independent experiments. **P < 0.01.
NFATc1 activation requires sustained calcium signaling, which is manifested as increased intracellular calcium concentrations related to prolonged calcium oscillations (12) rather than an acute and transient calcium flux. Despite extensive study, how RANKL induces sustained calcium signaling is not understood, although induction of expression of ITAM-associated β3 integrins and OSCAR receptors that engage matrix components likely plays a role (56). Our results suggest that decreased RBP-J expression after RANKL stimulation facilitates RANKL-induced sustained calcium signaling, but also highlight an important role for basal PLCγ2 and downstream signaling in osteoclast precursors prior to RANKL stimulation. Basal PLCγ2 activity in osteoclast precursors is likely maintained primarily by constitutive ligation of ITAM-associated receptors, based on our results and previous reports showing that DAP12, FcRγ, and PLCγ2 are basally phosphorylated, that basal calcium concentrations are high, and that SYK and

We showed previously that in the presence of intact ITAM-mediated costimulatory signals (29), RBP-J more effectively suppresses TNF-α–induced than RANKL-induced osteoclast differentiation and bone resorption. Our current study helps explain this observation by showing that RBP-J decreases basal PLCγ2/calcium signaling, which imposes a requirement for a strong activating signal, such as that provided by RANKL. As TNF-α induces much weaker calcium signaling than RANKL (51), activation of NFATc1 by TNF-α would require a higher level of basal PLCγ2/calcium signaling than activation by RANKL. This suggests a model where the level of basal PLCγ2/calcium signaling, which is determined by the balance between ITAM-mediated induction and RBP-J–mediated suppression (Figure 6I), determines whether stimulation of osteoclast precursors with factors such as RANKL or TNF-α induces sufficient calcium signaling to cross the threshold required to effectively activate NFATc1.

Figure 8. RBP-J deficiency enables TNF-α to induce inflammatory bone resorption in Dap12−/− mice. (A) Concentration of serum TRAP obtained from the indicated mice before and after 5-day application of TNF-α to the calvarial periosteum. *P < 0.05; **P < 0.01. n = 5 in each group. (B) μCT images of the surface of whole calvaria obtained from the indicated mice. Scale bar: 1 cm. (C) Quantification of resorptive pit numbers of calvaria obtained from the indicated mice after 5-day application of PBS or TNF-α to the calvarial periosteum. n = 5 in PBS group. n = at least 5 in TNF-α group. *P < 0.05; **P < 0.01.
calcium-dependent PYK2 are basally active in myeloid cells (21, 22, 24). In addition to constitutive ITAM-mediated costimulation, we found in this study that endogenous TGF-β signaling also contributes to basal expression and activity of PLCγ2 and downstream basal calcium signaling. Our results show that RBP-J suppresses basal PLCγ2 expression and function, thereby lowering intracellular calcium concentrations and restraining calcium oscillations, thus making it more difficult to activate NFATc1 (12).

The correlation between PLCγ2 expression and phosphorylation in control, Rbpflox/floxLysM-Cre+, Dap12−/−Ferc−/−, and TKO cells (Figure 4, D and E) suggests that the expression levels of PLCγ2 may contribute to the regulation of its activity. Although the regulation of PLCγ2 activity has been extensively studied, the mechanism by which PLCγ2 expression is regulated has not been previously explored. Our data identify a new regulatory loop whereby RBP-J restrains expression of TGF-βRI and thereby modulates cell responsiveness to TGF-β, which in turn regulates expression of PLCγ2 at the transcription level through binding of Smad2/3 to its upstream regulatory region. Suppression of TGF-β signaling by RBP-J in osteoclast precursors provides another piece of evidence of crossregulation of Notch pathway and TGF-β signaling (43–46). Although TGF-β was previously shown to increase intracellular calcium levels and activate downstream calcium signaling and PKC in other systems (47–49), our work, for what we believe is the first time, shows that in BMMs or osteoclast precursors, these effects are mediated by increased PLCγ2 expression and downstream calcium oscillations. Our results provide direct evidence that Plcg2 is a target of Smad2/3. Thus, our data shows that, in addition to ITAMs and the RANK pathway in osteoclast precursors or BMMs, TGF-β-Smad2/3 signaling also contributes to calcium signaling. Furthermore, the TGF-β-Smad2/3-induced PLCγ2/calcium oscillation pathway is restrained by RBP-J. The complex convergence of RANK, ITAM, and TGF-β signaling and its interaction with NOTCH/RBP-J signaling may enable cells to fine tune downstream PLCγ2/calcium signaling in response to various environmental conditions. TGF-β-induced PLCγ2/calcium signaling may explain in part the positive priming effects of TGF-β on osteoclast precursors for differentiation (41, 42). Our single cell analysis of calcium oscillations shows that only a subset of cells in osteoclastogenic cell cultures oscillate at a given time. This heterogeneity of calcium oscillations in osteoclast precursors may help explain the distinct differentiation kinetics of individual cells and is consistent with previous reports that only a subset of cells strongly activate NFATc1 (57).

FcγRI does not significantly affect physiological bone remodeling (18, 24). Our results show that FcγRI-mediated costimulation is dispensable for TNF-α-induced local calvarial bone resorption, which differs from a previous report that used a TNF-α transgenic arthritis model (58). The different results in these 2 models may be explained by differences in the duration of TNF-α stimulation, different concentrations of TNF-α, or differences between effects of systemic and local expression of TNF-α (58). This supports a context-dependent role of FcγRI in regulating pathological bone resorption in various conditions, a notion that is further supported by our findings that a role for FcγRI in TNF-induced bone resorption emerged under conditions of DAPI2 deficiency, as FcγRI was required for RBP-J to compensate for DAPI2 deficiency in the TKO mice. Although the compensation of DAPI2 function by RBP-J deficiency was almost complete in vivo, the multinucleated osteoclasts formed in vitro did not seem fully functional based on their crenated morphology and inefficient bone resorptive ability. This discrepancy between in vivo and in vitro osteoclastogenesis suggests that additional signals from the bony environment could promote osteoclast resorptive function in vivo (9, 18, 32). β3 Integrin plays an important role in cytoskeleton organization (32, 59). RBP-J deficiency significantly rescued β3 integrin expression in Dap12−/−Ferc−/− cells in response to RANKL, but its level was less than that in control cells (Figure 2C), which may partially explain the cellular “crenated” morphology without obvious actin ring formation in these TKO cells.

RBP-J is a key regulator of osteoclastogenesis and bone resorption. Our previous work showed that RBP-J suppresses osteoclast differentiation by attenuating positive signals that lead to c-FOS/BLIMP1/NFATc1 activation while augmenting the expression of the transcriptional repressor IRF8, which suppresses Nfatc1 expression. RBP-J did not significantly affect the expression of several other negative factors for osteoclastogenesis, such as BCL6, MAFB, and noncanonical NF-κB signaling component p100 (Supplemental Figure 15). The current study reveals that the mechanism by which RBP-J suppresses positive signals that promote osteoclastogenesis is attenuation of stimulatory signaling by suppression of PLCγ2 and downstream calcium-dependent signaling pathways. In contrast, RBP-J deficiency downregulated the transcriptional repressor IRF8 (29) independently of its effects on PLCγ2 and calcium signaling (B. Zhao, unpublished data). Thus, RBP-J functions as a key central regulator that modulates both ITAM-dependent and independent RANK/TNFR signaling during osteoclastogenesis. Our findings establishing a key role for RBP-J in physiological and pathological bone remodeling suggest that modulating RBP-J expression/activity has therapeutic implications for abnormal osteoclastogenesis and associated bone resorption.

Methods

Mice and analysis of bone phenotype. Rbpflox/flox (Rbpflox/floxLysM-Cre+) mice (29) and Dap12−/−, Ferc−/−, and Dap12−/−Ferc−/− mice (21) have been described previously. The extent of RBP-J deletion by LysM-Cre in vitro and in vivo was typically 70% to 80% (ref. 29 and Supplemental Figure 16). We deleted Rbpf in myeloid lineage osteoclast precursors in Dap12−/−, Ferc−/−, or Dap12−/−Ferc−/− mice by crossing these mice with Rbpflox/flox to generate Rbpflox/floxDap12−/−, Rbpflox/floxFerc−/−, or Rbpflox/floxDap12−/−Ferc−/− mice (referred to as TKO), respectively. The mice with Dap12−/− or Dap12−/−Ferc−/− genotypes also equivalently express LysM-Cre'. Age- and sex-matched mice with a LysM-Cre' genotype (referred to as control) were used as controls. We generated bone marrow–chimeric mouse by transplanting the bone marrows of control, Dap12−/−, Dap12−/−Ferc−/−, Rbpflox/floxDap12−/−, or TKO mice to 4-week-old male C57BL/6 mice that were obtained from The Jackson Laboratory and served as recipient mice. The method of bone marrow transplantation has been described before (60). We sacrificed chimeric mice 4 months after bone marrow transplantation. We used the protocol for in vivo analysis of TNF-α-induced inflammatory bone resorption as described before (29, 52). When the mice were sacrificed, serum was collected to determine TRAP concentration by using the Mouse TRAP Assay Kit (Immunodiagnostic Systems) according to the manufacturer.
er’s instructions. Femur and calvarial bones were subjected to sectioning, TRAP staining, and histological analysis (60). Bioquant Osteo II software was used for bone histomorphometry.

**Reagents.** Murine M-CSF, TNF-α, and sRANKL were purchased from Peprotech. Recombinant mouse TGF-β was from R&D systems. TGF-β receptor I inhibitor SB431542, PLCγ inhibitor U-73122, CaMKK inhibitor STO-609, and PYK2 inhibitor AG 17 were obtained from Millipore. Fluo-4 AM, Fura Red AM, and pluronic F127 were from Life Technologies.

**Cell culture.** Murine bone marrow cells were harvested from tibiae and femora, and cultured overnight in petri dishes (BD Biosciences) in α-MEM medium (Invitrogen) with 10% FBS (Invitrogen). Except where stated, CMG14-12 supernatant, which contained the equivalent of 20 ng/ml of recombinant M-CSF, was used as a source of M-CSF, as described (10, 61), in experiments. Nonadherent cells were then replated into tissue-culture dishes and cultured in the same medium for 3 days to obtain BMMs, which are capable of differentiating to osteoclasts, and thus were used as osteoclast precursors. The attached BMMS were scraped, seeded at a density of 4.5 × 10^4/cm², and cultured in α-MEM medium with 10% FBS and CMG14-12 supernatant for 1 day. Except where stated, the cells at this time point were used for the basal condition. The cells were then treated without or with RANKL (80 ng/ml) or TNF-α (40 ng/ml) for times indicated in the figure legends. Culture media were exchanged every 3 days. For osteoclast differentiation assays in 96-well plates, 4 wells per condition were used. TRAP staining was performed with an acid phosphatase leukocyte diagnostic kit (Sigma-Aldrich) in accordance with the manufacturer’s instructions.

**In vitro gene silencing.** Antisense inhibition using locked nucleic acid (LNA) technology from Exiqon was applied to silence gene expression in vitro. LNA oligonucleotides specifically targeting Tgfr1 and nontargeting control LNAs were from Exiqon and were transfected into murine BMMS using TransIT-TKO transfection reagent (Mirus) in accordance with the manufacturer’s instructions.

**Intracellular calcium measurements.** BMMS were plated in 96-well culture plates with 200 μl culture medium/well. The cells were cultured for 24 hours and labeled with Fluo-4 NW using the Fluo-4 NW Calcium Assay Kit (Molecular Probes). The intracellular calcium concentration was measured according to the manufacturer’s instruction.

**Calcium oscillation measurements.** Osteoclast precursors were cultured without or with exogenous TGF-β (10 ng/ml), or with 100 μM SB431542, an inhibitor blocking the kinase activity of TGF-β receptor I, in the presence of recombinant murine M-CSF (20 ng/ml) for 24 hours in glass-bottom culture dishes (MatTek Corporation) and subjected to calcium oscillation measurement, as previously described (18), with small modifications. Briefly, cells were loaded with 5 μM fluo-4 AM, 10 μM Fura Red AM, and 0.05% pluronic F127 and analyzed using confocal microscopy (Leica). Cells were excited at 488 nm, and emissions at 500 to 550 nm for fluo-4 and 580 to 680 nm for Fura Red were acquired simultaneously at 5-second intervals. The relative intracellular calcium levels in single cells were monitored for 300 seconds and indicated by the ratio of the fluorescence intensity of fluo-4 to that of Fura Red (referred to as [Ca++] index ratio in corresponding figure legends). Ionomycin (10 μM) was used to confirm cellular responsiveness to calcium influx for every experiment. At least 100 single cells in each condition were monitored, and each condition was repeated at least 3 times. The average amplitude of calcium oscillation in single cells was calculated using the TuneR and SeeWave packages from the R programming language (62, 63). Each cell trace was converted into a text file and used as input into a user-created function computer code, as follows: meanAmp <- function(x) {cell <- read.table(x); matrix <- as.matrix(cell); Wob <- Wave(matrix, samp.rate = .2); object <- meanspec(Wob,2, wL = 4, 2, plot = FALSE); x <- object[2]; return(mean(x[3: (wL/2)]))}, where n = number of time points in cell trace; wL must be an even number less than n.

Control BM MM cells cultured with M-CSF alone had small amplitudes of oscillation. For each experiment, oscillating cells were defined as having oscillation amplitudes greater than 1.25 times the median oscillation amplitude of the control conditions. The proportions of oscillating/nonoscillating cells in each condition were calculated, and their differences were statistically analyzed using the χ² test for independence (P < 0.05 and P < 0.01 were taken as statistically significant).

**Reverse transcription and real-time PCR.** Reverse transcription and real-time PCR were performed as previously described (60). The primers for real-time PCR were as follows: Nfatc1: 5′-CCCGT-CACATTCTGCTCACT-3′ and 5′-CAGATTAACCGCTGATACGCCT-3′; Prdm1: 5′-TTCTTTGTGTGTGTGTTTGGCACCTT-3′ and 5′-TTGGAACACCTCCTTTGAGATT-3′; Acp5: 5′-ACGCGTACTTGGGCTTC-3′ and 5′-TCTGGTGAGGCTCTGC-3′; Ctsk: 5′-AAGATATTGTGCTGCTTG-3′ and 5′-ATCGCTGC-GTCCCTCT-3′; Itgb3: 5′-CCCGGGGACTTTAATGAGACACTT-3′ and 5′-ACGGCCTCCAATCCACCATAC-3′; Cldn2: 5′-AGACCAGGACACAGCTTTG-3′ and 5′-GCCATATTGGCGAAAAGCAGC-3′; primary Pleg2: 5′-GCTCAAAACCGCTCTTCC-3′ and 5′-TGAGGGTTGTATGTTACA-3′; Tgfr1: 5′-TGCCAATAAC-GCAGCCTGTC-3′ and 5′-AATGAAAGGGCCGATCTAGTG-3′; Id1: 5′-AGTGTGACCCGCTGCTGCTACG-3′ and 5′-CAGATCTC-CACCTGCTGTCACT-3′; Id3: 5′-CAGTACACCCTGAACTACGCC-3′ and 5′-CCATTCTCGGAAAGCCAGC-3′; Calbn1a: 5′-GGAGATCCACAGCGATATCC-3′ and 5′-CAACTGCTGCT-GTCCAGGG-3′; Cdkn2b: 5′-AGCTGATGCTGTTGCTTGAAG-3′ and 5′-GATCCCAAGATTTCTCTTCG-3′; Smad7: 5′-TTCGGAGA-CAACAGGATGCGCTG-3′ and 5′-AGCCTTGTGAGGAAAC-CAGGGAA-3′; Gapdh: 5′-ATCAGAAGGTTGTGAAAGCA-3′ and 5′-AGACACCTGCTGCTCAGTGT3′.

**Immunoblot analysis.** Total cell extracts were obtained using lysis buffer containing 20 mM HEPES (pH 7.0), 300 mM NaCl, 10 mM KCl, 1 mM MgCl₂, 0.1% Triton X-100, 0.5 mM DTT, 20% glycerol, and 1× proteinase inhibitor cocktail (Roche). The cell membrane-permeable protease inhibitor Pefabloc (1 mM) was added immediately before harvesting cells. The protein concentration of extracts was quantified using the BCA Protein Assay Kit (Pierce). Cell lysates (10 μg/sample) were fractionated on 7.5% SDS-PAGE, transferred to Immobilon-P membranes (Millipore), and incubated with specific antibodies. Western Lightning plus-ECL (PerkinElmer) was used for detection. Densitometry was performed using ImageJ software (http://imagej.nih.gov/ij/). Phospho-PLCγ2, PLCγ2, phospho-PYK2, and phospho-SMAD2/3 antibodies were from Cell Signaling. c-FOS, BLIMP1, GAPDH, and p38α antibodies were from Santa Cruz Biotechnology Inc. NFATc1 and SMAD2/3 antibodies were from BD Biosciences, and β-tubulin antibody was from Abcam.

**ChIP assay.** ChIP assays were performed as previously described (29). Briefly, 5 to 10 × 10^6 mouse BMMS (2.5 × 10^5 cells/100 mm dish) per condition were treated as indicated in figure legends, and 50% of chromatin from each condition was used per immunoprecipita-
tion (IP). Cell fixation, lysis, and nuclear extraction were performed as described (64). A Branson Sonifier 250 with a microtip at a power output of 5 (equivalent to 12 W) was used. Samples were sonicated for 6 cycles at 20 seconds each, separated by 1 minute incubations on ice. Average DNA fragment size of 200 to 500 bp was confirmed by agarose gel electrophoresis. For IP, 4 μg per sample of antibodies against RNA polymerase II (Millipore), Smad2/3 (BD Biosciences), or corresponding control rabbit or mouse IgG (Santa Cruz Biotechnology) was used. Immunoprecipitated DNA was analyzed by quanti-
tative real-time PCR and normalized relative to 28S rRNA-encoding gene segments that reflect amounts of nonspecific background DNA precipitation in each reaction. The primers used to detect Pol II occupancy at the Plcg2 locus were as follows: 5′-GCTCAGAACACAGCAGCTTC-3′ and 5′-TTGGAGGGTTATGGGTACA-3′. The primers that amplify 28S RNA-encoding gene segments are as follows: 5′-GATCCCTTC-GATGTCGGCTCTTCCTATC-3′ and 5′-TTGGTCCTTTCTGCCAGTGAA-3′.

RNA-seq. Total RNA was extracted using RNeasy Mini Kit (QIA-
GEN) following the manufacturer’s instructions. RNA-seq libraries were constructed per the Illumina TrueSeq RNA Sample Preparation Kit. High-throughput sequencing was performed using the Illumina HiSeq 2500/1500. RNA-seq reads were aligned to the mouse genome (mm9, NCBI Build 37) using TopHat (65). Cufflinks (66) was sub-
sequently used to assemble the aligned reads into transcripts and then estimate the transcript abundances as RPKM (reads per kilo base per million) values. Mapped reads are an average of 37 million per library. Gene Set Enrichment Analysis (GSEA program, Broad Institute) was performed according to the program’s instructions. All original RNA-seq data were deposited in the NCBI’s Gene Expression Omnibus (GEO GSE53218).

Statistics. Statistical analysis was performed using Graphpad Prism software. One-tailed Student’s t test was applied when there were only 2 groups of samples. In the case of more than 2 groups of samples with 1 condition, 1-way ANOVA followed by Tukey’s post hoc test was used to calculate differences between any groups of samples. In the case of more than 2 groups of samples with more than 1 con-
tdition/treatment, 2-way ANOVA followed by Sidak’s multiple com-
parisons was used. The χ² test was used to calculate difference of proportions of oscillating/nonoscillating cells. P < 0.05 was taken as statistically significant; all data are presented as the mean ± SD.

Study approval. All mouse experiments were approved by the Institutional Animal Care and Use Committee of the Hospital for Special Surgery.

Acknowledgments

We thank Yingli Shang for help with sectioning and Stephen Doty for help with analysis of the histological sections. We thank Taka-
ko Negishi-Koga and Hiroshi Takayanagi for technical support for calcium oscillation experiments. We are grateful to Steven Gold-
ing, F. Patrick Ross, and Takenobu Katagiri for valuable discuss-

We thank Yingli Shang for help with sectioning and Stephen Doty for help with analysis of the histological sections. We thank Taka-
ko Negishi-Koga and Hiroshi Takayanagi for technical support for calcium oscillation experiments. We are grateful to Steven Gold-
ing, F. Patrick Ross, and Takenobu Katagiri for valuable discuss-

Address correspondence to: Baohong Zhao, Hospital for Special Surgery, Research Building 4th floor, 535 East 70th Street, New York, New York 10021, USA. Phone: 212.774.2772; E-mail: zhaob@hss.edu.

8. Agasi A, Takayanagi H, M. The molecular under-
9. Zhao B, Ishikawa LB. Negative regulation of osteoclastogenesis and bone resorption by cyto-
11. Faccio R, Takeshita S, Zallone A, Ross FP, Teite-
elbaum SL. C-Fms and the alphaβ3 integrin collaborate during osteoclast differentiation.

13. Ishikawa LB. A signal-switch hypothesis for cross-
15. Bezzubadica IS, Medzhirone R, Novel ITAM role of ITAM sig-
17. Negishi-Koga T, Takayanagi H. Calcium-ITAM signaling is an essential axis of osteoclast differen-
19. Mao D, Epplle H, Uthgenannt B, Novack DV, Fac-
22. Wang L, et al. ‘Tuning’ of type I interferon-
ized jak-STAT1 signaling by calcium-depend-
24. Mocsay A, et al. The immunomodulatory adapter proteins DAP12 and Fc receptor gamma-chain (FcγR) regulate development of functional osteo-
25. Kopan R, Ilagan MX. The canonical Notch signal-
pathway: unfolding the activation mecha-


42. Yasui T, et al. Regulation of RANKL-induced osteoclastogenesis by TGF-β through molecular interaction between Smad3 and Traf6. J Bone Miner Res. 2011;26(7):1447-1456.


