Osteopetrosis in TAK1-deficient mice owing to defective NF-κB and NOTCH signaling

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The MAP kinase TGFβ-activated kinase (TAK1) plays a crucial role in physiologic and pathologic cellular functions including cell survival, differentiation, apoptosis, inflammation, and oncogenesis. However, the entire repertoire of its mechanism of action has not been elucidated. Here, we found that ablation of *Tak1* in myeloid cells causes osteopetrosis in mice as a result of defective osteoclastogenesis. Mechanistically, *Tak1* deficiency correlated with increased NUMB-like (NUMBL) levels. Accordingly, forced expression of *Numbl* abrogated osteoclastogenesis whereas its deletion partially restored osteoclastogenesis and reversed the phenotype of *Tak1* deficiency. *Tak1* deletion also down-regulated Notch intracellular domain (NICD), but increased the levels of the transcription factor recombinant recognition sequence binding protein at Jκ site (RBPJ), consistent with NUMBL regulating notch signaling through degradation of NICD, a modulator of RBPJ. Accordingly, deletion of *Rbpj* partially corrected osteopetrosis in *Tak1*-deficient mice. Furthermore, expression of active IKK2 in RBPJ/TAK1-deficient cells significantly restored osteoclastogenesis, indicating that activation of NF-κB is essential for complete rescue of the pathway. Thus, we propose that TAK1 regulates osteoclastogenesis by integrating activation of NF-κB and derepression of NOTCH/RBPJ in myeloid cells through inhibition of NUMBL.

osteoclast | osteopetrosis | NUMBL | TAK1 | RBPJ

Osteoclasts (OCs) are required for normal skeletal development. Differentiation of OCs from their marrow progenitors requires receptor activator of NF-κB (RANK) ligand (RANKL) (1). Binding of RANKL to its cognate receptor on the cell surface of OC progenitors mobilizes adaptor and signaling proteins to the intracellular motif of RANK (2). This RANK signaling cluster recruits scaffold proteins such as NF-κB-essential modifier (NEMO)/inhibitor of NF-κB kinase-γ (IKKγ), which form a platform to recruit other proteins by using polyubiquitin chains. Among these key proteins are complexes containing Tab1, Tab2, and the MAP kinase TGF-β activated kinase-1 (TAK1), the role of which in osteoclastogenesis has been described (3–7). Additionally, IKK subunits IKK1 and IKK2 are recruited to this kinase complex and form the basic unit that activates downstream NF-κB signaling in signal- and cell-specific manners (8, 9).

The role of NF-κB molecules in osteoclastogenesis and skeletal development has been widely described (10, 11). In this regard, deletion of IKK1 and IKK2, inhibition of their kinase activities, or blocking of their binding to NEMO attenuates osteoclastogenesis (12–18). Similarly, inhibition of phosphorylation of IκB by blocking IKK assembly and activation preserves expression of the inhibitory protein, which in turn remains avidly bound to NF-κB subunits, thus abolishing their nuclear translocation and arresting osteoclastogenesis (19). Likewise, deletion or inhibition of NF-κB proximal mediators such as TNF receptor-associated factor-6 (TRAF6) and c-Src led to inhibition of NF-κB activity, and to abnormal or arrested osteoclastogenesis and subsequent osteopetrosis (20–22).

The details of the mechanisms facilitating TRAF6 induction of IKK complex signaling remain vague. However, it has been suggested that TAB/TAK1 complexes mediate TRAF6 induction of NF-κB (23–26). This process is dominated by posttranslational modifications, primarily formation of polyubiquitin chains that enable protein–protein interactions. Polyubiquitin staging facilitates cross-phosphorylation, subcellular localization, and stabilization of signals (3, 27). Conversely, signaling of protein complexes is downregulated by degradation of key proteins such as TRAF6 (28).

Recent reports suggested that NUMB/NUMB-like (NUMBL) proteins, first described as critical for cell fate determination (29, 30), regulate TRAF6 expression and stability (31), regulate NOTCH signaling, and regulate ubiquitination of specific substrates (32–34). NOTCH, in turn, through release of Notch intracellular domain (NICD), binds to the transcriptional factor recombinant recognition sequence binding protein at Jκ site (RBPJ) and modulates gene transcription (35). In this regard, the role of NOTCH/RBPJ signaling in OCs has been described (36, 37). In this study, we discovered that deletion of *Tak1* coincides with elevated levels of NUMBL and RBPJ and decreased expression of NICD, events that led to arrest of osteoclastogenesis. Consistently, overexpression of NUMBL in WT cells diminished expression of these proteins and blocked osteoclastogenesis. Conversely, reintroduction of TAK1 reinstated osteoclastogenesis. More interestingly, genetic ablation of NUMBL or RBPJ in TAK1-null cells restored osteoclastogenesis and rescued the bone defects in mice.

Results

Myeloid Deletion of TAK1 Leads to Osteopetrosis in Mice. To investigate the physiologic role of TAK1 in the skeleton, we

Significance

Skeletal anomalies are major health disparities resulting from dysregulation of bone homeostasis. Osteoclasts (OCs) are the principal bone resorbing and remodeling cells. The function of the OC relies on intricate signaling network dominated by NF-κB and MAP kinases. TGF-β activated kinase-1 (TAK1) is the proximal activator of these pathways and ultimately is a key target for regulating cellular functions. The role of TAK1 in physiologic and pathologic cellular functions has been widely described. However, the precise mechanism by which TAK1 regulates these functions remains enigmatic. We discovered a novel mechanism by which TAK1 regulates expression of the sensory proteins NUMB/NUMB-like and subsequent activation of Notch–recombinant recognition sequence binding protein at Jκ site (RBPJ) pathway in myeloid cells. We provide genetic evidence that dysregulation of this pathway leads to osteopetrosis.

conditionally deleted the *Tak1* gene by using mice carrying the *Tak1*-floxed gene (Fig. S1), which we crossed with various cre recombinase mice in which the enzyme is expressed at different stages of osteoclastogenesis under the lysozyme M or CD11b promoter (deletion in monocytes/OC progenitors), and cathepsin K (deletion in late stage of osteoclastogenesis). All *Tak1* mutant mice were born alive and survived ~4–6 wk. However, they were significantly smaller in size compared with their WT and heterozygous littermates. Gross examination indicated growth retardation and failed tooth eruption or malformed incisors (Fig. S1*A*, arrow) in all mouse strains as shown for the TAK1 conditional deletion using lysozyme M-cre (ΔLM). Given this phenotypic redundancy, all subsequent studies were conducted by using TAK1 conditional deletion using lysozyme M-cre (TAK1$^{\Delta LM}$). Histologic analysis revealed significantly reduced number of TRAP-positive cells, thicker/larger growth plates, and cartilage remnants in TAK1$^{\Delta LM}$ bones (Fig. 1*A*). Micro-CT scanning revealed changes in bone parameters in TAK1$^{\Delta LM}$ mice consistent with osteopetrosis. Specifically, quantification of bone parameters showed that volumetric bone mass was increased two to threefold in the KO mice compared with WT counterparts. Trabecular number and trabecular thickness were also increased whereas trabecular spacing was dramatically decreased (Fig. 1*B* and Fig. S1 *D* and *E*). These findings suggest that *Tak1*-deficient mice display osteopetrosis.

Deletion of TAK1 Hinders Differentiation and Signaling by OC Progenitors. The osteopetrotic phenotype of TAK1 mutant mice suggests that *Tak1* gene is essential for differentiation of myeloid progenitors into OC or OC function. To explore the former proposition, bone marrow macrophages (BMMs) were isolated from WT and TAK1$^{\Delta LM}$ mice and cultured in the presence of macrophage colony-stimulating factor (M-CSF) and RANKL. The vast majority of these cells did not survive as a result of lack of NF-κB activity. Cell survival was significantly rescued in the presence of TNF-α neutralizing antibodies (Fig. S2), an observation consistent with a recent report (7). Hence, TNF-α neutralizing antibody and isotype matching IgG (control) were used in all subsequent experiments. Examination of in vitro cultures clearly shows that OC differentiation from

Fig. 1. Tak1-deficient mice display an osteopetrotic phenotype. Tak1-floxed mice were crossed with the lysozyme-M (LM) cre mice as described in *Materials and Methods*. Mice (3-4 wk old; *n* = 8 per group) were killed, and long bones were processed for histology and stained with TRAP to visualize OCs (*A*). OC counts per bone area are presented (*Lower*). (*B*) Micro-CT analysis. Bone volume/total volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), and trabecular separation (Tb.Sp) parameters were collected (*P < 0.05, **P < 0.001).

TAK1$^{\Delta LM}$ cells was significantly blunted (Fig. 2*A*). Similar results were obtained using CD11b-cre (TAK1$^{\Delta CD}$) and Cathepsin-K-cre (TAK1$^{\Delta CK}$)-derived cells (Fig. S1*C*). Consistently, TAK1-null cells expressed low levels of the OC markers tartrate resistant acid phosphatase (TRAP), Cathepsin K, β3-integrin, and matrix metalloproteinase 9 (MMP9) compared with WT cells (Fig. 2*B*). This differentiation defect was corrected when WT TAK1 was reintroduced retrovirally in the TAK1-deficient cells, thus establishing specificity of the deletion (Fig. 2 *C* and *D* and Fig. S3). Next, we examined signal transduction pathways believed to be regulated by TAK1, namely NF-κB and MAPK. As expected, RANKL-stimulated phosphorylation/activation of IKKβ, p38, and JNK is blunted in the absence of TAK1 compared with WT cells (Fig. 2*E*). These observations confirm that *Tak1* deletion attenuates MAPK and NF-κB signaling and hampers OC differentiation.

TAK1 Regulates the Expression of NUMBL. To better clarify the mechanistic steps governing TAK1 action in bone, we examined other TAK1 partners. We discovered that expression levels of NUMBL, a TAB-TAK1 interacting partner (38), were elevated in TAK1$^{\Delta LM}$ cells (Fig. 3*A*). This expression was reduced upon introduction of WT-TAK1, but not the inactive form of TAK1, namely TAK1-K63W (Fig. 3*B*). Interestingly, the expression profile of NUMBL under these conditions inversely correlated with the adaptor protein TAB2 (Fig. 3*B*). Further analysis showed that NUMBL expression is regulated at the transcriptional and posttranslational levels. However, additional studies are required to elucidate the details of these mechanisms. These observations suggest that NUMBL is regulated by TAK1 and may modulate osteoclastogenesis. Indeed, virally expressed NUMBL (pMx-NUMBL) significantly blocked RANKL-induced osteoclastogenesis (Fig. 3 *C* and *D*) compared with GFP-infected cells. These observations suggest that defective osteoclastogenesis in the absence of TAK1 may be caused, at least in part, by elevated levels of NUMBL. Thus, we surmised that reduction of NUMBL levels might restore osteoclastogenesis by TAK1-null cells. To this end, we eliminated *Numb/Numbl* genes (both are redundant and have overlapping functions) from TAK1$^{\Delta LM}$ by crossing *Numb/Numbl* double KO mice with TAK1-floxed/cre+ to induce *Numb/Numbl* and T*ak1* myeloid conditional deletion (referred to as TAK1/N/Nl$^{\Delta LM}$ mice). We observed a significant increase in OC number in histological sections of long bones of the triple KO mice compared with TAK1$^{\Delta LM}$ alone (Fig. S4*A*, *Insets*; quantification in Fig. S4*A*, *Right*). This observation was further confirmed by ex vivo OC cultures derived from the various mouse genotypes, validating that deletion of *Numb/Numbl* significantly, but incompletely, reinstated osteoclastogenesis in *Tak1*-null cells (Fig. 3 *E* and *F*). Consistently, we observed no osteopetrotic phenotype in the triple KO mice compared with the TAK1$^{\Delta LM}$ mice (Fig. 3*G*, arrows; quantification in Fig. 3*H*). Taken together, these findings suggest that TAK1 regulates NUMBL expression and the abundance of NUMBL in the absence of TAK1 and TAB2 arrests osteoclastogenesis.

NUMBL Inhibits Osteoclastogenesis by Targeting the Notch–RBPJ Pathway. Earlier reports have suggested that NUMB/NUMBL mediates degradation of NICD (39, 40), which binds to and regulates the activity of RBPJ. Consistent with this notion, we found that levels of RBPJ are markedly elevated concomitant with decreased levels of NICD in TAK1$^{\Delta LM}$ cells (Fig. 4*A*). Notably, the robust expression of RBPJ appears RANKL-dependent. Buttressing regulation by NUMB/NUMBL, compound deletion of *Tak1/Numb/Numbl* reduced expression of RBPJ concurrent with rise in NICD expression (Fig. 4*B*). These observations suggest that elevated levels of RBPJ may contribute to inhibition of osteoclastogenesis and confirms that NUMB/NUMBL regulate this process. Indeed, retrovirally expressed

Fig. 2. Myeloid-specific deletion of TAK1 inhibits differentiation and signaling by OC progenitors. BMMs were isolated from TAK1$^{\Delta LM}$ and WT mice. Cells were plated with M-CSF and RANKL for 4 d and then fixed and TRAP-stained and counted (A). (B) PCR quantification of OC genes TRAP, Cathepsin-K, β3-integrin, and MMP9. (C and D) Retroviral expression of TAK1 in the TAK1$^{\Delta LM}$ cells (dark bars in D represent pMX-GFP, gray bars represent pMX-TAK1). (E) WT and TAK1$^{\Delta LM}$ were stimulated with RANKL for 30 min or left untreated. Cells were then lysed and subjected to Western blots using the various antibodies indicated. β-Actin was used as loading control. LM, lysozyme-M (*$P < 0.01$, **$P < 0.001$; Fig. S3).

RBPJ significantly arrested osteoclastogenesis in vitro (Fig. 4C). Conversely, osteoclastogenesis was enhanced in *Rbpj*-null cells compared with WT counterparts (Fig. 4D). In agreement with these findings, treatment of OC precursors with the γ-secretase inhibitor (GSI), which prevents NOTCH cleavage, indeed decreased intracellular levels of NICD (NICD/actin ratio; Fig. 4E), increased expression of RBPJ (RBPJ/actin ratio; Fig. 4E), and significantly arrested osteoclastogenesis (Fig. 4F).

RBPJ Is an Endogenous Repressor of Osteoclastogenesis and Is a Downstream Target of TAK1. To further address the endogenous role of TAK1–NICD–RBPJ axis in osteoclastogenesis, we generated TAK1/RBPJ$^{\Delta LM}$ double KO mice. Consistent with our in vitro observations, osteoclastogenesis partially recovered in TAK1/RBPJ$^{\Delta LM}$ double mutant cells (Fig. 5 *B* and *D* vs Fig. 5*A*; quantification in Fig. 5*E*). More compelling, examination of micro-CT images revealed significant reversal of the osteopetrotic phenotype in TAK1/RBPJ$^{\Delta LM}$ (Fig. 5*I*) compared with TAK1$^{\Delta LM}$ (Fig. 5*G*). These findings were further supported by quantitative measurements of bone parameters (Fig. 5 *J–M*) and by histologic evidence (Fig. S4*B*). Protein expression of TAK1 and RBPJ in these mice is depicted in Fig. S4*C*.

Activation of NF-κB Is Essential for Complete Rescue of Osteoclastogenesis. Despite significant rescue of the bone defect of TAK1$^{\Delta LM}$ mice in vivo by deletion of RBPJ or NUMB/NUMBL, rescue of in vitro osteoclastogenesis was only partial. Thus, we surmised that, in the absence of TAK1, the baseline activity of NF-κB is too low and hence insufficient to support in vitro survival and differentiation. We further postulated that resumption of osteoclastogenesis not only requires removal of suppression by deleting RBPJ but also require baseline activation of NF-κB. To test this

proposition, we transduced WT, TAK1$^{\Delta LM}$, and TAK1/RBPJ$^{\Delta LM}$ cells with constitutively active IKK2 (IKK2-SSEE), which is the immediate target of TAK1. The results confirm that activation of NF-κB recapitulates osteoclastogenesis by double KO cells [Fig. 5*S* (image) and Fig. 5*T* (OC counts)] to levels comparable to WT cells (Fig. 5 *N* and *T*). Thus, activation of NF-κB and inhibition of Notch signaling reinstates osteoclastogenesis in *Tak1*-deficient conditions.

Discussion

In this study, we show, in agreement with previous studies (6, 7), that the MAPK TAK1 is crucial for osteoclastogenesis and that its deletion leads to osteopetrosis in mice. This finding is consistent with the notion that TAK1 is the primary activator of IKK
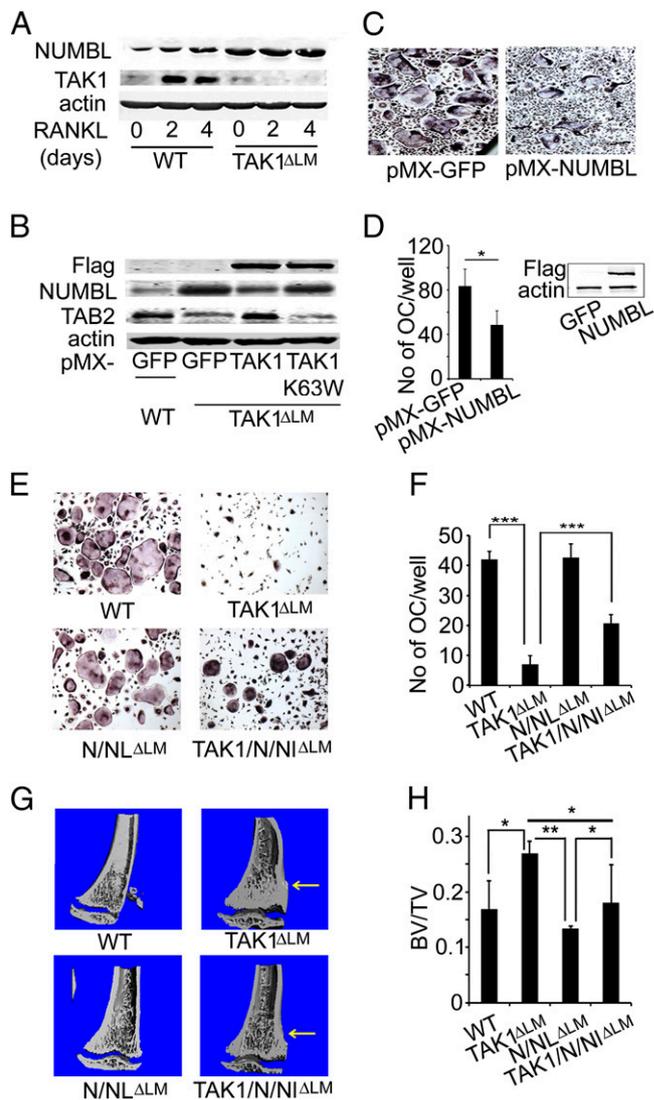
Fig. 3. Effect of expression or deletion of NUMBL on osteoclastogenesis and bone development. (A) WT and TAK1$^{\Delta LM}$ BMMs were treated with RANKL for 0, 2, or 4 d, lysed, and subjected to immunoblotting using the indicated antibodies. (B) WT and TAK1$^{\Delta LM}$ cells were transfected with pMX-GFP, pMX-TAK1, and pMX-TAK1K63W and plated with M-CSF and RANKL for 4 d, followed by cell lysis and immunoblotting using the indicated antibodies. (C and D) BMMs were infected with pMx-GFP or pMx-NUMBL and cultured for OC assay. Western blot represents virally expressed NUMBL fused to FLAG. (E and F) OCs derived from triple *Tak1/Numb/Numbl* KO (referred to as TAK1/N/NI$^{\Delta LM}$) compared with TAK1 and Numb/L (N/NL$^{\Delta LM}$) KO. (G and H) Micro-CT images and quantification from control and null mice described in E (n = 6 per group; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).
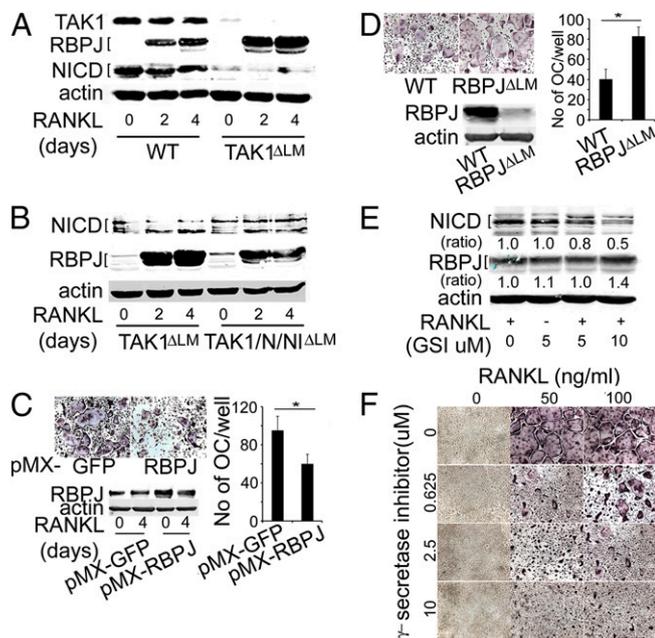
Fig. 4. Increased NUMBL in TAK1$^{\Delta LM}$ inhibits osteoclastogenesis by targeting the Notch–RBPJ pathway. (*A*) WT and TAK1$^{\Delta LM}$ BMMs were treated with RANKL for 0, 2, and 4 d, then lysed and subjected to immunoblots with the indicated antibodies. (*B*) Cells from TAK1cKO and TAK1/N/NI$^{\Delta LM}$ (triple KO) were cultured as in *A*, and lysates were subjected to Western blots as indicated. (*C*) BMMs were transfected with pMx-GFP and pMx-RBPJ and followed by OC culture and TRAP staining. OC photomicrograph quantification of OC counts per well area and Western blot of RBPJ expression are depicted. (*D*) OCs from WT and RBPJ$^{\Delta LM}$. (*E*) BMMs were treated with GSI and RANKL (4 d) as indicated. Cell lysates were then subjected to Western blots for NICD, RBPJ, and β-actin. Expression ratio of NICD/actin and RBPJ/actin are indicated. (*F*) WT BMM cells were treated with or without RANKL and GSI at different concentration. Photomicrographs of OC cultures at the various conditions indicated are depicted (*P < 0.01).

that regulate osteoclastogenesis and bone physiology. These include TAK1 regulation of NUMBL, which in turn modulates NOTCH/RPBJ and NF-κB signaling pathways.

To decipher the mechanism underlying diminished osteoclastogenesis and ensuing osteopetrosis in the absence of TAK1, we surveyed TAK1 potential signaling partners and identified the conserved proteins NUMB and NUMBL as potential candidates. First, we observed that RANKL regulates expression of NUMBL in OC precursors. Surprisingly, we found that expression of NUMBL protein is elevated in TAK1-null cells whereas expression of TAB2, which mutually interacts with NUMBL and TAK1, was diminished compared with WT cells. Notably, the kinase activity of TAK1 appears essential for these events, as reintroduction of WT but not catalytically inactive TAK1, restored TAB2, and inhibited NUMBL protein expression in TAK1-null cells. These observations suggest that TAK1 is a repressor of NUMBL. Our findings further suggest that elevated NUMBL levels and/or absence of TAK1 destabilize TAB2 protein, and that NUMBL is potential modulator of NF-κB signaling and OCs. This proposition is supported by recent evidence showing that (*i*) NUMB activates E3 ligase-mediated degradation of target proteins (34), (*ii*) NUMBL interacts with TAB2 and inhibit its binding (and binding of its partner TAK1) to TRAF6 (38), and (*iii*) NUMBL binds to and induces degradation of TRAF6 (31).

By using deletion of *numb/numbl* genes in the TAK1$^{\Delta LM}$ context, we provide compelling genetic evidence that NUMB/NUMBL proteins modulate TAK1-mediated osteoclastogenesis. These findings support our proposed model that elevated expression of NUMB/NUMBL arising from *Tak1* deletion plays a significant part in inhibiting osteoclastogenesis and that their deletion partially restores OCs. In this setting, whereas OC formation in vitro was partially restored, very significant OC formation in vivo and reduction of the osteopetrotic phenotype were evident. We suggest that incomplete reversal of the osteopetrotic phenotype is likely a result of additional contributing factors regulated by TAK1, which are independent of NUMB/NUMBL. In addition, it remains unclear how osteoclastogenesis and bone resorption resume in the absence of TAK1 under the conditions described here. However, we postulate that osteoclastogenesis observed in this triple KO mouse is likely to be TAK1-independent. This presumption is not unusual in light of our recent in vivo evidence that distal activation of the NF-κB pathway at the IKK2 level was sufficient to induce osteoclastogenesis and bone loss independent of proximal signals

and MAPKs. Indeed, we confirm that activation of downstream targets of TAK1, including IKK2, and the MAPKs p38 and JNK, is impaired in RANKL-treated TAK1-null OC progenitors. Importantly, we provide previously unidentified mechanistic details suggesting that TAK1 integrates multiple signaling pathways
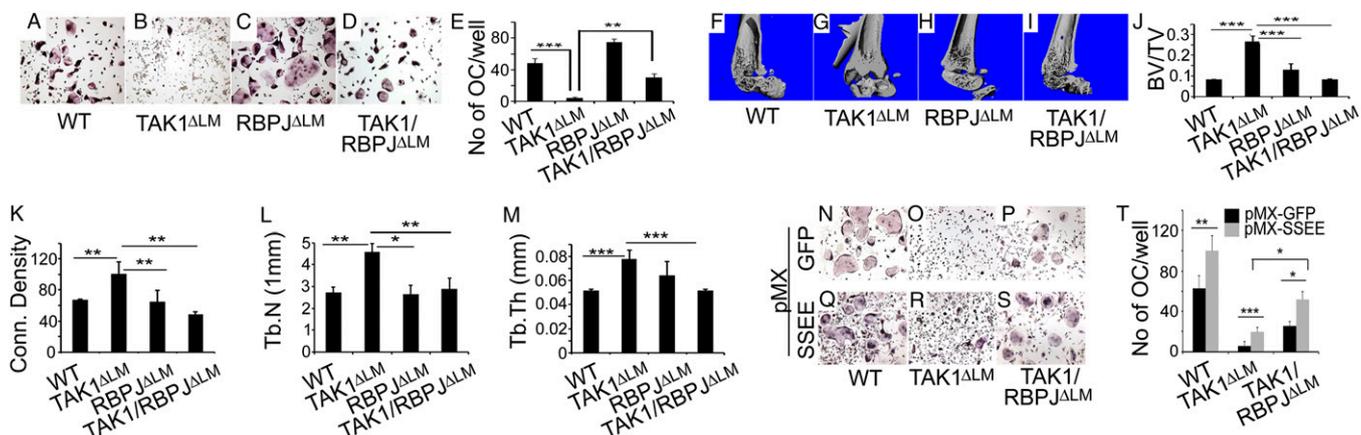
Fig. 5. Deletion of RBPJ in TAK1$^{\Delta LM}$ significantly rescues the TAK1$^{\Delta LM}$ osteopetrotic phenotype and simultaneous activation of NF-κB fully reinstates OCs. (*A–E*) osteoclastogenesis from WT, RBPJ$^{\Delta LM}$, TAK1$^{\Delta LM}$, and TAK1/RBPJ$^{\Delta LM}$. (*F–I*) Micro-CT scanning images and (*J–M*) the corresponding quantification parameters volumetric bone (i.e., BV/TV), connective density, trabecular numbers (Tb.N), trabecular thickness (Tb.Th.), and trabecular spacing (Tb.Sp). (*N–S*) WT, TAK1cKO and TAK1/RBPJ$^{\Delta LM}$ BMMs were transfected with pMx-GFP and pMx-SSEE (constitutively active IKK2) and subjected to osteoclastogenesis. OC photomicrographs along with quantification of TRAP-positive cells (*T*) are depicted (*P < 0.05, **P < 0.01, ***P < 0.001).

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such as receptor engagement and subsequent TAK1/NEMO complex assembly (41).

To further examine the mechanism by which NUMB/NUMBL restore OCs and reverse the osteopetrotic phenotype in TAK1-deficient mice, we identified NOTCH signaling as a prominent candidate. NUMB and NUMBL were identified as inhibitors of NOTCH signaling in flies (32), acting through interaction with NICD, recruitment of the E3 ligase Itch, polyubiquitination, and degradation of NICD (42). Under normal conditions, activation of NOTCH signaling entails cleavage of NOTCH into NICD, which in turn translocates to the nucleus and binds to and regulates transcriptional activity of RBPJ. We found that, under TAK1 deficiency, increased levels of NUMBL coincide with decrease expression of NICD and marked increase in RBPJ expression. This finding is consistent with the notion that RBPJ is an endogenous repressor of OC and in agreement with recent reports (36, 43). Indeed, we show that retroviral expression of RBPJ inhibits osteoclastogenesis, that basal osteoclastogenesis is elevated in mice lacking RBPJ, and, most importantly, that combined deletion of RBPJ in the TAK1-deficient mice significantly rescued OCs and reversed osteopetrosis. Furthermore, these findings are supported by our data that inhibition of NICD formation by γ-secretase inhibitors, arrests osteoclastogenesis, and elevates expression of RBPJ, hence buttressing the notion that regulation of this pathway by TAK1-NUMBL plays a central role in the osteopetrotic phenotype of the TAK1-deficient mice. Accordingly, we propose that TAK1 regulation of NUMBL, NOTCH, and RBPJ signaling in OC is a novel axis worthy of further investigation. We speculate that stimulation with RANKL activates TAK1, which, through activation of NOTCH, mobilizes and switches RBPJ to activator complexes. At the cessation of the signal, RBPJ reverts to the repressor complex and restrains osteoclastogenesis in a negative-feedback mode. In this regard, to our knowledge, we provide the first evidence that deletion of TAK1 leads to accumulation of RBPJ protein that in turn reinforces the RBPJ repressor complex. The precise mechanism leading to regulation of RBPJ protein expression and stability in this setting remains to be elucidated.

We noticed that, whereas deletion of NUMB/NUMBL or RBPJ nearly rescued the osteopetrotic phenotype in mice, parallel in vitro rescue experiments were only partial. We hypothesized that two elements are essential for full activation of the OC transcriptional machinery. First, suppression by high levels of RBPJ must be relieved. Second, basal activation by NF-κB target genes must be present. To address this proposition, we show that introduction of constitutively active form of IKK2, i.e., IKK2-SSEE, which overrides the need for TAK1, almost completely rescued osteoclastogenesis in TAK1-null cells in vitro. We surmised that the high degree of phenotypic rescue in vivo benefited from residual NF-κB activity in vivo, which was lacking in vitro. This is supported by previous evidence that established cross-transcriptional regulation of NFATc1 by RBPJ and NF-κB (44). In this regard, it is important to point out that RBPJ exerts its OC transcriptional suppression through binding to DNA motif overlapping the NF-κB binding site in the NFATc1 promoter. Hence, attenuation of DNA binding through NICD interaction rather than decreased protein expression is more important for inhibiting RBPJ suppression. This concept is supported by a recent finding wherein occupation of DNA binding sites and repression by RBPJ is regulated by NICD, which mediates exchange to activators (45).

Collectively, our current working model suggests that TAK1 is responsible for concurrent activation of NF-κB and suppression of NUMBL–NOTCH pathways (Fig. S5). Deletion of TAK1 results in NF-κB inactivation and simultaneous accumulation of NUMBL and subsequent decrease of cytosolic NICD. As a result of lower NICD levels, expression levels of RBPJ rise in nuclear repressor complexes and halt OC transcriptional activity. Deleting NUMBL or RBPJ concomitant with introducing NF-κB gain of function reinstates osteoclastogenesis and alleviates osteopetrosis. Thus, we propose the previously undescribed concept that TAK1 integrates multiple pathways to regulate osteoclastogenesis.

Materials and Methods

Animals. Approval for using animals was obtained from Washington University School of Medicine Institutional Animal Care and Use Committee prior to performing this study. Mice were housed at the Washington University School of Medicine barrier facility. *Tak1* floxed mice on a C57BL/6 background (46) were crossed with LysM-Cre mice to produce heterozygous mice. The TAK1-Cre heterozygous mice were further intercrossed to generate TAK1 homozygous null mice (TAK1/LysM:cre, or TAK1$^{\Delta LM}$). LysM-Cre and *numb/numbl* floxed mice were purchased from Jackson Laboratories. Raphael Kopan, Cincinnati Children's Hospital Medical Center, Cincinnati, provided *rbpj* floxed mice.

Cell Culture and Osteoclastogenesis. Murine OCs were prepared from bone marrow cells. Cells from bone marrow were cultured in α-MEM supplemented with 100 units/mL penicillin/streptomycin and 10% FBS (vol/vol) with 10 ng/mL M-CSF for 16 h to separate adherent cells from nonadherent cells. Nonadherent cells were harvested were used as enriched bone marrow–derived monocyte/macrophage precursors (i.e., BMMs). To generate OCs, BMMs were further cultured with M-CSF (20 ng/mL) and RANKL (50 ng/mL). After an additional 4 d of culture, cells were fixed and stained for TRAP by using TRAP-Leukocyte kit (Sigma). TRAP-positive cells containing more than three nuclei were considered multinucleated bona fide OCs.

Cloning Methods. To express the NUMBL into OCs and to study its role in osteoclastogenesis, the NUMBL gene was cloned from the American Type Culture Collection (ATCC) clone (NUMBL mouse cDNA clone ATCC 10698964; GenBank ID BC068116). The ATCC clone DNA was digested with EcoRI and NotI and ligated into similarly digested pMx-vector as previously described (41). The cloning of NUMBL into pMx-vector was confirmed by restriction/digestion analysis and expression of NUMBL protein was confirmed by immunoblot analysis using the anti-NUMBL (H-80) antibody (Santa Cruz). A similar approach was followed to clone and express, e.g., pMX-RBPJ, pMX-IKK2SSEE, pMX-TAK1, and pMX-GFP.

Retroviral infection. Retroviruses were generated by transfection of relevant constructs into Plat-E packaging cells with Fugene 6. After retroviral infection for 12–24 h and puromycin selection for 2 d, cells were cultured according to experimental conditions. Specific details of this method have been published (17, 41).

Additional description of methods is included in *SI Materials and Methods*.

Statistical Analysis. Statistical analyses were performed by using Student *t* test. Multiple treatments were analyzed by using one-way ANOVA followed by post hoc Newman–Keuls test of significance. Values are expressed as mean ± SD of at least three independent experiments. *P* values are indicated where applicable.

1. Abu-Amer Y (2005) Advances in osteoclast differentiation and function. *Curr Drug Targets Immune Endocr Metabol Disord* 5(3):347–355.
2. Darnay BG, Besse A, Poblenz AT, Lamothe B, Jacoby JJ (2007) TRAFs in RANK signaling. *Adv Exp Med Biol* 597:152–159.
3. Kanayama A, et al. (2004) TAB2 and TAB3 activate the NF-kappaB pathway through binding to polyubiquitin chains. *Mol Cell* 15(4):535–548.
4. Walsh MC, Kim GK, Maurizio PL, Molnar EE, Choi Y (2008) TRAF6 autoubiquitination-independent activation of the NFkappaB and MAPK pathways in response to IL-1 and RANKL. *PLoS ONE* 3(12):e4064.
5. Wang C, et al. (2001) TAK1 is a ubiquitin-dependent kinase of MKK and IKK. *Nature* 412(6844):346–351.
6. Huang H, et al. (2006) Osteoclast differentiation requires TAK1 and MKK6 for NFATc1 induction and NF-kappaB transactivation by RANKL. *Cell Death Differ* 13(11):1879–1891.
7. Lamothe B, Lai Y, Xie M, Schneider MD, Darnay BG (2013) TAK1 is essential for osteoclast differentiation and is an important modulator of cell death by apoptosis and necroptosis. *Mol Cell Biol* 33(3):582–595.
8. Hayden MS, Ghosh S (2004) Signaling to NF-kappaB. *Genes Dev* 18(18):2195–2224.

9. Perkins ND (2007) Integrating cell-signalling pathways with NF-kappaB and IKK function. *Nat Rev Mol Cell Biol* 8(1):49–62.
10. Abu-Amer Y, Darwech I, Otero J (2008) Role of the NF-kappaB axis in immune modulation of osteoclasts and bone loss. *Autoimmunity* 41(3):204–211.
11. Boyce BF, Yao Z, Xing L (2010) Functions of nuclear factor kappaB in bone. *Ann N Y Acad Sci* 1192:367–375.
12. Chaisson ML, et al. (2004) Osteoclast differentiation is impaired in the absence of inhibitor of kappa B kinase alpha. *J Biol Chem* 279(52):54841–54848.
13. Dai S, Hirayama T, Abbas S, Abu-Amer Y (2004) The IkappaB kinase (IKK) inhibitor, NEMO-binding domain peptide, blocks osteoclastogenesis and bone erosion in inflammatory arthritis. *J Biol Chem* 279(36):37219–37222.
14. Jimi E, Ghosh S (2005) Role of nuclear factor-kappaB in the immune system and bone. *Immunol Rev* 208:80–87.
15. Ruocco MG, et al. (2005) IkappaB kinase (IKK)beta, but not IKKalpha, is a critical mediator of osteoclast survival and is required for inflammation-induced bone loss. *J Exp Med* 201(10):1677–1687.
16. Clohisy JC, Yamanaka Y, Faccio R, Abu-Amer Y (2006) Inhibition of IKK activation, through sequestering NEMO, blocks PMMA-induced osteoclastogenesis and calvarial inflammatory osteolysis. *J Orthop Res* 24(7):1358–1365.
17. Otero JE, et al. (2008) Defective osteoclastogenesis by IKKbeta-null precursors is a result of receptor activator of NF-kappaB ligand (RANKL)-induced JNK-dependent apoptosis and impaired differentiation. *J Biol Chem* 283(36):24546–24553.
18. Darwech I, Otero J, Alhawagri M, Dai S, Abu-Amer Y (2009) Impediment of NEMO oligomerization inhibits osteoclastogenesis and osteolysis. *J Cell Biochem* 108(6):1337–1345.
19. Clohisy JC, Hirayama T, Frazier E, Han SK, Abu-Amer Y (2004) NF-kB signaling blockade abolishes implant particle-induced osteoclastogenesis. *J Orthop Res* 22(1):13–20.
20. Boyce BF, Yoneda T, Lowe C, Soriano P, Mundy GR (1992) Requirement of pp60c-src expression for osteoclasts to form ruffled borders and resorb bone in mice. *J Clin Invest* 90(4):1622–1627.
21. Naito A, et al. (1999) Severe osteopetrosis, defective interleukin-1 signalling and lymph node organogenesis in TRAF6-deficient mice. *Genes Cells* 4(6):353–362.
22. Lomaga MA, et al. (1999) TRAF6 deficiency results in osteopetrosis and defective interleukin-1, CD40, and LPS signaling. *Genes Dev* 13(8):1015–1024.
23. Landström M (2010) The TAK1-TRAF6 signalling pathway. *Int J Biochem Cell Biol* 42(5):585–589.
24. Lee SW, Han SI, Kim HH, Lee ZH (2002) TAK1-dependent activation of AP-1 and c-Jun N-terminal kinase by receptor activator of NF-kappaB. *J Biochem Mol Biol* 35(4):371–376.
25. Mizukami J, et al. (2002) Receptor activator of NF-kappaB ligand (RANKL) activates TAK1 mitogen-activated protein kinase kinase kinase through a signaling complex containing RANK, TAB2, and TRAF6. *Mol Cell Biol* 22(4):992–1000.
26. Takaesu G, et al. (2003) TAK1 is critical for IkappaB kinase-mediated activation of the NF-kappaB pathway. *J Mol Biol* 326(1):105–115.
27. Kawadler H, Yang X (2006) Lys63-linked polyubiquitin chains: Linking more than just ubiquitin. *Cancer Biol Ther* 5(10):1273–1274.
28. Chen ZJ (2005) Ubiquitin signalling in the NF-kappaB pathway. *Nat Cell Biol* 7(8):758–765.
29. Gulino A, Di Marcotullio L, Screpanti I (2010) The multiple functions of Numb. *Exp Cell Res* 316(6):900–906.
30. Nie J, et al. (2002) LNX functions as a RING type E3 ubiquitin ligase that targets the cell fate determinant Numb for ubiquitin-dependent degradation. *EMBO J* 21(1-2):93–102.
31. Zhou L, Ma Q, Shi H, Huo K (2010) NUMBL interacts with TRAF6 and promotes the degradation of TRAF6. *Biochem Biophys Res Commun* 392(3):409–414.
32. Beres BJ, et al. (2011) Numb regulates Notch1, but not Notch3, during myogenesis. *Mech Dev* 128(5-6):247–257.
33. Di Marcotullio L, et al. (2006) Numb is a suppressor of Hedgehog signalling and targets Gli1 for Itch-dependent ubiquitination. *Nat Cell Biol* 8(12):1415–1423.
34. Di Marcotullio L, et al. (2011) Numb activates the E3 ligase Itch to control Gli1 function through a novel degradation signal. *Oncogene* 30(1):65–76.
35. Mumm JS, Kopan R (2000) Notch signaling: From the outside in. *Dev Biol* 228(2):151–165.
36. Zhao B, Grimes SN, Li S, Hu X, Ivashkiv LB (2012) TNF-induced osteoclastogenesis and inflammatory bone resorption are inhibited by transcription factor RBP-J. *J Exp Med* 209(2):319–334.
37. Li S, et al. (2014) RBP-J imposes a requirement for ITAM-mediated costimulation of osteoclastogenesis. *J Clin Invest* 124(11):5057–73.
38. Ma Q, Zhou L, Shi H, Huo K (2008) NUMBL interacts with TAB2 and inhibits TNFalpha and IL-1beta-induced NF-kappaB activation. *Cell Signal* 20(6):1044–1051.
39. McGill MA, Dho SE, Weinmaster G, McGlade CJ (2009) Numb regulates post-endocytic trafficking and degradation of Notch1. *J Biol Chem* 284(39):26427–26438.
40. McGill MA, McGlade CJ (2003) Mammalian numb proteins promote Notch1 receptor ubiquitination and degradation of the Notch1 intracellular domain. *J Biol Chem* 278(25):23196–23203.
41. Otero JE, Dai S, Alhawagri MA, Darwech I, Abu-Amer Y (2010) IKKbeta activation is sufficient for RANK-independent osteoclast differentiation and osteolysis. *J Bone Miner Res* 25(6):1282–1294.
42. Wang H, Ouyang Y, Somers WG, Chia W, Lu B (2007) Polo inhibits progenitor self-renewal and regulates Numb asymmetry by phosphorylating Pon. *Nature* 449(7158):96–100.
43. Ma J, et al. (2013) Disruption of the transcription factor RBP-J results in osteopenia attributable to attenuated osteoclast differentiation. *Mol Biol Rep* 40(3):2097–2105.
44. Fukushima H, et al. (2008) The association of Notch2 and NF-kappaB accelerates RANKL-induced osteoclastogenesis. *Mol Cell Biol* 28(20):6402–6412.
45. Castel D, et al. (2013) Dynamic binding of RBPJ is determined by Notch signaling status. *Genes Dev* 27(9):1059–1071.
46. Xie M, et al. (2006) A pivotal role for endogenous TGF-beta-activated kinase-1 in the LKB1/AMP-activated protein kinase energy-sensor pathway. *Proc Natl Acad Sci USA* 103(46):17378–17383.

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