TGFβ-induced degradation of TRAF3 in mesenchymal progenitor cells causes age-related osteoporosis

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Inflammaging induces osteoporosis by promoting bone destruction and inhibiting bone formation. TRAF3 limits bone destruction by inhibiting RANKL-induced NF-κB signaling in osteoclast precursors. However, the role of TRAF3 in mesenchymal progenitor cells (MPCs) is unknown. Mice with TRAF3 deleted in MPCs develop early onset osteoporosis due to reduced bone formation and enhanced bone destruction. In young mice TRAF3 prevents β-catenin degradation in MPCs and maintains osteoblast formation. However, TRAF3 protein levels decrease in murine and human bone samples during aging when TGFβ1 is released from resorbing bone. TGFβ1 induces degradation of TRAF3 in murine MPCs and inhibits osteoblast formation through GSK-3β-mediated degradation of β-catenin. Thus, TRAF3 positively regulates MPC differentiation into osteoblasts. TRAF3 deletion in MPCs activated NF-κB RelA and RelB to promote RANKL expression and enhance bone destruction. We conclude that pharmacologic stabilization of TRAF3 during aging could treat/prevent age-related osteoporosis by inhibiting bone destruction and promoting bone formation.
Osteoporosis is a common disease of aging, characterized by low bone mass, resulting from increased bone resorption and reduced bone formation that lead to fragile bones and increased risk of fractures1-3. It is associated with sex steroid deficiency and low-grade chronic inflammation of aging (inflammaging)4,5. These are accompanied by increased production of pro-inflammatory cytokines, including TNF, IL-1, and IL-6,6,7, which increase the expression of RANKL, a TNF superfamily member that is expressed by osteoblastic and immune cells8,9, in bone marrow (BM) and is required for osteoclastogenesis9,10,11. RANKL activates NF-κB and other signaling pathways in osteoclast precursors (OCPs) through its receptor, RANK, and the adaptor protein, TNF receptor-associated factor 6 (TRAF6), to induce osteoclastogenesis10,11.

TRAF3 is also a TNF receptor family adaptor protein, but unlike TRAF6, it limits RANKL- and TNF-induced osteoclastogenesis12,13. It forms a complex in unstimulated B cells with the ubiquitin ligases, cellular inhibitors of apoptosis (cIAP1 and cIAP2), and TRAF2 to constitutively degrade NF-κB-inducing kinase (NIK)13 and thus it prevents processing of NF-κB p100 to p52 and activation of non-canonical signaling13. RANKL induces TRAF3 autophagolysosomal degradation in OCPs to promote osteoclastogenesis14,15. RANKL can also mediate osteoclastogenesis in vitro in the absence of TRAF615 by degrading TRAF3, despite reports that TRAF6 expression is required for RANKL-induced osteoclast formation in vitro14,16,17. In addition, mice with TRAF3 conditionally deleted in myeloid progenitor cells (which include OCPs) develop early onset osteoporosis due to increased bone resorption, indicating that TRAF3 expression in these cells limits age-related bone loss14. Importantly, prevention of TRAF3 degradation by the autophagolysosome inhibitor, chloroquine, inhibits RANKL-induced osteoclast formation in vitro and prevents PTH-induced osteoclastogenesis and ovariectomy-induced osteoporosis in mice14, suggesting that inhibition of TRAF3 degradation in OCPs could prevent osteoporosis in humans. However, the role, if any, of TRAF3 in mesenchymal or osteoblast lineage cells has not been investigated.

Osteoblasts are derived from mesenchymal progenitor cells (MPCs), which express Prx118,19. Although Prx1 is expressed predominantly in appendicular bones in embryos18, it is also expressed in developing vertebrae, and mice with global knockout of Prx1 have defects in the skull, limbs, and vertebrae18,19. Prx1Cre mice have been used to conditionally delete genes in MPCs to investigate their roles in skeletogenesis as well as in bone modeling and remodeling20. Here, we crossed Prx1Cre with Traf3fl/fl mice to delete TRAF3 in MPCs to determine if TRAF3 has a function in MPCs. These TRAF3 conditional knockout (cko) mice develop early onset osteoporosis due to a combination of increased bone resorption and decreased bone formation. We found that TRAF3 protein levels are lower in the bone and BM of old than young WT mice and, importantly, in samples of bone from older adults than from children. These findings suggest that prevention of TRAF3 degradation in bone cells could be a novel therapeutic approach to prevent age-related osteoporosis.

Results
TRAF3 cKO mice develop early onset osteoporosis. To determine if TRAF3 has a role in osteoblastic bone formation, we crossed Traf3fl/fl mice14,21 with Prx1Cre mice to generate Traf3fl/flPrx1cre (cko) mice with TRAF3 conditionally deleted in MPCs. The cKO mice appeared normal at birth and later in life, and trabecular bone volume (BV/TV) assessed by micro-CT in tibial metaphyses of 3-month (m)-old cKO mice was similar to that in Traf3fl/fl (WT) littermates (Fig. 1a). However, BV/TV values in tibial metaphyses of 9- and 15-m-old cKO mice were significantly lower than in WT mice (Fig. 1a). Similarly, vertebral BV/TV values were normal in 3-m-old cKO mice, but were significantly reduced in 9- and 15-m-old cKO mice compared with their respective WT littermates (Fig. 1b). As is typical during aging, BV/TV values in the tibiae and vertebrae of 15-m-old WT mice were lower than those of younger mice (Fig. 1a, b). We found no differences in the degree of bone loss between male and female cKO mice.

TRAF3 protein levels were lower in samples of femora and vertebrae from 15-m-old than from 3-m-old WT mice (Fig. 1c). They were barely detectable in femoral samples of the 3- and 15-m-old cKO mice, but were detectable at lower levels in vertebral samples from 3-m-old cKO than from WT mice (Fig. 1c), presumably reflecting the presence in these samples of large numbers of hematopoietic cells, which had largely been flushed from the femora. In contrast, TRAF3 levels were almost undetectable in the vertebral samples from the 15-m-old cKO mice (Fig. 1c). This reflects the reduction in TRAF3 levels in whole BM samples that we observed in WT mice during aging (Supplementary Fig. 1a). Although Prx1 also regulates chondroblast development and is expressed in craniofacial bones,20,22,23 we found that long bone growth plates and articular cartilage were histologically normal in young and adult cKO mice (Fig. 1d), which also had normal body length and craniofacial development and no features of osteoarthritis. Thus, TRAF3 does not play a significant role in MPCs in the regulation of skeletal modeling or bone mass during embryonic and postnatal development up to at least 3–4 months after birth.

Age-related reduction of bone formation in TRAF3 cKO mice. Very few trabeculae were present in the long bone metaphyses of 9- and 15-m-old cKO mice, reflecting the marked acceleration of age-related bone loss. Thus, we evaluated bone formation in vertebral sections of these mice because they had adequate numbers of trabeculae for analysis. Bone formation rates were normal in 3-m-old cKO mice, but they were significantly lower in 9-m-old cKO mice than in WT littermates (Fig. 1e). Similarly, mean trabecular osteoblast surface values were normal in 3-m-old cKO mice, but were significantly lower in 9-m-old cKO mice than in WT littermates (Fig. 1f). Osteoblast surface and bone formation values in 15-m-old cKO mice were similar to those in WT littermates because these values had decreased in the WT mice by this age (Fig. 1e, f), reflecting the reduction that typically occurs during aging. Consistent with these findings, serum levels of the bone formation marker, osteocalcin, were similar in 3-m-old WT and cKO mice and in 9-m-old WT mice, but they were significantly lower in 9-m-old cKO mice (Fig. 1g). However, values in 15-m-old WT mice were similar to those in 9- and 15-m-old cKO mice because values in the WT mice had decreased by this age, which is typical of aging (Fig. 1g).

Age-related increase in bone resorption in TRAF3 cKO mice. We assessed bone resorption parameters in vertebral sections of cKO mice and found that osteoclast numbers and surfaces were normal in 3-m-old mice, but were increased in 9- and 15-m-old cKO mice compared to WT littermates (Fig. 1h). Consistent with this, serum levels of the bone resorption marker, TRACP5b, were normal in 3-m-old cKO mice and were significantly increased in 9- and 15-m-old cKO mice (Fig. 1i). Thus, the early onset osteoporosis in the TRAF3 cKO mice is a result of decreased bone formation and increased bone resorption, which is similar to age-related osteoporosis in WT mice25 and humans.26 Although osteoclast number and activity generally are reduced with age in mice due to aging-associated low bone turnover, we found no...
significant differences in osteoclast numbers and surfaces in the vertebral sections or in serum TRACP5b levels among 3-, 9-, and 15-month-old WT mice (Fig. 1h, i). This may reflect the fact that 15-month-old WT mice are still not aged and are equivalent to ~50-year-old humans who still tend to have normal bone turnover marker levels.

**Age-related reduction in OB differentiation from cKO MPCs.**

To investigate the molecular mechanisms for the reduced bone formation in the cKO mice, we cultured BM cells to expand stromal cells, followed by treatment with osteoblast-inducing medium. Alkaline phosphatase (ALP)-positive colony formation from BM cells from 3-m-old cKO mice was similar to that
of cells from WT mice (Fig. 2a, b). In contrast, ALP⁺ colony formation from BM cells from 9-m-old cKO mice was significantly reduced (Fig. 2a, b). However, the total area of ALP⁺ and ALP⁻ colony formation from BM cells from 9-m-old cKO and WT BM cells was similar (Fig. 2b). Although ALP⁺ osteoblast differentiation from bone-derived MPCs (BdMPCs) from 3-m-old cKO mice was similar to that from WT littermate mice, it was significantly lower in cells from 9-m-old cKO mice than from WT littermates (Fig. 2c). In contrast, BdMPCs from 3- and 9-m-old cKO mice had faster growth rates than those of their respective littermate cells (Fig. 2d), which was supported by the findings that cKO BdMPCs had a higher S-phase cell population than control mice (Fig. 2e). The total area of cells from BM and BdMPCs was similar between cKO and WT in Fig. 2a, c because the cKO cells had reached confluence at this stage. These findings suggest that the reduced bone formation during aging in the mice with TRAF3 deleted in MPCs is mainly the result of reduced osteoblast differentiation and not reduced MPC proliferation and could be due to exposure to increasing concentrations of a factor(s), such as TGFβ, during aging.

TGFβ limits OB differentiation of MPCs from 9-m-old cKO mice. TGFβ1, one of the most abundant non-collagenous proteins in bone matrix, is released during bone resorption and activated by the acid environment under osteoclasts in resorption lacunae. Although TGFβ1 couples bone formation to resorption by recruiting MPCs, it actually inhibits osteoblast differentiation from these cells, and thus could mediate the inhibitory effects we observed in the cKO cells. We found that the concentrations of total (Fig. 2f) and the active form (Fig. 2g) of TGFβ1 in serum were significantly increased in 9-m-old cKO mice, but not in 3-m-old cKO mice, compared to their respective WT littermates. However, BdMPCs from 3- and 9-m-old cKO mice expressed levels of TGFβ1 similar to those in cells from their respective littermates (Supplementary Fig. 2), suggesting that these cells were not the source of the increased serum TGFβ1 levels. To investigate if the increased active TGFβ1 in the older cKO mice is responsible for the reduction in osteoblast differentiation, we treated BdMPCs from 3- and 9-m-old WT and cKO mice with TGFβ1 for 7 days followed by induction of osteoblast differentiation without TGFβ1. The area of ALP⁺ osteoblasts formed from BdMPCs from 3-m-old cKO mice was similar to that from cells from WT littermates (Fig. 2h), expressed as numbers (Oc.N) and surfaces (Oc.S) from the mice in (a). Mean ± SD (3-m group: n = 4, other groups: n = 6 biologically independent samples; **p < 0.01). Scale bar, 100 µm. 

Serum osteocalcin levels tested by ELISA from the 3-, 9-, and 15-m-old WT and cKO mice in (a). Mean ± SD (n = 8 biologically independent samples; **p < 0.01). h Representative images of TGFβ1-stained sections from 9-m-old WT and cKO mice, and histomorphometric analysis of osteoblast surfaces (OB.S/BS) from the mice in (a). Mean ± SD (3-m group: n = 4, other groups: n = 6 biologically independent samples; **p < 0.01). Scale bar, 100 µm. 

Representative H&E-stained images of vertebral sections from 9-m-old WT and cKO mice, and histomorphometric analysis of osteoclast numbers (Oc.N) and surfaces (Oc.S) from the mice in (a). Mean ± SD (3-m group: n = 5, other groups: n = 6 biologically independent samples; **p < 0.05). Scale bar, 100 µm. i Serum TRACP-5b levels tested by ELISA from the mice in (a). Mean ± SD (n = 8 biologically independent samples; **p < 0.05, **p < 0.01). All analyses done using one-way ANOVA with Tukey’s post-hoc test.

TGFβ1 degrades TRAF3 in bone during aging. TGFβ1 levels were significantly lower in samples of tibial metaphyses, femoral BM, and femoral cortical bone from 18- than from 3-m-old WT mice (Fig. 3a and Supplementary Fig. 1a & b). Importantly, they were significantly lower in samples of vertebral spinous processes removed surgically from older otherwise healthy adults than in those from children (Fig. 3b and Supplementary Fig. 1c), with no differences detected between males and females. In addition, using immunofluorescence we found that TGFβ1 was expressed strongly in osteoclast-expressing osteoblasts on trabecular and endosteal bone surfaces in decalcified vertebral samples from young adult mice (Fig. 3c). The numbers of these double-positive cells on bone surfaces were significantly lower in samples from 18-m-old WT mice (Fig. 3c). In addition, there were significantly fewer TRAF3⁺ cells in the BM of the old mice (Fig. 3c). These findings are consistent with a recent report that TGFβ3 levels are lower in circulating blood monocytes from older (>65-years-old) than from younger humans (20–30-years-old).

Since RANKL induces TFR3 degradation in myeloid progenitors to promote osteoclastogenesis, we next examined if factors that regulate osteoblast differentiation might degrade TRAF3 in MPCs. We found that TGFβ1, but not TNF, PTH, or BMP2, reduced TRAF3 protein levels in WT mouse MPCs (Fig. 3d). The levels of total TGFβ1 protein in bone samples (Fig. 3e) and of active TGFβ1 protein in BM, but not in serum (Fig. 3f), from 19-m-old WT mice were significantly higher than those in young (2.5-m-old) mice. Consistent with this, levels of the active form of TGFβ1 in vertebral samples from older adults (55–87-years-old) were higher than those in samples from children, although there was no difference in total TGFβ1 levels (Fig. 3g).

TGFβ1 induces TRAF3 ubiquitination and lysosomal degradation. TGFβ1 increased TRAF3 ubiquitination in MPCs (Fig. 3h). TFR3 is degraded in B cells by cIAP1 and cIAP2. We found that both cIAP1/2 and TRAF3 bound to the TGFβ receptor I (TGFβRI; Fig. 3i), and that cIAP1/2 bound to TRAF3 in MPCs (Fig. 3i). We also confirmed that TGFβRI was present in these lysates using immunoprecipitation and WB (Fig. 3i, bottom). TGFβ1 increased binding of TRAF3 and cIAP2 to TGFβRI and of cIAP2 to TRAF3 (Fig. 3i). Treatment of these cells with the IAP inhibitor, AT406, which degrades cIAP1 and cIAP2, reduced these interactions and almost completely abrogated binding of TRAF3 to TGFβRI (Fig. 3i), suggesting that TRAF3 binding to TGFβRI requires cIAP1/2. Previous studies have reported that phosphorylation of TGFβRI is associated with its ubiquitination. Our findings suggest that cIAP1/2 and TRAF3...
form a complex on the TGFβRI that results in TRAF3 ubiquitination and degradation. Interestingly, AT406 almost completely prevented TGFβ1 induction of cIAP binding to TRAF3 (Fig. 3i) since it markedly reduced cIAP protein levels in MPCs (Fig. 3j). However, AT406 did not completely prevent TGFβ1-induced inhibition of osteoblast differentiation (Fig. 3k), suggesting that an IAP-independent pathway may also be involved in TGFβ1-induced inhibition of osteoblast differentiation.

Treatment of BdMPCs from 3-m-old WT mice with TGFβ1 plus the lysosome inhibitor, chloroquine, significantly increased ubiquitinated TRAF3 (Fig. 3h), suggesting that chloroquine prevented TRAF3 degradation. The proteasome inhibitor, MG132, also increased the amount of ubiquitinated TRAF3, but to a much lesser extent (Fig. 3h), suggesting that TRAF3 is degraded predominantly by lysosomes in MPCs, similar to its degradation in OCPs14. Consistent with this, TGFβ1 increased the area of

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**Legend:**
- **a:** ALP and ALP & eosin images of WT and cKO MPCs at 3 months-old and 9 months-old.
- **b:** ALP+ Ar./total cell Ar. (%)
  - WT vs cKO: 3 months-old and 9 months-old.
- **c:** ALP+ Ar. (mm²/well) for WT and cKO MPCs at 3 months-old and 9 months-old.
- **d:** No. BdMPC/well (x10^5) for WT and cKO MPCs over 8 days.
- **e:** BrdU and 7AAD staining for WT and cKO MPCs at 3 months-old and 9 months-old.
- **f:** Serum total TGFβ1 (ng/ml) for WT and cKO MPCs at 3 months-old and 9 months-old.
- **g:** Serum active TGFβ1 (pg/ml) for WT and cKO MPCs at 3 months-old and 9 months-old.
- **h:** ALP+ OB Ar (mm²) for WT and cKO MPCs at 3 months-old and 9 months-old.
- **i:** ALP+/total cell Ar (%) for WT and cKO MPCs at 3 months-old and 9 months-old.
- **j:** ALP+ OB Ar (mm²) for WT and cKO MPCs at 3 months-old and 9 months-old.
- **k:** ALP+ OB Ar (mm²) for WT and cKO MPCs at 9 months-old.
- **l:** ALP+ OB Ar (mm²) for WT and cKO MPCs at 9 months-old.
- **m:** ALP+ OB Ar (mm²) for WT and cKO MPCs at 9 months-old.
co-localization of TRAF3 with the lysosome marker, LAMP2, in the cytoplasm of MPCs (Fig. 3l). Importantly, chloroquine inhibited TGFβ1-induced TRAF3 degradation (Fig. 3m) and dose-dependently prevented the inhibition of osteoblast differentiation induced by TGFβ1 (Fig. 3n) in WT cells. Chloroquine also reduced the inhibition of osteoblast differentiation induced by TGFβ1 in cKO cells, but only at the highest concentration tested (3000 nM), which supports lysosomal degradation of TRAF3 being a major, but not the only mechanism whereby TGFβ inhibits OB formation.

**TRAF3 maintains β-catenin activity in MPCs.** β-Catenin signaling regulates many crucial biological processes, including MPC fate and osteoblast differentiation35, while inactivation of β-catenin prevents MPC differentiation into osteoblasts36. Total β-catenin protein levels were lower in bone samples from young and adult TRAF3 cKO mice than from their respective WT controls (Fig. 4a). Consistent with this, treatment of WT MPCs with TGFβ1 for 2–4 days markedly reduced total β-catenin protein levels, associated with reduced TRAF3 protein levels (Fig. 4b). We also assessed the expression of β-catenin in nuclei of WT and cKO MPCs derived from neonatal calvarial cells infected with a control pMX-GFP or pMX-TRAF3 retrovirus using immunofluorescence (Fig. 4c). We found that the basal level of β-catenin, assessed as the area of nuclear staining, was lower in the cells from cKO than from WT mice (Fig. 4c), suggesting that activation of β-catenin is TRAF3-dependent. TGFβ1 reduced the area of β-catenin staining in nuclei in MPCs from both control virus-infected WT and cKO cells (Fig. 4c), suggesting that TGFβ1 also regulates β-catenin activation through a TRAF3-independent mechanism. Indeed, over-expression of TRAF3 blocked the reduction of nuclear β-catenin staining induced by TGFβ1 in WT and cKO cells (Fig. 4c), further confirming that activation of β-catenin is through TRAF3. We confirmed that TRAF3 retrovirus infection of calvarial cells caused over-expression of TRAF3 (Fig. 4d). However, over-expression of TRAF3 in unstimulated cKO cells did not restore the degree of β-catenin translocation to that observed in unstimulated WT cells, probably because GSK3β is constitutively activated in unstimulated cells to degrade β-catenin, and over-expression of TRAF3 does not stimulate OB differentiation via β-catenin.

**TRAF3 limits GSK3β activity to prevent β-catenin degradation.** β-Catenin is phosphorylated and degraded by GSK3β39. In general, GSK3β activity is increased by Tyr216 phosphorylation and reduced by Ser9 phosphorylation40. GSK3β Tyr216 phosphorylation was increased and Ser9 phosphorylation was reduced in TRAF3 cKO MPCs (Fig. 4e), with no difference in total GSK3β levels. We generated GSK3β plasmids in which Tyr216 and Ser9 were mutated such that they could not be phosphorylated (Y216m and S9m, respectively), and infected MPCs with them or WT GSK3β-expression vectors. Over-expression of these GSK3β constructs was confirmed by increased HA levels in the MPCs (Fig. 4f). Over-expression of mutated Tyr216-GSK3β (Y216m; as a dominant negative GSK3β) markedly reduced Tyr216 phosphorylation, associated with increased β-catenin in vehicle-treated cells. It also inhibited TGFβ1-induced degradation of β-catenin (Fig. 4f). In contrast, over-expression of mutated Ser9-GSK3β (S9m) had little effect on total β-catenin levels in either vehicle- or TGFβ1-treated cells compared to those in WT-GSK3β–infected cells (Fig. 4f). These findings suggest that TGFβ1-induced Tyr216 phosphorylation activates GSK3β to cause β-catenin degradation.

Consistent with the above findings, over-expression of TRAF3 in vehicle-treated cells decreased Tyr216 and increased Ser9 phosphorylation of GSK3β, associated with decreased phospho-β-catenin and increased total β-catenin (Fig. 4g). Over-expression of TRAF3 also reduced TGFβ1 induction of phospho-Tyr216 and phospho-β-catenin, and thus partly reversed TGFβ1-induced reduction of total β-catenin levels (Fig. 4g). Importantly, GSK3β directly associated with TGFβR1 (Fig. 4h), and over-expression of TRAF3 reduced TGFβ1-induced binding of GSK3β to TGFβR1. In particular, it reduced binding of Tyr216-phosphorylated GSK3β to the TGFβR1 (Fig. 4h, lower panel). In addition, the GSK3β inhibitor, SB-21676341 prevented TGFβ1-induced inhibition of OB differentiation from20 WT BdMPCs (Fig. 4i, j), and reduced the increase in protein levels of p-GSK3β (Tyr216) (Fig. 4k, l). The inhibitor also prevented TGFβ1-induced inhibition of OB differentiation from cKO cells (Fig. 4i, j), but this required higher concentrations of the inhibitor, reflecting the higher level of p-GSK3β (Tyr216) in these cells (Fig. 4k, l). In addition, over-expression of TRAF3 in WT MPCs increased the area of ALP+ cells (Fig. 4m, n).

TGFβ1 receptor signaling also regulates osteoblast differentiation by phosphorylating regulatory Smads, including Smad1, 2, 3, 5, and 9, which can form complexes with Smad4 to regulate target gene expression42. ReLB, a non-canonical NF-kB signaling protein regulated by TRAF3, represses TGFβ target gene expression by binding to the Smad2, 3, and 4 promoters in HEK 293T cells, and repression of these genes can be rescued by inhibition of Smad443. Therefore, we examined canonical TGFβ signaling in TRAF3 cKO MPCs and found 1-fold enhanced basal and TGFβ1-induced phosphorylation of Smad2 and 3 (Supplementary Fig. 3a & b), with normal levels of total Smad2 and 3.
led us to postulate that TRAF3 might negatively regulate expression of RANKL in osteoblastic cells. We co-cultured calvarial pre-osteoblasts with spleen cells from newborn mice in a standard co-culture assay to assess their osteoblastic osteoclastogenic potential. We found that cKO pre-osteoblasts induced significantly more osteoclasts from either cKO or WT spleen cells than WT calvarial cells, which was blocked by addition of RANK:Fc, indicating that TRAF3-deficient MPCs enhance osteoclast differentiation through RANKL. Indeed, levels of RANKL protein and mRNA in tibial metaphyseal bone were significantly higher in 3- and 9-m-old cKO than WT MPCs. However, levels of osteoprotegerin (OPG), a RANKL decoy receptor that inhibits RANKL-induced osteoclast formation, were also elevated in samples from 3-m-old cKO mice, resulting in a relatively normal RANKL/OPG ratio in these mice. Importantly, this ratio was increased in samples from 9-m-old cKO mice.

Activated NF-κB RelA and RelB promote RANKL expression by cKO MPCs. TRAF3, TRAF2, and cIAPs degrade NIK and negatively regulate lymphotoxin β receptor-mediated canonical and non-canonical NF-κB activation. TGFβ1 markedly
increased nuclear translocation of RelA and RelB in TRAF3 cKO MPCs (Fig. 5f), suggesting that TRAF3 might regulate RANKL expression in MPCs through them. We detected low levels of RelA and RelB in nuclear fractions of untreated WT MPCs (Fig. 5g). This is consistent with a low level of NF-κB signaling being required to maintain cell survival even in static conditions and massive TNF-induced necrosis of liver cells causing death of RelA−/− mice in utero.48 For this reason cells are often starved to clear NF-κB protein from nuclei to facilitate detection of small changes in their translocation49. TGFβ1 markedly increased levels of nuclear RelA and RelB in the MPCs (Fig. 5f). We identified three κB binding sites in the distal region of the murine RANKL promoter (Fig. 5g). ChIP assays showed RelA (Fig. 5h) and RelB (Fig. 5i) binding to these κB sites, which was markedly higher in cKO MPCs (Fig. 5j). Consistent with this, over-expression of either RelA (Fig. 6a) or RelB (Fig. 6b) in WT mouse calvaria-derived MPCs increased RANKL mRNA expression (Fig. 6c). However, the level of soluble RANKL in the culture medium was not increased (Fig. 6d). Notably, over-expression of either RelA or RelB increased membrane-bound RANKL protein levels (Fig. 6e), consistent with membrane-bound RANKL in osteoblasts regulating osteoclast differentiation.50

Perilacunar/canalicular remodeling is normal in TRAF3 cKO mice. TGFβ1 signaling in osteocytes regulates perilacunar/canalicular remodeling,51 which is reduced in osteocyte-specific TGFβRII conditional knockout mice,51 associated with reduced osteoclast numbers and expression of the resorption-related genes, Acp5, cathepsin K, Mmp2, 13 and 14, and slightly increased bone mass. Thus, the increase in levels of total TGFβ1 in old WT mouse tibiae or in active TGFβ1 in BM in old mice and in adult human bone samples might increase perilacunar remodeling. We did not observe changes in mRNA levels of Acp5, Mmp2, Mmp13, Mmp14, or Ctsk in 15-m-old cKO mice (Supplementary Fig. 4a), in protein expression levels of MMP13 or CTSK in sections of bones of 3–9-, or 15-m-old cKO mice, assessed using immunohistochemistry (Supplementary Fig. 4b–d), or in osteocyte lacunar area (Supplementary Fig. 4e), consistent with increased TGFβ1 in bone matrix of old mice being inactive and TGFβ1 activated in resorption lacunae by acid produced by osteoclasts likely not acting on osteocytes in the bone matrix.

Discussion

This is the first report of an important regulatory role for TRAF3 in MPCs in which it maintains their differentiation into osteoblasts and restrains their expression of RANKL to limit osteoclastogenesis during aging. In young mice, TRAF3 limits TGFβ1-induced GSK3β activation (through Tyr216 phosphorylation) and degradation of β-catenin in MPCs, which allows β-catenin to maintain osteoblast differentiation and induce OPG expression,52 which limits bone destruction. In contrast, as illustrated in Fig. 7, during aging, increased cytokine production in response to low-grade chronic inflammation56 increases RANKL expression by osteoblastic7 and immune cells.8 This leads to RANKL-induced TRAF3 degradation in OCPs14 and thus increased bone resorption through NF-κB signaling12,14. As a result, TGFβ is released from bone matrix and induces TRAF3 ubiquitination through cIAPs and subsequent lysosomal degradation in MPCs. Consequently, (1) GSK3β is activated to degrade β-catenin, resulting in inhibition of osteoblast differentiation and reduced expression of OPG;52 and (2) RelA and RelB induce RANKL expression to further enhance osteoclastogenesis and a self-amplifying cycle of bone destruction, TGFβ release, TRAF3 degradation, and NF-κB activation.

Importantly, the above model (and loss of this protective effect of TRAF3 in the cKO mice, with associated osteoporosis) is supported by significantly lower TRAF3 protein levels in bone from older adults than from children and in bone and BM from old than from young mice, associated with increased levels of active TGFβ1 in the BM of older mice and in the bone of older humans. A caveat with the human bone samples we used is that degenerative spinal conditions in older patients could have led to lower TRAF3 levels, particularly because degenerative arthritis is more common in aged humans.53 However, unlike vertebral bodies, the spinal processes we collected are not subject to articular wear and tear, a common cause of osteoarthritis.54 Thus, low TRAF3 levels in older subjects most likely reflect the effects of age-related osteoporosis and not osteoarthritis. These findings support our conclusion that the reduction of TRAF3 in hematopoietic cells, including OCPs, and in MPCs during aging results in enhanced bone resorption and reduced bone formation, respectively (Fig. 7).

The normal bone mass in 3–4-m-old cKO mice is intriguing, given that 9-m-old mice are osteoporotic. We attribute this to two functional changes in MPCs as a consequence of TRAF3 deficiency. First, although RANKL expression by MPCs is increased in 3-m-old cKO mice, OPG is also increased to the same extent because TRAF3 is unavailable to limit β-catenin-induced OPG expression52 and this prevents bone loss. In contrast, RANKL levels are higher than those of OPG in older cKO mice (Fig. 5d), and this drives increased bone resorption. To our knowledge, this
is the first report of RANKL expression being regulated directly by RelA and RelB in any cell type. It identifies another mechanism whereby NF-kB negatively regulates bone mass in addition to inducing osteoclast formation \(^{10,11}\) and inhibiting osteoblast formation by down-regulating Fra-1 \(^{39}\) and Runx2 \(^{29}\) expression in osteoblast precursors. Activation of the RANKL promoter is complicated because RANKL transcription can be activated by binding of 1,25(OH)\(_2\)VitD\(_3\) \(^{36}\) and PTH-induced CREB \(^{57}\) to both proximal and distal elements of its promoter in osteoblastic cells and by c-Fos at the distal D5 enhancer in activated T cells \(^{8}\). These findings overall suggest that the distal elements induce RANKL activation \(^{36}\), NF-kB also interacts with the transcriptional start site and the D5 enhancer near the Vitamin D response elements \(^{58}\). However, we did not find any kB binding sites within 3 kb from the murine RANKL promoter coding start site, but found three between 3 and 5.5 kb. Importantly, RelA and RelB bound functionally to each of these sites.

Second, cKO MPCs have a complex phenotype: increased proliferation in both young and older cKO mice, but their differentiation into osteoblasts is normal in young and impaired in
Fig. 4 Reduction in TRAF3 activates GSK-3β to impair β-catenin signaling. a WB of TRAF3 and β-catenin in long bones. b WB of TRAF3, β-catenin, and GAPDH in WT BdMPCs induced for OB differentiation with vehicle or TGFβ1. c IF and quantification of nuclear β-catenin and β-actin (cytoskeleton) in WT and cKO calvarial pre-OBs treated with vehicle or TGFβ1 for 48 h following pMX-GFP control or pMX-TRAF3 retrovirus infection. Mean ± SD (n = 4 biologically independent samples; *p < 0.05, **p < 0.01). Scale bar, 20 μm. d WB of TRAF3 and GAPDH in cells as in (c). e WB of TRAF3, phospho-GSK-3β (Tyr216/Ser9), and total GSK-3β in cortical bones from 12-m-old WT and TRAF3 cKO mice. f WB of β-catenin, phospho-GSK-3β (Ser9/Tyr216), total GSK-3β, HA, and GAPDH in WT calvarial pre-OBs treated with vehicle or TGFβ1 for 48 h following lentivirus infection with GFP, or HA-tagged WT, Ser9-mutated (59m) or Tyr216-mutated (Y216m) GSK-3β. g Protein levels of TRAF3, phospho-β-catenin, β-catenin, phospho-GSK-3β (Ser9/Tyr216), and total GSK-3β tested in WT calvarial pre-OBs treated with vehicle or TGFβ1 for 8 h following pMX-GFP or -TRAF3 retrovirus infection. h Cell lysates from (g) immunoprecipitated using anti-TGFβRII Ab followed by TGFβ3 and phospho-GSK-3β (Tyr216). i BDMPCs from 4-m-old WT and cKO mice treated with TGFβ1–/– GSK-3β inhibitor, SB-216763, for 7 days and stained for ALP activity. j Quantification of ALP+ cell areas. Mean ± SD (n = 3 biologically independent samples; *p < 0.05, **p < 0.01, ***p < 0.001 vs. vehicle; #p < 0.001 vs. TGFβ1 alone; one-way ANOVA with Tukey’s post-hoc test). k, l WB of phospho-GSK-3β (Tyr216) in WT and cKO BDMPCs treated with TGFβ1 plus SB-216763, and densitometry analysis was performed. (l). Mean ± SD (n = 3 biologically independent samples; *p < 0.05, **p < 0.01, ***p < 0.001 vs. vehicle; #p < 0.05, ##p < 0.01, ###p < 0.001 vs. TGFβ1 alone.). m PMX-GFP or -TRAF3 retrovirus-infected cells induced for OB differentiation for 5 days in 48-well plates and stained for ALP activity. n Quantification of ALP+ cell areas. Mean ± SD (n = 3 biologically independent samples; *p < 0.05; unpaired Student’s t test). All other analyses done using one-way ANOVA with Tukey’s post-hoc test. All experiments were repeated twice with similar results. TGFβ1 (1 ng/ml)

In older mice, associated with increased active TGFβ1 in serum in 9- m-old cKO mice. In addition, total Gβ1 levels are increased in the metaphyseal bone of 19-m-old WT mice, which also have increased active TGFβ1 levels in their BM. TGFβ1 promotes MPC proliferation, but it also inhibits their differentiation. Thus, we propose that the increased MPC proliferation in young cKO mice maintains a sufficient pool of cells to differentiate into osteoblasts and maintain bone mass, but increased release of TGFβ1 as the mice age limits MPC differentiation. This is the first report linking TRAF3 to TGFβ signaling in any cell type and points to possible important negative regulatory roles in TGFβ signaling in other cell types. TGFβRI indirectly associated with TRAF3 (Fig. 3j) by recruiting cIAP1 and 2, which resulted in TGFβ3 degradation followed by inactivation of β-catenin. However, our findings also show that pre-treatment with TGFβ1 prevents osteoblast differentiation from both WT and TRAF3 cKO MPCs, suggesting that TGFβ1 also inhibits bone formation by a TRAF3-dependent mechanism.

We found that basal and TGFβ1-stimulated levels of p-Smad 2 and 3 were mildly increased in MPCs from the cKO mice, suggesting that TRAF3 is also involved in canonical TGFβ1 signaling in these cells. Interestingly, loss of expression of Smad4 in MPCs causes stunted growth and spontaneous fractures in mice, associated with increased MPC proliferation, which is also increased in TRAF3 cKO MPCs. However, this Smad4 cKO phenotype is much more severe than that in our cKO mice and, although other Smad4 cKO mice also have osteoporosis, these mice later develop increased bone mass11,61, while targeted deletion of Smad3 in mice also results in osteopenia due to reduced bone formation, associated with increased osteocyte number.

Previous studies have linked TRAF6 to TGFβ-induced activation of p38/MAPK signaling, for example, in prostate cancer cell migration. Following treatment with TGFβ1, TRAF6 induced Lys63-linked polyubiquitination of the p85a subunit of PI3K and Lys63-linked polyubiquitination of TGFβ-activated kinase-1, independent of TGFβRI and II kinase activity. In addition, TGFβ1 enhances and is indispensable for RANKL-induced osteoclastogenesis in vitro through binding of Smad3 to a TRAF6-TAB1-TAK1 complex in OCPs, also independent of TGFβRI kinase activity.

TRAF3 has important regulatory roles in immune and other cell types that differ from those of other TRAFs, which generally promote NF-κB activation. For example, TRAF3 represses NF-κB non-canonical activation in OCPs and T and B cells. It also inhibits B cell survival, while some human B cell lymphomas and 4–12% of multiple myeloma cases have inactivating TRAF3 mutations, indicating a tumor suppressor role for TRAF3 in B cells. In addition, mice with TRAF3 deleted in myeloid cells develop early onset osteoporosis, inflammatory diseases, infections, and tumors, indicating that TRAF3 is an inflammation and tumor suppressor in myeloid cells. Thus, therapeutic approaches to maintain TRAF3 levels in immune cells during aging could prevent or reduce the incidence of several common diseases. Of note, chloroquine prevents ovariectomy-induced osteoporosis in mice and TRAF3 degradation was also prevented by an IAP inhibitor (Fig. 3k). We are currently investigating if prevention of TRAF3 degradation by long-term treatment of aging mice with CQ or IAP inhibitors can prevent age-related osteoporosis.
**Micro-CT and bone histomorphometric analysis.** Following our standard protocol for in vivo assessment of bone formation\(^{12,28}\), mice were given injections of calcine (10 mg/kg) 5 and 1 days before sacrifice. Right tibiae and T12–L2 vertebrae were fixed in 10% neutral buffered formalin for 2 days. Micro-CT scanning was performed on these specimens using a vivaCT 40 instrument (Scanco Medical) with a resolution of 10.5 µm in a 1 mm section of trabecular bone beneath the growth plates of tibia and in the entire trabecular bone in the first lumbar vertebrae.

These bone samples were then processed sequentially through 90% ethanol for 12 h, and then heated at 60 °C overnight to cure the plastic. 4 µm thick unstained sections were cut using a Shandon Finesse ME microtome and examined under fluorescence microscopy for calcine double labeling analysis. Left tibiae and L3–L5 vertebrae were fixed in 10% neutral buffered formalin for 2 days and processed in 70% ethanol for 2 days, decalcified in 10% EDTA for 14 days and embedded in paraffin. Dynamic parameters of bone formation on unstained plastic sections and static parameters of osteoblasts and osteoclasts on 4 µm thick H&E and TRAP-stained paraffin sections were blindly assessed by an investigator who was not involved in the sample collection and group assignment using an OsteoMeasure Image Analysis System (Osteometrics, Decatur, GA)^{12,28}.

**In vitro osteoblast differentiation assay.** To assess osteoblast differentiation capacity, 1 × 10^6 BM cells from WT and cKO mice were seeded in 12-well-plates with α-MEM containing 15% FBS for 5 days. This was followed by treatment with osteoblast differentiation medium (50 µg/ml ascorbic acid and 10 mM β-glycerophosphate\(^2^{28}\) in α-MEM) for 7 days, with a medium change on day 4. After 7 days, the cells were fixed in 10% neutral buffered formalin followed by ALP

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**Image 94x643 to 274x721**

**Fig. 5** RANKL expression is increased in TRAF3 cKO osteoblastic cells. a Calvarial pre-OBs and spleen cells isolated from 7-day-old WT or cKO pups, co-cultured with 10^{-8} M 1,25(OH)\(_2\)D\(_3\)/Vitamin D\(_3\)–/+ RANKL:Fc (1 µg/ml) for 7 days and TRAP-stained. b Osteoclast number per well (Oc.N/well) in (a) were counted. Mean ± SD (n = 4 biologically independent samples; *p < 0.05, **p < 0.01). c WB of TRAF3, OPG, RANKL, and GAPDH in protein lysates from tibial metaphyseal bone from 3- and 9-m-old WT and cKO mice. d Densitometry analysis of RANKL/OPG protein ratio in (c). Mean ± SD (n = 3 biologically independent samples; *p < 0.05). e RANKL mRNA expression in BdMPCs from 3- and 9-m-old WT and cKO mice. Mean ± SD (n = 3 biologically independent samples; *p < 0.05, **p < 0.01). f BdMPCs from 3-m-old WT and cKO mice treated with TGFβ1 (1 ng/ml) for 0, 0.5, and 8 h. WB of RelA, RelB, HDAC2, and GAPDH in nuclei and cytoplasm. g Scheme for mouse RANKL promoter analysis showing putative κB binding sites and primer design to test binding sites. h, i Sheared chromatin from WT and cKO BdMPCs was used to perform DNA IP using h RelA, I RelB Abs or IgG control. Real-time PCR performed using designed primers that contain the putative κB binding sites 1, 2, 3, or an unrelated site, normalized to the input. Mean ± SD; n = 3 biologically independent samples; *p < 0.05. j Sheared chromatin from WT (W) and cKO (K) BdMPCs used to perform DNA IP using RelA, RelB, or IgG control Abs. PCR performed using designed primers that contain the putative κB binding sites 1, 2, 3, and an un-related site. All analyses done using one-way ANOVA with Tukey’s post-hoc test. All the in vitro experiments repeated twice with similar results.
enhancing bone resorption. In addition, TRAF3 ubiquitination and subsequent lysosomal degradation in MPCs. As a result, both RelA and RelB are activated to promote RANKL production, further allowing β-catenin accumulation and nuclear translocation to maintain osteoblast differentiation and secretion of OPG52, which limits osteoclast formation.

During aging, TGF-β catenin to inhibit osteoblast differentiation and OPG secretion to promote osteoclast formation along with increased RANKL expression and bone loss. In young WT mice, TGF-β levels in bone, BM, and serum are not increased and TRAF3 is present to limit these processes.

To assess osteoblast precursor proliferation, BdMPCs were sequentially cultured to two 12-well-plates. On day 6, the cells were digested and seeded into four 6-well plates. Proliferation was assessed by counting cell numbers on days 1, 2, 4, 6, and 8. To assess cell cycle status, 5 × 10^5 BdMPCs from 3-m-old WT and cKO mice were counted after digestion with 0.2% of trypsin. The digested cells were passaged again and split 1:2. This procedure was repeated once to produce highly pure (>95%) BdMPCs.

To assess osteoblast precursor proliferation, BdMPCs were sequentially cultured from small to larger wells. Initially, 5 × 10^3 BdMPCs from 3- and 9-m-old WT and cKO mice were cultured in each well of a 24-well-plate for 4 days, and cell numbers were counted after digestion with 0.2% of trypsin. The digested cells were passaged again and split 1:2. This procedure was repeated twice with similar results.

**Fig. 6** RelA and RelB increase RANKL expression by MPCs. Calvarial pre-osteoblasts from 7-day-old WT mice were infected with GFP control, RelA, or RelB retroviruses for 48 h. a RelA and b RelB mRNA expression tested by real-time PCR to confirm successful over-expression. Mean ± SD (n = 3 biologically independent samples; **p < 0.01; unpaired Student’s t test). c RANKL mRNA expression tested by real-time PCR. Mean ± SD (n = 3; *p < 0.05, **p < 0.01; one-way ANOVA with Tukey’s post-hoc test). d Culture media collected from culture wells and RANKL protein levels measured by ELISA. Mean ± SD (n = 3 biologically independent samples; no significant difference; one-way ANOVA with Tukey’s post-hoc test). e Membrane-bound RANKL levels in 50,000 CD45-Sca-1+ MPCs tested by flow cytometry and expressed as mean fluorescence intensity (MFI). Average of 2 biologically independent samples from two individual experiments. All the in vitro experiments were repeated twice with similar results.

**Fig. 7** Model of TRAF3 degradation during aging leading to bone loss. (1) During aging, increased RANKL expressed by MPCs induces TRAF3 ubiquitination and subsequent lysosomal degradation in osteoclast precursors (OCP) to stimulate osteoclast formation and bone resorption through NF-κB1,2,14. As a result, increased amounts of TGFβ are released from bone matrix and activated in the acid environment in resorption lacunae. (2) Activated TGFβ induces TRAF3 ubiquitination and subsequent lysosomal degradation in MPCs. As a result, both RelA and RelB are activated to promote RANKL production, further enhancing bone resorption. In addition, (3) in MPCs, TRAF3 binds to the TGFβR and negatively regulates GSK-3β activity to prevent β-catenin degradation, allowing β-catenin accumulation and nuclear translocation to maintain osteoblast differentiation and secretion of OPG, which limits osteoclast formation. During aging, TGFβ1 degrades TRAF3 and phosphorylates Tyr216 to activate GSK-3β, resulting in degradation of β-catenin to inhibit osteoblast differentiation and OPG secretion to promote osteoclast formation along with increased RANKL expression and bone loss. In young WT mice, TGFβ levels in bone, BM, and serum are not increased and TRAF3 is present to limit these processes.
were collected from digestions 2 to 6; 5 × 10^3 cells calvarial cells were seeded in 96-well plates. Spleens were ground up in a 40 µm cell strainer to make a single cell suspension, then resuspended with red blood cell lysis buffer for 2 min. 10^6 spleen cells were added to the wells containing the calvarial cells and cultured in the presence of 10^{-8} M 1,25(OH)_{2}D_{3} for 7 days when large multinucleated cells are observed under inverted microscopy. TRAP staining was performed to evaluate osteoclast formation.

**Over-expression of TRAF3 and GSK-3β**

5 × 10^5 calvarial pre-osteoblasts from newborn WT mice were cultured overnight at 37°C with 5% CO₂ in a 100-mm dish, followed by treatment with 25% volume of pMx-GFP or pMx-TRAF3 retroviral supernatants generated by Plat-E packaging cells and 2 µg/ml polybrene. After 48 h later, the retroviral infection medium was changed with fresh cell culture medium (a-MEM containing 10% FBS) plus with PBS or TGFβ1 for 8 or 48 h. The culture medium was collected and the cells were gently washed twice with PBS. To detect protein phosphorylation, 400 µl protein lysis buffer (RAPI lysis buffer containing protease inhibitor cocktail; Roche; #11697498001, 1 mM phenylmethylsulfonyl fluoride (PMSF), 50 mM sodium fluoride, 0.2 mM sodium orthovanadate, and 10 µg/ml peptatin) was added to each 100-mm dish and the cell protein lysate was collected.

Similarly, cells seeded on chamber slides were fixed following retroviral infection and TGFβ1 treatment by 4% PFA at 37°C for 5 min, and β-actin (cysketalope) and β-catenin were detected by immunofluorescence staining. The human GSK-3β gene (NM_001146163.1) was cloned into the pYB70926-107His pre-osteoblast vector (Cyagen) with HA-tag. The DNA sequences of TCC for Ser9 and TAT for Tyr216 were point-mutated to GGG (Gly) and GTT (Val), respectively, using a GeneArt Site-Directed Mutagenesis Plus Kit (Life Technologies Corp., #A14604). The mutation was confirmed by DNA sequencing. The lentiviruses were packaged in HEK 293T cells (ATCC, #CRL-3216) using a Lentiviral Packaging Kit (OriginGene, #TR30037). As with pMx-TRAF3/GFP retroviral infection, the viral supernatants (25% v/v) were added to 5 × 10^5 cultured MPCs with 2 µg/ml polybrene overnight to over-express GFP control and WT or mutated GSK-3β followed by treatment with PBS or TGFβ1 for 48 h. Protein levels of WT, Ser9-, or Tyr216-phospho-GSK-3β, and β-catenin in these cells were tested by WB.

**Collection of human bone samples**

We followed a protocol with informed consent from all patients or their guardians that was approved by the Research Subjects Review Board of the University of Rochester Medical Center. These human studies were performed in adherence to the relevant ethical regulations (Declaration of Helsinki). We collected samples of vertebrae that were removed from pediatric and adult patients undergoing elective surgery to correct skeletal malformations or deformities. The vertebral specimens would typically have been discarded as part of the surgical procedure. The study was approved by the Research Subjects Review Board of the University of Rochester Medical Center. The above cell cultures were terminated by fixation with 4% paraformaldehyde for 5 min at 4°C, and cells were permeabilized with 0.1% Triton X-100 and blocked with 5% goat serum for 1 h at room temperature. The samples were incubated with the primary antibody overnight followed by treatment of vehicle or TGFβ1 (1 ng/ml) plus or minus 10 nM MG132 for 48 h.

**Immunofluorescence staining**

Paraflin-embedded sections (4 µm thick) of decalcified L2 vertebrae from 2- and 18-month-old C57BL/6j mice were double-immunostained using Abs to TRAF3 and osteocalcin (Ocn), and then treated with chloroquine (CQ; 50 µM) for 2 h followed by treatment with vehicle or TGFβ1 (1 ng/ml) plus CQ for 8 h, and expression of TRAF3 and LAMP2 was assessed in a minimum of 50 cells in 5 representative fields at ≥20 magnification in each of 4 chamber slides in each group. The above cell cultures were terminated by fixation with 4% paraformaldehyde for 5 min at 4°C, and cells were permeabilized with 0.1% Triton X-100 and blocked with 5% goat serum for 1 h at room temperature before incubation with primary Abs in a humid chamber at 4°C overnight. On the 2nd day, after 3 washes with PBS containing 0.05% Tween 20, Alexa Fluor 488- and 568-conjugated secondary Abs were added and incubated for 1 h at room temperature. The images were acquired in a minimum of 50 stained BdMPCs and calvarial cells were imaged in 5 representative fields in each of 4 chamber slides in each group using a ≥20 objective lens and a Zeiss fluorescence microscope. These fields were photographed and the areas of localization of TRAF3 and LAMP2 in the cytoplasm of BdMPCs and of β-catenin positive staining in nuclei of calvaria-derived cells were measured in the digital images using ImageJ image analysis system (NIH).

**ChIP assay**

Transcription factor binding sites within –8 kb before the murine RANKL coding start site were searched using TISEARCH software to identify putative xB binding sites. ChIP assays were performed to test for binding of RelA and RelB to each of the three xB binding sites, following our published procedure. Briefly, the sheared chromatin from WT and TRAF3 cKO BdMPCs that had been fixed with 1% formaldehyde was immunoprecipitated with Abs to RelA or RelB IgG as a negative control. The precipitated DNA was used as a template for PCR using primers specifically designed to amplify a segment of 120–150 bp containing the putative xB binding sites. The sequences of the primers were: site 1, forward 5′-TGCTGGGAGCTTGCTTCTT-3′ and reverse 5′-CCCTT GGGTTGATACG-3′; site 2, forward 5′-GGTCCTGGCTTGGTTGTCTACA TTA-3′ and reverse 5′-GGAGGAAAAACAGGTGTCCTT-3′; and site 3, forward 5′-GGTG TGGGACCTACATTGCC-3′ and reverse 5′-CCCCCATGATGGGATGATATGGC-3′. In addition, a pair of unrelated primers, forward 5′-AAGAAGGCTCAGTATG-3′ and reverse 5′-CCCCGAGGAGCCAGGGGAGAAAG-3′, was designed in the DNA region that is outside the 2.5 kb of the xB binding sites.

**Quantitative real-time PCR**

1 µg of total RNA extracted from MPCs and cortical bones was reversely transcribed to cDNA in a 20 µl reaction using an iScript cDNA Synthesis kit (Bio-Rad). The expression levels of Traf3 fl/fl and Acp5 were measured using an iCycler real-time PCR instrument (Bio-Rad) and normalized to the constitutively expressed genes Actb and Gapdh.
values were obtained by subtracting the GAPDH CT value from the corresponding CT value of specific genes. Primer sequences are as follows:

fl-CGCAGTGGTGTGAACGGATTTG-3′
fl−CTAAGCCCAACAGCTCT-3′
cathepsin K (ctk), forward, 5′−CAGGTCGCCAACAGAGTGAT-3′, and reverse, 5′−GAAGC ACCAACAGAGGAGAAG-3′; Mmp2, forward, 5′−AACGGCTGGGAAATCAGC-3′, and reverse, 5′−GTAAACAAGGCTTCATGGGG-3′; Mmp13, forward, 5′−GGGAAATCCTGGAAGATCTAC-3′, and reverse, 5′−TAAGGAGAGTTAAGCTATT-3′; Mmp14, forward, 5′−AGGAGACGGGATGCTACATC-3′, and reverse, 5′−GTCCTAGGGGTCAAGGA-3′; Gapdh, forward, 5′−GGTCGGGTGAAAGGGATTG-3′, and reverse, 5′−ATGAGCCCTTCCACAA-3′.

The relative abundance (ΔCT) of each gene was calculated by subtracting the GAPDH CT value from the corresponding CT value of specific genes, and ΔΔCT values were obtained by subtracting the ΔCT values of WT samples from cKO samples and then raised to the power 2 (2ΔΔCT) to yield fold-expression relative to the WT controls.

Flow cytometry. Calvarial pre-osteoblasts from 7-day-old WT mice were infected with GFP control, pMX-RelA-GFP or pMX-RelB-GFP retroviruses for 2 days before being harvested for FACS analysis. Briefly, the live cells were stained with anti-CD45-APC, anti-Scal-PECy7 and anti-RANKL-Biotin Abs in FACS buffer (2% FBS in PBS) at 4 °C for 30 min, followed by PE-Texas Red Streptavidin staining at 4 °C for 30 min. After the staining was completed, cells were kept in FACS buffer with DAPI and tested immediately. Around 50,000 CD45− live cells were gated out for further analysis of surface RANKL expression. To measure BrdU, 1 S-phase BdnPCs, a FITC BrdU flow kit (#559619) from BD Bioscience was used for cell staining. Briefly, cells were fixed and permeabilized using BD Cytofix/ Cytoperm buffer and permeabilization buffer plus, then digested in 300 µg/ml DNase I for 1 h at 37 °C. Each sample of cells was stained with 1 µl FITC-conjugated BrdU antibody in 50 µl BD Perm/Wash buffer for 20 min at room temperature and after washing once were resuspended in 20 µl 7-AAD. Stained cells were acquired using a flow cytometer (FACS LSR II; BD Biosciences). Flowjo software was used for data analysis.

Statistics. All results are given as the mean ± S.D. Variance was similar between groups for most parameters assessed. Comparisons between two groups were analyzed using Student’s two-tailed unpaired t-test and those among 3 or more groups using one-way analysis of variance followed by Tukey honestly significant difference test and those among 3 or more groups using one-way analysis of variance followed by Tukey’s post-hoc multiple comparisons. P values < 0.05 were considered statistically significant. Each experiment was repeated at least twice with similar results. The sample size for in vivo experiments is based on an un-paired t-test power analysis carried out by our statistician using SigmaStat Statistical Software. 5–8 mice were used in each group where bone parameters are being assessed to detect significant differences from controls with an alpha error of 5%. The power is 0.98, i.e., there is 98% chance of detecting a specific effect with 95% confidence when alpha = 0.05. No data were excluded from the analyses.

Data availability
All relevant data are available from the authors upon reasonable request.

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Author contributions

J.L. performed and analyzed the experiments shown in Figs. 1a–i, 2e–g, 3a–c, g–j, p, 4a–m, 5c–e, g–j, as well as Fig. 6 and wrote the first draft of the paper. A.A. performed and analyzed the experiments shown in Figs. 2a–d, h–m and 5b–k. J.L. generated the TRAF3 cKO mice and carried out initial skeletal analyses. X.Y. performed and analyzed the experiments shown in Fig. 3f, o, J.O.S. and A.M. provided human bone samples. Z.Y. conceived and coordinated the studies, performed and analyzed the experiments shown in Figs. 1a, b, d–f, h, 3e, 4g, 5a, b, and wrote the paper. B.F. supervised, conceived and coordinated the studies, and wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

Additional information

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