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TGF- β type II receptor phosphorylates PTH receptor to integrate bone remodelling signalling

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Abstract

Parathyroid hormone (PTH) regulates calcium homeostasis and bone metabolism by activating PTH type I receptor (PTH1R). Here we show that transforming growth factor (TGF)- β type II receptor (T β RII) forms an endocytic complex with PTH1R in response to PTH and regulates signalling by PTH and TGF- β . T β RII directly phosphorylates the PTH1R cytoplasmic domain, which modulates PTH-induced endocytosis of the PTH1R–T β RII complex. Deletion of T β RII in osteoblasts increases the cell-surface expression of PTH1R and augments PTH signalling. Conditional knockout of *T β RII* in osteoblasts in mice results in a high bone mass with increased trabecular bone and decreased cortical bone, similar to the bone phenotype in mice expressing a constitutively active PTH1R. Disruption of PTH signalling by injection of PTH(7–34) or ablation of PTH1R rescues the bone phenotype of *T β RII* knockout mice. These studies reveal a previously unrecognized function for T β RII and a mechanism for integration of PTH and local growth factor at the membrane receptor level.

The bones of most mammals are continuously formed and resorbed through a process called bone remodelling¹. The bone remodelling cycle is achieved through the coordinated activity of two cell types: osteoblasts, which deposit the calcified bone matrix; and osteoclasts, which resorb bone². Osteoclasts probably evolved as an adaptive mechanism to regulate the mineral-ion homeostasis of terrestrial vertebrates. PTH is secreted by the parathyroid glands, which also first emerged in terrestrial vertebrates, presumably to regulate bone remodelling by acting directly on osteoblasts and indirectly on the osteoclast¹. The interaction of PTH with locally osteotropic factors such as TGF- β and insulin-like growth factor (IGF)³, which are evolutionarily conserved in aquatic vertebrates^{4–7}, orchestrates an anabolic signalling network for the coupling of bone resorption and formation. However, the mechanisms responsible for the interaction of these osteotropic factors are still unclear.

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AUTHOR CONTRIBUTIONS

T.Q. performed most of the experiments, analysed data and prepared the manuscript. X.W. performed the most of the *in vivo* experiments and analysed *in vivo* data. F.Z. maintained the mice and collected tissue samples. C.T. provided suggestions for the project. M.W. prepared the manuscript. X.C. supervised the project and wrote most of the manuscript.

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On binding to PTH1R^{8,9}, a G-protein-coupled seven-transmembrane receptor (GPCR), PTH activates G_{α_s} and G_{α_q} , leading to the production of cyclic AMP, activating cAMP-dependent protein kinase (PKA) and stimulating phospholipase for the activation of protein kinase C (PKC)^{10–12}. Signalling by PTH through PKA and PKC is rapidly shut off in association with the endocytosis of PTH1R. Phosphorylation at the cytoplasmic domain of PTH1R is crucial for the recruitment of arrestin proteins, which are required for the endocytic process¹³.

TGF- β 1 is present abundantly in the bone matrix. Active TGF- β 1 released during osteoclastic bone resorption induces the migration of bone mesenchymal stem cells to couple bone resorption with formation¹⁴. TGF- β elicits its cellular response through the ligand-induced formation of a heteromeric complex containing TGF- β types I (T β RI) and II (T β RII) kinase receptors^{15–17}. T β RII is a constitutively active serine/threonine (S/T) kinase that transphosphorylates the GS motif of T β RI on ligand binding, resulting in subsequent phosphorylation of a subclass of intracellular signalling molecules called R-Smads. R-Smads then interact with Co-Smad (Smad4) and translocate into the nucleus, where they induce cellular responses by acting as transcription factors^{18–20}. Several lines of evidence have indicated that PTH and TGF- β work in concert to exert their physiological activities in bone. For example, PTH increases the concentration of TGF- β in bone³. In addition, PTH induces bone resorption by directly activating osteoblasts¹, which release osteotropic growth factors (including TGF- β) from the bone matrix. PTH requires TGF- β /Smad3 signalling to exert its anti-apoptotic effects in osteoblasts²¹. TGF- β has parathyroid hormone-related peptide (PTHrP)-dependent and PTHrP-independent effects on endochondral bone formation²². These factors may therefore work jointly to couple bone resorption to bone formation^{23,24}.

Endocytosis of growth factors and GPCRs is known to integrate different signalling pathways²⁵. We found that PTH induced the recruitment of T β RII as an endocytic activator. T β RII directly phosphorylated the cytoplasmic domain of PTH1R and facilitated PTH-induced endocytosis of the PTH1R–T β RII complex. In particular, the signalling of both receptors was coordinately regulated during endocytosis. Disruption of TGF- β in osteoblasts in mice increased the cell-surface expression of PTH1R and produced a bone phenotype that mimicked those seen in mice expressing a constitutively active PTH1R. These findings show a functional interaction between PTH and TGF- β receptors that integrates the activities of these two critical bone remodelling factors.

RESULTS

PTH induces endocytosis of T β RII

Endocytosis of seven-transmembrane receptors has been shown to integrate signals of different pathways. To test whether endocytosis of PTH1R coordinates the signals of PTH and TGF- β , we first examined the effect of PTH on internalization of T β RII. Flag-tagged T β RII was expressed in human embryonic kidney 293 (HEK293) cells or HEK293 cells stably expressing PTH1R (HEK293-PTH1R). In the absence of PTH, T β RII was present predominantly at the cell surface at 16 h post-transfection (Fig. 1a; Supplementary Information, Fig. S1a). When stimulated with PTH, T β RII was internalized in HEK293-PTH1R cells (Fig. 1a). To reveal PTH ligand, PTH was labelled with the red fluorophore tetramethylrhodamine (TMR) at Lys 13 (PTH^{TMR}). PTH^{TMR} bound only to cells expressing PTH1R and was internalized into intracellular vesicles within 30 min (Fig. 1b, top panels). Immunostaining showed that internalization of T β RII was induced by PTH^{TMR} in a time-dependent fashion, similarly to that of PTH^{TMR} (Fig. 1b, middle panels). In addition, the amount of cell-surface T β RII was decreased significantly in 30 min (Fig. 1c). In particular, the internalized T β RII was co-localized with PTH^{TMR} in the cytoplasmic vesicles or in

proximity to the membrane (Fig. 1b, bottom panels). Consistent with this observation, measurement of T β RII levels on the plasma membrane by cell-surface biotinylation showed that PTH decreased endogenous cell-surface T β RII by more than 80% in 20 min (Fig. 1d, e). In contrast, PTH^{TMR} did not induce endocytosis of IGF type I receptor (IGF1R) and bone morphogenetic protein type II receptor (BMPRII) (Supplementary Information, Fig. S1b). To assess the physical interaction between PTH and T β RII directly, we employed fluorescence resonance energy transfer (FRET) between PTH^{TMR} (acceptor) and T β RII fused with green fluorescent protein (T β RII^{GFP}, donor) (Fig. 1f; Supplementary Information, Fig. S2). The FRET efficiency determined by donor recovery after acceptor photobleaching was $10.57 \pm 2.1\%$ s.d., indicating that PTH recruited T β RII into close proximity despite the fact that the two fluorophores were separated by plasma membrane. The FRET efficiency between PTH and a mutant receptor (T β RII-K277R) devoid of kinase activity was less than 2.5%, suggesting that the kinase activity is involved in the interaction. Taken together, these results show that PTH induces PTH1R-dependent endocytosis of T β RII.

PTH induces the formation and internalization of T β RII–PTH1R complexes

To gain information on the nature of the physical interaction between PTH1R and T β RII, yellow fluorescent protein (YFP)-based protein-fragment complementation assay (PCA) was used^{26,27}. The YFP1 fragment was fused to the carboxy terminus of T β RII, and the YFP2 fragment was fused to the C terminus of PTH1R. The PCA strategy enables the visualization of protein–protein interactions in living cells based on the reconstitution of the YFP fluorophore when complementary YFP-labelled fragments come into close proximity (Fig. 2a). Expression of T β RII–YFP1 or PTH1R–YFP2 alone in the presence of PTH, or together without PTH, did not generate fluorescence (Fig. 2b, c). YFP fluorescence was induced on the plasma membrane when both plasmids were expressed in the presence of exogenous PTH, whereas no fluorescence was observed with exogenous TGF- β (Fig. 2b, c), indicating that PTH induces the interaction between T β RII and PTH1R. As expected, PTH^{TMR} was predominantly co-localized with the reconstituted YFP fluorophores at the cell membrane by 30 s and became visible as intracellular puncta by 5 min (Fig. 2d). In contrast, the interaction of PTH1R with T β RII-K277R was not seen (Fig. 2c, d), even though T β RII-K277R was well expressed (Supplementary Information, Fig. S3). The formation of the complex of PTH1R with T β RII was confirmed in immunoprecipitation experiments with overexpressed proteins (Fig. 2e) or endogenous proteins in primary osteoblasts (Fig. 2f). Again, no interaction between PTH1R and T β RII-K277R was observed (Fig. 2e). Because several GPCRs have been reported to form dimers or oligomers capable of signalling^{28,29}, we investigated whether interaction between PTH1R and T β RII was necessary for the dimerization or oligomerization of PTH1R. PCA was performed with PTH1R that had been fused with different YFP fragments at the same location on the cytoplasmic tail of PTH1R (Fig. 2g). PTH reconstituted YFP fluorescence by 30 s (Fig. 2h, upper panels) and PTH^{TMR} was predominantly co-localized with the YFP fluorescence (Fig. 2h, lower panels), indicating PTH-induced formation of PTH1R dimer or oligomer. Most of the T β RII was co-localized with the YFP fluorophores of PTH1R dimer/oligomer (Fig. 2i), suggesting that T β RII can be recruited by, but may not be essential for, the dimerization or oligomerization of PTH1R. In line with these observations, PTH induced the recruitment and co-localization of T β RII with β -arrestin (Fig. 3), an adaptor protein involved in PTH endocytosis. Taken together, these results demonstrate that PTH induces the interaction of T β RII with PTH1R and the internalization of T β RII–PTH1R as a complex.

Endocytosis of T β RII–PTH1R coordinates signalling of PTH and TGF- β

The results described above suggest that internalization of the PTH1R–T β RII complex regulates the signalling of PTH and TGF- β . Indeed, PTH decreased TGF- β -induced

phosphorylation and the nuclear accumulation of Smad2/Smad3 (Fig. 4a, b) and blunted the interaction of Smad2 with Smad4 (Fig. 4c). Expression of PTH1R inhibited TGF- β -induced transcriptional activity of a Smad-binding element (SBE) reporter, and PTH enhanced the inhibition (Fig. 4d; Supplementary Information, Fig. S4a). In addition, inhibition of endocytosis by knocking down β -arrestin-1/2 relieved the inhibitory effect of PTH on the phosphorylation of Smad2 (Fig. 4e). These results indicate that PTH attenuates TGF- β /Smad signalling by inducing the endocytosis of PTH1R–T β RII as a complex. Conversely, expression of T β RII decreased PTH-induced cAMP accumulation (Fig. 4f) and inhibited the PTH-induced phosphorylation of cAMP-response-element-binding protein (CREB) (Fig. 4g) and the transcriptional activity of a CREB responsive element (CRE) reporter (Fig. 4h; Supplementary Information, Fig. S4b). Moreover, expression of T β RII decreased the cell-surface concentration of PTH1R in a 125 I-PTH binding assay (Fig. 4i). The inhibitory effects of T β RII on PTH signalling were decreased by co-expression of T β RI (Supplementary Information, Fig. S5), probably as a result of decreased availability of T β RII through its interaction with T β RI (ref. 15). Indeed, T β RII-K277R also showed significantly decreased effects (Fig. 4f–i). These findings indicate that T β RII inhibits PTH signalling by inducing the endocytosis of PTH1R and decreasing cell-surface PTH1R. Taken together, these results indicate that PTH-induced endocytosis of PTH1R–T β RII modulates the signalling of both PTH and TGF- β .

T β RII directly phosphorylates PTH1R to modulate their endocytosis

We next investigated the mechanism by which PTH1R and T β RII are internalized as a complex. T β RII possesses an S/T kinase that phosphorylates the S/T cluster region (GS motif) of T β RI in the activation of the Smad signalling pathway¹⁷. Four S/T clusters were identified in the PTH1R cytoplasmic domain (cPTH1R) (Fig. 5a, upper panel). One of these is highly homologous with the GS motif of T β RI (Fig. 5a, lower panel). To examine whether the T β RII kinase phosphorylates PTH1R directly, we prepared a native T β RII kinase, a cytoplasmic domain of T β RII (cT β RII)³⁰, and a kinase-dead cT β RII-K277R, and then tested their potential phosphorylation activity with cPTH1R as a substrate. cT β RII showed auto-phosphorylation activity and phosphorylated cPTH1R (Fig. 5b), whereas cT β RII-K277R did not. To confirm these results, HEK293-PTH1R cells were transfected with T β RII or T β RII-K277R and labelled with 32 P. Immunoprecipitation of the cell lysates showed that phosphorylated PTH1R was significantly increased by expression of T β RII but not by expression of T β RII-K277R (Fig. 5c). Kinase assay for cPTH1R (residues 461–551) showed that the phosphorylation sites are within the region containing four S/T clusters (Fig. 5a, d).

To identify precisely which cluster was phosphorylated, we generated four individual mutations (M1–M4) for each of the S/T clusters (Fig. 5a). The phosphorylation of cPTH1R by T β RII was diminished by mutation (M2) of the S/T cluster, which is homologous with the GS motif of T β RI (Fig. 5e), indicating that the S/T cluster is required for the phosphorylation. Deletion or mutation of the region containing this GS-like cluster was previously shown to reduce receptor internalization markedly^{31,32}. T β RII kinase directly phosphorylated a synthesized peptide motif containing the GS-like cluster (Fig. 5f, upper panel). Mass spectrometry analysis identified singly and doubly phosphorylated species (Fig. 5f, lower panel), indicating that T β RII kinase is able to phosphorylate the region at more than one site simultaneously. Moreover, the M2 mutation and a constitutively activated mutation³³ of PTH1R significantly decreased the interaction with T β RII (Fig. 5g). Accordingly, the mutants relieved the inhibitory effect of PTH1R on TGF- β signalling (Fig. 5h) and showed increased CRE transcription activity (Supplementary Information, Fig. S6a). These results, together with the result that T β RII kinase decreased the cell-surface level of PTH1R other than the M2 mutant (Fig. 4i; Supplementary Information, Fig. S6b–d), indicate

that the kinase activity of T β RII modulates endocytosis of PTH1R by phosphorylation of the PTH1R cytoplasmic domain.

Knockout of *T β RII* in osteoblasts increases cell-surface expression of PTH1R and PTH signalling

If T β RII kinase activity is required for PTH-induced endocytosis of PTH1R in osteoblasts, we reasoned that elimination of T β RII would enhance the cell-surface expression of PTH1R and PTH signalling. T β RII was selectively deleted in osteoblasts by crossing the mice homozygous for floxed *T β RII* (*T β RII^{fllox/flox}*) with Cre-transgenic mice driven by an osteocalcin promoter (Supplementary Information, Fig. S7). This mutation resulted in upregulation of the cell-surface expression of PTH1R in osteoblasts, as assessed by a radioligand-binding assay (Fig. 6a). In accord with the increased PTH1R, basal cAMP accumulation was also increased (Fig. 6b). To investigate more precisely the effects of a loss of T β RII on osteoblasts, primary osteoblasts were isolated from mice homozygous for the *T β RII^{fllox/flox}* gene and then infected with adenoviral Cre to remove T β RII. T β RII-null osteoblasts (Δ T β RII) showed an increase in basal-phosphorylated CREB, close to the level observed in wild-type osteoblasts treated with PTH (Fig. 6c). In these cells, PTH no longer stimulated the phosphorylation of CREB, probably because of saturation of PTH1R/cAMP signalling. Consistently, in the T β RII-null osteoblasts, PTH no longer induced the phosphorylation of PTH1R (Fig. 6d) and PTH had no effect on the phosphorylation of extracellular-signal-regulated kinase (ERK) (Fig. 6c), which is activated through the endocytosis of PTH1R³⁴. These data indicate that the deletion of T β RII increases PTH signalling by disrupting PTH1R endocytosis, resulting in an increased cell-surface expression of PTH1R in *T β RII^{-/-}* mice.

Osteoblast-specific *T β RII* knockout mice show increased bone formation, similar to the bone phenotype of mice expressing constitutively active PTH1R

Mice lacking T β RII were viable and indistinguishable from wild-type littermates (*T β RII^{+/+}*) at birth. By 8 weeks of age, *T β RII^{-/-}* mice showed a marked increase in bone mass and bone mineral density compared with that of their wild-type littermates (Fig. 6e–h, j). Haematoxylin and eosin (H&E) staining and microcomputed tomography (μ CT) analysis revealed a substantial increase in trabeculation at the metaphyseal region of long bones (Fig. 6g, h, k), with a disorganized reticular pattern extending into the endosteal region such that the size of the bone marrow space was reduced. In contrast, cortical bone in the metaphyseal region was more porous than in controls and was reduced in volume (Fig. 6h, l). The thickness of cortical bone at diaphysis was also decreased (Fig. 6i, m). These data indicate that a loss of T β RII in osteoblasts affects the trabecular and cortical bone morphology differentially even though total bone mass is increased. These changes are strikingly similar to those of mice expressing a constitutively active PTH1R in cells of the osteoblastic lineage³⁵.

Because PTH1R mediates the anabolic effect of PTH in bone and an excess of PTH signalling is characterized by large numbers of osteoclasts and rapid bone turnover, we analysed the numbers of osteoblasts and osteoclasts at the trabecular and endosteal surfaces of *T β RII^{-/-}* mice by histomorphometry. Both parameters were substantially increased from 6 weeks to 12 weeks of age (Fig. 7a–d), except that the number of osteoclasts at trabeculae had returned to normal in mature mice at 12 weeks of age (Fig. 7c). Calcein double labelling of the *T β RII^{-/-}* mice (Fig. 7e) indicated a disorganized pattern at trabecular and endosteal surface, consistent with greatly induced bone turnover and decreased periosteal mineral apposition, a major determinant of cortical bone formation. Additionally, the concentrations of serum markers, including osteocalcin for bone formation (Fig. 7f) and the C-terminal telopeptide of type I collagen (CTX) (Fig. 7g) for bone resorption, were significantly

increased in *TβRII*^{-/-} mice relative to their wild-type littermates. Moreover, the expression level of the receptor activator of nuclear factor-κB ligand (RANKL) was significantly increased in the *TβRII*-null osteoblasts (Fig. 7h). These results show greatly increased bone remodelling in the *TβRII*^{-/-} mice. However, the effects are manifested differently in different bone compartments. In trabecular bone, the activity of osteoclasts seems to be overwhelmed by the robust osteoblast activity, particularly as the mice age. Conversely, at the endosteal surface, osteoclasts seem to remain active, which ultimately leads to cortical thinning, a phenomenon also seen in situations in which PTH1R is hyperactive.

Injection of PTH(7–34) or decrease in PTH1R expression rescues the bone phenotype of the *TβRII*^{-/-} mice

To determine whether the increased bone mass in *TβRII*^{-/-} mice was a result of stimulation of PTH signalling, we injected PTH(7–34), a PTH antagonist^{36–38}, subcutaneously into the *TβRII*^{-/-} mice. The bone phenotype of *TβRII*^{-/-} mice was rescued by increasing doses of PTH(7–34), as shown by reduced trabecular bone volume and increased cortical thickness (Fig. 8a (upper panels), b). Calcein double labelling showed that the highly enhanced incorporation of calcein in *TβRII*^{-/-} mice returned to a level comparable to that of their wild-type littermates (Fig. 8a, lower panels). Moreover, the high bone turnover in *TβRII*^{-/-} mice was suppressed by injection of PTH(7–34), as demonstrated by decreased serum levels of calcium, osteocalcin and CTX, and decreased numbers of osteoblasts and osteoclasts at the trabeculae, whereas the circulating PTH level was not significantly changed (Fig. 8c–h). These parameters indicate downregulated bone remodelling by antagonist-mediated disruption of PTH action. Finally, we ablated PTH1R in osteoblasts simultaneously with *TβRII* by crossing the *TβRII*^{-/-} mice with *PTH1R*^{+/-} mice. The osteoblast-specific *TβRII*^{-/-}/*PTH1R*^{+/-} mice showed a substantial decrease in bone formation compared with the *TβRII*^{-/-} mice, and produced a similar bone phenotype to that observed in *TβRII*^{-/-} mice with injection of PTH(7–34) (Fig. 8i, j). These results demonstrate that hyperactive PTH signalling through PTH1R leads to the bone phenotype observed in the *TβRII*^{-/-} mice.

DISCUSSION

Endocytosis of cell-surface receptors is a regulatory mechanism for the integration of different signalling pathways²⁵. We have shown that PTH induces an interaction between *TβRII* and PTH1R to facilitate their endocytosis as a complex. Phosphorylation of PTH1R by *TβRII* modulates endocytosis, leading to downregulation of PTH signalling. Similarly, the recruitment of *TβRII* to PTH1R by PTH dampens TGF-β signalling. Conversely, PTH signalling is stimulated in the *TβRII* knockout mice, producing a bone phenotype strikingly similar to that observed in animals bearing a constitutively active PTH1R. We therefore propose a working model in which PTH-induced endocytosis of a *TβRII*–PTH1R complex provides a platform for integrating the signals of TGF-β and PTH (Fig. 8k).

Activation of PTH1R differentially affects cortical and trabecular bone surfaces. Patients with Jansen's metaphyseal chondrodysplasia, caused by constitutively activating mutations of PTH1R, show ligand-independent cAMP accumulation, loss of cortical bone, and augmentation of trabecular bone³³. These bone phenotypes are reproduced in mice with osteoblastic expression of Jansen's PTH1R mutant³⁵. In contrast, *PTH1R* knockout mice show decreased trabecular bone and increased thickness of cortical bone³⁹. The skeletal abnormalities with constitutively activating PTH1R are similar to the phenotype in the *TβRII*^{-/-} mice. In particular, PTH ligand binding, cAMP production and CREB phosphorylation are increased in untreated *TβRII*^{-/-} osteoblasts. In addition, intermittent injection of PTH in *TβRII*^{-/-} mice no longer enhanced the bone phenotype exhibited in *TβRII*^{-/-} mice (data not shown). The sustained PTH1R signalling contributed to the cAMP activation in the *TβRII*^{-/-} mice, resulting in increased bone mass and the differential

regulation of trabecular versus cortical bone. The observation was validated by the rescue of bone phenotype of *TβRII*^{-/-} mice by the injection of PTH(7–34) or by the disruption of PTH1R expression. The bone phenotype of the *TβRII*^{-/-} mice is apparently not simply generated by the loss of Smad signalling, which regulates the mechanical properties of bone matrix^{40,41}. Indeed, TβRII is internalized dynamically (Supplementary Information, Fig. S8) and is a target of both PTH and TGF-β. Thus, TGF-β/Smad signalling probably occurs through the signalling cascade integrated in the effect of PTH on bone. Actually, PTH activates other signalling pathways such as the canonical Wnt signalling⁴².

PTH stimulates the bone remodelling process by regulating bone formation and bone resorption. PTH glands probably developed in terrestrial vertebrates to provide an additional ligand to activate the PTH1R, which evolved earlier in evolution to service locally produced PTHrP. The fact that PTH arrives at PTH1R through the systemic circulation provides a means for the rapid and integrated regulation of osteoblasts and osteoclasts in physiological settings of increased calcium demands. We further speculate that the mechanisms described in this study that involve interaction between PTH1R and TβRII could serve as a platform for integrating signals from local osteotropic factors for the rapid skeletal adjustments imposed by the increased demands for calcium and bone remodelling required of terrestrial vertebrates. Therefore, direct phosphorylation of the cytoplasmic domain of PTH1R by TβRII integrates PTH signalling for bone remodelling and TGF-β1 signalling for coupling bone resorption and formation^{14,43}. Finally, these findings raise the possibility of manipulating this pathway with pharmaceuticals that would more effectively couple osteoclast and osteoblast activity.

METHODS

Plasmids and antibodies

The TβRII-Flag/pcDNA3.0 and PTH1R-HA/pcDNA3.0 plasmids were cloned from human TβRII³⁰ and PTH1R⁴⁴. Zipper-YFP1/pcDNA3.1, zipper-YFP2/pcDNA3.1²⁶, and luciferase reporters SBE⁴⁵ and CRE (Stratagene, La Jolla, CA) have been described previously. PTH1R-HA was introduced into pMSCVneo retroviral expression vector (BD Biosciences, San Jose, CA) to generate pMSCV-PTH1R-HA. TβRII-YFP1, PTH1R-YFP1, and PTH1R-YFP2 were generated by replacing the zippers in zipper-YFP1 or zipper-YFP2. Cytoplasmic domain of TβRII (amino acids 192–566, cTβRII) and cytoplasmic domain of PTH1R (amino acids 461–593, cPTH1R) were fused with GST at N-terminals using pGEX-KG prokaryotic gene fusion vector (Pharmacia Corp., Pfizer Inc., New York, NY). The mutants TβRII-K277R-Flag, TβRII-K277R-YFP1, PTH1R-H223R-YFP2, PTH1R-M2-YFP2, PTH1R-H223R-HA, PTH1R-M2-HA, TβRII-K277R-GST, cPTH1R-M1-GST, cPTH1R-M2-GST, cPTH1R-M3-GST, and cPTH1R-M4-GST were generated by primer-mediated PCR mutagenesis and verified via DNA sequencing. Primary antibodies used for immunoblotting, immunoprecipitation, or immunostaining included mouse Flag antibody M2 (IF, 1:500; IB, 1:2000) (Sigma-Aldrich Corp., St. Louis, MO); mouse HA antibody 16B12 (IB, 1:2000) and rabbit PTH1R antibody PRB-640P (IB, 1:500) and PRB-630P (IB, 1:500) (Covance, Princeton, NJ); mouse TβRII antibody C-4 (IF, 1:250; IB, 1:500), rabbit CREB antibody (IB, 1:1000), and rabbit P-CREB antibody (IB, 1:1000) (Upstate Biotechnology, Inc., Lake Placid, NY); Rabbit PTH(1–84) antibody (IB, 1:500) (GeneTex, Inc., Irvine, CA); mouse ERK antibody C-16 (IB, 1:1000), mouse P-ERK antibody E-4 (IB, 1:800), and mouse Smad4 antibody B-8 (IB, 1:1000) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); mouse Smad2 antibody L16D3 (IB, 1:1000) and mouse P-Smad2 antibody (Ser465/467) (IB, 1:500) (Cell Signaling Technology, Danvers, MA).

Cells, transfection and reagents

The HEK293, C3H10T1/2 and UMR106 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in DMEM or α -minimal essential medium supplemented with 10% fetal bovine serum (FBS; Invitrogen Corp., Carlsbad, CA). HEK293-PTH1R cells were generated by stable expression of PTH1R-HA with pMSCV-neo retrovirus transduction. The transfection of DNA plasmids were performed with Lipofectamine reagent (Invitrogen Corp.). The knockdown of β -arrestins was performed with siRNAs against β -arrestin1 and β -arrestin2 (Santa Cruz Biotechnology, Inc.). Human PTH(1–34) (here termed PTH) and PTH(7–34) was purchased from Bachem, Inc. (Torrance, CA), and the radioligand [125 I]-[Nle-8,21,Tyr-34]-human PTH(1–34)-OH (here termed 125 I-PTH) was labelled at University of Alabama. [Lys-13(N^e-5-carboxy-TMR)]-PTH(1–34)-NH₂ (here termed PTH^{TMR}) and PTH1R small peptide (residues 476–506) was synthesized by Genemed Synthesis, Inc. (San Antonio, TX); 100 nM of PTH, PTH^{TMR}, 125 I-PTH or PTH(1–84), or 2 ng of TGF- β 1, was used for cell treatment.

Immunoprecipitation, immunoblotting analysis and luciferase reporter assay

The cells were lysed in IP buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate) containing protease inhibitors. The lysates were immunoprecipitated by incubation with the appropriate antibodies, followed by absorption on Protein G-Sepharose. The immunoprecipitates were separated by SDS-PAGE and blotted onto a nitrocellulose membrane. Immunoblots were characterized by using the SuperSignal West Femto Substrate system (Pierce, Thermo Fisher Scientific, Inc., Rockford, IL). To perform immunoblotting for T β RII-K277R, the cells were lysed in IP buffer with 0.5% SDS. Luciferase activities were assayed with the Dual-Luciferase Reporter Assay System (Promega Corp., Madison, WI).

T β RII trafficking

The cells expressing T β RII were classified into three types: first, those in which T β RII was dominantly present at the cell surface (membrane), second, those that located to the cell surface and vesicles in the cytosol (membrane/vesicle), and third, those that mainly localized to the cytosol as vesicles (vesicle). Endocytic trafficking was assessed as described⁴⁶. For cell-surface T β RII biotinylation, HEK293-PTH1R cells treated with PTH at different time points were suspended in PBS containing 1 mM Sulfo-NHS-SS-Biotin, and rocked at 37 °C for 30 min. After quenching of excess biotin, the cells were lysed; the cell lysates were then immunoprecipitated with anti-T β RII antibody and immunoblotted with anti-T β RII antibody (total T β RII) or streptavidin-tagged horseradish peroxidase (cell-surface T β RII). Chemiluminescent density was quantified with a Storm 860 scanner (Molecular Dynamics, Sunnyvale, CA). Values of cell-surface T β RII were normalized to total T β RII. The curve for cell-surface level was determined by comparing the values at different time points, with that at time zero being set to 100%. Data correspond to means $\pm$ s.d. from three independent experiments.

Ligand binding, receptor internalization, cAMP assay and real-time PCR

Ligand binding and receptor internalization were performed as described¹³, with minimal modifications. Cells grown in 35-mm six-well plates were incubated in serum-free DMEM for 1 h at 0 °C, followed by a 45-min incubation with the buffer containing 125 I-PTH (100,000 c.p.m. per well) as a radioligand. For the binding study, cell-associated 125 I-PTH was extracted with 0.8 M NaOH and quantified in a scintillation counter. For cAMP assays, cells grown in 35-mm six-well plates were stimulated with PTH at 37 °C for 15 min. Cellular cAMP was extracted and measured with the Biotrak enzymeimmunoassay system (GE Healthcare, Inc., Princeton, NJ). For real-time PCR, total RNA was isolated from

calvarial osteoblasts with TRIzol reagent (Invitrogen). cDNA synthesis was performed with TaqMan Reverse Transcription reagent (Applied Biosystems, Foster, CA). PCR was performed with TaqMan Gene Expression assays (Applied Biosystems) with the primer-probe set mm0041908-m1 for RANKL and mm99999915-g1 for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Values were normalized to *GAPDH* mRNA levels.

Fusion protein expression and kinase assay

The GST-tagged cytoplasmic domains of PTH1R (residues 461–593) and T β RII (residues 192–567) were expressed in BL21 (DE3) cells, then pulled down by glutathione-agarose beads and added into kinase reaction that contained 50 mM Tris-HCl pH 7.5, 10 mM MgCl₂ and 200 mCi of [γ -³²P]ATP. The products were analysed by 10% SDS-PAGE and the protein-associated radiolabel was determined by the Storm 860 scanner. Mass spectrometric analysis was performed at the Prometic Core Facility of Johns Hopkins University.

Confocal microscopy, FRET measurements and YFP-PCA analysis

Cells plated on glass coverslips were washed with cold PBS, fixed with 4% paraformaldehyde for 10 min, and then carefully permeabilized with 0.1% Triton X-100 for 6 $\pm$ 2 s.d. min. The cells were mounted with Vectashield medium (Vector Laboratories) and imaged with a Leica TCS SP1 or SP2 confocal microscope. FRET experiments were performed as described⁴⁷. In brief, HEK293-PTH1R cells were transfected with T β RII^{GFP} (donor) and then treated with PTH^{TMR} (acceptor) for 15 min. FRET efficiency between PTH^{TMR} and T β RII^{GFP} was determined by donor (GFP) recovery after acceptor (TMR) photobleaching. The T β RII-K277R^{GFP} expressed on the plasma membrane was examined as a control. The YFP-PCA analysis was performed as described previously^{26,27}. The results shown are means $\pm$ s.d. from four independent experiments.

Generation of conditional *T β RII* knockout mice and *PTH1R T β RII* double knockout mice

The genotype of transgenic mice was determined by PCR analyses of genomic DNA isolated from mouse tails. Mice with osteoblast-specific inactivation of T β RII (*T β RII*^{-/-}) were generated by crossing OC-Cre mice⁴⁸ with mice homozygous for a floxed T β RII allele⁴⁹. Genotyping for the Cre transgene was performed by PCR with the primers Cre 5' (5'-CAAATAGCCCTGGCAGAT-3') and Cre 3' (5'-TGATACAAGGGACATCTTCC-3'). The floxed T β RII allele was identified with the primers⁵⁰ lox1F (5'-TAAACAAGGTCCGGAGCCCA-3') and lox1R (5'-ACTTCTGCAAGAGGTCCCCT-3'). Recombination and subsequent loss of the floxed T β RII allele were analysed with primers lox1F and lox2R (5'-AGAGTGAAGCCGTGGTAGGT-3'). Mice with osteoblast-specific disruption of PTH1R (*PTH1R*^{-/-}) were generated by crossing OC-Cre mice with mice homozygous for a floxed PTH1R allele⁵¹. The mice with disruption of PTH1R in a *T β RII* knockout background (*T β RII*^{-/-}/*PTH1R*^{-/-}) were generated by crossing *PTH1R*^{+/-} mice with *T β RII*^{-/-} mice. The floxed PTH1R allele was analysed with the primers as described⁵¹. All mice were given a standard chow diet and water. Procedures involving mice were approved by the Institutional Animal Care and Use Program of the Johns Hopkins University.

Primary osteoblast isolation and adenovirus infection

Calvarial osteoblasts were isolated as described previously⁵². The cells from *T β RII*^{-/-} mice were cultured in osteogenic medium (0.1 M dexamethasone, 10 M glycerol phosphate, 0.05 M L-ascorbic acid in α -MEM) with 5% FBS for 3 days to ensure the deletion of T β RII through osteocalcin-driven expression of Cre. For adenovirus infection, monolayer osteoblasts were infected with Cre recombinase virus M1 (Ad-CreM1) or control adenovirus (Ad-GFP) (Vector Laboratories, Burlingame, CA) at a multiplicity of infection of 100 and

were harvested for immunoblotting analysis after 48 h. For treatment with PTH, calvarial osteoblasts were pre-cultured in osteogenic medium for 3 days.

Microcomputed tomography, radiographic analysis and histomorphometric analysis

Tibiae and femora obtained from mice were dissected free of soft tissue and analysed by a high-resolution μ CT imaging system, MicroCT-40 (Scanco, Southeastern, PA), with a voltage of 55 kilovolt peak (kVp) and a current of 109 μ A. Three-dimensional trabecular structural parameters, including bone mineral density, trabecular and cortical bone volume, and cortical thickness in the secondary spongiosa, were measured as described previously⁵³. Radiographs were taken with a Faxitron X-ray machine (Faxitron X-Ray LLC, Lincolnshire, IL), under constant conditions (19 kV, 10 s exposure). To examine the dynamic bone formation, two sequential doses of calcein (Sigma-Aldrich Corp.; 10 mg kg⁻¹ in 2% sodium bicarbonate in sterile saline) was injected into mice 3 and 9 days before they were killed. Calcein double labelling was then observed under a fluorescence microscope. The numbers of osteoblasts and osteoclasts were determined by quantitative histomorphometric analysis conducted in a blinded fashion with OsteoMeasure_{XP} software (OsteoMetrics, Inc., Decatur, GA). Four randomly selected visual fields per specimen, in three specimens per mouse in each group, were measured.

PTH(7–34) injection and serum analysis

PTH(7–34) or vehicle (equivalent volume of 1 mM acetic acid in sterile PBS) in a final volume of 100 μ l was given daily by subcutaneous injection for 4 weeks to 1-month-old male mice (three per group). Serum and bone were collected for levels of calcium, osteocalcin, CTX and PTH, and for mineralized bone histology and histomorphometry, respectively. Calcium was determined with a Calcium Colorimetric Assay Kit (BioVision, Inc., Mountain View, CA). Serum levels of intact PTH were measured with a mouse PTH enzyme-linked immunosorbent assay (ELISA) kit (Alpco Diagnostics, Salem, NH). Osteocalcin and CTX were determined with a mouse osteocalcin ELISA kit (Biomedical Technologies, Inc., Stoughton, MA) and a rat/mouse RatLaps ELISA kit (Nordic Bioscience Diagnostics, Herlev, Denmark), respectively.

Statistical analysis

Statistical differences between two groups of data were analysed with Student's *t*-test. Data are presented as means $\pm$ s.d.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

PTH induces endocytosis of TβRII. (a, b) PTH induces endocytosis of TβRII in cells expressing PTH1R. HEK293 cells or HEK293-PTH1R cells were transfected with TβRII-Flag for 16 h. The cells were then stimulated with PTH or PTH^{TMR} for the indicated periods and immunostained with anti-Flag antibody (green). TβRII is internalized after PTH treatment (a) and co-localized with PTH^{TMR} (red) during endocytosis (b). Scale bars, 10 μm. (c) Quantitative assessment of endocytic trafficking of TβRII induced by PTH. The cells were classified by expressing TβRII into three types (membrane, membrane/vesicle and vesicle) as described in Methods. (d, e) PTH decreases the endogenous TβRII level at the cell surface. HEK293-PTH1R cells were stimulated with PTH for the indicated periods and then biotinylated at the cell surface. The cell lysates were immunoprecipitated (IP) with anti-TβRII antibody, and the immunoprecipitates were probed with streptavidin-labelled horseradish peroxidase to detect cell-surface TβRII, and with anti-TβRII antibody to detect total TβRII (d). The ratio of cell-surface TβRII to total TβRII was determined with the value at time zero being set to 100% (e). (f) PTH recruits TβRII into close proximity. FRET was performed between PTH^{TMR} and TβRII^{GFP} or TβRII-K277R^{GFP}. The efficiency of FRET was determined by donor (GFP) recovery after acceptor (TMR) photobleaching. Two asterisks, $P < 0.01$ versus the unbleached area. Results in panels c, e and f are shown as the mean $\pm$ s.d. (panels c, e, $n = 3$; panel f, $n = 4$). Uncropped images of blots are shown in Supplementary Information, Fig. S9.

Figure 2.

PTH induces the formation and internalization of TβRII-PTH1R complexes. **(a)** Strategy of YFP-based PCA (YFP-PCA). **(b, c)** PTH, but not TGF-β, induces an interaction of PTH1R with TβRII as shown with YFP-PCA. HEK293 cells were transfected with indicated plasmids and then treated with PTH or TGF-β for 30 s. The reconstituted YFP fluorescence appears green. The cells were stained with 4,6-diamidino-2-phenylindole (DAPI) (blue)**(b)**, and YFP fluorescence was quantified by fluorimetry normalized to untransfected cells. Scale bars, 10 μm. Two asterisks, $P < 0.01$ versus untreated **(c)**. **(d)** Co-localization of PTH and the TβRII-PTH1R complex appears yellow. Scale bars, 10 μm. **(e, f)** TβRII specifically interacts with PTH1R in response to PTH in immunoprecipitation assays. HEK293 cells co-transfected with indicated plasmids **(e)** or primary calvarial osteoblasts **(f)** were treated with PTH for 30 min. Cell extracts were immunoprecipitated (IP) and immunoblotted (IB) with the indicated antibodies. The asterisk indicates that the cells were lysed in IP buffer with 0.5% SDS for immunoblotting. HA, haemagglutinin. **(g, h)** PTH induces the formation of PTH1R dimer/oligomer as shown with YFP-PCA illustrated in **g**. HEK293 cells co-expressing PTH1R-YFP1 and PTH1R-YFP2

were treated with PTH and then stained with DAPI (blue). Reconstituted YFP fluorescence on formation of the PTH1R dimer/oligomer appears green (**h**, upper panel). Co-localization of PTH^{TMR} (red) and the PTH1R dimer/oligomer (green) appears yellow (**h**, lower panel). Scale bars, 10 μ m. (**i**) T β RII is mainly co-localized with PTH-induced PTH1R dimer/oligomer. HEK293 cells co-expressing PTH1R–YFP1, PTH1R–YFP2 and T β RII–Flag were treated with PTH for 5 min and then immunostained with anti-Flag antibody. Reconstituted YFP fluorescence appears green, T β RII appears red, and co-localization of T β RII and the PTH1R dimer/oligomer appears yellow. Results in panels **c** are shown as the mean $\pm$ s.d. (n = 3). Uncropped images of blots are shown in Supplementary Information, Fig. S9.

Figure 3.

PTH induces recruitment and co-localization of TβRII with β-arrestin. **(a)** PTH^{TMR} (red)-induced recruitment of β-arrestin2-GFP (βarr-GFP; green) expressed in HEK293-PTH1R cells. The cells were treated with PTH^{TMR} for 30 min (right three panels) or were untreated (left panel). **(b)** PTH induces co-localization of TβRII with β-arrestin2-GFP. The HEK293-PTH1R cells co-transfected with TβRII-Flag and β-arrestin2-GFP were treated with PTH^{TMR} for 30 min. TβRII-Flag (blue) was immunostained with anti-Flag antibody. Co-localization of PTH^{TMR}, TβRII and β-arrestin2-GFP in the merged image appears white. Scale bars, 10 μm.

Figure 4.

Endocytosis of TβRII–PTH1R coordinates signalling of PTH and TGF-β. **(a–c)** PTH inhibits the TGF-β-induced phosphorylation of Smad2, the nuclear accumulation of phosphorylated Smad2/3 and the interaction between Smad2 and Smad4, as determined with immunoblotting (IB), immunostaining and immunoprecipitation (IP), respectively. C3H10T1/2 cells transduced with pMSCV-PTH1R–HA retrovirus were pretreated with PTH for 30 min, and then treated with TGF-β for 15 min for immunoblotting, or 30 min for immunostaining and for immunoprecipitation. **(d)** PTH represses TGF-β-induced SBE luciferase transcription activities in HEK293 cells. RLU, relative luciferase units. **(e)** Knocking down of β-arrestins eliminates the inhibitory effect of PTH on TGF-β-induced Smad2 phosphorylation in HEK293-PTH1R cells. **(f)** Expression of TβRII inhibits the PTH-induced accumulation of intracellular cAMP. UMR106 cells transfected with the indicated plasmids were stimulated with the indicated concentrations of PTH for 15 min. cAMP accumulation was detected, and the values were normalized to the level induced by 100 nM PTH, which was 74.0 ± 7.8 s.d. pmol per 35-mm well. Asterisk, $P < 0.05$ versus vector. **(g)** Expression of TβRII inhibits PTH-induced phosphorylation of CREB in UMR106 cells. The asterisk indicates that the cells were lysed in IP buffer with 0.5 % SDS for immunoblotting. **(h)** Expression of TβRII represses PTH-induced CRE luciferase transcription activities in UMR106 cells. RLU, luciferase units. **(i)** Expression of TβRII decreases the cell-surface PTH1R level. HEK293-PTH1R cells transfected with the indicated plasmids were treated with ¹²⁵I-PTH for 30 min. The amount of ¹²⁵I-PTH bound to the cell-surface PTH1R was determined with a scintillation counter. Asterisk, $P < 0.05$ versus vector; two asterisks, $P < 0.01$ versus vector. Results in panels **d**, **f**, **h** and **i** are shown as the mean $\pm$ s.d. ($n = 3$). Uncropped images of blots are shown in Supplementary Information, Fig. S9.

Figure 5.

TβRII directly phosphorylates PTH1R to modulate endocytosis of PTH1R/TβRII complex. (a) Amino acids in the PTH1R cytoplasmic domain from 461 to 593 contain four S/T clusters (underlined). Serines or threonines in these clusters (grey background) were replaced by alanines to generate four mutants (M1–M4) (upper panel). Residues 484–498 of PTH1R share conserved S/T residues with TβRI at residues 182–194 (lower panel). (b) TβRII kinase domain (cTβRII) directly phosphorylates the intracellular domain of PTH1R (cPTH1R) by *in vitro* kinase assay. Glutathione *S*-transferase (GST), cTβRII–GST or cTβRII–K277R–GST proteins alone (top panel), or in combination with PTH1R–GST (second panel), were pulled down by glutathione beads. The beads-bound proteins were incubated with [γ-³²P]ATP, and the protein-associated radiolabel was detected by phosphorimager analysis. The GST-tagged proteins were revealed by Coomassie brilliant blue staining (lower two panels). (c) Phosphorylation of PTH1R was increased by expression of TβRII, but not by expression of TβRII–K277R, as determined by *in vivo* kinase assay. HEK293-PTH1R cells were transfected with plasmids as indicated and labelled with [γ-³²P]ATP. The cell lysates were immunoprecipitated by anti-HA antibody, and the protein-associated radiolabel was detected by phosphorimager analysis. The asterisk indicates that the cells were lysed in IP buffer with 0.5% SDS for immunoblotting. (d) A fragment of PTH1R cytoplasmic domain containing residues 461–551, which contains four

S/T clusters (cPTH1R (461–551)), was sufficient for phosphorylation by cTβRII. Asterisk, false-positive band. **(e)** Mutation of the S/T cluster in residues 484–498 of cPTH1R (cPTH1R-M2) disrupted the phosphorylation of cPTH1R by cTβRII. **(f)** TβRII kinase directly phosphorylates a synthesized small peptide (residues 477–506) of PTH1R. The peptide was resolved on a 4–20% Tris/Tricine/SDS gel, and the peptide-associated radiolabel was revealed by phosphorimager analysis (upper right). Mass spectrometry analysis showed two peaks, at m/z 3,399.7 and 3,479.7, respectively (lower, arrows), different by 80 units (the mass of a phosphate group) and 160 units from m/z 3319.7 (the mass of the peptide). **(g, h)** The M2 mutation (PTH1R-M2–HA) or a constitutively activated mutation (PTH1R-H223R–HA) disrupted the interaction between TβRII and PTH1R **(g)** and the inhibitory effect of PTH1R on the TGF-β-induced Smad2 phosphorylation **(h)**. Uncropped images of blots are shown in Supplementary Information, Fig. S9.

Figure 6.

Conditional disruption of $T\beta RII$ in osteoblasts increases cell-surface expression of PTH1R and results in a bone phenotype consistent with overactivity of PTH1R. (a) Primary calvarial osteoblasts from $T\beta RII^{-/-}$ mice show increased binding of ^{125}I -PTH. (b) Primary calvarial osteoblasts from $T\beta RII^{-/-}$ mice show increased basal accumulation of cAMP. The cAMP level in $T\beta RII^{+/+}$ cells was 6.1 ± 5.3 s.d. pmol per 35-mm well. (c) Cre-mediated ablation of $T\beta RII$ in primary $T\beta RII^{fllox/fllox}$ calvarial osteoblast ($\Delta T\beta RII$) activates CREB phosphorylation independently of ligand. (d) PTH-induced phosphorylation of PTH1R was inhibited in $\Delta T\beta RII$ cells. WT, wild type. (e) Faxitron radiographs of lower limbs from 8-week-old $T\beta RII^{+/+}$ and $T\beta RII^{-/-}$ mice. (f) μCT images of femora. Scale bar, 1.0 mm. (g) Three-dimensional reconstruction of the proximal tibiae from μCT scans. Scale bar, 1.0 mm. (h) Micrographs from proximal tibiae stained with H&E. Scale bars, 0.5 mm. (i) Cross-sections of cortical bone at femora diaphysis from μCT scans (left two panels; scale bar, 200 μm) and H&E-stained sections of tibia diaphysis from $T\beta RII^{+/+}$ and $T\beta RII^{-/-}$ mice (right two panels; scale bar, 50 μm). (j–l) Quantitative μCT analysis at distal femora: bone mineral density measurements (j), trabecular bone volume (k) and cortical bone volume (l). (m) Quantitative μCT for cortical bone thickness at mid-diaphysis of femora. Data shown represent means $\pm$ s.d. for five animals per group. Asterisk, $P < 0.05$ versus $T\beta RII^{+/+}$; two asterisks, $P < 0.01$ versus $T\beta RII^{+/+}$. Uncropped images of blots are shown in Supplementary Information, Fig. S9.

Figure 7.

Conditional disruption of $T\beta RII$ in osteoblasts increases bone remodelling. (**a, b**) The number of osteoblasts was measured at the metaphyseal trabecular bone (**a**) and the endosteal cortical bone (**b**) of tibiae. The number of osteoblasts per bone perimeter (N.ob/B.Pm) is shown. (**c, d**) The number of osteoclasts was measured at the metaphyseal trabecular bone (**c**) and the endosteal cortical bone (**d**) of tibiae. The number of osteoclasts per bone perimeter (N.oc/B.Pm) was shown. (**e**) Calcein double labelling in the metaphyseal trabecular bone (upper panel; scale bar, 50 μ m) and the diaphyseal cortical bone (lower panel; scale bar, 25 μ m) of tibiae from 8-week-old $T\beta RII^{+/+}$ and $T\beta RII^{-/-}$ mice. (**f, g**) Eight-week-old $T\beta RII^{-/-}$ mice show increased osteocalcin (**f**) and CTX (**g**) in serum. (**h**) Basal mRNA level of RANKL was increased in $\Delta T\beta RII$ cells. Data shown represent means $\pm$ s.d. for five animals per group. Asterisk, $P < 0.05$ versus $T\beta RII^{+/+}$; two asterisks, $P < 0.01$ versus $T\beta RII^{+/+}$.

Figure 8.

Injection of PTH(7–34) or disruption of PTH1R in osteoblasts rescues the bone phenotype exhibited in the $T\beta RII^{-/-}$ mice. (a) 1-month-old $T\beta RII^{-/-}$ male mice were injected subcutaneously with vehicle or PTH(7–34) at indicated dosages each day for 4 weeks. Representative μ CT images of cross-sections at the distal femora (scale bar, 500 μm) (upper) and calcein double labelling in the metaphyseal trabecular bone at distal femora (scale bar, 25 μm) (lower) are shown. (b) Quantitative μ CT analysis at the distal femora. BV/TV indicates trabecular bone volume expressed as a percentage of tissue volume. (c–f) Quantitative analysis of the serum markers. Calcium (c), osteocalcin (d), CTX (e) and intact PTH (f) were measured in serum collected at the time at which the mice were killed. (g, h) Histomorphometric analysis of the number of osteoblasts (g) or osteoclasts (h) per bone perimeter at the metaphyseal trabecular bone of tibiae. (i, j) Seven-week-old $T\beta RII^{-/-}$ PTH1R $^{+/+}$ mice reproduced the bone phenotype observed in $T\beta RII^{-/-}$ mice with injection of PTH(7–34). BV/TV at the distal femora (i) and representative μ CT images of cross-sections at the distal femora (scale bar, 500 μm) (j; upper panels) and calcein double labelling in the metaphyseal trabecular bone at distal femora (scale bar, 25 μm) (j; lower panels) are shown. (k) Model of the integration of the PTH and TGF- β signalling pathway at the level of PTH1R and T β RII receptors through endocytosis. Data shown represent means $\pm$ s.d. for

three animals per group. Asterisk, $P < 0.05$ versus $T\beta RII^{-/-}$ with vehicle, or versus $T\beta RII^{-/-}/PTH1R^{+/+}$; two asterisks, $P < 0.01$ versus $T\beta RII^{-/-}$ with vehicle.