Sorting nexin 27 couples PTHR trafficking to retromer for signal regulation in osteoblasts during bone growth

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\textbf{ABSTRACT} The parathyroid hormone 1 receptor (PTHR) is central to the process of bone formation and remodeling. PTHR signaling requires receptor internalization into endosomes, which is then terminated by recycling or degradation. Here we show that sorting nexin 27 (SNX27) functions as an adaptor that couples PTHR to the retromer trafficking complex. SNX27 binds directly to the C-terminal PDZ-binding motif of PTHR, wiring it to retromer for endosomal sorting. The structure of SNX27 bound to the PTHR motif reveals a high-affinity interface involving conserved electrostatic interactions. Mechanistically, depletion of SNX27 or retromer augments intracellular PTHR signaling in endosomes. Osteoblasts genetically lacking SNX27 show similar disruptions in PTHR signaling and greatly reduced capacity for bone mineralization, contributing to profound skeletal deficits in SNX27-knockout mice. Taken together, our data support a critical role for SNX27-retromer mediated transport of PTHR in normal bone development.

\textbf{INTRODUCTION} Growth and refurbishment of the vertebrate skeleton are strictly dependent on the coordinated cross-talk between bone-resident cells and their ability to respond to external stimuli via cell surface signaling receptors (Kronenberg, 2003; Sims and Martin, 2014). The type 1 parathyroid hormone receptor (PTHR), a member of the class B G protein–coupled receptor (GPCR) family, is one of the best characterized and most important signaling receptors, modulating bone remodeling and mineral ion homeostasis (Gardella and Vilardaga, 2015). In mammals, PTHR is primarily present in bone cells and kidney proximal tubules, where it elicits its physiological function(s) in blood calcium and phosphate metabolism and tissue development through the endocrine and paracrine/autocrine actions of two distinct peptide ligands, PTH and PTH-related protein (PTHrP; McCauley and Martin, 2012; Gardella and Vilardaga, 2015). Not surprisingly, abnormal expression and/or function of PTHR correspond with severe bone dysmorphisms (termed skeletal dysplasia) and various metabolic syndromes in both humans and mice (Karaplis and Seeman, 2007; Kraenzlin and Meier, 2011). Specifically, disruption in PTHR signaling affects differentiation of cartilage-synthesizing chondrocytes and bone-forming osteoblasts (OBs), leading to alterations in postnatal growth and development of skeletal long bones. Intermittent injection of a PTH analogue is used in the clinical management of osteoporosis (Gardella and Vilardaga, 2015). In mammals, PTHR is primarily present in bone cells and kidney proximal tubules, where it elicits its physiological function(s) in blood calcium and phosphate metabolism and tissue development through the endocrine and paracrine/autocrine actions of two distinct peptide ligands, PTH and PTH-related protein (PTHrP; McCauley and Martin, 2012; Gardella and Vilardaga, 2015). Not surprisingly, abnormal expression and/or function of PTHR correspond with severe bone dysmorphisms (termed skeletal dysplasia) and various metabolic syndromes in both humans and mice (Karaplis and Seeman, 2007; Kraenzlin and Meier, 2011).

PTHR is released by the parathyroid gland in response to low serum calcium levels, which stimulate PTHR in OBs and activate parallel signaling pathways through trimeric G protein subunits G\textsubscript{12} and G\textsubscript{13}.
Gαq. The Gαq cascade activates phospholipase C, leading to inositol-(1,4,5)-trisphosphate production, elevated Ca2+, protein kinase C (PKC) activation, and subsequent mitogen-activated protein kinase (MAPK)-mediated cell proliferation. Gαq activates adenyl cyclases to produce cAMP, stimulating the protein kinase A (PKA) pathway, transcription factor activation, and production of receptor activator of NF-κB ligand (RANKL), eventually stimulating RANK-expressing osteoclasts (bone-degrading cells). These signaling cascades in OBs result in the phosphorylation of downstream targets, most notably cAMP-response element-binding protein (CREB), the kinase Akt, and MAPKs, including extracellular signal–regulated kinases ERK1/2. As with other GPCRs, PTHR-evoked cAMP production has long been believed to originate almost exclusively at the cell membrane, with signal termination achieved upon receptor phosphorylation via G protein receptor kinases, β-arrestin-mediated internalization into early endosomes, and lysosomal degradation, that is, “canonical” GPCR signaling (Lohse et al., 1992; Ferrari et al., 1999; Bisello et al., 2002; Chavun et al., 2002; Boussein et al., 2005). However, it is now known that cAMP accumulation persists long after PTHR is internalized into endosomes (Ferrandon et al., 2009). This “noncanonical” endosomal GPCR signaling involves an elaborate ensemble of proteins, which function to promote PTHR-mediated cAMP accumulation, including G proteins, β-arrestins, and small Rab GTPases (Rab5), which facilitate endocytosis and trafficking of the receptor to early endosomes (Vilardaga et al., 2012). Here cAMP generation persists until final signal arrest from the receptor upon PKA-driven intraluminal acidification and the recruitment of the retromer protein complex (Ferrandon et al., 2009; Feinstein et al., 2011; Gidon et al., 2014).

Retromer is an evolutionarily conserved endosomal protein complex composed of a trimeric core consisting of vacuolar protein sorting 26 (VPS26), VPS29, and VPS35 (Collins, 2008). Retromer plays a central role in endosomal membrane trafficking and is best recognized for regulating retrograde endosome-to–trans-Golgi network (TGN) trafficking of transmembrane cargoes (Seaman, 2012; Gallon and Cullen, 2015). Recently, however, it has been shown that retromer plays an equally important role in recycling of endocytosed protein to the plasma membrane (PM) via a complex with sorting nexin 27 (SNX27; Temkin et al., 2011; Steinberg et al., 2013). SNX27 is a member of a large family of proteins containing a membrane-binding phox homology domain that controls endosomal recruitment (Cullen and Korswagen, 2012; Teasdale and Collins, 2012). It also possesses a C-terminal 4.1/ezrin/moesin/radixin (FERM)–like domain that mediates binding to transmembrane cargoes containing F-x-N-P-x-Y sequences (Ghai et al., 2011, 2013) and a unique N-terminal postsynaptic density 95/discs large/zonula occludens-1 (PDZ) domain. The PDZ domain binds to type 1 PDZ-binding motifs (PDZbms) with the consensus [TS]-x-[φ] (where φ is a bulky hydrophobic amino acid) to mediate endosomal trafficking (Joubert et al., 2004; Lunn et al., 2007; Lauffer et al., 2010; Balana et al., 2011; Cai et al., 2011; Temkin et al., 2011; Valdes et al., 2011; Hayashi et al., 2012; Wang et al., 2013; Loo et al., 2014). SNX27 and retromer form a complex to mediate the trafficking of many PDZb-containing cargoes, and cargo interactions with SNX27 are allosterically enhanced by the retromer subunit VPS26 binding directly to the PDZ domain (Steinberg et al., 2013; Gallon et al., 2014). Although SNX27–retromer disruption has been associated with neuronal defects in mouse models and human disease (Muhammad et al., 2008; Cai et al., 2011; Vilarino-Guell et al., 2011; Zimprich et al., 2011; Wang et al., 2013, 2014; Loo et al., 2014; Damseh et al., 2015), the range of cargoes bound by the SNX27–retromer complex (Steinberg et al., 2013) points to a much broader physiological role.

Here we use a broad range of structural and cellular approaches combined with mouse knockout models to demonstrate that the SNX27–retromer complex plays a central role in PTHR signaling and retromer-mediated endosomal recycling during bone growth and remodeling in vivo. PTHR is coupled to the retromer complex via the structurally unique SNX27 PDZ domain. We show that the assembly of this complex occurs on endosomes in response to PTH stimulation, where it directs PTHR to retromer for recycling to the cell surface, and provide evidence that the PTHR-SNX27-retromer association is physiologically required to restrict PTH signaling in OBs, where disruption of SNX27 impairs OB activity and contributes to a severe growth and maturation deficit in the skeleton of SNX27-deficient mice. Thus we propose that SNX27 serves as an endosomal PDZ-cargo adaptor that links PTHR to the retromer trafficking complex to regulate PTHR signaling during postnatal bone development.

RESULTS

SNX27 PDZ domain interacts with PTHR in endosomes after PTH stimulation

The type 1 PDZ motif (T-TM-V-M) encoded at the C-terminus of PTHR is highly conserved across species and similar to other known SNX27–PDZ–binding proteins including the potassium channel Kir3.3, making PTHR a potential SNX27-PDZ–interacting cargo (Figure 1, A and B). To test this, we coexpressed C-terminal green fluorescent protein (GFP)–tagged full-length (FL) and mutant variants of SNX27 lacking either the entire PDZ-domain (ΔPDZ) or a single–amino acid point substitution (H114A) known to destabilize PDZ-associated SNX27 cargoes (Lauffer et al., 2010; Gallon et al., 2014) in a human embryonic kidney cell (HEK293) clone stably expressing myc-tagged PTHR (Figure 1, C and D) and communoprecipitated the resulting complexes using anti-GFP coated beads (GFP-TRAP). We chose HEK293 cells exogenously expressing PTHR as a model because they have been reliably used to study PTHR signaling and trafficking (Ferrandon et al., 2009; Feinstein et al., 2011). myc-PTHR bound to the full-length SNX27 (Figure 1E). In contrast, no detectable levels of PTHR were observed after its communoprecipitation with GFP alone (control). PTHR binding was reduced upon mutation of the SNX27 PDZ domain (H114A) and completely abolished after removal of the entire PDZ protein–interaction module (ΔPDZ; Figure 1E).

We next reasoned that if SNX27–PTHR interaction is physiologically important, then the two proteins should also colocalize to the same intracellular compartments in intact cells. Here PTH–PTHR ligand receptor complexes were visualized using N-terminally GFP-tagged (exon 2) PTHR (GFP-N–PTHR) in combination with a fluorescent tetramethylrhodamine (TMR)–labeled PTH(1–34) analogue (herein PTH-TMR), which has been routinely used as a probe to monitor PTH internalization and trafficking (Ferrandon et al., 2009; Qiu et al., 2010; Feinstein et al., 2011). When PTH-TMR was administered to a GFP-N–PTHR-HEK293 cell clone at steady state (0; 4°C, 10 min), PTH–PTHR complexes localized predominantly at the cell surface, with little to no fluorescence overlap with endogenous SNX27, which was restricted to a population of intracellular vesicles (Figure 1F). These vesicles were identified as endosomes by their colocalization with the early endosome marker early endosome antigen-1 (EEA1), small GTPase Rab5, and phosphatidylinositol 3-phosphate lipid sensor Fab1p/PTB/Vac1/EEA1 (FYVE; Supplemental Figures S1 and S2). Colocalization between SNX27 and GFP-N–PTHR on endosomes was evident within 5 min of PTH stimulation, with peak fluorescence overlap observed at 15–30 min poststimulation (Figure 1F). At 60 min poststimulation, a subset of SNX27-bearing endosomes overlapped, albeit partially, with GFP-N–PTHR at a perinuclear compartment. This
SNX27 interacts with internalized PTHR after activation. (A) Schematic drawing of PTHR, highlighting the PDZbm at the intracellular C-terminus. (B) Sequence conservation of the PDZbs of PTHR between species, together with published PDZbs of known SNX27-interacting partners β2AR and Kir3.3, using Jalview 2. (C, D) Schematic illustration and expression of C-terminally GFP-tagged SNX27 full-length (FL), PDZ truncation (∆PDZ), and H114A mutant proteins in HEK293 cells. Bar, 10 μm. (E) Coimmunoprecipitation of HEK293 expression of C-terminally GFP-tagged SNX27 full-length (FL), PDZ truncation (∆PDZ), and H114A mutant proteins in HEK293 cells. Bar, 10 μm. (F) Agonist and antagonist stimulation (min) of PTHR and SNX27 on endosomes in stable HEK293 cells expressing PTHR or PTHR ΔPDZbm and endogenous SNX27 in HEK293 cells. Bar, 10 μm. (G–I) Comparative colocalization analysis of internalized PTHR and SNX27 on endosomes in stable HEK293 cells expressing PTHR or PTHR ΔPDZbm at 15 min poststimulation. Bar, 10 μm. Inset, magnified boxed region. Bar, 1 μm. ***p < 0.001.

To explore further the spatiotemporal relationship between PTHR and SNX27 during agonist stimulation, we next coexpressed SNX27-mCherry and GFP-N-PTHR in HEK293 cells and followed their response to PTH (1-34) by time-lapse confocal microscopy. When stimulated with agonist (100 nM PTH for 15 min), GFP-N-PTHR was internalized into endosomes that colocalized with SNX27-mCherry and moved coordinately over time (Figure 2, A–D, and Supplemental Video S1). Of interest, PTHR internalization was observed most conspicuously at peripheral membrane ruffles in response to PTH and coincided with the colocalization of SNX27 with PTHR-bearing endosomes (Figure 2, E–G, and Supplemental Video S2). Although they occupy the same endosomes, SNX27 and PTHR signals were not completely superimposed immediately after PTH stimulation but instead spatially segregated along the outer limiting membrane, as confirmed by circumferential line-scan analysis (Figure 2B). At later time points, however (i.e., ~75 s after PTH stimulation), SNX27 and PTHR converged at common tubular protrusions emanating from the endosome surface (Figure 2, F and G). These protrusions were decorated with the recycling endosome marker Rab4b (Supplemental Figure S1) and were strikingly reminiscent of the retro-romer recycling tubules previously described for β2AR (Temkin et al., 2011). Taken together, these data indicate that PTHR forms a complex with SNX27 via its PDZ-domain, which requires both the PTH-induced internalization of PTHR and an intact PTHR PDZbm for SNX27–PTHR association on endosomes.

**SNX27 links PTHR to the retro-romer complex**

To define the precise step(s) in the PTHR sorting and/or delivery cascade in which SNX27 operates, we further mapped its intracellular trafficking itinerary in relation to established components of the PTHR endocytic machinery. Because SNX27 (Temkin et al., 2011) and PTHR (Feinstein et al., 2011) have been independently shown to traffic along retromer-associated recycling pathway(s), we first assessed for colocalization between SNX27–PTHR and retromer after PTH stimulation. We observed extensive colocalization between SNX27 (SNX27-GFP), PTHR (as monitored by PTHR-TMR), and retromer (here using antibodies against endogenous VPS35; Arighi et al., 2004) on endosomes 15 min after PTH stimulation (Figure 3A). This close overlap was verified by measuring the fluorescence intensity peaks between respective image channels by correlative line-scan analyses (Figure 3B). SNX27–retromer association appeared independent of the PTHR endocytosis channel, and was strikingly reminiscent of the retromer recycling tubules previously described for β2AR (Temkin et al., 2011). Taken together, these data indicate that PTHR forms a complex with SNX27 via its PDZ-domain, which requires both the PTH-induced internalization of PTHR and an intact PTHR PDZbm for SNX27–PTHR association on endosomes.

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whereas SNX27-retromer association remained stable throughout the stimulation period (Figure 3, C and D), consistent with the view that they form part of the same endocytic recycling complex (Steinberg et al., 2013; Gallon et al., 2014).

The PDZ-dependent SNX27–PTHR interaction, together with the striking colocalization observed between these binding partners and retromer, suggested that SNX27 serves as a physical platform to scaffold PTHR to the retromer complex. To assess this, we used isothermal titration calorimetry (ITC) to measure the binding of the putative C-terminal PTHR PDZbm to the SNX27 PDZ domain. We used a synthetic peptide representing the C-terminal eight residues of the PTHR (EEWETVM). In comparison to the type 1 PDZbms of terminal carboxyl group of Met56 establishes an array of hydrogen bonds with the backbone of the conserved GYGF residue stretch in SNX27, whereas Thr2 forms a hydrogen bond with SNX27 His114, explaining the requirement for serine or threonine at this position of the motif. In addition to intermolecular β-sheet main-chain contacts, PDZ recognition extends upstream of the canonical type I PDZ triplet and is mediated mainly by the two Glu5 and Glu5 residues. Glu5 is embraced between SNX27 Asn56 and Arg58, and Glu5 similarly forms a salt bridge with Arg58. This network of interactions cooperatively stabilizes the PTHR peptide in the PDZ cavity, burying a surface area of 470 Å² (PDBePiSA server, www.ebi.ac.uk), and provides a basis for strong and specific association (Figure 4C). The Asn56 and

FIGURE 2: Spatiotemporal interaction of PTHR with SNX27 after endocytosis. (A) Time-lapse confocal microscopy of live HEK293 cells stably expressing GFP-PTHR and SNX27-mCherry. Interaction between GFP-PTHR and SNX27-mCherry is shown at both the basal state (agonist naïve) and 15 min poststimulation with 100 nM PTH(1-34). Bar, 10 μm. (B) Inset from A showing the localization and circumferential line-scan analysis of SNX27-mCherry to PTHR-positive endosomal microdomains, starting at 0°. Bar, 1 μm. (C, D) Time-lapse dynamics of HEK293 cells stably expressing GFP-PTHR and SNX27-mCherry after stimulation with 100 nM PTH(1-34) across 5 min (C) and intensity plot over 300 s (D). (E, F) Mobilization of GFP-PTHR and SNX27-mCherry to endosomes after stimulation with 100 nM PTH(1-34) across 120 s. Bar, 1 μm. (F) Inset from boxed region showing the recruitment of SNX27-mCherry to tubular endosomal protrusions occupied by GFP-PTHR (arrow). Bar, 1 μm. (G) Fluorescence intensity tracking of colocalization between GFP-PTHR and SNX27-mCherry across 360 s after agonist stimulation.

GLUT1 and Kir3.3, two cargoes trafficked by the SNX27–retromer pathway (Steinberg et al., 2013), the PTHR PDZbm bound more tightly to the SNX27 PDZ domain (Kd = 6.3 μM, compared with 17 and 154 μM for Kir3.3 and GLUT1, respectively; Figure 4A). Of importance, this binding is allosterically enhanced upon association with VPS26A (Kd = 2.2 μM; enthalpic increase from ΔH = −13.7 to −19.7 kcal/mol), confirming that the SNX27–retromer complex physiologically forms a trimeric complex with PTHR. This allosteric enhancement is similar to what we previously observed for Kir3.3 (Gallon et al., 2014). By comparison, the PDZ binding–defective mutant of SNX27 (H114A) showed a dramatically reduced binding signal, as expected.

To investigate PTHR recognition at the atomic level, we next determined the crystal structure of the SNX27 PDZ domain bound to a PTHR peptide at ultrahigh resolution (0.95 Å; (Figure 4B and Table 1). The final model contains residues 40–135 for the SNX27 PDZ domain and 587–593 for the PTHR peptide (EEWETVM). Using the common nomenclature for PDZbm sequences, this corresponds to residues −6 to 0. High-quality electron density maps demonstrate that the PDZbm of PTHR binds in a similar orientation as in the previously published SNX27 PDZ–Kir3.3 complex (Balana et al., 2011). The PTHR peptide forms an antiparallel β-strand interaction with β6 in the SNX27 PDZ domain. Two glutamic acid side chains (Glu5 and Glu5) associate with a basic patch on the SNX27 surface, in particular forming an electrostatic interaction that encompasses the conserved Arg58 side chain of SNX27. This interaction could explain both the relatively high affinity of this peptide (and the similar Kir3.3; Gallon et al., 2014)) for SNX27 and the highly favorable enthalpy of association measured by ITC (Figure 4B). The Glu5 and Gin7 residues were not well ordered in our crystal, suggesting that these amino acids are not directly implicated in SNX27 recognition. In detail (Figure 4C), our structure confirms that the C-terminal PDZ triplet TVM is crucial for the interaction. The
Arg56 of SNX27 are conserved across species, as is the GYGF stretch that mediates binding to all PDZbm triplets (Ye and Zhang, 2013; Figure 4D). To confirm the structural basis of SNX27 binding to the PTHR, we mutated the PTHR sequence by either adding an Ala to the C-terminus or altering the Glu3 side chain to Ala. Both alterations abolished binding to the PTHR peptides, according to ITC (Figure 4A). Taken together, these data confirm that PTHR binds directly to the SNX27 PDZ domain and that this interaction site is located close to the VPS26-binding surface of SNX27 (Figure 4E) and is allosterically coupled to SNX27–VPS26 association, as for Glut1 and Kir3.3 (Gallon et al., 2014).

Depletion of SNX27–retromer causes sustained PTHR signaling

Because termination of cAMP generation from endosomal PTHR requires retromer (Feinstein et al., 2011) and the results described so far indicate that SNX27 is the molecular adaptor that directs PTHR to retromer, we next asked whether SNX27 also functions to restrict cAMP signaling initiated by the receptor. For this, we used plasmid-based short hairpin RNAs (shRNAs) against SNX27 and VPS35 to deplete SNX27 and retromer expression in PTHR-expressing HEK293 cells and assessed the effects on PTHR-evoked cAMP signaling by examining the phosphorylation of several downstream secondary messenger pathways, including CREB, AKT, and MAPK (pERK1/2). Transfection and knockdown (KD) efficiencies were monitored by visualizing the percentage of cells expressing the GFP-retromer KD cells using PTH-TRHR as a probe. Intriguingly, under resting conditions (i.e., temperature block 4°C, 10 min), we found that depletion of either SNX27 or VPS35 protein led to a reduction in PTH-PTHHR surface levels (up to ~50% as quantified by PTH-TRHR intensity) compared with cells expressing nontargeting control shRNA (Figure 5, D and E). Thus the elevated PTHR signaling observed in SNX27- or retromer-depleted cells did not originate from enhanced levels of receptor at the cell surface.

We next checked whether loss of either SNX27 or retromer routes PTH-PTHHR trafficking to a distinct endocytic compartment by monitoring the fate of internalized PTH-TRHR over various time points (up to 120 min) after PTH stimulation and washout in SNX27 and retromer-depleted cells in reference to the early endosome marker EEA-1. In PTH-stimulated PTHR-HEK293 cells expressing the nontargeting shRNA (unpublished data) or neighboring cells lacking expression of the SNX27 shRNA reporter, PTH-PTHHR consistently internalized into EEA-1-positive early endosomes at 5 min poststimulation (Figure 5, F and G) before entering VPS35-positive endosomes (typically within 10–30 min), with PTH-TRHR signal dissipating by 60–120 min poststimulation/washout, a period that coincided with repopulation of the receptor back to the PM (unpublished data). In comparison, whereas the low cell surface levels of PTH-PTHHR precluded the ability to accurately track internalized PTH-TRHR in SNX27 and retromer KD cells (Figure 5, D and F) at time points immediately after administration of the PTH analogue (5–30 min), we did, however, observe PTH-TRHR signal in vesicular clusters at the perinuclear

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region of SNX27 and retromer KD cells at later time points (≥60 min). These accumulated and were most conspicuous 120 min after PTH stimulation (Figure 5, F and H). These perinuclear clusters were distinct from early endosomes, as they lacked EEA-1 and were rarely observed in neighboring nontransfected or nontargeting shRNA control cells but were frequent in cells depleted of either SNX27 or VPS35 (Figure 5, F and H), implying that this was a general phenomenon upon disruption of the SNX27–retromer machinery. To reveal the nature of these PTH–PTHR-containing compartments, we performed immunostaining against various subcellular markers, including the lysosomal integral membrane protein (LAMP-1; Figure 5H), which identified this population of perinuclear vesicles as lysosomes. These data imply that down-regulation of SNX27 and retromer expression reduces PTHR surface levels, leads to an increase in sustained endosomal signaling, and stalls the entry of PTH into degradative lysosomes.

OBs lacking SNX27 display overactive PTH signaling and impaired mineralization activity in vitro

To confirm whether the observed disturbances in PTHR signaling in SNX27-retromer-depleted HEK293 cells translated physiologically, we further assessed the integrity of PTHR signaling in OBs (which normally express PTHR) isolated from SNX27-deficient (SNX27−/−) mice (Cai et al., 2011). First, we checked the functional consequence of SNX27 ablation on PTH-induced cAMP accumulation across a range of PTH concentrations. We observed a significant elevation in PTH-induced cAMP accumulation in OBs derived from bone marrow mesenchymal stem cells (BMSCs) from SNX27−/− mice compared with those from wild-type (WT) littermates (Figure 6A). We then probed cAMP-associated secondary messenger signaling pathways including pCREB, pAKT, and MAPK (pERK1/2), and found again that they were significantly increased in the early phase (5–30 min) of PTH stimulation (Figure 6, B–D) despite reduced cell surface PTH–PTHR levels (Supplemental Figure S5). Because PTHR also operates through the paracrine/autocrine ligand PTHrP in OBs (Miao et al., 2005), we also checked whether SNX27 deficiency altered PTHrP-induced PTHR signaling. In this instance, PTHrP(1–141) stimulation induced elevated CREB and AKT phosphorylation profiles (most evident between the 5- and 30-min time points) in OBs derived from SNX27−/− mice compared with WT controls (Figure 6B). Intriguingly, we noted that the intensity of CREB phosphorylation was consistently more pronounced in OBs stimulated with PTHrP than in those exposed to PTH over the 90-min stimulation period (Figure 6C), possibly reflecting structural differences between PTHrP(1–141) and PTH(1–34) peptides or distinctions in the activation/deactivation kinetics, as previously reported for these two ligand systems (Ferrandon et al., 2009). PTH signaling is critical to OB differentiation and bone anabolic function (Datta and Abou-Samra, 2009). Therefore, to study the cumulative effect of PTHR deregulation on the function of SNX27−/− OBs, we assessed their differentiation and bone formation activity in vitro. Whereas the number of alkaline phosphatase (ALP)–expressing OBs, we assessed their differentiation and bone formation activity in vitro. Whereas the number of alkaline phosphatase (ALP)–expressing OBs, we assessed their differentiation and bone formation activity in vitro.
Postnatal SNX27-knockout mice exhibit skeletal growth deficits and reduced bone mass

PTHR is a crucial regulator of postnatal bone development and remodeling. Because our studies identify PTHR as a novel SNX27-interacting cargo and a role for SNX27 in bone morphogenesis had not been ascribed, we further assessed the pathophysiological consequences of SNX27 ablation on the mouse skeleton. SNX27−/− mice die at 4 wk postpartum (Cai et al., 2011). Skeletal examination of P5 SNX27−/− mice by whole-mount preparations and three-dimensional (3D) micro–computed tomographic (μCT) reconstruction revealed overall impairment in skeletal growth in SNX27−/− mice (Figure 7, A and B). Reflecting this, SNX27−/− deficient mice were macroscopically smaller and exhibited shortening of limbs and tails and smaller skull sizes that were of reduced bone density compared with their WT and heterozygous littermates. These developmental disturbances extended to long bones (femurs and tibias), forelimbs (humerus, ulna and radius), and spines of 4-wk SNX2−/− mice (Figure 7, C–F). Detailed longitudinal reconstruction and assessment of microarchitectural bone parameters of femora and tibia isolated from 4-wk-old, sex-matched littermates revealed that bone trabeculation within the metaphyseal region was drastically reduced in SNX27−/− mice (Figure 8, A and C). In addition, severe cortical thinning was observed in cross-sectional views in long bones of SNX27−/− mice compared with their litterate controls (Figure 8B). These profound reductions in trabecular bone volume (BV/TV) and trabecular number (Tb.N.) and thickness (Tb.Th.) were also verified by histomorphometric analysis (Figure 8, D and E).

To distinguish whether the drastically reduced bone mass might result from enhanced bone resorption by osteoclasts or reduced bone formation by OBs, we analyzed the number of tartrate-resistant acid phosphatase (TRAP)–positive osteoclasts at the trabecular of SNX27−/− bones. We found that the osteoclast number per bone surface (OC.N/BS [mm]) was only marginally decreased in the bones of SNX27−/− mice compared with WT littermates (Figure 8, F and G). In comparison, histomorphometric assessment of the number of OBs occupying trabecular bone surfaces (OB.N) revealed that they were substantially reduced in the long bones of SNX27−/− mice (Figure 8, H and I). Reflecting this, the total deposition (OS/BS) and thickness (O.WI [mm]) of recently deposited unmineralized bone (osteoid) was equally reduced in SNX27−/− mice (Figure 8I). Therefore the decrease in bone mass in SNX27−/− deficient mice results from inadequate osteoblastic differentiation and bone formation and not enhanced bone resorption.

Finally, in addition to the observed microarchitectural changes in bone density, there was a conspicuous enlargement of the gap between the tibial and femoral diaphysis and epiphyseal (Figure 8A). Histological assessment of long bones revealed that this cartilaginous expansion arose from postnatal delays in the formation of the secondary ossification center and epiphyseal growth plate in SNX27−/− mice (Figure 8, D and J). Tibial and femoral growth plates of SNX27−/− mice are ~50% longer than in WT (Figure 8K), as confirmed by immunohistochemistry for the chondrocytic marker type II collagen (Figure 8J). Despite these anomalies, the characteristic columnar cellular organization and proteoglycan levels (periodic acid-Schiff [PAS])

TABLE 1: Summary of crystallographic structure determination statistics.

| A. Data collection | B. Refinement |
|--------------------|---------------|
| Wavelength (Å)     | 0.88560       |
| Space group        | P212121       |
| Cell dimensions    | a, b, c (Å)   |
|                    | 37.1, 48.6, 55.7 |
|                    | a, b, g (°)   |
|                    | 90, 90, 90    |
| Resolution (Å)     | 15.72–0.95 (0.97–0.95) |
| Rmerge             | 0.102 (1.70)  |
| Rmeas              | 0.112 (1.84)  |
| Rpim               | 0.046 (0.701) |
| <I>/αI             | 11.7 (1.6)    |
| Total number of reflections | 840,387 (41,355) |
| Total number of unique reflections | 64,036 (3111) |
| Completeness (%)   | 99.8 (100.0)  |
| Multiplicity       | 13.1 (13.3)   |
| Half-set correlation (CC1/2) | 0.994 (0.601) |
| Wilson B-factor    | 9.3           |

Highest-resolution shell is shown in parentheses.
and Safranin O staining) of the growth plate layers were generally well preserved. Viewed collectively, these data indicate that the maturation of the postnatal skeleton in SNX27-deficient mice is severely delayed due, at least in part, to growth plate abnormalities and reduced osteoblastic differentiation and bone formation.

**DISCUSSION**

Here we report multiple lines of evidence to indicate that SNX27 functions as an endosomal cargo adaptor for PTHR, a clinically important GPCR central to normal bone homeostasis and systemic calcium regulation. We show that SNX27 associates with endosomes bearing PTHR after agonist-induced internalization, where it binds directly to PTHR with high affinity and simultaneously scaffolds the receptor to the retromer complex. Recruitment of PTHR to SNX27-retromer–recycling tubules restricts PTH-evoked cAMP signaling, a prerequisite for PTHR signal regulation and function in OBs during postnatal bone growth and remodeling.

The spatiotemporal segregation of GPCR signaling in endosomes is now recognized as a key process in the cell's response to different stimuli (von Zastrow and Sorkin, 2007; Sorkin and von Zastrow, 2009). In the case of PTHR, it has been suggested that activation by PTH can promote sustained endosomal signaling but PTHrP cannot (Vilardaga et al., 2014). Several reports have shown that the endosome also acts as the site for PTHR signaling desensitization after PKA-mediated intraluminal acidification and recruitment of select endocytic machineries (Feinstein et al., 2011; Gidon et al., 2014; Vilardaga et al., 2014).

Our work confirms the central role of retromer in terminating cAMP production by PTHR. However, until now, the molecular basis for this was incompletely understood. It was suggested that retromer likely controlled retrograde trafficking of PTHR to the TGN (Feinstein et al., 2011) and might associate with PTHR via either the structural similarity of the VPS26 retromer subunit to β-arrestins (Shi et al., 2006; Collins, 2008; Aubry et al., 2009) or binding of an aromatic-containing FLN sequence to the retromer VPS35 subunit, although no direct interactions were shown (Feinstein et al., 2011; Vilardaga et al., 2014). Our results clearly show that SNX27 serves as an endosomal platform to integrate PTHR signal activation and retromer-mediated termination. We propose that SNX27 not only confers spatiotemporal control over endosomal PTHR signaling, but that it also directs PTHR to retromer for recycling back to the plasma membrane (possibly via the TGN), thus providing a mechanistic basis for SNX27/retromer–mediated signal attenuation (Figure 9).
FIGURE 6: SNX27 is required for PTHR signal termination in OBs and OB differentiation. (A) Cyclic AMP assay performed on SNX27 WT and SNX27−/− OBs stimulated for 10 min with increasing concentrations of PTH(1-34). (B, C) SNX27 WT and SNX27−/− OBs were stimulated with 100 nM PTH(1-34) (B) or 100 nM PTHrP(1-141) (C), for 15 min, followed by a washout. Signaling pathways (CREB, AKT, ERK) were assessed through Western blotting. (D) Densitometry quantification of protein bands from SNX27 WT and SNX27−/− Western blots (B) were conducted using Photoshop 2014 and expressed as a percentage with respect to their respective control. (E) ALP and alizarin red staining (ARS) of SNX27 WT and SNX27−/− BMSC-derived OBs cultured in vitro under mineralizing conditions (50 μg/ml l-ascorbic acid, 2 mM β-glycerophosphate, 10−8 M dexamethasone) for 21 d. (F) Quantification of ARS-positive nodules per square millimeter using ImageJ. (G) Calvarial OBs isolated from SNX27 WT and SNX27−/− were cultured under mineralizing conditions (+) for 21 d and stained with ALP or ARS. (H) mRNA expression from BMSC-derived SNX27 WT and SNX27−/− OBs was analyzed through quantitative PCR, using HMBS as a reference gene. *p < 0.05, **p < 0.01, and ***p < 0.001.
cascades upon PTHR stimulation, most evident at times that parallel receptor internalization into SNX27 and retromer-bearing endosomes in PTHR-expressing HEK293 cells. We further show that depletion of the SNX27 or retromer leads to a reduction in PTH-PTHR cell surface levels, presumably reflecting reduced recycling rates or mistrafficking of the receptor (e.g., into lysosomes) as observed for other SNX27 PDZbm-bearing cargoes (Steinberg et al., 2013; Gallon et al., 2014). In fact, during the course of this investigation, depletion of either SNX27 or retromer (VPS35) was independently shown to correlate with a 50% decrease in the rate of PTHR recycling at the cell surface following agonist stimulation (J. McGarvey and P. A. Friedman, personal communication).

Intriguingly, despite these reduced cell surface levels, PTH-induced PTHR signaling remains elevated upon SNX27 and retromer disruption. This implies that the levels of PTHR remaining on the cell surface are sufficient to elicit signal sensitization when ligands are added at saturating concentrations. It also implies that newly synthesized PTHR or compensatory retrieval mechanism(s) might help to maintain PTHR levels when SNX27–retromer expression becomes limited. For example, cytosolic NHERF proteins (NHERF1/2) contain PDZ module(s) that also bind to the PTHR PDZbm (Mahon et al., 2002; Sneddon et al., 2003) and have been reported to regulate the membrane retention, trafficking, and desensitization of PTHR (Wang et al., 2007, 2008; Ardura et al., 2011; Wheeler et al., 2011). Of interest, mice lacking NHERF1 (Shenolikar et al., 2002) and patients harboring NHERF1 mutations (Karim et al., 2008) manifest bone abnormalities (osteopenia and osteomalacia; Weinman et al., 2006; Liu et al., 2012) that are attributed, in part, to overactivated PTH.

As with other SNX27-interacting cargo, for example, Kir3.3 (Balana et al., 2011), our findings demonstrate that PTHR cargo recognition is achieved via its C-terminal PDZbm, which binds with high affinity to the N-terminal PDZ domain of SNX27. Of importance, the PDZ domain not only discriminates SNX27 from other SNX family members, but it also serves as an interaction module to simultaneously wire PTHR to the retromer core subunit VPS26. VPS26 binding additionally stabilizes and enhances the affinity of the SNX27 PDZ domain for the PTHR-PDZbm, a feature common to other SNX27–retromer–interacting cargo housing PDZbms (Gallon et al., 2014). Similar to Kir3.3 (PESE; Balana et al., 2011), the PTHR houses a four-amino acid stretch (EEWE) encoded immediately upstream of the canonical PDZbm triplet (TVM) that encompasses two acidic glutamate residues, at positions −3 and −5, that form a strong electrostatic interaction with the conserved Arg58 side chain of the SNX27 PDZ domain. Precisely how this linear amino acid stretch accounts for the preferential binding of affinity of the PTHR observed over other known SNX27 PDZbm-bearing cargo (Clairfeuille and Collins, unpublished data) forms the subject of our future studies.

Together with previous work describing a role for the retromer complex in endosomal PTHR trafficking (Feinstein et al., 2011), our morphological and signaling analyses best support a function for SNX27 as a molecular adaptor for recruiting PTHR into retromer-decorated tubules for endosome-to-cell surface recycling. SNX27/retromer-mediated transport thus attenuates sustained endosome-associated PTHR signaling. This conclusion is supported by our evidence that genetic ablation of SNX27 leads to enhanced cAMP accumulation and subsequent overactivation of downstream signaling cascades upon PTHR stimulation, most evident at times that parallel receptor internalization into SNX27 and retromer-bearing endosomes in PTHR-expressing HEK293 cells. We further show that depletion of the SNX27 or retromer leads to a reduction in PTH-PTHR cell surface levels, presumably reflecting reduced recycling rates or mistrafficking of the receptor (e.g., into lysosomes) as observed for other SNX27 PDZbm-bearing cargoes (Steinberg et al., 2013; Gallon et al., 2014). In fact, during the course of this investigation, depletion of either SNX27 or retromer (VPS35) was independently shown to correlate with a 50% decrease in the rate of PTHR recycling at the cell surface following agonist stimulation (J. McGarvey and P. A. Friedman, personal communication).

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FIGURE 7: Skeletal overview of SNX27-knockout mice. (A) Whole-mount skeletal preparations stained for Alcian blue (cartilage) and Alizarin red (bone). (B) μCT analysis of SNX27-knockout mice (SNX27−/−) compared with heterozygous (SNX27+/−) and WT littermates at P5. Top and side 3D μCT reconstructions of the skull reveal reduced bone density of cranial sutures in SNX27−/− mice. (C, D) Gross anatomy of SNX27 WT and SNX27−/− mice at 4 wk of age. (E) Measurements of the lengths of femurs and tibias from WT and SNX27−/− mice. (F, G) μCT surface reconstructions of forelimbs and spine from WT and SNX27−/− mice. Insets, detailed view of L2–L4. Scale bar, 1 mm. Values are mean ± SEM from six mice per group. ***p < 0.001.
the physiological importance of SNX27 to bone morphogenesis. Of interest, the skeletal dysmorphisms exhibited in SNX27-deficient mice bear some resemblance to those in humans (Schipani et al., 1995, 1996) and mice (Karaplis et al., 1994; Lanske et al., 1999; Calvi et al., 2001; Miao et al., 2002) with disruptions in PTHR and associated G protein–dependent signaling pathways (Weinstein et al., 2006; Wu et al., 2011). On one hand, the reduced cortical bone density, increased cAMP accumulation, overactivation of associated secondary messenger signaling cascades (pCREB, pAKT, and pERK1/2), along with the unregulated transcriptional expression of PTH-reponse genes observed in both unstimulated OBs and total bone, phenocopies those observed in osteoblastic cells from mice bearing constitutively active mutations in the PTHR gene (Calvi et al., 2001) and/or deletion of Gαs (Wu et al., 2011). On the other hand, the expansion of the cartilaginous growth plate (as opposed to its premature closure) clearly distinguish SNX27-deficient mice from those with disrupted PTHR signaling pathways alone (Calvi et al., 2001; signaling and impaired mineralization capacity in OBs (Liu et al., 2012), in keeping with the skeletal deficits observed in SNX27−/− mice in our study. The precise interrelationship that exists between PTHR, NHERFs, and SNX27-retromer is unclear, but it appears that at least two PDZ-dependent modes of PTHR trafficking can operate in vertebrates to modulate cell surface levels—one regulated by NHERF1 (Ardura et al., 2011) and the other presided over by SNX27, as shown in this study.

SNX27 disruption in humans (Damseh et al., 2015) and mice (Cai et al., 2011) manifests in severe growth and developmental disturbances (as shown here) and also correlates with net alterations in the expression of many critical neuronal proteins (Cai et al., 2011; Wang et al., 2013, 2014; Loo et al., 2014). Although SNX27 is expressed in many tissues (including bone; Chan and Pavlos, unpublished data), studies characterizing the effect of its disruption have been largely restricted to its role in the impairment of neuronal function. Our finding that SNX27 deficiency leads to severe skeletal dysplasia extends the physiological importance of SNX27 to bone morphogenesis. Of interest, the skeletal dysmorphisms exhibited in SNX27-deficient mice bear some resemblance to those in humans (Schipani et al., 1995, 1996) and mice (Karaplis et al., 1994; Lanske et al., 1999; Calvi et al., 2001; Miao et al., 2002) with disruptions in PTHR and associated G protein–dependent signaling pathways (Weinstein et al., 2006; Wu et al., 2011). On one hand, the reduced cortical bone density, increased cAMP accumulation, overactivation of associated secondary messenger signaling cascades (pCREB, pAKT, and pERK1/2), along with the unregulated transcriptional expression of PTH-reponse genes observed in both unstimulated OBs and total bone, phenocopies those observed in osteoblastic cells from mice bearing constitutively active mutations in the PTHR gene (Calvi et al., 2001) and/or deletion of Gαs (Wu et al., 2011). On the other hand, the expansion of the cartilaginous growth plate (as opposed to its premature closure) clearly distinguish SNX27-deficient mice from those with disrupted PTHR signaling pathways alone (Calvi et al., 2001;
Kobayashi et al., 2002; Miao et al., 2002; Hirai et al., 2011). Thus additional SNX27-signaling cargo must contribute to the bone phenotype. Indeed, the β2AR (Lauffer et al., 2010; Temkin et al., 2011), an established SNX27-binding GPCR, is functionally required for the anabolic action of PTH on bone (Hanyu et al., 2012). Receptors of other notable bone morphogens, including transforming growth factor β (TGFβ), BMPs, and Wnts, which interact with PTHR and traffic via the retromer recycling pathway, may similarly add to the net bone phenotype (Qiu et al., 2010; Harterink et al., 2011; Yin et al., 2013; Gleason et al., 2014). Irrespective of the exact number of cargoes involved, our studies clearly demonstrate that PTHR is a physiologically important SNX27-PDZ cargo with central roles in bone growth and remodeling.

Unraveling the fundamental mechanisms governing PTHR signaling has been a focus of intensive experimental and pharmaceutical research (McCauley and Martin, 2012; Gardella and Vilarodga, 2015). The identification of new molecules, such as SNX27, that directly modulate PTHR signaling, trafficking, and function may open up new avenues for the development of more effective therapeutic agents that are applicable not only to disorders of bone metabolism like osteoporosis but also extend to wider metabolic syndromes.

MATERIALS AND METHODS
Antibodies, peptides, and constructs
We used the following materials from the respective suppliers: phos- pho-CREB, phospho-AKT, pan-AKT (Cell Signaling Technology, Danvers, MA); PTHR, phosphor-ERK1/2, VPS35 (Santa Cruz Biotechnology, Dallas, TX); total-ERK1/2 (Promega, Madison, WI); EEA1 and TGN38 (BD Biosciences, Franklin Lakes, NJ); SNX27, Lamp1 (Abcam, Cambridge, United Kingdom); anti-Myc (Merck Millipore, Darmstadt, Germany); anti-GFP (Enzo Life Sciences, Farmingdale, NY); collagen II, α-tubulin, peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG), goat anti-rabbit IgG (Sigma-Aldrich, St. Louis, MO); Hoechst 33258 (Thermo Fisher Scientific, Waltham, MA). Human parathyroid hormone fragment PTH(1-34) was purchased from Sigma-Aldrich. A tetramethylrhodamine-labeled parathyroid hormone (PTH−TMR) by which TMR was added to the ε-amino group of Lys3 of PTH(1-34) was synthesized from Genesyn Synthesis (San Antonio, TX). PTHR(1-141) was generously provided by T. J. (Jack) Martin (St Vincent’s Institute of Medical Research, Melbourne, Australia). The PTHR synthetic peptides (QEEWETVM, QEEWATVM, and QEEWETVMA) used for ITC were from Genscript (Piscataway, NJ). Constructs were as follows: β-arrestin2-GFP (Addgene plasmid 35411), SNX27(FL)-GFP, SNX27ΔPDZ-GFP, and SNX27(FL)-mCherry (previously described in Loo et al., 2014); and SNX27H114A-GFP (generated using QuikChange Site-Directed Mutagenesis Kit, Agilent Technologies, Santa Clara, CA). Stable knockdown HEK293 cell lines were generated using a lentivirus shRNA system (Thermo Fisher Scientific) with pGIPZ shnontargeting (RHS4346), pGIPZ shSNX27 (V2LHS_237898), and pGIPZ shVPS35 (V2LHS_156301). GFP-Rab5 and GFP-Rab4b constructs were previously described (Pavlos et al., 2010). The mCherry-ML1N2 plasmid as described by Li et al. (2013) was generated synthetically by Genscript and subcloned into plmCherryC1. The mCherry-2xFYVE construct was generated by subcloning 2xFYVE from eGFP-2xFYVE into, as detailed in Kerr et al. (2010). For construction of GFP-NPTHR, the N-terminus of the PTHR (residues 61–101) were replaced with the enhanced GFP sequence preceded by the linker Arg-Leu-Ile-Ser-Gly-Ser according to the methods described in Castro et al. (2005). Untagged PTHR and PTHRΔPDZ2bm were synthesized by Genscript. Myc-PTHR was generated by OriGene Technologies (Rockville, MD).

Animals
The generation of SNX27−/− mice was described previously (Cai et al., 2011). This study was performed in strict accordance with the Animal Welfare Act 2002 (Western Australia) and requirements of the eighth (2013) edition of the Australian code for the care and use of animals for scientific purposes. All of the animals were handled according to institutional animal care protocols approved by the Animal Ethics Committee of the University of Western Australia (Approval No. RA/3/100/1399).

Cell culture and transfection
All cell culture products were purchased from Thermo Fisher Scientific. Primary mouse OBs were isolated from BMSCs and calvarial bone as described previously (Bakker and Klein-Nulend, 2012; Kular et al., 2015) and cultured in complete α-MEM (10% fetal bovine serum [FBS], 100 U/ml penicillin, 100 U/ml streptomycin). HEK293 cells were cultured in complete DMEM-Glutamax (10% FBS, 2 mM l-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin) and maintained in humidified conditions of 5% CO2 at 37°C. Cells were transfected using Lipofectamine LTX (Thermo Fisher Scientific) according to the manufacturer’s protocol. Stable cell lines were derived through antibiotic selection of neomycin/G418 at 500 μg/ml or puromycin at 2 μg/ml.

Immunoprecipitation
The respective plasmids were transfected into HEK293 cell line stably expressing Myc-PTHR, using Lipofectamine LTX according to manufacturer’s protocol. Cells were incubated for 48 h, followed by the addition of PTH(1-34) (100 nM) for 10 min. Cells were washed twice with ice-cold 1× phosphate-buffered saline (PBS) and lysed in nonde- natured extraction buffer (50 mM 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid [HEPES]–KOH, pH 7.2, 150 mM NaCl, 1 mM MgCl2, 0.5 mM ethylene glycol tetraacetic acid, 1 mM phenylmethylsulfonyl fluoride [PMSF], 1× complete protease inhibitor [CPI], 0.1% Triton X-100) on ice. Cell lysate was homogenized and centrifuged at 20,000 × g for 10 min at 4°C.
15,000 rpm for 15 min at 4°C. Cleared lysate was incubated with GFP-Trap-A beads (ChromoTek, Planegg-Martinsried, Germany) overnight at 4°C. Beads were washed in washing buffer (20 mM HEPES-KOH, pH 7.2, 150 mM NaCl, 1 mM MgCl₂, 1 mM PMSF, 0.5% Triton X-100), boiled in 2x nondenaturing loading buffer, and resolved by SDS-PAGE (12%).

**Immunofluorescence and time-lapse confocal microscopy**

Stable transfected HEK293 cells were cultured on poly-L-lysine-coated glass coverslips for 48 h and serum starved for 1 h before stimulation with PTH(1-34) (100 nM) or PTH⁻<sup>TMR</sup> (100 nM) over a time course of 0–120 min. After 15 min of stimulation, cells were washed twice with 1x PBS and replaced with complete medium. Cell surface PTH⁻<sup>TMR</sup> fluorescence was detected by subjecting cells to an ice block (4°C for 10 min) and washing and then fixing them (4% paraformaldehyde [PFA]). Cells were then permeabilized with 0.1% Triton X-100 and/or 0.1% saponin and stained with indicated antibodies. Samples were mounted in ProLong Gold Antifade (Thermo Fisher Scientific) and imaged using a Nikon A1Si confocal microscope equipped with a 10x (dry) lens and a 60x (oil) lens (Nikon, Tokyo, Japan). Time-lapse confocal microscopy was performed under controlled atmospheric conditions (37°C and 5% CO₂/air) in a Tokai Hit (Fujinomiya, Japan) Stage Top incubator (INUG2E-TIZ) as previously described (Ng et al., 2013).

**Protein expression, purification, and crystallization**

cDNAs of residues 9–327 of VPS26A (mouse) and 40–135 of SNX27 (rat) were cloned in a pMW172KanH6 plasmid downstream of a hexahistidine tag and in a pGEX4-T2, yielding an N-terminally GST-tagged protein. The QuikChange II Kit (Agilent Technologies) was used for site-directed mutagenesis. Proteins were expressed in *Escherichia coli* BL21 (DE3) strain overnight at 20°C and purified using affinity chromatography followed by gel filtration. VPS26A was purified on a nickel–nitrilotriacetic acid gravity column and eluted with 300 mM imidazole in buffer of 200 mM NaCl and 20 mM HEPES (pH 7). The SNX27 PDZ domain was purified on a glutathione–Sepharose gravity column and eluted after 3 h of thrombin cleavage in 200 mM NaCl/25 mM Tris (pH 8) buffer. Proteins were then gel filtered using a Sepharose S200 16/60 column attached to an AKTA system (GE Healthcare, Waukesha, WI). For crystallization, SNX27PDZ fractions were gel filtered into 20 mM Bis-Tris plus 30 mM NaCl (pH 6.5) buffer, pooled, and concentrated and then directly mixed together to a 2:1 M ratio of SNX27 PDZ domain to PTHR peptide, where the final SNX27 PDZ concentration was 25 mg/ml. Four 96-well crystallization hanging-drop screens were set up using a Mosquito Liquid Handling robot (TTP LabTech, Melbourn, United Kingdom) at 20°C. Optimized diffraction-quality crystals were obtained using the sitting drop vapor diffusion method and a buffer containing 0.1 M Bis/Tris (pH 5.5) and 2 M ammonium sulfate.

**Data collection and structure determination**

Data were collected at 100 K at beamline MX2 (Australian Synchrotron; Supplemental Table S1) and integrated and scaled with MOSFLM and SCALA. The structure was determined by molecular replacement using Phaser-MR (McCoy et al., 2007) with the SNX27 PDZ domain crystal structure as an input (Protein Data Bank [PDB code] 3QDO). A model was built using COOT (Emsley et al., 2010), followed by repeated refinement and model building with PHENIX. REFINES and COOT. All residues in the final model were built in accordance with Ramachandran statistics. The final structure was solved at 0.95-Å resolution and revealed electron density corresponding to all amino acids contained in the synthetic peptide but the Q<sup>7</sup> residue. All structural figures were generated using PyMOL (www.pymol.org).

**Isothermal titration calorimetry**

ITC experiments were performed on a MicroCal iTC200 instrument (Malvern, Malvern, United Kingdom) in 50 mM Tris (pH 8)/100 mM NaCl. The PTHR (OQEEWTVTM) peptide at a concentration of 0.875 mM was titrated into 50 μM SNX27 PDZ domain WT or H114A (supplemented with 50 μM VPS26A when required) protein solutions at 25°C. Data were processed using ORIGIN to extract the thermodynamic parameters ΔH and K<sub>a</sub> (1/K<sub>d</sub>) and the stoichiometry n. Here ΔG and ΔS were derived from ΔG = −RT ln K<sub>a</sub> and ΔG = ΔH − T ΔS.

**Histology and histomorphometry**

Lept proximal tibia and distal femur samples from 1-mo-old wild-type and knockout littermates were processed using a Leica TP1020 processor (Leica Biosystems, Nussloch, Germany) in preparation for either methyl methacrylate (MMA) or paraffin embedment according to standard protocol. Samples were sectioned using a Leica Biosystems RM2255 Automated Microtome for MMA-embedded samples or a Biocut 2035 at a thickness of 5 μm. Sections were stained for TRAP, Goldner’s trichrome, von Kossa, Safranin O, and PAS stains, and IHC was performed for collagen 2 according to standard protocols. Sections were then scanned using a Leica Biosystems Aperio ScanScope. Histomorphometric analysis was performed using BioQuant Osteo, version 13.2.6 (Bioquant Image Analysis, Nashville, TN).

**Mineralization and ALP assay**

Mineralization assays were conducted using 4 × 10<sup>4</sup> cells/well (24-well plate) in complete αMEM supplemented with osteogenic medium, that is, Control (complete αMEM with 50 μg/ml l-ascorbic acid and 2 mM β-glycerophosphate [Sigma-Aldrich]). Culture medium was replaced every other day and fixed after 21 d using 4% PFA. Bone nodules were stained using Alizarin Red S solution (Sigma-Aldrich), and bone nodule area (mm<sup>2</sup>) was quantified using ImageJ (National Institutes of Health, Bethesda, MD). ALP activity was visualized using a Leukocyte Alkaline Phosphatase Kit (Sigma-Aldrich).

**Cyclic AMP assay**

Primary OBs were seeded into 96-well plate at 6 × 10<sup>4</sup> cells/well 24 h before the assay. Cells were serum starved for 1 h before the addition of IBMX (200 μM) for 30 min. Cells were then stimulated with PTH(1-34) (100 nM) for 5 min, and assay was subsequently performed using Cyclic AMP XP<sup>TM</sup> Assay Kit (CellSignaling Technology) according to manufacturer’s protocol. Absorbance was read at 450 nm. Four independent experiments were conducted.

**Immunoblotting**

Cultured HEK293 stable PTHR-expressing clones and primary OBs were serum starved for 1 h before stimulation with PTH(1-34) (100 nM) from 0 to 90 min, after a washout after 15 min of stimulation. Cells were washed with ice-cold 1× PBS and lysed in 1× RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 1% sodium deoxycholate) supplemented with protease inhibitors: 1 mM PMSF (Sigma-Aldrich), 1 mM sodium orthovanadate, and 1× Complete Mini EDTA-free protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland). After ice incubation for 20 min, cell lysates were cleared by centrifugation at 15,000 rpm for 30 min at 4°C. A 10-μg amount of each protein sample was resolved by SDS-PAGE (8–12% polyacrylamide gels), and Western blot analyses were carried out with standard protocols using the indicated antibodies. Protein
bands were quantified by densitometry analysis using Photoshop 2014 (Adobe, San Jose, CA; data shown are representative of at least three independent experiments and expressed as mean ± SEM).

RNA and quantitative PCR

For quantifying gene expression, RNA samples were extracted using TRIzol (Thermo Fisher Scientific). First-strand synthesis was conducted using 1–2 μg of total RNA using SuperScript III RT Kit (Thermo Fisher Scientific) according to the manufacturer’s protocol. Quantitative PCR was performed using a SensiMix II Probe Kit (Bioline Reagents, London, United Kingdom) and the Universal ProbeLibrary (Roche Diagnostics) and analyzed using a CFX Connect Real-Time System and CFX Manager Software (Bio-Rad, Hercules, CA). Relative fold expression was normalized to β-actin (ACTB; TaqMan) and hydroxymethylbilane synthase (HMBS) sample controls using the primer sequences shown in Table 2.

**Skeletal staining and micro–computed tomography**

Killed P5 mice were skinned, eviscerated, and fixed in 90% ethanol for 7 d and prepared for whole-mount staining with Alcian Blue and Alizarin Red S according to the protocol detailed in Linz et al. (2015). Whole-mount μCT of P5 mice was performed using SkyScan 1176 (Bruker, Kontich, Belgium) at 45 kV and 556 mA with a pixel size of 17.7 μm. For 0.1-mo-old mice, μCT was performed on the left proximal tibia and distal femur metaphysis and diaphysis for trabecular and cortical bone analysis, respectively, using SkyScan 1174 (Bruker, Kontich, Belgium) at 45 kV and 556 mA with a pixel size of 6.1 μm. All images were reconstructed using the SkyScan NRecon program version 1.1 and analyzed using SkyScan CTAn software (Bruker).

**Statistical analysis and data presentation**

Results were statistically analyzed using a two-tailed t-test using Prism 5 (GraphPad Software, La Jolla, CA). All data shown are representative of at least three independent experiments and expressed as mean ± SEM.

**PDB accession codes**

Structural data are deposited in the PDB under accession number 428J. Raw diffraction data are available at the Diffraction Images Repository (http://xr-diffraction.imb.uq.edu.au).

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### TABLE 2: Primer sequences used in this work.

| Gene | Forward sequence | Reverse sequence | Accession number |
|------|------------------|------------------|------------------|
| ACTB | Taqman (#4352341E; Thermo Fisher Scientific) | | |
| ALP  | cggatctctgccaaacac | tcatgatctcgtgtgtaat | NM_007431.2 |
| RUNX2| cgtgtcaggaagctcttttt | ggtctaagctgcttcctct | NM_01146038.1 |
| OSX  | tgcctcattctgcttgcttc | agtcaggggaggtcag | NM_130458.3 |
| RANKL| tgaagacacactctgctcctg | ccccaaatggtgctgcttc | NM_011613.3 |
| PTH1R| gggctcacgtcgctcatct | tgtcttcattcggcattgg | NM_011199.2 |
| MEPE | tgaagacacactctgcttcctg | ccccaaatggtgctgcttc | NM_053172.2 |
| PHEX | gcgataagagtagatctgagg | tctggctcagctcctctcatctg | EF194891.1 |
| SNX27| ggagacacctctctgcttct | tccctggagacctctctctg | NM_029721.1 |
| HMBS | cagtgatgaaagatgggcaac | acagggacctggtgtaat | NM_013551.2 |
SNX27-retromer regulates PTHR signaling

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