Heterotrimeric G proteins in the control of parathyroid hormone actions

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Abstract

Parathyroid hormone (PTH) is a key regulator of skeletal physiology and calcium and phosphate homeostasis. It acts on bone and kidney to stimulate bone turnover, increase the circulating levels of 1,25 dihydroxyvitamin D and calcium and inhibit the reabsorption of phosphate from the glomerular filtrate. Dysregulated PTH actions contribute to or are the cause of several endocrine disorders. This calciotropic hormone exerts its actions via binding to the PTH/PTH-related peptide receptor (PTH1R), which couples to multiple heterotrimeric G proteins, including G_s and $G_{q/11}$. Genetic mutations affecting the activity or expression of the alpha-subunit of G_s, encoded by the *GNAS* complex locus, are responsible for several human diseases for which the clinical findings result, at least partly, from aberrant PTH signaling. Here, we review the bone and renal actions of PTH with respect to the different signaling pathways downstream of these G proteins, as well as the disorders caused by *GNAS* mutations.

Key Words
- PTH
- G proteins
- GNAS
- bone
- kidney

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Introduction

Parathyroid hormone (PTH) is an 84-amino acid peptide secreted from the chief cells of the parathyroid glands (Habener *et al*. 1984, Potts 2005). PTH is synthesized as a pre–pro hormone consisting of 115 amino acids. The pre-sequence (25 amino acids), which serves as the signal peptide necessary for the peptide's delivery across the membrane of the endoplasmic reticulum, and the pro-sequence (6 amino acids), which is thought to be necessary for efficient transport and proper folding, are removed prior to the secretion of the remaining 84-amino acid sequence, which makes up the mature PTH hormone (Kemper *et al*. 1974, Wiren *et al*. 1989). PTH secretion is regulated through the actions of various factors, including blood-ionized calcium, which acts directly via its own G protein-coupled receptor (Brown *et al*. 1993). Other regulators of PTH synthesis/secretion include 1,25-dihydroxyvitamin D, serum phosphate levels and the phosphaturic hormone fibroblast growth factor-23 (FGF23) (Silver *et al*. 1985, Moallem *et al*. 1998, Ben-Dov *et al*. 2007, Krajisnik *et al*. 2007).

The actions of PTH are critical for the maintenance of serum calcium and phosphate levels and contribute directly to bone turnover and remodeling. Consistent with these roles, PTH exerts its actions primarily in bone and kidney. It increases both bone formation and bone resorption via its actions on osteoblasts, but the net effect depends on the nature of PTH exposure. Intermittent PTH administration favors bone formation and, thus, has an anabolic effect on bone. This effect is utilized in the clinic for treating osteoporosis in postmenopausal

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women (Neer *et al.* 2001). Continuously elevated PTH levels, on the other hand, enhance bone resorption, as in patients with hyperparathyroidism (Habener *et al.* 1984, Potts 2005). Additionally, it has been shown that PTH directly stimulates the production of FGF23 in mature osteoblasts and osteocytes (Lavi-Moshayoff *et al.* 2010, Rhee *et al.* 2011*b*). In kidney, both proximal and distal parts of the nephron are PTH targets (Habener *et al.* 1984, Potts 2005). PTH enhances the reabsorption of calcium in the distal tubule, whereas it stimulates the synthesis of the active vitamin D metabolite 1,25 dihydroxyvitamin D ($1,25(OH)_2D$) and inhibits the reabsorption of phosphate in the proximal tubule. $1,25(OH)_2D$ stimulates the absorption of calcium in the intestine, and therefore, the overall outcome of PTH actions are an elevation in serum calcium and a reduction in serum phosphate levels. Impaired or excess actions of this calciotropic hormone lead to several endocrine diseases. Diminished PTH action results in hypocalcemia and reduced $1,25(OH)_2D$ levels with elevated serum phosphate, whereas excess PTH action causes hypercalcemia, hypophosphatemia and skeletal lesions that result from increased bone resorption.

PTH exerts its actions through the PTH/PTH-related peptide (PTHrP) receptor (PTH1R), which belongs to the family B G protein-coupled receptors (Jüppner *et al.* 1991). PTH1R couples to multiple different G proteins, including G_s and $G_{q/11}$ (Abou-Samra *et al.* 1991, Bringhurst *et al.* 1993) (Fig. 1). The amino terminal portion of PTH can also bind and activate another, closely related G protein-coupled receptor, termed PTH2R (Usdin *et al.* 1995); however, it is now known that the latter receptor is primarily for the actions of the neuropeptide tuberoinfundibular peptide of 39 residues (TIP39) (Usdin *et al.* 1999). As with other G protein-coupled receptors, stimulation of PTH1R by PTH induces a GDP–GTP exchange on the alpha-subunit of the heterotrimeric G protein (Bourne *et al.* 1991, Cabrera-Vera *et al.* 2003, Syrovatkina *et al.* 2016). GTP-bound alpha-subunit dissociates from the Gβγ subunits and becomes available for regulating the activities of specific effectors, such as adenylyl cyclases, certain phospholipases, potassium and calcium ion channels and src tyrosine kinase, which in turn generate various intracellular second messengers. The Gβγ complex also regulates a range of effector proteins, some of which are

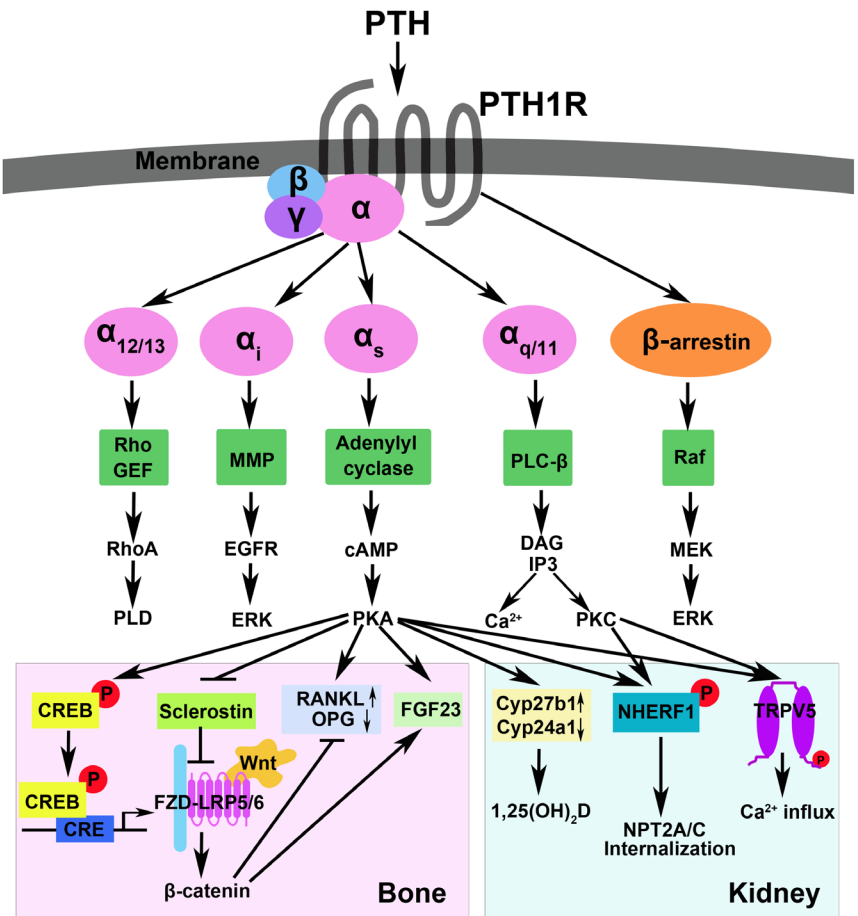

Figure 1
A summary diagram of G protein-mediated pathways activated by PTH.

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identical to those regulated by G alpha-subunits, such as adenylyl cyclases, phospholipase Cβ and certain potassium and calcium ion channels. The duration of G protein-mediated intracellular signaling is tightly controlled through the intrinsic GTP hydrolase activity of the alpha-subunit, which limits the half-life of the GTP-bound form. The GDP-bound alpha-subunit readily reassociates with the Gβγ subunits and thereby resumes an inactive conformation (Bourne *et al*. 1991, Cabrera-Vera *et al*. 2003, Syrovatkina *et al*. 2016). The G protein activation cycle is key to the actions of PTH, as well as numerous other hormones, neurotransmistters and autocrine/paracrine factors throughout the body. In this article, we review the G protein-dependent signaling pathways that mediate the different actions of PTH in bone and kidney. In humans, defects within the gene encoding the alpha-subunit of the stimulatory G protein (*GNAS*) are associated with various phenotypes that directly reflect altered PTH actions. Thus, we also review the *GNAS*-related diseases, particularly focusing on clinical features resulting from abnormal PTH signaling.

G_s/cAMP/PKA-mediated actions of PTH in bone

It has been shown that intermittent PTH treatment enhances the activation frequency of bone multicellular units and osteoblast surface, as well as increasing osteoblast numbers and activity (Shen *et al*. 1993, Boyce *et al*. 1996, Lane *et al*. 1996, Manolagas 2000). Studies have revealed various different mechanisms underlying the bone anabolic action of PTH. These include stimulation of osteoblast proliferation and differentiation, inhibition of osteoblast apoptosis and activation of quiescent lining cells (Dobnig & Turner 1997, Jilka *et al*. 1999, 2009, Iida-Klein *et al*. 2002, Bellido *et al*. 2003, Lindsay *et al*. 2006, Jilka 2007, Kim *et al*. 2012).

G_s is a ubiquitously expressed heterotrimeric protein mediating the actions of many endogenous ligands (Weinstein *et al*. 2001). Although several effectors of the G_s alpha-subunit (Gαs) have been described, by far the most extensively studied and, evidently, the most important Gαs effector is adenylyl cyclase, which catalyzes the synthesis of the ubiquitous second messenger cyclic AMP (cAMP). A major target of intracellular cAMP is the cAMP-dependent protein kinase (PKA), which phosphorylates a whole host of critical proteins to initiate specific cellular events.

PKA-dependent PTH action increases the expression levels of several osteoblast-specific genes, such as Runx2 (Franceschi & Xiao 2003), osteocalcin (Boguslawski *et al*. 2000) and matrix metalloproteinase 13 (Selvamurugan *et al*. 1998). These typically depend on the activation of AP1 (activator protein 1) family of transcription factors c-fos and c-jun via phosphorylation of cAMP response element-binding protein (CREB) (Clohisy *et al*. 1992, Pearman *et al*. 1996, McCauley *et al*. 1997), although other transcription factors are also involved. For example, a role for αNAC (nascent polypeptide-associated complex α-subunit) upon PKA phosphorylation has been described recently, particularly with respect to PTH-induced osteocalcin expression and the anabolic effect of PTH on bone (Pellicelli *et al*. 2014). PTH-induced changes in gene expression also involve intermediate kinases and phosphorylation events. A recent study using both *in vitro* and *in vivo* approaches has shown that p38 mitogen-activated protein kinase (MAPK) is an important mediator of PTH actions downstream of PKA and that ablation of this protein in osteoblasts (using osteocalcin–Cre) markedly impairs the osteoanabolic activity of PTH (Thouverey & Caverzasio 2016).

PTH signaling also cross-talks with the Wnt/β-catenin signaling to promote osteogenesis. Wnt/β-catenin signaling pathway is an important promoter of osteoblast differentiation and bone formation (Day *et al*. 2005, Hill *et al*. 2005, Rodda & McMahon 2006). PKA can phosphorylate and increase the stability of β-catenin (Guo *et al*. 2010*a*). PTH1R signaling has been shown to result in binding of the receptor to the Wnt co-receptor low-density lipoprotein receptor-related protein 6 (LRP6), phosphorylation of the latter and stabilization of β-catenin in osteoblasts (Wan *et al*. 2008). It has also been shown that PTH-induced cAMP/PKA signaling phosphorylates and, thereby inactivates glycogen synthase kinase 3 beta (GSK3β), thus promoting Wnt/β-catenin signaling (Suzuki *et al*. 2008). Furthermore, PTH acts on osteocytes to suppress the expression of sclerostin, an inhibitor of canonical Wnt signaling (Li *et al*. 2005, Semenov *et al*. 2005). PTH action on sclerostin is primarily through cAMP signaling (Keller & Kneissel 2005) and mediated by myocyte enhancer factor-2 (MEF2) transcriptional regulators (Leupin *et al*. 2007). Using the cAMP signaling pathway in osteoblasts, PTH also inhibits the expression of Dickkopf 1 (Dkk1) (Guo *et al*. 2010*a*), which is another Wnt pathway inhibitor (Li *et al*. 2006, Morvan *et al*. 2006).

PTH exposure also activates osteoclastogenesis through an indirect effect on stromal cells and/or mature osteoblasts by activation of the receptor activator of nuclear factor-κ B/RANK ligand (RANK/RANKL) system (Lacey *et al*. 1998, Quinn *et al*. 1998, Yasuda *et al*. 1998, Lee & Lorenzo 1999, Ma *et al*. 2001, Ben-awadh *et al*. 2014). RANKL is expressed

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on the surface of stromal cells and osteoblasts/osteocytes, and binds to its receptor, RANK, which is present on cells of the monocyte/macrophage lineage (Yasuda *et al*. 1998, Li *et al*. 2000*a*). Osteoclastogenesis is stimulated by exposure to macrophage colony-stimulating factor (M-CSF) and RANKL with simultaneous decrease in the expression of osteoprotegerin (OPG), a RANKL decoy ligand secreted from osteoblasts (Yasuda *et al*. 1998). PTH also inhibits OPG expression in early osteoblasts (Lee & Lorenzo 1999, Onyia *et al*. 2000, Huang *et al*. 2004). Stimulation of RANKL and inhibition of OPG expression by PTH also occurs primarily through the Gαs/cAMP signaling pathway, as shown in various studies using osteoblastic cells (Fu *et al*. 2002, Kondo *et al*. 2002, Lee & Lorenzo 2002).

Another action of PTH in bone is to stimulate the production of FGF23, an important phosphaturic hormone (Consortium *et al*. 2000, Shimada *et al*. 2001). Studies using mouse models and cultured cells demonstrated that PTH directly induces transcription of FGF23 in bone cells (Lavi-Moshayoff *et al*. 2010, Rhee *et al*. 2011*b*), in addition to an indirect action in the same regard by increasing the production of $1,25(OH)_2D$, which also stimulates FGF23 production (Collins *et al*. 2005, Kolek *et al*. 2005, Saito *et al*. 2005). Current evidence indicates that the effect of PTH on FGF23 production is dependent on Wnt/β-catenin signaling and occurs via the activation of the nuclear receptor-related 1 protein (Nurr1) downstream of the Gαs/cAMP pathway (Lavi-Moshayoff *et al*. 2010, Rhee *et al*. 2011*b*, Meir *et al*. 2014, Fan *et al*. 2016).

A constitutively active mutant form of PTH1R, identified in patients with Jansen metaphyseal chondrodysplasia (Schipani *et al*. 1995) results in profound increases in trabecular bone mass in mice when specifically expressed in osteoblasts or osteocytes (Calvi *et al*. 2001, O'Brien *et al*. 2008, Rhee *et al*. 2011*a*). *In vitro*, this mutant version of PTH1R predominantly activates G_s-dependent signaling pathways (Schipani *et al*. 1995). Accordingly, it has recently been shown that the increase in bone mass by constitutively active PTH1R depends on Gαs expression in the osteoblast lineage (Sinha *et al*. 2016). Moreover, in transgenic mice expressing a constitutively active PKA mutant in late osteoblasts and osteocytes, trabecular bone mass is increased and sclerostin expression is reduced (Kao *et al*. 2013). Expression of the same PKA mutant in mature osteoblasts also lead to increased bone mass with improved bone architecture and mechanical bone properties (Tascau *et al*. 2016). In addition, constitutive activation of Gαs signaling by an engineered G protein-coupled receptor in osteoblasts throughout embryogenesis results in a dramatic increase in trabecular bone volume in mice (Hsiao *et al*. 2008). However, if Gαs activation is delayed until birth, a much milder increase occurs in bone mass (Hsiao *et al*. 2010), and if activation is delayed until 4 weeks of age, no skeletal phenotype is detected (Hsiao *et al*. 2008). These studies highlight that the effect of PTH on bone mass utilizes the G_s signaling pathway and is subject to developmental stage-specific constraints.

The role of G_s signaling has been studied directly in various mouse models in which Gαs is ablated conditionally in different stages of osteoblast differentiation. Sakamoto and coworkers have reported that ablation of Gαs in differentiated osteoblasts (using Cre driven by the 2.3-kb fragment of the collagen Iα1 promoter) resulted in reduced formation of primary spongiosa and reduced trabecular bone volume but had increased cortical bone thickness, primarily due to a decrease in RANKL expression and osteoclastic bone resorption (Sakamoto *et al*. 2005). And these mice are born with subcutaneous edema and die soon after birth, features that have been observed in global Gαs-knockout models (Yu *et al*. 1998, Skinner *et al*. 2002, Chen *et al*. 2005, Germain-Lee *et al*. 2005).

Postnatal removal of Gαs in the osteoblast lineage (using a doxycycline-regulated Cre driven by the osterix promoter) reveals markedly reduced trabecular and cortical bone mass (Sinha *et al*. 2016). Moreover, the actions of intermittent PTH on trabecular bone are blunted *in vivo* in mice in which Gαs is ablated postnatally in the osteoblast lineage. However, despite Gαs deficiency, PTH is able to stimulate osteoblast differentiation and bone formation, suggesting that PTH exerts these specific actions via other G proteins. A role for $G_{q/11}$ signaling, however, appears unlikely, as PTH could stimulate bone formation robustly in knockin mice expressing a mutant PTH1R (DSEL mutant) deficient in coupling to $G_{q/11}$ (D/D mice) (Guo *et al*. 2010*b*, Sinha *et al*. 2016).

The constitutive deletion of Gαs using the osterix promoter-driven Cre ($Gαs^{OsxKO}$), which ablates this protein in early osteoblast lineage, also results in reduced trabecular bone in the primary spongiosa with decreased trabecular thickness and number, as well as increased trabecular spacing (Wu *et al*. 2011). $Gαs^{OsxKO}$ mice have severe osteoporosis due to impairment of both endochondral and intramembranous ossification. The marked decrease in osteoblast number is the most prominent pathology and one of the underlying mechanisms is attenuated Wnt signaling that results at least in part from increased expression of the Wnt inhibitors sclerostin and Dkk1. Additionally, osteogenic maturation is accelerated in

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mesenchymal progenitors committed to the osteoblast lineage, resulting in the depletion of osteoblasts and accumulation of osteocytes. However, the bone that is present in $G\alpha s^{OsxKO}$ mice is mainly woven, suggesting that G_s signaling plays an important role in the formation of orderly lamellar bone (Wu *et al*. 2011).

Gαs was also knocked out in late osteoblasts and osteocytes by using Dmp1-Cre (Fulzele *et al*. 2013). These mice show severe osteopenia, with decreased trabecular and cortical bone and diminished bone mineral density. Osteocyte density is elevated, but the lacunar–canalicular network was reduced and disorganized. Interestingly, these mice show increased myelopoiesis due to altered bone marrow microenvironment.

G_s/cAMP/PKA-mediated actions of PTH in kidney

Actions in the renal proximal tubule

PTH shows its renal effects by acting on both the proximal and the distal part of the nephron. PTH1R protein is expressed primarily in the epithelial cells of the proximal and distal tubules but not in the thin limbs of Henle, collecting ducts or glomeruli (Lupp *et al*. 2010).

PTH has an indirect calcemic effect at the renal proximal tubule (RPT) by increasing the circulating level of $1,25(OH)_2D$ (Rasmussen *et al*. 1972, Larkins *et al*. 1974). Although $1,25(OH)_2D$ synthesis is upregulated, its metabolism by 24-hydroxylation is reduced by the action of PTH (Trechsel *et al*. 1979). These PTH actions are mainly mediated by Gαs signaling, which induces the expression of the gene encoding 25-hydroxyvitamin D 1α hydroxylase (Cyp27b1) and destabilizes the transcript encoding vitamin D 24-hydroxylase (Cyp24a1) (Rasmussen *et al*. 1972, Larkins *et al*. 1974, Horiuchi *et al*. 1977, Henry 1985, Shigematsu *et al*. 1986, Brenza *et al*. 1998, Zierold *et al*. 2001).

PTH inhibits the reabsorption of phosphate from the glomerular filtrate in RPT by decreasing the abundance of sodium-phosphate co-transporters NPT2a and NPT2c on the apical membrane, thus enhancing renal phosphate excretion (Keusch *et al*. 1998, Pfister *et al*. 1998). Both G_s and $G_{q/11}$ signaling have roles in PTH-mediated phosphate reabsorption in RPT (Pfister *et al*. 1999, Traebert *et al*. 2000, Capuano *et al*. 2007, Segawa *et al*. 2007, Cunningham *et al*. 2009, Weinman *et al*. 2011). It has been well documented that G_s signaling has a role in the acute effects of PTH, whereas $G_{q/11}$ signaling is required for long-term PTH effects based on studies with PTH analogs that specifically activate the Gαs pathway and the DSEL-knockin mice (Guo *et al*. 2010*b*, Nagai *et al*. 2011). PTH1R interacts with Na(+)/H(+) exchanger regulatory factors (NHERF) 1 and 2 (Mahon *et al*. 2002). This interaction seems to play a critical role in determining G protein coupling, switching the G protein coupling preference of PTH1R toward $G_{q/11}$ from G_s. Further molecular studies have demonstrated that, although the interaction of PTH1R with NHERF1 enhances $G_{q/11}$ coupling without affecting Gi or G_s coupling, the interaction of PTH1R with NHERF2 alters coupling to these G proteins in a manner favoring activation of $G_{q/11}$ and reduction of cAMP generation, i.e. promoting Gi coupling and inhibiting G_s coupling (Wang *et al*. 2010). It has also been shown that NHERFs are critical in the membrane retention of PTH1R (Wang *et al*. 2007). Based on additional studies of NHERFs in kidney, it is clear that NHERF1 is required for the membrane targeting of NPT2a and that its ablation in mice results in phosphate wasting (Shenolikar *et al*. 2002). Phosphorylation of NHERF1, which occurs by both G_s and $G_{q/11}$-mediated pathways, dissociates Npt2a from NHERF1, thus allowing Npt2a internalization and lysosomal trafficking (Weinman *et al*. 2007, Wang *et al*. 2012).

Our group has recently investigated the specific role of Gαs in RPT by studying knockout mice in which Gαs is ablated conditionally using Cre recombinase driven by the promoter of type-2 sodium–glucose cotransporter ($G\alpha s^{Sglt2KO}$ mice) (Zhu *et al*. 2016). The Cre driven by this promoter is active in S1 and S2, but not in S3, segments of RPT. $G\alpha s^{Sglt2KO}$ mice are normophosphatemic but show hypocalcemia with reduced serum $1,25(OH)_2D$ and elevated serum PTH levels. In addition, PTH-induced elevation in urinary cAMP excretion is blunted, together with a mildly blunted reduction of serum phosphate in response to PTH. However, renal Cyp27b1 mRNA levels are normal at baseline and after PTH injection, renal Cyp27b1 mRNA increases markedly in these mice. This finding suggests that Gαs-independent signaling pathways play a role, at least partly, in the induction of Cyp27b1 by PTH. Consistent with this interpretation, PKC activation has been suggested to mediate the action of PTH in this regard (Janulis *et al*. 1992, Ro *et al*. 1992). Reduced serum $1,25(OH)_2D$ levels in $G\alpha s^{Sglt2KO}$ mice could be explained by elevated renal Cyp24a1 expression. As PTH regulates the stability of Cyp24a1 mRNA via a cAMP-dependent mechanism (Zierold *et al*. 2001), the elevation of Cyp24a1 expression likely reflects the PTH resistance. In addition, $G\alpha s^{Sglt2KO}$ mice show increased FGF23 expression in bone and a mildly elevated serum FGF23 (Zhu *et al*. 2016),

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which is a potent inducer of Cyp24a1 expression (Shimada *et al*. 2004). Of note, similar changes in serum biochemistries and renal expression levels of Cyp24a1 and Cyp27b1 are present in mice heterozygous for universal ablation of the maternal Gαs allele (Liu *et al*. 2011*a*, Zhu *et al*. 2016).

Actions in the renal distal tubule

PTH triggers cAMP signaling and is a major regulator of Ca reabsorption in the distal part of the nephron (Shareghi & Stoner 1978, Chabardes *et al*. 1980, Imai 1981, Lau & Bourdeau 1989, Shimizu *et al*. 1990, Friedman & Gesek 1993). Calcium is reabsorbed into the cell through the transient receptor potential vanilloid 5 and 6 (TRPV5 and TRPV6) (Hoenderop *et al*. 2003, de Groot *et al*. 2008). TRPV5 is exclusively expressed in the distal convoluted tubule and connecting tubule, whereas TRPV6 expression is more wide-spread, including the intestine (Hoenderop *et al*. 2001, Nijenhuis *et al*. 2003, van de Graaf *et al*. 2006). PTH shows reabsorptive activity of calcium by increasing cAMP generation in isolated perfused nephron segments (Friedman & Gesek 1995). However, it has also been shown that the calcium-reabsorptive role of PTH in distal tubule requires both PKA and PKC activation (Friedman *et al*. 1996). Moreover, cAMP-independent PTH action with the involvement of a phorbol ester-insensitive PKC isotype to stimulate calcium reabsorption in the connecting tubule and the cortical connecting duct has been postulated (Hoenderop *et al*. 1999). Activation of the cAMP–PKA pathway by PTH increases TRPV5-mediated calcium influx by enhancing the opening of a constant TRPV5 channel at the cell surface (de Groot *et al*. 2009). This PTH effect appears to involve, at least partly, inhibition of calmodulin binding to the C-terminus of TRPV5 (de Groot *et al*. 2011). Thus, both cAMP-dependent and -independent pathways seem to be involved in the distal tubular actions of PTH.

Human disorders caused by mutations in the gene encoding Gαs

Gαs is encoded by the *GNAS* complex locus, which gives rise to several other coding and non-coding transcripts (Fig. 2) (Kozasa *et al*. 1988, Ishikawa *et al*. 1990, Swaroop *et al*. 1991, Hayward *et al*. 1998*a*,*b*, Peters *et al*. 1999, Hayward & Bonthron 2000, Li *et al*. 2000*b*, Liu *et al*. 2000*b*, Wroe *et al*. 2000). Exons 1–13 encode Gαs, whereas NESP55 (Neuroendocrine secretory protein-55), XLαs and A/B transcripts use individual, upstream first exons that splice onto exons 2–13. *GNAS* also produces an antisense transcript (*GNAS*-AS1), which originates immediately upstream of the promoter for XLαs and spans the first exon of NESP55. In addition to this complexity in its transcriptional profile, *GNAS* is an imprinted locus. Although NESP55 is expressed exclusively from the maternal allele, XLαs, A/B and *GNAS*-AS1 are expressed exclusively from the paternal allele. The promoters of these monoallelic transcripts are located within differentially methylated regions (DMR), and the unmethylated promoter drives the transcription. In contrast, the Gαs promoter is not methylated and shows activity on both parental alleles, i.e. biallelic. Nevertheless, paternal Gαs expression is silenced in a small set of tissues, including

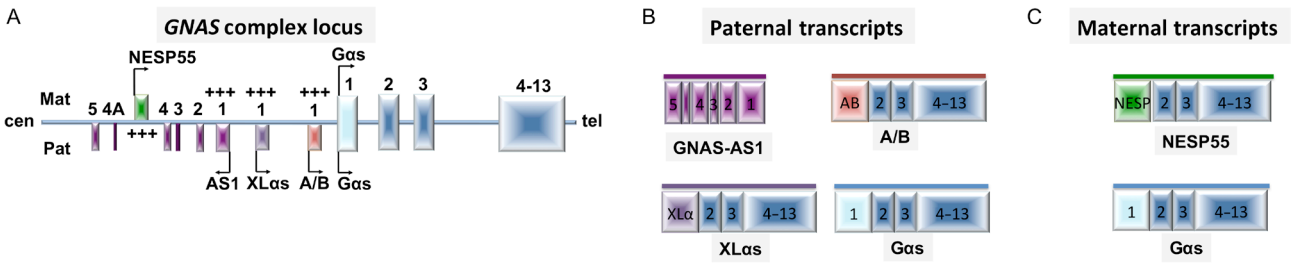

Figure 2
The *GNAS* complex locus and its gene products. (A) In addition to that driving Gαs expression, the locus contains four distinct promoters that are located within DMRs. The non-methylated promoter is active and drives the expression in a parent-of-origin-specific manner. Gαs promoter is non-methylated, and in most tissues, Gαs promoter is active on both parental alleles; however, the paternal Gαs allele is silenced, at least partially, in several tissues including the renal proximal tubule and the thyroid. Boxes and connecting lines represent the exons and introns, respectively. Maternal (mat) and paternal (pat) *GNAS* products are illustrated above and below the gene structure, respectively. '+++' indicates methylated DMR promoters either on the paternal allele (NESP55) or the maternal allele (XLαs, A/B and *GNAS*-AS1 exon 1). Arrows indicate direction of transcription. The figure is not drawn to scale. (B) and (C) paternally and maternally expressed *GNAS* products, respectively. Gαs transcripts, which use exons 1–13, are biallelically expressed in most tissues. Maternally expressed NESP55 and the paternally expressed XLαs and A/B originate from the sense strand and use distinct first exons that splice onto exons 2–13 of Gαs. Note that the portion of NESP55 transcript derived from exons 2 to 13 are within the 3′-untranslated region. Another transcript is also derived from the paternal *GNAS* allele, but this transcript is made from the antisense strand (*GNAS*–AS1 transcript).

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renal proximal tubule, thyroid, pituitary and certain parts of the brain (Yu *et al*. 1998, Hayward *et al*. 2001, Germain-Lee *et al*. 2002, Mantovani *et al*. 2002, Liu *et al*. 2003, Chen *et al*. 2009). This tissue-specific monoallelic expression of Gαs underlies the imprinted mode of inheritance observed for some of the phenotypes resulting from *GNAS* mutations (see below).

McCune–Albright syndrome and fibrous dysplasia of bone

Residues Arg201 and Gln227 of Gαs are crucial for the intrinsic GTPase activity, and thus, mutations at these sites result in a constitutively active Gαs mutant (Landis *et al*. 1989). By stimulating adenylyl cyclase, constitutively active Gαs causes overproduction of cAMP in a ligand-independent manner, promoting cellular responses that are normally mediated by cAMP signaling. Somatic Gαs mutations of this nature are found in various endocrine and non-endocrine tumors, such as growth hormone-secreting adenomas (Landis *et al*. 1989) (Table 1). The same Gαs mutant, most frequently with a substitution at residue 201 (95% of reported cases), is the cause of McCune–Albright syndrome (MAS) or isolated fibrous dysplasia of bone (FD) (Weinstein *et al*. 1991, Schwindinger *et al*. 1992, Alman *et al*. 1996). MAS is characterized by the presence of FD together with hyperpigmented skin lesions (café-au lait spots) and hyperactive endocrine organs including pituitary, thyroid and adrenal (Boyce & Collins 1993). *GNAS* mutations in FD/MAS are post-zygotically acquired, and therefore, the patients are mosaic (Weinstein *et al*. 1991, Lumbroso *et al*. 2004). The skin, bone and endocrine systems are the most frequently affected tissues in MAS, and this finding indicates that a mutation occurs early in embryogenesis, before the separation of the three germ layers. The phenotype of patients with FD/MAS depends on the extent of tissues containing the *GNAS* mutation and the role of G_s signaling in those tissues (Dumitrescu & Collins 2008).

FD is an unregularly growing excess bone, causing a localized increase in bone mass. Bone pathology shows spicules of woven bone and undermineralized bone matrix embedded in a mass of connective tissue made of abnormal, poorly differentiated stromal cells (Marie *et al*. 1997, Riminucci *et al*. 1997, 1999). This fibrosis is reminiscent of that seen in patients with severe primary or secondary hyperparathyroidism (Kumbasar *et al*. 2004). Likewise, mice expressing a constitutively active PTH1R mutant also display significant accumulation of fibroblastoid cells in the bone marrow (Calvi *et al*. 2001), indicating a major role for enhanced PTH actions in the pathogenesis of fibrous dysplasia. Increased bone resorption with enhanced osteoclastogenesis is another feature of FD, which is mediated by excess production of IL-6 (Yamamoto *et al*. 1996) and RANKL (Piersanti *et al*. 2010). The osteomalacia is related, at least partly, to excess production of FGF23, which leads to hypophosphatemia (Riminucci *et al*. 2003, Kobayashi *et al*. 2006). As mentioned previously, PTH was shown to stimulate FGF23 production via Gαs signaling, and thus, the enhanced FGF23 production is plausibly driven by the increased cAMP accumulation in the cells. Nevertheless, Gαs signaling has also been shown to contribute to the processing of FGF23 into its inactive fragments (Bhattacharyya *et al*. 2012). Moreover, it is conceivable that the excess FGF23 production is secondary to the aberrant differentiation of the stromal cells.

FD/MAS is a genetic but a non-inherited disease, as no germline inheritance of the Gαs mutation has thus far been reported. It is therefore postulated that activating Gαs mutations are incompatible with life and lead to embryonic lethality, unless the mutation-bearing and wild-type cells form a mosaic (Happle 1986). Recently, however, a transgenic mouse model expressing a constitutively active Gαs mutant has been described, in which the transgene was transmitted along multiple generations (Saggio *et al*. 2014), casting doubts about this hypothesis. Nevertheless, the development of FD lesions in this model occurs much later than expected based on the findings in humans, suggesting that there might be significant differences at the molecular level between this particular mouse model and human FD.

Albright's hereditary osteodystrophy and pseudohypoparathyroidism

Heterozygous inactivating mutations in Gαs-coding *GNAS* exons cause Albright's hereditary osteodystrophy (AHO), characterized by obesity with round face, short stature, brachydactyly, subcutaneous ossification and cognitive impairment (Albright *et al*. 1942, Patten *et al*. 1990, Weinstein *et al*. 1990) (Table 1). These patients also show resistance to PTH in the RPT with blunted urinary cAMP and phosphate excretion in response to exogenous PTH, termed pseudohypoparathyroidism (PHP) type-I (Albright *et al*. 1942, Chase *et al*. 1969). Hypocalcemia and hyperphosphatemia with elevated PTH levels are the typical biochemical features of PHP type-I. Only mutations on the maternal allele cause PTH resistance, owing to the silencing of the paternal Gαs allele in RPT

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Table 1 Diseases caused by genetic or epigenetic alterations of *GNAS*.

	Molecular defects	Parental origin	Hormonal abnormalities	Additional clinical features	Urinary cAMP and phosphate response to PTH	Erythrocyte $G_s\alpha$ activity
McCune Albright Syndrome (MAS) (OMIM #174800)	Gαs coding mutations Activating Affected residue Arg201	Post-zygotic Mosaic	Peripheral precocious puberty Thyrotoxicosis Pituitary gigantism Cushing's syndrome Hypophostamic rickets	POFD Café au-lait spot	NA[g]	NA
Isolated polyostotic fibrous dysplasia (POFD)	Gαs coding mutations Activating Affected residue Arg201	Somatic	No	No	NA	NA
Tumors	Gαs coding mutations Activating Affected residues Arg201, Gln227	Somatic	Pituitary tumor: growth hormone-secreting, ACTH secreting ACTH-independent macronodular adrenal hyperplasia, sex cord stromal tumor	No	NA	NA
PHP Ia (OMIM #103580)	Gαs coding mutations Inactivating	Maternal	PTH resistance TSH resistance Other hormone resistances (e.g., GHRH, gonadotrophins)	AHO	Blunted	Reduced
PHP Ic (OMIM #612462)	Gαs coding mutations[a] Receptor uncoupling	Maternal	PTH resistance TSH resistance Other hormone resistances (e.g., GHRH, gonadotrophins)	AHO	Blunted	Normal
PPHP (OMIM #612463)	Gαs coding mutations Inactivating	Paternal	No[b]	AHO	Normal	Reduced
POH (OMIM #166350)	Gαs coding mutations Inactivating	Paternal	No[c]	No[c]	Normal	Reduced
PHP Ib (OMIM #603233)	Methylation defects[f]	Maternal	PTH resistance TSH resistance	No[d]	Blunted	Normal[e]

[a]By definition, PHP-Ic is caused by mutations that are downstream of G_s; however, some cases were shown to carry Gαs-coding mutations within exon 13 affecting receptor coupling but not basal activity. [b]Mild hormone resistance has been detected in some PPHP cases. [c]Hormone resistance and/or AHO features were detected in few POH patients, in whom the mutation was maternal. [d]Some patients can have AHO features. [e]A study showed mildly diminished erythrocyte Gαs activity in a series of patients with *GNAS* methylation defects. [f]In familial cases, *GNAS* methylation defects are caused by microdeletions at either the neighboring *STX16* gene or at the NESP55 DMR; the cause of the methylation defects in some sporadic PHP-Ib cases is paternal uniparental disomy involving chromosome 20. [g]NA, not applicable.

of normal individuals by epigenetic mechanisms. Thus, a mutation affecting the maternal allele causes severe Gαs deficiency in RPT, whereas a paternal mutation does not alter the Gαs level significantly (Yu *et al*. 1998).

In some cases with AHO and PTH resistance, resistance to other hormones, such as TSH and gonadotropins, are also observed, reflecting the predominant maternal expression of Gαs in several other tissues, including thyroid and testes. This subtype of PHP is termed PHP type-Ia. Conversely, some patients display AHO features without developing any hormone resistance. This condition, termed pseudo-pseudohypoparathyroidism (PPHP), is often found in the same kindreds as PHP type-Ia. The Gαs mutation leads to PHP type-Ia upon maternal inheritance, whereas the same mutation causes PPHP (i.e. AHO alone) upon paternal inheritance (Davies and Hughes 1993). AHO features develop regardless of the parental origin of the mutation, with the exception of obesity and cognitive impairment, which appear to develop predominantly in patients with PHP-Ia rather than PPHP (Long *et al*. 2007, Mouallem *et al*. 2008).

Although subcutaneous ossification is not a rare finding in patients with heterozygous inactivating Gαs mutations, more extensive ossification has been detected in some patients carrying the same mutations, termed progressive osseous heteroplasia (POH). Patients with POH initially display ectopic, extra-skeletal heterotopic ossifications in skin and subcutaneous tissues, but these

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subsequently invade deep connective tissues and skeletal muscle (Kaplan & Shore 2000). It has been shown that POH has a mosaic distribution through the dermomyotomes, which is similar to the skin lesions observed in MAS (Cairns *et al*. 2013). Based on this finding, it has been suggested that a somatic mutation in a progenitor cell of somitic origin causes loss of heterozygosity at the *GNAS* locus, thus leading to POH (Cairns *et al*. 2013). Investigation of multiple kindreds with POH revealed that this disease is predominantly inherited through paternal Gαs mutations, suggesting that the disruption of one of paternally expressed *GNAS* products contributes to the pathogenesis (Shore *et al*. 2002). Recently, it was shown that activated hedgehog signaling is a major player in heterotopic ossification caused by inactivating *GNAS* mutations, suggesting that inhibition of this pathway could be a future drug target for POH (Regard *et al*. 2013).

Most AHO features could be attributed to diminished signaling downstream of PTH1R, which binds not only PTH but also PTH-related peptide (PTHrP), a paracrine factor that plays a crucial role in endochondral bone development (Karaplis *et al*. 1994). Brachydactyly (type E) and short stature are due to impaired PTHrP actions in endochondral bone formation, similar to that observed in patients with mutations in either HDAC4 or PTHLH (gene-encoding PTHrP) (Klopocki *et al*. 2010, Maass *et al*. 2010, Williams *et al*. 2010). Of note, these AHO features are also found, in a more severe manner, in patients carrying mutations in PRKAR1A or PDE4D, which encode the type 1A PKA regulatory subunit or the type 4D cAMP phosphodiesterase, respectively (Linglart *et al*. 2011, Lee *et al*. 2012, Michot *et al*. 2012), further highlighting the importance of reduced G_s signaling in the pathogenesis. On the other hand, evidence suggests that ectopic ossifications, at least those observed in patients with POH, may result from deficient Gαs signaling downstream of other receptors. Although ablation of Gαs in the mouse limb bud mesenchyme – through the use of Prx1–Cre – results in a severe phenotype resembling POH (Regard *et al*. 2013), ablation of PTH1R using the same approach does not lead to ectopic ossification (Fan *et al*. 2016).

Two additional subtypes of PHP type-I have been described. PHP-Ic is phenotypically identical to PHP-Ia (Table 1). The genetic defect may be downstream of Gαs in these cases, as Gαs activity appears normal in easily accessible cells from patients with PHP-Ic, unlike those from patients with PHP-Ia (Farfel *et al*. 1981). In several PHP-Ic cases, however, Gαs mutations within exon 13 – encoding the C-terminal portion – have been identified, and the mutant protein was shown to affect receptor coupling but not the basal activity (Linglart *et al*. 2002, 2006, Thiele *et al*. 2011). Thus, when Gαs activity is assessed in patients using direct stimulators of G_s rather than receptor agonists, those Gαs mutants appear functional, suggesting that the diagnosis of PHP-Ic, at least in some cases, may reflect the limitation of the Gαs activity assay employed.

Another subtype is PHP-Ib (Table 1), which refers to patients who present with PTH and, in some cases, mild TSH resistance in the absence of AHO features (Peterman & Garvey 1949, Reynolds *et al*. 1952). Several studies, however, have recently indicated that PHP-Ib patients can also have certain AHO features (de Nanclares *et al*. 2007, Mariot *et al*. 2008, Unluturk *et al*. 2008, Mantovani *et al*. 2010). PHP-Ib is caused by methylation changes within the *GNAS* locus (Liu *et al*. 2000*a*). The patients show loss of methylation at exon A/B with biallelic expression of the A/B transcript, whereas some cases display methylation defects at additional *GNAS* DMRs (Liu *et al*. 2000*a*, Bastepe *et al*. 2001*b*). Like in PHP-Ia, hormone resistance in PHP-Ib is inherited maternally (Jüppner *et al*. 1998). The loss of A/B methylation and/or derepressed A/B promoter activity on the maternal allele leads to silencing of Gαs *in cis*, and this silencing takes place in tissues where Gαs expression normally occurs, exclusively or predominantly, from the maternal allele, such as the renal proximal tubule and thyroid. Thus, Gαs expression in those tissues is suppressed on both *GNAS* alleles. Genetic changes that lead to these *GNAS* methylation abnormalities have been discovered in most familial cases with PHP-Ib and include various microdeletions either at the neighboring *STX16* locus, located ~200 kb centromeric to *GNAS*, or at the NESP55 DMR (Bastepe *et al*. 2003, 2005, Linglart *et al*. 2005, Chillambhi *et al*. 2010, Richard *et al*. 2012, Elli *et al*. 2014, Rezwan *et al*. 2015, Takatani *et al*. 2016). A recent study has also revealed a large genomic inversion that disrupts the *GNAS* locus as a cause of PHP-Ib (Grigelioniene *et al*. 2017). Sporadic PHP-Ib cases display broad methylation abnormalities within *GNAS*, including loss of A/B methylation, and in some of these cases, the underlying cause is paternal uniparental disomy involving chromosome 20 (Bastepe *et al*. 2001*a*).

$G_{q/11}$/PLC/PKC-mediated PTH actions in bone

In addition to G_s, the PTH1R couples to $G_{q/11}$-dependent activation of phospholipase C (PLC) (Abou-Samra *et al*. 1991, 1992, Bringhurst *et al*. 1993). Upon activation,

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$G_{q/11}$ leads to the generation of the second messengers inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) through the action of phospholipase C on the membrane phospholipids (Bourne *et al*. 1991, Cabrera-Vera *et al*. 2003, Syrovatkina *et al*. 2016). IP3 and DAG, in turn, increase intracellular Ca^{2+} and activate downstream protein kinase C (PKC) isozymes. PTH signaling to PLC is negatively regulated by the phosphorylation of PTH1R and PKA actions (Tawfeek & Abou-Samra 2008).

In osteosarcoma-derived osteoblast-like cells (e.g. UMR106 and ROS cells), PTH treatment results in the activation of $G_{q/11}$ signaling (Abou-Samra *et al*. 1991, 1992). This is evidenced by accumulation of IP3, indicating activation of phospholipase C. In primary osteoblastic cells from mouse calvaria, PTH also induces IP3 generation (Farndale *et al*. 1988). On the other hand, PLC-independent mechanisms for the activation of different PKC isozymes have also been suggested. One study showed that phospholipase D is necessary for the membrane translocation of PKC-alpha induced by PTH (1–34), as well as PTH (3–34), PTHrP and PTH (1–31) (Radeff *et al*. 2004). Another study indicated that PTH also activates PKC-delta via a PLC-independent pathway (Yang *et al*. 2006). Via activation of PKC, PTH increases osteoblast proliferation (Miao *et al*. 2001, Swarthout *et al*. 2001). On the other hand, it has also been shown that PKC-alpha plays an inhibitory role in osteoblast differentiation (Nakura *et al*. 2011). Overexpression of Gα11 in UMR106-01 significantly increases PTH1R coupling to PLC without altering the cAMP response to PTH, and it also enhances the effects of PTH stimulation on MMP13 expression (Cheung *et al*. 2005). In addition, PTH-induced regulation of insulin-like growth factor-binding protein-5 (IGFBP-5) gene expression appears to involve PKC-delta activation, in addition to PKA (Erclik & Mitchell 2002).

The *in vivo* role of $G_{q/11}$ signaling in mediating the action of PTH in bone is less well characterized than the role of G_s in the same regard. As discussed above, a knockin mouse model has been generated, expressing a mutated PTH1R (DSEL mutant) that can stimulate Gαs/cAMP/PKA signaling but not $G_{q/11}$/PLC/PKC signaling (Iida-Klein *et al*. 1997). The mice (D/D) exhibit delayed hypertrophic differentiation of chondrocytes and abnormalities in endochondral bone formation (Guo *et al*. 2002). These findings reflect impaired actions of PTHrP, demonstrating that the $G_{q/11}$ signaling opposes G_s signaling in growth plate chondrocytes (Bastepe *et al*. 2004). When fed a low-calcium diet or infused with PTH, D/D mice show significant differences from wild-type mice, including a diminished peritrabecular stromal cell response and reduced new bone formation (Guo *et al*. 2010*b*). Bone marrow cells and primary osteoblasts isolated from D/D mice also show attenuated colony formation and proliferation, but normal osteogenic differentiation ability (Guo *et al*. 2010*b*). These findings indicate that the PLC–PKC signaling pathway is essential for bone modeling and remodeling, as well as normal responses to PTH.

Transgenic mice with osteoblast-specific overexpression of a constitutively active Gαq mutant (using the 2.3-kb fragment of the Col1a1 promoter) exhibit osteopenia and decreased bone formation, and do not respond to PTH treatment regarding bone volume (Ogata *et al*. 2007). A study using transgenic mice that specifically overexpress native Gα11 in osteoblasts found similar effects. Bone formation or bone resorption remained unchanged, and no changes were observed in trabecular or cortical bone in Gα11 transgenic mice in response to PTH treatment (Dela Cruz *et al*. 2016). On the other hand, mice with osteoblast-specific ablation of Gαq/Gα11 exhibit, upon daily PTH injections, higher bone volume and bone turnover than the wild-type mice (Ogata *et al*. 2011). These results suggest Gαq/Gα11 signal inhibits bone development, osteogenic differentiation and PTH osteoanabolic action.

$G_{q/11}$/PLC/PKC-mediated PTH actions in kidney

The $G_{q/11}$ signaling pathway has also been implicated in the PTH-induced inhibition of phosphate reabsorption via its effects on the apical NPT2a cotransporter. It has been shown that addition of PTH, as well as a stable GTP analog, results in the accumulation of IP3 in canine renal cortical tubular cells, thus demonstrating activation of PLC (Coleman & Bilezikian 1990). In transfected LLCPK-1 porcine renal tubular cells, reduction of Gαq and/or Gα11 levels by the use of small-interfering RNA markedly diminishes the effect of PTH on PLC signaling, and phosphorylation of PLC-beta3 by a PKA-mediated mechanism dampens the level of PTH-induced IP3 generation (Tawfeek & Abou-Samra 2008). PTH-regulated phosphate transport has been shown to rely on PLC signaling in assays using LLCPK-1 cells (Bringhurst *et al*. 1993). Moreover, experiments using opossum kidney (OK) cells, a model of RPT, and proximal tubule basolateral membranes have demonstrated a role of phospholipase C/PKC in regulating renal actions of PTH (Dunlay & Hruska 1990, Azarani *et al*. 1995). Moreover, treatment with phorbol esters, a pharmacological activator of

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PKCs, in OK cells mimics the inhibitory effect of PTH and significantly suppresses the expression and activity of NPT2a (Cole *et al*. 1987, Malmström *et al*. 1988, Quamme *et al*. 1989*a*,*b*, Pfister *et al*. 1999). Similar results were observed *ex vivo* using isolated intact proximal tubules of mice treated with 1,2-dioctanoylglycerol (DOG), an activator of PKC pathway, and based on further analyses, it has been suggested that the PLC/PKC pathway predominantly regulates the actions of PTH in the apical membrane (Traebert *et al*. 2000). Impaired PTH-induced PLC stimulation in Nherf1-deficient mice, which fail to couple apical PTH1Rs to PLC/PKC, also suggests that apical PTH1R preferably couples to the PLC/PKC pathway (Capuano *et al*. 2007). In addition, studies with the D/D mice (see above) have provided further support for an important role for the PLC/PKC pathway in PTH-induced inhibition of renal phosphate reabsorption *in vivo*. Although wild-type mice show dramatically decreased serum phosphate levels in response to continuous infusion of PTH (1–34), the D/D mice display only a transient response (Guo *et al*. 2013). Thus, the $G_{q/11}$ pathway is essential for the normal renal actions of PTH on phosphate reabsorption.

It is important to note that $G_{q/11}$ proteins also play a crucial role in regulating PTH generation as the action of the calcium-sensing receptor is primarily mediated by these G proteins (Hofer & Brown 2003, Ward 2004). Parathyroid gland-specific Gαq/Gα11 double-knockout mice exhibit parathyroid hyperplasia, markedly increased serum calcium and PTH, severe delay in bone formation and resorption (Wettschureck *et al*. 2007). Similar findings are also present in transgenic mice in which the PTH promoter drives expression of the C-terminal peptide of Gαq, which specifically inhibits the latter (Pi *et al*. 2008). In humans, mutations in GNA11, which encodes Gα11, also correlate with dysregulated PTH levels. Inactivating mutations in GNA11 have been identified as a cause of familial hypocalciuric hypercalcemia type 2, whereas gain-of-function mutations in this gene were shown to be responsible for autosomal dominant hypocalcemia type 2 (Mannstadt *et al*. 2013, Nesbit *et al*. 2013, Li *et al*. 2014).

XLαs-mediated actions of PTH

XLαs is a large variant of Gαs derived from the imprinted *GNAS* complex locus (Kehlenbach *et al*. 1994). XLαs uses an alternative upstream promoter and a distinct first exon, but shares the same exons 2–13 with Gαs (Hayward *et al*. 1998*a*, Peters *et al*. 1999) (Fig. 2). Because nearly all exons are shared between XLαs and Gαs, XLαs is identical to Gαs over almost the entire amino acid sequence but contains a unique and much larger N terminus. As indicated previously, Gαs is expressed biallelically in most tissues, whereas the maternal allele of XLαs is silenced, i.e. XLαs is expressed exclusively from the paternal *GNAS* allele. XLαs is abundantly expressed in neuroendocrine tissues, particularly pituitary, and brain, its expression is also detectable in pancreas, kidney, bone and muscle (Kehlenbach *et al*. 1994, Pasolli *et al*. 2000, Pasolli & Huttner 2001, Liu *et al*. 2011*a*, Pignolo *et al*. 2011, Krechowec *et al*. 2012). Because Gαs and XLαs share the amino acid sequences encoded by exons 2–13, disease-causing *GNAS* mutations that are located in these exons affect both Gαs and XLαs when they are on the paternal allele. Thus, all mutations that are responsible for PPHP, except for those located in exon 1, disrupt XLαs activity. Moreover, when present on the paternal *GNAS* copy, all mutations found in patients with FD/MAS and multiple tumors, which are located in exons 8 or 9, affect XLαs activity. XLαs inactivation is specifically implicated in progressive osseous heteroplasia, which is predominantly, but not always, inherited paternally, and in severe intrauterine growth retardation observed in PPHP, whereas XLαs hyperactivity in the formation of thyroid and ovarian tumors in patients with MAS (Shore *et al*. 2002, Mariot *et al*. 2011, Richard *et al*. 2013). In addition, increased XLαs activity has been implicated in the development of 20q-amplified breast tumors (Garcia-Murillas *et al*. 2013).

Studies using transfected cells overexpressing XLαs suggest that it can mimic the action of Gαs with respect to PTH-induced cAMP generation (Bastepe *et al*. 2002, Linglart *et al*. 2006). XLαs, like Gαs, is localized to the plasma membrane at the basal state (Pasolli *et al*. 2000, Linglart *et al*. 2006). However, activated XLαs and Gαs traffic differently in XLαs- or Gαs-transfected cells. XLαs remains at the plasma membrane after PTH stimulation, whereas Gαs redistributes to the cytosol (Liu *et al*. 2011*b*). This property of XLαs is consistent with higher basal activity of GTPase-deficient XLαs mutants in transfected cells and may explain the finding that XLαs overexpression significantly prolongs the cAMP response induced by a typically short-acting PTH analog (M-PTH (1–14)) (Liu *et al*. 2011*b*). In addition, when XLαs is expressed ectopically in the renal proximal tubules in transgenic (rptXLαs) mice, overexpressed XLαs enhances the cellular responses downstream of Gαs signaling (Liu *et al*. 2011*a*). And transgenic overexpression of XLαs in renal proximal

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tubules partially rescues the PTH resistance phenotype of mice with ablation of maternal Gαs (Liu *et al*. 2011*a*).

Although XLαs displays a Gαs-like role when overexpressed, *in vivo* studies using XLαs-knockout (XLKO) mice suggest that XLαs has actions distinct from the actions of Gαs. Mice in which XLαs is ablated show poor adaptation to feeding, early postnatal lethality, hypermetabolism and reduced adiposity, and have defects in glucose and energy metabolism (Plagge *et al*. 2004). These phenotypes are similar to those observed in mice heterozygous for deleted paternal Gnas exon 2, in which the paternal alleles of both Gαs and XLαs have been knocked out (Yu *et al*. 1998, 2000, 2001). In humans, loss of function of paternal *GNAS* allele has been implicated to have feeding difficulties and defects in fat tissues that are reminiscent of the phenotypes of XLKO mice (Aldred *et al*. 2002, Genevieve *et al*. 2005, Richard *et al*. 2013). These phenotypes differ vastly from and are, by and large, opposite of those observed in mice heterozygous for Gαs ablation alone (Chen *et al*. 2005, Germain-Lee *et al*. 2005). We have recently found that at early postnatal age, XLKO mice exhibit hyperphosphatemia and hypocalcemia, together with increased serum PTH (He *et al*. 2015). We additionally found that basal and PTH-stimulated IP3 signaling and the abundances of PKCδ, PKCα and PKCζ are repressed in XLKO mice at postnatal day 2 and that overexpression of XLαs in transfected cells or in renal proximal tubules *in vivo* stimulates IP3 generation and PKC isozymes activation (He *et al*. 2015). These results demonstrate that XLαs can promote $G_{q/11}$ signaling to stimulate the PLC/PKC pathway *in vivo* and is required for the renal actions of PTH during early postnatal development.

Other G proteins in the actions of PTH

By using osteoblastic UMR106 cells, it has been demonstrated that PTH stimulates, in a RhoA-dependent manner, the activity of phospholipase D (PLD), an enzyme that hydrolyzes phosphatidylcholine to generate choline and the bioactive lipid phosphatidic acid (Singh *et al*. 2003). A subsequent study by the same group of investigators showed that PTH-induced PLD activation was downstream of $G_{12/13}$ activation (Singh *et al*. 2005). Although the overall significance of PTH1R coupling to $G_{12/13}$ remains to be clarified with respect to the physiological actions of PTH, experiments using antagonist minigenes in UMR106 cells identified a small set of genes that are regulated by PTH in a G_{12}-, but not G_s- or $G_{q/11}$-dependent manner, including Mmp8, Gadd45a and Foxa2 (Wang *et al*. 2011). In addition, based on studies using cultured cells, PLD1 and PLD2 have been found to regulate PTH1R endocytosis and trafficking (Garrido *et al*. 2009). It thus appears that $G_{12/13}$ mediates certain specific actions of PTH, as well as the regulation of PTH1R upon activation.

PTH has been shown to stimulate the MAP kinase signaling pathway in several different cell lines, such as OK cells, in a dose- and time-dependent manner (Verheijen & Defize 1997, Cole 1999, Lederer *et al*. 2000). These studies used various stimulators and inhibitors to differentially alter G_s/cAMP/PKA and $G_{q/11}$/PLC/PKC pathways, concluding that the effect of PTH occurs via both of those signaling pathways. One study also indicated that the PTH-induced activation of MAP kinase signaling, as judged by the phosphorylation of ERK1/2, was independent of ras activation (Verheijen & Defize 1997). It has also been shown, by using OK cells and various pharmacologic inhibitors, that ERK1/2 phosphorylation by PTH occurs in a biphasic manner, with the earlier phase mediated by tyrosine kinase and phosphatidylinositol-4,5-bisphosphate 3-kinase and the late phase dependent on protein kinase C (Lederer *et al*. 2000). In contrast, another study using human embryonic kidney 293 (HEK293) cells transiently transfected with the human PTH1R cDNA has subsequently determined that the PTH-induced ERK1/2 phosphorylation in these cells has an early phase that relies on G_s and $G_{q/11}$ pathways and a late phase that involves the action of beta-arrestin in a G protein-independent manner (Gesty-Palmer *et al*. 2006). The study also utilized a mouse embryonic fibroblast line in which exon 2 was ablated from both parental alleles of *Gnas* (Bastepe *et al*. 2002); these cells allowed identification of the ERK1/2 phosphorylation phase that depends on G_s/cAMP signaling (Gesty-Palmer *et al*. 2006). An involvement of epidermal growth factor has been suggested in the action of PTH as a stimulator of ERK1/2 phosphorylation in OK cells (Cole 1999). Further studies revealed that PTH can stimulate ERK1/2 signaling through the G_i signaling pathway, which leads to the stimulation of metalloprotease-mediated cleavage of membrane-bound heparin-binding fragment of epidermal growth factor (HB-EGF) and transactivation of the epidermal growth factor receptor (EGFR) (Ahmed *et al*. 2003, Sneddon *et al*. 2007). It has been shown that, although PTH (1–34) both activates and internalizes PTH1R in distal kidney cells, PTH (7–34) induces receptor endocytosis without inducing cAMP or PLC signaling pathways. On the other hand, PTH (1–31) activates the PTH1R without causing receptor internalization (Sneddon *et al*. 2003, 2004). By employing these ligands, it was then shown that

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PTH1R activation, but not internalization, is required for stimulation of MAP kinase signaling in distal kidney cells (Sneddon *et al*. 2007).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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