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MOLECULAR BIOLOGY OF SKELETAL DEVELOPMENT (T BELLIDO, SECTION EDITOR)

New PTH Signals Mediating Bone Anabolism

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Abstract
Purpose of Review Because of its clinical relevance, extensive work has been performed to uncover the signaling pathways modulated by parathyroid hormone (PTH). This review focuses on the recent identification of novel effectors of the anabolic effect of PTH in bone.
Recent Findings PTH-induced activation of PKA leads to inactivation of SIK2 and nuclear translocation of HDAC4/5. This inhibits MEF2C-dependent *Sost* expression. However, the phenotypic characterization of the HDAC4/5 double knockout mice shows normal anabolic response to intermittent PTH, supporting the notion that HDAC/*Sost*-independent mechanisms must exist. *Lrp6* and a member of the *Usp* gene family have been identified as novel targets of the transcriptional coregulator Nascent polypeptide-associated complex And Coregulator alpha (αNAC) downstream from PTH activation. We propose that the αNAC cascade is involved in the transmission of the anabolic PTH signal.
Summary Further deciphering the signaling pathways transducing the anabolic effects of PTH will allow to develop novel therapeutically relevant molecules.

Keywords PTH · Osteocytes · Histone DeAcetylases 4 and 5 · αNAC · LRP6

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Introduction

Parathyroid hormone (PTH), an 84-amino acid peptide hormone, is an important regulator of calcium homeostasis. The parathyroid gland senses low levels of circulating calcium and secretes PTH. The endocrine function of PTH is elicited by its direct effect on bone and kidneys and indirect effect on the gastrointestinal tract. High levels of PTH promote calcium and phosphorus release by activating osteoclast-mediated resorption of bone. At the level of the kidney, it acts to decrease calcium excretion and phosphorus reabsorption. The indirect effect of PTH is mediated by vitamin D activation, which in turn stimulates the absorption of dietary calcium and phosphorus from the gut [1, 2].

The profound effect of PTH on the skeleton shapes bone microarchitecture and modulates its strength. Chronic and excessive infusion of PTH has been associated with bone loss. Primary hyperparathyroidism (PHPT) is one classical example mimicked by continuous PTH administration, associated with osteopenia [3], accelerated bone loss [4], and enhanced bone turnover. However, intermittent administration of PTH at a low dosage promotes bone formation in a time interval referred to as the anabolic window [5]. This osteoanabolic effect is antagonized by the activation of bone resorption afterwards, yet the net effect is enhanced bone formation [6, 7]. While new therapeutics are in the pipeline, at the time of writing this review, the intermittent

treatment with the N-terminal fragment of PTH(1–34) is the only US Food and Drug Administration (FDA)-approved osteoanabolic therapy for postmenopausal osteoporosis [8].

PTH signaling is a common factor mediating the crosstalk among osteoblasts, osteocytes, and osteoclasts. At the cellular level, the biological effect of PTH is mediated through signaling cascades and downstream targets controlling proliferation, maturation, and differentiation events. Because of its clinical relevance, extensive work has been performed to uncover the key signaling pathways modulated by PTH. In this review, we will focus on the recent work describing the effectors of the anabolic effect of PTH in bone, with emphasis on the proposed role of the transcriptional coregulator Nascent polypeptide-associated complex And Coregulator alpha (αNAC). PTH mediates phosphorylation of αNAC at residue serine 99 through a Gαs-PKA-dependent pathway [9•]. This leads to nuclear translocation of αNAC. In the nucleus of differentiated osteoblasts, αNAC associates with transcription factors, components of the basic transcriptional machinery, and other partners to regulate the expression of target genes [9•, 10, 11, 12•].

PTH1R

PTH signals in cells by activating the parathyroid hormone receptor 1 (PTH1R), a seven-transmembrane domain G protein-coupled receptor (GPCR) that belongs to the class B secretin-like GPCR family [13]. In bone, chondrocytes, osteoblasts, and osteocytes express PTH1R [14]. In mice, deletion of *Pth1r* either globally or in the osteoblastic lineage (osteoblasts or osteocytes) impairs proper bone development and the capacity of bone tissue to respond to PTH treatment. Systemic deletion of *Pth1r* results in mild cortical thickening in specific regions of long bones accompanied by a more profound decrease of trabecular bone development [15]. Osteoblast-specific inactivation with the osteocalcin-driven Cre (OC-Cre) yields a similar phenotype [16].

A mild osteopenic phenotype has also been reported when *Pth1r* is deleted from osteocytes postnatally using a tamoxifen-inducible Dmp1-CRE driver [17]. However, different results were reported when *Pth1r* was constitutively deleted from osteocytes. These animals show an increased trabecular and cortical bone volume at 3 months of age, resulting from a low bone turnover state [18, 19••]. A significant finding, regardless of the steady-state bone phenotype of mice in which *Pth1r* is ablated in osteocytes, is that the anabolic response to PTH is blunted in those animals [18, 19••]. These results indicate the importance of the osteocyte cell population for PTH-induced bone gain responses.

Signaling Pathways

PTH1R is coupled to different G proteins including the Gαs, the Gαq11, and the Gαq12/13 subunits which respectively primarily mediate their intracellular effects through the activation of the adenylate cyclase (ADCY), the phospholipase C (PLC), and the Ras homolog gene family, member A (RHOA) [20, 21]. Within this wide range of signaling cascades activated by PTH, it has been demonstrated that its anabolic function is mainly mediated by the activation of the Gαs-dependent accumulation of cyclic AMP (cAMP) and activation of protein kinase A (PKA) and not by Gαq-mediated PLC activation. This was initially examined using various amino-terminal fragments of PTH that differentially activate Gαs- or Gαq-mediated signaling [22]. PTH(1–34) activates both cAMP production and PLC, while PTH(1–31) only activates cAMP synthesis [23–25]. On the other hand, PTH(3–38) only activates the PLC pathway [24, 25]. Anabolic effects were measured upon daily injections of PTH(1–34) and PTH(1–31), but not PTH(3–38) [26, 27]. These studies established that PTH-stimulated cAMP production is sufficient for mediating the anabolic effect, but activation of the PLC pathway is insufficient. These classical biochemistry studies have now been confirmed using mouse molecular genetics approaches and osteoblast-specific postnatal inactivation of Gαs [28, 29, 30••].

The phenotype of mice with osteoblasts specifically deficient for Gαs is mimicked by a mutation leading to cytoplasmic retention of the transcriptional coregulator αNAC, suggesting that Gαs and αNAC form part of a common genetic pathway [31, 32]. Confirming this hypothesis, we found that treatment of osteoblasts with PTH(1–34) or a PKA-selective activator leads to translocation of αNAC to the nucleus. αNAC was phosphorylated by PKA at residue serine 99 in vitro. Phospho-S99-αNAC accumulates in osteoblasts exposed to PTH(1–34) but not in treated cells expressing dominant negative PKA. Nuclear accumulation is abrogated by an S99A mutation but enhanced by a phosphomimetic residue (S99D). Chromatin immunoprecipitation (ChIP) analysis showed that PTH(1–34) treatment leads to accumulation of αNAC at the *osteocalcin* (*Bglap*) promoter [9•]. These data show that αNAC is a substrate of PKA following PTH signaling. We propose that αNAC acts as a downstream effector of the anabolic action of PTH in bone. We are currently testing the physiological relevance of this proposed mechanism using site-directed mutagenesis of αNAC at S99 in knock-in mice models.

The important role of the Gαs-dependent pathway in PTH-mediated bone acquisition was also confirmed using mice expressing a constitutively active PTH1R (caPTH1R) systemically. These mice show a drastic increase of trabecular bone volume [33], but this abnormal increase of bone volume is completely reversed when Gαs is postnatally deleted from

the osteoblasts [30••]. Interestingly, the same study also showed than even if the intermittent PTH (iPTH)-mediated increase of bone mass is blunted in mice lacking osteoblast expression of Gαs, PTH is still able to increase osteoblast numbers and bone formation rates [30••]. This discovery illustrates well the complexity of PTH signaling in bone cells. It also suggests than other pathways are required to work in collaboration with Gαs-dependent signalization to improve bone mass following PTH stimulation. One possibility would involve the β-arrestin 2-dependent pathway, which is a Gαs-independent cascade that has been shown to be involved in iPTH-induced bone formation [34–36].

The cAMP second messenger activates the PKA-dependent signaling cascade as well as a PKA-independent pathway that involves the Rap guanine nucleotide exchange factor (GEF) 3 and 4 (also called EPAC). While the physiological importance of the latter remains to be demonstrated in bone, the bone anabolic function of PKA has been confirmed using a transgenic approach [37, 38]. Forcing the expression of a constitutively active form of PKA (caPKA) in osteoblasts led to an important increase of the bone volume in long bones [38]. This phenotype is associated with an increase of bone turnover and aligns with the phenotype observed in mice expressing caPTH1R specifically in osteocytes [39, 40]. Interestingly, the increased bone volume observed when caPKA is expressed in osteoblasts is more dramatic than the increase observed when the transgene is only expressed in osteocytes [37, 38]. Here again, this difference suggests that the PKA axis is not the only one required to mediate the anabolic action of PTH on bone.

Coreceptors

Optimal activation of PTH1R by PTH involves its interaction with the LDL receptor-related protein 6 (LRP6) [41–44]. When *Lrp6* expression is disrupted in mice, the anabolic action of iPTH is blunted [42•]. It has been suggested that *N*-cadherin regulates the amount of LRP5/LRP6 complexes at the plasma membrane [45]. This indirectly negatively impacts the abundance of PTH1R/LRP6 complexes by affecting the LRP6 pool available for an interaction with PTH1R [43]. This model is supported by ablation of the *Cdh2* gene (coding for N-cadherin) in mice which increases membrane PTH1R/LRP6 complexes. This in turn promotes signaling downstream from PTH1R, enhancing bone formation following iPTH treatment [43, 46].

LRP6 availability and activity are mainly regulated via interaction with extracellular ligands and phosphorylation of its intracellular domain (reviewed in [47–49]). However, mechanisms regulating *Lrp6* transcription remain largely unknown. Kulkarni and colleagues [50] reported that *Lrp6* expression can be modulated by PTH, but the exact mechanism still needs to be determined. Recent data from our laboratory suggests that this regulation involves the transcriptional coregulator αNAC (unpublished).

In order to identify additional target genes affected by PTH-mediated αNAC phosphorylation, we performed ChIPSeq analysis against αNAC in MC3T3-E1 cells treated with vehicle or PTH(1–34). RNASeq was performed in parallel. Candidate genes that showed increased αNAC binding to their proximal promoter and increased expression following PTH(1–34) treatment were selected for further analysis. This strategy identified the *Lrp6* gene as a potential new αNAC target. ChIPSeq results were validated using conventional quantitative ChIP. Deletions and point mutation experiments in transient transfection assays confirmed that the αNAC regulation of *Lrp6* transcription requires a proximal promoter element; expression of the reporter was reduced by more than 50% when this element is deleted or mutated (not shown). This is the first study that characterizes transcriptional regulation of *Lrp6* gene expression. It identified a novel αNAC target gene induced by PTH signaling and suggests a putative "feed-forward" mechanism to prime subsequent PTH responses.

Anabolic Action of PTH and Wnt Signaling

PTH signaling activates the different nodes of the Wingless-related integration site (Wnt) signal transduction network in osteoblastic cells. Wnt signaling plays a significant role in bone metabolism. Once activated, it fates mesenchymal stem cells (MSCs) to osteogenic commitment, regulates pre-osteoblast proliferation, differentiation, and survival, and controls osteoclastic bone resorption by augmenting osteoprotegerin (OPG) production (reviewed in [51]). Wnt proteins bind to a dual-receptor complex of the frizzled (Fzd) receptor and the LRP5 and LRP6 coreceptors, stabilizing its main downstream effector β-catenin and triggering the transcription of Wnt target genes. Recent studies have shown that the anabolic effect of PTH can be altered by the Wnt/LRP6/β-catenin axis. As discussed before, PTH-bound PTH1R engages with LRP6 and activates β-catenin in osteoblasts and osteocytes [44] and this PTH-induced activation is completely abrogated in *Lrp6* deficient mice [42•]. This interaction appears to be exclusive to LRP6, as the loss of LRP5 does not affect the anabolic actions of intermittent PTH [52]. Although PTH activates β-catenin in osteoblasts [43, 53], studies using mice with β-catenin-deficient osteocytes revealed that the anabolic effects of PTH therapy in the trabecular compartment do not require osteocytic β-catenin [54].

The work of Li et al. [55] has improved our understanding of the role of T cells and other lineages in mediating PTH-induced bone anabolism. Their findings demonstrated that T cell-produced Wnt10b and its costimulatory molecule CD40

ligand (CD40L) can drive the anabolic effect of intermittent PTH on osteoblasts and bone mass [55, 56]. This information adds another level of complexity to PTH anabolism modalities and highlights the importance of investigating the interactions among osteoblasts, osteocytes, and T cells as a circular regulatory circuit of PTH anabolism. Uncertainty remains as to the identity of cytokines and Wnt modulators that play a multidirectional role mediating the connection among different PTH-responsive lineages.

An established mechanism by which PTH exerts its anabolic effect in bone is through the suppression of the sclerostin (SCL) gene (*SOST*). SCL is a potent Wnt antagonist, mainly secreted by osteocytes. *SOST* regulation by PTH remains a hot area for investigation by different research groups. Kramer and colleagues [57] reported contradictory evidence from studies in *Sost* transgenic and *Sost*-deficient mice [58, 59], raising questions about whether PTH-induced bone gain requires the downregulation of *Sost* or not. A recent study performed in osteocyte-specific *Sost* transgenic mice has resolved this discrepancy, presenting evidence that PTH can activate alternative anabolic pathways independently of *Sost*/SCL suppression [18].

We have reported that altered gene dosage for Gαs and αNAC in compound heterozygous mice ($G\alpha s^{ob+/-}$; $\alpha NAC^{+/-}$) results in reduced bone mass, increased numbers of osteocytes, and enhanced expression of *Sost* [9•]. This modulation of *Sost* expression levels was also observed in a different αNAC knock-in mouse model with a serine-to-alanine mutation at position 43 (S43A) that results in a decrease in nuclear αNAC [31]. Taken together, these data lead us to speculate about an important role for nuclear αNAC in *Sost* regulation during the transition stage from osteoblasts to osteocytes. It is likely that PTH regulates αNAC functions in osteocytes. Understanding the molecular mechanism by which αNAC regulates *Sost* levels in osteocytes will be insightful, and future studies will focus on the transcriptional partners of αNAC that may have a direct or indirect effect on *Sost* transcription.

PTH-Mediated Regulation of HDACs

In studying the regulation of *Sost* expression caused by PTH signaling, investigators have unveiled a role for myocyte enhancer factor 2C (MEF2C). Mice with specific inactivation of *Mef2c* in osteocytes have reduced *Sost* expression and increased bone mass [60]; targeted deletion of the *Sost* distal enhancer containing the MEF2C binding site yields a similar phenotype [61•]. PTH inhibits the transcription of the *Mef2c* gene [62, 63] and controls its binding to the upstream *Sost* enhancer through class IIa Histone DeAcetylases 4 and 5 (HDAC4/5) in osteocytes [64, 65]. Different classes of HDACs (I, IIa, IIb) are expressed in osteoblasts. PTH induces the nuclear translocation of HDAC5 and its binding to MEF2C, suppressing *Sost* expression [64]. Interestingly, this inhibitory mechanism governing *Sost* production has been shown to be exclusive to class IIa HDACs, whereas class I HDACs are involved in constitutive *Sost* expression [64].

Class IIa HDAC function is regulated via nucleocytoplasmic shuttling involving differential phosphorylation and protein-protein interactions, where phosphorylated class IIa HDACs are sequestered in the cytoplasm through interaction with 14-3-3 proteins [66]. Recent studies have identified the relevant kinases involved in this mechanism in osteocytes. Members of the salt inducible kinase (SIK) family phosphorylate HDAC4/5 to maintain them in the cytoplasm. Wein and colleagues [67••] have shown that PTH-induced activation of PKA leads to phosphorylation and inactivation of SIK2. This in turn permits dephosphorylation and subsequent nuclear translocation of HDAC4/5, thus inhibiting MEF2C-dependent *Sost* expression [67••]. The model is further supported by the demonstration that small molecule inhibitors of SIKs mimic the skeletal effects of PTH and increase bone formation and bone mass [67••]. This work is significant as it demonstrates the validity of further deciphering the signaling pathways transducing the anabolic effects of iPTH in order to develop novel therapeutically relevant molecules. Another important finding of the phenotypic characterization of the models used in the Wein et al. study is the observation that HDAC4/5 double knockout mice show normal anabolic response to intermittent PTH, supporting the notion that HDAC/SCL-independent mechanisms must exist [67••].

Basic Domain-Leucine Zipper Transcription Factors and PTH Signal Transduction

In response to PTH, the basic domain-leucine zipper (bZIP) transcription factor, cAMP-response element-binding protein (CREB), is phosphorylated [68]. It then stimulates the transcription of genes that encode for members of the AP-1 family of bZIP factors, *Fos* and *Jun* [68–70]. A recent study has linked the class III HDAC, Sirtuin 1 (SIRT1), to the PTH-mediated regulation of the *Mmp13* promoter. The mechanism involves the direct binding of SIRT1 to cJUN and this interaction at the AP-1 site within the *Mmp13* promoter blocks AP-1 activity and downregulates *Mmp13* [71]. Interestingly, the authors further showed that the deacetylation of cJUN by SIRT1 is cAMP dependent [71].

We have shown that αNAC functions as a transcriptional coregulator for the AP-1 family member, cJUN [72, 73]. Protein-protein interactions were detected between αNAC and cJUN, as well as between αNAC and the TATA-binding protein (TBP), and αNAC potentiates the transcriptional activity of cJUN [72, 73]. Additional studies from our lab have also shown that αNAC can recruit HDAC corepressors at specific gene promoters regulating their expression in a

promoter and cell-specific manner. Coimmunoprecipitation assays have detected complexes between the αNAC and the corepressors HDAC1 and HDAC3 in myoblasts and osteoblasts [12•]. Identifying the target promoter genes regulated by the αNAC-HDACs interaction in osteoblasts and osteocytes is of great interest. In this context, we can speculate a mechanistic model regulating *Sost* expression involving the interaction between αNAC and HDACs in osteocytes.

It has been shown that another bZIP transcription factor, activating transcription factor 4 (ATF4), plays a critical role in the anabolic actions of PTH in bone. ATF4 is a key regulator of osteoblast function [74–76]. The anabolic bone response to PTH is severely impaired in mice lacking ATF4; in these animals, PTH-stimulated osteoblast proliferation, survival, and differentiation are suppressed [77].

Our recent results show that αNAC does not exclusively function as a positive regulator of gene transcription, but rather as a docking platform for transcriptional activator or repressor complexes to control gene expression during mesenchymal differentiation [12•]. This dynamic role as an activator or repressor of gene transcription is controlled through posttranslational modification by covalent attachment of a Small Ubiquitin-like MOdifyer (SUMO). SUMOylation leads to transcriptional inhibition by providing a novel protein-protein interface, allowing interaction of the SUMOylated αNAC protein with transcriptional corepressors. The amino acid sequence of αNAC contains one copy of the composite "phospho-sumoyl switch" motif that couples sequential phosphorylation and SUMOylation [78]. We found that αNAC is selectively SUMOylated at lysine residue 127 within the motif and that SUMOylation is enhanced when a phosphomimetic mutation (serine to aspartic acid, S to D) is introduced at the nearby serine residue 132. The S132D, hyper-SUMOylated αNAC mutant, specifically interacts with the corepressor HDAC2 and enhances the inhibitory activity of the Factor Inhibiting ATF4-mediated Transcription (FIAT) on ATF4-mediated transcription from the *Bglap* gene promoter [11]. Considering the role of ATF4 in PTH-induced osteoblast proliferation, survival, and differentiation [77], these results suggest a further role for αNAC in the downstream events mediating the anabolic actions of PTH in bone. It has not escaped the authors that inhibition of the kinase that activates the αNAC phospho-sumoyl switch would prevent αNAC SUMOylation and its interaction with corepressors, in turn leading to increased gene expression in osteoblasts and osteocytes. This is a current intense focus of research in our laboratory.

Ubiquitination and PTH Anabolism

The recent findings on the role of HDACs in the regulation of *Sost* expression caused by PTH signaling is bound to spur future work on the upstream cascades regulating the stability, activity, and subcellular localization of HDACs. While it has been demonstrated that PTH signaling affects the activity of HDAC-regulating kinases [67••], it is highly probable that HDAC-ubiquitinating enzymes could affect their activity and abundance via stability and proteosomal degradation.

Protein ubiquitination is an important posttranslational modification that regulates a multitude of biological processes. These biological functions include cell cycle regulation, protein degradation, DNA repair, kinase signaling, and trafficking events. Deubiquitinating enzymes (DUBs) maintain a dynamic balance between ubiquitination and deubiquitination by removing ubiquitin moieties, thus affecting the stability and function of target proteins. Little is known about the role of the ubiquitin-specific peptidase (USP) family of enzymes in the regulation of bone mass, particularly in response to PTH stimulation. Alonso et al. [79] have reported that PTH can induce the expression of USP2, a PTH1R-specific deubiquitinating enzyme, thus modulating PTH1R sorting. Further work using MC3T3-E1 osteoblastic cells has shown that USP2 upregulation is involved in osteoblast proliferation following PTH treatment [80].

The genomic landscape encompasses about 58 different USPs, few of which have a clear assigned function and substrate. Recent studies have been oriented towards uncovering the upstream signals regulating the activity of those enzymes, their partner ligases, and their potential substrates. Our efforts to identify genes affected by PTH-mediated αNAC phosphorylation through ChIPSeq analysis against αNAC in PTH-treated MC3T3-E1 cells revealed a member of the USP family as a novel αNAC transcriptional target (unpublished). Inhibition of the expression of this USP family member using RNA knockdown appears to regulate mesenchymal cell lineage-making decisions and differentiation in response to PTH. We are pursuing this work by trying to uncover targets and partners of this USP family member using proteomics-based approaches.

Perspectives and Conclusion

Recent research focusing on the anabolic action of PTH has brought to attention the key roles of osteocytes in the transduction of the PTH signal. Future work will benefit from improved in vitro models of established osteocytic cell lines that have been recently described in the literature [65, 81•].

A role for class IIa HDACs has also been uncovered. Interestingly, deciphering the signaling events taking place between the ligand-receptor engagement and gene transcription responses has revealed the involvement of salt inducible kinases (Fig. 1). The demonstration that small molecule inhibitors of SIKs mimic the skeletal effects of PTH and increase bone formation and bone mass [67••] identifies potentially therapeutically relevant molecules. This may prove significant as the current formulation of iPTH is nearing the end of patent protection.

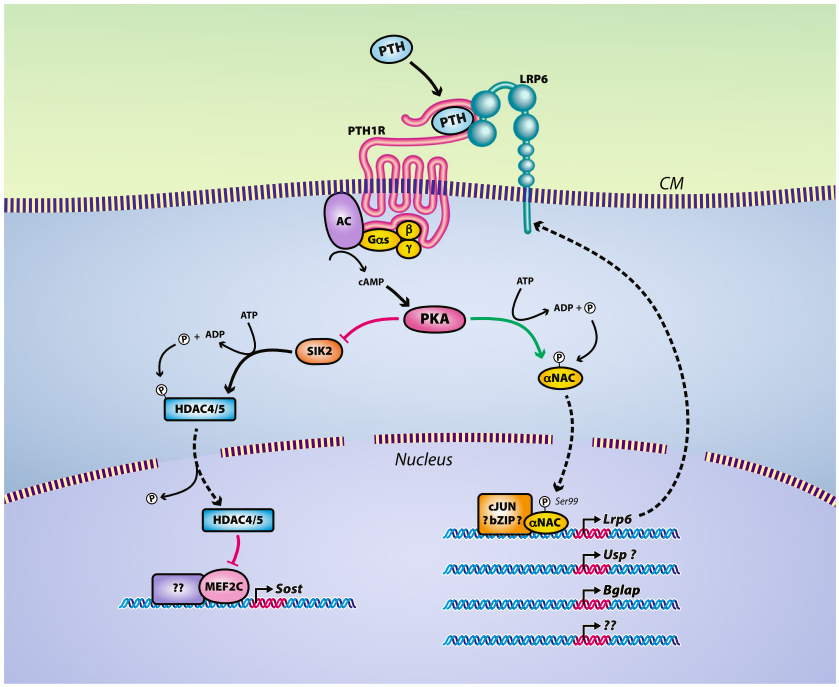

Fig. 1 Novel pathways downstream from PTH. Optimal activation of the PTH receptor 1 (PTH1R) by PTH involves its interaction with the LDL receptor related protein 6 (*LRP6*). PTH1R is coupled to Gαs leading to activation of adenylate cyclase (*AC*), accumulation of cyclic AMP (*cAMP*), and activation of protein kinase A (*PKA*). PTH-induced activation of PKA leads to inactivation of salt inducible kinase 2 (*SIK2*) and nuclear translocation of Histone DeAcetylases 4 and 5 (*HDAC4/5*). This inhibits myocyte enhancer factor 2C (*MEF2C*)-dependent sclerostin gene (*Sost*) expression. The PTH-Gαs-PKA pathway also mediates phosphorylation of the transcriptional coregulator Nascent polypeptide-associated complex And Coregulator alpha (αNAC) at residue serine 99 (*Ser99*). This leads to nuclear translocation of αNAC. *Lrp6* and a member of the ubiquitin-specific peptidase (*Usp*) gene family have been identified as novel targets of αNAC downstream from PTH activation, in addition to the characterized target osteocalcin (*Bglap*). This work also hints that additional members of the basic domain-leucine zipper (bZIP) family of transcription factors may interact with αNAC besides cJUN. The characterization of *Lrp6* as an αNAC target gene suggests a pathway that can amplify PTH responses by increasing expression of the coreceptor molecule

The power of mouse molecular genetics revealed that anabolic response to intermittent PTH also involves HDAC/SCL-independent mechanisms, since HDAC4/5 double knockout mice show normal bone gain when treated with iPTH [67••]. Some of these implicate Wnt ligands and modulators in non-bone cell types [55, 56]. We also propose that the cascade involving αNAC and the novel targets that we recently identified are involved in the transmission of the anabolic PTH signal. We will test that hypothesis using site-specific αNAC mutations in knock-in mice strains. We predict that mice sporting a mutation of the PKA phospho-acceptor site within the αNAC sequence should exhibit a blunted response to intermittent PTH treatment. Reciprocally, mice with a phosphomimetic residue at the corresponding position could turn out to be quite informative depending on the observed phenotype. An osteopenic phenotype associated with increased osteoclastogenesis would place αNAC within the cascade mediating the effects of constant infusion with PTH and suggest an implication of the coregulator in the control of RANKL expression. Alternatively, the mice could demonstrate an exuberant response to iPTH treatment.

Through the characterization of the phenotype of knock-in mice strains in which the phosphoacceptor residue controlling the phospho-sumoyl switch within the αNAC sequence has been mutated, we will demonstrate the physiological relevance of αNAC SUMOylation. Maintaining αNAC in a hyper-SUMOylated state (phosphomimetic mutation) should prevent it from acting as a positive regulator of gene transcription. The mutation should mimic some aspects of the phenotype of mice in which αNAC is excluded from the nucleus [9•, 31]. Thus, the phosphomimetic mutant mice should exhibit decreased *Bglap* gene expression, and mutant bones could be osteopenic with accelerated mineralization characterized by less osteoid tissue and a reduced volume of immature, woven-type bone showing poor lamellation. Mutant bones could have poor biomechanical properties. Endocrine dysfunction with impaired energy metabolism could result from inhibited *Bglap* expression. Preventing SUMOylation through mutation of the acceptor lysine or mutation of the phosphoacceptor residue within the phospho-sumoyl switch to a non-phosphorylatable residue should result in increased target gene expression. Bones from these mice could show increased bone volume with favorable biomechanical

properties, establishing a positive effect of reducing αNAC SUMOylation. All these mutant strains have been established in our laboratory and the analysis of their phenotypes is ongoing.

The characterization of *Lrp6* as an αNAC target gene further supports the notion that αNAC is involved as an effector of the PTH signal. The results are significant as they represent the first description of a mechanism regulating *Lrp6* transcription. As previously mentioned, it suggests a pathway that can amplify PTH responses by increasing expression of a coreceptor molecule (Fig. 1). Our analysis of the *Lrp6* promoter could also identify additional transcription factors interacting with αNAC, besides cJUN. Interestingly, the data to date points to distinct AP-1 family members and other bZIP factors.

Finally, the identification of a USP family member as an αNAC target downstream from the PTH-PKA signal spurs future studies to uncover ubiquitin modifiers that respond to PTH and affect key bone modulators of kinases and transcription factors. Such information will expand our knowledge of the PTH-mediated mechanisms regulating osteoblast proliferation and differentiation.

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Compliance with Ethical Standards

Conflict of Interest Hadla Hariri, Martin Pellicelli, and René St-Arnaud declare no potential conflicts of interest.

Human and Animal Rights and Informed Consent All reported studies/experiments with animal subjects performed by the authors have been previously published and complied with all applicable ethical standards (McGill University Institutional Animal Care and Use Committee and Canadian Council on Animal Care).

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- • Of importance
- •• Of major importance

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