

Autotaxin negatively regulates parathyroid hormone-induced osteogenesis through autocrine inhibition of β -catenin signaling

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Article

Keywords:

Posted Date: March 13th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-8853584/v1>

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Additional Declarations: There is no conflict of interest

Abstract

Autotaxin (ATX/ENPP2) is a lysophospholipase D responsible for lysophosphatidic acid (LPA) production from lysophosphatidylcholine in biological fluids. LPA controls bone metabolism through interplay between specific LPA receptors. However, because *Enpp2* deletion causes embryonic lethality, ATX functions within the osteoblast cell lineage and its contributions to bone growth remain undefined. This study shows that expression of *Enpp2* is upregulated during osteoblastogenesis and reveals *Enpp2* as a target gene of the cAMP signaling pathway. The bone anabolic molecule parathyroid hormone 1–34 fragment (PTH) further increases *Enpp2* expression in osteoblasts. *Enpp2* expression was significantly higher in bone of transgenic Col1-PPR mice expressing constitutively active PTHR1. PTH treatment elevated circulating ATX levels in mice and hypoparathyroidism patients under PTH therapy. Conditional deletion of *Enpp2* in osteoblastic cells (ObDATX) enhanced increased trabecular bone mass and osteoblast activity in response to PTH. Transcriptomic analysis revealed upregulation of bone- and Wnt-related genes in ObDATX mice treated with PTH. *In vitro*, pharmacological ATX inhibition stimulated osteogenesis, and both pharmacological inhibition and genetic deletion of ATX induced spontaneous accumulation of active β -catenin and transcription of Wnt/ β -catenin target genes. These results reveal that ATX as a new negative feed-back regulator of PTH1-34-induced osteogenesis by blocking the Wnt/ β -catenin canonical pathway. These data provide insight to a better understanding of mechanism of bone response to PTH and offer a new therapeutic strategy to enhance bone anabolism.

Introduction

Autotaxin (ATX) is a lysophospholipase D (LysoPLD) that catalyzes the production of lysophosphatidic acid (LPA) from lysophosphatidylcholine (LPC)¹. LPA is a bioactive lipid exhibiting growth factor-like activity, interacting with a variety of G protein-coupled receptors (GPCRs) expressed in numerous cell types and tissues². These include bone, where LPA regulates the activities of bone cells such as osteoclasts, osteoblasts, and osteocytes^{3–6}. The genetic deletion of ATX (*Enpp2*) in mice results in embryonic lethality at E9.5 due to defective vascular morphogenesis and the failure of neural tube closure⁷. Consequently, the role of ATX in bone development and late postnatal period remain uncharacterized.

ATX is predominantly expressed in adipose tissue⁸, the central nervous system⁹, the placenta¹⁰, and lymph nodes¹¹. Furthermore, ATX is detectable in various biological fluids, including plasma, urine, seminal fluid and cerebrospinal fluid¹². Murine studies have demonstrated that ATX concentration in the bloodstream is tightly regulated, with a relatively short half-life of approximately two minutes¹³. These biodynamic properties suggest that ATX homeostasis in the circulation may have a critical yet undefined physiological role. Conversely, ATX has been implicated in multiple pathological processes (inflammation¹⁴, fibrosis^{15,16}, regeneration¹⁷ and disease conditions (cardiovascular¹⁸, obesity¹⁹, multiple sclerosis²⁰, liver steatosis²¹ and cancer^{22–24}). In addition, inhibition or loss of ATX prevents bone erosions in mice with arthritis⁵.

Bone is a highly dynamic tissue whose integrity is preserved throughout life via two distinct processes: skeletal modeling during growth and bone remodeling in adulthood²⁵. These processes are tightly controlled by the coordinated activity of osteoblasts, which mediate bone formation, and osteoclasts, which regulate bone resorption²⁶. Balance disruption in activities of these cell populations underlie the pathogenesis of bone diseases, which represent the most prevalent disorders in the population, with hormonal deprivation and aging being well-established contributors to osteoporosis development^{25,27}. Additionally, autoimmune processes and chronic inflammation represent secondary causes of osteoporosis in rheumatoid arthritis²⁸. Hence, enhanced bone remodeling induced by endocrine disorders, treatment or cancer promotes the fracture risk and fragility fractures^{29–33}.

Currently, the most used bone-targeted therapies focus on inhibiting bone catabolism, primarily through bisphosphonates and denosumab, which act on osteoclast-mediated bone resorption^{34,35}. However, anabolic therapies aimed at stimulating bone formation remain limited. The most notable options include the parathyroid hormone fragment PTH1-34 (Teriparatide, TRPT) and the anti-sclerostin monoclonal antibody romosozumab³⁶. Nevertheless, the clinical application of these therapies entails several constraints. These include the need of daily or even twice daily injections for TRPT. Also, increased risk of osteosarcoma associated with TRPT after prolonged administration were observed in rat models, thus limiting current therapies in human to only two years³⁷. In addition, cardiovascular risks were linked to romosozumab treatment³⁸. Furthermore, the transition phase following the cessation of anabolic therapies is a critical aspect of osteoporosis management, as switching to antiresorptive agents is essential for maintaining gains in bone mineral density and minimizing subsequent fracture risk³⁹.

It is well established that ATX concentration in serum declines with age in children and adults^{40,41}. Our recent findings revealed a correlation between serum ATX levels and bone remodeling markers (CTX, PAL, OCN) in a normal pediatric cohort, suggesting potential links between ATX expression and osteoblast activity⁴⁰. However, the specific role of ATX in osteogenesis and the underlying molecular mechanisms remain unknown.

In this study, we demonstrate that ATX is upregulated during osteoblastogenesis and that *Enpp2* is a target gene for the cAMP signaling pathway. This regulation promotes expression of ATX in PTH-treated osteoblasts. Moreover, PTH increased the circulating levels of ATX in mice and in children suffering from hypoparathyroidism. Conditional knock-out mice with postnatal inactivation of *Enpp2* in osteoblastic lineage cells (ObDATX) showed that osteoblast-derived ATX is not essential for physiological bone mass acquisition. Remarkably, ObDATX mice exhibited significantly higher trabecular bone mass and osteoblastic activity under PTH treatment suggesting that ATX acts as an endogenous autocrine inhibitor of PTH-induced osteogenesis. Both RNA sequencing analysis and *in vitro* experiments confirmed that ATX inhibition enhances osteogenesis by promoting spontaneous activation of Wnt/ β -catenin signaling pathway.

Our study showed that ATX loss or inhibition promotes PTH-induced bone formation by activating the β -catenin/Wnt pathway. Targeting ATX may represent a new strategy to improve the potency of TRPT as anabolic agent in adults and children.

Results

Enpp2 is a target gene of PTH.

To investigate the potential role of ATX in osteoblast activity, we analyze the time-course expression during osteoblastogenesis. We first quantified *Enpp2* (ATX gene) expression throughout the osteoblastogenesis process using the MC3T3 cell line (Fig. 1A) and calvaria primary osteoblasts (COBs) (Fig. 1B) cultured under osteogenic conditions. In both cell models, *Enpp2* expression followed a bell-shape curve during the course of osteoblastogenesis. These results suggest that ATX may act as a modulator of osteoblastogenesis. Moreover, LPA exerts a complex activity on bone tissue as revealed by both a pro- and an anti-anabolic action pending on which type of LPA receptor involved, LPA₁³ or LPA₄⁴². Therefore, the contribution of ATX in bone anabolism is unknown.

PTH1-34/TRPT is currently the most widely used agent among bone anabolic therapies³⁶. Therefore, we checked if ATX expression could be modulated by PTH. *In vitro*, PTH1-34 induces *Enpp2* expression in a dose-depended manner in MSCs at day 7 of osteoblastogenesis (Fig. 1C). *In vivo*, we detected significantly elevated *Enpp2* expression in the total bone of transgenic mice expressing constitutively active PTHR1 under the Col1 promoter (Fig. 1D). PTH1-34 also upregulates ATX protein synthesis by MC3T3 at day 4 of culture under osteogenic condition (Fig. 1E). PTH activates PTH receptor 1 (PTHR1) leading to activation of the G α s/adenylate cyclase/cAMP pathway⁴³. To better investigate the signaling pathway of *Enpp2* activation, we analyzed the effect of modulators of the cAMP pathway. Stimulation of MC3T3 at day 4 of osteoblastogenesis with forskolin (an adenylate cyclase activator) resulted in a significant increase in *Enpp2* expression and ATX protein production (Fig. 1E). Such observation was also found in MSCs at day 7 of osteoblast differentiation in PTH-treated cells in presence of forskolin and IBMX (a PDE inhibitor) (Fig. 1F). Altogether, these findings indicate that *Enpp2* is a target gene of the cAMP pathway, providing a potential mechanistic explanation for PTH1-34 action.

Consistently, plasma concentration of ATX increased 4 hours after systemic administration of PTH in mice (Fig. 1G). Because TRPT is the standard of care in hypoparathyroidism⁴⁴, we measured ATX concentration in the serum of pediatric hypoparathyroidism patients before and after treatment with TRPT. TRPT significantly increased serum ATX levels 4 hours post-treatment, with 6 out of 7 patients exhibiting responsiveness to TRPT-induced increased serum ATX serum (Fig. 1H). These findings provide both preclinical and clinical evidence supporting upregulation of ATX by PTH *in vivo*.

Our results suggest an effect of PTH on *Enpp2* expression in bone cells and in mice. We therefore asked if the increased ATX levels could be the result of the direct activation of osteoblasts by PTH.

Osteoblast-derived ATX is not essential for postnatal bone mass acquisition.

To further assess the role of ATX in postnatal bone development, we generated mice with inducible (Tet-off system) postnatal inactivation of *Enpp2* in osteoblastic lineage cells (ObDATX). This was achieved by crossing *Osx-Cre:GFP^{+/-}* mice with *Enpp2^{fllox/fllox}* animals. *Osx-Cre:GFP^{+/-}* mice served as control group (CTRL) for all experiments. To implement this model, the breeders were maintained on a doxycycline-supplemented diet (food and water), throughout the gestation, with supplementation discontinued at weaning. First, mesenchymal stromal cells (MSCs) were collected from ObDATX and CTRL animals between 6 and 8 weeks of age, which were subjected to osteogenic culture conditions. Osterix (*Osx*) expression pattern showed the expected peak at day 7 of osteoblast differentiation (Fig. 2A), whereas *Enpp2* consecutive knockdown was observed with a delay at day14 of osteoblastogenesis (Fig. 2B). *In vivo*, *Enpp2* expression in the bone of ObDATX mice was significantly reduced by fourfold compared to CTRL mice, confirming the efficiency of gene deletion in osteoblast lineage cells (Fig. 2C). ObDATX animals exhibited no significant changes in growth (body size, body weight) between 1 and 6 months of age or in bone mass gain (BMD, BV/TV, cortical thickness) during this period either in females (Supplementary Fig. 1A-1F) or males (Supplementary Fig. 1G-1K).

Osteoblast-derived ATX is an autocrine inhibitor of PTH-induced anabolic action.

Firstly, we followed up during three weeks the effect of intermittent PTH treatment on bone mass acquisition in ObDATX and CTRL mice using microcomputed tomography (μ CT) analysis (Supplementary Fig. 2A-2c). Unexpectedly, ObDATX mice exhibited a significant greater increase in trabecular bone mass (BV/TV) compared to CTRL mice after one week of PTH treatment. After two and three weeks, the maximum threshold of bone formation was reached as revealed by non-statistically significant differences in BV/TV values between the two groups (Supplementary Fig. 2A-2C).

We then investigated this effect at an early time-point in a second set of experiments stopped at day 7, enabling both μ CT (Fig. 2D-2H) and histomorphometry (Fig. 2I-2O) analyses. The greater increase in BV/TV and trabecular thickness (TbTh) values quantified by μ CT at day 7 in ObDATX mice compared to CTRL mice (Fig. 2E, 2F), were further confirmed at the histological level using Goldner's trichrome staining on bone tissue sections (Fig. 2I-2K). These results indicate that the lack of ATX in osteoblasts amplifies bone formation induced by PTH. To better characterize the level of bone formation, we assessed the dynamic parameters of bone formation. Animals received a double injection of calcein (yellow) and alizarin (red) four and one day before sacrifice, respectively (Fig. 2L). Histological analysis revealed a significant increase in the mineral apposition rate (MAR), mineral surface (MS) and bone formation rate (BFR) in the trabecular bone of ObDATX mice compared to CTRL mice, indicating enhanced osteoblastic activity *in vivo* (Fig. 2M-2O). These results suggest that osteoblast-derived ATX acts as an endogenous inhibitor of PTH-induced bone formation.

Inhibition of osteoblast-derived ATX in vivo increases PTH-induced expression of bone-associated genes.

To gain deeper insight into the molecular mechanisms underlying the anti PTH osteogenic action of ATX, we focused our analysis on the expression of several molecular pathways involved in bone formation. Total RNA was extracted from long bones from CTRL and ObΔATX mice treated daily with PTH for 7 days and subjected to bulk RNA sequencing. Principal component analysis revealed a clear separation between the two groups, identifying 582 upregulated and 661 downregulated genes in ObΔATX mice compared to CTRL (Fig. 3A, 3B). Gene set enrichment analysis (GSEA) of these differentially expressed genes highlighted significant enrichment of pathways related to bone and cartilage biology, including "Bone Mineralization", "Bone Morphogenesis", and "Endochondral Bone Morphogenesis", which exhibited the highest q-values in ObΔATX mice (Fig. 3C). Bone anabolic effects of PTH relies on the increase of the bone remodeling process involving bone-resorbing osteoclasts and bone-forming osteoblasts, ultimately leading to a net gain in bone mass⁴⁵. Consistently, key osteoblast markers (*Bglap*, *Bglap2*, *Spp1*) and osteoclast-associated genes (*Ctsk*, *Acp5*) were significantly more upregulated in ObΔATX mice than in CTRL, alongside 26 additional bone-associated genes (Fig. 3D), suggesting that inhibition of ATX in osteoblastic lineage cells may potentiate bone remodeling.

Inhibition of osteoblast-derived ATX in vivo increases PTH-induced Wnt-linked pathway.

RNA-seq analysis further revealed that the differentially expressed genes were predominantly enriched in the Wnt signaling pathway (Fig. 3E). Notably, both positive regulators of Wnt signaling (*Metn1*, *Cgref1*, *Mfap2*, *Fhl2*, *Bambi*)^{46–50} and negative regulators (*Wif1*, *Timp1*, *Dkk1*)^{51–53} were upregulated in ObΔATX mice treated with PTH (Fig. 3F). This suggests that increase in bone mass may result from a net amplification of Wnt/β-catenin signaling, integrating both intrinsic activating and inhibitory signals.

ATX inhibition promotes the Wnt/β-Cat pathway in mesenchymal stromal cells.

Since the transcriptomic analysis showed a regulation of the Wnt pathways, we focused our attention on the Wnt/β-Cat pathway, given its role as the master regulator of osteogenesis. Pharmacological inhibition of ATX by the inhibitor PF8380 in MSC obtained from control mice induced spontaneous accumulation of active β-Cat, as demonstrated by Western blot analysis (Fig. 4A). This was associated with a higher increase in the proportion of nuclear β-Cat positive cells, accounting for 42% of the total, as determined by immunofluorescence imaging (Fig. 4B). Additionally, spontaneous accumulation of β-Cat was observed in early osteoblast progenitors following treatment with both PF8380 and PTH (Fig. 4C, 4D). Moreover, PF8380 treatment led to the spontaneous transcription of Wnt/β-Cat target genes, including *TCF7*, *Axin2* and *LEF*, in both MSCs (Fig. 4E) and MSC-derived osteoblast progenitors (Fig. 4F). This evidence supports the hypothesis that blocking ATX activity with PF8380 activates the Wnt/β-Cat pathway. To further investigate this hypothesis at the genetic level, we isolated MSCs from *Enpp2*^{flox/flox} mice and treated them with adenoviral particles encoding Cre recombinase fused to RFP (Ad.RFP/Cre), thereby enabling *in vitro* deletion of ATX gene (Fig. 4G). Notably, inhibition of ATX expression in MSCs also induced spontaneous transcription of Wnt/β-Cat target genes *TCF7*, *Axin2* and *C-Myc* (Fig. 4G). These findings indicate that blocking ATX activity, either at enzymatic or gene expression level, promote the Wnt/β-Cat signaling pathway in MSCs.

Altered production of LPA in the context of genetic or pharmacological ATX inhibition eventually interferes with LPA receptor intracellular signaling pathways⁵⁴. Recent studies reported that the LPA receptor subtype-4 (LPA₄) regulates osteogenic differentiation of progenitor cells via inhibition of β -Cat signaling⁵⁵. Here, *Lpar4* silencing mimicked the effects of ATX inhibition in wild type MSCs by promoting spontaneous transcription of Wnt/ β -Cat target genes *TCF7*, *Axin2* and *C-Myc* (Fig. 4H). These findings suggest that LPA₄ and ATX participate in interconnected pathways during osteogenesis.

Pharmacological ATX inhibition enhances osteoblastogenesis.

We evaluated the effects of ATX inhibition and PTH on the early stages of osteoblastogenesis. Wild-type MSCs were cultured under osteogenic conditions and treated or not with the type 1 ATX inhibitor BMP22 and PTH from day 0 to day 7. PTH treatment resulted in the accelerated onset of ALP activity (Supplementary Fig. 3A, 3B) and mineralization (Supplementary Fig. 3C, 3D), as evidenced by a significant 2-fold and 2.2-fold increase in ALP and alizarin staining, respectively. Notably, BMP22, when administered independently, also demonstrated the capacity to accelerate the onset of ALP activity (Supplementary Fig. 3A, 3B) and mineralization (Supplementary Fig. 3C, 3D) to a similar extent as PTH. The combination of both drugs did not result in a further increase in staining, likely because the maximum effect was already being achieved when the molecules were provided individually. These findings confirm that pharmacological ATX inhibition promotes osteoblast differentiation.

Collectively, our results indicate that ATX acts as an autocrine factor upregulated by PTH in osteoblastic lineage cells, from MSCs to mature osteoblasts. ATX constrains PTH-induced osteogenesis through the production of LPA and the subsequent inhibition of the Wnt/ β -Cat signaling pathway. Blocking ATX expression or enzymatic activity enhances osteogenesis mediated by PTH.

Discussion

This study demonstrates that the inhibition of ATX lysophospholipase D activity enhances PTH-induced osteogenesis via the activation of the Wnt/ β -catenin signaling pathway. Current anabolic bone therapies are effective. However, their clinical application is constrained by significant limitations, particularly regarding treatment duration due to potential adverse effects. Notably, TRPT has been associated with increased risk of osteosarcoma,³⁷ while romosozumab has raised concerns due to cardiovascular adverse events³⁸. Therefore, the development of novel therapies to enhance bone anabolism is of critical importance.

Osteogenesis is regulated by complex molecular mechanisms involving multipotent mesenchymal progenitor cells, which also give rise to chondrocytes, adipocytes and myocytes. The ATX/LPA axis plays a crucial role in chondrocyte differentiation⁵⁶, which is essential for the proper cartilage formation, and in myocyte differentiation, which is crucial for muscle repair¹⁷. Our findings revealed that conditional inhibition of ATX in osteoblastic lineage cells does not induce postnatal phenotypic alterations in mice under normal growth conditions. However, these animals exhibit an enhanced high-bone-mass

phenotype when subjected to a PTH-induced bone anabolic challenge, suggesting that ATX functions as an autocrine inhibitor of bone formation. Conditional knock-out mice for ATX within the adipose tissue using *aP2-Cre* animals revealed a similar lack of phenotypic alterations when fed a normal chow⁵⁷. In contrast, when fed a high-fat diet, these animals developed significant metabolic impairments, including increased fat mass, adipocyte size and glucose tolerance⁵⁷. This suggests that ATX serves as an endogenous inhibitor of obesity. Collectively, these findings suggest a dichotomous role for ATX in mesenchymal lineage cells, with a physiological function in cartilage development and muscle repair, and a pathological role in high-fat diet-induced obesity and in PTH-induced bone anabolism.

Several growth factors and cytokines, including TNF α , IL-6, IL-23 and IFN α as well as hypoxia, regulate *Enpp2* expression via the activation of transcription factors such as NFAT1, AP-1, SP1, Stat3, Hoxa13, NF κ B, and Hif2 α .^{58–63} Our study demonstrates that *Enpp2* is also a target gene for the adenylate cyclase (AC)/cAMP signaling pathway. The *Enpp2* promoter contains a cAMP-responsive element binding protein/activator protein-1 (CREB/AP-1)-like motif, supporting the hypothesis of gene transcription could be induced by an increase in intracellular cAMP and activation of PKA down-stream signaling⁶³. However, it has yet to be determined whether *Enpp2* transcription in osteoblastic lineage cells depends on CREB or on exchange proteins directly activated by cAMP (EPACs)⁶⁴. Nevertheless, the activation of PTHR1, a canonical G protein-coupled receptor linked to the G α S/AC/cAMP pathway in osteoblastic lineage cells, likely accounts for the observed upregulation of ATX by PTH *in vitro* and *in vivo*.

Global deletion of *Lpar1* and *Lpar4* has been shown to exert opposite effects on bone development. This hypothesis is supported by evidence indicating that LPA regulates osteoblast differentiation through an interplay between these two receptors, with *Lpar1*-KO animals exhibiting an osteoporotic phenotype, whereas *Lpar4*-KO mice display osteopetrosis⁴². Recent molecular characterization of LPA₄'s inhibitory role in osteogenic differentiation of progenitor cells revealed its repressive activity on β -Cat signaling⁵⁵. Our findings indicate that inhibition of ATX activity also promotes β -Cat signaling, suggesting that LPA₄ and ATX engage in common signaling pathways during osteoblastic differentiation. ATX generates LPA, which functions as a universal ligand for all six LPA receptors subtypes⁵⁴. However, due to its additional role as a docking molecule for LPA, ATX facilitates selective LPA receptors signaling, preferentially activating the P2Y-type receptors (LPA_{4–6}) over the EDG-type receptors (LPA_{1–3})⁵⁴. LPA₁ and LPA₄ are the primary EDG and P2Y types LPA receptors, respectively, expressed in MSCs, osteoblasts and bone tissue⁴². These findings strongly suggest that ATX impairs PTH-induced osteogenesis via LPA₄.

PTH plays a pivotal role in maintaining calcium homeostasis. Intermittent administration of PTH (iPTH) stimulates bone formation, whereas continuous infusion promotes bone resorption⁶⁵. It is noteworthy that PTH treatment also triggers negative feedback mechanisms, as evidenced by the internalization of the TGF β type II receptor as well as down-regulation of PTH/PTH-related protein receptor gene expression, which attenuates respectively TGF β and PTH signaling, and reduces osteogenesis^{66,67}. Furthermore, TRPT has been shown to elevate serum DKK1 levels in post-menopausal osteoporotic

patients, potentially leading to a negative feedback regulation of bone anabolism⁶⁸. DKK1 is a negative regulator of Wnt/ β -Cat signaling but is also a target of the β -Cat/TCF pathway providing an autocrine negative feedback mechanism to avoid excessive Wnt/ β -Cat activation⁵³ and likely bone formation. Our transcriptomic analysis in the bone of ObDATX mice treated with PTH confirmed increased expression of Wnt-linked genes including DKK1. Our findings also indicate that PTH up-regulates ATX, constituting an additional negative feedback mechanism counteracting its anabolic activity. Moreover, PTH inhibits *Sost* expression, resulting in reduced serum sclerostin levels, increased osteoblastic activity, and enhanced bone anabolism⁶⁹. Our study demonstrates that inhibiting ATX activity enhances the Wnt/ β -Cat pathway, thereby potentiating PTH-induced osteogenesis. PTH-induced osteogenesis involves the activation of a complex intracellular network, including the G α_s /AD/cAMP/Protein Kinase A (PKA) pathway, which activates the β -Cat⁷⁰. However, additional signals pathways may be required to fully activate the Wnt/ β -Cat pathway and mediate the anabolic effects of PTH. This has been previously demonstrated by the role of T-cell-derived Wnt10b, which mediates the sclerostin-independent bone anabolic activity of intermittent PTH treatment⁷¹. The combination of intermittent PTH treatment with an anti-sclerostin antibody has been proposed as a potential more effective therapeutic strategy than either treatment alone⁷¹. However, this hypothesis has yet to be tested in a clinical setting.

Collectively our findings introduce the novel concept that PTH-induced ATX up-regulation may act as a regulatory rheostat on osteoblast activity, preventing excessive bone synthesis. Combined intermittent PTH treatment with pharmacological ATX inhibitors may offer enhanced therapeutic benefits compared to current strategies. This approach could either increase bone formation under the current TRPT dosing regimen or allow a reduction in TRPT doses in the long-term treatment of patients with hypoparathyroidism or other bone fragility disorders.

Materials and Methods

Population

All patients and parents/legal guardians were informed of the study and did not object to participation. The study was registered under ClinicalTrials.gov Identifier NCT04671719 and approved by a French ethics committee (CPP Sud Méditerranée, registration number 20.12.02.42053). Children and teenagers aged 10 to 15 years with hypoparathyroidism were prospectively recruited from Reference Centers for Rare Diseases rare diseases of the metabolism of calcium and phosphate at the Femme Mère Enfant Hospital, Lyon 1 University (Bron, France) and Bicêtre Hospital, Paris-Saclay University (Paris, France) between February 2021 and February 2023. Exclusion criteria were as follows: children weighing less than 8 kg, patients with liver disease, individuals in emergency situations, and parental/legal guardian objection to participation in the study. Data collection was as followed. Demographic and physical baseline data were collected during the recruitment consultation by experienced physicians: age, sex, height, weight, BMI, pubertal stage (Tanner), blood pressure, ongoing treatments (calcium, phosphate, and vitamin D supplements, teriparatide), history of fractures, age at diagnosis, etiology of

hypoparathyroidism, and teriparatide doses ($\mu\text{g/kg/day}$). The following biological data were also collected at baseline (H0) and 4 hours after teriparatide injection (H4): urea, creatinine, renal function estimated using the Schwartz formula (eGFR), calcium (mmol/L), phosphate (mmol/L), PTH (ng/L), 25-OH vitamin D (nmol/L), 1,25-(OH) $_2$ vitamin D $_3$ (pmol/L), FGF23 C-ter (RU/mL), CTX (pg/mL), and ALP (IU/L).

Mice.

Mice with a specific deficiency of *Enpp2* in the osteoblastic cell lineage (Ob Δ ATX) were generated by using the Cre/loxP strategy. *Enpp2*^{fl/fl} mice were kindly provided by Dr. Moolenaar (NKI, Amsterdam, Netherlands) (21). These mice were crossed with BALB/c heterozygous mice expressing Cre-recombinase driven by the osterix promoter (Osx1-GFP::Cre/+ mice) obtained from Dr. Andrew P. McMahon, Harvard University, Cambridge, USA ⁷². Osx1-GFP::Cre/+ mice were used as control (CTRL). Bone microarchitecture of female and male mice was analysed every 4 weeks by μ CT (see technical details below) from week 4 until week 20 (males) or 24 (females). Five-week-old male CTRL and Ob Δ ATX mice were treated subcutaneously daily with PTH1-34 (80 $\mu\text{g/kg}$) for 21 days (protocol 1) or 7 days (protocol 2) at which time animals were euthanized. Bone microarchitecture was assessed by μ CT starting from day 0 and weekly until the end of the protocols. Mice were housed in pathogen-free conditions in the Animal Care Facility Lyon-Est Conventional and Specific Pathogen Free (ALECS, University of Lyon, SFR Santé Lyon Est) in accordance with the Guide for the Care and Use of Laboratory Animals of the European Council and under the supervision of the authorized investigators. All protocols involving animal experimentations were approved by the Animal Care and Ethics Committee of the Université Claude Bernard Lyon 1 and the French Ministère de l'Enseignement Supérieur (CEEA-55; application number APAFIS#3913-201512120903612 v5).

Blood samples and ATX measurements with ELISA assays.

Fasting blood samples were collected in the morning during follow-up consultations at baseline (H0) and 4 hours (H4) after teriparatide subcutaneous injection. Circulating ATX concentrations were measured in patient serum using an ATX sandwich ELISA kit (K-5600, batch #XCM100421, Echelon Biosciences Inc®, Salt Lake City, USA), with sera diluted to 1/50 as recommended by the manufacturer. Absorbances were read at a wavelength of 450 nm using a Thermo Scientific Multiskan EX spectrophotometer (ThermoFisher®), and ATX concentrations were determined from a standard curve with a coefficient of determination (R^2) ≥ 0.98 .

Cell culture and reagents

MC3T3-E1 cells (Sigma-Aldrich) were cultured in Eagle's minimum essential medium (aMEM; Sigma-Aldrich) containing 2 mM L-glutamine (Sigma), 50 U/ml penicillin (Sigma), 50 $\mu\text{g/ml}$ streptomycin (Sigma), and 10% fetal calf serum (FCS). Primary osteoprogenitor (COB) cells were isolated from calvariae of neonatal (5–6 days old) mice of both genotypes by sequential enzymatic digestion with type

1 collagenase (Sigma). After mice sacrifice, the calvariae were dissected out, cleaned, cut along the sagittal suture and pooled. The fragments were then digested at 37°C, under gentle stirring. After centrifugation, calvaria cells were collected and plated in T75 flasks in α MEM containing 2 mM L-glutamine, 50 U/ml penicillin, 50 μ g/ml streptomycin, and 10% FCS. After 24 h, attached cells were trypsinized and plated (5000 cells/cm²) in 6 well plates. Mouse bone mesenchymal stromal (MSC) cells were isolated from the bone marrow of femurs and tibias of mice as previously described⁷³. Cells were maintained in α MEM with 10% (v/v) FBS, 50 U/ml penicillin and 50 μ g/ml streptomycin at 37°C. In all cases, cultures were grown at 37°C under 5% CO₂. For induction of osteogenic differentiation, cells were seeded and cultured until they reached confluence. The medium was then supplemented (day 0) with ascorbic acid (50 μ g/ml) and β -glycerophosphate (10 mM) for up to 21 days (Sigma France). Cells were treated overnight with 20nM teriparatide (TRPT, Lilly France) or 50 μ M forskolin or 600 μ M of 3-Isobutyl-1-méthylxanthine (IBMX), both from Sigma France, or with autotaxin inhibitors 10nM PF8380 and 1 μ M BMP22 obtained from Sigma and Avanti Research, respectively.

Lpar4 knock down.

SiRNA LPA4 (ENPP2) (ref E048995-00-0005, Dharmacon USA) were used according manufacturer protocol. Briefly, cells were seeded and cultured until they reached 70% of confluence in 12 wells plate. After cells were washed, 750 μ l of serum and antibiotic free α MEM medium were added. A pre warmed preparation of lipofectamine (1 μ l, Dharmacon, USA), SiRNA LPA4 or Non-targeting Control Pool (: D-001910- 10-05 Dharmacon usa) (25 μ M) and 200 μ l of α MEM medium was added overnight. The day after cells was washed two time and 10% FBS α MEM medium was added for 48h until RNA isolation.

In vitro Cre recombination

Primary osteoblastic cells harvested from *Enpp2^{fl/fl}* mice and cultivated until 50 to 60% confluency were infected with viral particles Cre Adeno-associated virus or GFP Adeno-associated virus (5000 MOI) and Polybrene (Sigma-Aldrich, Darmstadt, Germany) at 4 μ g/ml for 48 hours, Then cells was washed two time and 10% FBS α MEM medium was added for 48h until RNA isolation

Western Blot

After cell treatment, cells were lysed with lysis buffer (RIPA, orthovanadate, protease inhibitor), and protein quantity was evaluated using a BCA protein dosage kit (Thermo Fisher). 10 μ g of the sample in Laemmli buffer was separated by SDS–polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. Membranes were incubated with primary antibodies overnight at 4°C, followed by secondary antibody incubation at room temperature for 1 hour. Detection was performed using Clarity Western ECL Substrate (Bio-Rad, Hercules, CA, USA), and chemiluminescent signals were detected with the Azure 300 Imaging System. Proteins were analyzed with antibodies recognizing Autotaxin (Cayman, dilution 1/250), active β -catenin (Abcam ab246504, 1/1000), Actin (sc-

47778 Santa Cruz, 1/5000) and secondary antibody coupled to the HRP enzyme (Invitrogen, USA 10179202, 1/10,000).

Immunocytochemistry Fluorescence

Cells were cultured on cover glasses and fixed with 4% (wt/vol) formaldehyde. Cultures were blocked with 3% (wt/vol) BSA for 60 min and incubated with primary antibodies (rabbit anti-HIF1 α , mouse anti- β -catenin, active β -catenin; Abcam) for 1 hour. Cultures were then incubated for 60 min with Alexa dye 488-conjugated secondary antibodies. DNA was stained with DAPI and observed using an Axio Observer Z1 (Zeiss).

Quantitative RT-PCR

Total RNA from cell cultures and from powdered whole bone was extracted using Trizol (Invitrogen AB) and the Nucleospin RNAII kit (Macherey-Nagel). Complementary DNA were synthesized by reverse transcription using the iScript cDNA Synthesis kit (Biorad) according to the manufacturers' instructions. Expression of target genes was quantified by qRT-PCR using the Biorad CFX Connect Real Time PCR Detection System with the iTaq Universal SYBR Green Supermix (Biorad) and sets of specific primers (Table 1). Quantifications were normalized to *RPL32a* values and calculated as relative expression using the $2^{(-\Delta\Delta C(T))}$ method (27). Data were expressed as fold increase compared to control. Primer sequences (designated as f, forward, and as r, reverse) were as follows: *Axin2* (f, 5'-TGACTCTCCTTCCAGATCCCA-3'; r, 5'-TGCCCACACTAGGCTGACA-3'), *C-myc* (f, 5'-ATGCCCCTCAACGTGAACTTC-3'; r, 5'-CGCAACATAGGATGGAGAGCA-3'), *Enpp2* (f, 5'-GCCCTGATGTCCGTGTATCT-3'; r, 5'-CGTTTGAAGGCAGGGTACAT-3'), *LEF* (f, 5'-AGAAATGAGAGCGAATGTCGTAG - 3'; r, 5'-CTTTGCACGTTGGGAAGGA - 3'), *Lpar4* (f, 5'-CACATATAAGGATGGAGTCGC-3'; r, 5'-GTCAACTCAACAGAAGAGGC-3'), *OPG* (f, 5'-GGGCGTTACCTGGAGATCG-3'; r, 5'-GAGAAGAACCCATCTGGACATTT-3'), *Osx* (f, 5'-ATGGCGTCCTCTCTGCTTG-3'; r, 5'-TGAAAGGTCAGCGTATGGCTT-3'),

Table 1
Characteristics of children with hypoparathyroidism from the
GNAS-AUTAX cohort.

Age (years), mean (min-max). N = 7	12 (10–15)
Female sex, n (%)	5 (71%)
Calcemia H0 (mmol/L), mean $\pm$ SD	1.81 $\pm$ 0.11
Calcemia H4 (mmol/L), mean $\pm$ SD	2.17 $\pm$ 0.28
Phosphate H0 (mmol/L), mean $\pm$ SD	1.79 $\pm$ 0.32
Phosphate H4 (mmol/L), mean $\pm$ SD	1,67 $\pm$ 0.28
Schwartz eGFR (mL/min), mean $\pm$ SD	97.7 $\pm$ 10.7
25-OH vitamin D (nmol/L), mean $\pm$ SD	73.71 $\pm$ 16.94
1,25 di OH vitamin D (pmol/L), mean $\pm$ SD	178.46 $\pm$ 118.5
FGF 23 C-ter (Ru/mL), mean $\pm$ SD	108.83 $\pm$ 47.16
Total ALP (U/L), mean $\pm$ SD	293 $\pm$ 68.16
CTX (pg/mL), mean $\pm$ SD	2521 $\pm$ 910.28

RankL (f, 5'-CCAGCTATGATGGAAGGCTCA-3'; r, 5'-GAGAAGAACCCATCTGGACATTT-3'), *RPL32a* (f, 5'-GGATCCCTCCACCCTATGACA-3'; r, 5'-AGCCGAACAACCTTGAGAGC-3'), *TCF7* (f, 5'-ACAGTGCTCTAGGCTGTCC - 3'; r, 5'-CCACCTGAGAATGTTGGTGCT-3').

Bulk RNA sequencing

RNA-seq was performed in triplicate for the following conditions: Total RNA from long bones of 3 wild type (n = 3) and 4 Ob Δ ATX (n = 4) mice treated with PTH1-34 was extracted using Trizol (Invitrogen AB) and the Nucleospin RNAII kit (Macherey-Nagel), and transferred for sequencing to IntegraGen SA Co. (Evry, France). Libraries were prepared with the NEBNext Ultra II directional RNA library prep kit for the Illumina protocol according to the supplier's recommendations. Briefly, the successive stages of this protocol are the purification of PolyA containing mRNA molecules using poly-T oligo attached magnetic beads from 1 μ g of total RNA (with the magnetic mRNA isolation kit from New England Biolabs), fragmentation using divalent cations under elevated temperature to obtain approximately 300–base pair (bp) pieces, double-strand cDNA synthesis, and, last, Illumina adapter ligation and cDNA library amplification by PCR for sequencing. Sequencing then involves paired-end 100-bp reads with Illumina NovaSeq. Image analysis and base calling involved using Illumina Real-Time Analysis (3.4.4) with default parameters. For quantification of gene expression, STAR was used to obtain the number of reads associated with each gene in the Gencode vM24 annotation (restricted to protein-coding genes, antisense, and lincRNAs). Raw counts for each sample were imported into R statistical software. The

extracted count matrix was normalized for library size and coding length of genes to compute fragments per kilobase of transcript per million mapped reads (FPKM) expression levels. For the analysis, the Bioconductor edgeR package was used to import raw counts into R statistical software and compute normalized log2 counts per million mapped reads by using weighted trimmed mean of M values as a normalization procedure. The normalized expression matrix from the 1000 most variant genes (based on SD) was used to classify the samples according to their gene expression patterns by using principal components analysis (PCA), hierarchical clustering, and consensus clustering. PCA involved the FactoMineR::PCA function with “ncp = 10, scale.unit = FALSE” parameters. Hierarchical clustering involved the stats::hclust function (with Euclidean distance and ward.D method). Consensus clustering involved the Consensus ClusterPlus::ConsensusClusterPlus function to examine the stability of the clusters. We established consensus partitions of the dataset in K clusters (for K = 2, 3 to 8) on the basis of 1000 resampling iterations (80% of genes and 80% of sample) of hierarchical clustering, with Euclidean distance and ward.D method. Then, the cumulative distribution functions (CDFs) of the consensus matrices were used to determine the optimal number of clusters (e.g., K = 3), considering both the shape of the functions and the area under the CDF curves. tSNE analysis involved using the Bioconductor Rtsne package applied with the PCA object (theta = 0.0, perplexity = 30, and max_iter = 1000). Differential expression analysis was carried out using the Bioconductor edgeR package to import raw counts into R statistical software. Differential expression analysis involved using the Bioconductor limma package and the voom transformation. To improve the statistical power of the analysis, only genes expressed in at least one sample ($\text{FPKM} \geq 1$) were considered. A qval threshold ≤ 0.05 and a minimum fold change 1.2 were used to define differentially expressed genes.

Micro-Computed Tomography (μ CT).

Three-dimensional (3D) microarchitecture of the distal metaphyseal femur and cortical midshaft, and of the proximal metaphyseal tibia were carried out using a Skyscan 1176 micro-CT scanner (Skyscan Inc.). The X-ray excitation voltage was set to 50 kV with a current of 500 mA. A 0.5 mm aluminum filter was used to reduce beam-hardening artifacts. Samples were scanned in 70% ethanol with a fixed voxel size of 9.08 μm . Section images were reconstructed with NRecon software (version 1.6.1.8, Skyscan). The region of interest to delineate trabecular bone was drawn manually away from the endocortical surface, starting at 0.3mm of underneath the growth plate and ending at 1.3mm. For cortical analysis, 0.5mm on either sides of the femur midshaft were reconstructed. The global threshold was set at 0.394 g HA/cm³. Three-dimensional modeling and analysis of bone, vertebra length and bone volume to tissue volume (BV/TV) were obtained with the CTAn (version 1.9) and CTVol (version 2.0) softwares.

Histology

For histology and histomorphometry, long bones CRTL and ObDATX mice were isolated, were fixed (3.7% paraformaldehyde in PBS), dehydrated in acetone and embedded in methylmethacrylate. Tissue samples were cut into 5 μm sections and stained with Goldner's trichrome. Bone Morpho software (Exploranova, La Rochelle, France) was used to quantify the bone volume/tissue volume (BV/TV) ratio and the

thickness of bone trabeculae (TbTh). Calcein and alizarin fluorescent labeling of mineralizing bone surfaces were visualized in unstained sections and used for the measurement of trabecular bone formation rate (BFR, in $\mu\text{m}^3/\mu\text{m}^2/\text{day}$), taken as the product of the mineral apposition rate (MAR, $\mu\text{m}/\text{day}$, measured from the distance between two calcein and alizarin labels) and the mineralizing surfaces (MS/BS, the fraction of the bone surface which was labeled with calcein, in %)

Statistical analyses

Differences between groups were determined by One-way analysis of variance (ANOVA) followed by a Turkey's multiple comparison test, or Two-way ANOVA followed by a Sidák's multiple comparisons test, or Wilcoxon Signed Rank Test using GraphPad Prism version 10.6.1 software. Single comparisons were carried out using the non-parametric Mann-Whitney test.

$P < 0.05$ was considered statistically significant.

Declarations

Data availability

Raw data from RNA-seq and spatial transcriptomics have been deposited in the NCBI's Gene Expression Omnibus (GEO) database (accession numbers GSE316514). All other relevant data from this study are available from the corresponding authors upon reasonable request.

Conflict of interest

The authors declare no conflict of interest

Supplementary informations

Supplementary informations accompanies the manuscript on the *Bone Research* website <http://www.nature.com/boneres>

Fundings:

This work was supported by the ANR grant BoneTAX (Grant N° ANR-20-CE14-00036) (OP), the French society of rheumatology, Filière OSCAR and the DREAMER/Novartis program (MAL), the French Ministère de l'Enseignement Supérieur et de la Recherche (LD) and the Université Claude Bernard Lyon 1 (CA).

Author contributions:

Conducting experiments: CA, CI, EH, GJ, IMG, LB, LD, MAL, SP, OP. Designing research studies: EH, IMG, MCS, OP. Acquiring data: CA, CI, EH, GJ, IMG, LB, LD, MAL, SP, OP. Analyzing data: CA, EH, GJ, IMG, LD,

Acknowledgements

The authors thank Dr. Marion David for scientific advices in analysing ATX expression and Dr. Luc Malaval for providing primary COB cell samples. The authors thank Fanny Bourguillaut and Diane Platel for technical assistance. The author also thank the members of Animalerie Lyon Est Conventiionnelle et SPF (Animal Care Facilities Lyon-Est Conventional and Specific Pathogen Free, ALECS, University of Lyon, SFR Santé Lyon Est) for animal housing and care and the members of the Centre d'Imagerie Quantitative Lyon-Est platform (CIQLE, Lyon-Est Quantitative Imaging Center) from the Universite Claude Bernard Lyon 1 and SFR Santé Lyon Est for histology experiments.

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Figures

Fig. 1

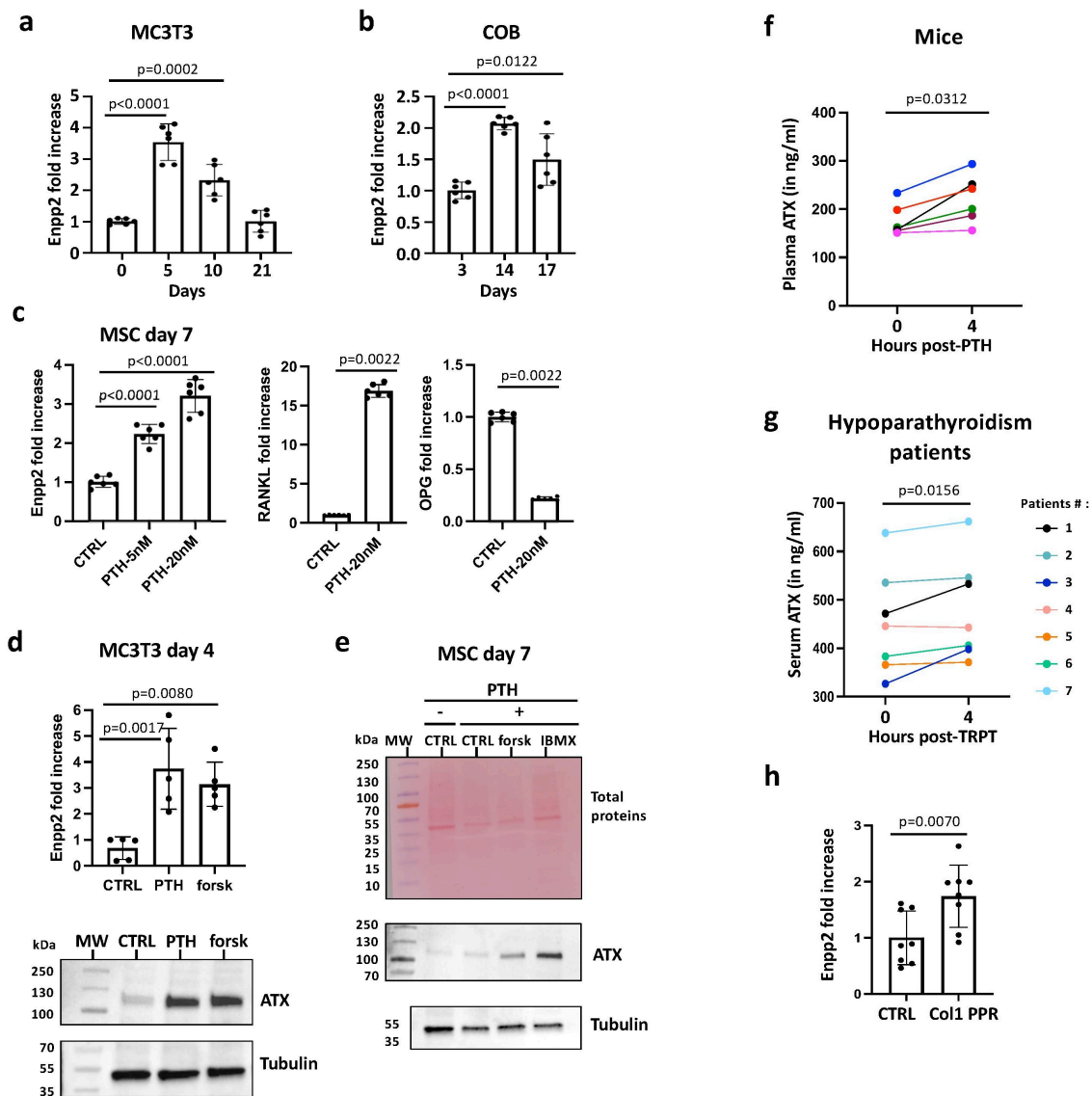


Figure 1

ATX is expressed in osteoblast and up-regulated by PTH *in vitro* and *in vivo*.

Enpp2 mRNA expression profile during osteoblastogenesis in **A)** MC3T3 osteoblast cell line and **B)** in new born mice calvaria derived primary osteoblasts (COB). Data from time course of *Enpp2* expression were reported as fold increase compared to day 0 and day 3 for MC3T3 and COB, respectively. Values are the mean $\pm$ SD of three replicates from two independent experiments. Data were analyzed by One-way ANOVA and Turkey's multiple comparison tests. **C)** *Enpp2* mRNA expression profile in MSC at day 7 in absence (CTRL) or presence of PTH1-34 (5nM or 20nM, left panel). Data were reported as fold increase compared to CTRL. Values are the mean $\pm$ SD of three replicates from two independent

experiments. Statistical significance was assessed using One-way ANOVA and Turkey's multiple comparison tests. **D)** *Enpp2* mRNA expression in the bone of wild type (CTRL, n=8) and Col1 PPR (n=8) mice. Statistical significance was assessed using Mann Whitney tests. **E)** *Enpp2* mRNA expression profile in MC3T3 at day 4 in absence (CTRL) or presence of PTH1-34 (20nM) or forskolin (forsk, 50μM) (upper panel). Data were reported as fold increase compared to CTRL. Values are the mean ± SD of n=5 replicates from two independent experiments. Statistical significance was assessed using One-way ANOVA and Turkey's multiple comparison tests. Western blotting detection of ATX (lower panel) in MC3T3 at day 4 in presence of PTH1-34 (20ng/ml) and forskoline (forsk, 50μM) overnight. Tubulin detection was used as protein loading control. **F)** Western blotting detection of ATX (middle panel) in MSC at day 7 in presence of PTH1-34 supplemented or not with forsk and IBMX (600μM) overnight. Ponceau's red staining of the whole membrane (upper panel) and tubulin detection (lower panel) were used as protein loading control. Quantification of circulating ATX levels by ELISA at baseline (H0) and 4 hours (H4) after PTH1-34 injection in **G)** mouse plasma (n=6) and **H)** in the serum of 7 hypoparathyroidism children treated with TRPT. Statistical analysis by Wilcoxon matched-pairs signed rank test.

Fig. 2

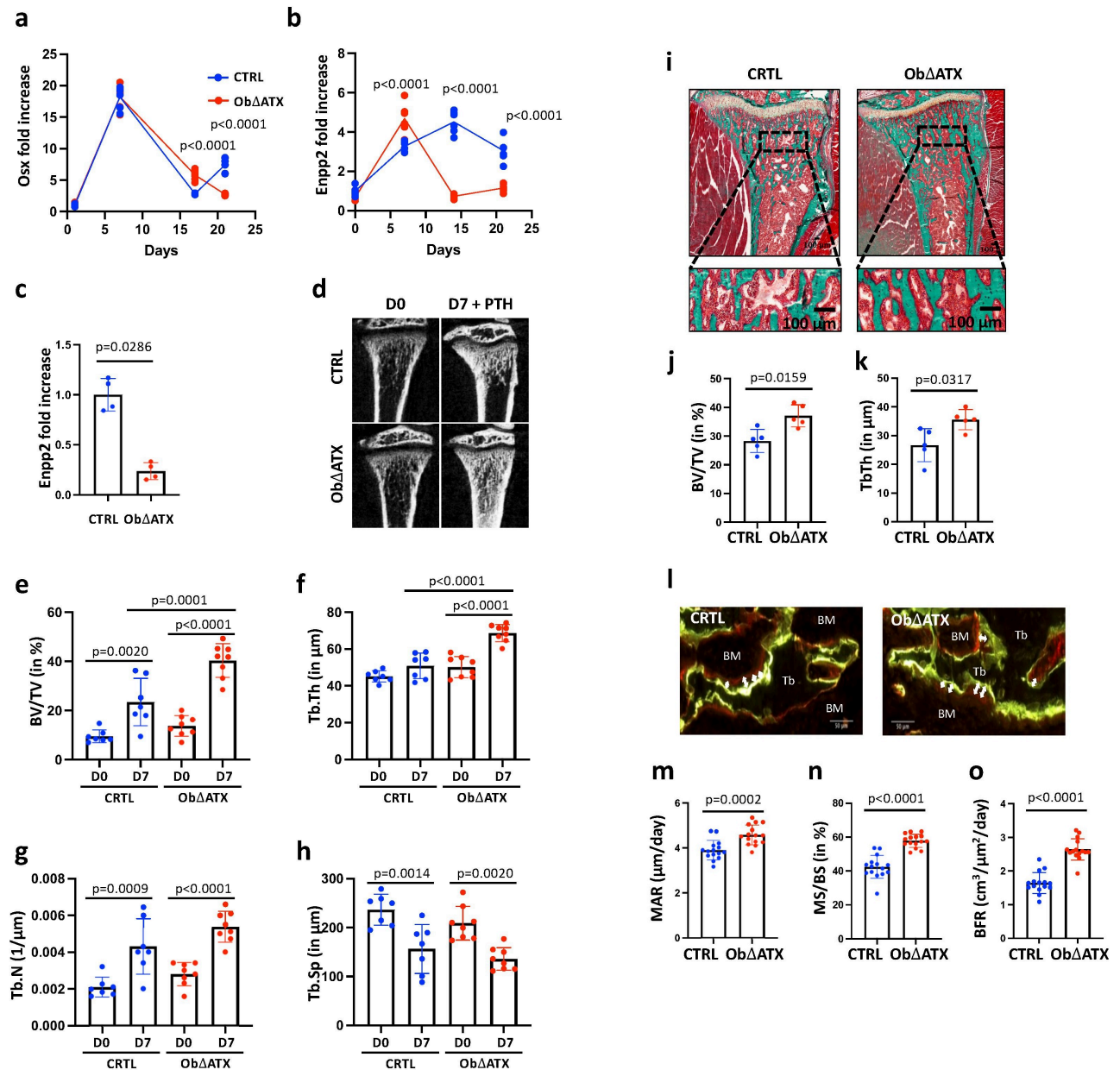


Figure 2

Deletion of *Enpp2* in osteoblastic lineage cells enhances PTH1-34-induced bone mass acquisition.

A) *Osx* and B) *Enpp2* mRNA expression patterns along osteoblast differentiation from MSC isolated from Control (CTRL, blue line, n=6) or conditional osteoblast mutant (ObDATX, red line, n=6) mice displaying postnatal inactivation of *Enpp2* driven by *Osx* promoter, showing *Osx* peak at day 7 in both cell cultures and specific down-regulation of *Enpp2* at day 14 following *Osx* activation. Data represent the mean $\pm$ SD from two independent experiments. Statistical significance was assessed using Two-way ANOVA and Sidák's multiple comparison tests. **C) *Enpp2* mRNA expression** in bone compartment depleted of

bone marrow. *Enpp2* mRNA was significantly decreased reflecting efficient osteoblast *Enpp2* knockout. Data represent mean $\pm$ SD from n=6 animals per group. Statistical significance was assessed using Mann-Whitney test. **D)** Representative three-dimensional reconstructions from in vivo microcomputed tomography (μ CT) of distal tibias from CTRL or ObDATX 4 week old mice before (D0) and after daily treatment with PTH1-34 at 80 μ g/kg for one week (D7) showing increased trabecular bone mass after PTH treatment for both genotypes. Bar chart of trabecular bone mass quantification showing comparisons values in **E)** % of trabecular Bone Volume over Tissue (BV/TV), and in micro architecture parameters of **F)** trabecular thickness, **G)** trabecular number and **H)** trabecular spacing of CTRL (n=7) and ObDATX (n=8) mice. Data represent mean $\pm$ SD. Statistical significance was assessed using One-way ANOVA and Turkey's multiple comparison tests. **I)** Representative Goldner stained histological sections of tibias in CTRL and ObDATX mice at day 7 after daily treatment with PTH1-34 showing increased bone mass in the mutant bone (bar : 100 μ m) quantified by histomorphometric analysis of **J)** BV/TV (in %) and **K)** mean of trabecular thickness (in μ m). Data represent mean $\pm$ SD of CTRL (n=5) and ObDATX (n=5) mice. Statistical significance was assessed using Mann Whitney tests. **L)** Representative images of unstained sections captured by epifluorescence microscopy showing tibias *in vivo* calcein (green) /alizarin (red) staining of CTRL and ObDATX mice at day 7 after daily treatment with PTH1-34 (bar : 50 μ m; BM: bone marrow; Tb: bone trabeculae). Bar charts reporting the measures of **M)** MAR (mineral apposition rate μ m/day), **N)** MS/BS (mineralizing surfaces over the bone surface in %) and **O)** BFR (trabecular bone formation rate μ m³/ μ m²/day). Values correspond to 5 independent sections from 3 animals in each group. Data represent mean $\pm$ SD of CTRL (n=15) and ObDATX (n=15). Statistical significance was assessed using Mann Whitney tests.

Fig. 3

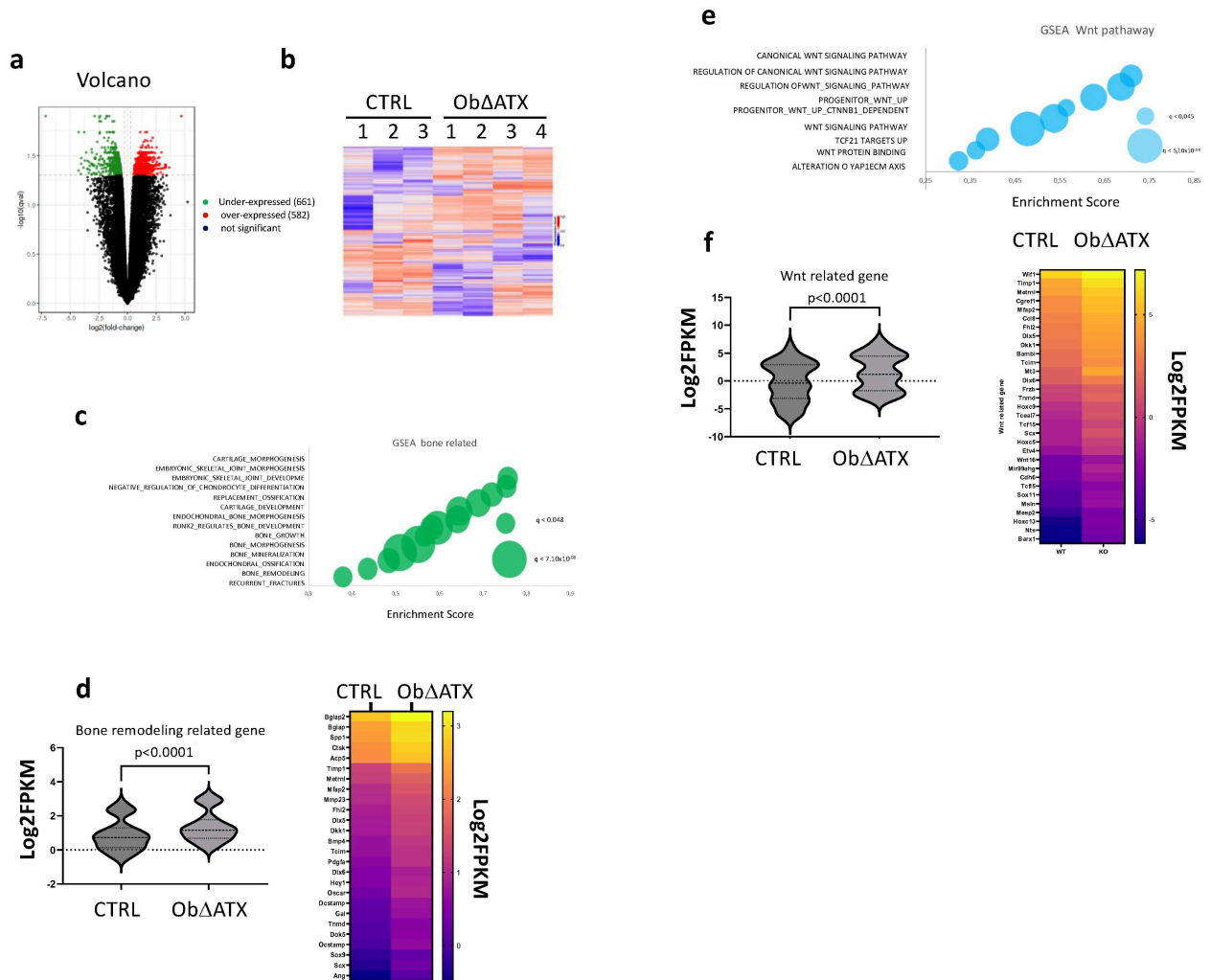


Figure 3

Deletion of *Enpp2* in osteoblastic lineage cells enhances PTH-induced expression of bone-associated gene and Wnt-linked pathway.

A) Volcano plots for the differentially expressed genes (DEGs), ct and ObΔ ATX. Numbers are given for the upregulated (582, red plots) and downregulated (661, green plots) DEGs. **B)** hierarchical clustering showed with a Heat map of differential gene expression between the Control (CTRL) and ATX osteoblastic-deleted bone cell (ObΔATX) groups. **C)** bone remodeling GSEA terms enriched in ObΔATX vs CTRL bone cells. The x-axis represents the enrichment score, y-axis is the bone remodeling GSEA terms enriched. The size of the dot indicates adjusted q-values. **D)** violin plot and heat map bone remodeling gene expression. Violin plot of mRNA expression (FPKM values) for ct and ObΔATX bone cells, Log2 transformation was used. Heatmap of normalized FPKM of DEGs Associated to bone remodeling in CTRL and ObΔATX bone marrow cells, Log2 transformation was used. **E)** wnt signaling GSEA terms enriched in ObΔ ATX vs CT bone cells. The x-axis represents the enrichment score, y-axis is

the wnt signaling GSEA terms enriched. The size of the dot indicates adjusted q-values. **F)** Violin plot and heat map of wnt canonical gene expression Violin plot of mRNA expression (FPKM values) for ct and ObΔATX bone cells, Log2 transformation was used. Heatmap of normalized FPKM of DEGs Associated to wnt signaling in CTRL and ObΔATX bone cells, Log2 transformation was used.

Fig. 4

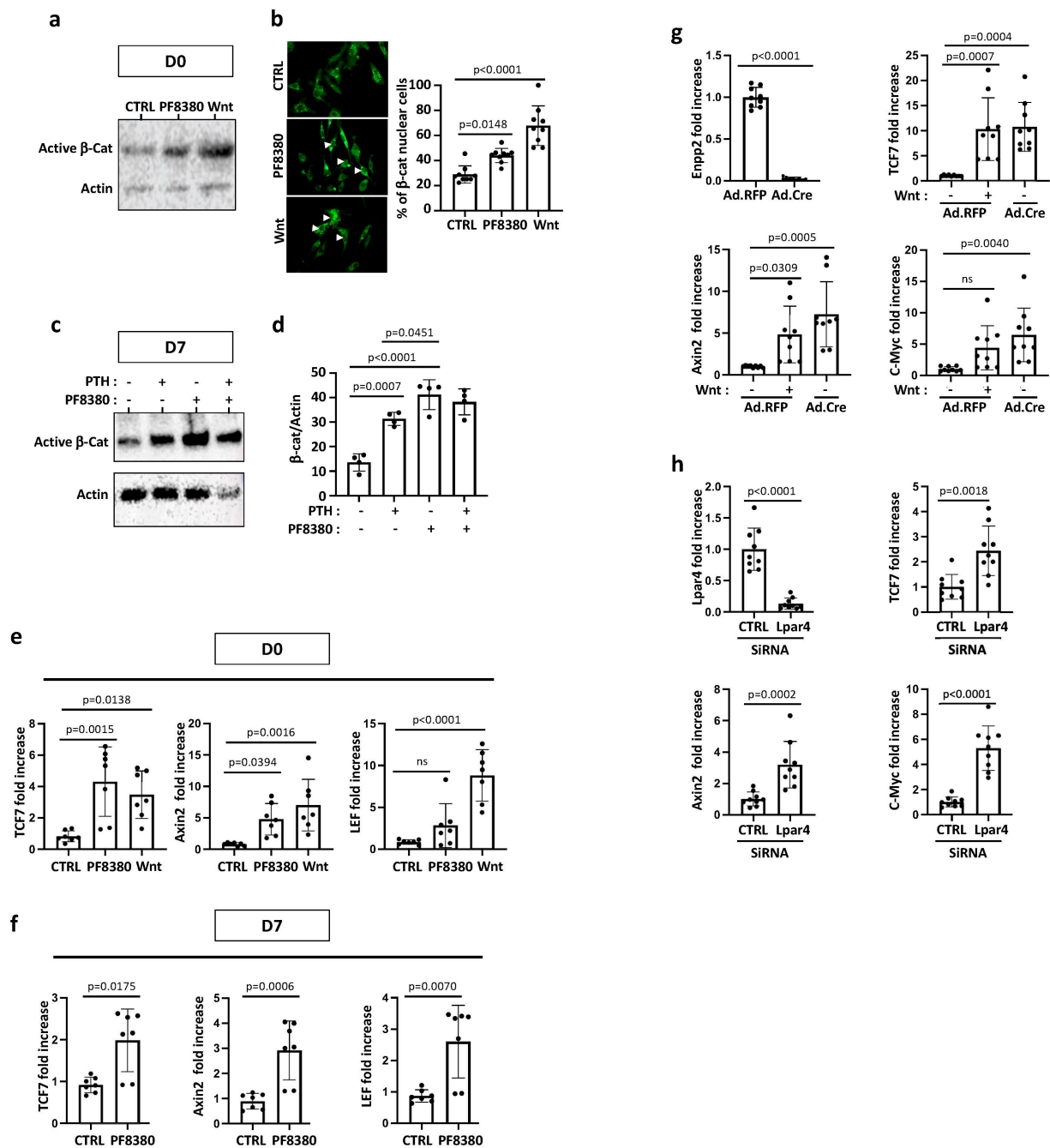


Figure 4
Inhibition of ATX activity promotes the Wnt/b-Cat pathway in mesenchymal stromal cells.

A) Evaluation of active β -catenin expression by Western blot in osteoblastic primary cells treated for 24h with 50% of DMSO (CTRL), PF8380 10 μ M (ATX inhibitor), or Wnt conditioned medium (Wnt, positive control). Actin evaluation serves as a loading control. **B)** Immunostaining of active β -catenin (green) at 40X magnification on primary osteoblastic cells treated for 24h with 50% of Wnt, PF8380 or CTRL. Nuclear-positive active β -catenin was evaluated by counting. Values represents the mean $\pm$ -SD of 5 different fields for 9 independent assays. Statistical significance was assessed using One-way ANOVA and Turkey's multiple comparison tests. **C)** Evaluation of active β -catenin expression by Western blot in osteoblastic primary cells after 7 days of osteoblastic differentiation, treated for 24h with PTH1-34 (PTH), PF8380, or PTH + PF8380. Tubulin evaluation serves as a loading control. **D)** Quantification of Western blot intensity expressed by the ratio of β -catenin/Tubulin. The values represent the mean $\pm$ -SD of 4 independent assays. Statistical significance was assessed using One-way ANOVA and Turkey's multiple comparison tests. **E)** RT-qPCR analysis of Wnt canonical gene targets in osteoblastic primary cells treated for 24h with DMSO, PF8380 or Wnt. *TCF7*, *Axin2* and *LEF* expression were expressed as the fold increase from $\Delta\Delta$ Ct of CTRL using *RPL 13a* as the reference gene. The values represent the mean $\pm$ -SD of 3 independent assays. Statistical significance was assessed using One-way ANOVA and Turkey's multiple comparison tests. **F)** RT-qPCR analysis of Wnt canonical gene targets in osteoblastic primary cells after 7 days of osteoblastic differentiation, treated for 24h with DMSO (CTRL) and PF8380. *TCF7*, *Axin2* and *LEF* expression were expressed as described above. The values represent the mean $\pm$ -SD of 3 independent assays. Statistical significance was assessed using Mann-Whitney test. **G)** Evaluation of *Enpp2* and Wnt canonical gene target expression (*TCF7*, *Axin2* and *C-Myc*) in osteoblastic primary cells harvested from *Enpp2*^{flox/flox} mice after transfection with adenoviral particles coding for red fluorescent protein (RFP) or CRE recombinase protein (Cre). Data were expressed as the fold increase from $\Delta\Delta$ Ct of Ad.RFP value using *RPL 13a* as the reference gene. The values represent the mean $\pm$ -SD of 3 independent assays. Statistical significance was assessed using Mann-Whitney test for Ad.RFP versus Ad.CRE and using One-way ANOVA and Turkey's multiple comparison tests for *TCF7*, *Axin2* and *C-Myc*. **h)** Evaluation of *Lpar4* and Wnt canonical gene target expression in osteoblastic primary cells transfected with CTRL siRNA or siRNA against *Lpar4*. Gene expressions were expressed as the fold increase from $\Delta\Delta$ Ct of CTRL value using *RPL 13a* as the reference gene. The values represent the mean $\pm$ -SD of 3 independent assays. Statistical significance was assessed using Mann-Whitney test.

Supplementary Files

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