Increased Ca\(^{2+}\) signaling through Ca\(_{V}1.2\) promotes bone formation and prevents estrogen deficiency–induced bone loss

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Introduction

Ca\(_{V}1.2\), an L-type voltage–gated Ca\(^{2+}\) channel, plays essential roles in many excitable cells, including
neurons, smooth and cardiac muscle cells, and endocrine cells (1). Upon membrane depolarization, Ca\(^{2+}\)
influx through the activated Ca\(_{V}1.2\) channel triggers a wide range of physiological processes, such as exci-
tation-contraction coupling in cardiac muscle cells, graded contraction in smooth muscle cells of the vas-
culature and gut, hormone secretion in endocrine tissue, postsynaptic responses in various neurons, and
activation of gene expression paradigms across a variety of tissues. Common to all of these examples is
that the excitable cells in which they occur support frequent and dynamic changes in membrane potential
that facilitate activation of the voltage-gated Ca\(_{V}1.2\) channel. In contrast, roles for Ca\(_{V}1.2\) signaling in non-
exciteable cells are not well understood, but a gain-of-function mutation (G406R) in the human CACNA1C
gene, encoding the pore-forming \(a_{1C}\) subunit of Ca\(_{V}1.2\), causes Timothy syndrome (TS) (2) and reveals
prominent but previously unappreciated roles for Ca\(_{V}1.2\) in nonexcitable tissues.

While the prevalence of osteoporosis is growing rapidly with population aging, therapeutic options
remain limited. Here, we identify potentially novel roles for Ca\(_{V}1.2\) L-type voltage–gated Ca\(^{2+}\)
channels in osteogenesis and exploit a transgenic gain-of-function mutant Ca\(_{V}1.2\) to stem bone
loss in ovariectomized female mice. We show that endogenous Ca\(_{V}1.2\) is expressed in developing
bone within proliferating chondrocytes and osteoblasts. Using primary BM stromal cell (BMSC)
cultures, we found that Ca\(^{2+}\) influx through Ca\(_{V}1.2\) activates osteogenic transcriptional programs and
promotes mineralization. We used Prx1\(^{-}\), Col2a1\(^{-}\), or Col1a1-Cre drivers to express an inactivation-
deficient Ca\(_{V}1.2\) mutant in chondrogenic and/or osteogenic precursors in vivo and found that the
resulting increased Ca\(^{2+}\) influx markedly thickened bone not only by promoting osteogenesis, but
also by inhibiting osteoclast activity through increased osteoprotegerin secretion from osteoblasts.
Activating the Ca\(_{V}1.2\) mutant in osteoblasts at the time of ovariectomy stemmed bone loss.
Together, these data highlight roles for Ca\(_{V}1.2\) in bone and demonstrate the potential dual anabolic
and anticatabolic therapeutic actions of tissue-specific Ca\(_{V}1.2\) activation in osteoblasts.
In a previous study, increased Ca\(^{2+}\) influx through the TS-mutant Ca\(_{\text{v}}\)\(_{1.2}\) (Ca\(_{\text{v}}\)\(_{1.2}^{\text{TS}}\)) channel in mandibular chondrocytes was shown in zebrafish and mouse models to recapitulate the macrosdontia observed in TS patients (4). As the mandible is unique among skeletal elements in the skull to derive from endochondral ossification, we hypothesized that Ca\(_{\text{v}}\)\(_{1.2}\) may influence bone development more broadly. Indeed, while plasma membranes of bone cells (e.g., osteoblasts) have not been shown to prominently display the voltage-dependent changes that control Ca\(_{\text{v}}\)\(_{1.2}\) activity in excitable tissue, earlier reports identified functional L-type Ca\(^{2+}\) channels, mainly Ca\(_{\text{v}}\)\(_{1.2}\), in human mesenchymal stem cells from BM cultured in osteogenic medium (5), in osteosarcoma cell lines (6), and in a rat osteoblast-like cell line (7). Further, Ca\(_{\text{v}}\)\(_{1.2}\) was shown in an osteosarcoma cell line to be the primary site for Ca\(^{2+}\) influx, which promoted osteoblast differentiation (8, 9). In addition, Ca\(_{\text{v}}\)\(_{1.2}\) expression and channel activity were regulated by calciotropic hormones such as 1,25(OH)\(_2\)D\(_3\) (8, 10) and parathyroid hormone (11), suggesting a functional contribution of Ca\(_{\text{v}}\)\(_{1.2}\) L-type Ca\(^{2+}\) channel to bone development. More recently, a mutation in \(\text{CACNA1C}\) causing reduced current density through Ca\(_{\text{v}}\)\(_{1.2}\) was associated with osteopenia (12).

Because Ca\(_{\text{v}}\)\(_{1.2}\) is a well-characterized pharmacological target, for which antagonists are widely used to treat hypertension, angina, and cardiac arrhythmias, we set out to explore Ca\(_{\text{v}}\)\(_{1.2}\) contributions to bone development and the consequent therapeutic potential of modulating Ca\(_{\text{v}}\)\(_{1.2}\) activity in bone. We characterized the expression profile of Ca\(_{\text{v}}\)\(_{1.2}\) during bone development and exploited Ca\(_{\text{v}}\)\(_{1.2}^{\text{TS}}\) transgenic mouse models to show that the gain-of-function Ca\(_{\text{v}}\)\(_{1.2}^{\text{TS}}\) promotes bone formation both during development and postnatally. We provide evidence that the augmented bone mass results from increased osteoblast differentiation and decreased osteoclast formation. With this foundation, we then demonstrated that activation of Ca\(_{\text{v}}\)\(_{1.2}^{\text{TS}}\) expression in osteoblasts reduced bone loss in an ovariectomy-induced (OVX-induced) osteoporosis mouse model.

**Results**

**\(\text{Ca}_{\text{v}}\)\(_{1.2}\) is expressed during mouse bone development.** To determine whether Ca\(_{\text{v}}\)\(_{1.2}\) contributes to bone development, we first examined endogenous Ca\(_{\text{v}}\)\(_{1.2}\) expression in developing limbs by using a Ca\(_{\text{v}}\)\(_{1.2}\) reporter mouse (B6.129P2-Cacna1ctm1Dgen/J, Ca\(_{\text{v}}\)\(_{1.2}^{\text{TS}}\)) in which a lacZ cassette (with a nuclear localization signal) disrupts the \(\text{Cacna1c}\) locus. While complete KO of \(\text{Cacna1c}\) is embryonic lethal due to inadequate cardiac output, Ca\(_{\text{v}}\)\(_{1.2}^{\text{TS}}\) mice are viable and fertile without obvious differences in any aspect of development (13) and only minor differences in morphology (4). The lacZ expression in Ca\(_{\text{v}}\)\(_{1.2}^{\text{TS}}\) mice thereby provides an accurate picture of the temporospatial Ca\(_{\text{v}}\)\(_{1.2}\) expression pattern during development and definitive identification of Ca\(_{\text{v}}\)\(_{1.2}\)-expressing cells. We performed X-gal staining on frozen sections of long bones from early postnatal mice (P10), in which we observed strong staining in the resting and proliferating chondrocytes, but we did not detect staining in the hypertrophic chondrocytes (Figure 1, A–C). Expression was also strong in the perichondrium/periosteum (Figure 1, A and D), and extensive staining appeared in the lining cells of trabecular bones underneath the growth plate (Figure 1, A and E). At a later prenatal stage (P18), we also observed significant staining in the endosteum (Figure 1F). This staining pattern suggested that at least some of Ca\(_{\text{v}}\)\(_{1.2}\) resides in osteoblast progenitors, which we further tested by examining expression in primary BM stromal cells (BMSCs) isolated from adult Ca\(_{\text{v}}\)\(_{1.2}^{\text{TS}}\)/lacZ mice. Ca\(_{\text{v}}\)\(_{1.2}\) expression as indicated by lacZ staining in these BMSCs (Figure 1G) — along with previously reported RNA sequencing data showing Ca\(_{\text{v}}\)\(_{1.2}\) expression in osteoblasts and osteoblast progenitors (14), but not in the osteoclast lineage (15) — suggest important roles for Ca\(_{\text{v}}\)\(_{1.2}\) in skeletogenesis.

**Pharmacological inhibition of Ca\(_{\text{v}}\)\(_{1.2}\) channel activity decreased osteoblast differentiation.** Based on the above expression patterns, we next asked whether Ca\(^{2+}\) influx through endogenous Ca\(_{\text{v}}\)\(_{1.2}\) affected osteoblast differentiation. We performed an in vitro osteogenesis assay with BMSCs in the absence and presence of 2 different specific L-type Ca\(^{2+}\) channel blockers, diltiazem and nifedipine, applied to the cultures 12 hours before adding differentiation media. Cells were then cultured with the channel blocker or their diluents throughout the differentiation process. After 14 days of culture in osteogenic media, BMSCs treated with a channel blocker displayed substantially reduced mineralized nodule formation, as shown by von Kossa staining (Figure 1H). Quantitative PCR (qPCR) revealed that, in cells treated with the channel blocker, there was a marked decrease in transcripts of several osteoblast markers, including \(\text{Alpl, Ibsp,}\) and \(\text{Bglap}\) (Figure 1I). Together, these data suggest that Ca\(^{2+}\) signaling through endogenous Ca\(_{\text{v}}\)\(_{1.2}\) regulates osteogenesis.
Increased Ca\(^{2+}\) influx through Ca\(_{\text{v}}\)\(_{1.2}\) promotes bone formation in vivo. To explore whether Ca\(^{2+}\) signaling through Ca\(_{\text{v}}\)\(_{1.2}\) in developing bone affects skeletogenesis in vivo, we exploited a transgenic mouse line in which a silenced TS mutant Ca\(_{\text{v}}\)\(_{1.2}\) Ca\(_{\text{c}}\text{a}_{\text{1c}}\) cDNA (Ca\(_{\text{v}}\)\(_{1.2}\)TS) as a control had been knocked into the Rosa26 locus (Rosa26-Ca\(_{\text{v}}\)\(_{1.2}\)TS and Rosa26-Ca\(_{\text{v}}\)\(_{1.2}\)WT, respectively). Expression of these cDNAs can be activated in a tissue-specific manner upon Cre recombinase–mediated excision of an upstream stop codon (16). We activated Ca\(_{\text{v}}\)\(_{1.2}\)TS expression with Prxl-Cre targeting early mesenchymal stem cells (17), Col2a1-Cre targeting osteochondral progenitors (18), or Col1a1-Cre targeting more mature osteoblast-lineage cells (19). At 6 weeks of age, mice in which Ca\(_{\text{v}}\)\(_{1.2}\)TS expression was driven by Prxl-Cre showed profoundly increased bone mass in the appendicular skeleton and in the skull but not the axial skeleton — consistent with the restricted expression pattern of Prxl (20) — as detected by radiography and \(\mu\)CT when compared with their Cre\(^{-}\) control littermates (Figure 2, A, C, and E). The combined distal femur cortical and trabecular bone volume (BV/TV) in Prxl-Cre;Ca\(_{\text{v}}\)\(_{1.2}\)TS mice was increased by > 2-fold compared with controls at 6 weeks of age (Figure 2D). Ca\(_{\text{v}}\)\(_{1.2}\)TS expression driven by either Col2a1-Cre or Col1a1-Cre produced an increased bone mass phenotype similar to that observed in Prxl-Cre;Ca\(_{\text{v}}\)\(_{1.2}\)TS mice (Supplemental Figure 1, A, B, D and E; supplemental material available online with this article; https://doi.org/10.1172/jci.insight.95512DS1). Compared with the appendicular-restricted phenotype in the Prxl-Cre;Ca\(_{\text{v}}\)\(_{1.2}\)TS mice, we observed increased bone mass in both the appendicular and axial skeleton (data not shown) in the Col2a1-Cre;Ca\(_{\text{v}}\)\(_{1.2}\)TS and Col1a1-Cre;Ca\(_{\text{v}}\)\(_{1.2}\)TS mice, however. These results are consistent with the broad expression pattern of Col2a1-Cre and Col1a1-Cre throughout the skeleton. The high bone mass phenotype persisted at least to 12 months of age (data not shown) and resulted in almost complete obliteration of the BM cavity in long bones. Consistent with obliteration of the BM cavity and predicted extramedullary hematopoiesis, the Ca\(_{\text{v}}\)\(_{1.2}\)TS mutant mice exhibited marked splenomegaly (Supplemental Figure 1, G–J). This latter observation suggests that expression of the noninactivating Ca\(_{\text{v}}\)\(_{1.2}\)TS mutant channel — not just overexpression of Ca\(_{\text{v}}\)\(_{1.2}\) — promotes increased bone mass in vivo. Alican Blue Hematoxylin/Orange G (ABH/OG) staining confirmed that both primary and secondary ossification centers of Ca\(_{\text{v}}\)\(_{1.2}\)TS mutant mice were occupied with excessive bone (Figure 2F and Supplemental Figure 1, C and F). Extensive Alican Blue–stained cartilage remnant was detected in the primary spongiosa under the growth plate of mutant mice (Figure 2F and Supplemental Figure 1, C and F). Moreover, the...
mutant bones lacked sculpting (Figure 2C and Supplemental Figure 1, A and D), indicating osteoclast defects (see below for further analysis).

Since the Prx1-Cre driver activated Ca\textsubscript{v}1.2\textsuperscript{TS} expression during skeletal development, we next assessed whether we were able to detect the increase in bone mass in embryonic stage. Indeed, at P0, histology confirmed that Prx1-Cre;Ca\textsubscript{v}1.2\textsuperscript{TS} femurs possessed a thicker bone collar and higher bone mass in the presumptive marrow cavity compared with littermate controls (Figure 2, G and H).

Increased Ca\textsuperscript{2+} influx through Ca\textsubscript{v}1.2 promotes osteoblast differentiation. To investigate further whether enhanced osteoblast function contributed to the increased bone mass, we performed dynamic histomorphometric analysis using calcein and alizarin red double labeling. Extensive green and red labeling was observed in the Prx1-Cre;Ca\textsubscript{v}1.2\textsuperscript{TS} mutant femur, in stark contrast to that in the control, indicating more active bone formation in Ca\textsubscript{v}1.2\textsuperscript{TS}-expressing mice (Figure 3, A and B). A similar result was observed in Col2a-Cre;Ca\textsubscript{v}1.2\textsuperscript{TS} mutant femurs with calcein double labeling (Supplemental Figure 3, A and B).

To test the role of Ca\textsubscript{v}1.2\textsuperscript{TS} in osteogenesis directly, we performed osteoblast differentiation assays in vitro by isolating BMSCs from Rosa26-Ca\textsubscript{v}1.2\textsuperscript{TS} adult mice and infecting the cultured cells with adenovirus expressing either Cre recombinase (to remove the floxed STOP codon) or GFP as a control. von Kossa staining revealed widespread formation of mineralized nodules in Ca\textsubscript{v}1.2\textsuperscript{TS} expressing (infected with Cre) BMSCs after 10 days of differentiation, a time when there was limited mineralization in the control (infected with GFP) BMSCs (Figure 3C). We explored the mechanisms driving the accelerated mineralization in BMSCs expressing Ca\textsubscript{v}1.2\textsuperscript{TS} with qPCR. This revealed markedly upregulated mRNA expression of 2 master osteogenic transcription factors, Runx2 and Sp7, in Ca\textsubscript{v}1.2\textsuperscript{TS}-expressing BMSCs (Cre-infected) compared with control cells (GFP-infected) 48 hours after exposure to osteogenic differentiation media (Figure 3D). The increase persisted for the ensuing 15 days. Similarly, expression of Alpl, Ibsp, and Bglap transcripts was also elevated within 48 hours of differentiation in Ca\textsubscript{v}1.2\textsuperscript{TS}-expressing BMSCs and remained significantly higher (compared with control) over the following 15 days (Figure 3D). Thus, these results indicate that expression of Ca\textsubscript{v}1.2\textsuperscript{TS} accelerated osteoblast differentiation of BMSCs in vitro.
CaV1.2TS channel blocks osteoclast differentiation. The lack of sculpting of CaV1.2TS mutant long bones and the excessive cartilage remnant in the mutant primary spongiosa prompted us to hypothesize that osteoclast defects also contributed to the increased bone mass in CaV1.2TS mutant bones. To test this hypothesis, we performed tartrate-resistant acid phosphatase (TRAP) staining on femurs from 6-week-old mice. We observed substantially fewer osteoclasts in CaV1.2TS-expressing mice compared with littermate controls (Figure 4A and Supplemental Figure 3C). Quantitative analysis showed that osteoclast number per tissue area (Oc. N/T. Ar) was decreased by 55% and osteoclast area per tissue area (Oc. Ar/T. Ar) decreased by 54% in Prx1-Cre;CaV1.2TS mice (Figure 4B). A similar phenotype was also observed in Col2a1-Cre;CaV1.2TS mice (Supplemental Figure 3, D and E), indicating reduced osteoclastogenesis in CaV1.2TS mutant mice.

As Prx1-Cre, Col2a1-Cre, and Col1a1-Cre do not target the osteoclast lineage, we hypothesize that CaV1.2TS interfered with osteoblast-mediated osteoclastogenesis through the osteoprotegerin/RANKL (OPG/RANKL) pathway. We tested this by measuring serum OPG, an inhibitor of RANKL, and observed a 34% increase of OPG in Prx1-Cre;CaV1.2TS and a 27% increase in Col2a1-Cre;CaV1.2TS transgenic mice.
We further examined whether CaV1.2TS affects the Rankl/Opg (Tnfs11/Tnfrsf11b) mRNA expression ratio in primary BMSC cultures after activating CaV1.2TS expression and found — consistent with the in vivo data — a 61% decrease in CaV1.2TS-expressing BMSCs (Figure 4D). Additionally, we cocultured BM-derived macrophages (BMMs) with CaV1.2TS-expressing or WT calvarial osteoblasts in the presence of 1,25-dihydroxyvitamin D3 and prostaglandin E2 and observed less TRAP-positive osteoclasts with osteoblasts expressing the CaV1.2TS than with WT osteoblasts (Figure 4, E and F), suggesting that CaV1.2TS expressed in cells of osteoblast lineage blocks osteoclastogenesis.

Postnatal expression of CaV1.2TS channel in Sp7-lineage cells enhances accrual of trabecular bone and prevents estrogen deficiency–induced bone loss. Having shown that CaV1.2TS expression enhances bone formation, we tested whether we could exploit CaV1.2TS expression to stimulate bone formation postnatally. We generated a doxycycline-inducible Sp7-Cre;CaV1.2TS line by crossing the floxed-STOP Rosa26-CaV1.2TS with an inducible Sp7-Cre recombinase (Sp7-tTA;TetO-EGFP-Cre) (21). We blocked Cre expression with doxycycline-impregnated food until mice were 2 months old, then we removed doxycycline to activate CaV1.2TS and analyzed the long bones 8 weeks later. μCT 3-D reconstruction of the distal femur metaphysis revealed a marked increase in trabecular bone in CaV1.2TS-expressing mice (Supplemental Figure 4A). Quantitative measurement showed that BV/TV was increased by 56% in Sp7-Cre;CaV1.2TS male mice after activating CaV1.2TS expression for 8 weeks. This increase in bone acquisition was characterized by significant increase in trabecular thickness (Tb.Th) and trabecular number (Tb.N), whereas trabecular bone separation (Tb.Sp) was unaffected (Supplemental Figure 4B).

We then tested whether expression of CaV1.2TS in postnatal bone could prevent estrogen deficiency–induced bone loss in female mice. We performed OVX on 2-month-old Sp7-Cre;CaV1.2TS mice or their Cre−;CaV1.2TS littermate controls and induced CaV1.2TS expression by removing doxycycline at the time of surgery. Simultaneous sham operation on the control (Cre−;CaV1.2TS littermates) allowed us to monitor the effects of OVX in the absence of activating CaV1.2TS. At 4 months old, the growth plates of the distal femur and the length of the humerus were...
normal in these mice (Supplemental Figure 5), suggesting that depleting estrogen or activating CaV1.2TS expression at the 2-month-old time point does not affect the growth plate and the longitudinal growth of long bones. In the Cre controls subject to OVX, we observed noticeable trabecular bone loss (compared with sham controls) 8 weeks after OVX (Figure 5A). Quantitative measurements showed that 43% of BV/TV was lost in control mice after OVX (Figure 5B), consistent with less trabecular bones shown in H&E staining (Figure 5C). Compared with sham-operated control mice, the parameter Tb.N decreased and Tb.Sp increased after OVX surgery (Figure 5B), indicating that OVX can induce loss of trabecular bone by perforation and division, as seen in previous OVX experiments (22, 23). Dynamic histomorphometric analysis showed that bone formation rate (BFR) was decreased by 71%, mineral apposition rate (MAR) was decreased by 49%, and the percentage of mineralizing surface over bone surface (MS/BS) was decreased by 38% in the control group after OVX (Figure 5, D and E), indicating reduced osteoblast function after estrogen deficiency at 2 months of age. In contrast, activation of CaV1.2TS (by removing doxycycline) at the time of surgery effectively mitigated bone loss and maintained bone mass after OVX (BV/TV, 4.25% ± 0.54%) to the same level as that in the sham-operated controls (BV/TV, 4.49% ± 0.33%) (Figure 5B). Consistent with these data, we confirmed by H&E staining that OVX CaV1.2TS mice maintained trabecular bone in femurs similar to control sham mice and stemmed trabecular bone loss compared with OVX control mice (Figure 5C). The maintenance of bone mass after OVX in the CaV1.2TS-expressing bones was in part due to sustained osteoblast function, as dynamic histomorphometric analysis showed that BFR, MAR, and MS/BS were unaffected in CaV1.2TS mutant femurs after OVX compared with control sham mice and were significantly higher than control OVX mice (Figure 5, D and E).
Discussion

This study revealed unexpected roles for the Ca\textsubscript{\textsubscript{\textalpha\textbeta\textgamma}} voltage-gated Ca\textsuperscript{2+} channel in bone development and remodeling and uncovered consequences of increased Ca\textsuperscript{2+} influx through the channels. Using conditional mouse models and the BMSC culture system, we demonstrated that expression of a transgenic Ca\textsubscript{\textalpha\textbeta\textgamma} mutant channel promotes bone formation and inhibits bone resorption during skeletal development, homeostasis, and osteoporotic contexts. Actions of voltage-gated L-type Ca\textsuperscript{2+} channels were previously reported in osteosarcoma cell lines (6, 8, 24), and a previous immunohistochemical study suggested the presence of Ca\textsubscript{\textalpha\textbeta\textgamma} in developing bones (25). Our data, exploiting a definitive Ca\textsubscript{\textalpha\textbeta\textgamma} signal from a mouse in which lacZ was knocked into the Cacna1c gene, reveal the endogenous Ca\textsubscript{\textalpha\textbeta\textgamma} expression pattern and avoid nonspecific signals that can arise from detection by antibodies. With this approach, we observed prominent endogenous Ca\textsubscript{\textalpha\textbeta\textgamma} expression in the developing perichondrium/periosteum, endosteum, and growth plate, which all give rise to osteoprogenitors. We did not detect Ca\textsubscript{\textalpha\textbeta\textgamma} in the marrow cavity, as was suggested by one study using an immunohistochemical approach (25) but not by another (26). Further, we observed the consequences of activating Ca\textsubscript{\textalpha\textbeta\textgamma} expression in vivo. Thus, these data extend previous findings for a Ca\textsubscript{\textalpha\textbeta\textgamma} role in mandibular development (4) and highlight additional unexpected roles for Ca\textsubscript{\textalpha\textbeta\textgamma} channels in nonexcitable tissue.

It is intriguing that, in the growth plate, we observed Ca\textsubscript{\textalpha\textbeta\textgamma} expression in the proliferating chondrocytes but not hypertrophic chondrocytes. One resulting hypothesis is that termination of Ca\textsubscript{\textalpha\textbeta\textgamma} expression (and the resulting downstream Ca\textsuperscript{2+} signaling) is required for the transition of proliferating chondrocytes to a hypertrophic state. Consistent with this hypothesis is our observation that bones expressing Ca\textsubscript{\textalpha\textbeta\textgamma} are shorter: in Ca\textsubscript{\textalpha\textbeta\textgamma}-expressing mice in which Ca\textsubscript{\textalpha\textbeta\textgamma} was driven by Prx1-Cre or Col2a1-Cre — both of which target chondrocyte lineages — a delay in chondrocyte maturation may reduce the ultimate length of the long bones.

Because these mouse models all employed Cre recombinases that drove Ca\textsubscript{\textalpha\textbeta\textgamma} expression in osteoblast lineages, and not in osteoclasts or their precursors, we conclude that the observed antiresorptive responses resulted from an increase in the Rankl/Opg expression ratio and OPG secretion and consequent extrinsic inhibition of osteoclastogenesis. This is consistent with previous data showing that Ca\textsubscript{\textalpha\textbeta\textgamma} regulates OPG secretion (27) and the well-defined roles for Ca\textsubscript{\textalpha\textbeta\textgamma} channels in promoting hormone secretion from endocrine tissue. Moreover, our lacZ expression data showed that Ca\textsubscript{\textalpha\textbeta\textgamma} is expressed within bone tissue where osteoblast precursor cells reside, and previous RNA-seq data showed no expression of Ca\textsubscript{\textalpha\textbeta\textgamma} in murine BMMs or during osteoclast differentiation (15).

Our data showing upregulation of 2 master osteogenic transcription factors, Runx2 and Sp7, after expression of Ca\textsubscript{\textalpha\textbeta\textgamma} suggest that increased Ca\textsuperscript{2+} influx through the noninactivating mutant Ca\textsubscript{\textalpha\textbeta\textgamma} channel augments signaling cascades that promote osteoblast differentiation and mineralization. Moreover, the reduced mineralization observed in BMSC cultures treated with Ca\textsubscript{\textalpha\textbeta\textgamma} blockers highlights the physiologic contribution of Ca\textsuperscript{2+} influx through endogenous Ca\textsubscript{\textalpha\textbeta\textgamma} channels in osteoblast precursors. Nevertheless, we cannot exclude the possibility that Ca\textsuperscript{2+}-independent signaling through Ca\textsubscript{\textalpha\textbeta\textgamma} contributes to the Ca\textsubscript{\textalpha\textbeta\textgamma}-induced increase in bone formation. Two previous studies reported that the mutant Ca\textsubscript{\textalpha\textbeta\textgamma} channel activates Ca\textsuperscript{2+}-independent downstream signaling pathways in neurons or hair follicles, as suggested by experiments showing that expression of a “pore-dead” Ca\textsubscript{\textalpha\textbeta\textgamma} channel recapitulated the Ca\textsubscript{\textalpha\textbeta\textgamma} channel effects (28, 29). Based on observations regarding the role of Ca\textsubscript{\textalpha\textbeta\textgamma} in mandibular development — in which a Ca\textsuperscript{2+}-permeant WT or Ca\textsubscript{\textalpha\textbeta\textgamma} mutant channel, but not a pore-dead channel, successfully rescued abnormal development after Ca\textsubscript{\textalpha\textbeta\textgamma} knockdown — we propose that Ca\textsuperscript{2+} influx through Ca\textsubscript{\textalpha\textbeta\textgamma} is essential for bone development.

Our data showing that activating a transgenic, overexpressed Ca\textsubscript{\textalpha\textbeta\textgamma} channel during embryogenesis or postnatally increases bone mass and prevents bone loss secondary to estrogen deficiency do not likely have implications for TS patients, who have a mutant, endogenous CACNA1C locus present throughout development and beyond. Rather, the consistent results obtained by activating a Ca\textsubscript{\textalpha\textbeta\textgamma} channel specifically with Prx1-Cre, Col2a1-Cre, or Col1a1-Cre suggest that increasing Ca\textsuperscript{2+} influx through a noninactivating Ca\textsubscript{\textalpha\textbeta\textgamma} channel in any osteoblast lineage cell promotes bone formation by increasing osteoblast differentiation and inhibiting osteoclast differentiation. The dual effect of transgenic Ca\textsubscript{\textalpha\textbeta\textgamma} suggests a possible therapeutic strategy for osteoporotic or osteopenic conditions. The majority of current osteoporosis treatments are antiresorptive agents that reduce osteoclast bone resorption, such as estrogens, bisphosphonates, and calcitonin (30, 31). These antiresorptives (especially bisphosphonates) are associated with gastrointestinal problems, osteonecrosis of
the jaw, and atypical femur fractures or fragility fractures. Therefore, our approach here is more in line with anabolic therapies such as recombinant human parathyroid hormone (rhPTH) (32) and more recently developed agents demonstrating dual roles (e.g., romosozumab) that show increasing promise (33). Further, the increased bone accrual postnatally observed with the inducible Sp7-Cre model highlights the potential for the development of bone-targeted CaV1.2 agonists, starting with lead compounds such as BayK-8644 or FPL 64176, for anabolic and anticatabolic skeletal effects, as observed in our CaV1.2TS mice.

Methods

Experimental mouse models. CaV1.2+/LacZ, Rosa26-CaV1.2TS, and Rosa26-CaV1.2WT mouse lines have been described previously (4, 16). Homozygous Rosa26-CaV1.2TS or Rosa26-CaV1.2WT mice were crossed with the transgenic Ptx1-Cre (17), Col2α1-Cre (18), or 2.3Colα1-Cre (19) mice. All skeletal analyses were performed on 6-week-old mice unless otherwise specified. For serum OPG assay (R&D Systems), serum was collected from 6-week-old mice without fasting following the manufacturer’s instructions.

To generate inducible mouse models for investigating postnatal bone formation and remodeling, we crossed Rosa26-CaV1.2TS mice with Sp7-Cre (B6.Cg-Tg [Sp7-tTA, tetO-EGFP/cre] 1Amc/J; The Jackson Laboratory) (21). Breeders and their pups were maintained on a rodent diet containing 625 mg/kg doxycycline (Harlan) to suppress Cre recombinase expression in the embryonic and early postnatal stages. Chow was replaced with regular rodent diet (no doxycycline) to induce Cre expression when mice were 2 months old. For OVX experiments, OVX or sham operation were performed on 2-month-old female mutant (Sp7-Cre;CaV1.2TS) and control (Cre;CaV1.2TS) mice under isofluorane anesthesia. The doxycycline diet was replaced with regular rodent diet on the same day of OVX or sham surgery. Tissue was collected for analysis 8 weeks after OVX.

Chemicals. All chemicals were obtained from MilliporeSigma unless otherwise indicated.

X-gal staining. X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining was performed on frozen tissue sections. Forelimbs or hindlimbs were fixed in 4% paraformaldehyde on ice for 20 minutes (embryos) or 1 hour (postnatal stage), decalcified with 14% EDTA at 4°C, processed to 30% sucrose, and snap-frozen embedded with OCT compound (Sakura Finetek). Frozen sections (10 μm–thick) were refixed in ice-cold 1× PBS with 2% paraformaldehyde, 0.2% glutaraldehyde, 2 mM MgCl2, 0.2% Tween-20, and 30% sucrose for 10 minutes and stained with X-gal staining solution (5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 1 mg/ml X-gal, 2 mM MgCl2, 0.1% sodium deoxycholate, and 0.2% IGEPAL CA-630) in dark for 24–48 hours at 37°C. Specimens were counterstained with Nuclear Fast Red. For X-gal staining on BMSCs, the staining was performed for 48 hours at 37°C.

X-ray radiography and μCT analysis of mice. X-ray radiographic images of the hindlimbs were acquired using a Faxitron X-ray system (Faxitron Biopics) at 31 kV for 9 seconds. For μCT scanning, the specimens were fixed in 10% neutral buffered formalin and then fitted in a 50 ml centrifuge tube with 70% ethanol. Images were obtained with a Scanco VivaCT 80 scanner (Scanco Medical AG) set to 55 kVp and 145 μA, voxel size 10.4 μm. To quantify trabecular bone parameters, 50 μCT slices (~0.5 mm total) immediately below the distal growth plate of the femur were analyzed.

Double labeling and cryosectioning. For dynamic histomorphometry, mice were injected i.p. with calcine and alizarin-3-methyliminodiacetic acid (MilliporeSigma) at 20 mg/kg and 40 mg/kg, respectively, on days 8 and 2 prior to euthanasia. Femurs were extracted and fixed in 10% neutral buffered formalin for 24 hours. After washing with 1× PBS, nondecalcified femurs were processed to 30% sucrose and OCT embedding, and they were sectioned at 10 μm using Leica Cryostat equipped with CryoJane (Leica). The slides were counterstained with DAPI. Dynamic histomorphometry was performed with OsteoMeasure commercial software.

Histology and TRAP staining. For histology, decalcified samples were processed for paraffin embedding and then sectioned at 6-μm thickness. H&E, ABH/OG, and TRAP staining were performed following standard protocols.

Mouse BMSC isolation and culture, viral infection, and osteoblast differentiation. BMSCs were isolated aseptically from the femurs and tibias of 6- to 10-week-old Rosa26-CaV1.2TS or WT mice and were plated in α-MEM without ascorbic acid (Thermo Fisher Scientific) with 15% FBS (HyClone) and 1% penicillin/streptomycin (Invitrogen) at 37°C in a 5% CO2 humidified incubator. After 48 hours of adhesion, the media was changed to remove nonadherent cells. BMSCs reached confluence after an additional 4 days of culture with a single media change. Cells were passaged with 0.25% trypsin-EDTA digestion (Invitrogen) and reseeded at 3 × 10^5 cells/6-well or 1 × 10^5 cells/12-well for mineralization assays.
For viral infections, cells at 70% confluence were infected with adenovirus expressing either GFP or Cre-recombinase (Baylor College of Medicine, Houston, Texas, USA) at a multiplicity of infection (MOI) of 50 in serum-containing medium for 24 hours. Seventy-two hours after viral infection, BMSCs were cultured in osteogenic media (α-MEM containing 15% FBS, 1% penicillin/streptomycin, 50 μg/ml L-ascorbic acid [MilliporeSigma] and 10 mM β-glycerophosphate [MilliporeSigma]). For von Kossa staining, cells were fixed in methanol on ice for 20 minutes, rinsed with distilled water, and exposed to 365 nm ultraviolet light in 5% silver nitrate solution (MilliporeSigma) for 2 minutes.

Coculture osteoclastogenesis assay. Primary calvarial osteoblasts were isolated from P0 to P2 Cre;CaV1.2Tm (control) or Sp7-Cre;CaV1.2Tm pups. The calvaria were digested in 1× PBS containing 0.18% collagenase P (Roche Diagnostics) for 10 minutes at 37°C with vigorous shaking. The digestion was repeated 4 times, and the cells isolated from the last 3 digestions were combined. The isolated osteoblasts (5 × 10⁴ cells/well) were seeded in a 96-well plate with 200 μl of α-MEM containing 15% FBS and 1% penicillin/streptomycin in 5% CO₂ at 37°C 2 days before starting the coculture. Twenty-four hours after adhesion, the medium was changed to osteogenic media. BMMs were isolated from BM of WT C57BL/6J mice and cultured on a 100-mm culture dish after lysis of the RBC with α-MEM containing 15% FBS, 1% penicillin/streptomycin, and 5 ng/ml of M-CSF for 24 hours. Nonadherent cells were collected, washed once with 1× PBS, resuspended in osteogenic media with addition of 10 nM of 1,25-dihydroxyvitamin D₃ and 1 μM of prostaglandin E₂, and added to the wells of the 96-well plate containing the calvarial osteoblasts. The coculture was observed for 6–9 days, and half of the media was changed every other day until the appearance of giant osteoclasts. To visualize osteoclasts, cells were fixed and processed for TRAP staining.

Real-time PCR. Total RNA was isolated from BMSCs using RNeasy mini kit (Qiagen). Total RNA (1 μg) was reverse transcribed to cDNA using iScript Reverse Transcription Supermix (Bio-Rad). qPCR was performed using SYBR green Supermix (Bio-Rad). Gene expression was first normalized to GAPDH and then normalized to control samples. The primers for Runx2, Sp7, Alpl, Bglap, and Ihsp were previously described (34). The primers for Gapdh are: forward, 5′- AGTAAACACCGAGGGGG - 3′; reverse, 5′- GGTCTGATCACAGGGCATGG - 3′. The primers for Opg (Tnfsf11b) are: forward, 5′- CCGAGGACCACAATGAACAACT - 3′; reverse, 5′- CTGGGTGTGGTCCATCCATGATG - 3′. The primers for Rankl (Tnfsf11) are: forward, 5′- CTGGGCAAGATCTCTAACATGA - 3′; reverse, 5′- GGTACGCTTCCCGATGTTTC - 3′.

Statistics. Statistical analyses were performed using GraphPad Prism 7.0. Two-tailed unpaired t tests were used for 2 conditions, and 1-way ANOVA with Tukey’s post-test analysis was applied for multipe comparisons. P < 0.05 was considered statistically significant. Data are represented as mean ± SD from n ≥ 3 experiments. qPCR assays were performed with 3 independent RNA isolates. Viral transfections were repeated n ≥ 3. Fold changes were calculated by dividing the value of the treatment group by the value of the control group at the same time point. Increasing or decreasing percentage changes were calculated by dividing the value of difference between the treatment group and control group by the value of the control group and then multiplying 100.

Study approval. All mouse protocols were approved by the IACUC at Duke University.

Author contributions
CC designed research, conducted experiments, acquired data, analyzed data, and wrote the manuscript; YR conducted experiments; ASB designed research, conducted experiments, acquired data, and analyzed data; AJM conducted experiments; DR conducted experiments; SHM acquired data and analyzed data; CC designed research, conducted experiments, acquired data, analyzed data, and wrote the manuscript.

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