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Trpv6 mediates intestinal calcium absorption during calcium restriction and contributes to bone homeostasis

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Abstract

Energy-dependent intestinal calcium absorption is important for the maintenance of calcium and bone homeostasis, especially when dietary calcium supply is restricted. The active form of vitamin D, 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃], is a crucial regulator of this process and increases the expression of the transient receptor potential vanilloid 6 (*Trpv6*) calcium channel that mediates calcium transfer across the intestinal apical membrane. Genetic inactivation of *Trpv6* in mice (*Trpv6*^{-/-}) showed, however, that TRPV6 is redundant for intestinal calcium absorption when dietary calcium content is normal/high and passive diffusion likely contributes to maintain normal serum calcium levels. At the other hand, *Trpv6* inactivation impaired the increase in intestinal calcium transport following calcium restriction, however without resulting in hypocalcemia. A possible explanation is that normocalcemia is maintained at the expense of bone homeostasis, a hypothesis investigated in this study.

In this study, we thoroughly analyzed the bone phenotype of *Trpv6*^{-/-} mice receiving a normal (~1%) or low (~0.02%) calcium diet from weaning onwards using micro-computed tomography, histomorphometry and serum parameters.

When dietary supply of calcium is normal, *Trpv6* inactivation did not affect growth plate morphology, bone mass and remodeling parameters in young adult or ageing mice. Restricting dietary calcium had no effect on serum calcium levels and resulted in a comparable reduction in bone mass accrual in *Trpv6*^{+/+} and *Trpv6*^{-/-} mice (-35% and 45% respectively). This decrease in bone mass was associated with a similar increase in bone resorption, whereas serum osteocalcin levels and the amount of unmineralized bone matrix were only significantly increased in *Trpv6*^{-/-} mice.

Taken together, our findings indicate that TRPV6 contributes to intestinal calcium transport when dietary calcium supply is limited and in this condition indirectly regulates bone formation and/or mineralization.

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Keywords

intestinal calcium absorption; TRPV6; 1,25(OH)₂D₃; bone remodeling; hyperosteoidosis

Introduction

The maintenance of normal serum calcium levels is vital to several cellular functions, including cell metabolism, neurotransmission and muscle contraction. Calcium is also a crucial component of the mineral phase within the skeletal tissues and hereby provides structure and strength to skeletal elements. The major source for calcium acquisition is intestinal absorption, a pathway controlled by 1,25(OH)₂ vitamin D₃ [1,25(OH)₂D₃], the active form of vitamin D. Indeed, 1,25(OH)₂D₃ treatment increases, whereas systemic inactivation of the vitamin D receptor (*Vdr*) impairs intestinal calcium absorption [1]. In line herewith, the consequences of inactivation of the *Vdr* in humans and mice are hypocalcemia, hyperparathyroidism, hypophosphatemia and bone pathology including rickets and osteomalacia [2–4]. Since dietary calcium supplementation can correct this phenotype, the 1,25(OH)₂D₃-mediated intestinal calcium absorption is suggested to be important during normal to low calcium intake [5–7]. These findings also led to the model that two pathways are contributing to intestinal calcium absorption: a passive, paracellular pathway of calcium absorption operating when dietary supply of calcium is high and a 1,25(OH)₂D₃-dependent, active calcium transport that predominates when calcium intake declines.

The active transcellular pathway of intestinal calcium absorption is considered to comprise three constituent processes: luminal calcium is first transferred across the apical brush-border by calcium channels of the transient receptor potential vanilloid (TRPV) family, especially TRPV6; next, intracellular calcium diffusion is facilitated by its binding to the calcium binding proteins, calbindins (especially Calbindin-D_{9k}), and finally calcium is extruded across the basolateral membrane by an energy-requiring process [8]. Evidence for this model is provided by gene expression analysis showing increased expression of *Trpv6* and *Calbindin-D_{9k}* after 1,25(OH)₂D₃ treatment or during low dietary calcium intake when 1,25(OH)₂D₃-mediated calcium transport gains importance. Consistent herewith, *Trpv6* and *Calbindin-D_{9k}* mRNA levels are decreased in *Vdr*^{−/−} mice [1,9].

Recent genetic studies question, however, the critical role of TRPV6 and Calbindin-D_{9k} in (1,25(OH)₂D₃-mediated) intestinal calcium absorption. Indeed, duodenal calcium absorption is not impaired in *Trpv6*^{−/−} mice and *Calbindin-D_{9k}/Trpv6* double null mice fed a normal calcium diet. At the other hand, the increase in duodenal calcium transport in response to a low calcium diet, is less pronounced in the *Trpv6*^{−/−} mice and *Calbindin-D_{9k}/Trpv6* double null compared to wild-type mice, suggesting a defined but limited contribution of TRPV6 to intestinal calcium absorption [10,11]. These minor changes in intestinal calcium absorption in *Trpv6*^{−/−} mice may explain their normal serum calcium levels. Accordingly, the phenotype of *Trpv6*^{−/−} mice is rather mild and in sharp contrast with that of *Vdr*^{−/−} mice which display, even during normal calcium intake, a manifest decrease in intestinal calcium absorption resulting in altered calcium, phosphate and hormonal levels as well as in major bone and growth plate abnormalities [1,4].

The normocalcemia in *Trpv6*^{−/−} mice might however as well be due to compensatory mechanisms. Indeed, parathyroid hormone (PTH) and 1,25(OH)₂D₃ serum levels are increased and these hormones may affect calcium handling in kidney or bone [10,11]. A small decrease in bone mass, measured by dual energy X-ray absorptiometry (DXA) has been reported in *Trpv6*^{−/−} mice [11]. We performed a detailed analysis of the bone phenotype of *Trpv6*^{−/−} mice and show that the increase in unmineralized bone matrix was more pronounced in *Trpv6*^{−/−}

mice in response to a low calcium diet. These data suggest that TRPV6-mediated calcium absorption indirectly contributes to normal bone homeostasis when dietary calcium is limited.

Materials and methods

Animals

Trpv6^{+/-} mice were bred to obtain *Trpv6*^{-/-} mice and *Trpv6*^{+/+} littermates and genotyping was performed as described [11]. Mice were exposed to a 12 h light, 12 h dark cycle and had unlimited access to food and water. All procedures were approved by the Ethical Committee of the Katholieke Universiteit Leuven and University of Medicine and Dentistry of New Jersey.

In vivo study protocol

Mice received a standard rodent chow diet containing 1.0% calcium and 0.7% phosphate (Teklad diet 92309). Mice were sacrificed at 8-weeks (wks) or 5-months of age; serum and bones were isolated and processed as described [1,12]. To assess the effect of calcium restriction, a low calcium diet (0.02% calcium, 0.4% phosphate, 2200 IU vitamin D₂/kg; Teklad diet 86162) was given from weaning on, until sacrifice at 8-wks of age. For dynamic histomorphometry, animals received i.p. injection of calcein (16 mg/kg; Sigma-Aldrich) 4 and 1 days prior to sacrifice.

Serum and urine analysis

Calcium and phosphate levels in serum were analyzed by SYNCHRON Clinical Systems (Beckman Coulter). Levels of osteocalcin and 1,25(OH)₂D were measured using an in house [13] and ¹²⁵I 1,25(OH)₂D (Diasorin) RIA. Serum PTH (Immutopics) and C-telopeptide (CTx; Nordic Bioscience Diagnostics A/S) levels were determined by ELISA.

Histology and histomorphometry

Left tibiae were fixed in Burkhardt's solution, embedded undecalcified in methyl methacrylate, and sectioned at 4 µm. The sections were stained according to von Kossa or Goldner to assess mineralized bone and unmineralized bone matrix respectively. Unstained sections were used to analyze calcein labeling. Histomorphometric analysis was done as described [14,15], using a Zeiss Axiovert microscope and an Axiovision (v6.1.0) image analyzing system, and expressed according to the American Society for Bone and Mineral Research standardized histomorphometry nomenclature [16].

Bone mineral density (BMD) and morphometry

Micro-computed tomography (µCT) analysis of the left tibiae was performed *ex vivo* using a high resolution SkyScan 1172 system (50 kV, 200 µA, 0.5 mm aluminum filter). Serial tomographs, reconstructed from raw data using the cone-beam reconstruction software (NRecon, v.1.4.4.0; Skyscan), were used to compute trabecular and cortical parameters, respectively from the metaphyseal and mid-diaphyseal area. A bone standard, scanned by µCT, was used for the calibration of bone density measurements.

In vitro osteoblast and osteoclast cultures

Bone marrow stromal cells (BMSC) were isolated and cultured overnight in DMEM containing 10% fetal calf serum (FCS). After removal of non-adherent cells, cells were cultured until confluence. Primary osteoblasts were isolated from calvariae of neonatal mice by sequential digestion with 0.1% collagenase II and 0.2% dispase (Invitrogen), and cells of the second to fifth fraction were pooled as described [14]. At confluence, osteoblastic differentiation was stimulated in all cell types by addition of 50 µg/ml ascorbic acid, 10 mM β-glycerophosphate and 10⁻⁸ M dexamethasone (Sigma-Aldrich; osteogenic medium) for 10 days.

For *in vitro* osteoclast formation, bone marrow cells were plated overnight in α -MEM containing 10% FCS and 10 ng/ml M-CSF (R&D Systems). Non-adherent cells were harvested and plated in α -MEM containing 20 ng/ml M-CSF and 100 ng/ml RANKL (Peprotech) (day 1) and medium was changed on day 3. At day 6, RNA was isolated to quantify *Trpv6* expression.

Isolation of RNA and quantitative real time-PCR (qRT-PCR)

To assess *Trpv6* mRNA expression, total RNA of duodenum, kidney, femur and thyroid/parathyroid glands of 8-wks-old wild-type mice and growth plate chondrocytes of neonatal mice, was extracted with TRIzol (Invitrogen) followed by phenol/chloroform purification. RNA of cultured osteoblasts, bone marrow stromal cells and osteoclasts was isolated using the RNeasy minikit (QIAGEN). cDNA was synthesized using reverse transcriptase SuperScript II RT (Invitrogen). qRT-PCR was performed on the 7500 Fast Real-Time PCR System (Applied Biosystems), using specific forward and reverse oligonucleotide primers and probes for *Trpv6* (forward: ATC CGC CGC TAT GCA CA; reverse: AGT TTT TCT CCT GAA TCT TTT TCC AA; probe: TTC CAG CAA CAA GAT GGC CTC TAC TCT GA) and hypoxanthine-guanine phosphoribosyltransferase (*Hprt1*) (forward: TTA TCA GAC TGA AGA GCT ACT GTA ATG ATC; reverse: TTA CCA GTG TCA ATT ATA TCT TCA ACA ATC; probe: TGA GAG ATC ATC TCC ACC AAT AAC TTT TAT GTC CC) [1,17]. Gene expression of *Trpv6* was normalized for the expression of *Hprt1*.

Statistics

Quantified results are expressed as the mean $\pm$ SEM. Comparison between 2 groups was done by 2-sided, 2-sample Student's t-test. Multiple groups were analyzed by two-way ANOVA followed by Fisher's LSD Multiple Comparison test using the statistical software NCSS 2000 (Number Cruncher Statistical Systems, Kaysville, UT). Differences were considered significant at $P < 0.05$, and marked with symbols (*) as indicated, increasing symbol numbers representing $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

Results

Bone mass is normal in *Trpv6*^{-/-} mice on a 1% calcium diet

As reported, serum calcium and phosphate levels are not altered in *Trpv6*^{-/-} mice receiving a normal to high calcium diet. To verify that TRPV6 is mainly involved in intestinal calcium absorption, we quantified expression levels in several tissues of wild-type mice by qRT-PCR. Among the organs contributing to calcium and phosphate homeostasis, *Trpv6* is mainly expressed in the intestine and almost absent in the kidneys, parathyroid/thyroid glands, bone, growth plate and cultured osteoblasts and osteoclasts (on average 1% vs intestine) (Fig. 1). These data suggest that TRPV6-mediated intestinal calcium absorption is not critical when dietary calcium supply is normal or high. At the other hand, PTH and 1,25(OH)₂D₃ levels are significantly increased in *Trpv6*^{-/-} mice [10,11], a finding that may suggest that compensatory mechanisms, acting at the skeleton and/or kidney, contribute to preserve normal serum calcium and phosphate levels. We could, however, not detect any difference in the urinary calcium/creatinine levels between the genotypes (data not shown), which is in accordance with the reported unaltered gene expression of *Trpv5* and *Calbindin-D_{9k}* [10]. These findings indicate that renal calcium handling was not altered in *Trpv6*^{-/-} mice.

Next, we thoroughly analyzed the bone parameters of young adult male *Trpv6*^{-/-} mice (8-wk-old), receiving a standard rodent chow diet from weaning onwards. The general appearance and structure of the tibia did not differ between the genotypes, as analyzed by μ CT (Fig. 2A). Trabecular parameters, including bone volume (Fig. 2B), number (Fig. 2C) and thickness (Fig. 2D), as well as cortical characteristics, including thickness (Fig. 2E) and porosity (Fig. 2F), were comparable between male littermates. Accordingly, trabecular bone volume, quantified

on von Kossa-stained histological sections of the tibia, was not different between genotypes (Figs. 2G and H). Similar results were obtained in female mice (data not shown).

Furthermore, histological analysis of Goldner-stained sections showed that the organization (Fig. 2I) and width (Fig. 2J) of the tibial growth plate was not affected by *Trpv6* inactivation, features that are manifestly altered in rickets caused by 1,25(OH)₂D₃ deficiency or resistance.

Intestinal calcium absorption decreases with age, which predisposes the elderly to hyperparathyroidism and osteoporosis [18]. We therefore investigated bone parameters in 5-months-old female mice by μ CT analysis. Bone structure was comparable between genotypes (Fig. 3A) and quantification revealed that trabecular bone volume (Fig. 3B) number (Fig. 3C) and thickness (Fig. 3D) were similar in *Trpv6*^{+/+} and *Trpv6*^{-/-} mice. Also cortical parameters, including the thickness (Fig. 3E) and porosity (Fig. 3F), were not altered by *Trpv6* inactivation in 5-months old mice. Accordingly, the decrease in bone mass with age was comparable in both genotypes: % BV/TV decreased from 6.4 ± 0.3 at 8-wks of age to 5.0 ± 0.3 in 5-months old *Trpv6*^{+/+} mice ($P < 0.01$, $n=9$) and from 5.9 ± 0.3 at 8-wks of age to 4.4 ± 0.5 in 5-months old *Trpv6*^{-/-} mice ($P < 0.05$, $n=9$).

These data indicate that TRPV6 inactivation does not impair bone mass in young adult or ageing mice.

***Trpv6* deficiency does not alter bone remodeling during normal calcium intake**

Bone mass is the result from balanced rates of bone resorption and formation and both these processes may be altered equally without a net effect on bone volume. Therefore, bone remodeling was assessed in more detail in 8-wk-old mice. Serum levels of C-terminal telopeptides (CTX), which are degradation products of type I collagen released during bone resorption, were comparable between genotypes, suggesting normal osteoclast activity in *Trpv6*^{-/-} mice (Fig. 4A).

In addition, also bone formation remained unchanged in *Trpv6*^{-/-} mice, indicated by normal serum levels of osteocalcin, a protein secreted by mineralizing osteoblasts (Fig. 4B). Histological analysis of Goldner-stained tibia sections of *Trpv6*^{+/+} and *Trpv6*^{-/-} mice revealed no major difference in the distribution of unmineralized bone matrix lining the trabecular bone (Fig. 4C). Quantification demonstrated that osteoid abundance (OS/BS; Fig. 4D) and thickness (Fig. 4E) were similar in the two genotypes. Dynamic bone formation parameters were assessed by repeated injection of calcein, respectively four and one day before sacrifice (Fig. 4F). The mineralizing surface (Fig. 4G), mineral apposition rate (Fig. 4H), bone formation rate (Fig. 4I) as well as the mineralization lag time (Fig. 4J) was comparable between genotypes, suggesting normal osteoblast function and number. Taken together, inactivation of *Trpv6* had no effect on bone mass or bone remodeling when mice were fed a normal calcium diet.

Dietary calcium restriction impairs bone mass accrual equally in *Trpv6*^{+/+} and *Trpv6*^{-/-} mice

Active intestinal calcium transport gains importance when dietary calcium supply is low, a process in which TRPV6 is supposed to be involved. As recently demonstrated, *Trpv6* inactivation impaired active intestinal calcium absorption under these conditions, although a substantial level of calcium transport was still detectable [10,11]. Bone parameters were therefore analyzed in *Trpv6*^{+/+} and *Trpv6*^{-/-} mice challenged with a low calcium diet (0.02% calcium) from weaning on, and sacrificed at 8-wks of age. We observed no differences in serum calcium and phosphate levels between *Trpv6*^{+/+} and *Trpv6*^{-/-} mice on the low calcium diet and they were comparable to the serum levels of mice receiving a 1% calcium diet (Figs. 5A and B). Dietary calcium restriction increased PTH and 1,25(OH)₂D serum levels in *Trpv6*^{+/+} mice. As previously described, PTH and 1,25(OH)₂D levels were already elevated in

Trpv6^{-/-} mice during normal calcium intake, and only 1,25(OH)₂D was additionally increased in response to a low calcium diet (Figs. 5C and D).

Low calcium intake resulted in a severe reduction in bone mass, as illustrated by μ CT analysis of the tibia, and this decrease appeared similar in both genotypes (Fig. 6A). Indeed, quantitative analysis showed that trabecular bone volume was equally decreased in *Trpv6*^{+/+} and *Trpv6*^{-/-} mice receiving a low calcium diet (Fig. 6B). The reduced bone mass was mainly due to a decline in trabecular number and a slight but non-significant decrease in trabecular thickness (Figs. 6C and D).

Cortical parameters were also affected by dietary calcium restriction, evidenced by a decrease in cortical thickness and an increase in cortical porosity. Likewise to the other observations, the response to the low calcium diet was comparable in *Trpv6*^{+/+} and *Trpv6*^{-/-} mice (Figs. 6E and F). The cortical bone mineral density (BMD) was not altered by calcium restriction or *Trpv6* inactivation (Fig. 6G). Of note, the morphology of the growth plate was not manifestly changed by the dietary calcium content, and did not differ between genotypes (Fig. 6H).

Thus, when fed a low calcium diet, bone gain was impaired in *Trpv6*^{+/+} and *Trpv6*^{-/-} mice, likely as an attempt to maintain normal serum calcium levels. Despite the impaired intestinal calcium transport, *Trpv6* inactivation did not aggravate the decrease in bone mass.

Bone turnover is increased in *Trpv6*^{-/-} mice during calcium restriction

Parallel to the analysis in mice receiving a high calcium diet, the effect of combined *Trpv6* inactivation and calcium restriction on bone remodeling was further assessed. In response to the low calcium diet, osteoid surface increased in both genotypes, as evidenced by Goldner staining (Fig. 7A). Quantitative analysis showed, however, that the increase in osteoid surface was only significant in the *Trpv6*^{-/-} mice compared to *Trpv6*^{-/-} mice on a high calcium diet (160%, $p < 0.01$), whereas a less pronounced effect was observed in *Trpv6*^{+/+} mice (120%; non-significant, Fig. 7B). Osteoid thickness increased only slightly during calcium restriction and the increase was comparable between genotypes (Fig. 7C).

Bone turnover parameters in serum were further investigated. Calcium restriction stimulated osteoblast activity, evidenced by increased serum osteocalcin levels. The induction in serum osteocalcin levels was 2.1-fold in *Trpv6*^{-/-} mice, but only 1.5-fold in *Trpv6*^{+/+} mice, a finding that is in accordance with the more pronounced presence of unmineralized bone matrix (Fig. 7D). Serum CTx levels were increased in both genotypes following calcium restriction, with a tendency to a more marked induction in *Trpv6*^{-/-} mice. These data indicate that bone resorption is increased in both genotypes when calcium intake declines (Fig. 7E).

Taken together, the impaired bone mass accrual under low dietary calcium conditions was comparable in *Trpv6*^{+/+} and *Trpv6*^{-/-} mice, but *Trpv6* inactivation lead to more pronounced hyperosteoridosis.

Discussion

Efficient intestinal calcium absorption is required to maintain normal serum calcium levels, as well as normal bone mass and mineralization. When dietary supply of calcium is limited, active intestinal calcium transport becomes thus crucial. Genetic studies have shown that the apical calcium channel, TRPV6, is involved in the energy-dependent duodenal calcium absorption [10]. We report in this study that TRPV6-mediated intestinal calcium absorption prevents hyperosteoridosis when dietary calcium is restricted, whereas this calcium channel is redundant during normal dietary calcium supply.

Decreased calcium intake results in enhanced active intestinal calcium transport [1], which guarantees that the total amount of absorbed calcium remains within the normal range. Prolonged dietary calcium restriction, however, leads to severe bone loss and osteoid excess associated with hypocalcemia, hypophosphatemia and secondary hyperparathyroidism [19], underscoring the limited adaptability of the intestinal calcium absorption as well as its importance for calcium and bone homeostasis. The finding that dietary calcium is less efficiently absorbed in *Trpv6* null mice receiving a low calcium diet [10], prompted us to investigate whether *Trpv6* inactivation has an impact on calcium and bone homeostasis. Serum calcium and phosphate levels, however, remained normal in *Trpv6*^{-/-} mice during calcium restriction, suggesting adequate activation of compensatory mechanisms, most likely in response to the increased PTH and 1,25(OH)₂D serum levels [10,11]. The decrease in bone mass accrual was also similar between genotypes and resulted from increased bone resorption outpacing enhanced bone formation. On the other hand, the increase in osteoblast activity and osteoid abundance was more pronounced in *Trpv6* null mice, suggesting enhanced bone formation and/or defective matrix mineralization, likely to ensure normal serum calcium levels. These data indicate that TRPV6 is an important component of the transcellular calcium absorption pathway and is required to guarantee sufficient calcium absorption in conditions of dietary calcium restriction, in order to maintain proper bone mineralization.

Though TRPV6 is considered to be mainly involved in intestinal calcium absorption, inactivation of *Trpv6* in other cell types may theoretically account for the bone phenotype in *Trpv6* null mice. Compared to the gene expression of *Trpv6* in the duodenum, *Trpv6* levels were substantially lower and almost absent in bone, chondrocytes, osteoblasts and osteoclasts of wild-type mice. These data are in agreement with previous studies reporting that *Trpv6* is detectable in bone and bone cells [20–22], but more prominently expressed in the enterocytes of duodenum, proximal jejunum and cecum, as well as in prostate, brain and placenta in man [23,24] and rodents [21,22,25,26]. Taken together, these observations suggest that the bone phenotype in *Trpv6* null mice is likely the result of impaired intestinal calcium uptake in response to low dietary calcium, rather than being the consequence of cell autonomous defects in bone cells.

Trpv6 inactivation thus reduces the ability to efficiently adapt to a low calcium diet, features that are also observed during aging [27]. Indeed, the capacity of the intestine to increase active calcium absorption declines in the elderly, a defect which is mainly attributed to 1,25(OH)₂D₃-resistance and which is often associated with secondary hyperparathyroidism and bone loss, but without leading to hypocalcemia [18,28–31]. Similar aging characteristics are observed in animals [32,33] and this age-related decrease in calcium transport is associated with a reduced expression of *Trpv6* and *Calbindin-D_{9k}* in the intestine [34,35]. The present study indicates that the decline in bone mass with age is not (solely) due to lower TRPV6 levels, since bone parameters were not affected by *Trpv6* ablation in aging mice. When, in addition, the dietary supply of calcium is low, as is often the case in elderly people, reduced TRPV6 levels do contribute to the skeletal response.

1,25(OH)₂D₃ is one of the main regulators of active intestinal calcium absorption and increases gene transcription of several calcium transporters including *Trpv6*, *Calbindin-D_{9k}*, *Calbindin-D_{28k}* and *PMCA_{1b}* [1,9,36]. TRPV6 has been considered to be a critical downstream effector of 1,25(OH)₂D₃ action on intestinal calcium absorption, since its expression was manifestly and consistently induced in conditions associated with high 1,25(OH)₂D₃ levels [1,36]. However, the mild phenotype of *Trpv6*^{-/-} mice questions the crucial role of TRPV6 in 1,25(OH)₂D₃-mediated intestinal calcium absorption. Indeed, lack of 1,25(OH)₂D₃ activity in mice, due to inactivating mutations in *CYP27b1* or *Vdr*, manifestly impairs active intestinal calcium transport even during normal calcium intake. Although this reduced calcium absorption is associated with decreased *Trpv6* levels, intestinal calcium transport is not

impaired in *Trpv6* null mice receiving a similar diet [1,9,10]. In addition, $1,25(\text{OH})_2\text{D}_3$ treatment increases active calcium absorption in *Trpv6*^{-/-} as much as in *Trpv6*^{+/+} mice, measured by the everted gut sac assay [10] or oral gavage [37]. The finding that intestinal calcium absorption was only moderately affected by *Trpv6* inactivation likely explains that bone abnormalities were less pronounced compared to *Vdr* or *CYP27b1* null mice. The latter suffer from severe rickets even during normal calcium intake [3,4] whereas in *Trpv6* null mice osteoid excess was only observed during calcium restriction and rachitic growth plate abnormalities were never detected. Taken together, these observations indicate that TRPV6 is not critical for the $1,25(\text{OH})_2\text{D}_3$ -mediated increase in intestinal calcium absorption, but does contribute to the calcium absorptive process during calcium restriction.

The finding that TRPV6 is not essential for the maintenance of serum calcium levels during normal/high calcium intake [10,11], nor for bone morphology and remodeling, also indicates that other molecules contribute to active intestinal calcium absorption. These factors can then compensate for the deficiency in TRPV6, providing an explanation for the modest effects of TRPV6 deletion on intestinal calcium absorption. However, no increase was detected in the expression of the currently identified components of the transcellular pathway, such as *Trpv5*, *Calbindin-D9k*, *Calbindin-D28k* and *PMCA1b* in *Trpv6* null mice [10,11]. Recent studies suggest that $1,25(\text{OH})_2\text{D}_3$ stimulates intestinal calcium absorption via regulation of the paracellular pathway, at least *in vitro* [38]. In agreement herewith, gene array analysis showed that $1,25(\text{OH})_2\text{D}_3$ treatment of rats decreased the expression of several cell adhesion molecules in the duodenum, such as *claudin 3* and *cadherin 17* [39] whereas *claudin 2* and *claudin 12*, components of the tight junction complexes, are upregulated by $1,25(\text{OH})_2\text{D}_3$ and contribute to the $1,25(\text{OH})_2\text{D}_3$ -induced stimulation of the paracellular calcium absorption *in vitro* [40]. Thus, $1,25(\text{OH})_2\text{D}_3$ stimulates active intestinal calcium absorption not only via a transcellular, but also via a paracellular pathway, although *in vivo* genetic evidence is still lacking. We observed a small increase in *claudin 12* (+10%, $P < 0.05$) gene expression in the duodenum of *Trpv6*^{-/-} mice, but *claudin 2* mRNA expression did not differ significantly between genotypes (data not shown). *Cadherin 17* was also shown to be decreased in *Trpv6*^{-/-} mice [10]. These data suggest that other transport mechanisms, still to be largely identified, compensate for the loss of TRPV6, which might explain the rather mild phenotype of *Trpv6* null mice.

In summary, this study demonstrates that TRPV6 is redundant for intestinal calcium absorption during normal calcium intake, and is therefore not necessary for normal bone volume, morphology and remodeling when dietary calcium supply is sufficient. TRPV6 does contribute to the energy-dependent pathway of intestinal calcium absorption and is essential to ensure adequate calcium absorption during dietary calcium deprivation, thereby avoiding excessive bone turnover and impaired bone mineralization. These findings also underscore the essential role of adequate dietary calcium absorption for bone volume and mineralization.

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Fig. 1. *Trpv6* is predominantly expressed in the duodenum

Trpv6 mRNA expression in duodenum, kidney, parathyroid/thyroid gland, femur, growth plate chondrocytes, *in vitro* differentiated osteoclasts, bone marrow stromal cells (BMSC) and calvarial osteoblasts of wild-type mice. *Trpv6* mRNA levels are corrected for *Hprt1*; n=2.

Fig. 2. Bone mass and morphology are comparable in young adult *Trpv6*^{-/-} and *Trpv6*^{+/+} mice
 (A) Representative cross-sectional 3-D μ CT images of the tibia of 8-wk-old male *Trpv6*^{+/+} and *Trpv6*^{-/-} mice on a normal calcium (1%) diet.
 (B-E) Quantification of the trabecular bone volume (BV/TV) (B), trabecular number (Tb. N) (C), trabecular thickness (Tb. Th) (D), cortical thickness (Ct. Th) (E) and cortical porosity (Ct. poro) (F) on μ CT images. n = 4–5.
 (G-H) Von Kossa staining of tibia (G) and histomorphometric analysis of trabecular bone volume (BV/TV) (H) at 8-wks of age. Scale bar: 200 μ m; n = 7.
 (I-J) Goldner-stained sections demonstrating normal organization of the tibial growth plate (I) and quantification of the growth plate width (J). Scale bar: 100 μ m; n = 3.

Fig. 3. Bone mass remains normal with ageing in *Trpv6*^{-/-} mice

(A) Representative μ CT images of the tibia of 5-months-old female *Trpv6*^{+/+} and *Trpv6*^{-/-} mice receiving a normal calcium (1%) diet.

(B-F) Quantification of the trabecular bone volume (BV/TV) (B), trabecular number (Tb. N) (C), trabecular thickness (Tb. Th) (D), cortical thickness (Ct. Th) (E) and cortical porosity (Ct. poro) (F) by μ CT. n = 6–10.

Fig. 4. Bone remodeling is not affected by *Trpv6* inactivation

(A-B) Serum C-terminal telopeptide (CTx) (A) and osteocalcin levels (B) in 8-wk-old male *Trpv6*^{+/+} and *Trpv6*^{-/-} mice. n = 8–10.

(C-E) Representative Goldner-stained tibia sections of 8-wk-old mice showing osteoid matrix in dark pink (arrow head, C). Quantification of the osteoid surface (OS/BS) (D) and thickness (O. Th) (E). Scale bar: 10 μm; n = 4.

(F-J) Calcein labeling demonstrating comparable distance between double labels in both genotypes (F) and histomorphometric analysis of the mineralizing surface (MS) (G), mineral apposition rate (MAR) (H), bone formation rate (BFR/BS) (I) and the mineralization lag time (Mlt) (J). Scale bar: 25 μm; n = 4.

Fig. 5. *Trpv6* inactivation combined with calcium restriction does not affect serum calcium and phosphate levels
 (A-D) Serum calcium (A), phosphate (B), PTH (C) and 1,25(OH)₂D (D) levels in 8-wk-old mice fed a normal (1%) or a low (0.02%) calcium diet from weaning on. n = 4–5. * P<0.05, *** P<0.001 as indicated (2-way ANOVA followed by Fisher's LSD multiple-comparison test).

Fig. 6. Low calcium intake induces comparable bone loss in *Trpv6*^{-/-} and *Trpv6*^{+/+} mice
 (A) Representative cross-sectional 3-D μCT images of tibiae of 8-wk-old *Trpv6*^{+/+} and *Trpv6*^{-/-} mice on a normal or low calcium diet started from weaning on.
 (B-G) Quantification of the trabecular bone volume (BV/TV) (B), trabecular number (Tb. N) (C), trabecular thickness (Tb. Th) (D), cortical thickness (Ct. Th) (E), cortical porosity (Ct. poro) (F) and cortical bone mineral density (Ct. BMD) (G) of *Trpv6*^{+/+} and *Trpv6*^{-/-} mice on a normal (1%) or low calcium diet by μCT. n = 5–8. * P<0.05, ** P<0.01 within genotypes (2-way ANOVA followed by Fisher's LSD multiple comparison test).
 (H) Goldner-stained sections, showing normal organization of the tibial growth plate of *Trpv6*^{+/+} and *Trpv6*^{-/-} mice, fed a low calcium diet. Scale bar: 100 μm.

Fig. 7. Bone formation is increased in *Trpv6*^{-/-} mice under low dietary calcium conditions
 (A) Goldner-stained tibia sections of 8-wk-old *Trpv6*^{+/+} and *Trpv6*^{-/-} mice on a normal (1%) or low (0.02%) calcium diet given from weaning on, showing osteoid matrix in dark pink (arrow head). Scale bar: 50 μ m.
 (B-C) Histomorphometric analysis of the osteoid surface (OS/BS) (B) and thickness (O. Th) (C). n = 5.
 (D-E) Serum osteocalcin (D) and serum C-terminal telopeptide (CTx) levels (E). n = 5-8. * P<0.05, *** P<0.001 within genotypes (2-way ANOVA followed by Fisher's LSD multiple comparison test).