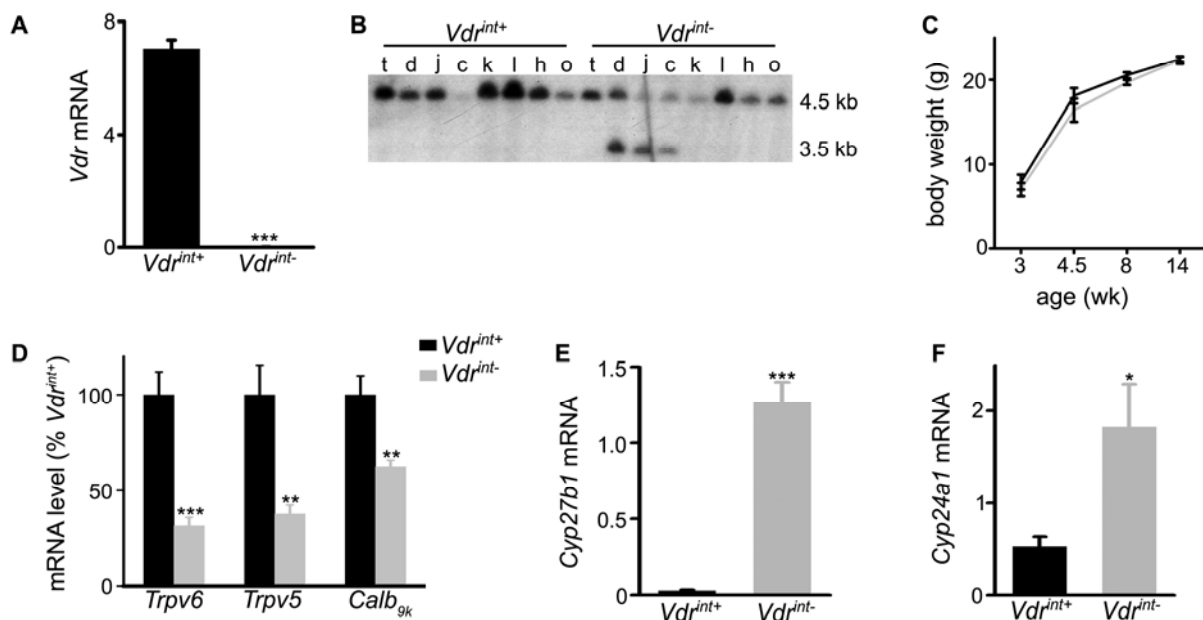


SUPPLEMENTAL DATA:

'NORMOCALCEMIA IS MAINTAINED IN MICE UNDER CONDITIONS OF CALCIUM MALABSORPTION BY VITAMIN D-INDUCED INHIBITION OF BONE MINERALIZATION' BY LIEBEN ET AL.

SUPPLEMENTAL FIGURES

SUPPLEMENTAL FIGURE 1



Supplemental Figure 1. Inactivation of the *Vdr* in the intestine of *Vdr*^{int-} mice.

(A) qRT-PCR analysis of *Vdr* mRNA levels in duodenum. n=8.

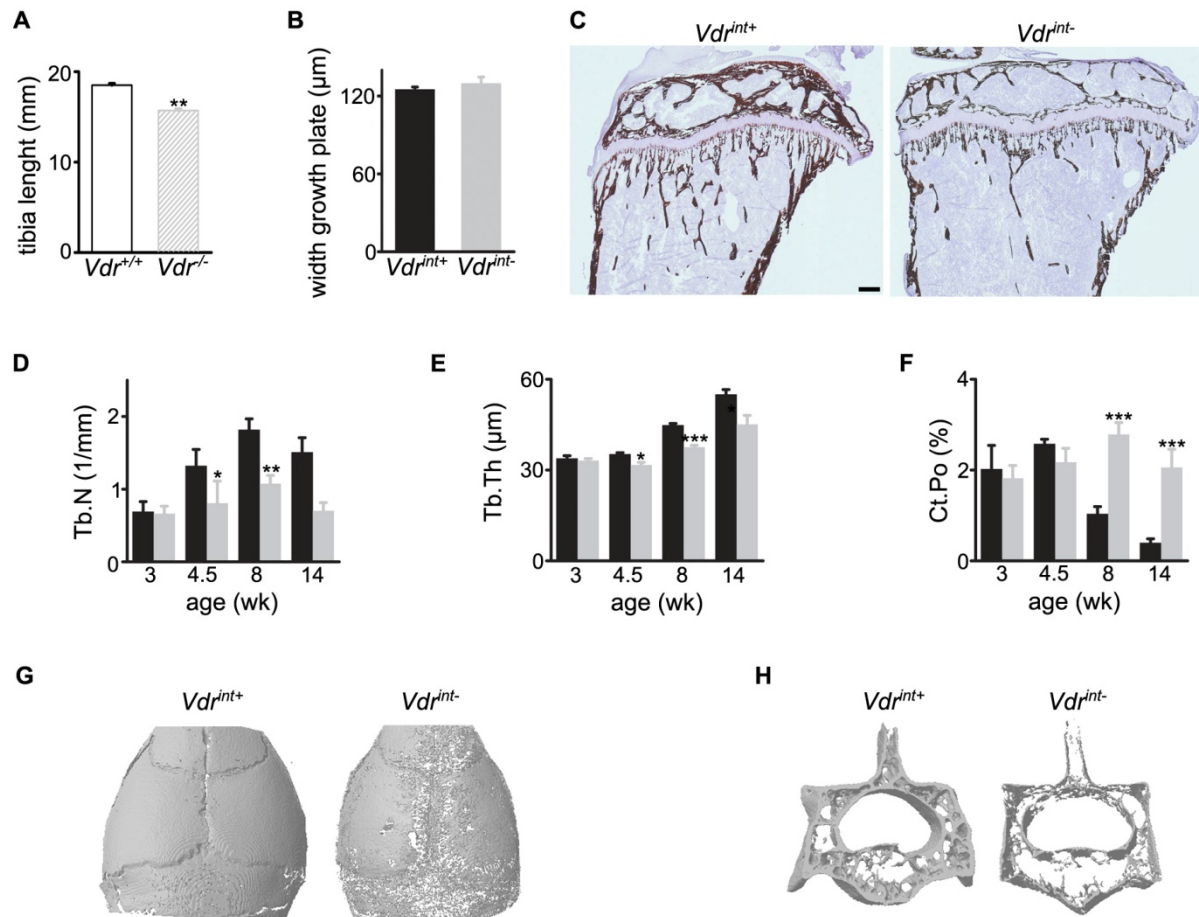
(B) Southern blot analysis of *Sacl*-digested genomic DNA isolated from tail (t), duodenum (d), jejunum (j), colon (c), kidney (k), liver (l), heart (h) and cultured calvarial osteoblasts (o), demonstrating excision of exon 2 (resulting in 3.5 kb band) in the intestine of *Vdr*^{int-} mice.

(C) Body weight at different ages. n=10.

(D) Duodenal mRNA levels of *Trpv6*, *Trpv5* and *Calbindin-D*_{9k} (*Calb*_{9k}, also known as *S100g*) by qRT-PCR, with *Vdr*^{int+} value set at 100%. n=9.

(E-F) Renal mRNA levels of *Cyp27b1* (E) and *Cyp24a1* (F) assessed by qRT-PCR. n=8.

SUPPLEMENTAL FIGURE 2



Supplemental Figure 2. $Vdr^{int-/-}$ mice develop severe osteopenia.

(A) Tibia length of $Vdr^{+/+}$ and $Vdr^{int-/-}$ mice. n=6.

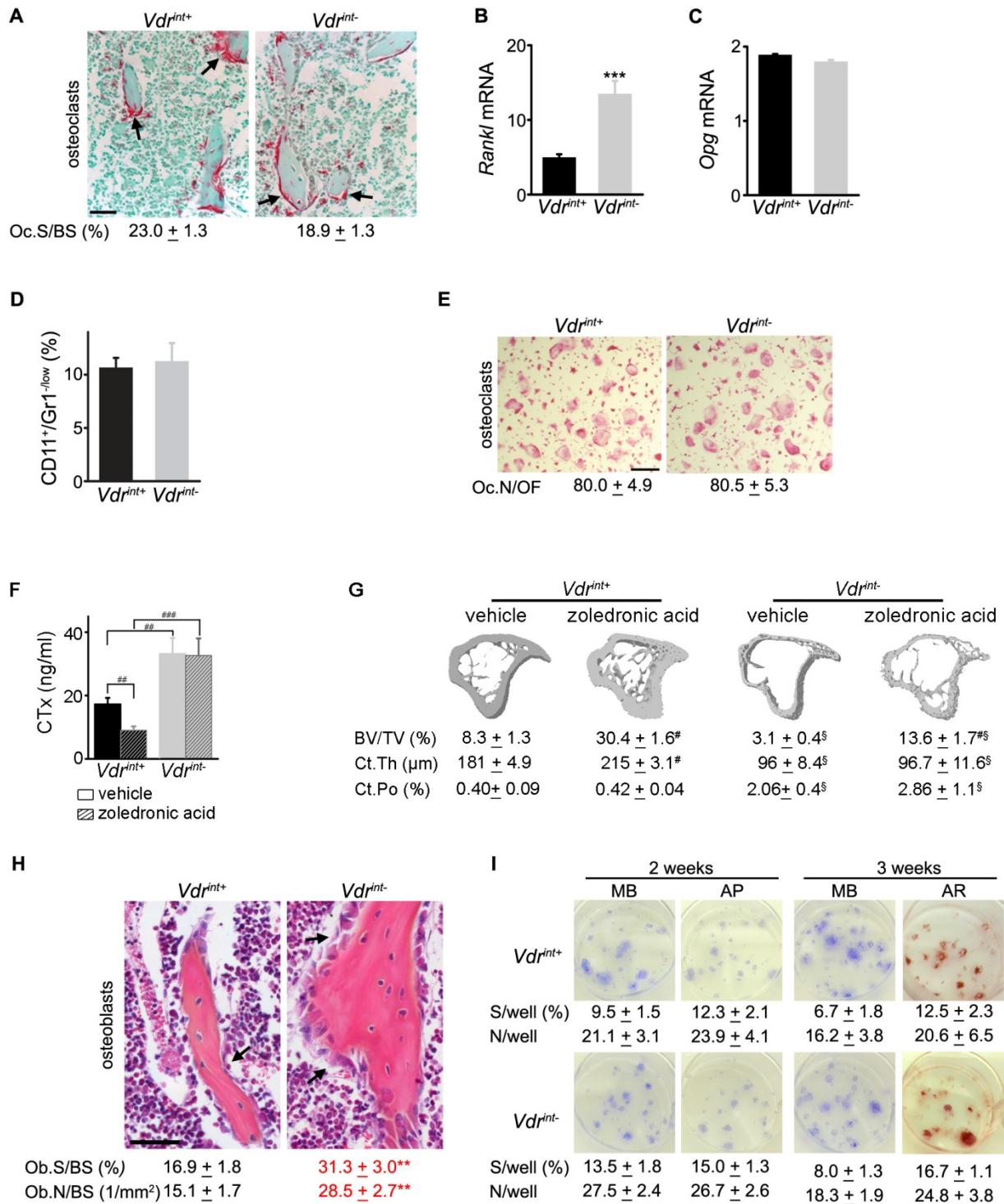
(B) Width of the tibial growth plate. n=7.

(C) Von Kossa-stained tibia sections. Scale bar: 200 μ m.

(D-F) μ CT quantification of trabecular number (Tb.N; D), thickness (Tb.Th; E), and cortical porosity (Ct.Po, F). n=9.

(G-H) 3D μ CT images of calvariae (G) and L4 vertebrae (H).

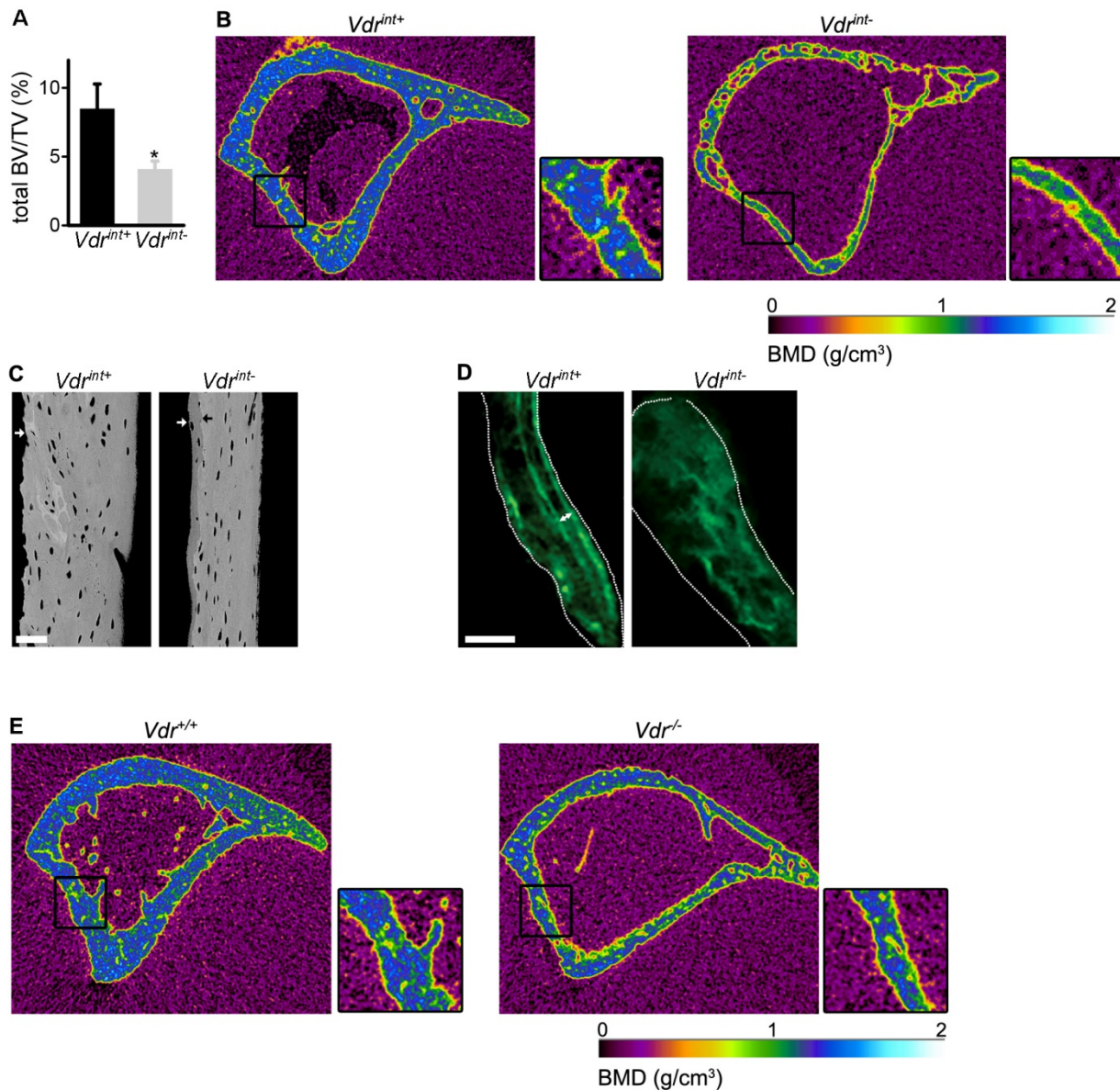
SUPPLEMENTAL FIGURE 3



Supplemental Figure 3. Bone remodeling is increased in *Vdr^{int-}* mice.

- (A) Visualization of osteoclasts (arrow) on the trabeculae by TRAP staining and quantification of the osteoclast surface (Oc.S/BS). Scale bar: 50 μ m; n=5.
- (B-C) mRNA expression of *Rankl* (B) and *Opg* (C) in the femur by qRT-PCR. n=8.
- (D) FACS analysis of the relative number of osteoclast precursors (CD11b⁺/Gr-1^{low}) in the bone marrow. n= 5-6.
- (E) TRAP staining of in vitro differentiated osteoclasts and quantification of TRAP-positive multinucleated cells (nuclei>3) per 3 optical fields (Oc.N/OF). Scale bar: 50 μ m.
- (F) Serum CTx levels in 14-wk old mice after treatment with vehicle or zoledronic acid for 6 weeks. n=4-8; ### P<0.001, ## P<0.01 as indicated.
- (G) Cross-sectional 3D μ CT images of the tibia and quantification of the trabecular bone volume (BV/TV), cortical thickness (Ct.Th) and cortical porosity (Ct.Po) in 14-wk old mice after treatment with vehicle or zoledronic acid for 6 weeks. Significant differences (P<0.05) are indicated as: # vs. vehicle of the same genotype, § vs. *Vdr^{int+}* on the same treatment protocol (two-way ANOVA followed by Fisher's LSD multiple-comparison test); n=4-8.
- (H) H&E staining demonstrates the increased abundance of cuboidal osteoblasts on the trabeculae in *Vdr^{int-}* mice (arrow). Quantification of the osteoblast surface (Ob.S/BS) and number (Ob.N/BS). n=4; scale bar: 50 μ m.
- (I) Methylene blue (MB), alkaline phosphatase (AP) and alizarin red (AR) staining of in vitro differentiated bone marrow stromal cells, cultured in osteogenic conditions for 2 and 3 wk. Quantification of the surface (S/well) and number (N/well) of colonies per well. n=10.

SUPPLEMENTAL FIGURE 4



Supplemental Figure 4. Bone mineralization is impaired in *Vdr^{int-/-}* mice.

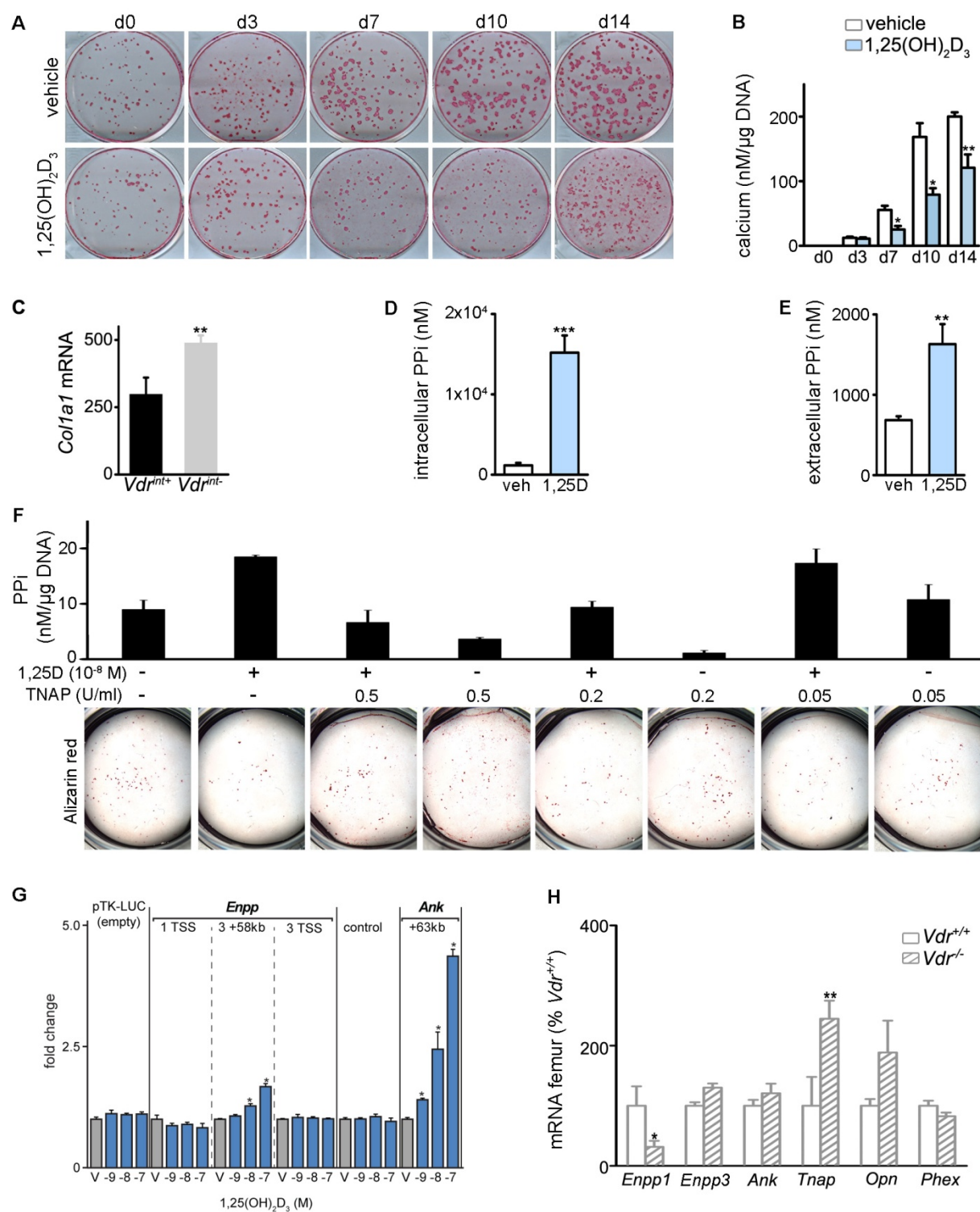
(A) Total trabecular bone volume, including the mineralized and unmineralized bone volume quantified on Goldner-stained sections. n=6; * P<0.05 vs. *Vdr^{+/+}* mice.

(B and E) Cross-sectional 3D μ CT images (magnifications of the boxed area) with the color scaling reflecting differences in bone mineral density.

(C) BSE image of the cortex shows hypomineralization of the periosteal bone in *Vdr^{int-/-}* mice at 4.5-wk of age, illustrated by the darker region between the white and black arrows. Scale bar: 50 μ m.

(D) Sequential injection of calcein shows distinct double labels in the trabecular bone of *Vdr^{int+/+}* mice (double arrow), but diffuse labeling in *Vdr^{int-/-}* mice. Scale bar: 25 μ m.

SUPPLEMENTAL FIGURE 5



Supplemental Figure 5. 1,25(OH)₂D₃ suppresses matrix mineralization in MC3T3-E1 cultures.

(A-B) Mineralization of MC3T3-E1 cultures after osteogenic differentiation for 3 wk followed by treatment with vehicle or 10⁻⁸ M 1,25(OH)₂D₃ for 0, 3, 7, 10 or 14 days (d), assessed by Alizarin red staining (A) and quantification of calcium deposition corrected for DNA content (B, n=3).

(C) mRNA expression in the femur by qRT-PCR. n=8.

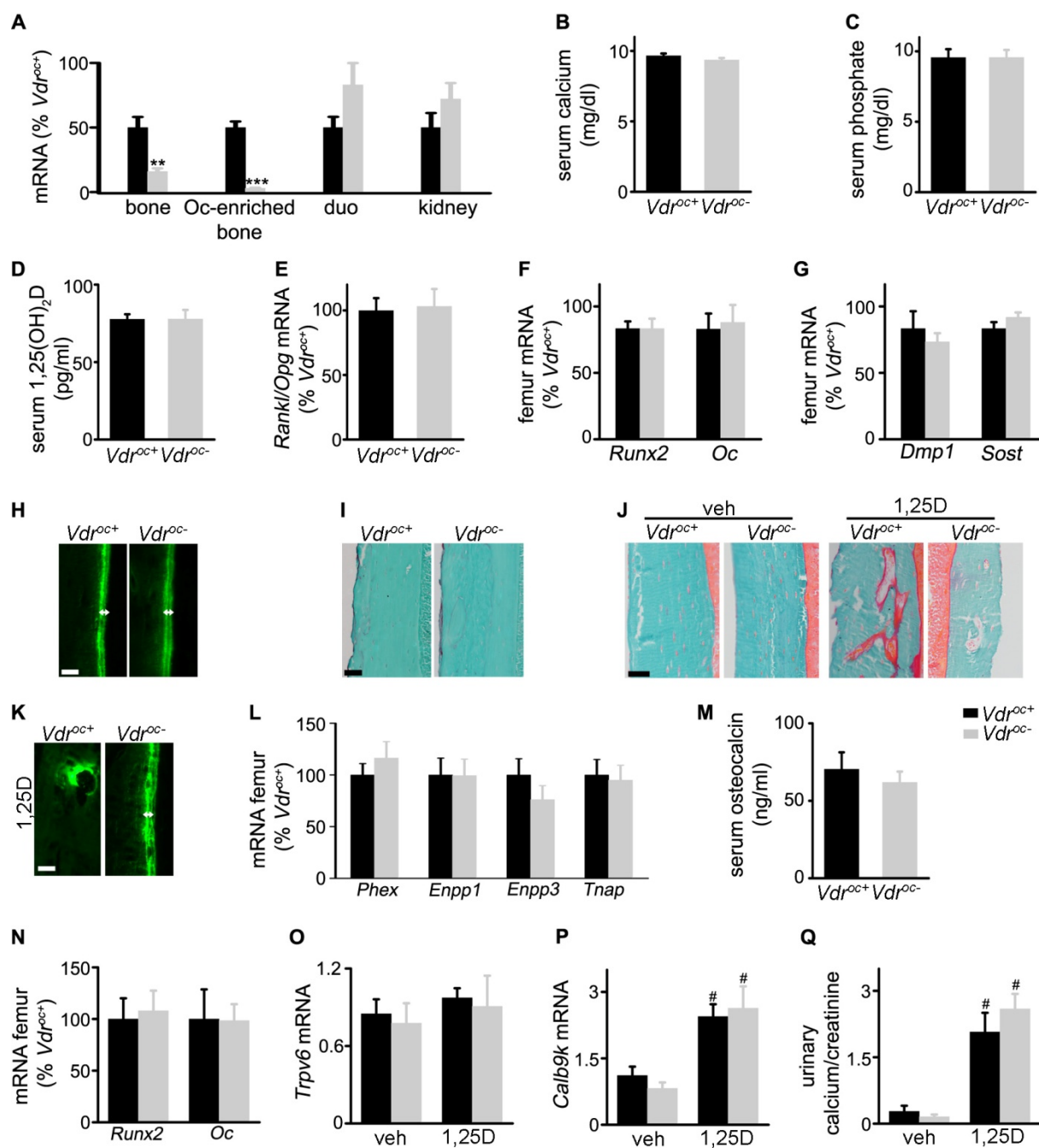
(D-E) PPI levels in MC3T3-E1 cell lysates (intracellular, D) and conditioned medium (extracellular, E) after treatment with vehicle (veh) or 10⁻⁸ M 1,25(OH)₂D₃ (D3) for 72 hours, and corrected for DNA content. n=5.

(F) Quantification of PPI levels in conditioned medium, corrected for DNA content, and visualization of mineralization by Alizarin red staining in differentiated *Vdr*^{+/+} osteoblasts following treatment for 4 days with vehicle (veh) or 10⁻⁸ M 1,25(OH)₂D₃ (D3) in combination with different concentrations of TNAP as depicted. n=3.

(G) The luciferase activity, corrected for β-galactosidase, in vehicle- or 1,25(OH)₂D₃-treated (16h) MC3T3-E1 cells transfected with a luciferase-reporter including the TSS of *Enpp1* (1 TSS), the +58 kb peak and the 3 TSS of/in *Enpp3*, a control sequence or the +63 kb peak of Ank. n=6; * P<0.05 vs. vehicle-treatment within each construct.

(H) Femoral mRNA levels analyzed by qRT-PCR. n=8.

SUPPLEMENTAL FIGURE 6



Supplemental Figure 6: Phenotype of non-treated and 1,25(OH)₂D₃-treated *Vdr*^{OC-} mice.

(A) *Vdr* mRNA expression, analyzed by qRT-PCR, in different tissues of *Vdr*^{OC+} and *Vdr*^{OC-} mice. Oc, osteocyte; duo, duodenum; n=4.

(B-D) Serum calcium (B, n=8), phosphate (C, n=8) and 1,25(OH)₂D (D, n=5) levels of *Vdr*^{OC+} and *Vdr*^{OC-} mice.

(E-G) qRT-PCR analysis of the mRNA expression in the femur of *Vdr*^{OC+} and *Vdr*^{OC-} mice, showing the Rankl/Opg ratio (E), osteoblastic (F), and osteocytic (G) differentiation markers. n=8.

(H and K) Calcein labeling in non-treated (H) and 1,25(OH)₂D₃-treated (K) mice, showing defective mineral incorporation in the cortex of 1,25(OH)₂D₃-treated *Vdr*^{OC+} mice.

(I) TRAP staining of the cortex of *Vdr*^{OC+} and *Vdr*^{OC-} mice.

(J) Goldner staining of the cortex of vehicle- and 1,25(OH)₂D₃-treated mice, showing excessive osteoid in *Vdr*^{OC+} mice.

(L and N) qRT-PCR analysis of the mRNA expression in the femur of 1,25(OH)₂D₃-treated *Vdr*^{OC+} and *Vdr*^{OC-} mice, showing mineralization inhibitors (L) and osteoblastic differentiation markers (N). n=6.

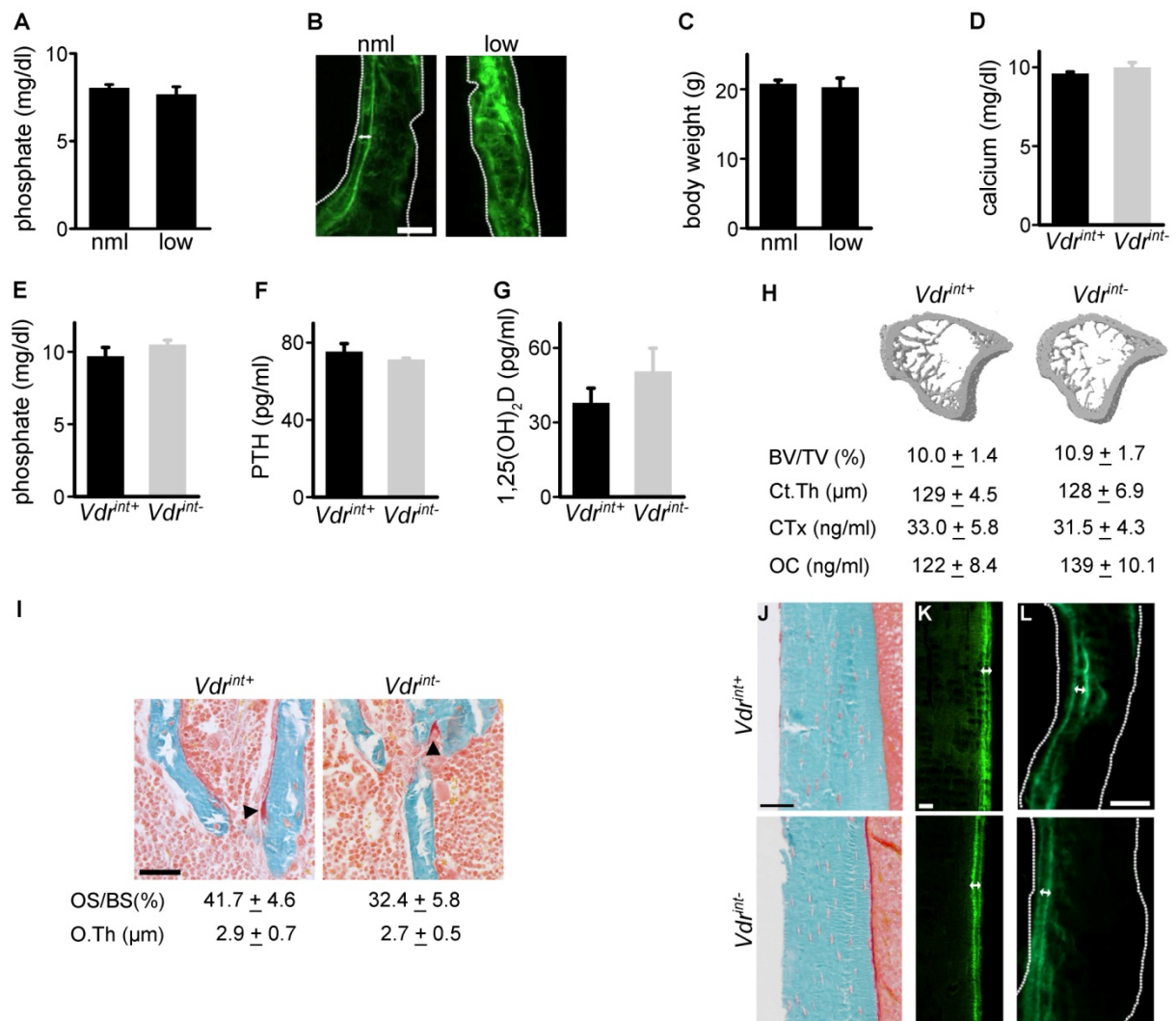
(M) Serum osteocalcin levels in 1,25(OH)₂D₃-treated *Vdr*^{OC+} and *Vdr*^{OC-} mice. n=5.

(O-P) mRNA expression in the duodenum of vehicle and 1,25(OH)₂D₃-treated *Vdr*^{OC+} and *Vdr*^{OC-} mice, analyzed by qRT-PCR. n=6.

(Q) Urinary calcium levels of vehicle and 1,25(OH)₂D₃-treated *Vdr*^{OC+} and *Vdr*^{OC-} mice, corrected for creatinine. n=5.

Significant differences (P<0.05) are indicated as # vs. vehicle of the same genotype (two-way ANOVA followed by Fisher's LSD multiple-comparison test); scale bar: 50μm; 1,25D, 1,25(OH)₂D₃.

SUPPLEMENTAL FIGURE 7



Supplemental Figure 7. Dietary calcium manipulation mimics or prevents the *Vdr^{int-}* phenotype.

(A) Serum phosphate levels in *Vdr^{int+}* mice fed a normal or a low calcium diet from weaning on. n=6-8.

(B) Histological image of the trabeculae following sequential calcein injection, shows that dietary calcium restriction (low) results in diffuse label incorporation. Scale bar: 25 μ m.

(C) Body weight of *Vdr^{int+}* mice fed a normal or a low calcium diet from weaning on. n=6-8.

(D-G) Serum calcium (D), phosphate (E), PTH (F), and 1,25(OH)₂D (G) levels in *Vdr^{int+}* and *Vdr^{int-}* mice fed a high calcium diet from weaning on. n=3-5.

(H) Quantitative μ CT analysis of the tibia of *Vdr^{int+}* and *Vdr^{int-}* mice fed a rescue diet from weaning on and measurement of serum CTx and osteocalcin levels. n=6-8.

(I-J) Goldner staining of the trabeculae (I) and cortex (J) of *Vdr^{int+}* and *Vdr^{int-}* mice fed a rescue diet from weaning on. Quantification of the osteoid surface (OS/BS) and width (O.Th). n=6; scale bar: 50 μ m.

(K-L) Histological images of the cortex (K, scale bar: 10 μ m) and trabeculae (L, scale bar: 25 μ m) following sequential calcein injection in *Vdr^{int+}* and *Vdr^{int-}* mice fed a rescue diet from weaning on.

nml, normal calcium diet; low, low calcium diet.

SUPPLEMENTAL TABLES

Table S1: Oligonucleotide sequences used in qRT-PCR to quantify gene expression

Gene Name	Gene Symbol	Accession Number	*	Sequence
Progressive ankylosis	<i>Ank</i>	NM_020332	F	5'- TCG CTG ACG CTC TGT TTT GT -3'
			R	5'- GGC AAA GTC CAC TCC AAT GAT AT -3'
			P	5'- TTC TGG ACC CCC AAC GTC TCT GAG AA -3'
Calbindin-D _{9k}	<i>Calb-D_{9k}</i> (S100g)	NM_009789	F	5'- CCT GCA GAA ATG AAG AGC ATT TT -3'
			R	5'- CTC CAT CGC CAT TCT TAT CCA -3'
			P	5'- CAA AAA TAT GCA GCC AAG GAA GGC GA -3'
Collagen type 1	<i>Col1a1</i>	NM_007742	F	5'- TGT CCC AAC CCC CAA AGA C -3'
			R	5'- CCC TCG ACT CCT ACA TCT TCT GA -3'
			P	5'- ACG TAT TCT TCC GGG CAG AAA GCA CA -3'
Cytochrom P450, family 24a1	<i>Cyp24a1</i>	NM_009996	F	5'- TGC GGA TTT CCT TTG TGA TAT TTA T -3'
			R	5'- ATT GTA GAG AAT CCA CAT CAA GCT GTT -3'
			P	5'- TCT TTC CAA GAA AGA ACT GTA CGC TGC TGT CAC -3'
Cytochrom P450, family 27b1	<i>Cyp27b1</i>	NM_010009	F	5'- ATG TTT GCC TTT GCC CAG AG -3'
			R	5'- GAC GGC ATA TCC TCC TCA GG -3'
			P	5'- CCC TGG TTC CTC ATC GCA GCT TCA -3'
Ectonucleotide pyrophosphatase phosphodiesterase 1	<i>Enpp1</i>	NM_008813	F	5'- AAG CGC TTA CAC TTC GCT AAA AG -3'
			R	5'- TGA TGG ATT CAA CGC AAG TTG -3'
			P	5'- AGC CCC TGA CCT TCT ACC TGG ACC CTC -3'
Ectonucleotide pyrophosphatase phosphodiesterase 3	<i>Enpp3</i>	NM_338425		Mm01193731_m1 (Applied Biosystems)
Hypoxanthine-guanine phosphoribosyltransferase	<i>Hprt</i>	NM_013556	F	5'- TTA TCA GAC TGA AGA GCT ACT GTA ATG ATC -3'
			R	5'- TTA CCA GTG TCA ATT ATA TCT TCA ACA ATC -3'
			P	5'- TGA GAG ATC ATC TCC ACC AAT AAC TTT TAT GTC CC -3'
Osteocalcin	<i>Oc</i> (<i>Bglap</i>)	NM_001037939	F	5'- GGC CCT GAG TCT GAC AAA GC -3'
			R	5'- GCT CGT CAC AAG CAG GGT TAA -3'
			P	5'- ACA GAC TCC GGC GCT ACC TTG GAG C -3'

Gene Name	Gene Symbol	Accession Number	*	Sequence
Osteoprotegerin	<i>Opg</i> (<i>Tnfrsf11b</i>)	NM_008764	F	5'- GAA GGG CGT TAC CTG GAG ATC -3'
			R	5'- CTG AAT TAG CAG GAG GCC AAA T -3'
			P	5'- TCA CCT GAG AAG AAC CCA TCT GGA CAT TTT -3'
Osteopontin	<i>Opn</i> (<i>Spp1</i>)	NM_001204201	F	5'- CCC ATC TCA GAA GCA GAA TCT CC -3'
			R	5'- TTC ATC CGA GTC CAC AGA ATC C -3'
			P	5'- AAG CAA TTC CAA TGA AAG CCA TGA CCA CAT -3'
Phosphate regulating gene with homologies to endopeptidases on the X	<i>Phex</i>	NM_011077	F	5'- GGA TAT TAG GTG CCG AGA GGA A -3'
			R	5'- TAC CCT TGA GAA TTC TAG CCA TCT ATA TT -3'
			P	5'- TTA TTC CAG AAT TCC AAA CCT CAG CAG GC -3'
Receptor activator of nuclear factor NF- κ B ligand	<i>Rankl</i> (<i>Tnfsf11</i>)	NM_011613	F	5'- CAT TTG CAC ACC TCA CCA TCA -3'
			R	5'- TTG CTT AAC GTC ATG TTA GAG ATC TTG -3'
			P	5'- TCG GGT TCC CAT AAA GTC ACT CTG TCC TCT T -3'
Runt related transcription factor 2	<i>Runx2</i>	NM_001145920	F	5'- TAC CAG CCA CCG AGA CCA A -3'
			R	5'- AGA GGC TGT TTG ACG CCA TAG -3'
			P	5'- CTT GTG CCC TCT GTT GTA AAT ACT GCT TGC A -3'
Sclerostin	<i>Sost</i>	NM_024449	F	5'- CCT GAG AAC AAC CAG ACC ATG A -3'
			R	5'- TGG GCC GTC TGT CAG GAA -3'
			P	5'- TCC GAG TAC AGC TGC CGC GAG C -3'
tissue non-specific alkaline phosphatase	<i>Tnap</i> (<i>Alpl</i>)	NM_007431	F	5'- CGC ACG CGA TGC AAC A -3'
			R	5'- CGG ACT TCC CAG CAT CCT T -3'
			P	5'- CAC TCA GGG CAA TGA GGT CAC ATC CA -3'
Transient receptor potential vanilloid channel 6	<i>Trpv6</i>	NM_022413	F	5'- ATC CGC CGC TAT GCA CA -3'
			R	5'- AGT TTT TCT CCT GAA TCT TTT TCC AA -3'
			P	5'- TTC CAG CAA CAA GAT GGC CTC TAC TCT GA -3'
Vitamin D receptor	<i>Vdr</i>	NM_009504	F	5'- GGA TCT GTG GAG TGT GTG GAG ACC -3'
			R	5'- CTT CAT CAT GCC AAT GTC CAC GCA G -3'
			P	5'- CTG TTC ACC TGC CCC TTC AAT GGA GAT -3'

* F, forward primer; R, reverse primer; P, probe

Table S2: Oligonucleotide sequences used in qRT-PCR to validate ChIP-seq data

Regulatory sequences	*	Sequence
TSS of <i>Enpp1</i>	F	5'-TGG CTT CCT GTC TGA TTT CGG GC-3'
	R	5'-AGC GAT TCC AGT CCA GTC CTC GT-3'
+58kb peak in <i>Enpp3</i>	F	5'-GGC CAG CTT TTG TCC TTT GCA CG-3'
	R	5'-AGA GGT GGC ACA ACG GCT TCC TT-3'
TSS of <i>Enpp3</i>	F	5'-AAG CCT AAG TGG GAA GGC AGG CA-3'
	R	5'-AGC ATT AGC CAC AGA GGA GCC CA-3'
TSS of <i>Ank</i>	F	5'-GGA GCA TCC CAG AAC CCT AGT CCA-3'
	R	5'-TTC GGG GAC AAC CGA GCT GGA AA-3'
+63kb peak in <i>Ank</i>	F	5'-TGG TCA CAC CCA TAT AGC CCC AGT-3'
	R	5'-CAA ACA GAC AGA TGT GGG CGG GA-3'
Control region	F	5'-GAA AGC GTC CTT GCT GCT TGC CT-3'
	R	5'-ACC AGA CCA AGA CAC CAA CAT GCC-3'

* F, forward primer; R, reverse primer

Table S3: Oligonucleotide sequences used to amplify regulatory sequences from BAC DNA

Regulatory sequences	Genomic localization	*	Sequence
TSS of <i>Enpp1</i>	Chromosome 10	F	5'- GCG ACA AAG CTT TGT AGG TGT TGG GGT CTT TGG CAG G -3'
	24431608-24432208 bp	R	5'- GCT CCC GGA TCC CGG TGT CCG GCC TTT GAG ACC TCG G -3'
+58kb peak in <i>Enpp3</i>	Chromosome 10	F	5'- CAG CAT AAG CTT CAT TTA TTT TGG CTG GTT AAT TTA C--3'
	24497990-24498590	R	5'- CAG GGT GGA TCC TTC TGA GAG CCA AAA ATG TTC CTT C -3'
TSS of <i>Enpp3</i>	Chromosome 10	F	5'- GCC ACC AAG CTT GTA AGA CTA AAA TGA ACA GTT TTG C -3'
	24555701-24556301 bp	R	5'- ATG ATG GGA TCC TGG TGC TTA GGA GAT AAG AAG TGA C -3'
+63kb peak in <i>Ank</i>	Chromosome 15	F	5'- CAT ACA AAG CTT AGA AAC AAG GTT CTT TCA GCC TTT C -3'
	27459585-27460185	R	5'- AAC GTG GGA TCC AGG AAG GAG CAC AGC ATG TCT GTA AG -3'
Control region	Chromosome 10	F	5'- TTT CTG AAG CTT CAA AGT AAA TTT ATA ATT ATA CAG -3'
	24456853-24457453 bp	R	5'- AAA CAG GGA TCC AGG CCA CCT GGA AAT AAC AGA CCC -3'

* F, forward primer; R, reverse primer

SUPPLEMENTAL METHODS

Serum, urine and bone biochemistry

Calcium and phosphate levels in serum, incinerated bone and cell lysates were analyzed by SYNCHRON Clinical Systems (Beckman Coulter) and corrected for dry weight (bone), or DNA (cell lysates). Ionized calcium levels were assessed on the ABL725 blood gas analyzer (Radiometer Medical ApS) directly after sampling of heparinized arterial blood (PICO50 radiometer, Radiometer Medical). Levels of osteocalcin and 1,25(OH)₂D were measured using an in house (1) and ¹²⁵I 1,25(OH)₂D (Diasorin) RIA kit respectively. Serum PTH (Immutopics), FGF23 (Kainos Laboratories Inc.) and CTx (Nordic Bioscience Diagnostics A/S) levels were determined by ELISA.

Bone mass measurement and histological analysis

Left tibiae were fixed in Burkhardt's solution and embedded undecalcified in methyl methacrylate (MMA). MMA sections (10 µm) were stained according to the von Kossa and Goldner method or left unstained for dynamic histomorphometry. Right tibiae were fixed in 2% paraformaldehyde, decalcified in 0.5 M EDTA (pH 7.4)/PBS prior to dehydration, embedded in paraffin, and sectioned at 5 µm. Osteoclasts were visualized on paraffin sections reacted for tartrate resistant acid phosphatase (TRAP) activity (2), and osteoblast parameters were quantified on H&E stained sections. To analyze dynamic bone parameters, calcein (16 mg/kg BW; Sigma-Aldrich) was i.p. injected 4 days and 1 day prior to sacrifice. Histomorphometric analysis was done as previously described (3,4), and expressed according to the American Society for Bone and Mineral Research standardized histomorphometry nomenclature (5).

Micro-computed tomography (µCT) analysis of the tibiae was performed ex vivo using the high resolution SkyScan 1172 system (50 kV, 200 µA, 0.5 mm Al 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 was used for the calibration of bone density measurements. Measurements are performed according to the guidelines of the American Society for Bone and Mineral Research (6).

The concentration of calcium and phosphate surrounding the osteocytes and the mineral content of the mid-diaphyseal cortices was visualized and semi-quantified on polished, gold-coated MMA-embedded tibia sections using an environmental scanning electron microscope (Philips XL30 ESEM FEG) (7). Backscattered electron (BSE) images were used to visualize bone mineral content variations and energy dispersive X-ray micro-analysis (EDAX) was used to measure elemental calcium and phosphor (the latter as representative element for phosphate) by point analyses and line scans.

RNA extraction

Total RNA of tissues and cell cultures was extracted as previously described (4). The osteocyte-enriched bone fraction was generated by removal of the bone marrow, and sequential collagenase A and EDTA digestions of the long bones.

In vitro osteoblast and osteoclast cultures

To assess in vitro osteoblast differentiation and mineralization, CFU fibroblast (CFU-F) and CFU osteoblast (CFU-OB) in isolated bone marrow cells cultures were quantified following 14 and 21 days culture period in osteogenic conditions (50 µg/ml ascorbic acid, 10 mM β-glycerophosphate, 10^{-8} M dexamethasone) and staining for methylene blue, alkaline phosphatase and Alizarin red. To address cell autonomous defects in osteoclastogenesis, non-adhesive hematopoietic cells, isolated from bone marrow, were cultured in α-MEM containing 20 ng/ml M-CSF and 100 ng/ml RANKL (Peprotech EC) (day 1) and TRAP staining was performed at day 6. To investigate the effect of $1,25(\text{OH})_2\text{D}_3$ on mineralization, the osteoblastic MC3T3-E1 cells (Riken Cell Bank, Tsukuba, Japan) were first differentiated in osteogenic medium for 21 days. After this period, differentiated cells were treated with 10^{-8} M $1,25(\text{OH})_2\text{D}_3$ for 72 hours when pyrophosphate (PPi) levels were determined, or for the indicated time points when mineralization was analyzed by Alizarin red staining or by quantification of total calcium levels. All quantifications were performed on 2-3 wells per group, and repeated 2 to 4 times (4).

ChIP, ChIP-seq and analysis of enhancer activity

ChIP using VDR, RXR and non-specific IgG antibodies was performed as described previously (8). The isolated DNA (Input) and the precipitated DNA (ChIP-DNA) was then subjected to qRT-PCR with the primers listed in Supplemental Table 2.

ChIP-seq analysis was performed as described (9). The tag density for each factor was normalized to 1×10^7 tags and displayed using the UCSC genome browser.

To confirm the responsiveness of the regulatory sequences to $1,25(\text{OH})_2\text{D}_3$, luciferase-reporter construct were generated by cloning the appropriate mouse DNA fragments (400-600bp) obtained through DNA amplification of BAC DNA with the primers listed in Supplemental Table 3, into the pTK-LUC+ vector. MC3T3-E1 cells were transfected as described (10) and analyzed for luciferase and β-galactosidase activity 16 hours after treatment with vehicle or $1,25(\text{OH})_2\text{D}_3$. Luciferase activity was normalized to β-galactosidase activity.

Flow cytometry

The number of osteoclast precursors in the bone marrow of 8-wk old mice was determined by flow cytometry using R-PE-conjugated anti-CD11b and FITC-conjugated anti-Gr-1 antibodies as described (11). Osteoclast precursors were identified as the CD11b⁺/Gr1^{-/low} cell population.

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