Supplemental Data

Intestinal Clock System Regulates Skeletal Homeostasis

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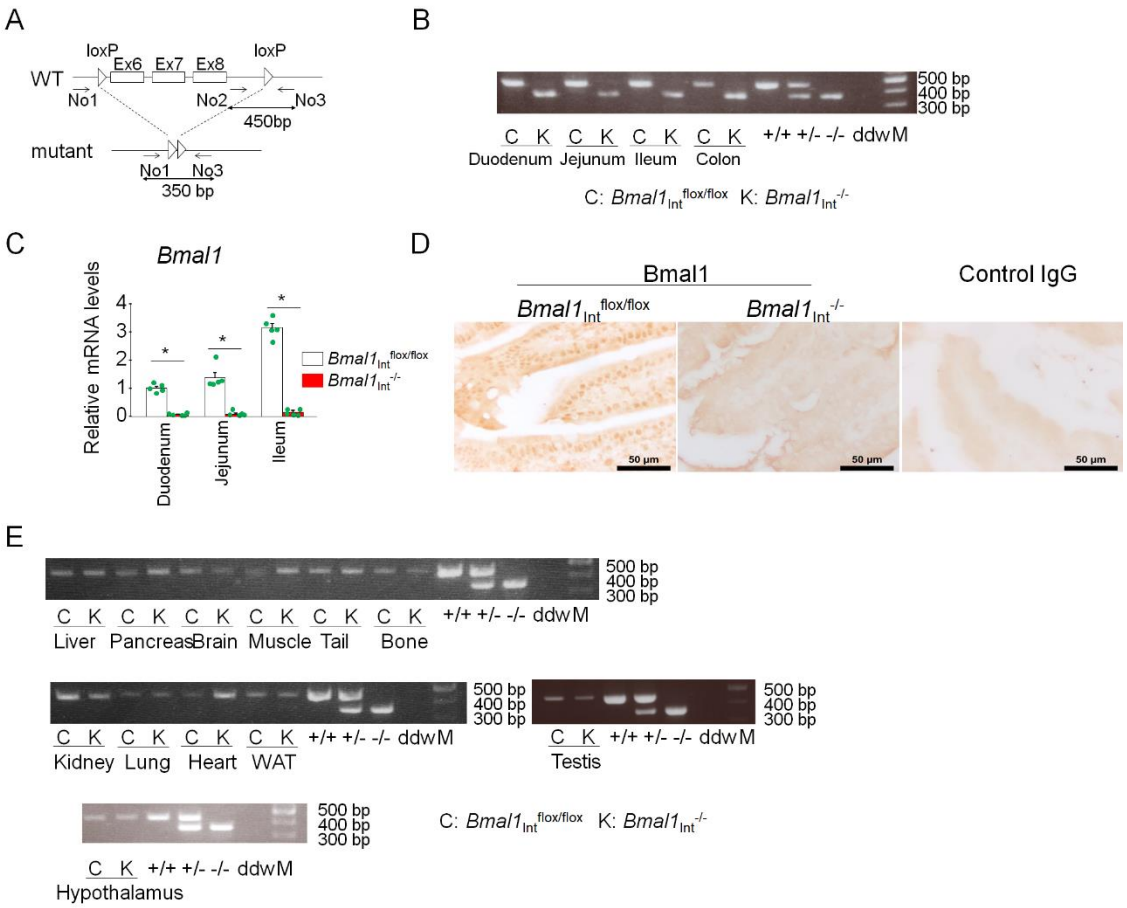
Supplemental Figure 1



Supplemental Figure 1. Generation of *Bmal1*$_{Int}^{-/-}$ mice.

A. Schematic model of the generation of *Bmal1*$_{Int}^{-/-}$ mice.

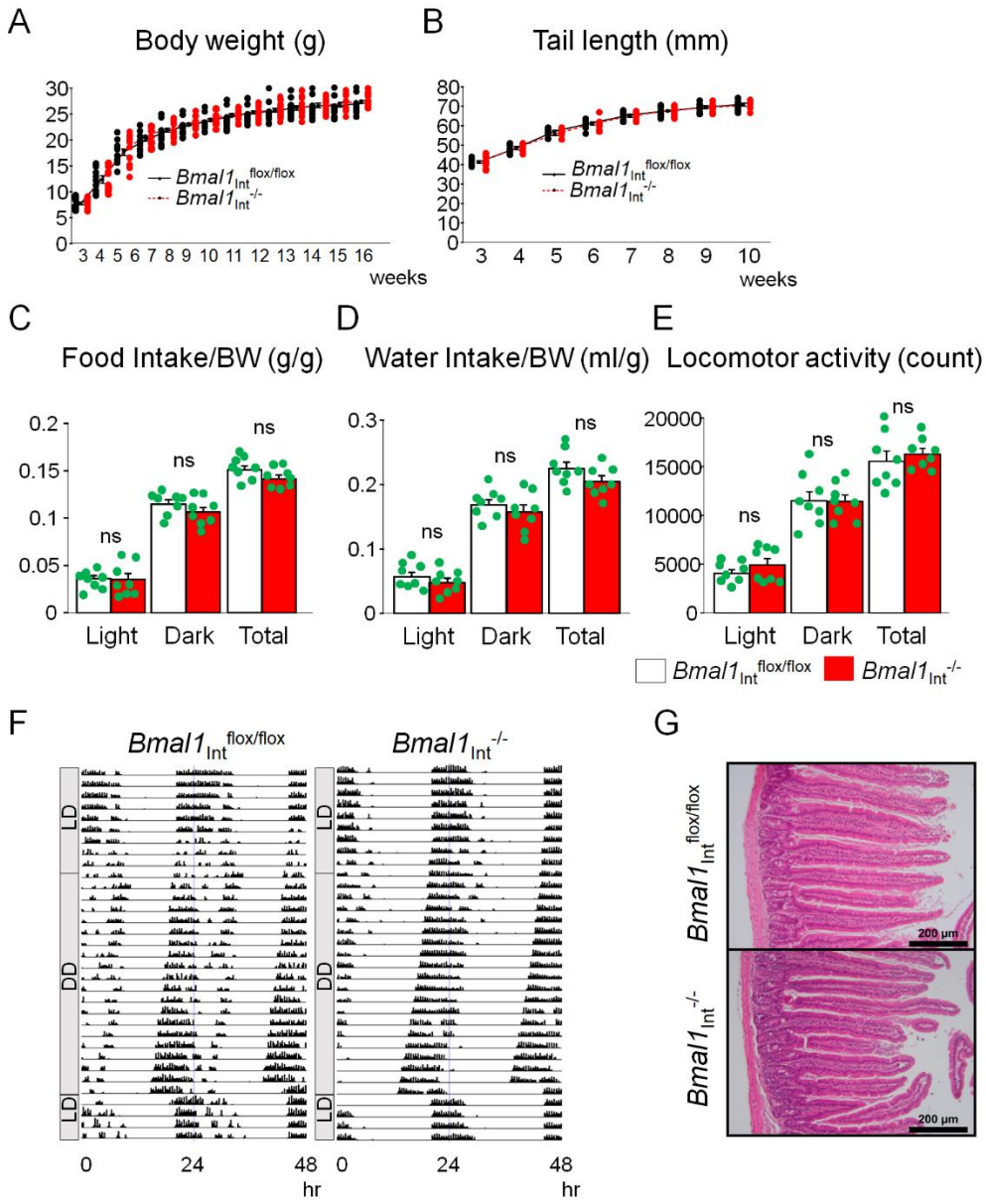
B. Intestinal villi were obtained from the duodenum, jejunum, ileum, and colon of 8-week-old *Bmal1*$_{Int}^{flox/flox}$ mice or *Bmal1*$_{Int}^{-/-}$ mice, and genomic DNA was extracted. The excision of *Bmal1* was confirmed by a PCR analysis using the specific primers shown in Supplemental Figure 1A.

C. Intestinal villi were obtained from the duodenum, jejunum, ileum, and colon at ZT2 and RNA was extracted. The expression of *Bmal1* was analyzed by real-time RT-PCR (N=5).

D. The duodenum was collected at ZT2 and the expression of Bmal1 was evaluated by immunohistochemistry. A representative of 3 independent experiments is shown.

E. Genomic DNA was extracted from the tissues as indicated. No significant excision of *Bmal1* occurred in extra-intestinal tissues.

Supplemental Figure 2



Supplemental Figure 2. Phenotypic analyses of *Bmal1*$_{Int}^{-/-}$ mice.

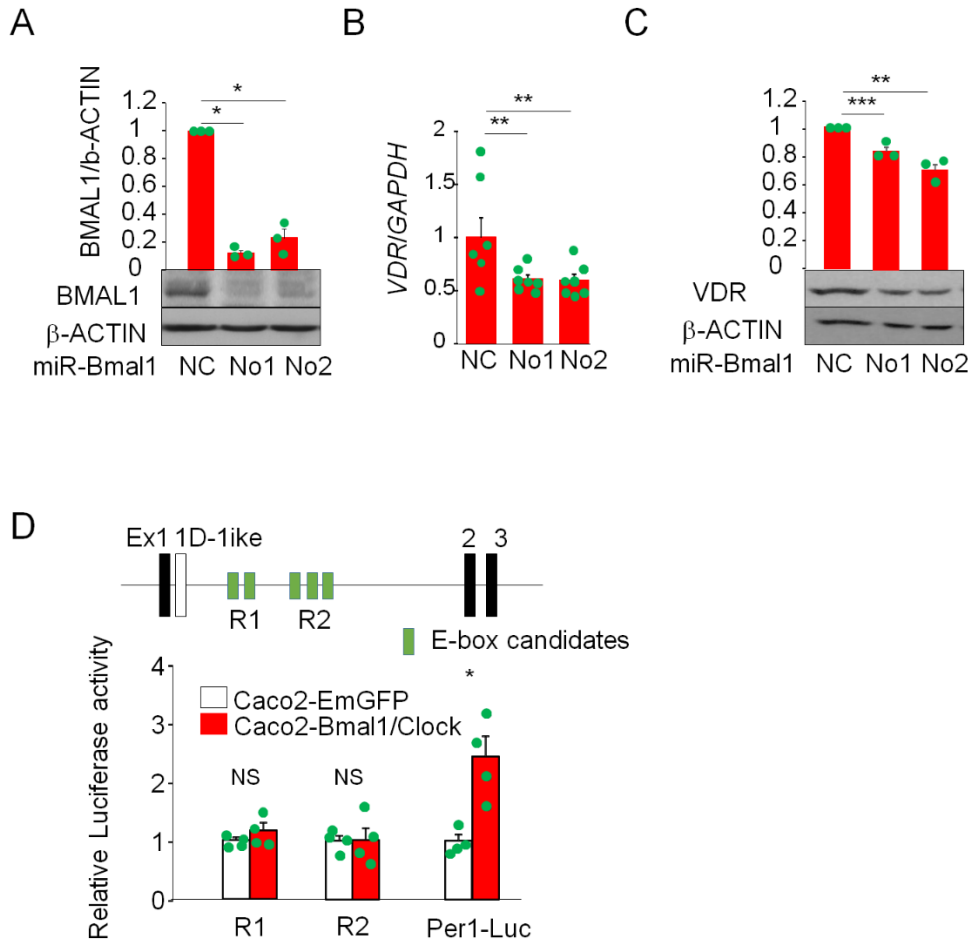
A and B. Body weight (A) and tail length (B) were measured weekly. No significant differences were observed between *Bmal1*$_{Int}^{flox/flox}$ mice or *Bmal1*$_{Int}^{-/-}$ mice (N=11).

C-E. Food intake (C), water intake (D), and locomotor activity (E) were measured in 8-week-old *Bmal1*$_{Int}^{flox/flox}$ mice or *Bmal1*$_{Int}^{-/-}$ mice (N=8). NS: not significant.

F. An actogram was double plotted for 8-week-old mice. Figure 1B was graphically modified based on Supplemental Figure 2F.

G. Hematoxylin and Eosin staining of the duodenum of 8-week-old mice. A representative of 3 independent experiments is shown.

Supplemental Figure 3

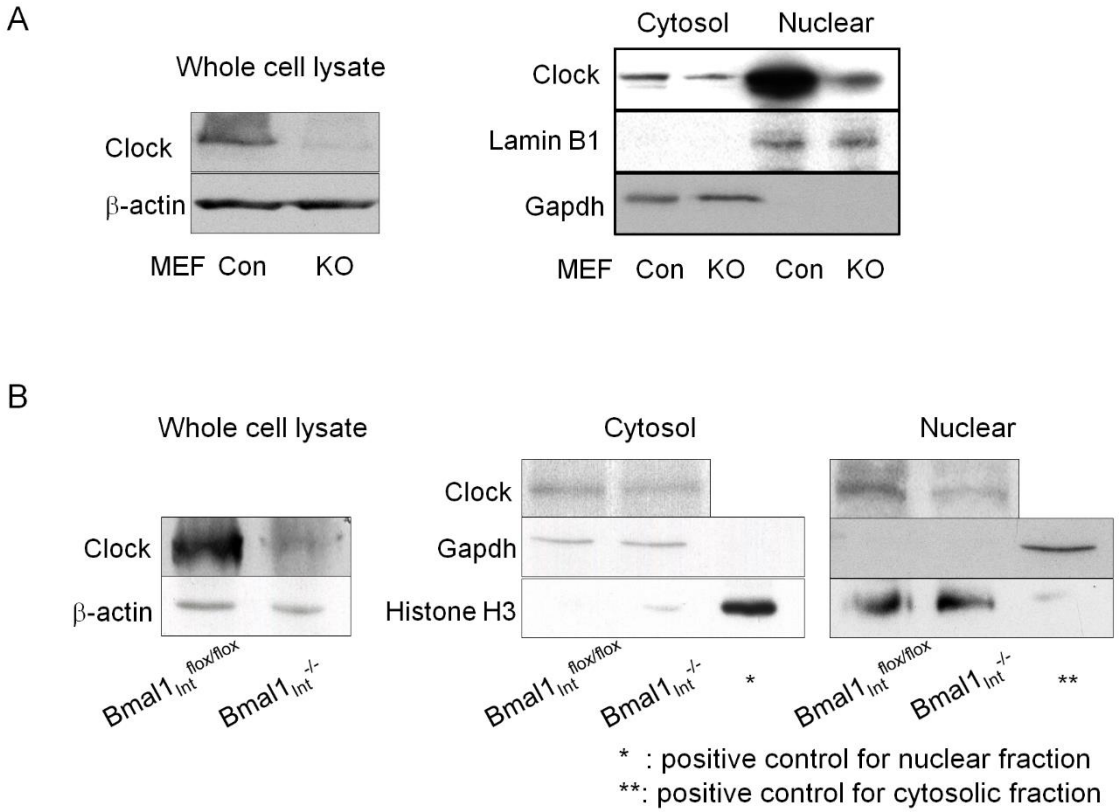


Supplemental Figure 3. Functional E-boxes are absent in the *Vdr* gene.

A-C. The expression of BMAL1 was knocked down in Caco2 cells (A) and the expression of *VDR* and VDR was assessed by real-time RT-PCR (N=6)(B) and western blot analysis (N=3)(C), respectively. *; $p<0.01$, **; $p<0.05$ by one-way ANOVA.

D. A luciferase assay using vectors containing the R1 or R2 region in intron 1 of the Vdr gene was performed in Caco2 cells stably expressing EmGFP or mBmal1/mClock. The Per1-Luc vector was used as a positive control for the experiment (N=4). *; $p<0.05$, by Student's *t*-test. NS; not significant.

Supplemental Figure 4

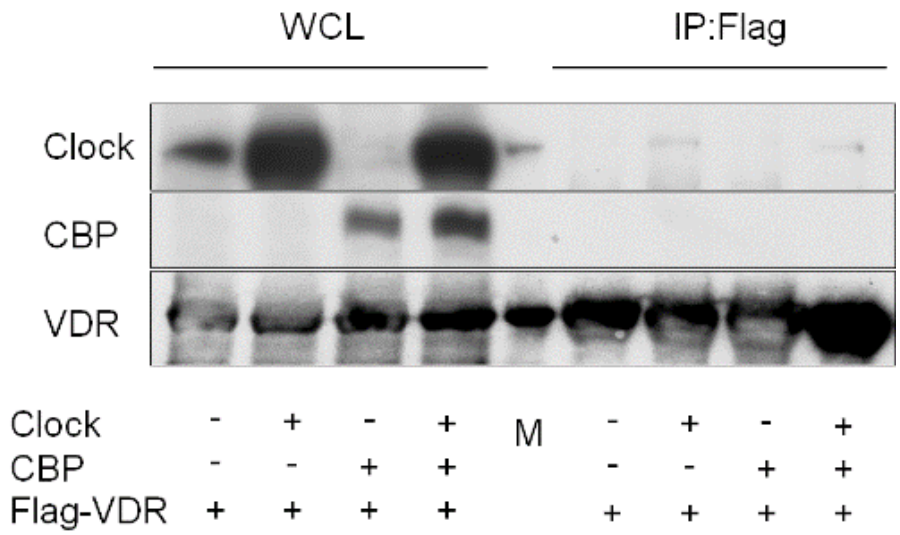


Supplemental Figure 4. Nuclear Clock expression is decreased in *Bmal1*$_{Int}^{-/-}$ mice.

A. Whole cell lysate, cytosolic and nuclear proteins were collected from MEFs isolated from mice globally lacking *Bmal1* gene (KO) or *Bmal1*$_{Int}^{flox/flox}$ mice (Con), and the expression of Clock was analyzed by a Western blot analysis. A representative of 3 independent experiments is shown.

B. Whole cell lysate, cytosolic and nuclear proteins were collected from the villi of the duodenum from *Bmal1*$_{Int}^{flox/flox}$ mice or *Bmal1*$_{Int}^{-/-}$ mice at ZT 8, and the expression of Clock was assessed by a Western blot analysis. A representative of 3 independent experiments is shown.

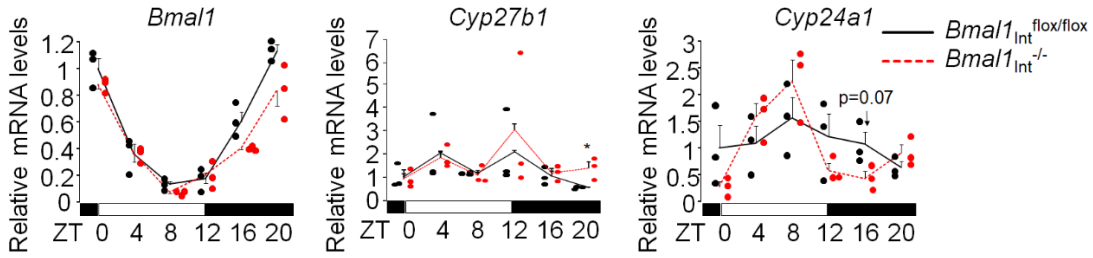
Supplemental Figure 5



Supplemental Figure 5. CBP is not recruited to Clock/VDR protein complex.

HEK293T cells were transfected with vectors containing Clock, CBP, and/or Flag-VDR. Thirty-six hours after transfection, cells were treated with 10^{-8} M of $1,25(OH)_2D$ overnight and immunoprecipitation using anti- Flag antibodies was followed by a Western blot analysis for Clock or CBP. WCL: whole cell lysate, M: marker.

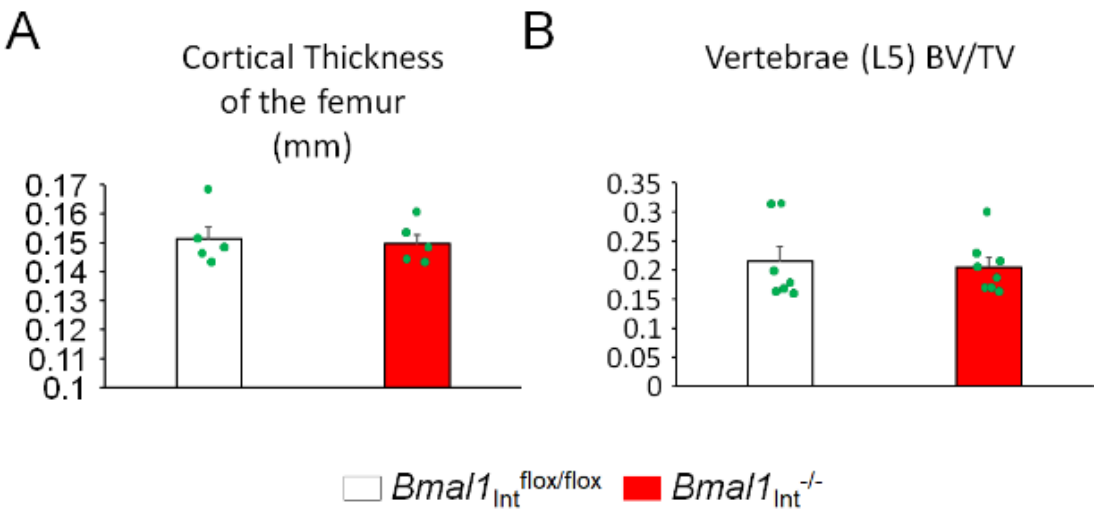
Supplemental Figure 6



Supplemental Figure 6. Expression profile of *Cyp27b1* and *Cyp24a1* in the kidney is altered in *Bmal1*$_{Int}^{-/-}$ mice.

Expression profiles of genes of interests in the kidney of 8-week-old mice (N=3). *; $p<0.05$, *Bmal1*$_{Int}^{flox/flox}$ *vs Bmal1*$_{Int}^{-/-}$ at indicated time points by Student's *t*-test.

Supplemental Figure 7

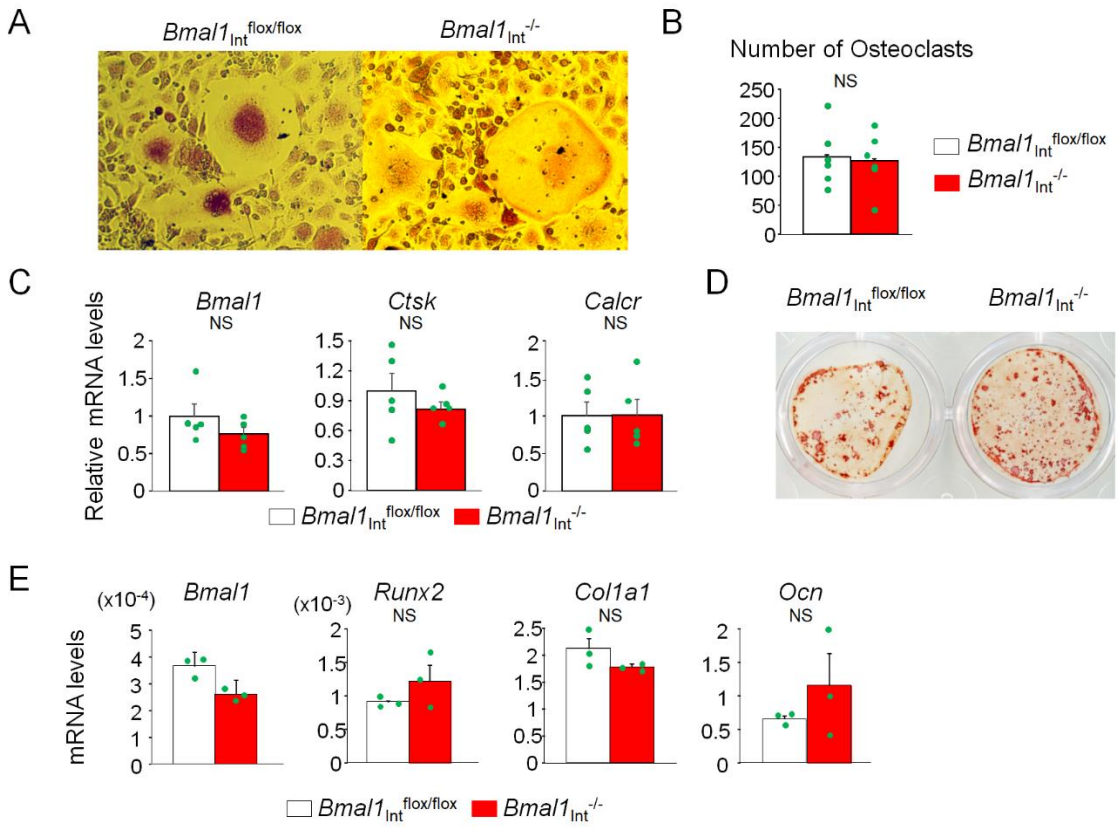


Supplemental Figure 7. The lack of Bmal1 in the intestine does not affect the microarchitecture of cortical bone of the femur and trabecular bone of the lumbar vertebrae.

A. Femurs were collected at 16 weeks old, and microCT analysis was performed to evaluate cortical thickness (N=5).

B. Lumbar vertebrae (L5) were collected at 16 weeks old, and trabecular bone architecture was analyzed by microCT (N=7).

Supplemental Figure 8

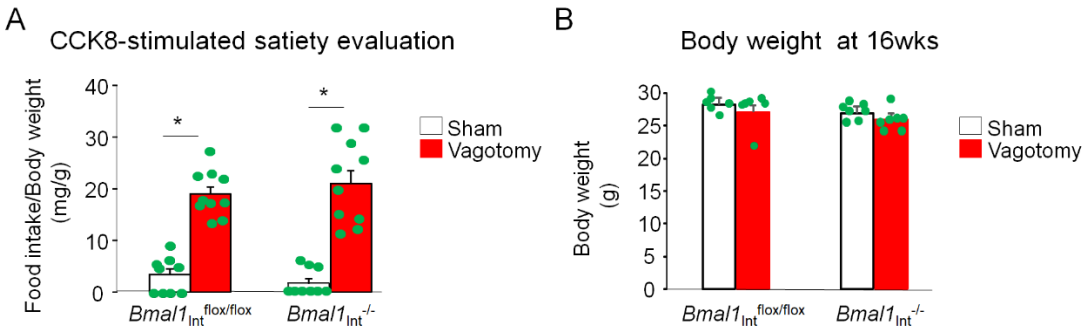


Supplemental Figure 8. *In vitro* osteoblast and osteoclast differentiation assays reveal that the lack of Bmal1 in osteoblast or osteoclast precursor cells does not affect their differentiation capacity.

A-C. Osteoclast precursor cells were obtained from the spleens of 8-week-old *Bmal1*$_{Int}^{flox/flox}$ mice or *Bmal1*$_{Int}^{-/-}$ mice, and treated with osteoclast differentiation media for 9 days. Osteoclasts were visualized by TRAP staining (A) and the number of osteoclasts containing 3 or more nuclei was calculated (N=6) (B). The expression of *Bmal1*, *Ctsk*, and *Calcr* was assessed by real-time RT-PCR (N=5) (C). NS: not significant.

D-E. Preosteoblasts were obtained from the calvariae of 7-10-day-old *Bmal1*$_{Int}^{flox/flox}$ mice or *Bmal1*$_{Int}^{-/-}$ mice, and treated with osteoblast differentiation media for 10 days. Mineral apposition was visualized by Alizarin Red staining D). The expression of *Bmal1*, *Runx2*, *Col1a1*, and *Ocn* was assessed by real-time RT-PCR (N=3)(E). NS: not significant.

Supplemental Figure 9



Supplemental Figure 9. Vagotomy is efficiently performed both *in* $Bmal1_{Int}^{flox/flox}$ mice and $Bmal1_{Int}^{-/-}$ mice.

A. The efficacy of vagotomy was evaluated by the CCK8-stimulated satiety method. An intraperitoneal injection of CCK8 suppressed food intake in sham-operated $Bmal1_{Int}^{flox/flox}$ mice and $Bmal1_{Int}^{-/-}$ mice, but not in vagotomized mice (N=9-10). *; $p<0.01$, by Student's *t*-test.

B. Vagotomy did not affect the body weights of $Bmal1_{Int}^{flox/flox}$ mice or $Bmal1_{Int}^{-/-}$ mice (N=6-7).

Supplemental Methods

Genotyping

The excision of the floxed allele was confirmed based on a PCR analysis using genomic DNA as previously described (1). Briefly, as shown in Supplemental Figure S1A, the 280-bp *Bmal1*-excised allele was amplified using forward primer No.1 (5′- GGGGATTTCCATCTGTGTTTAC-3′) and reverse primer No.3 (5′- CTCATCTGCTTATCTGCTCTGGGG -3′), whereas amplification of the *Bmal1*-unexcised allele (250 bp) was performed using forward primer No.2 (5′- TCAACGTTGTTTCGATGCTCGTG-3′) and reverse primer No.3.

Cell Culture

Caco-2 cells (ATCC) were purchased from ATCC and maintained in Eagle's Minimum Essential Medium supplemented with 20% fetal bovine serum and 1% of NEAA. HEK293T cells (ATCC) were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum. Mouse embryonic fibroblasts (MEFs) were isolated from mice globally lacking the *Bmal1* gene or their littermate controls as previously reported (2). In brief, the extraembryonic membranes and viscera were removed from embryos 14.5 days post-coitus, minced, and incubated with 0.25% trypsin-EDTA for 5 minutes at 37°C. The supernatant after precipitation by gravity was used as MEFs. MEFs were used within 2 passages.

In vitro knockdown experiments

Knockdown experiments for BMAL1 in Caco-2 cells were performed based on the adenovirus-mediated expression of the microRNA (miRNA) system using the BLOCK-iT™ Pol II miR RNAi Expression Vector Kit with EmGFP (Invitrogen, Thermo Fisher Scientific). The adenovirus was prepared using the ViraPower™ Adenovirus Expression System (Invitrogen, Thermo Fisher Scientific) according to the manufacturer's protocol.

In vitro overexpression experiments

The forced expression of mBmal1 and mClock in Caco-2 cells was performed using lentiviral vectors. The cDNA of mBmal1 or mClock was inserted into a TOPO pENTR vector using the pENTR Directional TOPO cloning kit and recombined with the CSII-EF-RfA–IRES-puro vector

(a kind gift from Dr. Hiroyuki Miyoshi (RIKEN BRC, Ibaraki, Japan)) with the LR recombination reaction system (Invitrogen). A lentivirus was infected into Caco-2 cells in the presence of 5 μg/ml polybrene (Sigma) and cells expressing the gene of interest were selected by puromycin (1 μg/ml).

***In vivo* chromatin immunoprecipitation assays**

Control and *Bmal1*$_{Int}^{-/-}$ mice at 8–9 weeks of age were intraperitoneally injected at ZT4 or ZT16 with either vehicle (Propylene Glycol)(Wako Pure Chemical Industries, Ltd., Osaka, Japan) or 1,25-$(OH)_2D_3$ (EMD Millipore) at a dose of 10 ng/g of body weight, and 4 hours after the injection (ZT8 or ZT20), the intestinal villi from 7 cm of the proximal duodenum and jejunum were scraped by coverslips and homogenized in nuclear extraction buffer (10 mM HEPES-NaOH, 10 mM KCl, 0.1 mM EDTA, 1 mM orthovanadate, and protease inhibitor cocktail (Complete TM; Roche)), and the precipitates after centrifugation (5 min at 700×*g*) were used as the nuclear fraction. Samples were subjected to a crosslinking reaction for 15 min with 1% formaldehyde in PBS, and the fixative was neutralized by adding glycine at a final concentration of 125 mM. Following centrifugation (5 min at 700×*g*), the pellet was washed twice with nuclear extraction buffer and sonicated in IP buffer (20 mM HEPES-NaOH, 137 mM NaCl, 1 mM EDTA, 5% glycerol, 1% Triton X-100, 1.67 mM $MgCl_2$, 1 mM orthovanadate, and protease inhibitor cocktail (Complete TM; Roche) [pH 7.8]) supplemented with 1% sodium dodecyl sulfate (SDS) (Bioruptor, cycle conditions; on/off: 10 s/20 s, 15 cycles). The supernatant was collected after centrifugation (20,000×*g* at 4°C for 30 min) and diluted in IP buffer (final concentration: 0.1% SDS). Samples were then pre-incubated with Dynabeads protein A (Thermo Fisher Scientific) at 4°C for 60 min with gentle rotation and the precleared supernatant was incubated with an anti-Vdr antibody (Cell Signaling, #12550), anti-Clock antibody (Abcam, #ab3517), or anti-Acetyl-histone H4 (Merck Millipore, #06-866) antibody. After the overnight incubation at 4°C, Dynabeads protein A was then added to the mixture at 4°C for 4 h, and the beads were sequentially washed as follows: once in IP buffer, once in IP buffer supplemented with 500 mM NaCl, once in LiCl buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl [pH8.0]), and twice in TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]). Genomic DNA was purified from chromatin using a DNA purification kit (Qiagen). A quantitative PCR analysis using specific primers that detect the VDRE or E-boxes in the genes of interest was performed. DNA obtained from precleared samples was used as the input.

In vitro chromatin immunoprecipitation assays

The expression vector containing the histone acetyltransferase (HAT) activity-dead Clock mutant was generated based on a previous study (3) using QuikChange II XL (Agilent Technologies, Santa Clara, CA) according to the manufacturer's protocol. HEK293T cells were transfected with the pcDNA3.1-mBmal1-V5 vector, pSG5-hVDR vector, and/or pFlag vectors containing wild-type (WT)-mClock (WT-Clock) or HAT activity-dead mClock (MT-Clock) using the PEI method. Six hours after the treatment with 10^{-8}M of 1,25-$(OH)_2D_3$, cells were subjected to a crosslinking reaction for 15 min with 1% formaldehyde in PBS, and the fixative was neutralized by adding glycine at a final concentration of 125 mM. After rinsing twice with PBS, cells were solubilized with nuclear extraction buffer and sonicated in IP buffer supplemented with 1% sodium dodecyl sulfate (SDS). Chromatin immunoprecipitation was performed as described above.

Luciferase Assay

VDRE-mediated Vdr transcriptional activity was analyzed using the luciferase vector (pGV-P2-$(DR3)_2$) containing 2 tandem repeats of canonical DR3 (direct repeat spaced by three nucleotides). HEK293T cells were transfected with pGV-P2-$(DR3)_2$ in combination with the pSG5-hVDR expression vector, pcDNA3.1-mBmal1-V5 expression vector, and/or pSG5-mClock-myc expression vector, as indicated in the figure, and 24 hours after transfection, cells were treated with 10^{-8} M of 1,25-$(OH)_2D_3$ overnight, followed by the assessment of luciferase activity using specific substrates in a luminometer. In order to evaluate whether E-boxes at the mouse *Vdr* gene were functional, the first intron (R1; Chr15:97732426-97731130, R2; Chr15:97730380-97728020) containing the candidate region for E-Boxes was amplified from the BAC clone (B6Ng01-143J09, RIKEN, Japan) and luciferase vectors were prepared by subcloning the corresponding PCR products into pGL4.26[luc2/minP/Hygro] (pGL4.26-R1 or R2)(Promega, Madison, WI) vectors digested with *Eco*RV using T4 DNA Ligase. HEK293T cells were transfected with the pGL4.26-Vdr-R1 or R2, pcDNA3.1-mBmal1-V5, and pSG5-mClock-myc expression vectors using the PEI method and transfection luciferase activity was assessed 48 hours later. The luciferase vector containing the *Per1* promoter region, which possesses functional E-boxes, was used as a positive control in experiments. Transfection efficiency was corrected by co-transfection with the TK-*Renilla* luciferase construct (Promega). Transient transfection was

performed using polyethyleneimine (PEI) (PEY-Max, Polyscience Inc., #24765). In brief, 0.5 μg of DNA in 25 μl of HBSS was mixed with 1 μl of 1 mg/ml PEI-Max, vortexed, left to stand at RT for 15 minutes, and then added to each well of a 24-well plate. The total amount of DNA in each well was equalized using an empty vector. The luciferase assay was performed in duplicate according to the protocol of the dual-luciferase reporter assay system (Promega).

Isolation of nuclear proteins

In the cell fractionation of MEFs, cells were solubilized in hypotonic lysis buffer (10 mM Hepes (pH 7.8), 10 mM KCl, 0.1 mM EDTA (pH 8.0), and a protease inhibitor cocktail (Complete TM; Roche Diagnostics Mannheim, Germany)), and centrifuged at 12,000×*g* for 20 minutes. The supernatant was used as the cytosolic fraction. The pellets were then lysed with hypertonic buffer (50 mM Hepes (pH 7.8), 420 mM KCl, 0.1 mM EDTA (pH8.0), 5 mM $MgCl_2$, 2% glycerol, and a protease inhibitor cocktail (Complete TM; Roche Diagnostics Mannheim, Germany)) and supernatants collected after centrifugation (12,000×*g* for 20 minutes) were used as the nuclear fraction. The isolation of nuclear proteins from the villi of the duodenum was performed using the ReadyPrep™ Protein Extraction kit (BioRad, #163-2089).

***In vitro* osteoclastogenesis of osteoclast precursors from the spleen**

Spleens were collected from 8-week-old mice, minced in culture media (αMEM supplemented with 10% FCS), and filtrated using a 100-μm nylon mesh. Suspended spleen cells were added to a total volume of 8 ml of culture media and centrifuged for 5 minutes. The pellet was treated with 2 ml of RBC lysis buffer (Sigma R7757 Hybri Max), and washed twice with culture media. The pellet after centrifugation (1000 rpm for 5 minutes) was redissolved in 10 ml of culture media containing 25 ng/ml of macrophage colony-stimulating factor (M-CSF) (PeproTech, Inc.) seeded at 20×10^6 cells/75-cm^2 flask, and incubated overnight. Culture media containing non-adherent cells were collected and the pellet after centrifugation (1000 rpm for 5 minutes) was used as osteoclast precursor cells. The induction of osteoclastogenesis was performed by incubating cells with 50 ng/ml of M-CSF and 100 ng/ml of soluble receptor activator of nuclear factor-κB ligand (sRANKL) (PeproTech, Inc.). Osteoclasts were stained with a tartrate-resistant acid phosphatase (TRAP) staining kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan. #294-67001). The number of osteoclasts containing 3 or more nuclei was counted as multinucleated osteoclasts.

***In vitro* osteoblastogenesis of calvarial osteoblasts**

Neonatal calvarial osteoblasts were collected from 7-10-day-old pups as previously described (4). In brief, calvariae were dissected and digested 5 times with collagenase (2.5 mg/ml) (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 0.05% trypsin/EDTA. Cells released from fractions between digest 2 through 5 were collected as primary calvarial osteoblasts and maintained in DMEM supplemented with 10% FCS and non-essential amino acids. Osteoblastogenesis of primary calvarial osteoblasts was induced by incubating cells with 4 mM of β-glycerophosphate and 50 μg/ml of ascorbic acid in αMEM with 10% FCS. Mineral apposition was evaluated by Alizarin Red staining.

RNA isolation and quantitative real-time PCR

A 2-cm piece of the duodenum proximal to the pyloric junction was dissected and villi were scraped by coverslips. Total RNA was isolated using TRIzol extraction (Invitrogen, Thermo Fisher Scientific) and the RNeasy MiniElute Cleanup kit (Qiagen) according to the manufacturers' instructions. Genomic DNA was removed from RNA samples using the DNase treatment. cDNA was generated using the SuperScript II First-Strand Synthesis SuperMix Kit (Invitrogen, Thermo Fisher Scientific) according to the manufacturer's protocols. Quantitative real-time PCR was performed using a 7300 Real-time PCR system or StepOnePlus™ Real-time PCR system (Applied Biosystems). TaqMan Gene Expression Assays for *Gapdh*, *Cryptochrome1*, *Rankl*, *Cyp27b1*, *Cyp24a1*, *Trpv6*, and *Tph1* were purchased from Applied Biosystems. Primer sequences for *Bmal1*, *Clock*, *Per1*, *Rev-erbα*, *Dbp*, *Cabp9k*, *Ncx1*, *Ocn*, *Ctsk*, *VDR*, and *BMAL1* are available upon request. *Gapdh* was used as an internal standard control gene for all quantifications.

Western blot analysis

In the preparation of whole cell lysates, cells were solubilized in RIPA buffer (1% Triton, 1% Na deoxycholate, 0.1% SDS, 150 mM NaCl, 10 mM Tris-Cl (pH 7.4), 5 mM EDTA, 1 mM orthovanadate, and protease inhibitor cocktail (Complete TM; Roche Diagnostics Mannheim, Germany). Equal amounts of protein were separated by SDS-PAGE and transferred electrophoretically to PVDF membranes. Membranes were blocked in BlockAce reagent

(Dainippon Pharmaceuticals, Osaka, Japan) or Blocking-one P reagent (Nacalai Tesque, Kyoto, Japan), immunoblotted with anti-Bmal1 (Abcam, #ab93806), anti-cMyc (Santa Cruz Biotechnology, #sc-789), anti-V5 (Invitrogen, # 46-0705), or anti-Flag (Sigma, #F3165) and developed with horseradish peroxidase-coupled secondary antibodies, followed by enhancement with a chemiluminescence (ECL) detection system (GE Healthcare).

Co-immunoprecipitation

The transfection of expression vectors in HEK293T cells was performed using PEI as described above. Cells were solubilized in NP-40 buffer (5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, 10% glycerol, and 10 mM Tris-HCl, pH 8.0) containing a protease inhibitor cocktail (Complete TM, EDTA-free; Roche Diagnostics) and centrifuged. Supernatants were precleared with protein A/G-Sepharose (Santa Cruz Biotechnology) at 4°C for 2 hours, followed by immunoprecipitation with an anti-Flag antibody (Sigma, #F3165) at 4°C overnight. Samples were washed five times with PBS containing a protease inhibitor cocktail and then subjected to a Western blot analysis.

MicroCT analysis

Microarchitecture of the midshaft cortical bone of the femur and of the trabecular bone in the lumbar 5 (L5) vertebral body, was evaluated by microCT (resolution 10 μm, VivaCT-40; Scanco Medical AG, Bassersdorf, Switzerland). The midpoint of each femur, with an isotropic pixel size of 21 μm and slice thickness of 21 μm, was evaluated for cortical bone analysis. Bone area (BA), total cross-sectional area (TA), BA/TA and cortical thickness (Ct.Th) were calculated. Approximately 0.3 mm below the cranial and above the caudal growth plate of trabecular bone in L5 vertebrae was analyzed. Measurements included bone volume/total volume, trabecular number (Tb.N.), trabecular thickness (Tb.Th.), and trabecular spacing. All scans were analyzed using manufacturer software (version 4.05; Scanco Medical AG).

Monitoring of food and water intakes and locomotor activity

Food and water intakes were recorded every 10 min in 8-week-old mice using a cFDM-300 system (Melquest, Toyama, Japan). Locomotor activity was evaluated using high-density-arranged infrared sensors (The SCANET MV-40, MELQUEST, Toyama). Data were analyzed

using Feedam software (Melquest, Toyama City, Japan).

Histological analysis

Tissue samples were harvested and fixed in 10% buffered formalin, and paraffin-embedded samples were then prepared. Hematoxylin and Eosin (H&E) staining was performed according to conventional methods.

Immunohistochemistry

Tissue samples were collected, fixed in 10% buffered formalin, and paraffin-embedded. In Bmal1 and Vdr staining, antigen retrieval was performed following deparaffinization and rehydration using citrate buffer at 95ºC for 60 min and endogenous peroxidase activity was quenched. After blocking, sections were incubated with an anti-*Bmal1* antibody (Abcam, #ab93806) or anti-*Vdr* antibody (Cell Signaling, #12550) at 4°C overnight, followed by an incubation with a HRP-labeled polymer, which was conjugated with secondary antibodies and visualized with 3, 3'-diaminobenzidine using the EnVision+ System (Dako). For PGP9.5 staining, sections were incubated with an anti-PGP9.5 antibody (Abcam, #ab8189) at 4°C overnight, followed by visualization using Alexa Fluor® 488-conjugated secondary antibodies (Invitrogen). Phalloidin (Alexa Fluor 555 phalloidin, A34055, Invitrogen) and DAPI (340-07971, DOJINDO) was used to stain F-actin and the nucleus, respectively. Images were captured by conforcal microscopy (TCS SP8, Leica Microsystems).

Measurement of serum parameters

Serum PINP, CTX and PTH levels were measured using RAT/Mouse PINP EIA (IDS), RatLaps™ ELISA (MBL Life Science) and the mouse intact PTH ELISA kit (Immutopics), respectively, following the manufacturers' instructions. Serum and urinary phosphate and urinary creatinine levels were measured using Phospha-C test Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan, #270-49801) and LabAssay™ Creatinine (Wako Pure Chemical Industries, Ltd., Osaka, Japan, #290-65901), respectively, following the manufacturers' instructions. The excretion fraction of Ca (EFCa) was calculated as follows: EFCa=Urine-Ca × Serum-Cre/Serum-Ca × Urine-Cre. Urinary adrenaline levels were measured using the Adrenaline/Epinephrine EIA Kit

(RE 592 51) (IBL Co., Ltd. #82001) according to the manufacturer’s instructions.

Supplemental References

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