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***Dmp1Cre*-directed knockdown of PTHrP in murine decidua is associated with a life-long increase in bone mass, width, and strength in male progeny**

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Abstract

Parathyroid hormone related-protein (PTHrP) is a pleiotropic regulator of tissue homeostasis. In bone, *Dmp1Cre*-targeted PTHrP deletion in osteocytes causes osteopenia and impaired cortical strength. We report here that this outcome depends on parental genotype. In contrast to our previous report using mice bred from *Dmp1Cre.Pthlh^{f/w}* parents, adult (16 week old and 26 week old) *Dmp1Cre.Pthlh^{f/f}* mice from homozygous parents (*Dmp1Cre.Pthlh^{f/f(hom)}*) have stronger bones, with 40% more trabecular bone mass and 30% greater femoral width than controls. This increase in bone size was observed as early as 12 days of age, when greater bone width was also found in male and female *Dmp1Cre.Pthlh^{f/f(hom)}* mice, but not in gene-matched mice from heterozygous parents. This suggested a maternal influence on skeletal size prior to weaning. Although *Dmp1Cre* has previously been reported to cause gene recombination in mammary gland, milk PTHrP protein levels were normal. The wide-bone phenotype was also noted *in utero*: *Dmp1Cre.Pthlh^{f/f(hom)}* embryonic femora were more mineralized and wider than controls. Closer examination revealed that *Dmp1Cre* caused PTHrP recombination in placenta, and in the maternal-derived decidual layer that resides between the placenta and the uterus. Decidua from mothers of *Dmp1Cre.Pthlh^{f/f(hom)}* mice also exhibited lower PTHrP levels by immunohistochemistry and were smaller than controls. We conclude that *Dmp1Cre* leads to gene recombination in decidua, and that decidual PTHrP might, through an influence on decidual cells, limit embryonic bone radial growth. This suggests a maternal-derived developmental origin of adult bone strength.

Keywords: PTHrP, genetic animal models, developmental modeling, molecular pathways - development

Introduction

Bone size and geometry are among the factors determining bone strength ⁽¹⁾. During skeletal development, bone grows in both the longitudinal and radial axes. Longitudinal growth is mediated by chondrocytes at the growth plates, where hypertrophic chondrocytes cease dividing, enlarge and eventually mineralize surrounding matrix ⁽²⁾. Simultaneously, expansion of bone diameter (termed “radial growth”) balances bone length and width. Although longitudinal growth has been studied widely, little is known about the signaling pathways orchestrating radial growth ⁽³⁾.

Parathyroid hormone-related protein (PTHrP, gene name: *Pthlh*) is produced by many tissues, and acts locally to maintain their physiological function ^(4,5). Global knockout of PTHrP is neonatal lethal and causes widespread skeletal defects including reduced bone length, due largely to PTHrP’s role in promoting chondrocyte maturation ⁽⁶⁾. Heterozygous *Pthlh* deletion causes osteopenia in adult mice ⁽⁷⁾. This is likely due to a requirement for local PTHrP production by the osteoblast lineage to promote bone formation in adults during bone remodeling. Such a conclusion was established by studies in genetically altered mice: *Pthlh* knockdown targeted to osteoblasts (*Col1(2.3kb)Cre.Pthlh^{f/f}*) ⁽⁷⁾ or to osteocytes (*Dmp1(10kb)Cre*) ⁽⁸⁾ both caused low bone formation and osteopenia in adulthood. Osteocytes were found to produce full-length PTHrP ⁽⁸⁾, which acts through the PTH receptor to stimulate prolonged signaling in osteoblasts and osteocytes ⁽⁹⁾.

Here we report an additional effect of PTHrP on bone structure. This arose from an unexpected finding when, in continuing our previous study ⁽⁸⁾, we sought to assess the effect of *Dmp1Cre*-targeted knockdown of PTHrP in osteocytes in older mice. To limit mouse wastage, we changed our breeding strategy to use cousin-bred homozygous breeding pairs, as we have in previous mouse models, to facilitate generation of large numbers of mice ^(10,11). To our surprise, adult male PTHrP-deficient mice generated from homozygous breeders (denoted *Dmp1Cre.Pthlh^{f/f(hom)}*) exhibited an opposing phenotype to our previous work, in which mice were generated from heterozygous breeders (denoted *Dmp1Cre.Pthlh^{f/f(het)}*) ⁽⁸⁾. Adult male *Dmp1Cre.Pthlh^{f/f(hom)}* mice had high trabecular bone mass, and wide long bones, but normal body weight, and normal bone length. Since this was a profound and reproducible phenotype, we sought to determine the source of this difference.

Although we previously reported that *Dmp1Cre* can lead to gene recombination in the mammary gland ⁽⁸⁾, there was no alteration in milk PTHrP levels in *Dmp1Cre.Pthlh^{f/f(het)}* dams. However, suckling male and female *Dmp1Cre.Pthlh^{f/f(hom)}* mice both exhibited the wide bone phenotype. We traced the phenotype back to fetal development and found it was associated with recombination of PTHrP in decidua in mothers of *Dmp1Cre.Pthlh^{f/f(hom)}* mice, which also displayed low PTHrP levels in decidua basalis and reduced decidual size. This implies that PTHrP from the decidua may limit bone radial

growth in male and female mice, and that this has life-long effects on skeletal size in males, that override the previously described effects of endogenous PTHrP deletion in osteocytes⁽⁸⁾. This has significant implications for bone development, sex-differences in bone growth, and for breeding strategies used with *Dmp1Cre*-targeted mouse models.

Materials and Methods

Animal experiments and assessment of recombination

Dmp1Cre.Pthlh^{f/f(het)} mice have been described previously ⁽⁸⁾ and were bred from *Dmp1Cre* (Tg(*Dmp1-cre*)^{1jqfe}) mice (containing the *Dmp1* 10-kb promoter region) provided by Lynda Bonewald (University of Kansas, Kansas City, USA) ⁽¹²⁾, and *Pthlh*-flox (*Pthlh^{tm1Ack}*) mice by Andrew Karaplis (McGill University, Montreal) ⁽¹³⁾ with LoxP sites spanning *Pthlh* exon III ⁽⁸⁾. St. Vincent's Health Animal Ethics Committee approved all animal procedures, which were conducted in accordance with the Australian code for the care and use of animals for scientific research.

Two breeding strategies were used in this study (Figure 1A, 4A). Initially, mice hemizygous for *Dmp1Cre* were crossed with *Pthlh^{f/f}* mice to generate *Dmp1Cre.Pthlh^{f/w}* breeders. These were used to generate hemizygous-bred PTHrP deficient (*Dmp1Cre.Pthlh^{f/f(het)}*) and *Dmp1Cre* mice, as in our previous study ⁽⁸⁾ (Figure 1A, shown in blue). *Dmp1Cre.Pthlh^{f/f(hom)}* mice were generated from breeding pairs that were both *Dmp1Cre.Pthlh^{f/f(het)}* (Figure 1A, shown in red). Cousin-matched *Dmp1Cre* mice were bred to generate homozygous-bred *Dmp1Cre* cousin controls (denoted *Dmp1Cre^(hom)*). Adult mice were collected at 14 weeks (male only), and at 26 weeks (both male and female) after an *in vivo* micro-CT scan at 16 weeks of age. Sample sizes used were based on our previous studies; no explicit power analysis was used.

12 day old pups were collected from both homozygous and heterozygous breeders. For the milk collection, mice were mated at 6 weeks of age, and after the mice became pregnant for the first time, males were removed. At day 12 of the (first) lactation, dams were anesthetized and injected with 1 IU of oxytocin (Sigma) ⁽¹⁴⁾. After 5 minutes, milk was collected and then kept at -80°C.

Dmp1Cre.Pthlh^{f/f(hom)} and *Dmp1Cre^(hom)* embryos and matching placenta/decidua were collected (4-7 litters/genotype) at embryonic day (E)18.5 of first pregnancy, and *Dmp1Cre.Pthlh^{f/f(het)}* embryos, placenta and decidua were collected at E17.5 of first pregnancy. The sex of embryos was determined by PCR ⁽¹⁵⁾. All mice were group housed at the St Vincent's BioResources Centre, in a 12 h light and dark cycle and provided commercial food (Barastoc Irradiated Mouse Cubes, ID102108) and water *ad libitum*. Terminal blood samples were collected by cardiac puncture exsanguination and sera kept at -80°C.

To assess *Pthlh* recombination, tissues were collected from various organs including marrow-flushed femora and tibiae, calvaria, hearts, spleens, brains, kidneys, muscles,

mammary glands, and uteri of 6-week-old female *Dmp1Cre.Pthlh^{ff(hom)}* mice, and at E17.5 embryos were collected, placenta removed, and adjacent decidua were dissected from the placental tissue from *Dmp1Cre.Pthlh^{ff(hom)}*, *Dmp1Cre.Pthlh^{ff(het)}* and *Dmp1Cre^(hom)* embryos. Genomic DNA (gDNA) was extracted using Isolate II genomic DNA kit (Bioline, London, UK). PCR was performed using primers designed to detect native and recombined DNA as described previously ⁽⁸⁾. Investigators were blinded to animal genotypes during animal handling and tissue collection.

Micro-computed tomography (micro-CT)

Micro-CT was carried out on samples from E17.5, E18.5, 12 days, 14 and 26 weeks of age. The observer was blinded to genotype and sex of all samples at the time of analysis. 26 week old mice were also anaesthetized and scanned by *in vivo* micro-CT at 16 weeks of age. After collection, embryos were fixed in 95% ethanol for 5 days. Femora of 14 and 26 week old mice, and tibiae of 12 day old mice were fixed overnight in 4% paraformaldehyde at 4°C, then stored in 70% ethanol until further analysis. Femoral and tibial morphology and microarchitecture were assessed using the Skyscan 1076 (E18.5, 12 days, 14 and 26 weeks of age) or 1276 (E17.5) micro-CT system (Bruker, Aartselaar, Belgium), as described previously ⁽¹⁶⁾ with the following modifications.

For micro-CT analysis at E17.5 and E18.5, embryos were scanned at 55 kV and 200 mA, and 48 kV and 208 mA, respectively. Projections were acquired over a pixel size of 5 µm and 9 µm, respectively. Image slices were then reconstructed by NRecon (Bruker, version 1.7.1.0) with beam-hardening correction of 35%, ring artifact correction of 6, smoothing of 1, and defect pixel masking of 50%. The length of mineralized bone was measured in each femur. Femoral cortical structure was analyzed at three sites, based on the extent of mineralized femur: i) 20% of the mineralized length distal to the proximal end of the mineralized region (metaphysis; Top 20%); ii) Midshaft (Mid); iii) 20% of the mineralized length proximal to the distal end of the mineralized region (Bottom 20%). Automatic adaptive thresholding was used for each sample.

Tibiae from 12 day old mice were scanned at 37 kV and 228 mA. Regions of interest (ROI) commenced at a distance equal to 30% of the tibial length down the growth plate and an ROI of 10% of the tibial length was analyzed. The lower adaptive threshold limit used for cortical analysis was equivalent to 0.58 g/mm³ Calcium hydroxyapatite (CaHA).

Femora from 14, 16 and 26 week old mice were scanned at 45 kV and 220 mA. For trabecular and cortical analyses, ROI commenced at a distance equal to 7.5% or 30%, respectively, of the total femur length proximal to the distal end of the femur; for each, an ROI of 15% of the total femur length was analyzed. For 14 week old mice, the lower adaptive threshold limits for trabecular and cortical analysis were equivalent to 0.34 g/mm³ and 0.75 g/mm³ CaHA, respectively. For 16 week old mice, the lower adaptive threshold for trabecular and cortical analysis were equivalent to 0.30 g/mm³ and 0.64 g/mm³ CaHA, respectively. For 26 week old mice, the lower adaptive threshold for

trabecular and cortical analysis were equivalent to 0.33 g/mm³ and 0.76 g/mm³ CaHA, respectively. For trabecular analysis in the 5th lumbar vertebrae (L5), an ROI of half the height of the bone (vertically centered) with a diameter 2/3 the width of the vertebral body was analyzed.

Histomorphometry

Tibiae from 14 week old mice were embedded in methylmethacrylate and sectioned at 5 μ m thickness for histomorphometric analysis, as previously described ^(17,18). The observer was blinded to genotype and sex of all samples during analysis. To determine bone formation rates, calcein was injected intraperitoneally (20 mg/kg) at 7 and 2 days before tissue collection. Sections were stained with Toluidine blue or Xylenol orange, as described ⁽¹⁹⁾. Static and dynamic histomorphometry of trabecular bone surfaces was carried out in the secondary spongiosa of the proximal tibia, commencing 370 microns distal to the growth plate, using the OsteoMeasure system (Osteometrics Inc., USA).

Three-point bending test

Mechanical properties of femora were derived from three-point bending tests using a Bose Biodynamic 5500 Test Instrument (Bose, DE, USA), as described previously ⁽²⁰⁾. The observer was blinded to genotype and sex of all samples during analysis. Once whole-bone properties were determined, tissue-level mechanical properties were calculated using micro-CT-based measurements of the mid-shaft dimensions ⁽⁸⁾.

Biochemical assays

Cross-linked C-telopeptides of type I collagen (CTX1) were measured in duplicate with the IDS RatLaps enzyme immunoassay (Abacus, Berkeley, CA, USA). Serum levels of procollagen type 1 N propeptide (P1NP) were measured in duplicate using IDS Rat/Mouse PINP EIA kit (Abacus, Berkeley, CA, USA). Both were measured in serum collected from mice fasted overnight.

To measure milk PTHrP content, amino-terminal PTHrP RIA was carried out as previously described, with a sensitivity of 2 pM ⁽²¹⁾. Milk was diluted 1:500 in assay buffer prior to measurement of PTHrP. Milk PTHrP levels were also bioassayed as the cAMP generated in response to treatment of UMR106-01 cells, using PTH(1-34)-induced cAMP response as a standard curve ⁽²²⁾. Replicate cell cultures in 24-well plates were incubated in cell culture medium with 1 mM isobutylmethylxanthine (IBMX) added. After treatment for 12 mins with 1:8 diluted milk samples, cAMP was measured by removing medium and adding acidified ethanol, drying, reconstituting in assay buffer and cAMP assay as described ⁽²³⁾. cAMP was then corrected for total protein content of the milk, measured by Pierce BCA protein assay kit (Thermo Fisher Scientific). For this, milk was diluted 1:400 in PBS and absorbance was measured at OD562nm using the Polarstar Optima+ and a bovine serum albumin standard curve.

Embryo skeletal staining

Alcian blue and Alizarin red S staining was carried out on E18.5 embryos, as described

previously ⁽²⁴⁾. Embryos were fixed in 95% ethanol for 5 days after skin removal. Remnant skin and viscera were dissected as much as possible, followed by defatting in acetone for 2 days. Thereafter, they were stained for 4 days at 40 °C in freshly prepared staining solution: 0.3% Alcian blue in 70% ethanol - 1 volume; 0.1% alizarin red S in 95% ethanol - 1 volume; glacial acetic acid - 1 volume; 70% ethanol - 17 volumes. After washing in distilled water for 2 hours, they were cleared with 2% potassium hydroxide (KOH) for 2 days. Afterwards, they were put in 20% Glycerol in 1% KOH until skeletons were clearly visible, then successively placed into 50%, 80% and 100% glycerol solutions in 1% KOH for 2 days each. Femoral length was determined by measuring the distance between femoral head and distal end through a dissecting microscope, and an average of right and left femur lengths in each embryo was calculated.

Immunohistochemistry and histology

Immunohistochemistry was carried out as described previously ^(25,26) on paraffin-embedded placenta/decidua (collected at E17.5) and the medial and lateral sides of tibial sections from 12 week old male *Dmp1Cre.Pthlh^{ff(hom)}* mice using goat rabbit anti-PTHrP (1:1000, R87, generated against PTHrP(1-14) ⁽²⁷⁾. The observer was blinded to genotype of all samples during analysis. Embryonic and adult tibial samples were decalcified in EDTA and embedded as previously described ^(17,18). Embryonic tibiae were stained with toluidine blue. Placental/decidual samples were fixed overnight in 4% paraformaldehyde at 4°C, stored in 70% ethanol, and embedded in paraffin wax until further analysis. Sections (5µm) were taken onto Superfrost Plus slides, dewaxed in Histoclear (National Diagnostics, Atlanta, GA), and rehydrated in graded ethanols. Endogenous peroxidase was blocked for 30 min in 2% H₂O₂ in methanol. After rinsing with 0.05 M phosphate buffered saline (PBS), samples were blocked with TNB (Renaissance TSA indirect (Tyramide Signal Amplification) PerkinElmer Life Sciences cat no- NEL700) for 60 min. PTHrP antibody (made against PTHrP (1-14) in house R87) 1:1000 or rabbit IgG (negative control) was applied for 2 hr at room temperature in a humid chamber. A secondary antibody (swine anti-rabbit, Dako) was applied for 30 min at 1:300, followed by streptavidin horseradish peroxidase (Dako) 1:300 in the same blocking solution for 30 min. PTHrP staining was visualized with diaminobenzidine kit (Dako) and counterstained with Mayer's hematoxylin. Samples were rinsed in PBS between each step. PTHrP-DAB positive regions within the decidua and spongiotrophoblast zones were quantified with MetaMorph® image analysis software (Molecular Devices, San Jose, CA). The decidua and spongiotrophoblast zones were manually defined. Color thresholding was applied and compared to negative controls. Integrated morphometry analysis was used to quantify DAB intensity, and frequency parameters.

Statistical analysis

Statistically significant differences were determined by one-way or two-way ANOVA followed by Sidak's post-hoc test or Fishers LSD posthoc test (uncorrected), as indicated in all figure and table legends. Student's t-test was used where only one comparison was being made. To analyze PTHrP positive areas in decidua/placentae, area under the curves

were compared by Student's t-test. p values are shown where $p < 0.1$; $p < 0.05$ was considered statistically significant.

Results

Adult male *Dmp1Cre.Pthlh^{ff(hom)}* mice have a high trabecular bone mass set-point, reached before 14 weeks of age

We first confirmed that adult *Dmp1Cre.Pthlh^{ff(hom)}* mice (i.e. mice bred from parents expressing *Dmp1Cre* and homozygous for the *Pthlh^{ff}* genotype, Figure 1A) exhibited the same pattern of gene recombination as in our earlier study of *Dmp1Cre.Pthlh^{ff(het)}* mice ⁽⁸⁾. We observed *Pthlh* gene recombination in bone (femur/tibia, and calvaria), mammary glands, brain, and muscle (Figure 1B). No recombination was detected in uterus, heart, liver or kidney. When assessed by immunohistochemistry, the proportion of PTHrP positive osteocytes was significantly reduced by approximately 20% (Figure 1C). This pattern of recombination, and level of knockdown, accorded with that previously described in *Dmp1Cre.Pthlh^{ff(het)}* mice ⁽⁸⁾.

In contrast to our previous experiments showing osteopenia in 12 week old *Dmp1Cre.Pthlh^{ff(het)}* mice ⁽⁸⁾, adult male *Dmp1Cre.Pthlh^{ff(hom)}* mice had greater trabecular bone volume than age- and sex-matched controls (Figure 1D-H). At 14, 16 and 26 weeks of age, male *Dmp1Cre.Pthlh^{ff(hom)}* mice had significantly higher trabecular bone volume (Figure 1D) and trabecular number (Figure 1E) than age- and sex-matched controls. This phenotype was stable; the proportional difference in trabecular bone volume and number between genotypes was the same at all three time points assessed: i.e. trabecular bone volume and number were at a constant ~140% and ~133% of sex-matched male controls. Trabecular thickness was slightly higher in 14 week old *Dmp1Cre.Pthlh^{ff(hom)}* mice compared to controls, but remained unchanged in 16- and 26 week old *Dmp1Cre.Pthlh^{ff(hom)}* mice compared to sex- and age-matched controls (Figure 1F). Trabecular separation was ~18% lower than controls in male *Dmp1Cre.Pthlh^{ff(hom)}* mice, and was statistically significant only at the age of 14 weeks (Figure 1G).

When representative images were generated to show this difference in trabecular bone mass (Figure 1I), we noted that male *Dmp1Cre.Pthlh^{ff(hom)}* mice also had wider bones; when measured in the trabecular region of analysis; metaphyseal periosteal perimeter was significantly higher in male *Dmp1Cre.Pthlh^{ff(hom)}* mice at all three time points (Figure 1H), confirming the wider bone phenotype of these mice. When *Dmp1Cre.Pthlh^{ff(het)}* mice were bred and aged to 16 and 26 weeks, no significant difference in trabecular structure or metaphyseal bone perimeter was detected at either age between *Dmp1Cre.Pthlh^{ff(het)}* mice and their controls (Supplemental Figure 1). These indicate that male *Dmp1Cre.Pthlh^{ff(hom)}* mice have a different phenotype to *Dmp1Cre.Pthlh^{ff(het)}* mice ⁽⁸⁾, although they have the same genotype, showing that parental genotype influences trabecular bone mass and bone radial growth.

The high trabecular bone mass in *Dmp1Cre.Pthlh^{f/f(hom)}* mice was sex-specific. Female *Dmp1Cre.Pthlh^{f/f(hom)}* mice showed no significant difference in trabecular bone volume, trabecular number, or metaphyseal periosteal perimeter compared to *Dmp1Cre^(hom)* controls (Figure 1D-H). They did have lower trabecular separation at 16 weeks (Figure 1G), implying a mild and transient elevation in trabecular bone mass.

To confirm this trabecular phenotype in homozygous-bred mice, trabecular bone structure was studied at a second anatomical region, 5th lumbar (L5) vertebrae. Similar to long bones, vertebrae of 14 week old male *Dmp1Cre.Pthlh^{f/f(hom)}* had higher trabecular bone volume and trabecular number than controls (Table 1). Trabecular separation was lower in male *Dmp1Cre.Pthlh^{f/f(hom)}* mice than controls, and there was no significant difference in trabecular thickness (Table 1). This confirmed the trabecular phenotype was not restricted to a single anatomical location.

Although trabecular bone mass was greater in male 14 week old *Dmp1Cre.Pthlh^{f/f(hom)}* mice, dynamic histomorphometry revealed no difference in any bone formation or resorption parameters compared to controls (Table 2). In addition, no significant difference was detected in serum levels of the bone formation or resorption markers, P1NP and CTX1, of 14 week old male *Dmp1Cre.Pthlh^{f/f(hom)}* mice compared to controls (Table 2). This, and the similar proportion of elevation in trabecular bone mass at 14, 16 and 26 weeks suggest that the high trabecular bone mass arose before 14 weeks of age, and has reached a greater adult set-point for “peak bone mass” than controls.

Adult male *Dmp1Cre.Pthlh^{f/f(hom)}* mice have a wide bone phenotype that leads to greater bone strength

Since metaphyseal bone width was greater in adult male *Dmp1Cre.Pthlh^{f/f(hom)}* mice than controls, we analyzed femoral cortical bone structure in more detail (Figure 2A). Although femoral length was not different between *Dmp1Cre.Pthlh^{f/f(hom)}* mice and *Dmp1Cre^(hom)* controls (Figure 2B), adult male *Dmp1Cre.Pthlh^{f/f(hom)}* femora were wider in both anteroposterior and mediolateral dimensions compared to controls at 14, 16 and 26 weeks (Figure 2C,D). The greater femoral width in male *Dmp1Cre.Pthlh^{f/f(hom)}* mice was in proportion: the ratio of anteroposterior to mediolateral widths was not different in these mice compared to controls at any time point (data not shown). Adult male *Dmp1Cre.Pthlh^{f/f(hom)}* mice exhibited greater marrow and cortical area, and greater periosteal and endocortical perimeters, compared to age- and sex-matched controls (Figure 2E-I). Although cortical diameter was greater, this was balanced on the endocortical and periosteal surfaces, as there was no significant difference in cortical thickness (Figure 2G). As in trabecular bone, the greater cortical bone width phenotype was stable, showing a similar proportional difference compared to controls at all three time points.

Female *Dmp1Cre.Pthlh^{ff(hom)}* mice showed no significant difference in cortical bone size or shape compared to age-matched *Dmp1Cre^(hom)* mice at 16 or 26 weeks of age (Figure 2).

Heterozygous-bred female *Dmp1Cre.Pthlh^{ff(het)}* mice at 16 and 26 weeks of age did not exhibit any significant changes in cortical dimensions compared to *Dmp1Cre^(het)* controls (Supplemental Figure 2A-I), nor in marrow area (Supplemental Figure 2E). At 16 weeks, male mice showed a small (<10%) transient elevation in anteroposterior width, cortical area, cortical thickness, endocortical perimeter and periosteal circumference, but no change in mediolateral width; this was no longer detected at 26 weeks (Supplemental Figure 2A-I).

Since greater bone width can be associated with greater bone strength if the material properties are maintained, we carried out three-point bending tests. Femora from 26 week old male *Dmp1Cre.Pthlh^{ff(hom)}* mice could withstand higher loads than age-matched *Dmp1Cre^(hom)* controls, reaching a higher ultimate force (Figure 3A,B) and failure force (Table 3) before breaking. There was no significant difference in ultimate displacement (Figure 3C). When corrected for bone size, both 14- and 26-week-old male *Dmp1Cre.Pthlh^{ff(hom)}* femora showed lower ultimate stress and yield stress, compared to controls (Figure 3D-F), suggesting a material defect. 14 week old male *Dmp1Cre.Pthlh^{ff(hom)}* femora had higher ultimate and failure strain than controls (Table 3). 26 week old male *Dmp1Cre.Pthlh^{ff(hom)}* femora had lower failure stress (Figure 3G), and reduced toughness and elastic modulus (Table 3) compared to sex- and age-matched controls. These changes reflect differences in the material properties of the cortex that increase compliance. It suggests that the increase in geometry is an adaptive mechanism to maintain structural properties.

While ultimate force, failure force, ultimate stress, yield stress, failure stress and toughness were modified in the male mice, these parameters were not changed in females (Figure 3 and Table 3). Surprisingly, femora from female *Dmp1Cre.Pthlh^{ff(hom)}* mice achieved a greater yield force and displacement compared to age- and sex-matched controls (Table 3), suggesting a higher elastic deformation. When corrected for bone size, 26 week old female *Dmp1Cre.Pthlh^{ff(hom)}* femora had higher yield strain and lower elastic modulus (Table 3), suggesting, like in the male bone, a more flexible material than controls.

Femora from 26 week old heterozygous-bred *Dmp1Cre.Pthlh^{ff(het)}* mice did not show any significant difference in mechanical properties compared to *Dmp1Cre^(het)* controls (Supplemental Figure 3).

The wide bone phenotype is present at 12 days of age in male and female *Dmp1Cre.Pthlh^{ff(hom)}* mice

Since we previously observed recombination of PTHrP in the mammary glands of *Dmp1Cre.Pthlh^{f/f(het)}* mice ⁽⁸⁾, and deletion of maternal mammary PTHrP is reported to lead to greater bone mass in progeny at 12 days of age ⁽²⁸⁾, we sought to determine whether bone mass was modified in 12 day old *Dmp1Cre.Pthlh^{f/f(hom)}* mice, and whether maternal milk PTHrP content was reduced. 12 day old mice were assessed from both heterozygous (*Dmp1Cre.Pthlh^{f/f(het)}*) (Figure 4A) and homozygous (*Dmp1Cre.Pthlh^{f/f(hom)}*) (Figure 1A) breeding strategies.

While no significant differences in cortical dimensions were detected in tibiae from male or female *Dmp1Cre.Pthlh^{f/f(het)}* mice (from heterozygous breeders), *Dmp1Cre.Pthlh^{f/f(hom)}* mice (from homozygous breeders) exhibited greater tibial width at 12 days of age (Figure 4B-I) with no difference in tibial length (Figure 4C). Both male and female *Dmp1Cre.Pthlh^{f/f(hom)}* mice had significantly greater tibial width in the anteroposterior and mediolateral direction, compared to sex-matched *Dmp1Cre^(hom)* (Figure 4B,D,E). Male and female *Dmp1Cre.Pthlh^{f/f(hom)}* mice also showed greater tibial marrow area (Figure 4G), cortical area (Figure 4H), and periosteal perimeter (Figure 4I) compared to sex-matched cousin-bred *Dmp1Cre^(hom)* controls. Female *Dmp1Cre.Pthlh^{f/f(hom)}* also had greater cortical thickness than female *Dmp1Cre^(hom)* controls (Figure 4F), although it should be noted that sample numbers were low.

Although PTHrP gene recombination was detected in mammary tissue from *Dmp1Cre.Pthlh^{f/f(het)}* mice ⁽⁸⁾, milk PTHrP levels, measured either by radioimmunoassay or bioassay, and milk protein levels were not significantly altered in *Dmp1Cre.Pthlh^{f/f(het)}* mice compared to controls (Table 4).

The *Dmp1Cre.Pthlh^{f/f(hom)}* wide-bone phenotype exists *in utero*

Since no change in milk PTHrP could explain the phenotype at 12 days of age, we determined whether the phenotype existed *in utero* by assessing embryonic bone size. Consistent with our observations at 12 days, embryonic *Dmp1Cre.Pthlh^{f/f(hom)}* femora (E18.5) were wider in both anteroposterior and mediolateral dimensions, and exhibited a higher moment of inertia compared to *Dmp1Cre^(hom)* controls (Figure 5A, E-J). Greater bone area and tissue area of *Dmp1Cre.Pthlh^{f/f(hom)}* embryos confirmed that their femora are wider than controls (Supplemental Figure 4). This greater bone width was observed along the full length of the bone, including both metaphysis and the diaphysis.

The length of the mineralized portion of *Dmp1Cre.Pthlh^{f/f(hom)}* femora, measured by micro-CT, was also greater than *Dmp1Cre^(hom)* controls (Figure 5B). Micro-computed tomography cannot detect the full length of the bone at this age, as the cartilage ends are not yet mineralized prior to birth. Total femoral length measured in Alizarin Red/Alcian Blue stained samples showed no difference between *Dmp1Cre.Pthlh^{f/f(hom)}* and *Dmp1Cre^(hom)* controls (Figure 5C,D), suggesting that *Dmp1Cre.Pthlh^{f/f(hom)}* embryos have normal bone length, but accelerated mineralization *in utero*. Indeed, this was also

indicated by shortened proliferating and hypertrophic zones in sections of the proximal tibiae (Figure 5K).

Consistent with a lack of change in overall bone growth, there were no significant differences in body weight, placental weight, or body to placental weight ratios between *Dmp1Cre.Pthlh^{f/f(hom)}* embryos and *Dmp1Cre^(hom)* controls (Supplemental Figure 6A-C). This suggests the increased bone width of *Dmp1Cre.Pthlh^{f/f(hom)}* embryos is not caused by changes in placental efficiency.

Heterozygous-bred *Dmp1Cre.Pthlh^{f/f(het)}* embryos did not show any significant difference in the mineralized length compared to *Dmp1Cre^(het)* controls (Supplemental Figure 5A), nor in the bone width or structure (Supplemental Figure 5B-K). No alteration in weight was observed in *Dmp1Cre.Pthlh^{f/f(het)}* embryos, although *Dmp1Cre.Pthlh^{f/f(het)}* placentae were significantly smaller than littermate *Dmp1Cre.Pthlh^{w/w(het)}* (Supplemental Figure 6D-F).

Low PTHrP levels and modified cell morphology in *Dmp1Cre.Pthlh^{f/f}* decidua

Since we observed increased bone width *in utero* in *Dmp1Cre.Pthlh^{f/f(hom)}* mice, and PTHrP is produced by uterus and decidua (29-32), we sought to determine whether PTHrP expression is modified in placenta or decidua from *Dmp1Cre.Pthlh^{f/f(hom)}* mice.

We tested *Pthlh* recombination in decidua (which is maternal-derived) and placenta (which is embryo-derived) collected from *Dmp1Cre.Pthlh^{f/f(hom)}* embryos (from *Dmp1Cre.Pthlh^{f/f}* dams), *Dmp1Cre.Pthlh^{f/f(het)}* embryos (from *Dmp1Cre.Pthlh^{f/w}* dams), and *Dmp1Cre^(hom)* controls (from *Dmp1Cre* dams) (Figure 6A). Genomic excision was observed in placenta from *Dmp1Cre.Pthlh^{f/f(hom)}* embryos and in the adjacent decidua from their dams (*Dmp1Cre.Pthlh^{f/f}*). In *Dmp1Cre.Pthlh^{f/f(het)}* embryos, we also observed recombination in placenta, and a lower degree of *Pthlh* recombination in decidua from their *Dmp1Cre.Pthlh^{f/w}* dams. These results showed that *Pthlh* DNA recombination occurred in placenta of *Dmp1Cre.Pthlh^{f/f}* embryos (from both *Dmp1Cre.Pthlh^{f/w}* and *Dmp1Cre.Pthlh^{f/f}* parents). The recombination also occurs in decidua of *Dmp1Cre.Pthlh^{f/f}* parents and at lower levels in *Dmp1Cre.Pthlh^{f/w}* mice. The low level of recombination in *Dmp1Cre.Pthlh^{f/w}* mice may explain the wider bones observed in 12 day old *Dmp1Cre^(het)* mice compared with 12 day old *Dmp1Cre^(hom)* mice, since *Dmp1Cre^(het)* mice would have undergone embryonic development in a *Dmp1Cre.Pthlh^{f/w}* maternal environment.

We then sought to localize PTHrP protein and quantify the level of knockdown in placenta and adjacent decidua from *Dmp1Cre.Pthlh^{f/f(hom)}* and *Dmp1Cre.Pthlh^{f/f(het)}* embryos. In embryos from homozygous dams, PTHrP staining of decidua and placenta showed positive staining for PTHrP in both the decidua and the spongiotrophoblast layer (junctional zone) of the placenta (Figure 6B). No PTHrP was detected in the placental

labyrinth zone of control or *Dmp1Cre.Pthlh^{f/f(hom)}* embryos. PTHrP staining was notably less in decidua from mothers of *Dmp1Cre.Pthlh^{f/f(hom)}* mice compared with control decidua (from mothers of *Dmp1Cre^(hom)* mice) (Figure 6C,D), and this was significant when quantified. There was no difference in PTHrP staining in the spongiotrophoblast zone of the adjacent placenta (Figure 6D).

We also examined the morphology of decidua from samples adjacent to *Dmp1Cre.Pthlh^{f/f(hom)}* placenta. The decidual cells from mothers of *Dmp1Cre.Pthlh^{f/f(hom)}* embryos had less hypertrophic, vacuolated cells than *Dmp1Cre^(hom)* decidua, and exhibited a smoother, more compact cobblestone appearance (Figure 6E,F). Total decidual area was significantly less in samples from mothers of *Dmp1Cre.Pthlh^{f/f(hom)}* embryos than *Dmp1Cre^(hom)* (Figure 6G), but the area of the spongiotrophoblast zone was not significantly modified (Figure 6G). This suggests PTHrP may act locally within the decidua to promote decidual cell maturation, and this may cause increased bone width growth of *Dmp1Cre.Pthlh^{f/f(hom)}* mice *in utero*.

The reduction in decidual size, and reduced PTHrP staining were both specific to *Dmp1Cre.Pthlh^{f/f(hom)}* samples. PTHrP staining levels were not altered in decidua adjacent to *Dmp1Cre.Pthlh^{f/f(het)}* placenta collected from *Dmp1Cre.Pthlh^{f/w}* mothers, compared to staining in decidua adjacent to littermate *Dmp1Cre.Pthlh^{w/w(het)}* placenta (Figure 6H). There was also no change in decidual size in decidua adjacent to *Dmp1Cre.Pthlh^{f/f(het)}* placenta compared to littermate *Dmp1Cre.Pthlh^{w/w(het)}* controls (Figure 6I).

Discussion

This study identifies a potential effect of maternal PTHrP on progeny bone mass, shape and strength. *Dmp1Cre*-mediated recombination and reduced PTHrP was observed in decidua, and adjacent developing embryos exhibited increased embryonic bone radial growth and mineralization. This wide bone phenotype was observed in both male and female mice at 12 days of age, and was sustained until at least 6 months of age in male mice (Figure 7). These effects on bone size, trabecular bone mass, and bone strength dominate over the previously reported effects of the *Dmp1Cre*-targeted reduction of endogenous PTHrP in osteocytes to suppress bone formation and reduce trabecular bone mass of young adult mice ⁽⁸⁾. This indicates an additional role for PTHrP in maternal physiology and skeletal development.

The greater bone width along the full bone length in *Dmp1Cre.Pthlh^{f/f(hom)}* embryos and adult male *Dmp1Cre.Pthlh^{f/f(hom)}* mice suggests that the maternal influence on bone widening determines radial expansion of the embryonic cartilage anlagen, the early cartilage model of the developing bone, rather than inducing periosteal apposition at the diaphysis. Maternally-derived PTHrP might limit radial expansion of chondrocytes during cartilage anlage development, and may suppress signaling pathways that promote radial expansion of chondrocytes at growth plate. This contrasts with actions of embryo-derived PTHrP from the placenta ⁽⁶⁾ and cartilage ^(33,34) to stimulate longitudinal bone growth. Although longitudinal bone growth has been studied widely, very little is known about signaling pathways orchestrating radial growth. There are two non-mutually-exclusive theories describing how bone radial width is determined: the “mechanostat” theory suggests that bone size and shape are adapt to mechanical strain ^(35,36), while the “sizostat” theory suggests a set of genes regulates bone width to reach a pre-programmed setting ⁽³⁷⁾. Although different genomic markers have been correlated with bone size and bone shape ⁽³⁸⁾, no specific genes or molecular pathways have yet been described as major determinants of cortical bone diameter. Our data suggests that decidual PTHrP is a determinant for the cortical width sizostat.

These data are also consistent Frost’s mechanostat model (33, 34) which could explain most, if not all, of the observed skeletal phenotype, including the embryonic bone and the trabecular compartment. The concept is that skeletal adaptation is driven by strain. When tissue strains increase above a threshold, they stimulate adaptive modelling to increase mass or change geometry to reduce local tissue strains. We observed reduced failure stress, and elastic modulus in the *Dmp1Cre.Pthlh^{f/f(hom)}* mice, which increased yield and ultimate strain. That is, the bone material is more compliant, allowing threshold strains to be achieved at lower magnitudes of applied force. We propose that this stimulates an adaptive response to increase bone size, maintaining structural properties (e.g. breaking force and stiffness). While we do not have mechanical properties for the embryonic bone, cartilage also responds to mechanical loads ⁽³⁹⁾. Altered material properties in earlier development would be consistent with increased radial growth and accelerated mineralization to reduce deformation of the cartilaginous anlage. Increased trabecular

mass also reflects mechanical adaptation to a more compliant material.

Although both male and female *Dmp1Cre.Pthlh^{ff(hom)}* mice had wider long bones at 12 days of age, this phenotype was retained through to adulthood only in males, suggesting that the mechanisms controlling continued radial growth and bone width are sex-dependent. Although placental nutrition has sexually dimorphic effects on embryo growth⁽⁴⁰⁾, we did not observe sex differences in this study until after 12 days, again emphasizing that the wide bone phenotype is unlikely to relate to placental nutrition. Post-pubertal sex differences in cortical diameter are common to all mammals⁽⁴⁰⁻⁴⁹⁾, with females having narrower bones than males, however the molecular mechanisms driving this sexual dimorphism remain largely unknown; this mouse model may therefore shed new light on the mechanisms that contribute to this sexual dimorphism. The retention of this phenotype in males, but not females, suggests that hormonal changes at puberty in females may slow their radial growth. While most studies investigating sexual dimorphism in murine bone width have focused on periosteal growth at the diaphysis, our results suggest that differences between males and females in cortical width might also arise from radial expansion of the growth plate. Estradiol is known to slow longitudinal growth: a previous study has shown that ovariectomy increased tibial length and increased chondrocyte proliferation⁽⁵⁰⁾, and 17beta-estradiol treatment of 26-day-old female and male rats led to shorter tibial length and an early reduction in growth plate longitudinal width⁽⁵⁰⁾. Testosterone also affects chondrocytes: local injection of testosterone into the tibial epiphyseal growth plate of castrated growing male rats significantly increased epiphyseal growth plate length⁽⁵¹⁾. Furthermore, while the perinatal testosterone surge is required for adult bone length, bone width is determined by post-pubertal testosterone⁽¹⁸⁾. The cellular and intracellular pathways by which estradiol and/or testosterone differentially affect growth plate radial growth in control and *Dmp1Cre.Pthlh^{ff(hom)}* mice remains to be investigated.

In *Dmp1Cre.Pthlh^{ff(hom)}* mice, greater cortical width was not associated with greater total bone length. To our knowledge, this is the first evidence of changes in bone diameter independent of cortical thickness, longitudinal growth, and total body weight gain. We have recently reviewed the literature on this topic⁽⁵²⁾, and noted that all previously reported mouse models with developmental changes in bone diameter also showed other skeletal defects such as reduced bone length, or altered cortical thickness^(18,53-55). For example, mice lacking the endogenous nuclear localization sequence and C-terminus of PTHrP displayed retarded growth with lower body weight and total skeletal size at the age of 2 weeks⁽⁵⁴⁾, while Insulin-like growth factor I null (*Igf1^{-/-}*) mice displayed smaller body size, shortened femoral length and reduced cortical thickness compared to wild type littermates^(55,56). Having altered radial, but not longitudinal, growth makes the *Dmp1Cre.Pthlh^{ff(hom)}* mouse an appealing model for studying specific mechanisms underlying radial bone growth.

Although we detected PTHrP recombination in the mammary glands⁽⁸⁾, milk PTHrP

levels were not significantly modified, and the wide-bone phenotype predated the commencement of suckling, indicating that a change in mammary supply of PTHrP is unlikely to cause the wide-bone phenotype we observed. We had thought that this may have been a possibility since we previously noted *Dmp1Cre*-driven PTHrP recombination in the mammary gland⁽⁸⁾, and suckling pups from mice lacking PTHrP in the milk supply (*BLG-Cre/PTHrP^{lox/-}*) had higher ash calcium content, indicating greater bone mass, compared to controls at day 12 of lactation⁽²⁸⁾. The normal levels of PTHrP in milk in mothers of *Dmp1Cre.Pthlh^{f/f(hom)}* mice suggests that the mammary cells expressing *Dmp1Cre* are not the same cells (mammary epithelial cells) that secrete PTHrP to the milk⁽⁵⁷⁾, and are different to those targeted by the *BLG-Cre/PTHrP^{lox/-}* model^(28,58). Another possibility is that the level of PTHrP recombination was too low in mammary tissues to modify milk PTHrP production.

The role of decidual PTHrP has not previously been investigated, although it was shown in the 1990s that uterine PTHrP is required for viable embryogenesis⁽³²⁾. PTHrP is produced within the decidua, myometrium, amnion, and chorion, where it dilates the uterine and placental vasculature and inhibits myometrial contractions⁽⁵⁹⁻⁶¹⁾. Here we showed PTHrP is expressed by both the decidual layer and spongiotrophoblast layer (or junctional zone), but not the labyrinth zone. *Dmp1Cre*-directed *Pthlh* recombination was detected in decidua from *Dmp1Cre.Pthlh^{f/f}* (mothers of *Dmp1Cre.Pthlh^{f/f(hom)}* mice) and at lower levels in *Dmp1Cre.Pthlh^{f/w}* (mothers of *Dmp1Cre.Pthlh^{f/f(het)}* mice) mothers. Although no changes in PTHrP protein levels were detected in decidua or placenta of *Dmp1Cre.Pthlh^{f/f(het)}* mice, decidua from mothers of *Dmp1Cre.Pthlh^{f/f(hom)}* mice had significantly less PTHrP staining. Decidua from mothers of *Dmp1Cre.Pthlh^{f/f(hom)}* mice were also smaller in size and decidual cells showed less vacuolation and hypertrophy. The smaller decidua and reduced size of decidual cells in mothers of *Dmp1Cre.Pthlh^{f/f(hom)}* mice was a surprising finding in this study. This may reflect reduced decidual cell differentiation, since decidual cells become enlarged and vacuolated during decidualization^(62,63). However, since our observations are made at day E18.5, well after decidualization, it may reflect decidual cell atrophy in mothers of *Dmp1Cre.Pthlh^{f/f(hom)}* mice. Previous studies reported that PTHrP repressed decidualization of human uterine fibroblast cells⁽³¹⁾, that rat intrauterine injection of PTH/PTHrP receptor antagonist increased decidualization⁽³²⁾, and *Pthlh* mRNA levels are downregulated during decidualization in primary endometrial stromal cells⁽⁶⁴⁾, we suggest the reduced size of decidual cells at day E17.5 is more likely to reflect early commencement of atrophy. How this might drive the increased radial growth and mineralization in the *Dmp1Cre.Pthlh^{f/f(hom)}* embryo remains to be established.

This is, to our knowledge, the first report that *Dmp1-Cre* targets decidua, which is a transient uterine tissue. Although the *Dmp1-Cre* mouse is widely used as an osteocyte and late-osteoblast conditional knockout mouse, multiple additional tissues are now known to be targeted. Use of *Dmp1-Cre*-driven reporters showed expression in kidney, brain, skeletal muscle, and some bone marrow cells⁽⁶⁵⁾, and we recently reported *Dmp1-Cre*

driven recombination of *Pthlh* in the mammary gland, muscle and brain ⁽⁸⁾, but *Dmp1-Cre* recombination of *Pthlh* did not occur in the non-pregnant uterus ⁽⁸⁾. The expression of *Dmp1-Cre* in decidua has major implications for the design of experimental breeding, and for reporting the breeding strategy used in experiments utilizing *Dmp1Cre* for gene deletion. However, this clearly depends on the function of the targeted gene. For example, although gp130, and its inhibitor protein SOCS3, are expressed in murine decidua ^(66,67), studies using homozygous-bred *Dmp1Cre.gp130^{ff}* mice ⁽¹⁰⁾ and *Dmp1Cre.Socs3^{ff}* mice ^(11,68) showed no significant change in their phenotypes compared to heterozygous-bred mice of the same genotype ^(11,69).

The influence of maternal PTHrP in restricting bone width contrasts with the role of endogenous PTHrP from the embryo-derived portion of the placenta, which promotes placental calcium transport and skeletal development, as indicated by reduced bone length in *Pthlh* null embryos ⁽⁶⁾. Although decidua and placenta provide nutrition to promote embryonic and placental weight gain *in utero* ⁽⁷⁰⁾, and these effects influence adult health, including bone mass ⁽⁷¹⁾, there was no change in total embryo or placental weight in *Dmp1Cre.Pthlh^{ff(hom)}* embryos. This suggests maternal contributors to general embryonic nutrition, such as uteroplacental blood flow and nutrient transport across the placenta controlled by (for example) growth hormone, IGF-I, insulin, and glucocorticoids, are unlikely to contribute to the phenotype we observed. The influence of decidual PTHrP on growth also appears to be specific to the skeleton. The alteration in the morphological features of decidua might be associated with changes in its function, resulting in changes in skeletal development. Whether decidual PTHrP, like embryonic placental PTHrP regulates placental calcium transport or fetal PTH levels ^(72,73) or other local regulators of bone development, which are known to regulate fetal bone development ⁽⁷⁴⁾, or it acts systematically to regulate bone radial growth remains unknown.

Unfortunately, with the current tools available, it is not possible to carry out decidua-specific deletion of PTHrP to provide a clear answer to this question. The Cre mouse typically used to drive gene deletion in the decidua is a progesterone receptor Cre (*Pgr-Cre*). This induces recombination in decidua because its main target tissue is the uterus ⁽⁷⁵⁾. However, earlier work showed that use of a PTHrP antagonist in the uterus prevents embryo implantation ⁽³²⁾. This would make it impossible to assess the bone phenotype of embryos derived from these mothers as they would be resorbed well before osteogenesis. *Pgr-Cre* is also expressed in ovary, oviduct, and pituitary gland which may also have an impact, ⁽⁷⁵⁾. Unfortunately, a second *Pgr-Cre* model has been reported to be expressed in osteoblasts ⁽⁷⁶⁾, which would further complicate interpretation in this context. Finally, it is important to note that the decidua is only partially derived from uterine tissue; it also contains a high proportion of bone-marrow derived cells which differentiate into both haemopoietic and non-haemopoietic decidual cells ⁽⁷⁷⁻⁷⁹⁾. There are no Cre mice available to specifically target these populations within the decidua.

The decidua contains a wide range of cell types. This includes decidual parenchymal

(stromal) cells, macrophages, dendritic cells, specialized NK cells, T cells, and innate lymphoid cells. Their function in placentation and embryo implantation have been the main focus of study, but many of the cytokines produced by these cells could influence embryonic bone development. Some examples include cytokines like angiopoietin-1 and 2, VEGFA, TGF β , IL-6, TNF α , IL-8 and chemokines such as CXCL1, CXCL9, CXCL10, and CCL5, which each have been reported to regulate vascularization and / or chondrocyte differentiation⁽⁸⁰⁻⁸⁵⁾.

PTHrP is produced by multiple organs and cell types and has a wide range of disparate functions ⁽⁵⁾. The phenotype we observe here appears to override the effect we previously described of low trabecular bone mass with PTHrP knockdown in osteocytes⁽⁸⁾. This is surprising, since the progeny described here, as would be expected, also exhibited PTHrP knockdown in osteocytes, but did not exhibit low trabecular bone mass. Indeed, in this study the heterozygous-bred PTHrP knockdown mice, which were only two weeks older than in our original study, did not exhibit low trabecular bone mass, suggesting that the earlier phenotype was both more mild, and transient. We suggest that developmental changes in bone width have a more significant, and longer-lasting effect on bone structure than the effect of PTHrP in osteoblasts and osteocytes. Greater bone width during development may also predispose the mice towards developing a greater trabecular bone mass. This is similar to the emergence of sex differences in the skeleton: male mice have wider bones and greater trabecular bone mass than females. A relationship between trabecular and cortical bone mass has previously been noted in human cortical development, where girls and women with fewer and thinner trabeculae have smaller cortices⁽⁸⁶⁾.

In conclusion, *Dmp1Cre* leads to recombination in maternal decidua, and reduced PTHrP levels in decidua may limit trabecular bone mass, bone geometry and strength, not only of neonatal progeny, but also of adult male progeny. *Dmp1Cre.Pthlh^{ff(hom)}* embryos had accelerated skeletal development, with more mineralized and wider femora at E18.5. Although this effect was observed in both males and females in neonates, it was retained through to adulthood only in male mice. This indicates that maternal PTHrP may limit bone growth, in a way that has a life-long influence on bone mass, shape and strength in male progeny.

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Figure Legends:

Figure 1: Breeding strategy, PTHrP recombination and high trabecular bone mass in 14, 16 and 26 week old male *Dmp1Cre.Pthlh^{f/f(hom)}* mice. **A:** Schematic showing breeding strategy and data collection points. **B:** PTHrP recombination in tissues from 6 week old *Dmp1Cre.Pthlh^{f/f(hom)}* mice. Upper band indicates wild type *Pthlh* allele, while lower band indicates a recombined *Pthlh* allele. **C:** PTHrP knockdown in osteocytes in tibiae from 14 week old male *Dmp1Cre.Pthlh^{f/f(hom)}* mice. **D-H:** Trabecular structure of *Dmp1Cre.Pthlh^{f/f(hom)}* distal femoral primary spongiosa analyzed by micro-CT in male mice (m) at 14, 16 and 26 weeks of age, and in female mice (f) at 16 and 26 weeks of age. Trabecular bone volume, trabecular number, trabecular thickness, trabecular separation, and metaphyseal periosteal perimeter are shown as mean (dot), interquartile range (box), median (line) and range; n=9-10/group; p values shown where p<0.1 for comparisons with sex- and age-matched *Dmp1Cre^(hom)* by two-way ANOVA (16 and 26 weeks old) and Student's t-test (14 weeks old). **I:** Representative micro-CT images of trabecular bone in the distal femoral primary spongiosa of 26-week old male mice, showing density (scale above images), and raw cross-sectional images of the metaphysis (i), metaphyseal diaphysis (ii) and diaphysis (iii), showing a difference in bone size, and projection of trabecular bone into the lower metaphysis in *Dmp1Cre.Pthlh^{f/f(hom)}* samples.

Figure 2: Wide bone phenotype of adult male *Dmp1Cre.Pthlh^{f/f(hom)}* mice. **A:** Schematic showing measurement regions. Bone length (**B**) and cortical dimensions of male (m) *Dmp1Cre.Pthlh^{f/f(hom)}* and *Dmp1Cre^(hom)* mice at 14, 16 and 26 weeks of age, and female mice (f) at 16 and 26 weeks of age. Anteroposterior (**C**) and mediolateral (**D**) width, measured by micro-CT at the midshaft. **E-I** Femoral marrow area (**E**), cortical bone area (**F**), thickness (**G**), and both endocortical (**H**) and periosteal (**I**) perimeter were analyzed in cortical region of interest by micro-CT. Data are shown as mean (dot), interquartile range (box), median (line) and range; p values shown where p<0.1 for comparisons with sex- and age-matched *Dmp1Cre^(hom)* by two-way ANOVA (16 and 26 weeks old) and Student's t-test (14 weeks old).

Figure 3: Greater ultimate strength in 26-week old and less stress in 14 and 26-week old male *Dmp1Cre.Pthlh^{f/f(hom)}* femora measured by three-point bending tests. **A:** Representative load-deformation curves of 26-week old male *Dmp1Cre.Pthlh^{f/f(hom)}* and *Dmp1Cre^(hom)* bones. Greater ultimate force (**B**) and normal ultimate deformation (**C**) in femora from 26-week old males; no change in 14 week old males and 26 week old females. **D:** Representative stress-strain curves of 26-week old male *Dmp1Cre.Pthlh^{f/f(hom)}* and *Dmp1Cre^(hom)* bones, after correction in each sample based on anteroposterior and mediolateral dimensions (shown in Figure 2). Also shown are ultimate stress (**E**), yield stress (**F**), and failure stress (**G**). Data shown as mean (dot), interquartile range (box), median (line) and range, n=9-10/group. p values shown where p<0.1 for comparisons with compared to age- and sex-matched controls by two-way ANOVA (16 and 26 weeks old) and Student's t-test (14 weeks old).

Figure 4: Wide bone phenotype of 12 day old male and female *Dmp1Cre.Pthlh^{f/f(hom)}* mice. **A:** Schematic diagram showing heterozygous breeding strategy. **B:** Representative micro-CT images of cortical bone of 12 day-old *Dmp1Cre.Pthlh^{f/f(hom)}* tibiae. **C-I:** Tibial cortical structure of 12 day old *Dmp1Cre.Pthlh^{f/f}* mice from heterozygous (het) and homozygous (hom) breeders compared to their respective *Dmp1Cre* controls. Shown are tibial length (**C**), anteroposterior (**D**) and mediolateral (**E**) width, cortical thickness (**F**), marrow area (**G**), cortical area (**H**) and periosteal perimeter (**I**). Data is shown as mean $\pm$ SEM with individual data points, p values shown where $p < 0.1$ for comparisons with age- and sex-matched controls by two-way ANOVA.

Figure 5: Accelerated bone development of E18.5 *Dmp1Cre.Pthlh^{f/f(hom)}* femora. **A)** representative micro-CT images of *Dmp1Cre.Pthlh^{f/f(hom)}* and *Dmp1Cre^(hom)* femora at E18.5, showing bone mineral density. **B)** Femoral mineralized length (measured by micro-CT), **C,D)** total femoral length (measured in Alizarin Red/Alcian Blue stained samples, shown in panel D, with solid lines indicating bone length, and dashed lines indicating length of the mineralized portion), **E-J:** Mean polar moment of inertia (**E, H**), mediolateral (**F, I**), and anteroposterior widths (**G, J**) of *Dmp1Cre.Pthlh^{f/f(hom)}* and *Dmp1Cre^(hom)*, measured by micro-CT at three different locations shown in panel A: at 20% of the mineralized length distal to the proximal end of the mineralized region (Top 20%), at the midshaft (Mid), and at 20% of the mineralized length proximal to the distal end of the mineralized region (Bottom 20%). Data is shown as mean $\pm$ SEM with individual data points, p values shown where $p < 0.1$ for comparisons with controls by two-way ANOVA (**B-J**). **K:** Representative Toluidine Blue stained sections from E18.5 *Dmp1Cre* control and *Dmp1Cre.Pthlh^{f/f(hom)}* proximal tibiae (2 samples per genotype) showing the truncated proliferating and hypertrophic zones.

Figure 6: Decreased decidual PTHrP and impaired decidualization adjacent to *Dmp1Cre.Pthlh^{f/f(hom)}* placenta at E17.5. **(A)** PCR of DNA from placenta (P) and adjacent decidua (D) from two each of *Dmp1Cre^(hom)*, *Dmp1Cre.Pthlh^{f/f(het)}* and *Dmp1Cre.Pthlh^{f/f(hom)}* embryos at E17.5. Upper band indicates wild type *Pthlh* DNA, while lower band indicates recombined *Pthlh*. **(B-F)** Immunostaining for PTHrP in samples of placenta and decidua from *Dmp1Cre.Pthlh^{f/f(hom)}* and *Dmp1Cre^(hom)* embryos at E17.5; IgG control staining was measured in images of both zones. Decidua (d), spongiotrophoblast (sp) and labyrinth (lb) zones are shown in low power images (**B,C**). Scale bar = 1 mm. Frequency of PTHrP stained objects segregated by staining intensity in the spongiotrophoblast layer and decidua from *Dmp1Cre.Pthlh^{f/f(hom)}* and *Dmp1Cre^(hom)* embryos (**D**). **(E,F)** High power images of decidua. Scale bar = 20 micron. **(G)** Quantitation of total decidual area and spongiotrophoblast area from decidua adjacent to *Dmp1Cre.Pthlh^{f/f(hom)}* and *Dmp1Cre^(hom)* embryos; mean $\pm$ SEM with individual data points, **(H)** Quantitation of PTHrP immunostaining in decidua and spongiotrophoblast zone in decidua adjacent to *Dmp1Cre.Pthlh^{f/f(het)}* and *Dmp1Cre^(het)* placenta. **(I)** Quantitation of total decidual area and spongiotrophoblast area from decidua adjacent to *Dmp1Cre.Pthlh^{f/f(het)}* and *Dmp1Cre^(het)*

embryos; mean $\pm$ SEM with individual data points; p values shown where $p < 0.1$ for comparisons with determined by one-way ANOVA.

Figure 7: Maternal PTHrP determines bone width in progeny. Mice from breeders heterozygous for PTHrP (*Dmp1Cre.Pthlh^{f/w}* breeders) had normal bone size (length and width) compared to their sex- and age-matched controls, but lower adult trabecular bone mass. Decidual PTHrP may limit fetal skeletal development and radial growth, independent of longitudinal growth. Mothers of *Dmp1Cre.Pthlh^{f/f(hom)}* mice (which are *Dmp1Cre.Pthlh^{f/f(het)}*) had lower levels of decidual PTHrP, leading to wider long bones in *Dmp1Cre.Pthlh^{f/f(hom)}* progeny. This phenotype was observed not only in embryos and neonatal mice, but also in adult male mice. Adult male mice also showed high trabecular bone mass compared to their sex-matched *Dmp1Cre* controls.

Table 1. Trabecular structure of L5 (5th lumbar vertebrae) from 14 week old male *Dmp1Cre.Pthlh^{f/f(hom)}* and *Dmp1Cre^(hom)* controls by microcomputed tomography. Values are mean $\pm$ SEM. n=9-10/group; p values shown where $p < 0.1$ vs *Dmp1Cre^(hom)* by Student's t-test.

	<i>Dmp1Cre^(hom)</i>	<i>Dmp1Cre.Pthlh^{f/f(hom)}</i>
Trabecular bone volume/TV (%)	17.84 $\pm$ 0.89	21.32 $\pm$ 1.16 (p=0.030)
Trabecular number (/mm)	3.20 $\pm$ 0.13	3.70 $\pm$ 0.15 (p=0.025)
Trabecular thickness (μ m)	55.6 $\pm$ 1.3	57.3 $\pm$ 1.0
Trabecular separation (μ m)	203.5 $\pm$ 4.9	185.4 $\pm$ 6.4 (p=0.039)

Table 2 Histomorphometry of tibia and serum biochemical data of 14 week-old male *Dmp1Cre.Pthlh^{f/f(hom)}* and *Dmp1Cre^(hom)* controls. Histomorphometry was measured in the distal tibial metaphyseal secondary spongiosa. Values are mean $\pm$ SEM. n=7-10/group. Abbreviations: BV: bone volume; BS: bone surface; B. Pm: bone perimeter; N.Ob: osteoblast numbers; B.Pm: bone perimeter; N.Oc: osteoclast numbers; Oc.Pm: osteoclast perimeter; P1NP: Procollagen type 1 N-terminal propeptide; CTX1: C-telopeptide of type 1 collagen.

	<i>Dmp1Cre^(hom)</i>	<i>Dmp1Cre.Pthlh^{f/f(hom)}</i>
Osteoid surface/BS (%)	6.16 $\pm$ 1.85	6.47 $\pm$ 1.83
Osteoid thickness (μ m)	0.90 $\pm$ 0.11	1.20 $\pm$ 0.23
Osteoblast surface/BS (%)	7.83 $\pm$ 2.46	9.02 $\pm$ 2.55
Osteoblast numbers (N.Ob/B.Pm) (N/mm)	6.72 $\pm$ 2.09	7.77 $\pm$ 2.16
Osteoclast surface/BS (%)	3.77 $\pm$ 0.38	3.86 $\pm$ 0.44
Osteoclast numbers (N.Oc/B.Pm) (N/mm)	1.79 $\pm$ 0.29	1.93 $\pm$ 0.23
N.Oc/Oc.Pm (N/mm)	46.60 $\pm$ 6.18	49.79 $\pm$ 1.50
Single-labeled mineralizing surface (sL.S/BS) (%)	22.4 $\pm$ 2.4	20.8 $\pm$ 3.8
Double-labeled mineralizing surface (dL.S/BS) (%)	12.9 $\pm$ 2.9	15.9 $\pm$ 4.2
Mineralizing surface/BS (%)	24.2 $\pm$ 2.0	26.3 $\pm$ 3.3
Mineral apposition rate (μ m/day)	1.09 $\pm$ 0.08	1.05 $\pm$ 0.15
Bone formation rate/BV (%/day)	1.30 $\pm$ 0.10	1.41 $\pm$ 0.32
P1NP (ng/ml)	29.6 $\pm$ 2.3	36.6 $\pm$ 4.1
CTX1 (ng/ml)	25.4 $\pm$ 1.8	21.5 $\pm$ 1.6

Table 3. Additional strength data from three point bending test on femora from 14 and 26 week old *Dmp1Cre.Pthlh^{f/f(hom)}* (f/f) and *Dmp1Cre^(hom)* (w/w) mice. Values are mean $\pm$ SEM. n=9-10/group; p values shown where p<0.1 for comparisons with sex- and age-matched w/w by Student's t-test (14 weeks old) and two-way ANOVA (26 weeks old) with uncorrected Fishers LSD post-hoc test.

	14 week old			26 week old				
	Male w/w	f/f		Male w/w	f/f		Female w/w	f/f
Moment of inertia (mm ⁴)	0.76 ± 0.06	1.16 ± 0.10	p=0.002	0.62 ± 0.05	1.16 ± 0.09	p<0.001	0.59 ± 0.03	0.67 ± 0.03
Whole bone parameters								
Yield force (N)	12.64 ± 0.63	13.85 ± 0.94		11.50 ± 0.44	13.71 ± 0.96		12.61 ± 0.84	15.73 ± 1.61 p=0.043
Yield displacement (µm)	436.9 ± 56.2	454.4 ± 43.2		157.4 ± 13.5	161.3 ± 15.2		132.4 ± 11.5	190.8 ± 25.5 p=0.022
Post-yield displacement (µm)	298.4 ± 53.6	388.9 ± 44.4		293.7 ± 20.1	238.3 ± 36.9		200.0 ± 38.9	154.2 ± 28.9
Failure force (N)	14.26 ± 1.63	15.88 ± 1.21		14.99 ± 0.47	18.25 ± 0.73	p=0.013	18.04 ± 0.97	20.06 ± 1.18
Stiffness (N/mm)	49.8 ± 7.0	66.9 ± 7.3	p=0.055	92.33 ± 4.95	111.06 ± 9.77	p=0.059	114.02 ± 4.07	106.27 ± 6.93
Tissue level parameters								
Ultimate strain (%)	5.31 ± 0.46	6.81 ± 0.47	p=0.035	6.84 ± 0.45	8.13 ± 0.85		5.97 ± 0.29	7.17 ± 0.50
Yield strain (%)	4.23 ± 0.50	4.77 ± 0.46		3.30 ± 0.28	3.81 ± 0.34		2.88 ± 0.24	4.25 ± 0.55 p=0.013
Post-yield strain (%)	2.91 ± 0.50	4.07 ± 0.46	p=0.054	6.15 ± 0.41	5.69 ± 0.92		4.38 ± 0.86	3.43 ± 0.64
Failure strain (%)	7.15 ± 0.29	8.84 ± 0.54	p=0.008	9.44 ± 0.40	9.50 ± 1.02		7.26 ± 0.75	7.68 ± 0.52
Toughness (mJ/mm ³)	1.33 ± 0.10	1.29 ± 0.11		1.74 ± 0.13	1.13 ± 0.13	p=0.006	1.53 ± 0.19	1.42 ± 0.13
Elastic modulus (MPa)	1009.4 ± 136.4	852.4 ± 73.2		692.1 ± 48.4	450.0 ± 47.2	p=0.002	891.5 ± 57.2	718.9 ± 49.5 p=0.021

Table 4. Biochemical analysis of milk samples. PTHrP content was measured by radioimmunoassay (RIA) in milk samples from mothers of *Dmp1Cre.Pthlh^{f/f(hom)}* and *Dmp1Cre^(hom)* mice on day 12 of lactation. PTHrP equivalent concentration was measured by measuring cAMP response to milk treatment of UMR106-01 cells (bioassay). Data shown as mean $\pm$ SEM. n=7/group. No significant differences relating to genotype were detected by Student's t-test.

Mothers of:	<i>Dmp1Cre^(hom)</i>	<i>Dmp1Cre.Pthlh^{f/f(hom)}</i>
PTHrP (pmol)/milk protein (g) by RIA	64.54 $\pm$ 13.53	45.53 $\pm$ 5.12
PTHrP (pmol)/milk protein (g) by bioassay	581.68 $\pm$ 43.57	582.80 $\pm$ 90.97
Milk protein (g/L)	100.42 $\pm$ 4.16	99.28 $\pm$ 7.61

Legends to Supplemental Figures

Supplemental Figure 1: No difference in trabecular structure or metaphyseal diameter in femora from *Dmp1Cre.Pthlh^{f/f(het)}* mice and littermate *Dmp1Cre^(het)* controls. Trabecular structure of distal femoral primary spongiosa analyzed by micro-CT in male and female mice at 16 and 26 weeks of age. Trabecular bone volume, trabecular number, trabecular thickness, trabecular separation, and metaphyseal periosteal perimeter are shown as mean (dot), interquartile range (box), median (line) and range; n=9-10/group. No significant differences associated with genotype were detected (two-way ANOVA). Y-axes are drawn to match those of Figure 1 to allow comparison with *Dmp1Cre.Pthlh^{f/f(hom)}* mice. Breeding strategy is shown in Figure 4A.

Supplemental Figure 2: Cortical bone structure in femora from *Dmp1Cre.Pthlh^{f/f(het)}* mice and littermate *Dmp1Cre^(het)* controls. **A:** Schematic showing measurement regions (note, this is identical to Figure 2A, and is reproduced here for convenience). **B:** Femoral length of male and female mice at 16 and 26 weeks of age. Anteroposterior (**C**) and mediolateral (**D**) width, measured by micro-CT at the midshaft. **E-I:** Femoral marrow area (**E**), cortical bone area (**F**), thickness (**G**), and both endocortical (**H**) and periosteal (**I**) perimeter were analyzed in cortical ROI by micro-CT. Data are shown as mean (dot), interquartile range (box), median (line) and range; n=9-10/group. p values shown where p<0.1 for comparisons with sex- and age-matched *Dmp1Cre^(het)* by two-way ANOVA. Y-axes are drawn to match those of Figure 2 to allow comparison with *Dmp1Cre.Pthlh^{f/f(hom)}* mice. Breeding strategy is shown in Figure 4A.

Supplemental Figure 3: No change in bone strength measured by three-point bending tests in heterozygous-bred *Dmp1Cre.Pthlh^{f/f(het)}* femora compared to littermate *Dmp1Cre^(het)* controls at 26 weeks of age. Shown are ultimate force (**A**), ultimate deformation (**B**), ultimate stress (**C**), yield stress (**D**), and failure stress (**E**). Data shown as mean (dot), interquartile range (box), median (line) and range, n=9-10/group. No significant differences relating to genotype were detected by two-way ANOVA.

Supplemental Figure 4: Additional micro-CT data of E18.5 *Dmp1Cre.Pthlh^{f/f(hom)}* embryos. Bone area and tissue area were analyzed by micro-CT in three different regions of femora: 20% of the mineralized length distal to the proximal end of the mineralized region (Top 20%), midshaft (Mid), and 20% of the mineralized length proximal to the distal end of the mineralized region (Bottom 20%); see Figure 5A. Data is shown as mean ± SEM with individual data points p values shown where p<0.1 for comparisons with controls by two-way ANOVA.

Supplemental Figure 5: Bone structure of E17.5 *Dmp1Cre.Pthlh^{f/f(het)}* femora. Mineralized femoral length (**A**), mediolateral width (**B,H**), anteroposterior width (**C,I**), mean polar moment of inertia (**D,G**), bone area (**E,J**), and tissue area (**F,K**) of *Dmp1Cre.Pthlh^{f/f(het)}* and *Dmp1Cre^(het)* embryos measured by micro-CT in three different regions of femora: 20% of the mineralized length distal to the proximal end of the

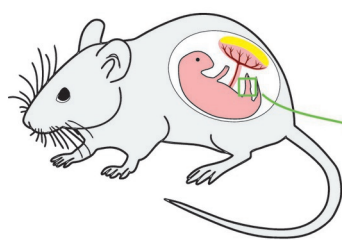
mineralized region (Top 20%), midshaft (Mid), and 20% of the mineralized length proximal to the distal end of the mineralized region (Bottom 20%). Data is shown as mean $\pm$ SEM with individual data points. No significant differences relating to genotype were detected by two-way ANOVA.

Supplemental Figure 6: Embryo weight, placental weight, and decidual PTHrP of *Dmp1Cre.Pthlh^{f/f(hom)}* (A-C) and *Dmp1Cre.Pthlh^{f/f(het)}* (D-F) mice. Embryo weight (A,D), placental weight (B,E) and embryo to placental weight ratio (C,F) of *Dmp1Cre.Pthlh^{f/f(het)}* and *Dmp1Cre^(het)* embryos at E17.5; mean $\pm$ SEM with individual data points. No significant differences relating to genotype were detected by two-way ANOVA.

Figure 7

Heterozygous breeders

Dmp1Cre.Pthlh^{f/w}



embryo

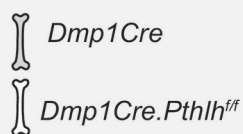
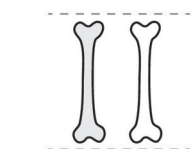


Offspring age

E18.5

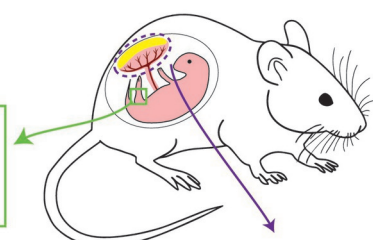
12 days

adult

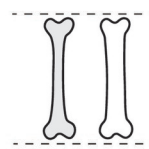
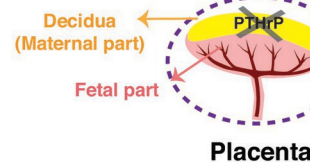
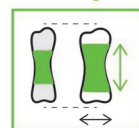


Homozygous breeders

Dmp1Cre.Pthlh^{f/f}



embryo

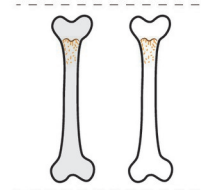


Male



Maintenance

Female

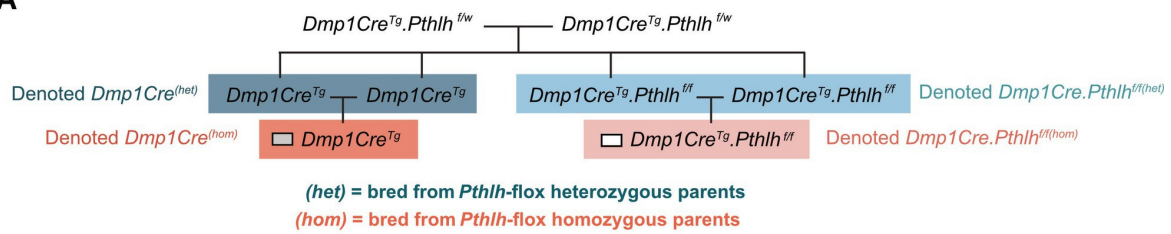


Recovery

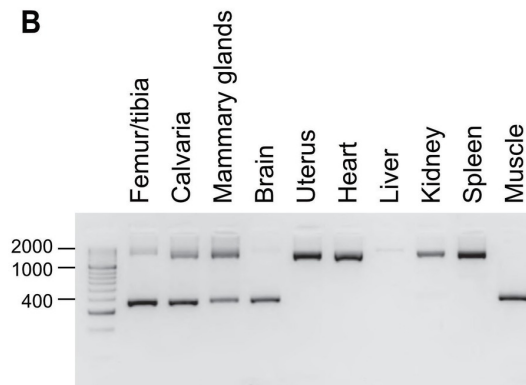
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Figure 1

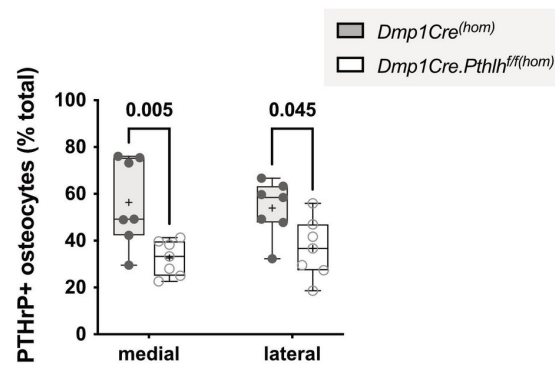
A



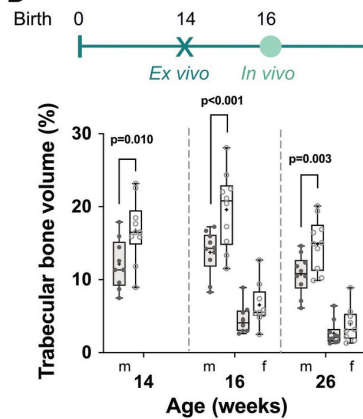
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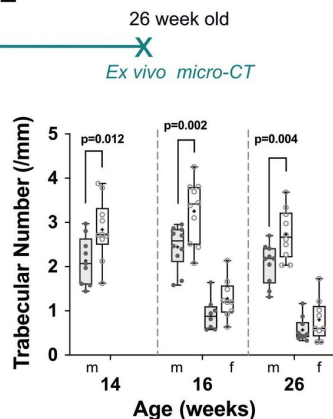
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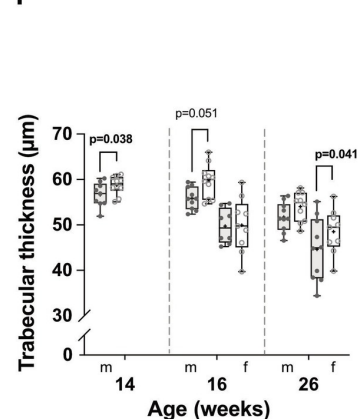
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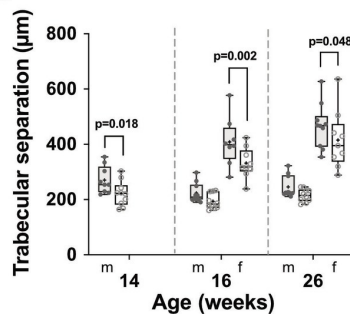
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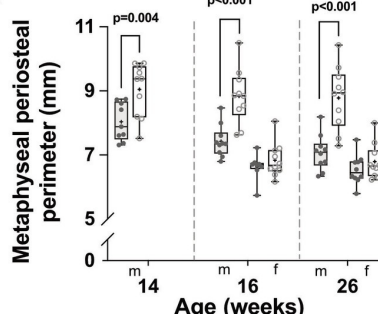
F



G



H



I

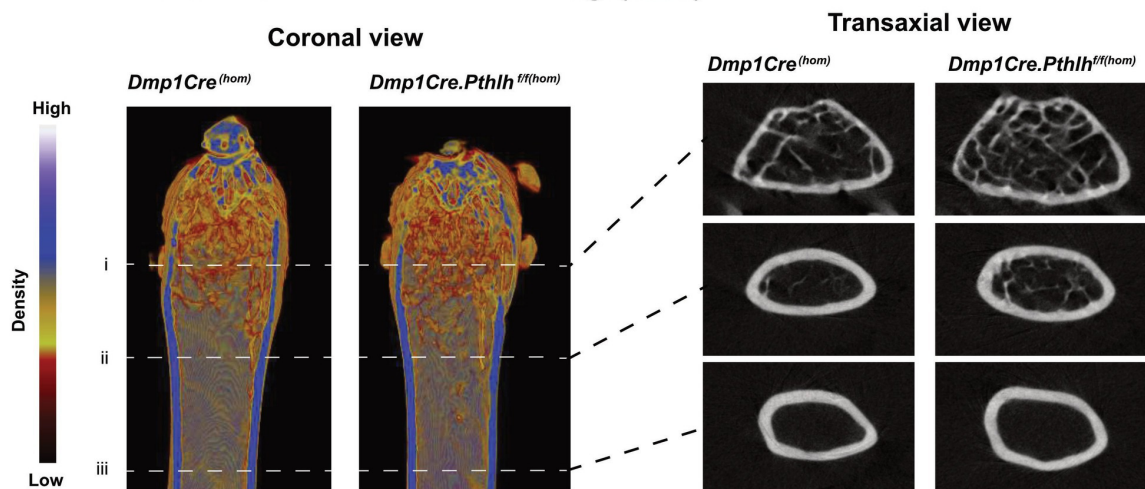
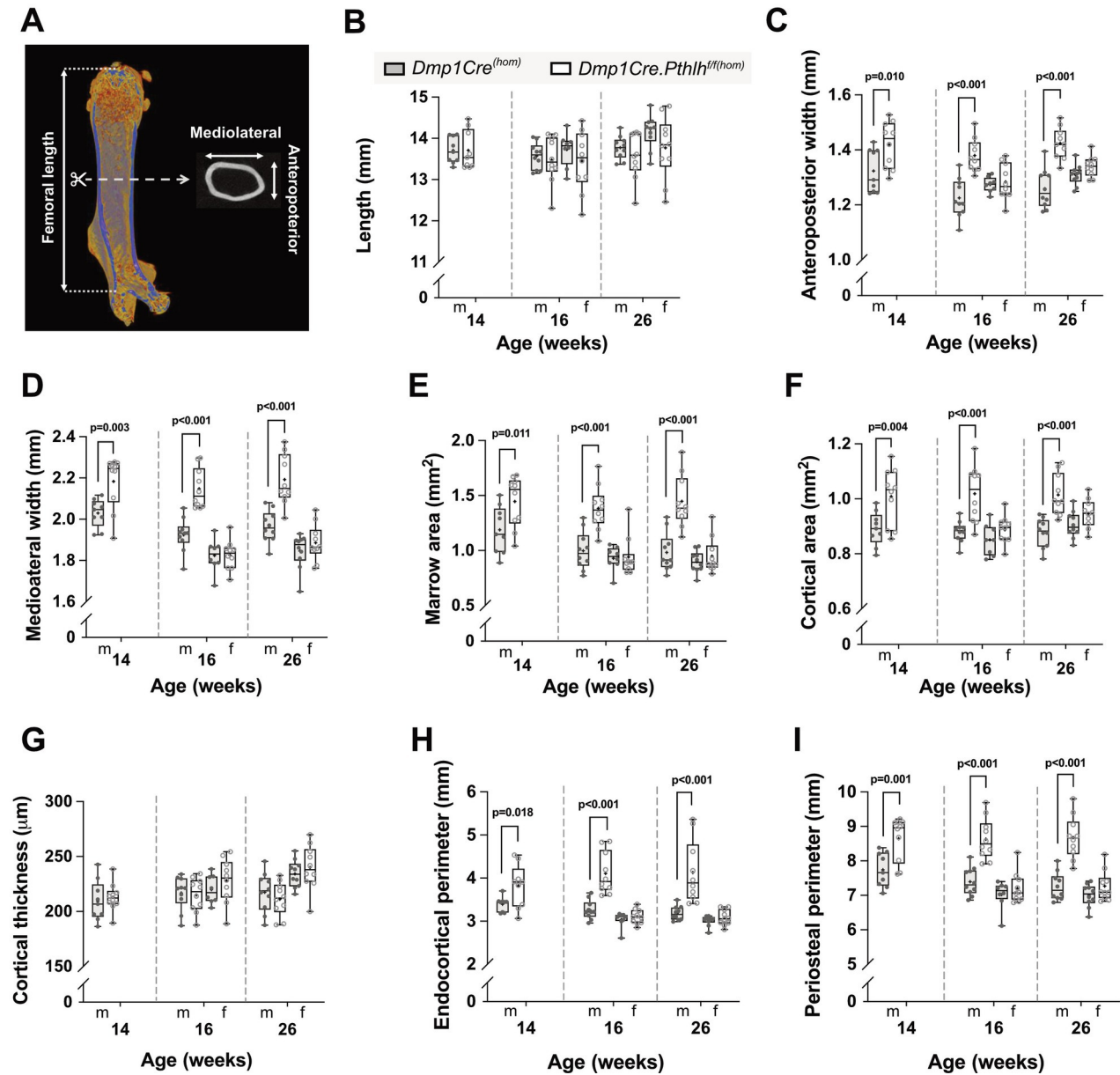
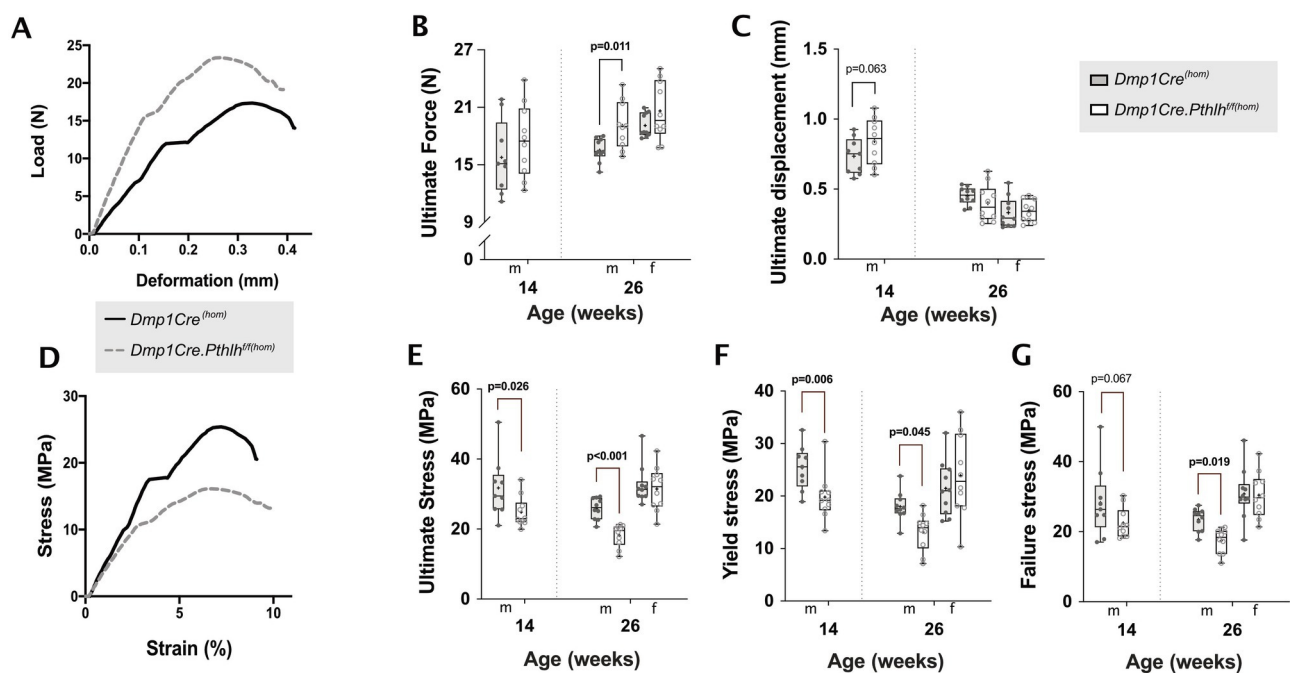


Figure 2



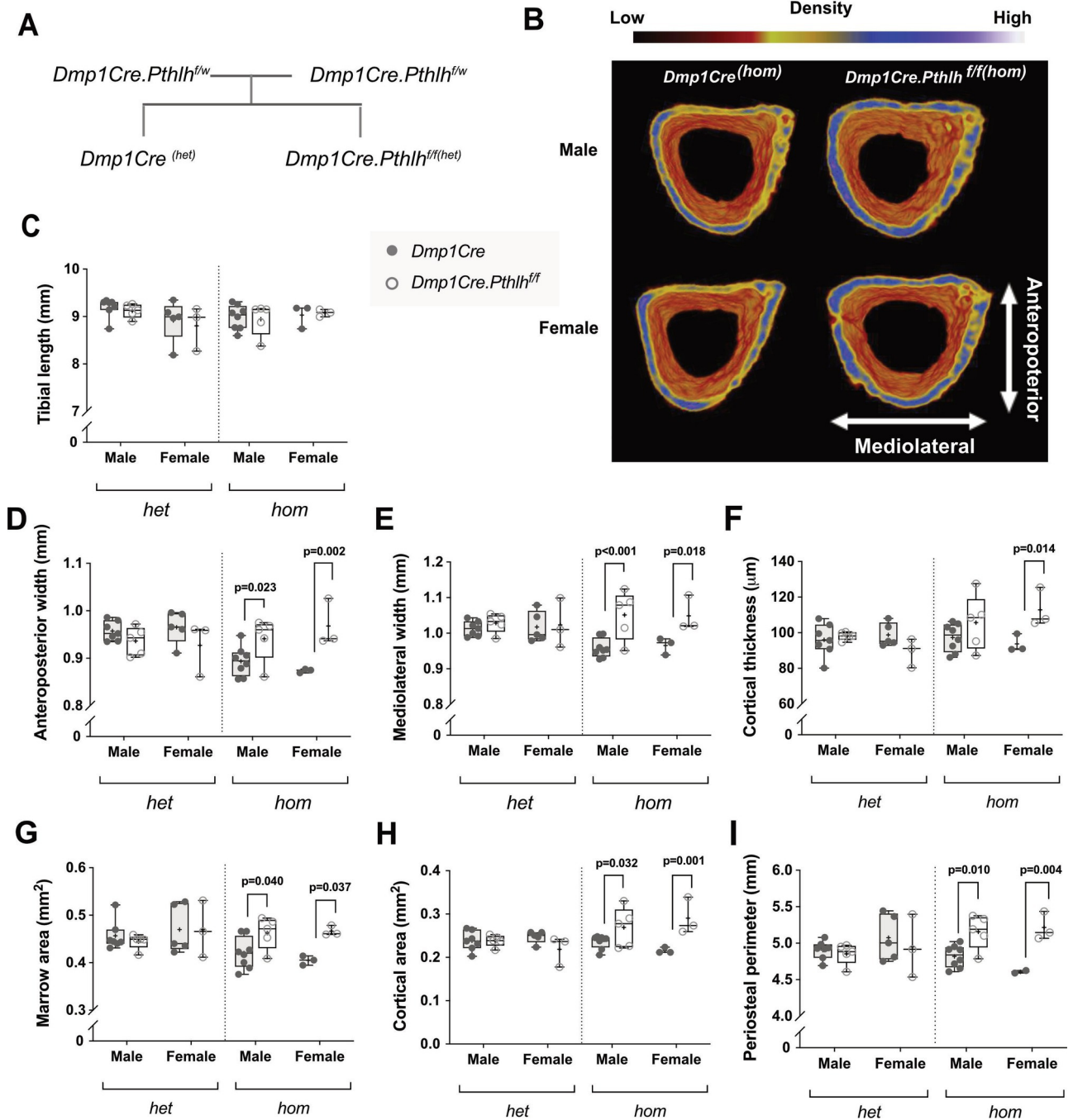
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Figure 3



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Figure 4



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Figure 5

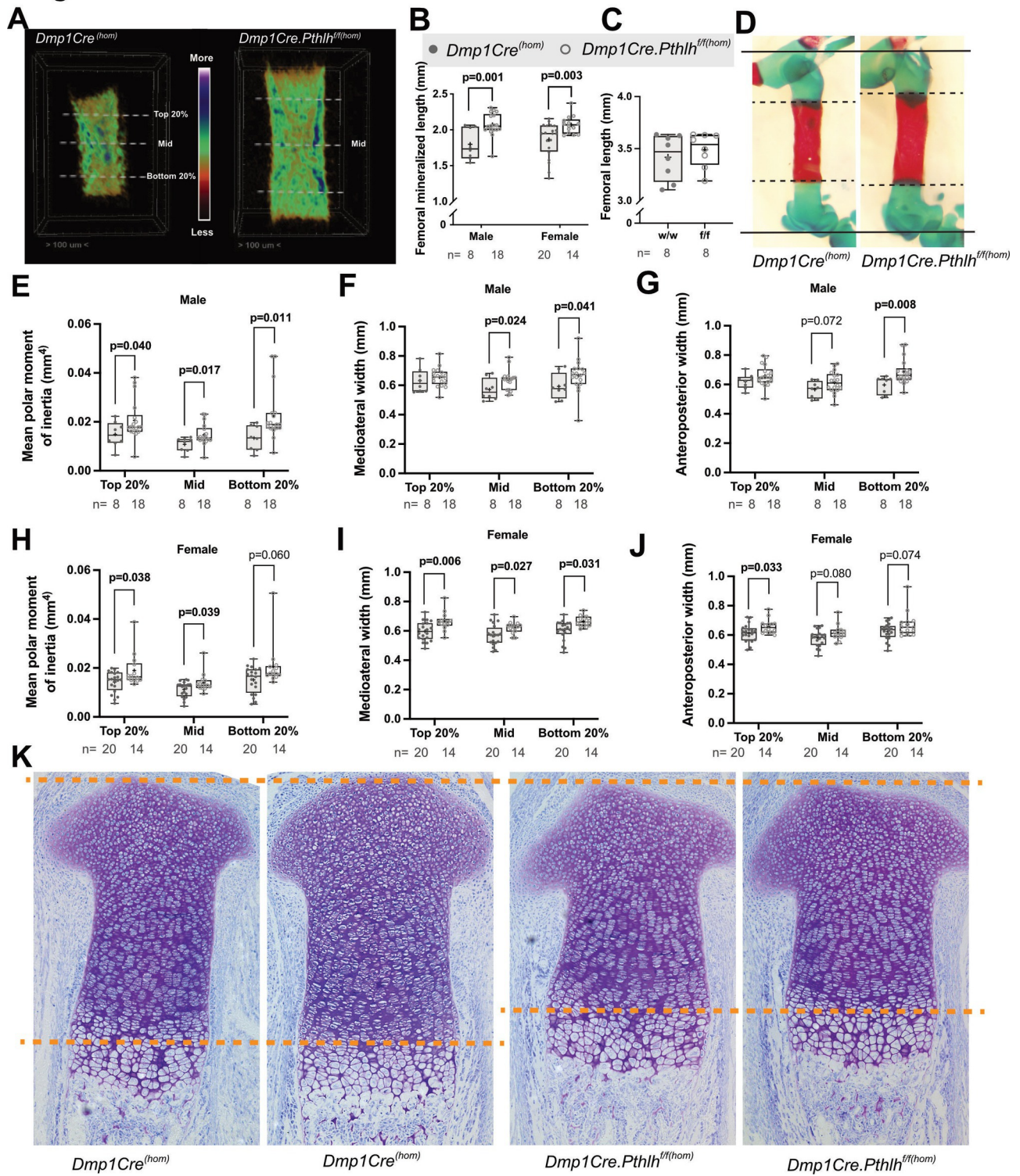
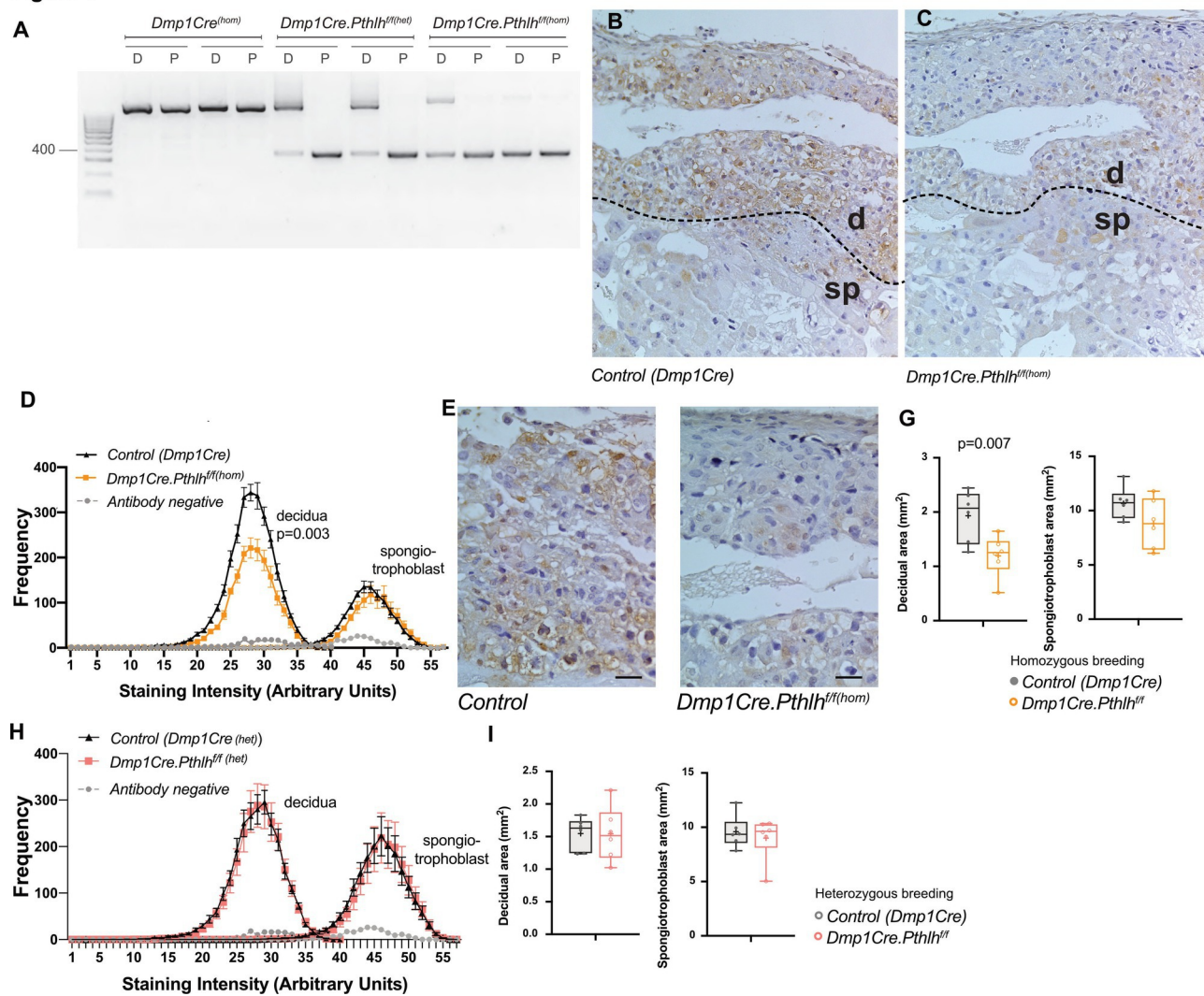
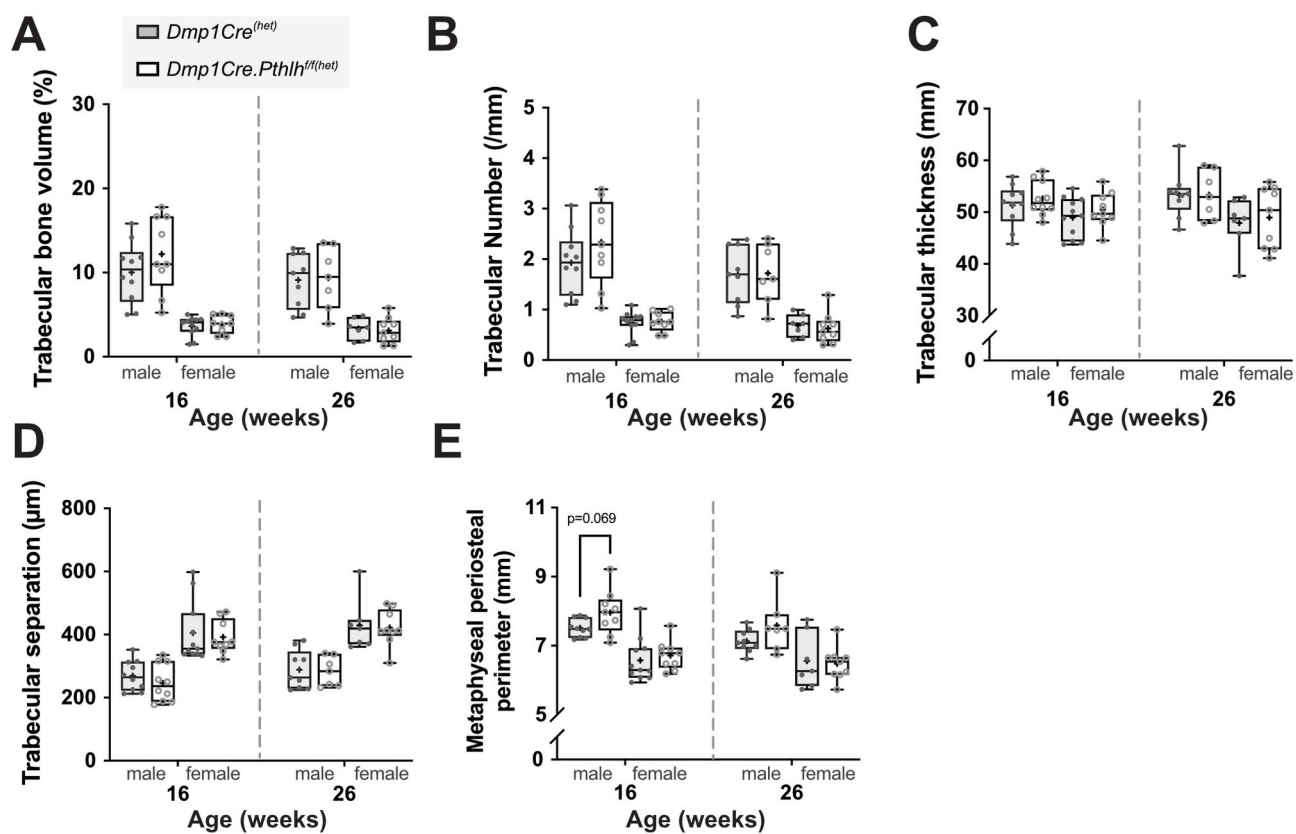


Figure 6



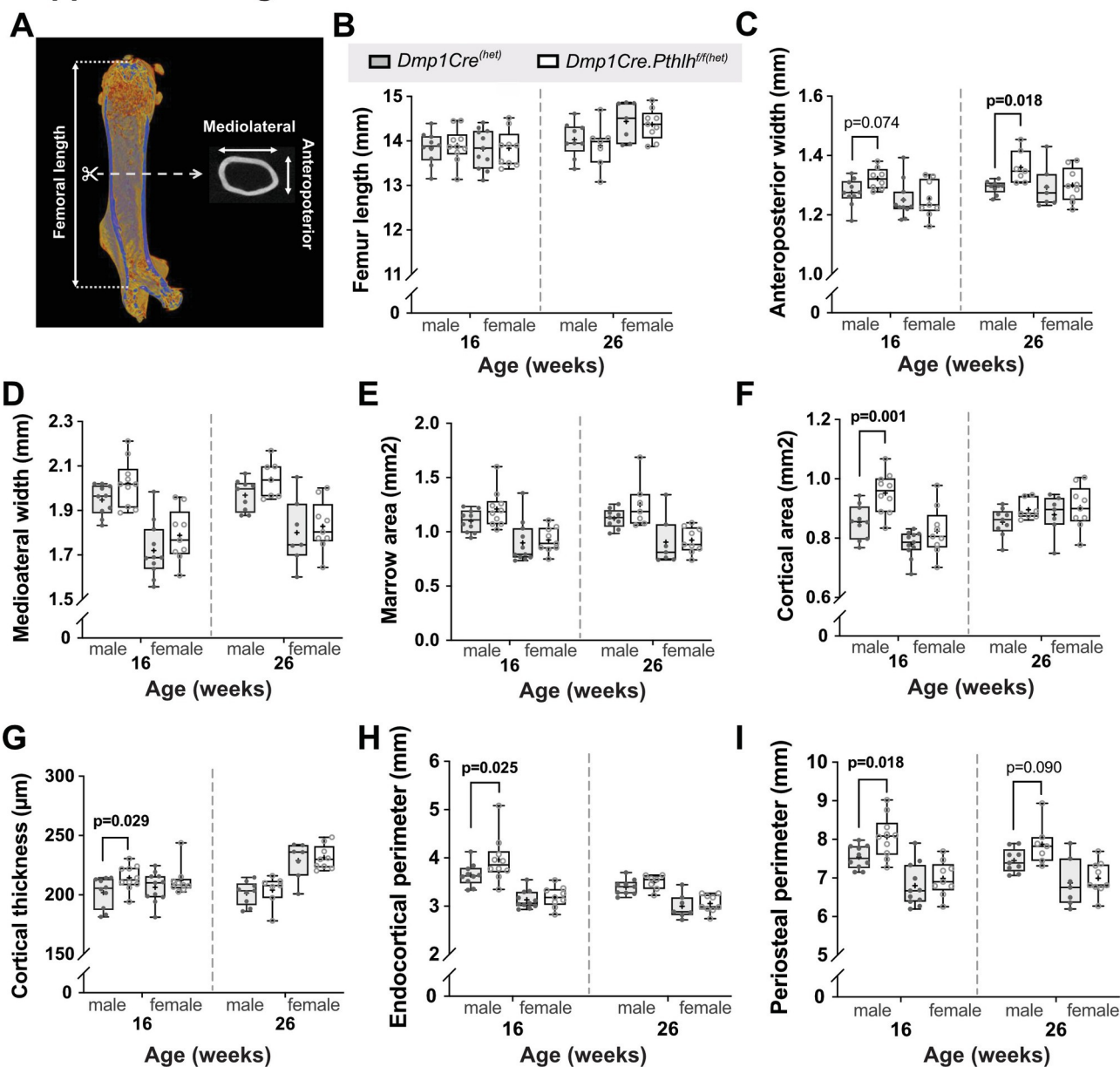
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Supplemental Figure 1



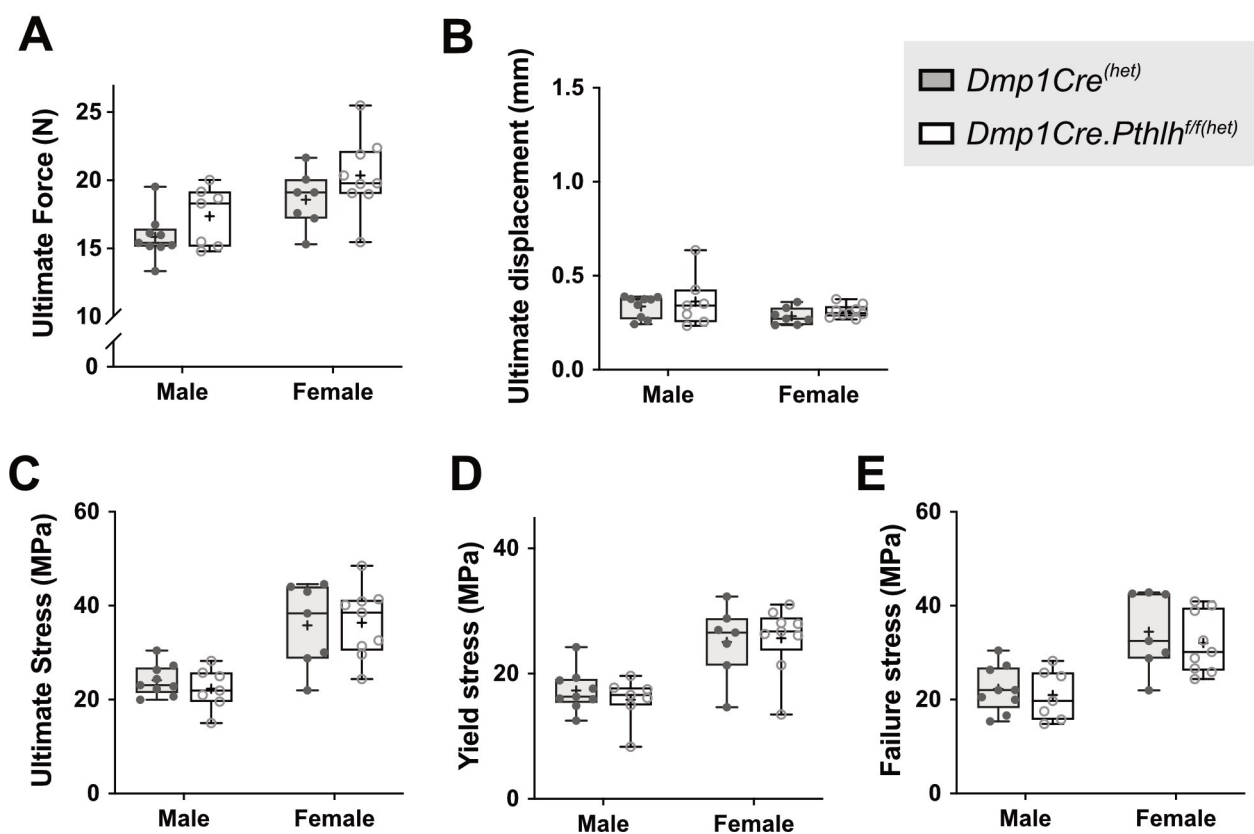
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Supplemental Figure 2



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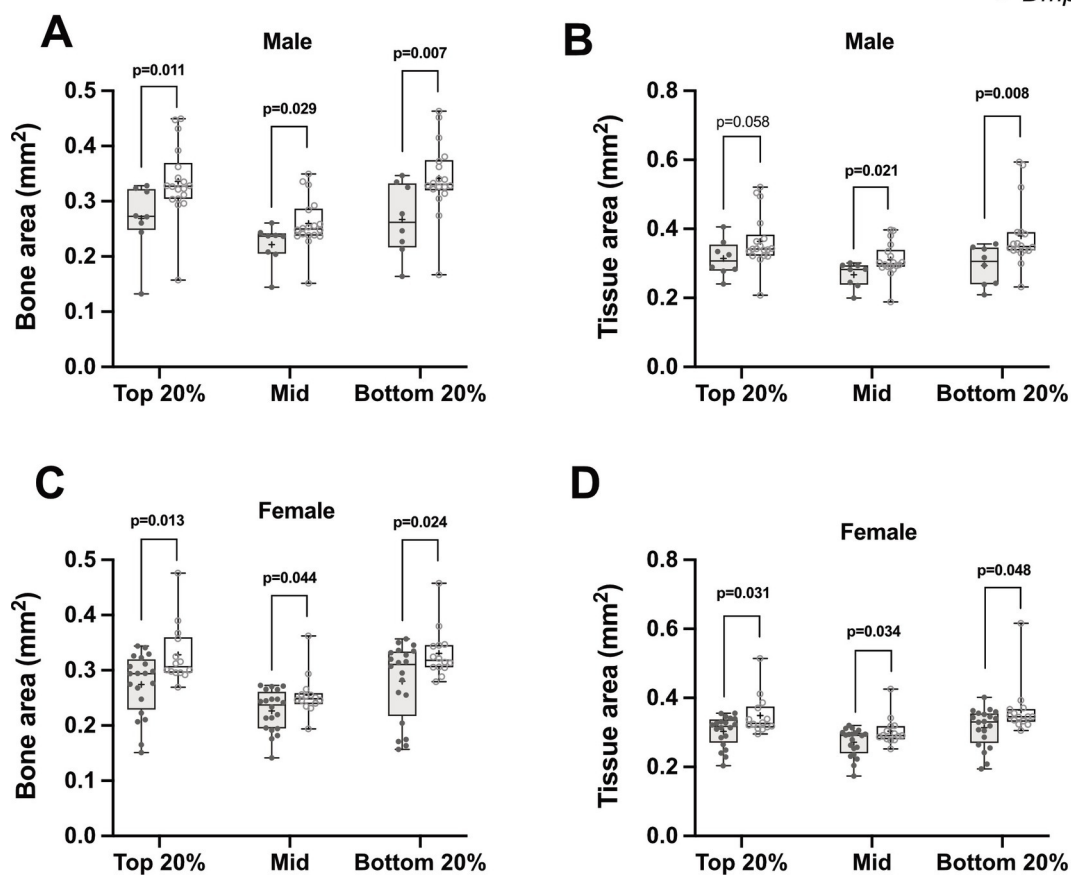
Supplemental Figure 3



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Supplemental Figure 4

- *Dmp1Cre^(hom)*
- *Dmp1Cre.Pthlh^{f/f}(hom)*



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Supplemental Figure 5

