Lrp5 functions in bone to regulate bone mass

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Author contributions:

YC created and performed studies on the HBM mice, and measured serum serotonin levels by competitive ELISA. PJN performed radiographic imaging and biomechanical testing on the HBM mice. BTM contributed to the serotonin and Tph1 qRT-PCR measurements in HBM and Lrp5 knockout mice. CRZ performed multiple studies using the Lrp5<sup>f</sup> mice. NA performed studies on the Tph1<sup>−/−</sup> mice, and with SM measured whole blood serotonin levels from HBM and Lrp5 knockout mice by HPLC. DRR generated the Lrp5<sup>f</sup> strain and ZZ participated in conditional inactivation of this allele using different Cre transgenes. CMJ performed the Prx1::Cre experiments. RB, FM, and QY organized studies on Lrp5 and Tph1 knockout mice and the mouse pharmacology experiment. HG and JAG organized the rat pharmacology experiment. RAC, XH, MB, DRP, QL, BZ, BOW, AGR, and MLW designed experiments, and provided reagents and financial support. MLW prepared the first draft of the manuscript. All co-authors contributed detailed methods and results, revised and approved the manuscript.

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**Detailed Methods**

*Mice with conditional Lrp5 HBM and conditional Lrp5 knockout alleles*

To create *Lrp5* HBM alleles we performed homologous recombination in the 129/Sv hybrid R1 ES cell line\(^1\). The targeting vector consisted of a 2.5 kb 5’ targeting arm containing genomic DNA from *Lrp5* intron 2, and a 3’ targeting arm containing 6.9 kb of genomic DNA extending from *Lrp5* intron 2 to intron 4 (Fig. 1a). We performed site directed mutagenesis to create two different HBM-associated mutations, either p.G171V (which is evolutionarily conserved, equivalent to residue 170 in the mouse, and encoded within exon 3) or p.A214V (which is evolutionarily conserved, equivalent to residue 213 in the mouse, and encoded within exon 3), within the 3’ targeting arm. In order to select for correctly targeted clones we included a floxed neomycin-resistance cassette (Neo\(^R\)) in the 5’ targeting arm that is transcribed in reverse orientation to the *Lrp5* transcript and included a thymidine kinase (TK) selection cassette at the end of the 3’ targeting arm (Fig. 1a). We electroporated and selected ES cells using standard methods. We confirmed correct targeting by Southern blot using 5’ and 3’ flanking probes and confirmed that the HBM mutations were retained within correctly targeted clones by PCR amplification and sequencing of exon 3. We then used correctly targeted ES cells to generate knockin mice. We bred knockin mice that retained Neo\(^R\) and also generated mice in which we excised the neo-cassette using an EIIa::Cre transgenic mouse strain that expresses Cre-recombinase during early embryonic development (Jax Labs stock # 003724).

Another study inserted an *Lrp5* HBM cDNA containing a carboxyl-terminal FLAG-tag into the 1st exon of the endogenous *Lrp5* locus\(^2\). This strategy may have altered Lrp5 function in two ways. First, the epitope tag may have disrupted the ability of Lrp5 to transduce Wnt signaling, since we had previously reported impaired Wnt signaling when LRP5 was carboxyl-terminal tagged with a myc-epitope\(^3\). Second, the insertion of the cDNA, which was not designed to undergo splicing, may not have been strongly expressed. For example, Kelly et al. (2004) were unable to detect expression of a LacZ cDNA they had inserted into the first coding exon of *Lrp5*\(^4\). The level of expression of *Lrp5* knockin alleles is important, since prior studies in mice that were haploinsufficient for *Lrp5*\(^5,6\) or that over-expressed wild-type *Lrp5*\(^7\) indicate that bone mass can be affected by the amount of *Lrp5* mRNA that is expressed.

To create a conditional *Lrp5* loss-of-function allele, we performed homologous recombination in the CJ7 ES cell line\(^8\). The targeting vector consisted of a ~6.7 kb 5’ targeting arm containing genomic DNA from *Lrp5* intron 1 extending into intron 2 and a 3’ targeting arm containing ~2.0 kb of genomic DNA extending from *Lrp5* intron 2 (Figure 3a). We placed LoxP sites in the same orientation ~1600 bp upstream of exon 2 and ~800 bp downstream of exon 2. In order to select for correctly targeted clones we included an FRT-flanked neomycin-resistance cassette (Neo\(^R\)) in the 5’ targeting arm that is transcribed in reverse orientation to the *Lrp5* transcript and included a diptheria toxin selection cassette at the end of the 3’ targeting arm (Figure 3a). We electroporated and selected ES cells using standard methods. Homologous recombinants were sought by a PCR-based strategy and we confirmed correct targeting by Southern blot using 5’ and 3’ flanking probes. We also confirmed that the LoxP sites were retained within correctly targeted clones by PCR amplification and sequencing. Correctly targeted ES cells were used to generate floxed mice. We removed the neo-cassette from the targeted allele by breeding mice with FLPeR mice, a 129 derived strain that broadly expresses flipase driven by the ROSA26 locus\(^9\). We bred mice that retain the floxed exon 2 and also generated mice in which we excised exon 2 using the Cmv::Cre transgenic mouse strain that expresses Cre-recombinase during early embryonic development\(^10\).

In the remainder of the methods we use the following nomenclature for the *Lrp5* alleles described in the manuscript: *Lrp5*\(^5\) or (\(+\)) for the wild-type allele, *Lrp5*\(^–\) or (\(–\)) for loss-of-function alleles, some of which have been previously described\(^5,11,12\), *Lrp5*\(^5\) or (f) for the conditional loss-of-function allele, *Lrp5*\(^\text{neoG171V}\) or (G\(\text{X}\)) for the knockin p.G171V allele that contains Neo\(^R\), *Lrp5*\(^\text{G171V}\) or (G) for the HBM p.G171V allele in which Neo\(^R\) has been Cre-excised, *Lrp5*\(^\text{neoA214V}\) or (A\(\text{X}\)) for the knockin p.A214V allele that contains Neo\(^R\), and *Lrp5*\(^\text{A214V}\) or (A) for the HBM p.A214V allele in which Neo\(^R\) has been Cre-excised.

For the *Lrp5* HBM mutant mice, we developed a PCR based genotyping assay to distinguish wild-type alleles, knockin alleles that contain Neo\(^R\), and knockin alleles that lack Neo\(^R\) (Fig. 1a). Briefly, forward primer
P1 (5’-AGT ACT GGC TGG CAC AGA-3’) is located within intron 2 upstream of the site of the neomycin selection cassette insertion, reverse primer P2 (5’ -GTC AGT TTC ATA GCC TGA- 3’) is located within the neomycin selection cassette, and reverse primer P3 (5’-CAG GCT GCC CTT GCA GAT-3’) is located in intron 2 downstream of the site of neo-cassette insertion. Because the P2 primer anneals to DNA within the neo-cassette, P1/P2 only generates its 320-bp amplimer in Lrp5 alleles that contain the neo-cassette. Primer pair P1/P3 generates a 250-bp amplimer from the wild-type allele and does not efficiently amplify across the allele containing the neo-cassette. However, after Cre-mediated excision of the neo-cassette, primer pair P1/P3 can generate a 400-bp amplimer that retains the LoxP site (Fig. 1c).

We employed a similar PCR-based strategy to distinguish wild-type, Lrp5f, and Lrp5– alleles for the Lrp5 conditional knockout mice. Briefly forward primer AS-5 (5’ TGC TCT TTC ATG CCC TCA GTG TA) is located in intron 1 upstream of the 5’ LoxP site, forward primer AS-WT (TCT TGT AGC ACC CAG GAC CAT C) is located in intron 2 upstream of the 3’ LoxP site, and reverse primer AS-3 (5’ CAC CAC AGC CAA CAG TCA CAG A) is located in intron 2 downstream of the 3’ Lox P site (Fig. 3a). AS-WT/AS-3 will generate a 282 bp amplimer for wild-type alleles and a 404 bp amplimer for Lrp5f alleles. AS-5/AS-3 are too far apart to efficiently amplify wild-type or floxed alleles, but following Cre-mediated excision of exon 2 will generate a 534 bp amplimer (Fig. 3b).

**Tissue-specific expression of Lrp5 HBM alleles and creation of Lrp5 knockout alleles**

As noted above, we bred mice with the Lrp5neoG171V and the Lrp5neoA214V allele to EIIa::Cre mice to excise NeoR during early embryonic development, thereby generating lines of mice in which all cells lack the neo-cassette. We used three other Cre expressing mouse lines to excise NeoR in a cell-type-specific or tissue-specific manner. We excised NeoR in maturing osteoblasts and osteocytes with a Dmp1::Cre mouse strain 13. We excised NeoR in intestinal epithelial cells using a Villin::Cre mouse strain 14. We excised NeoR in early limb bud mesenchyme using a Prx1::Cre mouse strain 15. We determined whether excision occurred by extracting DNA from long bone and duodenum, and performing PCR amplification with primers P1, P2, and P3.

We bred mice with the Lrp5f allele to Cmv::Cre mice to generate mice in which all cells lack Lrp5. We knocked out Lrp5 in maturing osteoblasts and osteocytes with a Dmp1::Cre mouse strain 13. We knocked out Lrp5 in intestinal cells by breeding mice with Lrp5f alleles to Villin::Cre mice 14. We determined whether excision occurred by extracting DNA from long bone, duodenum, and other tissues, and performing PCR amplification with primers AS-WT, AS-5, and AS-3. We also determined whether Villin::Cre was expressed in the intestinal stem cell of the duodenum by crossing a Villin::Cre mouse to the Rosa26mT/mG reporter mouse strain 16 to obtain double heterozygous offspring. The duodenum of the offspring was recovered, embedded in OCT, and cryosectioned at 10 µm thickness. The sections were fixed in 4% paraformaldehyde for 5 minutes, stained with 4’,6-diamidino-2-phenylindole to identify cell nuclei, and imaged with fluorescence microscopy.

An earlier study 2 utilized a 2.3Col1a1::Cre driver that is expressed earlier during osteoblastic differentiation 17 to conditionally alter Lrp5 activity. It is possible that Lrp6 might fully compensate for Lrp5 if the latter gene’s expression is altered at earlier stages of osteoblast differentiation compared to later stages. However, this would not explain the differences in outcome between our study and the earlier study 2 when Lrp5 activity was altered in the intestine. Although we used a different Villin::Cre driver line, it drove efficient expression of Cre-recombinase in the intestinal epithelial stem cells that give rise to the serotonin producing enterochromaffin cells (Supplementary Fig. S5). For any Cre-expressing transgenic animal, it is possible that Cre expressed at low levels in tissues other than its intended site of expression is responsible for the resultant phenotype. The use of PCR-based recombination assays and the advent of newer mouse models for monitoring Cre-recombination (e.g., 16) have increased the sensitivity for identifying unexpected sites where transgenes are expressed. For example, we have found that the Dmp1::Cre transgene is expressed at low levels in some non-bone tissues (Supplementary Fig. S5A), although not in the duodenum (Fig. 2d and Fig. 3c). Unanticipated sites of Cre expression could also have occurred for the 2.3Col1a1::Cre and Villin::Cre transgenes used in the earlier study 2.

**PCR based assessment of Cre-recombination**
DNA was recovered from ear punches as previously described \(^8\), and from femur bone, duodenum, and other tissues of mice using the DNeasy kit (Qiagen). Briefly, the proximal and distal ends of the femur were removed and the marrow flushed out with sterile saline. The femur was decalcified in 10% EDTA, pH 7.4 for 2 days at 4°C prior to DNA extraction. The duodenum was defined as the first cm after the pyloric sphincter. It was rinsed with sterile saline and homogenized prior to DNA extraction. PCR Primers P1, P2, P3 and AS-5, AS-WT, AS-3, were used to amplify DNA that had been extracted from conditional *Lrp5* HBM and conditional *Lrp5* knockout mice, respectively.

**Northern blot analysis of *Lrp5* expression in mouse long bone**

Intact mouse femurs and tibias, including marrow, were pulverized in liquid nitrogen and total RNA was extracted using TRIzol (Invitrogen). Ten \(\mu\text{g}\) of this bone-derived total RNA from each mouse was used for northern blotting. *Lrp5* mRNA expression was assessed by hybridizing the blot with a 500-bp cDNA fragment corresponding to exons 2 to 4 of mouse *Lrp5*. Following detection of *Lrp5* mRNA, the blot was then hybridized with a 316 bp cDNA fragment from *Gapdh* (Ambion, Cat # AM7431), whose mRNA detection served as a loading control.

**Genetic backgrounds of experimental animals used in this study**

Since several of the mice reported in this study were generated and independently maintained in different labs, the background strains of the mice used for each figure included in this paper are specified below. Littermates and other closely related mice were used as controls in most experiments. When an experiment did not employ littermates or closely related mice as controls, the source of the control mice is specifically indicated.

1. (Figure 1) *Lrp5* \(_G\) and *Lrp5* \(_A\) knockin alleles were created with the 129F1 hybrid R1 ES cell line. EIIa::Cre mice have been maintained on a C57BL/6J background.
2. (Figure 1) Bone measurements in mice that were heterozygous and homozygous for *Lrp5* \(_G\) and *Lrp5* \(_A\) knockin alleles were performed on mice with mixed 129S1/SvIMJ and C57BL/6J backgrounds.
3. (Figure 2) Bone measurements in mice with conditional activation of *Lrp5* \(_G\) and *Lrp5* \(_A\) knockin alleles using Dmp1::Cre were performed on offspring of mice that had been backcrossed for greater than 7 generations to 129S1/SvIMJ (Jax Labs stock # 002448). Dmp1::Cre mice have a mixed CD-1 and 129 background.
4. (Figure 2) Bone measurements in mice with conditional activation of *Lrp5* \(_G\) and *Lrp5* \(_A\) knockin alleles using Villin::Cre were performed on offspring of mice that had been backcrossed for greater than 7 generations to C57BL/6J mice (Jax Labs stock # 000664). Villin::Cre mice have been maintained on a C57BL/6J genetic background.
5. (Figure 2) Bone measurements in mice with conditional activation of *Lrp5* \(_A\) knockin alleles using Prx1::Cre were performed on offspring of mice that had been backcrossed for greater than 7 generations to C57BL/6J mice (Jax Labs stock # 000664). Prx1::Cre mice have been maintained on a C57BL/6J genetic background.
6. (Figure 3) *Lrp5*\(^{f}\) mice were created with the CJ7 ES cell line derived from 129S1/SvImJ mice. Germine transmission was obtained by crossing chimeric mice with C57BL/6J females. Neo\(^R\) was deleted from the targeted *Lrp5*\(^{f}\) allele by crossing with FLPeR mice \(^9\), which are on a 129/Sv background. Cmv::Cre mice \(^10\) have been maintained on a mixed FVB/N/SJL background.
7. (Figure 3) Bone measurements in mice with global inactivation of the *Lrp5*\(^{f}\) allele and their non-inactivated littermates were performed on mice with mixed 129/C57BL/6J/FVB/N/SJL backgrounds.
8. (Figure 3) Bone measurements on mice with conditional inactivation of *Lrp5*\(^{f}\) using Dmp1::Cre and their non-inactivated littermates were performed on mice with 129/C57BL/6J/FVB/N background. Dmp1::Cre mice have a mixed CD-1 and 129 background.
9. (Figure 3) Bone measurements on mice with conditional inactivation of *Lrp5*\(^{f}\) using Villin::Cre and their non-inactivated littermates were performed on mice with a C57BL/6J background. Villin::Cre mice have a C57BL/6J background.
10. (Figure 4) Whole blood serotonin measurements following the HPLC method of Tenner and colleagues were performed on Lrp5 knockout, WT, and G/G mice that had been backcrossed onto the C57BL/6J background and on Lrp5 knockout, WT, and +/-A mice on a mixed 129S1/SvIMJ/C57BL/6J background.

11. (Figure 4) Whole blood serotonin and intestine-extracted serotonin measurements following the HPLC method of Liu and colleagues, along with bone measurements following the methods of Iwaniec and colleagues, were performed on Lrp5 knockout and WT littermate controls maintained on a mixed 129SvEvBrd and C57BL/6J-TyrBrd background. These Lrp5 knockout mice exhibit the identical eye phenotype reported by others (data not shown).

12. (Figure 4) Quantitative RT-PCR for Tph1 transcript levels were performed on Lrp5 knockout, WT, and +/-A mice on a mixed 129S1/SvIMJ/C57BL/6J background. These mice were maintained in the same animal facility, were all more than 3-months-old, but were not littermates.

13. (Figure 4) Quantitative RT-PCR for Tph1 transcript levels were performed on female littermate Lrp5 knockout and WT mice on a mixed 129SvEvBrd and C57BL/6J-TyrBrd background. These mice were 50 to 57 weeks old, with Lrp5 knockout mice having low bone mass in the 5th lumbar vertebra, femur midshaft and femoral neck.

14. (Figure 5) Bone measurements were performed on Tph1–/– mice that had been backcrossed to C57BL/6 and FVB/N for greater than 7 generations. Age-matched and strain-matched, but not littermate, WT mice maintained at the same facility were used as controls. Measurements were performed using the methods of Sawakami and colleagues.

15. (Figure 5) Bone measurements in male Tph1–/– (disruption of exon 3 in male mice at 4 months of age and disruption of exons 4 to 7 in male and female mice at 7 months of age) and WT littermate controls maintained on a mixed 129SvEvBrd and C57BL/6J-TyrBrd background were performed following the methods of Iwaniec and colleagues.

16. (Figure 6) For LP-923941 pharmacology studies female C57BL/6 mice and Wistar rats were examined.

17. (Supplementary Figure S7) Serum serotonin measurements utilizing the kit described in an earlier study were performed on Lrp5 knockout, WT, and Lrp5 +/-G and Lrp5 +/-A mice on a mixed 129S1/SvIMJ/C57BL/6J background. These mice were maintained in the same animal facility and were all more than 3-months-old, but were not littermates.

Histomorphometry
Four to five 8-wk-old male mice of each genotype were injected with calcein, 8 and 2 days prior to sacrifice as previously described. Undecalcified left femurs were fixed in 4% neutral-buffered formaldehyde for 48 hours, dehydrated in graded alcohol and embedded in methylmethacrylate. One 20 µm coronal section from the distal femur of each mouse was analyzed using the OsteoMeasure Analysis System (OsteoMetrics). Mineralizing surface (MS/BS; %), mineral apposition rate (MAR; µm/day), and bone formation rate (BFR; µm3/µm2/year) were measured in the distal femur metaphyseal trabecular bone as previously described.

Measurement of whole blood serotonin, serum serotonin, tissue serotonin, and Tph1 mRNA expression, and LacZ and Axin2 mRNA expression.
Whole blood serotonin measures were performed in different cohorts of mice using one of two methods. The method of Tenner et al. (2008) was used for the HBM, Lrp5 knockouts, and their controls. Briefly, the mice were placed under deep anaesthesia using isoflurane. Three hundred µl of blood was then collected by cardiac puncture into a syringe containing 100 µl sodium heparin (300U/ml). The heparinized blood was then placed into a chilled Eppendorf tube containing 10 µl of 70% perchloric acid and 5 µl of ascorbic acid (10 mg/ml), vortexed for 10 seconds, and centrifuged at 4°C, 17,000 x g for 25 minutes. The supernatant was transferred to a new tube, frozen at -80°C, and transported on dry ice. Serotonin measurements were performed using HPLC with fluorometric detection as previously described. For serum serotonin measures, blood was collected from the retro-orbital sinus and permitted to clot at room temperature for 30 minutes. Serum was recovered by centrifugation at 4°C, 17,000 x g for 25 minutes and then assayed by
competitive ELISA (Fitzgerald Industries International) following the manufacturer’s recommendation. The method of Liu et al (2008) was employed for whole blood measurements from Lrp5+/− and Lrp5+/+ littermate controls, except that blood was collected from the retro-orbital sinus of non-anaesthetized mice. Briefly, blood was collected into Capiject tubes containing dipotassium EDTA (Terumo Medical Corp.) and mixed rigorously with 9 volumes of solution containing 0.5M trichloroacetic acid and 0.05M sodium ascorbate. The samples were then filtered through a GF/B filter (Whatman) by centrifugation at 650 x g for 5 minutes at 4°C and analyzed by an HPLC fluorometric method as previously described. Serotonin was extracted from different segments of intestine and measured as previously described.

Quantitative RT-PCR determinations of intestinal Tph1 expression were performed independently in two laboratories. For Lrp5+/−, Lrp5+/+, and Lrp5+/A214V mice on a mixed 129S1/SvIMJ/C57BL/6J background 2.5 cm (~50 mg) of proximal intestine containing the duodenum was excised, opened, washed in PBS, placed in Trizol reagent, and frozen at -80°C. Total RNA was subsequently prepared from each individual duodenum and cDNA was synthesized from 2 µg of RNA using SuperScript First-Strand (Invitrogen). Relative gene expression of Tph1 (F: 5’-TTTCCATCCGTCCTGTGG-3’; R: 5’-TCATCTTCTCCTTTGCATCTTC-3’) normalized to endogenous Gapdh (F: 5’-GCCAAAAGGGTCATCATCTC-3’; R: 5’-CCTGCTTCACCACCTCTTG-3’) was measured in triplicate using Power SYBR Green PCR Master Mix (Applied Biosystems) on a 7300 Real-Time System (Applied Biosystems) and analyzed using the manufacturer’s software to render the expression levels in linear format.

Peripheral dual-energy X-ray absorptiometry (DEXA)

Live mice were anesthetized with isoflurane and placed in a prone position, with limbs outstretched, on a PIXImus dual energy x-ray absorptiometer (GE Lunar). For the longitudinal studies on HBM mice, whole-body scans were collected serially, beginning at 4.5-wk-old and extending bi-weekly until 16.5-wk-old. Whole body scans were collected in 12-wk-old mice with Lrp5−/− and controls, formalin-preserved mouse carcasses were studied. The mouse carcasses were positioned and scanned as described above. For a second strain of Tph1−/− and littermate control mice, whole bone scans were collected in 17-wk-old mice. From the whole body scans, areal bone mineral density (aBMD) and bone mineral content (BMC) were calculated for the post-cranial skeleton, for the lumbar spine (L3-L5, inclusive), and for individual bones using the manufacturer’s analysis software.
The distal femoral metaphyses and the 5th lumbar vertebrae (L5) were scanned ex vivo on one of three desktop µCTs (µCT 20, Scanco Medical AG, Bassersdorf, Switzerland; µCT 40, Scanco Medical AG, Bassersdorf, Switzerland; SkyScan 1172; SkyScan Ltd, Kontich, Belgium) to measure 3-dimensional morphometric properties in the cortical and trabecular bone. The same instrument was used when scanning mutant and wild-type bones from a particular cross to ensure comparability. For scanning the HBM and the control mice, a standardized region of the distal femoral metaphysis, measuring 2.76 mm in height, was scanned at 10-13 µm resolution. For scanning the L5 vertebrae from these mice the central 1.0 mm of the vertebral body was scanned at 10 µm resolution. The distal femoral and L5 vertebral scan stacks were manually segmented to isolate trabecular bone, from which the following parameters were calculated as previously described: bone volume fraction (bone volume per unit total volume; BV/TV), trabecular number (Tb.N), separation (Tb.Sp), and thickness (Tb.Th). The same distal femur algorithm was employed for scanning the Lrp5 knockout and Tph1–/– mice and their littermate controls; the L5 vertebral bodies from these mice were scanned at 8 µm voxel dimension and consisted of images between the growth plates. The starting and ending slices contained equal amounts of primary and secondary spongiosa, and along with the ~ 300 slices between them were used to calculate trabecular bone parameters.

**Biomechanical testing**

Seventeen-wk-old mice were sacrificed and the left femur was dissected free of soft tissues, wrapped in saline-soaked gauze, and frozen at -20°C until testing. Once all samples were collected, the femurs were brought to room temperature slowly (~1.5h) in a saline bath. Femurs were positioned posterior side down across the two lower supports of a three-point bending rig, mounted in a Bose Electroforce 3200 electromagnetic test machine, which has a force resolution of 0.01N. The femurs were loaded to failure in monotonic compression using a crosshead speed of 0.2 mm/s, during which force and displacement measurements were collected every 0.005s. From the force versus displacement curves, ultimate force (in N), yield force (in N), stiffness (in N/mm), and energy to failure (in mJ) were calculated using standard equations. Prior to testing, the length of each femur was measured to the nearest 0.1 mm, along the diaphyseal axis, using digital calipers.

**Characterization of Tph1–/– mice**

Three separate lines of Tph1 knockout mice were studied. Two are published, one from MDC, Berlin, Germany, and the second from Lexicon Pharmaceutical’s, Inc. A third knockout, also from Lexicon, lacks exons 4 to 7 is unpublished. Intestinal serotonin content is reduced by over 95% in all 3 knockout lines. Mice maintained at the MDC in Berlin were euthanized by overdose of isoflurane, and trunks, purified of skin and inner organs were fixed in 4% PFA for the further µCT analysis and DEXA scanning of the spine. Local German authorities approved the studies with standards corresponding to those prescribed by the American Physiological Society. Mice maintained at Lexicon Pharmaceuticals, Incorporated (The Woodlands, Texas) were euthanized with CO2.

**Pharmacological inhibition of gut Tph1 activity**

C57BL/6 mice underwent bilateral ovariectomy or sham surgery at 16-wks-old and were treated for 6 weeks starting at 67-weeks-old with LP-923941. Successful ovariectomy surgery was confirmed by a 72% reduction in uterine wet weight, a 27% increase in body weight, and a 12% increase in midshaft femur marrow cavity area. Each of these parameters is affected in a similar manner in chronically OVX rats. A preliminary 7-day dose-response study in 9-wk-old female C57BL/6 mice (Figure 7A) showed LP-923941 given at a dose of 250 mg/kg by daily oral gavage decreased serotonin contents by 85% in the jejunum, 84% in the ileum, 58% in the duodenum, and greater than 35% in both the proximal and distal colon. There was no reduction in brain serotonin content, consistent with the observation that this compound does not cross the blood-brain barrier.

For the 6 week mouse pharmacology study, LP-923941 was given by daily oral gavage at a dose of 250 mg/kg in a vehicle of 10% propylene glycol. As a positive control that stimulates bone formation, teriparatide, (hPTH 1-34, Bachem) was given daily by subcutaneous injection at a dose of 80 µg/kg in a vehicle of 20 mM.
NaH2PO4 in 0.9% saline. Both sham-surgery and ovariectomized mice were given vehicles, LP-923941 or teriparatide. Half of the control mice received LP-923941 vehicle by oral gavage and half received teriparatide vehicle by injection. These two subsets of control mice did not show differences in any measured parameter and were combined for analyses. At the conclusion of the study, whole blood and intestinal segments were analyzed for serotonin contents and ex vivo µCT analyses were performed on cortical bone in the midshaft femur and cancellous bone in the 5th lumbar vertebral body. Since the quantity of cancellous bone in the distal femur metaphysis is minimal in aged female C57BL/6 mice, this bone site was not examined. Methods were identical to those employed for analyzing Lrp5 knockout mice. For the PINP assay, blood was collected in heparinized capillary tubes from non-anesthetized mice by retro-orbital bleeding. This blood was immediately diluted 5-fold into the assay buffer, centrifuged, and the supernatant frozen until analysis. PINP was measured in singlicate using a Rat/Mouse PINP EIA (Immunodiagnostic Systems).

Wistar rats were treated with 50 or 250 mg/kg LP-923941 (vehicle of 10% propylene glycol) by daily oral gavage for 6 weeks starting 5 weeks after bilateral ovariectomy or sham surgery at 12 to 15 weeks of age, body weight 225-250 gram. As a positive control teriparatide, in a vehicle of neutral PBS containing 2% rat serum, was administered subcutaneously at a dose of 80 µg/kg. Successful ovariectomy surgery was confirmed by a 76% reduction in uterine wet weight. LP-923941 exposure was confirmed weekly, 2 hours after the daily dose, and found to be approximately 800 nM in intact and OVX rats throughout the 6-week treatment period. Intestinal serotonin contents, after extraction with 0.1% formic acid and 0.05M sodium ascorbate, were determined by HPLC on a SeQuant SIC HILIC (50 x 2.1) column (Merck SeQuant AB, Umea, Sweden). Trabecular bone volume (BV/TV) was measured in the 5th lumbar vertebral body and distal femur metaphysis with a SkyScan 1076 µCT scanner (SkyScan Ltd, Kontich, Belgium).

**Statistical analyses**

Longitudinal data, comprising serial DEXA scans and body weight measurements, were tested for differences among genotypes using single classification repeated measures ANOVA. Differences among genotypes in cross-sectional measurements (e.g. µCT data, biomechanical properties, serotonin measurements) were tested for significance using single classification ANOVA, followed by the Sheffe post-hoc comparison to probe pairwise comparisons in the event that the omnibus ANOVA was significant. These statistical calculations were performed in StatView 5.0 (SAS Inc., Durham, NC) for Windows. For the pharmacology study, two- or three-factor ANOVAs were performed using SPSS for Windows, version 11.5.0 (SPSS, Chicago, IL). For all tests, the experiment-wise error rate was set at $\alpha=0.05$.

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SUPPLEMENTARY DATA
Figure S1: Characterization of mice with HBM Lrp5 alleles (A) Graphs depicting the body mass measured using a standard laboratory scale in cohorts of female (left) and male (right) WT (+/+), littersmate with knockin Lrp5 HBM mutations (p.A214V HBM knocking mutation represented in upper panels (+/A and A/A); p.G171V HBM knocking mutation represented in lower panels (+/G and G/G)). (B) Graphs depicting whole body bone mineral content (BMC) measured via DEXA in cohorts of female (left) and male (right) Lrp5 WT, +/A, +/G and G/G mice. * indicates a significant difference ($p < 0.05$) when compared to WT mice, whereas # indicates a significant difference ($p < 0.05$) when compared to (+/A and +/G) mice. (C) Graphs depicting mRNA expression in 16-week-old Lrp5 WT and A/A mice as determined by qRT-PCR. (left panel) Normalized LacZ transcript levels femur cortex RNA from atGal negative (-) mice and in Lrp5 WT and A/A atGal positive (+) mice with Rplp2 transcript serving as an internal control (right panel) Normalized Axin2 transcript levels in Lrp5 WT and A/A mice, with Rplp2 transcript serving as an internal control. The mean LacZ or Axin2 transcript levels for female Lrp5 WT mice is set at 100%. The numbers of mice studied ($n =$) are indicated, as are error bars equal to 1 s.d. Asterisks (*) indicate a significant difference ($p < 0.05$) compared to WT mice of the same sex.
Figure S2: Characterization of mice with Neo<sup>R</sup>-containing HBM Lrp5 alleles. Graphs depicting trabecular bone architectural properties in the distal femur (upper row) and the 5<sup>th</sup> lumbar vertebra (lower row) in WT mice (+/+ ) and mice with Neo<sup>R</sup>-containing HBM Lrp5 alleles (+/A<sub>N</sub> and +/G<sub>N</sub>). No significant differences were observed.
Figure S3: μCT reconstructions of a 0.8 mm span of the distal femoral metaphysis in male mice with inherited and conditionally-activated HBM alleles. (A) Reconstructions from WT mice and mice heterozygous for a Neoβ-containing HBM allele (+/AN) are similar in bone mass and structure. (B) Reconstructions in mice with and without Neoβ-containing HBM alleles (+/AN and +/A, respectively), and with (+) and without (-) the Dmp1::Cre transgene or the Villin::Cre transgene are depicted. Note that trabecular and cortical bone mass in mice with the AN allele increases when the Dmp1-transgene is present (top row) to levels comparable to those seen in mice with the inherited HBM allele (+/A) (third column). In contrast, there is no difference in trabecular and cortical bone mass in (+/AN) mice when the Villin-Cre transgene is present (bottom row). The bones shown are from mice that exhibited the median values for distal femoral BV/TV within each genotype combination, and are therefore representative of the groups.
Figure S4: Photomicrographs of non-demineralized histologic sections from the distal femurs in 8-wk-old female mice with inherited and conditionally activated HBM alleles. (A) MacNeal's tetrachrome staining, countered with Von Kossa staining, reveals increased cortical and trabecular bone area (black staining) in mice with the inherited HBM allele (+/A) and in mice with the NeoR-containing HBM allele (+/AN) and the Dmp1::Cre transgene, compared to mice with the NeoR-containing HBM allele (+/AN) but not the Dmp1::Cre transgene. Also note the increased prevalence of plump, active osteoblasts (darker blue cells at the tips of the green arrowheads) in mice with the inherited HBM allele and in mice with the NeoR-containing HBM allele and the Dmp1::Cre transgene, compared to mice with the NeoR-containing HBM allele but not the Dmp1::Cre transgene. The images in the lower panels are higher magnification images of the areas indicated by the red box in the upper panels. (B) Sections from the same mice depicted in panel A, prepared and photographed to reveal dual fluorochrome labeling, show increased labeling and increased inter-label distance (distance between the red arrowheads) in mice with the inherited HBM allele and in mice with the NeoR-containing HBM allele and the Dmp1::Cre transgene, compared to mice with the NeoR-containing HBM allele but not the Dmp1::Cre transgene. The images in the lower panels are higher magnification images of the areas indicated by the red box in the upper panels.
Figure S5: Conditional inactivation of Lrp5 using Dmp1::Cre and Villin::Cre (A) Photographs of agarose gels containing PCR amplimers derived from mouse genomic DNA extracted from a variety of tissues in four 9-wk-old male littermates that are homozygous for the Lrp5<sup>f/f</sup> allele. Two littermates were Dmp1::Cre transgene negative (top two panels) and two were Dmp1::Cre transgene positive (bottom two panels). Among the Dmp1::Cre-positive littermates, Cre-mediated excision of the floxed Lrp5 allele (530 bp band) occurred in cortical bone and to a lesser extent in skeletal muscle (gastrocnemius), colon, and cerebrum. (B) Representative μCT reconstructions of a 0.8 mm span of the distal femoral metaphysis in 16-wk-old female and male mice, illustrating the low bone mass phenotype (cortical thinning and trabecular bone paucity) generated when Lrp5 is inactivated in Dmp1::Cre-expressing cells. (C) Fluorescence microscope images from the duodenum of a mouse carrying the Villin::Cre transgene and the Rosa<sup>26<sub>TmG</sub></sup> allele. The Rosa<sup>26<sub>TmG</sub></sup> allele expresses Tomato fluorescent protein, until Cre-mediated recombination causes Green fluorescent protein (GFP) expression. DAPI staining indicates the locations of all cell nuclei. Note that Villin::Cre produces GFP expression in all epithelial cells, which include enterochromaffin cells, and not in non-epithelial cells. (D) Representative μCT reconstructions of a 0.8 mm span of the distal femoral metaphysis in 9-wk-old female mice illustrating the similarity in phenotype among Lrp5<sup>f/f</sup> mice with or without the Villin::Cre transgene.
Figure S6: Conditional activation of HBM alleles using Prx1::Cre.  (A) Representative μCT scan reconstructions from 12-wk-old female mice with and without Neo^R^-containing HBM alleles (+/A_N and +/-A, respectively), and with (+) and without (-) the Prx1::Cre transgene. Note the findings in the midshaft femur cortical bone and the distal femur trabecular bone in mice with the A_N allele and the Prx1::Cre transgene are comparable to those findings seen in mice with the inherited HBM allele (+/A). In contrast, the Prx1::Cre transgene has no effect on the 5th lumbar vertebral trabecular bone mass. (B) Table of trabecular bone architectural properties in mice with and without Neo^R^-containing HBM alleles (+/A_N and +/-A, respectively), and with (+) and without (-) the Prx1::Cre transgene. In mice with the Neo^R^-containing HBM allele (+/A_N), presence of the Prx1::Cre transgene significantly increases distal femoral trabecular bone architectural properties, but has no effect on vertebral trabecular bone architectural properties. The increase in femoral trabecular bone properties is comparable to that seen in mice with the inherited HBM allele (+/A). (C) Photograph of an agarose gel containing PCR amplifiers derived from mouse genomic DNA extracted from marrow-flushed tibia, cleaned and washed vertebral body, and duodenum. PCR amplifiers correspond to the sizes expected from the targeting strategy depicted in Fig. 1a. Mice with +/-A genotypes produce amplifiers corresponding to the WT allele (arrowhead) and the HBM allele (arrow), whereas mice with +/-A_N or +/-G_N genotypes produce amplifiers corresponding to the wild-type allele (arrowhead) and the Neo^R^-containing HBM allele (double arrowhead). Mice that are double heterozygotes for the Neo^R^-containing HBM allele and the Prx1::Cre transgene produce additional amplifiers corresponding to wild-type and HBM alleles when DNA from tibia is used as template, indicating that Cre-recombination occurred in that bone. In contrast there is no evidence that Cre-recombination occurred in the vertebral body or in the duodenum, indicating that non-specific Cre activity did not occur in the spine or the duodenum.

### Table: µCT-based trabecular bone phenotype measurements in mice harboring heterozygous, conditionally-activated (A_N) or globally active (A) A214V high bone mass alleles at the Lrp5 locus, with or without Prx1-driven expression of Cre recombinase to activate the conditional HBM alleles^a

| 5th Lumbar µCT | +/A_N | +/A |  
|----------------|-------|-----|  
| Tb.N (1/mm)    | 5.31 ±0.31 | 4.70 ±0.34 | 4.55 ±0.33 | 4.68 ±0.34 |  
| Tb.Th (μm)     | 72.1 ±5.3 | 99.1 ±19.4 | 104.7 ±9.4 | 102.5 ±16.8 |  
| Tb.Sp (μm)     | 293.2 ±57.0 | 172.0 ±27.2 | 176.9 ±25.8 | 170.0 ±23.9 |  
| Tb.BMC (mg)    | 0.48 ±0.48 | 1.33 ±0.47 | 1.60 ±0.40 | 1.43 ±0.39 |  

Values presented are mean ± SD.

^aIndicates significantly different from Cre-negative +/-A_N control mice

# B

### Table: µCT-based trabecular bone phenotype measurements in mice harboring heterozygous, conditionally-activated (A_N) or globally active (A) A214V high bone mass alleles at the Lrp5 locus, with or without Prx1-driven expression of Cre recombinase to activate the conditional HBM alleles^a

| Lrp5 Alleles | Prx1::Cre transgene | +/A_N | +/A |  
|--------------|---------------------|-------|-----|  
| Distal Femur µCT |  
| Tb.N (1/mm)    | 3.57 ±0.45 | 3.81 ±0.48 | 4.84 ±0.34 | 5.24 ±0.40 |  
| Tb.Th (μm)     | 82.0 ±5.8 | 74.3 ±9.2 | 104.5 ±15.9 | 97.6 ±11.9 |  
| Tb.Sp (μm)     | 286.6 ±45.4 | 269.3 ±42.0 | 217.1 ±15.7 | 191.8 ±23.9 |  
| Tb.BMC (mg)    | 0.15 ±0.05 | 0.15 ±0.03 | 0.31 ±0.07 | 0.32 ±0.06 |  

Values presented are mean ± SD.

^aIndicates significantly different from Cre-negative +/-A_N control mice
Figure S7: Serum serotonin (5HT) in mice with different Lrp5 genotypes. (A) Graphs depicting serum 5HT levels measured by competitive ELISA in 3-mo-old Lrp5 WT (+/+) and knockout (-/-) mice on a 129Sv/J background, and in Lrp5 WT and heterozygous HBM (+/A and +/G) mice on a mixed 129Sv/C57BL/6J background. (B) Graphs depicting serum 5HT levels measured by HPLC in 3-mo-old Lrp5 WT and knockout littermates on a mixed 129SvEvBrd/C57BL/6J-Tyr^c-Brd background. Serum serotonin levels did not differ between mice with different Lrp5 genotypes.
Figure S8: Bone mass following pharmacologic inhibition of Tph1. μCT reconstructions of the 5th lumbar vertebrae in female sham operated or ovariectomized (OVX) mice treated with vehicle, the Tph1 inhibitor LP-923914, or teriparatide (hPTH 1-34). Note that LP-923941 had no effect on bone mass, whereas teriparatide increased bone mass in both sham and OVX mice. The bones shown are from mice that exhibited the median values for vertebral BV/TV within each treatment group, and are therefore representative of the groups.
Figure S9: (A) Graphs depicting the final body weights (left panel) and final uterine weights (right panel) of sham (SHM) and ovariectomized (OVX) rats after having been treated daily for 6 weeks with vehicle, LP-923941 (50 or 250 mg/kg/day), or teriparatide (PTH, 80 μg/kg/day). (B) μCT reconstructions of the 5th lumbar vertebrae in female sham operated or ovariectomized (OVX) rats treated with vehicle-alone (0 mg/kg/day), the Tph1 inhibitor LP-923914 (50 or 250 mg/kg/day), or teriparatide (hPTH 1-34, 80 μg/kg/day). Note that LP-923941 had no effect on bone mass in OVX rats, whereas teriparatide increased bone mass in OVX rats. The bones shown are from rats that exhibited the median values for vertebral BV/TV within each treatment group, and are therefore representative of the groups.
Figure S10: Model by which LRP5 mutations in osteocytes affect bone mass. (A) Endogenous inhibitors, such as SOST and DKK1, normally bind LRP5 and block Wnt-mediated LRP5 signaling. Mechanical stress reduces SOST and DKK1 expression, thereby relieving LRP5 inhibition. (B) Mutations that cause a lack of LRP5 reduce the osteocyte's ability to participate in Wnt-mediated signal transduction. This loss may be partially compensated by LRP6, a closely related paralog of LRP5. (C) LRP5 HBM mutations in the 4th (e.g., G171V) or 5th (e.g., A214V) blade of the 1st β-propeller (yellow bands on the blades, indicated by the arrow) reduce the ability of endogenous LRP5 inhibitors such as SOST and DKK1 to bind LRP5, thereby enabling Wnt-mediated signaling in the absence, or at reduced levels, of mechanical stress.
### TABLE S1: Bone phenotype measurements in wild type and knockin mice harboring high bone mass alleles at the Lrp5 locus

| Mutation/Parameter | WT | Heterozygous | Homozygous | WT | Heterozygous | Homozygous |
|--------------------|----|--------------|------------|----|--------------|------------|
| **A214V mutation** |    |              |            |    |              |            |
| 5th Lumbar µCT     |    |              |            |    |              |            |
| Tb.N (1/mm)        | 2.91 ±0.50 | 5.35 ±0.45* | 5.62 ±0.48* | 3.20 ±0.50 | 4.43 ±0.38* | 4.16 ±0.56* |
| Tb.Th (µm)         | 68.0 ±2.5  | 89.6 ±3.4*   | 92.0 ±6.0*  | 68.5 ±5.4  | 104.1 ±8.3* | 100.6 ±9.1* |
| Tb.Sp (µm)         | 253.1 ±27.8 | 169.9 ±16.8* | 154.5 ±15.3* | 212.7 ±21.7 | 162.6 ±20.1* | 170.5 ±32.3* |
| Distal Femur µCT   |    |              |            |    |              |            |
| Tb.N (1/mm)        | 1.12 ±0.35 | 3.67 ±0.45*  | 4.21 ±0.39## | 1.80 ±0.48 | 3.98 ±0.40* | 3.69 ±0.60* |
| Tb.Th (µm)         | 57.5 ±4.0  | 104.6 ±6.1*  | 121.6 ±11.1*# | 68.5 ±6.4  | 98.9 ±4.9*  | 96.2 ±5.9*  |
| Tb.Sp (µm)         | 283.7 ±25.3 | 178.0 ±18.3* | 141.7 ±21.8## | 279.4 ±51.3 | 171.6 ±17.7* | 188.2 ±32.4* |
| Midshaft Femur Strength |     |              |            |    |              |            |
| Stiffness (N/mm)   | 84.9 ±9.8  | 112.8 ±9.4*  | 123.0 ±8.5* | 87.4 ±6.9  | 130.8 ±12.0* | 134.1 ±10.9* |
| Yield Force (N)    | 14.5 ±2.2  | 22.5 ±3.4    | 31.0 ±10.0*# | 19.5 ±2.6  | 36.9 ±4.6*  | 39.9 ±7.0*  |
| Postyield Disp. (µm) | 285.9 ±68.6 | 353.1 ±79.8  | 357.8 ±153.5 | 292.6 ±72.2 | 316.9 ±98.3 | 405.5 ±66.2## |
| Femur Length       | 16.31 ±0.29 | 16.61 ±0.44  | 16.94 ±0.24* | 16.36 ±0.26 | 17.04 ±0.32* | 17.31 ±0.43* |
| Midshaft Femur µCT |    |              |            |    |              |            |
| Tt.Ar (mm²)        | 1.86 ±0.19 | 2.09 ±0.17*  | 2.32 ±0.09## | 2.33 ±0.14 | 2.75 ±0.23* | 3.10 ±0.26## |
| Ct.Ar (mm²)        | 0.97 ±0.07 | 1.33 ±0.10*  | 1.44 ±0.09## | 1.18 ±0.08 | 1.88 ±0.22* | 1.87 ±0.20*  |
| Md.Ar (mm²)        | 0.89 ±0.13 | 0.76 ±0.08*  | 0.88 ±0.05## | 1.15 ±0.13 | 0.86 ±0.08* | 1.21 ±0.16*  |
| Ct.Th (mm)         | 0.25 ±0.01 | 0.34 ±0.02*  | 0.34 ±0.02*  | 0.26 ±0.02 | 0.42 ±0.04* | 0.36 ±0.02## |
| l_{min} (mm⁴)      | 0.16 ±0.03 | 0.23 ±0.04*  | 0.30 ±0.03## | 0.25 ±0.03 | 0.42 ±0.07* | 0.50 ±0.08## |
| l_{max} (mm⁴)      | 0.28 ±0.05 | 0.40 ±0.06*  | 0.45 ±0.03## | 0.42 ±0.05 | 0.72 ±0.14* | 0.84 ±0.18## |
| **G171V mutation** |    |              |            |    |              |            |
| 5th Lumbar µCT     |    |              |            |    |              |            |
| Tb.N (1/mm)        | 3.65 ±0.67 | 4.83 ±0.39*  | 5.00 ±0.38* | 2.81 ±0.49 | 3.78 ±0.17* | 4.67 ±0.21## |
| Tb.Th (µm)         | 65.0 ±1.9  | 83.0 ±4.4*   | 82.6 ±6.0*  | 67.6 ±4.9  | 82.9 ±7.9*  | 90.7 ±9.7*  |
| Tb.Sp (µm)         | 279.4 ±37.6 | 197.8 ±22.7* | 193.5 ±26.9* | 279.9 ±35.7 | 212.4 ±24.5* | 161.5 ±16.7## |
| Distal Femur µCT   |    |              |            |    |              |            |
| Tb.N (1/mm)        | 1.37 ±0.30 | 3.85 ±0.36*  | 4.22 ±0.46* | 1.57 ±0.29 | 3.63 ±0.46* | 4.55 ±0.48## |
| Tb.Th (µm)         | 57.7 ±4.4  | 89.9 ±3.4*   | 97.3 ±14.3* | 59.7 ±5.2  | 81.1 ±5.6*  | 90.2 ±5.7##  |
| Tb.Sp (µm)         | 275.4 ±27.0 | 179.0 ±13.8* | 155.5 ±24.7* | 272.8 ±26.0 | 178.7 ±16.5* | 148.9 ±16.3## |
| Midshaft Femur Strength |     |              |            |    |              |            |
| Stiffness (N/mm)   | 86.2 ±7.7  | 106.0 ±8.0*  | 110.4 ±12.8* | 86.4 ±6.3  | 93.9 ±9.7  | 115.1 ±10.6## |
| Yield Force (N)    | 16.1 ±1.9  | 22.7 ±1.4*   | 26.5 ±6.5*  | 17.5 ±2.5  | 21.7 ±2.7  | 28.9 ±6.1##  |
| Postyield Disp. (µm) | 182.1 ±63.3 | 236.6 ±96.7  | 250.3 ±119.1 | 124.4 ±79.0 | 214.1 ±55.2* | 269.3 ±108.6* |
| Femur Length       | 16.00 ±0.17 | 16.16 ±0.34  | 16.03 ±0.46 | 15.98 ±0.36 | 16.03 ±0.19 | 16.33 ±0.32 |
| Midshaft Femur µCT |    |              |            |    |              |            |
| Tt.Ar (mm²)        | 1.68 ±0.12 | 1.82 ±0.17   | 1.93 ±0.15* | 1.90 ±0.15 | 1.76 ±0.09  | 2.19 ±0.17## |
| Ct.Ar (mm²)        | 0.97 ±0.05 | 1.13 ±0.08*  | 1.26 ±0.14## | 1.08 ±0.08 | 1.16 ±0.10  | 1.49 ±0.19## |
| Md.Ar (mm²)        | 0.71 ±0.11 | 0.69 ±0.10   | 0.67 ±0.04  | 0.82 ±0.08 | 0.60 ±0.04* | 0.71 ±0.11*  |
| Ct.Th (mm)         | 0.27 ±0.02 | 0.31 ±0.01*  | 0.34 ±0.03## | 0.28 ±0.01 | 0.33 ±0.03* | 0.37 ±0.05## |
| l_{min} (mm⁴)      | 0.16 ±0.02 | 0.19 ±0.03   | 0.21 ±0.04* | 0.18 ±0.03 | 0.17 ±0.02  | 0.27 ±0.05## |
| l_{max} (mm⁴)      | 0.21 ±0.03 | 0.27 ±0.04*  | 0.33 ±0.05## | 0.30 ±0.05 | 0.29 ±0.05  | 0.44 ±0.08## |

AValues presented are mean ± SD.

*indicates significantly different from +/+ control

#indicates significantly different from heterozygous mutant
TABLE S2: µCT-based trabecular bone phenotype measurements in mice harboring heterozygous, conditionally-activated (AN or GN) or globally active (A or G) high bone mass alleles at the Lrp5 locus, in conjunction with gut-driven or bone-driven expression of Cre recombinase to activate the conditional HBM alleles\textsuperscript{a}

| Lrp5 Alleles: | +/A\textsubscript{N} | +/A | +/G\textsubscript{N} | +/G |
|--------------|----------------|-----|----------------|-----|
| Cre transgene: | - | + | - | + |

**Villin driver for Cre**

| Distal Femur µCT | | | |
|------------------|------------------|------------------|------------------|
| Tb.N (1/mm) | | | |
| Females | 2.86 ±0.06 | 2.89 ±0.27 | 4.10 ±0.23* | 4.04 ±0.37* |
| Males | 3.82 ±0.16 | 4.10 ±0.39 | 4.97 ±0.28* | 4.96 ±0.46* |
| Tb.Th (µm) | | | |
| Females | 66.5 ±5.1 | 63.0 ±5.6 | 96.0 ±7.2* | 93.6 ±5.2* |
| Males | 81.5 ±4.8 | 83.5 ±10.5 | 104.5 ±8.3* | 110.3 ±14.9* |
| Tb.Sp (µm) | | | |
| Females | 344.8 ±7.72 | 345.1 ±37.5 | 209.2 ±19.2* | 220.3 ±26.5* |
| Males | 254.4 ±11.9 | 234.8 ±24.3 | 164.1 ±15.1* | 167.2 ±30.7* |

**5\textsuperscript{th} Lumbar µCT**

| Tb.N (1/mm) | | | |
| Females | 3.91 ±0.3 | 3.89 ±0.26 | 4.57 ±0.57* | 4.25 ±0.48 |
| Males | 4.43 ±0.27 | 4.36 ±0.52 | 5.11 ±0.73 | 5.08 ±0.48* |
| Tb.Th (µm) | | | |
| Females | 80.7 ±5.7 | 82.8 ±5.8 | 110.5 ±4.3* | 111.4 ±6.4* |
| Males | 97.5 ±19.1 | 89.9 ±15.6 | 127.3 ±29.7 | 136.6 ±20.4* |
| Tb.Sp (µm) | | | |
| Females | 243.0 ±23.8 | 246.4 ±20.4 | 213.3 ±36.9 | 234.0 ±35.5 |
| Males | 208.6 ±16.1 | 213.7 ±33.5 | 180.6 ±35.4 | 182.4 ±24.3 |

**Dmp1 driver for Cre**

| Distal Femur µCT | | | |
|------------------|------------------|------------------|------------------|
| Tb.N (1/mm) | | | |
| Females | 2.91 ±0.63 | 4.50 ±0.52* | 4.16 ±0.58* | 4.45 ±0.37* |
| Males | 3.44 ±0.63 | 4.87 ±0.39* | 4.83 ±0.39* | 4.90 ±0.44* |
| Tb.Th (µm) | | | |
| Females | 68.8 ±5.1 | 91.2 ±8.4* | 83.9 ±9.2* | 87.6 ±10.3* |
| Males | 76.3 ±11.4 | 91.7 ±13.8* | 88.5 ±13.8 | 91.1 ±10.9* |
| Tb.Sp (µm) | | | |
| Females | 358.6 ±79.2 | 193.8 ±41.8* | 221.0 ±47.5* | 197.0 ±29.6* |
| Males | 300.2 ±46.2 | 183.8 ±23.7* | 184.5 ±24.5* | 180.6 ±29.3* |

**5\textsuperscript{th} Lumbar µCT**

| Tb.N (1/mm) | | | |
| Females | 3.59 ±0.62 | 4.47 ±0.56* | 4.74 ±0.61* | 4.56 ±0.36* |
| Males | 4.10 ±0.54 | 5.13 ±0.56* | 5.04 ±0.45* | 5.19 ±0.30* |
| Tb.Th (µm) | | | |
| Females | 88.9 ±12.5 | 103.8 ±11.2* | 110.6 ±18.8* | 104.4 ±15.0* |
| Males | 95.3 ±18.2 | 128.1 ±28.9 | 128.3 ±31.6 | 137.8 ±29.2* |
| Tb.Sp (µm) | | | |
| Females | 291.9 ±49.4 | 232.4 ±36.0 | 212.9 ±39.5* | 229.6 ±20.6* |
| Males | 250.1 ±30.5 | 194.1 ±22.9* | 199.3 ±23.3* | 184.4 ±17.3* |

\textsuperscript{a}Values presented are mean ± SD.

*Indicates significantly different from Cre-negative +/A\textsubscript{N} or +/G\textsubscript{N} control mice
### TABLE S3: Bone phenotype measurements in Tph1 mutant mice

| Age/Strain/Parameter | Tph1+/+ | Tph1−/− | % difference |
|----------------------|---------|---------|--------------|
| 4 mo. old, FVB/N bkgrnd (DEXA) |         |         |              |
| spine BMC (mg)       | 21.4 ± 1.0 | 21.4 ± 3.0 | 0%; p = 0.98 |
| 5th Lumbar µCT       |         |         |              |
| Tb.N (1/mm)          | 3.20 ±0.46 | 3.47 ±0.41 | +8.4%; p = 0.31 |
| Tb.Th (µm)           | 68.4 ± 6.8 | 68.4 ± 10.4 | 0%; p = 0.99 |
| Tb.Sp (µm)           | 331.3 ±49.1 | 299.2 ±39.3 | -9.7%; p = 0.99 |
| Distal Femur µCT     |         |         |              |
| Tb.N (1/mm)          | 1.10 ±0.16 | 1.65 ±0.30 | +50.0%; p = 0.003 |
| Tb.Th (µm)           | 64.5 ±9.0 | 64.4 ±6.8 | -0.2%; p = 0.98 |
| Tb.Sp (µm)           | 203.3 ±22.9 | 148.6 ±26.7 | -26.9%; p = 0.002 |
| 6 mo. old, C57BL/6 bkgrnd (DEXA) |         |         |              |
| spine BMC (mg)       | 21.7 ± 3.9 | 22.9 ± 4.2 | +5.5%; p = 0.60 |
| 5th Lumbar µCT       |         |         |              |
| Tb.N (1/mm)          | 3.96 ±0.44 | 3.35 ±0.55 | -15.4%; p = 0.07 |
| Tb.Th (µm)           | 65.6 ±5.6 | 68.5 ±8.0 | +4.4%; p = 0.08 |
| Tb.Sp (µm)           | 245.0 ±30.8 | 307.1 ±65.2 | +25.3%; p = 0.51 |
| Distal Femur µCT     |         |         |              |
| Tb.N (1/mm)          | 1.33 ±0.41 | 1.08 ±0.47 | -18.8%; p = 0.30 |
| Tb.Th (µm)           | 64.2 ±10.4 | 60.8 ±13.4 | -5.3%; p = 0.62 |
| Tb.Sp (µm)           | 171.1 ±35.0 | 229.3 ±86.4 | +34.0%; p = 0.14 |
| 4 mo. old, 129+C57BL/6 bkgrnd (DEXA) |         |         |              |
| spine BMC (mg)       | ---ND--- | ---ND--- | ---ND---     |
| 5th Lumbar µCT       |         |         |              |
| Tb.N (1/mm)          | 5.37 ±0.17 | 5.48 ±0.14 | +2.7%; p = 0.64 |
| Tb.Th (µm)           | 48.1 ±1.1 | 49.9 ±1.2 | +3.7%; p = 0.27 |
| Tb.Sp (µm)           | ---ND--- | ---ND--- | ---ND---     |
| Distal Femur µCT     |         |         |              |
| Tb.N (1/mm)          | 4.00 ±0.40 | 4.10 ±0.40 | +2.5%; p = 0.48 |
| Tb.Th (µm)           | 48.0 ±0.7 | 48.0 ±0.6 | 0%; p = 0.98 |
| Tb.Sp (µm)           | ---ND--- | ---ND--- | ---ND---     |

AValues presented are mean ± SD.

BND denotes data not available.

CSignificant changes associated with mutation are indicated in bold.
Table S4: Enzymatic and Cell-Based Potencies of LP-923941 and Related Compounds

| Compound                        | IC50 Value (Human TPH1) | IC50 Value (Rat RBL Cells) | IC50 Value (Human BON Cells) |
|---------------------------------|--------------------------|----------------------------|------------------------------|
| LP-533401 (enantiomer mixture)  | 2.41 µM                  | 336 nM                     | 11.6 µM                      |
| LP-923941 (active enantiomer)   | 1.24 µM                  | 165 nM                     | 4.0 µM                       |
| LP-660417 (inactive enantiomer) | 13.1 µM                  | 3,378 nM                   | 246 µM                       |