ISCI, Volume *20*

Supplemental Information

Lrp4 Mediates Bone Homeostasis and Mechanotransduction through Interaction with Sclerostin *In Vivo*

Whitney A. Bullock, April M. Hoggatt, Daniel J. Horan, Andrew J. Elmendorf, Amy Y. Sato, Teresita Bellido, Gabriela G. Loots, Fredrick M. Pavalko, and Alexander G. Robling

Lrp4: +/+ KI/KI

A

B

C

Figure S1

Figure S1. Lrp4KI mice display supernumerary teeth and altered molar cusp patterns. Related to Figure 2. (A) µCT reconstructions from the occlusal view of the maxillary incisors illustrating a set of secondary incisors (blue arrow) distolingual to the primary set in WT (+/+) and $Lrp4^{KI}$ (KI/KI) mice. (B and C) Close-up view of the mandibular dentition reveal extra incisors of varying development, and additional molar teeth on the distal end and lingual edge of the tooth row (blue arrows). The molars also exhibit altered cusp pattern compared to those seen in the WT mice.

Table S1. Radiographic and biomechanical analysis of female Lrp4 WT, HET and KI mice. Related to Figure 2.

	Wild-type (WT)	**R1170W/+ (HET)**	**R1170W/R1170W (KI)**
Whole Body BMC (18 wk; g)	0.49 ± 0.1	0.47 ± 0.1	0.77 ± 0.1
Body Weight (18 wk; g)	22.5 ± 1.3	22.1 ± 1.0	21.6 ± 2.2
Femur length (mm)	15.2 ± 0.5	15.0 ± 0.2	15.3 ± 0.5
Femur µCT			
Tb.N (1/mm)	2.16 ± 0.35	1.88 ± 0.30 *	2.84 ± 0.29 *
Tb.Th (mm)	0.04 ± 0.01	0.04 ± 0.01	0.08 ± 0.01 *
Tb.Sp (mm)	0.47 ± 0.08	0.55 ± 0.09 *	0.34 ± 0.05 *
Tb.BMD (mg/cm^3)	868 ± 40	879 ± 37	924 ± 34 *
Tb.BMC (mg)	0.06 ± 0.12	0.02 ± 0.01	0.41 ± 0.18 *
Ct.BMD (mg/cm^3)	1.08 ± 0.02	1.08 ± 0.01	1.09 ± 0.01 *
Ct.BMC (mg)	2.59 ± 0.42	2.60 ± 0.09	3.97 ± 0.70 *
5th Lumbar µCT			
Tb.N (1/mm)	3.79 ± 0.8	3.69 ± 1.0	5.93 ± 1.1 *
Tb.Th (mm)	0.05 ± 0.01	0.05 ± 0.01	0.08 ± 0.02 *
Tb.Sp (mm)	0.26 ± 0.04	0.27 ± 0.05	0.17 ± .05 *
Tb.BMD (mg/cm^3)	871 ± 10	878 ± 15	871 ± 19
Tb.BMC (mg)	0.28 ± 0.19	0.26 ± 0.14	0.79 ± 0.27 *
Femur 3 point bending			
Stiffness (N/mm)	70.2 ± 12.2	67.9 ± 21.9	108.6 ± 2.6 *
Energy to F_U (mJ)	3.38 ± 1.3	4.10 ± 1.3	9.35 ± 1.6 *
Energy to F_F (mJ)	10.1 ± 2.6	9.6 ± 2.9	15.3 ± 4.7 *

*$p<0.05$ for comparison to WT mice, using one-way ANOVA followed by Fisher's PLSD post hoc tests.

Table S2. Radiographic and biomechanical analysis of male Lrp4 WT, HET and KI mice. Related to Figure 2.

	Wild-type (WT)	**R1170W/+ (HET)**	**R1170W/R1170W (KI)**
Whole Body BMD (18 wk; g/cm^2)	70.1 ± 7.3	75.0 ± 8.0	91.6 ± 12.6*
Whole Body BMC (18 wk; g)	0.54 ± 0.1	0.57 ± 0.1	0.76 ± 0.2*
Body Weight (18 wk; g)	28.3 ± 0.8	27.68 ± 3.2	26.34 ± 2.2
Femur length (mm)	15.4 ± 0.5	15.1 ± 0.2	15.2 ± 0.2
Femur µCT			
Tb.BV/TV (unitless)	0.046 ± 0.02	0.046 ± 0.02	0.166 ± 0.05*
Tb.N (1/mm)	3.193 ± 0.34	2.960 ± 0.27	3.722 ± 0.31*
Tb.Th (mm)	0.05 ± 0.00	0.05 ± 0.01	0.07 ± 0.01*
Tb.Sp (mm)	0.31 ± 0.03	0.34 ± 0.03	0.25 ± 0.03*
Tb.BMD (mg/cm^3)	887.3 ± 8.7	887.1 ± 21.6	901.5 ± 15.0*
Tb.BMC (mg)	0.16 ± 0.10	0.17 ± 0.08	0.67 ± 0.23*
Ct.BMD (mg/cm^3)	1.06 ± 0.01	1.06 ± 0.00	1.07 ± 0.01*
Ct.BMC (mg)	2.43 ± 0.23	2.57 ± 0.21	3.79 ± 0.28*
Ct.Th (mm)	0.188 ± 0.01	0.195 ± 0.01	0.259 ± 0.02*
5th Lumbar µCT			
Tb.BV/TV (unitless)	0.20 ± 0.03	0.18 ± 0.03	0.37 ± 0.15*
Tb.N (1/mm)	4.75 ± 0.22	4.45 ± 0.23*	5.63 ± 1.15*
Tb.Th (mm)	0.47 ± 0.01	0.048 ± 0.00	0.06 ± 0.02*
Tb.Sp (mm)	0.20 ± 0.01	0.21 ± 0.013*	0.18 ± 0.06*
Tb.BMD (mg/cm^3)	881.7 ± 6.3	882.5 ± 9.5	870.9 ± 23.5
Tb.BMC (mg)	0.342 ± 0.07	0.289 ± 0.07	0.586 ± 0.24*
Femur 3 point bending			
Ultimate Force (N)	15.7 ± 2.9	16.9 ± 2.7	32.2 ± 4.4*
Stiffness (N/mm)	56.9 ± 18.7	79.9 ± 20.8*	102.7 ± 51.2*
Energy to F_U (mJ)	4.94 ± 1.5	4.92 ± 1.2	11.96 ± 3.7*
Energy to F_F (mJ)	8.2 ± 2.8	11.7 ± 3.0*	16.6 ± 3.5 *
Cranial Dimensions			
Skull Thickness (µm)	0.201 ± 0.08	----	0.295 ± 0.05 *
Foramen Ovale Area (µm)	0.337 ± 0.18	----	0.273 ± 0.11

*$p<0.05$ for comparison to WT mice, using one-way ANOVA followed by Fisher's PLSD post hoc tests.

Table S3. Radiographic analysis and serum measurements in WT and Lrp4-KI female mice with and without a Dmp1-hSost transgene. Related to Figure 4.

	WT / NTG	WT / Dmp1-hSost	KI / NTG	KI / Dmp1-hSost
Whole Body BMC (17 wk; g)	0.473 ± 0.02	0.446 ± 0.04*	0.610 ± .0.07	0.639 ± 0.09
Femur µCT				
Tb.N (1/mm)#@	2.11 ± 0.36	1.24 ± 0.46*	2.42 ± 0.39	1.99 ± 0.48
Tb.Th (mm) #	0.038 ± 0.006	0.046 ± 0.013	0.067 ±0.010	0.063 ± 0.014
Tb.Sp (mm) #@†	0.49 ± 0.10	0.88 ± 0.26*	0.41 ± 0.07	0.53 ± 0.15
Tb.BMD (mg/cm^3) #	923.7 ± 24.6	926.1 ± 31.7	948.9 ± 20.1	939.3 ± 18.2
Tb.BMC (mg) #@	0.05 ± 0.02	0.01 ± 0.01*	0.28 ± 0.14	0.17 ± 0.11
Ct.BMD (mg/cm^3) #	1.07 ± 0.01	1.06 ± 0.02	1.09 ± 0.02	1.08 ± 0.02
Ct.BMC (mg) #	2.44 ± 0.14	2.35 ± 0.14	3.56 ± 0.34	3.54 ± 0.46
5th Lumbar µCT				
Tb.N (1/mm) #	3.72 ± 0.2	2.41 ± 0.8	5.41 ± 2.5	5.13 ± 1.2
Tb.Th (mm) #	0.048 ± 0.003	0.056 ± 0.007	0.083 ± 0.011	0.085 ± 0.021
Tb.Sp (mm) #†	0.263 ± 0.16	0.440 ± 0.12*	0.254 ± 0.23	0.204 ± 0.06
Tb.BMD (mg/cm^3) #	849.6 ± 14.1	863.1 ± 29.0	875.5 ± 3.83	877.9 ± 23.6
Tb.BMC (mg) #	0.237 ± 0.02	0.133 ± 0.06	0.617 ± 0.36	0.703 ± 0.27
Serum sclerostin (pg/µL) #	148.3 ± 19	131.2 ± 32	700.3 ± 291	778.8 ± 335

Symbols indicate significance of the main effects and interaction (#=Lrp4 genotype $p<0.05$; @=Dmp1-hSost $p<0.05$; †=interaction $p<0.05$). When at least one term was significant, Fisher's PLSD post-hoc tests were conducted and are indicated as *$p<0.05$.

Table S4. Radiographic analysis and serum measurements in WT and Lrp4-KI male mice with and without a Dmp1-hSost transgene. Related to Figure 4.

	WT / NTG	WT / Dmp1-hSost	KI / NTG	KI / Dmp1-hSost
Whole Body BMC (17 wk; g)	0.55 ± 0.10	0.55 ± 0.10	0.68 ± 0.08	0.72 ± 0.13
Whole Body BMD (17 wk; g/cm^2)	72.3 ± 8.6	73.2 ± 8.6	85.1 ± 7.3	89.9 ± 10.2
Femur µCT				
Tb.BV/TV (unitless) #†	0.08 ±0.04	0.05 ± 0.02*	0.12 ± 0.03	0.17 ± 0.04*
Tb.N (1/mm) #@†	3.34 ± 0.68	1.98 ± 0.25*	3.32 ± 0.27	3.56 ± 0.32
Tb.Th (mm) #@	0.050 ± 0.01	0.058 ± 0.01*	0.067 ± 0.00	0.072 ± 0.00
Tb.Sp (mm) #@†	0.307 ± 0.06	0.514 ± 0.06*	0.287 ± 0.03	0.266 ± 0.03
Tb.BMD (mg/cm^3) #†	916.5 ± 19.6	925.0 ± 19.1*	925.9 ± 9.8	936.1 ± 14.7*
Tb.BMC (mg) #†	0.345 ± 0.19	0.184 ± 0.10	0.471 ± 0.13	0.793 ± 0.32*
Ct.BMD (mg/cm^3) #	1.05 ± 0.03	1.04 ± 0.03	1.07 ± 0.01	1.08 ± 0.02
Ct.BMC (mg) #	2.58 ± 0.51	2.53 ± 0.19	3.47 ± 0.17	3.84 ± 0.47
Ct.Th (mm) #	0.198 ± 0.03	0.198 ± 0.01	0.243 ± 0.01	0.244 ± 0.03
5th Lumbar µCT				
Tb.BT/TV (unitless) #@	0.24 ± 0.09	0.08 ± 0.01*	0.43 ± 0.05	0.40 ± 0.14
Tb.N (1/mm) #@†	4.99 ± 0.5	2.09 ± 0.3*	6.37 ± 0.7	5.29 ± 1.4
Tb.Th (mm) #	0.051 ± 0.01	0.060 ± 0.01	0.072 ± 0.00	0.078 ± 0.01
Tb.Sp (mm) #@†	0.190 ± 0.02	0.479 ± 0.07*	0.141 ± 0.03	0.198 ± 0.11
Tb.BMD (mg/cm^3)	874.5 ± 14.1	871.7 ± 11.3	856.4 ± 8.0	870.2 ± 17.3
Tb.BMC (mg) #†	0.40 ± 0.16	0.13 ± 0.03*	0.58 ± 0.02	0.59 ± 0.22
Serum sclerostin (pg/µL) #	143.3 ± 37	121.6 ± 23*	1007 ± 173	993.4 ± 109

Symbols indicate significance of the main effects and interaction (#=Lrp4 genotype p<0.05; @=Dmp1-hSost p<0.05; †=interaction p<0.05). When at least one term was significant, Fisher's PLSD post-hoc tests were conducted and are indicated as *p<0.05

Table S5. Histomorphometric analysis of cortical bone changes and serum sclerostin levels in WT and Lrp4-KI mice treated with sclerostin antibody. Related to Figure 5.

	WT / Veh	**WT / Scl-mAb**	**KI / Veh**	**KI / Scl-mAb**
Ec.MS/BS (%)$^{@†}$	70.8±4.0	97.9±3.0*	82.9±9.2	92.1±3.6
Ec.MAR (μm/day)	9.16±1.3	8.95±0.7	10.57±2.3	9.54±1.7
Ec. BFR/BS ($\mu m^3/\mu m^2$/yr)	2381.7±451.8	3194.8±225.5	3160.3±542.0	3192.0±457.0
Serum sclerostin (pg/μL) $^{\#@†}$	207.7±194.4	2971.5±44.7*	780.0±310.2	2942.1±45.0*

Symbols indicate significance of the main effects and interaction (#=Lrp4 genotype p<0.05; @=Scl-mAb p<0.05; †=interaction p<0.05). When at least one term was significant, Fisher's PLSD post-hoc tests were conducted and are indicated as *p<0.05

Table S6. Histomorphometric analysis of cortical bone changes from muscle paralysis in WT and Lrp4-KI mice. Related to Figure 6

	WT + Saline	**WT + Botox**	**KI + Saline**	**KI + Botox**
Ps. MS/BS (%)[@]	65.9 ± 25.5	13.9 ± 9.6*	46.9 ± 16.5	12.3 ± 4.3*
Ps. MAR (μm/day) [@]	0.54 ± 0.17	0.0 ± 0.0*	0.56 ± 0.16	0.30 ± 0.22
Ps. BFR/BS ($\mu m^3/\mu m^2$/day) [@]	134.4 ± 79	0.0 ± 0.0*	93.1 ± 30	15.8 ± 14*

Symbols indicate significance of the main effects and interaction (@=Scl-mAb $p<0.05$). When at least one term was significant, Fisher's PLSD post-hoc tests were conducted and are indicated as *$p<0.05$

Transparent Methods

CONTACT FOR REAGANT AND RESOURCE SHARING

Further information and requests for resources and reagants should be directed to and will be fulfilled by the Lead Contact, Alexander Robling (arobling@iupui.edu).

EXPERIMENTAL MODELS AND SUBJECT DETAILS

Animals

Two different engineered mouse models were used. The first is a new knock-in model of a human patient with an Lrp4 mutation who presented with a sclerosteosis-like phenotype (Leupin et al., 2011). The orthologous mutation was generated in mice using a Crispr/cas9 approach (Fig. 1A). Briefly, a 135bp donor oligo was knocked into the 3' half of Lrp4 exon 25 and adjacent intron. The donor oligo contained a c.3508C→T mutation (plus several local nonsense mutations to facilitate genotyping) that results in an Arg1170Trp substitution (referred to as Lrp4^{KI}). The second engineered model—^{8kb}Dmp1-hSOST transgenic mice—was utilized to generate overexpression of Sost in bone tissue (Tu et al., 2012). Briefly, a 12-kb DNA fragment containing 8 kb of the 5'-flanking region, the first exon, the first intron, and 17 bp of exon 2 of the murine Dmp1 gene was used to drive expression of a human SOST cDNA. The Dmp1-hSOST mice and Lrp4-R1170W knock-in mice were on a fixed C57Bl/6J background. Male and female mice used in experiments ranged from 4-18 weeks of age. All mice were maintained on-site in accordance with Indiana University IACUC procedures.

METHOD DETAILS

Radiographic imaging

Whole-body DEXA scans were collected on isolfuorane-anesthetized mice using a Faxitron UltraFocus^{DXA} x-ray densitometer (Faxitron Bioptics, Inc., Tucson, AZ). All mice were scanned between the ages of 4 to 18 weeks (longitudinal studies) or 3 days prior to the start of the experimental period and again immediately before euthanasia (disuse and antibody studies). From the whole-body scans, areal bone mineral density (BMD) and bone mineral content (BMC) were calculated for the whole body (head and tail excluded) or limbs, depending on the study, using the Faxitron ROI tools.

Collection of serial tibial pQCT measurements in live mice is described elsewhere (Robling et al., 2016). Briefly, a single slice through the proximal tibia, located 4 mm distal to the intracondryl eminance, was collected on isoflurane-anesthetized mice using a Stratec x-ray µScope (Stratec Inc.) at 70-µm resolution. Slices were analyzed for bone mineral content and density using Stratec software in peel mode 2.

After sacrifice, femora or tibiae were scanned, reconstructed, and analyzed on a Scanco µCT-35 desktop microcomputed tomographer (Scanco Medical AG, Brüttisellen Switzerland) as previously described (Niziolek et al., 2015). Briefly, samples were scanned at 10-µm resolution (femur, tibia and vertebra) or 20-µm resolution (skull), 50-kV peak tube potential and 151-ms integration time. Standard output parameters related to cancellous and cortical bone mass, geometry, and architecture were measured and reported (Bouxsein et al., 2010).

Mice received injections of demeclocycline (80 mg/kg), alizarin complexone (20 mg/mL) and calcein (10 mg/kg) at timepoints outlined in individual experiments. After µCT scanning, the fixed femora or tibiae were dehydrated in graded ethanols, cleared in xylene, and embedded in methylmethacrylate. Thick sections were cut from the midshaft using a diamond-embedded wafering saw. Sections were ground and polished to ~30 µm, mounted and coverslipped, then digitally imaged on a fluorescent microscope. Periosteal and endocortical bone formation parameters were calculated by measuring the extent of unlabeled perimeter (nL.Pm), single-labeled perimeter (sL.Pm), double-labeled perimeter (dL.Pm), and the area between the double labeling (dL.Ar) with Image-Pro Plus software (MediaCybernetics Inc., Gaithersburg, MD). The derived histomorphometric parameters mineralizing surface (MS/BS), mineral apposition rate (MAR), and bone formation rate (BFR/BS) were calculated using standard procedures (Dempster et al., 2013).

Molecular Mechanisms

Whole blood samples were collected from wild-type, heterozygous and homozygous Lrp4 mutant mice via cheek bleeding at 8 weeks of age. Approximately 150 µL of blood was collected into serum separator tubes (BD Microtainer), allowed to clot at room temperature for 20 minutes, then centrifuged at 10,000*g* for 1 minute. Serum was removed and stored at −80°C

until all samples were collected and assayed together. Serum sclerostin and C-terminial telopeptide were measured by commercially available ELISA kits (Mouse/Rat SOST Quantikine ELISA, R&D Systems; RatLabs CTX-I EIA, iDS) according to the manufacturer's instructions. Serum samples were measured in duplicate and averaged.

In vivo muscle function in Lrp4 mutant mice was evaluated using the 1305A Whole Mouse/Rat Test System (Aurora Scientific Inc., Aurora, ON, Canada) (Organ et al., 2016). Electrodes were inserted subcutaneously near the tibial nerve in anesthetized mice, which were positioned in the instrument to allow ankle dorsiflexion force quantification. Electrode placement and stimulation current were adjusted to achieve the maximum twitch response and then increased to ~35 mA for plantarflexion to ensure supramaximal stimulation of the muscle fibers. The maximum isometric torque (N/m) was recorded for stimulation frequencies between 25 and 300 Hz, with a pulse width of 0.2 ms and strain duration of 200 ms. Data were recorded using the Dynamic Muscle Control/Data Acquisition and Dynamic Muscle Control Data Analysis programs (Aurora Scientific Inc.).

Immediately after sacrifice, mouse femur, tibia and fibula were dissected, stripped of soft tissue, and flushed to remove bone marrow. The remaining cortical bone tissue was immediately snap frozen, pulverized into powder, and solubilized in 800 µL of 4X SDS-PAGE sample buffer. The extract was heated at 95°C for 5 min, centrifuged at 14,000 x g for 10 min, and the supernatant was retained to run directly on gels.

Approximately 20 µg of protein from each sample was run on a 6 % (Lrp4) or 4-12% (sclerostin) polyacrylamide gradient gel (GenScript). Separated proteins were transferred to nitrocellulose overnight, stained with Ponceau-S to visualize total protein and loading consistency. Membranes were blocked in 5% milk, then reacted with primary and HRP-conjugated secondary antibodies. Bound HRP was reacted with ECL reagent (Amersham, ECL Prime Reagent). Blots were imaged using an iBrightCL1000 (Invitrogen).

Eight-week-old female mice (Lrp4$^{+/+}$ or Lrp4$^{KI/KI}$) were randomized to receive twice-weekly subcutaneous injections of either sclerostin neutralizing antibody (Scl-mAb) at 25 mg/kg or vehicle control for the next 4 weeks (n = 8 mice per genotype and treatment group). Scl-mAb

doses were adjusted weekly on the basis of body mass measurement. Animals were euthanized by CO_2 inhalation when 12 weeks old, followed by tissue harvest.

Parameters related to whole bone strength were measured using 3-point bending tests (Cui et al., 2011). Each femur was loaded to failure in monotonic compression on a TestResources R-series controller, during which force and displacement were collected every 0.01 seconds. From the force/displacement curves, ultimate force and energy to failure were calculated using standard equations (Turner and Burr, 1993).

Forty 12-week-old male mice were used for the Botox experiments, comprising 20 mice of each genotype (i.e., 20 $Lrp4^{KI}$ and 20 wild-type). Each genotype was further divided into control (saline-injected) and Botox-treated mice (n = 10/group). The right hindlimb musculature (quadriceps, triceps surae, tibialis anterior, hamstrings) was injected with a total of 20 µL of Botulinum Toxin A (Botox; Allergan Inc., Irvine, CA), while the left hindlimb musculature was left alone and served as an internal control. Control mice received 20 µL injections of saline in the right hindlimb in an identical fashion as the Botox-treated mice. The injections (both Botox and saline) were repeated one week later to ensure paralysis in the Botox-treated group -. Botox efficacy was qualitatively evaluated for each mouse every 3-4 days, based on the inability of the treated mice to use the limb in normal cage locomotion.

DATA AND SOFTWARE AVAILABILITY

Original data can be found at http://dx.doi.org/doi:10.17632/9jm7ftmnsb.1

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were conducted with SigmaPlot. Statistical details of each experiment can be found in the figure legends. Statistical significance was taken at $p<0.05$. Two-tailed distributions were used for all analyses. Data are presented as means ± SEM.