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Loading-Induced Bone Formation is Mediated by *Wnt1* Induction in Osteoblast-Lineage Cells

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Abstract

Mechanical loading on the skeleton stimulates bone formation. Although the exact mechanism underlying this process remains unknown, a growing body of evidence indicates that the Wnt signaling pathway is necessary for the skeletal response to loading. Recently, we showed that Wnts produced by osteoblast lineage cells mediate the osteo-anabolic response to tibial loading in adult mice. Here, we report that *Wnt1* specifically plays a crucial role in mediating the mechano-adaptive response to loading. Independent of loading, short-term loss of *Wnt1* in the *Osx*-lineage resulted in decreased cortical bone area in the tibias of 5-month old mice. In females, strain-matched loading enhanced periosteal bone formation in *Wnt1*^{F/F} controls, but not in *Wnt1*^{F/F}; *Osx*CreERT2 knockouts. In males, strain-matched loading increased periosteal bone formation in both control and knockout mice; however, the periosteal relative bone formation rate was 65% lower in *Wnt1* knockouts versus controls. Together, these findings show that *Wnt1* supports adult bone homeostasis and mediates the bone anabolic response to mechanical loading.

Introduction

Compressive mechanical loading on the skeleton stimulates bone formation. Although the exact mechanism underlying this process remains unknown, a growing body of evidence indicates that the Wnt signaling pathway plays a role in loading-induced bone formation. For example, loading was found to suppress expression of Wnt antagonist Sclerostin in the bone (Robling, et al. 2008), and in an ulnar loading model, Tu et al. subsequently showed that the downregulation of this antagonist was in fact necessary for the bone anabolic response to loading (Tu, et al. 2012). Furthermore, mice lacking Wnt co-receptor Lrp5 exhibit a diminished bone anabolic response to loading (Zhao, et al., 2013), as do mice treated with a drug that blocks Wnt ligand secretion (Lawson, et al. 2021).

Recently, we showed that blocking Wnt ligand secretion in the *Osx*-lineage cells of skeletally mature mice reduced the osteo-anabolic response to loading. Using *OsxCreERT2* to target osteoblast lineage cells, 5-month old *WlsF/F*; *OsxCreERT2* mice were tamoxifen-treated to inactivate *Wntless* – the Wnt-specific transport receptor responsible for shuttling Wnt ligands to the cell surface for exocytosis – to collectively block the secretion of all Wnt ligands. In a tibial loading model, we showed that the periosteal response to loading was 65% lower in *Wls* knockouts relative to *OsxCreERT2*-negative controls (Lawson, et al. 2021). This study was congruent with a wider body of evidence implicating Wnt signaling in the skeletal response to loading, and furthermore showed that Wnt ligands produced by osteoblast-lineage cells were important mediators of loading-induced bone formation.

Gene expression studies have aimed to characterize the transcriptional signatures produced by bone anabolic loading. In our lab and others, RNASeq analysis of cortical bone samples showed that *Wnt1* and *Wnt7b* were differentially expressed in loaded vs non-loaded

bones (Kelly et al., 2016; Chermide-Scabbo, et al. 2020; Harris and Silva, 2021), suggesting that these Wnt ligands in particular may be necessary for the skeletal response to loading. Furthermore, age-related declines in the skeletal response to loading are associated with dampened upregulation of *Wnt1* and *Wnt7b* in the bone (Holguin, et al., 2016), while *in vivo* overexpression of *Wnt1* and *Wnt7b* in the osteo-lineage is linked to increased bone mass in mice (Joeng, et al. 2017; Song, et al. 2020). Based on these studies, we hypothesized that loading-induced *Wnt1* and *7b* upregulation in the bone is necessary for the bone anabolic response to loading. In this communication, we report that blocking Wnt1 upregulation in the osteo-lineage via Wnt1 deletion in *Osx*-lineage cells blunts the bone anabolic response to skeletal loading.

Methods

Mice

Maintenance and genotyping. All animal studies were approved by the Washington University IACUC. Mice were derived from breeders originally gifted by Drs. Henry Kronenberg (OsxCreERT2 [Maes, et al, 2007]) and Brendan Lee (Wnt1 floxed [Joeng, et al, 2017]) or obtained from Jackson Labs (Wnt7b^{C3} floxed; B6; 129X1-Wnt7b^{tm2Amc}/J; stock number 008467). Experimental mice were generated by breeding Wnt1F/F dams to Wnt1F/F; OsxCreERT2 sires (for Wnt1 single conditional mutants), or Wnt1F/F; Wnt7bF/F dams to Wnt1F/F; Wnt7F/F; OsxCreERT2 sires (for Wnt1/7b double conditional mutants). Mice were weaned at 21 days and housed up to five per cage with *ad libitum* access to normal chow and water. Mice were genotyped by Transnetyx (Transnetyx, USA) using tail snip DNA with probes for wild-type Wnt1 (*Wnt1-1 WT*), conditional Wnt1 (*L1L2-Bact-P MD*), wild-type Wnt7b (*Wnt7b-2 WT*), conditional Wnt7b (*Wnt7b-2 FL*), and OsxCreERT2 (*Cre*). Mice were euthanized by CO₂ asphyxiation.

Tamoxifen induction. Gene deletion was induced in 20 to 22 week old mice. Tamoxifen was dissolved in corn oil to a final concentration of 10mg/ml, and delivered by oral gavage for 5 consecutive days at a dose of 50mg/kg/day. The first day of dosing was “Day 1” in the experimental timeline. Tamoxifen-treated Wnt1F/F and Wnt1F/F; Wnt7bF/F (cre-negative) mice served as genotype controls for *Wnt1* and *Wnt1/7b* knockout experiments, respectively.

Knockdown validation

Bone-specific deletion was confirmed by DNA recombination PCR and knockdown efficiency was evaluated by RT-qPCR (see Gene Expression below for RT-qPCR methods). Validation

experiments were performed 3 weeks after the first tamoxifen dose, on Day 22. Mice used for validation experiments were not subjected to tibial loading.

Recombination PCR. In preparation for DNA recombination analysis, skeletal and extra-skeletal tissues were crushed manually with mortar and pestle, then digested overnight in Proteinase K. Genomic DNA was isolated using a commercially available kit from Qiagen (DNeasy). 5'-CTGCCCAGCTGGGTTTCTACTACG-3' (FOR) and 5'-ACCAGCTGCAGACTCTTGAATCCG-3' (REV) were used to amplify wild-type Wnt1 DNA from the intact/conditional allele (800bp). 5'-AGTGAGCTAGTACGGGGTCC-3' (FOR) and 5'-AGGACCATGAACTGATGGCG-3' (REV) were used to amplify the modified/recombined Wnt1 locus, which produces a Wnt1 null allele (368bp). 5'-TGACAGAGGATGGGGAGAAG-3' (FOR) and 5'-GGTCTTTCCAAGGGTGGTCT-3' (REV) were used to amplify wild-type Wnt7b DNA from the intact locus. 5'-GAGGAAGTCAGGCAGGTGTC-3' (FOR) and 5'-TATCCCACCGATACGCAAAC-3' (REV) were used to amplify the modified/mutant Wnt7b allele (Rajagopal, et al. 2008). PCR reactions were run separately then combined and resolved by electrophoresis on a 2.5% agarose gel.

Knockdown efficiency qPCR. PrimeTime assay Mm.PT.58.30187381 (Integrated DNA Technologies, USA) was used to assay *Wnt1* expression. These primers amplify a region spanning exons 2 and 3, which is deleted in knockout tissues. Deletion of exons 2-3 produces a Wnt1 null allele; thus, qPCR data reflects the abundance of wild-type *Wnt1* mRNA in the bone. *Wnt7b* was assayed using primers spanning exons 2-3: FOR: 3'-CGGGCAAGAAGTCCGAGTAG-5'; REV: 3'-GCGACGAGAAAAGTCGATGC-5'. Exon 3 is deleted in the Wnt7b^{C3} conditional mouse.

Tibial MicroCT

Sequential microCT scans of the tibia were used to evaluate the effects of short-term *Wnt1* and *Wnt1/7b* deletion on cortical bone morphometry. Tibias were scanned at the mid-diaphysis 24-48 hours before the start of tamoxifen induction (Day 0), followed by a second scan approximately 3 weeks later on Day 22. Under anesthesia (1-3% isoflurane), the left tibia was pulled straight and a 2.1mm region centered 5mm proximal to the distal TFJ was scanned on a vivaCT40 with settings optimized for bone (70 kVp, 115 μ A, 300 ms integration, 0.4 sigma, 1 support, 1000 projections, 10.5 μ m/voxel) (Scanco Medical, Switzerland). Post-scans were completed on mice in situ after sacrifice. For all scans a 1.05mm region in the center of the scan was contoured in the Scanco software and evaluated for cortical bone area, total area, cortical thickness, polar moment of inertia, and tissue mineral density.

Tibial loading

Starting on Day 22 after tamoxifen induction, mice were subjected to an in vivo tibial loading regimen to stimulate lamellar cortical bone formation as previously described (Sun et al., 2019; Main et al., 2020). Briefly, the right tibias of Isoflurane-anesthetized mice were positioned vertically between two fixtures and an ElectroPuls E1000 instrument (Instron, USA) was used to apply cyclic axial compression (60 cycles/day, 4Hz) on the right tibia for 5 consecutive days. Contralateral left tibias served as non-loaded controls. Strain gage analysis was performed on mice that were sacrificed 3 weeks after tamoxifen induction (Day 22). The right tibia was dissected to remove muscle and cleaned of soft tissue before a single-element strain gage was glued to the antero-medial surface 5mm proximal to the distal tibiofibular junction (TFJ). The tibia was axially loaded on a material testing machine (Dynamite 8841) at a peak-to-peak force ranging from -2N to -8N for 12 cycles at 4Hz to mimic

loading. LabView data acquisition software was used to collect data, which was analyzed to calculate the force-strain relationship equation in each group. Strain-matched vs force-matched loading. Force-strain relationship equations for each group were used to define the loading force necessary to engender a peak target strain of approximately -3000 μ e, which was found to induce a lamellar bone formation response in Wnt1F/F males and females (Suppl). Strain-matched loading thus entailed the use of loading forces, which sometimes differed between genotype groups, to engender a peak target strain of approximately -3000 μ e at the tibial mid-diaphysis in all groups. In males, this was -10.5N for both genotype groups. In females, strain-matched loading required -9.2N and -5.4N in control and knockout mice, respectively. An additional force-matched female knockout group (ie -9.2N) was analyzed to determine if increasing strain magnitude could restore the loading response.

Dynamic histomorphometry

In preparation for bone formation analysis by dynamic histomorphometry, mice were loaded for 5 days, then given calcein and alizarin to label sites of active mineralization. Calcein (10 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) and alizarin (30 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) were given by IP injection on Days 26 and 31, respectively, followed by sacrifice on Day 33 (see timeline, Fig 4A). Bilateral tibias loaded and non-loaded controls) were de-hydrated through ethanol and embedded in MMA plastic as described previously. Fluorescent transverse sections of the diaphysis (5mm proximal to the distal TFJ) were captured on a Leica confocal microscope. Bioquant analysis software was used to calculate periosteal (Ps) and endocortical (Ec) MS/BS, MAR, and BFR/BS (Dempster, et al, 2012). Relative bone formation rate (rBFR/BS) was calculated as [loaded – non-loaded]. Relative (*r*) values were used as a net measure of the effect of loading within each animal.

Gene Expression

Gene expression was analyzed 3 weeks after tamoxifen induction, 4-hr after the 5th bout of loading.

RNAScope in situ hybridization. Tibias were fixed for 24 hours in 10% neutral buffered formalin (NBF), de-calcified in 14% EDTA, and submitted to the Musculoskeletal Histology and Morphometry Core (Washington University in St Louis, USA) for paraffin embedding and sectioning. Bones were sectioned to a thickness of 5µm through the transverse plane approximately 5mm distal to the tibial plateau. Hybridization was performed using the HybEZ II Hybridization System (ACDBio) in conjunction with the RNAScope 2.5HD Assay-BROWN kit. Probes for murine Wnt1, Bmp2, and Bglap were purchased from ACDBio (Catalog No. 401091, 406661, and 441211). Sections were counterstained with Hematoxylin and imaged on a Hamamatsu NanoZoomer slide scanning system at the Alafi Neuroimaging Laboratory (Washington University in St. Louis, USA). Expression was quantified by scoring cells within the ROI (site of peak compressive strain) as negative, low, medium, or high for *Wnt1* expression. Two-sided Chi-square test was used for statistical analysis.

RT-qPCR. Bilateral tibias were collected and snap-frozen in liquid nitrogen as described previously (Lawson, et al 2021; Holguin et al). Briefly, tibias were dissected out and soft tissues were removed from the bone surface using forceps, leaving the periosteum intact. Tibias were cut 2mm below the tibial plateau and 1mm below the tibio-fibular junction to remove the epiphysis and distal tibia. The remainder, consisting largely of compact cortical bone, was spun at 13,000rpm for 30s to remove bone marrow. Bones were pulverized with a Mikro Dismembrator, lysed in Trizol reagent, and phase separated with chloroform reagent. Total RNA was purified using RNeasy Total RNA kit (Qiagen), and cDNA was prepared from samples with

RIN > 6.0 using the iScript cDNA Synthesis Kit (BioRad). Gene expression was analyzed with SYBR-based reagents on a StepOne Plus Machine (Applied Biosystems), and reported as a relative expression value (2^{-dCT}) relative to reference gene *Tbp*.

Data Analysis

The main effects of genotype (control vs Wnt1Δ or Wnt1/7bΔ) and loading (non-loaded vs loaded) and their interaction were analyzed by 2-factor ANOVA, and *post hoc* Sidak's multiple comparisons test was used for pairwise comparisons (Prism 7.0, San Diego, CA, USA; GraphPad Software, Inc., La Jolla, CA, USA). In this analysis the significance of the interaction ("Int") term is crucial because it indicates whether the effect of loading differed between genotype groups. One-factor ANOVA was used for outcomes where loading was not a factor (eg knockdown efficiency, rBFR/BS). Significance was defined at p<0.05. Individual data points and the mean ± standard deviation are plotted.

Results

Tibial loading induces *Wnt1* expression in osteocytes

Tibial loading induces *Wnt1* expression in bone (Kelly et al, 2016; Holguin et al, 2016; Harris and Silva, 2021). To identify the source of *Wnt1* in response to loading, *in situ* hybridization was used to evaluate *Wnt1* RNA expression in the loaded and non-loaded tibias of *Wnt1^{F/F}* control mice after 5 days of loading (Fig 1A). In loaded tibias *Wnt1* expression was detected in 39.3% ($\pm$ 14.8%) of the osteocytes at the site of peak compressive strain, while in non-loaded control tibias, *Wnt1* expression was detected in only 3.8% ($\pm$ 2.8%) of the osteocytes in the same region of interest ($p < 0.0001$, chi-square test) (Fig 1B). In contrast, little expression was observed in the bone lining cells of either loaded or non-loaded bones after 5 days of loading (Figure S1).

Figure 1. Tibial loading increased *Wnt1* expression in the bone. (A) Tibial *Wnt1* expression was analyzed by RNAScope *in situ* hybridization after 5 days of loading. Results representative of n=4. (B) Osteocytes were scored for *Wnt1* expression at the site of peak compressive strain (lower panels). Data was analyzed with Chi-square test. Significance was defined as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

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An inducible Cre/LoxP approach was used to delete *Wnt1* in the *Osx*-lineage cells of 5-month old mice

To investigate the role of *Wnt1* in the skeletal response to loading, a tamoxifen-inducible Cre/LoxP approach was used to conditionally inactivate *Wnt1* in the osteoblast-lineage cells of 22 month old mice. Mice homozygous for the floxed conditional *Wnt1* allele (Joeng et al. 2017) were dosed with tamoxifen by oral gavage for 5 consecutive days to induce exon 2-3 deletion. Tamoxifen-treated *Wnt1*^{F/F} mice (Cre-negative) served as genotype controls.

Gene deletion was confirmed on Day 22 by DNA recombination PCR and knockdown efficiency testing by RT-qPCR (Fig 2A). At sacrifice, left and right tibias were collected from each mouse. Extra-skeletal tissues from a *Wnt1*^{F/F}; *Osx*CreERT2 mouse were also collected to assess the specificity of gene deletion. Recombination PCR indicated that deletion was specific to bone, and that no deletion occurred in the extra-skeletal tissues surveyed (Fig 2B). PCR analysis also confirmed that bone-specific DNA deletion occurred in 5/5 of the knockout mice tested, but not in any of the controls tested (Fig 2C). Knockdown efficiency testing by qPCR indicated that *Wnt1* mRNA expression was 78% lower in cortical bones of knockouts relative to controls (Fig 2D).

Figure 2. *Wnt1* was conditionally deleted in the *Osx*-expressing cells of adult mice. (A) Five-month old *Wnt1*^{F/F} (control) and *Wnt1*^{F/F}; *Osx*^{CreERT2/+} (knockout) mice were dosed with tamoxifen to induce *Wnt1* deletion in the osteoblast lineage. (B-C) DNA Recombination PCR indicated that *Wnt1* deletion was specific to the tibias of *Osx*^{CreERT2}-positive mice. (D) Tibial *Wnt1* RNA expression was 78% lower in *Wnt1*^Δ mice compared to controls. Individual data points and the mean ± std deviation are shown. n=3-6/group.

Bone area decreased after short-term *Wnt1* deletion

To evaluate the effects of short-term *Wnt1* deletion on cortical bone morphometry, tibias were serially scanned by in vivo microCT 3 weeks apart (Fig 3A).

At baseline, there were no differences in cortical morphology between control and *Wnt1* knockout mice, consistent with no baseline phenotype prior to induction of gene deletion. But, 22 days after initial tamoxifen dosing, in both males and females the cortical area and cortical thickness were lower in *Wnt1* knockout mice than in controls, and in females pMOI was also lower in knockouts (Fig. 3B, 3D, 3F). These changes are consistent with a reduction in cortical area in *Wnt1* knockouts from day 0 to 22, whereas cortical area did not change in controls. Similarly, total area was reduced significantly from day 0 to 22 in female knockouts, and marginally in male knockouts ($p=0.123$), whereas it did not change in control mice. In slight contrast, cortical thickness of increased from day 0 to 22 in control mice of both sexes, while in controls it was marginally reduced ($p=0.055$, males) or unchanged ($p>0.05$, females). Therefore, *Wnt1* deletion for only 3 weeks led to reduced cortical bone mass, due either to loss of bone or reduced accrual of bone.

Figure 3. Cortical bone area decreased after short-term *Wnt1* deletion. The tibias of 5-month old male and female mice were serially scanned to determine the effects of short-term (3 week) *Wnt1* deletion in skeletally mature mice. Tibias were scanned before tamoxifen induction on Day 0, and again on Day 22. Individual data points and the mean $\pm$ std deviation are shown. n=3-6/group.

Loading-induced periosteal bone formation is impaired in *Wnt1* knockouts

Next, to evaluate the role of *Wnt1* in the loading response, an in vivo tibial loading strategy was used to induce bone formation. Three weeks following 5 days of tamoxifen treatment, mice were subjected to daily compressive loading on the right tibia for 5 consecutive days and dosed with calcein and alizarin to label newly mineralizing bone (Fig 4A). Based on *a priori* strain analysis, the majority of mice were subjected to strain-matched loading to a peak target strain of approximately -3000. Preliminary analysis indicated that -3000 $\mu\epsilon$ elicited a robust lameller bone formation response in *Wnt1*F/F controls (Suppl).

In male mice, tibial loading enhanced indices of periosteal (Ps) bone formation in both control and *Wnt1* knockout groups, but the response to loading was diminished in knockouts. Two-way ANOVA indicated a significant effect of loading (main effect) for periosteal bone formation indices, and a significant (or trending) interaction between loading and genotype, indicating that the effect of load was different in control versus *Wnt1* knockout mice (Fig. 4B-D). In controls, Ps.MS/BS, Ps.MAR, and Ps.BFR/BS were 4, 2.5, and 9.6-fold higher, respectively, in loaded vs non-loaded limbs ($p<0.01$) (Fig 4C-4E). By comparison, loading increased Ps.MS/BS, Ps.MAR, and Ps.BFR/BS by only 1.9, 1.4, and 2.8-fold, respectively, in knockouts. We also calculated the relative bone formation rate (rBFR/BS), defined as BFR/BS_{Loaded} minus $BFR/BS_{Non-Loaded}$, as a measure of loading-induced bone formation. Periosteal rBFR/BS was 65% lower in knockouts relative to controls ($p<0.05$; Fig 4I), which further indicates that the periosteal response to loading was impaired by the loss of *Wnt1*.

Figure 4. Loading-induced periosteal bone formation was blunted in Wnt1 knockout males. (A) Timeline for bone formation analysis by dynamic histomorphometry. Mice received calcein and alizarin by IP injection on days 26 and 31, respectively. (B-D) Periosteal bone formation indices in mice loaded to -10.5N (-2800ue). Loading enhanced periosteal bone formation in both groups, albeit to a lesser degree in knockouts. (E) Relative bone formation rate (rBFR/BS), defined as $BFR/BS_{Loaded} - BFR/BS_{Non-Loaded}$, was used as an index of loading-induced bone formation.

In females, we observed similar negative effects of *Wnt1* deletion on the periosteal response to loading (Fig 5). First, comparing mice loaded to a similar peak strain (~3000 $\mu\epsilon$; “strain matched”), loading stimulated increases in periosteal bone formation in control but not knockout (*Wnt1* Δ^1) mice. In controls, Ps.MS/BS, Ps.MAR and Ps.BFR/BS were 2.8, 3.5 and 8.3-fold higher, respectively, in the loaded vs non-loaded bones ($p \leq 0.0001$) (Fig 5B-5D). By comparison, in knockout females loading-associated changes in Ps.MS/BS, Ps.MAR, and Ps.BFR/BS were marginal and not significant. Moreover, periosteal relative bone formation rate (rBFR/BS) was 94% lower in knockouts relative to controls after strain-matched loading ($p < 0.0001$; Fig 5H).

To achieve a strain-matched stimulus of ~3000 $\mu\epsilon$, the force applied to female *Wnt1* knockout mice was 40% less than the force applied to control mice (-5.4N vs. -9.2N; Fig S2), which is consistent with difference in bone morphology (lower Ct.Ar and pMOI in knockouts; Fig 3B, F). Because this level of stimulus did not induce a significant loading response in female knockouts (*Wnt1* Δ^{SM} group), we asked if a higher magnitude stimulus might induce a response. Thus, we subjected a second cohort of knockout females (*Wnt1* Δ^{FM}) to -9.2N to achieve a “force match” (compared to control); this engendered an estimated ~5600 $\mu\epsilon$ peak strain (Fig S2), a level that typically induces a woven bone response (Holguin, et al. 2014).

Knockout females subjected to force-matched loading did display a significant periosteal loading response. Ps.MS/BS, Ps.MAR, and Ps.BFR/BS were 1.8, 2.4 and 4-fold higher, respectively, in loaded vs non-loaded limbs (Fig 5B-5D). Notably, these values were nominally less than the respective fold-increases in control mice (2.8, 3.5 and 8.3), and Ps.MAR and Ps.BFR/BS were significantly less in loaded limbs of force-matched knockout females compared to controls. Moreover, periosteal rBFR/BS was 56% lower in knockouts relative to controls after

308 force-matched loading ($p<0.01$; Fig 5D). Therefore, despite being loaded to 85% higher peak
309 strain, *Wnt1* knockout females still had a blunted periosteal response to loading.

Figure 5. Loading-induced periosteal bone formation was blunted in *Wnt1* knockout females. Loading-induced periosteal bone formation was reduced in *Wnt1* knockouts. Female controls were loaded to -9.2N to engender a peak target strain of -3040 μe . Knockouts were subjected to strain-matched loading (SM) or force-matched loading (FM) by applying either -5.4N or -9.2N, respectively.

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Finally, in contrast to the robust loading response elicited on the periosteal surface of control mice, tibial loading did not induce a strong endocortical bone formation response. In males, indices of endocortical bone formation were not significantly greater in loaded compared to non-loaded limbs, for both control and knockout mice (Figure S4). In female control mice, loading did stimulate a modest increase in Ec.MAR ($p<0.05$, loaded vs. non-loaded) and Ec.BFR/BS ($p=0.052$), but not in strain-matched *Wnt1* knockout mice (Figure S5). Force-matched loading did induce a significant increase in Ec.MAR and Ec.BFR in knockout females, with values in loaded limbs not different from the loaded limbs of control mice.

Induction of osteogenic genes was diminished in the bones of *Wnt1* knockouts

Gene expression analysis was performed 4-hr after the 5th bout of loading. Congruent with previous reports {Holguin et al, 2016; Harris and Silva, 2021}, we found that loading potently induced *Wnt1* expression in the bone (Fig 6A). RT-qPCR analysis indicated that *Wnt1* expression was 6.6-fold higher in the loaded vs non-loaded tibias of *Wnt1*^{F/F} controls ($p<0.0001$). In contrast, loading had no effect on the abundance of *Wnt1* mRNA in the tibias of *Wnt1* knockouts ($p=0.999$) (Fig 6A), consistent with bone-targeted knockout.

Expression of genes associated with osteo-induction, osteoblast maturation, and matrix synthesis were also analyzed. RNAScope *in situ* hybridization showed that loading increased *Bmp2* expression in osteocytes and *Bglap* expression on the bone surface (Suppl). These findings were supported by qPCR, which showed that *Bmp2* and *Bglap* were 1.8 and 3.7-fold higher, respectively, in the loaded vs non-loaded bones of *Wnt1*^{F/F} controls ($p<0.001$) (Fig 6B, 6D). In contrast, loading did not significantly increase *Bmp2* or *Bglap* expression in knockouts. Similarly, loading increased expression of the matrix gene *Colla1* in the tibias of control mice

334 (5.5-fold higher in loaded vs non-loaded, $p<0.001$), while in knockouts *Col1a1* did not increase
335 with loading (Fig 6C).

Figure 6. Loading-induced upregulation of bone formation genes was impaired in *Wnt1* knockouts. The right tibias of anesthetized mice were loaded to a target strain of -3000ue; left tibias served as non-loaded controls. (A) *Wnt1* induction was observed in control but not knockout animals. (B-D) Loading-induced upregulation of *Bmp2*, *Col1a1*, and *Bglap* was diminished in *Wnt1* cKO mice. n=4-5 per sex in each group for a total of n=9/genotype. Two-factor ANOVA was used to evaluate the effect of tibial loading (“Load”) and genotype (“Gen”), and their interaction (“Int”), on gene expression.

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Finally, we assayed several reported Wnt-target genes. Neither loading nor genotype had a significant effect on the expression of *Axin2* or *Nkd2* in the bone (Suppl). In contrast, gene expression analysis by two-factor ANOVA revealed that loading had a modest but significant effect on the expression of *Opg*. In controls, *Opg* was 1.4-fold higher in loaded vs non-loaded limbs ($p=0.077$), while in knockouts *Opg* was only 1.2-fold higher ($p=0.482$) (Suppl).

In sum, these results indicate that *Wnt1* induction in *Osx*-lineage cells is required for the bone anabolic effects of tibial loading.

The skeletal phenotype caused by *Wnt1* deletion was recapitulated in *Wnt1/7b* knockout mice

Because *Wnt7b* is osteo-anabolic (Song, et al. 2020) and is induced in the bone by loading (Holguin et al. 2016), we asked whether the phenotypes observed in *Wnt1* knockouts would be exacerbated by also targeting *Wnt7b*. The cortical bone phenotype observed in *Wnt1* Δ mice was recapitulated in *Wnt1/7b* Δ mice (*Wnt1*F/F; *Wnt7b*F/F; *OsxCreERT2*). In brief, relative to *Wnt1*F/F; *Wnt7b*F/F controls, the bone morphology of *Wnt1/7b* Δ mice was not different on Day 0 (prior to tamoxifen induction), but on Day 22 cortical bone area, cortical thickness, and pMOI were less in *Wnt1/7b* Δ mice (Figure S8).

Loading-induced periosteal bone formation was also impaired in *Wnt1/7b* double knockouts, similar to *Wnt1* single knockouts. In controls, loading significantly enhanced Ps.MS/BS and Ps.MAR, resulting in an overall increase in Ps.BFR/BS of 11 and 5.6-fold in the loaded bones of male and female mice, respectively, compared to non-loaded controls ($p<0.0001$; Figure S9). By comparison, loading did not significantly increase bone formation indices in *Wnt1/7b* knockouts. Consequently, the relative bone formation rate (rBFR/BS) in *Wnt1/7b* Δ mice was 90% lower in males and 86% lower in females compared to their respective

controls. These data further support the finding that *Wnt1* is a crucial mediator of loading-induced bone formation.

Discussion

Axial loading on the skeleton enhances bone mass by stimulating bone formation. Although the exact mechanism underlying this process remain unknown, a growing body of evidence indicates that the Wnt signaling pathway plays a role in regulating this process. For example, mice with mutations that impede Wnt secretion in *Osx*-lineage cells exhibit diminished bone anabolic responses to tibial loading.

An important caveat, however, is that the skeletal response to bone anabolic loading declines with age. Tibial loading, for example, enhances periosteal bone formation in adult mice of different ages; however, the magnitude of the osteogenic response to loading is significantly lower in the bones of aged 22-month old mice relative to young adult 5-month old mice (Holguin, et al. 2014). In an effort to establish a basis for these differences, Chermide-Scabbo et al performed RNASeq experiments in 5 and 22-month C57 old mice to compare the transcriptional responses to axial tibial loading after 1, 3, or 5 days of loading. In both age groups, *Wnt1* emerged as a top 10 upregulated gene on days 1 and 3 (Chermide-Scabbo et al. 2020). The fold-change difference for *Wnt1* in 5-month old mice was 5.8 and 6.7 on days 1 and 3, respectively, while in 22-month old mice the fold-change difference for *Wnt1* was 1.4 and 2.3. Thus, bone anabolic loading increased *Wnt1* in both age groups, albeit to a lesser degree in aged mice – concomitant with a diminished osteogenic response to loading. Together, these findings establish a link between osteo-inductive loading and *Wnt1* induction in the bone.

To determine the role of *Wnt1* in the osteo-anabolic response to loading, a tamoxifen-inducible knockout strategy was used to inactivate *Wnt1* in the osteoblast lineage cells of 5-month old mice. To determine the effects of *Wnt1* deletion on the loading response, mice were subjected to a compressive loading regimen on the right tibia to model loading-induced bone formation.

Tibial loading induces Wnt1 in osteocytes

Transcriptional profiling studies from our lab and others have identified *Wnt1* as a mechano-responsive gene (Kelly et al. 2016; Chermside-Scabbo et al. 2020; Harris and Silva, 2021). RNASeq libraries prepared from the bones of 10 week old mice subjected to a single bout of loading showed that *Wnt1* was highly enriched in the loaded vs non-loaded tibias 3 and 24-hrs after loading (Kelly, et al). A single bout of loading in 5-month old mice increased *Wnt1* expression in the tibia by nearly 6-fold (Chermside-Scabbo, et al. 2020). Using laser capture microscopy, Harris et al isolated osteocyte vs osteoblast-enriched populations from the loaded and non-loaded bones of wild-type mice and found that *Wnt1* was highly enriched in loaded bones, particularly in samples that were enriched for osteocytes (Harris, et al. 2021).

Our data were congruent with these previous studies. Gene expression analysis indicated that *Wnt1* was 6.6-fold higher in the loaded vs non-loaded bones of *Wnt1*^{F/F} mice after 5 bouts of loading ($p < 0.0001$) (Fig 6A). Additionally, histological analysis by RNAScope *in situ* hybridization showed that *Wnt1* induction occurred primarily in the intra-cortical region, in osteocytes (vs bone lining cells, ie osteoblasts) (Fig 1A). RNAScope analysis further revealed that loading increased not only the total percentage of cells that were *Wnt1*-positive, but also the abundance of *Wnt1* RNA within those cells (Fig 1B). Together, these data show that *in vivo* tibial loading induces *Wnt1* in the bone, particularly in osteocytes.

Wnt1 upregulation was required for a robust osteo-inductive transcriptional response to tibial loading

OsxCreERT2 was used to inactivate *Wnt1* in the Osx-lineage cells of 5-month old mice. To achieve this, tamoxifen dissolved in corn oil was delivered by oral gavage for 5 days. We previously used a similar dosing strategy for Cre reporter analysis in 5-month old OsxCreERT2; Rosa-Ai9 mice, which showed robust TdTomato reporter expression on the bone surface (osteoblasts) and throughout the cortical bone (osteocytes) within days of tamoxifen treatment (Lawson, et al. 2021) – indicating that OsxCreERT2 targets both osteoblasts and osteocytes in the bones of adult mice.

OsxCreERT2-mediated *Wnt1* deletion completely neutralized the effect of loading on *Wnt1* expression in the bone. While *Wnt1* expression increased 6.6-fold in the tibiae of *Wnt1*F/F controls ($p < 0.0001$), *Wnt1* expression remained unchanged in the loaded vs non-loaded bones of *Wnt1* knockouts ($p = 0.999$) (Fig 6A). Loading-induced *Colla1*, *Bmp2*, and *Bglap* upregulation was similarly robust and significant in controls but not in knockouts, indicating that *Wnt1* was required for the activation of these osteogenic markers.

Wnt1 regulates the periosteal response to skeletal loading

Wnt1 inactivation in the Osx-lineage limited the periosteal response to loading. In males, strain-matched loading increased Ps.BFR/BS in all mice, independent of genotype. However, the relative bone formation rate – which reflects the overall magnitude of the loading response – showed that the periosteal response to loading was markedly blunted in knockouts (Fig 4E). Similarly, in females strain-matched loading increased Ps.BFR/BS in controls but not in knockouts. Ps.rBFR/BS showed that the periosteal response was blunted by a significant margin in *Wnt1* knockouts, even when knockouts were subjected to a greater strain stimulus (Fig 5D).

These data show that *Wnt1* plays a crucial role in mediating the bone anabolic effects of skeletal loading.

These findings are congruent with a report by Wang, et al (2021) that inactivating *Wnt1* in the *Osx*-lineage impaired periosteal bone growth in young adult mice. Using a Dox-repressible system, Wang et al. showed that inactivating *Wnt1* in the *Osx*-lineage of 4-week old mice produced measurable deficits in the skeleton 4 and 8 weeks later. Analysis by dynamic histomorphometry revealed that the skeletal deficits observed in 12-week old *Wnt1* knockouts was due to reduced periosteal bone formation, while changes to endocortical bone formation were not significant (Wang, et al. 2021).

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References

- Chermside-Scabbo CJ, Harris TL, Brodt MD, Braenne I, Zhang B, Farber CR, Silva MJ. Old Mice Have Less Transcriptional Activation But Similar Periosteal Cell Proliferation Compared to Young-Adult Mice in Response to in vivo Mechanical Loading. *J Bone Miner Res.* 2020 Sep;35(9):1751-1764. doi: 10.1002/jbmr.4031. Epub 2020 Jun 1. PMID: 32311160; PMCID: PMC7486279.
- Dempster DW, Compston JE, Drezner MK, Glorieux FH, Kanis JA, Malluche H, Meunier PJ, Ott SM, Recker RR, Parfitt AM. Standardized nomenclature, symbols, and units for bone histomorphometry: a 2012 update of the report of the ASBMR Histomorphometry Nomenclature Committee. *J Bone Miner Res.* 2013 Jan;28(1):2-17. doi: 10.1002/jbmr.1805. PMID: 23197339; PMCID: PMC3672237.
- Harris TL, Silva MJ. Gene expression of intracortical bone demonstrates loading-induced increases in Wnt1 and Ngf and inhibition of bone remodeling processes. *Bone.* 2021 Sep;150:116019. doi: 10.1016/j.bone.2021.116019. Epub 2021 May 21. PMID: 34023542; PMCID: PMC8408835.
- Holguin N, Brodt MD, Silva MJ. Activation of Wnt Signaling by Mechanical Loading Is Impaired in the Bone of Old Mice. *J Bone Miner Res.* 2016 Dec;31(12):2215-2226. doi: 10.1002/jbmr.2900. Epub 2016 Sep 7. PMID: 27357062; PMCID: PMC5397287.
- Joeng KS, Lee YC, Lim J, Chen Y, Jiang MM, Munivez E, Ambrose C, Lee BH. Osteocyte-specific WNT1 regulates osteoblast function during bone homeostasis. *J Clin Invest.* 2017 Jun 30;127(7):2678-2688. doi: 10.1172/JCI92617. Epub 2017 Jun 19. PMID: 28628032; PMCID: PMC5490765.

- 470 Kelly NH, Schimenti JC, Ross FP, van der Meulen MC. Transcriptional profiling of cortical
471 versus cancellous bone from mechanically-loaded murine tibiae reveals differential gene
472 expression. *Bone*. 2016 May;86:22-9. doi: 10.1016/j.bone.2016.02.007. Epub 2016 Feb
473 12. PMID: 26876048; PMCID: PMC4833881.
- 474 Lawson LY, Brodt MD, Migotsky N, Chermide-Scabbo CJ, Palaniappan R, Silva MJ.
475 Osteoblast-Specific Wnt Secretion Is Required for Skeletal Homeostasis and Loading-
476 Induced Bone Formation in Adult Mice. *J Bone Miner Res*. 2021 Sep 20. doi:
477 10.1002/jbmr.4445. Epub ahead of print. PMID: 34542191.
- 478 Luther J, Yorgan TA, Rolvien T, Ulsamer L, Koehne T, Liao N, Keller D, Vollersen N, Teufel S,
479 Neven M, Peters S, Schweizer M, Trumpp A, Rosigkeit S, Bockamp E, Mundlos S,
480 Kornak U, Oheim R, Amling M, Schinke T, David JP. Wnt1 is an Lrp5-independent
481 bone-anabolic Wnt ligand. *Sci Transl Med*. 2018 Nov 7;10(466):eaau7137. doi:
482 10.1126/scitranslmed.aau7137. PMID: 30404864.
- 483 Maes C, Kobayashi T, Kronenberg HM. A novel transgenic mouse model to study the osteoblast
484 lineage in vivo. *Ann N Y Acad Sci*. 2007 Nov;1116:149-64. doi:
485 10.1196/annals.1402.060. PMID: 18083926.
- 486 McKenzie JA, Silva MJ. Comparing histological, vascular and molecular responses associated
487 with woven and lamellar bone formation induced by mechanical loading in the rat ulna.
488 *Bone*. 2011 Feb;48(2):250-8. doi: 10.1016/j.bone.2010.09.005. Epub 2010 Sep 22.
489 PMID: 20849995; PMCID: PMC3021598.
- 490 Rajagopal J, Carroll TJ, Guseh JS, Bores SA, Blank LJ, Anderson WJ, Yu J, Zhou Q, McMahon
491 AP, Melton DA. Wnt7b stimulates embryonic lung growth by coordinately increasing the

replication of epithelium and mesenchyme. *Development*. 2008 May;135(9):1625-34.
doi: 10.1242/dev.015495. Epub 2008 Mar 26. PMID: 18367557; PMCID: PMC2556060.

Schreibvogel S, Kuchibhotla V, Knaus P, Duda GN, Petersen A. Load-induced osteogenic differentiation of mesenchymal stromal cells is caused by mechano-regulated autocrine signaling. *J Tissue Eng Regen Med*. 2019 Nov;13(11):1992-2008. doi: 10.1002/term.2948. Epub 2019 Aug 13. PMID: 31359634.

Song D, He G, Song F, Wang Z, Liu X, Liao L, Ni J, Silva MJ, Long F. Inducible expression of Wnt7b promotes bone formation in aged mice and enhances fracture healing. *Bone Res*. 2020 Feb 3;8:4. doi: 10.1038/s41413-019-0081-8. PMID: 32047703; PMCID: PMC6997361.

Wang F, Rummukainen P, Heino TJ, Kiviranta R. Osteoblastic Wnt1 regulates periosteal bone formation in adult mice. *Bone*. 2021 Feb;143:115754. doi: 10.1016/j.bone.2020.115754. Epub 2020 Nov 13. PMID: 33189914.

Figures

Figure 1. Tibial loading increased *Wnt1* mRNA expression in the bone. (A) Tibial *Wnt1* expression was analyzed by RNAScope *in situ* hybridization after 5 days of loading. Results representative of n=4. (B) Osteocytes were scored for *Wnt1* expression at the site of peak compressive strain (lower panels). Data was analyzed with Chi-square test. Significance was defined as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

Figure 2. *Wnt1* was conditionally deleted in the *Osx*-expressing cells of adult mice. (A) Twenty to 22-month old *Wnt1*^{F/F} (control) and *Wnt1*^{F/F}; *Osx*^{CreERT2/+} (knockout) mice were dosed with tamoxifen to induce *Wnt1* deletion in the osteoblast lineage. (B-C) DNA Recombination PCR indicated that *Wnt1* exon 2-3 deletion was specific to the tibias of *Osx*CreERT2-positive mice. (D) Tibial *Wnt1* RNA expression was 78% lower in *Wnt1*Δ mice compared to controls. Individual data points and the mean ± std deviation are shown. n=3-6/group.

Figure 3. Cortical bone area decreased after short-term *Wnt1* deletion. The tibias of 5-month old male and female mice were serially scanned to evaluate the effects of short-term (3 week) *Wnt1* deletion in skeletally mature mice. Tibias were scanned before tamoxifen induction on Day 0, and again on Day 22. Individual data points and the mean ± std deviation are shown. n=3-6/group.

Figure 4. Loading-induced periosteal bone formation was blunted in *Wnt1* knockout males. (A) Timeline for bone formation analysis by dynamic histomorphometry. Mice received calcein and alizarin by IP injection on days 26 and 31, respectively. (B-D) Periosteal bone formation indices in mice loaded to -10.5N (-2800ue). Loading enhanced periosteal bone formation in both groups, albeit to a lesser degree in knockouts. (E) Relative bone formation rate (rBFR/BS),

defined as BFR/BS_{Loaded} minus $BFR/BS_{\text{Non-Loaded}}$, was used as an index of loading-induced bone formation.

Figure 5. Loading-induced periosteal bone formation was blunted in *Wnt1* knockout females. Loading-induced periosteal bone formation was reduced in *Wnt1* knockouts. Female controls were loaded to -9.2N to engender a peak target strain of -3040 $\mu\epsilon$. Knockouts were subjected to strain-matched loading (SM) or force-matched loading (FM) by applying either -5.4N or -9.2N, respectively.

Figure 6. Loading-induced upregulation of bone formation genes was impaired in *Wnt1* knockouts. The right tibias of anesthetized mice were loaded to a target strain of -3000 $\mu\epsilon$; left tibias served as non-loaded controls. (A) *Wnt1* induction was observed in control but not knockout animals. (B-D) Loading-induced upregulation of *Bmp2*, *Colla1*, and *Bglap* was diminished in *Wnt1* cKO mice. n=4-5 per sex in each group for a total of n=9/genotype. Two-factor ANOVA was used to evaluate the effect of tibial loading (“Load”) and genotype (“Gen”), and their interaction (“Int”), on gene expression.

Figure S1. *Wnt1* mRNA expression in a loaded tibia after 5 days of loading. *Wnt1* was observed in osteocytes on both the tensile and compressive sides of the bone, including osteocytes near the bone surface (red arrowheads). *Wnt1* expression was not observed in the periosteal layer (white arrowheads). Results representative of n=4.

Figure S2. Strain gage analysis was used to experimentally determine the force-strain relationships in male (A) and female (B) mice 3 weeks after the start of tamoxifen induction. In males, strain-matched loading was achieved by loading controls and knockouts to -10.5N, which engendered a peak target strain of -2800 $\mu\epsilon$ in both genotypes. In females, strain-matched (SM)

loading was achieved by loading controls to -9.2N (-3040μE) and knockouts to -5.4N (-3200μE).

Female knockouts in the force-matched (FM) group were loaded to -9.2N, which engendered a peak strain of -5600μE.

Figure S3. *Wnt1*^{F/F} mice were loaded to a peak target strain of approximately -3000μE (-10.5N males; -9.2N females) to stimulate lamellar bone formation. A *Wnt1*^{F/F} female loaded to -5600ue (-15N) had a woven bone formation response to loading. Scale bars represent 500um.

Figure S4. Endocortical bone formation indices in males loaded to -10.5N (-2800ue). Independent of genotype, loading had a negligible effect on endocortical bone formation.

Figure S5. Endocortical bone formation indices in females.

Figure S6. Supplemental in situ hybridization. Loading increased *Bmp2* expression in osteocytes, while *Bglap* and Bmp target gene *Id1* were increased on the bone surface.

Figure S7. Wnt target gene expression. RNAScope (top) and qPCR (bottom) were used to analyze Wnt target gene expression in the tibia after 5 days of loading. RNAScope results representative of n=3 (2 controls, 1 KO)

Figure S8. Deletion of *Wnt1* and *Wnt7b* in the *Osx*-lineage of 5-month old mice. *Wnt1*^{F/F}; *Wnt7b*^{F/F}; *OsxCreERT2* (*Wnt1/7b* knockout) mice were tamoxifen-treated at 5-months old to induce *Wnt1* and *Wnt7b* deletion in the *Osx*-lineage. DNA recombination PCR was performed on Day 22 to confirm bone-specific deletion of *Wnt1* (exon 2-3) and *Wnt7b* (exon 1) in *Wnt1/7b* knockouts. Serial microCT scans were used to evaluate the combined effect of *Wnt1/7b* deletion (*Wnt1/7b*Δ) over a 3 week period. Tamoxifen-treated cre-negative *Wnt1*^{F/F}; *Wnt7b*^{F/F} mice served as control.

571 **Figure S9.** Loading-induced bone formation was reduced in *Wnt1/7b* double conditional
572 knockouts. Dynamic histo-morphometry was used to analyze bone formation in *Wnt1F/F*;
573 *Wnt7bF/F*; *OsxCreERT2* (*Wnt1/7bΔ*) males (top) and females (bottom).

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575 Supplemental Figures

Wnt1 RNAScope

Suppl-1. *Wnt1* expression in a loaded tibia after 5 days of loading. *Wnt1* was observed in osteocytes on both the tensile and compressive sides of the bone, including osteocytes near the bone surface (red arrowheads). *Wnt1* expression was not observed in the periosteal layer (white arrowheads). Results representative of n=4.

576

Suppl-2. Strain gage analysis was used to experimentally determine the force-strain relationships in male (A) and female (B) mice 3 weeks after tamoxifen induction. In males, strain-matched loading was achieved by loading controls and knockouts to -10.5N, which engendered a peak target strain of -2800ue in both genotypes. In females, strain-matched (SM) loading was achieved by loading controls to -9.2N (-3040ue) and knockouts to -5.4N (-3200). Female knockouts in the force-matched (FM) group were subjected to a peak target strain of -5600ue.

Suppl-3. $Wnt1^{F/F}$ mice were loaded to a peak target strain of approximately $-3000\mu\epsilon$ ($-10.5N$ males; $-9.2N$ females) to stimulate lamellar bone formation. A $Wnt1^{F/F}$ female loaded to $-5600\mu\epsilon$ ($-15N$) had a woven bone formation response to loading. Scale bars represent $500\mu m$.

578

Suppl-4. Endocortical bone formation indices in males loaded to -10.5N (-2800ue). Independent of genotype, loading had a negligible effect on endocortical bone formation.

Suppl-5. Endocortical bone formation indices in females after 5 days of tibial loading.

Suppl-6. Supplemental RNAScope gene expression data. Results representative of n=3.

Suppl-7. Wnt target gene expression. RNAScope (top) and qPCR (bottom) were used to analyze Wnt target gene expression in the tibia after 5 days of loading. RNAScope results representative of n=3 (2 controls, 1 KO)

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Suppl-8. Deletion of *Wnt1* and *Wnt7b* in the *Osx*-lineage of 5-month old mice. *Wnt1*F/F; *Wnt7b*F/F; *Osx*CreERT2 (*Wnt1/7b* knockout) mice were tamoxifen-treated at 5-months old to induce *Wnt1* and *Wnt7b* deletion in the *Osx*-lineage. DNA recombination PCR was performed on Day 22 to confirm bone-specific deletion of *Wnt1* (exon 2-3) and *Wnt7b* (exon 1) in *Wnt1/7b* knockouts. Sequential microCT scans were used to evaluate the effect of *Wnt1/7b* deletion (*Wnt1/7b* Δ) over a 3 week period. Tamoxifen-treated cre-negative *Wnt1*F/F; *Wnt7b*F/F mice served as control.

Suppl-9. Loading-induced bone formation was reduced in Wnt1/7b double conditional knockouts. Dynamic histo-morphometry was used to analyze bone formation in Wnt1F/F; Wnt7bF/F; OsxCreERT2 (Wnt1/7bΔ) males (top) and females (bottom).