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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 Wnt1F/F controls, but not in Wnt1F/F; OsxCreERT2 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.

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Author Contributions

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Keywords

Osteoblasts; Osteocytes; Bone; Osteogenesis; Mechanobiology; Wnt Signaling Pathway

Introduction

Mechanical loading on the skeleton stimulates bone formation. Although the mechanisms underlying this process are not fully defined, a growing body of evidence indicates that the Wnt signaling pathway plays a role in loading-induced bone formation. For example, ulnar loading suppresses expression of Wnt antagonist Sclerostin, and downregulation of this antagonist is in fact necessary for the bone anabolic response to loading[1–2]. Furthermore, mice lacking Wnt co-receptor Lrp5 exhibit a diminished bone anabolic response to loading, whereas mice with high-bone mass (HBM) mutations in Lrp5 have enhanced responses to loading[3–5].

Wnt signaling initiates when one of 19 Wnt ligands ('Wnts') binds to Frizzled and Lrp5/6 co-receptors[6]. Recently, we reported that blocking Wnts secretion in the Osx-expressing cells of skeletally mature mice reduced the osteo-anabolic response to loading[7]. OsxCreERT2; $Wls^{F/F}$; mice were tamoxifen-treated to inactivate Wntless – the Wnt-specific transport receptor responsible for shuttling Wnt ligands to the cell surface for exocytosis[8] – thereby blocking the secretion of all Wnts from osteoblasts and osteocytes. We showed that the periosteal response to tibial loading was 65% lower in Wls knockouts relative to WlsF/F controls, indicating that Wnts produced by osteoblast-lineage cells are important mediators of loading-induced bone formation[7].

Gene expression studies have aimed to identify the key factors that mediate loading-induced bone formation. 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, suggesting that these Wnt ligands in particular may be necessary for the skeletal response to loading[9–11]. Furthermore, age-related declines in the skeletal response to loading are associated with dampened upregulation of *Wnt1* and *Wnt7b* in the bone, while *in vivo* overexpression of *Wnt1* and *Wnt7b* in the osteo-lineage causes increased bone mass[12–14]. Based on these studies, we hypothesized that loading-induced upregulation of *Wnt1* and *Wnt7b* in osteoblasts is necessary for the bone anabolic response to loading. In this communication, we report that blocking *Wnt1* upregulation in the osteoblast-lineage via *Wnt1* deletion in Osx-expressing cells blunts the bone anabolic response to skeletal loading in mice.

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[15]), Brendan Lee (Wnt1 floxed[13]), 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 OsxCreERT2; Wnt1F/F sires (for *Wnt1* single conditional mutants), or Wnt1F/F; Wnt7bF/F dams to OsxCreERT2; Wnt1F/F; Wnt7F/F 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 CO_2 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

Tissue-specific gene deletion was confirmed by DNA recombination PCR, and RNA 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 (cortical bone from tibia) and extra-skeletal tissues were collected from tamoxifen-treated control and knockout mice. 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’-ACCAGCTGCAGACTCTTGGAATCCG-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 as described previously[16]. PCR reactions were run separately, then combined and resolved by electrophoresis on a 2.5% agarose gel.

Knockdown efficiency qPCR.—Tibias were dissected and processed for knockdown efficiency testing as described under *Gene Expression* (RT-qPCR). PrimeTime assay Mm.PT.58.30187381 (Integrated DNA Technologies, USA) was used to assay *Wnt1* expression in the cortical bone. 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’-CGGGCAAGAACTCCGAGTAG-5’; REV: 3’-GCGACGAGAAAAGTCGATGC-5’. Exon 3 is deleted in the $Wnt7b^{C3}$ conditional mouse.

Tibial MicroCT

Sequential *in vivo* μCT 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 positioned for scanning, and a 2.1 mm region centered 5 mm proximal to the distal TFJ was scanned on a vivaCT40 with settings optimized for bone (70 kVp, 115 μA, 300 ms integration, 0.4 sigma, 1 support, 1000 projections, 10.5 μm/voxel) (Scanco Medical, Switzerland). Post-scans were completed on mice *in situ* after sacrifice. For all scans a 1.05 mm 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 periosteal lamellar bone formation as previously described[17–18]. 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 exposed and cleaned of soft tissue before a single-element strain gage was glued to the antero-medial surface 5 mm 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 −2 N to −8 N for 12 cycles at 4 Hz. LabView data acquisition software was used to collect data, which was analyzed by linear regression to calculate the force-strain equation for each sex-genotype group (Fig S2).

Strain-matched and force-matched loading.—Force-strain equations were used to define the loading force necessary to engender a target strain of approximately −3000 με in each group. In males, this required a loading force of −10.5 N for both genotypes. In females, strain-matched loading required −9.2 N and −5.4 N in control and knockout mice, respectively. These forces were found to induce a robust lamellar bone formation response in Wnt1F/F males and females (Fig S3). An additional cohort of *Wnt1* knockout females were loaded to −9.2 N to provide a force-matched comparison with control females. In force-matched knockout females, −9.2 N was estimated to engender a peak strain of −5600 με (Fig S2).

Dynamic histomorphometry

Bone formation was analyzed by dynamic histomorphometry. As outlined in Figures 4 and 5, mice were loaded for 5 days, then calcein (10 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) and alizarin (30 mg/kg; Sigma-Aldrich) were given by IP injection on days 26 and 31, respectively, to label sites of active mineralization. Mice were sacrificed on day 33. Bilateral tibias (loaded and non-loaded) were de-hydrated through ethanol and embedded in

MMA plastic as described previously[19]. Transverse sections from the mid-diaphysis (5mm proximal to the TFJ) were used to capture 20 μm z-stack images on a Leica confocal microscope (Leica DMi8) for analysis. Bioquant analysis software was used to calculate periosteal (Ps) and endocortical (Ec) bone formation indices, including % mineralizing surface (MS/BS), mineral apposition rate (MAR), and bone formation rate (BFR/BS) [20]. Relative bone formation rates (rBFR/BS) were calculated as [*loaded – non-loaded*] in each animal. Thus, relative (*r*) values were used as a net measure of the effect of loading within each animal.

Gene Expression

Gene expression was analyzed by *in situ* hybridization and RT-qPCR on day 26, as outlined in Figure 6.

RNAScope in situ hybridization.—*In situ* hybridization was used to analyze gene expression in cre-negative Wnt1F/F and Wnt7bF/F male and female control mice. 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 5 μm through the transverse plane approximately 5 mm 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*, *Bglap, Axin2, Opg, and Id1,* and *Wnt7b* were purchased from ACDBio (Catalog No. 401091, 406661, 441211, 488961, 322335, and 401131). Sections were hematoxylin-counterstained and imaged on a Hamamatsu NanoZoomer slide scanning system at the Alafi Neuroimaging Laboratory (Washington University in St. Louis, USA). *Wnt1* RNAScope was quantified by scoring cells at the site of peak compressive strain as negative, low, medium, or high for *Wnt1* expression. Chi-square test was used for statistical analysis. *Wnt1* RNAScope analysis was performed in n=4 mice, including 2 males and 2 females.

RT-qPCR.—Bilateral tibias were processed for RT-qPCR as described previously[7, 12]. Briefly, forceps were used to remove soft tissues from the bone surface, leaving the periosteum intact. Tibias were cut 2 mm below the tibial plateau and 1 mm below the tibio-fibrular junction to remove the epiphysis and distal tibia. The remainder – consisting largely of compact cortical bone – was spun at 13,000 rpm for 30 seconds to remove bone marrow. Bones were snap-frozen in liquid nitrogen, pulverized with a Mikro Dismembrator, lysed in Trizol, then 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 ($2^{-\Delta CT}$), relative to reference gene *Tbp*. Gene expression analysis in males and females showed that loading had a significant or near-significant effect on osteogenic marker expression in Wnt1F/F control mice, irrespective of sex, but not in Wnt1F/F; OsxCreERT2 knockout males and females (Fig S10). Thus – because combining gene expression data from males and females did not impact how the results were

interpreted – we chose to pool the data and present the findings as 4 panels instead of 8 in Figure 6.

Statistical Analysis

The main effects of genotype (knockout vs control) and loading (loaded vs non-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, 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 $p<0.05$, with trends noted at $0.05< p <0.10$. 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[9, 11–12]. To identify the source of Wnt1 ligand expression in the bone, *in situ* hybridization was used to analyze *Wnt1* RNA expression in the tibias of cre-negative Wnt1F/F and Wnt7bF/F mice after 5 days of tibial loading (Fig 1A). In loaded bones, *Wnt1* RNA was detected in 39.3% (± 14.8%) of the osteocytes at the site of peak compressive strain, while in non-loaded bones *Wnt1* expression was detected in only 3.8% (± 2.8%) of the osteocytes in the same region of interest ($p<0.0001$, chi-square test) (Fig 1B). *Wnt1* expression was not detected by *in situ* hybridization on the bone surface (ie in osteoblasts) of either loaded or non-loaded bones (Fig S1).

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 bone anabolic response to loading, an inducible Cre/LoxP approach was used to conditionally inactivate *Wnt1* in the osteoblast-lineage cells of 5-month old mice. Gene deletion was confirmed on day 22 by DNA recombination PCR, and RT-qPCR was used to evaluate *Wnt1* knockdown efficiency in the bone (Fig 2A). Recombination analysis using deletion-specific primers indicated that *Wnt1* deletion was bone-specific; no off-target effects were observed in any of the extra-skeletal tissues surveyed (Fig 2B–C). Additionally, bone-specific deletion occurred in 5/5 knockout mice tested, but not in any (0/4) of the controls (Fig 2D). Finally, mRNA knockdown testing showed that tibial *Wnt1* expression was 78% lower in *Wnt1* knockouts compared to controls on day 22 (Fig 2E).

Bone area decreased after short-term Wnt1 deletion

To evaluate the effects of short-term *Wnt1* deletion on cortical bone morphometry, tibias were analyzed by *in vivo* μCT before (day 0) and after tamoxifen induction (day 22) (Fig 3, top panel). At baseline (day 0) no differences in cortical bone morphology were detected in control vs *Wnt1* knockout mice, consistent with no phenotype prior to inducing gene deletion. However, between days 0 and 22, Ct.Ar decreased 11.6% and 8.6% in male and

female knockouts, respectively (Fig 3B). Ct.Th also decreased between days 0 and 22 in male knockouts (−6.3%, p=0.055) while in female knockouts pMOI decreased 17.7%, consistent with a reduction in Tt.Ar and Ct.Ar between days 0 and 22 (Fig 3A, 3D, 3F). In contrast, an increase in Ct.Ar and Ct.Th was observed in Wnt1F/F controls – likely due to the anabolic effects of tamoxifen[21].

In sum, *Wnt1* deletion for only 3 weeks led to reduced cortical bone mass in adult mice, due either to loss of bone or reduced accrual of bone.

Loading-induced periosteal bone formation is impaired in Wnt1 knockouts

Next, to evaluate the role of *Wnt1* in the loading response, *in vivo* tibial loading was used to induce cortical bone formation, starting on day 22. Based on *a priori* strain analysis, mice were subjected to strain-matched loading to a peak target strain of approximately −3000 με for bone formation and gene expression analyses (Fig S2). Preliminary findings showed that this stimulus elicited a robust lamellar bone formation response in Wnt1F/F mice (Fig S3).

Ps bone formation, males—In males, tibial loading increased periosteal (Ps) bone formation in both groups, but the response to loading was blunted in *Wnt1* knockouts. Two-factor ANOVA analysis indicated that the main effect of loading on Ps.MS/BS, Ps.MAR, and Ps.BFR/BS was significant (Fig 4A–C). Additionally, a significant (or near-significant) loading-genotype interaction was detected for Ps.MS/BS, Ps.MAR, and Ps.BFR/BS, indicating that the response to loading was different in control vs *Wnt1* knockout mice.

In Wnt1F/F males, Ps.MS/BS and Ps.MAR were 4 and 2.5-fold higher in the loaded vs non-loaded limbs, resulting in an overall 9.6-fold increase in Ps.BFR/BS (Fig 4A–C). By comparison, in knockouts Ps.MS/BS and Ps.MAR were 1.9 and 1.4-fold higher in the loaded vs non-loaded limbs, resulting in a 2.8-fold increase in Ps.BFR/BS. A comparison of Ps.BFR/BS in the loaded tibias of control vs knockout mice showed that Ps.BFR/BS was statistically different in control vs knockout mice (p<0.05), indicating that *Wnt1* was important for the skeletal response to loading (Fig 4C). Additionally, we calculated the *relative* bone formation rate (rBFR/BS), defined as BFR/BS_{Loaded} *minus* $BFR/BS_{Non\text{-}Loaded}$, as a measure of loading-induced bone formation. This analysis indicated that the periosteal response to loading was 65% lower in *Wnt1* knockouts relative to controls (Fig 4D).

Ps bone formation, females—The bone anabolic response to loading was similarly blunted in *Wnt1* knockout females. To achieve strain-matched loading in females, Wnt1F/F and Wnt1F/F; OsxCreERT2 mice were loaded to −9.2 N and −5.4 N, respectively (Fig S2). In Wnt1F/F females, Ps.MS/BS and Ps.MAR were 2.9 and 3.5-fold higher in the loaded vs non-loaded limbs, resulting in a 8.3-fold increase in Ps.BFR/BS (Fig 5A–C). In contrast, Ps.MS/BS, Ps.MAR, and Ps.BFR/BS were only negligibly affected by loading in strain-matched knockouts (Fig 5A–C). Ps.rBFR/BS indicated that in females, the periosteal response to loading was diminished 94% in *Wnt1* knockouts relative to strain-matched controls (Fig 5D).

Force-matched loading in females—To achieve a strain-matched stimulus of −3000 με in females, the force applied to *Wnt1* knockouts (−5.4 N) was 40% less than the force required in controls (−9.2 N), consistent with the finding that pMOI was different in control vs knockout females on day 22 (Fig 3F). Because this level of stimulus did not induce a significant loading response in Wnt1F/F; OsxCreERT2 females ($Wnt1\Delta^{SM}$ group), we asked if a higher magnitude stimulus might induce a response. To address this, a second cohort of knockout females was subjected to 'force-matched' loading ($Wnt1\Delta^{FM}$ group, Figs 4A–D). In Wnt1F/F; OsxCreERT2 females, −9.2 N was estimated to engender a peak strain of −5600 με (Fig S2), a level that typically induces a woven bone response[12] (Fig S3); in knockout females −9.2 N induced lamellar bone formation.

In force-matched knockouts, Ps.MS/BS, Ps.MAR, and Ps.BFR/BS were 1.8, 2.4, and 4-fold higher in the loaded vs non-loaded limbs ($p<0.0001$), indicating that tibial loading stimulated Ps bone formation (Fig 5A–C). Notably, these values were nominally less than the respective fold-increases in control mice (2.9, 3.5 and 8.3), and a comparison of Ps.MAR and Ps.BFR/BS in the loaded limbs of control vs knockout mice showed that both indices were significantly lower in knockouts (Fig 5A–C). Finally, relative to the control group Ps.rBFR/BS was 39% lower in *Wnt1* knockouts after force-matched loading (1.91 vs 1.17 $\mu m^3/\mu m^2$/day in control vs knockout mice; Fig 5D). Therefore, despite being loaded to 85% greater peak strain than control, *Wnt1* knockout females still had a diminished response to loading.

Endocortical bone formation—In contrast to the strong anabolic response elicited on the periosteal surface in Wnt1F/F mice, tibial loading did not induce a strong endocortical response (Figs S4 and S5) – consistent with the lower values of strain engendered on this surface. In males, neither loading nor genotype had an effect on Ec.MS/BS, Ec.MAR, or Ec.BFR/BS, and Ec.rBFR/BS indicated that endocortical bone formation was not different in control vs knockout mice (Fig S4). In females, a significant or near-significant increase in Ec.MAR and Ec.BFR/BS was observed in controls and force-matched knockouts, but not in strain-matched knockouts (Fig S5). Loading-induced endocortical bone formation was not different in control vs force-matched knockouts (Fig S5D).

In sum, these findings show that *Wnt1* was required for the bone anabolic response to skeletal loading, particularly for periosteal bone formation.

Loading-induced upregulation of bone formation genes was diminished in Wnt1 knockouts

To better understand the role of *Wnt1* in loading-induced bone formation, gene expression analysis was performed after 5 days of loading, on day 26 (Fig 6, top panel). Gene expression was analyzed by RT-qPCR (control vs knockout mice) and by RNAScope *in situ* hybridization (control mice only).

Congruent with previous reports[11–12], we found that loading potently induced *Wnt1* mRNA 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 Wnt1F/F controls (Fig 6A). In contrast, loading had no effect on the abundance of *Wnt1* mRNA in the tibias of Wnt1F/F;

OsxCreERT2 knockouts (loaded vs non-loaded, p=0.999), consistent with bone-targeted knockout.

Genes associated with osteo-induction, osteoblast differentiation, and matrix synthesis were also analyzed. RNAScope *in situ* hybridization showed that loading increased *Bmp2* expression in osteocytes and *Bglap* expression in osteoblasts (Fig S6B, S6D). 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 Wnt1F/F mice (Fig 6B, 6D). In contrast, *Bmp2* and *Bglap* were not significantly different in the loaded vs non-loaded bones of *Wnt1* knockouts. Loading also significantly increased *Col1a1* expression – a marker of bone matrix synthesis – in the tibias of control but not knockout mice (Fig 6C).

Wnt pathway-related genes were also analyzed by qPCR and *in situ* hybridization. Neither loading nor genotype were found to have a significant effect on the expression of *Axin2* or *Nkd2* (Figs S6E, S7A–B). In contrast, *Opg* was regulated by loading but not genotype (main effect of loading, $p<0.05$). *Opg* expression was 1.4 and 1.2-fold higher in the loaded vs non-loaded limbs of control and knockout mice, respectively (Fig S7C). *In situ* analysis showed that loading induced *Opg* expression in osteocytes (Fig S6F). Gene expression findings also showed that *Sost* and *Dkk1* were not different in the loaded limbs of control vs knockout mice, indicating that the diminished loading response observed in knockouts was not due to elevated expression of these Wnt antagonists in the bone (Fig S7D–E). Finally, *Wnt1* knockout mice had a marginally lower loading-induced induction of *Wnt7b*, while *Wnt16* was unaffected by loading or genotype (Fig S7F–G). Analysis by *in situ* hybridization showed that *Wnt7b* mRNA was expressed in osteocytes (Fig S6A)

In sum, these findings show that *Wnt1* plays a crucial role in mediating the bone anabolic response to mechanical loading.

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

Because *Wnt7b* is osteo-anabolic[14] and is induced in the bone by loading[12], we asked whether the phenotypes observed in *Wnt1* knockouts would be exacerbated by also targeting *Wnt7b*. We confirmed DNA recombination of *Wnt1* and *Wnt7b* genes in the tibias of *Wnt1/7b* knockout mice (tamoxifen-treated Wnt1F/F; Wnt7bF/F; OsxCreERT2) (Fig.S8A–B). We also confirmed knockdown of *Wnt1* (−63% in the loaded limbs of *Wnt1/7b* knockouts vs controls, p=0.083) and *Wnt7b* (−87% in the loaded limbs of knockout vs control mice, p=0.194) at the mRNA level (Fig S9A–B).

The cortical bone phenotype observed in *Wnt1* knockouts was recapitulated in *Wnt1/7b* knockouts. Before gene deletion (day 0) cortical bone morphology was not different between control vs *Wnt1/7b* knockout mice (Fig S8). However, Ct.Ar and pMOI decreased between days 0 and 22 in *Wnt1/7b* knockout mice, but increased in Wnt1F/F; Wnt7bF/F controls (Fig S8D, S8H). Ct.Th was not significantly different on days 0 and 22 in *Wnt1/7b* knockouts, but was significantly higher on day 22 relative to day 0 in controls (Fig S9F). We also found that Me.Ar decreased and TMD increased significantly between days 0 and 22 in control but not knockout mice (Fig S8E, S8G).

Finally, when *Wnt1/7b* knockouts and their controls were subjected to strain-matched loading (approximately −3000 με), *Wnt1/7b* knockouts exhibited an impaired response to loading, similar to *Wnt1* single knockouts. In male and female controls, loading increased Ps.MS/BS and Ps.MAR significantly ($p<0.01$), resulting in an overall 5.6 to 11-fold increase in Ps.BFR/BS ($p<0.0001$; Fig S9C–D). In contrast, loading generally did not have a significant effect on bone formation indices in *Wnt1/7b* knockouts. Analysis of *relative* bone formation rates showed that the periosteal response to loading was reduced 86-90% in *Wnt1/7b* knockouts compared to sex-matched controls.

Discussion

Mechanical loading on the skeleton stimulates bone formation. To investigate the role of *Wnt1* in the skeletal response to loading, we tamoxifen-dosed 5-month old OsxCreERT2; Wnt1F/F mice to inactivate *Wnt1* in the osteoblast lineage, then subjected mice to *in vivo* tibial loading 21 days later. Over 3 weeks, Ct.Ar decreased 9-12% in *Wnt1* knockouts, concomitant with a 78% reduction in tibial *Wnt1* expression (Figs 2E, 3B). In controls, loading increased *Wnt1* expression in the bone ($p<0.0001$) and *in situ* hybridization showed that this induction occurred in osteocytes (Figs 1A, 6A). Loading-induced *Wnt1* upregulation was associated with a 8 to 10-fold increase in Ps.BFR/BS in Wnt1F/F mice (Figs 4D, 5D). In knockouts, Osx-targeted *Wnt1* deletion blocked *Wnt1* upregulation (Fig 6A), leading to a 65 to 94% lower periosteal bone formation response (Figs 4E, 5D). Together, these findings show that *Wnt1* is important for homeostasis of the adult skeleton, as well as for the bone anabolic response to mechanical loading.

Loading increases Wnt1 expression in osteocytes

Whole-bone transcriptional profiling studies have shown that mechanical loading increases the expression of Wnt family genes – including *Wnt1* and *Wnt7b* – in the bone[9–10]. Subsequently, Harris and Silva used laser capture microdissection to isolate intracortical bone samples from loaded and non-loaded bones of C57Bl/6 mice, and showed that *Wnt1* in these osteocyte-enriched samples was upregulated by loading[11]. Congruent with these results, our gene expression analyses showed that *Wnt1* was 6.6-fold higher in the loaded vs non-loaded bones of Wnt1F/F mice after 5 days of loading (Fig 6A). Additionally, analysis by RNAscope® *in situ* hybridization showed that *Wnt1* induction occurred primarily in osteocytes embedded in the intra-cortical matrix rather than in cells lining the bone surface (i.e., osteoblasts) (Figs 1A and S1). Analysis of *in situ* hybridization further revealed that loading increased not only the total percentage of *Wnt1*-positive cells in the bone, but also the magnitude of *Wnt1* expression within those cells (Fig 1B). Together, these data show that mechanical loading stimulates *Wnt1* expression in the bone, particularly in osteocytes.

Loading-induced bone formation is reduced in Wnt1 knockouts

OsxCreERT2 was used to inactivate *Wnt1* in the Osx-lineage cells of 5-month old mice. Previously, we used a similar tamoxifen 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 within the cortical bone (osteocytes) within days of

tamoxifen treatment – indicating that OsxCreERT2 targets both osteoblasts and osteocytes in the bones of adult mice[7].

OsxCreERT2-mediated *Wnt1* deletion completely neutralized the effect of loading on *Wnt1* expression in the bone (Figs 6A and S10A). While tibial *Wnt1* expression increased 6.6-fold in loaded bones of Wnt1F/F controls, *Wnt1* expression remained unchanged in the loaded vs non-loaded limbs of *Wnt1* knockouts. qPCR analysis in male and female mice showed that although the gene expression response to loading was generally more robust in Wnt1F/F control females compared to Wnt1F/F males, the increase in *Wnt1*, *Bmp2*, *Col1a1*, and *Bglap* expression in response to loading was significant or near-significant in both sexes (Fig S10). These results were congruent with the finding that the bone formation response was more pronounced in Wnt1F/F females compared to Wnt1F/F males (Figs 4D and 5D). Meanwhile, the induction of *Wnt1*, *Bmp2*, *Col1a1*, and *Bglap* in response to loading was reduced in knockouts, irrespective of sex, indicating that loading-induced *Wnt1* expression as required for the upregulation of *Bmp2*, *Col1a1*, and *Bglap* in the bone. In combination with our *in situ* hybridization findings, these results suggest that loading stimulates osteoblast differentiation/activity on the bone surface (*Bglap*, Fig S6D) by inducing *Wnt1* ligand expression in osteocytes (Figs 1 and S1).

At the tissue level, *Wnt1* inactivation in the Osx-lineage reduced the periosteal response to loading. In males, periosteal bone formation rate was higher in the loaded vs non-loaded bones of 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 65% lower in knockouts (Fig 4D). In females, strain-matched loading increased all indices of periosteal bone formation in control but not knockout mice (Fig 5A–C). Compared to Wnt1F/F controls, the periosteal response to loading (i.e., Ps.rBFR/BS) was 94% lower in strain-matched *Wnt1* knockouts (Fig 5D). Moreover, analysis of a force-matched cohort (−9.2 N, *Wnt1* knockout females) showed that although a loading response could be elicited with greater strain magnitude (−5600 με vs −3000 με), the periosteal response to loading was still 39% less in knockouts vs controls (Fig 5D). Together, these results demonstrate that *Wnt1* is required for loading-induced periosteal bone formation.

Bone area decreased in Wnt1 knockouts after 21 days deletion

Independent of loading, short-term *Wnt1* deletion (3 weeks) in the Osx-lineage was associated with decreased bone area and cortical thickness (Fig 3B, 3D). These findings are congruent with a report by Wang, et al. which showed that deleting *Wnt1* in the Osx-lineage impaired periosteal bone growth in young 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 of distal tibias from 12 week old mice revealed that these deficits were caused by a reduction in periosteal bone formation, while changes to endocortical bone formation were not significant[22].

Similar findings were reported by Joeng, et al. and Luther, et al. Mice lacking *Wnt1* in late osteoblasts (Dmp1Cre; Wnt1F/F) had skeletal deficits – including reduced Ct.Th, Tb.N, and Tb.Th – 2 months after birth[13], while mice lacking *Wnt1* in osteo-progenitors

(Runx2Cre; Wnt1F/F) exhibited BV/TV and Ct.Th deficits in the femur at 24 weeks, leading to increased risk of spontaneous fractures[23]. Hypomorphic *Wnt1* variants have also been linked to low bone mass and spontaneous fractures in mice[24]. Our study extends these findings by showing that short-term *Wnt1* deletion in adults leads to bone loss. Thus, osteoblast/osteocyte *Wnt1* is required for adult bone homeostasis.

The skeletal response to bone anabolic loading declines with age[25–27]. For example, tibial loading enhances periosteal bone formation in adult mice of different ages; however, the magnitude of the osteogenic response to loading is significantly lower in aged 22-month old vs. young adult 5-month old mice[12]. To understand the basis for these differences, Chermside-Scabbo, et al. used RNASeq to characterize the transcriptional responses to loading in 5 and 22-month old C57Bl/6 mice after 1, 3, and 5 days of loading[10]. In both age groups, *Wnt1* emerged as a top 10 upregulated gene on days 1 and 3. 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, loading increased *Wnt1* in both age groups, albeit to a lesser degree in aged mice – concomitant with a diminished osteogenic response to loading. Taken together, these findings suggest that diminished induction of *Wnt1* by loading may contribute to reduced loading-induced bone formation in the bones of aged mice.

The bone anabolic function of *Wnt1* in the Osx-lineage is further supported by our findings in *Wnt1/7b* double conditional knockout mice, which exhibited cortical bone deficits 21 days post-deletion (Fig S8D). Moreover, like *Wnt1* knockouts, *Wnt1/7b* knockouts did not exhibit a loading-induced increase in *Wnt1* expression and had a blunted periosteal response to loading (Fig S9). In general, the magnitude of the deficits in *Wnt1/7b* knockouts were comparable to those in *Wnt1* knockouts. Importantly, while we did see evidence of *Wnt7b* gene recombination in *Wnt1/7b* double knockout mice (Fig. S8B), we did not confirm significant knockdown of *Wnt7b* at the mRNA level (Fig. S9B). Thus, we are not able to infer whether *Wnt7b* has a functional role in loading-induced bone formation. Additional studies, perhaps using a different *Wnt7b* floxed mouse, are needed to address this question. Nonetheless, the consistent deficits in loading-induced bone formation in *Wnt1* and *Wnt1/7b* knockout mice show that in two different mouse lines, *Wnt1* in the Osx-lineage was essential for the response to loading.

In conclusion, our findings support a growing body of evidence that *Wnt1* is essential for bone homeostasis in the adult skeleton. In particular, we showed that mechanical loading induced *Wnt1* expression in osteocytes, and that loading-induced *Wnt1* expression in the Osx-lineage was required for the periosteal bone formation response to tibial loading.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Conflict of Interest Statement

LYL, NM, CCS, JTS, KJ, and BL have no financial or non-financial competing interests to disclose. MJS is on the editorial board at *Bone*, *Journal of Orthopaedic Research*, and *Calcified Tissue International*, and served on the board of directors at the Orthopaedic Research Society (2018-2022). RC serves as Editor-in-Chief for *Journal of Bone and Mineral Research* and has received research support from Ultragenyx.

Data Availability Statement

The data that support the findings of this study are openly available in Biorxiv at https://doi.org/10.1101/2022.02.28.482178.

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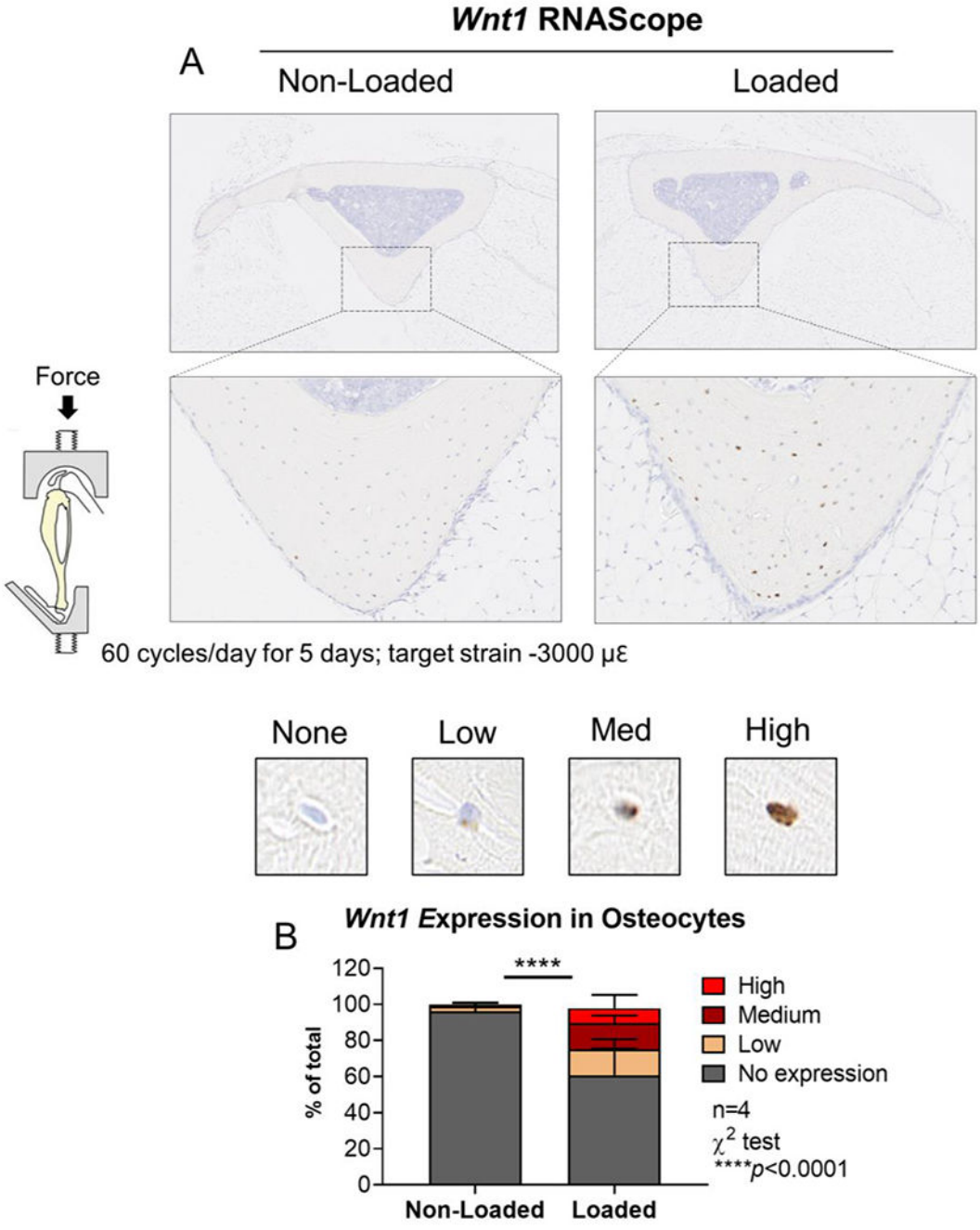

Figure 1. Tibial loading increased *Wnt1* expression in the bone.

Wnt1 mRNA expression was analyzed by RNAScope *in situ* hybridization after 5 days of loading. (A) Tibial *Wnt1* expression was induced by loading in a Wnt1F/F male. Results representative of n=4. (B) Osteocytes were scored for *Wnt1* expression at the site of peak compressive strain (lower panels).

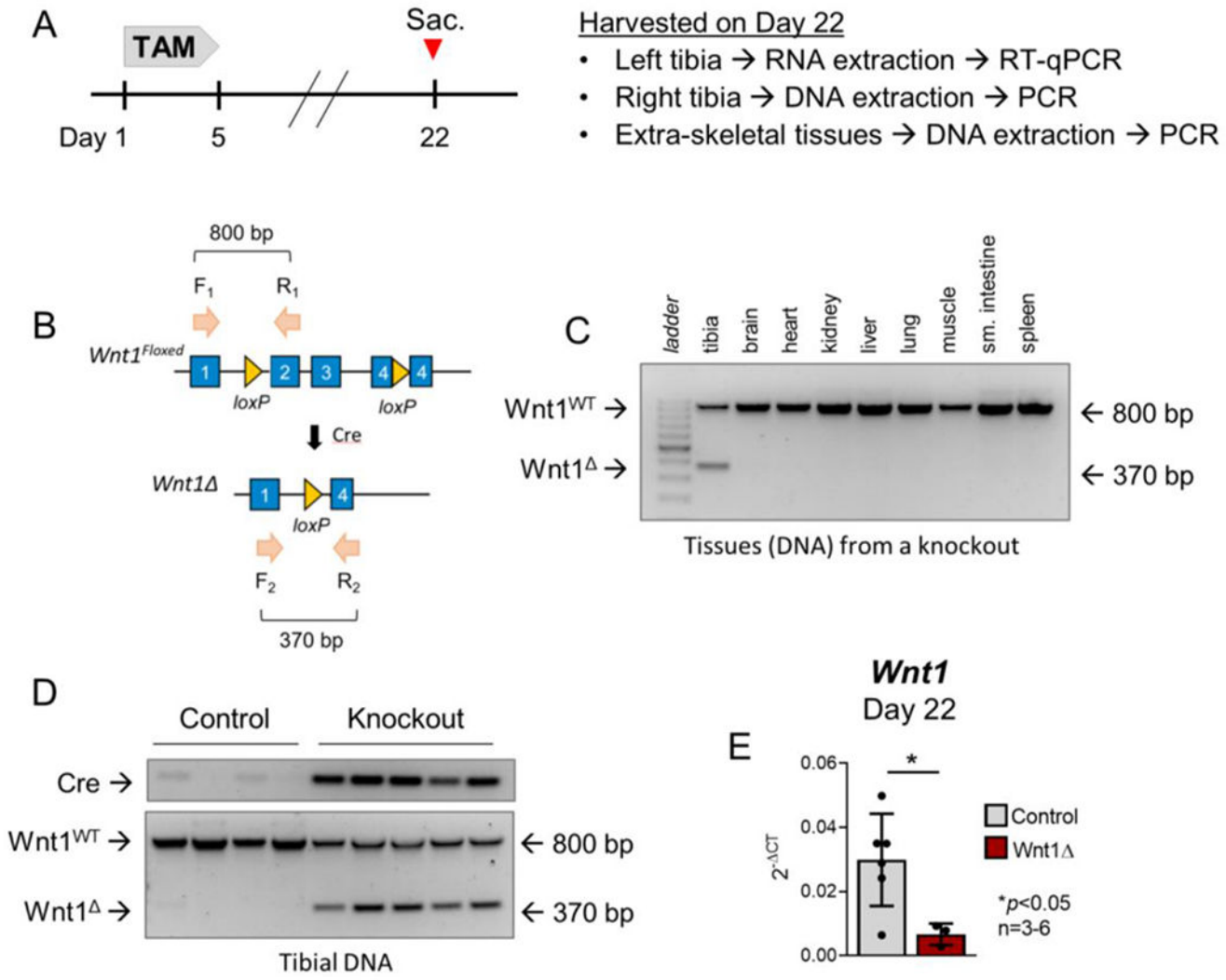

Figure 2. *Wnt1* was conditionally deleted in the Osx-expressing cells of adult mice.

(A) Five-month old Wnt1F/F (control) and OsxCreERT2; Wnt1F/F (knockout) mice were dosed with tamoxifen to induce *Wnt1* deletion in the osteoblast lineage. (B-D) DNA recombination analysis indicated that *Wnt1* (exon 2-4) deletion was specific to the tibias of OsxCreERT2-positive Wnt1F/F mice. (E) Tibial *Wnt1* RNA expression was 78% lower in *Wnt1* knockouts compared to controls. Individual data points and the mean ± std deviation are shown. n=3-6/group.

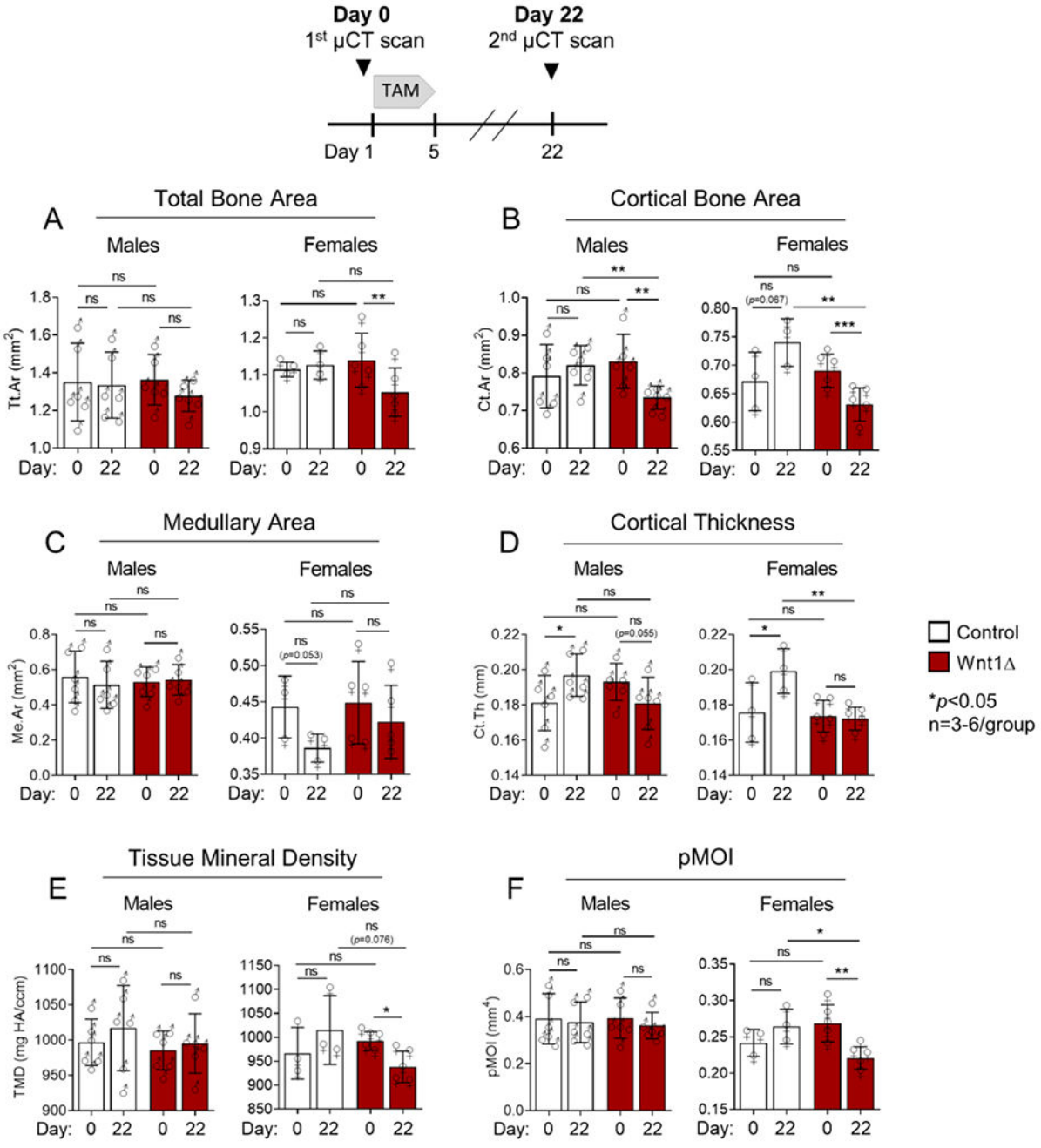

Figure 3. Cortical bone area decreased after short-term *Wnt1* deletion.

The tibias of naïve male and female mice were serially scanned to determine the effects of short-term *Wnt1* deletion on cortical bone morphometry. Tibias were µCT 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.

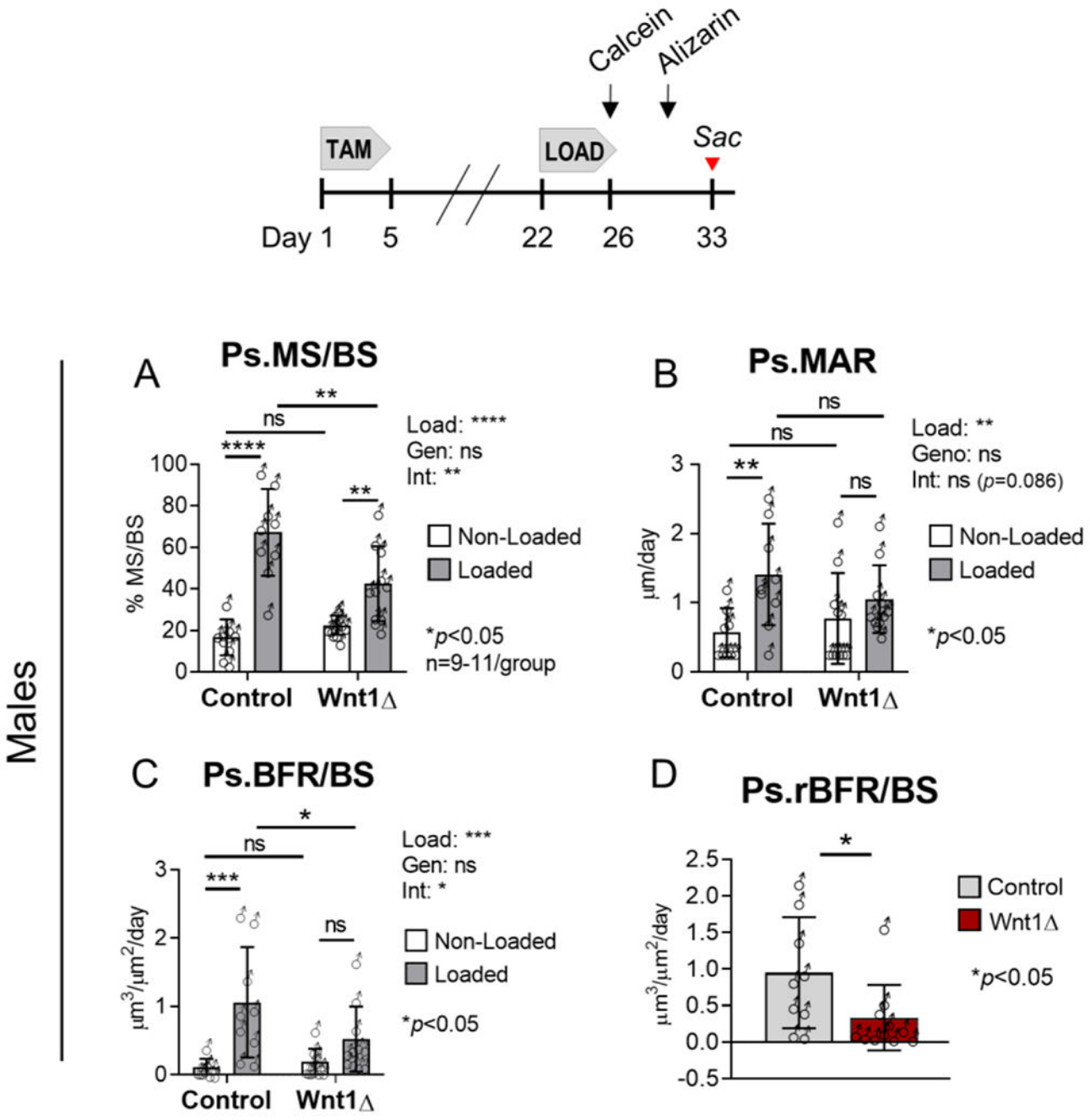

Figure 4. Loading-induced periosteal bone formation was blunted in *Wnt1* knockout males.

Dynamic histomorphometry was used to analyze bone formation after 5 days of strain-matched loading. (A-C) Periosteal bone formation indices in males subjected to strain-matched loading (approximately −3000 με). Loading increased periosteal bone formation in both groups, albeit to a lesser degree in *Wnt1* knockouts. (D) Relative bone formation rate (rBFR/BS), used as a net index of loading-induced bone formation, was 65% less in *Wnt1* knockouts relative to controls. n=9-11/genotype.

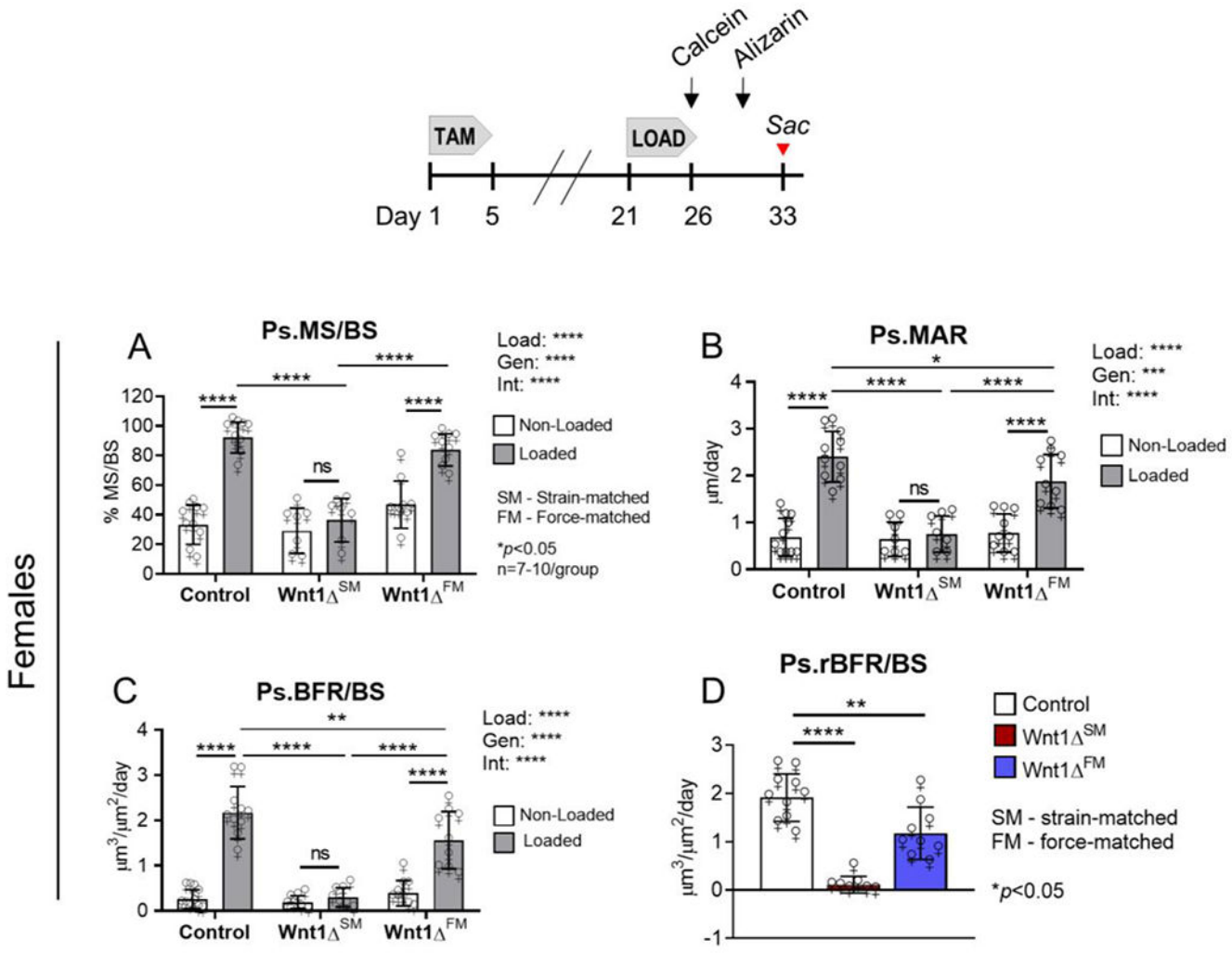

Figure 5. Loading-induced periosteal bone formation was blunted in *Wnt1* knockout females.

(A-D) Control females were loaded to −9.2 N to engender a peak target strain of −3040 με. Strain-matched (SM) knockouts were loaded to −5.4 N to achieve a similar strain (−3200 με), while force-matched (FM) knockouts were loaded to −9.2 N (approximately −5600 με). (D) Comparison of the relative bone formation rates showed that the periosteal response to loading was 39% lower in force-matched knockouts relative to controls. n=7-10/genotype.

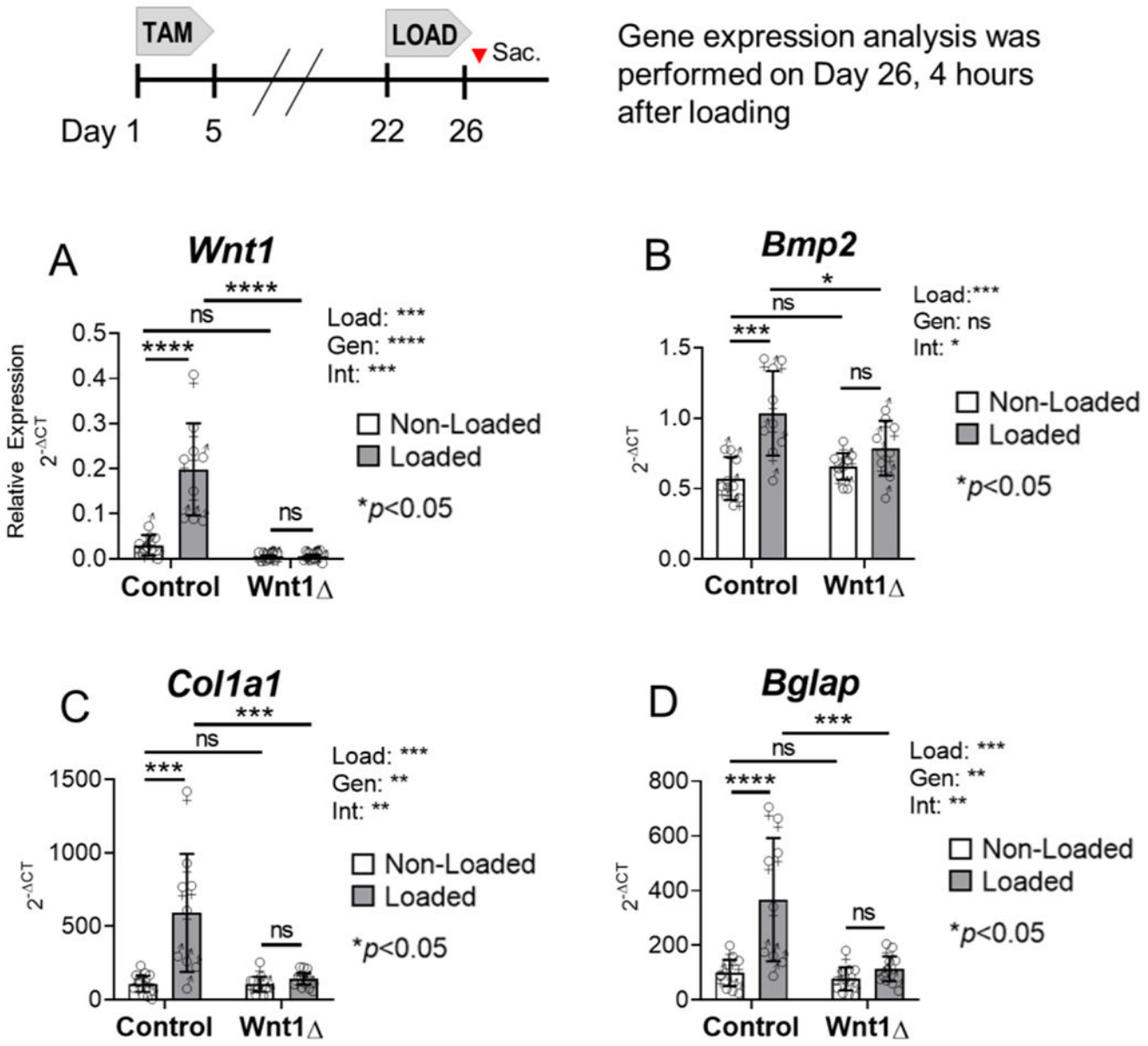

Figure 6. Loading-induced upregulation of bone formation genes was impaired in *Wnt1* knockouts.

(A) Tibial loading potently induced *Wnt1* expression in the bones of control but not knockout mice. (B-D) Loading-induced *Bmp2*, *Col1a1*, and *Bglap* upregulation was blunted in *Wnt1* knockouts relative to strain-matched controls. n=4-5 per sex per genotype 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.