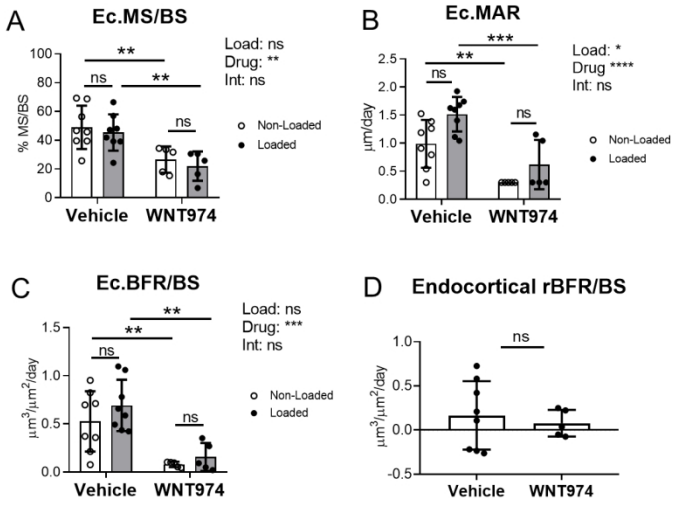

Supplemental Figure S1. Endocortical bone formation indices in wild-type C57Bl/6 mice treated with Porcupine inhibitor WNT974. Loading had a negligible effect on endocortical bone formation in both groups. Bars depict mean ± SD, with individual data points shown (n=5-8). Two-factor ANOVA (A-C) or one-factor ANOVA (D); *p<0.05, ns=not significant (p>0.05).

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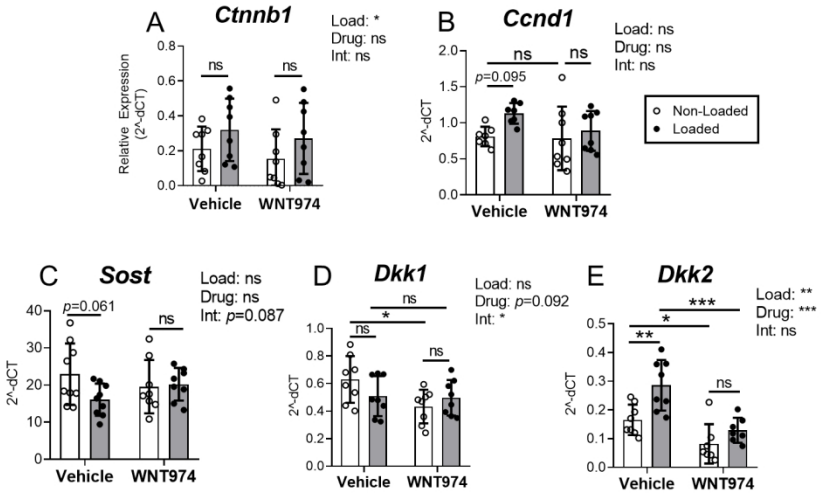

Supplemental Figure S2. Tibial gene expression was analyzed by qPCR to evaluate the effect of 5 days of loading ("Load"), WNT974 treatment ("Drug"), and their interaction ("Int") using two-factor ANOVA with Sidak's multiple comparisons test. Bars depict mean ± SD, with individual data points shown (n=8-10). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns=not significant (p>0.05). Data analysis as described in Figure 3.

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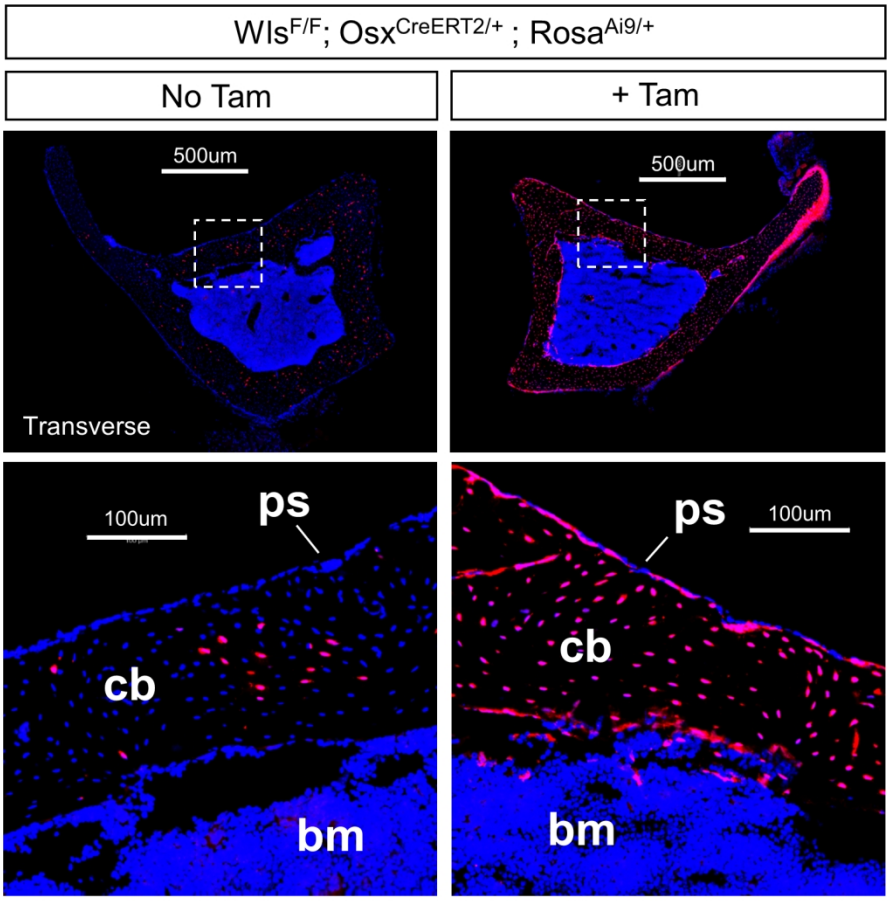

Supplemental Figure S3. Cre reporter expression in the tibia. A fluorescent Cre-reporter was bred into the transgenic WIs colony to generate WIs$^{F/F}$; Osx$^{CreERT2/+}$;Rosa$^{Ai9/+}$ mice, which were treated with tamoxifen (Tam) to delete *Wls* and to survey tdTomato/Ai9 reporter fluorescence in the tibia. tdTomato expression was observed throughout the cortical bone (cb) and in the periosteum (ps) of tamoxifen-treated mice. Some tdTomato-positive cells were also observed in negative control cortical bones of mice that never received tamoxifen. ps=periosteum, cb=cortical bone, bm=bone marrow. Results are comparable to recently published results from our lab (Zannit and Silva, 2019).

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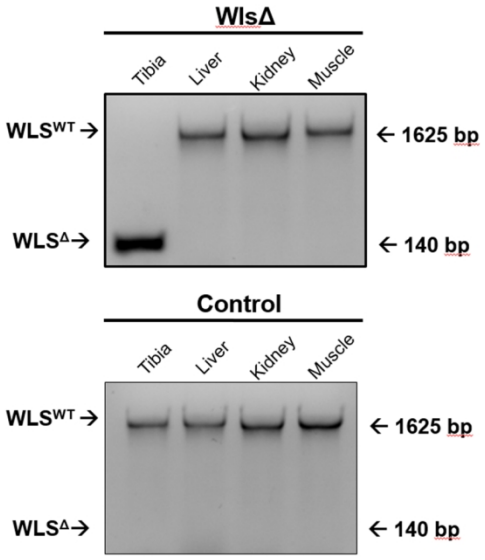

Supplemental Figure S4. DNA recombination PCR was used to evaluate the specificity of Wls deletion. Primers up- and downstream of the floxed locus (*Wls* exon 1) were used to amplify wild-type (1625bp) and recombined (150bp) DNA. Recombination at the locus and excision of exon 1 removes the ATG start site, rendering a *Wls* null allele (WLSΔ). DNA recombination was observed in the bones of Wls knockout mice but not in the bones of control mice. No DNA recombination was observed in any of the extra-skeletal tissues surveyed from either knockout or control mice. Results are representative of n=3 Wls cKO and 1 control.

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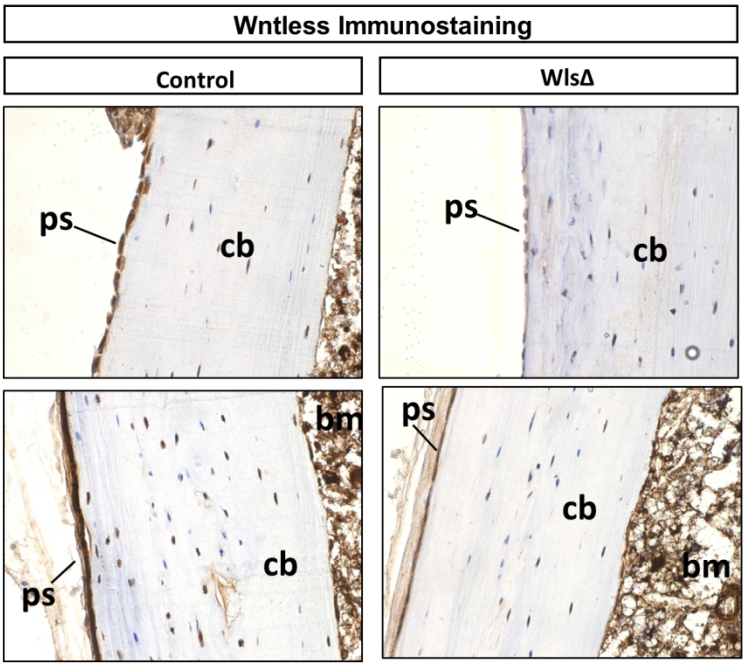

Supplemental Figure S5. Wntless immunohistochemistry. Tibial sections from control and knockout mice were incubated with an antibody specific for mouse Wntless (aka Gpr177) then counterstained with Hematoxylin (blue). Relative to control tissues (left two panels), there were fewer Wntless-positive cells (brown puncta) throughout the cortical bone (cb) and periosteum (ps) of *Wls* knockout bones. Tibias were sectioned through the sagittal plane and imaged at 40x. ps=periosteum, cb=cortical bone, bm=bone marrow. Representative of n=3/genotype.

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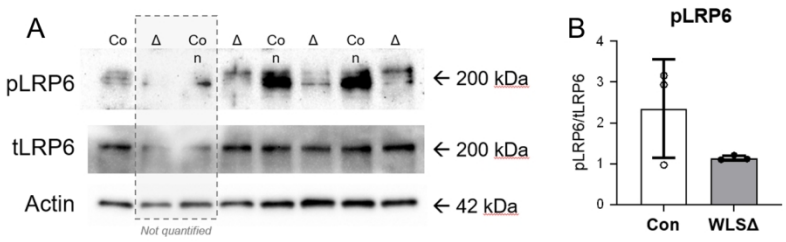

Supplemental Figure S6. Western blotting showed diminished Wnt signaling in $WLS^{F/F}$;$Osx^{CreERT2}$ (Δ) compared to $WLS^{F/F}$ control (Con) mice. LRP5/6 are transmembrane co-receptors for Wnt ligands that are required for canonical Wnt/β-catenin signaling. Upon stimulation with Wnt ligands, LRP6 is phosphorylated at multiple sites. For this experiment, mice were gavaged with tamoxifen for 3 days, had 2 days of clearance, were sacrificed on what would have been day one of loading to assay Wnt signaling at the start of the loading. Tibias were stripped of muscle, cut at the distal tibiofibular junction (TFJ) and 2mm distal to the tibial plateau, centrifuged to remove the bone marrow, and flushed with ice-cold PBS. Protein was extracted with scissors on ice in 150µL of RIPA buffer (CST #9806) spiked with protease and phosphatase inhibitors (ThermoFisher #78440). After 25min on ice with vortexing every 5min, supernatant was collected, and ~20µg of protein was run on an Any kD gel (Bio-Rad #4569033). Proteins were transferred onto a PVDF membrane (wet transfer with buffer containing 20% methanol) at 4°C for 85min at constant 75V. The membrane was blocked with 2.5% BSA (Sigma #10735086001) in tris-buffered saline with 1% Tween-20 (TBS-T) for 1hr. The membrane was cut at 75kDa and incubated with the respective primary antibodies (1:1000) for pLRP6 (CST #2568) and β-actin (CST #8457) at 4°C overnight. Membranes were washed with TBS-T (3x10min), incubated with HRP-conjugated anti-rabbit secondary antibody (1:2000, Sigma GENA934) in 2.5% BSA for 1hr. After washing, ECL substrate (Bio-Rad 1705062) was used to image the membrane on the Bio-Rad ChemiDoc XRS+ platform. After acquisition, the membrane was stripped for 8min with (Theremo #46430) and probed for LRP6 (CST #3395), as described. Band intensity was quantified in ImageJ.

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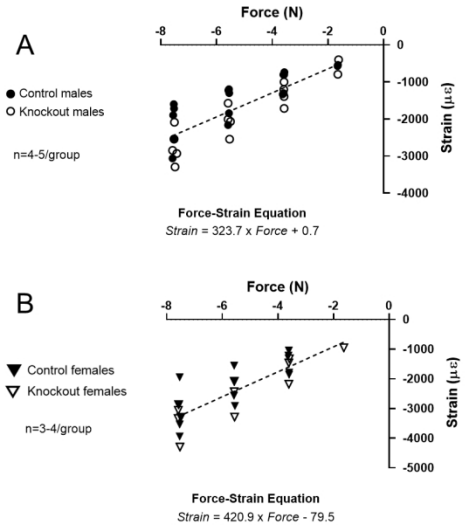

Supplemental Figure S7. Force-strain relationships in 5-month old male (A) and female (B) $Wls^{F/F}$ control and $OsxCreERT2;Wls^{F/F}$ cKO mice (WLSΔ). Males and females were loaded to -11N and -8N, respectively, which engendered an estimated peak compressive strain of -3500 με. Regression lines were similar between control and knockout mice of each gender, and thus they were pooled for a single sex-specific regression. Males had a lower slope (stiffer) and thus required a higher force to generate an equivalent target strain compared to females.

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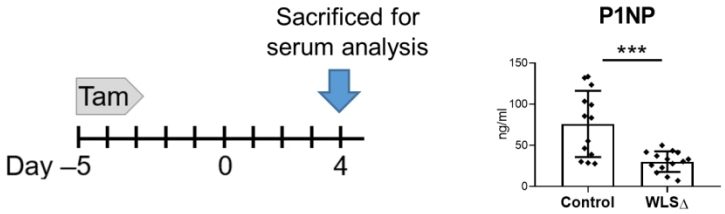

Supplemental Figure S8. Serum P1NP was 60% lower in Wls cKO mice compared to controls (n=13-14). Data represent both sexes. Bars depict mean ± SD, with individual data points shown (n=5-8 per sex per treatment group). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns=not significant (p>0.05).

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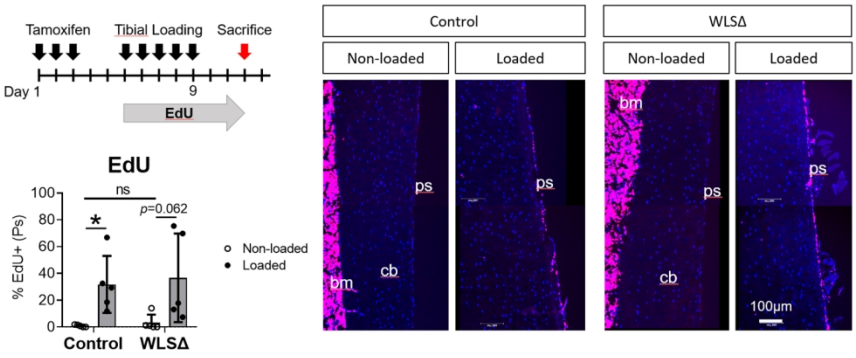

Supplemental Figure S9. Loading-induced proliferation on the cortical bone surface was observed in both control and Wls cKO (WLSΔ) mice. (A) Mice were loaded for 5 days and EdU was administered in the drinking water to label proliferative cells. (B) Representative DAPI/EdU merged images from the non-loaded and loaded tibias of control and knockout mice shown. (C) ps=periosteum, cb=cortical bone, bm=bone marrow. Bars depict mean ± SD, with individual data points shown (n=5), *p<0.05.

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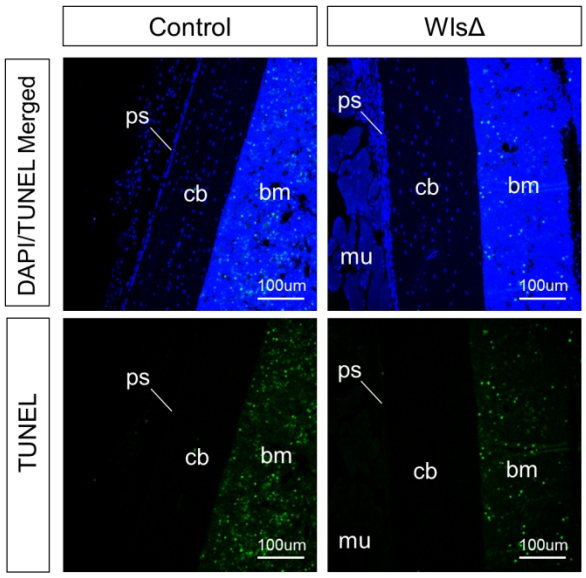

Supplemental Figure S10. The TUNEL assay was used to assess cell death in the non-loaded and loaded tibias of control and WIs cKO (WLSΔ) mice after 5 days of loading. TUNEL staining was comparable in the non-loaded (shown) and loaded (not shown) tibias of both groups. Results are representative of n=3 non-loaded and 3 loaded tibias from each genotype. cb=cortical bone, bm=bone marrow, mu=muscle, ps=periosteum.

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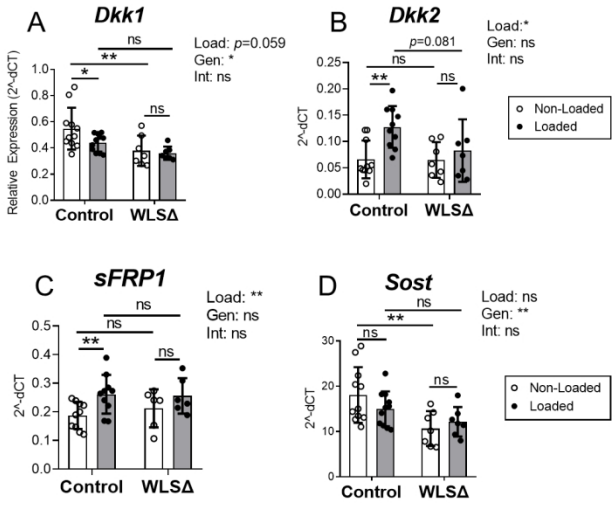

Supplemental Figure S11. Expression of Wnt pathway inhibitors in the tibia after 5 days of loading. Gene expression was evaluated by qPCR 4-hrs after the 5th bout of loading (n=7-11). *p<0.05, **p<0.01, ns=not significant (p>0.05). Data analysis as described in Figure 3.

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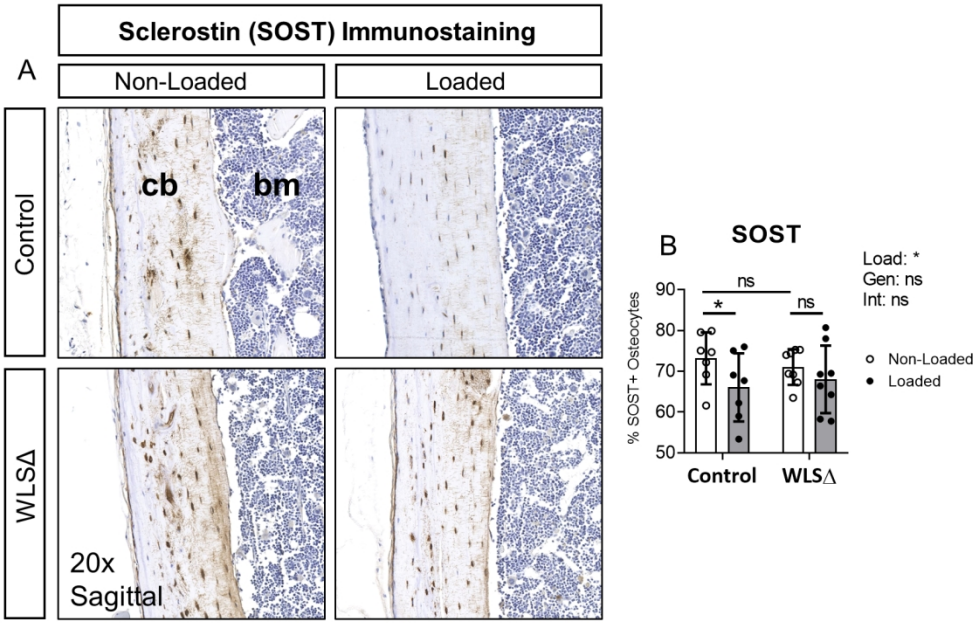

Supplemental Figure S12. Sclerostin immunohistochemistry. An antibody specific to mouse Sclerostin was used to analyze Sclerostin protein expression in the tibias of control and WIs cKO (WLSΔ) mice. Representative images of Sost staining in the non-loaded (left) and loaded (right) tibias of control (top) and WIs cKO (bottom) mice shown (A). Percent SOST+ osteocytes was calculated as the ratio between Sost+ osteocytes (brown puncta) to total osteocytes (blue puncta) at the mid-diaphysis (B). Bars depict mean ± SD, with individual data points shown (n=7-8) *p<0.05. Data analysis as described in Figure 3.

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