Figures and figure supplements

 Reactivation of a developmental Bmp2 signaling center is required for therapeutic control of the murine periosteal niche

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Figure 1. Osteoprogenitor-derived Bmp2 couples length to width in the appendicular skeleton. (a,b) Representative 3D reconstructions of the murine femur using microcomputed tomography (microCT). (c) Femoral length or (d) femoral width at mid-diaphysis, presented as mean ± s.d. with n = 8–20.
bones per age per genotype. *p<0.05, **p<0.005, or ***p<0.0005 vs. age-matched Bmp2 Prx1-cKO cohort. (e,g) Representative toluidine blue histology at the mid-diaphysis of the forelimb. (f,h) MicroCT analysis of total cross-sectional bone tissue area presented as mean ± s.d. with n = 4. *p>0.05. (i,j) Cross-sectional composition of cortical bone in the radius of newborn (n = 6–9 per genotype) or 2 week-old mice (n = 3–9 per genotype) (see Materials and methods). Abbreviations: PC, perichondrium; PO, periosteum. (k,l,m) Representative toluidine blue histology at the mid-diaphysis of indicated skeletal elements. (n) Length or (o) width of embryonic day 15 metatarsals, measured following 1 or 5 days of ex vivo culture. Mean ± s.d. with n = 6–12 where **p<0.005, ****p<0.0005. (p,q,r) Femur width mean ± s.d. with n = 6–12 where *p<0.05. (s) Minimum moment of inertia in P14 femur, calculated by microCT (n = 4) shown as mean ± s.d. where ****p<0.0005. (t) Toluidine blue histology revealing cortical microcracks in the humerus of Bmp2 Prx1-cKO mice at 4 weeks of age. (u) X-ray images showing representative bowing of the radius and ulna of Bmp2 Prx1-cKO mice in the absence of frank fractures. Statistical analyses were performed using two-tailed Student’s t-test.

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Figure 1—figure supplement 1. BMP2 acts downstream of IGF-1 pathway in the periosteum. (a) Persistence of IGF1 + cells in Bmp2 Prx1-cKO periosteum. Transverse sections of the radius and ulna were imaged in brightfield following immunostaining to visualize cells expressing IGF-1. (b) Elisa analysis demonstrates that circulating levels of IGF-1 are not statistically reduced in Bmp2 Prx1-cKO mice.

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Figure 1—figure supplement 2. Skeletal phenotype analysis of Bmp2<sup>Flox/Flox</sup>; Col1a1-Cre mice shows that loss of Bmp2 in mature osteoblasts does not cause a periosteal growth defect. (a,b) Alizarin red and alcian blue whole mount staining of (a) forelimbs and (b) hindlimbs from at postnatal day 14 mice. (c,d) Representative toluidine blue histology at the mid-diaphysis of the (c) forelimb or (c) femur at postnatal day 14. (e) X-ray imaging shows that Bmp2 Col1a1-cKO reach peak adult body size with no evidence of spontaneous fractures. (f) Length and width remain coupled at postnatal day 14 following ablation of Bmp2 in mature osteoblasts.

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Figure 1—figure supplement 3. Skeletal phenotype analysis of Bmp2^{Flox/Flox}, Prx1-Cre mice reveals architectural abnormalities compounded by material defects. Bone mass analyzed in the femur of juvenile (2 week-old) mice by microcomputed tomography (microCT). (a) X-ray imaging shows that Bmp2 Prx1-cKO reach peak adult body size despite slender bones. (b) Trabecular bone at the distal metaphysis and (c) cortical bone at the mid-diaphysis of the femur visualized by 3D reconstructions. Images represent the group mean and are shown to scale. (d–h) Dynamic histomorphometry assessing bone formation rate as a function of bone surface (BFR/BS) at (e) endosteal versus (f) periosteal surfaces, or mineral apposition rate (MAR) at
(g) Endosteal versus (h) periosteal surfaces. n = 4, presented as mean ± s.d. where *p<0.05 vs. age-matched Bmp2<sup>F/F</sup> littermates. (i) Scanning electron microscopy of cross-sections at the femoral mid-diaphysis. (j) Residual cartilage islands and increased osteocyte density in the femur of Bmp2 Prx1-<sup>cKO</sup> mice visualized by safranin O and nuclear red staining. (k,l) Picrosirius red and hematoxylin stain, followed by polarized light microscopy reveal accumulation of disorganized collagen extracellular matrix in the femur of Bmp2 cKO mice. (m–p) Dynamic histomorphometry assessing osteoid thickness (OTH) at (m) endosteal versus (n) periosteal surfaces, or mineralization lag time (MLT) at (o) endosteal versus (p) periosteal surfaces. n = 4, presented as mean ± s.d. where *p<0.05 vs. age-matched Bmp2<sup>F/F</sup> littermates. DOI: https://doi.org/10.7554/eLife.42386.005
Figure 2. Robust versus quiescent states of Bmp2 expression reflect active versus homeostatic states of periosteal bone growth. LacZ staining on tissues from mice expressing beta-galactosidase from one allele of the endogenous Bmp2 locus (Bmp2<sup>lacZ/+</sup>). (a) Lateral view of a whole mount E13.5 Bmp2<sup>lacZ/+</sup> mouse embryo, representative of other Bmp2<sup>lacZ/+</sup> littermates. (b,c) Longitudinal sections through the (b) forelimb or (c) hindlimb of E14.5 Bmp2<sup>lacZ/+</sup> mouse embryos. (d,e) Longitudinal sections of (d) digits or (e) radius/ulna of newborn Bmp2<sup>lacZ/+</sup> mice. (f–l) Longitudinal sections through cortical femoral bone from (f) 7 day-old, (g) 14 day-old, (h) 21 day-old or (i) 6 month-old Bmp2<sup>lacZ/+</sup> mice. Abbreviations: PO (periosteum), CB (cortical bone), or MA (marrow) in brackets. (j–l) Standardized fractures were established in femurs of Bmp2<sup>lacZ/+</sup> and WT littermate mice (n = 3). (k,l) LacZ staining 3 days post-fracture in (k) Bmp2<sup>lacZ/+</sup> or (l) negative control WT mice.

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**UC Davis Knockout Mouse Repository (KOMP)**

**Velocigene Project ID 12406**

**Method**
ES cell clone microinjected

**Gene**
Bmp2

**Chromosome**
2

**Deletion Start**
133,554,429

**Deletion End**
133,554,764

**Deletion Size**
336

**Genome Build**
38

**MGI ID**
MGI:88177

**NCBI Gene ID**
12156

**BAC ID**
62H24

**BAC Library**
bMQ

**Cassette**
ZEN-Ub1

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**Figure 2—figure supplement 1.** Schematic of the Bmp2<sup>lacZ</sup> knock-in allele. Homologous recombination was used to replace the first 336 coding nucleotides of murine Bmp2 with non-membrane targeted bacterial beta-galactosidase. (a) Specifics of allele construction from the UC Davis Knock Out

**Figure 2—figure supplement 1 continued on next page**
Mouse Project repository, Velocigene project ID 12406. (b) Schematic of the targeting strategy and the ZEN-Ub1 replacement cassette. (c) Genomic view of targeted locus visualized with the publicly-available Ensembl genome browser (www.ensembl.org).
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Figure 2—figure supplement 2. Bmp2 expression domain during periosteal bone growth. (a) LacZ staining on tissues from mice expressing beta-galactosidase from the endogenous Bmp2 locus (Bmp2^lacZ/±). Longitudinal sections through the (a) forelimb of an E14.5 Bmp2^lacZ/± mouse embryo, (b) Figure 2—figure supplement 2 continued on next page
Figure 2—figure supplement 2 continued

forelimb of a newborn Bmp2<sup>lacZ/+</sup> mouse, or (c) humerus of a 2 week-old Bmp2<sup>lacZ/+</sup> mouse. Black bar indicates LacZ expression domain in the PO (periosteum), CB (cortical bone), or MA (marrow) in brackets.

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Figure 3. Robust versus quiescent states of periosteal BMP signaling reflect active versus homeostatic states of periosteal bone growth. Fluorescent and brightfield microscopy on tissues from mice with transgenic expression of enhanced green fluorescent protein (gfp) under the control of a minimal fragment of the Id1 promoter with pan-BMP response elements (BRE:gfp). (a) GFP (green) and DAPI (blue) imaging on sagittal (left) or frontal (right) cryosections of the hand plate from E13.5 BRE:gfp embryos. (b,c) Serial sections through the forelimb of newborn BRE:gfp mice were analyzed by Von Kossa staining for mineralized tissue, Alcian Blue staining for cartilage, or DAPI counterstaining of GFP expression domains. (d,e) Toluidine blue or GFP/DAPI imaging on sagittal cryosections through the femur of (d,e) 2 week-old or (f) 6 month-old BRE:gfp mice. White arrowheads, GFP in the bone collar surrounding the growth plate cartilage; red arrowheads, GFP+ cells in growth plate; red dotted lines demarcate GFP+ cells of the (2c) bone collar or (2e) innermost layer of the periosteum. Abbreviations: PO, periosteum; CB, cortical bone; TB, trabecular bone; Ma, Marrow. For all timepoints, \( n \geq 3 \) histological sections were examined from equivalent skeletal sites of multiple littermate mice.

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Figure 4. Bmp2 is dispensable for development and maintenance of the periosteum. (a,b) The periosteum forms during development and is maintained in postnatal life in Bmp2 Prx1-cKO mice. Sagittal sections of the femur from mice were stained with picrosirius red and hematoxylin, and imaged by brightfield (left panels) or polarized light (right panels) microscopy. Images shown are representative of femurs harvested from littermates at ages (a) embryonic day 17.5, (b) postnatal day 0, or (c) postnatal day 14. Arrowheads point to the outer collagen-rich canopy of periosteum in Bmp2 Prx1-cKO mice.

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Figure 5. *Bmp2* is essential for periosteal BMP signaling and periosteal expression of BMP target genes. (a, b) Loss of phospho-Smad1/5+ cells in *Bmp2* Prx1-cKO periosteum. Transverse serial sections of the radius and ulna from (a) newborn and (b) 2 week-old mice, imaged in brightfield following toluidine blue stain to visualize skeletal tissue (left panels) or by fluorescence microscopy following DAPI and immunostaining to visualize cells with phospho-activated Smads1/5 (right panels). Black boxes on left panels indicate regions expanded in two right panels. (c) Loss of ID1+ cells in *Bmp2* Prx1-cKO periosteum. Transverse sections of the radius and ulna were imaged in brightfield following immunostaining to visualize cells expressing the BMP target gene, *Id1*. Black boxes on left panels indicate regions expanded in right panels. (d) Toluidine blue and in situ hybridization for *Col1a1* in cross-sections of the radius/ulna from 2 week-old *Bmp2* Prx1-cKO mice. Abbreviations: BC, bone collar/perichondrium; EO, endosteum; PO, periosteum; Ma, marrow. For all timepoints, n ≥ 3 histological sections were examined from equivalent skeletal sites of multiple littermate mice. DOI: https://doi.org/10.7554/eLife.42386.013
Figure 6. Bmp2 acts downstream of intermittent parathyroid hormone treatment in the juvenile periosteum. Intermittent PTH\textsubscript{1-34} therapy does not rescue periosteal growth in juvenile Bmp2 Prx1-cKO mice. (a) Juvenile mice were given intermittent PTH\textsubscript{1-34} therapy (100 mg/kg, subcutaneous) for 14 days. (b) Longitudinal sections of the femur stained with toluidine blue to visualize trabecular bone architecture. (c) Transverse sections of the radius and ulna stained with toluidine blue to visualize cortical bone architecture. (d–g) Bone mass analyzed in the femur by microcomputed tomography (microCT). (d) Trabecular bone at the distal metaphysis and (e) cortical bone at the mid-diaphysis of the femur visualized by 3D reconstructions. Images represent the group mean and are shown to scale. (f) Ratio of bone volume (BV) to trabecular volume (TV). (g) Total cross-sectional area at the mid-diaphysis. Quantitative microCT data presented as mean ± s.d. where *p<0.05 vs. matched genotype vehicle control and #p<0.05 vs. WT vehicle control (n = 4–5 per group). (h,i) Dynamic histomorphometry assessing bone formation rate as a function of bone surface (BFR/BS) at (h) endosteal versus (i) periosteal surfaces. (j,k) Dynamic histomorphometry assessing mineral apposition rate (MAR) at (j) endosteal versus (k) periosteal surfaces. Dynamic histomorphometry (n = 4–5 per group) presented as mean ± s.d. where *p<0.05. BFR\textsuperscript{PO}-value=0.0503) vs. matched genotype vehicle control and Figure 6 continued on next page.
#p<0.05 vs. WT vehicle control. (l,m) Elisa analysis measuring circulating (l) serum phosphate and (m) serum calcium in juvenile mice treated with intermittent PTH_{1-34} presented as mean ± s.d. where *p<0.05 vs. vehicle-treated Bmp2^{F/F} or **p<0.05 vehicle-treated Bmp2 Prx1-cKO littermates. (n) Transverse sections of the radius/ulna with immunostaining to visualize cells expressing the BMP target gene, ID3. Abbreviations: PO, periosteum; Ma, marrow. n ≥ 3 histological sections were examined from multiple mice per cohort.

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Figure 7. Bmp2 acts downstream of intermittent parathyroid hormone treatment in the adult periosteum. Intermittent PTH$_{1-34}$ therapy does not improve biomechanical stability or fracture repair in adult Bmp2 Prx1-cKO mice. (a) Adult mice were given intermittent PTH$_{1-34}$ therapy (100 mg/kg, subcutaneous) for (b–g) 14 days or (h) 21 days. (b–d) Quantitative microCT and biomechanical analysis on adult mice treated 2 weeks with PTH$_{1-34}$ ($n = 3$–$5$), presented as mean ± s.d. where *$p<0.05$ vs. matched genotype vehicle control. (b) Ratio of bone volume (BV) to trabecular volume (TV). (c) Peak moment as a function of I$_{MIN}$/C$_{MIN}$. (d) Predicted minimum moment of inertia. (e–h) Incidence of femoral or forelimb fractures in adult mice treated (e–g) two weeks ($n = 4$–$5$ per group) or (h) 21 days ($n = 8$ per group) with PTH$_{1-34}$. Biomechanics are reported as mean ± s.d., where $P$-value was calculated using 1-way ANOVA and post-test Newman Keuls. All data points were included in the analysis. Remaining group comparisons were made by 2-way ANOVA and Tukey’s multiple comparison tests.

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**Figure 8.** Bmp2 acts downstream of sclerostin neutralizing antibody in the periosteum. (a–c) Haploinsufficiency of Dkk1 does not rescue periosteal growth in juvenile Bmp2 Prx1-cKO mice. Femoral bone mass was analyzed by microCT in juvenile mice (two weeks-old). (b) Transverse sections of the femur mid-diaphysis were visualized by 3D reconstructions. Images represent the group mean and are shown to scale. (c) Calculated areal moment of inertia. n = 6–8 shown as mean ± s.d. **p<0.005 compared using 2-way ANOVA. (d–l) Pharmacologic activation of Wnt pathway does not rescue periosteal growth or fracture repair in adult Bmp2 Prx1-cKO mice. WT or Bmp2 Prx1-cKO mice (13 weeks-old) were treated with sclerostin neutralizing antibody (SOST-ab, 20 mg/kg, two times/week for 2 weeks, subcutaneous). Femoral bone mass was analyzed by microCT. (e) Trabecular bone at the distal metaphysis and (f) cortical bone at the mid-diaphysis of the femur visualized by 3D reconstructions. Images represent the group mean. Scale bars, 1 mm. (f,h–j) Quantitative microCT data presented as mean ± s.d. *p<0.05, ***p<0.005, or ****p<0.00005. vs. matched genotype vehicle control (n = 4–8 per group), compared using 2-way ANOVA and Tukey multiple comparisons test. (f) Ratio of bone volume (BV) to trabecular volume (TV) at the distal femoral metaphysis. (h) Total cross-sectional area at the mid-diaphysis. (i) Polar moment of inertia at the mid-diaphysis. (j) Minimum moment of inertia at the mid-diaphysis. (k,l) X-ray imaging revealing non-union forelimb fractures in mice treated two weeks with SOST-ab (n = 17–24 wrists per group).

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Figure 9. Bmp2 acts downstream of sclerostin neutralizing antibody to reactivate the developmental periosteal BMP signaling center. (a,b) PTH$_{1-34}$ and SOST-ab activate BMP signaling in bone and alkaline phosphatase activity in WT mice. Adult WT mice (4 months-old) were given a single injection of PTH$_{1-34}$ (100 mg/kg, subcutaneous) or SOST-ab (20 mg/kg, subcutaneous). (a) Immunoblot for total and phospho-activated Smads1/5 in marrow-free bone tissue. Experiment was repeated twice. (b) Alkaline phosphatase activity (red) counterstained with DAPI (blue) were imaged on longitudinal sections of the femur ($n = 2–3$ mice per group). (c–e) SOST-ab activates BMP signaling in the periosteum in a Bmp2-dependent manner. BRE:gfP and a Cre-dependent tdTomato reporter were bred onto a Bmp2 Prx1-cKO background. (c–d) BRE:gfP (green) and (e) tdTomato (red) was visualized in femurs of adult mice (4 months-old) given 2 injections of (c, top) saline, (c, bottom) 2 injections of SOST-ab (20 mg/kg, subcutaneous) and sacrificed 72 hr after the first injection or (d–e) 4 injections of SOST-ab (20 mg/kg, two times/week for 2 weeks, subcutaneous). Abbreviations: Ma, marrow; CB, cortical bone; PO, periosteum.

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Figure 9—figure supplement 1. Magnified images of BRE-gfp expression in cortical bone following SOST-ab treatment. SOST-ab activates BMP signaling in the periosteum in a Bmp2-dependent manner. BRE:gfp and a Cre-dependent tdTomatoFlox reporter were bred onto a Bmp2 Prx1-cKO background. GFP (green) and DAPI (blue) were imaged on longitudinal sections of the femur. (a) BRE:gfp was visualized in adult mice (4 months-old) given 2 injections of SOST-ab (20 mg/kg) and sacrificed 72 hr after the first injection.

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Figure 9—figure supplement 2. BRE-gfp expression relative to Prx1-Cre; TdTomato^Flox/Flox^ lineage, with separated fluorescent channels. (a–c) SOST-ab activates BMP signaling in the periosteum in a Bmp2-dependent manner. BRE-gfp and a Cre-dependent tdTomato reporter were bred onto a Bmp2^+/Flox^ line in the context of an overall BRE:gfp^Tg/+ background.
Figure 9—figure supplement 2 continued

Ptxl-cKO background. GFP (green), tdTomato (RFP), and DAPI (blue) were imaged on longitudinal sections of the femur. BRE: GFP or tdTomato were visualized in adult mice after two weeks of SOST-ab (20 mg/kg, two times/week for 2 weeks, subcutaneous).

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Figure 10. Deposition of mineralized bone matrix is optimal when BMP2 and canonical Wnt signaling are balanced. (a,b) Primary periosteal cells isolated from 4 week-old Bmp2^{F/F} and Bmp2^{F/F}; Prx1-Cre mice were analyzed by QPCR in three repeat experiments. Fold change mRNA is reported as mean ± s.d. compared by two-tailed student’s t-test where p*<0.001 vs. Bmp2^{F/F} cells. BMP4 expression was at the limit of detection. Primary BMSC were differentiated in osteogenic medium (OM) plus recombinant growth factors. (c) Calcified matrix was assessed on day 10 by alizarin red staining (2.5X, brightfield). Cultures were performed in duplicate using pooled cell populations from n = 2 Bmp2^{F/F} or n = 4 Bmp2^{F/F}; Prx1-Cre mice. (d) Quantification of alizarin red in (c). Error bars represent distribution of two independent experiments. (e) Primary BMSC cells from n = 3 Bmp2^{F/F} and n = 4 Bmp2^{F/F}; Prx1-Cre mice were differentiated as non-pooled cultures in OM plus recombinant growth factors as indicated. QPCR analysis on day three was reported as mean ± s.d. compared by two-tailed student’s t-test where P*<0.001 vs. Bmp2^{F/F} cells in OM; P#<0.001 vs. Bmp2^{F/F}; Prx1-Cre cells in OM; n = number of independent cultures per condition.

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Figure 11. Bmp2 is a direct target gene of canonical Wnt pathway in osteoblast progenitors. (a) Cartoon summarizing discovery of canonical WNT target genes by anti-Bcl9 chromatin immunoprecipitation from E10.5 mouse limb buds, deep sequencing, and bioinformatic analysis of peaks. 2099 high-confidence peaks passed statistical threshold in two independent experiments, with significant enrichment at genetic loci associated with (b) limb, skeletal, and bone development as well as Wnt, TGF-beta, Shh, and Hippo signaling pathways. High-confidence peaks surrounding genetic loci for (c) Axin2, (d) Lef1, (e) Bmp2, and (f) Grhl3. Bioinformatic and statistical analysis are described in methods.

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Figure 11—figure supplement 1. Bmp2 is upregulated by canonical Wnt pathway in osteoblast progenitors. (a) Immortalized mouse E13.5 limb bud cells were cultured in OM ± recombinant Wnt3a (40 ng/ml). QPCR analysis for Bmp2 was performed at 24 hr, 48 hr, and 6d and expressed as fold change from untreated cells.
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Figure 12. Bmp2 acts downstream of canonical Wnt pathway to specify Sp7+/Col1A1+ osteoblasts. (a) Cartoon summarizing regulatory analysis of Sp7 locus by chromatin immunoprecipitation of H3-acetyl-K14, Smad1, endogenous Grhl3, or transiently expressed V5-GRHL3 from chromatin of immortalized E13.5 limb bud cell cultures grown in presence of recombinant BMP2 or Noggin. (b) QPCR analysis revealed BMP-dependent enrichment of these proteins at a putative consensus-binding motif for Grhl3 located at a highly conserved genomic region ~13 kb upstream of the Sp7 transcription start site. Data reflect mean±distribution in two independent experiments.

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Figure 13. Developmental, reparative, and therapeutic signals converge on Bmp2 to specify osteoblasts in the periosteal niche. (a) A proposed hierarchical and regionalized gene regulatory network, summarizing the source and identity of signals that regulate transcription of Bmp2 for downstream specification of Sp7+/Col1A1+ osteoblasts in the periosteal niche. Boxed regions, biological compartments; Boxed text, inputs evaluated in this study; Double arrows in a link, communication across territories; Flat footpads ending at genetic loci, transcriptional inhibition; Pointed footpads ending at genetic loci, transcriptional induction; Diamonds under footpads, evidence of direct cis-regulatory interaction provided by our study; White bubbles, protein/protein interactions. Made with Biotapestry.org.

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