Targeting skeletal endothelium to ameliorate bone loss

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Recent studies have identified a specialized subset of CD31hiEMCNhi (CD31hiEMCNhi) vascular endothelium that positively regulates bone formation. However, it remains unclear how CD31hiEMCNhi endothelium levels are coupled to anabolic bone formation. Mice with an osteoblast-specific deletion of Shn3, which have markedly elevated bone formation, demonstrated an increase in CD31hiEMCNhi endothelium. Transcriptomic analysis identified SLIT3 as an osteoblast-derived, SHN3-regulated proangiogenic factor. Genetic deletion of Slit3 reduced skeletal CD31hiEMCNhi endothelium, resulted in low bone mass because of impaired bone formation and partially reversed the high bone mass phenotype of Shn3−/− mice. This coupling between osteoblasts and CD31hiEMCNhi endothelium is essential for bone healing, as shown by defective fracture repair in SLIT3-mutant mice and enhanced fracture repair in SHN3-mutant mice. Finally, administration of recombinant SLIT3 both enhanced bone fracture healing and counteracted bone loss in a mouse model of postmenopausal osteoporosis. Thus, drugs that target the SLIT3 pathway may represent a new approach for vascular-targeted osteoanabolic therapy to treat bone loss.
Results

SHN3 acts in osteoblasts to regulate CD31hiEMCNhi endothelium.

To address our hypothesis that osteoblasts are able to coordinate levels of osteogenic CD31hiEMCNhi endothelium to maintain bone formation capacity, we assessed CD31hiEMCNhi endothelium levels in a mouse strain displaying augmented postnatal bone formation, namely, Shn3−/− mice. We found that CD31 and EMCN double-positive endothelium was present in the marrow immediately beneath the growth plate and was considerably higher in Shn3−/− mice than in wild-type (WT) controls. This effect was bone specific because, relative to littermate controls, Shn3−/− mice displayed normal levels of EMCN+ vessels in other organs, including heart, brain, lung and kidney (Supplementary Fig. 1a). This vessel phenotype was present in neonatal Shn3−/− mice and thus preceded the appearance of the high bone mass phenotype (Supplementary Fig. 1b). Flow cytometry also confirmed greater amounts of CD31hiEMCNhi vascular endothelium in the bones of Shn3−/− mice than in WT littermate controls (Fig. 1c,f and Supplementary Fig. 1c). Taken together, these observations suggest that SHN3 regulates levels of skeletal CD31hiEMCNhi endothelium in addition to its role in regulating osteoblast activity.

Because SHN3 acts in a cell-intrinsic manner to regulate bone formation by osteoblasts, we reasoned that SHN3 also acts in osteoblasts to control levels of CD31hiEMCNhi endothelium. To test this directly, we bred Shn3+/− mice to a cre-deleter strain targeting osteoblast progenitors, OSX-cre, and to a cre-deleter strain targeting mature osteoblasts, DMP1-cre. Both Shn3+/− and Shn3+/+ male mice exhibited a similar degree of higher bone mass relative to littermate Shn3+/− controls, including greater cortical bone thickness, largely recapitulating the characteristic bone phenotype of Shn3−/− mice (Fig. 2a,b and Supplementary Fig. 2a–c). On the basis of this, we selected the Shn3+/+ strain for further study because it implicates a more restricted subpopulation in any phenotypes observed16,17. Histomorphometric analysis confirmed that the high bone mass phenotype of Shn3+/+ mice was due to augmented osteoblast-mediated bone formation predominantly on the endosteal surface (Fig. 2d,e and Supplementary Fig. 2d,e). Analysis of skeletal CD31hiEMCNhi endothelium by both immunostaining and flow cytometry demonstrated that Shn3+/+ mice displayed higher levels of this subset of endothelial cells relative to littermate Shn3+/− controls in a similar manner as seen in Shn3−/− mice (Fig. 2f–h).

To further confirm that the function of SHN3 to regulate skeletal CD31hiEMCNhi vascular endothelium maps to osteoblasts, we generated endothelial cell–specific Shn3-deficient mice (Shn3ocn−/− mice) using the Cdh5 (VE-cadherin)-Cre. Despite observing efficient deletion of Shn3 in BM endothelial cells (Supplementary Fig. 2i), levels of CD31hiEMCNhi endothelial cells were unchanged in Shn3ocn−/− mice relative to littermate Shn3+/+ controls (Supplementary Fig. 2j–m). Accordingly, Shn3ocn−/− mice also displayed normal bone mass relative to littermate Shn3+/+ controls (Supplementary Fig. 2n). Taken together with the Shn3+/− and Shn3+/+ phenotype, we conclude that osteoblasts regulate skeletal CD31hiEMCNhi vascular endothelium in an SHN3-dependent manner and that SHN3 does not act directly in endothelial cells to regulate skeletal phenotypes.

To determine whether osteoblasts continuously participate in this regulation or whether this process is limited to embryonic development, we intercrossed Shn3+/− mice with osteocalcin-CreERT mice expressing a tamoxifen-activated Cre recombinase in mature osteoblasts under the control of the osteocalcin promoter (Shn3ocnEMCN−/− mice).

Fig. 1 | Shn3−/− mice have higher levels of CD31hiEMCNhi endothelium. a,b. Representative confocal images (n = 4 total images per group) of 2-week-old Shn3+/− and Shn3−/− male mouse femurs stained with CD31 (a) or EMCN (b) (red) and DAPI (blue). Growth plate and cortical bone are marked with a dashed line. Scale bars, 300 μm (top); 50 μm (bottom). c. Quantification of relative CD31hi (top) and EMCNhi vessel area (bottom) in the BM cavity of the femur sections in 2-week-old Shn3+/− and Shn3−/− male mice (n = 4 per group). d. Representative images (n = 3 total images per group) of CD31 (green) and EMCN (red) dual-immunostained femur sections from the femur in 2-week-old male mice. The growth plate is marked. Scale bars, 100 μm. e,f. Representative flow cytometry plots (e) with quantification (f) of CD31hiEMCNhi endothelial cells from the femurs of 2-week-old Shn3+/− and Shn3−/− mice (n = 4 per group). Values represent mean ± s.e.m. **P < 0.01; ***P < 0.001 by an unpaired two-tailed Student’s t-test in all panels.
Fig. 2 | Ablation of Shn3 in osteoblasts enhances osteogenesis and angiogenesis in vivo. a, Representative μCT images of the trabecular bone in the distal femur (left) and bone volume/total volume (BV/TV) (right) in Shn3f/f (n = 7) and Shn3dmp1 (n = 8) male mice at 8 weeks of age. Scale bars, 1 mm. b, Representative histomorphometric images of the L3 vertebrae (left) and BV/TV (right) in Shn3f/f (n = 6) and Shn3dmp1 (n = 4) male mice at 8 weeks of age. Scale bars, 300 μm. MAR and BFR/BS: 300 μm/mm²/year). osteoclast number/bone perimeter (No.Oc./B.Pm) and osteoblast surface/bone surface (Ob.S/BS (%)) are shown. Scale bars, 300 μm. MAR and BFR/BS: n = 6 per group; No.Oc./B. Pm and Ob.S/BS: n = 5 per group. c, Representative confocal images (n = 5 total images per group) of femur sections from 2-week-old Shn3f/f and Shn3dmp1 male mice with EMCN (red) and CD31 (green). Growth plate is marked with a dashed line. Scale bars, 100 μm. f, Representative flow cytometry plots (f) and relative frequency of CD31EMCNendothelial cells (g) from the femurs of 2-week-old male Shn3dmp1 (n = 6) and Shn3f/f (n = 5) mice. h, Representative μCT images of the trabecular bone in the distal femur metaphysis (h) and relative quantitative analysis of BV/TV (i) in Shn3f/f (oil = 7; tamoxifen = 7) and Shn3dmp1 male mice (oil = 6; tamoxifen = 8). Analysis was performed 12 weeks after tamoxifen injection into 4-week-old mice. Scale bars, 1 mm. j, Representative confocal images (n = 3 total images per group) of femur sections from Shn3f/f and Shn3dmp1 male mice at 4 weeks after tamoxifen injection into 4-week-old mice. Growth plate is marked with a dashed line. Scale bars, 300 μm. k, Representative confocal images (k) and relative frequency of CD31EMCNendothelial cells (l) from the femurs of Shn3f/f and Shn3dmp1 male mice (n = 5 per group), 6 weeks after tamoxifen injection into 4-week-old mice. Values represent mean ± s.e.m. *P < 0.05; **P < 0.01; ***P < 0.001 by an unpaired two-tailed Student’s t-test (a,b,d,g,i) or by one-way ANOVA followed by a Dunnett’s test (i).
Cre-mediated deletion was induced with tamoxifen, and the resulting skeletal and vascular phenotypes were analyzed. Relative to either tamoxifen treatment of Shn3<sup>WT</sup> mice, tamoxifen treatment of Shn3<sup>−/−</sup> mice resulted in higher levels of both CD31<sup>hi</sup>EMCN<sup>hi</sup> endothelial cells and trabecular bone mass and cortical bone thickness, with the change in CD31<sup>hi</sup>EMCN<sup>hi</sup> endothelium preceding detectable effects on bone mass (Fig. 2h–l and Supplementary Fig. 2o). Thus, mature osteoblasts continuously participate in tuning the levels of CD31<sup>hi</sup>EMCN<sup>hi</sup> endothelium in bone, and this coupling is not solely a developmental phenomenon.

**SHN3 regulates Slit3 expression in osteoblasts.** To investigate whether the regulation of marrow angiogenesis by SHN3 is a direct effect of osteoblasts on endothelial cells, we harvested conditioned medium from WT and Shn3<sup>−/−</sup> primary osteoblasts and introduced it to cultures of primary BM-derived endothelial cells. Conditioned medium from Shn3<sup>−/−</sup> primary osteoblasts displayed an enhanced ability relative to conditioned medium from WT osteoblasts to induce endothelial migration and capillary tube formation, suggesting that the relevant mediator is a soluble osteoblast-derived factor (Fig. 3a–d). To identify potential proangiogenic factors regulated by SHN3 in osteoblasts, we performed RNA-sequencing (RNA-seq) transcriptional profiling. Gene Ontology (GO) analysis demonstrated that the set of differentially expressed genes (DEGs) in Shn3<sup>−/−</sup> versus WT osteoblasts is enriched for genes mediating angiogenesis in addition to the expected enrichment for genes involved with bone development and mineralization (Fig. 3e). Examination of the expression of a number of proangiogenic factors revealed that only SLIT3, a soluble axonal chemorepellent recently shown to have angiogenic functions, showed substantially greater levels in Shn3<sup>−/−</sup> osteoblasts (Fig. 3f)–h). Further expression analysis confirmed robust Slit3 expression in osteoblasts and demonstrated negligible Slit3 expression in osteoclasts (Supplementary Fig. 3a,b). Similarly, immunofluorescence for beta-galactosidase in Slit3<sup>−/−</sup> mice<sup>31</sup> bearing a targeted insertion of an LacZ cassette into the Slit3 locus identified SLIT3 expression in cells adjacent to the bone surface consistent with osteoblasts (Supplementary Fig. 3c). Similarly, human mesenchymal stromal cell–derived osteoblasts demonstrated enhanced SLIT3 expression in osteoclasts (Supplementary Fig. 3d). To verify that Slit3 expression is augmented in Slit3<sup>−/−</sup> osteoblasts relative to WT controls, we used three complementary approaches. First, real-time PCR was used to validate the higher levels of Slit3 observed by RNA-seq (Fig. 3g). Second, overexpression or knockdown of Shn3 in human mesenchymal stem cell (hMSC)–derived osteoblasts demonstrated, respectively, a suppression or an enhancement of both mRNA and protein levels of Slit3 (Fig. 3h,i). Moreover, overexpression of Shn3 in murine primary osteoblasts also dramatically reduced SLIT3 expression (Supplementary Fig. 3e). Lastly, ELISA demonstrated threefold higher SLIT3 secretion in conditioned medium from Shn3<sup>−/−</sup> osteoblasts relative to WT controls (Fig. 3j). Thus, SHN3 is a negative regulator of SLIT3 expression in osteoblasts.

Recent studies have shown that proosteoclast–derived PDGF-BB is able to induce CD31<sup>hi</sup>EMCN<sup>hi</sup> endothelium in bone<sup>1</sup>. We were unable to detect PDGF-BB secretion by osteoblasts, and negligible Pdgfb mRNA was observed in both WT and Shn3<sup>−/−</sup> osteoblasts (Fig. 3f and Supplementary Fig. 3f). In addition, serum PDGF-BB levels were unaltered in Shn3<sup>−/−</sup> mice (Supplementary Fig. 3f). Thus, we do not observe evidence of regulation of PDGF-BB by SHN3.

SHN3 acts predominantly by regulating ERK activity, as mice bearing a knockin of a mutation in three amino acids comprising the ERK interacting motif (Shn3<sup>MKI</sup> mice) in SHN3 largely recapitulate the high bone mass phenotype of Shn3<sup>−/−</sup> mice<sup>1</sup>. Slit3 levels were also higher in primary Shn3<sup>MKI</sup> osteoblasts relative to WT controls (Fig. 3k), and treatment with the ERK pathway inhibitor trametinib reduced Slit3 expression in both hMSC–derived osteoblasts and murine primary calvarial osteoblasts relative to vehicle (Fig. 3l and Supplementary Fig. 3g). Consistent with these observations, Shn3<sup>MKI</sup> mice also displayed greater levels of CD31<sup>hi</sup>EMCN<sup>hi</sup> skeletal endothelium, a phenotype similar to that seen in Shn3<sup>−/−</sup> mice (Fig. 3m). Thus, SHN3 regulates SLIT3 expression and CD31<sup>hi</sup>EMCN<sup>hi</sup> endothelium levels via its ability to bind and regulate ERK.

**SLIT3 acts downstream of SHN3 to promote CD31<sup>hi</sup>EMCN<sup>hi</sup> endothelium formation and bone formation in vivo.** To determine whether SLIT3 contributes to the regulation of BM endothelium by osteoblasts in vitro, we conducted a dose–response curve for SLIT3 treatment as described in prior studies<sup>1,2,10</sup>. BM endothelial progenitor outgrowth cells (EPOCs) treated with recombinant SLIT3 displayed enhanced migration and tube formation relative to a vehicle control (Fig. 4a,b and Supplementary Fig. 4a). In addition, SLIT3 resulted in modestly higher levels of proliferation in BM-derived EPOCs, consistent with the activity of SLIT3 observed in nonskeletal vascular endothelium<sup>1</sup> (Supplementary Fig. 4b). SLIT1 and SLIT2 displayed a similar ability to promote tube formation relative to vehicle, consistent with observations that each of the SLITs displays a similar capacity for ROBO binding and activation<sup>22,23</sup> (Supplementary Fig. 4c,d). Next, conditioned medium was collected from WT and Shn3<sup>−/−</sup> osteoblasts, and placed on BM-derived endothelial cells together with an anti-SLIT3 blocking antibody or an isotype control. Enhanced tube formation was seen with conditioned medium from SHN3-deficient osteoblasts, and treatment with an anti-SLIT3 blocking antibody abrogated this effect (Fig. 4c,d). Investigation of the signaling pathways downstream of SLIT3 in BM endothelial cells demonstrated activation of ERK mitogen-activated protein kinase and Hippo signaling, pathways known to participate in angiogenesis (Supplementary Fig. 4e–g). Interestingly, we also noted that treatment of BM-derived endothelial cells with SLIT3 enhanced the acquisition of a CD31<sup>hi</sup>EMCN<sup>hi</sup> surface immunophenotype in culture (Supplementary Fig. 4f). Similarly, systemic administration of SLIT3 resulted in greater levels of skeletal CD31<sup>hi</sup>EMCN<sup>hi</sup> endothelium production in vivo (Supplementary Fig. 4g). In addition, SLIT2 augmented formation of CD31<sup>hi</sup>EMCN<sup>hi</sup> endothelium in vivo relative to vehicle, suggesting that other SLITs are also capable of eliciting this response (Supplementary Fig. 4h). Thus, SLIT3 is both necessary and sufficient to mediate osteogenesis by regulating production of CD31<sup>hi</sup>EMCN<sup>hi</sup> endothelium.

To assess whether SLIT3 regulates CD31<sup>hi</sup>EMCN<sup>hi</sup> endothelium under physiological conditions, we performed immunofluorescence and flow cytometry on Slit3<sup>−/−</sup> mice, with both approaches revealing a reduction in CD31<sup>hi</sup>EMCN<sup>hi</sup> skeletal endothelium (Fig. 4e–h). Concurrent with this reduction in CD31<sup>hi</sup>EMCN<sup>hi</sup> endothelium, Slit3<sup>−/−</sup> mice showed a substantial reduction in both total bone mass and cortical bone thickness relative to WT littermates (Fig. 4i–k). Consistent with prior reports<sup>1</sup>, SLit3<sup>−/−</sup> mice displayed a decrease in bone weight at 1 month of age, with body weight normalization occurring by 3 months of age (Supplementary Fig. 5a). Thus, osteopenia could be observed even at ages where the weight of Slit3<sup>−/−</sup> mice was indistinguishable from that of littermate controls. This osteopenia was due to reduced osteoblast activity, because bone formation was reduced without substantial alterations in osteoclast numbers or serum levels of cross-linked C-telopeptide of type I collagen (CTX), a marker of osteoclast activity (Fig. 4l,m and Supplementary Fig. 5b,c). To evaluate whether this reduction in bone formation was due to cell-intrinsic defects in osteoblast activity, we examined the differentiation of SLIT3-deficient osteoblasts. Mineralization activity, alkaline phosphatase (ALP) induction, and induction of characteristic osteoblast transcripts were all intact or even slightly enhanced in the absence of SLIT3 (Supplementary Fig. 5d–f). Furthermore, treatment of osteoblasts with recombinant SLIT3 did not enhance osteoblast activation relative to vehicle, nor did treatment with a ROBO1–Fc fusion that blocks SLIT signaling.
Fig. 3 | Inhibition of Shn3 enhances Slit3 expression in osteoblasts. a,b, Representative images (a) and relative quantification (b) of a transwell migration assay of BM-derived EPOCs. n = 5 per group. BLM, basal medium; CM, conditioned medium. c,d, Representative images (c) and relative quantification of tube branch numbers (d) of a Matrigel tube formation assay with EPOCs. n = 5 per group. BLM, basal medium; CM, conditioned medium. e, GO enrichment analysis of genes differentially expressed in Shn3−/− osteoblasts relative to Shn3+/− osteoblasts. The numbers on the bars are significance values based on the hypergeometric test. f, Proangiogenic gene expression in primary Shn3+/− and Shn3−/− osteoblasts. g, Real-time PCR of Slit3 expression in Shn3+/− and Shn3−/− osteoblasts. The units on the color scale are normalized read counts from the DESeq2 R package. h, mRNA (left) and protein (right) levels of Slit3 in human mesenchymal stromal cells (hMSCs) expressing a GFP-targeting control (shGFP) or Shn3-targeted (shShn3) shRNAs cultured under osteogenic conditions. n = 4 per group. i, mRNA (left) and protein (right) levels of Slit3 in hMSCs overexpressing a vector control or Shn3 cultured under osteogenic conditions. n = 4 per group. j, Results from ELISA for SLIT3 secretion by Shn3+/− and Shn3−/− osteoblasts. n = 6 per group. k, Real-time PCR analysis of Slit3 in Shn3+/− and Shn3+/− osteoblasts (n = 4). l, Real-time PCR analysis of Slit3 in hMSCs treated with trametinib (TTNB). n = 6 per group. m, Representative confocal images (n = 3 total images per group) of CD31 (green) and EMCN (red) immunostained sections from the femurs of 2-week-old Shn3+/− and Shn3+/− male mice. Growth plate is marked with a dashed line. Scale bars, 100 μm. Values represent mean ± s.e.m. *P < 0.05; **P < 0.01; ***P < 0.001 by an unpaired two-tailed Student’s t-test (g.h.k) or one-way ANOVA followed by a Dunnett’s test (b.d.l). All immunoblots are cropped from the original.

impair osteoblast differentiation (Supplementary Fig. 5g.h). Thus, the ability of Slit3 to enhance bone formation in vivo is not attributable to the direct effects of SLIT3 on osteoblasts, which is consistent with the model that SLIT3 directly augments levels of CD31+EMCN+ endothelium to augment bone formation. To confirm whether osteoblasts represent the key cellular source of SLIT3 controlling levels of skeletal CD31+EMCN+ endothelium and bone mass accrual in vivo, we generated mice bearing a conditional Slit3 floxed allele (Slit3eo mice) and validated their deletion capacity (Supplementary Fig. 6a). We bred Slit3eo mice to cre-deleter strains targeting either osteoblast progenitors via OSX-cre or mature osteoblasts via DMP1-cre. Osteoblast-specific deletion of Slit3 in vivo recapitulated both the impaired CD31+EMCN+ endothelium levels and the osteopenia phenotypes observed in Slit3−/− mice (Fig. 5a–d and Supplementary Fig. 6b–e). Furthermore, neither neuron-specific deletion of Slit3 by using Synapsin-cre nor specific deletion of
**Fig. 4 | Slit3<sup>−/−</sup> mice have reduced skeletal vasculature and bone mass in vivo.** a-d, Representative images (a,c) and relative quantification of tube branch numbers (b,d) in a Matrigel tube formation assay with EPOCs. n = 5 per group. Scale bars, 200 μm. e,f, Representative confocal images of CD31 or EMCN (red) and DAPI (blue) immunostained sections (e) and relative quantification (f) of CD31<sup>+</sup> or EMCN<sup>+</sup> vessel area in the BM cavity of the femurs from male Slit3<sup>−/−</sup> and Slit3<sup>−/−</sup> mice at 2 weeks of age. n = 4 per group. Growth plate and cortical bone are marked with a dashed line. Scale bars, 300 μm. g,h, Representative confocal images (n = 3 total images per group) of CD31 (green) and EMCN (red) immunostained bone sections (g), flow cytometry dot plots (h, left) and relative frequency of CD31<sup>+</sup>/EMCN<sup>+</sup> endothelial cells (h, right) of the femur in 2-week-old Shn3<sup>−/−</sup>/Slit3<sup>−/−</sup> (n = 5), Shn3<sup>−/−</sup>/Slit3<sup>+/−</sup> (n = 3), Shn3<sup>−/−</sup>/Slit3<sup>+/+</sup> (n = 5) and Shn3<sup>−/−</sup>/Slit3<sup>−/−</sup> mice (n = 5). Growth plate is marked with a dashed line. Scale bars, 100 μm. i, Representative histological images (left) and relative BV/TV (middle) of the L3 vertebra (Slit3<sup>−/−</sup> = 9, Slit3<sup>+/−</sup> = 7) and cortical thickness (right) of distal femoral metaphyseal regions (Slit3<sup>−/−</sup> = 7, Slit3<sup>+/−</sup> = 6) at 6 weeks of age. Scale bars, 500 μm. j, Representative μCT images of the trabecular bone in the distal femur metaphysis (left) and relative BV/TV analysis of 6-week-old Shn3<sup>−/−</sup>/Slit3<sup>−/−</sup> (n = 7), Shn3<sup>−/−</sup>/Slit3<sup>+/−</sup> (n = 7), Shn3<sup>−/−</sup>/Slit3<sup>−/−</sup> (n = 7) and Shn3<sup>−/−</sup>/Slit3<sup>−/−</sup> mice (n = 6) (right). Scale bar, 1 mm. k, Representative μCT images of the trabecular bone in the distal femur metaphysis (left), relative trabecular BV/TV analysis (middle) and cortical bone thickness (right) in the femoral midshaft from male Slit3<sup>−/−</sup> mice (n = 8) and Slit3<sup>−/−</sup> mice (n = 5) at 12 weeks of age. Scale bars, 1 mm. l,m, Representative images of calcein labeling (l) and relative histomorphometric quantification (m) of MAR and BFR/BS in 6-week-old Shn3<sup>−/−</sup>/Slit3<sup>−/−</sup> (n = 5), Shn3<sup>−/−</sup>/Slit3<sup>−/−</sup> (n = 6), Shn3<sup>−/−</sup>/Slit3<sup>−/−</sup> (n = 4) and Shn3<sup>−/−</sup>/Slit3<sup>−/−</sup> male mice (n = 4). Scale bars, 100 μm. Values represent mean ± s.e.m. *P < 0.05; **P < 0.01; ***P < 0.001 by an unpaired two-tailed Student’s t-test (f,i,k) or one-way ANOVA followed by a Dunnett’s test (b,d,h,j).

Slit3 in endothelial cells by using Cdh5-cre in vivo led to detectable bone loss (Supplementary Fig. 6f,g). Thus, osteoblasts are the key functional source of SLIT3 influencing skeletal biology.

We next questioned whether the enhanced production of SLIT3 by SHN3-deficient osteoblasts contributes to the high bone mass phenotype of SHN3-deficient mice. To address this, we performed a genetic interaction study by intercrossing Shn3<sup>−/−</sup> and Slit3<sup>−/−</sup> mice. As shown in Fig. 4, an epistatic genetic interaction was observed between Shn3- and Slit3-null alleles, because the higher bone formation, trabecular bone mass, and CD31<sup>+</sup>/EMCN<sup>+</sup> endothelial cell levels in Shn3<sup>−/−</sup> mice were partially reversed in Shn3<sup>−/−</sup>/Slit3<sup>−/−</sup> mice. Thus, SLIT3 contributes to both the high bone mass phenotype and enhanced CD31<sup>+</sup>/EMCN<sup>+</sup> endothelium phenotype of SHN3-deficient mice in vivo. In addition, a genetic interaction similar...
to that observed with the Shn3 and Slit3 germline null alleles was recapitulated using conditional deletion of Shn3 and Slit3 alleles in osteoblasts using Osx-cre (Supplementary Fig. 7a,b). Thus, the interaction between Shn3 and Slit3 alleles to regulate bone mass is intrinsic to osteoblasts. Taken together, these results imply that SLIT3 production is used by osteoblasts to condition their environment through angiogenesis to be conducive for bone formation.

**ROBO1 is a key receptor for SLIT3 on marrow endothelial cells.** Because SLITs are known to signal through ROBO1–ROBO4, we next explored which ROBO receptors might be acting in endothelial cells to mediate the response to SLIT3. CD31\(^{hi}\)EMCN\(^{hi}\) skeletal endothelial cells were isolated by FACS and subjected to RNA-seq transcriptome analysis, revealing that Robo1 and Robo4 are the predominant ROBO family receptors expressed (Supplementary Fig. 8a). Immunofluorescence for beta-galactosidase in the bones of Robo1\(^{–/–}\) mice bearing a knockin of an LacZ cassette into the Robo1 locus confirmed that Robo1 expression is present in endothelium near the growth plate, a localization consistent with CD31\(^{hi}\)EMCN\(^{hi}\) endothelial cells (Supplementary Fig. 3c). We also characterized ROBO expression in other skeletal cell types, indicating that osteoblasts express Robo1 and Robo2, whereas osteoclasts show negligible expression of ROBO family members (Supplementary Fig. 8b,c). Given the expression of Robo1 and Robo4 in CD31\(^{hi}\)EMCN\(^{hi}\) endothelial cells, the skeletal phenotype of Robo1\(^{–/–}\) and Robo4\(^{–/–}\) mice was examined. Robo1\(^{–/–}\), but not Robo4\(^{–/–}\), mice display low trabecular bone mass in long bones relative to WT controls (Fig. 5e and Supplementary Fig. 8d,e), and Robo1\(^{–/–}\) mice also showed lower levels of CD31\(^{hi}\)EMCN\(^{hi}\) skeletal endothelium (Fig. 5f and Supplementary Fig. 8f). Robo2 expression was not detected in either sorted CD31\(^{hi}\)EMCN\(^{hi}\) skeletal endothelial cells or cultured BM-derived endothelial cells, and Robo2 expression was not induced by either germline Robo1 deficiency or shRNA-mediated Robo1 knockdown (Supplementary Fig. 8g–i). Thus, no evidence of ROBO2-mediated compensation for ROBO1 loss of function was observed.

Next, the mechanism of SLIT3-mediated effects on BM endothelial cells was investigated. Knockdown of Robo1 in BM-derived endothelial cells impaired their response to SLIT3 relative to an irrelevant shRNA control as determined by both their tube formation capacity and phosphorylation of the hippo pathway signaling intermediate YAP (Supplementary Fig. 9a,b). YAP has been reported to play a crucial role in endothelial cell migration and tube formation\(^{25,27}\). Consistent with this, relative to an irrelevant shRNA control, shRNA-mediated knockdown of YAP considerably reduced the ability of SLIT3, but not FGF2, to induce tube formation in BM-derived endothelial cells, indicating that YAP acts downstream of the SLIT3–ROBO1 pathway to control tube formation (Supplementary Fig. 9c,d). Taken together, ROBO1 is a key receptor controlling both endothelial cell responses to SLIT3 and overall bone mass accrual.

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**Fig. 5 | Osteoblast-derived Slit3 controls osteogenesis and CD31\(^{hi}\)EMCN\(^{hi}\) endothelium via ROBO1. a,b,** Representative confocal images \((n = 3\) total images per group\) of CD31 (red) and DAPI (blue) (a) or CD31 (green), EMCN (red) and DAPI stained (blue) (b) femur sections in 2-week-old OSX Cre and Slit3\(^{osx}\) male mice. Growth plate and cortical bone are marked with a dashed line. Scale bars, 100 \(\mu m\). c, Representative \(\mu\)CT images of the trabecular bone in the distal femur metaphysis (left) and relative BV/TV analysis (right) of 3-week-old OSX Cre and Slit3\(^{osx}\) male mice. Scale bar, 1 mm. \(n = 6\) per group. d, Representative \(\mu\)CT images of the femoral midshaft (left) and relative cortical bone thickness analysis (right) of 3-week-old OSX Cre and Slit3\(^{osx}\) male mice. Scale bar, 1 mm. \(n = 6\) per group. e, Representative \(\mu\)CT images of the trabecular bone in the distal femur metaphysis (left) and relative BV/TV analysis (right) of 12-week-old Robo1\(^{+/–}\) \((n = 4)\) and Robo1\(^{–/–}\) female mice \((n = 5)\). Scale bars, 1 mm. f, Representative confocal images \((n = 3\) total images per group\) of CD31 (green) and EMCN (red) with DAPI stained (blue) femur sections from 2-week-old Robo1\(^{+/–}\) and Robo1\(^{–/–}\) mice. Growth plate is marked with a dashed line. Scale bars, 100 \(\mu m\). Values represent mean \(\pm\) s.e.m. \(^*P < 0.05; ^{**}P < 0.001\), by an unpaired two-tailed Student’s t-test in all panels.
An SHN3/SLIT3 osteoblast–endothelium coupling pathway plays an essential role in fracture healing. Given that bone repair is accompanied by extensive elaboration of new blood vessels, we hypothesized that the SHN3–SLIT3 pathway-mediated communication between osteoblasts and endothelial cells may be vital for bone fracture healing. In support of this hypothesis, immunohistochemical analysis of human fracture callus tissue demonstrated robust expression of SLIT3 in osteoblasts and the presence of CD31+ endothelium in physical proximity to osteoblasts (Supplementary Fig. 10a,b). An open femoral midshaft fracture model was established and used to study bone healing in Shn3−/−, Slit3−/−, Shn3−/−Slit3−/− and WT control mice. Twenty-one days postfracture, we performed micro computed tomography (μCT) and histology analysis and found that fracture healing was enhanced in Shn3−/− mice. Relative to WT controls, the fracture site in Shn3−/− mice displayed extensive bridging with mature lamellar bone that had already remodeled to contain narrow elements within the newly formed callus tissue. In contrast, deletion of Slit3 led to complete non-union and arrest of fracture healing with only trace amounts of cartilaginous callus present relative to WT controls (Fig. 6a–c). Consistent with the results of the bone mass genetic interaction studies, SLIT3 deficiency largely reversed the enhanced healing phenotype of SHN3-deficient mice. These observations of enhanced and impaired healing in SHN3-deficient and SLIT3-deficient mice, respectively, translated into improved or impaired biomechanical properties of the callus, as shown by the load to failure or maximum load sustained across the fracture site. Consistent with observations in basal bone mass phenotypes, Shn3−/− mice displayed enhanced angiogenesis throughout the callus relative to WT controls, and this phenotype was reversed by SLIT3 deficiency (Fig. 6d,e and Supplementary Fig. 10c,d). Thus, the coupling between osteoblasts and vascular endothelium mediated by the SHN3–SLIT3 pathway is essential for fracture healing.

SLIT3 has therapeutic effects in models of fracture healing and postmenopausal osteoporosis. Because SLIT3-mediated crosstalk between osteoblasts and CD31+EMCN+ endothelium is an important regulator of bone mass accrual and bone fracture healing, we hypothesized that administration of exogenous SLIT3 may have therapeutic effects to promote bone formation and regeneration. To examine this, we administered recombinant SLIT3 twice weekly via intravenous (i.v.) injection in 5-week-old male mice concurrent with performing an open femoral midshaft fracture. After 21 days of treatment, μCT and histological analysis showed an enhancement of bone fracture healing in SLIT3-treated mice (Fig. 6f,g). The bone volume in the callus area was greater by almost twofold relative to controls (Fig. 6h).

Furthermore, vascularization of the fracture callus was also augmented by SLIT3 administration relative to vehicle (Fig. 6i and Supplementary Fig. 10e). Finally, biomechanical testing demonstrated that SLIT3 treatment considerably enhanced the maximum load sustained and stiffness of the fracture callus, demonstrating improvements in clinically meaningful endpoints (Fig. 6j). Notably, examination of vascular morphology did not detect alterations in lung, heart, kidney or retina, and no changes in brain ultrastructure were present after SLIT3 administration (Supplementary Fig. 11a–d). Thus, under this dosing strategy and with regard to these endpoints, the effects of SLIT3 were specific to bone.

Nevertheless, because SLIT3 can affect a wide variety of tissues, it is important to anticipate potential extraskeletal toxicities of SLIT3 therapy. As a proof-of-principle strategy to avoid potential extraskeletal toxicities, local delivery of SLIT3 into a fracture site was achieved with a SLIT3-loaded collagen sponge. This approach recapitulated the effects of systemic SLIT3 delivery in promoting fracture healing as judged by improved mineralization and biomechanical properties of the fracture callus and augmented formation of CD31+EMCN+ endothelium relative to either no sponge or implantation of a sponge without SLIT3 (Fig. 6k,l and Supplementary Fig. 11e). Local delivery of SLIT3 also did not impact non-skeletal vascular abundance or morphology or brain ultrastructure (Supplementary Fig. 11f–h).

Given the therapeutic effects of SLIT3 in a fracture model, we next examined whether systemic SLIT3 administration can protect from bone loss in the murine ovariectomy (OVX) model of postmenopausal osteoporosis. Successful OVX was confirmed 2 months after OVX by the presence of both osteopenia and uterine atrophy (Fig. 6m and Supplementary Fig. 12a,b). First, the ability of SLIT3 to prevent bone loss was examined in this model. Mice were treated with i.v. injection of SLIT3 or vehicle twice weekly initiated 2 weeks after OVX. μCT analysis showed that SLIT3 administration substantially counteracted OVX-induced bone loss as shown by greater trabecular bone volume/total volume (BV/TV) and cortical thickness (Fig. 6m and Supplementary Fig. 12c). Because the effects of SLIT3 were comparable with the effects of parathyroid hormone (PTH) treatment, this suggests that the magnitude of SLIT3 effect is clinically significant. Moreover, both SLIT3 and PTH administration reversed the attenuation of endosteal CD31+EMCN+ endothelium occurring after OVX and similarly rescued endosteal bone formation (Supplementary Fig. 12d–f). To further investigate the therapeutic activity of SLIT3, we examined the ability of SLIT3 to promote bone formation after the onset of OVX-induced osteopenia. Mice were treated with i.v. injection of SLIT3 or vehicle twice weekly initiated 8 weeks after OVX surgery. SLIT3 administration substantially reversed bone loss as shown by
greater trabecular BV/TV and cortical thickness (Supplementary Fig. 12g,h). Furthermore, SLIT3 administration considerably augmented the amount of CD31 hiEMCN hi endothelial cells in this context (Supplementary Fig. 12i,j). Taken together, these results provide proof of principle that SLIT3 may have clinical utility to enhance fracture healing and to treat disorders of low bone mass such as postmenopausal osteoporosis.

Discussion

Even though bone formation is mediated solely by osteoblasts, it is likely that many other tissue types present in bone, such as vascular endothelium or autonomic and sensory nerves, contribute to creating a conducive milieu for bone formation 1,2,29–31. To the degree that the creation of a local osteogenic milieu should be coordinated with the cell-intrinsic matrix production capacity of osteoblasts,
it would be mechanistically attractive for osteoblasts to regulate their own matrix production alongside the activities of these supporting cell types. However, this remains a poorly understood facet of bone physiology. In this study, we hypothesized that mice with extreme increases in bone formation represent an opportunity to identify how osteoblasts regulate supporting tissue types in bone to create a pro-osteogenic milieu. In particular, we used the greatly enhanced bone formation phenotype of mice lacking the adaptor protein Shn3 to identify that osteoblast-derived SLIT3 enhances levels of an osteogenic subtype of vascular endothelium in bone, CD31EMCN endothelium. Accordingly, mice lacking SLIT3 or the known SLIT receptor ROBO1 display reduction in both the levels of marrow CD31EMCN endothelium and basal bone mass.

Support for osteoblasts being a key source of SLIT3 in bone includes the observation of robust and specific SLIT3 expression in osteoblasts, without appreciable SLIT3 expression in osteoclasts or CD31EMCN endothelium. Conditional deletion of Shn3 in late-stage osteoblasts enhanced SLIT3 expression and CD31EMCN endothelium levels, and osteoblast-derived conditioned medium enhanced BM endothelial tube formation in a SLIT3-dependent manner. Moreover, genetic interaction studies demonstrated that the CD31EMCN endothelium-promoting and enhanced bone formation effects of Shn3 deficiency are partially SLIT3 dependent. This rescue of the Shn3 phenotype is not a generic property of crossing Shn3 to a mouse with low bone mass, because crossing the Shn3−/− mouse strain to the osteopenic Rsk2−/− strain did not substantially alter the Shn3−/− phenotype. Thus, the epistatic interaction between Shn3 and Slit3 alleles in both regulation of bone mass and CD31EMCN endothelium levels provides in vivo genetic evidence that SLIT3 is a critical effector downstream of SHN3, although it does not preclude the existence of additional effectors.

In addition, ROBO1-deficient, but not ROBO4-deficient, mice show an osteopenic phenotype, and accordingly ROBO1 knockdown partially blocks BM endothelial responses to SLIT3. Although the phenotype of ROBO1-deficient mice and the in vitro studies indicate that it is a key receptor of SLIT signals in the regulation of bone mass accrual, it cannot be excluded that other SLIT-ROBO members similarly contribute in either an independent or a redundant manner.

Flow cytometry analysis of CD31EMCN endothelium suggests that this is a relatively rare population of cells, with only very limited numbers of cells present relative to other hematopoietic or mesenchymal lineages. This raises the question of how such a small population can exert such a large effect on organ physiology. One possible explanation is that the highest density of CD31EMCN endothelium is observed at very active sites of bone formation, such as the primary spongiosum immediately adjacent to the growth plate of an actively growing long bone, and within this site CD31EMCN endothelium is observed to be in close physical proximity with osteoblast-lineage cells. This physical proximity between CD31EMCN endothelium and the osteoblast lineage cells they support may act to amplify each other’s physiological effects. Further work is needed to clarify the nature and mediators of these interactions beyond SLIT3. In addition, much remains to be learned about properties that define CD31EMCN endothelium, including how this population relates to other endothelial cell types present in bone.

Given the evidence that fracture healing is accompanied by extensive elaboration of new blood vessels, the role of SHN3/SLIT3-mediated coupling between osteogenesis and CD31EMCN endothelium was explored in bone regeneration and found to be critically important. Impaired fracture healing is observed in elderly patients, patients with systemic vascular diseases such as diabetes, patients with inflammatory disorders or chronic infection, or patients with large traumatic bone defects. For these classes of patients, a single bone fracture often results in many years of pain, severely impaired mobility and numerous attempts at surgical management of their fracture. From this perspective, developing a means for medical therapy to promote fracture healing is urgently needed. Interestingly, the phenotypes observed with disruption of the SHN3/SLIT3 axis may extend beyond simply promoting more bone formation, because the fracture callus observed in SHN3-deficient mice was markedly more mature, including displaying overall more mature lamellar bone in addition to enhanced recruitment of hematopoietic elements to the callus. Conversely, SLIT3-deficient mice displayed an arrest at early stages of fracture callus maturation, displaying a lack of propagation of the mineralization sites on either side of the callus.

In this article, we also provide proof of principle for the potential of exogenous SLIT3 to promote bone fracture healing and prevent bone loss in a model of postmenopausal osteoporosis. Notably, these findings contrast with a prior in vitro study suggesting that SLIT2 suppresses osteoblast differentiation in vitro. This result more broadly suggests that agents that target bone vascular may represent a novel class of bone anabolics and raises the possibility that vascular-targeted anabolics may have a synergistic or complementary effect when used in combination with an osteoblast-targeted anabolic such as a PTH analogue or an anti-SOST antibody. Development of new categories of bone anabolic agents is especially important given the current limitations on the maximum duration of therapy with PTH-based anabolic agents. Likewise, in light of increasing evidence establishing that osteoporosis drugs can be used in a sequential or combination manner to obtain superior clinical outcomes, having therapeutic access to a larger diversity of anabolic pathways is highly desirable. Notably, because the pharmacokinetics and pharmacodynamics of SLIT3 in vivo are not currently known, further enhancement of the magnitude of SLIT3 effect may be possible with optimization of dosing and delivery strategies.

Methods

Methods, including statements of data availability and any associated accession codes and references, are available at https://doi.org/10.1038/s41591-018-0020-z.

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Author contributions

R.X. and M.B.G designed the experimental plan. R.X. executed most experiments. A.Y., Z.W., X.Y., J.K., N.L., Y.L., A.W., Z.Z., J.-H.S., J.M.B. and K.I. assisted with mouse studies. C.Z. performed RNA-seq analysis. M.P.B., B.Z. and J.-H.S. assisted with osteoclast samples. C.Z. performed RNA-seq analysis. M.P.B., B.Z. and J.-H.S. assisted with osteoclast studies. R.X. and M.B.G wrote the manuscript. M.B.G and L.H.G. supervised the project.

Competing interests

L.H.G. is on the board of directors of and holds equity in the GlaxoSmithKline and Citigroup Biomedical Imaging Core, Weill Cornell Microscopy and Image Analysis and Flow Cytometry Core Facilities for technical support.

Additional information

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mice were all previously reported, with parameters4. CT analysis was conducted on a Scanco Medical µ subcommittee on animal care (Institutional Animal Care and Use Committee). handled according to protocols approved by the Weill Cornell Medical College were used for all experiments. (Sigma) to 1-month-old mice once a day for 5 consecutive days. Littermate controls and Osteocalcin-CreERT mice were mated with expressing Flp recombinase. Slit3 floxed mice were backcrossed with C57BL/6j mice for eight generations. Transgenic mice expressing Cre recombinase under control of the cdh5 promoter (cdh5-Cre) , osterix promoter (osx-Cre), dmp1 promoter (dmp1-Cre) and Osteocalcin-CreERT mice were mated with Slit3 floxed or Slit3 floxed mice to obtain various Shn3−/− mice being a generous gift from Dr. Marc Tessier-Lavigne4,7,8,14,19,36. To generate Slit3 floxed mice, we obtained from the International Mouse Phenotyping Consortium the SLIT3-F08 mouse embryonic stem cell line in which exon 8 is flanked by loxp sites. After validation, F08 EC cells were injected into C57BL/6j blastocysts, and the derived chimeras displaying germine transmissions were selected for further breeding. The LacZ and nestin-actin assays were monitored by intercrossing with transgenic mice expressing Flp recombinase. Slit3 floxed mice were backcrossed with C57BL/6j mice for eight generations. Immunofluorescence, histology and histomorphometry. For immunofluorescence, fresh bone dissected and soft tissues from WT mice and mutant mice were collected and immediately fixed in ice-cold 4% paraformaldehyde solution for overnight. Decalcification was specially carried out with 0.5 M EDTA at 4 °C with constant shaking for bone samples from mice to obtain various mineralization, 1% penicillin/streptomycin, 1% HEPES and 1% non-essential amino acids, and differentiated with ascorbic acid and β-glycerophosphate. Conditioned medium was collected from culture of primary osteoblasts and stored at −80 °C. hMSCs were cultured and differentiated into osteoblasts using a commercial kit (Cyagen). All results were routinely tested by two independent experimenters. For staining of extracellular matrix mineralization, cells were fixed with 10% neutral-buffered formalin and stained with alizarin red. Mineralization activity was measured by colorimetric analysis. For ALP activity, osteoblasts were fixed with 10% neutral formalin buffer and stained with the solution containing fast blue and naphthol (Sigma-Aldrich). Alternatively, osteoblasts were incubated with tetrafluoro-4-fluorooanilidine blue solution, washed and incubated with solutions containing 6.5 mM NaN3, 18.5 mM NaHCO3, 2 mM MgCl2 and phosphate substrate (Sigma-Aldrich). ALP activity was measured by a spectrophotometer (Thermo). Osteoclast culture and differentiation. Murine BM-derived osteoclasts were isolated from 5-day-old mice by triple collagenase/Dispase II digestion. Cells were cultured in α-MEM medium (Gibco) containing 10% FBS, 2 mM l-glutamine, 1% penicillin/streptomycin, 1% HEPES and 1% non-essential amino acids, and differentiated with ascorbic acid and β-glycerophosphate. Conditioned medium was collected from culture of primary osteoblasts and stored at −80 °C. hMSCs were cultured and differentiated into osteoblasts using a commercial kit (Cyagen). All results were routinely tested by two independent experimenters. For staining of extracellular matrix mineralization, cells were fixed with 10% neutral-buffered formalin and stained with alizarin red. Mineralization activity was measured by colorimetric analysis. For ALP activity, osteoblasts were fixed with 10% neutral formalin buffer and stained with the solution containing fast blue and naphthol (Sigma-Aldrich). Alternatively, osteoblasts were incubated with tetrafluoro-4-fluorooanilidine blue solution, washed and incubated with solutions containing 6.5 mM NaN3, 18.5 mM NaHCO3, 2 mM MgCl2 and phosphate substrate (Sigma-Aldrich). ALP activity was measured by a spectrophotometer (Thermo). Osteoclast culture and differentiation. Murine BM were flushed from the femur and tibia of mice and cultured in petri dishes in α-MEM medium with 10% FBS. BM mononuclear cells were removed from BM culture dishes and cultured in the same medium for 3 d to obtain osteoclast precursors. The osteoclast precursors then differentiated into osteoclasts in the presence of human RANKL (50 ng ml−1; PeproTech) and M-CSF for 3 d. Peripheral blood mononuclear cells from the whole blood of healthy volunteers were isolated by density gradient centrifugation using Ficoll (Invitrogen, Carlsbad, CA, USA). Cells were stained with antibodies against CD146 (eBioscience), FITC-conjugated CD45 (35-0451-81; BD Biosciences) for 45 min on ice. After washing, cells were resuspended in PBS (pH 7.2) with 2 mM EDTA and 1 μg ml−1 DAPI (live/dead exclusion) for analysis on an LSRII flow cytometer system (BD Biosciences) cytometer and analyzed using FlowJo software (Tree Star). Cell sorting was performed with a FACS Aria III cell sorter (Becton Dickinson) at Weill Cornell. Cells with exclusion of DAPI+ cells and doublets. The strategy to sort CD31+EMCN+ endothelial cells is diagrammed in Supplementary Fig. 1c. Endothelial cell culture and functional assays. Mouse BM-derived late-stage EPOCs were obtained from BioChain (7030031) and cultured in growth medium (α-MEM with 10% FBS in the presence of 50 ng ml−1 M-CSF). Mouse BM mononuclear cells were cultured and differentiated into osteoclast precursors in the form of a single linear scratch made with a yellow pipette tip. After gently washing the well twice, cells were cultured in medium with SLIT3 or vehicle. At 6 and 12 h after injury, cells were stained with 0.5% crystal violet and photographed. The width of the wound area was quantitatively evaluated using ImageJ (http://rsb.info.nih.gov/ij/download.html). Endothelial cell proliferation assays were conducted in 12-well plates precoated with gelatin (STEMCELL Technologies). A total of 3× 104 cells per well were plated overnight and stimulated with a wound in the form of a single linear scratch made with a yellow pipette tip. After gently washing the well twice, cells were cultured in medium with SLIT3 or vehicle. At 6 and 12 h after injury, cells were stained with 0.5% crystal violet and photographed. The width of the wound area was quantitatively evaluated using ImageJ (http://rsb.info.nih.gov/ij/download.html). Endothelial cell proliferation assays were conducted in 12-well plates precoated with gelatin (STEMCELL Technologies). EPOCs (3× 105 cells per well) were seeded in the medium with a serial dilution of α-MEM with vehicle or vehicle plus 10 ng ml−1 M-CSF. At 1, 24, 48 and 72 h, cells were incubated with tetrafluoro-4-fluorooanilidine blue solution (Thermo Fisher), and the supernatant was evaluated with a spectrophotometer (Thermo) (five wells for each condition). Endothelial cell tube formation assay was conducted in 96-well plates precoated with Matrigel (BD). After 1-h serum starvation, EPOCs (3× 105 cells per well) were seeded in conditioned medium dilution or control medium on precoated Matrigel in plates precoated with 0.5% FBS in 5-h incubation. In five wells for each condition).
Quantitative real-time PCR analysis. Total RNA was extracted using TRIzol reagent (Invitrogen) or RNeasy Mini Kit (Qiagen), and reverse transcription was performed with the High-Capacity cDNA Reverse Transcription Kit from Applied Biosystems according to the manufacturer's instructions. We performed quantitative analysis of gene expression using SYBR Green PCR Master Mix (Applied Biosystems) with the Mx3000P real-time PCR system (Agilent Technologies). Hprt expression was used as an internal control. The primers used for PCR are described in Supplementary Table 1.

RNA sequencing and analysis. Reads were aligned to the mm9 mouse transcripts using STAR (version 2.3.0e)\(^*\) with default parameters, and resulting bam files were sorted and indexed using samtools. Gene counts were obtained by applying feature counts (version 1.4.3)\(^*\) to sorted bam files, and only unique-mapping reads were used. Genes without any expression counts in any sample were discarded. The DESeq2 (version 1.10.1) package\(^*\) was used to normalize gene count data and then detect DEGs between mutant mice and control groups with false discovery rate < 0.1 and absolute log2 fold change > 0.5. Mosaic version 1.1 was used to retrieve GO information for all genes of the mouse genome\(^*\). We performed functional analysis on DEGs with DAVID\(^*\) (version 6.7), and biological process GO terms with enrichment \(P < 0.05\) were selected as overrepresented functions.

Western blot analysis. We performed Western blot analysis according to a previously described standard protocol\(^*\). Primary antibodies were specific for SLIT3 (1:500; AF3629; R&D Systems), ROBO1 (1:500; ab7279; Abcam), ROBO2 (1:100; ab75014; Abcam), YAP (1:1,000; 4912; Cell Signaling), p-YAP (1:1,000; 4911; Cell Signaling), AKT (1:1,000; 4691; Cell Signaling), p-AKT (1:1,000; 4690; Cell Signaling), ERK (1:1,000; 9102; Cell Signaling), p-ERK (1:1,000; 9101; Cell Signaling), and beta-actin (1:5,000; sc-47778; Santa Cruz) or Hsp90 (1:100; sc-13119; Santa Cruz). Secondary anti-mouse/rabbit HRP-conjugated antibodies were subsequently applied.

ELISA analysis. We analyzed SLIT3 ELISA (Lifespan LS-F7173) and CTX ELISA (Lifespan LS-F12349) by using a kit. All ELISA assays were run according to the manufacturer's instructions.

Bone fracture model. All surgical procedures were performed under isoflurane anesthesia via nosecone. Surgical sites were sterilized by a betadine/iodide/isopropanol prep after hair removal by a clipper with a #40 blade and depilation cream (Natural Hair Removal, Natural). After fracture surgery, the visceral lining or muscle was sutured with absorbable Ethicon Vicryl sutures (Cat #95057-014; VWR) before closing the wound clips that were then removed 2 weeks postoperatively. Animals received intraperitoneal Buprenex (0.5 mg kg\(^{-1}\)) and oral meloxicam (2.0 mg kg\(^{-1}\)) as analgesia before surgery and once every 24 h post surgery for 3 days. All surgical procedures are approved by the Institutional Animal Care and Use Committee of Weill Cornell Medical College (Protocol 2012-0005).

Bone fracture was done following previously described protocols with modifications\(^*\). In brief, after anesthesia and surgical site sterilization, an incision above the right anterolateral femur was made. The femur and patella were then removed, and a single cut was made in the middle of the femoral diaphysis using a Dremel saw with a diamond thin cutting wheel (Cat. #100230-724; VWR). A blunt depilatory cream (Nair). After surgery, the visceral lining or muscle was sutured with absorbable Ethicon Vicryl sutures (Cat #95057-014; VWR) before closing the skin with wound clips that were then removed 2 weeks postoperatively. Animals received intraperitoneal Buprenex (0.5 mg kg\(^{-1}\)) and oral meloxicam (2.0 mg kg\(^{-1}\)) as analgesia before surgery and once every 24 h post surgery for 3 days. All surgical procedures are approved by the Institutional Animal Care and Use Committee of Weill Cornell Medical College (Protocol 2012-0005).

Bone fracture was done following previously described protocols with modifications\(^*\). In brief, after anesthesia and surgical site sterilization, an incision above the right anterolateral femur was made. The femur and patella were then removed, and a single cut was made in the middle of the femoral diaphysis using a Dremel saw with a diamond thin cutting wheel (Cat. #100230-724; VWR). A blunt 25-gauge needle was then inserted into the marrow space through the patellar groove into the femur cavity. The needle was then removed, and a single cut was made in the middle of the femoral diaphysis using a Dremel saw with a diamond thin cutting wheel (Cat. #100230-724; VWR). A blunt 25-gauge needle was then inserted into the marrow space through the patellar groove into the femur cavity. The needle was then trimmed to avoid it from protruding into the patella–femoral joint space. Muscle was then placed over the posterior surface on the lower supports, spaced 9.9 mm apart. The upper supports were spaced 3.3 mm. Load was applied at a rate of 0.1 mm s\(^{-1}\) until failure occurred. The failure load (N) and bending stiffness (N mm\(^{-1}\)) were calculated from the force–displacement curves and the four-point dimensions.

Human bone callus collection. The project was approved by the Ethics Committee of Shaoxing People’s Hospital (No. 080), and the protocol was carried out in accordance with approved guidelines. Preoperative informed consent was obtained from each patient. From January 2010 to June 2014, bone callus samples were obtained from patients undergoing surgical treatment in the Department of Orthopedics of Shaoxing People’s Hospital. Callus was collected from patients who required surgical treatment for failure of skeletal traction.

Inclusion criteria were as follows: (1) surgeries after failure of conservative treatment or external fixation were performed temporarily before open reduction and plate fixation for long bone fractures; (2) secondary surgeries after failure of internal fixation, including loosened or broken plates or screws, bent or broken intramedullary nails, and fracture angulation and aversion abnormalities; and (3) secondary surgeries for hypertrophic non-union. Exclusion criteria were as follows: (1) fracture complicated with microbial infection; (2) fracture complicated with brain injury; (3) bone tumors; (4) systemic bone-related diseases; and (5) patients treated with hormones, steroids, vitamin D and calcium. Further demographic information has been published elsewhere\(^*\).

Statistical methods. All data were presented as the mean ± s.e.m. Sample sizes were calculated on the assumption that a 30% difference in the parameters measured would be considered biologically significant with an estimate of sigma of 10–20% of the expected mean. All ELISA methods and CTX ELISA subsequently applied. Where applicable, animals were randomized to treatments versus control groups. Statistical methods are indicated in the figure legends. The GraphPad PRISM software (v6.0a; GraphPad, La Jolla, CA, USA) was used for statistical analysis. A P value < 0.05 was considered statistically significant: *P < 0.05; **P < 0.01; ***P < 0.001.

Reporting Summary. Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

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Life Sciences Reporting Summary

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### Experimental design

1. **Sample size**
   
   Describe how sample size was determined.

   Generally, sample sizes were calculated on the assumption that a 30% difference in the parameters measured would be considered biologically significant with an estimate of sigma of 10-20% of the expected mean. Alpha and Beta were set to the standard values of .05 and 0.8, respectively.

2. **Data exclusions**
   
   Describe any data exclusions.

   No animals or samples were excluded from analysis.

3. **Replication**
   
   Describe the measures taken to verify the reproducibility of the experimental findings.

   Experiments were repeated 2 times to ensure reproducibility except where otherwise indicated in the figure legends.

4. **Randomization**
   
   Describe how samples/organisms/participants were allocated into experimental groups.

   For all experiments utilizing SLIT3 treatment of mice, mice were randomized to SLIT3 or vehicle treatment. Where applicable, mice were randomized to treatment with tamoxifen or vehicle.

5. **Blinding**
   
   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

   Investigators performing uCT or histomorphometry analysis were blinded to the genotype and treatment group of each sample.

   Note: all in vivo studies must report how sample size was determined and whether blinding and randomization were used.

6. **Statistical parameters**

   For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

   n/a Confirmed

   - The exact sample size \((n)\) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
   - A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
   - A statement indicating how many times each experiment was replicated
   - The statistical test(s) used and whether they are one- or two-sided
     
     Only common tests should be described solely by name; describe more complex techniques in the Methods section.
   - A description of any assumptions or corrections, such as an adjustment for multiple comparisons
   - Test values indicating whether an effect is present
     
     Provide confidence intervals or give results of significance tests (e.g. P values) as exact values whenever appropriate and with effect sizes noted.
   - A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
   - Clearly defined error bars in all relevant figure captions (with explicit mention of central tendency and variation)

   *See the web collection on statistics for biologists for further resources and guidance.*
7. Software

Describe the software used to analyze the data in this study.

GraphPad Prism and Microsoft Excel

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a third party.

There are no restrictions

SLIT3 (1:500; R&D Systems, AF3629), ROBO1 (1:500; Abcam, ab7279), ROBO2 (1:1000; Abcam, ab75014), YAP (1:1,000; Cell Signaling, 4912), p-YAP (1:1,000; Cell signaling, 4911), AKT (1:1,000; Cell Signaling, 4691), p-AKT (1:1,000; Cell Signaling, 4060), ERK (1:1,000; Cell Signaling, 9102), p-ERK (1:1,000; Cell Signaling, 9101) and beta-actin (1:5,000; sc-47778, Santa Cruz) or Hsp90 (1:1000; sc-13119, Santa Cruz), Purified Rat Anti-Mouse CD16/CD32 (BD Biosciences), APC-conjugated EMCN antibody (ebioscience 50-5851-80), PE-conjugated CD31 (ebioscience 12-0311-81), FITC-conjugated CD45 (Tonbo 35-0451), APC/Cy7-conjugated Ter119 (Biolegend 116223) and PerCP-Cy7-conjugated CD146 (BD Biosciences 562231), CD31 (553370, BD Pharmigen, 1:100), CD31 conjugated to Alexa Fluor 488 (FAB3628G, R&D Systems, 1:50), Endomucin (sc-65495, Santa Cruz, 1:100) or Beta Galactosidase antibody (GTX77365, Gene Tex, 1:100), EMCN (sc-65495, Santa Cruz, 1:200) and human Slit3 (ab198726, Abcam 1:50). SLIT3 antibodies were experimentally validated in data provided to reviewers. Other antibodies were validated by the manufacturer.

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

Only primary cells were used for this study.

b. Describe the method of cell line authentication used.

No transformed cell lines were used for this study.

c. Report whether the cell lines were tested for mycoplasma contamination.

Yes, all cell lines were routinely tested for mycoplasma contamination.

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

No commonly misidentified cell lines were used for this study.
Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide all relevant details on animals and/or animal-derived materials used in the study.

Shn3-/- (BALB/c), Shn3KI/KI (C57BL/6j), Shn3 floxed allele (C57BL/6j), Slit3-/- (BALB/c), Robo1-/- (ICR) and Robo4-/- (C57BL/6j) mice were all previously reported, with Robo1-/- mice being a generous gift from Dr. Marc Tessier-Lavigne 4,7,8,14,19,40. To generate Slit3 floxed mice, the SLIT3-F08 mouse embryonic stem (ES) cell line in which exon 8 is flanked by loxP sites was obtained from International Mouse Phenotyping Consortium (IMPC). After validation, F08 EC cells were injected into C57BL/6j blastocysts, and the derived chimeras displaying germline transmissions were selected for further breeding. The LacZ and neo cassettes were removed by intercrossing with transgenic mice expressing Flp recombinase. Slit3 floxed mice were backcrossed with C57BL/6j mice for 8 generations.

Transgenic mice expressing Cre recombinase under control of the cdh5 promoter (cdh5-Cre) 41, osterix promoter (osx-Cre) 42, dmp1 promoter (dmp1-Cre) 43 and Osteocalcin-CreERT mice44 were mated with Shn3 floxed mice or Slit3 floxed mice to obtain various Shn3 or Slit3 conditional KO mouse. For postnatal activation of CreERT, 100mg/kg tamoxifen (Sigma) in corn oil (Sigma) was intraperitoneally injected to 1 month-old mice once a day for five consecutive days. Littermate controls were utilized for all experiments.

All animals were maintained in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were handled according to protocols approved by the Weill Cornell Medical College subcommittee on animal care (IACUC).

For the prophylactic OVX model, 12-week-old female mice (JAX, C57BL/6j) were anesthetized and bilaterally ovariectomized or sham operated. Ovariectomized mice were given twice weekly intravenous injections of 1mg per kg body weight of SLIT3 or vehicle or daily sc injections of 80ug/kg PTH (1-34) for 6 weeks starting 2 weeks after ovariectomy. For the model where SLIT3 was delivered in a therapeutic manner after osteopenia onset, OVX was performed in 12 week old mice, mice were observed for 8 weeks post-OVX to allow for onset of osteopenia, and then mice were treated with SLIT3 (1mg/kg) or vehicle for 6 further weeks.

For fracture model, 5 week old mice of the indicated genotypes underwent fracture as described in the methods section.

For all experiments, group sizes and other cohort details are indicated in figure legends.

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

This information is described in the following publication: Han W, He W, Yang W, Li J, Yang Z, Lu X, Qin A, Qian Y. The osteogenic potential of human bone callus. Sci Rep. 2016 Oct 31;6:36330.