Induction of osteogenesis by bone-targeted Notch activation

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Declining bone mass is associated with aging and osteoporosis, a disease characterized by progressive weakening of the skeleton and increased fracture incidence. Growth and lifelong homeostasis of bone rely on interactions between different cell types including vascular cells and mesenchymal stromal cells (MSCs). As these interactions involve Notch signaling, we have explored whether treatment with secreted Notch ligand proteins can enhance osteogenesis in adult mice. We show that a bone-targeting, high affinity version of the ligand Delta-like 4, termed Dll4_(E12), induces bone formation in male mice without causing adverse effects in other organs, which are known to rely on intact Notch signaling. Due to lower bone surface and thereby reduced retention of Dll4_(E12), the same approach failed to promote osteogenesis in female and ovariectomized mice but strongly enhanced trabecular bone formation in combination with parathyroid hormone. Single cell analysis of stromal cells indicates that Dll4_(E12) primarily acts on MSCs and has comparably minor effects on osteoblasts, endothelial cells or chondrocytes. We propose that activation of Notch signaling by bone-targeted fusion proteins might be therapeutically useful and can avoid detrimental effects in Notch-dependent processes in other organs.

INTRODUCTION

Osteoporosis is the most common disease affecting the skeletal system in humans and characterized by low bone mass, reduced mineral density and disarranged bone microarchitecture. This reduces bone strength and increases the risk of fractures, which leads to secondary health problems and increased mortality^{1,2}. The skeletal system is undergoing lifelong remodeling mediated by bone-forming osteoblasts and bone-resorptive osteoclasts³. Disbalance between bone formation and resorption can lead to osteopenia, the loss of bone density, and frequently progresses into osteoporosis⁴. Anti-osteoporosis therapies are either anabolic, by enhancing osteoblast activity, or anti-resorptive, involving the inhibition of osteoclast activity. FDA approved osteoclast inhibitors include bisphosphonates, such as Alendronate, Risedronate, Ibandronate and Zoledronic acid⁵⁻⁸ but also the peptide hormone calcitonin⁹ and the female sex hormone estrogen¹⁰, whereas a peptide fragment of parathyroid hormone (PTH1-34) and monoclonal antibodies inhibiting sclerostin are used in the clinic to increase bone formation¹¹⁻¹⁴. Apart from specific drawbacks of individual drugs, systemic administration generally facilitates the emergence of adverse side effects, which could be potentially avoided by directing therapeutic agents to bone. Examples include L-Asp-hexapeptide conjugated-estradiol (E2)^{15,16} and conjugates of prostaglandin E2 and bisphosphonate¹⁷. These drugs contain targeting moieties made of either synthetic bisphosphonates or repeats of negatively-charged glutamate (Glu) or aspartate (Asp) amino acid residues, which show high affinity binding to hydroxyapatite, a main component of mineralized bone¹⁸. Apart from enrichment in the skeletal system, the modified drugs show reduced plasma retention, which limits adverse effects after administration^{15,16,19}. However, the efficacy of these drugs still requires clinical validation and there is still high demand for novel and effective anti-osteoporosis drugs.

Notch signaling is an evolutionary conserved pathway with numerous functional roles including the regulation of osteogenesis. In mammals, there are 4 Notch receptors, namely Notch 1-4, and 5 ligands belonging either to the Jagged/Serrate (Jag1, Jag2) or the Delta-like subfamily (Dll1, Dll3 and Dll4)²⁰. As both receptors and ligands are transmembrane proteins, their interaction requires cell-cell contact and triggers ligand internalization, which is necessary for Notch receptor activation²¹⁻²³. Soluble fragments containing the ligand extracellular domain can bind Notch but fail to induce receptor activation and thereby effectively act as antagonists^{21,22}. In skeletal development, Notch signaling in limb mesenchyme controls the maintenance and proliferation of mesenchymal progenitor cells and prevents premature osteoblastic differentiation^{24,25}. Context-specific Notch signaling also plays important roles in osteoclast differentiation and function²⁶. Notch activity in bone marrow (BM) macrophages inhibits commitment to osteoclast differentiation, but it enhances the maturation and function of committed osteoclast precursors^{27,28}. Notch is generally a negative regulator of endothelial cell (EC) proliferation and vascular growth, but exerts the opposite function in bone and promotes the formation of type H vessels. This capillary subtype is associated with osteoblast lineage cells and enhances osteogenesis by providing growth factors and other signals^{23,29-31}.

The reports above suggest that Notch activation, e.g. through the administration of exogenous ligand molecules, might have the capacity to enhance bone formation. Given the many functional roles of Notch signaling in different organs and processes, it would be desirable to preferentially direct soluble Notch ligands to bone and thereby limit potentially harmful side effects elsewhere in the body. Here, we report the generation of a soluble, bone-targeted fusion protein, termed Dll4_(E12), combining the extracellular domain (ECD) of Delta-like 4 (Dll4), several point mutations to increase affinity for Notch receptors³², and a negatively charged poly-aspartate peptide motif (Asp_{8x}) mediating binding to hydroxyapatite. We show that Dll4_(E12) increases bone formation in male adult mice without inducing adverse

effects in other organs that have been previously linked to compromised Notch function. The same approach fails to enhance osteogenesis in female and ovariectomized mice, which we attribute to the lower bone surface and reduced retention of bone-targeted ligand in these animals. In contrast, bone formation in response to PTH is strongly enhanced by Dll4_(E12) in female mice. Based on the histological analysis of Dll4_(E12)-treated tissue samples as well as immunohistological and single cell RNA sequencing (scRNA-seq) analysis of bone stromal cell populations, we conclude that Dll4_(E12) is safe and can be used to activate Notch signaling in bone mesenchymal stromal cells leading to enhanced osteogenesis.

RESULTS

Generation and characterization of soluble, bone-binding Dll4

Notch signaling plays important roles in the maintenance of mesenchymal progenitor cells and the coupling of angiogenesis and osteogenesis in the developing skeletal system^{23-25,29}. To explore whether Notch activation might be therapeutically useful to enhance bone formation in the adult organism, we generated a fusion construct comprising the ECD of Dll4, a critical Notch ligand in ECs, a cassette of poly-histidine epitope tags (His_{6x}) enabling protein purification and detection, and a carboxyterminal stretch of 8 aspartic acid residues (Asp_{8x}) mediating binding to hydroxyapatite (Dll4-Asp_{8x}) (Figure 1A). Expression and molecular weight of the secreted protein was validated by expression in HEK293 cells followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the culture supernatant (Figure 1-figure supplement 1A). Previous work has established that soluble Notch ligands lack the capacity to activate their corresponding receptors and act as inhibitors blocking the interaction between endogenous, membrane-anchored Delta/Jagged ligands and Notch receptors^{21,22}. As expected, Dll4-Asp_{8x} inhibits the expression of established Notch target genes in confluent human umbilical vascular endothelial cells

(HUVECs) *in vitro* (Figure 1-figure supplement 1B). When culture dishes were pre-coated with poly-L-Lysine enabling the binding of negatively charged molecules, Dll4-Asp_{8x} acts as an activator and induces the transcription of Notch target genes in sub-confluent HUVECs (Figure 1-figure supplement 1C). These data indicate that Dll4-Asp_{8x} can interact with Notch receptors and that immobilization via the Asp_{8x} motif enables Notch activation in cultured cells.

For *in vivo* experiments in mice, a cDNA encoding Dll4-Asp_{8x} was cloned into the vector *pLIVE*, which mediates constitutive protein expression in liver after hydrodynamic tail vein injection²⁷. Immunohistological characterization of sectioned *pLIVE-Dll4-Asp_{8x}*-treated femurs isolated from male mice at 2 days post-injection (dpi) and 3 weeks post-injection (wpi) shows strong Dll4 signals at trabecular bone, the endosteum lining the inner surface of compact bone, and around distal vessel buds in proximity of the growth plate. In contrast, no comparable signals are seen in samples from male control animals (Figure 1-figure supplement 2A-C and Figure 1-figure supplement 3A). These data confirm the successful expression of recombinant Dll4-Asp_{8x} *in vivo* and binding of the fusion protein to bone. However, Dll4-Asp_{8x}-treated bone samples at 3 wpi do not show overt alterations in Osterix⁺ osteoblast lineage cells and deposition of the matrix proteins Osteopontin (Opn) and Collagen I alpha 1 chain (Colla1) (Figure 1-figure supplement 3B).

Generation of a high affinity Dll4 fusion protein

To address the possibility that Dll4-Asp_{8x} does not achieve sufficient levels of Notch activation in bone, we also generated a high affinity variant of this protein by introducing multiple point mutations into the Dll4 ECD, which were previously reported to enhance Notch binding and signaling³² (Figure 1A and Figure 1-figure supplement 4A). Following expression in HEK293 cells, SDS-PAGE confirmed the correct molecular weight of the resulting Dll4_(E12) version of Dll4-Asp_{8x} in culture supernatants (Figure 1-figure supplement

4B). Purified and soluble Dll4_(E12) inhibits endogenous Notch signaling in cultured HUVECs similar to Dll4-Asp_{8x}, whereas Dll4_(E12) in combination with poly-L-Lysine pre-coating induces the upregulation of Notch target genes (Figure 1-figure supplement 4C and D). To overexpress Dll4_(E12) *in vivo*, a cDNA encoding Dll4_(E12) was cloned into the *pLIVE* vector and the resulting *pLIVE-Dll4_(E12)* construct was administered to male mice via hydrodynamic tail vein injection. Western blot analysis of liver lysates confirmed the expression of Dll4_(E12) at 3 wpi (Figure 1-figure supplement 4E). Consistent with the hydroxyapatite-binding properties of Dll4-Asp_{8x}, immunohistological analysis of femurs confirm the accumulation of Dll4_(E12) at the surface of trabecular and cortical bone (Figure 1B). Notably, histological analysis also reveals a strong increase in the Colla1-positive area in *pLIVE-Dll4_(E12)*-injected mice (Figure 1C). Micro-computed tomography (μ -CT) and histomorphometric analysis show significant increases in bone mass and density (bone volume over total volume; BV/TV), connectivity density and thickness of trabeculae and osteoblast surface in *pLIVE-Dll4_(E12)*-injected tibiae (Figure 1D, E). However, no significant changes are observed in trabecular separation, trabecular number and erosion surface (Figure 1E). *In vivo* double calcein labeling further argues for enhanced bone formation in mice treated with Dll4_(E12) (Figure 1F). Together, these results show that key parameters of bone quality can be enhanced by expression of a soluble, hydroxyapatite-binding version of Dll4 with high affinity for Notch receptors.

Lack of adverse biological effects of Dll4_(E12) treatment

The Notch pathway is involved in a wide range of biological processes and, accordingly, Notch inhibition *in vivo* can have serious pathological effects. Considering that circulating Dll4_(E12) released by liver cells might act as an antagonist of endogenous Notch signaling interactions, we examined several different organs for Dll4 immunoreactivity and the appearance of pathological alterations. While liver hepatocytes show the expected expression of Dll4_(E12) (Figure 1-figure supplement 5A), no increase in Dll4 immunoreactivity

indicating binding of Dll4_(E12) was seen in spleen or lung (Figure 1-figure supplement 5B and C). In small intestine, Notch inhibition has been linked to toxicity by impairing the formation and distribution of goblet cells²⁸. However, intestinal crypts from *pLIVE-Dll4*_(E12)-injected mice show no goblet cell metaplasia (Figure 2A). While chronic Dll4 blockade was shown to cause sinusoidal vessel dilation in liver³³, histological analysis reveals no differences between *pLIVE-Dll4*_(E12) and control livers (Figure 2B). This result is particularly remarkable, as liver is the source of Dll4_(E12) protein expression after hydrodynamic tail vein injection. Dll4-mediated Notch1 activation is essential for thymic T cell development²⁸, but flow cytometry shows comparable numbers of CD4⁺ and CD8⁺ double positive T cells in thymi from control and *pLIVE-Dll4*_(E12)-injected mice (Figure 2C, D). While it has been proposed that Dll4-triggered Notch signaling modulates inflammatory responses³⁴, inflammatory cytokines are also not significantly increased in plasma from *pLIVE-Dll4*_(E12)-injected mice (Figure 2-figure supplement 1). Notch inhibition was also shown to compromise the barrier function of vascular endothelium³⁰, but the extravasation of fluorescent, Texas-Red-labeled Dextran is not increased in femurs from *pLIVE-Dll4*_(E12)-injected mice (Figure 2E, F). Furthermore, there is no significant difference in Texas-Red Dextran extravasation in other organs investigated, namely liver, spleen and lung (Figure 2-figure supplement 2A-C). The sum of these results indicates that Dll4_(E12) causes no adverse systemic effects that are known to result from Notch inhibition.

Effects of recombinant Dll4_(E12) on bone and vasculature

Next, we performed a more detailed analysis of the alterations in *pLIVE-Dll4*_(E12) femur at 3 wpi. While Osterix⁺ (OSX⁺) osteoblasts are strikingly increased relative to control samples, there is a significant reduction in the number of Runx2⁺ osteoprogenitors (Figure 3A, B and Figure 3-figure supplement 1). Dll4_(E12) treatment was also performed on *Tg(Hey1-EGFP)^{ID40Gsat}* mice, which express enhanced green fluorescent protein (GFP) under the

control of *Hey1*, a Notch target gene. *Dll4*_(E12) strongly increases the number of perivascular GFP⁺ cells in the metaphysis relative to control animals (Figure 3-figure supplement 1). Notably, these cells are located in close proximity to OSX⁺ osteoblasts but lack strong nuclear OSX immunostaining. Vessel-associated *Hey1*-expressing cells in *Dll4*_(E12)-treated mice are also found in the transition zone interconnecting the metaphyseal and diaphyseal vasculature, whereas no GFP⁺ cells are found in BM (Figure 3-figure supplement 1). In addition to the increase in OSX⁺ cells, *Dll4*_(E12) treatment enhances the expression of major non-collagenous proteins involved in bone matrix organization and deposition, namely Osteopontin and Osteocalcin (Figure 3C). Similarly, immunostaining of the receptor tyrosine kinase PDGFR β and the proteoglycan NG2, both markers of mesenchymal stromal cells, are significantly increased in the *pLIVE-Dll4*_(E12) metaphysis (Figure 3-figure supplement 2A, B). Arguing against increased bone turnover involving higher bone resorption, *Dll4*_(E12) treatment does not lead to significant alterations in the number of V-ATPase⁺ osteoclasts (Figure 3D). Furthermore, immunohistological analysis reveals that adipocyte number and the expression of critical chondrocyte markers, such as Aggrecan (*Acan*) and the transcription factor Sox9, are not significantly altered by recombinant *Dll4*_(E12) (Figure 3E and Figure 3-figure supplement 2C, D).

To assess whether blood vessels in long bone are altered by treatment with *Dll4*_(E12), we examined the expression of some known vascular makers such as Endomucin (*Emcn*) and VEGFR3 in femoral sections. Low VEGFR3 immunostaining was previously shown to mark type H vessels columns that are associated with OSX⁺ osteoprogenitors in the metaphysis, whereas vessel buds in direct proximity of growth plate chondrocytes and the sinusoidal (type L) vasculature of the bone marrow show high anti-VEGFR3 signal³¹. Notably, the region with VEGFR3^{low} (type H) *Emcn*⁺ vasculature and OSX⁺ cells is significantly increased in the metaphysis of *pLIVE-Dll4*_(E12)-injected mice relative to control (Figure 4A-C). Notch signaling in bone ECs was previously shown to lead to an expansion of type H vasculature

and increased osteogenesis, but it also promotes artery formation²³. However, the abundance of arteries and small arterioles is not significantly altered by Dll4_(E12) (Figure 4D).

Bone formation is not increased by Jag1-Asp_{8x} and Jag1_(JV1)

Homotypic signaling interactions between Notch receptors and the ligand Jagged1 in osteochondral progenitors negatively regulate the pool of these progenitors but also promote bone formation^{35,36}. Moreover, Delta and Jagged ligands have distinct, sometimes opposite biological functions in certain processes such as retinal angiogenesis or pancreas development^{37,38}. To address whether exogenous Jagged1 has the capacity to enhance bone formation, we generated a soluble Jag1-Asp_{8x} fusion protein in which, analogous to Dll4-Asp_{8x}, the transmembrane and intracellular domain are replaced by His_{6x} and Asp_{8x} sequence motifs. In parallel, a high affinity variant of Jag1-Asp_{8x}, termed Jag1_(JV1), was generated by replacing several amino acid residues in the ligand ECD, as previously reported³⁹ (Figure 5A). Jag1-Asp_{8x} and Jag1_(JV1) proteins were purified from HEK293 cell culture supernatants. SDS-PAGE and Western blotting confirmed the correct molecular weight of the purified proteins (Figure 5-figure supplement 1A-C). Soluble Jag1_(JV1) inhibits *DLL4*, *HEY1*, *HEY2* and *EFNB2*, known targets of Notch signaling, in cultured HUVECs, whereas immobilized Jag1_(JV1) induces upregulation of Notch target genes at higher levels compared to Jag1-Asp_{8x} stimulation (Figure 5-figure supplement 1D and E).

To overexpress these recombinant proteins *in vivo*, cDNAs of *Jag1-Asp_{8x}* and *Jag1_(JV1)*, respectively, were cloned into *pLIVE* vector. Following hydrodynamic tail vein injection of male mice, both Jag1-Asp_{8x} and Jag1_(JV1) are readily detectable at bone surfaces in sections, but Jag1-Asp_{8x} is also more broadly detected throughout the marrow cavity (Figure 5-figure supplement 2A, B). Neither Jag1-Asp_{8x} nor Jag1_(JV1), however, induce appreciable alterations in Colla1 deposition (Figure 5B). Micro-computed tomography (μ-CT) analysis reveals a slight but significant increase in trabecular bone thickness in *pLIVE-Jag1_(JV1)*-injected mice,

whereas bone volume over total volume (BV/TV), connectivity density, trabecular number and trabecular separation are not altered significantly (Figure 5C). These results argue that only Dll4_(E12) is able to stimulate substantial osteogenesis in adult mice, while Dll4-Asp_{8x}, Jag1-Asp_{8x} and Jag1_(JV1) lack this capacity.

Sex-specific effects of Dll4_(E12) on bone formation

Next, we investigated whether Dll4_(E12) treatment provides any beneficial effects in the ovariectomy model of postmenopausal osteoporosis. In comparison to sham controls, ovariectomized female mice show significant reduction of trabecular bone, which is not improved by Dll4_(E12) expression (Figure 6A-D). Similarly, Colla1 and PDGFR β immunoreactivity is not significantly altered by Dll4_(E12) (Figure 6E-F).

Given that bone surface is substantially lower in adult female mice relative to age-matched males (Figure 6G and Figure 6-figure supplement 1A, B), we reasoned that Dll4_(E12) binding might depend on the sex of animals. Indeed, endogenous Dll4 immunoreactivity is already significantly lower in control females relative to males and the strong increase in femur sections from Dll4_(E12)-expressing males is not mirrored by female samples (Figure 6-figure supplement 1C, D). Together, these findings indicate that the biological effect of Dll4_(E12) depends on the available bone surface, suggesting that this approach might not be beneficial in settings with low bone mass.

Anabolic therapy is a clinical approach for the treatment of osteoporosis, aiming at increase in bone mass. We therefore explored whether Dll4_(E12) can be beneficial in female mice if used in combination with PTH. Indeed, hydrodynamic tail vein injection of female mice with *pLIVE-Dll4_(E12)* followed by daily PTH1-34 administration over a period of 3 weeks leads to a profound increase in Dll4 immunoreactivity in femoral sections (Figure 7-figure supplement 1A, B). This effect is mirrored by significant increases in Colla1, PDGFR β and Emcn immunostaining in response to combined Dll4_(E12) and PTH1-34 treatment (Figure

7-figure supplement 1C-E). Furthermore, the combination of Dll4_(E12) and PTH1-34 increases the abundance of OSX⁺ osteoprogenitors and the length of the metaphysis in adult females, whereas the Osteopontin⁺ area is not significantly enhanced relative to PTH1-34 alone (Figure 7-figure supplement 2A, B).

Analysis of Dll4_(E12) and PTH1-34 treated femurs by μ -CT shows that the changes above are accompanied by profound increases in trabecular bone and, in particular, connectivity density and bone surface relative to control females or separate administration of either Dll4_(E12) or PTH1-34 (Figure 7A-G). In contrast, the beneficial effects of PTH treatment on cortical bone are not enhanced by Dll4_(E12) (Figure 7H-K). These findings indicate that Dll4_(E12) can enhance trabecular bone formation in response to anabolic therapy.

Analysis of Dll4_(E12)-induced processes at single cell resolution

Next, we analyzed long bones from *pLIVE-Dll4_(E12)* and control-injected males at 3 wpi by scRNA-seq in order to identify the stromal cell populations mediating the response to Dll4_(E12). Following tissue dissociation, hematopoietic cells were depleted from single cell suspensions with magnetic activated cell sorting (MACS) and the remaining stromal single cells were captured and barcoded with the BD Rhapsody Express Single-Cell Analysis System (Figure 8A). We sequenced approximately 20,000 cells from long bones of four *pLIVE-Dll4_(E12)* and control-treated mice. The resulting cell libraries were filtered to remove barcode artefacts and low-quality cells using stringent parameters for low library complexity, mitochondrial gene overrepresentation, cell doublet probability and low gene expression to ensure that downstream analysis was not affected by technical artefacts (Figure 8-figure supplement 1A-D). A total of 7750 Dll4_(E12) cells and 9265 control cells passed filtering and were carried forward for further analysis. To investigate the cell diversity in the Dll4_(E12) and control group, we performed clustering of cells based on scaled expression profiles (Louvain algorithm), combined with manual clustering annotation using known and de-novo identified

marker genes. We identified 5 distinct cell populations in both *pLIVE-Dll4*_(E12) and control-injected bone: MSCs, chondrocytes, ECs, osteoblasts and smooth muscle cells (SMCs) (Figure 8B-E). For each population, we then identified all genes that were differentially expressed between the *pLIVE-Dll4*_(E12) and control samples (Wilcoxon Rank Sum Test). Due to their small number, SMCs were omitted from this analysis. Data analysis reveals that EC and osteoblast populations show no or only minor alterations in response to *Dll4*_(E12). Consistent with this finding, expression of EC markers (*Pecam1*), regulators of vascular growth (*Vegfr2/Kdr*), and of venous (*Nr2f2*, *Vegfr3/Flt4*) or arterial specification (*Sox17*, *Efnb2*) are not altered in the *Dll4*_(E12) group (Figure 8F, I). Expression of Notch pathway ligands, receptors and downstream targets is also only marginally affected in endothelium of the *pLIVE-Dll4*_(E12)-injected mice (Figure 8-figure supplement 2A, B).

Notably, MSCs and chondrocytes show the strongest response to overexpressed *Dll4*_(E12), although chondrocytes display a comparably smaller number of differentially expressed genes (Figure 8G, H). *Dll4*_(E12) chondrocytes show normal expression of critical transcripts including the transcription factor *Sox9* and the proteoglycan core protein Aggrecan (*Acan*) (Figure 8J), consistent with the results from the immunostaining of bone sections (Figure 3-figure supplement 2C). In contrast, the alpha1 subunit of type X collagen (*Col10a1*), which is mainly expressed by hypertrophic chondrocytes in the growth plate, is upregulated after *Dll4*_(E12) treatment ($P=3.97e-32$) (Figure 8J).

In the MSC population, representative markers such as transcripts for the receptor tyrosine kinase PDGFR β (*Pdgfrb*), a critical regulator of MSC proliferation, and Leptin receptor (*Lepr*), a marker of BM stromal cells, are not altered (Figure 8K). However, known pro-osteogenic genes like *Igf2* encoding insulin-like growth factor 2 (ref. 40) are upregulated ($P=9.52e-121$), whereas transcripts associated with negative regulation of osteogenesis are downregulated. The latter includes the transcript for Insulin-like growth factor-binding protein 3 (*Igfbp3*) ($P=2.80e-166$), a secreted protein that binds insulin-like growth factors and limits

their bioavailability⁴⁰, and matrix Gla (γ -carboxyglutamate) protein (*Mgp*) ($P=1.11\text{e-}159$), a negative regulator of calcification⁴¹ (Figure 8H and Figure 8-figure supplement 2C). MSCs from *Dll4*_(E12)-treated mice showed also increased expression of *Angptl4* ($P=9.82\text{e-}132$), which encodes angiopoietin-like 4, a positive regulator of osteogenesis that is highly expressed at bone fracture sites but is also a stimulator of osteoclast-mediated bone resorption^{42,43} (Figure 8H and Figure 8-figure supplement 2C). Transcripts for the proteoglycan PRG4, a positive regulator of skeletogenesis and parathyroid hormone-mediated bone formation⁴⁴, are also elevated in *Dll4*_(E12) MSCs ($P=8.39\text{e-}112$) (Figure 8H and Figure 8-figure supplement 2C).

Gene Ontology (GO) analyses (hypergeometric test) of each cell population reveals an enrichment of GO terms for morphogenesis, development, vascular growth, and ossification among differentially expressed genes in the MSC population and, to much smaller extent, in chondrocytes (Figure 8-figure supplement 2D). Analysis of endogenous Notch receptor (*Notch1-4*) and ligand (*Dll1*, *Dll4* and *Jag1*, *Jag2*) expression in *Dll4*_(E12)-treated stromal cells, however, indicates no alterations except the downregulation of *Notch3* in MSCs ($P=1.10\text{e-}64$) (Figure 8-figure supplement 2A, B). While *Dll4*_(E12) results in a strong increase of *OSX*⁺ cells *in vivo*, scRNA-seq analysis reveals very limited gene expression changes in osteoblasts (Figure 8-figure supplement 2E). Together, these data indicate that the pro-osteogenic effects of *Dll4*_(E12) are mediated by MSCs.

***Dll4*_(E12) effects within the bone MSC subpopulations**

Bone MSCs are highly heterogeneous, found in different locations and can be subdivided based on marker gene expression⁴⁵⁻⁴⁸. Sub-clustering (Louvain algorithm) of the MSC population in our scRNA-seq data identifies 3 distinct subpopulations (Figure 9A-C). Diaphyseal MSCs (dpMSCs) show high expression of *Lepr* and *Kitl*, the gene encoding stem cell factor, the ligand of c-Kit receptor (Figure 9A-C and E). Metaphyseal MSCs (mpMSCs)

characterized by expression of markers associated with osteogenesis, namely of the transcription factor Osterix (encoded by the gene *Sp7*), the secreted extracellular matrix protein periostin (*Postn*), and the transcriptional repressor and Notch pathway gene *Heyl* (Figure 9A-C and E). In addition, we find a small population of fibroblast-like cells, which express the proteoglycan Decorin (*Dcn*) and the glycosyl phosphatidylinositol-anchored cell surface protein Sca-1 (*Ly6a*) (Figure 9A-C). Notably, dpMSCs represent 85-86% of total MSCs in both the Dll4_(E12) and control group (Figure 9A, B). Most of genes that are differentially expressed in Dll4_(E12)-treated samples are also found in the dpMSC subpopulation. This includes the upregulated genes *Igf2* (P=5.39e-88) and *Prg4* (P=3.05e-90), which are known to promote osteogenesis, and the downregulated genes *Igfbp3* (P=1.69e-156) and *Mgp* (P=9.76e-148), which are inhibitors of bone formation (Figure 9D, E). Furthermore, UMAP projections show a significant increase in the fraction of *Sp7/Osterix*-expressing cells in the dpMSC population (Figure 9F). Consistent with the *Heyl-EGFP* reporter results (Figure 3-figure supplement 1A), *Heyl* expression labels mpMSCs and a small fraction of dpMSCs. The latter might, as the *Tg(Heyl-EGFP)^{ID40Gsat}* reporter expression suggests (Figure 3-figure supplement 1A), represent cells in the transition zone between the metaphysis and diaphysis.

The scRNA-seq data show no significant increase in total *Heyl*⁺ MSCs, which is presumably due to the low expression and therefore limited detection of this transcript (Figure 9F). Taken together, our results support that Dll4_(E12) treatment promotes osteogenesis through effects in MSCs and, in particular, the dpMSC population. In contrast, molecular changes are limited in the EC and osteoblast populations, suggesting that these cells are not exposed or unresponsive to Dll4_(E12).

DISCUSSION

Notch signaling has multiple important functional roles in the skeletal system, including the maintenance and expansion of the mesenchymal progenitor cell pool and the coupling of angiogenesis and osteogenesis^{23-25,29,38,49}. Interactions between Dll4 and Notch receptors, predominantly Notch1 but also Notch4, negatively control EC proliferation and angiogenic growth of the vasculature in most organs. In bone, however, Notch signaling promotes angiogenesis and triggers the expansion of type H vessels, which, in turn, results in increased osteogenesis^{23,50}. To promote bone angiogenesis and osteogenesis, we have designed synthetic Notch ligands containing poly-Asp peptide motifs, which have been previously shown to bind different regions in long bone including the primary spongiosa in proximity of the growth plate^{51,52}, a region containing a high amount of angiogenic vessels. It also has been shown that an Asp_{6x} peptide preferentially binds to areas of bone resorption, whereas a different motif, (AspSerSer)_{6x}, targets bone-forming surfaces⁵³. Future work will have to address whether other bone surface-binding moieties will endow Notch ligands with distinct and perhaps even more potent biological properties due to the activation of different target cell populations. Our scRNA-seq analysis shows, for example, that the Asp_{8x} motif in Dll4_(E12) leads to very limited changes in bone ECs. This might reflect that the spatial distance between the bone surface and ECs precludes sufficient physical contact between hydroxyapatite-bound Dll4_(E12) and endothelial Notch receptors.

Nevertheless, we found that Dll4_(E12) can trigger an increase in trabecular bone, whereas similar effects were not seen for Dll4-Asp_{8x}, Jag1-Asp_{8x} or Jag1_(JV1). The underlying reasons for this difference are not clear, but it is possible that only Dll4_(E12) accomplishes a critical level of Notch activation required for bone formation in adult male mice. This should, however, not lead to conclusions about the functional roles of endogenous Notch ligand expression. In fact, numerous reports have shown that Jagged1 can regulate osteogenic differentiation of bone mesenchymal stromal cells and the ligand is also highly upregulated during fracture repair^{13,54-56}.

Notch signaling was shown to inhibit chondrocyte differentiation by suppressing the expression of the transcription factor Sox9, a known regulator of chondrogenesis⁵⁷. Genetic disruption of Notch signaling in embryonic limb bud mesenchyme using *Prx1-Cre* transgenic mice led to the accumulation of hypertrophic chondrocytes in the growth plate, whereas chondrocyte proliferation was strongly reduced²⁴. Our findings show that Dll4_(E12) treatment of adult mice increases the expression of *Col10a1* in chondrocytes, which encodes the alpha chain of type X collagen, a short chain collagen expressed by hypertrophic chondrocytes that is essential for growth plate development and mineralization of trabecular bone⁵⁸. The absence of appreciable accumulation of Dll4_(E12) inside the growth plate, however, suggests that these alterations might be indirect. It was also shown that conditional activation of Notch signaling in osteocytes increases bone formation and mineralization, which is sufficient for the rescue of both age-associated and ovariectomy-induced bone loss³⁶. While our work does not exclude anabolic effects of Dll4_(E12) through the activation of Notch signaling in osteocytes, these cells were not recovered in our scRNA-seq data presumably due to the conditions of tissue dissociation.

Instead, our results indicate that Dll4_(E12) activates Notch signaling primarily in immature MSCs and leads to the expansion of this population and, as shown previously²⁴, reduced Runx2 expression. It is also fully consistent with previous findings showing that genetic approaches leading to Notch activation in mesenchymal progenitors suppress differentiation and promote the expansion of these cells^{24,25,59}. In this context, it is presumably highly advantageous that Dll4_(E12)-mediated Notch activation is, in contrast to irreversible genetic modifications, transient and does not suppress osteoblastic differentiation, presumably because cells are not permanently exposed to the bone surface-bound recombinant protein. This is indicated by the absence of Hey1-GFP signal in OSX⁺ cells but also the absence of major transcriptional alterations in osteoblasts.

MSCs are a heterogeneous population of stromal cells, which includes cells with progenitor properties, colony forming capacity *ex vivo*, and the potential to generate bone, fat, fibroblasts, and other cell types^{47,60-62}. MSCs are likely to include comparably rare mesenchymal stem and progenitor cells characterized by the ability to give rise to multiple differentiated cell types in a clonal fashion^{60,61,63,64}. It might appear surprising that our scRNA-seq data show the strongest gene expression changes in diaphyseal MSCs, but previous work has established that vessel-associated mesenchymal stromal cells in the diaphysis can give rise to bone-forming cells^{47,48,65}. In fact, expansion of the metaphyseal region in response to Dll4_(E12) might well involve the incorporation of dpMSCs from the adjacent transition zone and marrow. Taken together, we propose that the pro-osteogenic capacity of Dll4_(E12) is primarily mediated by Notch-controlled expansion of immature MSCs. It is also feasible that bone formation is enhanced through the release of secreted signaling molecules acting in a paracrine fashion, which might apply to the alterations seen in chondrocytes.

Our study not only proves that the administration of exogenous Notch ligand can induce bone formation in adult mice, the results also show that this treatment does not lead to adverse side effects. Circulating Dll4_(E12) might act as an antagonist and therefore disrupt critical endogenous Notch-ligand interactions in multiple organs, which might lead to defects in small intestine, liver or T cell development that are known to be caused by Notch inhibition^{33,66,67}. The absence of such defects might reflect efficient retention of Dll4_(E12) fusion protein in bone and thereby low bioavailability in other organs. It is, nevertheless, remarkable that even liver, the site of Dll4_(E12) expression, does not show overt morphological alterations.

Together, our findings establish that bone-targeting and binding-optimized Dll4 ligand can be used to stimulate osteogenesis in adult mice without adverse side effects. We propose

that Dll4_(E12) might serve as a promising example for the design of future anti-osteoporosis drugs.

MATERIALS AND METHODS

Plasmid Construction

To generate the Dll4-Asp_{8x} soluble protein containing the Dll4 ECD fused to 6x His and 8x Asp peptide sequences, a cDNA fragment encoding amino acids 1-530 of murine Dll4 (<https://www.uniprot.org/uniprot/Q9JI71>) was amplified via PCR using the following oligonucleotide primers: *Dll4(ECD)-BamHI-Fwd*: 5'-ACTACATATAGGATCCaccctaggattgctccagg-3' and *Dll4(ECD)-PolyD.XhoI-Rev*: 5'-CTTATGATCTCGAGGtagtcgtcgtcgtcgtcgtcgtcgtcgtgatggatgatgatctgttctgttttcagaggacgc-3'.

To generate the plasmid encoding the affinity variant Dll4_(E12), which contains several point mutations in the ECD⁵⁹, the plasmid encoding Dll4-Asp_{8x} was modified with the QuickChange Lightning Multi Site-Directed Mutagenesis Kit (Cat#201515, Agilent Technologies) and the following primers: *Dll4-g1242a*: 5'-gctgcggggtccagcatcttccagc-3', *Dll4-t1479c*: 5'-cctctgcagttgccctcaatttcacctggc-3', *Dll4-a1513t*: 5'-ggaaccttctcactcatcatccaagcttggcac-3', *Dll4-a1587t*: 5'-aaactctctcatcagccaattcatcatccaaggctc-3', *Dll4-c1740t*: 5'-gcaagaagcgcgatgactactcggacattatgag-3', *Dll4-t1777c*: 5'-cagatggcagcccgctcgtcctgcc-3', *Dll4-a1803g*: 5'-ctggcacagtactccccagtcagcccg-3'.

The generation of the Jag1-Asp_{8x} soluble protein containing the Jag1 ECD fused to 6x His and 8x Asp peptide sequences involved a strategy analogous to the construction of Dll4-Asp_{8x}. A cDNA covering amino acid residues 1-1067 of the murine Jagged1 ECD (<https://www.uniprot.org/uniprot/Q9QXX0>) was amplified with the following oligonucleotide primers: *Jagged1(ECD)-BamHI-Fwd*: 5'-

ACTACATATAGGATCCgccgcagcgcgatcgcgtcccccacg-3' and *Jagged1(ECD)-XhoI-PolyD-Rev*:
5'-CTTATGATCTCGAGGtagtcgtcgtcgtcgtcgtcgtcgtcgtgatggatggatgatctgttctgttttcagaggac
gc-3'. Site-directed mutagenesis was used to generate Jag1_(JV1), a high affinity version of the
Jag1 ECD due to several point mutations³⁹, linked to 6xHis and 8xAsp peptides with the
following primers *Jagged1-c260g&t261g*: 5'-gtatcagtcgccgcgtcagggccggggga-3', *Jagged1-c95t*:
5'-ggtgtgcggggccttgggtcagttg-3', *Jagged1-c202g*: 5'-cgcaagtgcaccggcgacgagtgt
g-3', *Jagged1-g214a*: 5'-gcaccGgcgacgagtgtatactacttcaaagtg-3', *Jagged1-a545g&g546a*: 5'-
attgcccacttcgagtatcgaatccgagtgcctgtgatg-3'.

All PCR reactions were carried out using PrimeSTAR Max DNA Polymerase (R045B,
Takara). The resulting PCR fragments were digested by restriction enzymes BamHI and XhoI
(New England Biolabs) and the resulting fragments were inserted into the *in vivo*
overexpression vector *pLIVE* (MIR-5420, Mirus) for *in vivo* studies and into the expression
vector *pcDNA3.1* (Invitrogen) for experiments in cultured cells.

HEK293 cell transfection and His-tagged protein purification

For overexpressing Dll4 or Jag1 recombinant proteins, 4x 10⁶ HEK293 cells were
plated per 10 cm dish. 20 hours later medium was refreshed and after 3 hours cells in each
dish were transfected with 18µg plasmid DNA of *pcDNA3.1-Dll4-Asp_{8x}*, *pcDNA3.1-Dll4_(E12)*,
pcDNA3.1-FRT-Jag1(ECD)-His_(6x)-Asp_{8x} and *pcDNA3.1-FRT-Jag1(ECD)_(JV1)-His_(6x)-Asp_{8x}*
using the Calphos Mammalian Transfection kit (631312, Clontech631312), following the
manufacturer's instructions. 16 hours later Opti-MEM culture medium (31985-047, Gibco)
was added to the cells.

Culture supernatants were collected at 24 hours post-transfection and clarified by
centrifugation at 300x g for 15 min at 4°C. For whole cell lysate samples, cells were washed
twice with PBS and 1 mM PMSF and incubated with RIPA modified buffer (20 mmol·L⁻¹
Tris HCl, pH 8.0, 150 mmol·L⁻¹ NaCl, 0.5% Triton X-100, 0.1% SDS, 0.1% Na-DOC, 2

mmol·L⁻¹ EDTA, cOmplete ULTRA (05892970001, Roche) and phosphatase inhibitor cocktail set V (524629, EMD Millipore)) for 20 min at 4°C. Lysates were further sonicated, spun down at 4°C for 10 min at full speed and protein concentration was quantified using BCA Protein Assay Kit (23225, Pierce).

For the purification of His-tagged recombinant proteins from supernatants, Ni-NTA agarose resin (302010, QIAGEN) packed in disposable polystyrene columns (29922, Thermo Scientific) were used. Binding of recombinant proteins to Ni-NTA resin was done in a buffer containing 20 mmol·L⁻¹ Tris-HCl, 150 mmol·L⁻¹ NaCl, and 4 mmol·L⁻¹ imidazole at pH 7.4. After the passage of lysates, beads were washed with wash buffer containing 20 mmol·L⁻¹ Tris-HCl, 150 mmol·L⁻¹ NaCl, and 20 mmol·L⁻¹ imidazole at pH 7.4 before His-tagged proteins were eluted into elution buffer containing 20 mmol·L⁻¹ Tris-HCl, 150 mmol·L⁻¹ NaCl, and 500 mmol·L⁻¹ imidazole, pH 7.4.

To remove imidazole, eluted proteins were dialyzed using Pierce Slide-A-Lyzer 10K MWCO Dialysis Cassettes (66380, Thermo Scientific) in dialysis buffer (20 mmol·L⁻¹ Tris-HCl, 150 mmol·L⁻¹ NaCl at pH 7.4.). To adjust the loading volume, 500µl of supernatant, flow-through and wash solution were concentrated to 30µl using Amicon Ultra- 0.5 Centrifugal Filter Unit with 3KDa cutoff (UFC500396, Millipore). After quantitation, whole cell lysates, concentrated protein solutions and eluted recombinant proteins were mixed with SDS loading buffer and boiled for 10 min. Samples were run on 8% SDS-PAGE gel and visualized by SimplyBlueTM SafeStain (LC6060, Invitrogen). Target proteins were detected by comparison with protein standard markers.

HUVEC cell culture and Dll4 or Jag1 treatment *in vitro*

For Dll4 experiments, HUVECs (ThermoFisher, C0035C) were cultured in MEM200 medium (M200500, ThermoFisher) supplemented with LSGS (S00310, Thermofisher), 2% FCS (631106, Clontech) and 1% penicillin-streptomycin solution (P4333, SIGMA). For Jag1

experiments HUVECs (ThermoFisher, C0035C) were cultured in EBM2 medium (33156, Lonza) supplemented with EGM2 bullet kit (CC-4176, Lonza). HUVECs were grown in a humidified atmosphere with 5% CO₂ at 37°C. Cells were not tested for mycoplasma contamination. For Notch activation assays with immobilized Dll4 or Jag1 recombinant proteins, poly-L-lysine (P6282, SIGMA) coating was used in combination with Dll4-Asp_{8x}, Dll4_(E12), Jag1-Asp_{8x} or Jag1_(JV1). Corning 60mm x 15mm culture dishes (430196, Corning) were pre-incubated with 0.2mg of poly-L-lysine in 0.1 M sodium borate buffer (pH=8.5) overnight at 37°C in the cell culture incubator (Heracell™ 240i, Thermo Fisher). Dishes were washed 4 times with H₂O followed by 4 times additional washes with PBS. Following incubation with 4µg of Dll4, Jag1 recombinant proteins or BSA in 2 ml PBS for 12 hours at 4°C and 4 washes with PBS, each culture dish was seeded with 2.2 x 10⁵ cells 4 ml complete medium and cultured for 16h.

For Notch inhibition assays, 4.5 x 10⁵ cells per well were cultured in Corning 6-well plates pre-coated with 0.1% Gelatin. The final volume of M200 + LSGS or EGM2 medium was adjusted to 2 ml and 20µg of Dll4 recombinant, 35µg of Jag1 recombinant proteins or BSA protein was added to confluent HUVECs for 8h at 37 °C.

Quantitative RT-PCR

For the analysis of gene expression, total RNA from HUVECs was collected directly from the culture dish with 350µl RLT plus lysis buffer (1053393, Qiagen) followed by immediate RNA extraction using RNeasy Plus Micro Kit (Qiagen, 74034). Complementary DNA was generated with 500ng RNA per reaction iScript cDNA Synthesis Kit (170-8890, BIO-RAD). Quantitative PCR (qPCR) was performed on a CFX96 Touch Real-Time PCR Detection System (BIO-RAD). The following FAM-conjugated TaqMan gene expression probes (ThermoFisher) were used in combination with SsoAdvanced Universal Probes Supermix (BIO-RAD): *DLL4* (Hs00184092_m1), *HEY1* (Hs00232618_m1), *HEY2*

540 (Hs00232622_m1), *HES1* (Hs00172878_m1), *EFNB2* (Hs00187950_m1), *NRARP*
541 (Hs01104102_s1). VIC-conjugated *ACTB* (4352935) or VIC-conjugated *GAPDH*
542 (44326317E) were used to normalize gene expression. HUVECs with at least 3 independent
543 stimulation experiments for each gene were analyzed to obtain the relative expression
544 differences using the $2^{-\Delta\Delta C_t}$ method.

545

546 **Mice and *in vivo* experiments**

547 C57BL/6J and *Tg(Hey1-EGFP)^{ID40Gsat}* males or C57BL/6J at the age of 8 weeks were
548 used for hydrodynamic tail vein injection. Animals were anesthetized with 3.75% isoflurane
549 and injected with 5μg/g (plasmid/body weight) plasmid suspended in TransIT-EE
550 hydrodynamic delivery solution (MIR5340, Mirus) according to the manufacturer's
551 instruction. The appropriate amount of plasmid was suspended in an injection volume of 10%
552 of the body weight and injected into each individual mouse via the tail vein in 5-7s as
553 previously reported⁶⁸.

554 For the ovariectomy (OVX) osteoporosis model, 8-week-old C57BL/6J female mice
555 were anesthetized by intraperitoneally injection of ketamine hydrochloride (Ketavet; 100
556 mg/kg body weight) and Xylazine (Rompun; 16 mg/kg body weight) in sterile PBS. Once
557 animals had entered the tolerance phase without foot reflexes, a small incision was made
558 along the dorsal midline, the abdominopelvic cavity was opened and ovaries were removed
559 bilaterally. The skin was closed by metal clips. After that, analgesic Carprofen (Rimadyl; 4
560 mg/kg body weight) was applied subcutaneously. Mice in the sham group underwent the
561 same procedure without removal of ovaries. 4 weeks after the surgery, mice received *pLIVE-*
562 *Dll4(E12)* or vehicle by hydrodynamic tail vein injection. 3 weeks later, mice were analyzed
563 and the successful ovariectomy was confirmed by the reduction of uterus size.

564 For PTH and *pLIVE-Dll4(E12)* administration, 8-week-old C57BL/6J females were
565 divided randomly into 4 groups: vehicle control, *pLIVE-Dll4(E12)*, PTH, and PTH plus

pLIVE-Dll4(E12). Following *pLIVE-Dll4(E12)* injection at day 0, PTH (1-34) (human) acetate salt (Bachem) was injected subcutaneously every day from day 1 to day 20 at a dose 100 mg/kg body weight, diluted in sterilized PBS. Mice were analyzed at day 21.

All animals were housed at the Max Planck Institute for Molecular Biomedicine and protocols were approved by animal ethics committees with permissions (Az 81-02.04.2019.A114 and Az 81-02.04.2020.A416) granted by the Landesamt für Natur, Umwelt und Verbraucherschutz (LANUV) of North Rhine-Westphalia.

Bone sample preparation and immunohistochemistry

Freshly isolated femurs were collected from plasmid and vehicle-injected mice and immediately fixed in 4% ice-cold paraformaldehyde solution for 24 h. Decalcification was carried out with 0.5 M EDTA (pH 8.0) at 4°C with constant shaking on a horizontal shaker for 48h. Next, decalcified bones were immersed into 20% sucrose and 2% polyvinylpyrrolidone (PVP) for 24h. Finally, tissues were embedded and frozen in embedding solution containing 8% porcine gelatin together with 20% sucrose and 2% PVP. Cryosections of 80-100µm thickness were generated with low-profile disposable blades 819 (LEICA) on a Leica CM2050 cryostat.

For immunostaining, bone sections were air-dried, rehydrated in PBS, permeabilized for 30 min with 0.3% Triton X-100 PBS and blocked in PBS containing 5% donkey serum at room temperature for 30 min. Blocked sections were incubated with primary antibodies diluted in 5% donkey serum in PBS overnight at 4°C. After primary antibody incubation, sections were washed with PBS for three times and incubated with appropriate Alexa-Fluor-conjugated secondary antibodies (1:400) diluted in PBS for overnight at 4°C. Nuclei were counterstained with DAPI after secondary antibody incubation. Sections were washed four times with PBS before mounting with FluoroMount-G (0100-01, Southern Biotech). Finally, the slides were air-dried and sealed with nail polish.

The following primary antibodies were used: rat anti-Endomucin (SC-65495, Santa Cruz, 1:100), rat anti-PECAM-1 (553370, Pharmingen), rabbit anti-Osterix (Abcam, ab22552, 1:1000), rat anti-Runx2 (MAB2006, R&D, 1:50), rabbit anti-Collagen type I (AB675P, Millipore, 1:200), goat anti-Osteopontin (AF808, R&D Systems, 1:100), rabbit anti-Osteocalcin (LS-C17044, LifeSpan BioSciences, 1:100), goat anti-Dll4 (AF1389, R&D Systems, 1:100), goat anti-Jag1 (J4127, Sigma, 1:100), goat anti-PDGFR β (AF1042, R&D, 1:100), rabbit anti-NG2 (AB5320, Millipore), goat anti-BCAM (AF8299, R&D Systems, 1:50), rabbit anti-ATP6V1B1 + ATP6V1B2 (ab200839, Abcam), rabbit anti-Aggrecan (AB1031, Millipore), goat anti-Sox9 (AF3075, R&D Systems), rabbit anti-Perilipin (9349, Cell Signaling).

Secondary antibodies were: donkey anti-rat IgG conjugated to AF594 (A21209, Invitrogen), donkey anti-goat IgG conjugated to AF647 (A21447, Invitrogen), donkey anti-rabbit IgG conjugated to AF488 (A21206, Invitrogen), donkey anti-rabbit IgG conjugated to AF594 (A21207, Invitrogen), donkey anti-rat IgG conjugated to 647 (712-605-153, Jackson ImmunoResearch), donkey anti-goat IgG conjugated to AF488 (A11055, Invitrogen), donkey anti-rabbit IgG conjugated to AF647 (A31573, Invitrogen), donkey anti-rat IgG conjugated to AF488 (A21208, Invitrogen), and donkey anti-goat IgG conjugated to AF647 (A32849, ThermoScientific).

Histology of liver and intestine

The dissected intestinal tract was flushed gently with cold PBS followed by a flush with 4% PFA. The intestine was fixed at 4°C for 16h. The Median lobe of liver was dissected and fixed at 4°C for 16h. Formalin-fixed and paraffin-embedded tissues were sectioned at 7 μ m thickness. Histochemical identification of intestinal cell types was performed with Alcian blue and Nuclear Fast Red double staining. Liver sections were stained with Hematoxylin and Eosin. Images were taken with Zeiss Axio Imager M1 microscope.

618

619 **Immunohistochemistry of lung**

620 Lung sample preparation and immunohistochemistry were performed as previously
621 described⁶⁹. To expose the heart and lungs, the chest cavity of anesthetized mice was opened.
622 Through the right ventricle with manual pressure, a warm (37 °C) solution of 6% gelatin/PBS
623 (G1890, Sigma) was gently perfused. After 15 minutes exposure to ice-cold paper tissue, the
624 ventral trachea was cannulated with an intravenous catheter tube that was secured by tying a
625 suture around the trachea. The lungs were inflated to full capacity by gently injecting warm
626 (37 °C) 1% low gelling agarose/PBS (A4018, Sigma). After 20 minutes exposure to ice-cold
627 paper tissue, the lungs were placed in 2% paraformaldehyde solution (PFA/PBS, 4 °C, P6148,
628 Sigma) for 30 min. After washing with cold PBS for 30 min, the lung lobes were sliced
629 (150 µm) using vibrating blade microtome (VT1200, Leica). Lung slices were fixed in
630 4%PFA/PBS at 4 °C for 1 h, washed thoroughly in PBS and incubated twice in PBS for
631 30 min at room temperature. Lung slices were blocked (5% donkey serum, 0.5% Triton X-100
632 in PBS) overnight at 4 °C. Next, sections were treated with primary antibodies (goat anti-
633 CD31 (FAB3628, R&D Systems, 1:100), rat anti-CD31 (553370, Pharmingen, 1:100), goat
634 anti-DLL4 (R&D Systems; AF1389, 1:100)) in blocking solution over night at 4 °C.
635 Following four washes with PBST (10 min each), sections were incubated with secondary
636 antibodies (donkey anti-goat IgG AF488 (A11055, Invitrogen, 1:500), donkey anti-goat IgG
637 AF647 (A21447, Thermo Fisher, 1:500), donkey anti-rat IgG AF647 (A21247, Invitrogen,
638 1:500)) in blocking solution over night at 4 °C. Nuclei were counterstained with DAPI
639 (D9542, Sigma, 2 µg/ml). After four wash steps with PBS, sections were mounted using
640 FluoroMount-G (Southern Biotech) under cover slips.

641

642 **Analysis of inflammatory cytokines and thymic T cells**

Peripheral Blood was collected into anticoagulant EDTA-treated Microvettes (20.1341, Sarstedt) for blood collection. Cells were removed from plasma by centrifugation for 15min at 2,000x g using an ice-cold refrigerated centrifuge. Cytokines levels were measured from the resulting supernatant with LEGENDplex™ Mouse Inflammation Panel (13-plex) with V-bottom plates (740446, Biolegend). Analysis and quantification were performed according to the manufacturer's protocol. Data analysis was performed using software provided by Biolegend. Manual gating was used to define beads A and B, while an automatic gating strategy was used to gate individual cytokine in APC-PE plot.

Mice were euthanized for analysis at the age of 11 weeks. To prepare single cell suspensions of thymocytes, individual thymi were placed in pre-wet 70µm cell strainers immersed in ice-cold 2%FCS/PBS in petri dishes for gentle disruption with the end of a 5ml syringe plunger. Thymocytes were stained with rat-anti-CD45-FITC (11-0451, eBioscience), rat-anti-CD8-Biotin (13-0081, eBioscience) and rat-anti-CD4-APC (17-0042-82, eBioscience) followed by staining with Streptavidin PE/Cy7 (SA1012, Thermo Scientific). Flow cytometry was performed using a FACS Aria Fusion with FACSDiva (BD Biosciences).

Analysis of vascular leakage

Adult mice were injected with 1mg of 70 kDa Dextran, Texas Red™ (Lysine fixable, D1864, Molecular Probes) dissolved in 200µl of PBS via tail vein injection. Femur, liver, kidney and lung were harvested from Dextran and PBS-injected mice after 15min and subjected to sample preparation and staining as describe above for bone samples.

Micro-CT analysis and histomorphometry

Tibiae were collected and attached soft tissue was removed thoroughly prior to fixation in 4% paraformaldehyde overnight at 4°C. Fixed samples were analyzed with micro computed tomography (µCT 50) by Scanco Medical AG, Switzerland. A voxel size of 6µm

was chosen in all three spatial dimensions. For each sample, 500 slices were evaluated covering a total of 3mm, X-ray voltage was 70kVp, intensity 86μA, and integration time 1000ms, frame averaging 1.

Calcein double labelling was performed as reported previously⁷⁰ with minor modifications to calculate bone formation rate (BFR) and mineral apposition rate (MAR). Briefly, mice were intraperitoneally injected with 10mg kg⁻¹ calcein (Sigma, C0875) dissolved in 2% sodium bicarbonate solution at the tenth day and third day before euthanasia. Bones were fixed in 4% PFA for 2d at room temperature followed by transfer to 70% ethanol. Fixed bones were incubated with 5% potassium hydroxide for 96h at room temperature. Processed bones were embedded in bone embedding solution (8% porcine gelatin in the presence of 20% sucrose and 2% PVP). Cryosections were taken at 20μm using low-profile blades on a Leica CM2050 cryostat. Single plane images were acquired from the sections using an LSM 880 confocal microscope (Carl Zeiss). Representative images show trabecular bone. Mineral apposition rate and bone formation rate were calculated from trabecular bone in the metaphysis.

Single cell sequencing of bone marrow stromal cells

Femurs and tibiae were harvested 3 weeks post-injection from adult mice at the age of 11 weeks. Bones were crushed by pestle and mortar with ice-cold 2% FCS/PBS and followed by 30 min digestion at 37 °C with collagenase cocktail by mixing with 2 mg/ml of collagenase IV (17104-019, Gibco), 2 mg/ml collagenase I (17100-017, Gibco), and 10 U/ml of Dispase (17105-041) in a 1:1:1 ratio. The resulting single cell suspension was subjected to depletion of lineage-positive hematopoietic cells with following reagents from Miltenyi Biotec: Lineage Cell Depletion Kit (130-090-858), CD45 Microbeads (130-052-301), CD117 Microbeads (130-091-224), Ter-119 Microbeads (130-049-901), and CD71-biotin (Biolegend, 113803) in combination with Anti-Biotin UltraPure MicroBeads (Miltenyi Biotec, 130-105-

637). Lineage-depleted stromal cells were loaded into a BD Rhapsody™ Cartridge for capturing single cells for whole transcriptome according to the manufacturer's instructions. scRNA libraries were prepared with The BD Rhapsody Whole Transcriptome Analysis (WTA) Amplification kit. DNA sequencing was performed on a NextSeq500 (Illumina).

scRNA-seq bioinformatics and data access

FASTQ files from BD Rhapsody WTA and sequencing were first trimmed for adapters and Phred score (>20) using TrimGalore! (0.6.4), discarding reads shorter than 66bp after trimming. The first mate was then split into barcode and UMI using the pattern described in the BD Rhapsody Bioinformatics Handbook (revision 6.0, Figure 3), allowing up to 2 mismatches in each of the fixed sequences L1 and L2. A barcode whitelist was created by counting the occurrences of each of the three CLS sequences, choosing those that occur at least 1000 times, and forming every possible combination of CLS1-CLS2-CLS3. Reads were mapped using Salmon alevin (1.1.0) with the vM22 Gencode transcripts and genomic decoy sequences, including the above whitelist for barcode correction. Automatic filtering by Salmon alevin was disabled, and Alevin count matrices were used for downstream analyses. Count matrices were loaded into R using tximport (1.1.14). Empty wells were identified using DropletUtils (1.6.1) with an FDR cutoff of 0.001. Cells classified as empty wells or with less than 500 expressed genes, and genes with signal in less than 10 cells were removed from the analysis. Doublets were identified using scDblFinder (1.0.0) with default parameters and subsequently removed. Filtered count matrices were imported into Seurat (3.1.3). Mitochondrial gene contribution was estimated using Seurat's PercentageFeatureSet, and cells with more than 20% mitochondrial reads were removed. Expression counts were normalized using scran normalize (1.14.6). Control and treatment samples were analyzed together as an integrated Seurat dataset. The data was scaled using Seurat's ScaleData

function and variable features were selected by FindVariableFeatures (nFeatures=2000, selection.method='vst').

Main celltype clusters were identified using Louvain clustering with a resolution parameter of 0.05, using the top 50 principal components (PCs). Marker genes were identified using Seurat's FindAllMarkers function with default settings, and cells identified as hematopoietic were removed as contaminants. Marker expression was plotted using DoHeatmap on the top 10 marker genes in each cluster. Dimensionality reduction plots are based on UMAP projections using Seurat's RunUMAP function.

For the MSC population subclustering, we first applied stricter empty droplet filtering (FDR=0) to remove low complexity subpopulations. The expression values were re-scaled and variable feature selection was repeated. We then used the top 6 PCs for Louvain clustering at a resolution of 0.05, and UMAP projection. Marker genes were identified using Seurat's FindAllMarkers function with default settings, and marker expression was plotted using DoHeatmap on the top 5 marker genes in each cluster.

Differentially expressed genes were identified separately for each cluster, using Seurat's FindMarkers function with default settings (Wilcoxon Rank Sum Test) on the Dll4_(E12)-treated and control samples. Scaled expression violin plots are based on Seurat's RNA assay and the "data" slot. Each violin is scaled to the width of the plotting window.

Gene Ontology (GO) analysis was performed using GOstats (2.52.0) hypergeometric testing. P-values were multiple testing corrected using the Bonferroni method.

scRNA-seq data have been deposited in the GEO functional genomics data repository under the accession number GSE152285. Data can be accessed via <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE152285> with the token mzcbbwwqgptidxmh.

Western blots

Half of the median liver lobe was dissected from plasmid and vehicle injected mice and snap frozen in liquid nitrogen. Liver samples were homogenized in lysis buffer (50 mM Tris-HCl pH 7.4, 1 mM EDTA, 1% Triton X-100, 0.2% Na-DOC, 0.2% SDS, cOmplete ULTRA Proteinase inhibitor 1x (Roche, 05892970001), Phosphatase Inhibitor Cocktail Set V (524632-1SET, Merck), 1 mM PMSF) with TissueLyser LT (QIAGEN) and clarified by centrifugation by 20000 x g for 20 min at 4°C. Protein concentration in lysates was measured using PierceTM BCATM Protein-Assay kit (23225, Pierce). Soluble supernatants were prepared in SDS-PAGE sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting after loading 40µg of total liver lysate. Signal was detected using horseradish peroxidase-conjugated secondary antibodies followed by ECL Prime Western Blotting Detection Reagents (RPN2232, GE Healthcare). Primary antibodies: goat anti-Dll4 (AF1389, R&D, 1:200), rabbit anti-GAPDH (AM4300, Ambion, 1:1000). Secondary antibodies: goat anti-rabbit IgG, HRP-linked whole Ab (7074, Cell Signaling, 1: 15000), donkey anti-goat IgG, HRP-linked whole Ab (ABIN1536502, antibody online, 1:15000).

0.5µg recombinant Jag1 recombinant proteins were prepared in SDS-PAGE sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis, followed by immunoblotting. Primary antibodies used are mouse anti-His tag (Zymed; 372900 1:200) and goat anti-Jag1 (Sigma; J4127 1:500). Secondary antibodies used are sheep anti-mouse IgG 656G, HRP-linked whole Ab (HG-Healthcare, NA931, 1:40000) and Peroxidase AffiniPure bovine anti - goat IgG (H+L) (Jackson Immuno Research, 805-035-180, 1:15000).

Image acquisition, processing and statistical analysis

Confocal image acquisition was performed using confocal microscope LSM880 (Zeiss). Z-stacks of images were processed and 3D reconstructed with Imaris software (version 9.50, Bitplane). Image J (NIH) and Illustrator (Adobe) software were used for image

processing. Quantifications of vascular and bone related parameters were performed with Fiji software on high resolution images.

For quantification of Osterix⁺ and Runx2⁺ cells, a region of 400 µm from growth plate towards the caudal region was selected in images of the metaphysis. Osteoclast surface/bone surface (Oc. S/BS; %) and osteoclast number/bone perimeter (No. Oc./B. Pm) were calculated based on ATP6V1B1 + ATP6V1B2 staining of bone sections. VEGFR3^{low} area was calculated based on VEGFR3 staining and normalized to the total selected area. Adipocyte cell number was calculated based on Perilipin staining of bone sections. Artery number was calculated based on BCAM staining

Coll1a1, PDGFRb, Dll4, Endomucin, OPN areas were calculated based on the corresponding stainings and were normalized to DAPI area. The length of metaphysis was calculated as the mean of three values: length of left, right and middle regions of metaphysis.

All images shown are representative for the respective staining in several experiments. Within one experiment laser excitation and confocal scanner detection were the same. All images shown in the figures are maximum intensity projections and are representative of at least three mice analyzed for each condition unless stated otherwise.

Statistical analysis was performed with Graphpad Prism software. All data are presented as mean ± s.e.m or mean ± SD. Unpaired two tailed Student's t-test, Mann-Whitney U test, one-way and two-way ANOVA were used to determine statistical significance, as indicated in the legends. All experiments were performed independently at least three times and respective data were used for statistical analysis. Sample sizes for each experiment are described in the respective figure legends. No randomization or blinding was used and no animals were excluded from analysis. Several independent experiments were performed to guarantee reproducibility of findings.

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Figure Legends

Figure 1. Recombinant Dll4_(E12) increases bone formation *in vivo*

(A) Schematic diagram showing the domain organization of murine Dll4 full length protein and recombinant Dll4-Asp_{8x} and Dll4_(E12) fusion proteins. The latter contain 6x His epitope tags (green boxes) and negatively charged peptides consisting of 8 Asp residues (8x Asp; blue boxes) instead of the transmembrane (TM) domain and cytoplasmic region (CR) of Dll4. Missense mutations were introduced in the MNL (modulus at the N-terminus of Notch ligands) and DSL (Delta-Serrate-Lin) domains to generate Dll4_(E12) with increased Notch

binding affinity. The resulting amino acid replacements are highlighted in red. SP, signal peptide.

(B) Representative overview and high-magnification confocal images of Dll4 staining (green) on the femurs of *pLIVE-Dll4_(E12)* and control-injected mice at the age of 11 weeks. Nuclei, DAPI (blue). Images on the right show higher magnifications of insets in metaphysis (1), bone marrow (2) and cortical bone (3).

(C) Tile scan confocal images showing Colla1 staining (green) in sections from *pLIVE-Dll4_(E12)* and control femur. Nuclei, DAPI (blue).

(D) Representative 3D reconstruction of μ CT measurements for tibial metaphysis of 11-week-old *pLIVE-Dll4_(E12)*- and control-injected mice.

(E) Bone parameters measured by μ CT analyses: bone volume/total volume (BV/TV) in percentage, trabecular thickness in millimeters, connectivity density in 1 per cubic millimeter, trabeculae number in 1 per millimeter, trabecular separation in millimeters and trabecular number in 1 per millimeter. Data represent mean $\pm$ s.e.m. (n=5 mice, except for trabecular number with n=4 mice), (p-values determined by unpaired t test with Welch's correction).

Graphs at the bottom represent quantitation of eroded surface over bone surface (ES/BS) in percentage and osteoblast surface over total bone surface (Ob.S/BS) in percentage, calculated from histological HE-stained bone sections. Data represent mean $\pm$ s.e.m. (n=5 mice), (p-values determined by Mann Whitney U test).

(F) Representative images of calcein double labeling (7-day time interval) in mineralized sections of the distal femur confirm increased bone formation after *pLIVE-Dll4_(E12)* injection. Quantification of mineral apposition rate (MAR) and bone formation rate (BFR) (right panels). Data represent mean $\pm$ s.e.m. (n=5 mice), (p-values determined by unpaired t test with Welch's correction).

Figure 1-source data 1. Source data for Figure 1E and F.

Figure 1-figure supplement 1. Generation of Dll4-Asp_{8x} recombinant proteins

(A) SDS-PAGE analysis of purified Dll4-Asp_{8x} recombinant proteins. From left to right, protein molecular mass marker (Marker), extract of untransfected HEK293 cells (Cells (-)) OR *pcDNA3.1-Dll4-Asp_{8x}* transfected cells (Cells (+)), supernatant after culture of untransfected (Supernatant (-)) OR *pcDNA3.1-Dll4-Asp₈* transfected cells (Supernatant (+)), flow through after loading of Supernatant (+) on His-tag protein purification column (Flow-through), wash buffer (Wash), elute (Elute), elute after dialysis (Elute (AD)) and Opti-MEM culture medium (Medium) were loaded.

(B, C) RT-qPCR analysis of *DLL4*, *HEY1*, *HEY2*, *HES1*, *EFNB2*, and *NRARP* transcripts in confluent HUVECs stimulated with soluble (B) or in sub-confluent HUVECs stimulated with immobilized Dll4-Asp_{8x} or BSA with poly-L-lysine coating (C). Data represent mean $\pm$ s.e.m. (n=4 for (B) and n=6 for (C)), (p-values determined by multiple unpaired t test with Welch's correction).

Figure 1-supplement 1-source data 1. Source data for Figure 1-figure supplement 1B, C.

Figure 1-supplement 1-source data 2. Images of uncropped blots and gels with the relevant bands labeled for Figure 1-figure supplement 1, Figure 1-figure supplement 4, and Figure 5-figure supplement 1.

Figure 1-supplement 1-source data 3. ZIP file containing the full raw unedited blots and gels for Figure 1-figure supplement 1, Figure 1-figure supplement 4, and Figure 5-figure supplement 1.

Figure 1-figure supplement 2. Dll4-Asp_{8x} detection in bone

(A, B) Confocal images showing Dll4 (cyan) and Emcn (red) staining in femoral metaphysis (A) and endosteum and cortical bone (B) of *pLIVE-Dll4-Asp_{8x}* and control-injected 11-week-old mice. Nuclei, DAPI (blue).

(C) Higher magnification images showing pronounced Dll4 signal (cyan, arrows) in proximity of Emcn⁺ (red) distal vessels in the *pLIVE-Dll4-Asp_{8x}* metaphysis. Nuclei, DAPI (blue).

Figure 1-figure supplement 3. Bone formation is not increased by Dll4-Asp_{8x}

(A) Tile scan images of Dll4 (cyan) staining in sections of *pLIVE-Dll4-Asp_{8x}* and control femur. Nuclei, DAPI (blue). Images on the right show higher magnifications of insets in metaphysis (1), bone marrow (2) and cortical bone (3).

(B) OSX, Osteopontin and Col1a1 (green) immunostaining, as indicated, in *pLIVE-Dll4-Asp_{8x}* and control femur. Nuclei, DAPI (blue).

Figure 1-figure supplement 4. Generation of Dll4_(E12)

(A) Diagram showing the amino acid sequence of mouse and rat Dll4 ECD. Amino acid replacements shown in red were introduced into the murine sequence for the generation of Dll4_(E12) with increased Notch binding affinity.

(B) SDS-PAGE analysis of purified Dll4_(E12) recombinant protein. From left to right, protein molecular mass marker (Marker), supernatant after culture of untransfected (Supernatant (-)) OR *pcDNA3.1-Dll4_(E12)*-transfected HEK293 cells (Supernatant (+)), flow-through after loading of Supernatant (+) on His-tag protein purification column (Flow-through), wash buffer (Wash), elute (Elute), elute after dialysis (Elute (AD)), and Opti-MEM culture medium (Medium) were loaded.

(C, D) RT-qPCR analysis of *DLL4*, *HEY1*, *HEY2*, *HES1*, *EFNB2*, and *NRARP* transcripts in confluent HUVECs stimulated with soluble (C) or in sub-confluent HUVECs stimulated with

immobilized Dll4_(E12), Dll4-Asp_{8x} and BSA by poly-L-lysine coating (**D**). Data represent mean $\pm$ s.e.m. (n=3 for (**C**) and n=4 for (**D**)), (p-values determined by ordinary one-way ANOVA with Tukey's multiple comparison test).

(**E**) Western blot analysis of Dll4_(E12) protein expression in liver lysate of *pLIVE-Dll4_(E12)* and control-injected mice. GAPDH is used as loading control.

Figure 1-supplement 4-source data 1. Source data for Figure 1-figure supplement 1C, D.

Figure 1-figure supplement 5. Dll4_(E12) immunoreactivity *in vivo*

(**A-C**) Representative confocal images showing Dll4_(E12) immunoreactivity after *pLIVE-Dll4_(E12)* or control injection in liver (**A**), spleen (**B**) and lung (**C**). Dll4_(E12) signal (green) is prominent in liver hepatocytes but undetectable in spleen and lung. Note absence of overt changes in the vasculature (red) of all three organs. Nuclei (DAPI, blue).

Figure 2. Notch-dependent processes are not blocked by Dll4_(E12) *in vivo*

(**A**) Alcian blue and Nuclear fast red double staining of small intestine. Secretory goblet cells are labeled by Alcian blue.

(**B**) Hematoxylin and eosin staining of liver sections of 11-week-old *pLIVE-Dll4_(E12)* and control-injected mice.

(**C, D**) Analysis of CD4⁺/CD8⁺ cells from *pLIVE-Dll4_(E12)* and control thymi by flow cytometry (**C**) and corresponding quantification (**D**). Data represent mean $\pm$ s.e.m. (n=3 mice), (p-values determined by unpaired t test with Welch's correction).

(**E**) Confocal images showing comparable extravasation of fluorescent Dextran (70kD) in femur of *pLIVE-Dll4_(E12)* and control-injected mice.

(**F**) Quantitation of Texas-Red-labeled Dextran extravasation in femoral metaphysis and diaphysis of *pLIVE-Dll4_(E12)* and control-injected mice. Data represent mean $\pm$ s.e.m. (n=5

mice), (adjusted p-values determined by two-way ANOVA with Tukey's multiple comparison test).

Figure 2-source data 1. Source data for Figure 2D, F

Figure 2-figure supplement 1. Inflammatory cytokines are not induced by Dll4_(E12)

Quantification of inflammatory cytokines in the plasma of *pLIVE-Dll4_(E12)*- and control-injected mice. Data represent mean $\pm$ s.e.m. (n=3 mice), (p-values determined by multiple unpaired t test with Welch correction).

Figure 2-supplement 1-source data 1. Source data for Figure 2-figure supplement 1

Figure 2-figure supplement 2. Dll4_(E12) does not induce vascular leakage

(A-C) Representative confocal images and corresponding quantitation showing comparable extravasation of Texas-red-coupled fluorescent Dextran (purple) in liver (A), spleen (B) and lung (C) of *pLIVE-Dll4_(E12)* and control-injected mice. Vasculature in liver and spleen (A, B) has been immunostained for Emcn (green) and in lung (C) for CD31 (green). Data represent mean $\pm$ s.e.m. (n=5 mice), (p-values determined by unpaired t test with Welch's correction).

Figure 2-supplement 2-source data 1. Source data for Figure 2-figure supplement 2A-C

Figure 3. Recombinant Dll4_(E12) increases osteogenesis

(A) Tile scan confocal images of OSX staining (green) in femurs from *pLIVE-Dll4_(E12)* and control-injected mice. Nuclei, DAPI (blue). Graph shows quantitation of OSX⁺ cells. Data represent mean $\pm$ s.e.m. (n=4 mice), (p-values determined by two-tailed unpaired t test).

1103 (B) Runx2 staining (green) of *pLIVE-Dll4*_(E12) and control femurs. Nuclei, DAPI (blue).
1104 Graph shows quantitation of Runx2⁺ cells. Data represent mean ± s.e.m. (n=4 mice), (p-values
1105 determined by two-tailed unpaired t test).

1106 (C) Representative images showing Osteopontin (Opn) and Osteocalcin staining (green) in
1107 *pLIVE-Dll4*_(E12) and control femur. Nuclei, DAPI (blue). Data represent mean ± s.e.m. (n=3
1108 mice), (p-values determined by unpaired t test with Welch's correction).

1109 (D) Tile scan images of ATP6V1B1 + ATP6V1B2 (V-ATPase) staining (green) in *pLIVE-*
1110 *Dll4*_(E12) and control femur. Nuclei, DAPI (blue). Graphs show quantification of osteoclast
1111 surface/bone surface (Os. S/B S) and osteoclast number/bone perimeter (No. Oc./B. Pm).
1112 Data represent mean ± s.e.m. (n=4 mice), (p-values determined by unpaired t test with
1113 Welch's correction).

1114 (E) Tile scan images of Perilipin staining (green) in *pLIVE-Dll4*_(E12) and control femurs.
1115 Nuclei, DAPI (blue). Graph shows quantitation of Perilipin⁺ adipocytes. Data represent mean
1116 ± s.e.m. (n=3 mice), (p-values determined by unpaired t test with Welch's correction).

1117

1118 **Figure 3-source data 1.** Source data for Figure 3A-E

1119

1120 **Figure 3-figure supplement 1. Activation of the *Hey1-EGFP* reporter by *Dll4*_(E12)**

1121 Expression of the *Tg(Hey1-EGFP)*^{ID40Gsat} reporter (GFP, green) in 11-week-old *pLIVE-*
1122 *Dll4*_(E12) and control femur. Note that *Dll4*_(E12) increases the number of OSX⁺ cells (cyan) and
1123 leads to induction of GFP expression in the metaphysis but also in the adjacent transition zone.
1124 GFP⁺ cells show no or weak nuclear OSX signals (arrows). Vessels, Emcn (red). Nuclei,
1125 DAPI (blue).

1126

1127 **Figure 3-figure supplement 2. Effect of *Dll4*_(E12) on metaphyseal MSCs and chondrocytes**

(A, B) Tile scan confocal images showing that PDGFR β (A) and NG2 (B) immunostaining in the adult metaphysis is increased by recombinant Dll4_(E12). Nuclei, DAPI (blue).

Graph shows quantitation of PDGFR β area/DAPI area (A) and NG2 area/DAPI area in percentage. Data represent mean $\pm$ s.e.m. (n=3 mice), (p-values determined by unpaired t test with Welch's correction).

(C, D) Representative confocal images of chondrocyte staining in adult metaphysis showing that ACAN⁺ (C) and SOX9⁺ (D) chondrocytes are not affected by *pLIVE-Dll4*_(E12) injection. Nuclei, DAPI (blue). Graphs shows quantitation of ACAN⁺ cells number (C) and SOX9⁺ cells number (D). Data represent mean $\pm$ s.e.m. (n=3 mice), (p-values determined by unpaired t test with Welch's correction).

Figure 3-supplement 2-source data 1. Source data for Figure 3-figure supplement 2A-D

Figure 4. Effect of Dll4_(E12) on bone vasculature

(A) Tile scan images of Emcn (red), VEGFR3 (green) and OSX (cyan) stained sections of *pLIVE-Dll4*_(E12) and control femur at 11 weeks of age. Dashed line indicates the border of VEGFR3^{high} and VEGFR3^{low} area.

(B) Higher magnifications of insets in (A) showing the metaphysis close to growth plate (1' and 2') and bone marrow close to transition zone (1'' and 2''). Stainings show VEGFR3 (green) and OSX (cyan). Note increase in OSX⁺ cells and presence of OSX-stained nuclei close to VEGFR3^{high} vessels after Dll4_(E12) treatment.

(C) Quantification of VEGFR3^{low} area per region of interest (ROI) in the metaphysis. Data represent mean $\pm$ s.e.m. (n=5 mice), (p-values determined by unpaired t test with Welch's correction).

(D) Tile scan images of BCAM (green) stained sections of *pLIVE-Dll4*_(E12) and control femur. Graph shows quantification of the number of BCAM⁺ arteries per region of interest (ROI) in

the metaphysis. Data represent mean $\pm$ s.e.m. (n=3 mice), (p-values determined by unpaired t test with Welch's correction).

Figure 4-source data 1. Source data for Figure 4C, D

Figure 5. Bone formation is not changed by recombinant Jag1-Asp_{8x} and Jag1_(JV1).

(A) Schematic diagram showing the domain organization of murine Jag1 full length protein and recombinant Jag1-Asp_{8x} and Jag1_(JV1) fusion proteins. Fusion proteins contain 6x His epitope tag (green boxes) and Asp_{8x} negatively charged peptide motif (blue boxes) instead of the transmembrane (TM) domain and cytoplasmic region (CR) of Jag1. Missense mutations were introduced in the calcium-binding C2 and DSL (Delta-Serrate-Lin) domains to generate Jag1_(JV1) with increased Notch binding affinity. The resulting amino acid replacements are highlighted in red. SP, signal peptide.

(B) Tile scan images of Col1a1 (green) staining on the femur sections of control, *pLIVE-Jag1-Asp_{8x}* and *pLIVE-Jag1_(JV1)*-injected mice at the age of 11 weeks. Nuclei, DAPI (blue).

(C) Representative 3D reconstruction from μ CT measurements of tibial metaphysis of *pLIVE-Jag1_(JV1)* and control-injected mice. Diagrams show bone parameters measured in μ CT analyses: bone volume/total volume (BV/TV) in percentage, trabecular thickness in millimeters, connectivity density in 1 per cubic millimeter, trabeculae number in 1 per millimeter and trabecular separation in millimeters. Data represent mean $\pm$ s.e.m. (n=5 mice), p-values determined by unpaired t test with Welch's correction).

Figure 5-source data 1. Source data for Figure 5C

Figure 5-figure supplement 1. Generation of Jag1-Asp_{8x} and Jag1_(JV1) recombinant proteins

1180 (A) SDS-PAGE analysis of purified Jag1-Asp_{8x} recombinant protein. From left to right,
1181 protein molecular mass marker (Marker), extract of untransfected HEK293 cells (Cells (-))
1182 OR *pcDNA3.1-Jag1-Asp_{8x}* transfected cells (Cells (+)), supernatant after culture of
1183 untransfected (Supernatant (-)) OR *pcDNA3.1-Jag1-Asp_{8x}* transfected cells (Supernatant (+)),
1184 flow through after loading of Supernatant (+) on His-tag protein purification column (Flow-
1185 through), wash buffer (Wash), elution of Jag1-Asp_{8x} (Elute) and Opti-MEM culture medium
1186 (Medium) were loaded.

1187 (A, B) SDS-PAGE analysis of purified Jag1-Asp_{8x} (A) and Jag1_(JV1) (B) recombinant proteins.
1188 From left to right, protein molecular mass marker (Marker), extract of untransfected (Cells (-))
1189 or transfected HEK293 cells (Cells (+)), supernatant after culture of untransfected
1190 (Supernatant (-)) or transfected cells (Supernatant (+)), flow through after loading of
1191 Supernatant (+) on His-tag protein purification column (Flow-through), wash buffer (Wash),
1192 elution of recombinant Jag1 (Elute) and Opti-MEM culture medium (Medium) were loaded.

1193 (C) Western blot analysis of eluted Jag1-Asp_{8x} and Jag1_(JV1) recombinant proteins used for *in*
1194 *vitro* stimulation experiments (D, E). Proteins detected at the expected molecular weight with
1195 anti-Jag1 and anti-His1 antibodies.

1196 (D, E) RT-qPCR analysis of human (HS) *DLL4*, *HEY1*, *HEY2*, *HES1*, *EFNB2*, and *NRARP*
1197 transcripts in confluent HUVECs after stimulation with soluble (D) or immobilized (poly-L-
1198 lysine coating) Jag1-Asp_{8x}, Jag1_(JV1) and BSA (E). Data represent mean ± s.e.m. (n=3), (p-
1199 values determined by ordinary one-way ANOVA with Tukey's multiple comparison test).

1200

1201 **Figure 5-supplement 1-source data 1.** Source data for Figure 5-figure supplement 1D, E

1202

1203 **Figure 5-figure supplement 2. Detection of Jag1-Asp_{8x} and Jag1_(JV1) in the bone**

1204 (A) Tile scan confocal images of Jag1 (cyan) immunostaining in sections from control,
1205 *pLIVE-Jag1-Asp_{8x}* and *pLIVE-Jag1_(JV1)* femur. Nuclei, DAPI (blue).

(B) Representative higher magnification images of CD31 (green) and Jag1 (cyan) staining in the metaphysis of control, *pLIVE-Jag1-Asp_{8x}* and *pLIVE-Jag1_(JV1)*-injected mice. Nuclei, DAPI (blue).

Figure 6. Bone formation is not increased by Dll4_(E12) in ovariectomized female mice

(A, B) Representative 3D μ -CT images of trabecular bone in the distal tibial metaphysis (A) and cortical bone in the mid-tibial diaphysis (B) of sham or ovariectomized mice treated with vehicle or *pLIVE-Dll4_(E12)*.

(C) Bone parameters measured by μ -CT analysis of trabecular bone volume/total volume, trabecular connectivity density, trabecular thickness, trabecular separation, bone surface, and trabecular number in the distal tibial metaphysis. Data represent mean $\pm$ SD. (n=5 mice), (p-values determined by two-way ANOVA with Tukey's multiple comparisons test).

(D) Quantitation of μ -CT analysis of cortical bone consistency and cortical thickness in the mid-tibial diaphysis. Data represent mean $\pm$ SD. (n=5 mice), (p-values determined by two-way ANOVA with Tukey's multiple comparisons test).

(E, F) Tile scan confocal images showing Colla1 staining (green; E) and PDGFR β staining (green; F) in femoral sections from ovariectomized female mice treated with vehicle or *pLIVE-Dll4_(E12)*. Nuclei, DAPI (blue). Graphs show relative ratio (percentage) of Colla1 area/DAPI area and PDGFR β area/DAPI area. Data represent mean $\pm$ SD. (n=5 mice), (p-values determined by two-tailed unpaired t test (E) with Welch's correction (F)).

(G) Representative μ -CT images of trabecular bone in the distal tibial metaphysis of male, female sham, and ovariectomized mice. Graphs show quantitation of the trabecular bone volume/total volume, trabecular number, and bone surface. Data represent mean $\pm$ SD. (n=5 mice), (p-values determined by one-way ANOVA followed by Sidak's multiple comparisons test).

Figure 6-source data 1. Source data for Figure 6C-G

Figure 6 – figure supplement 1. Reduced Dll4_(E12) immunoreactivity and trabecular bone in female mice compared to male mice.

(A, B) Representative overview confocal images of Colla1 staining (green) in the femurs of wild-type male and female mice treated with vehicle (A) or *pLIVE-Dll4_(E12)* (B). Nuclei, DAPI (blue). Data represent mean $\pm$ SD. (n=5-7 mice), (p-values determined by two-tailed unpaired *t*-test (control) or unpaired *t* test).

(C, D) Representative overview confocal images of Dll4 staining (green) in the femurs of wild-type male and female mice treated with vehicle (C) or *pLIVE-Dll4_(E12)* (D). Nuclei, DAPI (blue).

Diagrams represent percentage of Dll4 area/DAPI area. Data represent mean $\pm$ SD. (n=5-7 mice), (p-values determined by two-tailed unpaired *t*-test (control) or unpaired *t* test with Welch's correction (*Dll4_(E12)*)).

Figure 6-supplement 1-source data 1. Source data for Figure 6-figure supplement 1A-D

Figure 7. Synergistic action of recombinant Dll4_(E12) and PTH.

(A) Representative μ -CT images of trabecular bone in the distal femoral metaphysis of female wild-type mice treated with vehicle, *pLIVE-Dll4_(E12)*, PTH or the combination of both for 3 weeks.

(B–G) Quantitative analysis of μ -CT data on trabecular bone volume/total volume (B), trabecular connectivity density (C), trabecular number (D), bone surface (E), trabecular thickness (F), trabecular separation (G) in the distal femoral metaphysis. Data represent mean $\pm$ SD. (n=5 mice), (p-values determined by one-way ANOVAs (F, G) followed by Sidak's

multiple comparisons test or Brown-Forsythe and Welch ANOVAs (**B, C, D, E**) followed by Dunnett's T3 multiple comparisons test).

(**H**) Representative μ -CT images of cortical bone in the female midfemoral diaphysis.

(**I – K**) Graphs from μ -CT analysis of the cortical bone volume/total volume (**I**), cortical thickness (**J**), and cortical porosity (**K**) in the midfemoral diaphysis. Data represent mean $\pm$ SD. (n=5 mice), (p-values determined using one-way ANOVAs followed by Sidak's multiple comparisons test).

Figure 7-source data 1. Source data for Figure 7B-G, I-K

Figure 7 – figure supplement 1. Effects of combined PTH and Dll4_(E12) administration

(**A**) Time course of *pLIVE-Dll4_(E12)* and PTH administration to adult wild-type female mice.

(**B-E**) Representative overview confocal images and corresponding quantification of immunostainings for Dll4 (**B**, green), Colla1 (**C**, green), PDGFR β (**D**, green), Endomucin (**E**, red) in the femurs treated with vehicle, *pLIVE-Dll4_(E12)*, PTH or the combination of both. Nuclei, DAPI (blue). Data represent mean $\pm$ SD. (n=4-5 mice), Statistical significance was assessed using one-way ANOVAs (**B, C, D**) followed by Sidak's multiple comparisons test or Brown-Forsythe and Welch ANOVA (**E**) followed by Dunnett's T3 multiple comparisons test.

Figure 7-supplement 1-source data 1. Source data for Figure 7-figure supplement 1B-E

Figure 7 – figure supplement 2. Synergistic effects of Dll4_(E12) and PTH on osteogenesis

Representative overview confocal images and corresponding quantifications for OSX (**A**, white) and OPN (**B**, green) immunostaining in wild-type female femur after treatment with vehicle, *pLIVE-Dll4_(E12)*, PTH or the combination of both. Nuclei, DAPI (blue). Data

represent mean $\pm$ SD. (n=5 mice), (p-values determined using one-way ANOVA followed by Sidak's multiple comparisons test).

Figure 7-supplement 1-source data 1. Source data for Figure 7-figure supplement 2A, B

Figure 8. scRNA-seq analysis of control and *Dll4*_(E12)-treated bone

(A) Overview of the sample processing and scRNA-seq procedure.

(B) UMAP projection of all cells in *pLIVE-Dll4*_(E12) and control, colored by Louvain clusters. ECs, MSCs, SMCs, chondrocytes, and osteoblasts are indicated.

(C) UMAP projection of all cells colored by experimental condition (green=control, purple=*Dll4*_(E12)).

(D, E) Barplot showing absolute numbers of cells (D) and scaled expression heatmap of the top 10 marker genes (E) for each of the clusters shown in (B).

(F-H) Differential expression volcano plots showing $-\log_{10}$ (adjusted P-value) against average log-fold change of *pLIVE-Dll4*_(E12) vs. control in ECs (F), chondrocytes (G) and MSCs (H). Genes with an adjusted P-values smaller than $1e-10$ are colored in red.

(I-K) Selected cell-population relevant gene expression shown as violin (density) plots for EC (I), chondrocyte (J) and MSC (K) subpopulations.

Figure 8-source data 1. Source data for Figure 8F-H

Figure 8-figure supplement 1. scRNA quality control statistics

(A-D) Quality control plots. In each panel, the top plot shows distribution of count depths, middle plot shows knee plot, and the bottom plot shows cumulative count depth plotted as a function of cell barcodes sorted by count depth. Plot show control sample before filtering (A),

control sample after filtering **(B)**, *pLIVE-Dll4_(E12)* sample before filtering **(C)**, and *pLIVE-Dll4_(E12)* sample after filtering **(D)**.

Figure 8-figure supplement 2. Extended analysis of differentially expressed genes

(A) Differential expression volcano plots showing $-\log_{10}(\text{adjusted P-value})$ against average log-fold change of *pLIVE-Dll4_(E12)* vs. control for Notch pathway related genes (parent GO term “Notch signaling pathway” GO:0007219) for EC, chondrocyte, osteoblast, and MSC populations.

(B) Heatmap of the average expression log fold-change of selected Notch pathway genes with a P-value $< 1e-10$ and log fold-change > 0.2 across the four major cell populations.

(C) Heatmap of the average expression log fold-change of genes with a P-value $< 1e-10$ and log fold-change > 0.2 across the four major cell populations.

(D) Dot plot of Gene Ontology (GO) analysis results showing all enriched GO terms with a P-value below $1e-5$ across all four major cell populations. Color encodes hypergeometric test P-value, size of each dot is proportional to the Odds-Ratio (OR).

(E) Differential expression volcano plot for the osteoblast population showing $-\log_{10}(\text{adjusted P-value})$ against average log-fold change of *pLIVE-Dll4_(E12)* vs. control. Genes with an adjusted P-value less than $1e-10$ are colored in red.

Figure 8-figure supplement 2-source data 1. Source data for Figure 8-figure supplement 2A-E

Figure 9. scRNA-seq analysis of MSC subclusters

(A) UMAP projection of MSCs in both control and *pLIVE-Dll4_(E12)* samples colored by Louvain clusters. Metaphyseal mpMSCs, diaphyseal dpMSCs and fibroblasts are indicated.

(B) Barplot showing absolute numbers of cells per sample (left), and scaled expression heatmap of the top 10 marker genes (right) in each of the clusters shown in (A).

(C) UMAP projection of all cells colored by experimental condition green=control, purple= $Dll4_{(E12)}$.

(D) Differential expression volcano plots showing $-\log_{10}(\text{adjusted P-value})$ against average log-fold change of treatment/control for dp MSCs (left) and mp MSCs (right). Genes with an adjusted P-value less than $1e-10$ are colored in red.

(E) Violin plots showing selected differentially expressed genes in the three MSC subpopulations. Each violin is split along the vertical axis into control and $pLIVE-Dll4_{(E12)}$.

(F) UMAP projections of control and $Dll4_{(E12)}$ -treated cells colored for the expression of *Sp7* and *Hey1*.

(G) Heatmap of the average expression log fold-change of selected Notch pathway genes with a P-value $< 1e-10$ and log fold-change > 0.2 in dpMSCs and mpMSCs.

Figure 9-source data 1. Source data for Figure 9D-G

Key Resources Table

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
strain, strain background (<i>Mus musculus</i> , C57BL/6JRj)	WT	Janvier Labs		
genetic reagent (<i>Mus musculus</i>)	<i>Tg(Hey1-EGFP)^{ID40Gsat}</i>	GENSAT	MGI:4847129	

recombinant DNA reagent	pcDNA3.1-Dll4(ECD)-His(6x)-Asp(8x) (plasmid)	This paper		Dll4-Asp _(8x)
recombinant DNA reagent	pcDNA3.1-Dll4(ECD)-Variant- His(6x)-Asp(8x) (plasmid)	This paper		Dll4 _(E12)
recombinant DNA reagent	pLIVE-Dll4(ECD)-His(6x)-Asp(8x) (plasmid)	This paper		<i>pLIVE-Dll4-Asp(8x)</i>
recombinant DNA reagent	pLIVE-Dll4(ECD) Variant- His(6x)-Asp(8x) (plasmid)	This paper		<i>pLIVE-Dll4(E12)</i>
recombinant DNA reagent	pcDNA3.1- Jag1 (ECD)- His(6x)-Asp(8x) (plasmid)	This paper		Jag1- Asp _(8x)
recombinant DNA reagent	pcDNA3.1- Jag1 (ECD)- JV1- His(6x)-Asp(8x) (plasmid)	This paper		Jag1 _(JV1)
recombinant DNA reagent	pLIVE- Jag1 (ECD)- His(6x)-Asp(8x) (plasmid)	This paper		<i>pLIVE-Jag1- Asp(8x)</i>
recombinant DNA reagent	pLIVE- Jag1 (ECD)- JV1- His(6x)-Asp(8x) (plasmid)	This paper		<i>pLIVE-Jag1(JV1)</i>
cell line (<i>Homo-sapiens</i>)	Human Umbilical Vein Endothelial Cells (HUVEC)	ThermoFisher	Cat# C0035C	Cell identity and absence of mycoplasma contamination or human pathogens were certified by the supplier
cell line (<i>Homo-sapiens</i>)	Human Embryonal kidney -293 (HEK293)	DSMZ	Cat# ACC 305	Cell identity and absence of mycoplasma contamination were certified by the supplier

antibody	anti-Endomucin (Rat, monoclonal)	Santa Cruz	Cat# SC-65495	IF (1:100)
antibody	anti-PECAM-1 (Rat, monoclonal)	Pharmigen	Cat# 553370	IF (1:100)
antibody	anti-CD31 (Goat, polyclonal)	R&D Systems	Cat# FAB3628	IF (1:100)
antibody	anti-Pdgfr β (Goat, polyclonal)	R&D Systems	Cat# AF1042	IF (1:100)
antibody	anti-NG2 (Rabbit, polyclonal)	Millipore	Cat# AB5320	IF (1:100)
antibody	anti-BCAM (Goat, polyclonal)	R&D Systems	Cat# AF8299	IF (1:50)
antibody	anti-ATP6V1B1 + ATP6V1B2 (Rabbit, polyclonal)	Abcam	Cat# ab200839	IF (1:100)
antibody	anti-Aggrecan (Rabbit, polyclonal)	Millipore	Cat# AB1031	IF (1:100)
antibody	anti-Sox9 (Goat, polyclonal)	R&D Systems	Cat# AF3075	IF (1:100)
antibody	anti-Perilipin (Rabbit, polyclonal)	Cell Signaling	Cat# 9349	IF (1:100)
antibody	anti-Osterix (Rabbit, polyclonal)	Abcam	Cat# ab22552	IF (1:1000)

antibody	anti-Collagen Type I (Rabbit, polyclonal)	Millipore	Cat# AB765P	IF (1:200)
antibody	anti-Osteopontin (Goat, polyclonal)	R&D Systems	Cat# AF808	IF (1:100)
antibody	anti-Osteocalcin (Rabbit, polyclonal)	LifeSpan BioSciences	Cat# LS- C17044	IF (1:100)
antibody	anti-Runx2 (Rabbit, monoclonal)	R&D Systems	Cat# MAB2006	IF (1:50)
antibody	anti-Dll4 (Goat, polyclonal)	R&D Systems	Cat# AF1389	IF (1:100) WB (1:200)
antibody	anti-Jag1 (Goat, polyclonal)	Sigma	Cat# J4127	IF (1:100) WB (1:500)
antibody	anti-GAPDH (Rabbit, monoclonal)	Ambion	Cat# AM4300	WB (1:1000)
antibody	anti-CD45-FITC (Rat, monoclonal)	eBioscience	Cat# 11-0451	
antibody	anti-CD8-Biotin (Rat, monoclonal)	eBioscience	Cat# 13-0081	
antibody	anti-CD4-APC (Rat, monoclonal)	eBioscience	Cat# 17-0042- 82	
antibody	Streptavidin PE/Cy7	Thermo Scientific	Cat# SA1012	

antibody	Lineage Cell Depletion Kit	Miltenyi Biotec	Cat# 130-090-858	
antibody	CD45 Microbeads	Miltenyi Biotec	Cat# 130-052-301	
antibody	CD117 Microbeads	Miltenyi Biotec	Cat# 130-091-224	
antibody	Ter-119 Microbeads	Miltenyi Biotec	Cat# 130-049-901	
antibody	anti-rabbit Alexa Fluor-488 (Donkey, polyclonal)	Invitrogen	Cat# A21206	IF (1:500)
antibody	anti-rabbit Alexa Fluor-594 (Donkey, polyclonal)	Invitrogen	Cat# A21207	IF (1:500)
antibody	anti-rabbit Alexa Fluor-647 (Donkey, polyclonal)	Invitrogen	Cat# A31573	IF (1:500)
antibody	anti-rat Alexa Fluor-488 (Donkey, polyclonal)	Invitrogen	Cat# A21208	IF (1:500)
antibody	anti-rat Alexa Fluor-594 (Donkey, polyclonal)	Invitrogen	Cat# A21209	IF (1:500)
antibody	anti-rat Alexa Fluor-647 (Donkey, polyclonal)	Jackson Immuno Research	Cat# 712-605-153	IF (1:500)
antibody	anti-rat Alexa Fluor-647 (Donkey, polyclonal)	Invitrogen	Cat# A21247	IF (1:500)

antibody	anti-goat Alexa Fluor-488 (Donkey, polyclonal)	Invitrogen	Cat# A11055	IF (1:500)
antibody	anti-goat Alexa Fluor-647 (Donkey, polyclonal)	Invitrogen	Cat# A21447	IF (1:500)
antibody	anti-goat Alexa Fluor-647 (Donkey, polyclonal)	ThermoScientific	Cat# A32849	IF (1:500)
antibody	anti-rabbit IgG HRP-linked (Goat)	Cell Signaling	Cat# 7074	WB (1:15000)
antibody	anti-goat IgG HRP-linked (Donkey)	Antibody online	Cat# ABIN1536502	WB (1:15000)
antibody	anti-mouse IgG 656G HRP-linked (Sheep)	GE-Healthcare	Cat# NA931	WB (1:40000)
antibody	anti-goat IgG (H+L) Peroxidase AffiniPure Bovine	Jackson Immuno Research	Cat# 805-035-180	WB (1:15000)
sequence-based reagent	Human <i>GAPDH</i> Endogenous Control (VIC™/MGB probe, primer limited)	Applied Biosystems	Cat# 4326317E	TaqMan probe Hs99999905_m1
sequence-based reagent	Human <i>ACTB</i> Endogenous Control (VIC™/MGB probe, primer limited)	Applied Biosystems	Cat# 4326315E	TaqMan probe Hs99999903_m1
sequence-based reagent	Human <i>HEY1</i> TaqMan™ Gene Expression Assay (FAM)	Applied Biosystems	Cat# 4331182	TaqMan probe Hs00232618_m1
sequence-based reagent	Human <i>DLL4</i> TaqMan™ Gene Expression Assay (FAM)	Applied Biosystems	Cat# 4331182	TaqMan probe Hs00184092_m1

sequence-based reagent	Human <i>HEY2</i> TaqMan™ Gene Expression Assay (FAM)	Applied Biosystems	Cat# 4331182	TaqMan probe Hs00232622_m1
sequence-based reagent	Human <i>HES1</i> TaqMan™ Gene Expression Assay (FAM)	Applied Biosystems	Cat# 4331182	TaqMan probe Hs00172878_m1
sequence-based reagent	Human <i>EFNB2</i> TaqMan™ Gene Expression Assay (FAM)	Applied Biosystems	Cat# 4331182	TaqMan probe Hs00187950_m1
sequence-based reagent	Human <i>NRARP</i> TaqMan™ Gene Expression Assay (FAM)	Applied Biosystems	Cat# 4331182	TaqMan probe Hs01104102_s1
peptide, recombinant protein	Recombinant Human PTH (1-34)	BACHEM	Cat# H-4835-GMP, 4033364	
commercial assay or kit	BCA Protein Assay Kit	Pierce	Cat# 23225	
commercial assay or kit	RNeasy Plus Micro Kit	QIAGEN	Cat# 74034	
commercial assay or kit	iScript cDNA Synthesis Kit	BIO-RAD	Cat# 170-8891	
commercial assay or kit	SsoAdvanced Universal Probes Supermix	BIO-RAD	Cat# 172-5284	
commercial assay or kit	LEGENDplex™ Mouse Inflammation Panel (13-plex) with V-bottom plates	BioLegend	Cat# 740446	
commercial assay or kit	Anticoagulant EDTA-treated Microvettes	Sarstedt	Cat# 20.1341	
commercial assay or kit	BD Rhapsody Whole Transcriptome Analysis (WTA) Amplification kit	BD Biosciences	Cat# 633801	

commercial assay or kit	Agencourt AMPure XP magnetic beads	Beckman Coulter Life Sciences	Cat# A638880	
commercial assay or kit	Disposable polystyrene columns	Thermo Scientific	Cat# 29922	
commercial assay or kit	Amicon Ultra- 0.5 Centrifugal Filter	Millipore	Cat# UFC500396	
commercial assay or kit	Pierce Slide-A-Lyzer 10K MWCO Dialysis Cassettes	Thermo Scientific	Cat# 66380	
chemical compound, drug	Ni-NTA agarose resin	Qiagen	Cat# 302010	
chemical compound, drug	Sucrose	Sigma	Cat# S0389	
chemical compound, drug	cOmplete ULTRA Tablets Protease Inhibitor Cocktail	Roche	Cat# 05892970001	
chemical compound, drug	phosphatase inhibitor cocktail set V	EMD Millipore	Cat# 524629	
chemical compound, drug	SimplyBlue™ SafeStain	Invitrogen	Cat# LC6060	
chemical compound, drug	Gelatine	Sigma	Cat# G1890	
chemical compound, drug	Polyvinylpyrrolidone	Sigma	Cat# P5288	
chemical compound, drug	Trypsin-EDTA solution	Sigma	Cat# T3924	

chemical compound, drug	Paraformaldehyde	Sigma	Cat# P6148	
chemical compound, drug	70 kDa Dextran, Texas Red™ Lysine fixable	Molecular Probes	Cat# D1864	
chemical compound, drug	Calcein	Sigma	Cat# C0875	
chemical compound, drug	Fluoromount-G	Southern Biotech	Cat# 0100-01	
chemical compound, drug	ECL™ Prime Western Blotting Detection Reagent	GE-Healthcare	Cat# RPN2236	
chemical compound, drug	4-hydroxy tamoxifen	Sigma	Cat# H7904	
chemical compound, drug	Hematoxinilin	Sigma	Cat# MHS16	
chemical compound, drug	Dimethyl sulfoxide	Sigma	Cat# D8418	
chemical compound, drug	MEM200 endothelial cells medium	ThermoFisher	Cat# M200500	
chemical compound, drug	LSGS	ThermoFisher	Cat# S00310	
chemical compound, drug	EBM-2 endothelial cells medium	Lonza	Cat# CC-3156	

chemical compound, drug	EGM-2 Single Quots	Lonza	Cat# CC-4176	
chemical compound, drug	Opti-MEM	Gibco	Cat# 31985-047	
chemical compound, drug	poly-L-lysine	Sigma	Cat# P6282	
chemical compound, drug	HEPES	Sigma	Cat# H3537	
chemical compound, drug	Ketamine	Zoetis	Cat# 344771	
chemical compound, drug	Rompum	Bayer healthcare	Cat# D-51368	
chemical compound, drug	Collagenase I	Gibco	Cat# 17100-017	
chemical compound, drug	Collagenase IV	Gibco	Cat# 17104-019	
chemical compound, drug	Dispase	Gibco	Cat# 17105-041	
software, algorithm	ImageJ (v2.0.0 Fiji)	Schindelin et al., 2012		
software, algorithm	Volocity (v6.3)	Perkin Elmer		

software, algorithm	Illustrator (vCC2018)	Adobe		
software, algorithm	GraphPad Prism7	GraphPad Software		
software, algorithm	FlowJo (v10.3)	BD Life Sciences		
other	DAPI stain	Sigma	Cat# D9542	(1mg/ml)

1350

1351

1352

1353

A **B** **C**

A

Metaphysis

DAPI Emcn Dll4

B

Diaphysis

DAPI Emcn Dll4

C

Ctrl

DAPI Emcn Dll4

Dll4-Asp_{8x}

DAPI Emcn Dll4

Xu, Dinh et al., Figure 1-figure supplement 2_R1

Xu, Dinh et al., Figure 1-figure supplement 3_R1

A

DLL4_MOUSE	0	MTPASRSACRWALLLLAVLWPQQRAAGSGIFQLRLQEFVNQRGMLANGQSCPEGCRTFFR	60
DLL4_RAT	0	MTPGSRACRWALLLLAVLWPQQRAAGSGIFQLRLQEFANERGMLANGRPCEGCRTFFR	60
		* G28S MNNL	
DLL4_MOUSE	61	ICLKHFQATFSEGPCTFGNVSTPVLGTNSFVVRDKNSGSGRNPLQLPFNFTWPGTFSNLN	120
DLL4_RAT	61	ICLKHYQATFSEGPCTFGNVSTPVLGTNSFVIRDKNSGSGRNPLQLPFNFTWPGTFSNLN	120
		MNNL	* F107L * N118I
DLL4_MOUSE	121	QAWHTPGDDLRLPETSPGNSLSQIIIGSLAVGKIWRTEQNDTLTRLSYSYRVICSDNY	180
DLL4_RAT	121	QAWHTPGDDLRLPETSPGNSLSQIIIGSLAVGKNWKSDEQNTTLTRLRYSYRVVCSNY	180
		MNNL	DSL
DLL4_MOUSE	181	YGESCSRLCKKRDDHFGHYECQPDGSLSCLPGWTKGYCDQPICLSGCHEQNGYCSKPDEC	240
DLL4_RAT	181	YGDSCSRLCKKRDDHFGHYECQPDGSLSCLPGWTKGYCDQPICLSGCHEQNGYCSKPDEC	240
		* H194Y DSL	* L206P * K215E EGF-like1

B

C

Soluble Dll4-Asp_{8x} and Dll4_(E12)

D

Immobilized Dll4-Asp_{8x} and Dll4_(E12)

E

Xu, Dinh et al., Figure 1- figure supplement 5_R1

Xu, Dinh et al., Figure 2-figure supplement 1_R1

Xu, Dinh et al., Figure 2- figure supplement 2_R1

A

Control

DII4_(E12)

DAPI PDGFRβ

DAPI PDGFRβ

B

Control

DII4_(E12)

DAPI NG2

DAPI NG2

C

Control

DII4_(E12)

DAPI ACAN

DAPI ACAN

D

Control

DII4_(E12)

DAPI SOX9

DAPI SOX9

A **B** **C** **D**

A **B** **C**

D Soluble Jag1-Asp_{8x} and Jag1_(JV1)

E Immobilized Jag1-Asp_{8x} and Jag1_(JV1)

Xu, Dinh et al., Figure 6-figure supplement 1_R1

A **B** **C** **D** **E**

Xu, Dinh et al., Figure 7-figure supplement 2_R1

A**Control (unfiltered)** **B****Control (filtered)** **C****Dll4_(E12) (unfiltered)** **D****Dll4_(E12) (filtered)**

Xu, Dinh et al., Figure 8-figure supplement 2_R1

Xu, Dinh et al., Figure 9_R1