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4 Supplementary Materials for

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6 **MMP14-deficient extracellular matrix niche causes skeletal progenitor**
7 **depletion and immune-mediated bone loss**

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18 **This PDF file includes:**

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20 Material and methods
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24 **Other Supplementary Materials for this manuscript include the following:**

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26 Movies S1 to S2
27 Data S1 to S7 (Excel files)
28

Material and methods

Micro-CT Analysis

Mouse tibiae were dissected, fixed in 4% paraformaldehyde overnight and stored in 1x phosphate-buffered saline (PBS) at 4 °C. Micro-CT imaging was performed using the CT scanner (Skyscan 1076; Bruker, Kontich, Belgium) at a resolution of 9 µm. Tibia were scanned at an isotropic voxel size of 3.5 µm and 7 µm, respectively, with peak tube voltage of 55 kV and current of 0.145 mA. Trabecular bone parameters were measured in the proximal metaphysis of the tibia by using the Dataviewer, CTan and CTvol (Bruker, Kontich, Belgium). The region of interest was selected from below the proximal growth plate where the epiphyseal cap structure completely disappeared and continued for 100 slices toward the distal end of the tibia. Contours were drawn manually a few voxels away from the endocortical surface to define trabecular bone in the metaphysis. Cortical bone parameters were measured by analyzing 100 slices in mid-diaphysis tibia.

ELISAs

Serum P1NP, CTX1, and SOST levels were determined using the Mouse Procollagen type 1 N-terminal peptide (P1NP) ELISA Kit (MyBioSource, Cat. No: MBS021201), the Mouse CTX1 ELISA Kit (Novus Biologicals, Cat. No: NBP2-69074), and the mouse/rat SOST/Sclerostin Quantikine ELISA Kit (R&D Systems, Cat. No: MSST00), respectively.

Label retention experiment

Mmp14 flox; C10Cre; Td; R26-M2rtTA; TetOP-H2B-GFP mice were subjected to doxycycline (Dox) by using Dox diets (625mg/kg, Teklad Custom Diet TD.01306, Envigo, Madison, WI) from 4 weeks-old for 6 weeks to achieve widespread acquisition of green fluorescence. Subsequently,

mice were checked for the retention of H2B-GFP in the absence of Dox at defined intervals (16wks) after the end of Dox administration. Animals that were never exposed to dox were included in all experiments as one of the controls.

Osteoblast culture

Human osteoblastic cells Saos-2 cells (ATCC HTB-85) was cultured in modified Eagle's medium α (MEM α) supplemented with 10% fetal bovine serum, 100 units/mL of penicillin, and 100 μ g/mL of streptomycin, and maintained at 37 °C in a humidified atmosphere of 5% CO₂ in air. Osteoblastic differentiation will be induced by co-treatment of vitamin C (250 μ M) and dexamethasone (20 nM). No contamination of the cells from yeast, fungi, gram-positive or gram-negative bacteria were found using the Cell Culture Contamination Detection Kit (Molecular Probes).

Wes procedure

Wes analysis was performed on a Wes system (ProteinSimple, product number 004-600) according to the manufacturer's instructions using a 12-230 kDa Separation Module (ProteinSimple SM-W001 or SM-W002) and either the Anti-Rabbit Detection Module (ProteinSimple DM-001) or the Anti-Mouse Detection Module (ProteinSimple DM-002), depending on the primary antibody used. In brief, protein samples were diluted to three appropriate concentrations (0.02, 0.2, and 2 μ g/ μ L) in sample buffer, then mixed with Fluorescent Master Mix and heated at 95 °C for 5 min. The samples, blocking reagent (antibody diluent), primary antibodies (in antibody diluent), HRP-conjugated secondary antibodies and chemiluminescent substrate were pipetted into the plate (part of Separation Module). Instrument default settings were used: stacking and separation at 475 V

for 30 min; blocking reagent for 5 min, primary and secondary antibody both for 30 min; Luminol/peroxide chemiluminescence detection for ~15 min.

Cell viability assay

The cell viability was measured by MTT assay. Saos-2 cells and Saos-2-Mmp14KO cells were cultured in 96-well plate. After 24, 72, 120 hours, MTT (Invitrogen, Cat. No: V13154) in 1x PBS at a final concentration of 0.5 mg/mL was applied onto cells for 4 hours. After the solution was removed, the purple precipitate inside the cells was re-suspended in DMSO and then measured at 570 nm absorbance. The cell viability was calculated as percentage of absorbance value of control (Saos-2 cells); while the value of control was considered as 100%.

Flow cytometry analysis

Cells were stained using antibody cocktails. Antibodies and secondary reagents are listed in Table 1. Analysis was performed on a FACS Aria SORP instrument (BD Biosciences). All experiments included unstained controls for establishing gates derived from non-fluorescent mice, and single-color controls generated using periosteal or bone marrow cells. Some experiments included fluorophores minus one (FMO) control to assist with gating. Dead cells were excluded in most experiments using Zombie Aqua™ Fixable Viability Kit. Acquisition was performed using FACSDiva software and analysis was performed in FlowJo (BD Biosciences).

Differential gene expression.

We utilized Seurat package's differential gene expression function for identification of differentially expressed genes among the different cell states with a log2 fold change of 0.58 or

greater using the Wilcoxon rank sum test (Butler et al., 2018). The adjusted p values are based on the Bonferroni correction using all genes in the dataset (Seurat default). Combined cell states analyse to generate differentially expressed genes used the Wilcoxon rank sum test with a false discovery rate cut off of less than 1% and visualized as volcano plots. Differentially expressed genes (DEGs) between het and hom groups were tested for gene ontology (GO) enrichment and Kyoto encyclopedia of genes and genomes (KEGG) pathway analysis using the EnrichR.

Spatial transcriptomics

Spatial transcriptomics was conducted using the Visium Spatial Gene Expression System (10× Genomics, Pleasanton, CA). This technique makes use of gene expression slides coated in poly-T primers and encoded with a unique spatial barcode. Tibia from P16 mice were harvested, fresh frozen in OCT, then stored at -80°C. Samples were sectioned at -25°C at a thickness of 10 µm. Optimization and gene expression assays were carried out according to manufacturer's protocol. Briefly, slides were fixed in -20°C methanol, dried with isopropanol, and stained with H&E. A tile scan image of all reaction areas was generated using Akoya Vectra Polaris Imaging System (Akoya Biosciences). For tissue optimization, enzymatic permeabilization was conducted for 10-50 min, followed by first-strand cDNA synthesis with fluorescent nucleotides. The images were taken using Nikon Ti2-E widefield imaging (Nikon, Tokyo), and an optimal permeabilization time of 20 min was determined by visual inspection to maximize mRNA recovery while at the same time minimizing diffusion.

For gene expression, initial workflow was consistent with optimization. Library preparation, clean-up, and indexing were conducted using standard procedures. Samples were subjected to pair-ended sequencing using an Illumina HiSeq. Alignment and demultiplexing were conducted using the

SpaceRanger pipeline with subsequent analyses conducted using Seurat in R. The unique spatial barcode is used to determine the spatial location of origin for each transcript, registered to the original hematoxylin and eosin (H&E) stained image. Pathway analysis was conducted using EnrichR. Pathway scoring was determined using the AddModuleScore function in Seurat using gene lists obtained from validated KEGG pathways and published literature. Spatial feature plots represent a representative image, while all quantitative measures represent the cumulative data of multiple digits (4 het control and 4 hom). To determine DEGs between genes/modules in spatial, the Wilcox method of `stat_compare_means` function from the R package `ggpubr` was used.

Cell-cell interactions

For detecting cellular communications mediated by ligand-receptor interactions, Giotto was applied to SpatialScope outputs following the protocol outlined at https://rubd.github.io/Giotto_site/articles/tut14_giotto_signaling.html. Specifically, Giotto first performed preprocessing to remove low-quality genes/cells and create a spatial network connecting single cells using Delaunay triangulation. Next, the '`spatCellCellcom`' function was executed to analyze the ligand-receptor signaling, with the `spatial_network_name` parameter set as `Delaunay_network`. Subsequently, the top and most reliable ligand-receptor signaling interactions were selected based on the following threshold criteria: $p_{adj} < 0.25$, $abs(log_2fc) > 0.1$, $lig_nr > 10$, $rec_nr > 10$, $lig_expr > 0.5$, and $rec_expr > 0.5$. For the raw Slide-seq data, cell-cell interactions were detected by assuming each spot to represent a single cell, with the cell type determined by the majority proportion. The subsequent ligand-receptor signaling analysis performed by Giotto followed the same methodology.

Mass spectrometry analysis

Desalted peptides were applied onto a 30-cm column (inner diameter: 75 microns; packed in-house with ReproSil-Pur C18-AQ 1.9-micron beads, Dr. Maisch GmbH) at 60°C using the autosampler of the Thermo Easy-nLC 1200 (ThermoFisher). Eluted peptides were directly sprayed onto the timsTOF Pro (Bruker Daltonics). Peptides were loaded at 400 nl/min in buffer A (0.1% FA) and a percentage of buffer B (80% acetonitrile, 0.1% FA), ramped from 5% to 25% over 90 minutes, followed by a ramp to 35% over 30 minutes, a ramp to 58% over 5 minutes, and finally to 95% over the next 5 minutes, and maintained at 95% for another 5 minutes.

TimsControl was used to perform data acquisition on the timsTOF Pro. The mass spectrometer was set to data-dependent PASEF mode, performing one survey TIMS-MS and ten PASEF MS/MS scans per acquisition cycle. The analysis was conducted in a mass scan range from 100-1700 m/z and an ion mobility range from $1/K_0 = 0.85 \text{ Vs cm}^{-2}$ to 1.35 Vs cm^{-2} using equal ion accumulation and a dual TIMS analyzer ramp time of 100 ms each at a spectra rate of 9.43 Hz. Suitable precursor ions for MS/MS analysis were isolated in a window of 2 Th for $m/z < 700$ and 3 Th for $m/z > 700$ by rapidly switching the quadrupole position in sync with the elution of precursors from the TIMS instrument. The collision energy was decreased as a function of ion mobility from 45 eV for $1/K_0 = 1.3 \text{ Vs cm}^{-2}$ to 27 eV for 0.85 Vs cm^{-2} . Collision energies were linearly interpolated between these two $1/K_0$ values and maintained constantly above and below these base points. A polygon filter mask was used to eliminate singly charged precursor ions, and additional m/z and ion mobility information was employed for 'dynamic exclusion' to avoid re-sequencing of precursors that reached a 'target value' of 14500 a.u.

Raw data were processed using the MaxQuant computational platform¹⁰ (version 1.6.7.0 or 2.0.1.0) with Orbitrap or TimsTOF data using standard settings. The peak list was searched against

the Uniprot database of Mus Musculus (55466 entries, July 2020). Cysteine carbamidomethylation was set as a static modification, and methionine oxidation, deamidation, and N-terminal acetylation were set as variable modifications. The match-between-run option was enabled, and proteins were quantified across samples using the label-free quantification algorithm in MaxQuant, which generates label-free quantification (LFQ) intensities. Perseus software¹⁰ (v.1.6.15.0) was used to perform statistical analysis on the LFQ data. LFQ intensities were log₂-transformed. The significance of differences in the cell surface proteome and cell secretome between cell lines was estimated using Perseus' Volcano plot function, and the adjusted p-value was calculated using a two-sided permuted t-test (250 randomizations, FDR=0.05, and S0=0.1). To compare differential protein enrichment, a volcano plot comparing WT and mutant cells for different groups was first done. Visualization of the data was done using R scripts with the Tidyverse and ggrepel packages.

Co-culture and live cell imaging

Saos-2-Mmp14KO cells or control Saos-2 cells were cultured in α -MEM complete medium and seeded in chambered coverslip (Ibidi GmbH, Germany) that used for in immunofluorescence and high-end microscopy. Neutrophils were isolated from the whole bone marrow of 8-week-old GFP mice as described under the “Cell migration assay” and added to osteoblast monolayers in co-culture medium. Co-cultures were maintained at 37°C/5% CO₂ in a live-cell imaging chamber (Tokai Hit, Bala Cynwyd, PA). Time-lapse imaging was performed on a Nikon Ti2-E microscope equipped with phase-contrast and fluorescence filters. Images were acquired every 1 minute for 2 hours using NIS-Elements software.

189 **Data and materials availability**

190 The scRNAseq and 10x Visium spatial transcriptomic raw data supporting this study were
191 deposited on NCBI GEO with accession number PRJNA1392193. All data are available in the
192 main text or the supplementary materials.

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Supplementary Figures

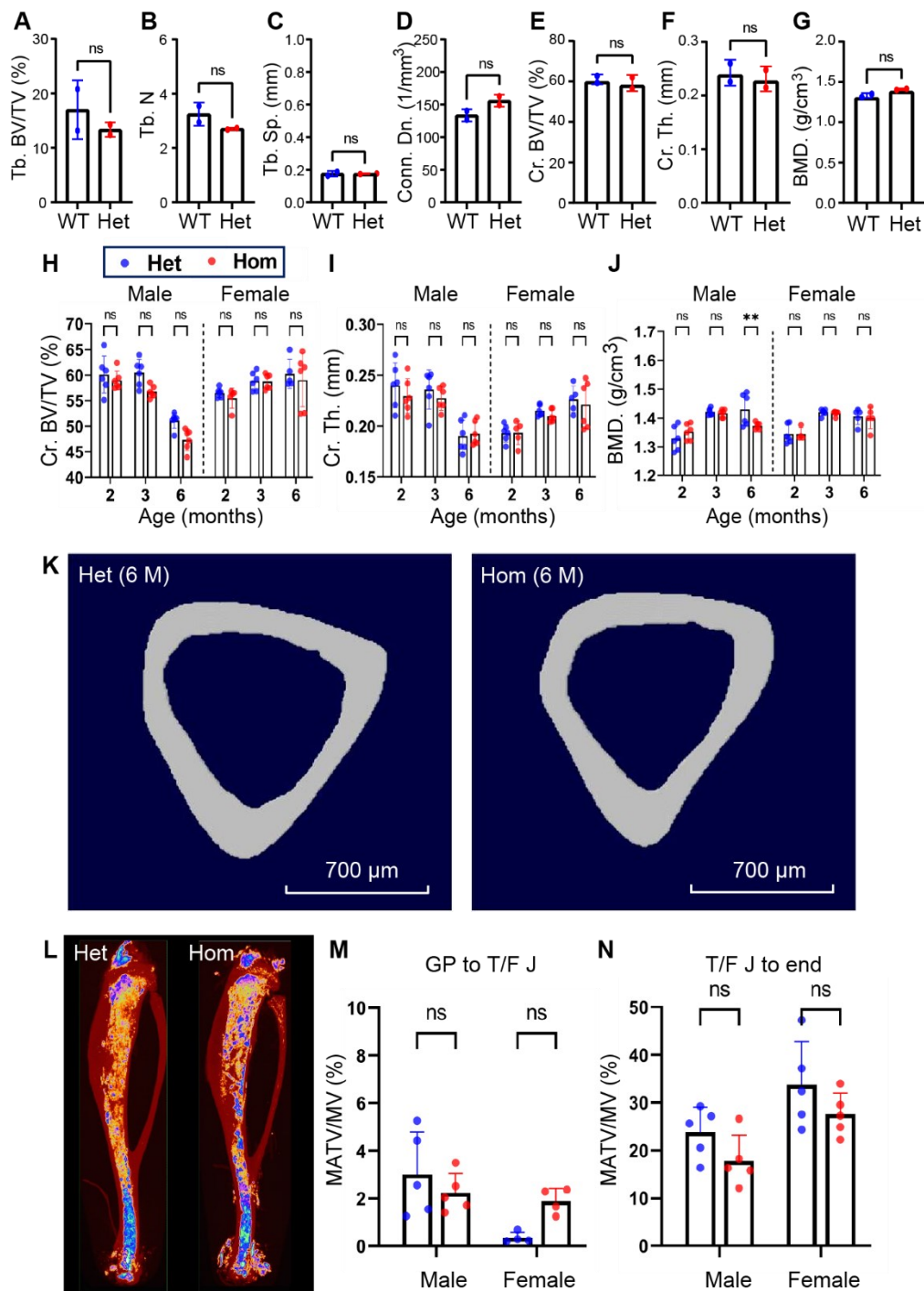
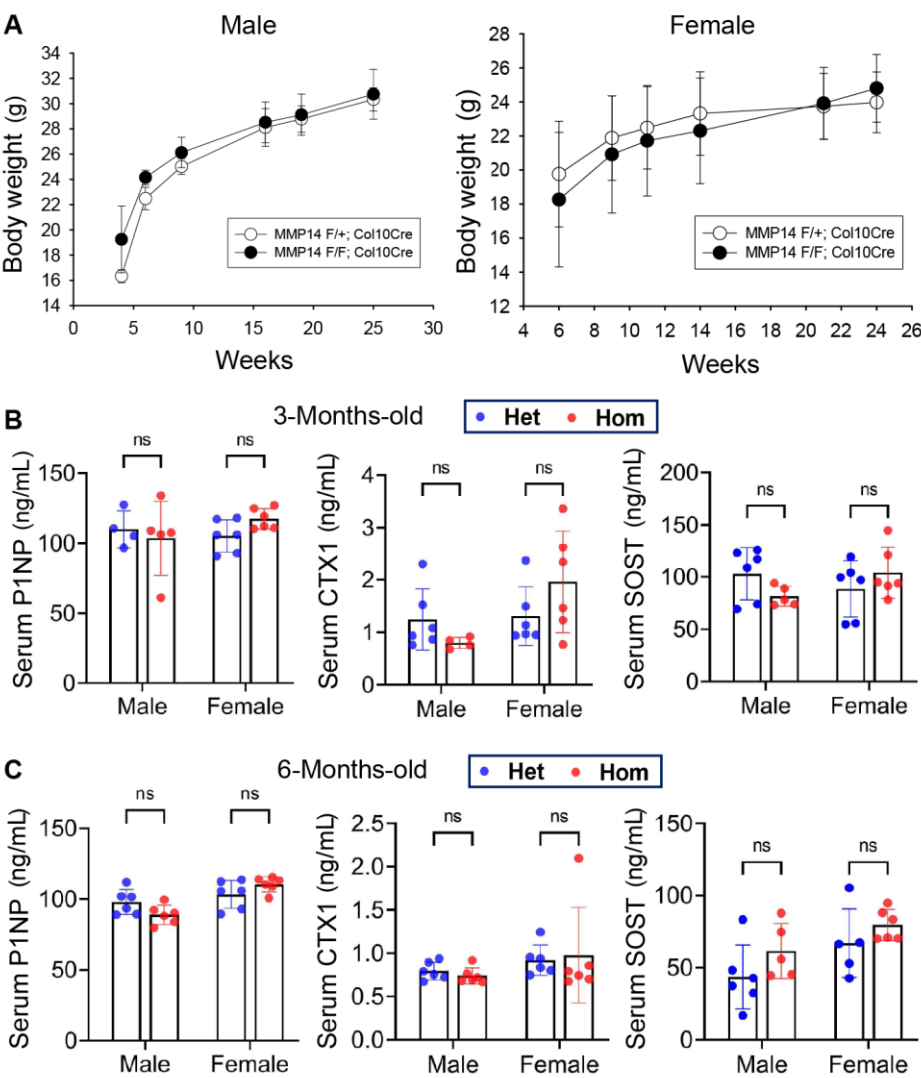


Fig. S1. Removal of MMP14 specifically in HC did not affect cortical bone and bone marrow adipocyte volume.

(A-G) Heterozygote *Mmp14*^{ΔHC} mice (Het) exhibited no bone phenotype compared to wild-type (WT) controls. Micro-CT analysis of the trabecular bone volume/total volume (A), trabecular number (B), trabecular spacing (C), and trabecular connectivity density (D) in the proximal tibia metaphysis of WT control (C10Cre; Td; Col1-GFP) and *Mmp14*^{F/+}; C10Cre; Td; Col1-GFP (Het) mice at the age of 2 months. **(E-G)** Micro-CT analysis of cortical bone volume/total volume (E), cortical thickness (F), and cortical bone mineral density (G) in the middle of proximal tibia metaphysis of WT and Het mice at the age of 2 months. A-G represent data from n = 2 male mice per genotype. All data represent mean ± SD. Statistical significance was assessed using t-test (ns, not significant). **(H-J)** Micro-CT analysis of cortical bone volume/total volume (A), cortical thickness (B), and cortical bone mineral density (C) in the middle of proximal tibia metaphysis of *Mmp14*^{F/F}; C10Cre; Td; Col1-GFP and control (*Mmp14*^{F/+}; C10Cre; Td; Col1-GFP) mice at the age of 2-6 months. **(K)** Representative micro-CT images of cortical bone in the middle proximal tibia metaphysis of male *Mmp14*^{F/F}; C10Cre; Td; Col1-GFP and control mice. **(L)** Representative osmium-stained tibiae scanned with Micro-CT of male *Mmp14*^{F/F}; C10Cre; Td; Col1-GFP and control mice. Marrow fat showed in blue colour, decalcified bone overlaid in light red colour. **(M-N)** Quantification of bone marrow adipocyte volume at the region of growth plate to tibia/fibula junction (GP to T/F J, figure F), and tibia/fibula junction to the end of the bone (T/F J to end, Figure G).



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Fig. S2. MMP14 F/F; Col10a1-Cre mice had grossly normal bone metabolism.

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(A) Body mass of 4-weeks to 5-months-old mice (n = 5-6 mice per genotype, total, from at least

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three independent experiments for all data. (B) Serum P1NP levels, serum CTX1 levels and serum

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SOST levels in 3-months old *Mmp14*^{F/F}; C10Cre; Td; Col1-GFP mice and littermate control. (C)

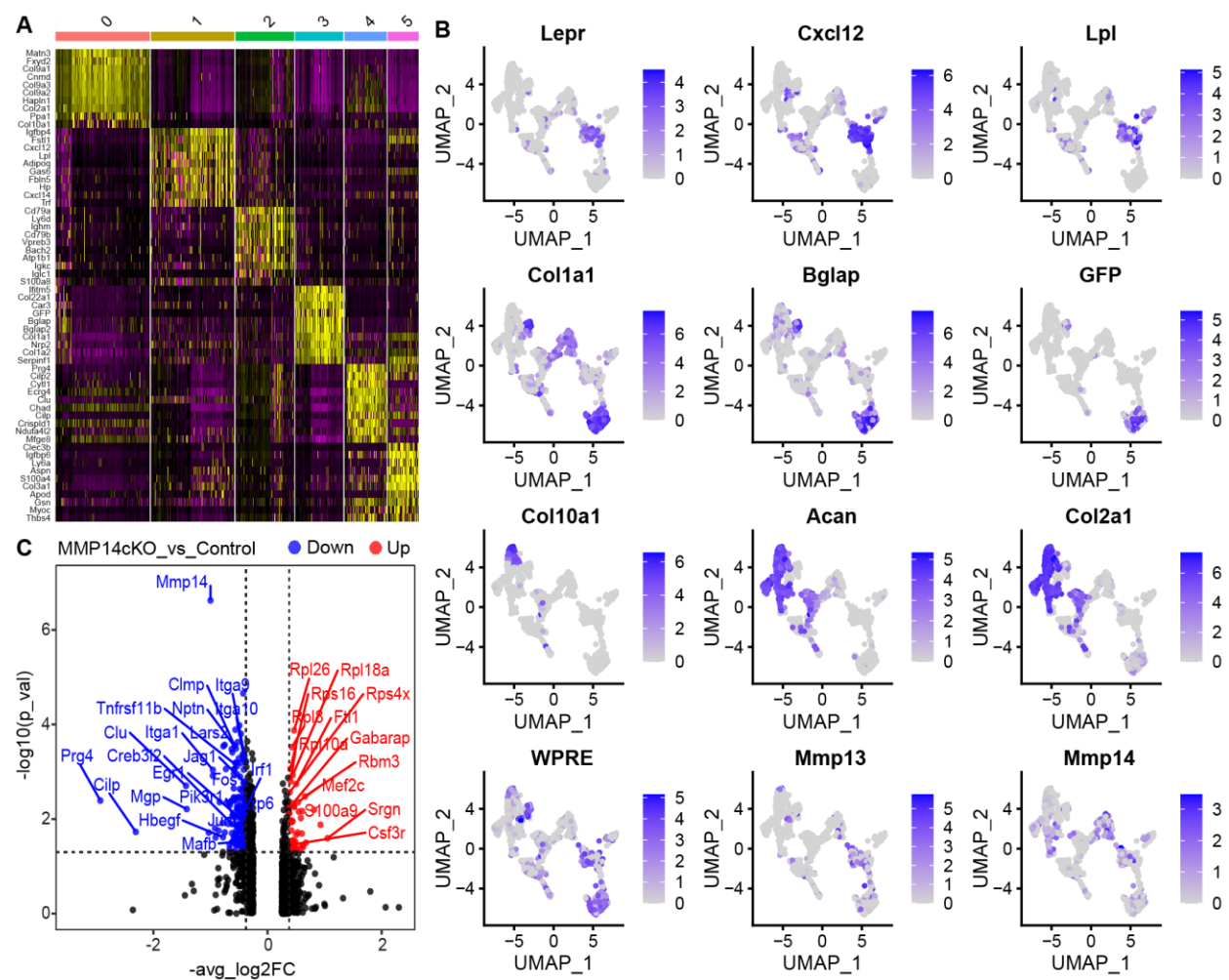
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Serum P1NP levels, serum CTX1 levels and serum SOST levels in 6-months old *Mmp14*^{F/F};

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C10Cre; Td; Col1-GFP mice and littermate control. (n = 6 males and 6 females in each group).

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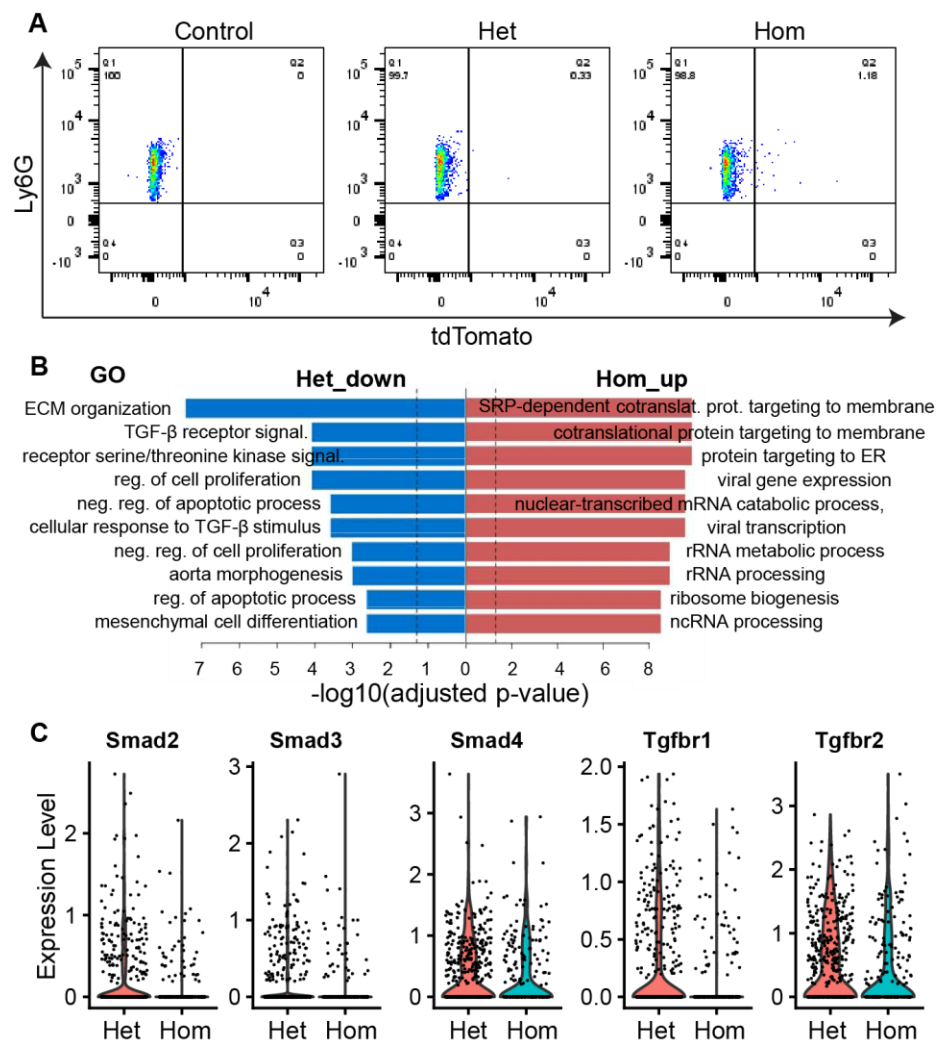
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Fig. S3. Identification of marker genes for cell annotation and DEGs using scRNA-Seq data
(A) Heatmap shows the scaled expression patterns of the top 10 marker genes in each cell type for figure 2E. The color keys from dark to yellow indicate relative expression levels from low to high.
(B) Feature plot showing expression of *WPRE*, *Mmp13*, *Mmp14*, and marker genes for SSPC (*Lepr*, *Cxcl12* and *Lpl*), osteoblast (*Col1a1*, *Bglap*, *GFP*), hypertrophic chondrocytes (*Col10a1*), chondrocytes (*Acan*, *Col2a1*). (C) Volcano plot showing the DEGs between the het versus hom samples in the chondrogenic and osteogenic cell populations. Red dots represent up-regulated DEGs, blue dots represent down-regulated DEGs, and black dots represent unchanged DEGs.



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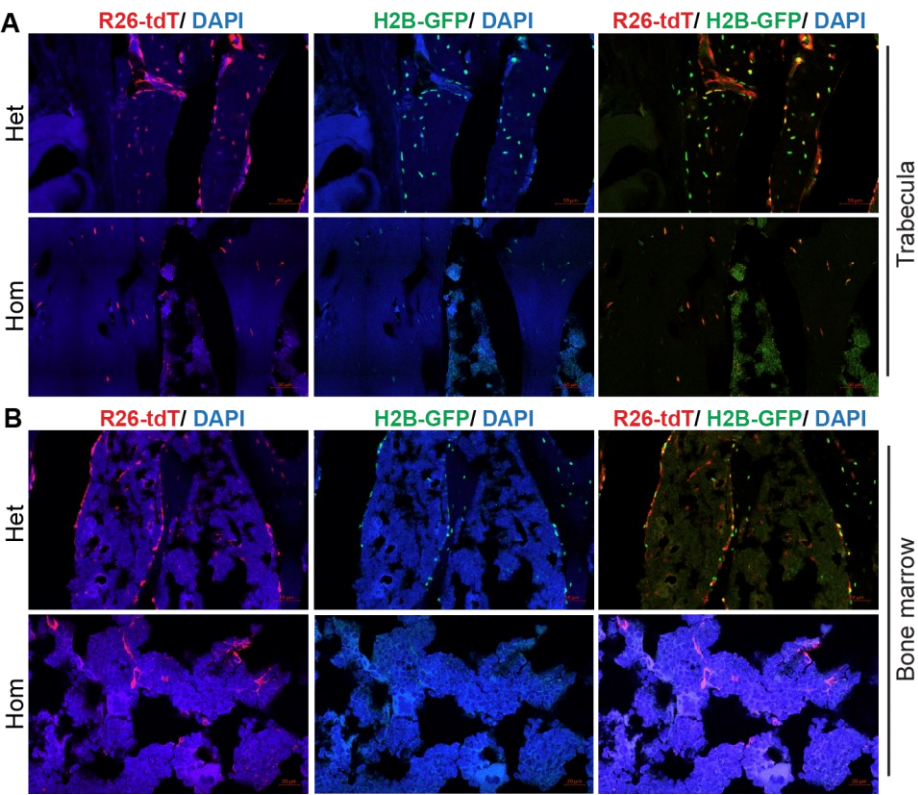
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Fig. S4. Mmp14 loss in HC descendants leads to reduction in TGF- β signaling in trabecular bone, as well as inducing tdT⁺ neutrophil-like cells.

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(A) Flow cytometry of bone marrow from 3-months old wild type (WT) mice, het mice (Mmp14F/+; Col10a1-Cre; R26-tdT), and hom mice (Mmp14F/F; Col10a1-Cre; R26-tdT). The x-axis indicates the tdTomato signal, while the y-axis represents the Ly6G signal (a marker for neutrophils). (B) GO enrichment analysis of biological processes for up- and down-regulated genes between het versus hom samples. (C) Expression of transcriptional target genes (*Smad2*, *Smad3*, *Smad4*, *Tgfr1*, and *Tgfr2*) of the TGF- β signaling pathway in the Mmp14 cKO trabecular bone compared to the littermate control.

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Fig. S5. Mmp14 in HC-descendants is required for the maintenance of SSPC.

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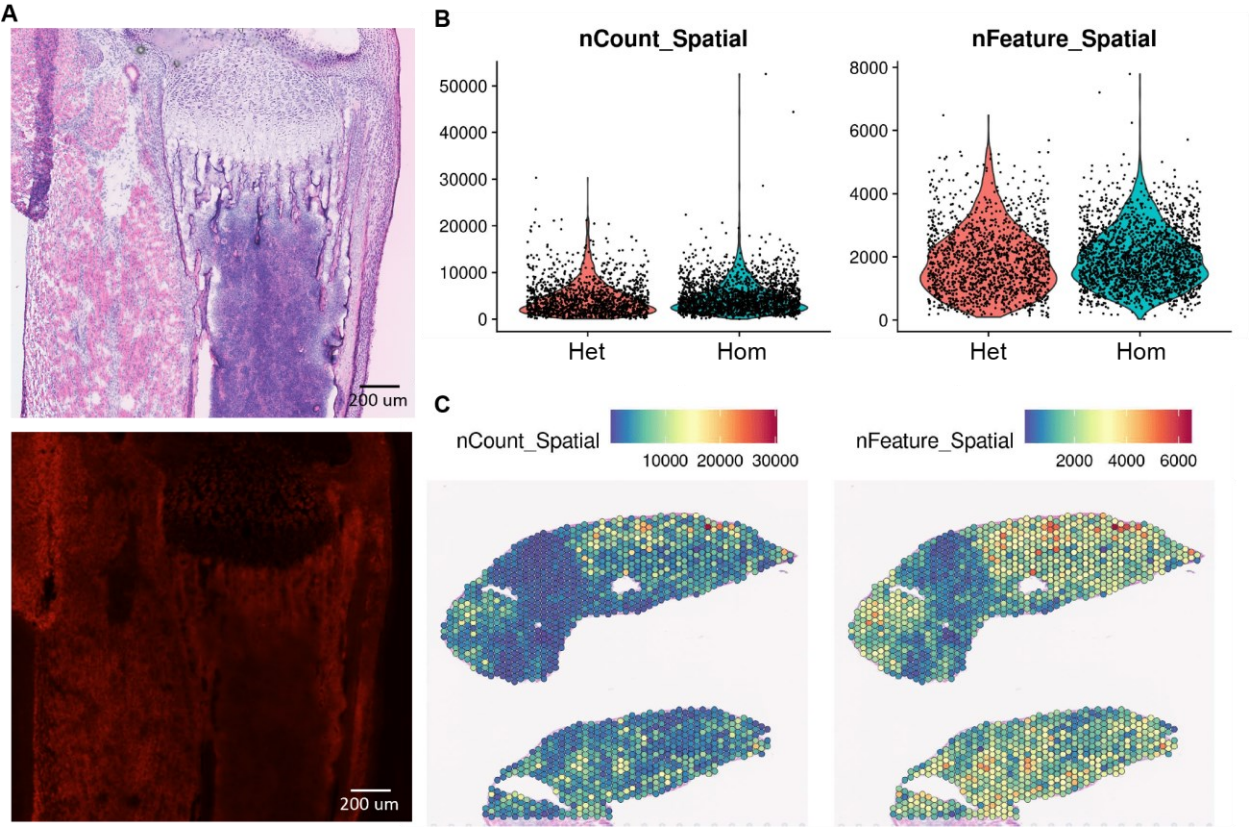
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Immunofluorescence staining of tdTomato (Red) and GFP (Green), counterstained with DAPI (blue), on trabecula (A) and bone marrow (B) of proximal tibia sections from H2B-GFP labelling experiment as indicated in Fig.3, N=5. The genotype of Het mice: Mmp14F/+; Col10a1-Cre; R26-tdT; R26-M2rtTA+/-; TetO-H2B-GFP+/-; and Hom mice: Mmp14F/F; Col10a1-Cre; R26-tdT; R26-M2rtTA+/-; TetO-H2B-GFP+/-.

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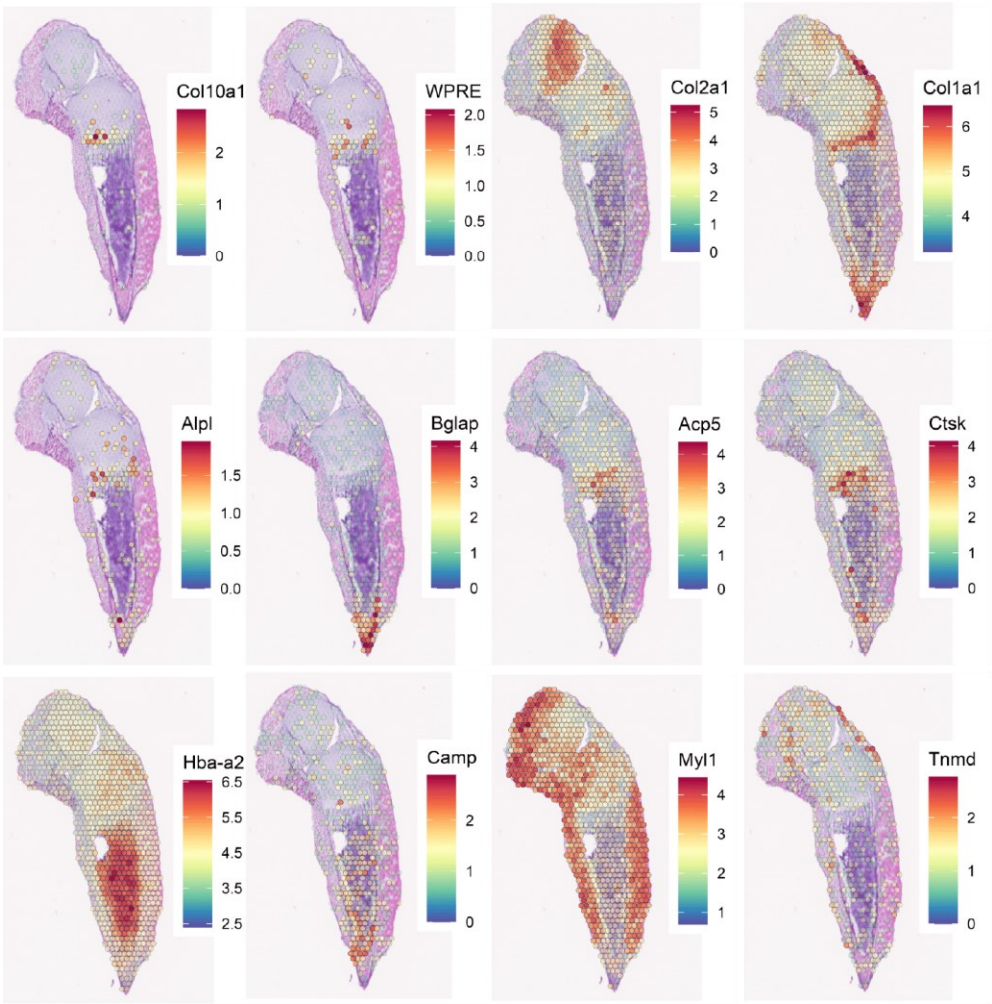


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Fig. S6. Transcriptional overview profiling of the mouse tibia using spatial transcriptomics.
(A) Fresh frozen samples are sectioned onto slides, subjected to a modified hematoxylin and eosin (H&E) and imaged. Optimization of enzymatic permeabilization, visualized through incorporation of fluorescent nucleotides during first strand synthesis of captured mRNAs. Spatial barcodes are used to map each transcript back to its 2D position of origin, registered to the original H&E image.
(B) Violin plot showing the counts and features of mRNA molecules in the het and hom samples.
(C) Spatial distribution of counts and features of mRNA molecules.

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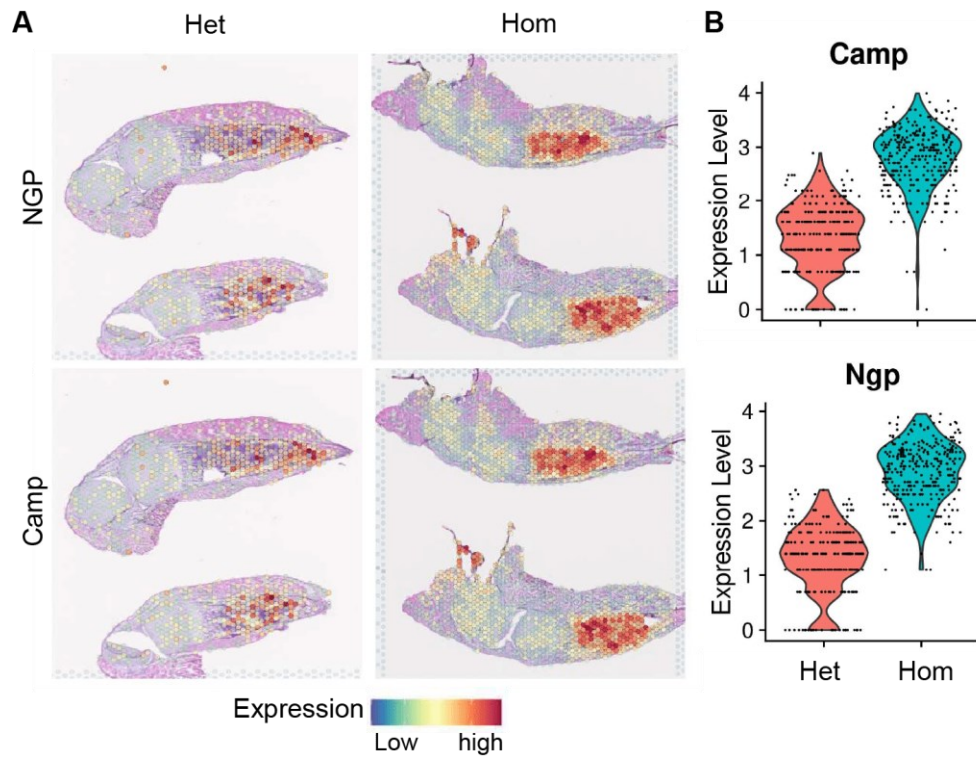
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276 **Fig. S7. Marker genes for spatial clusters show enriched expression within the spatially**
277 **defined cortical bone, chondrocyte, muscle, bone marrow, ligament, and HC-TB regions.**
278 Representative spatial gene expression of hypertrophic chondrocyte (*Col10a1*), chondrocyte
279 (*Col2a1*), bone (*Bglap2*), bone marrow (*Hba-a2*, *Camp*), muscle (*Myl1*), and ligament (*Tnmd*).
280 The color keys from blue to red indicate relative expression levels from low to high.

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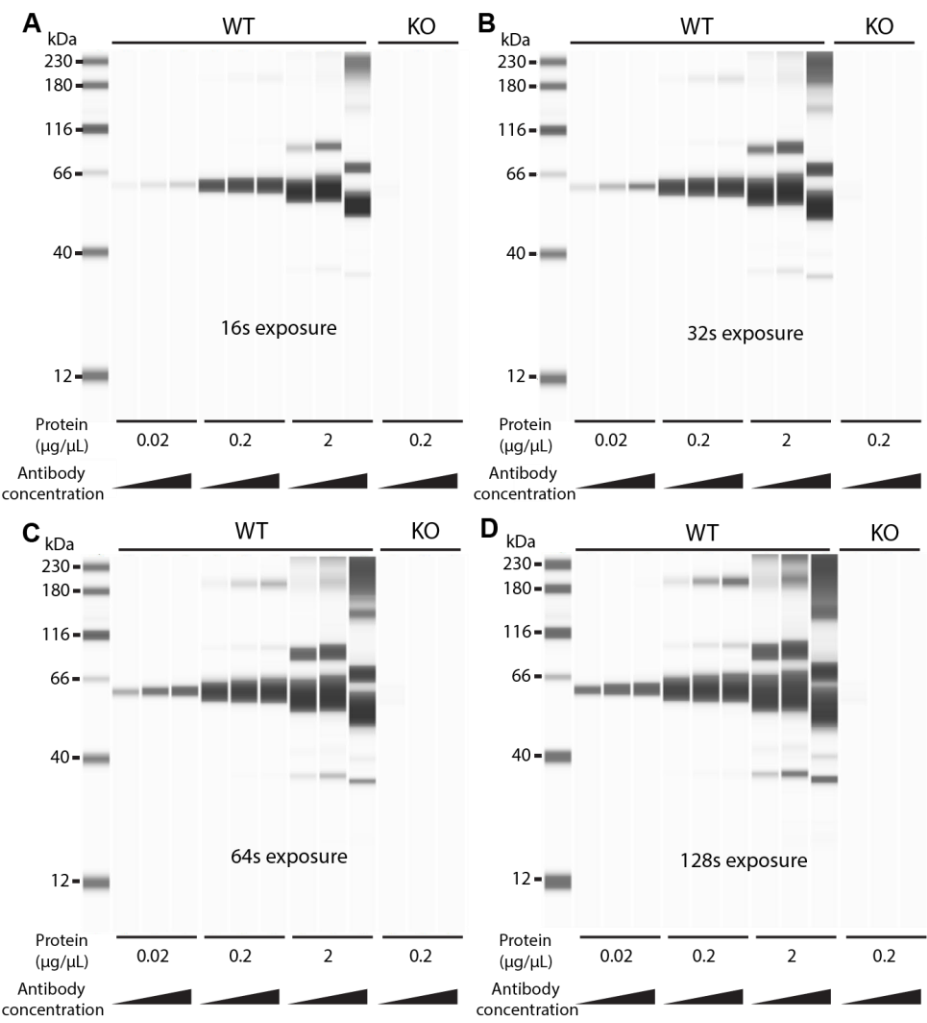
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284 **Fig. S8. Significant activation of neutrophils in the bone marrow of MMP14 F/F; Col10a1-**
 285 **Cre mice at P6.**

286 (A) Representative spatial gene expression of *Ngp* and *Camp* in bone marrow region. The color
 287 keys from blue to red indicate relative expression levels from low to high. (B) Violin plot showing
 288 the gene expression of *Ngp* and *Camp* in bone marrow region.
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293 **Fig. S9. Validation of *MMP14* knockout in human osteoblastic Saos-2 cells.**

294 Western blot analysis confirming successful *MMP14* knockout via CRISPR-Cas9-mediated
295 mutagenesis in the generated Saos-2-MMP14KO cell line. Wild-type (WT) Saos-2 cells served as
296 a positive control. Blots were exposed for 16 (A), 32 (B), 64 (C), and 128 (D) seconds to
297 demonstrate signal specificity and absence of MMP14 protein (~66 kDa) in the knockout.
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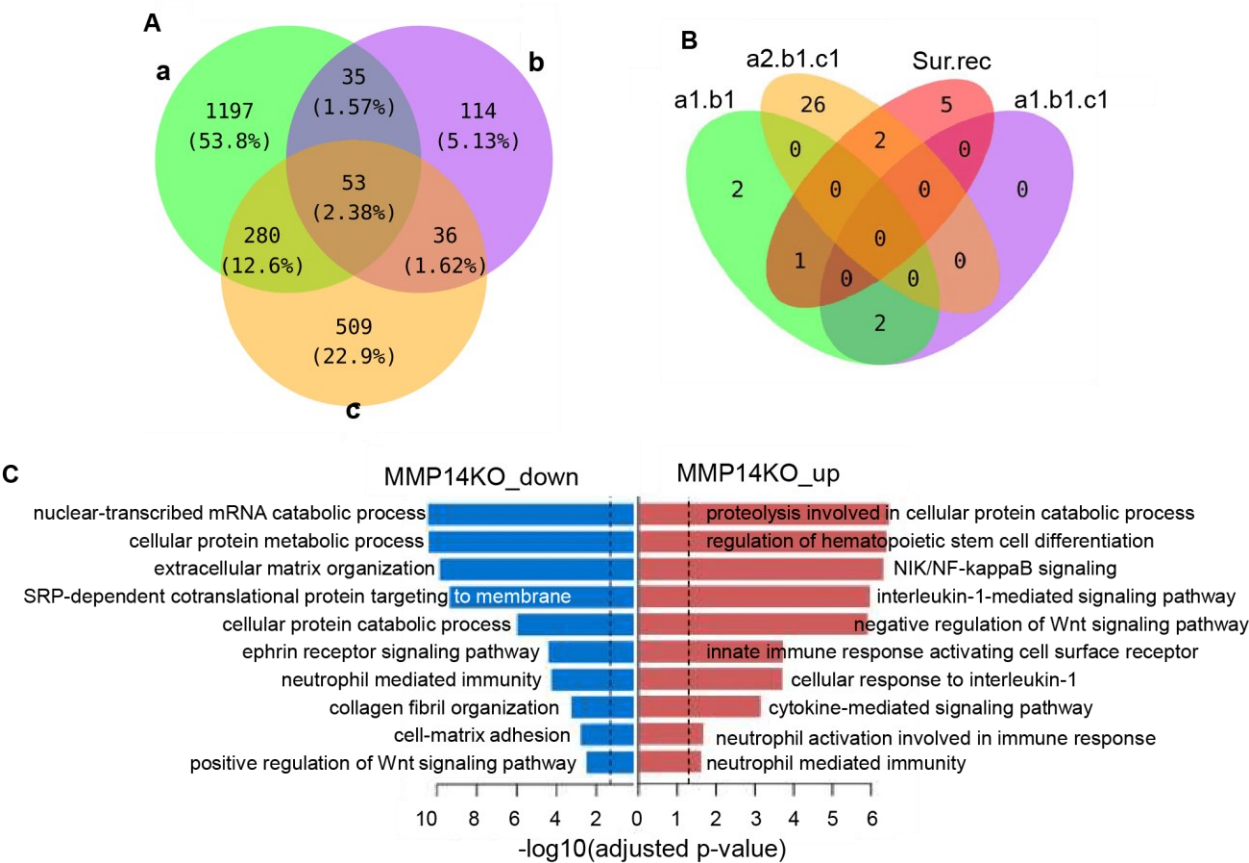


Fig. S10. Identification of Down-Regulated Surface Receptors among Differentially Expressed Proteins (DEPs)

(A) Venn diagram of the DEPs of secretomes (marked by a, green color), surface proteins (marked by b, purple color), and total proteins (marked by c, orange color) from Saos-2-Mmp14KO cells and control Saos-2 cells, respectively. (B) Venn diagram illustrating the overlap between the following four protein sets: (i) surface receptor; (ii) a1.b1: common DEPs that were down-regulated (marked by 1) in the secretome and surface proteins; (iii) a2.b1.c1: common DEPs that were up-regulated (marked by 2) in the secretome, while down-regulated in the surface and total proteins; (iv) a1.b1.c1: common DEPs that were down-regulated in the secretome, surface and total proteins. (C) GO enrichment analysis of biological processes for down- and up-regulated DEPs between Saos-2-Mmp14KO cells and control Saos-2 cells.

317 **Table S1. Genotyping Primers.**

318 The following primers were used for genotyping:

Gene	Primers	
<i>Mmp14^{F/F}</i>	S	5'- TAG AAC CTA GTG AGA GCG TG-3'
	AS	5'- ACC TAC ACT GTA GAA CTC AG-3'
	AS	5'- ATC TGC GTG TTC GAA TTC GC-3'
<i>Col10a1Cre</i>	Cre1	5'- GGA CAT GTT CAG GGA TCG CCA GGC G-3'
	Cre2	5'- GCA TAA CCA GTG AAA CAG CAT TGC TG-3'
	scrcolx-R	5'- TAC TGA CCT GTC GTT TCC -3'
	ColX_Cre F	5'- CAG ATC AGT GAT GGG CTA TGT G -3'
<i>EGFP</i>	OEGFP	5'- GTT CTT CTG CTT GTC GGC CA -3'
	OFP	5'- ATG GTG AGC AAG GGC GAG GA -3'
<i>Rosa26 LSL-tdTomato</i>	Wt-F	5'- AAG GGA GCT GCA GTG GAG TA -3'
	Wt-R	5'- CCG AAA ATC TGT GGG AAG TC -3'
	CAG-tdTomR	5'- GGC ATT AAA GCA GCG TAT CC -3'
	CAG-tdTomF	5'- CTG TTC CTG TAC GGC ATG G -3'
R26-M2rtTA	Rosa 5'	5'- GAG TTC TCT GCT GCC TCC TG -3'
	rtTA 3'	5'- AAG ACC GCG AAG AGT TTG TC -3'
	wt	5'- AAG TAG TGC AGC CCG GAG TA -3'
TetOP-H2B-GFP	JAX11029-mutF	5'- GCT CGT TTA GTG AAC CGT CAG -3'
	JAX25360-wtR	5'- TGG TTT CTT TGG GCT AGA GG -3'
	JAX31015-wtF	5'- CCA TCC CAA CAA TAC ATC ACA -3'
	JAX39486-mutR	5'- GGA CCT GCT TCA GAA CCT TG -3'

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320 **Movie S1.**
321 Live cell imaging for WT Saos2 osteoblastic cells co-culture with GFP positive neutrophils.
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323 **Movie S2.**
324 Live cell imaging for mutant Saos2 osteoblastic cells (Saos2-Mmp14KO cells) co-culture with
325 GFP positive neutrophils.
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327 **Data S1. (separate file)**
328 Top10 marker genes and cellular cluster statistics in each cell type for the heatmap in Fig 2 B.
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330 **Data S2. (separate file)**
331 Top10 marker genes and cellular cluster statistics in each cell type for the heatmap in Fig. S3 A.
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333 **Data S3. (separate file)**
334 DEGs lists and statistics for the volcano plot in Fig. S3 C.
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336 **Data S4. (separate file)**
337 Go term lists and statistics for Fig. S4 B.
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339 **Data S5. (separate file)**
340 DEGs lists and statistics for the volcano plot in Fig. 4 D.
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342 **Data S6. (separate file)**
343 Go term lists and statistics for Fig. 4 F.
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345 **Data S7. (separate file)**
346 Go term lists and statistics for Fig. S10 C.