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Dear Dr.

First of all, we would like to thank you for your invitation and help. We submit our manuscript entitled "Skeletal Phenotype Analysis of a conditional Stat3 Deletion Mice Model" by Yiling Yang, Qianye Chen, Siru Zhou, Xinyi Gong, Hongyuan Xu, Yueyang Hong, Qinggang Dai, and myself for your consideration for publication in *THE JOURNAL OF VISUALIZED EXPERIMENTS*.

Transgenic mouse models are powerful for understanding the critical genes controlling osteoclast differentiation and activity, and for studying the mechanism and pharmaceutical treatments of osteoporosis. Cathepsin K (Ctsk) -Cre mice have been widely used for functional studies of osteoclasts. The signal transducer and activator of transcription 3 (STAT3) is relevant in bone homeostasis, but its role in osteoclasts in vivo remains poorly defined. In this protocol describes a canonical method to understand the critical genes controlling osteoclasts activity in vivo, and some modified techniques for analyzing skeletal phenotype. I briefly summarize our protocols:

1. Micro-CT scanning and three-dimensional reconstruction implied increased bone mass in the conditional knockout mice.
2. Histological analysis is the best intuitive method to detect bone metabolism. H&E staining represents histomorphology of bone tissue and the suitable angle and depth of continuous sections.
3. Calcein and alizarin red double staining shown the osteogenesis activity of bone.
4. Tartrate-resistant acid phosphatase (TRAP) staining were used to detect the osteoclasts activity.

In short, this protocol describes a canonical method to understand the critical genes controlling osteoclasts activity in vivo, and some modified techniques for analyzing skeletal phenotype.

Thank you in advance for the opportunity to have this manuscript reviewed for publication in *THE JOURNAL OF VISUALIZED EXPERIMENTS*. This paper has been read and approved by all of its participating authors and has not been submitted for publication elsewhere. For reasons of conflict, we respectfully ask that appropriate reviewers with expertise in bone homeostasis and dental research would include Drs. Huang Li, Medical School of Nanjing University, Juan Li, West China School of Stomatology, Sichuan University, and Xue Feng, The Fourth Military Medical University.

Thank you once again, and please do not hesitate to contact me with any questions or comments at any point along the review process.

Sincerely,
Lingyong Jiang

TITLE:

Skeletal Phenotype Analysis of a Conditional *Stat3* Deletion Mouse Model

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KEYWORDS:

skeletal phenotype, bone metabolism, labeling, osteoclasts, STAT3, transgenic mice

SUMMARY:

This protocol describes a canonical method to understand the critical genes controlling osteoclast activity in vivo. This method uses a transgenic mouse model and some canonical techniques to analyze skeletal phenotype.

ABSTRACT:

Transgenic mouse models are powerful for understanding the critical genes controlling osteoclast differentiation and activity, and for studying mechanisms and pharmaceutical treatments of osteoporosis. *Cathepsin K (Ctsk)-Cre* mice have been widely used for functional studies of

osteoclasts. The signal transducer and activator of transcription 3 (STAT3) is relevant in bone homeostasis, but its role in osteoclasts in vivo remains poorly defined. To provide the in vivo evidence that STAT3 participates in osteoclast differentiation and bone metabolism, we generated an osteoclast-specific *Stat3* deletion mouse model (*Stat3 $^{fl/fl}$; Ctsk-Cre*) and analyzed its skeletal phenotype. Micro-CT scanning and 3D reconstruction implied increased bone mass in the conditional knockout mice. H&E staining, calcein and alizarin red double staining, and tartrate-resistant acid phosphatase (TRAP) staining were performed to detect bone metabolism. In short, this protocol describes some canonical methods and techniques to analyze skeletal phenotype and to study the critical genes controlling osteoclast activity in vivo.

INTRODUCTION:

Skeletal bone is the main load-bearing organ of the human body and is under pressure from both the internal and external environment during walking and exercise[1]. Throughout one's life, bones continuously go through self-renewal, which is balanced by osteoblasts and osteoclasts. The process of osteoclasts clearing old bones and osteoblasts forming new bone maintains the homeostasis and mechanical function of the skeletal system[2]. Disturbance in the balance may induce bone metabolic diseases, such as osteoporosis. Osteoporosis, which is caused by excess osteoclastic activity, is globally prevalent and causes substantial economic losses to society[2-4]. According to the limited number of drugs available for osteoporosis treatment and their risk of adverse effects[4], it is important to unveil the details of osteoclast formation and activity.

Osteoclasts derived from the monocyte/macrophage hematopoietic lineage have multiple nuclei (may have 2 to 50 nuclei) and are large (usually greater than 100 µm in diameter)[2]. Although the exploration of mechanisms and the screening of drugs for osteoclastic disorders have been widely improved via in vitro osteoclast culture, the complicated organic reactions make in vivo evidence indispensable for the targeted therapy. Due to genetic and pathophysiological similarities between mice and humans, genetically engineered mouse models are commonly used for studying the mechanisms and the pharmaceutical treatments of human disease in vivo[6]. The Cre-loxP system is a widely-used technology for mouse gene editing and has enabled researchers to investigate gene functions in a tissue-/cell-specific manner[5]. Cathepsin K (CSTK) is a cysteine protease secreted by osteoclasts that can degrade bone collagen[8]. It is well accepted that CTSK is selectively expressed in mature osteoclasts; therefore, *Ctsk-Cre* mice are considered to be a useful tool for functional studies of osteoclasts and has been used[6].

The signal transducer and activator of transcription (STAT) family is classical and highly significant in immunity and cancer progression and development[7,8]. Among seven STATs, STAT3 is reported to be the most relevant to bone homeostasis[9,10]. Several in vivo studies have reported that specific inactivation of STAT3 in osteoblasts decreases bone formation[9,10]. Nevertheless, solid evidence regarding the participation of STAT3 in osteoclast formation and bone metabolism in vivo is still limited. Recently, we provided in vivo evidence with an osteoclast-specific *Stat3* deletion mouse model (*Stat3 $^{fl/fl}$; Ctsk-Cre,* hereafter called *Stat3^{Ctsk}*) that STAT3 participates in osteoclast differentiation and bone metabolism[11]. In the present study, we describe the methods and protocols that we used to analyze the changes in bone mass, bone histomorphology, and bone anabolism and catabolism of the *Stat3^{Ctsk}* mice in order to study the influence of osteoclast-

specific STAT3 deletion on bone homeostasis.

PROTOCOL:

All methods relating to the animals described here were approved by the Institutional Animal Care and Use Committee (IACUC) of Shanghai Jiaotong University School of Medicine.

1. Breeding of osteoclast specific *Stat3* deletion mice

NOTE: *Stat3*$^{fl/fl}$ mice were obtained commercially. *Ctsk-Cre* mice were provided by S. Kato (University of Tokyo, Tokyo, Japan[12]). The mice were bred and maintained under specific pathogen-free (SPF) conditions in the institutional animal facility under standardized conditions.

1.1. Pair a sexually mature male mouse with two female mice of the same age. After 18 days, check daily checks for newborns. Separate the pregnant female mice and keep it alone if needed. Change male mice among different breeding cages if the female mice were not pregnant within 1 month of the pairing.

1.2. Cross *Stat3*$^{fl/fl}$ mice to *Ctsk-Cre* mice (F0). Clip the tails for genotyping and keep the male *Stat3*$^{fl/+}$*; Ctsk-Cre* mice until they are sexually mature, which is around 6 weeks of age (F1). Use the following primers: Stat3 F-TTGACCTGTGCTCCTACAAAAA; Stat3 R-CCCTAGATTAGGCCAGCACA; Ctsk-cre F-GAACGCACTGATTTCGACCA; Ctsk-cre R-GCTAACCAGCGTTTTCGTTC.

1.3. Cross 6-week-old male *Stat3*$^{fl/+}$*; Ctsk-Cre* mice with female *Stat3*$^{fl/fl}$ mice. Clip the tails for genotyping and keep the male *Stat3* $^{fl/fl}$*; Ctsk-Cre* mice until they are sexually mature, which is around 6 weeks of age (F2).

1.4. Cross 6-week-old male *Stat3* $^{fl/fl}$*; Ctsk-Cre* mice with female *Stat3*$^{fl/fl}$ mice (F3). Clip the tails for genotyping and replace the old breeding mice with younger mice in time (F3+N).

2. Specimen collection

2.1. Euthanize six pairs of 8-week old male *Stat3*$^{fl/fl}$ and *Stat3*^{Ctsk} littermate mice separately with carbon dioxide asphyxiation.

NOTE: The CO_2 flow rate displaces 30% of the cage volume per minute (e.g., for 45 cm x 30 cm x 30 cm cage the CO_2 flow rate is 40 L/min).

2.2. Place the mice in a supine position. Dislocate the bilateral hip joints gently by hand. Use ophthalmic scissors to cut off the skin vertically from the distal tibia and then dissect the entire skin from the hind limb.

2.3. Cut off the articular ligament of the right hip joint and knee joint with scissors to separate the hind limb. Cut the trochanter and the junction of the fibula and then immerse the hind limb in 4% paraformaldehyde. Keep the right hind limbs for step 3. Appropriately cut the bone at both

ends to fully immerse and fix the bone marrow with 4% paraformaldehyde.

2.4. Cut the articular ligament of the left hip joint and knee joint with scissors, gently remove the soft tissue, and carefully separate the tibia and femur. Immerse the tibia and femur separately in 75% ethanol. Keep the femora for step 4 and tibiae for step 6. Ensure to keep the trochanter intact.

3. Paraffin section preparation

3.1. Fix the right hind limb in 4% paraformaldehyde at 4 °C for 48 h.

3.2. Decalcify: Gently wash the specimens with 1x PBS for 10 min 3 times. Decalcify the specimens in 15% EDTA (150 g EDTA in 800 mL of ddH_2O and 100 mL of 10x PBS) with an ultrasonic decalcifier for 3 to 4 weeks until the bones can be bent. Replace with fresh decalcifying fluid every other day.

3.3. Gently wash the specimens 3x with 1x PBS and then immerse them in 75% ethanol at 4 °C overnight.

3.4. Dehydrate: On the second day, sequentially immerse specimens in 95% ethanol, 100% ethanol, and xylene, each for 1 h twice.

3.5. Immerse specimens in 1/2 xylene 1/2 paraffin for 30 min. Immerse the specimens in paraffin at 65 °C overnight.

3.6. Embed: Select a suitable embedding tank for embedding. Place the tibia uniformly underneath. Place the femur and tibia at a 90° angle. After the paraffin has fully cooled and solidified, remove it from the embedding tank. Number the specimens and store them at -20 °C overnight.

3.7. Cut 5 µm thick sections continuously using the microtome. Cut 20–40 sections. Spread the sections on 37 °C water, adhere them to microscope slides, and bake at 42 °C overnight.

4. Micro-CT scanning and analysis

4.1. Scan the left femora with a micro-CT scanner. Resolution: 10 µm; Voltage: 70 kV; Current: 114 µA; Fliter: 0.5 mm Al; Rotation step: 0.5°.

4.2. Reconstruct 3D images of the cortical bone and trabecular bone using the scanner's supporting software following manufacturer's instruction. ROIs are in a total 1 mm width of trabecular bone close to the distal growth plate and in a total 1 mm wide section of cortical bone in the middle of the femora.

4.3. Calculate the quantitative microarchitecture parameters: bone mineral density (BMD),

bone volume fraction (BV/TV), trabecular thickness (Tb.Th.), trabecular number (Tb.N.), trabecular separation (Tb.Sp.), and cortical bone thickness (Ct.Th.).

5. TRAP staining

5.1. Bake the paraffin sections at 65 °C for 30 min.

5.2. Dewax: Immerse the sections in xylene for 10 min. Perform this step 3x with fresh xylene.

5.3. Rehydrate: Immerse the sections sequentially in 100% ethanol, 95% ethanol, 70% ethanol, and ddH_2O, each for 5 min twice.

5.4. Prepare the staining solution using the TRAP staining kit following the manufacturer's instructions and warm to 37 °C.

NOTE: TRAP staining solution should be freshly prepared immediately before every assay.

5.5. Add 50–100 µL staining solution to each sample and incubate in a 37 °C humid chamber for 20–30 min. Check the staining status of the osteoclasts under a light microscope every 5 min until red multinucleated osteoclasts can be seen. End the reaction with ddH_2O.

5.6. Counterstain in hematoxylin solution for 30 s. Create a stable blue color by immersion in 1% ammonia solution for 1 min. Rinse in slowly running tap water.

5.7. Mount the sections with coverslips using neutral balsam and dry overnight.

5.8. Capture 3–5 fields of interest by a microscope. Analyze the trabecular perimeter by Image J: measure the length of scale bar (Ls) using the '**straight line**' tool as L1, then measure the length of trabecular perimeter using the '**segmented line**' tool as L2, the physical length (Lp)= Ls*L2 /L1). Count the number of TRAP-positive cells with more than three nuclei.

6. Calcein and alizarin red double labeling

6.1. Specimen preparation: Intraperitoneally inject 20 mg/kg calcein (1 mg/mL in 2% $NaHCO_3$ solution) on day 0, and 25 mg/kg alizarin red S (AL, 2 mg/mL in H_2O) on day 4. Sacrifice mice on day 7. Carefully disassociate the tibiae and fix in 4% paraformaldehyde at 4 °C for 48 h.

6.2. Dehydrate: After fixation, gently wash the tibiae 3x with 1x PBS. Sequentially immerse the specimens in 95% ethanol, 100% ethanol, and xylene for 5 min twice separately.

6.3. Immerse the specimens in acetone for 12 h, in 1/2 acetone 1/2 resin for 2 h, and in pure resin in a drying oven overnight.

NOTE: The resin was prepared by commercially available resin (e.g., Embed 812 Resin) according

to the manufacturer's instructions.

6.4. Embed: Add pure resin into a suitable silica gel embedding tank and place the specimens gently to avoid bubbles. Polymerize the resin in a drying oven at 60 °C for 48 h.

6.5. Cut the specimens into 5 µm thick sections continuously with a rotary microtome. Store the rest of the samples with desiccant at room temperature.

6.6. Adhere the sections with tweezers in a drop of 75% alcohol. Mount the sections with coverslips using neutral balsam. Capture red and green fluorescence labeling with a fluorescence microscope.

6.7. Measure the width between two labeling lines (Ir.L.Wi), single-labeled trabecular perimeter (sL.Pm), double-labeled trabecular perimeter (dL.Pm), and total trabecular perimeter (Tb.Pm). Calculate the mineral apposition rate (MAR) and bone formation rate (BFR/BS). MAR = Ir.L.Wi /interval days. BFR/BS= (dL.Pm±sL.Pm/2)*MAR/Tb.Pm*100 %.

REPRESENTATIVE RESULTS:

Using the present protocol, osteoclast specific *Stat3* deletion mice were generated to study the influence of STAT3 deletion on osteoclast differentiation. *Stat3*^{Ctsk} mice and their wildtype (WT) littermates were bred and kept after genotyping. Bone marrow macrophages were isolated and cultured into osteoclasts, and STAT3 deletion in *Stat3*^{Ctsk} mice was demonstrated (**Figure 1**).

Femora reconstruction and quantitative analysis by micro-CT indicated that the bone mass of the *Stat3*^{Ctsk} mice was increased compared with WT mice (**Figure 2**).

Histomorphology of the femora from WT and *Stat3*^{Ctsk} mice was examined via H&E staining (**Figure 3**).

Osteoclastogenic activity in the mice was detected by TRAP staining. Osteoclasts were TRAP^{+} (wine red or purple) cells with multiple nuclei and huge size (**Figure 4A**). The number of TRAP^{+} osteoclasts was lower in the *Stat3*^{Ctsk} mice compared with the WT mice (**Figure 4B**), which indicates that STAT3 deficiency impaired osteoclast formation.

Osteogenesis in mice is represented by the mineral apposition rate (MAR) and was measured by calcein and alizarin red double labeling (**Figure 5**). Calcein and alizarin red were sequentially intraperitoneally injected. Therefore, the area between the calcein (green) and alizarin red (red) fluorescence lines represents newly formed bone over four days. As shown in **Figure 5**, deleted STAT3 in osteoclasts did not influence bone anabolism.

FIGURE AND TABLE LEGENDS:

Figure 1: Illustration of osteoclast specific *Stat3* deletion mouse model generation and genetically engineered mice. (**A**) Schematic diagram of *Stat3* deletion in cathepsin K (Ctsk)-

expressing osteoclasts via the Cre-loxP system. (**B**) Breeding progress of osteoclast specific *Stat3* deletion mice. *Stat3*$^{fl/fl}$ mice were crossed to *Ctsk-Cre* mice (F0) to generate heterozygous *Stat3*$^{fl/+}$*; Ctsk-Cre* mice (F1). Male *Stat3*$^{fl/+}$*; Ctsk-Cre* mice were kept and crossed to female *Stat3*$^{fl/fl}$ mice to generate homozygous *Stat3*$^{fl/fl}$*; Ctsk-Cre* mice (F2). Male *Stat3*$^{fl/fl}$*; Ctsk-Cre* mice were kept and crossed to female *Stat3*$^{fl/fl}$ mice to generate *Stat3*$^{fl/fl}$*; Ctsk-Cre* mice and *Stat3*$^{fl/fl}$ mice. (**C**) Genotyping for various genotypes of mice. *Stat3* mutant: 187 bp, *Stat3* wildtype: 146 bp, Cre: 200 bp. (**D**) Western blot of STAT3 in osteoclasts cultured with bone marrow macrophages from *Stat3*$^{fl/fl}$ and *Stat3*^{Ctsk} mice.

Figure 2: Three-dimensional reconstruction and quantitative microarchitecture parameter analysis in femora of mice using micro-CT scanning. (**A**) Three-dimensionally reconstructed micro-CT images of trabecular bone of femora from 8-week-old WT and *Stat3*^{Ctsk} mice. The region of interest (ROI) was in a total 1 mm width of trabecular bone close to the distal growth plate. (**B**) 3D reconstructed micro-CT images of cortical bone of femora from 8-week-old WT and *Stat3*^{Ctsk} mice. ROI was in a total 1 mm wide section of cortical bone from the middle of the femora. (C–H) Quantitative microarchitecture parameters of micro-CT: bone mineral density (BMD), bone volume fraction (BV/TV), trabecular thickness (Tb.Th.), trabecular number (Tb.N.), trabecular separation (Tb.Sp.), and cortical bone thickness (Ct.Th.). Error bars represent the mean ± SD, n=4, *P<0.05.

Figure 3: Histomorphology of femora from WT and *Stat3*^{Ctsk} mice exhibited via H&E staining. M-shaped dotted curve indicates a cartilage layer. Relative high-power field of cortical bone is boxed with dotted lines and exhibited below. Relative high-power field of trabecular bone is circled with dotted lines and exhibited at the bottom.

Figure 4: Osteoclastogenesis in femora of mice represented via TRAP staining. (**A**) TRAP staining of femora from 8-week-old WT and *Stat3*^{Ctsk} mice. TRAP^{+} multinucleated osteoclasts are indicated by black triangles. Relative high-power field of osteoclasts circled with dotted lines are exhibited below, demonstrating multiple nuclei and a huge size. (**B**) The numbers of TRAP^{+} multinucleated osteoclasts were counted. Error bars represent the mean ± SD, n=5, *P<0.05.

Figure 5: Osteogenesis in femora of mice represented via calcein and alizarin red double labeling. (**A**) Seven-week-old mice were sequentially injected with calcein on day 0, with alizarin red on day 4, and then sacrificed on day 7. The area between calcein (green) and alizarin red (red) fluorescence lines represent newly formed cortical bone over four days. (**B**) Mineral apposition rate (MAR) and bone formation rate (BFR/BS) calculated by the distance of the two lines represent the osteogenic activity of the cortical bone. (**C**) The area between the calcein (green) and alizarin red (red) fluorescence lines represent newly formed trabecular bone in four days. (**D**) MAR and BFR/BS represent the osteogenic activity of the trabecular bone. Error bars represent the mean ± SD, n=5, *P<0.05.

DISCUSSION:

Genetically engineered mouse models are commonly used for studying the mechanism and pharmaceutical treatment of human disease[13]. *Ctsk-Cre* mice have been widely used for

functional studies of osteoclasts[6]. The present study described the protocols of the methods to analyze skeletal phenotype and to study the critical genes controlling osteoclast activity in vivo.

Histological analysis is the best intuitive method to detect bone metabolism. And the quality of the paraffin sections is the base of the histological analysis. Sufficient time for full fixation and decalcification of the bone tissue is extremely essential since bone tissues can be fragmented. Researchers focusing on femora and tibiae should place the femur and tibia at a 90° angle in the embedding step. In order to conduct reliable and high-quality histological analysis, we chose sagittal paraffin sections in which the cartilage layer was symmetrical and showed a clear M-shaped line in H&E staining, which represents the suitable angle and depth of those continuous sections. On the other hand, if you need to observe the knee joints and adjacent articular ligament, the femur and tibia should be placed at a 120° angle.

TRAP has served for decades as a biochemical marker for osteoclast function[14], since it is predominantly expressed in osteoclasts[15,16]. Two substrates are usually used to assess acid phosphatase activity. Naphthol-ASBI phosphate (N-ASBI-P) is an excellent substrate for the osteoclast-specific TRAP isoform 5b. However, para-nitrophenyl phosphate (pNPP) can also be hydrolyzed by nontype 5 TRAPs[17]. Therefore, the naphthol-ASBI phosphoric acid-pararosaniline method for histochemical demonstration of TRAP is highly specific in tissue sections[17-19]. Macrophage phosphatase (acid phosphatase) has a pH optimum of 5.0–6.0, at which it is mostly tartrate resistant[20]. Thus, we recommend that in the protocol of TRAP staining assay, tissue sections re-stained by hematoxylin should not be decolored with hydrochloric acid. Importantly, although osteoblasts and osteocytes close to bone remodeling areas also express TRAP[21], only huge TRAP-positive cells with more than three nuclei were considered as osteoclasts. In this study, *Stat3*^{Ctsk} mice showed inhibited osteoclast activity (**Figure 4**).

Calcein is a fluorochrome (green fluorescence) that is widely used to indicate skeletal growth during calcification. It can bind to calcium and be incorporated into the newly formed calcium carbonate crystals[22]. Similarly, calcein, alizarin red (red fluorescence), and tetracycline (yellow fluorescence) were built into the bone to estimate growth from the time of exposure[23,24]. Many studies in the past have used single fluorochromes (usually calcein) to label bone, but observers can be easily confused between the newly formed bone and old bone, especially in irregular trabecular bone. Therefore, we suggest that researchers inject two different types of fluorochromes into mice to distinguish the newer bone from the older bone. According to our tests, calcein coupled with alizarin might achieve the most sufficient and enduring contrast. In this study, calcein-alizarin red labeling was used to detect the osteogenic rate of bone tissue and it turned out that osteoblast activity was not influenced in the *Stat3*^{Ctsk} mice (**Figure 5**).

In short, to understand the critical genes controlling osteoclast activity, this protocol describes a canonical method to generate a transgenic mouse model. This protocol also describes some typical techniques for analyzing the skeletal phenotype. In this study, the deletion of STAT3 impaired osteoclast formation and increased bone mass. These techniques may be interesting for those who are new to skeletal tissue research. However, more characteristics of skeleton system are concerned for further study, such as mechanical property[25]. And we always need to

keep eyes on the development of new techniques.

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DISCLOSURES:

The authors have nothing to disclose.

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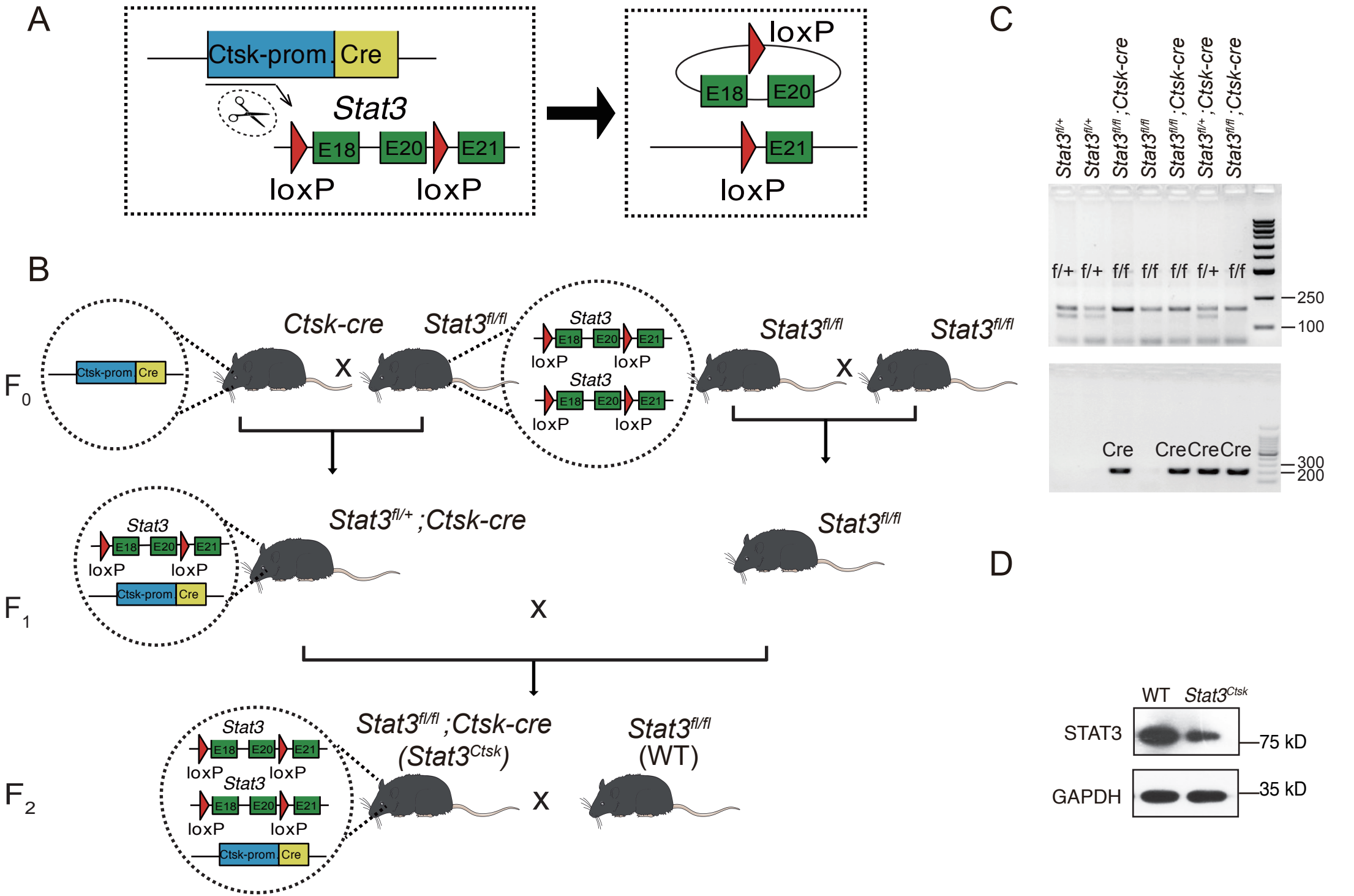
25. Sun, J. et al. Histone demethylase LSD1 regulates bone mass by controlling WNT7B and BMP2 signaling in osteoblasts. *Bone Research.* **6**, 14-14 (2018).

Figure 1



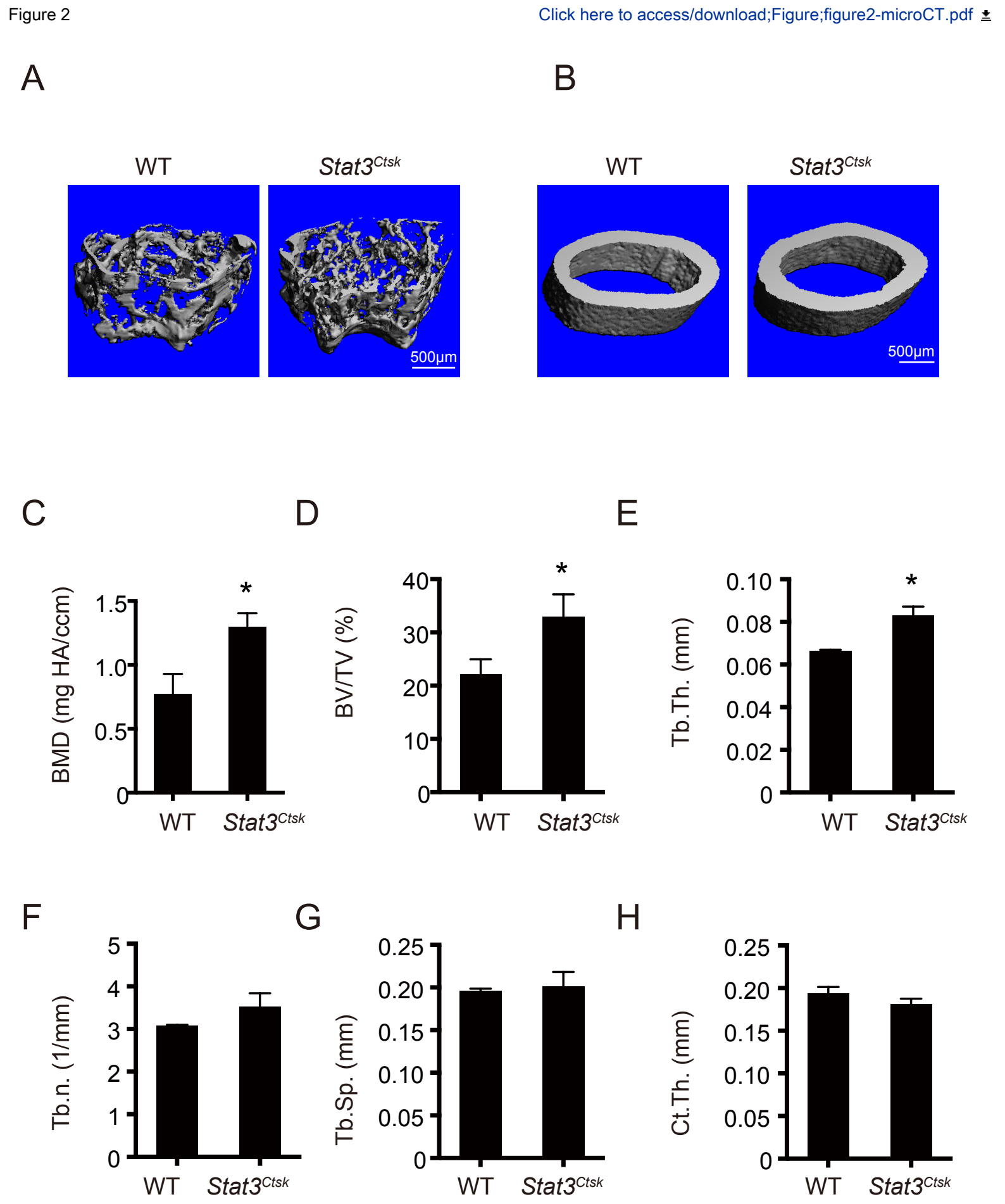
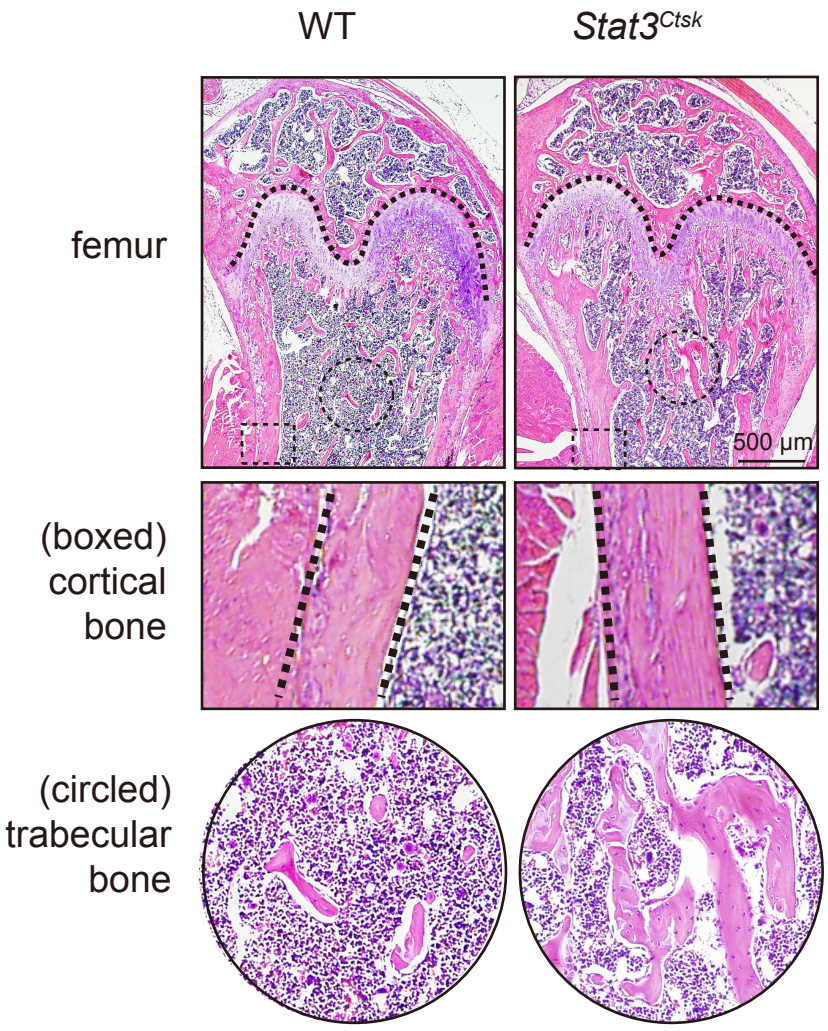


Figure 3



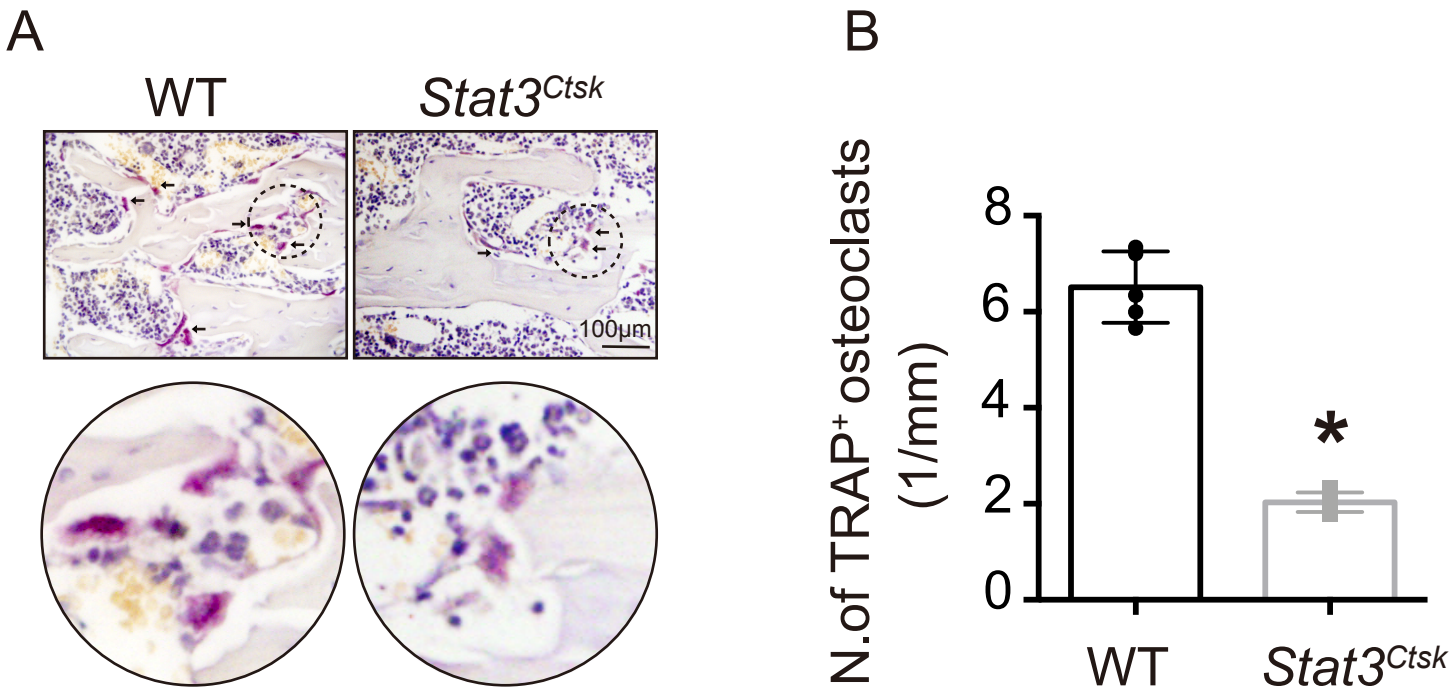


Figure 5

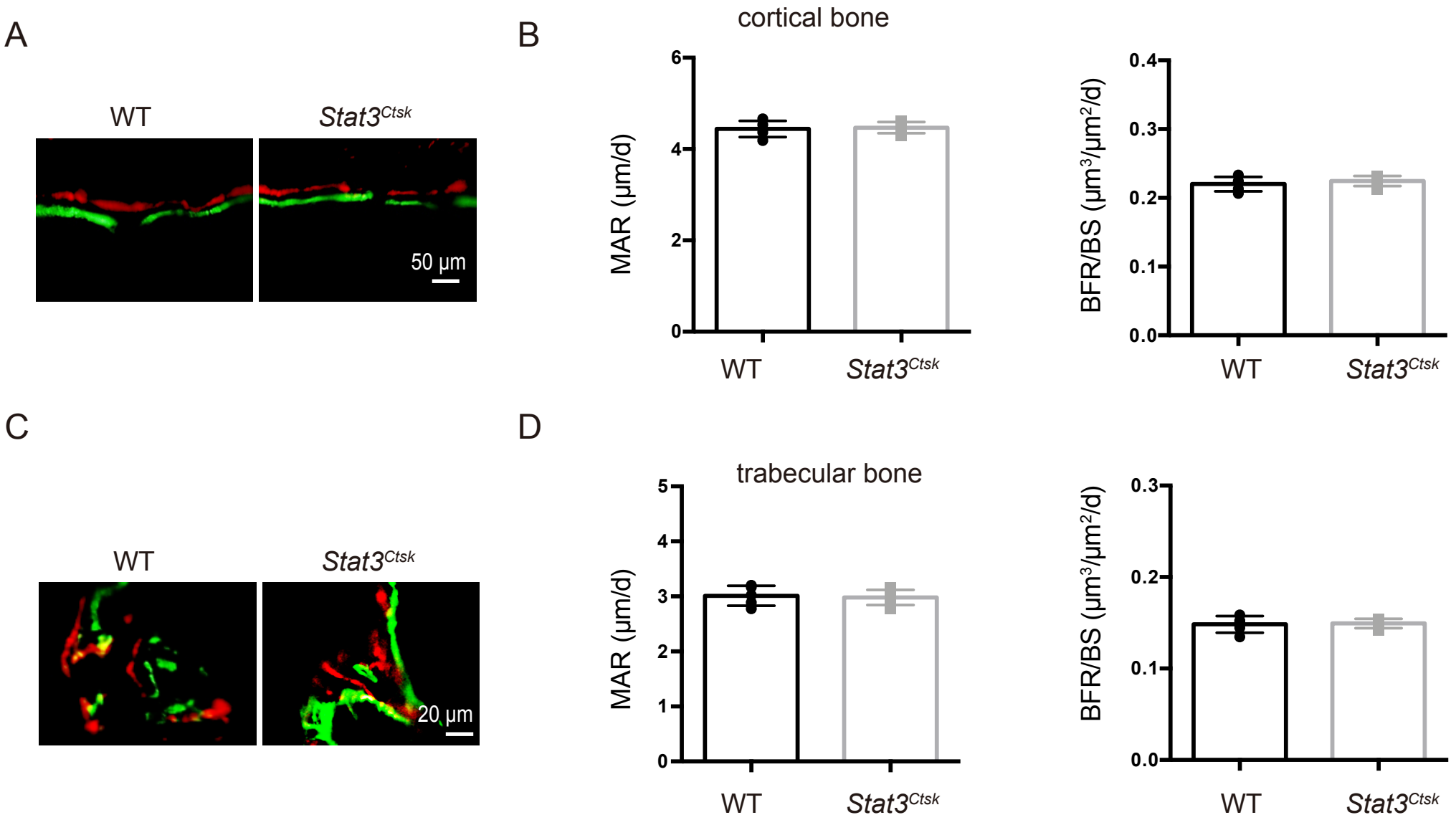


Table of Materials

Click here to access/download;Table of Materials;materials.xlsx

Name	Company	Catalog Number	Comments
4% Paraformaldehyde solution	Sangon biotech Co., Ltd.	E672002	
Acetone	Shanghai Experimental Reagent Co., Ltd.	80000360	
Alizarin	Sigma-Aldrich	A5533	
Ammonia solution	Shanghai Experimental Reagent Co., Ltd.		
Calcein	Sigma-Aldrich	C0875	
Ctsk-Cre mice			a gift from
DDSA	Electron Microscopy Sciences	13710	
DeCa RapidlyDecalcifier	Pro-Cure	DX1100	
DMP-30	Electron Microscopy Sciences	13600	
EDTA	Shanghai Experimental Reagent Co., Ltd.	60-00-4	
EMBED 812 RESIN	Electron Microscopy Sciences	14900	
fluorescence microscope	Olympus	IX73	
Hematoxylin solution	Beyotime Biotechanology	C0107	
Micro-CT	Scanco Medical AG	µCT 80	
NaHCO3	Shanghai Experimental Reagent Co., Ltd.	10018918	
Neutral balsam	Sangon biotech Co., Ltd.	E675007	
NMA	Electron Microscopy Sciences	19000	
Paraffin	Sangon biotech Co., Ltd.	A601889	
rotary microtome	Leica	RM2265	
Stat3$^{fl/fl}$ mice	GemPharmatech Co., Ltd	D000527	
TRAP staining kit	Sigma-Aldrich	387A	
xylene	Shanghai Experimental Reagent Co., Ltd.	1330-20-7	

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S. Kato, University of Tokyo, Tokyo, Japan

Editorial comments:

1. The editor has formatted the manuscript to match the journal's style. Please retain and use the attached file for revision.

Yes.

2. The manuscript needs thorough proofreading, please employ professional copyediting services.

We have employed professional copyediting services.

3. Please address specific comments marked in the manuscript.

Yes.

4. Please reword lines 71--75, 82-84, 184-186 as it matches with the previously published literature.

We have reworded them.

5. Once done please ensure that the highlight is no more than 2.75 pages including headings and spacings.

Yes.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

OK

Major Concerns:

None

Thanks for the reviewer’s careful examination and kind suggestions. We really appreciate for your review.

Minor Concerns:

The authors have improved the manuscript and addressed most issues raised by the reviewers. Despite all this, there are still numerous typos and English grammar issues. It would be advisable to get Editorial assistance for English grammar and style. Here are below a few minor comments:

Line 82: authors should delete '...or dampened osteoblastic activity:'

Thanks, we have deleted it.

Line 104: Stat3fl/fl mice were purchased.

Thanks, we have corrected it.

Lines 106-110: The written text should be revisited. Many English grammar and syntax errors.

Thanks, we have polished it.

Line 112-133: not clear why male mice are kept until 6 weeks old for breeding. Confusing. Simply mention that male mice are kept until sexual maturity at around 6 weeks of age.
Thanks, we have corrected it.

Line 125: Indicate what is the CO2 flow rate.
Thanks, we have added an example to explain the distinct flow rate.

Line 134: replace 'destroy' with 'cut'
Thanks, we have corrected it.

Line 137: replace 'disassociate' with 'separate'.
Thanks, we have corrected it.

Line 145: indicate how long are each of the PBS washes (10min? 20min?)
We washed specimens with PBS for 10mins 3 times and we had added it.

Lines 153-154: indicate how long are each of the dehydration steps (10min? 20min?).
As we mentioned, each step was repeated “for 1 h twice respectively”.

Line 199: 'running tap water'.
Thanks, we have corrected it.

Line 203: a proper link to the software should be given for instructions how to use.
Yes, we have added details in line 204-207 (5.8.).

Line 245: replace 'exhibited' with 'examined'.
Thanks, we have corrected it.

Reviewer #2:
The authors have addressed majority of my concerns. However, some concerns were not addressed properly. The purpose of the rebuttal letter is to provide a satisfactory answer to the concerns/questions raised. It may not be always sufficient to mention "we have made the change", without mentioning the page numbers where the changes were made. Please answer the following to the point with reference to the page numbers in the manuscript:
Thanks for the reviewer’s kind suggestions. We would add the specific page numbers in rebuttal letter this time. But the line numbers in the PDF version you downloaded seem to be different with those in the Word version I uploaded.

3) MicroCT conditions should be more detailed. Which filter was used and at what rotation images were captured should be indicated.
Yes, we have added details in line 170-171 (4.1.) in page 3.

7) Describe how histomorphometric analyses e.g. mineral apposition rate (MAR) and bone formation rate (BFR) were determined. Please include a detailed description of the procedure and software used.
Yes, we have added details in line 231-234 (6.7.) in page 6.

8) Mention how many fields were analyzed for osteoclast counting and which software was used for this. Please indicate the page number in the manuscript where this information has been added.
Yes, we have added details in line 203-204 (5.5.) in page 4.

10) I am curious to know what is "M-mode cartilage layer" mentioned in the initial version of the manuscript
The "M-mode cartilage layer" we mentioned was not a professional term. A high-quality histological sagittal paraffin section should contain symmetrical bone tissue. According to our experiments, the curve of cartilage layer was clear and identifiable in HE staining, or even could be straightly observed without any staining methods. Therefore, we would adjust the angle of paraffin while cutting according to the shape of cartilage layer until it looked like an “M”.

11) The authors should better justify how the methodologies described are different from many published literature. Labeling using two or even three dyes have been reported earlier, this is nothing novel. However, if no video of the process is available then duly acknowledge the papers first reported this technique.
Thanks for the reviewer’s suggestions. It’s exactly our purpose to describe a routine system of skeletal research and to make it visible for those who are new to skeletal tissue research. About the labeling colors, according to our tests, calcein coupled with alizarin might achieve most sufficient and enduring contrast, which might be a useful reference.