



HOKKAIDO UNIVERSITY

Title	Differential osteoblastic activity in primary metaphyseal trabecular and secondary trabecular of c-fos deficient mice
Author(s)	Yamamoto, Tomomaya; Abe, Miki; Hongo, Hiromi et al.
Citation	Journal of oral biosciences, 65(4), 265-272 https://doi.org/10.1016/j.job.2023.08.002
Issue Date	2023-12
Doc URL	https://hdl.handle.net/2115/93556
Rights	©2023. This manuscript version is made available under the CC-BY-NC-ND 4.0 license https://creativecommons.org/licenses/by-nc-nd/4.0/
Rights(URL)	https://creativecommons.org/licenses/by-nc-nd/4.0/
Type	journal article
File Information	2023_Yamamoto&Abe.pdf



Differential osteoblastic activity in primary metaphyseal trabecular and secondary trabeculae of *c-fos* deficient mice

Tomomaya Yamamoto^{a,b#}, Miki Abe^{a#}, Hiromi Hongo^a, Haruhi Maruoka^a, Hirona Yoshino^a,
Mai Haraguchi-Kitakamae^{a,c}, Nobuyuki Udagawa^d, Minqi Li^e, Norio Amizuka^a, Tomoka
Hasegawa^{a§}

^a Developmental Biology of Hard Tissue, Faculty of Dental Medicine, Hokkaido University,
Sapporo, Japan, ^b Northern Army Medical Unit, Camp Makomanai, Japan Ground Self-
Defense Forces, Sapporo, Japan, ^c Division of Craniofacial Development and Tissue Biology,
Graduate School of Dentistry, Tohoku University, Sendai, Japan, ^d Department of Oral
Biochemistry, Matsumoto Dental University, Shiojiri, Japan, ^e Center of Osteoporosis and
Bone Mineral Research, Department of Bone Metabolism, School of Stomatology, Shandong
University, Jinan, China

[#] These authors made equal contributions.

Running title: Osteoblastic activity in *c-fos*^{-/-} mice

§ Address for correspondence to

Tomoka Hasegawa DDS, PhD.
Developmental Biology of Hard Tissue,
Graduate School of Dental Medicine and Faculty of Dental Medicine,
Hokkaido University
Kita-13, Nishi-7, Kita-Ku, Sapporo, Japan
Tel/Fax: +81-11-706-4226
E-mail: hasegawa@den.hokudai.ac.jp

ABSTRACT

Objectives: It has been highlighted that osteoblastic activities in remodeling-based bone formation are coupled with osteoclastic bone resorption, while those in modeling-based bone formation are independent of osteoclasts. This study aimed to verify whether modeling-based bone formation occurs even in the absence of osteoclasts.

Methods: Eight-week-old male *wild-type* and *c-fos*^{-/-} mice employed histochemical analyses in this study. Histochemical analyses were conducted on primary trabeculae near the chondro-osseous junction (COJ), a site of modeling-based bone formation, and the secondary trabeculae, a site of remodeling-based bone formation, in the femora and tibiae of mice.

Results: Immunoreactivity of alkaline phosphatase (ALP), a marker of osteoblastic lineages, was observed on the *wild-type* metaphyseal trabeculae, while ALP was scattered throughout the *c-fos*^{-/-} femora. PHOSPHO1, an enzyme involved in matrix vesicle-mediated mineralization, was predominantly detected not only in the primary trabeculae but also as short lines of osteoblasts in the secondary trabeculae of the *wild-type* mice. In contrast, *c-fos*^{-/-} femora showed several patches of PHOSPHO1 positivity in the primary trabeculae but hardly any in the secondary trabeculae. Consistently, calcein labeling was observed at the primary trabeculae close to the COJ in both *wild-type* and *c-fos*^{-/-} mice; however, calcein labeling in the secondary trabeculae was only detected in *wild-type* mice, not in *c-fos*^{-/-} mice. Transmission electron microscopic observation demonstrated abundant rough endoplasmic reticulum in the osteoblasts of the secondary trabeculae in *wild-type* mice, but not in *c-fos*^{-/-} mice.

Conclusions: Osteoblastic activities at the sites of modeling-based bone formation might be maintained even in the absence of osteoclasts.

250 words

Key words: modeling, bone remodeling, osteoblast, osteoclast, c-fos

1. Introduction

In order to form mineralized new bone, osteoblasts secrete abundant bone matrix proteins, including collagen fibrils, as well as matrix vesicles, which are extracellular small vesicles that initiate bone mineralization [1, 2]. Membrane transporters and enzymes such as Ca^{2+} -ATPase, tissue nonspecific alkaline phosphatase (ALP; an enzyme synthesizing monophosphate ions from pyrophosphates), ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1; an enzyme producing pyrophosphates), and phosphoethanolamine/phosphocholine phosphatase 1 (PHOSPHO1; an enzyme hydrolyzing phosphocholine and phosphoethanolamine to release phosphate ions within the matrix vesicles) are localized on osteoblasts and/or matrix vesicles to increase local concentrations of inorganic phosphate (PO_4^{3-}) and calcium (Ca^{2+}) ions in the bone-forming areas [3-5]. Abundant PO_4^{3-} and Ca^{2+} flow into the matrix vesicles to nucleate and grow calcium phosphate crystals inside the vesicles, which are then released to form mineralized nodules and subsequent collagen mineralization. Recently, we reported that ALP is broadly distributed in mature osteoblasts and preosteoblasts, whereas ENPP1 is localized not only in mature osteoblasts but also in osteocytes embedded in bone [6]. PHOSPHO1, in relation to ALP, appears to be specifically expressed in mineralizing mature osteoblasts [7]. Additionally, calcein/tetracycline labeling has often been used to visualize mineral deposition in bone [8]. Thus, ALP is adequate to show the broad distribution of osteoblastic cells, while calcein labeling and PHOSPHO1 are hallmarks of mineral deposition and mineralizing mature osteoblasts, respectively.

The currently considered mechanisms of bone formation can be divided into two different histological manners: remodeling-based bone formation and modeling-based bone formation [9]. In sites of bone remodeling, such as secondary trabeculae, bone resorption and formation are well-coupled in a normal state. Therefore, many researchers have previously assumed that osteoclasts secrete osteoblast-activating molecules currently known as "coupling factors" to activate osteoblasts [10, 11]. Accordingly, it has been hypothesized that the presence of osteoclasts is essential for osteoblast activation [12-14]. This hypothesis was strengthened by the report that the anabolic effect of intermittent administration of parathyroid hormone (PTH) was not observed in osteoclast-deficient *c-fos*^{-/-} mice [15]. C-fos plays an important role in osteoclastogenesis at a very early stage during the differentiation of hematopoietic precursors into the osteoclast lineage [16-18]. *C-src*^{-/-} mice, in which osteoclasts are present but unable to resorb bone matrix due to a lack of ruffled borders, show many cement lines indicative of newly

formed bone on the old bone matrix, as well as cell-to-cell contact between the ruffled borderless osteoclasts and mature osteoblasts [19]. Therefore, remodeling-based bone formation appears to be influenced by the presence of osteoclasts rather than osteoclastic bone resorption.

In contrast to bone remodeling, bone modeling changes the size and shapes of bone, and is believed to be independent of osteoclastic activity, implying that bone formation and resorption are not coupled. For instance, the chondro-osseous junction (COJ) is the site of the longitudinal growth of the primary trabeculae, suggesting it as the site of bone modeling [2, 20, 21]. Osteoblasts in the bone modeling site may be activated by different mechanisms compared to bone remodeling, but these mechanisms are still under discussion.

Therefore, it would be valuable to clarify the histological differences in osteoblast activity between bone remodeling and bone modeling sites. This study aimed to determine whether modeling-based bone formation takes place in the primary trabeculae close to COJ, while remodeling-based bone formation occurs in the secondary trabeculae, by histochemically examining *c-fos*^{-/-} mice, which lack osteoclasts.

2. Materials and Methods

2.1. Animals and tissue preparation

Eight-week-old mice homozygous for gene depletion of *c-fos* and age-matched *wild-type* littermates (n = 6 per group; kindly provided by Dr. Udagawa from Matsumoto Dental University) were used in this study. All animal experiments were conducted following the Guidelines for Animal Experimentation of Hokkaido University and Matsumoto Dental University.

For bone labeling, mice were subcutaneously injected with a 0.1% calcein solution at a dose of 20 mg/kg, 72 and 24 h before euthanizing. The mice were fixed with 4% paraformaldehyde in a 0.1 M cacodylate buffer (pH 7.4) through the left ventricle under anesthesia induced by an intraperitoneal injection of a combination of anesthetic agents: 0.3 mg/kg of medetomidine, 4.0 mg/kg of midazolam, and 5.0 mg/kg of butorphanol. Right femora were removed and immediately immersed in the same fixative for 24 h. These specimens were then decalcified with 10% ethylenediaminetetraacetic disodium salt (EDTA-2Na) for 2 months at 4 °C before they were embedded in paraffin. Sagittal sections of 5 µm thickness were cut parallel to the longitudinal axis of the bone. All sections used for subsequent immunohistochemistry were photographed using a Nikon Eclipse E800 microscope (Nikon

Instruments Inc., Tokyo, Japan), and light microscopic images were acquired using a digital camera (Nikon DXM1200C, Nikon). For bone Villanueva staining, right tibiae were immersed in 70% ethanol for 18 h prior to being embedded in methyl methacrylate.

For ultrastructural observation using transmission electron microscope (TEM), left femora and tibiae were immediately immersed in a mixture containing 2% paraformaldehyde and 2.5% glutaraldehyde diluted in a 0.067 M cacodylate buffer (pH 7.4) for 24 h at 4 °C. Left tibiae were decalcified with 4.13% EDTA-2Na for 2 months at 4 °C, while femora were not decalcified. The femoral and tibial specimens were post-fixed with 1% OsO₄ for 8 h at 4 °C, dehydrated with ascending concentrations of acetone, and embedded in epoxy resin (Epon 812, TAAB, Berkshire, UK). Ultrathin sections obtained from decalcified specimens were prepared using an ultramicrotome (Sorvall MT-5000; Ivan Sorvall, Inc., Norwalk, CT) and stained with uranyl acetate and lead citrate for TEM observation (Hitachi H-7100, Hitachi Co., Tokyo, Japan) at an accelerating voltage of 80 kV.

2.2. Enzyme histochemistry for tartrate-resistant acid phosphatase (TRAP)

Tartrate-resistant acid phosphatase (TRAP) was detected as previously described [22]. Briefly, dewaxed paraffin sections were rinsed with PBS and incubated in a mixture of 2.5 mg of naphthol AS-BI phosphate (Sigma-Aldrich Co. LLC, St. Louis, MS), 18 mg of red violet LB (Sigma) salt, and 100 mM L (+) tartaric acid (0.76 g; Nacalai Tesque, Inc., Kyoto, Japan) diluted in 30 mL of a 0.1 M sodium acetate buffer (pH 5.0) for 15 min at 37 °C.

2.3. Immunohistochemistry for ALP, PHOSPHO1, ENPP1, RANKL, and RANK

Dewaxed paraffin sections were examined for ALP, PHOSPHO1, ENPP1, Receptor activator of NF- κ B (RANK), and RANK ligand (RANKL) as previously described [23]. In brief, the sections were immersed in 0.3% H₂O₂ in phosphate-buffered saline (PBS) for 30 min to block endogenous peroxidase activity. To reduce nonspecific binding, the sections were treated with 1% bovine serum albumin (Seologicals Proteins Inc. Kankakee, IL) in PBS (1% BSA-PBS) for 20 min. The sections were incubated with rabbit polyclonal antiserum against ALP [24] diluted at 1:300 or rabbit polyclonal anti-PHOSPHO1 (Q8TCT1; Cusabio Technology Llc., Houston, TX) diluted at 1:100 with 1% BSA-PBS at room temperature (RT) for 2 h and then incubated with HRP-conjugated goat anti-rabbit IgG (P0399; DakoCytomation, Glostrup, Denmark) at a dilution of 1:100 for 1 h at RT. For the detection of ENPP1, RANKL, and RANK,

the serial sections were reacted with goat anti-ENPP1 (EB06948; Everest Biotech Ltd., Oxfordshire, UK) at a dilution of 1:200, with goat anti-RANKL (AF462; R&D systems, Inc., Minneapolis, MN) at a dilution of 1:100, or with goat anti-RANK (AF692, R&D systems, Inc., Minneapolis, MN) at a dilution of 1:100 at RT for 2 h. Subsequently, the sections were incubated with HRP-conjugated anti-goat IgG (A201PS; American Qualex Scientific Products, Inc., San Clemente, CA) at a dilution of 1:100. The sections were treated with 3,3'-diaminobenzidine tetrahydrochloride to visualize the immunoreaction.

2.4. Von Kossa staining

Von Kossa staining was performed as described previously [25]. Briefly, undecalcified semi-thin epoxy resin sections were incubated with a 5% aqueous solution of silver nitrate (Wako Pure Chemical Industries Ltd) for 60 min at RT under sunlight until a dark brown color developed. The sections were then rinsed with distilled water and incubated with a 5% sodium thiosulfate solution (Wako Pure Chemical Industries) for 5 min. Subsequently, the sections were faintly stained with toluidine blue.

3. Results

3.1. Bone formation on the primary and secondary trabeculae in the femora of wild-type and *c-fos*^{-/-} mice

The femora of *wild-type* mice showed numerous trabeculae and bone marrow tissue in the metaphyses and diaphyses (**Fig. 1A**), whereas *c-fos*^{-/-} femora exhibited abundant trabeculae throughout the metaphyses and diaphyses (**Fig. 1C**). Unlike the *wild-type* mice (**Fig. 1B**), TRAP-positive osteoclasts were absent in *c-fos*^{-/-} mice (**Fig. 1D**).

In the metaphyseal primary trabeculae, calcein labeling indicative of bone mineral deposition was observed on the bone surface at the primary trabeculae near the COJ in both *wild-type* (**Fig. 1E, I**) and *c-fos*^{-/-} mice (**Fig. 1G, K**). *Wild-type* mice exhibited several calcein-positive regions on the secondary trabeculae (**Fig. 1F, J**), whereas no detectable calcein labeling was found in the secondary trabeculae of *c-fos*^{-/-} mice (**Fig. 1H, L**). Von Kossa staining revealed mineralized bone matrices extending from the COJ in both *wild-type* (**Fig. 1M**) and *c-fos*^{-/-} mice (**Fig. 1O**). In the region of the secondary trabeculae, *wild-type* mice showed mineralized trabeculae with smooth bone surfaces (**Fig. 1N**), while the *c-fos*^{-/-} mice exhibited irregularly scalloped bone surfaces (**Fig. 1P**). These observations indicate that, even in the absence of

osteoclasts, osteoblasts can synthesize mineralized bone matrix at the primary trabeculae adjacent to the COJ, which is a site of bone modeling-based bone formation, but not in the secondary trabeculae associated with remodeling-based bone formation.

3.2. Immunolocalization of ALP, PHOSPHO1, and ENPP1, and TEM observation on the osteoblasts of primary and secondary trabeculae

Next, we examined the distribution of osteoblasts positive for ALP, PHOSPHO1, and ENPP1 in both *wild-type* and *c-fos*^{-/-} mice. In the *wild-type* mice, ALP-positive osteoblasts were broadly distributed in the metaphyses (**Fig. 2A**), and PHOSPHO1-reactive osteoblasts were mainly found in the region of the primary trabeculae near the COJ, with shorter lines observed in the regions of the secondary trabeculae (**Fig. 2B**). In contrast, *c-fos*^{-/-} femora exhibited numerous small islets of ALP-positive osteoblasts within the metaphyseal trabeculae (**Fig. 2C**). Patchy islets of PHOSPHO1-reactive osteoblasts were present in the primary trabeculae near the COJ, and a considerable number of islets were observed in the secondary trabeculae of *c-fos*^{-/-} mice (**Fig. 2D**).

At higher magnification, mature osteoblasts located on the primary trabeculae displayed intense immunoreactivity for ALP and PHOSPHO1, and slight immunoreactivity for ENPP1 in the *wild-type* mice (**Fig. 2E, F, M**). *C-fos*^{-/-} mice also exhibited immunoreactivity for ALP and PHOSPHO1, with relatively weak ENPP1 immunoreactivity (**Fig. 2G, H, O**). In contrast, in the secondary trabeculae, *wild-type* mice showed osteoblasts immunopositive for ALP and PHOSPHO1, but with little ENPP1 reactivity (**Fig. 2I, J, N**). Conversely, *c-fos*^{-/-} mice exhibited very weak ALP signal and minimal PHOSPHO1 and ENPP1 reactivities (**Fig. 2K, L, P**).

When observed at higher resolution using epoxy resin-embedded specimens and ultrathin sections using TEM, cuboidal mature osteoblasts with well-developed rough endoplasmic reticulum (rER) were observed on the primary trabeculae in both *wild-type* (**Fig. 3A, C**) and *c-fos*^{-/-} mice (**Fig. 3B, D**). In contrast, the secondary trabeculae of *wild-type* mice displayed plump osteoblasts with well-developed rER (**Fig. 3E, G**), while the *c-fos*^{-/-} trabeculae showed flattened osteoblast-like cells with few rER (**Fig. 3F, H**).

3.3. Immunolocalization of RANKL and RANK on the primary and secondary trabeculae

Finally, we examined the immunolocalization of RANKL and RANK, which are known to play a pivotal role in the interaction between osteoblasts and osteoclasts under normal conditions.

The immunoreactivity of RANKL was observed on the osteoblasts located on the primary trabeculae in both *wild-type* and *c-fos*^{-/-} mice (**Fig. 4A, C**). In the secondary trabeculae, the RANKL immunoreactivity was much weaker in *c-fos*^{-/-} mice compared to *wild-type* mice (**Compare Fig. 4E, G**). In contrast to RANKL, RANK-positive osteoclasts were present in the primary trabeculae of *wild-type* mice but absent in *c-fos*^{-/-} mice (**Fig. 4B, D**). Although *wild-type* mice displayed RANK-positive osteoclasts on the secondary trabeculae, *c-fos*^{-/-} mice did not exhibit detectable RANK-positive osteoclasts (**Fig. 4F, H**).

4. Discussion

In this study, we demonstrated the differential osteoblastic activity at the chondro-osseous junction and in the secondary metaphyseal trabeculae of femora from *wild-type* mice and *c-fos*^{-/-} mice. Our findings indicate that even in the absence of osteoclasts, osteoblasts are activated to undergo modeling-based bone formation.

To assess osteoblastic activity in bone mineralization, we utilized immunohistochemistry for ALP and PHOSPHO1, which are enzymes involved in providing monophosphate ions during bone mineralization. ALP synthesizes monophosphate ions from pyrophosphate and is expressed on the cell membrane of preosteoblasts and osteoblasts [26-31]. On the other hand, PHOSPHO1 is an enzyme that supplies monophosphate ions within matrix vesicles, suggesting that PHOSPHO1-positive osteoblasts induce matrix vesicle-mediated mineralization in bone [32-34]. This study revealed the deposition of calcein labeling and strong immunoreactivities of ALP and PHOSPHO1 on plump osteoblasts in the primary trabeculae, not only in *wild-type* mice but also in *c-fos*^{-/-} mice. These histological findings suggest activated osteoblastic bone formation even in the absence of osteoclasts in the bone modeling region.

In contrast, in the secondary trabeculae, where remodeling-based bone formation occurs, osteoblasts in *c-fos*^{-/-} mice appeared flattened and showed less intense immunoreactivity of ALP, with no immunoreaction of PHOSPHO1 compared to *wild-type* mice. Consistently, calcein labeling indicative of mineralization in bone was not observed in the corresponding area. These findings suggest that osteoblasts at bone remodeling sites may require the presence of osteoclasts to be actively involved in bone formation.

The RANK/RANKL signaling pathway plays a pivotal role in osteoclast differentiation [35]. In this study, histochemical analyses indicated that *c-fos*^{-/-} mice did not exhibit RANK-reactive cells, suggesting that c-fos signaling acts in the early stage of hematopoietic cells

before RANK/RANKL signaling and that no osteoclast progenitor cells are present. A recent study has reported that vesicular RANK, produced by osteoclasts, binds to RANKL in osteoblasts and promotes bone formation by activating RANKL reverse signaling [36]. According to their findings on the reverse signaling between RANK and RANKL [36], RANKL expression may also be reduced in the absence of RANK expression. However, many RANKL-positive osteoblasts were observed in the primary trabeculae of *c-fos*^{-/-} mice, as shown in **Fig. 4C**. We hypothesize that osteoblastic activity, including RANKL expression in the *c-fos*^{-/-} primary trabeculae, may be induced by other factors that is involved in bone remodeling. Therefore, osteoblasts in the *c-fos*^{-/-} primary trabeculae near the chondro-osseous junction appear to be activated even in the absence of osteoclasts or the reverse signaling between RANK and RANKL.

5. Conclusions

In conclusion, our findings suggest that osteoblasts in the bone modeling region may be activated through different mechanisms at bone remodeling region. At the sites of modeling-based bone formation, osteoblastic activities and bone formation are maintained even in the absence of osteoclasts.

Funding

This study was partially supported by the Grants-in Aid for Scientific Research of Japan Society for the Promotion of Science (JSPS, 19K10040, 22K09911 to Hasegawa T).

Ethical statement

The study followed the principles for animal care and research use set by Hokkaido University.

Authors' contributions

Yamamoto T and Abe M are the main researchers and equally contributed to this work, specifically in conducting immunohistochemistry and TEM observation experiments. Hasegawa T served as the project leader, organizing collaborators and providing the overall research framework. Udagawa N provided the *c-fos*^{-/-} mice. Hongo H, Maruoka H, Yoshino H, and Haraguchi-Kitakamae M contributed to the experimental work, including the preparation

of paraffin samples. Amizuka N participated in the discussions and assisted in the preparation of the manuscript. All authors have read and approved the final version of the manuscript.

Conflict of interest

No potential conflicts of interest exist.

References

1. Anderson HC. Vesicles associated with calcification in the matrix of epiphyseal cartilage. *J Cell Biol* 1969; 41: 59–72.
2. Ozawa H, Hoshi K, Amizuka N. Current concepts of bone mineralization. *J Oral Biosci* 2008; 50(1): 1–14.
3. Hasegawa T, Yamamoto T, Tsuchiya E, Hongo H, Tsuboi K, Kudo A, et al. Ultrastructural and biochemical aspects of matrix vesicle-mediated mineralization. *Jpn Dent Sci Rev* 2017; 53(2): 34–45.
4. Hasegawa T. Ultrastructure and biological function of matrix vesicles in bone mineralization. *Histochem Cell Biol* 2018; 149(4): 289–304.
5. Hasegawa T, Hongo H, Yamamoto T, Abe M, Yoshino H, Haraguchi-Kitakamae M, et al. Matrix Vesicle-Mediated Mineralization and Osteocytic Regulation of Bone Mineralization. *Int J Mol Sci* 2022; 23(17): 9941.
6. Yamamoto T, Hasegawa T, Mae T, Hongo H, Yamamoto T, Abe M, et al. Comparative immunolocalization of tissue nonspecific alkaline phosphatase and ectonucleotide pyrophosphatase/phosphodiesterase 1 in murine bone. *J Oral Biosci* 2021; 63(3): 259–264.
7. Mae T, Hasegawa T, Hongo H, Yamamoto T, Zhao S, Li M, et al. Immunolocalization of enzymes/membrane transporters related to bone mineralization in the metaphyses of the

- long bones of parathyroid-hormone-administered mice. *Medicina (Kaunas)* 2023; 59(6): 1179.
8. Parfitt AM, Drezner MK, Glorieux FH, Kanis JA, Malluche H, Meunier PJ, et al. Bone histomorphometry: standardization of nomenclature, symbols, and units. Report of the ASBMR Histomorphometry Nomenclature Committee. *J Bone Miner Res* 1987; 2: 595–610.
 9. Dempster DW, Zhou H, Recker RR, Brown JP, C Recknor CP, Lewiecki EM, et al. Remodeling- and modeling-based bone formation with teriparatide versus denosumab: A longitudinal analysis from baseline to 3 months in the AVA study. *J Bone Miner Res* 2018; 33(2):298–306.
 10. Frost HM. Tetracycline-based histological analysis of bone remodeling. *Calcif Tissue Res* 1969; 3: 211–237.
 11. Hattner R, Epker BN, Frost HM. Suggested sequential mode of control of changes in cell behaviour in adult bone remodelling. *Nature* 1965; 206: 489–490.
 12. Nakamura M, Udagawa N, Matsuura S, Mogi M, Nakamura H, Horiuchi H, et al. Osteoprotegerin regulates bone formation through a coupling mechanism with bone resorption. *Endocrinology* 2003; 144(12): 5441–5449.
 13. Sims NA, Jenkins BJ, Quinn JM, Nakamura A, Glatt M, Gillespie MT, et al. Glycoprotein 130 regulates bone turnover and bone size by distinct downstream signaling pathways. *J Clin Invest* 2004; 113(3): 379–389.
 14. Martin TJ, Sims NA. Osteoclast-derived activity in the coupling of bone formation to resorption. *Trends Mol Med* 2005; 11(2): 76–81.
 15. Luiz de Freitas PH, Li M, Ninomiya T, Nakamura M, Ubaidus S, Oda K, et al. Intermittent PTH administration stimulates pre-osteoblastic proliferation without leading to enhanced

- bone formation in osteoclast-less *c-fos*(-/-) mice. *J Bone Miner Res* 2009; 24(9): 1586–1597.
16. Wang ZQ, Ovitt C, Grigoriadis AE, Mohle-Steinlein U, Ruther U, Wagner EF. Bone and haematopoietic defects in mice lacking *c-fos*. *Nature* 1992; 360: 741–745.
 17. Grigoriadis AE, Wang ZQ, Cecchini MG, Hofstetter W, Felix R, Fleisch HA, et al. *c-Fos*: a key regulator of osteoclast-macrophage lineage determination and bone remodeling. *Science* 1994; 266: 443–448.
 18. Felix R, Hofstetter W, Cecchini MG. Recent developments in the understanding of the pathophysiology of osteopetrosis. *Eur J Endocrinol* 1996; 134: 143–156.
 19. Toraya H, Hasegawa T, Sakagami N, Tsuchiya E, Kudo A, Zhao S, et al. Histochemical assessment for osteoblastic activity coupled with dysfunctional osteoclasts in *c-src* deficient mice. *Biomed Res* 2017; 38(2): 123–134.
 20. Amizuka N, Hasegawa T, Oda K, Luiz de Freitas PH, Hoshi K, Li M, et al. Histology of epiphyseal cartilage calcification and endochondral ossification. *Front Biosci* 2012; 4: 2085–2100.
 21. Hasegawa T. Ultrastructure and biological function of matrix vesicles in bone mineralization. *Histochem Cell Biol*. 2018; 149(4): 289–304.
 22. Hasegawa T, Yamamoto T, Sakai S, Miyamoto Y, Hongo H, Qiu Z, et al. Histological effects of the combined administration of eldecacitol and a parathyroid hormone in the metaphyseal trabeculae of ovariectomized rats. *J Histochem Cytochem* 2019; 67(3):169–184.
 23. Yamamoto T, Hasegawa T, Sasaki M, Hongo H, Tsuboi K, Shimizu T, et al. Frequency of teriparatide administration affects the histological pattern of bone formation in young adult

- male mice. *Endocrinology* 2016; 157: 2604–2620.
24. Oda K, Amaya Y, Fukushi-Irie M, Kinameri Y, Ohsuye K, Kubota I, et al. A general method for rapid purification of soluble versions of glycosylphosphatidylinositol-anchored proteins expressed in insect cells: An application for human tissue-nonspecific alkaline phosphatase. *J Biochem* 1999; 126: 694–699.
 25. Hasegawa T, Tokunaga S, Yamamoto T, Sakai M, Hongo H, Kawata T, et al. Evocalcet rescues secondary hyperparathyroidism-driven cortical porosity in CKD male rats. *Endocrinology* 2023; 164(4): bqad022.
 26. Matsuzawa T, Anderson HC. Phosphatases of epiphyseal cartilage studied by electron microscopic cytochemical methods. *J Histochem Cytochem* 1971; 19: 801–808.
 27. de Bernard B, Bianco P, Bonucci E, Costantini M, Lunazzi GC, Martinuzzi P, et al. Biochemical and immunohistochemical evidence that in cartilage an alkaline phosphatase is a Ca^{2+} -binding glycoprotein. *J Cell Biol* 1986; 103: 1615–1623.
 28. Waymire KG, Mahuren JD, Jaje JM, Guilarte TR, Coburn SP, MacGregor GR. Mice lacking tissue nonspecific alkaline phosphatase die from seizures due to defective metabolism of vitamin B-6. *Nat Genet* 1995; 11:45–51.
 29. Hoshi K, Amizuka N, Oda K, Ikehara Y, Ozawa H. Immunolocalization of tissue nonspecific alkaline phosphatase in mice. *Histochem Cell Biol* 1997; 107: 183–191.
 30. Anderson HC, Sipe JB, Hessle L, Dhanyamraju R, Atti E, Camacho NP, et al. Impaired calcification around matrix vesicles of growth plate and bone in alkaline phosphatase-deficient mice. *Am J Pathol* 2004; 164: 841–847.
 31. Murshed M, Harmey D, Millán JL, McKee MD, Karsenty G. Unique coexpression in osteoblasts of broadly expressed genes accounts for the spatial restriction of ECM mineralization to bone. *Genes Dev* 2005; 19: 1093–1104.

32. Stewart AJ, Roberts SJ, Seawright E, Davey MG, Fleming RH, Farquharson C. The presence of PHOSPHO1 in matrix vesicles and its developmental expression prior to skeletal mineralization. *Bone* 2006; 39: 1000–1007.
33. Roberts S, Narisawa S, Harmey D, Millán JL, Farquharson C. Functional involvement of PHOSPHO1 in matrix vesicle-mediated skeletal mineralization. *J Bone Miner Res* 2007; 22(4): 617–627.
34. Yadav MC, Bottini M, Cory E, Bhattacharya K, Kuss P, Narisawa S, et al. Skeletal mineralization deficits and impaired biogenesis and function of chondrocyte-derived matrix vesicles in *Phospho1*($-/-$) and *Phospho1*/*Pit1* double-knockout mice. *J Bone Miner Res* 2016; 31(6):1275–1286.
35. Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinosaki M, Mochizuki S, et al. Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proc Natl Acad Sci USA* 1998; 95(7):3597–3602.
36. Ikebuchi, Y. Aoki S, Honma M, Hayashi M, Sugamori Y, Khan M, et al. (2018) Coupling of bone resorption and formation by RANKL reverse signalling. *Nature* 2018; 561: 195–200.

Figure legends

Figure 1. TRAP staining, calcein labeling, and von Kossa staining in the femora of *wild-type* and *c-fos* $^{-/-}$ mice

H-E staining displays trabecular bones extending from the growth plate (GP) in the *wild-type* femur (panel A), whereas *c-fos* $^{-/-}$ femur exhibited abundant trabeculae throughout the metaphysis (meta) and diaphysis (dia; panel C). Despite the presence of TRAP-positive osteoclasts in *wild-type* mice (red, panel B), *c-fos* $^{-/-}$ mice lacked TRAP-positive osteoclasts

(panel D). Panels E–L are calcein labeling in the metaphyseal primary trabeculae (E, G, I, K) and the secondary trabeculae (F, H, J, L) of the *wild-type* (E, F, I, J) and *c-fos*^{-/-} mice (G, H, K, L). Panels I–L are highly-magnified versions of images shown in panels E–H, respectively. In the metaphyseal primary trabeculae, at a higher magnification, calcein labeling was observed in the newly-formed bone in both *wild-type* (green, arrows in panel I) and *c-fos*^{-/-} mice (green, arrows in panel K). *Wild-type* mice also exhibited calcein labeling in the secondary trabeculae close to the diaphysis (arrows in panel J), whereas no detectable calcein labeling was observed in the corresponding area in *c-fos*^{-/-} mice (panel L). Von Kossa staining demonstrates well mineralization as indicated by the black color in both the primary and secondary trabeculae of *wild-type* (panels M–N) and *c-fos*^{-/-} mice (panels O–P). Scale bars, 500 μm (A–D), 100 μm (E–H), and 30 μm (I–P).

Figure 2. Immunolocalization of ALP, PHOSPHO1, and ENPP1 in the primary trabeculae of *wild-type* and *c-fos*^{-/-} mice

ALP and PHOSPHO1 immunohistochemistry of the femora of *wild-type* (panels A, B) and *c-fos*^{-/-} mice (panels C, D). Panels E–L are highly-magnified images of A–D; the images of the primary trabeculae (panels E–H) and the secondary trabeculae (panels I–L) were derived from the femora of the *wild-type* and *c-fos*^{-/-} mice. In *wild-type* mice, many ALP-positive cells (brown) were observed on both the primary and secondary trabeculae (panels A, E, I), while PHOSPHO1 reactivity was mainly seen in the region of the primary trabeculae rather than the secondary trabeculae close to the diaphysis (panels B, F, J). Some linear PHOSPHO1 immunoreactivity on the secondary trabeculae indicated plump mature osteoblasts (B, arrow in J). In contrast, in *c-fos*^{-/-} mice, many small islets indicative of ALP (brown) were scattered throughout the femur (panel C), and ALP-positive osteoblasts appeared cuboidal in shape in the primary trabeculae (panel G), but very flattened in the secondary trabeculae (arrow in panel K). Mature PHOSPHO1-reactive osteoblasts were found on the *c-fos*^{-/-} primary trabeculae (panels D, H); however, PHOSPHO1-positive cells could be hardly seen on the *c-fos*^{-/-} secondary trabeculae (panel L). In contrast to ALP and PHOSPHO1, ENPP1 immunoreactivity was faint in osteoblasts of the primary trabeculae (panels M, O), but not in the secondary trabeculae (panels N, P) of *wild-type* (panels M, N) and *c-fos*^{-/-} mice (panels O, P). Meta, metaphysis; epi, epiphysis; dia, diaphysis.

Scale bars, 100 μm (A–D) and 30 μm (E–P).

Figure 3. Light microscopic images with high resolution and TEM observations of osteoblasts in the *wild-type* and *c-fos*^{-/-} mice

Toluidine blue staining demonstrates numerous cuboidal osteoblasts, indicative of the active form of osteoblasts (ob), located on the bone surface in the primary trabeculae of *wild-type* (panel A) and *c-fos*^{-/-} mice (panel B). TEM images reveal the development of rough endoplasmic reticulum (rER) in these osteoblasts in both *wild-type* (panel C) and *c-fos*^{-/-} specimens (panel D). In contrast, the secondary trabeculae of *c-fos*^{-/-} mice exhibit only flattened osteoblasts (panel F), whereas the *wild-type* secondary trabeculae exhibit plump osteoblasts (panel E). TEM observation demonstrates abundant rER in the cuboidal, mature osteoblasts in the *wild-type* mice (panel G), but only a few rER in osteoblast-like cells in the *c-fos*^{-/-} mice (panel H). BV, blood vessels.

Scale bars, 15 μ m (A, B, E, F) and 2 μ m (C, D, G, H).

Figure 4. Immunolocalization of RANKL and RANK in the femur of *wild-type* and *c-fos*^{-/-} mice

In the *wild-type* mice, RANKL-positive osteoblasts (ob; brown in panels A, E) and RANK-reactive osteoclasts (oc; brown in panels B, F) are observed in the primary and secondary trabeculae. *C-fos*^{-/-} specimens exhibit an intense immunoreactivity on osteoblasts in the primary trabeculae (panel C) and weak RANKL-reactive osteoblasts in the secondary trabecular bone (panel G). In contrast, no RANK-immunoreactive cells are seen in the primary (panel D) and secondary trabeculae (panel H) of *c-fos*^{-/-} mice.

Scale bars, 40 μ m.

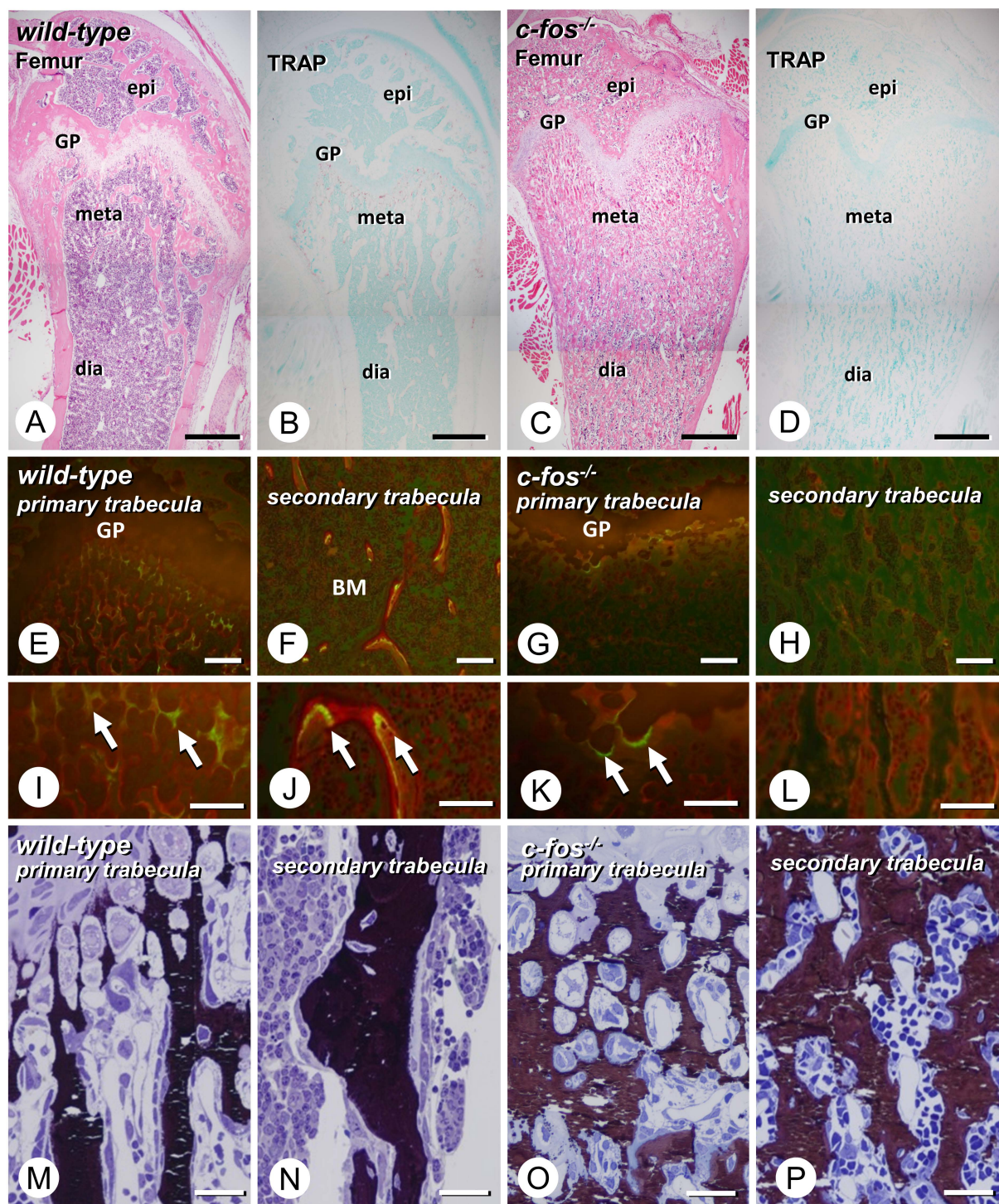


Fig. 1

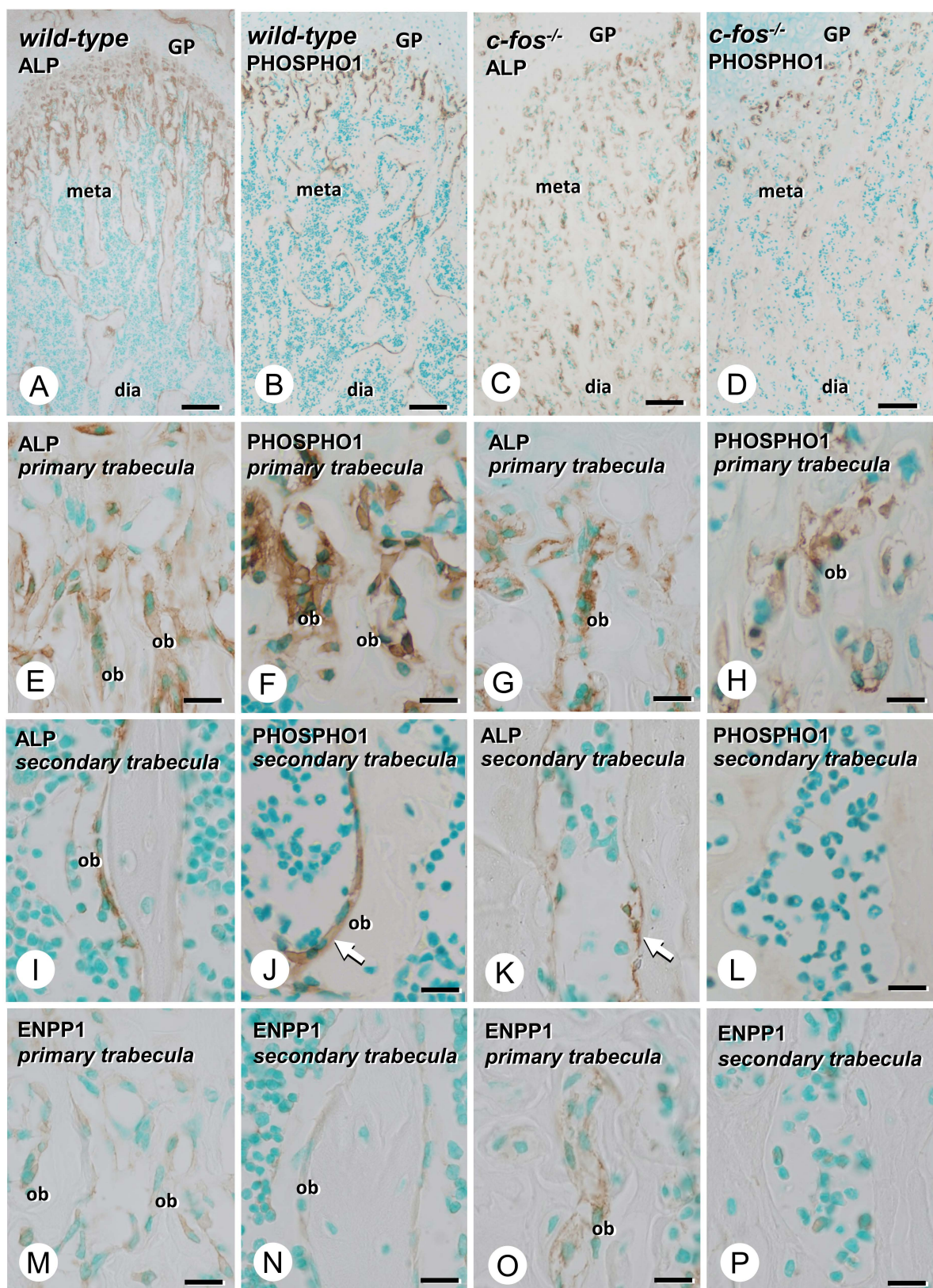


Fig. 2

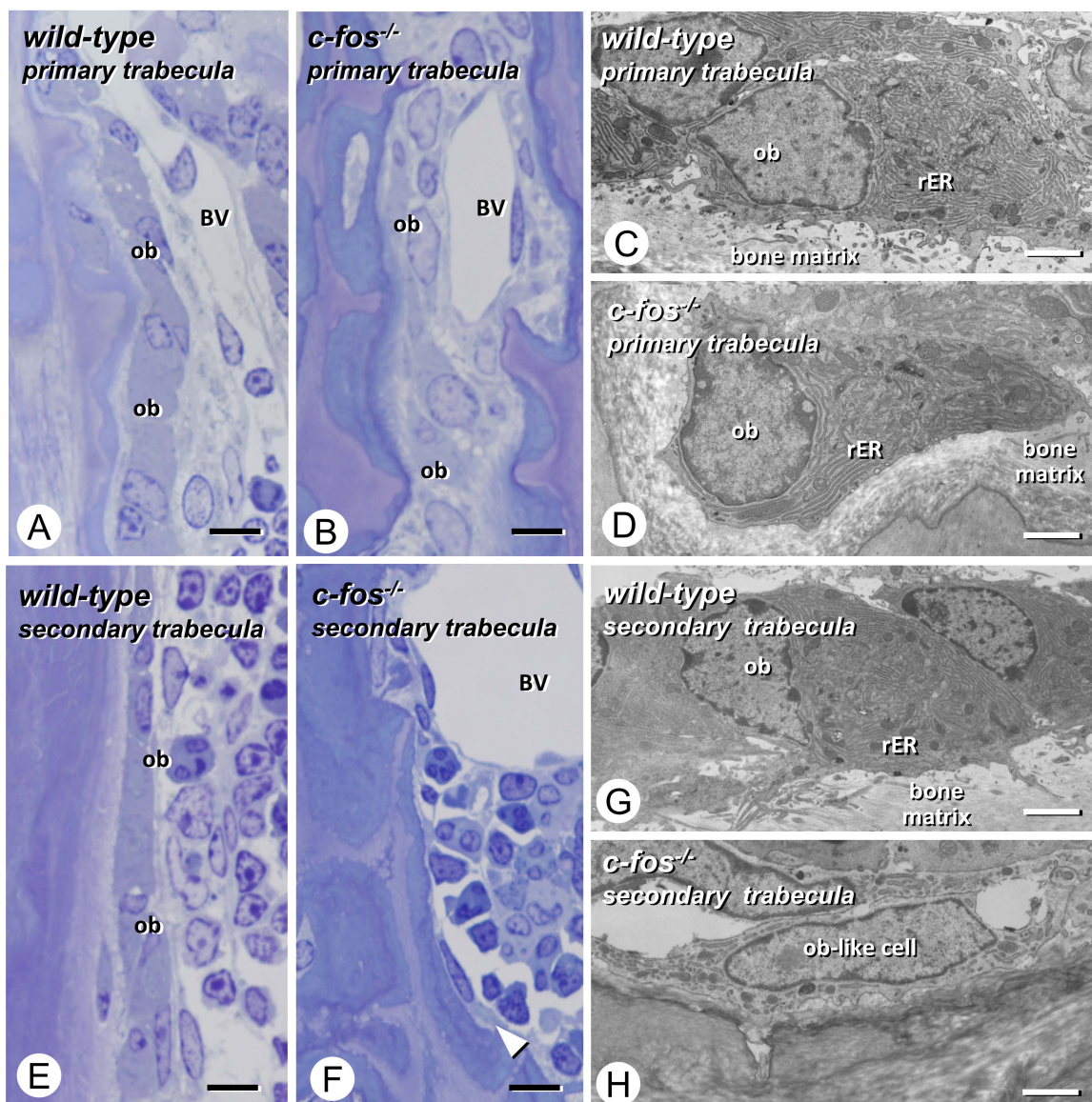


Fig. 3

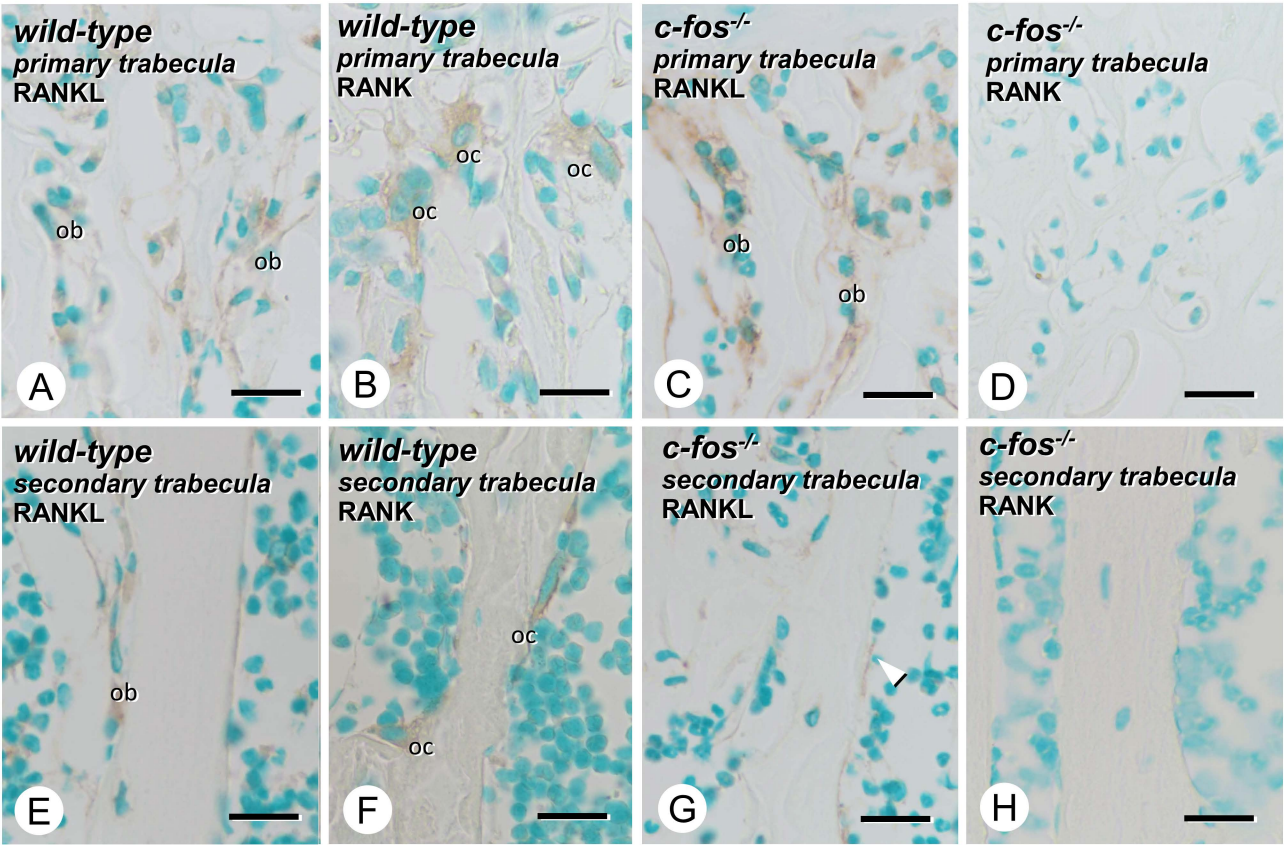


Fig. 4