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Supplementary Materials for

Osteoblastic lysosome plays a central role in mineralization

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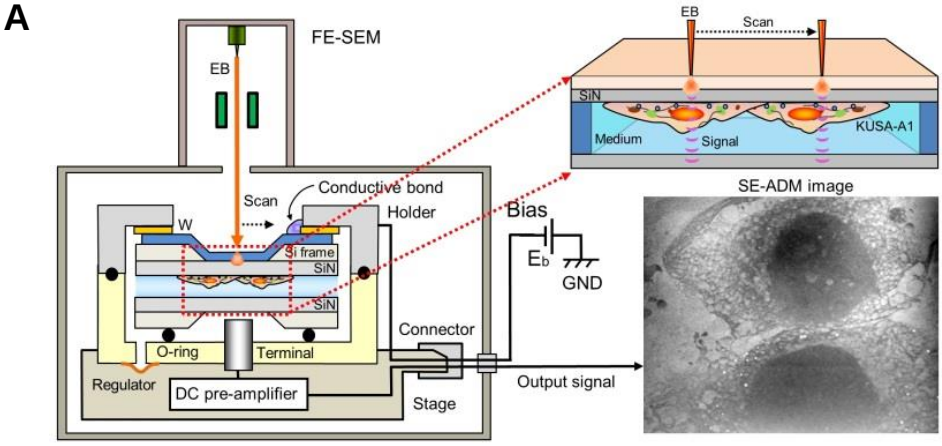
The PDF file includes:

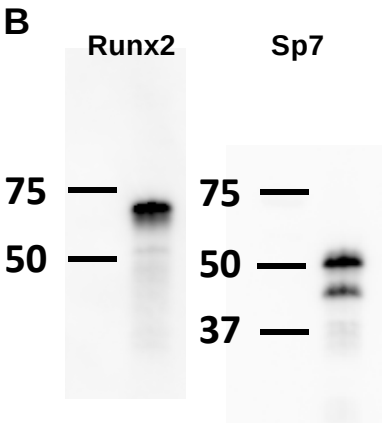
Other Supplementary Material for this manuscript includes the following:

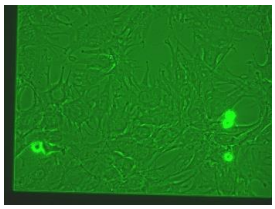
(available at advances.sciencemag.org/cgi/content/full/5/7/eaax0672/DC1)

Movie S1 (.mp4 format). Time-lapse SRM movie of calcein labeled osteoblasts.

Fig.S1


A
FE-SEM
EB
Scan
Conductive bond
Holder
W
Si frame
SiN
SiN
O-ring
Terminal
Connector
Regulator
DC pre-amplifier
Stage
Bias
E_b
GND
Output signal
EB
Scan
SiN
Medium
Signal
KUSA-A1
SE-ADM image


B
Runx2
Sp7
75
50
75
50
37


D


C
Osteogenic day3
Normal
Osteogenic

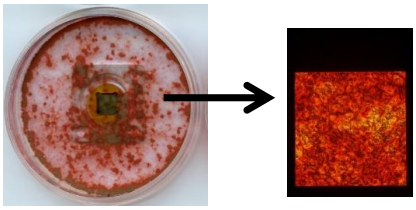

E

Fig. S1. SE-ADM observation of osteoblasts.
(A) High-resolution SE-ADM set up for osteoblast observation. Liquid-sample holder including osteoblasts is mounted on the pre-amplifier-attached stage, which is introduced into the specimen SEM chamber. The scanning electron beam is applied to the W-coated SiN film at a low acceleration voltage. Measurement terminal under the holder detects electrical signals through liquid specimens. Clear intracellular structures are visible (right image). (B) Expression of osteoblast transcription factors confirmed through Western blotting. Predicted molecular size for Runx2 and Sp7 are 55–62 and 46 kDa, respectively. The KUSA-A1 cells stably expressed these transcription factors without any stimulation. (C) Calcified nodule formation by osteoblasts. Cells were cultured with or without osteogenic medium. Osteoblasts produced calcified nodules when cultured in osteogenic media. Representative Alizarin Red S staining of 3-day culture in osteogenic media. (D) Representative phase-contrast image of osteoblasts cultured on SiN film. (E) Representative Alizarin Red S staining of osteoblasts cultured in SE-ADM culture dish holder. The center of the holder showed SiN film with cells positive for staining.

Fig.S2

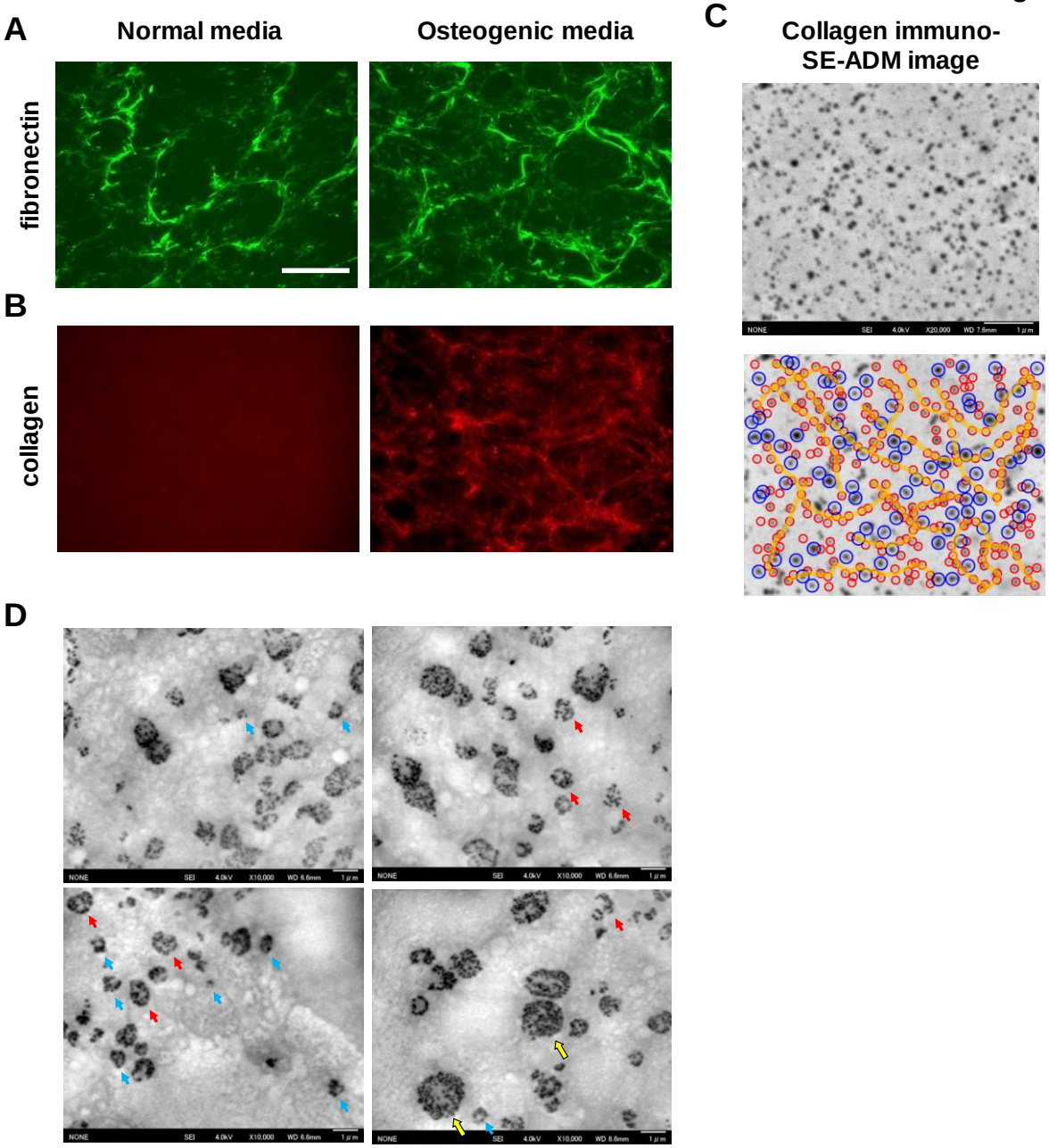
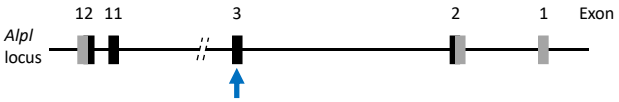

A
Normal media
Osteogenic media
fibronectin
B
collagen
C
Collagen immuno-
SE-ADM image
D

Fig. S2. Extracellular matrix analysis and SE-ADM observation of osteoblasts.

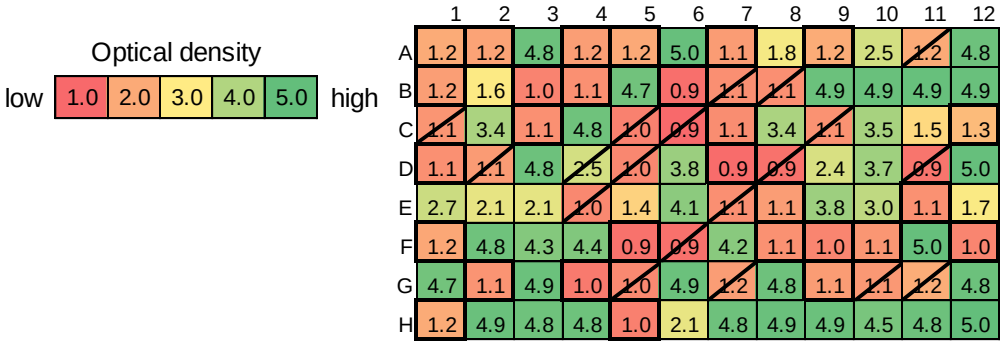
(A, B) Immunocytochemistry analysis of fibronectin (green) and type I collagen (red). Cells were cultured with or without osteogenic medium. More fibrous network of both extracellular matrices was evident when cells were cultured in osteogenic media. (C) SE-ADM image of osteoblasts particles and collagen immune-labeled 60 nm gold colloid. Cells were stained with anti-mouse collagen antibody and 60 nm gold colloid-conjugated anti-rabbit IgG and observed by SE-ADM. Image analysis of osteoblast particles and 60 nm gold colloid-conjugated collagen. The blue and red circles were osteoblasts particles and 60 nm gold colloids, respectively. The 60-nm gold colloids were connected, which may exhibit collagen fibers (orange lines), and the osteoblast particles localized between the fibers. (D) Representative high-resolution SE-ADM images. High-resolution MVBs images of four different areas were obtained using cells cultured in osteogenic medium for 7 h at 10,000 magnifications. MVBs of various sizes were dispersed in a whole cell area. Yellow arrows indicate large MVBs shown in Fig. 1G. Blue and red arrows indicate various size of MVBs shown in Fig. 1H and I. Scale bars represent 50 μm in (A, B).

Fig. S3

A



B



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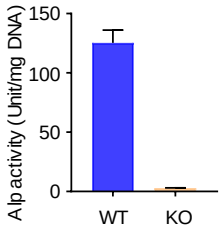
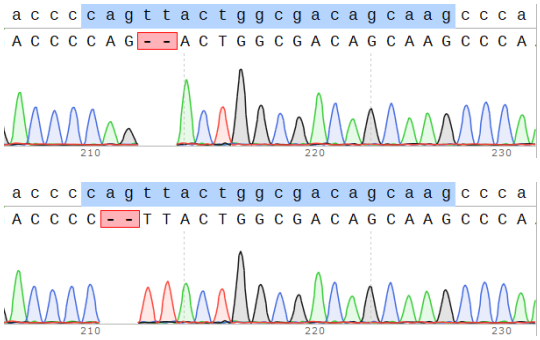


D



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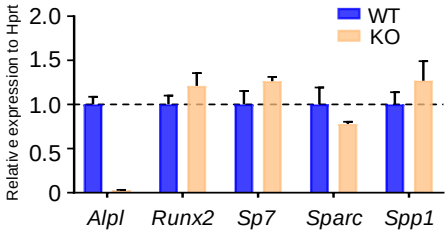

F

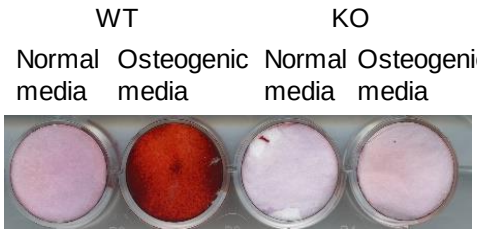

Fig. S3. Generation and characterization of *Alpl* knockout osteoblasts.

(A) Schematic illustrating the *Alpl* gene. gRNA targeting exon 3 was used for CRISPR-Cas9-mediated KO of *Alpl*. (B) Screening of *Alpl* KO clones by measuring supernatant alkaline phosphatase activity. Raw data of absorbance for each well of a 96-well plate are shown; wells without colonies labeled with diagonal line. Higher value (green) suggested high alkaline phosphatase activity, whereas lower value (red) without a diagonal line suggested the well contained an *Alpl* KO clone. (C) Measurement of intracellular alkaline phosphatase activity of wildtype (WT) and *Alpl* KO cells. Activity was completely diminished in *Alpl* KO cells. N = 3 replicates per cell line. Error bars represent mean ± S.D. (D) Sequence analysis of one *Alpl* KO clone. Two base deletions in each allele of *Alpl* were detected. (E) Baseline expressions of osteoblast differentiation genes. Quantitative reverse transcription polymerase chain reaction to compare osteoblastic mRNA expression without culture in osteogenic media. N = 3 replicates per cell line. Error bars represent mean ± S.D. (F) Calcified nodule formation, on Alizarin Red S staining. WT cells and *Alpl* KO cells were cultured with or without osteogenic medium for 5 days. WT osteoblasts produced calcified nodules when cultured in osteogenic media. In contrast, *Alpl* KO cells produced no calcified nodules.

Fig. S4

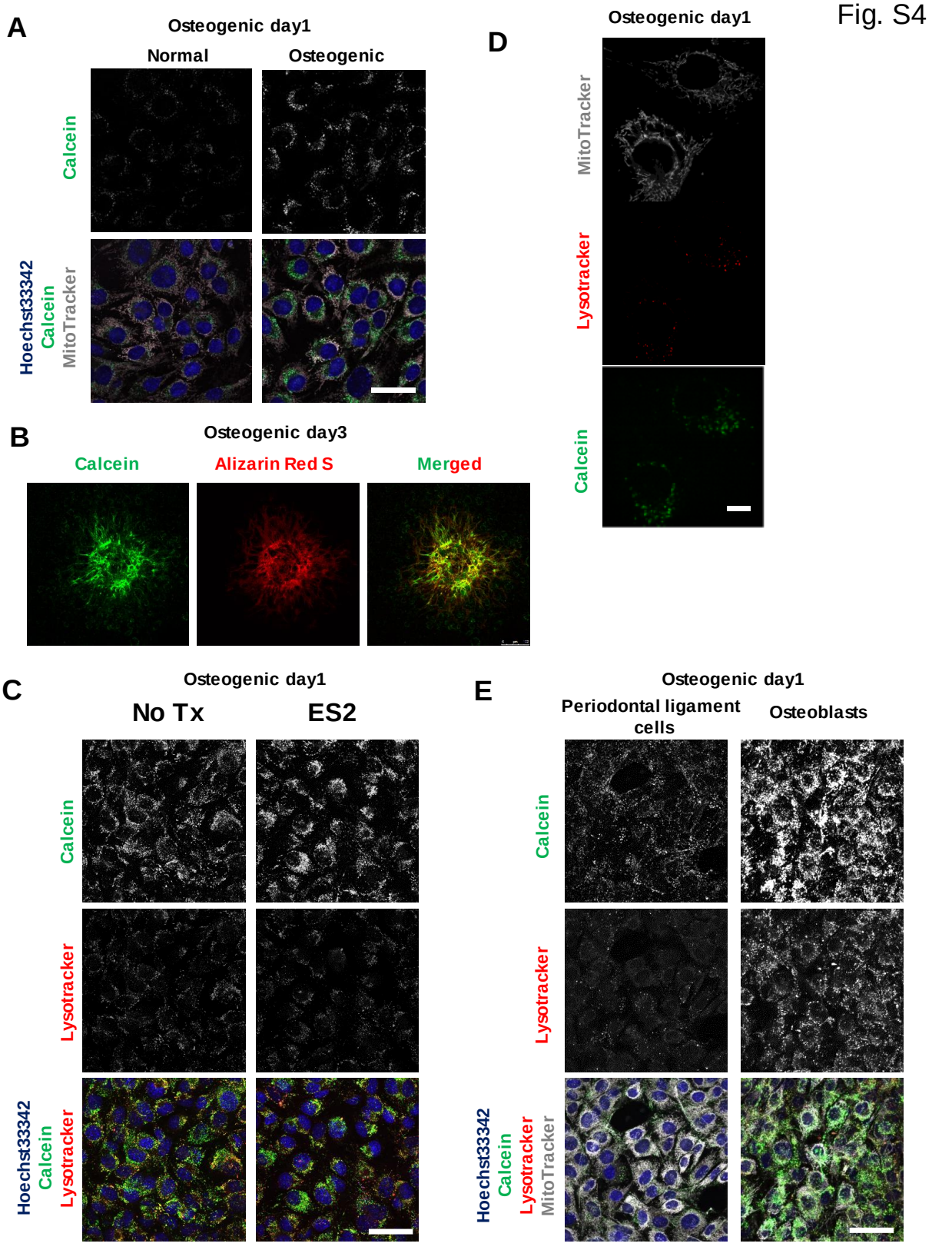
A
Osteogenic day1
Normal
Osteogenic
Calcein
Hoechst33342
Calcein
MitoTracker
B
Osteogenic day3
Calcein
Alizarin Red S
Merged
C
Osteogenic day1
No Tx
ES2
Calcein
Lysotracker
Hoechst33342
Calcein
Lysotracker
D
Osteogenic day1
MitoTracker
Lysotracker
Calcein
E
Osteogenic day1
Periodontal ligament cells
Osteoblasts
Calcein
Lysotracker
Hoechst33342
Calcein
Lysotracker
MitoTracker

Fig. S4. Lysosomal exocytosis inhibitor blocked secretion of calcein-positive vesicles.

(A) Calcein labeling of osteoblasts in normal or osteogenic media. Cultured cells were stained with organelle dyes. Representative confocal fluorescent images. (B) Representative images of Alizarin Red S staining without fixation. The cells were cultured in osteogenic medium with calcein for 3 days and stained with Alizarin Red S solution. Calcein-positive calcified nodules were visualized under confocal microscopy. (C) Representative confocal images of 40 nM ES2-treated osteoblasts. The cells were cultured in osteogenic media containing ES2 for 1 day and stained with Hoechst 33342, Lysotracker, and Mitotracker. The treated cells showed intracellular accumulation of calcein signals and lysosomes. (D) Representative 3D-SIM image by SpinSR10 spinning disk confocal super resolution microscope. Cells were cultured in osteogenic medium with calcein for 1 day and stained with Lysotracker and Mitotracker. (E) Representative confocal images of primary mouse periodontal ligament cells with osteogenic potential. The cells were cultured in osteogenic medium with calcein for 1 day and stained with Lysotracker, Mitotracker, and Hoechst 33342. Scale bars represent 50 μm in (A, C, E), 10 μm in (D). Tx, treatment.