Ezh2 knockout in mesenchymal cells causes enamel hyper-mineralization

Yoshifumi Kobayashi, Angela Quispe-Salcedo, Sanika Bodas, Satoko Matsumura, Erhao Li, Richard Johnson, Siva Nadimpalli, Marwa Choudhury, Daniel H. Fine, Amel Dudakovic, Andre J. van Wijnen, and Emi Shimizu

Supplementary protocols

Tissue preparation

Maxillae were collected *en bloc* from 3-week-old mice and were immersed in 10% neutral buffered formalin overnight at 4°C. Following demineralization in 10% EDTA solution for 5~6 weeks at 4°C, the samples were dehydrated through a series of ethanol washes, embedded in paraffin, and cut into 4 μm sagittal sections using a microtome (CUT 4055, Olympus, USA). The paraffin sections were mounted on coated glass slides (Fisherbrand, Superfrost Plus, Fisher Scientific, Pittsburgh, PA, USA) and were processed for hematoxylin and eosin staining (H&E, Sigma-Aldrich, St. Louis, Missouri, USA).

Scanning electron microscopy (SEM)

Tissue preparation and embedding of samples were performed according to the protocols and recommendations of Goldman et al. [1]. Briefly, all the fixed, dehydrated maxillae were mounted in self-curing EPON resin and cross-sectioned using a diamond saw at the eruption site of incisors, followed by polishing and etching with 1% nitric acid for 45 seconds. Images were captured using a variable pressure SEM (Carl Zeiss Supra 35VP, Oberkochen, Germany) at 20 kV, without sputter coat.

Histology

To prepare paraffin sections, mice maxillae were fixed in 4% paraformaldehyde and were decalcified in 10% EDTA. After paraffin embedding, the samples were sliced longitudinally. To prepare frozen sections, the heads were fixed in 4% paraformaldehyde and decalcified in 10% EDTA. Following embedding, the samples were cut sagittally. Immunohistochemical staining was performed using the ABC staining system (Santa Cruz Biotechnology), according to the manufacturer's instructions. The sections were incubated with anti-ameloblastin (sc-50534, Santa Cruz Biotechnology) or anti-KLK4 (ab71234, Abcam) antibodies at 4°C, followed by labelling with HRP-conjugated anti-IgG antibody. Staining was completed following a 5-min incubation with 3,3'-diaminobenzidine (DAB).

Quantitative Real-Time PCR

Total RNA from cultured cells or lower incisors was isolated using the TRIzol (Thermo Fisher Scientific, USA) method, and reverse transcribed to complementary DNA (cDNA) with TaqMan Reverse Transcription Reagents (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. The sequences were amplified by adding complementary DNA to the polymerase chain reaction (PCR) mixture containing each primer (Appendix Table 1) and Platinum SYBR Green qPCR SuperMix uracil-DNA glycosylase (UDG) (Thermo Fisher Scientific, USA). The reactions were pre-incubated at 50°C for 2 minutes for decontamination of deoxyuridine (dU)-containing DNA by UDG and then incubated at 95°C for 2 minutes to inactivate UDG and activate Taq. The PCR program continued 46 cycles of denaturation at 95°C for 15 seconds. annealing at 60°C for 30 seconds and elongation at 72°C for 30 seconds. The primer sequences were shown in

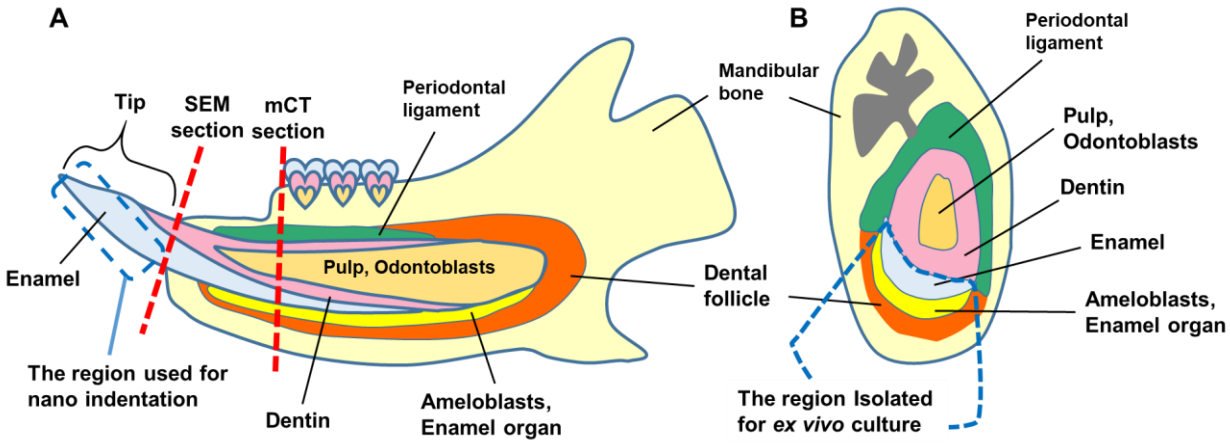
Supplementary Table S1. All data were normalized by Ct value of beta-actin gene expression from the same sample.

Supplementary Table S1. Primer sequences used for RT-qPCR

Gene	Forward	Reverse
Ambn	5'- AAAATGAAGGGCCTGATCCT-3'	5'- GCAAGCTTCCCAACTGTCTC-3'
Amelx	5'- ACCAGAGCATGATAAGGCAGCC-3'	5'- TGATGAGGCTGAAGGGTGTGAC-3'
Mmp20	5'- GAAGTGGCTGAACGAGGCATTG-3'	5'- TTGTCCGTGGAGGACCTTGCAT-3'
Klk4	5'- CCTGCCTAGTCTCTGGTTGG-3'	5'- TGAGGTGGTACACAGGGTCA-3'
Orai1	5'- GTTACTCCGAGGTGATGAGCCT-3'	5'- AGCTGGACTTCCACCATCGCTA-3'
Orai2	5'- ACTGTCCTGGAGGAAGCTCTAC-3'	5'- TACTTGGTCTCCAGCTGCACCT-3'
Orai3	5'- TCAAAGCCTCCAGCCGCACATC-3'	5'- GCTACTAACACAGTGGTGCAGG-3'
Actb	5'- TCCTCCTG AGCGCAAGTACTCT-3'	5'- CGGACTCATCGTACTCCTGCTT-3'

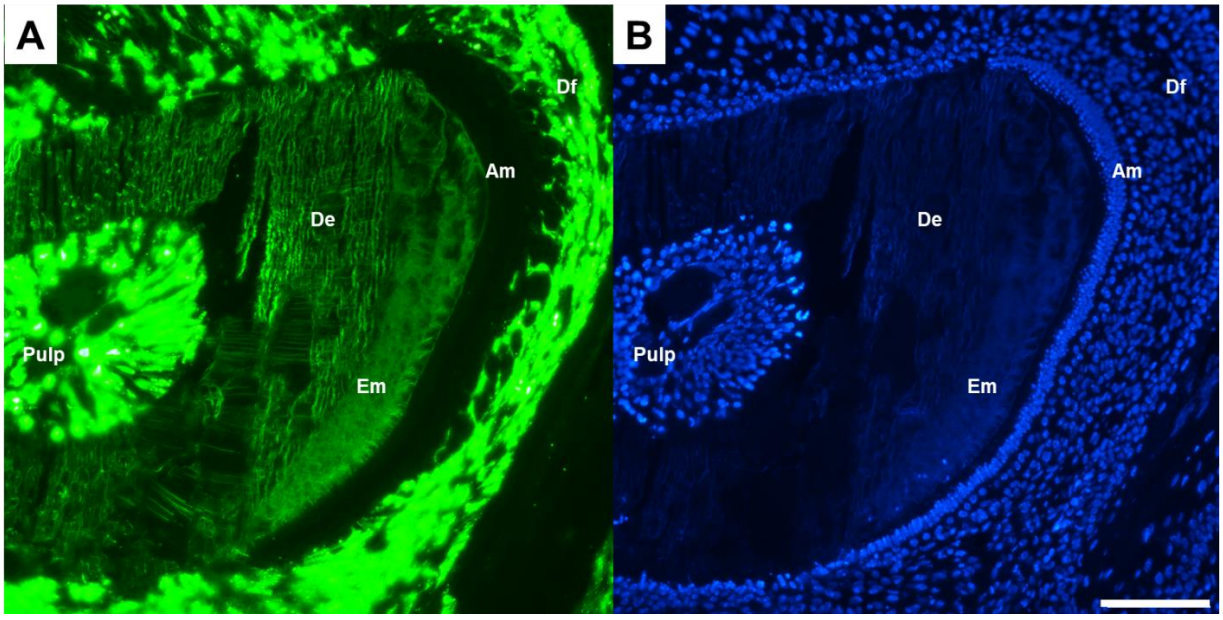
Supplementary figures

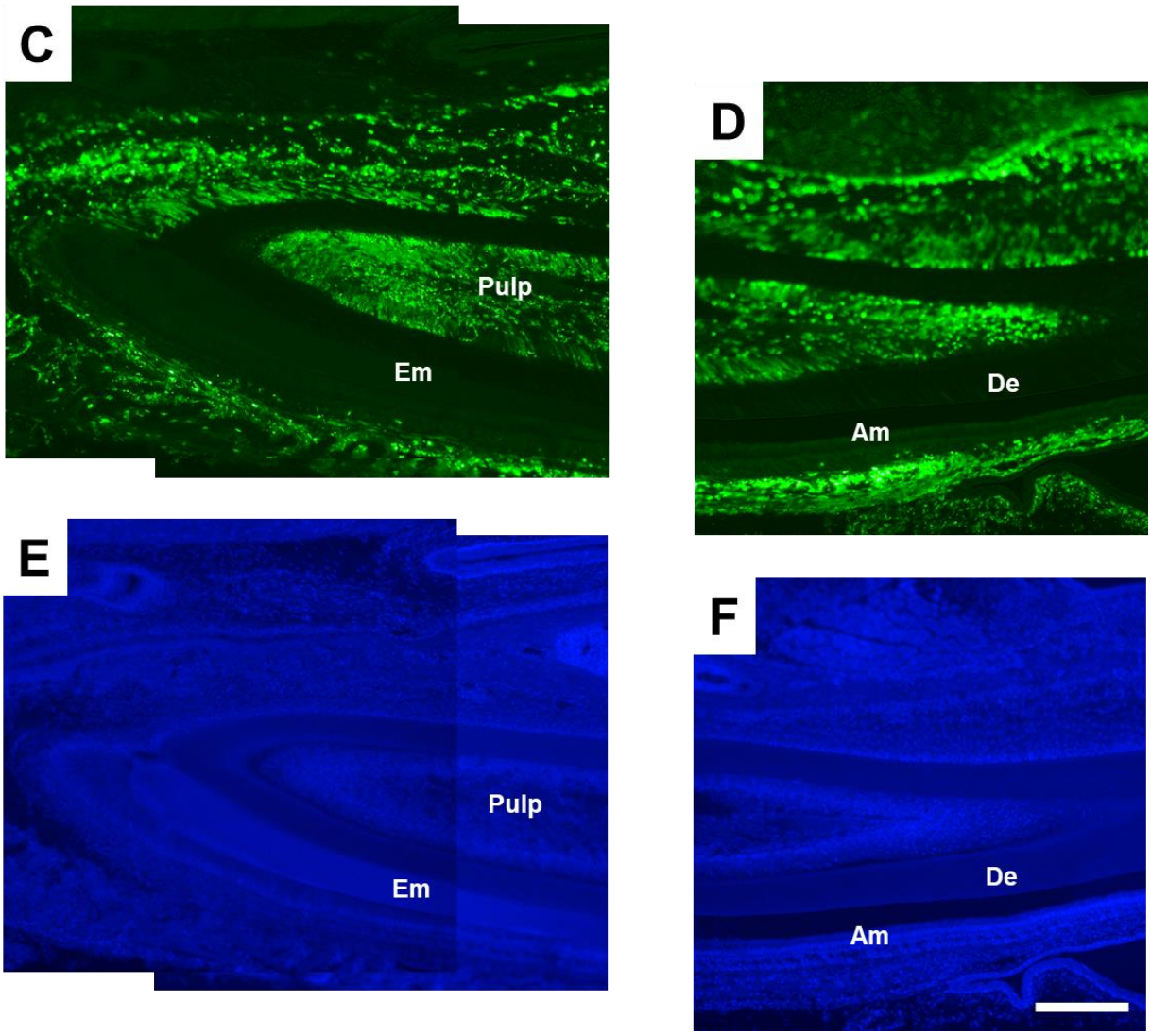
Supplementary fig. S1



Supplementary fig. S1. Schematic illustration of mouse mandibular incisor, showing (**A**) a sagittal-section and (**B**) a cross-section image.

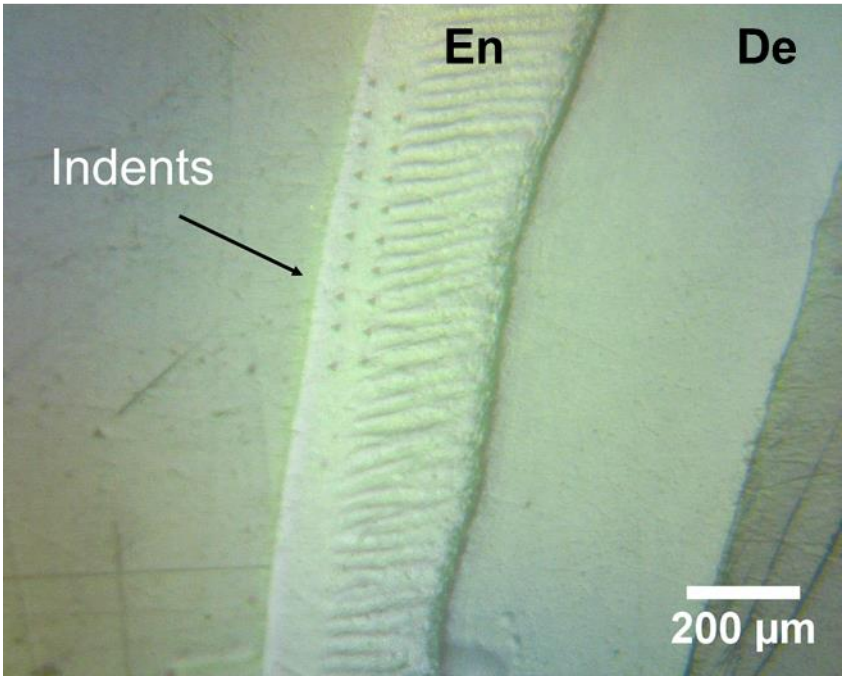
Supplementary fig. S2





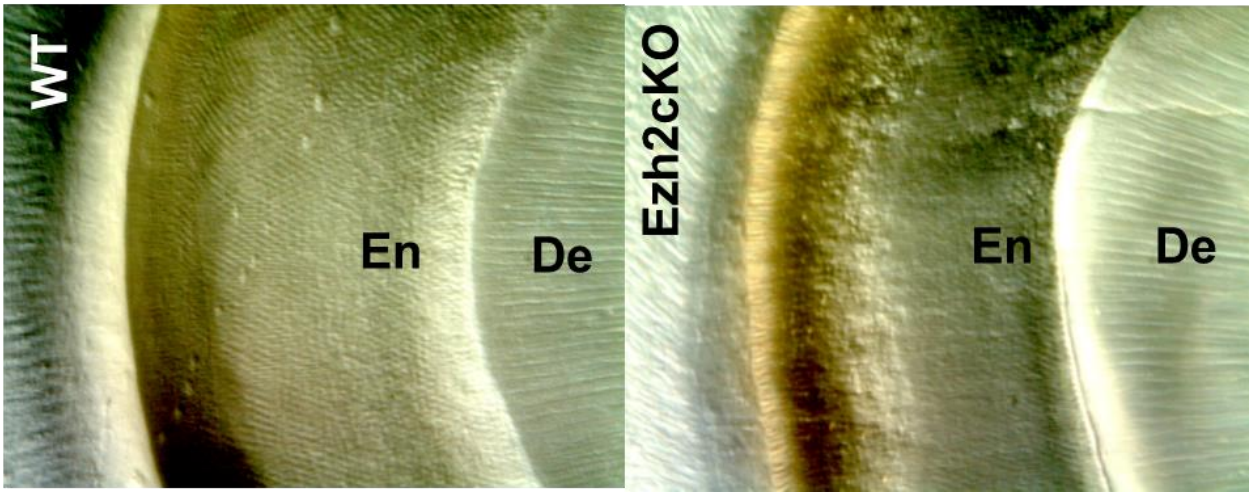
Supplementary fig. S2. Cre expression indicated by green fluorescence in an incisor with the surrounding tissues from *Prrx1*-Cre^{+}: Ai6 mouse. (**A**) ZsGreen and (**B**) DAPI stain image from an undecalcified frozen cross-section are shown. Scale bar = 100 µm. (**C-F**) A decalcified sagittal section of the same incisor showing (**C, E**) the apical and (**D, F**) the rostral regions. (**C, D**) ZsGreen; (**E, F**) DAPI stain. Am, Em, and De indicate ameloblast, enamel matrix, and dentin, respectively. Scale bar = 200 µm.

Supplementary fig. S3.



Supplementary fig. S3. Microscopic image showing a representative example of nanoindentation. En and De indicate enamel and dentin, respectively.

Supplementary fig. S4.



Supplementary fig. S4. Microscopic cross section images of the incisor from the wild type (WT) or *Ezh2* knockout (Ezh2KO) mice. Magnification × 400. En and De indicate enamel and dentin, respectively. Enamel hyper-mineralization was analyzed with an optical microscope in cross sections of the incisor from wild type and Ezh2cKO mice (3 weeks old). Although no significant change was observed in their dentin tubule structure, enamel from cKO mice did not exhibit a clear rod structure.

Reference

[1] H.M. Goldman, A. Blayvas, A. Boyde, P.G. Howell, J.G. Clement, T.G. Bromage, Correlative light and backscattered electron microscopy of bone--part II: automated image analysis, Scanning 22 (2000) 337-344. 10.1002/sca.4950220601.