

Supplementary Information

Npp1 prevents external tooth root resorption by regulation of cervical cementum integrity

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Supplementary Methods

Tissue preparation, immunohistochemistry (IHC) and histomorphometry

For histologic analysis, the dissected mandibles were fixed in 4% paraformaldehyde and decalcified in 10% EDTA for 4 weeks at 4°C. The decalcified tissues were dehydrated through a graded ethanol series, embedded in paraffin, and sectioned at 5-μm thickness. Slides were stained with H-E. For immunohistochemical staining, sections were treated with 3% hydrogen peroxide and incubated with rabbit polyclonal antibodies as described. The Histostain Plus Rabbit Primary DAB kit (Zymed Laboratories, San Francisco, CA, USA) were used following the manufacturers' instructions. Cervical cementum thickness was measured as described ¹.

RNA preparation and real-time qPCR

Total RNA was prepared using AccuPrep[®] Universal RNA Extraction kit (K-3140, Bioneer, Daejeon, South Korea) according to the manufacturer's instructions, and cDNA was synthesized from 3 μg of total RNA using Superscript II reverse transcriptase (100004925, Invitrogen, Carlsbad, CA, USA). Real-time qPCR was performed using specific primer sets listed in Supplementary Table S1 as described previously ².

Nucleoside triphosphate pyrophosphohydrolase (NTPPPHase) activity and quantitation of extracellular ATP

To measure the activity of total NTPPPHase, cells were incubated in phenol red-free medium for 2 hours with 1 mM thymidine 5' monophosphate p-nitrophenyl ester sodium (T4510, Sigma-Aldrich, St. Louis, MO, USA) solution at 37 °C. After incubation, 80 μl of supernatant for each condition was transferred to a 96 well plate containing 80 μl of 0.1 N NaOH. Absorbance was recorded at a wavelength of 410 nm. Extracellular ATP levels were

measured using ATP assay kit (ab83355, Abcam, Cambridge, MA, USA).

Western blot analysis and enzyme-linked immunosorbent assay (ELISA)

Western blot analysis was performed using specific antibodies against non-phosphorylated active β -catenin (8814, Cell Signaling), β -catenin (RB9035, Thermo Scientific), GSK-3 β (9315, Cell Signaling), phosphor-GSK-3 α/β (9331, Cell Signaling), protein kinase A (4782, Cell Signaling Technology, Danvers, MA, USA), CREB (sc-377154, Santa Cruz Biotechnology), phosphorylated-CREB (p-CREB; sc-81486, Santa Cruz Biotechnology) and β -actin (sc-1616R, Santa Cruz Biotechnology) as described previously ³. For ELISA, the production of Rankl was measured in the whole cell lysate and conditioned media from the cell culture using the ELISA kit (LS-F5674, LSBio, Seattle, WA, USA) following the manufacturers' instructions.

Mineralization induction and alizarin red staining

Cells at 95% confluency were cultured in the medium supplemented with 2% fetal bovine serum, 50 μ g/ml ascorbic acid (Sigma Aldrich), and 10 mM β -glycerophosphate (Sigma Aldrich), for 7 days. Mineral nodule formation was then observed by staining the cells with 40 mM alizarin red S (pH 4.2) after fixation with 4% PFA for 10 minutes. The amount of alizarin red S that bound to the minerals was quantified by destaining the samples in 10 mM sodium phosphate containing 10% cetylpyridinium chloride (pH 7.0) for 15 minutes at room temperature. The amount of alizarin red S in the destaining solution was measured at OD 562 nm.

Alkaline phosphatase (ALP) activity and staining

Cells were cultured with or without SAG in OM for 48 hours. Alkaline phosphatase activity was quantitated using an assay based on the hydrolysis of p-nitrophenylphosphate (p-NPP) to p-nitrophenol (p-NP). Briefly, cell layers were washed twice with ice-cold PBS and lysed in 50 mM Tris-HCl buffer (pH 7.0) containing 1% (v/v) Triton X-100 (Sigma Aldrich) and 1 mM PMSF (Sigma Aldrich). Whole cell lysates were assayed by adding 1 mg/ml of pNPP substrate in 0.1 M glycine buffer (pH 10.4) containing 1 mM ZnCl₂ (Sigma Aldrich) and 1 mM MgCl₂ (Sigma Aldrich) to each tube for 15 min at 37 °C. Reactions were stopped by adding NaOH (final concentration 0.6 N), and the absorbance of each lysate was measured spectrophotometrically at 405 nm. Enzyme activity was normalized to total protein content and expressed using the fold change. For ALP staining, cells were stained with a Leukocyte Alkaline Phosphatase kit (Sigma Aldrich) according to the manufacturer's protocol.

Supplementary Table S1. Primer sequences for real-time qPCR

Gene	Sense	Antisense
<i>Rankl</i>	ATGAAAGGAGGGAGCACGAA	GTACGTCGCATCTTGATCCG
<i>Opg</i>	ACGGACAGCTGGCACACCAG	CTCACACACTCGGTTGTGGG
<i>Bsp</i>	AAAGTGAAGGAAAGCGACGA	G TTCCTTCTGCACCTGCTTC
<i>Trap</i>	AGACCCAGACCCTGAACACC	CGCCCAAGAAAGCTCTACCTAA
<i>CtsK</i>	CACGGCAAAGGCAGCTAAAT	CCATAGCCCACCACCAACAC
<i>Dmp1</i>	AGTGAGTCATCAGAAGAAAGTCAAGC	CTATACTGGCCTCTGTCTAGCC
<i>Opn</i>	CCCGGTGAAAGTGACTGATTC	ATGGCTTTCATTGGAATTGC
<i>Gapdh</i>	TGCCCAGAACATCATCCCT	GGTCCTCAGTGTAGCCCAAG

Legends for Supplementary Figures

Supplementary Figure S1. Npp1 preserves less-mineralized cementum at cervix. ALP activity with ALP staining and mineralization ability by Alizarin red S staining were analyzed with OCCM-30 cells with *shEnpp1* and *shNC* differentiated for 7 days.

Supplementary Figure S2. Functional Npp1 preserves anti-resorptive cervical cementum by regulation of cervical cementum integrity. (a) In physiological state, the PPi-generating enzyme activity of Npp1 using ATP is important for preventing hydroxyapatite (HA) crystal formation on the surface of cervical cementum. Through inorganic pyrophosphate (PPi), Npp1 suppresses β -catenin activity that regulates non-collagenous matrix apposition. Hence, functional Npp1 preserves thin and less-mineralized cementum integrity and supports anti-resorptive property of cervical cementum through ATP homeostasis. (b) Disruption of Npp1 function leads to shortage of PPi and consequently induces HA formation with higher Tnap

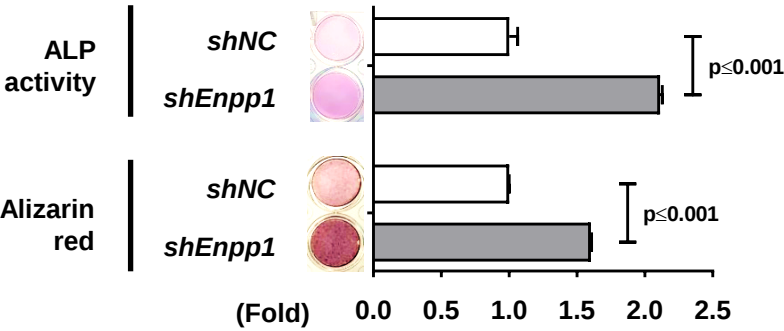
activity. In addition, the shortage of PPi induces β -catenin stabilization and results in RGD-rich matrix accumulation on the surface of cervical cementum. This ectopic formation of cementum is susceptible to resorption by increased expression of Rankl through redundant extracellular ATP.

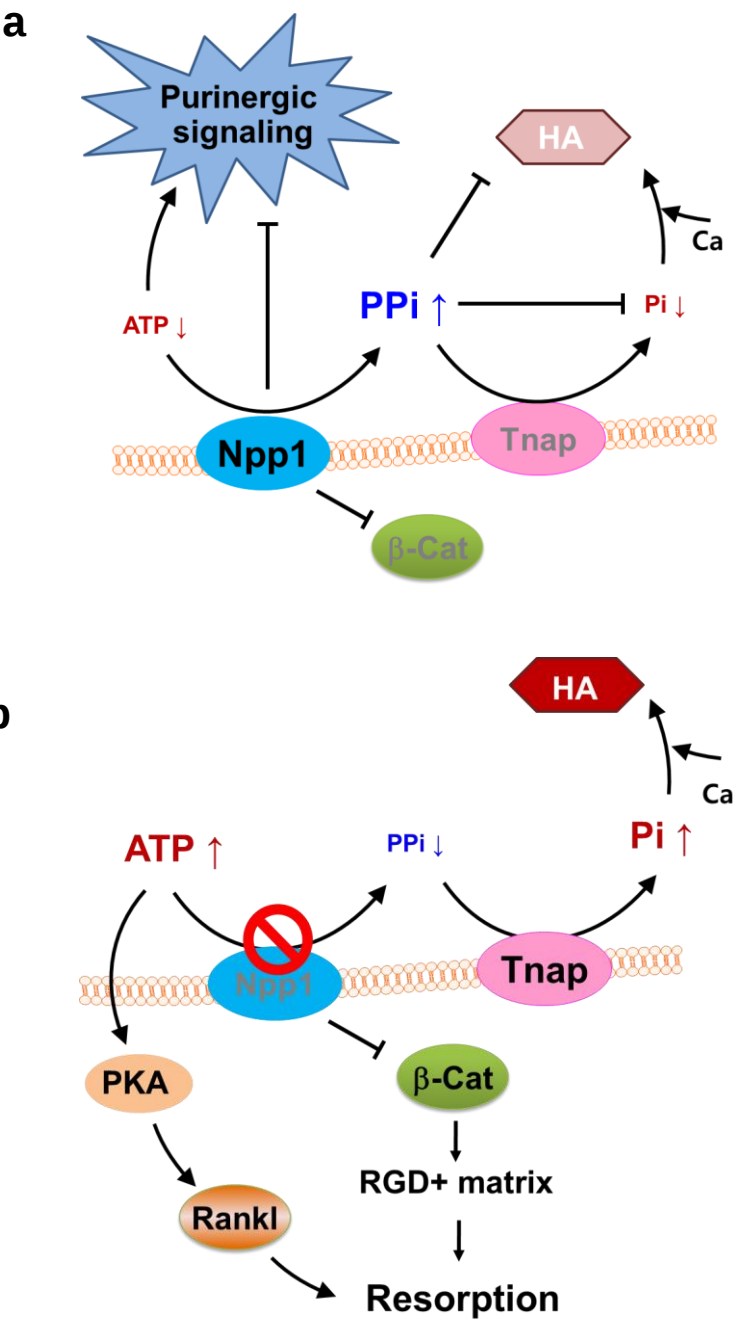
Supplementary Figure S3. Original full-size blots of Figure 3a and 5d. (a) The following antibodies were used: non-phosphorylated active β -catenin (Act. β -Cat, 1:2000), β -catenin (β -Cat, 1:2000), Gsk3 β (1:1000), phosphor-Gsk-3 α/β (p-GSK3 α/β , 1:1000) and β -Actin (1:2000). (b) Protein kinase A (PKA, 1:1000), CREB (1:500), phosphorylated-CREB (p-CREB, 1:200) and β -Actin (1:2000).

References

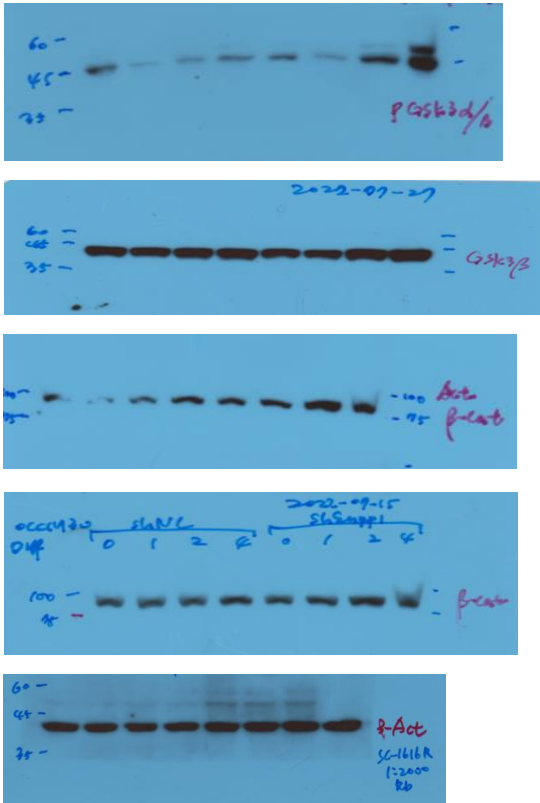
- 1 Choi, H. *et al.* A Reciprocal Interaction between beta-Catenin and Osterix in Cementogenesis. *Sci Rep* **7**, 8160, doi:10.1038/s41598-017-08607-5 (2017).
- 2 Choi, H. *et al.* TGF-beta Signaling Regulates Cementum Formation through Osterix Expression. *Sci Rep* **6**, 26046, doi:10.1038/srep26046 (2016).
- 3 Choi, H., Liu, Y., Jeong, J. K., Kim, T. H. & Cho, E. S. Antagonistic interactions between osterix and pyrophosphate during cementum formation. *Bone* **125**, 8-15, doi:10.1016/j.bone.2019.05.001 (2019).

Supplementary Figure S1





a



b

