Supplemental Methods and Materials

Animals

Axin2$^{LacZ/+}$ (#11809809) and Axin2$^{CreERT2/+}$; R26R$^{mTmG/+}$ (#018867 and #007576) mice were obtained from Jackson Labs. To induce Cre expression in Axin2$^{CreERT2/+}$; R26R$^{mTmG/+}$ mice, tamoxifen (4 mg/25 g body weight) was delivered intraperitoneally for 3 consecutive days; animals were then sacrificed at indicated time points. In this strain, delivery of tamoxifen instigates a recombination event wherein cells that are responsive to endogenous Wnt signaling starts to express green fluorescent protein (GFP). Subsequently, any descendants arising from this initial population of Wnt-responsive cells are also labeled with GFP. This strategy allowed us to unambiguously determine whether Wnt-responsive cells in the dentin-pulp complex declined with age.

Daβcat^{Ot} mice were generated by crossing dentin matrix acidic phosphoprotein 1(DMP1)-8kb-Cre mice with Catnb$^{lox(ex3)}$ mice, in which LoxP sites flank exon 3 that encodes for β-catenin degradation (1, 2). DMP1-8kb-Cre$^{+/-}$ mice were crossed with Catnb$^{lox(ex3)/lox(ex3)}$ mice to generate Catnb$^{lox(ex3)/+}$; DMP1-8kb-Cre$^{+/-}$ (daβcat^{Ot} mutant mice) and Catnb$^{lox(ex3)/+}$ mice (daβcat^{Ot} control mice). The daβcat^{Ot} control mice are indistinguishable from the wild-type C57BL/6 mice and used as littermate controls in the current study. Mice expressing green fluorescent protein (GFP) in osteocytes (DMP1^{GFP}) (3, 4) were also used in this study.

Xgal staining

To detect β-galactosidase activity, sections were fixed with 0.2% glutaraldehyde/PBS for 15 min and then washed 3 times with wash buffer containing 0.005% Nonidet P-40, 0.01% sodium deoxycholate, 2 mM $MgCl_2$/PBS. Tissue sections were stained overnight at 37℃ in a staining solution containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$, and 1 mg/ml Xgal (Thermo Fisher). Sections were rinsed 2 times in PBS, dehydrated in a graded ethanol series and cleared in CitriSolv, then mounted with Permount (5, 6).

μCT analyses

Micro-computed (μCT) topographies were performed using a SkyScan 1176 scanner (SkyScan, Bruker, Belgium) at a 5 μm resolution. Scanning was done at 45 kV, 556 mA. Samples were reconstructed and segmented with ScanIP. CT Analyzer software (version 1.02; SkyScan) was employed for morphometric quantification.

Immunohistochemistry

Primary antibodies and their dilutions are as follows: anti-Osterix (ab22552, Abcam, Cambridge, UK), anti-CTNNB1 (β-catenin) antibody (BD Biosciences, New Jersey, USA), anti-Nestin (ab6142, Abcam, Cambridge, UK), anti-Lef1 (2230S, Cell Signaling Technology, USA), anti-GFP (2956, Cell Signaling Technology), and

anti-DMP1 (ab103203, Abcam, USA). In all cases, the negative controls were performed at the same time using PBS to substitute primary antibody. Secondary antibodies are biotinylated goat anti-rabbit IgG antibody (BA-1000, Vector Lab, Burlingame, USA) and biotinylated horse anti-mouse IgG antibody (BA-2000, Vector Lab). The staining was visualized by ABC peroxidase standard staining kit (32020, Thermo Fisher Scientific, Rockford, USA) and DAB peroxidase substrate kit (SK4100, Vector Lab). For GFP staining, nickel solution was added to the DAB substrate to enhance the signaling, therefore, the GFP^{+ve} cells were dark purple. For β-catenin staining, fast green was used for counterstaining. Tissue sections were photographed using a Leica digital imaging system.

Histomorphometry

Histomorphometric measurements were performed to measure the areas of dentin and pulp. From four separate timepoints, three mice were analyzed, and from each mouse, six tissue sections were chosen for analysis. The dentin, the pulp, and the dentin+pulp areas were calculated by image J on each tissue section. The area of interest was specified as either the dentin or the pulp, and this value was then divided by total area occupied by dentin+pulp.

References:

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