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Supplemental Information

Regulation of Mesenchymal Stem to Transit-Amplifying Cell Transition in the Continuously Growing Mouse Incisor

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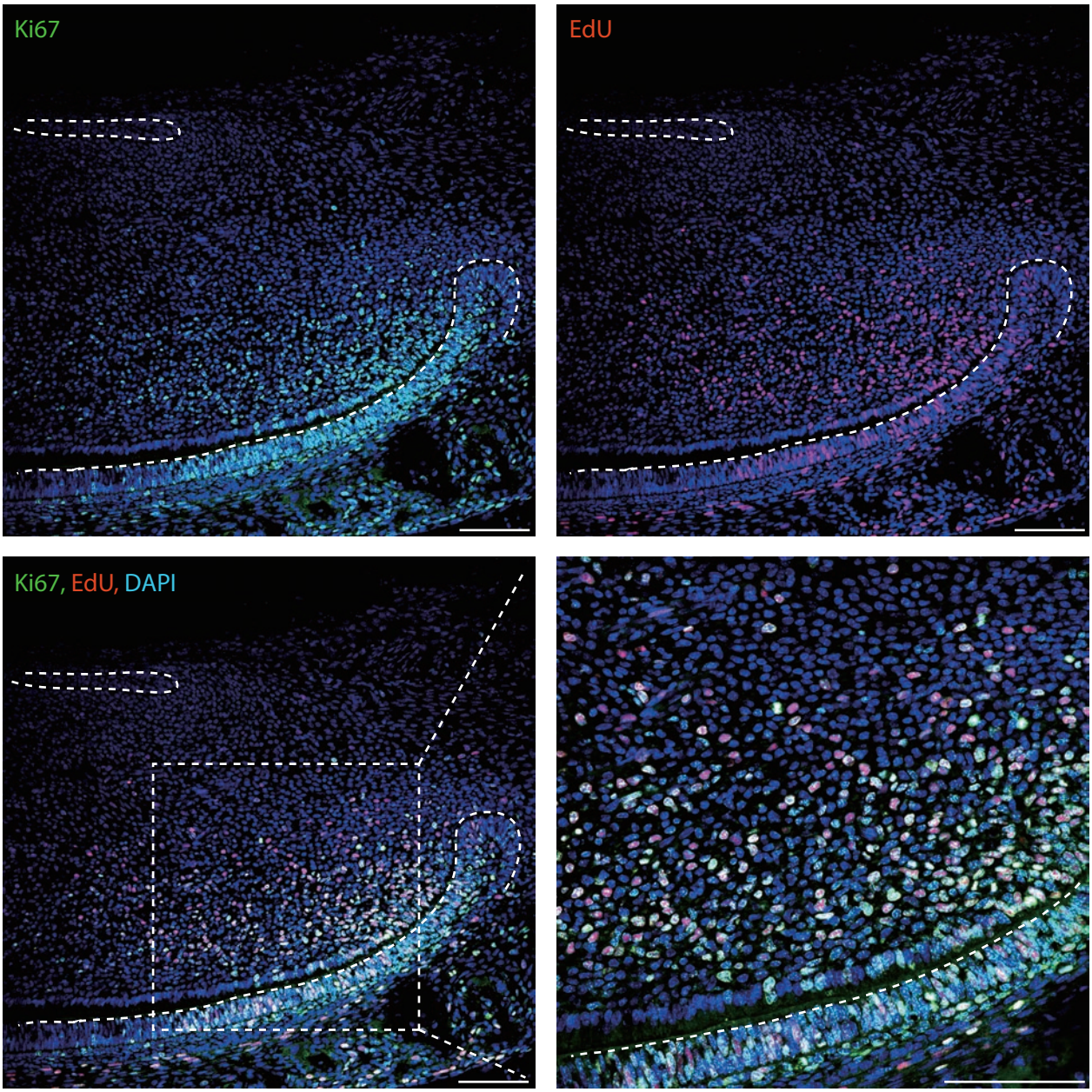

Figure S1. Identification of the TAC region by the cell proliferation marker Ki67 and EdU labelled cells. Related to Figure 1A. Double staining of mouse incisor sagittal sections showing co-localization of Ki67 with 16-24 hours chased EdU+ cells in the TAC region indicative of EdU+ fast cycling TACs. n>=5 mice. Bar is 100 μ m.

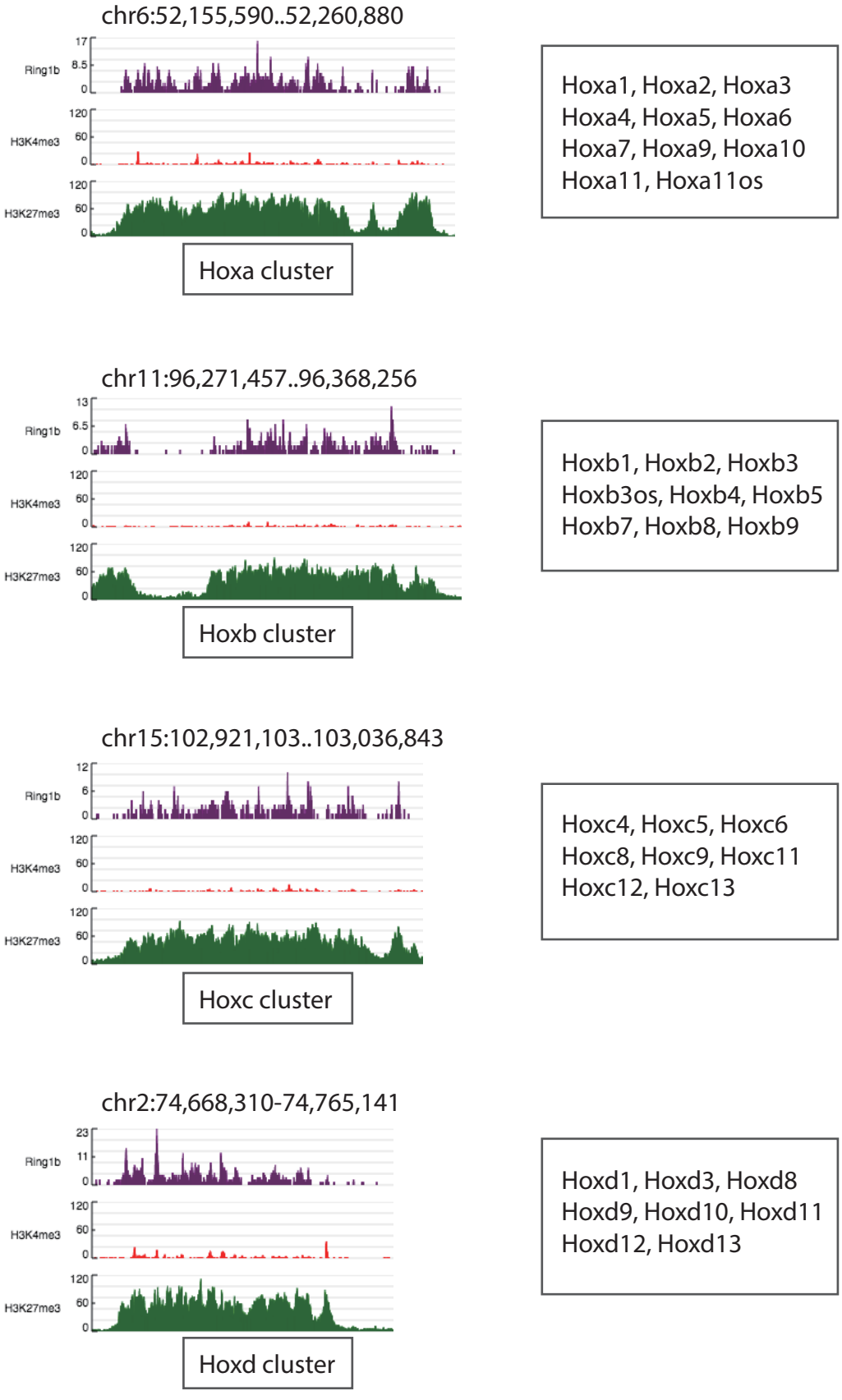

Figure S2. ChIP-seq identifies Hox gene clusters as Ring1b binding loci in TACs. Related to Figure 2A. Genomic views showing Hox clusters co-marked by Ring1b and H3K27me3 but no enrichment with H3K4me3 (left panel). List of Hox genes revealed on ChIP-seq datasets in TACs.

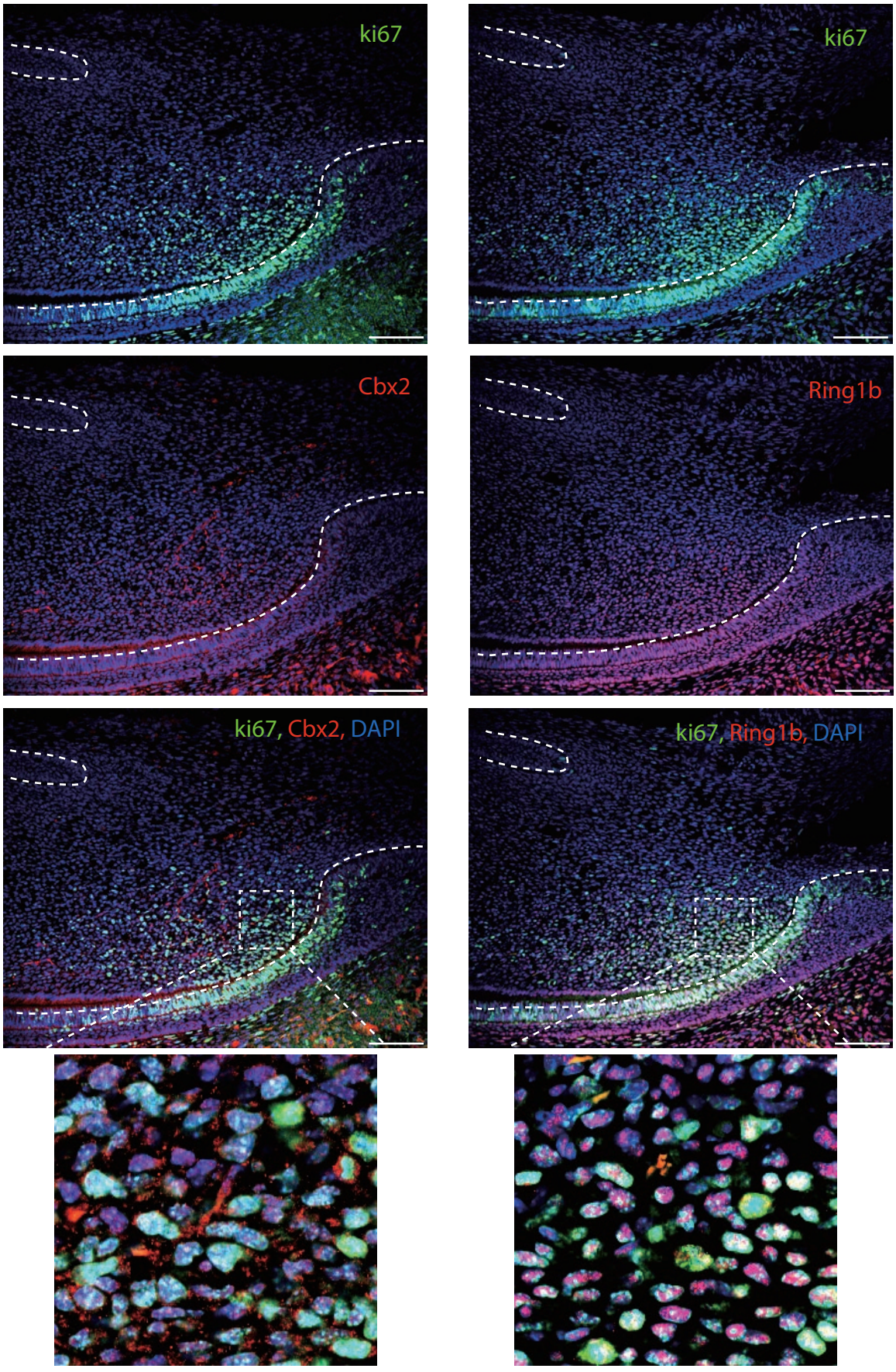

Figure S3. Co-localization of Cbx2 and Ring1b in the mouse dental pulp. Related to Figure 2E-G. Double immuno-staining of Cbx2 and Ring1b both showed co-localization with Ki67 in the TAC region on sequential sections of mouse incisors indicating co-localization of Cbx2 and Ring1b. n>=5 mice per group. Bar is 100 μ m.

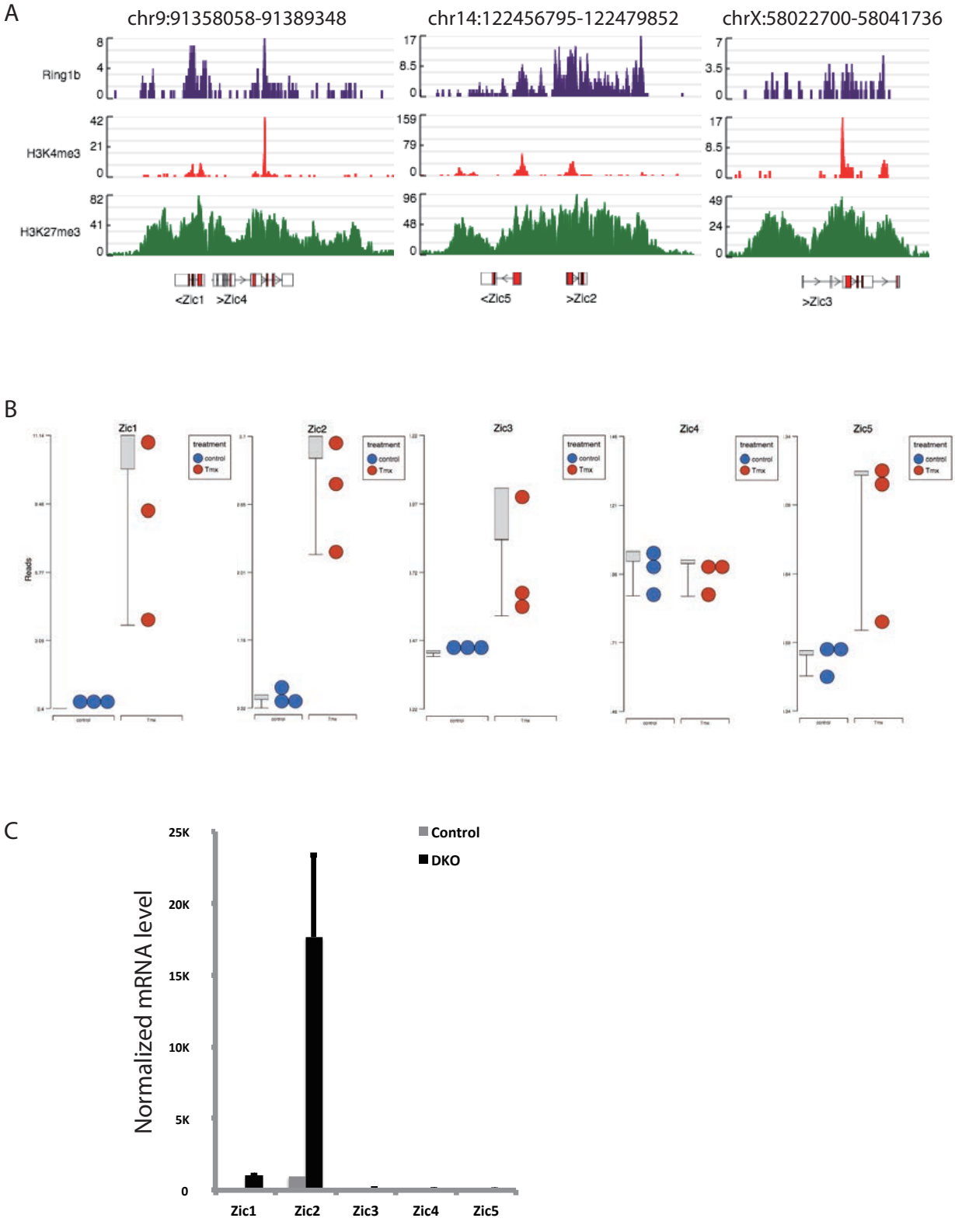

Figure S4. Genomic profiles of Zic family members in TACs. Related to Figure 4E. (A) Genome browser snapshots of Zic1-5 binding profiles. Zic1/2 shows co-occupation with Ring1b and H3K27me3, while other Zic family members also showed binding with H3K4me3. (B) Dot plots showed only Zic1/2 significantly up-regulated following deletion of Ring1b (n=3, $P<0.05$), whereas Zic3, Zic4 and Zic5 showed no significant difference (n=3, $P>0.05$) on microarray datasets. (C) Validation of Zic expression following loss of Ring1b by qPCR confirmed that Zic1/2 were significantly up-regulated compared with other Zic family members (n>=3, $P<0.001$ by Student's t-test). Data presented as mean ± S.E.M.

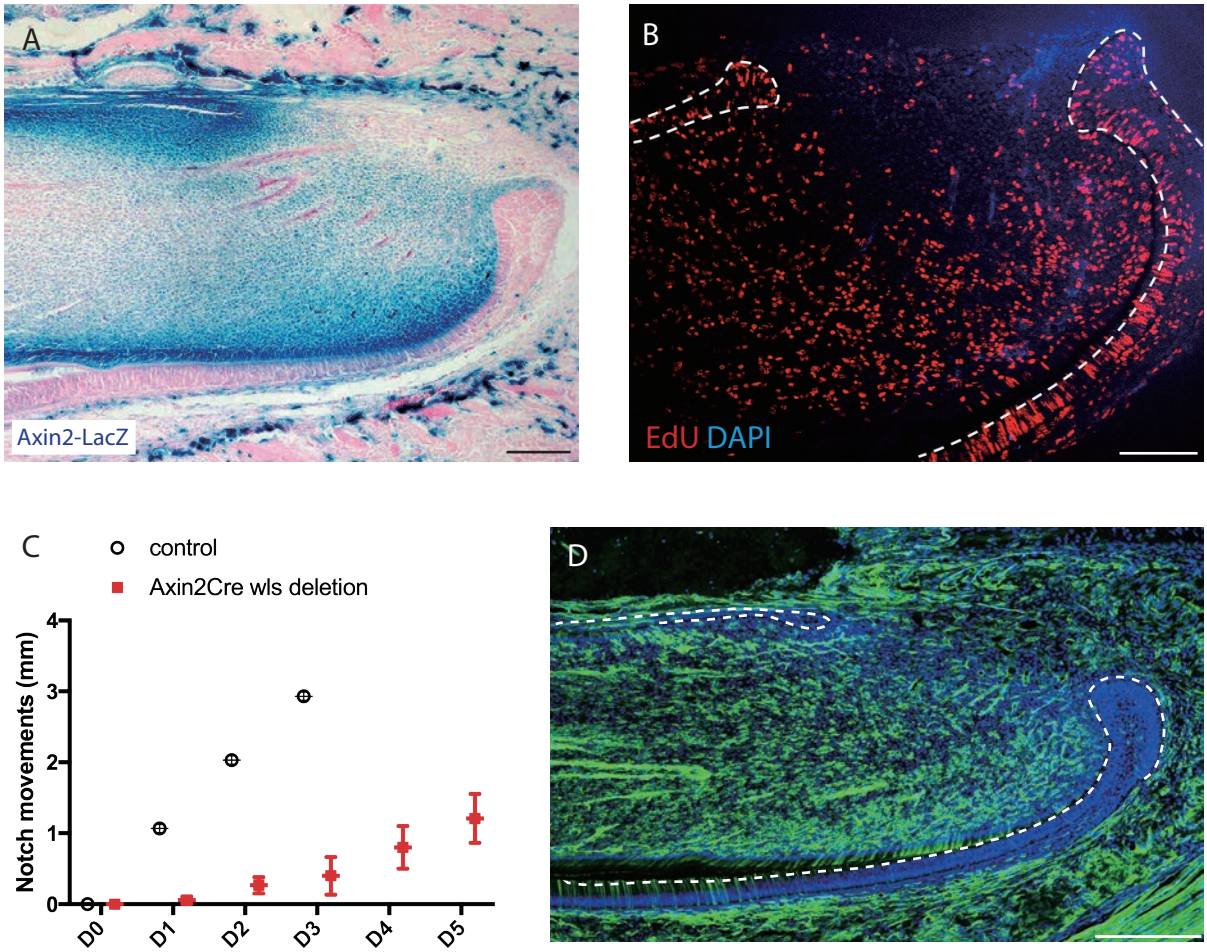

Figure S5. Axin2 expressed in TACs in the mouse incisor. Related to Figure 5 and Figure 6D.
(A) Sagittal section of Axin2LacZ mouse incisor stained for beta-galactosidase (LacZ) activity using X-gal showing Axin2 expression in the TAC region. (B) EdU+ cells detected in the TAC region after 16-24 hours chasing. n>=5 mice. (C) Comparison of growth rates. After one week of three doses of tamoxifen, notches were made 0.8-0.9 mm above the incisor gingiva at day 0 (D0) in Axin2cre;wls cko/cko and control mice. Notch movements were measured every day for five days. Notches in control incisors reach the tip after 3 days, while the notches in Axin2cre;wls cko/cko incisors only reach about 1/3 of full length incisor by day 5. n=3 and P<0.01 by Student's t-test. (D) Efficiency of Cre recombinase in the mouse incisor. PcagCreERT2 mice crossed with the mTmG reporter mouse line. Mouse incisors were harvested after two doses of tamoxifen (5 mg/30 g body weight) showed widespread location of GFP+ cells in mesenchyme. n=4 mice. Bar is 250 μ m.

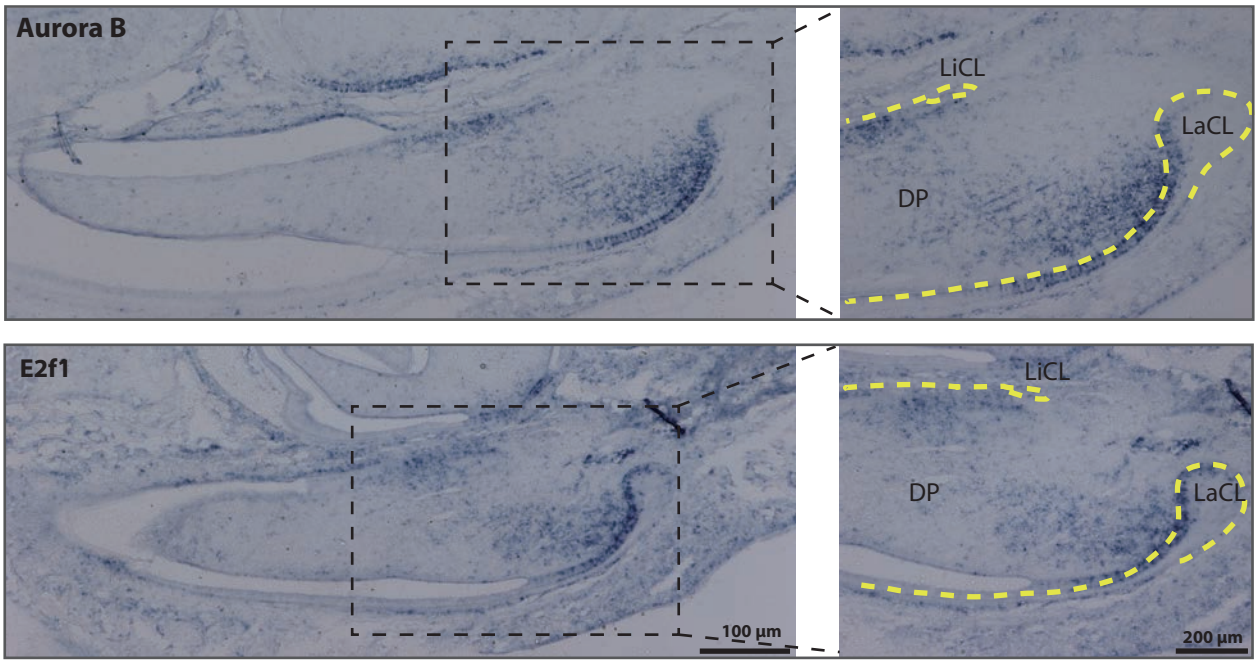

Figure S6. In situ hybridisation identifies Aurora B and E2f1 in TACs. Related to Figure 3E.
Aurora B and a positive cell cycle regulator E2f1 are detected mainly in the incisor mesenchyme between the labial and lingual aspects of the cervical loop where TACs are located. n>=3 mice per group. DP is for Dental Pulp, LiCL is for Lingual Cervical Loop and LaCL is for Labial Cervical Loop.

Table S2. List of real-time PCR primers. Related to Figure 3I, 3J and Figure 4D, 4F.

Primer name	Primer sequencing
Axin2-Forward	gagagtgagcggcagagc
Axin2-Reverse	cggctgactcgttctcct
Beta-actin-Forward	ctaaggccaaccgtgaaaag
Beta-actin-Reverse	accagaggcatacagggaca
Beta-catenin-Forward	ttcctatgggaacagtcgaag
Beta-catenin-Reverse	ttgtattgttactcctcgaccaaa
Cdc6-Forward	ctgtttcaggagacatccgtaa
Cdc6-Reverse	tcctgacatccgactccac
Cdc7-Forward	gcggacgtcctaacttctgt
Cdc7-Reverse	gcttccactacgcacgact
Cdc45-Forward	ggcaagaacttgaaactgcat
Cdc45-Reverse	cactggcctgtgggtatca
Cdkn2a-Forward	cgtaccccgattcaggtg
Cdkn2a-Reverse	accagcgtgtccaggaag
CyclinD1-Forward	tttctttccagagtcatcaagtgt
CyclinD1-Reverse	tgactccagaagggcttcaa
CyclinE2-Forward	cgagctgtggagggtctg
CyclinE2-Reverse	aaacggctactgcgtcttga
E2f1-Forward	tgccaagaagtccaagaatca
E2f1-Reverse	cttcaagccgcttaccaatc
Myc-Forward	cctagtgctgcatgaggaga
Myc-Reverse	tccacagacaccacatcaattt
Twist1-Forward	agctacgccttctccgtct
Twist1-Reverse	tccttctctggaaacaatgaca
Zic1-Forward	aacctcaagatccacaaaagga
Zic1-Reverse	cctcgaactcgcacttgaa
Zic2-Forward	gatccacaaaagaactcatacagg
Zic2-Reverse	cttcttcctgtcgctgctgt
Zic3-Forward	cctgcgcaaacacatgaa
Zic3-Reverse	ctatagcgggtggagtggaa
Zic4-Forward	gtggagcagggctcaaac
Zic4-Reverse	tggtgtccacagctgctact
Zic5-Forward	cactgccaccaacagtgg
Zic5-Reverse	aggacgaagtccctgctgt

Table S3. Plasmids used for Digoxigenin-labelled RNA probes. Related to Figure S6.

Name	Vector	5' clone site	3' clone site	Anti-sense probe	Sense probe	IMAG ID
Aurora B	pT7T3D-PacI	EcoRI	NotI	T3	T7	1226941
E2f1	pSPORT1	SalI	NotI	Sp6	T7	934181

Supplemental Experimental Procedures:

EdU / BrdU incorporation and staining

EdU was detected by Click-iT EdU Alexa Fluor 647 Imaging kit (Invitrogen C10340) according to the protocol. BrdU was detected by anti-BrdU antibody (ab6326, Abcam) 1:100 followed by secondary antibody donkey anti-Rat IgG (H+L) Alexa Fluor 594 (Invitrogen) 1:400 prior to DAPI staining for nuclei and cover-slipped for microscopy.

Immunofluorescence

Immunofluorescence staining used standard protocols on 12 µm sagittal cryosections of mouse incisors. Anti-mouse Ring1b antibody (Active motif #39663, 1:100), anti-Rabbit Ring1b (ab101273, Abcam, 1:500), anti-Rabbit Ki67 antibody (Abcam ab15580, 1:100) and anti-Rabbit CBX2 antibody (Abcam ab184968, 1:100) were used as primary antibodies. Goat anti-mouse IgG (H+L) Alexa Fluor 488, Donkey anti-rabbit IgG (H+L) Alexa Fluor 488, Alexa Fluor 594 and Alexa Fluor 647 (invitrogen, 1:400) were used as secondary antibodies. Hoechst 33342 (Invitrogen 62249, 1:500) used for DNA staining. Slides were then mounted using glycerol based antifade Citifluor™ AF1 (Citifluor Ltd., AF1-100) and cover slips added.

Cytospin

Flow sorted EdU+ cells were collected and re-suspended as 100 µl aliquots in 2% BSA in PBS before loading into a Shandon Single Cytofunnel. Cells were then forced to separate and deposited as a monolayer on slides to preserve the cellular integrity using Shandon Cytospin 3 Centrifuge at 1350 rpm for 5 minutes. Slides were then post-fixated, permeablized and immunostained with primary and secondary antibodies followed by DNA staining with Hoechst33342 prior to coverslips being added according to the standard protocol.

ChIP seq

Primary incisor pulp cells were isolated from 80 incisors for each set of ChIP-Seq. Cells were cross-linked with 1% formaldeyde at room temperature for 12 minutes and then quenched with 0.125 M glycine for another 5 minutes. Cells were suspended in 100 µl saponin-based permeablization buffer and labelled EdU by Click-iT® EdU Alexa Fluor® 647 Flow Cytometry Assay Kit (Thermo Fisher Scientific, C10424). FACS sorted EdU+ cells were collected and sonicated to yield chromatin at 100-500bp. Low-input ChIP was performed by

standard procedures using antibodies from Active motif (Ring1b antibody, 39663; H3K4me3, 39915; H3K27me3, 39155).

Two replicate ChIPseq experiments for Ring1b were pooled for the input sample. Total number of reads were over 40 million for each ChIPseq. The unique alignments without duplicate reads (final tags) were less than 1 million for Ring1b ChIPseq. Peaks were determined using the MACS peak finding algorithm. Using a cutoff of p-value = 1e-6, 3938 peaks were identified. A total of 6.2 million final tags were obtained for H3K4me3 and 13.6 million for H3K27me3. Peaks were determined using the SICER algorithm at a cutoff of E-value = 1 and a Gap parameter of 600 bp. 14,361 H3K4me3 enriched regions and 11,341 H3K27me3 enriched regions were identified respectively.

Western blots

Mouse incisor pulp tissues were disrupted by a hand rotor homogenizer and protein was extracted using cell lysis buffer (CLB) (10 mM Tris pH8.0, 10 mM NaCl, 0.2%NP40) followed by nuclear lysis buffer (NLB) (50 mM Tris pH8.1, 10 mM EDTA, 1%SDS). Equal volume of 2x SDS loading buffer (100 mM TrisHCL PH6.8, 4% SDS, 12% Glycerol, 2% β-mercapitalethanol, 0.008% bromophenol blue) was added to 30µg protein followed by incubation at 95° C for 3 minutes before loading onto SDS-PAGE gels and wet transfer to nitrocellulose membranes. Anti-Ring1b (Active motif 39663, 1:500) and anti-H3K27me3 antibodies (Diagenode pAb-069-050, 1:500) were used to detect protein expression levels and anti-Lamin B1 antibody (Abcam, Ab16048, 1:1000) used as an internal loading control. Peroxidase-conjugated Affinipure Goat-anti Rabbit IgG (H+L) (Jackson immunoResearch, 111-035-003, 1:3000) and Peroxidase-conjugated Affinipure Goat-anti mouse IgG (H+L) (Jackson immunoResearch 111-035-003, 1:3000) were used as secondary antibodies against primary antibodies prior to ECL (GE Healthcare Life Science, RPN2232) detection.

Co-immunoprecipitation

Protein was extracted from mouse incisor pulp tissue using RIPA buffer (150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and 50 mM Tris, pH 8.0) supplemented with protease inhibitor cocktail (Roche). The protein extract was precleared with 35 µl protein A/G Plus-Agarose beads (Santa Cruz, sc-2003) and immunoprecipitated with 3µg antibody of anti-H3K27me3 (Diagenode, pAb-069-050), anti-H2AK119ub (Cell signalling, #8240), anti-Ring1b (Active motif, 39663), 3µg anti-mouse IgG (PEPROTECH, 500-M00) was used as a

control, together with 35 µl protein A/G Plus-Agarose beads incubated at 4° C for 2 hours on a rotator. Beads were pelleted by centrifuge at 13000 rpm for 1minute. Equal volume of 2x SDS loading buffer (100 mM TrisHCL PH6.8, 4% SDS, 12% Glycerol, 2% β-mercaptoethanol, 0.008% bromophenol blue) was added to the bead pellets and protein complexes were eluted by incubation at 95° C for 3 minutes before loading onto SDS-PAGE gels and wet transfered to nitrocellulose membranes. Primary antibody anti-Ring1b and secondary antibody Peroxidase-conjugated Affinipure Goat-anti mouse IgG (H+L) were used to detect the interaction proteins in the precipitated protein complex followed by ECL detection.

Quantitative Real-time PCR

Total RNA was extracted from mouse dental pulp tissue using RNeasy Mini Kit (Qigen 74104), and purified by Ambion DNA-free DNA Removal Kit (Invitrogen, AM1906). cDNA was then synthesised using MMLV Reverse Transcriptase (Promega, 9PIM170). Both cDNA without MMLV reverse transcriptase and RNase-free water with MMLV reverse transcriptase were used as negative controls. For each sample, 1 µg cDNA was used for qPCR reaction with the LightCycler 480 SYBR Green I Master (Roche, 04707516001) using LightCycler 480 system qPCR platform (Roche, 05015278001). All primers used in the experiments are listed in Table S2. Data were analysed with $2^{-\Delta\Delta ct}$ methods. All ct values were normalized with β-actin levels as internal controls. Standard deviations were calculated from biological triplicate samples and were represented as error bars.

Cryosection preparation

Moue incisor samples were fixed in 4% PFA in PBS for 24-48 hours at 4° C and decalcified in 10-19% EDTA for 4 weeks. Samples were then sucrose cryoprotected by incubation with 30% sucrose until samples sunk to the bottom followed by incubation with half of 30% sucrose and half of OCT compound (VWR, 361603E) before embedding in OCT. Cryosectioning of samples at 10-12 um thickness was carried by Cryostat Microtome (Bright, OTF5000). Sections were stored at -80 ° C prior to staining.

LacZ staining

Frozen sections were post-fixed in 0.2% glutaraldehyde and permeabilized in 0.05% Tween20 (Sigma Aldrich) in PBS and then incubated in X-gal solution (Thermo scientific) overnight at 37° C for LacZ staining. Fast red was used for counterstaining.

Tunel assay

Frozen incisor sections were post-fixed in 4% PFA then permeabilized in 0.1M Sodium Citrate buffer with PH 6.0 (11.5% 0.1M citric acid monohydrate and 88.5% 0.1M Trisodium Citrate dihydrate) on ice. The sections were incubated in Tunnel reaction mixture at 37° C for 60 minutes according to the protocol (In Situ Cell Death Detection Kit, Fluorescein 11684795910 Roche) and counterstained with Hoechest33342 prior to coverslip. Fluorescein was detected by Confocoal microscopy (Leica TCS SP5) with an argon laser at 488nm excitation.

Digoxigenin-labelled section in situ hybridization

In situ hybridization (ISH) for detection of mouse Aurora B and E2f1 mRNA expression was performed on 12 μm cryo-sections of mouse incisors following the standard procedures. Briefly, 10 μg plasmid (Table S3) was linearized by 5' and 3' clone site restriction enzymes for sense and anti-sense probes. 1μg linearized DNA was used for DIG-labelling (Roche) and RNA probes were synthesised at 37° C for 2 hours followed by 2 μl DNase I incubation for 15 minutes at 37° C. Probes were then purified by SigmaSpin post-Reaction Clean-up Colums (S0185-70EA). Sections were fixed in 4% paraformaldehyde and hybridized with 1μl digoxigenin-labelled sense and antisense probes. Sections were treated with RNase-A or treated with the sense probe are used as negative controls. Images were taken on a Zeiss Axioskop 2 microscope equipped with a Zeiss AxioCam camera (Carl Zeiss).

Mice information

Wild type CD1 mice were obtained from CRL (Charles River Laboratory, UK). Mutant Ring1a and Ring1b floxed alleles were generated as described previously (Lapthanasupkul et al., 2012; Cales et al., 2008; and del Mar Lorente et al., 2000). $Ring1a^{-/-}$;$Ring1b^{fl/fl}$ compound mice were crossed with $pCAG^{CreERT2}$ transgenic mice to generate Ring1a-/-;Ring1bfl/fl ;Rosa26::CreERT2 mice. Ring1a-/-;Ring1bcko/cko mice were obtained by injecting 4-hydroxy tamoxifen (OHT) (40mg/kg body weight) and corn oil as control for 2 days before being scarified.

Axin2CreERT2/+ mouse line was described previously (Van Amerongen et al, 2012). A total of 14 mice were divided into 4 groups and Cre recombination was activated by 3 doses of Tamoxifen (Sigma Aldrich) injections (5 mg/30 g body weight) and corn oil as control. Mice then collected at 1, 3, 7, 14 and 28 days following the last injection of tamoxifen.

Ubiquitous $Pcag^{CreERT2}$ mouse line was crossed with $Wls^{fl/fl}$ mouse line (Carpenter et al, 2010). A total of 6 mice were divided in to 2 groups. The first group were treated with 3 doses of tamoxifen injections to activate cre recombination. The other groups were treated with 3 doses of corn oil injections as control. Both groups were collected 7 days post injection. Cre activity was analysed by crossing $Pcag^{CreERT2}$ with the R26R-mTmG reporter mice (Figure S5).