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# Ring1a/b polycomb proteins regulate the mesenchymal stem cell niche in continuously growing incisors

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ABSTRACT

Rodent incisors are capable of growing continuously and the renewal of dental epithelium giving rise to enamel-forming ameloblasts and dental mesenchyme giving rise to dentin-forming odontoblasts and pulp cells is achieved by stem cells residing at their proximal ends. Although the dental epithelial stem cell niche (cervical loop) is well characterized, little is known about the dental mesenchymal stem cell niche. Ring1a/b are the core Polycomb repressive complex1 (PRC1) components that have recently also been found in a protein complex with BcoR (Bcl-6 interacting corepressor) and Fbxl10. During mouse incisor development, we found that genes encoding members of the PRC1 complex are strongly expressed in the incisor apical mesenchyme in an area that contains the cells with the highest proliferation rate in the tooth pulp, consistent with a location for transit amplifying cells. Analysis of *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ mice showed that loss of Ring1a/b postnatally results in defective cervical loops and disturbances of enamel and dentin formation in continuously growing incisors. To further characterize the defect found in *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ mice, we demonstrated that cell proliferation is dramatically reduced in the apical mesenchyme and cervical loop epithelium of *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ incisors in comparison to *Ring1a*$^{-/-}$;*Ring1b*$^{fl/fl}$ *cre-* incisors. Fgf signaling and downstream targets that have been previously shown to be important in the maintenance of the dental epithelial stem cell compartment in the cervical loop are downregulated in *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ incisors. In addition, expression of other genes of the PRC1 complex is also altered. We also identified an essential postnatal requirement for Ring1 proteins in molar root formation. These results show that the PRC1 complex regulates the transit amplifying cell compartment of the dental mesenchymal stem cell niche and cell differentiation in developing mouse incisors and is required for molar root formation.



## Introduction

Rodent incisors including mouse incisors differ from molars as they are capable of continuously growing throughout the lifetime of the animal. These incisors grow and erupt continuously in order to compensate for functional attrition that constantly occurs at their incisal edges as the mouse feeds. Whereas molars have an obvious crown and root axis, mouse incisors have no conventional crown or root, rather the labial, enamel-covered surface is equivalent to a crown and the lingual, enamel-free surface to a root (Ohazama et al., 2010; Tummers and Thesleff, 2008; Tummers et al., 2007). The most proximal end of the incisor is open to provide a channel for blood and nerve supplies. Since the incisors continue grow, the renewal of tooth-forming cells including enamel-forming ameloblasts and dentin-forming odontoblasts is required and is achieved by stem cells residing at their open proximal (apical) ends (Harada et al., 1999; Smith and Warshawsky, 1975). The mouse incisor is therefore an interesting model to study the regulation of dental epithelial and mesenchymal stem cells in the same organ. It has been proposed that stem cells reside in specific compartments called 'stem cell niches' that provide essential signals required for their function and maintenance. Besides the stem cells themselves, components of the niche are thought to include supporting cells, extracellular matrix as well as neurovascular tissue. Communication among the cells inside the niche via signaling molecules is crucial to accomplish the balance between self-renewal and differentiation of stem cells (Jones and Wagers, 2008; Schofield, 1978). Additionally, the stem cell niche also protects stem cells from depletion and protects the host from excessive proliferation of stem cells (Scadden, 2006).

Early stages of mouse incisor embryonic development are similar to those of molars but when the incisor buds reach the cap stage

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they rotate anteroposteriorly. Subsequently, at the bell stage (E16.5) the epithelial compartments of the apex form a special structure called " the cervical loop". Histologically, the cervical loop comprises a central core of star-shaped cells called stellate reticulum that are surrounded by a layer of epithelial cells (Fig. 1A). The cervical loop at the labial aspect that is responsible for the continuous generation of ameloblast precursors, is larger than the one at the lingual aspect that does not generate ameloblasts and contains a larger number of stellate reticulum cells. It has been shown that these stellate reticulum cells contain slowly dividing stem cells that subsequently undergo asymmetric cell division. As these stem cells proliferate, one daughter cell remains within the niche as an undifferentiated stem cell whereas the other daughter cell is displaced away from the niche, enters a zone of transit-amplifying cells (TA cells) and differentiates into ameloblast (Harada et al., 1999). In addition to molecular signals in the cervical loop epithelium, it has been proposed that mesenchymal signals are also of importance and direct the continuous proliferation of epithelial progenitor cells. Members of Fibroblast growth factors (FGFs) family, *Fgf10* and *Fgf3*, are found to be expressed in the incisor mesenchyme and their receptors, including *Fgfr1b* and *Fgfr2b* are expressed adjacently in the dental epithelium (Harada et al., 1999). The potential role of *Fgf10* in the maintenance of the stem cell compartment is further confirmed by a hypoplastic cervical loop and a decreased growth rate of the dental epithelium in *Fgf10* null mice during late stages of incisor development. Additionally, *in vitro* experiments using an anti-FgF10 neutralizing antibody showed that functional disturbance of Fgf10 results in apoptosis of cervical loop cells (Harada et al., 2002). A similar phenotype was observed by epithelial-specific deletion of Fgfr2 in the cervical loop confirming that Fgf signals from the mesenchyme are required for the development and maintenance of the cervical loop stem cell niche (Lin et al., 2009).

The precise location of the mesenchymal stem cell niche in the incisor is unclear although the mesenchymal stem cells (MSCs) are generally believed to be located in the apical end mesenchyme, close to the cervical loops, since the growth and differentiation of the incisor always initiates at the apical end then extends towards the incisal end. Feng et al. (2011) suggested dual origins of dental pulp mesenchymal stem cells during incisor growth and repair, one of which located in the apical dental mesenchyme tissue (Feng et al., 2011). This was also confirmed by another study, using consecutive 5-bromo-2-deoxyuridine (BrdU) administration followed by a chase period, which showed a label-retaining slow-cycling stem cell population located in the very apical end of the incisor dental pulp mesenchyme (Seidel et al., 2010).

Polycomb Group (PcG) proteins were first described as repressors of *Hox* genes in *Drosophila melanogaster*. Mutations of these proteins in flies result in homeotic transformation of one body segment into the identity of another (Lewis, 1978). A number of studies in flies and mammals demonstrated that most PcG proteins are not classic DNA binding proteins but present as large multimeric protein complexes and exist as heterogeneous complexes of varying compositions. This existence is thought to result from a distinction of target genes for these complexes and also contributes to different functions of each complex (Satijn et al., 1997). It has been shown that the function of PcG complexes is to maintain the transcriptional repression of target genes by binding to the chromatin and inducing higher-order chromatin structure (Ringrose and Paro, 2004; Schuettengruber et al., 2007). Aside from the role in the control of body plan and segmentation, accumulating studies also revealed crucial functions of PcG proteins in the maintenance of embryonic and adult stem cells, control of cell proliferation, cancer development, genomic imprinting and X-chromosme inactivation (Delaval and Feil, 2004; Heard, 2005; Sparmann and van Lohuizen, 2006).

In mammals, two distinct PcG complexes have been extensively studied: Polycomb Repressive Complex (PRC) 1 and PRC2. While the PRC1 is essential for stable maintenance of gene repression by preventing nucleosome remodeling, PRC2 is involved in the initiation of gene repression and functions as a histone methyltransferase that specifically methylates lysine 27 of histone H3 (H3K27) in nucleosomes (Cao et al., 2002; Schwartz and Pirrotta, 2007; Valk-Lingbeek et al., 2004). Mammalian PRC1 consists of orthologs of *Drosophila* Polycomb (Cbx2, Cbx4, Cbx6, Cbx7 and Cbx8), Posterior sex combs (Mel18, Bmi1, Nspc1/Pcgf1 and MBLR), dRing (Ring1a and Ring1b) and Polyhomeotics (Phc1, Phc2 and Phc3). The core PRC2 consists of Suz12, Eed, Ezh1 and Ezh2 (Ringrose and Paro, 2004; Schwartz and Pirrotta, 2007). Among the core PRC1, Ring1a and Ring1b have been shown to possess E3 ubiquitin ligase activity for histone H2A that plays an important role in PcG-mediated silencing (de Napoles et al., 2004; Wang et al., 2004). Apart from the PRC1, Ring1a/b have further been identified in other protein complexes with BCoR and Fbxl10/Kdm2R (Gearhart et al., 2006; Sanchez et al., 2007).

Emerging evidence has demonstrated possible roles of Ring1b in the control of cell proliferation as well as the maintenance of ES cells. Using *Ring1a/b* knockout mouse ES cells, Ring1a/b have been shown to be required for the maintenance of ES cell identity by silencing the genes that govern differentiation of ES cells. It has been demonstrated that transcriptional repression mediated by Ring1a/b is Oct3/4-dependent. Moreover, in the presence of enforced expression of the differentiation inducer, *Gata6*, Ring1a/b target genes become derepressed and the Ring1a/b binding is also significantly reduced. These results indicate that Ring1a/b act downstream of the core transcriptional regulatory circuit to regulate ES cell self-renewal (Endoh et al., 2008). Subsequent studies further supported the essential role of Ring1b in stable maintenance of mouse ES cells. In order to maintain undifferentiated ES cells, Ring1b is needed to silence a particular subset of genes, which are co-occupied by ES cell regulators including Oct4 and Nanog. These Ring1b target genes also possess bivalent histone marks with CpG-rich promoters, and include developmental transcriptional factors, morphogens and cell surface markers (van der Stoop et al., 2008).

We provide here evidence that PRC1 gene expression localizes to a population of cells distal to the predicted location of the mesenchymal stem cells that have characteristics of transit amplifying cells and that the Ring1 components of the PRC1 complex are essential for proliferation and differentiation of these cells.

We show that in addition to providing precursors for the continuous replacement of mesenchymal cells during incisor growth, Ring1 proteins are essential for maintaining expression of Fgfs that act to regulate the adjacent epithelial stem cell niche, the labial cervical loop. In addition we also identify a role for Ring1 proteins in supporting normal molar root development.

## Results

### *Genes encoding proteins of the PRC1 complex are expressed in highly proliferative cells in the apical mesenchyme in continuously growing incisors*

Whole-mount *in situ* hybridization analysis showed that at post natal stages all of the genes encoding proteins of the PRC1 complex were expressed in the dental mesenchyme adjacent to the labial and lingual cervical loop epithelium of the mouse mandibular (Figs. 1 and 2) and maxillary (data not shown) incisors. *Ring1a* and its homolog, *Ring1b*, were expressed in the apical mesenchyme of the incisors (Fig. 1B,B′,C,C′; Fig. 2 A,A′, B,B′). Ring1b was also expressed in cells of the dental follicle and some patchy expression was evidence in the epithelial cells in the area of preameloblast formation (Fig. 2 B′). Expression of *Nspc1/Pcgf1* was observed in the apical mesenchyme

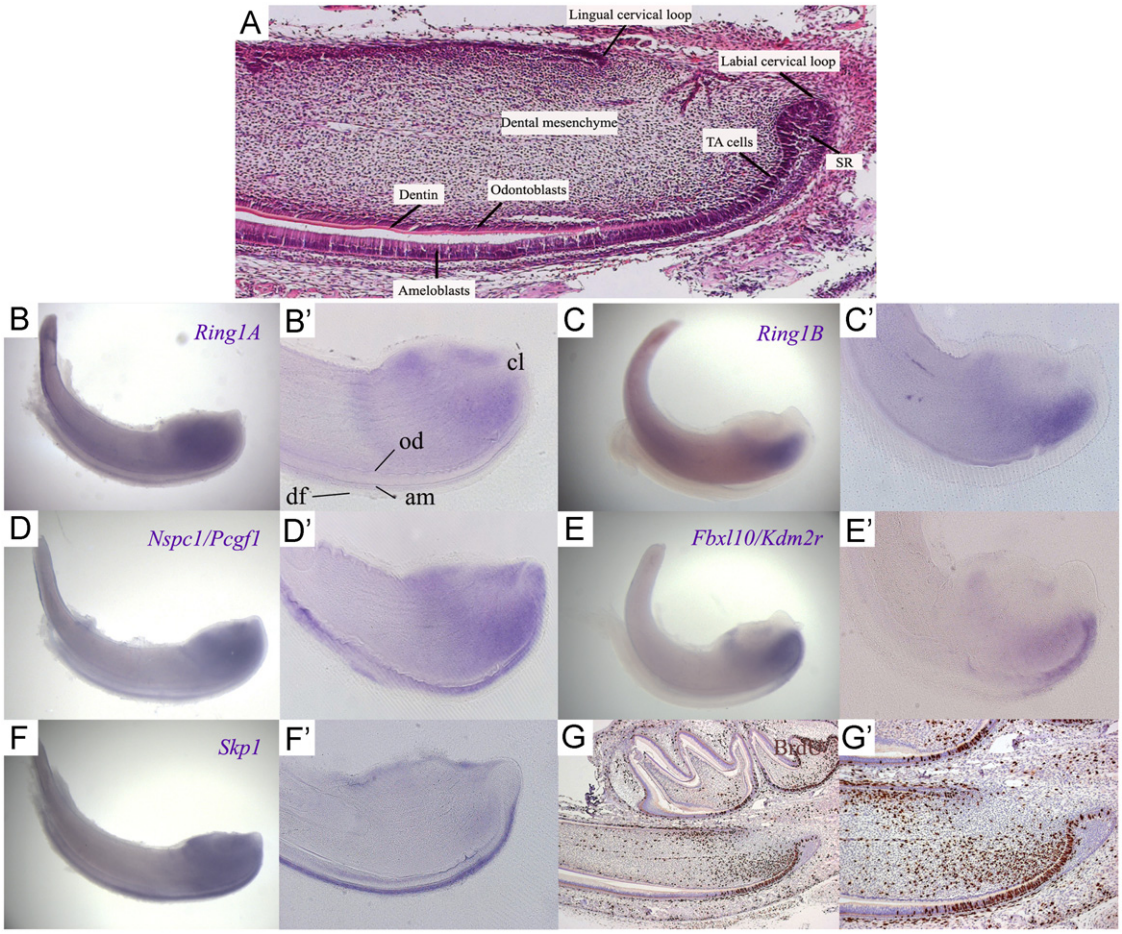


**Fig. 1.** Co-expression of genes encoding proteins of the PRC1 complex and cell proliferation marker BrdU in 2 day-old mouse mandibular incisor apical mesenchyme. (A) The apical end of incisor consists of cervical loops at both lingual and labial sides surrounding the dental mesenchyme. The labial cervical loop contains a stellate reticulum (SR) core surrounded by dental epithelium. TA, transit-amplifying. (B, B′, C, C′) *Ring1a* and *Ring1b* are strongly expressed in the dental mesenchyme close to both labial and lingual cervical loops of the incisor and in the area of cells with high rates of proliferation (TA cells). (D, D′) *Nspc1* is expressed in the dental mesenchyme near labial and lingual cervical loops, and in the transit-ampifying cells of the dental epithelium. (E, E′) *Fbxl10* expression is notable in the labial mesenchyme and the transit-ampifying cells of the dental epithelium. (F, F′) *Skp1* is weakly expressed in the mesenchyme and more highly expressed in cells of the dental follicle (df) of the incisor germ and the ameloblasts (Am). (G, G′) BrdU expression indicates that highly proliferative cells were located predominantly in the dental mesenchyme near labial and lingual cervical loops, and in also the transit-amplifying cells of the dental epithelium.

and part of the dental epithelium and follicle of the mouse incisor (Fig. 1D,D′; Fig. 2C,C′). *Fbxl10/Kdm2r* showed a similar but weaker expression compared to the expression of *Nspc1* (Fig. 1E,E′). *Skp1* expression was observed weakly in apical mesenchyme and in cells of the dental follicle covering the apical part of the developing incisor as well as the enamel-forming ameloblasts (Fig. 1F,F′; Fig. 2D,D′).

Many stem cell populations contain slow-cycling cells that produce progenitor cells that are rapidly dividing, often called transit-amplifying (TA) cells. We analysed the proliferation characteristics of the apical mesenchymal cells by administration of a single short pulse of synthetic nucleoside analogue, BrdU and located labeled cells using immunohistochemistry. BrdU+ve rapid-dividing cells were located in mesenchymal cells at the apical end as previously shown in a position that closely matched the location of PRC1 expressing cells, distal to label retaining cells (Fig. 1G,G′), suggesting that these cells are transit amplifying cells (Harada et al., 1999; 2002;Seidel et al., 2010).

### *Ring1a$^{-/-}$;Ring1b$^{cko/cko}$ mice display incisors with defective cervical loops and abnormal enamel and dentin formation*

In order to determine the roles of Ring1 proteins in incisors, postnatal conditional inactivation using tamoxifen-inducible Cre was used. Since *Ring1a$^{-/-}$* mice survive and are fertile, *Ring1b$^{fl/fl}$* mice were crossed with *Ring1a$^{-/-}$* and double homozygotes then crossed with *Rosa26::CreERT2* transgenic mice. Administration of tamoxifen between postnatal days 9 to 13 created double *Ring1a/b* loss of function animals that were analysed at P17.

Morphology of mouse maxillary and mandibular incisors was examined using microCT scanning and analysis revealed that P17 *Ring1a$^{-/-}$;Ring1b$^{cko/cko}$* incisors (Fig. 3B) had a similar gross morphology to *Ring1a$^{-/-}$;Ring1b$^{fl/fl}$cre-* incisors but were obviously shorter (Fig. 3A). In order to evaluate the incisor length, measurement analysis of incisor length (indicated by dotted lines in Fig. 3A and B) was carried out as described in the Materials and Methods section. The mean lengths of mandibular incisors of *Ring1a$^{-/-}$;Ring1b$^{cko/cko}$* mice were 6576 μm (Fig. 3B), whereas those of *Ring1a$^{-/-}$;Ring1b$^{fl/fl}$cre-* mice were 8085 μm (Fig. 3A). Statistical analysis by *t*-test additionally revealed that mandibular incisors (asterisks in Fig. 3C; $P<0.01$) of *Ring1a$^{-/-}$;Ring1b$^{cko/cko}$* mice ($n=8$) were significantly shorter than those of *Ring1a$^{-/-}$; Ring1b$^{fl/fl}$ cre-* incisors ($n=6$).

Histological examination revealed that P17 *Ring1a$^{-/-}$;Ring1b$^{cko/cko}$* incisors had abnormal cervical loops at their apical ends (Fig. 4B5, Fig. 2S), compared to *Ring1a$^{-/-}$;Ring1b$^{fl/fl}$ cre-* incisors (Fig. 4D5, Fig. 2S). Differentiation of odontoblasts and ameloblasts

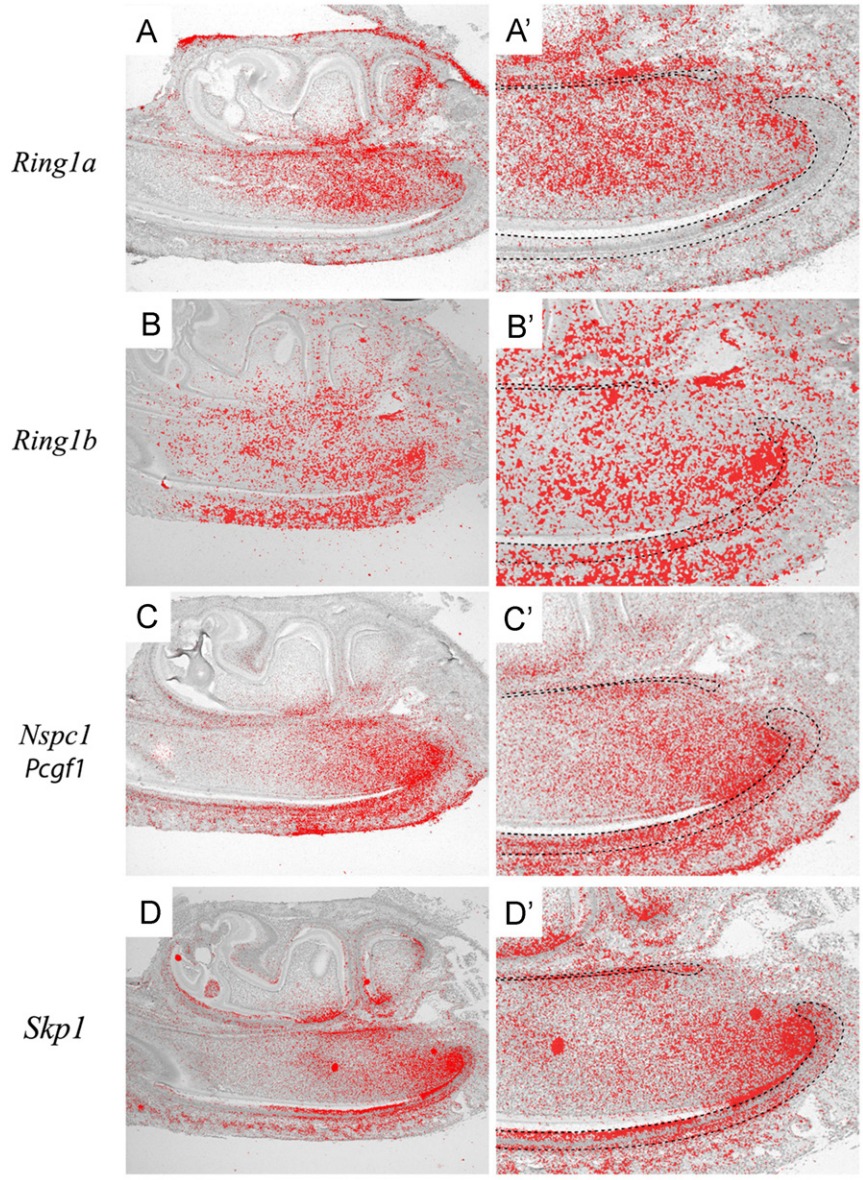


**Fig. 2.** Radioactive *in situ* hybridisation of PRC1 complex genes. Radioactive *in situ* hybridisation for *Ring1* (*A, A′*), *Ring1b B, B′*), *Nspc1/Pcgf1*(*C, C′*) and *Skp1* (*D, D′*) on sagittal sections of P5 mandibular incisors (A, A′). Silver grains were false coloured in red cervical loop outlined by black dashes. All four genes are expressed in mesenchymal cells between the lingual and labial aspects of the epithelial cervical loop. (For interpretation of the references to color in this figure legend, the reader is reffered to the web version of this article.)

was disrupted in *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ along most of the incisor length (Fig. 4D1–D4), subsequently leading to disturbances in enamel and dentin formation, compared to *Ring1a*$^{-/-}$;*Ring1b*$^{fl/fl}$*cre*- incisors (Fig. 4B1–B4). While in *Ring1a*$^{-/-}$;*Ring1b*$^{fl/fl}$*cre*- incisors the odontoblasts and ameloblasts were elongated and highly polarized (Fig. 4B1–B4), in *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ incisors they were more round in shape and had no nuclear polarization (Fig. 4D1–D4). No evidence of any odontoblast and ameloblast differentiation was observed at the inner side of the labial cervical loop in *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ incisors (Fig. 4D4), compared to the same region of *Ring1a*$^{-/-}$;*Ring1b*$^{fl/fl}$*cre*- incisors (Fig. 4B4). Furthermore, in *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ incisors, the tips of labial cervical loop were still present but they appeared smaller (Fig. 4D5) than those of *Ring1a*$^{-/-}$;*Ring1b*$^{fl/fl}$ *cre*- incisors (Fig. 4B5). MicroCT and histological examination of *Ring1a*$^{-/-}$;*Ring1b*$^{fl/fl}$*cre*- incisors showed them to be indistinguishable from wild type (data not shown).

### *Loss of Ring1a and Ring1b leads to down-regulation of genes important for enamel and dentin formation*

Abnormal odontoblasts and ameloblasts observed in *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ incisors were further analysed by *in situ* hybridization analysis of genes known to be markers of functional odontoblasts and ameloblasts, including *Dentin sialophosphoprotein* (*Dspp*), *Amelogenin* and *Shh*. *Dspp*, normally expressed in odontoblasts and newly differentiated ameloblasts (Begue-Kirn et al., 1998), was found to be down-regulated in *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ incisors (Fig. 5C), compared to wild type (WT) incisors (Fig. 5A) and *Ring1a*$^{-/-}$;*Ring1b*$^{fl/fl}$*cre*- incisors (Fig. 5B). *Amelogenin*, a gene encoding the major structural protein of enamel matrix and expressed in functional ameloblasts (Zeichner-David et al., 1995), was absent in *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ incisors (Fig. 5F), in comparison to WT (Fig. 5D) and *Ring1a*$^{-/-}$;*Ring1b*$^{fl/fl}$*cre*- incisors (Fig. 4E). Furthermore, *Shh* which normally marks pre-ameloblasts (Bitgood and McMahon,

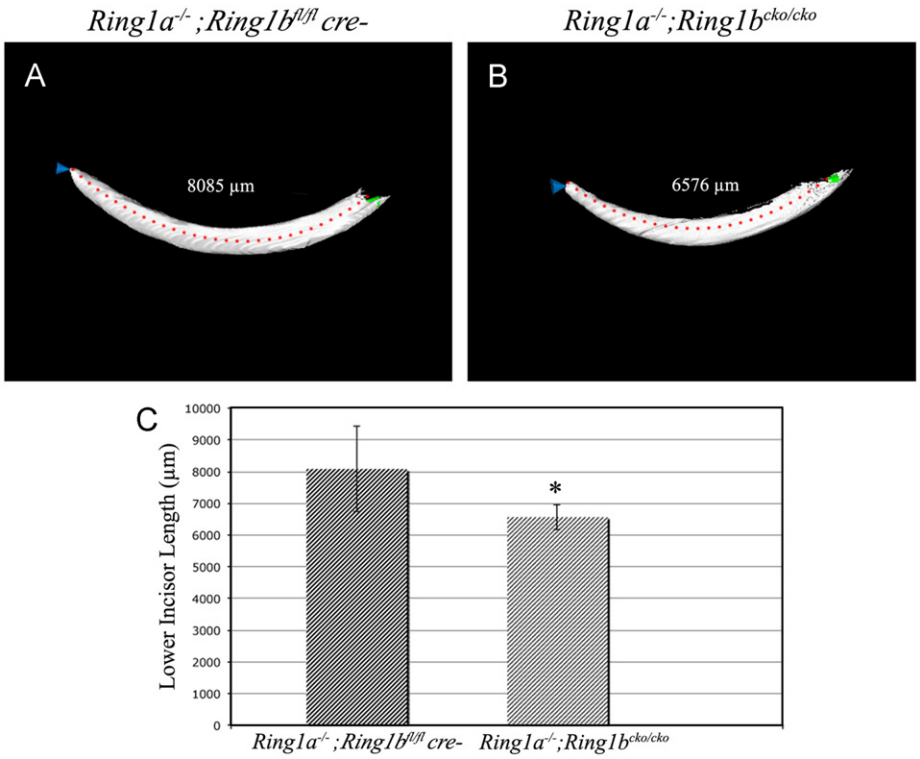


**Fig. 3.** MicroCT anaylsis of P17 *Ring1a*$^{-/-}$;*Ring1b*$^{fl/fl}$*cre-* and *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ incisors. (A) *Ring1a*$^{-/-}$;*Ring1b*$^{fl/fl}$*cre-* shows a normal shape of mouse incisor. (B) Incisor of *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ mouse is shorter than that of *Ring1a*$^{-/-}$;*Ring1b* $^{fl/fl}$*cre-*. Red dots in (A) and (B) indicate the measurement points used to determine the incisor length in MicorCT scans. (C) Mean incisor length ± standard deviation (SD) of mandibular (lower) incisors of *Ring1a*$^{-/-}$;*Ring1b*$^{fl/fl}$*cre-* mice ($n=6$) and *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ mice ($n=8$). Asterisk (*) indicates values significantly different between incisor length of *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ mice and that of *Ring1a*$^{-/-}$;*Ring1b* $^{fl/fl}$*cre-* mice according to Student's *t* test ($P < 0.01$). (For interpretation of the references to color in this figure legend, the reader is reffered to the web version of this article.)

1995), was also downregulated in *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ incisors (Fig. 5I), compared to WT (Fig. 5G) and *Ring1a*$^{-/-}$;*Ring1b*$^{fl/fl}$*cre-* incisors (Fig. 5H). In all these cases, gene expression was maintained in the most distally-located cells at a reduced level and was significantly reduced or absent from proximal cells (Fig. 5C, F, I). These results additionally confirmed the defects previously observed in odontoblasts and ameloblasts in histological sections.

### *Absence of Ring1a and Ring1b results in reduced cell proliferation in the apical mesenchyme and cervical loop epithelium of the continuously growing mouse incisor*

To further investigate the defects found in *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ incisors, cell proliferation analysis was carried out. Cell proliferation was analysed by immunohistochemistry with an antibody that detects mitosis, Phospho-histone H3(PH3). Immunohistochemical staining showed that mitotically active cells were markedly reduced in the apical mesenchyme adjacent to labial and lingual cervical loops and in the epithelial cells of the labial cervical loops of *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ incisors (Fig. 5L) in comparison to WT (Fig. 5J) and *Ring1a*$^{-/-}$;*Ring1b*$^{fl/fl}$*cre-* incisor (Fig. 5K). This suggests that Ring1a and Ring1b, either directly or indirectly (possibly by an affect on the mesenchymal stem cells) are required for mitosis of the dental mesenchymal stem/progenitor cells that constantly give rise to dentin-forming odontoblasts and also the cervical loop epithelium, giving rise to enamel-forming ameloblasts.

### *Loss of Ring1a and Ring1b results in down-regulation of Fgf signaling in the apical end of continuously growing incisors*

Fgf signaling has previously been shown to be important for the maintenance of the epithelial stem cell compartment in the cervical loop of continuously growing mouse incisors. Among members of the Fgf family, several Fgfs including *Fgf3*, *Fgf10* and *Fgf9* have been found to be involved in the development of mouse incisors. The cervical loop has been found to be absent in *Fgf10* null mice (Harada et al., 2002) while expression of *Fgf3* in the dental mesenchyme was shown to stimulate epithelial stem cell proliferation (Wang et al., 2007). Radioactive *in situ* hybridization revealed that *Fgf10*, normally expressed in both labial and lingual mesenchyme, was down-regulated in both *Ring1a*$^{-/-}$;*Ring1b*$^{fl/fl}$ *cre-* (Fig. 6B) and *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ (Fig. 6C) incisors in comparison to WT incisors (Fig. 6A). *Fgf3*, which is restrictedly expressed in the labial mesenchyme was down-regulated in *Ring1a*$^{-/-}$;*Ring1b*$^{fl/fl}$*cre-* (Fig. 6E) and completely absent in *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ (Fig. 6F) incisors in comparison to WT incisors (Fig. 6D). Analysis of direct downstream targets of Fgf signaling including *Pea3* and *Erm* (also known as *Etv4* and *Etv5*, respectively) (Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001) was also performed. Expression of *Pea3* and *Erm*, normally detected in the epithelial cervical loops and adjacent mesenchyme (Fig. 6G and J), were markedly down-regulated in *Ring1a*$^{-/-}$;*Ring1b*$^{fl/fl}$*cre-* (Fig. 6H and K) and completely absent in *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ (Fig. 6I and L) incisors in comparison to WT incisors (Fig. 6G and J). This suggests that *Ring1a* and *Ring1b* regulate Fgf signaling in developing mouse incisors.

### *Loss of Ring1a and Ring1b leads to alterations of expression of genes encoding the PRC1 complex*

Expression analysis of genes encoding members of the PRC1 complex including *Nspc1/Pegf1* and *Fbxl10/Kdm2r*, was further examined in the absence of *Ring1a* and *Ring1b*. Interestingly, *Nspc1/Pcgf1* *m*RNA was shown to be unaffected in both

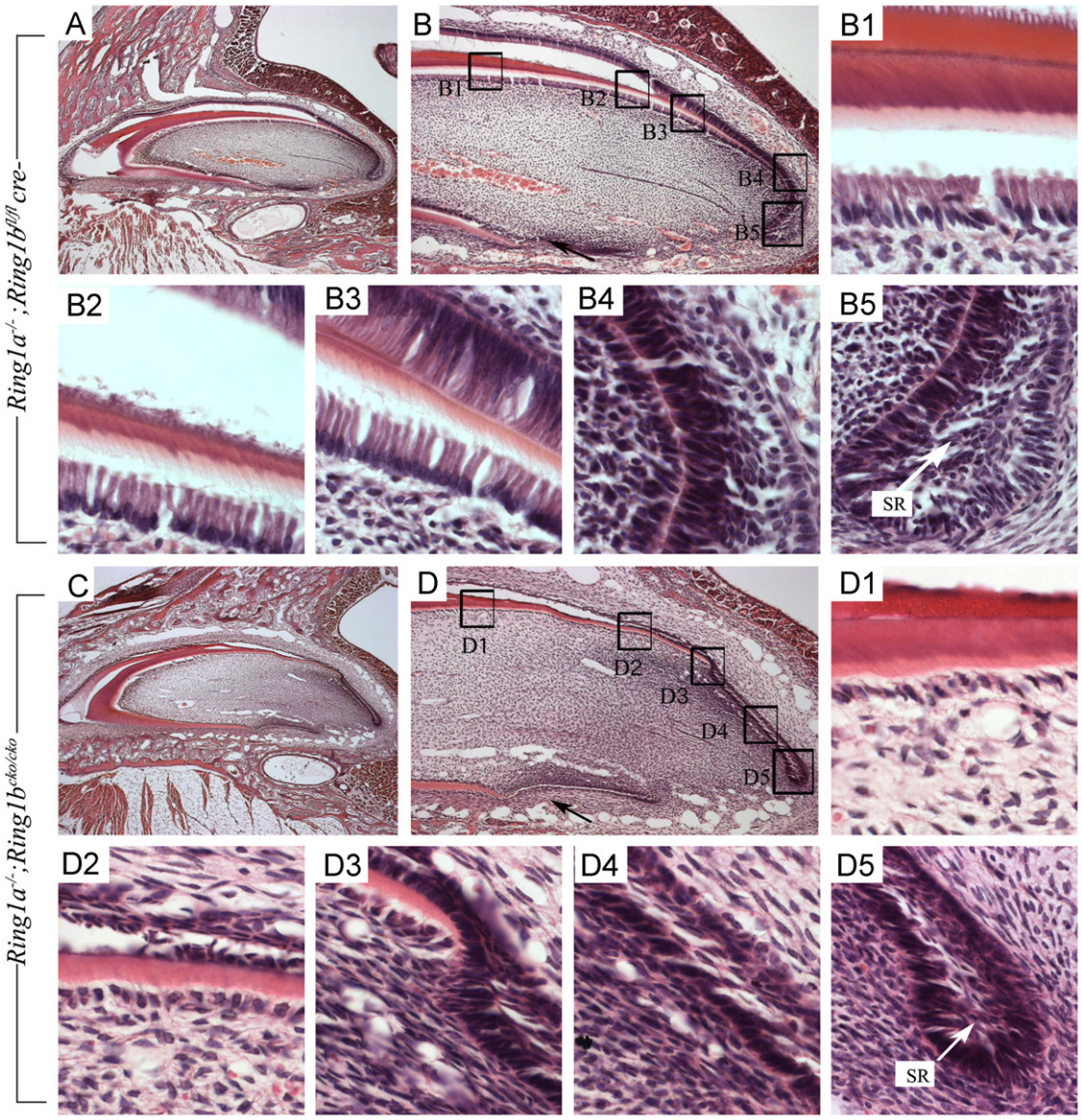


**Fig. 4.** Hematoxylin and eosin stained sagittal sections of a maxillary incisor of 17 day-old *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ and *Ring1a*$^{-/-}$;*Ring1b*$^{fl/fl}$*cre*- mice. (A, B) *Ring1a*$^{-/-}$;*Ring1b* $^{fl/fl}$ *cre*- incisors show normal incisor development. (B1–B5) Higher power views of boxed regions in (B) showing normal odontoblast and ameloblast differentiation (B4), labial cervical loop containing a core of stellate reticulum (SR) in *Ring1a*$^{-/-}$;*Ring1b*$^{fl/fl}$ *cre*- mice (B5). (C, D) *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ incisors show abnormal development. (D1–D4) Higher power views of regions boxed in (D) showing abnormal morphology of odontoblasts and ameloblasts. (D5) Higher magnification of the black box in (D) shows small labial cervical loop containing a few cells of stellate reticulum (SR) in *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ mice.

*Ring1a*$^{-/-}$;*Ring1b*$^{fl/fl}$*cre*- (Fig. 6N) and *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ (Fig. 6O) incisors compared to WT incisors (Fig. 6M), while *Fbxl10/Kdmr2* expression appeared to be down-regulated only in *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ (Fig. 6R) but not in *Ring1a*$^{-/-}$;*Ring1b*$^{fl/fl}$*cre*- (Fig. 6Q) incisors compared to WT incisors (Fig. 6P). These data show that Ring1a and Ring1b possibly regulate transcription of members of the PRC1 complex including Fbxl10/Kdmr2.

### *Ring1a and Ring1b are required for the development of molar roots*

The apical incisor phenotype observed in *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ mice prompted us to examine PRC1 gene expression and postnatal development of molars. The key postnatal developmental event is the formation of tooth roots. Expression of *Ring1a, Ring1b, Nspc1, Fbxl10 and Skp1* were all found to be localized in postnatal molars in apical areas at the early stages of root formation, particularly in root odontoblasts (Fig. 7). Micro CT analysis of first molars in P17 postnatal *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ mice (Fig. 8 B and D) revealed a lack of root formation compared to cre- controls (Fig. 8 A and C). Histological sections of the developing roots identified abnormal root odontoblasts that were small and non-polarised in the *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ molars (Fig. 9 B′) in comparison to the *cre*- molars (Fig. 9 A′).

In order to begin to understand the molecular consequences of loss of Ring proteins on root formation we investigated the expression of *Bmp4* since this has been linked to the formation of Hertwigs epithelial root sheath formation during root development (Hosoya et al., 2008; Yamashrio et al., 2003). Expression of *Bmp4* was highly restricted to developing root odontoblasts in wild type molars but was more widely distributed in mesenchymal cells in the area of arrested root formation in *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ (Fig. 9C and D, respectively). This suggests that restriction of BMP signaling to specific areas of root formation involves Ring proteins and in their absence expression becomes more widespread and as a result interferes with the normal development of root odontoblasts.

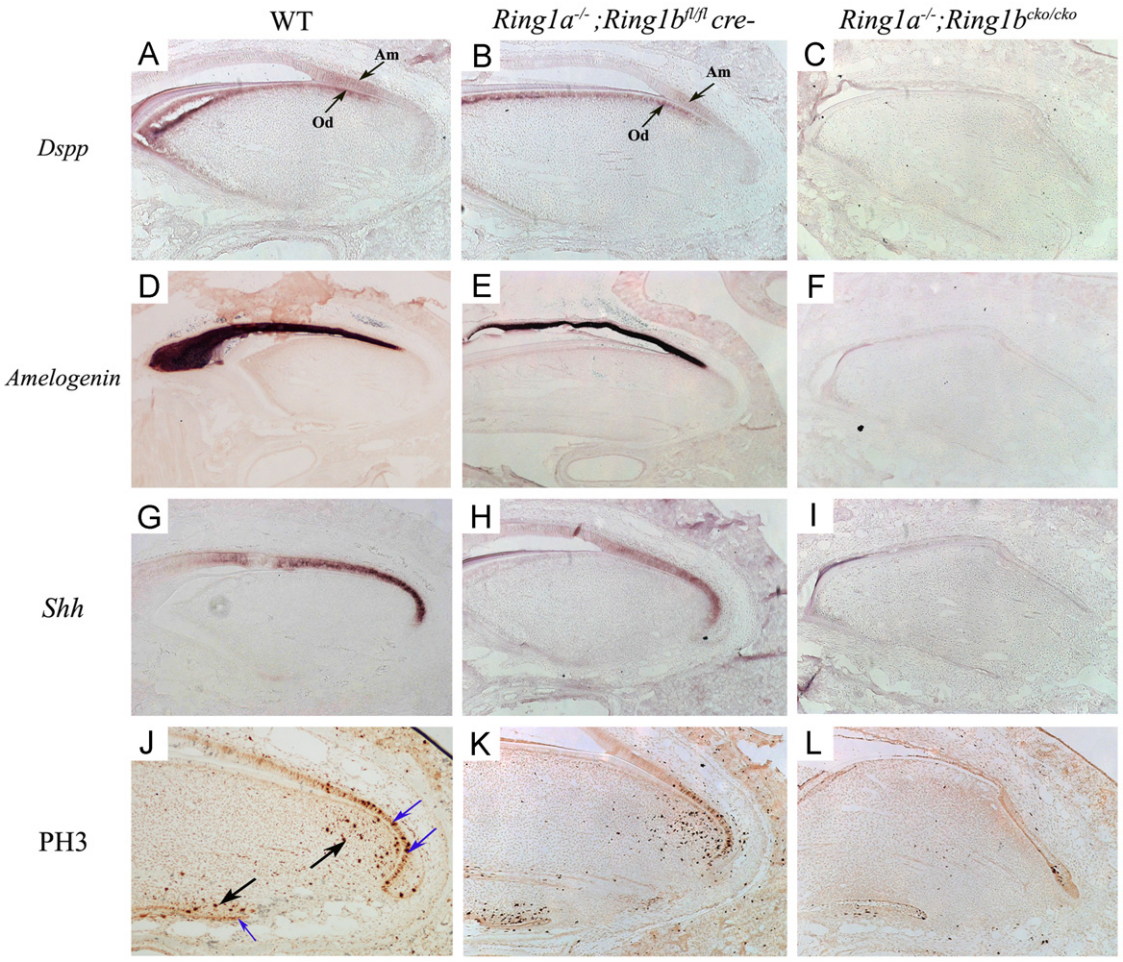


**Fig. 5.** Expression analysis of *Dspp*, *Amelogenin* and *Shh* and Phopho-histone H3 (PH3) in 17 day-old maxillary incisors of wild-type, *Ring1a*$^{-/-}$;*Ring1b*$^{fl/fl}$*cre*- and *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ mice (sagittal sections). (A and B) *Dspp* expression in the odontoblasts (od) and differentiated ameloblasts (am) of WT control and *Ring1a*$^{-/-}$;*Ring1b*$^{fl/fl}$*cre*- incisors. (C) Down-regulation of *Dspp* in *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ incisor. (D and E) Expression of *Amelogenin* in functional ameloblasts in WT and *Ring1a*$^{-/-}$;*Ring1b*$^{fl/fl}$*cre*- incisor, respectively. (F) *Amelogenin* expression is down-regulated in *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ incisor. (G and H) Strong expression of *Shh* is observed in pre-ameloblasts in WT and *Ring1a*$^{-/-}$;*Ring1b*$^{fl/fl}$*cre*- incisor, respectively. (I) *Shh* transcript is absent in *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ incisor. Immunohistochemistry against Phopho-histone H3 (PH3) shows a significant decrease of cell proliferation in 17 day-old *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ incisor (L). A number of mitotic cells are identified in the apical mesenchyme (black arrows) and inside the cervical loop epithelium (blue arrows) in WT (J) and *Ring1a*$^{-/-}$;*Ring1b*$^{fl/fl}$*cre*- incisor (K). (L) A minimal staining of mitotic cells is noted in the apical end of *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ incisor. (For interpretation of the references to color in this figure legend, the reader is reffered to the web version of this article.)

## Discussion

### *Ring1a/b regulate a mesenchymal stem cell niche in developing incisors*

Whilst *Ring1a*$^{-/-}$;*Ring1b*$^{fl/fl}$*cre*- mice do not exhibit any incisor phenotype, mice lacking both *Ring1a* and *Ring1b* postnatally display abnormal incisor development with an impairment of continuous growth. Furthermore, the lack of any incisor abnormalities in Ring1a$^{+/-}$;*Ring1b*$^{cko/cko}$, indicates a redundancy between Ring1a and Ring1b function during incisor development. However this redundancy is not complete since although *Ring1a*$^{-/-}$;*Ring1b*$^{fl/fl}$*cre*- incisors show no phenotypic abnormalities, they do show molecular changes in FGF activity. It has been shown that on the inactive X chromosome, the ubiquitination of histone H2A was retained in cells lacking Ring1a or Ring1b but not in cells lacking both (de Napoles et al., 2004). In addition, *Ring1b* single-knockout ES cells appeared normal whereas *Ring1a/b* double-knockout ES cells progressively lost ES cell characteristics after tamoxifen treatment to inactivate *Ring1b* (Endoh et al., 2008). It is possible that the absence of a tooth phenotype in *Ring1a*$^{+/-}$;*Ring1b*$^{cko/cko}$ is due to a compensatory role of the remaining copy of *Ring1a* for *Ring1b*. The compensatory role of Ring1a for Ring1b has previously been suggested owing to an increased expression of Ring1a protein in *Ring1b*-null ES cells (Endoh et al. 2008).

Rodent incisors are capable of erupting throughout their lifetime since the constant attrition at the incisal edge is compensated by the renewal of tooth-forming tissues at the apex of incisors deeply embedded in the jawbone. Our results show that the morphology of the whole cervical loop is remarkably abnormal in the P17 *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ mice, in comparison to the cervical loop of *Ring1a*$^{-/-}$;*Ring1b*$^{fl/fl}$*cre*- mice. The mice used in this study exhibited a level of Ring1b protein that progressively decreased after 48 h of tamoxifen treatment. These mice were injected with treated tamoxifen at P9 and P13 and sacrificed at P17, therefore they were in the presence of tamoxifen for a maximum of 8 day. Since there would likely be a delay of around 24h for the drug to start working, the maximum period during which Ring1b inactivation could take place in postnatal tissues was thus approximately 7 day. Our measurement analysis showed that the growth of *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ incisors was arrested and the incisor lengths of *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ mice were significantly shorter than those of *Ring1a*$^{-/-}$;*Ring1b*$^{fl/fl}$*cre*- mice. It has

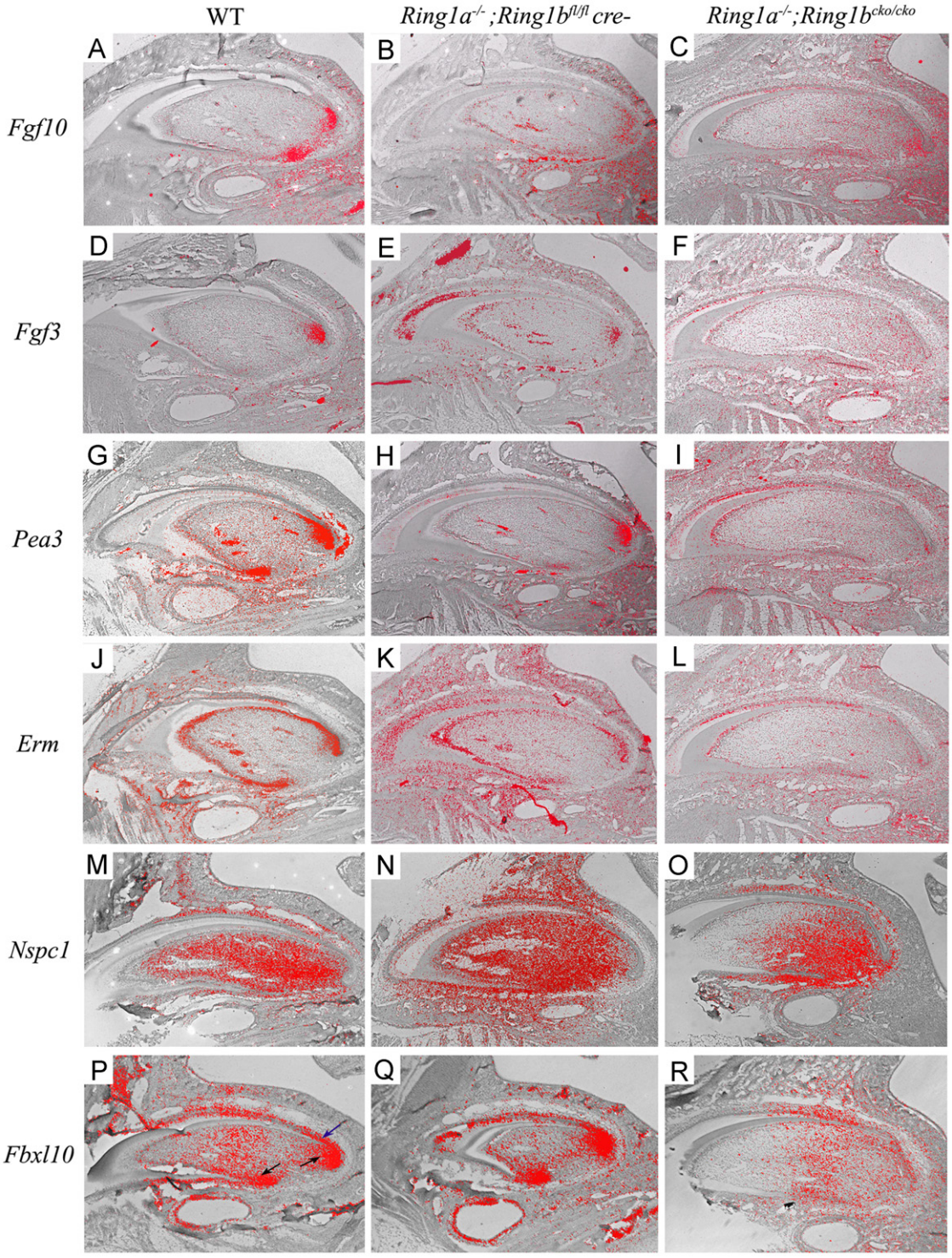


**Fig. 6.** Fgf signaling and transcripts encoding proteins of the PRC1complex are down-regulated in the apical end of *Ring1a*$^{-/-}$;*Ring1b*$^{fl/fl}$ *cre-* and completely absent in *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ incisors. (A-R) *In situ* hybridization analysis using 35$^{S}$ probes on paraffin sections of P17 WT, *Ring1a*$^{-/-}$;*Ring1b*$^{fl/fl}$*cre-* and *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ incisors (red color represents expression). (A) *Fgf10* expression is noted in both labial and lingual mesenchyme in a WT mouse incisor. (D) *Fgf3* is expressed only in the labial mesenchyme in a WT mouse incisor. *Fgf10* (B) and *Fgf3* (E) are down-regulated in *Ring1a*$^{-/-}$;*Ring1b*$^{fl/fl}$*cre-* and absent in *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ incisors (C and F). Expression of *Erm* (G) and *Pea3* (J) in WT incisors. (H, K) *Erm* and *Pea3* transcript is decreased in *Ring1a*$^{-/-}$;*Ring1b*$^{fl/fl}$*cre-* (H and K) and missing in *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ incisors (I and L). (M) High expression of *BCoR* is noticed in the labial and lingual mesenchyme (arrows) in WT mouse incisors. (N, O) (M) Expression of *Nspc1* is widespread in the dental mesenchyme of WT incisors. (N, O) Expression pattern of *Nspc1* is unchanged in *Ring1a*$^{-/-}$;*Ring1b*$^{fl/fl}$*cre-* (N) and *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ (O) incisors. (P) Strong *Fbxl10* expression is noted in the labial and lingual dental mesenchyme (black arrows) as well as part of the dental epithelium (blue arrow) in WT incisors. (Q) *Fbxl10* expression is unaffected in *Ring1a*$^{-/-}$;*Ring1b*$^{fl/fl}$*cre-* incisors. (R) Down-regulation of *Fbxl10* is clearly recognized in *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ incisors. (For interpretation of the references to color in this figure legend, the reader is reffered to the web version of this article.)

been demonstrated that the eruption rate of unimpeded mandibular mouse incisors is approximately 400 μm per day (Ness, 1965) and the difference of the mean lengths of *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ and cre- mandibular incisors was 1500 μm, thus they were slightly longer than what we expected if the growth of these incisors was halted for 7 day. This suggests that either the *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ incisors

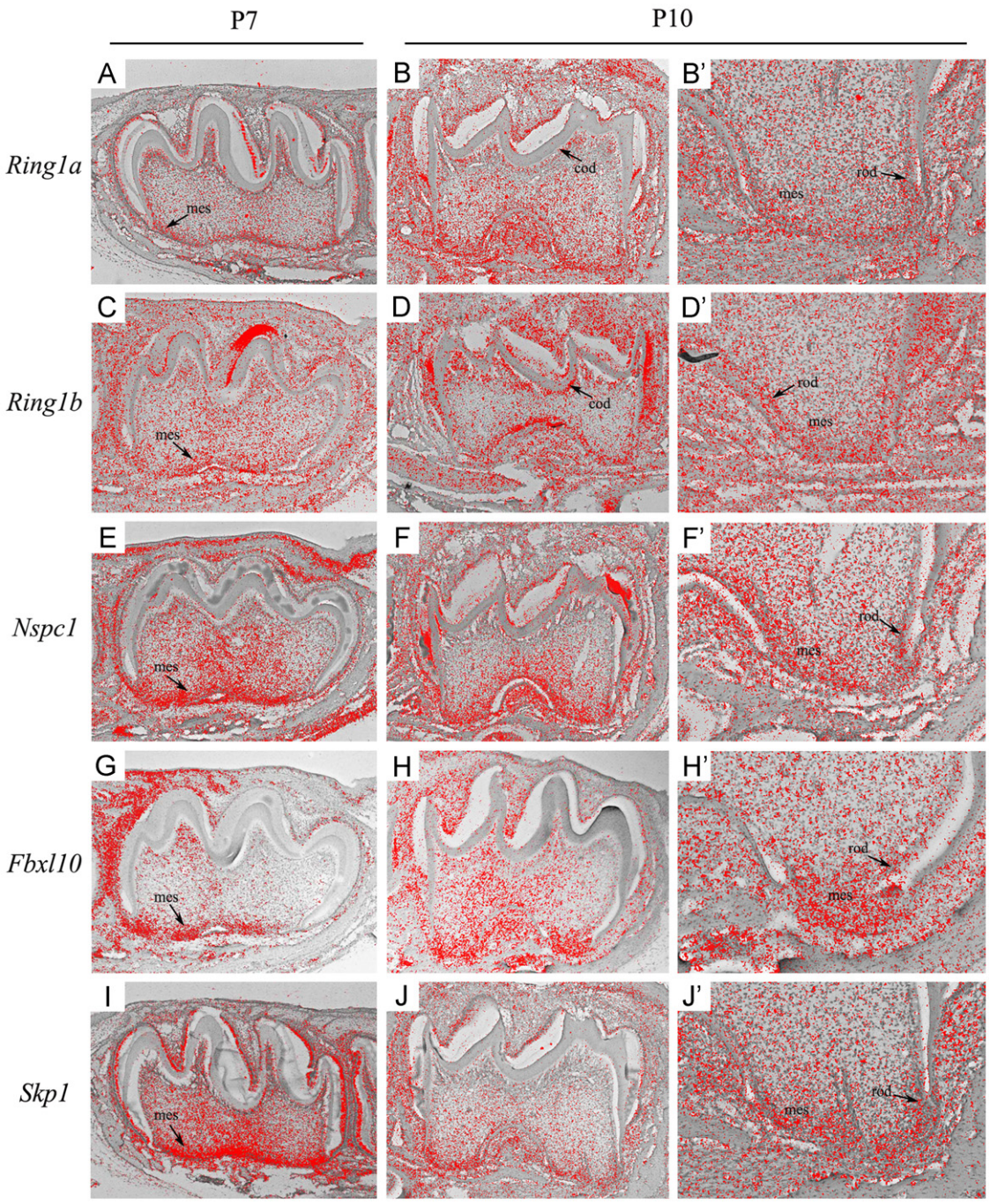


**Fig. 7.** Expression of genes encoding proteins of the PRC1 complex during postnatal molar development. (A, C, E, G, I) At P7, *Ring1a*, *Ring1b*, *Nspc1*, *Fbxl10* and *Skp1* are all expressed in the dental mesenchyme (mes) at the cervical area of developing molar roots (B, D, F, H, J) At P10, their expression is still maintained in the apical mesenchyme with a weaker expression in the mesenchyme of the coronal pulp. (B′, D′, F′, H′, J′) High magnification views of the apical region of one of the developing molar roots as shown in B, D, F, H and J, respectively. Expression of genes encoding members of the PRC1 complex is present in the apical mesenchyme (mes) and in the root odontoblasts (rod).

were still capable of growing at a lower rate, or that tamoxifen-induced cre-mediated excision may only be fully effective for approximately 4 day.

When comparing the cervical loops of *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ and *Ring1a*$^{-/-}$;*Ring1b*$^{fl/fl}$*cre*- mice, the secretion of enamel and dentin as well as the differentiation of ameloblasts and odontoblasts that normally appear as an increasing gradient from the apical end towards the incisal end were absent in the double-knockout mice. It is interesting to note that further in the incisal area, the odontoblasts facing the dentin matrix and the ameloblasts facing an empty space of enamel left by histological processing also appeared abnormal. These ameloblasts and odontoblasts were round in shape and had lost their nuclear polarization, suggesting that disruption may have occurred when these cells were differentiating and that they could not achieve terminal differentiation. Other possibilities are that soon after Ring1b was depleted, these cells became atrophic, suggesting an additional maintenance role or were unable to complete their normal rounds of cell division. It is known from tissue recombination experiments that functional differentiation of ameloblasts relies

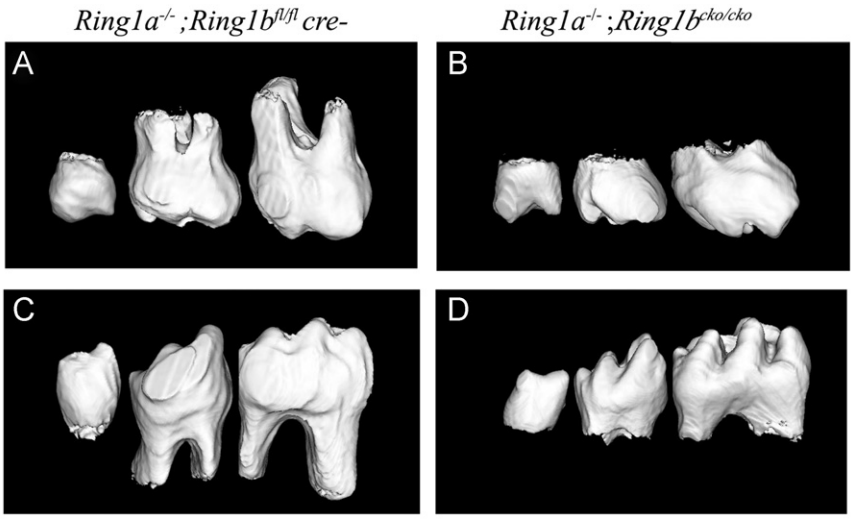


**Fig. 8.** MicroCT analysis of 17 day-old *Ring1a*$^{-/-}$;*Ring1b*$^{fl/fl}$*cre-* and *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ molars. (A, C) *Ring1a*$^{-/-}$;*Ring1b*$^{fl/fl}$*cre-* mice show normal maxillary (A) and mandibular (C) molar root development. (B, D) *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ incisor mice exhibit very short maxillary (B) and mandibular (D) molar roots.

on epithelial-mesenchymal interactions and requires a contact with dentin matrix to trigger differentiation (Karcher-Djuricic et al., 1985; Zeichner-David et al., 1995). The disruption of enamel-forming ameloblasts in *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ incisors could be due to a lack of dentin matrix secretion that results from aberrant odontoblasts in these double mutant mice. It is therefore possible that that the inability of mesenchymal cells to differentiate into odontoblasts is a cause of the odontoblast defects observed in *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ mice and that the abnormal ameloblast differentiation is a secondary consequence of this. Alternatively, ameloblast differentiation may be impaired as a consequence of a requirement for Ring proteins in mesenchymal transit amplifying cells for maintenance of the epithelial stem cell niche (see below) or the patchy expression of Ring1b in pre-ameloblasts may have a functional role. The aberrancy of odontoblasts and ameloblasts observed in *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ incisors was further confirmed by down-regulation of functional molecular markers of these cells, including *Dspp* in odontoblasts (D'Souza et al., 1997) and *Amelogenin* in ameloblasts (Zeichner-David et al., 1995). In addition, *Shh* which is expressed in early ameloblasts (Bitgood and McMahon, 1995) was also reduced from the dental epithelium of *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ incisors. The reduced expression of these genes in the mutant incisors correlated with abnormal cell morphology and since cells were more abnormal closer to the proximal (cervical loop) end, this would indicate that temporal effects of tamoxifen administration leading to incomplete recombination at early time points. Thus abnormal cell differentiation was thus more extensive that reduction of growth rate. An alternative explanation that cannot be excluded is that loss of Ring proteins has more long-range effects on cell differentiation.

The reduced cell proliferation noted in the apical dental mesenchyme close to both labial and lingual cervical loop epithelium where the differentiation of odontoblast progenitors takes place suggests that *Ring1a/b* proteins are required either directly or indirectly) for proliferation of the dental mesenchymal cells giving rise to dentin-forming odontoblasts. This finding is in agreement with previous studies that have shown that loss of Ring1a/b causes proliferation defects in ES cells (Endoh et al., 2008). Apart from the dental mesenchyme, a dramatically decrease in cell proliferation was also noted in the transit-amplifying cells of the cervical loop epithelium. This finding suggests that *Ring1a/b* regulates proliferative signals not only within the dental mesenchyme but also from the dental mesenchyme to the cervical loop epithelium. The most likely explanation for this is that *Ring1a/b* directly regulates a signaling molecule in the dental epithelium that controls epithelial cell proliferation (see below).

All these findings indicate that *Ring1a/b* double-knockout mutant incisors have lost the ability to grow continuously, highlighting the essential role of *Ring1a/b* in the regulation of the continuous growth of mouse incisors. We propose that *Ring1a*/b act in the dental mesenchymal stem cell micoenvironment (niche) in developing mouse incisors and that the loss of *Ring1a/b* therefore leads to a failure of the immediate progeny of mesenchymal stem cells (TA cells) to undergo proliferation and differentiation into odontoblast precursors, subsequently resulting in an arrest of the continuous growth of mouse incisors.

### *Ring1a/b regulate Fgf signaling in continuously growing mouse incisors*

The importance of *Ring1a/b* during incisor development was further illustrated by a down-regulation of Fgf signaling in *Ring1a*$^{-/-}$;*Ring1b*$^{fl/fl}$*cre-* and *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ incisors. Notably, Fgf signaling appeared to be reduced in both *Ring1a*$^{-/-}$;*Ring1b*$^{fl/fl}$*cre-* and *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ incisors, with a bigger decrease in the latter. Prior evidence has revealed that Fgf signaling plays essential roles during the development of mouse incisors, in particular *Fgf3* and *Fgf10*, which are restrictedly expressed in the dental mesenchyme underlying the rapidly proliferating cells of the inner enamel epithelium. Fgf10 has been shown to be a signal necessary for the maintenance of the epithelial stem cell niche residing in the cervical loop, owing to the hypoplastic cervical loops observed in *Fgf10*$^{-/-}$ mice (Harada et al., 2002). *Fgf3*$^{-/-}$ mice display abnormal enamel and *Fgf3*$^{-/-}$;*Fgf10*$^{+/-}$ mice exhibit very thin or no enamel (Wang et al., 2007). It is interesting to note that despite the down-regulation of *Fgf10* in *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ incisors, the tips of cervical loops, containing putative epithelial stem cells, do not become hypoplastic as seen in *Fgf10*$^{-/-}$ mice. They only appear slightly smaller than those in *Ring1a*$^{-/-}$;*Ring1b*$^{fl/fl}$*cre-* incisors. This may be explained by the fact that *Fgf10* was not completely absent in the dental mesenchyme surrounding the entire tip of cervical loops and that this residual *Fgf10* expression is sufficient to sustain the tips of cervical loops. Another possibility might be that *Ring1b* in these double-knockout mice was conditionally

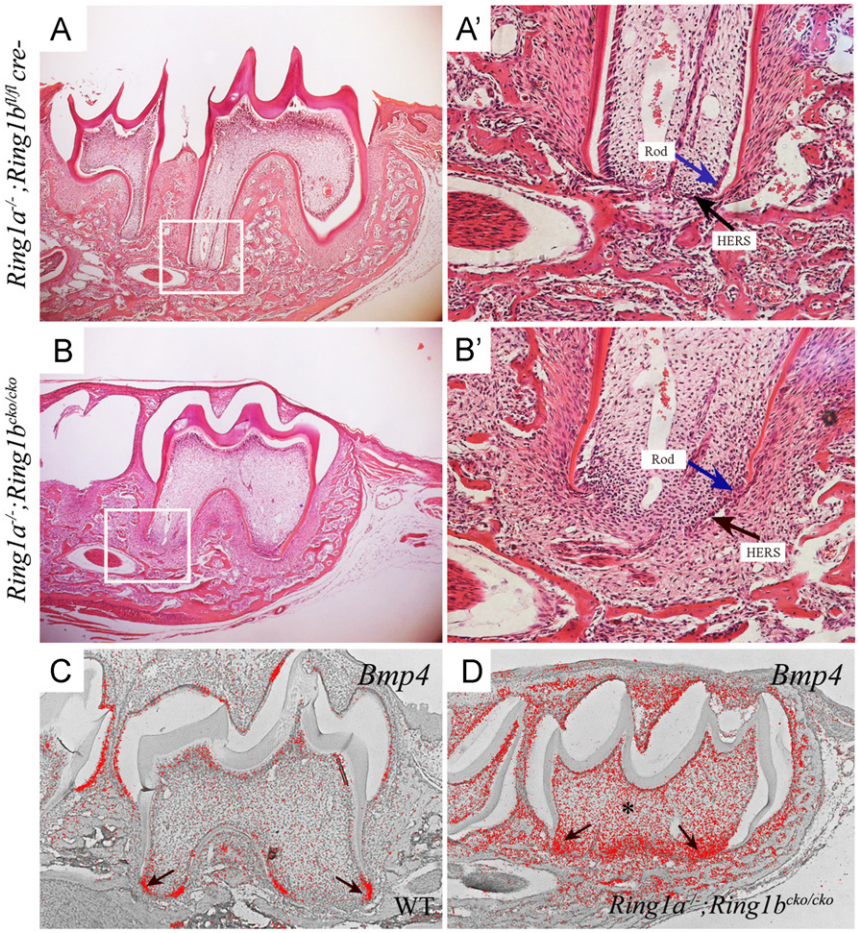


**Fig. 9.** Histology characteristics and Bmp signaling is altered in the mandibluar molar of *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ mice. (A-B′) Hematoxylin and eosin stained sections of a P17 mandibular molar of and *Ring1a*$^{-/-}$;*Ring1b*$^{fl/fl}$*cre*- and *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ mice. (A and B) *Ring1a*$^{-/-}$;*Ring1b*$^{fl/fl}$*cre*- maxillary mandibular (A, box) molars show normal root development whereas very short roots are observed in *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ mandibular (B, box) molars. (A′) Higher magnification of the white box in (A) shows normal root odontoblasts (Rod) differentiating adjacent to Hertwig's epithelial root sheath (HERS) in *Ring1a*$^{-/-}$;*Ring1b*$^{fl/fl}$*cre*- molar. (B′) Higher magnification of the white box in (B) shows aberrant odontoblast differentiation (blue arrow) close to Hertwig's epithelial root sheath (HERS). (C, D) *In situ* hybridization analysis of *Bmp4* expression using $35^S$ probes on paraffin sections of P11 WT and cre+ developing first molars (red color represents expression). (C) Normal expression of *Bmp4* in early odontoblasts (arrows) in WT developing first molars. (D) Expression of *Bmp4* is upregulated in *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ developing molars with ectopic expression in the dental pulp (asterisk). (For interpretation of the references to color in this figure legend, the reader is reffered to the web version of this article.)

inactivated for a short period of time that perhaps was not sufficient to cause an overt phenotype on the tips of cervical loops. Since the asymmetric expression of *Fgf3* on the labial aspect has been shown to stimulate epithelial stem cell proliferation and since enamel defects are observed in both *Fgf3*$^{-/-}$ and *Fgf3*$^{-/-}$;*Fgf10*$^{+/-}$ mice (Wang et al., 2007), it is possible that the down-regulation of both *Fgf3* and *Fgf10* in *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ incisors is likely to be a cause of reduced cell proliferation in the dental epithelium of the cervical loop, leading to the disruption of ameloblast formation. Among the members of Fgf ligands known to be present at the apex of mouse incisors, *Fgf9*, which is normally expressed in the limited area of TA cells or pre-ameloblasts in the labial epithelium was not investigated. However, it has been shown that mesenchymal *Fgf3* and *Fgf10* are targets of epithelial *Fgf9* (Klein et al., 2008; Yokohama-Tamaki et al., 2007).

The impairment of Fgf activity in *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ incisors was confirmed by the down-regulation of *Pea3* and *Erm*. *Pea3* and *Erm* belong to the Erythroblastoma Twenty-Six (ETS) family of transcription factors that are involved in a variety of transcriptional regulation events during growth and development, including proliferation, differentiation and oncogenic transformation (Monte et al., 1994; Wasylyk et al., 1998; Xin et al., 1992). Both genes have been demonstrated to be direct targets of Fgf signaling, based upon the closely related expression domains and the susceptibility to Fgf signaling interference (O'Hagan and Hassell, 1998; Roehl and Nusslein-Volhard, 2001). Besides being downstream targets of Fgf signaling, particular functions of these genes have not been described, however, it has been suggested that the close connection of *Erm* and *Pea3* transcription to Fgf signaling may serve to integrate Fgf signaling with other signals (Raible and Brand, 2001). Whether this is the case for the continuous growth of mouse incisors is an interesting issue for future research. Reduced Fgf signaling in *Ring1a*$^{-/-}$;*Ring1b*$^{fl/fl}$*cre*- incisors did not result in an obvious phenotype but does suggest that both Ring1a and Ring1b are required for maintaining endogenous levels of FGF expression indicates that these mice have a molecular abnormality and that *Ring1a* itself is essential for the regulation of Fgf signaling.

### *Ring1a/b regulate genes encoding proteins of the PRC1 complex*

The transcriptional alteration of protein members of the PRC1 complex observed in *Ring1a*$^{-/-}$;*Ring1b*$^{fl/fl}$*cre*- and *Ring1a*$^{-/-}$;*Ring1b*$^{cko/cko}$ incisors provides evidence that members of the

PCR1 complex including *Fbxl10* are targets of the complex during the continuous growth of mouse incisors. The importance of *Ring1*, particularly *Ring1b*, on the transcription levels of other members of the same protein complex has previously been demonstrated in PRC1. Loss of Ring1b in ES cells leads to a down-regulation of *Phc1/Mph1* and an up-regulation of *Bmi1*, *Mpc2*, *Rypb* and *Phc2*. However, these altered transcription levels do not seem to be entirely reflected in the protein levels, suggesting a distinct post-translation regulation of PRC1 members by Ring1b (Leeb and Wutz, 2007; van der Stoop et al., 2008).

Transcription of *Fbxl10* appears to be regulated by Ring1b but not Ring1a during mouse incisor development, indicating distinct interactions within this complex. *Nspc1/Pcgf1* expression was not altered in the absence of either *Ring1a* or *Ring1a/b*. The down-regulation of *Fbxl10/Kdm2r* expression observed only in *Ring1a*$^{-/-}$*;Ring1b*$^{cko/cko}$ incisors but not in cre- incisors suggests that only *Ring1b* may regulate *Fbxl10/Kdm2r* during the development of mouse incisors. It is likely that this is a direct interaction since i*n vitro* protein binding assay has shown that Ring1b protein is able to directly interact with Fbxl10/Kdm2r (Gearhart et al., 2006; Sanchez et al., 2007).

The evidence we present here identifies a population of dental pulp mesenchymal cells that are highly proliferative, express PRC1 proteins and have characteristics of cell progenitors (TA cells) in normal tooth growth and in response to tooth damage. The dental mesenchymal stem cell niche in rodent incisors thus provides an attractive, easily visualized and manipulated, experimental system to study the molecular characteristics and behaviour of mesenchymal and epithelial stem cells in an adult organ.

### *Ring1a/b also regulate molar root formation*

The severely impaired development of molar roots following postnatal loss of Ring1 proteins is consistent with aspects of the changes observed in incisors in these mutants. Molar root odontoblasts were very small, non-polarised and unorganized in the developing roots of *Ring1a*$^{-/-}$*;Ring1b*$^{cko/cko}$ mice, similar features to those in the incisors. A molecular downstream consequence of loss of Ring proteins is a change in the spatial expression of Bmp4 from being restricted to developing root odontoblasts to being widely expressed in undifferentiated root mesenchyme cells. Repression of BMP4 expression in cells other than root odontoblasts may thus be one possible function of PRC1 complex in regulating molar root formation.

## Materials and methods

### *Production of mouse lines*

Mutant Ring1a and Ring1b floxed alleles were generated as described previously (Cales et al., 2008; del Mar Lorente et al., 2000). Compound *Ring1a*$^{-/-}$*;Ring1b*$^{fl/fl}$ mice were obtained by crossing the *Ring1a*$^{-/-}$ mice with the *Ring1b*$^{fl/fl}$ mice. To accomplish conditional inactivation of *Ring1b in vivo*, the *Ring1a*$^{-/-}$*;Ring1b*$^{fl/fl}$ compound mice were crossed with *Rosa26::CreERT2* transgenic mice to generate *Ring1a*$^{-/-}$*;Ring1b*$^{fl/fl}$*;Rosa26::CreERT2* mice. The *Rosa26::CreERT2* transgenic mice were produced by inserting a tamoxifen-inducible CreER fusion protein gene into the ubiquitously expressed *Rosa26* gene (Seibler et al., 2003). Conditional deletion of *Ring1b* was carried out at the desired stage of postnatal life by 4-hydroxy tamoxifen (OHT) treatment (40 mg/kg body weight). *Ring1a*$^{-/-}$*;Ring1b*$^{cko/cko}$ mice were obtained by injecting OHT at P9 and P13 to inactivate Ring1b and the *Ring1a*$^{-/-}$*;Ring1b*$^{cko/cko}$ sacrificed at P17. The efficiency of tamoxifen-induced Cre expression to delete Ring1b was confirmed by *in situ* hybridisation of P17 incisors (Fig. 1S).

### *BrdU administration*

To detect rapidly dividing cells, 50 mg/kg body weight BrdU was administered intraperitoneally to wild type postnatal day 2 (P2) pups. Pups were subsequently sacrificed 2 h later and processed through histology and immunohistochemistry analysis.

### *Gene expression analysis*

Whole-mount digoxigenin-labelled *in situ* hybridization was carried out according to Shamim et al. (1998). Digoxigenin-labelled section *in situ* hybridization was carried as previously described (Nakatomi et al., 2006). Radioactive section *in situ* hybridization using $^{35}$S UTP radiolabelled riboprobes was performed on 8-μm sections as described previously (Wilkinson, 1982).

### *MicroCT analysis*

Mouse heads were scanned using a GE Locus SP microCT scanner to produce 14 μm voxel size volumes. After scanning, Explore Microview software program (GE) was used for visualization and analysis. Mouse teeth were characterised by generating three dimensional reconstructions and three dimensional isosurfaces of mouse teeth were then produced. For measurement of mouse incisor length, the locations of the centre points of incisors were identified on every 5 cross-sections from the most incisal to apical end. The position of each centre point was identified on the micro CT planes as three co-ordinates ($x$,$y$,$z$). The distance between every two points (($x_1$,$y_1$,$z_1$) and ($x_2$,$y_2$,$z_2$)), was then calculated using the formula derived from the three dimensional version of the Pythgorean theorem (Distance$=\sqrt{(x_2-x_1)^2+(y_2-y_1)^2+(z_2-z_1)^2}$). The length of the curved incisor was calculated from the sum of all the distances between each dot.

### *Immunohistochemistry*

Sections were incubated with antibody to Phospho-Histone H3 (Upstate, 06–570) or BrdU (Abcam, ab6326) following heat-based antigen retrieval. To perform peroxidase visualization for the biotin conjugated antibody, the sections were incubated in ABC solution (Vectastain kit, Vector, PK-6101). The colour reaction was then developed by applying DAB solution (0.5 mg/ml DAB and 0.1% $H_2O_2$) onto the sections or using a DAB peroxidase substrate kit (Vector, SK-4100).

## Summary

Stem cells are found in many adult organs where they provide a source of cells needed for tissue growth or to replace cells lost as a result of tissue damage. Adult human teeth do not grow and have only a limited ability to repair following damage, however, the incisors of rodents grow continuously to accommodate wear and all the specialised cells of the tooth must be continuously replaced. This is achieved by stem cell populations at the base of the tooth that provide sources of cells to replace all mesenchymal and epithelium-derived tooth cells. Whereas the incisor epithelial stem cell niche is well characterised, the mesenchymal stem cell niche is poorly understood. We have identified genes that are required for incisor tooth growth and cell differentiation that belong the PRC1 complex that is known to be essential for maintenance of embryonic stem cells. We how that PRC1 genes are expressed in the mesenchymal transit amplifying cells and are essential for mesenchymal cell proliferation and also for

expression of signals from the mesenchyme that regulate the epithelial stem cell niche.

## Acknowledgements

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2012.04.029.

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