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STEM CELLS AND REGENERATION

RESEARCH ARTICLE

Pitx2-Sox2-Lef1 interactions specify progenitor oral/dental epithelial cell signaling centers

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ABSTRACT

Epithelial signaling centers control epithelial invagination and organ development, but how these centers are specified remains unclear. We report that *Pitx2* (the first transcriptional marker for tooth development) controls the embryonic formation and patterning of epithelial signaling centers during incisor development. We demonstrate using *Krt14*^{Cre}/*Pitx2*$^{flox/flox}$ (*Pitx2*^{cKO}) and *Rosa26*^{CreERT}/*Pitx2*$^{flox/flox}$ mice that loss of *Pitx2* delays epithelial invagination, and decreases progenitor cell proliferation and dental epithelium cell differentiation. Developmentally, *Pitx2* regulates formation of the Sox2^{+} labial cervical loop (LaCL) stem cell niche in concert with two signaling centers: the initiation knot and enamel knot. The loss of *Pitx2* disrupted the patterning of these two signaling centers, resulting in tooth arrest at E14.5. Mechanistically, Pitx2 transcriptional activity and DNA binding is inhibited by Sox2, and this interaction controls gene expression in specific Sox2 and Pitx2 co-expression progenitor cell domains. We demonstrate new transcriptional mechanisms regulating signaling centers by *Pitx2*, *Sox2*, *Lef1* and *Irx1*.

KEY WORDS: Dental epithelial stem cells, Pitx2, Shh, Signaling centers, Sox2, Transcriptional regulation, Stem cell niche, Craniofacial/tooth development, Irx1, Enamel knot

INTRODUCTION

Pituitary homeobox 2 (*Pitx2*), is a crucial transcription factor that regulates the asymmetrical development of organs (Logan et al., 1998; Piedra et al., 1998; Ryan et al., 1998; Yoshioka et al., 1998) and is crucial for tooth development. Mutations in the *PITX2* gene have been found in individuals with Axenfeld-Rieger syndrome (ARS) who have developmental defects of the eyes, abdomen and teeth, indicating that it contributes to the development of these organs (Amendt et al., 1998; Semina et al., 1996). Mice with a general *Pitx2* knockout are embryonic lethal with defects in the heart, lung, body wall and teeth, further demonstrating that *Pitx2* plays an essential role in controlling organogenesis during development (Lin et al., 1999). In the case of teeth, the general knockout of *Pitx2* leads to an arrest in development at the bud stage, involving downregulation of fibroblast growth factor 8 (*Fgf8*) expression in the dental epithelium and changes in the distribution of bone morphogenetic protein 4 (*Bmp4*) in the adjacent mesenchyme (Gage et al., 1999a; Lin et al., 1999; Lu et al., 1999). Since these mice die at early stages of embryonic development, the details of the cellular and molecular mechanisms by which *Pitx2* controls early odontogenesis are unknown. However, using transgenic mice overexpressing a repressor of Pitx2, we have demonstrated that *Pitx2* plays a role in dental epithelial cell differentiation into ameloblasts (Cao et al., 2013; Li et al., 2013). Although *Pitx2* has long been considered as a master regulator of the transcriptional hierarchy in early tooth development, including stem cells (Pispa and Thesleff, 2003; Tucker and Sharpe, 2004), its specific role in cell differentiation and signaling has not been investigated due to embryonic lethality of global *Pitx2* knockout mice. Therefore, we investigated the specific role of *Pitx2* using a conditional knockout approach.

During embryonic development, stem-cell specification, and the proliferation and differentiation of transit-amplifying cells (TAs), which are a population in transition between stem cells and differentiated cells, are keys to organogenesis (Hsu et al., 2014). Organs such as teeth, hair follicles and mammary glands are derived from surface ectodermal cells via processes coordinated by interactions between the epithelium and mesenchyme (Jiménez-Rojo et al., 2012). As soon as the epithelial ectoderm receives signals from adjacent mesenchyme within a determined region, it forms a placode or a localized thickening of epithelial cells (Fig. 1B). Teeth develop from two epithelial cell populations within the dental placode: Sox2^{+} cells and Lef1^{+} cells (Sanz-Navarro et al., 2018; Sun et al., 2016) (Fig. 1B). The Sox2^{+} cells are the progenitors of various epithelial cell types that form bud- and cap-stage teeth, and the Lef1^{+} cells that are present during the placode, bud and cap stages produce growth factors that control tooth morphology. Ablation of *Sox2* in the developing tooth leads to impaired proliferation of dental epithelial stem cells (DESCs) during the early stages of tooth development and prevents the renewal of DESCs in the adult (Sanz-Navarro et al., 2018; Sun et al., 2016). The ablation of *Lef1* in the dental epithelium also leads to severe developmental defects of the teeth, causing an arrest during the transition from bud to cap stage (Sasaki et al., 2005; van Genderen et al., 1994). We have recently shown that conditional overexpression of *Lef1* in the dental epithelium results in a new stem cell compartment and lack of dental epithelial cell differentiation (Sun et al., 2016). Furthermore, *Pitx2* regulates *Sox2* and *Lef1* expression in the epithelium.

Tooth morphogenesis is stimulated by growth factors Fgf, Wnt, Shh and Bmp, which are produced by a cluster of cells known as the dental epithelial signaling center and by adjacent dental mesenchyme (Balic and Thesleff, 2015). Effective function of the epithelial signaling centers is crucial for tooth morphogenesis. There are two signaling centers, the initiation knot (IK), and enamel

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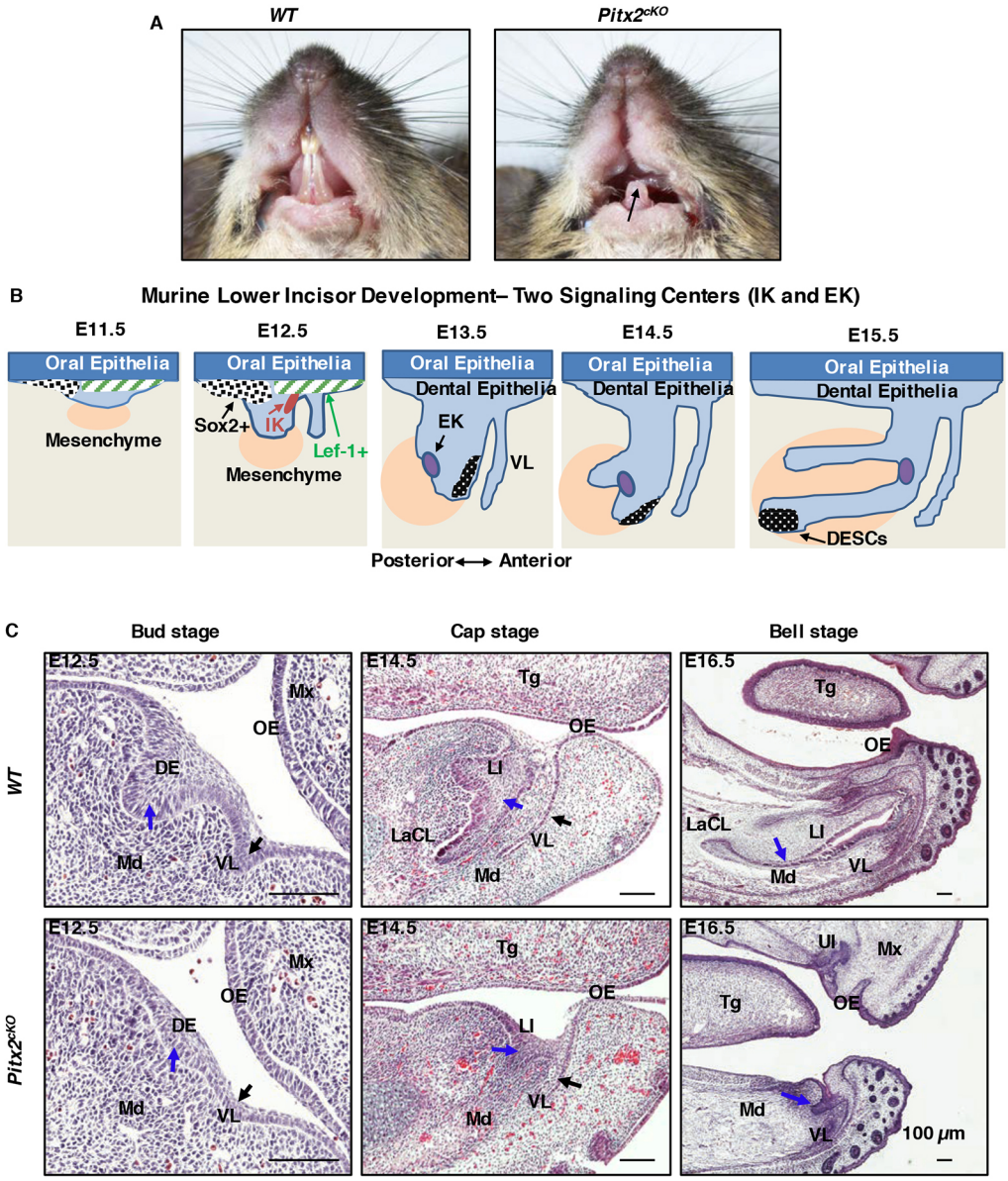

Fig. 1. Dental development is delayed at bud stage and disrupted at the cap stage in *Pitx2*^{cKO} embryos. (A) Representative images of weaning-age mice, showing the gum (black arrow) but an absence of teeth in *Pitx2*^{cKO} mice. (B) Murine lower incisor development and location of the two signaling centers (IK, initiation knot; EK, enamel knot). (C) Representative Hematoxylin and Eosin images of bud- (E12.5), cap- (E14.5) and bell- (E16.5) stage lower incisor morphogenesis. Development of the lower incisors of *Pitx2*^{cKO} embryos is delayed with respect to invagination of the dental epithelium (DE, blue arrows) and vestibular lamina (VL, black arrows, E12.5 and E14.5). No typical lower incisors were found in E14.5 and E16.5 *Pitx2*^{cKO} embryos. DE, dental epithelium; LaCL, labial cervical loop; DESC, dental epithelial stem cell; VL, vestibular lamina; Md, mandible; Mx, maxillary; LI, lower incisor; UI, upper incisor; OE, oral epithelium; Tg, tongue.

knot (EK), and the size of the IK signaling center correlates with that of the entire tooth at each stage (Ahtiainen et al., 2016) (Fig. 1B). Specifically, an epithelial signaling center at the cap stage (E13.5), the EK, is crucial for tooth morphogenesis during this stage. The EK is derived from the cells toward the posterior region of the tooth bud; it is not derived from the IK signaling center at bud stage (E12.5) (Ahtiainen et al., 2016; Du et al., 2017). Formation of the EK has been reported to be regulated by αE-cadherin-mediated restriction of YAP/TAZ activity in a cluster of non-proliferative cells (Li et al., 2016). We have also shown that Pitx2 regulates p21, which is localized to the EK during development (Cao et al., 2010a). However, further details of the mechanisms controlling dental epithelial signaling centers are not available.

Here, we report on our investigation of the mechanisms of *Pitx2* function at the cellular and molecular levels during early stages of tooth development in mice. We found that conditional knockout of *Pitx2* in the dental epithelium at approximately embryonic day 10 (E10.0) causes the disruption of tooth development. Pitx2 is necessary for the formation of the signaling centers, and the deletion of *Pitx2* in the dental epithelium results in an arrest of tooth development. Pitx2 interacts with Sox2 and Lef1 to differentially regulate genes that form the early IK and later the EK. Pitx2 regulates the expression of *Irx1*- and *Shh*-expressing cells to initiate DESC differentiation and tooth growth at the bud stage.

RESULTS

Pitx2 specifies the oral/dental epithelial cells and dental lamina to start the dental developmental program

To determine the cellular and molecular mechanisms underlying *Pitx2* specification of the early stages of tooth development, we conditionally knocked out *Pitx2* (*Pitx2*^{cKO}) in the oral and dental epithelia by crossing *Pitx2*$^{flox/flox}$ mice to the *keratin14-Cre* recombinase (*Krt14*^{Cre}) mouse line. The *Pitx2*^{flox} mice were previously generated, with *loxP* sites flanking the fourth exon of the *Pitx2* gene (Gage et al., 1999b). We tested the recombinase activity of *Krt14*^{Cre} line by crossing these mice with *Rosa26*^{GFP} mice, in which active Cre recombinase can initiate the expression of green fluorescent protein (GFP). Immunostaining of *K14*^{Cre}*Rosa26*^{GFP} mice for GFP at E11.5 and E14.5 showed GFP expression in the oral

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and dental epithelia starting at E11.5 (Fig. S1A). The *Pitx2*^{cKO} mice survived to weaning stage (postnatal day 21) and their overall body size was significantly smaller than that of their *Pitx2*$^{flox/flox}$ (WT) counterparts (Fig. S1B,C). In addition, the *Pitx2*^{cKO} mice completely lacked teeth (Fig. 1A, Fig. S1D).

The development of teeth (lower incisor) begins with a localized thickening of the oral epithelia at E11.5 with the neural crest-derived mesenchyme condensing around the invaginating epithelia (Fig. 1B). The placode stage (E10.5-11.5) is denoted by juxtaposed expression of *Sox2* (posterior region) and *Lef1* (anterior region) (Sun et al., 2016). At E12.5 (bud stage) the first signaling center or initiation knot (IK) is formed (red region, Fig. 1B). At E13.5, the EK has formed and expresses *Lef1*, *Shh* and *p21* (*Cdkn1a*). Furthermore, the Sox^{2+} cells, which are located first in the posterior region, have transitioned to a group of Sox^{2+} cells toward the anterior region (black speckled region, Fig. 1B) in the expanding tooth bud and adjacent to a transient structure termed the vestibular lamina (VL); thus, the enamel knot is more posterior. At E14.5 (the cap stage), the DESC niche is forming and condensed to a smaller region; at E15.5, both centers are separated and the EK will eventually be lost as the incisor develops. However, because the rodent lower incisor continuously grows, the DESCs in the labial cervical loop will provide the progenitor cells needed for growth of the incisor as it wears down due to gnawing and eating by the mouse and to the asymmetric nature of enamel deposition.

To identify the tissue-specific defects that result in toothless *Pitx2*^{cKO} mice, we examined *Pitx2*^{cKO} embryos at various stages of development and found early dental developmental defects (Fig. 1C, Fig. S1E). Specifically, *Pitx2*^{cKO} embryos had smaller bud stage teeth and undetectable cap and bell stage teeth compared with wild type (Fig. 1C). In addition, invagination of both the dental epithelium and vestibular lamina were delayed in the lower incisors of *Pitx2*^{cKO} embryos (Fig. 1C). We noticed similar defects in the developing molars of *Pitx2*^{cKO} embryos. Although the initiation of molar development was not affected, morphogenesis was arrested at a very early stage of tooth development (Fig. S1E).

Epithelial cell proliferation was decreased in *Pitx2*^{cKO} embryos

The disruption of odontogenesis in *Pitx2*^{cKO} embryos could be due to either increased cell death or decreased cell proliferation. Analyses of cell death using cleaved caspase 3 as a marker, in E12.5 and E14.5 wild-type and *Pitx2*^{cKO} embryos, did not reveal a significant increase in apoptosis (Fig. S2). We next performed a 5-bromo-2′-deoxyuridine (BrdU) incorporation assay to determine whether the rate of cell proliferation was reduced in *Pitx2*^{cKO} versus wild-type tooth buds. BrdU was administered to pregnant mice at E11.5 and E12.5, and the embryos were harvested 2 h after BrdU injection. Immunostaining for BrdU revealed that cell proliferation was significantly reduced in the lower incisors of *Pitx2*^{cKO} embryos at E12.5 but not at E11.5 (Fig. 2A,C). The IK is shown as non-proliferating cells in the E12.5 wild-type incisor (Fig. 2A, IK, white arrow). Staining of E14.5 wild-type and *Pitx2*^{cKO} lower incisors for Ki-67 showed that, whereas all dental epithelial cells outside the EK were positive in wild-type mice, fewer cells were positive for Ki-67 in the *Pitx2*^{cKO} lower incisors (Fig. 2B,D). BrdU incorporation was used to identify DNA replication in cells and staining for the Ki-67 protein marks cell proliferation. We used both assays to validate cell proliferation. Thus, the absence of an EK structure in the E14.5 *Pitx2*^{cKO} lower incisors (Fig. 2B) resulted in lack of necessary signals for these cells to proliferate and invaginate.

Label-retaining assays were performed, delivering 5-chloro-2-deoxyuridine (CIdU) and 5-iodo-2-deoxyuridine (IdU) to wild-type and *Pitx2*^{cKO} embryos at different time points (Fig. 2E). CIdU was injected into pregnant females at E12.5, and IdU was injected into pregnant females at E14.5 and E16.5, 2 h before the embryos were harvested. In wild-type mice, the LaCL region of the lower incisor tooth germ contained cells positive for IdU (green fluorescence) at both E14.5 and E16.5; however, the lower incisors contained less CIdU (red fluorescence staining) at the later versus earlier stage (Fig. 2E, top panels). The CldU-retaining cells have moved out of the LaCL to populate the ameloblast layer and are fully differentiated cells. In the *Pitx2*^{cKO} group, the lower incisors contained fewer IdU cells and more cells that retained the red fluorescent label (Fig. 2E, bottom panels).

Generation of a 3-dimensional model of bud-stage lower incisor tooth germs of E12.5 wild-type and *Pitx2*^{cKO} serial sagittal sections revealed that, in comparison with wild-type counterparts, the tooth germs in the *Pitx2*^{cKO} group were shorter along the labio-lingual axis and thinner at the widest point of the mediolateral axis (Fig. 2F). Based on these findings, we concluded that, in the *Pitx2*^{cKO} lower incisor tooth germs, the epithelial progenitors were not actively proliferating, resulting in a smaller tooth germ structure.

Pitx2 is required for DESC differentiation

Given that tooth morphogenesis was disrupted in *Pitx2*^{cKO} mice, we performed double immunostaining for Sox2 and Lef1 expression in sagittal sections of bud- (E12.5) and cap- (E14.5) stage lower incisors to determine the specific cell populations affected by *Pitx2* ablation (Fig. 3A). In the bud-stage (E12.5) of lower incisor germs of the wild-type embryos, Sox2 was posteriorly expressed, whereas Lef1 was anteriorly expressed, marking a cell population that appears to be the IK and the adjacent mesenchyme. In E12.5 *Pitx2*^{cKO} sections, the Sox2^{+} cell population was smaller and the position of the IK marked by Lef1 expression was shifted to the bottom of the bud (Fig. 3A, white arrow). In cap-stage (E14.5) lower incisors of wild-type embryos, Sox2^{+} cells were concentrated in the labial cervical loops (LaCL) and Lef1^{+} cells in the enamel knot (EK) (white arrow) (Jernvall and Thesleff, 2000). However, in the *Pitx2*^{cKO} incisors at this stage, the Sox2^{+} cells did not segregate to the LaCL and Lef1^{+} cells were present in the posterior region (Fig. 3A, white arrow). We conclude that tooth development was not just delayed, but also impaired, in E14.5 *Pitx2*^{cKO} embryos, based on the changes in Sox2 and Lef1 expression, and the organization of the IK and EK signaling centers as well as a lack of a well-defined LaCL.

A lack of differentiation in *Pitx2*^{cKO} embryos was further supported by reduced *Sox9* expression. In wild-type incisors of E12.5 embryos, Sox9 was expressed in the region adjacent to the oral cavity that lacked Sox2 expression (Fig. 3B, top panel, white arrow), and at E14.5, progenitors of the stellate reticulum (SR) and stratum intermedium (SI) showed Sox9 expression (Fig. 3B, top panel). In the *Pitx2*^{cKO} lower incisors, by contrast, at E12.5 the Sox9-positive signal (Sox9^{+}) was reduced (Fig. 3B, bottom panel), and at E14.5 only a few Sox9^{+} cells were present in either the oral or dental epithelium (Fig. 3B, bottom panel). Statistical analysis of the number of Sox9^{+} cells revealed that they were significantly reduced in the lower incisor tooth germs of *Pitx2*^{cKO} mice at both E12.5 and E14.5 (Fig. 3C). Because Sox9 is associated with dental epithelial differentiation, these data demonstrate a reduction in differentiation of progenitor cells regulated by *Pitx2*.

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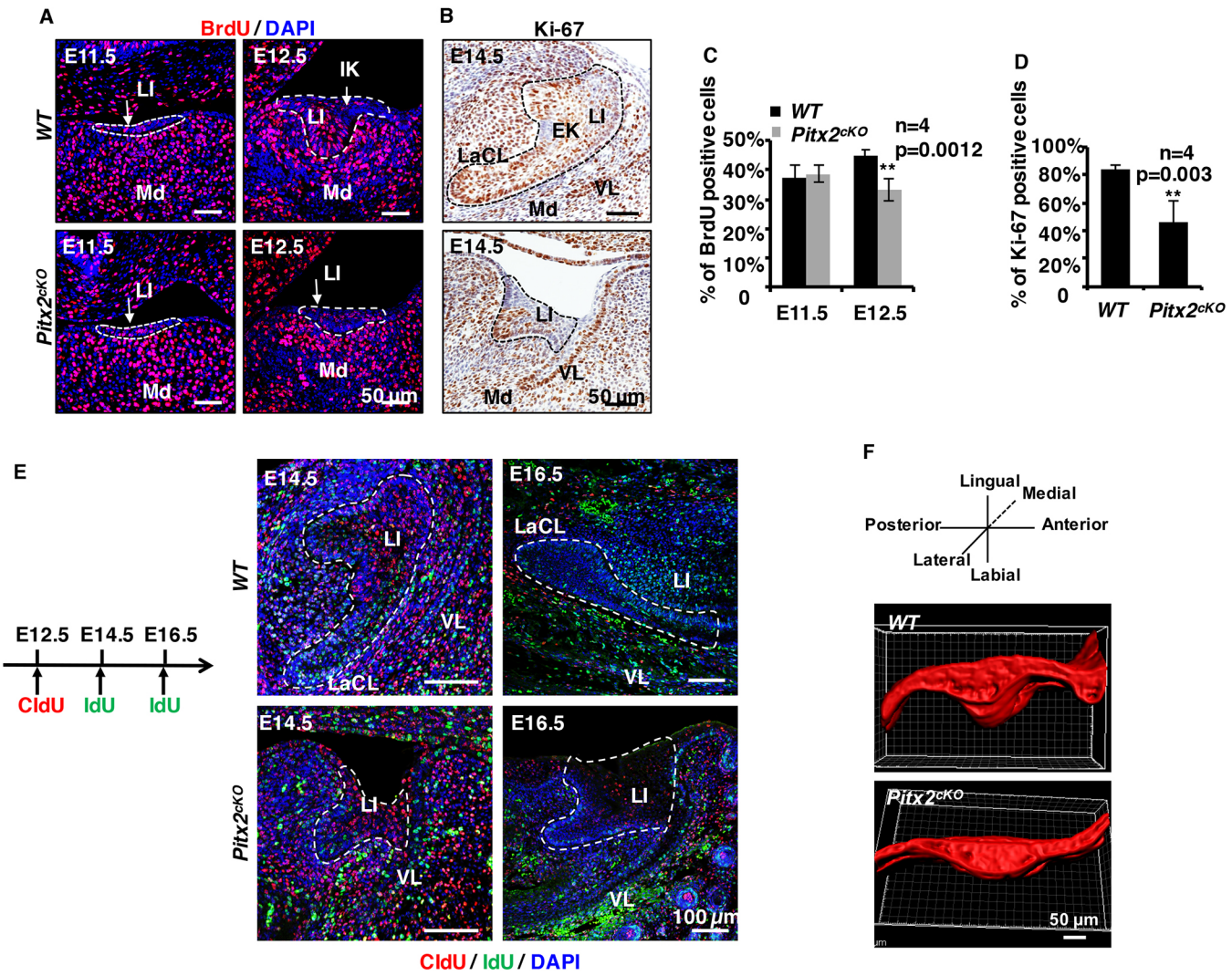

Fig. 2. Cell proliferation is decreased in *Pitx2*^{cKO} lower incisors. (A) Representative images showing immunofluorescence staining of BrdU incorporation in E11.5 and E12.5 mice. Regions outlined with dashed lines represent lower incisors. DAPI represents nuclei. (B) Immunostaining for Ki-67 in E14.5 mice. Regions outlined in black represent lower incisors. (C) Quantitation of the percentage of BrdU^{+} cells in regions outlined in A. (D) Quantitation of the percentage of Ki-67^{+} cells in regions outlined in B. Data are mean±s.e.m. (E) Representative images showing immunofluorescence staining of CldU and IdU in lower incisors from label retaining assay, following CldU injection at 48 (E14.5) or 96 (E16.5) h prior to embryo harvesting, and IdU injection 2 h before embryo harvesting. Regions outlined by dashed lines represent developing lower incisors. (F) 3-Dimension model of lower incisors in wild-type and *Pitx2*^{cKO} embryos at E12.5. Models were generated using the Imaris software, based on stacking of series sections of lower incisors. LI, lower incisor; EK, enamel knot; IK, initiation knot; LaCL, labial cervical loop; Md, mandible; VL, vestibular lamina. *n*=4, ***P*<0.01.

Pitx2 regulates Yap expression in the invaginating epithelial cells

Genetic studies have established that the Hippo pathway plays a crucial role in organ size, controlling cell number by modulating cell proliferation and apoptosis (Buttitta and Edgar, 2007; Hamaratoglu et al., 2006; Huang et al., 2005; Lai et al., 2005; Wu et al., 2003; Zhao et al., 2008a). The unphosphorylated (i.e. active) forms of YAP and TAZ associate with transcription factors (TFs) of the TEAD/TEF family in the nucleus, activating the expression of target genes and thereby promoting cell proliferation and inhibiting apoptosis (Cao et al., 2008; Zhao et al., 2008a,b). We have shown recently that Pitx2 interacts with Yap to regulate gene expression, and both Yap and Pitx2 share DNA-binding sites on gene promoters, indicating that they regulate multiple genes together in the Hippo signaling pathway (Tao et al., 2016). We asked whether unphosphorylated Yap and/or phosphorylated Yap (P-Yap) were affected in the *Pitx2*^{cKO} incisors. Immunostaining for Yap demonstrated that the *Pitx2*^{cKO} embryos had decreased Yap at E12.5 and E14.5 compared with the *Pitx2*^{Flox} controls (Fig. 4). There was little difference in the expression of P-Yap at E12.5 between the *Pitx2*^{cKO} and control embryos (Fig. 4B). However, at E14.5, P-Yap was not detected in the *Pitx2*^{cKO} lower incisor (Fig. 4D). Interestingly, the EK did not show expression of Yap in the control embryos (Fig. 4C). This is consistent with an earlier report showing that the EK contains low levels of nuclear Yap (Li et al., 2016). Thus, in addition to decreased cell proliferation in the epithelial compartment of the *Pitx2*^{cKO} lower incisor, the embryos also showed decreased Yap, consistent with the role of Pitx2 in regulating Yap interactions and a Hippo gene regulatory network. The disruption of Hippo signaling also contributes to the lack of cell proliferation and cell signaling.

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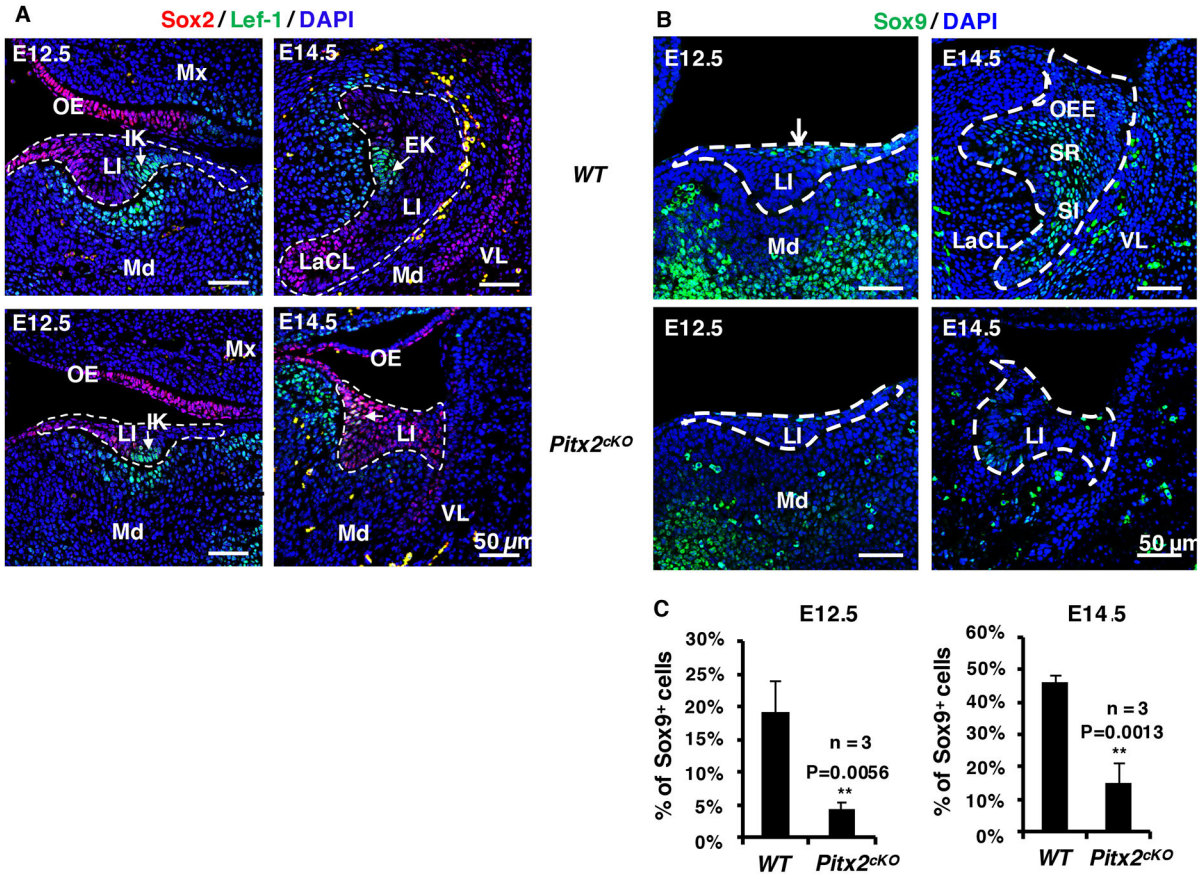

Fig. 3. Pitx2 controls DESC differentiation. (A) Sox2 and Lef1 immunofluorescence signal in lower incisors (outlined with dashes) at E12.5 and E14.5. DAPI staining represents nuclei. (B) Representative images showing immunofluorescence staining of Sox9 in bud- (E12.5) and cap- (E14.5) stage teeth. Arrow indicates Sox9 signal in cells lacking Sox2 expression in bud-stage lower incisor in wild-type mouse. Regions outlined by dashes show bud- and cap-stage teeth. (C) Quantitation of Sox9$^+$ cells in the lower incisors in B and C. Data are mean±s.e.m. The percentage of Sox9$^+$ cells is significantly lower in bud stage (n=3, P=0.0056) and cap stage (n=3, P=0.0013) *Pitx2*^{cKO} lower incisors compared with wild-type lower incisors. LI, lower incisor; LaCL, labial cervical loop; VL, vestibular lamina; Md, mandible; Mx, maxilla; EK, enamel knot; IK, initiation knot; OE, oral epithelium; OEE, outer enamel epithelium; SI, stratum intermedium; SR, stellate reticular. n=3, **P<0.01.

Pitx2 regulates adult enamel homeostasis

We next investigated whether Pitx2 was essential for growth of the lower incisors in the adult and during homeostasis, using an inducible *Pitx2* knockout mouse model. We crossed *Pitx2*$^{flox/flox}$ mice with *Rosa26*^{CreERT} to generate *Rosa26*^{CreERT}*Pitx2*$^{flox/flox}$ (*Pitx2*^{icKO}) mice. We allowed the *Rosa26*^{CreERT}*Pitx2*$^{flox/+}$ (*Pitx2*^{iHet}) and *Pitx2*^{icKO} progeny to mature and then injected them with tamoxifen (TAM) to ablate *Pitx2* expression (Fig. S3A). Eight days after TAM injection, the left lower incisor of each mouse was clipped and the difference in length of the left and right lower incisors was measured and recorded (Fig. S3B); 3.5 days later, the difference in length between the lower incisors of each mouse was recorded. In these experiments, inducible deletion of *Pitx2* did not affect growth of the adult lower incisor (Fig. S3B,C), although levels of *Pitx2* transcripts in tissues surrounding the cervical loop region were significantly lower in the TAM-treated *Pitx2*^{icKO} versus *Pitx2*^{iHet} mice (Fig. S3D). We next analyzed the enamel layer, under a scanning electron microscope, for dental enamel defects and found disruption of enamel rod organization in the *Pitx2*^{icKO} but not *Pitx2*^{iHet} mice (Fig. S3E). Thus, in adult mice lower incisors, after the signaling centers have been patterned and LaCL (DESCs) formed, *Pitx2* is not required for the proliferation of adult DESCs but is required for dental epithelial cell differentiation and amelogenesis during homeostasis (Cao et al., 2013, 2010b; Li et al., 2013).

Pitx2 specifies cells that form the EK during embryogenesis and cells expressing *Shh*

We next set out to identify the molecular mechanism by which *Pitx2* regulates tooth morphogenesis during early stages of tooth development by investigating the localization of β-catenin, and the expression of *Fgf8* and *Bmp4* transcript levels. The immunostaining for β-catenin in bud-stage lower incisor tooth germs showed more membrane-associated β-catenin in wild-type compared with *Pitx2*^{cKO} lower incisors (Fig. S4). Interestingly, the epithelial cells in the *Pitx2*^{cKO} at E12.5 appeared less polarized compared with wild type. To determine whether β-catenin nuclear localization was impaired in the E12.5 *Pitx2*^{cKO} embryos, sections were stained using the PY489-β-catenin antibody and co-stained for E-cadherin (Fig. S4B). Nuclear β-catenin was observed in the WT invaginating tooth bud, but was decreased in the *Pitx2*^{cKO} tooth bud (Fig. S4B). Furthermore, E-cadherin expression revealed that cells were less polarized in the *Pitx2*^{cKO} tooth germ compared with wild type (Fig. S4B). These data suggest that a decrease in polarized cells and nuclear β-catenin affect epithelial invagination.

The *in situ* hybridization for *Fgf8* and *Bmp4* transcripts revealed that *Fgf8* levels were decreased in *Pitx2*^{cKO} incisors compared with wild-type embryos, and *Bmp4* transcript levels were slightly decreased (Fig. S5). In addition, assessment of the levels of *Bmp2*, *Shh* and *Wnt10b* transcripts (*in situ* hybridization), and the

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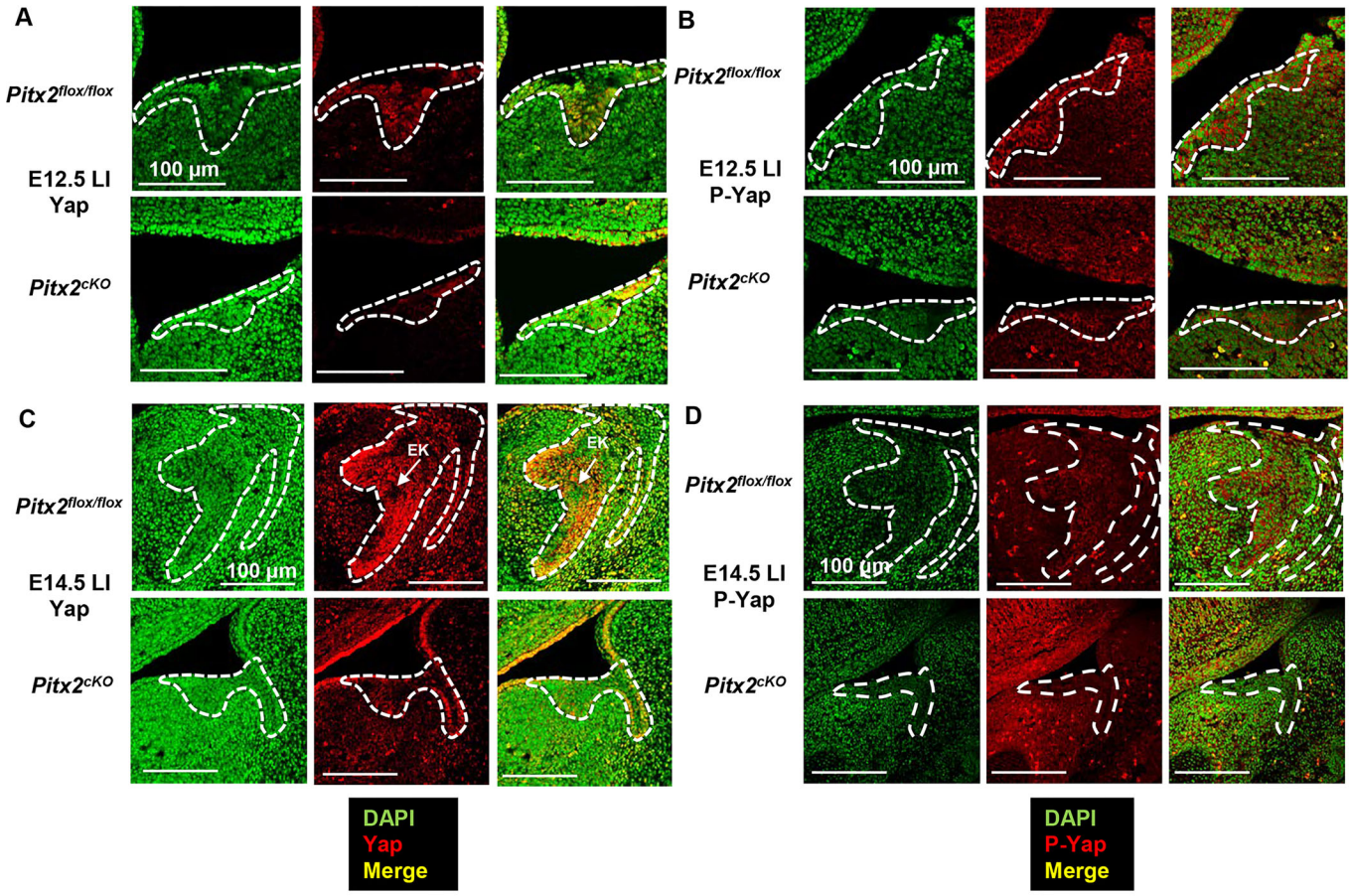

Fig. 4. Pitx2 regulates P-Yap but not Yap in the epithelial signaling centers during early incisor morphogenesis. (A,B) Immunofluorescence (IF) staining of Yap (A) and P-Yap (B), in E12.5 lower incisors of *Pitx2*$^{flox/flox}$ (control) and *Pitx2*^{cKO} embryos. (C,D) IF staining for Yap (C) and P-Yap (D) in E14.5 lower incisors of *Pitx2*$^{flox/flox}$ and *Pitx2*^{cKO} embryos. Yap is absent from the EK in the control embryos at E14.5. EK, enamel knot.

p21 protein (immunostaining) in the signaling centers of E12.5 incisors showed that only *Shh* was reduced in the *Pitx2*^{cKO} (Fig. 5A). There is a consensus Pitx2-binding site 3505 bp upstream of the *Shh* transcription start site, allowing Pitx2 to activate *Shh* expression. The expression domains of *Shh* and p21 mark the IK at E12.5 (Ahtiainen et al., 2016). The expression of *Fgf4*, *Shh* and *Wnt10b* transcripts, and immunostaining of p21, in E14.5 (cap-stage) lower incisors revealed positive signals for *Fgf4*, *Shh* and *Wnt10b* in wild-type mice but not in their *Pitx2*^{cKO} counterparts (Fig. 5B). Moreover, there were very few p21-positive cells in the posterior region of the *Pitx2*^{cKO} lower incisor (Fig. 5B). Taken together with the immunostaining results for the early EK marker Lef1 (Fig. 3A) and p21 (Jernvall et al., 1998; Jernvall and Thesleff, 2000), and the *in situ* hybridization results for the mature EK markers *Fgf4*, *Shh* and *Wnt10b* (Fig. 5B) in E14.5 lower incisors, these results led us to conclude that formation of the EK was disrupted in the *Pitx2*^{cKO} lower incisors.

Given that *Shh* levels were reduced in the dental epithelial signaling centers of *Pitx2*^{cKO} lower incisors, we tested the hypothesis that downstream targets of the *Shh* signaling pathway were downregulated. We show that *Gli1* expression was decreased in E12.5 and E14.5 *Pitx2*^{cKO} lower incisors compared with wild type (Fig. 5C). Cyclin D2, another downstream target of the *Shh* signaling pathway, was also decreased in E12.5 *Pitx2*^{cKO} lower incisors (Fig. 5C); interestingly, cyclin D2 marks the wild-type IK (Fig. 5C, yellow arrow). Notably, the cyclin D2 reduction was also apparent in the Sox2^{+} cell population and appeared to be decreased compared with those in the wild-type group in cells of the IK (Fig. 5C). These findings support the conclusion that *Pitx2* regulates cells expressing *Shh*, *p21* and *Ccnd2* in the IK dental epithelial signaling center at E12.5.

Pitx2 directly regulates the expression of *Irx1* in the epithelium

Recently, we reported that *Irx1* regulates lung progenitor cell differentiation and marks alveolar type 2 cells; in dental development, it also marks cells of the stratum intermedium (SI) and outer enamel epithelium (OEE) (Yu et al., 2017). In a previous RNA-seq analysis, we found that *Irx1* levels were higher in craniofacial tissues of *Pitx2c*-overexpressing transgenic mice compared with wild-type embryos. E14.5 tooth germs were checked for co-expression of Pitx2 and Irx1. While Irx1 expression is highest in the OEE and dental epithelium, Irx1 is also expressed at low levels in the EK, and both Pitx2 and Irx1 are co-expressed in the OEE and dental epithelium (Fig. S7). However, Irx1 was not expressed in the *Pitx2*^{cKO} incisor. In addition, a *Pitx2*-binding site was identified 1.6 kb upstream of the *Irx1* transcription start site (Fig. 6A). We therefore investigated, both *in vitro* and *in vivo*, the possibility that Pitx2 regulates *Irx1* transcription. Chromatin immunoprecipitation (ChIP) analysis revealed that endogenous Pitx2 protein binds the *Irx1* promoter in LS-8 cells (Fig. 6A,B). Pitx2 and Irx1 directly interact with each other, as shown by co-immunoprecipitation assays (Fig. 6C). LS-8 epithelial cells endogenously express Pitx2 and overexpress Irx1; the Pitx2

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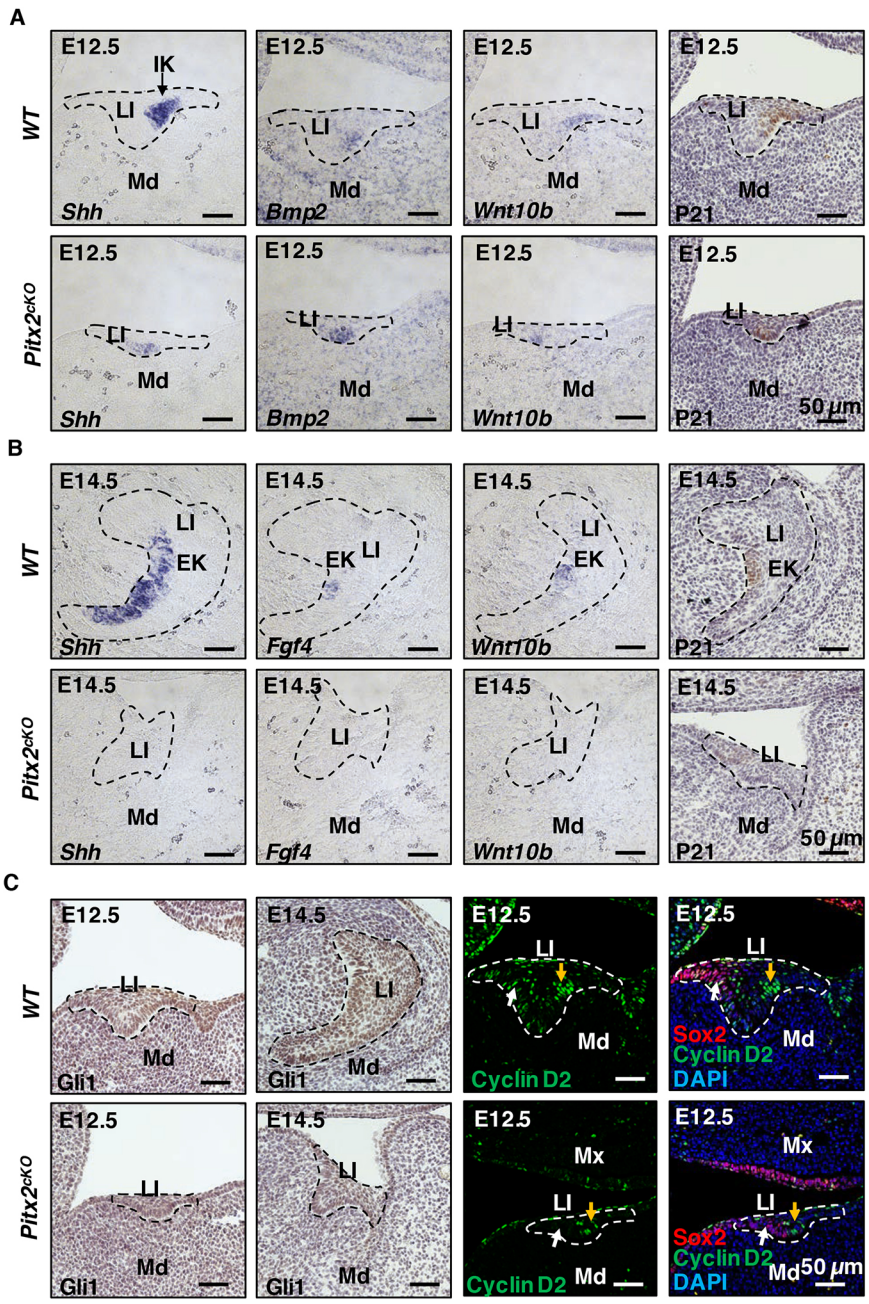

Fig. 5. *Pitx2* regulates enamel knot formation during early incisor development. (A) The expression of the *Shh*, *Bmp2* and *Wnt10b* mRNAs (*in situ* hybridization) and p21 protein (immunohistochemistry) in E12.5 wild-type and *Pitx2*^{cKO} embryos. (B) The expression of *Shh*, *Fgf4* and *Wnt10b* mRNAs (*in situ* hybridization) and p21 protein (immunohistochemistry) in E14.5 wild-type and *Pitx2*^{cKO} embryos. (C) The expression of Gli1, Sox2 and cyclin D2 protein in wild-type and *Pitx2*^{cKO} E12.5 and E14.5 embryos. Regions outlined in dashes represent developing lower incisors at E12.5 and E14.5. Yellow arrows indicate dental epithelial signaling centers; white arrows indicate regions with Sox2 expression. LI, lower incisors; EK, enamel knot; IK, initiation knot; Md, mandible; Mx, maxilla.

antibody was used to pull down the Pitx2-Irx1 protein complex. We did not perform an IP experiment using the Irx1 antibody to pull down Pitx2 as LS-8 cells do not endogenously express high levels of Irx1. In a gel-shift assay, Pitx2 protein bound to the *Irx1* promoter probe with the Pitx2-binding motif (Fig. 6D), and a luciferase assay showed that Pitx2 activated an *Irx1* promoter construct in LS-8 cells (Fig. 6E). Finally, immunostaining for Irx1 in lower incisors revealed that protein levels were lower in E12.5 and E14.5 *Pitx2*^{cKO} embryos than in their wild-type counterparts (Fig. 6F). Taken together, these data show that Pitx2 directly regulates *Irx1* transcription during tooth development.

A new molecular mechanism for Sox2 and Pitx2 controlling gene expression

Interestingly, we found that the Sox2 protein can inhibit Pitx2 protein binding to the *Irx1* promoter in a dose-dependent manner (Fig. 6D) and negatively regulates the transcriptional activity of Pitx2 in LS-8 cells (Fig. 6E). We also demonstrated this regulation in mice by immunostaining for Irx1 and Sox2 protein in the lower incisors. Sox2 and Irx1 were colocalized in the posterior part of the dental epithelium in E12.5 wild-type lower incisors and then were expressed in distinct cell populations (Sox2 in LaCL and Irx1 in OEE & SR) in E14.5 wild-type lower incisors (Fig. 6F). Irx1 was relatively higher in E14.5 lower incisor tooth germs compared with E12.5 lower incisor tooth germs, indicating that the lack of Sox2 expression in OEE cells at E14.5 allowed Pitx2 to activate *Irx1* expression. Sox2 also inhibits the synergistic activation of the *Lef1* promoter by Pitx2 and Lef1 in a dose-responsive mechanism (Fig. S6). These new molecular mechanisms demonstrate that Sox2 inhibits Pitx2 transcriptional activity and *Lef1* expression to maintain the Sox2^{+} domain cells such as the LaCL stem cell niche (Sun et al., 2016). However, *Sox2* is not expressed in the EK

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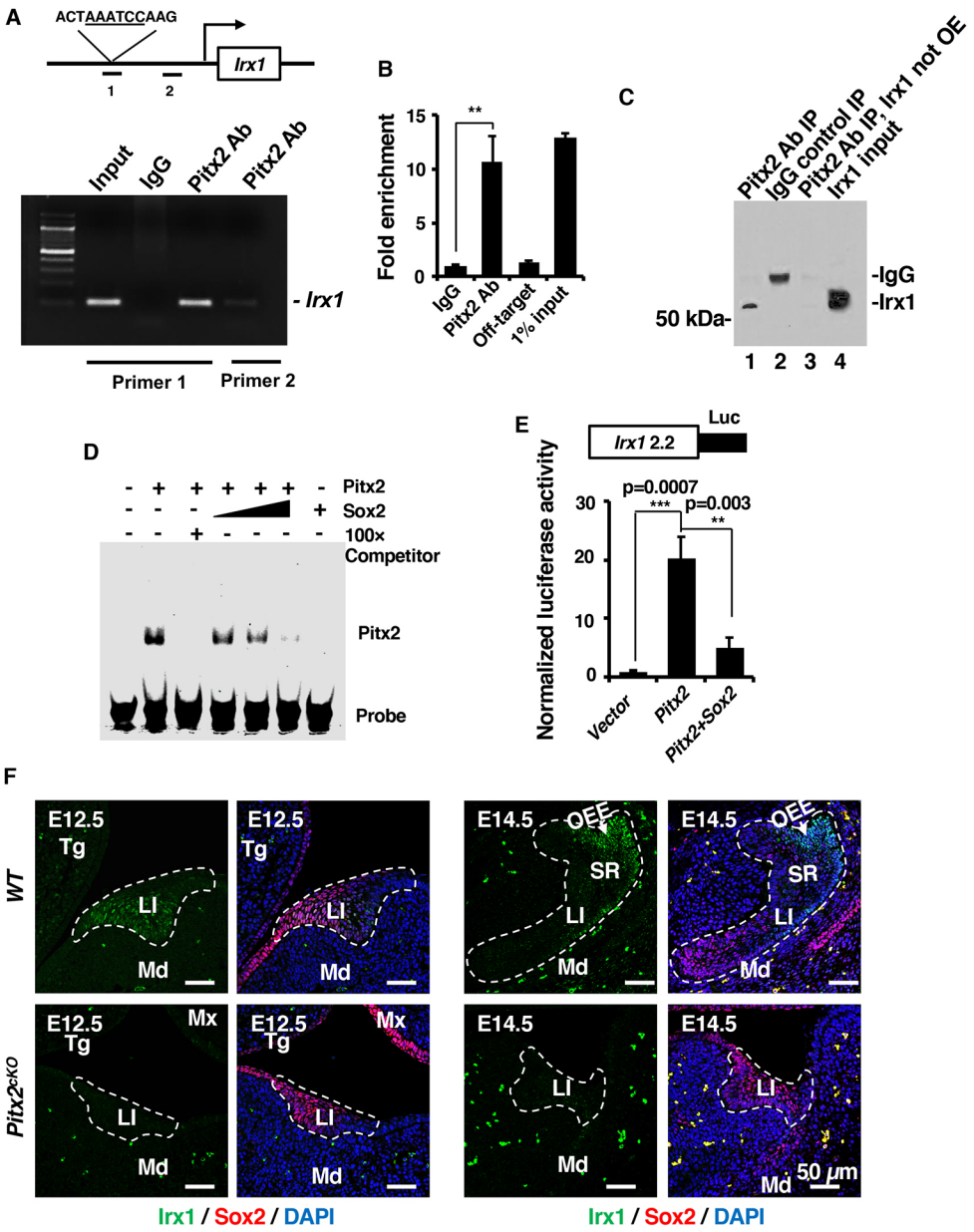

Fig. 6. Pitx2 activates the expression of *Irx1* modulated by a direct interaction with Sox2. (A) Schematic of the *Irx1* promoter including the Pitx2-binding motif and primers flanking the Pitx2-binding site (1) and control DNA site (2). (B) Quantitative RT-PCR analysis of ChIP assays with Pitx2 antibody performed in LS-8 cells. (C) Co-immunoprecipitation of Pitx2 and Irx1 proteins in LS-8 oral epithelial cells. LS-8 cells endogenously express Pitx2 and Irx1 was overexpressed and the protein complex was pulled down using a Pitx2 antibody (lane 1); IgG control (lane 2); Pitx2 antibody immunoprecipitation without overexpressing (OE) Irx1 (lane 3); Irx1 input (lane 4). (D) EMSA of Pitx2 protein with IRDye-labeled oligonucleotides from the *Irx1* promoter. (E) Schematic of 2.2 kb *Irx1* promoter in luciferase reporter construct and bar chart showing luciferase activity in LS-8 cells co-transfected with *Pitx2*, *Sox2* or vector-only plasmids. Luciferase activity is shown as mean fold activation normalized to luciferase activity in the vector group. (F) Representative images of immunostaining of Irx1 in E12.5 and E14.5 wild-type and $Pitx2^{cKO}$ embryos. Regions outlined with dashes represent developing lower incisors at E12.5 and E14.5. LI, lower incisors; OEE, outer enamel epithelium; SR, stellate reticular; Md, mandible; Tg, tongue. Data are mean±s.e.m. $^{**}P<0.01$; $^{***}P<0.001$.

signaling center, which expresses *Lef1*. Pitx2 expression is required to pattern the cells for each signaling center; Lef1 expression is later restricted to the EK and Sox2 expression is restricted to the LaCL stem cell niche.

DISCUSSION

Pitx2 is the earliest transcriptional marker for tooth development, and is required for the differentiation of stem cells and progenitors in several organs (Gage et al., 1999a; Lin et al., 1999; Lu et al., 1999; Mucchielli et al., 1997). In this paper, we show that Pitx2 regulates development of the signaling centers within the dental epithelium during tooth development. Furthermore, Pitx2 regulates the cells expressing *Shh* and promotes the formation of the enamel knot (EK) during the cap stage of tooth development. Pitx2 acts as a checkpoint for formation of the signaling centers and later for DESC differentiation to positively affect the accumulation of rapidly proliferating transit-amplifying cells during the early stages of tooth development. We have previously shown that Pitx2 is expressed in the DESCs of the murine lower incisor LaCL and the transit amplifying cells (Cao et al., 2013). In addition to controlling signals from the dental epithelial signaling centers (IK and EK), we show a new mechanism whereby Sox2 interacts with Pitx2 to negatively regulate *Irx1* transcription in $Sox2^{+}$ cell lineages, thereby contributing to the early stages of tooth morphogenesis. These types of protein interactions directly regulate and control gene expression in specific cells of the developing tooth organ.

Pitx2 regulates early progenitor cell proliferation in the developing tooth

Interestingly, cell proliferation in $Pitx2^{cKO}$ lower incisors was not affected at E11.5; however, it was significantly decreased at later embryonic stages, indicating that Pitx2 influences cell proliferation during embryonic tooth development. If Pitx2 regulated cell cycle progression directly, cell proliferation should have been downregulated in the $Pitx2^{cKO}$ lower incisor tooth germs at all stages of development. It is possible that the E11.5 and E12.5 dental epithelium of these mice retained sufficient functional Pitx2 protein to maintain cell proliferation, despite the fact *Pitx2* transcripts were

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ablated at E11.5 by *Krt14*^{Cre} (demonstrated by GFP immunostaining in *Rosa26*^{GPF} mice). However, it is more likely that Pitx2 regulates early progenitor cell proliferation in the developing tooth, and that the reduced cell proliferation in these mice at bud and cap stages is a consequence of an arrest of stem-cell proliferation and differentiation. In the tooth, stem cells give rise to TAs, which rapidly proliferate. In the lower incisors of E14.5 (cap stage) *Pitx2*^{cKO} embryos, Sox2^{+} stem cells failed to differentiate into such cells. In addition, the IK and EK did not form in *Pitx2*^{cKO} lower incisors, and given that the dental epithelial signaling centers provide Shh, Fgf4, Bmp2 and Wnt10b for the proliferation and differentiation of dental epithelial cells, it is reasonable to propose that cell proliferation is significantly downregulated in E14.5 *Pitx2*^{cKO} lower incisors due to a lack of an EK dental epithelial signaling center.

The fact that we did not observe a difference in growth of the lower incisor in the inducible *Pitx2* knockout indicates that Pitx2 is not required for this process in adult mice after development of the signaling centers has taken place. Interestingly, in individuals with Axenfeld-Rieger syndrome (ARS) with mutations in *PITX2*, their surviving teeth are brittle and fall out (Amendt et al., 2000). Our new data support a role for Pitx2 in late stage enamel formation, supporting a mechanism for brittle teeth in individuals with ARS. Furthermore, our demonstration that Pitx2 directly regulates the expression of *Irx1*, a gene that regulates the differentiation of Sox2^{+} stem cells into outer enamel epithelial cells (Yu et al., 2017), leads us to conclude that Pitx2 controls the differentiation of dental epithelial progenitor cells during tooth morphogenesis at later stages and not proliferation. We speculate that, in the continuously growing murine lower incisor during homeostasis, *Sox2* expression and other factors, such as *Shh*, *Bmi1*, *Lrig* and *Tbx1*, regulate the stem cell and transient-amplifying cell proliferation (Gao et al., 2015), rather than *Pitx2*. However, during incisor homeostasis, a recent report clearly demonstrated that, upon injury, repair of the incisor relied upon cells from the SI that repopulated the ameloblast layer to facilitate rapid tooth repair (Sharir et al., 2019). With these new data and the fact that Pitx2 and Irx1 expression is shifted to the ameloblasts, OEE and SI during homeostasis, Pitx2 and Irx1 may have a unique role in this repair process.

Pitx2 expression is required for the formation of the IK and EK signaling centers

The IK is a transient structure that does not express Sox2 but is derived from Sox2^{+} cells located in the posterior region of the placode (Du et al., 2017). The IK does express Shh, Bmp2, Wnt10b and p21 (Ahtiainen et al., 2016), also shown in this report, but also cyclin D2 and Lef1. In the *Pitx2*^{cKO} E12.5 embryos, the IK appears to be positioned in the middle of the tooth bud with reduced expression of the IK markers. The attenuation of Wnt signaling may be required for the clearance of the IK (Ahtiainen et al., 2016) and Lef1 expression is absent from the IK region after E12.5 and reappears in a *de novo* activation to mark the EK at E13.5.

The altered expression of *Shh*, *Bmp2*, *Fgf4* and *Wnt10b* suggests that *Pitx2* regulates the development of the EK at the cap stage in *Pitx2*^{cKO} embryos. Furthermore, *p21* and *Lef1* expression, early markers of dental epithelial signaling centers in the teeth (Jernvall and Thesleff, 2000; Keränen et al., 1999), revealed a small cell population (IK) that is positioned toward the anterior end of the developing tooth bud. This cell population (Sox2^{-} cells) does not represent progenitors of the EK, as the incisor EK cells are not derived from the IK (Ahtiainen et al., 2016; Du et al., 2017). The EK has been reported to be induced from *de novo* progenitor cells in the tooth bud (Ahtiainen et al., 2016; Du et al., 2017). Thus, Pitx2 may be involved in initiating the development of the EK, as we have shown that Pitx2 activates *Lef1* and *p21* expression (Amen et al., 2007; Cao et al., 2010a). Furthermore, the EK structure is not formed in the *Pitx2*^{cKO} embryos. Given that formation of the EK is the key event in the transition from bud to cap stage, the unique function of Pitx2 makes this transcription factor an extremely important determinant of tooth development.

The *Shh* signaling pathway plays crucial roles during the early stages of tooth development. *Shh* is expressed in the EK of both molars and incisors, and the BMP-Shh signaling network has been reported to control the fate of Sox2^{+} epithelial stem cells in molars (Li et al., 2015). In the tooth, *Shh* knockout leads to severe disruption of morphogenesis, leading to smaller teeth and disorganization of both the ameloblast and odontoblast layers (Dassule et al., 2000). Notably, overexpression of *Shh* in the dental epithelium also led to an arrest of tooth development at the bud stage (Cobourne et al., 2009), indicating that tooth development is very sensitive to Shh levels. The demonstration that *Shh* expression in the lower incisors of E12.5 *Pitx2*^{cKO} mice was decreased, causing reduced invagination of the dental epithelium, delays in the formation of bud stage teeth and an arrest in the differentiation of Sox2^{+} epithelial stem cells is consistent with the known role for *Shh* in tooth development. Moreover, Gli1 is transcriptionally activated by *Shh* in mice (Hynes et al., 1997), and *Gli1* was downregulated in *Pitx2*^{cKO} lower incisor tooth germs, which is consistent with the other effects on *Shh* signaling. In addition, tooth development is arrested at the bud stage when both *Gli2* and *Gli3* are knocked out in mice (Hardcastle et al., 1998), which is a phenotype similar to the *Pitx2*^{cKO} mice.

Sox2 inhibition of Pitx2 DNA binding regulates Pitx2 activation of Lef1 and Irx1 to maintain the signaling centers

Pitx2 operates in a gene regulatory network involving Sox2^{+} and Sox2^{-} cells. Sox2 protein in turn interacts with Pitx2 and represses Pitx2 transcriptional activity, providing a unique cellular and molecular mechanism of *Pitx2*-mediated regulation. Here, we show that this mechanism allows the formation of the signaling centers and DESCs, as well as their differentiation during tooth development.

Interestingly, the expression domains of Sox2, Lef1 and Irx1 overlap with Pitx2 during early tooth development. This raises the question of how *Sox2*, *Lef1* and *Irx1* are differentially expressed and regulated by Pitx2? We have previously shown that Pitx2 regulates *Sox2* and *Lef1* expression (Amen et al., 2007; Sun et al., 2016; Vadlamudi et al., 2005). Furthermore, we have demonstrated that Hmgn2, a small high-mobility group protein, can directly interact with Pitx2 to remove it from DNA and inhibit Pitx2 transcriptional activity (Amen et al., 2008). Sox2 contains a Hmg domain that represses Pitx2 transcriptional activation of Sox2, Lef1 and Pitx2 auto-regulation (Sun et al., 2016). We show, in this report, that Sox2 can inhibit Pitx2 from binding the *Irx1* promoter as a direct mechanism to control Pitx2 regulation of *Irx1* in a Sox2 expression domain during development. We have shown previously that Pitx2 directly interacts with Lef1 to synergistically activate the *Lef1* promoter (Vadlamudi et al., 2005). As a mechanism to regulate Lef1 in the signaling centers, we show a dose response relationship between Sox2-mediated inhibition of the synergistic activation of the *Lef1* promoter by Pitx2-Lef1. Thus, Sox2 controls the transcriptional activity of Pitx2 and represses Pitx2 activation of *Lef1*, *Pitx2*, *Irx1* and other factors regulated by Pitx2. Therefore, in Sox2 expression domains, it acts to inhibit Pitx2-mediated

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transcriptional activation, but outside the Sox2 domains (the IK and EK), Pitx2 then activates a gene regulatory network required for IK and EK formation, and progenitor cell differentiation. A potential mechanism for the clearance of the IK after E12.5 resides in the growing and changing cell population expressing Sox2. As the Sox2$^+$ cells transition from the posterior region of the placode and early tooth bud to the LaCL at E14.5, Sox2 expression would attenuate Pitx2 transcriptional activity, which would result in decreased *Lef1*, *p21* and *Shh* expression. This would clear the IK structure and favor the re-establishment of the EK away from the Sox2$^+$ cell domain. Sox2 expression domains are also regulated and maintained by other mechanisms, such as signaling factors and microRNAs. Because Lef1 and Sox2 expression domains appear to be nonoverlapping, Lef1 and Shh with other factors may also be controlling Sox2 expression (or lack of) in the IK and EK.

In summary, we have identified a novel molecular mechanism whereby Pitx2 regulates the expression of *Irx1* and cells expressing *Shh* to control the early stages of tooth morphogenesis. Pitx2 is expressed throughout the placode, bud and cap stage during tooth development (Cao et al., 2013; Hjalt et al., 2000). Specifically, our data show that Pitx2 regulates the differentiation of progenitors of the signaling centers. At the E12.5 bud stage, Pitx2 promotes *Lef1*, *p21* and *Shh* expression within the IK signaling center of the lower incisor, and these cells do not express Sox2. At the same time, Pitx2 directly promotes *Irx1* transcription and at later stages differentiation to Sox9$^+$ cells and other cell types. At the cap stage, Pitx2 directly regulates *Irx1* transcription in progenitors of the outer enamel epithelium, stratum intermedium and the stellate reticulum, promoting their differentiation. At this stage, Pitx2 is essential for the formation of the EK: the signaling center within the cap-stage lower incisors (Fig. 7). Sox2 directly interacts with Pitx2 to attenuate Pitx2 transcriptional activity and activation of *Sox2*, *Irx1*, *Shh* and *Lef-1*, when these two proteins share domains in the developing incisor. We speculate that this is a method to control Sox2$^+$ cells and separate those cells from the other types of cells expressing Pitx2. Our findings further demonstrate that Pitx2 is crucial for the communication between the DESCs and the epithelial signaling centers, suggesting that it is a master regulator of tooth development.

MATERIALS AND METHODS

Mouse lines and embryonic staging

Mouse maintenance and mouse-related procedures were performed using protocols approved by the Institutional Animal Care and Use Committee of the University of Iowa. The *Pitx2$^{flox/flox}$* mouse strain has been previously described (Gage et al., 1999b) and was a generous gift from the laboratory of James F. Martin (Baylor College of Medicine, Houston, TX, USA). The *K14^{Cre}* mouse strain has also been described previously (Cao et al., 2010b; Dassule et al., 2000). The *Rosa26^{CreERT}* [B6.129-*Gt(ROSA)26Sor$^{tm1(cre/ERT2)Tyj}$*/J] (stock number 008463) and *Rosa26^{GPF}* [B6.129(Cg)-*Gt(ROSA)26Sor$^{tm4(ACTB-tdTomato,-EGFP)Luo}$*/J] (stock number 007676) mouse strains originated from the Jackson Laboratory, and were generous gifts from the laboratory of John Engelhardt (The University of Iowa, Iowa, USA). Mice were genotyped using the primers listed in Table S1 following the standard genotyping protocol. Embryos were staged by checking for vaginal plugs in the crossed females, with the day of vaginal plug formation defined as E0.5.

Antibodies and reagents

The following primary antibodies were used for immunostaining: anti-IRX1 (Sigma, HPA043160, 1:100-1:300), anti-Lef-1 (Cell Signaling, 2230, 1:150), anti-Sox2 (R&D Systems, AF2018, 1:150), anti-Sox9 (Millipore, AB5535, 1:400), anti-GFP (Abcam, ab290, 1:1000), anti-Ki-67 (Abcam, ab15580, 1:500), anti-cleaved caspase-3 (Cell Signaling, 9661, 1:500), anti-BrdU (Abcam, ab6326, 1:1000), anti-CIdU (Accurate Chemical, OBT0030, 1:250), anti-IdU (Roche, 11170376001, 1:250), anti-p21 (BD Pharmingen, 556430, 1:100), anti-PY489-β-catenin (DSHB, University of Iowa), anti-β-catenin (Upstate, 06-734, 1:250), anti-Pitx2 (R&D Systems, AF7388, 1:500), anti-Myc (Cell Signaling, 9B11, 1:500) and anti-cyclin D2 (Santa Cruz, sc-593, 1:400). The following secondary antibodies were used for immunostaining: Alexa Fluor488 donkey anti-rabbit IgG (Invitrogen, A21206, 1:500), Alexa Fluor488 donkey anti-mouse IgG (Invitrogen, A21202, 1:500) and Alexa Fluor594 donkey anti-mouse IgG (Invitrogen, A21203, 1:500). Other staining reagents used were: DAPI (Invitrogen, D1306, 1 µg/ml), BrdU (Invitrogen, 00-0103), CIdU (Sigma, C6891) and IdU (Sigma, 17125).

Cloning, transient transfection and luciferase assay

A 2200 bp *Irx1* promoter containing the Pitx2-binding site was cloned and inserted into the pTK-luc vector. The *LEF-1* promoter has been previously described (Amen et al., 2007). Standard transient transfection by electroporation and luciferase assay were carried out in the ameloblast-derived LS-8 line (Chen, 1992) according to the methods previously described (Cao et al., 2013). LS-8 cultures (Chen, 1992) were seeded in

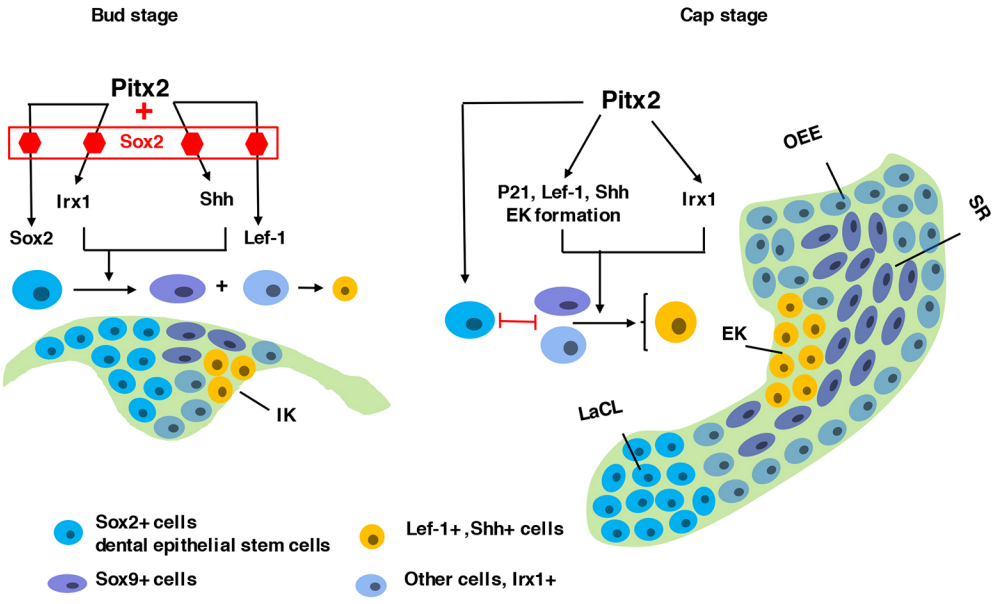

Fig. 7. Models of the Pitx2 molecular mechanisms regulating the early stages of tooth development. At bud stage, Pitx2 promotes *Shh* expression within the signaling center (Lef1$^+$ cells) of the lower incisor, and secretion of Shh stimulates both the proliferation and differentiation of Sox2$^+$ stem cells. At the same time, Pitx2 directly promotes *Irx1* transcription in Sox2$^+$-derived cells, thereby promoting their differentiation into Sox9$^+$ cells and other cells. At cap stage, Pitx2 directly regulates *Irx1* transcription in progenitors of the outer enamel epithelium and the stellate reticular, promoting their differentiation. At this stage, Pitx2 is also essential for the formation of the enamel knot, the signaling center within the cap-stage lower incisors. EK, enamel knot; IK, initiation knot; LaCL, labial cervical loop; OEE, outer enamel epithelium; SR, stellate reticular.

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flasks or plates in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum, and fed at least 24 h prior to the experiments.

Histological staining, immunostaining and imaging

Mouse embryos were harvested and washed with ice-cold 1× PBS, then fixed with 4% PFA and washed with 1× PBS three times (10 min each). Embryos were then taken through a standard dehydration and paraffin-embedding process. Sections of 5-8 µm were cut and then subjected to staining with either standard Hematoxylin and Eosin (H&E) or antibodies. For immunostaining, sections were incubated in citrate buffer (pH=6.0) in a 100°C water bath for 20 min, blocked with 10% donkey serum and incubated with primary antibodies overnight in 4°C. They were washed with 1× PBS, incubated with Alexa Fluor488 and 594 secondary antibodies, and stained with DAPI. Images were captured using a Nikon Eclipse microscope or a Zeiss 700 confocal microscope.

BrdU labeling and CIdU/IdU labeling assay

BrdU, CIdU and IdU were administered to pregnant females by intraperitoneal injection at different time points. Following the standard tissue processing steps described above, sections were treated with 2 M HCl for 40 min and neutralized with 1× PBS. They were then treated with citrate buffer and subjected to standard immunostaining as described above. Details of the methods used for IdU and CIdU double staining have been described previously (Sun et al., 2016).

In situ hybridization

PFA-fixed paraffin-embedded tissue sections were used for *in situ* hybridization. Embryo sections were cut into segments of 8 µm, and subsequently prepared as described previously (Gregorieff and Clevers, 2015). Briefly, sections were processed by a standard dewaxing and rehydration protocol, and rinsed twice in DEPC-treated water. Sections were next treated with 0.2 N HCl for 15 min at room temperature and incubated with proteinase K in 1× PBS buffer. Sections were then washed with 1× PBS quickly, and post-fixed for 5 min with 4% PFA. They were then treated with fresh acetic anhydride solution twice, and rinsed with 5× SSC buffer (pH 7.5). Sections were incubated with hybridization solution at 65°C for prehybridization, and then with hybridization solution containing 500 ng/ml of digoxigenin-labeled probes at 62-68°C for 36 h. Sections were washed with 50% formamide/2× SSC buffer (pH 4.5) at 65°C, blocked with blocking buffer and incubated with sheep anti-digoxigenin antibody (Roche, 1:2000) overnight at 4°C. Last, sections were incubated with NBT/BCIP solution, and images were taken using a bright-field microscope. The digoxigenin-labeled probes were generated using a DIG RNA Labeling Kit (Roche, 11175025910). Primers used to generate probes are listed in Table S2.

Lower incisor growth assay and scanning electron microscopy

Pitx2^{iHet} and *Pitx2*^{icKO} mice at ∼40 days of age received daily peritoneal injections of tamoxifen (2 mg/10 g body weight) for 10 days. After 8 days of injection, the left lower incisor was clipped and the difference in length with the contralateral incisor was measured using a caliper. Tamoxifen was injected daily for an additional 2 days, and at day 3.5 post-clipping, the mice were sacrificed and the teeth were measured again.

For analysis of the enamel, incisors were isolated by hemimandibular section. All incisors were fixed in 4% paraformaldehyde overnight, after which they were rinsed three times. One of the incisors from each mouse was immediately subjected to gradient alcohol dehydration, whereas the other was prepared for analysis of a cross-section. Specifically, the incisors were fractured at the level of the gingival margin, with the line of fracture parallel to the enamel surface, after which hydrochloric acid was applied to the enamel for 30 s and samples were rinsed three times. In both sample sets, following alcohol dehydration, critical-point drying was performed by submerging the sample in hexamethyldisilazane (HMDS) for 1 h and drying overnight. Samples were mounted on stubs and sputter-coated with gold/palladium alloy. Gross morphology of the complete incisor was assessed using a Hitachi S-4800 microscope in secondary electron mode, at 3 kV and an emission current of 7400 nA. Enamel cross-sections from fractured teeth were imaged in secondary electron mode, at 5 Kv and an emission current of 10,600 nA. All images were taken under high vacuum. The working distance ranged from 8 mm to 10.8 mm.

Chromatin immunoprecipitation assay (ChIP) and IP

The procedure for the ChIP assay has been described previously (Sun et al., 2016), and the ChIP Assay Kit (Zymo Research, Zymo-Spin ChIP Kit, D5210) was used for the analysis. LS-8 cells were washed twice with ice-cold 1× PBS solution and crosslinked (1% formaldehyde, room temperature, 7 min). They were then subjected to three rounds of sonication (6 s duration, 25% of maximum amplitude), which caused lysis and the shearing of genomic DNA into ∼200-1000 bp fragments. DNA and protein complexes were then immunoprecipitated using 5 µg Pitx2 antibody (Capra Sciences, PA-1023). In the negative control, the same amount of normal rabbit IgG replaced the Pitx2 antibody. Irx1 primers used for PCR were as follows: primer 1 forward, 5′-CAAGAAGAGGTCACAATTAGGAGCT-3′; primer 1 reverse, 5′-CTTGG-AATGTCAGGACCTGCTTT-3′; primer 2 forward, 5′-TCGTGGGAGACTCAAAGACAGG-3′; primer 2 reverse, 5′-AGAC-GCGGAGAGTCAACACGA-3′. All PCR products were visualized on a 1.5% agarose gel, and their identities were confirmed by sequencing. LS8 cells were transfected with an *Irx1* overexpression plasmid in pCDNA3.1 or empty pcDNA3.1 using PEI. Transfected cells were harvested, suspended in IP lysis buffer, sonicated and incubated with 1 µg of Pitx2 or normal rabbit IgG in the presence of Protein A Magnabeads (Fisher Scientific) for 2 h. The samples were then washed, resuspended in 4× Laemmli buffer and probed on a western blot. Myc-tag antibody listed above (1:200) was used for detection and an IgG-binding-depleted anti mouse-HRP was used (Abcam, ab131368, 1:4000).

Electrophoretic mobility shift assay (EMSA)

Fluorescent dye-labeled probe and purified mouse proteins were used for EMSA according to the protocol from the Odyssey Infrared EMSA Kit (LI-COR, P/N: 829-07910). Oligonucleotides from a region of the *Irx1*-promoter including a Pitx2-binding motif were generated by the Iowa Institute of Human Genetics of The University of Iowa. The sequences were: 5′-/IRdye700/TACCCTCCAGACTAAATCCAAGCAGGAAAGC-AG-3′ and 5′-TACCCTCCAGACTAAATCCAAGCAGGAAAGCAG-3′. Complementary oligonucleotides were annealed and 50 nM of probe was added into the binding reaction. The purified mouse Pitx2c and Sox2 proteins were prepared as previously described (Tao et al., 2016), and 5 µg of Pitx2c and 0-10 µg of Sox2 were added to the binding reaction. After 20-30 min of incubation at room temperature, 2 µl of 10× Orange Loading Dye was added to the 20 µl EMSA reaction. The entire binding reaction was then loaded onto a 6% native polyacrylamide gel and run until the dye reached the bottom of the gel. Finally, the gel was scanned using an Odyssey scanner.

3-Dimensional reconstruction

Serial sagittal sections from E12.5 wild-type and *Pitx2*^{cKO} lower incisors were stained with DAPI, and serial images were generated using a Zeiss 700 confocal microscope. Then images were automatically aligned using the StackReg plug-in for ImageJ, and the final 3-dimensional reconstructions were rendered using the Imaris software (Bitplane).

Statistical analysis

For each condition, a minimum of three experiments were carried out and data are presented as mean±s.e.m. An independent two-tailed *t*-test was used to determine the significance of differences between wild-type and *Pitx2*^{cKO} groups.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: B.A.A.; Methodology: Z.S., Y.S., M.S., S.E., H.C., M.L.P., B.A.A.; Validation: W.Y., Z.S., Y.S., M.S., S.E.; Formal analysis: W.Y., Z.S., Y.S., M.S., S.R.V., S.E., H.C., M.L.P., B.A.A.; Investigation: B.A.A.; Resources: B.A.A.; Data curation: W.Y., Z.S., Y.S., M.S., S.E., M.L.P.; Writing - original draft: W.Y., Z.S., S.R.V., S.E., H.C., B.A.A.; Writing - review & editing: W.Y., S.R.V., H.C., M.P., B.A.A.; Supervision: B.A.A.; Project administration: B.A.A.; Funding acquisition: B.A.A.

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