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Epithelial YAP1 Regulates Ameloblast Differentiation through SHH/FGF Signaling

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Abstract

Enamel produced by ameloblasts derived from the oral epithelium is the hardest and most mineralized tissue. Developmental enamel defects have significant clinical implications, such as enamel hypoplasia or opacities, increased tooth sensitivity, and impaired mastication. *Yap1* is a transcriptional coactivator that has been shown to be involved in regulating cell proliferation and differentiation. Although *Yap1* has been reported to play an important role in tooth development, the mechanism by which *Yap1* regulates enamel formation and ameloblast differentiation remains unclear. In this study, we report that ablation of *Yap1* in the dental epithelium using *Pitx2*^{Cre} leads to the defect of enamel formation. In the *Yap1*^{Pitx2Cre} mutant incisors, the expression of *Amelx*, *Ambn*, and *Mmp20* was greatly reduced in ameloblasts, indicating a defect in ameloblast differentiation. The proliferation of epithelial and mesenchymal cells was significantly reduced in *Yap1*^{Pitx2Cre} mutant incisors; however, there was no significant difference in apoptosis between wild type and mutant. Transcriptome analysis and in situ hybridization identified that *Shh*, *Ptch1*, *Fgf3*, *Fgf10*, *Etv4*, and *Etv5* were significantly downregulated after *Yap1* deletion. In the labial cervical loop of *Yap1*^{Pitx2Cre} mutant incisors, both SHH signaling and FGF signaling were significantly decreased. Furthermore, our results suggest that FGF signaling is regulated by SHH. SHH treatment induces *Fgf3* expression in vitro, and activation of the hedgehog pathway upregulates FGF signaling in vivo. Overexpression of *Ihh* attenuates enamel formation and cell proliferation defects caused by *Yap1* deletion, confirming the genetic integration of Hedgehog signaling. In summary, our study shows that epithelial *Yap1* may regulate ameloblast differentiation by modulating SHH/FGF signaling.

Keywords: tooth, enamel, epithelial-mesenchymal interaction, signal pathway, transcription factor, development biology

Introduction

The tooth is a complex ectodermal organ composed of soft vascular-dominated pulp, dentin, cementum, and enamel. Dental enamel, generated by ameloblasts, is the hardest tissue in vertebrates. Enamel developmental disorders can lead to dentin sensitivity, enamel disintegration, and decreased masticatory efficiency. The development of enamel is accompanied by the differentiation of ameloblasts, the secretion and mineralization of enamel matrix (Yu and Klein 2020). Dental epithelial cells undergo morphological changes during differentiation, including cytoplasmic elongation and polarization (Gan et al. 2020). The interaction between ectodermal epithelial cells and neural crest mesenchymal cells regulates the differentiation of epithelial cells into functional ameloblasts (Klein et al. 2008; Seidel et al. 2010; Balic 2019; Alghadeer et al. 2023).

Various signaling pathways, such as WNT, FGF, BMP, NOTCH, and Hedgehog, are involved in regulating the development and function of ameloblasts (Klein et al. 2008; Felszeghy et al. 2010; Liu et al. 2010; Seidel et al. 2010; Du et al. 2018). *Shh* is highly expressed in preameloblasts and secretory ameloblasts. SHH can induce upregulation of *Amelx* and *Ambn*, the main structural components of enamel, in ameloblasts (Dassule et al. 2000). The differentiation of ameloblasts can be inhibited by SHH antibody treatment (Zhang et al. 2008). Conditional knockout of *Smo* or *Shh* in dental epithelial cells leads to defects in cell proliferation and ameloblast development (Dassule et al. 2000; Gritli-Linde et al. 2002). *Fgf3* knockout mice have defects in enamel formation. Furthermore, *Fgf3* and *Fgf10* double knockout mice have very thin or missing enamel (Wang et al. 2007). *Fgf3* and *Fgf10* act through *Fgfr2b*, which is expressed in mouse dental epithelial cells (Kettunen et al. 2007). Deletion of *Fgfr2b* resulted in a significant reduction in tooth epithelial cell

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proliferation and cession of tooth development before the bud stage (Kettunen et al. 2007).

As a transcriptional coactivator, *Yap1* plays an important role in regulating cell proliferation, differentiation, and apoptosis (Li and Li 2016; Lin et al. 2017; Goodwin et al. 2020; Yang et al. 2023). Previous data have shown that Yes-associated protein 1 (YAP1) is highly expressed in the dental epithelium cells of incisors and molars, suggesting that YAP1 may be involved in regulating the differentiation process of ameloblasts. (Li et al. 2011; Liu et al. 2015). To investigate the function of YAP1 in ameloblast differentiation, we used $Pitx2^{Cre}$ mice to specifically knockout *Yap1* in dental epithelial cells. Our results indicate that $Yap1^{Pitx2Cre}$ mutant mice exhibit severe enamel developmental defects, and the SHH and FGF signaling pathways are affected. This study aims to investigate the molecular mechanism by which YAP1 regulates ameloblast differentiation.

Results

Yap1 Mutant Mice Exhibited Defects in Incisor and Molar Development

The results of in situ hybridization showed that *Yap1* was highly expressed in the incisor epithelial cells from E14.5 to E17.5 (Fig. 1A), suggesting that *Yap1* might play an important role in the differentiation of ameloblasts. To determine the function of *Yap1* in the ameloblast differentiation, we specifically deleted *Yap1* from dental epithelial cells using $Pitx2^{Cre}$ mice. Immunofluorescence analysis showed significantly reduced YAP1 expression in the incisors of $Yap1^{Pitx2Cre}$ mutant mice at E17.5 (Fig. 1F). Both male and female mice heterozygous for the *Yap1* mutation were normal and fertile. The mutant alleles ($Yap1^{Pitx2Cre}$) were born normally according to the Mendelian ratio. The $Yap1^{Pitx2Cre}$ mutants have white incisors with blunt tips, unlike wild-type incisors that are yellow and sharp (Fig. 1B, C). Micro–computed tomography analysis of the incisors and molars of $Yap1^{Pitx2Cre}$ mutant embryos showed a significant reduction in enamel (100% penetration) (Fig. 1B′, 1b, 1b′, 1C′, 1c, 1c′). Hematoxylin–eosin staining of sagittal paraffin sections of the incisors showed that the enamel layer of the mutant mice was much thinner than of the wild-type mice (Fig. 1D, 1d, 1E, 1e). The enamel thickness of the $Yap1^{Pitx2Cre}$ mutants was only one-third that of the wild-type littermates (Fig. 1G). This suggests that *Yap1* gene deletion leads to defective enamel formation.

Yap1 Deletion Leads to Defects in Ameloblast and Odontoblast Differentiation

In the secretory stage, the ameloblasts become polarized with the nuclei aligned at the apical side of the cells (Fig. 1d, black arrow). However, such polarization was not observed in the incisors of *Yap1* mutants ($Yap1^{Pitx2Cre}$), and the mutant epithelium was flattened at the secretory stage (Fig. 1e, black arrow). These findings clearly demonstrate that the differentiation of ameloblasts is disrupted due to the deletion of *Yap1*. Defective ameloblast differentiation was also confirmed by the expression of *Amelx*, *Ambn*, and *Mmp20*. Amelogenin (AMELX) and ameloblastin (AMBN) secreted by ameloblasts are important for enamel mineralization (Catón and Tucker 2009). Matrix metallopeptidase 20 (MMP20) is expressed in both odontoblasts and ameloblasts and is required for enamel maturation (Yamakoshi et al. 2006; Shintani et al. 2007). The expression of these genes was analyzed by in situ hybridization and immunofluorescence in the incisors and molars of $Yap1^{Pitx2Cre}$ mutant mice (Fig. 2A–C, Appendix Fig. 1). In the mutants, the expression of *Amelx*, *Ambn*, and *Mmp20* were all dramatically reduced.

Tooth development relies on interacting signaling pathways in the epithelial and mesenchymal cells. $Pitx2^{Cre}$ mice exhibited Cre activity in the mandibular epithelium as early as E10.5 (St Amand et al. 2000). Therefore, the deletion of *Yap1* in the dental epithelium may affect epithelial–mesenchymal interactions. The odontoblasts are derived from the neural crest–derived mesenchymal cells. To investigate whether there are defects in odontoblast development in mutant embryos, immunofluorescence was used to detect the expression of odontoblast differentiation marker NESTIN. NESTIN expression in the incisor (Fig. 2A) of mutant mice is much lower than that in the wild-type mice, suggesting a defect in odontoblast development. All of these results indicate that the deletion of the *Yap1* gene leads to defects not only in ameloblast differentiation but also in odontoblast differentiation.

Yap1 Deletion Leads to Impaired Cell Proliferation

To investigate the cellular mechanisms of enamel defects in *Yap1* mutants, we examined cell proliferation using 5-bromodeoxyuridine (BrdU) labeling. Immunofluorescence results showed a significant reduction of BrdU-positive epithelial and mesenchymal cells in the lingual cervical loop (liCL) and labial cervical loop (laCL) of the incisors at postnatal day 42 (P42) in $Yap1^{Pitx2Cre}$ mutant embryos compared with the wild type (Fig. 2D, E). Consistent with this, Ki67 labeling was also reduced in $Yap1^{Pitx2Cre}$ mutant incisor epithelial and mesenchymal cells (Appendix Fig. 3). The decrease in cell proliferation was also observed in the epithelial and mesenchymal cells of mutant incisors from E14.5 to E17.5 (Appendix Fig. 2). To determine whether enamel defects are due to abnormal cell apoptosis, we performed terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays on sagittal sections of mutant incisors. There was no significant difference in apoptosis between the $Yap1^{Pitx2Cre}$ and wild-type embryonic incisors at postnatal day 42 (P42) (Fig. 2F) and E14.5–E15.5 (Appendix Fig. 4), suggesting that there is no abnormal cell apoptosis after *Yap1* ablation. The results showed that YAP1 was crucial to cell proliferation during the development of incisors.

Yap1 Deletion Leads to Downregulation of SHH and FGF Signaling

YAP1 ablation caused impaired ameloblast and odontoblast differentiation on the labial side. At the cap stage around E14.5,

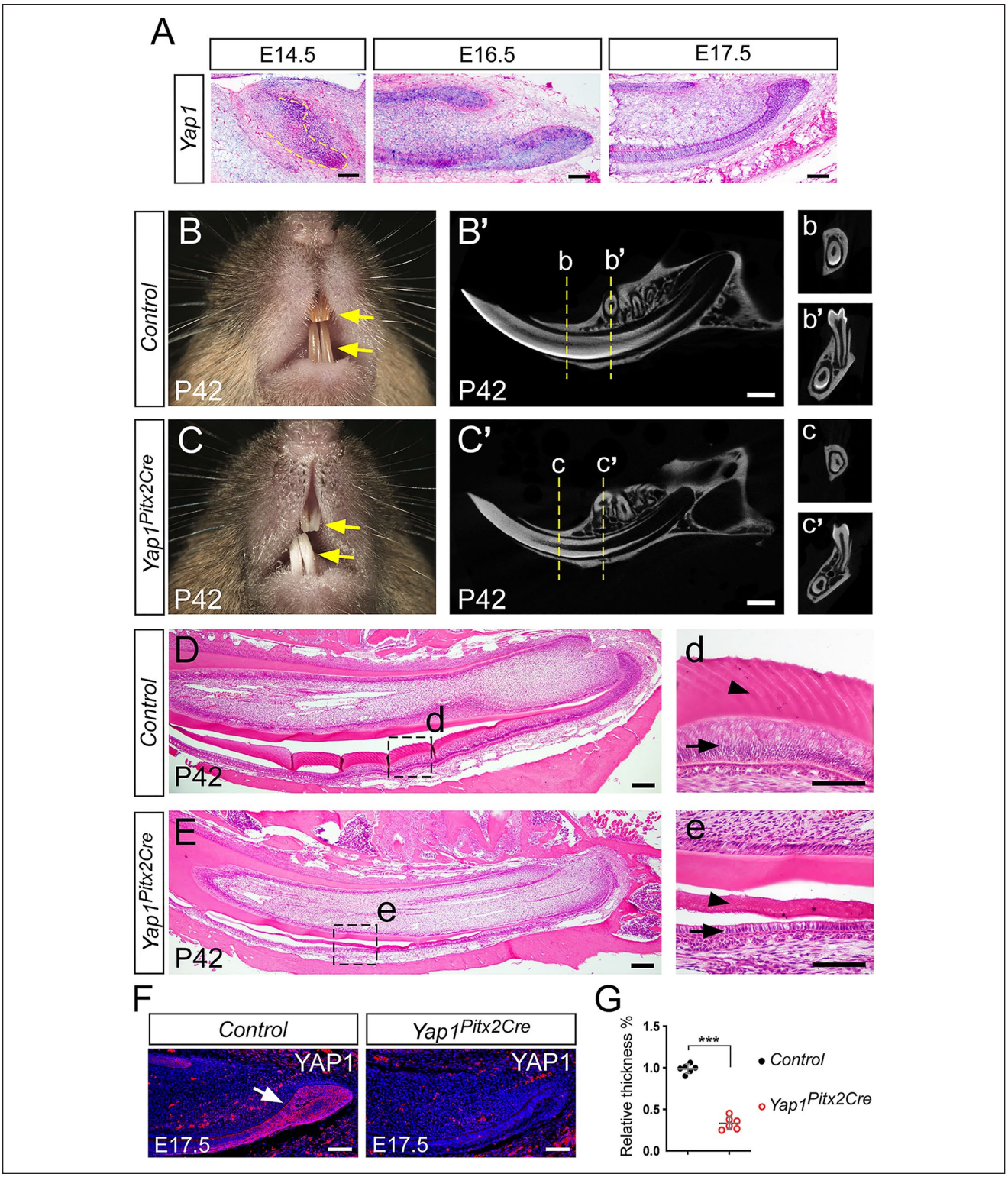

Figure 1. Dental epithelial inactivation of *Yap1* leads to enamel defects. (**A**) In situ hybridization shows expression of *Yap1* in the incisors at E14.5, E16.5, and E17.5. The yellow dashed line shows the boundary between the dental epithelium and mesenchyme of tooth germs. Comparison of control ($Pitx2^{Cre}$) (**B**) and mutant ($Yap1^{Pitx2Cre}$) (**C**) adult mouse tooth ($n=5$). Mutant maxillary and mandibular incisors are white and have blunt tips (yellow arrows). Micro–computed tomography (micro-CT) analysis of control ($Pitx2^{Cre}$) (**B′**) and mutant ($Yap1^{Pitx2Cre}$) (**C′**) adult mouse tooth in the sagittal view ($n=3$). Micro-CT shows the cross view of incisor (**b**, **c**) and molar (**b′**, **c′**) of control and mutant. Hematoxylin and eosin staining of control and mutant incisors in the sagittal view (**D**, **E**, **d**, **e**) at postnatal day 42 (P42) ($n=5$). Black arrowheads show the enamel matrix on the labial side. Black arrows show the ameloblasts on the labial side. (**F**) Immunofluorescence shows reduced YAP1 expression (white arrow) in mutant ($Yap1^{Pitx2Cre}$) and control ($Pitx2^{Cre}$) incisors (red arrowheads) at E17.5 ($n=4$). (**G**) Quantification of the enamel thickness between control and mutant ($n=6$). Scale bars: 1 mm (B, C), 200 µm (D, E), 100 µm (A, d, e, F).

asymmetrical labial–lingual cervical loops begin to form (Catón and Tucker 2009). The *Yap1*^{Pitx2Cre} mutant incisors also showed a significant decrease in cell proliferation at E14.5 (Appendix Fig. 2). To gain insight into the molecular mechanisms by which YAP1 regulates ameloblast differentiation, we analyzed the gene expression profiles of E14.5 mandibular incisors of mutant (*Yap1*^{Pitx2Cre}) and wild-type (*Pitx2*^{Cre}) embryos using RNA-Seq. In parallel comparisons, we uncovered dozens of protein-coding genes that were differentially expressed in the mutant versus the control (≥1.5 fold, <5% false discovery rate; Fig. 3A, B, Appendix Table 1). Among them, multiple genes known to be critical for ameloblast differentiation decreased significantly. Quantitative reverse transcription polymerase chain reaction confirmed the downregulation of *Shh*, *Ptch1*, *Fgf3*, *Fgf10*, *Etv4*, and *Etv5* in the mutant's mandibular incisor (Fig. 3C). The in situ hybridization results further demonstrated a reduction in the expression of *Etv5* and *Lef1* within the mandibular incisors of the mutant (Fig. 3D, E).

Shh, a member of the vertebrate Hedgehog family, is strongly expressed in preameloblasts and secretory ameloblasts. During tooth development, SHH signaling plays an important role in epithelial cells and epithelial–mesenchymal interactions. It has been reported that *Shh* expression is regulated by the YAP1 transcription factor during heterotopic ossification (Cong et al. 2021), lung development (Isago et al. 2020), and notochord formation (Cheng et al. 2023). The results of in situ hybridization also showed that *Shh* expression was decreased in the mutant's mandibular incisor epithelial cells (Fig. 4A) and molar epithelial cells (Appendix Fig. 5). It is known that YAP1 does not contain a DNA-binding domain, and the TEADs act as the major mediators of YAP1 coactivator activity. The consensus DNA binding sequence for TEAD is GGAATG. Two potential binding sites for the TEAD transcription factor were found in the *Shh* promoter (Fig. 4C). ChIP analysis was performed and confirmed that YAP1 can bind to the sites (Fig. 4D). The results indicate that YAP1 can directly regulate the expression of *Shh* by binding to the promoter region of *Shh*.

Fgf3 Expression Was Regulated by Shh in the Incisor

SHH is a glycoprotein secreted by epithelial cells and regulates epithelial mesenchymal interactions. Downregulation of mesenchymal cell proliferation indicates that *Shh* may affect the expression of genes in the mesenchyme. The expression of *Ptch1*, the target gene of Hedgehog signaling, was decreased in the mesenchyme of the mutant (*Yap1*^{Pitx2Cre}), suggesting that the activity of Hedgehog signaling in the mesenchyme of mutant was reduced (Fig. 5A, Appendix Fig. 6). Considering the important role of SHH signaling in epithelial–mesenchymal interactions, *Yap1* may further affect gene expression in mesenchymal cells through Hedgehog signaling.

Fibroblast growth factor signaling regulates not only the development of ameloblasts but also the differentiation of odontoblasts. Consistent with the RNAseq results, in situ hybridization showed reduced expression of *Fgf3*, *Fgf10*, and the FGF signaling target gene *Etv5* in the mutant incisors (Fig. 5A). *Fgf3* and *Fgf10*, which play important roles in enamel formation and cell proliferation, are expressed in the labial mesenchymal cells (Wang et al. 2007). The expression of *Fgf3* and *Fgf10* was downregulated in the mesenchyme of the mutant incisors (Figs. 4B, 5A). In addition, decreased expression of *Fgf3* and *Fgf10* was also observed in the molars (Appendix Fig. 6). We hypothesize that epithelial secreted SHH regulated the expression of *Fgf3* or *Fgf10* in the mesenchymal cells. To test our hypothesis, we treated mutant (*Yap1*^{Pitx2Cre}) incisors with SHH protein in an in vitro organ culture system. The results of real-time quantitative polymerase chain reaction (PCR) showed that SHH treatment could significantly induce the expression of *Fgf3* in the mutant incisors (Fig. 4E). Whole-mount in situ hybridization showed that SHH-saturated beads strongly induced *Fgf3* expression in the mesenchyme of the mutant incisors (Fig. 4F). All of the results indicate that FGF signaling activity in the incisors is regulated by *Shh*. The SHH/FGF signaling may mediate the effects of YAP1 on the differentiation of ameloblasts and odontoblasts.

Activation of Hedgehog Signaling Rescues Enamel Defects Caused by Yap1 Ablation

To verify our hypothesis that *Yap1* regulates ameloblast differentiation through Hedgehog signaling, a conditional transgenic allele (Tg-*pmes-Ihh*) was used to activate Hedgehog signaling in dental epithelial cells (Appendix Fig. 7). IHH can activate the same canonical Hedgehog signaling pathway as SHH (Varjosalo and Taipale 2008). Hematoxylin–eosin staining of sagittal paraffin sections of the P0 mouse incisors showed that the enamel layer was absent in the mutant mice, whereas it was present in the *Yap1*^{Pitx2Cre}; Tg-*pmes-Ihh* mice, suggesting restoration of enamel expression after *Ihh* overexpression (Fig. 5A). *Amelx* expression was also partially rescued in the ameloblasts of the incisors in *Yap1*^{Pitx2Cre}; Tg-*pmes-Ihh* embryos (Fig. 5A). The expression of *Ptch1* was increased in the mesenchyme of *Yap1*^{Pitx2Cre}; Tg-*pmes-Ihh* compared with *Yap1*^{Pitx2Cre} embryos (Fig. 5A), indicating that transgenic IHH as a secreted ligand functioned in the mesenchyme. Expression of *Fgf3* and *Etv5*, a marker gene for FGF signaling activity, was also upregulated in *Yap1*^{Pitx2Cre}; Tg-*pmes-Ihh* incisors, suggesting that FGF signaling activity was also restored (Fig. 5A). The defect of cell proliferation was rescued in *Yap1*^{Pitx2Cre}; Tg-*pmes-Ihh* compared with that in the *Yap1*^{Pitx2Cre} embryos (Fig. 5B–D). Proliferation of LaCL and LiCL epithelium cells was significantly increased in *Yap1*^{Pitx2Cre}; Tg-*pmes-Ihh* embryos compared with *Yap1*^{Pitx2Cre} mutants. Proliferation of liCL and laCL mesenchymal cells was also highly upregulated in *Yap1*^{Pitx2Cre}; Tg-*pmes-Ihh* embryos. The results indicate that activation of the Hedgehog pathway can rescue the cell proliferation defects in *Yap1*^{Pitx2Cre} incisors. Overexpression of *Ihh* rescued enamel and cell proliferation defects due to *Yap1* ablation, confirming the genetic integration of Hedgehog signaling. The results also confirmed that FGF signaling was regulated by Hedgehog signaling in the incisor.

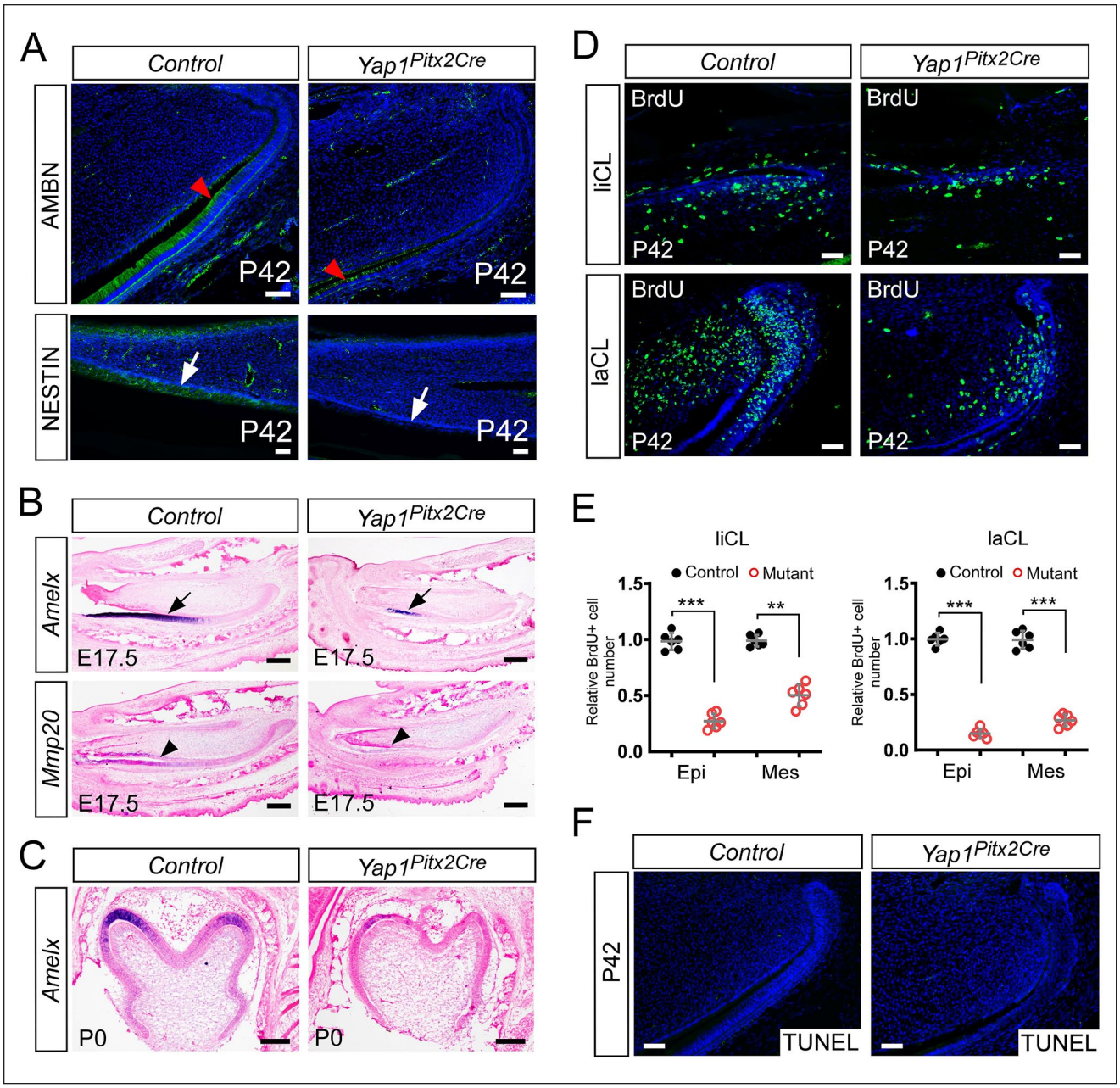

Figure 2. Deletion of *Yap1* in dental epithelial cells results in defects of cell proliferation in the mandibular incisors. (**A**) Immunofluorescence shows reduced AMBN and NESTIN expression in adult mutant ($Yap1^{Pitx2Cre}$) and control ($Pitx2^{Cre}$) incisors (red arrowheads) ($n=4$). The red arrowheads show the ameloblasts on the labial side. The white arrows show the odontoblasts. (**B**) In situ hybridization shows reduced *Amelx* (black arrows) and *Mmp20* (black arrowheads) on the labial side of mutant ($Yap1^{Pitx2Cre}$) and control ($Pitx2^{Cre}$) incisors at E17.5 ($n=5$). (**C**) In situ hybridization shows reduced *Amelx* in mutant ($Yap1^{Pitx2Cre}$) and control ($Pitx2^{Cre}$) molars at P0 ($n=5$). (**D**) BrdU labeling assays show the cell proliferation in the incisors of mutant ($Yap1^{Pitx2Cre}$) and control ($Pitx2^{Cre}$) at P42 ($n=5$). (**E**) Quantification of the relative epithelial (Epi) and mesenchymal (Mes) proliferation determined by the BrdU incorporation assay between control and mutant ($n=6$). (**F**) TUNEL staining shows the cell apoptosis in the mandibular incisors of $Yap1^{Pitx2Cre}$ and control ($Pitx2^{Cre}$) ($n=5$). liCL, lingual cervical loop; laCL, labial cervical loop. Error bars represent standard deviations. $^{*}P<0.05$. $^{**}P<0.01$. $^{***}P<0.001$. Scale bars: 100 µm.

Discussion

Studies in animals and humans have identified many genes that play important roles in ameloblast differentiation (Bei 2009; Alghadeer et al. 2023). In this study, enamel defect was found in $Yap1^{Pitx2Cre}$ mutant embryos, indicating that YAP1 is critical for the enamel formation and ameloblast differentiation. In $Yap1^{Pitx2Cre}$ mutants, SHH and FGF signaling activities were significantly downregulated. Our results suggest that YAP1 regulates ameloblast differentiation by modulating SHH and FGF signaling.

YAP1, a key molecule in the Hippo signaling pathway, acts as a transcriptional co-activator to regulate downstream gene expression (Liu et al. 2014; Yoshikawa et al. 2015). When the

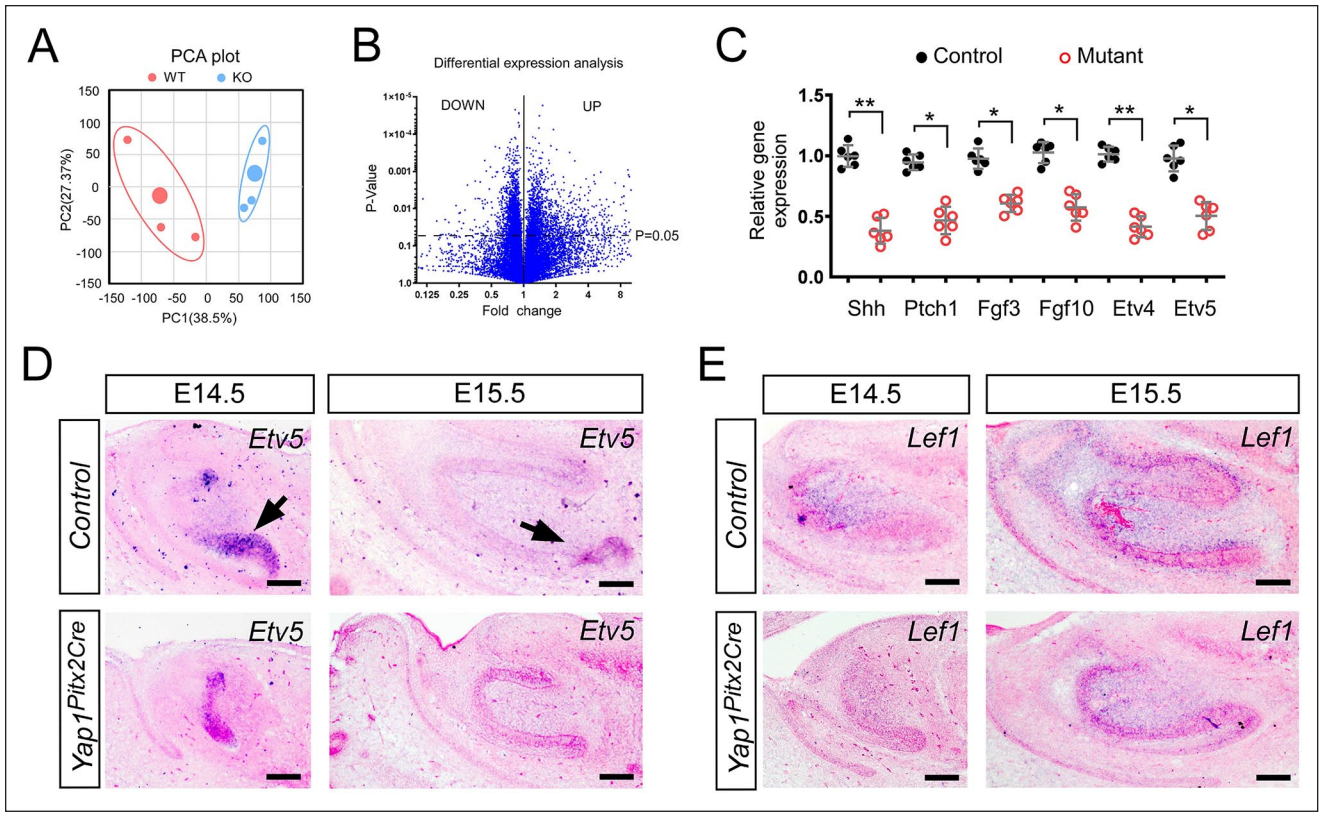

Figure 3. Differentially expressed genes in the mutant mandibular incisors. (**A**) Principal component analysis plot of RNA-seq samples of control (wild-type; WT) and mutant (knockout; KO). (**B**) Volcano plot of RNA-seq data showing the gene expression differences between the mandibular incisors of *Yap1*^{Pitx2Cre} and control (*Pitx2*^{Cre}). (**C**) Quantitative reverse transcription polymerase chain reaction demonstrates the transcription of *Shh*, *Ptch1*, *Fgf3*, *Fgf10*, *Etv4*, and *Etv5* in the incisors of control (*Pitx2*^{Cre}) and *Yap1*^{Pitx2Cre} at E14.5 ($n=6$). (**D**) In situ hybridization shows reduced *Etv5* expression (black arrows) in the incisors of control (*Pitx2*^{Cre}) and *Yap1*^{Pitx2Cre} at E14.5 and E15.5 ($n=3$). (**E**) In situ hybridization shows reduced *Lef1* expression in the incisors of control (*Pitx2*^{Cre}) and *Yap1*^{Pitx2Cre} at E14.5 and E15.5 ($n=3$). *$P<0.05$. **$P<0.01$. Scale bars: 100 µm.

Hippo pathway is inhibited, unphosphorylated YAP1 enters the nucleus and forms a complex with the TEAD family of transcription factors, thereby inducing the expression of target genes. *Yap1* is expressed in both tooth dental epithelial and mesenchymal cells (Li et al. 2011; Li and Li 2016). Overexpression of *Yap1* in the dental epithelium resulted in deformed tooth morphogenesis and enamel knot patterning (Liu et al. 2014). Knockdown of the *Yap1* in the epithelium using K14-Cre mice can lead to smaller tooth germs and reduced proliferation of dental epithelial cells (Liu et al. 2015). However, *Yap1*^{K14Cre} mice abort or die shortly after birth due to dehydration, and there are no reports on the function of the *Yap1* gene in enamel formation (Liu et al. 2015).

In this study, *Pitx2*^{Cre} mice were used to conditionally remove the *Yap1* gene from dental epithelial cells. *Pitx2* is one of the earliest epithelial markers in tooth development, and the Cre recombinase activity can be detected in dental epithelial cells as early as E10.5. *Yap1*^{Pitx2Cre} mutant mice are viable and have normal fertility but exhibit enamel defects. *Yap1*^{Pitx2Cre} mutant embryos showed significantly less enamel than that of wild type. Reduced expression of *Ambn*, *Amelx*, and *Mmp20* was observed in mutant ameloblasts. Mutants have smaller and shorter incisors than the wild type. *Yap1* mutant (*Yap1*^{Pitx2Cre}) incisor epithelial and mesenchymal cell proliferation was significantly reduced. This suggests that *Yap1* is important for ameloblast differentiation and cell proliferation.

Enamel is the only calcified tissue derived from epithelial cells in mammals. The continuous growing mouse incisor is a good model for studying the spatial and temporal regulation of ameloblast differentiation. *Shh* is strongly expressed in ameloblasts during the prosecretory and secretory stages and is a key cell-autonomous factor in ameloblast differentiation (Takahashi et al. 2007). Deletion of *Shh* in dental epithelial cells results in developmental defects of ameloblasts (Dassule et al. 2000). SHH can induce upregulation of *Amelx* and *Ambn*, the main structural components of enamel, in ameloblasts (Dassule et al. 2000). YAP1 has been reported to regulate *Shh* expression in lung epithelial cells and the neural tube (Isago et al. 2020; Cheng et al. 2023). Our results suggest that *Shh* is also a downstream target gene of YAP1 in dental epithelial cells. We hypothesized that *Shh* acts downstream of *Yap1* to mediate the interaction between the epithelium and the mesenchyme. Cell proliferation defects were also rescued in *Yap1*^{Pitx2Cre}; Tg-*pmes-Ihh* mice. Rescue experiments activating Hedgehog signaling in vivo confirmed the involvement of Hedgehog signaling.

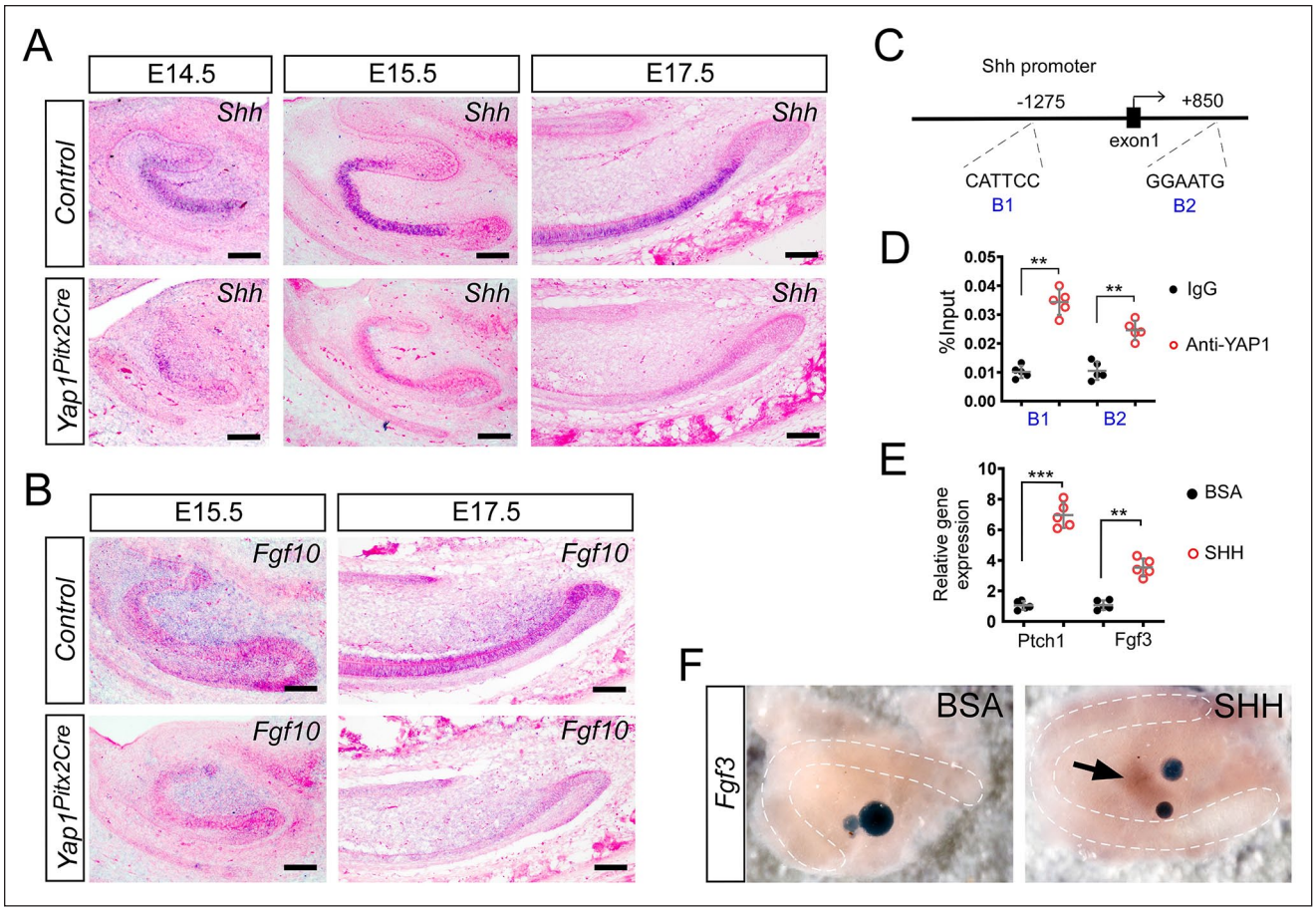

Figure 4. *Fgf3* expression is regulated by *Shh* in the incisor. (**A**) In situ hybridization shows reduced *Shh* expression in the incisors of control (*Pitx2*^{Cre}) and *Yap1*^{Pitx2Cre} at E14.5, E15.5, and E17.5 ($n=6$). (**B**) In situ hybridization shows reduced *Fgf10* expression in the mutant (*Yap1*^{Pitx2Cre}) and control (*Pitx2*^{Cre}) incisors at E15.5 and E17.5 ($n=3$). (**C**) Schematic representation of the *Shh* promoter sequence. The binding sites for TEAD are highlighted B1 and B2. (**D**) Quantitative analysis of ChIP assays are performed by real-time polymerase chain reaction (PCR) ($n=5$). (**E**) Quantitative reverse transcription PCR demonstrates that SHH treatment induced the expression of *Fgf3* in the incisor cultures ($n=5$). (**F**) Effect of SHH protein on *Fgf3* expression in the mutant (*Yap1*^{Pitx2Cre}) incisors ($n=4$). An SHH-saturated (1 μg/μL), but not bovine serum albumin (BSA) saturated, bead induces the expression of *Fgf3* (black arrow). The dashed lines indicate the epithelium. Error bars represent standard deviations. *$P<0.05$. **$P<0.01$. ***$P<0.001$. Scale bars: 100 μm.

The FGF signaling pathway plays an important role in regulating the proliferation of dental epithelial cells and ameloblast development. Dysregulation of FGF signaling can lead to tooth agenesis and enamel defects in humans. Apert and Crouzon syndrome are caused by gain- or loss-of-function mutations in *FGFR2*, respectively (Reardon et al. 1994; Wilkie et al. 1995). Autosomal recessive congenital deafness with labyrinthine aplasia, microtia, and microdontia is caused by homozygous or compound heterozygous mutations in *FGF3* (Sensi et al. 2011). In the absence of *Fgf3* and *Fgf10*, the enamel layer of the lower incisor was very thin or absent, and cell proliferation in the cervical loop was significantly reduced (Wang et al. 2007). Our data showed that SHH can regulate *Fgf3* expression in the culture system. Moreover, the expression of *Fgf3* and *Etv5* was also rescued. All of these data suggest that YAP1 affects enamel formation by regulating SHH/FGF signaling in the incisor. Whether *Yap1* regulates molar enamel formation through the same pathway needs to be further investigated because of the different signaling regulation of molar and incisor development.

In summary, our data indicate that *Yap1* plays a critical role in the differentiation of ameloblasts and cell proliferation. Both SHH and FGF signaling were downregulated after *Yap1* ablation. FGF signaling was regulated by Hedgehog signaling in the incisor. Overexpression of *Ihh* attenuates enamel formation and cell proliferation defects caused by *Yap1* deletion. Epithelial YAP1 may regulate ameloblast differentiation by tuning SHH/FGF signaling.

Materials and Methods

Animals

All mouse experimental protocols were approved by the Hangzhou Normal University Animal Care and Use Committee (HNUAC-2023238) and conformed to the updated ARRIVE

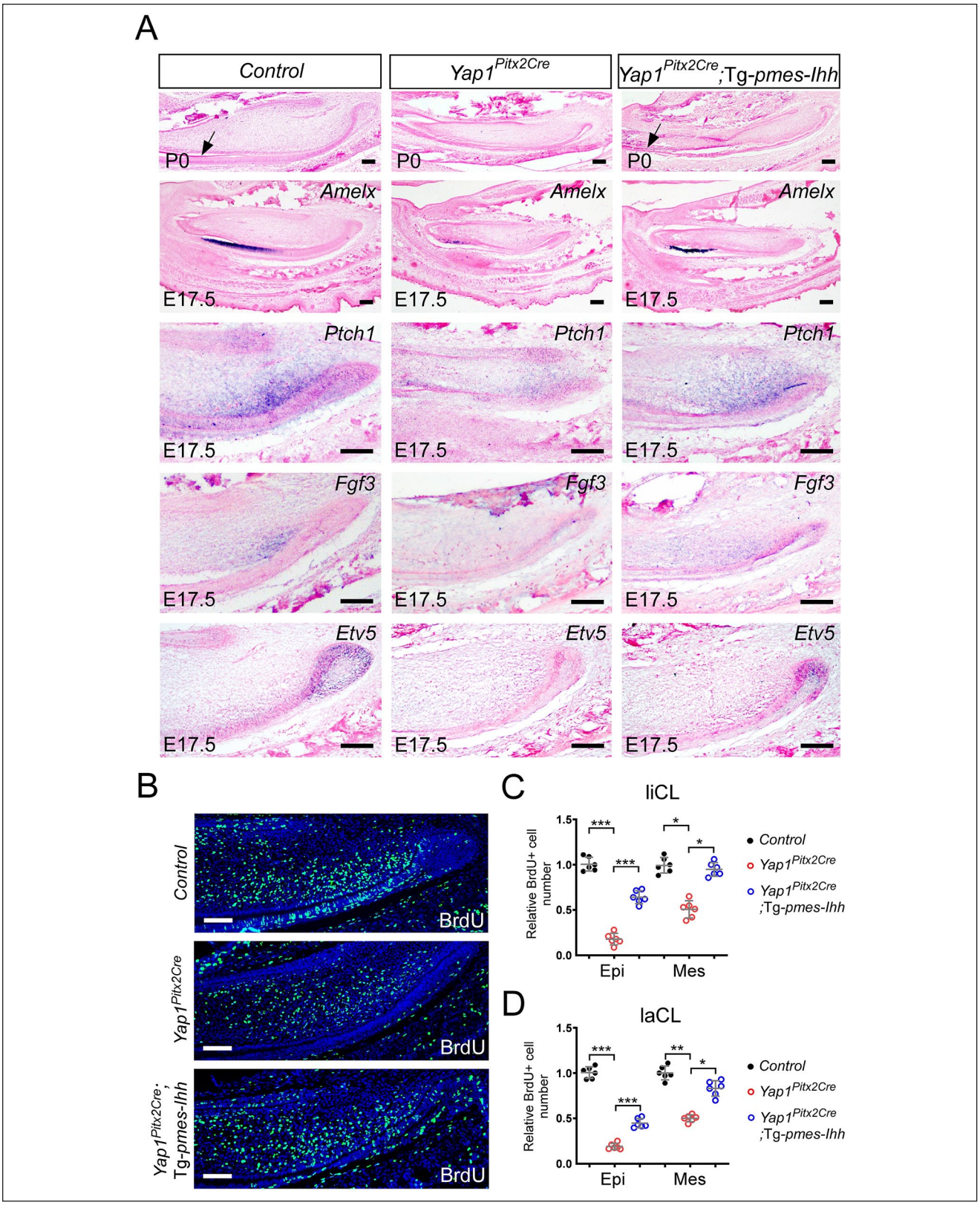

Figure 5. Reactivation of Hedgehog signaling rescues enamel defects in *Yap1*^{Pitx2Cre} mutant embryos. (**A**) Hematoxylin and eosin staining and in situ hybridization show the expression of *Amelx*, *Ptch1*, *Fgf3*, and *Etv5* in control (*Pitx2*^{Cre}), *Yap1*^{Pitx2Cre}, *and Yap1*^{Pitx2Cre}; Tg-*pmes-Ihh* embryos ($n = 5$). Black arrows show the enamel matrix on the labial side. (**B**) BrdU labeling assays show that the level of cell proliferation in the incisor of control (*Pitx2*^{Cre}), *Yap1*^{Pitx2Cre}, and *Yap1*^{Pitx2Cre}; Tg-*pmes-Ihh* embryos at E17.5 ($n = 5$). (**C**) Comparison of the relative proliferation of BrdU-labeled cells in the lingual cervical loop (liCL) of control (*Pitx2*^{Cre}), *Yap1*^{Pitx2Cre}, and *Yap1*^{Pitx2Cre}; Tg-*pmes-Ihh* incisors ($n = 6$). (**D**) Comparison of the relative proliferation of BrdU-labeled cells in the labial cervical loop (laCL) of control (*Pitx2*^{Cre}), *Yap1*^{Pitx2Cre}, and *Yap1*^{Pitx2Cre}; Tg-*pmes-Ihh* incisors ($n = 6$). Scale bars: 200 µm.

2.0 guidelines. *Pitx2*^{Cre} and *Yap1*^{flox} mouse strains were purchased from the Mutant Mouse Resource and Research Center and Jackson Laboratory, respectively. Construction of Tg-*pmes-Ihh* conditional transgenic mice has been described previously (Yang et al. 2016). The morning that a plug was discovered is considered to be day 0.5 of pregnancy (E0.5).

BrdU Labeling and TUNEL Assays

5-bromodeoxyuridine (BrdU) incorporation was used to analyze cell proliferation (Huang et al. 2023). Statistical significance was calculated using the Student's *t* test. Apoptotic cells were detected by TUNEL assay. The In Situ Cell Death Detection kit (Roche) was used according to the manufacturer's protocol. The protocols were described in the Appendix Materials and Methods.

Immunofluorescence and Histological Analysis

Embryos were collected at defined developmental stages and then fixed in 4% paraformaldehyde (PFA) for 30 min at 4 °C. The samples were dehydrated in a graded series of 50%, 70%, 80%, 90%, and 100% ethanol; cleared with xylene; and embedded in paraffin. Sections were stained with hematoxylin and eosin as described previously (Chen et al. 2021). The protocols are described in the Appendix Materials and Methods.

In Situ Hybridization

In situ hybridization using a section of embryonic samples was performed as previously described (Li et al. 2019). Embryos were collected at defined developmental stages and fixed in freshly made 4% PFA overnight at 4 °C. Samples were then dehydrated in a graded series of alcohols and embedded in paraffin. After treatment with proteinase K, sections (12 µm) were incubated overnight at 65 °C with a hybridization solution containing denatured RNA probes. Labeled nonradioactive RNA probes were produced by in vitro transcription using a DIG RNA labeling kit (Roche).

RNA-seq Analysis and Quantitative Real-Time PCR

RNA-seq was performed using total RNA extracted from E14.5 embryos. All the raw data generated from RNA-seq were deposited in BIGD (GSA: CRA021267), which is publicly accessible at https://ngdc.cncb.ac.cn/gsa. The protocols are described in the Appendix Materials and Methods.

In Vitro Organ Cultures

Incisors were dissected from E15.5 mutant embryos (*Yap1*^{Pitx2Cre}) and placed on a Nuclepore Track-Etch membrane (0.2-µm pore size) in TROWELL organ culture in a chemical defined medium as described previously (Zhang et al. 2022). The protocols are described in the Appendix Materials and Methods.

Statistical Analyses

All data are presented as means ± standard error of the mean. Student's *t* test was used to test differences among data sets. *P* values of less than 0.05 were regarded as statistically significant.

Author Contributions

Y. Zheng, Y. Qu, H. Liu, contributed to data acquisition and interpretation, critically revised the manuscript; H. Huang, J. Li, M. Qiu, contributed to data analysis, critically revised the manuscript; F. Li, contributed to conception, design, data analysis and interpretation, drafted and critically revised the manuscript. All authors gave their final approval and agree to be accountable for all aspects of the work.

Declaration of Conflicting Interests

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