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Tbx1 Regulates Progenitor Cell Proliferation in the Dental Epithelium by Modulating PITX2 Activation of p21

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Abstract

Tbx1$^{-/-}$ mice present with phenotypic effects observed in DiGeorge syndrome patients however, the molecular mechanisms of *Tbx1* regulating craniofacial and tooth development are unclear. Analyses of the *Tbx1* null mice reveal incisor microdontia, small cervical loops and BrdU labeling reveals a defect in epithelial cell proliferation. Furthermore, *Tbx1* null mice molars are lacking normal cusp morphology. Interestingly, p21 (associated with cell cycle arrest) is up regulated in the dental epithelium of *Tbx1*$^{-/-}$ embryos. These data suggest that Tbx1 inhibits p21 expression to allow for cell proliferation in the dental epithelial cervical loop, however Tbx1 does not directly regulate p21 expression. A new molecular mechanism has been identified where Tbx1 inhibits Pitx2 transcriptional activity and decreases the expression of Pitx2 target genes, p21, Lef-1 and Pitx2c. p21 protein is increased in PITX2C transgenic mouse embryo fibroblasts (MEF) and chromatin immunoprecipitation assays demonstrate endogenous Pitx2 binding to the p21 promoter. Tbx1 attenuates PITX2 activation of endogenous p21 expression and *Tbx1* null MEFs reveal increased Pitx2a and activation of Pitx2c isoform expression. Tbx1 physically interacts with the PITX2 C-terminus and represses PITX2 transcriptional activation of the p21, LEF-1, and Pitx2c promoters. *Tbx1*$^{-/+}$/*Pitx2*$^{-/+}$ double heterozygous mice present with an extra premolar-like tooth revealing a genetic interaction between these factors. The ability of Tbx1 to repress PITX2 activation of *p21* may promote cell proliferation. In addition, PITX2 regulation of *p21* reveals a new role for PITX2 in repressing cell proliferation. These data demonstrate new functional mechanisms for *Tbx1* in tooth morphogenesis and provide a molecular basis for craniofacial defects in DiGeorge syndrome patients.

Keywords

Tbx1; PITX2; p21; DiGeorge syndrome; Tooth development

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INTRODUCTION

DiGeorge syndrome is the most common genetic deletion syndrome in humans. It is a complex developmental disorder associated with congenital heart disease, craniofacial abnormalities, thymic and parathyroid defects (Dodson et al., 1969; Goldberg et al., 1993; Shprintzen et al., 1978). Many of the structures affected in this syndrome are derived from the pharyngeal system. Through chromosome engineering, gene targeting in mice (Jerome and Papaioannou, 2001; Lindsay et al., 1999; Lindsay et al., 2001; Merscher et al., 2001) and mutation screen in human patients (Paylor et al., 2006; Yagi et al., 2003), *TBX1* has been associated with this syndrome. *Tbx1* homozygous loss-of-function mice recapitulate most, if not all, of the pharyngeal system derived defects associated with DiGeorge syndrome. Many human syndromes such as DiGeorge and Axenfeld-Rieger syndrome (ARS) that have tooth defects also have heart and other defects (Fukui et al., 2000; Hjalt and Semina, 2005). The molecular mechanisms of Tbx1 in tooth and craniofacial development are the focus of this study.

T-box factors (like Tbx1) are transcription factors containing a conserved DNA binding domain termed the T-box, a 180 amino acid DNA binding domain (Bollag et al., 1994; Naiche et al., 2005). The first T-box factor identified was Brachyury and there are now 17 members of the T-box members identified in humans and mice (Naiche et al., 2005). T-box factors are involved in many developmental processes and signaling pathways including heart and tooth development. T-box factors can activate or repress transcription and some contain both activation and repression domains in their C-terminal tails (Kispert, 1995; Stennard et al., 2003). Furthermore, T-box factors interact with other transcription factors to regulate gene expression (Naiche et al., 2005).

The tooth development process is an excellent model to study molecular mechanisms involved in organogenesis. This process involves sequential and reciprocal interaction between the oral ectoderm and neural crest derived mesenchyme (Thesleff and Sharpe, 1997). Tooth development proceeds through a series of stages starting with initiation stage and followed with bud, cap and bell stages (for reviews (Alappat et al., 2005; Peters and Balling, 1999; Thesleff, 2003; Tucker and Sharpe, 2004). The invaginating (ectoderm) tooth bud encircles the condensing (neural crest) mesenchyme. A transient primary enamel knot appears in the dental epithelium, which marks the bud to cap stage (E14–15) transition. The inner dental epithelium derived pre-ameloblasts later differentiate into enamel secreting ameloblasts. During bell stage (E16–18) the inner dental epithelium and the dental papilla are juxtapositioned and, separated by a basement membrane. The inner dental epithelium and the basement membrane regulate the differentiation of odontoblasts (Lesot et al., 2001). This differentiation is initiated where the dental epithelium folds to form enamel knot structures (Bei et al., 2000; Thesleff et al., 2001). The enamel knots are signaling centers that regulate tooth morphogenesis and molar cusp patterns (Jernvall et al., 1994; Thesleff et al., 2001). Furthermore, p21 a cyclin-dependent kinase inhibitor is expressed in the dental epithelium and enamel knot and is known to inhibit cell proliferation (Jernvall et al., 1998; Weber et al., 2008). However, the transcription factors regulating p21 expression nor are the molecular mechanisms of p21 in regulating epithelial cell proliferation and differentiation known.

The cervical loop comprises a region of the lingual and labial epithelium that contain progenitor cells giving rise to four cell lineages; the inner enamel epithelium, stratum intermedium, stellate reticulum and the outer enamel epithelium (Harada et al., 1999; Harada and Ohshima, 2004; Harada et al., 2002). The cervical loop structures are present in both molars and incisors and are required for the proliferation, differentiation and growth of both types of teeth. The apical end of the rodent incisor or "apical bud" has been proposed to

be a special epithelial component for the stem cell niche that gives rise to progenitor cells of the cervical loop (Harada and Ohshima, 2004). These cells then populate the incisor and molar tooth germs and proliferate and differentiate in a gradient from less differentiated at the lingual side to the fully differentiated cells at the labial side as morphogenesis progresses. The molecular mechanisms controlling the proliferation and differentiation of cells in the cervical loops are not known as well as the transcription factors involved in this process.

Because *Tbx1* has an important role during development of pharyngeal system derivatives, we wanted to determine the molecular mechanisms of *Tbx1* function during tooth development. *Tbx1* expression has been reported in incisors and molars throughout the dental epithelium including the cervical loop and enamel knot (Caton et al., 2009; Mitsiadis et al., 2008; Zoupa et al., 2006). We report that *Tbx1* homozygous loss-of-function mice have defective dental progenitor epithelial cells proliferation. The proliferation defects are associated with an increase in p21 expression in the dental epithelium and cervical loop. *Pitx2*, a homeobox transcription factor and *Tbx1* expression patterns overlap during tooth development. Pitx2 activates endogenous p21 expression, which is antagonized by Tbx1. Tbx1 physically interacts with PITX2 to repress PITX2 transcriptional activity and PITX2 target genes. Interestingly, Tbx1 inhibits the endogenous auto-regulation of the Pitx2c isoform by Pitx2a. A genetic interaction between these two factors was revealed in the $Tbx1^{-/+}/Pitx2^{-/+}$ heterozygous mice with the development of an extra premolar-like tooth. These data provide new molecular mechanisms for Tbx1 and Pitx2 in regulating dental progenitor cell proliferation and tooth morphogenesis.

MATERIALS and METHODS

Animals

Heterozygous *Tbx1* mice carrying the lacZ knock-in allele $Tbx1^{tm1Bld}$ (here referred to as $Tbx1^{+/-}$) were obtained as described previously (Lindsay et al., 2001). Mutants were maintained and analyzed on a C57BL/6-129SvEvBrd (129S5) mixed genetic background and were crossed to each other or wild type mice of the same genetic background. The $Pitx2^{-/-}$ mice have been previously reported (Lu et al., 1999) and maintained and analyzed on a C57BL/6-129SvEvBrd (129S5) mixed genetic background and were crossed to each other or wild type mice of the same genetic background. The K14-PITX2C transgenic mice have been reported (Venugopalan et al., 2008). MEF's were obtained from E14.5 mutant and transgenic mice embryos. Embryos were collected at various time points, considering the day of observation of a vaginal plug to be embryonic day (E) 0.5. Mice and embryos were genotyped by PCR of DNA extracted from tail biopsies or yolk sacs, respectively using previously published PCR primer pairs (Lindsay et al., 2001).

Histology

Mouse embryos or heads were dissected in phosphate-buffered saline (PBS), and fixed with 4% paraformaldehyde-PBS solution for 4 hours. Following fixation, samples were dehydrated through graded ethanol, embedded in paraffin wax and sectioned (7 μm). Sections were stained with Hematoxylin and Eosin. All sections were visualized using a Nikon Eclipse 80i microscope with low noise fluorescence imaging capability or a Nikon SMZ800 Stereomicroscope with imaging. All image acquistion and analyses were done using an imaging workstation with NIS-Elements.

Detection of β-galactosidase (LacZ) activities

Whole embryos or heads were stained for β-galactosidase activity according to standard procedures. Embryos were fixed for 30–60 min at RT in 0.2% glutaraldehyde in PBS. Fixed

embryos were washed three times in rinse solution (0.005% Nonidet P-40 and 0.01% sodium deoxycholate in PBS) and stained overnight at room temperature using standard staining solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$, 0.4% X-gal in PBS). The next morning, samples were rinsed in PBS and then photographed. After that samples were post-fixed in 4% formaldehyde, they were dehydrated through graded ethanol, embedded in paraffin wax and sectioned. Sections were cut at 12-μm thickness and lightly counter-stained with eosin.

Immunohistochemistry

Tissue was prepared by 4% formaldehyde fixation of whole embryos or heads,which were paraffin wax embedded and sectioned at a thickness of 7 μm. The antigens were retrieved by autoclaving in Tris-HCl buffer (pH 9.0) for 5 minutes. Rabbit anti-P21 (Santa Cruz) was diluted 1:50 in TBS/0.1% Triton X-100/5% goat serum/1% BSA, Rabbit anti-AMELOGENIN (Santa Cruz) was diluted 1:500 in TBS/0.1% Triton X-100/5% goat serum/ 1% BSA. Primary antibody incubated overnight at 4 °C and detected with a biotinylated goat anti-rabbit IgG conjugate (1:200; Vector lab), avidin-biotin complex formation (Vector lab) and AEC Staining Kit (Sigma).

BrdU labeling

BrdU was injected into the pregnant mouse (50 μg/g of body weight) 2 h prior to harvesting embryos. Samples were embedded as described above. Sections were incubated for 5 minutes in 3% H_2O_2, microwaved 10 minutes in 10 mM Citrate Buffer (pH 6.0), hydrolyzed for 60 minutes in 2N HCl, neutralized for 10 minutes in 0.1 M sodium borate, rinsed, blocked for 1 hour in 10% goat serum, and immunostained with rat anti-BrdU antibody (1:250, Abcam). *Tbx1*$^{-/-}$ and wild type sections were placed on the same slide and processed together and equally for identical time periods. We sectioned two separate embryos for each genotype. For every 10 sections we collected two serial sections for BrdU and H&E staining, respectively. For quantitation we count at least 3 sections from each embryo.

Expression constructs, cell culture, transient transfection, luciferase and β-galactosidase assays

The *Tbx1* expression plasmid has been previously described (Xu et al., 2004). The PITX2 plasmids have been previously described (Amen et al., 2007). The human LEF-1 and p21 promoters have been previously described (Nakano et al., 1997; Vadlamudi et al., 2005). The 3.0 kb mouse Pitx2c 5' flanking genomic sequence was cloned into the luciferase vector using HindIII and BamH1 restriction enzymes. CHO and LS-8 cells (Chen et al., 1992) were cultured in DMEM supplemented with 5% or 10% fetal bovine serum (FBS) and penicillin/ streptomycin and transfected by electroporation. Cultures were fed 24 h prior to transfection, resuspended in PBS and mixed with 2.5 μg of expression plasmids, 5 μg of reporter plasmid and 0.5 μg of SV-40 β-galactosidase plasmid. Electroporation of CHO cells were performed at 360 V and 950 microfarads (μF) (Gene Pulser XL, Bio-Rad). LS-8 cells were transfected by electroporation as previously described (Green et al., 2001). Transfected cells were incubated for 24 h in 60 mm culture dishes and fed with 10% FBS and DMEM and then lysed and assayed for reporter activities and protein content by Bradford assay (Bio-Rad). Luciferase was measured using reagents from Promega. β-galactosidase was measured using the Galacto-Light Plus reagents (Tropix Inc.). All luciferase activities were normalized to β-galactosidase activity. All plasmids were double-banded CsCl purified.

Expression of endogenous or transiently expressed PITX2, and Tbx1 proteins were demonstrated using the PITX2 P2R10 antibody (Hjalt et al., 2000), or Tbx1 antibodies (Zymed). Approximately 10–40 μg of transfected cell lysates were analyzed in Western

blots. Following SDS gel electrophoresis, the proteins were transferred to PVDF filters (Millipore), immunoblotted and detected using specific antibodies and ECL reagents from GE HealthCare.

Immunoprecipitation assays

Approximately 24 h after cell transfection with *Tbx1* and *PITX2*, CHO cells were rinsed with 1 ml of PBS, and then incubated with 1 ml ice cold RIPA buffer for 15 m at 4°C. Cells were harvested and disrupted by repeated aspiration through a 25-gauge needle attached to a 1 ml syringe. The lysates were then incubated on ice for 30 min. Cellular debris was pelleted by centrifugation at 10,000× g for 10 min at 4°C. An aliquot of lysate was saved for analysis as input control. Supernatant was transferred to a fresh 1.5 ml microfuge tube on ice and pre-cleared using the Pre-clearing Matrix C-mouse (ExactaCruz C, Santa Cruz Biotechnology) for 30 min at 4°C. Matrix was removed by brief centrifugation and supernatant transferred to a new tube. An IP antibody-IP matrix complex was prepared as per manufacturer's instructions using primary anti-Tbx1 antibody (Zymed). The IP antibody-IP matrix complex was incubated with the pre-cleared cell lysate at 4°C for 12 hr. After incubation the lysate was centrifuged to pellet the IP matrix. The matrix was washed three times with PBS and resuspended in 15 μl of dH2O and 3 μl 6× SDS loading dye. Samples were boiled for 5 min and resolved on a 10% polyacylamide gel. A Western blot assay was used with PITX2 antibody and HRP conjugated ExactaCruz reagent to detect immunoprecipitated proteins.

Expression and purification of GST-Tbx1 fusion proteins

Tbx1 was PCR amplified from a cDNA clone as described and ligated into pGEX6P-2 GST vector (Amersham Pharmacia Biotech, Piscataway, NJ) (Amendt et al., 1999; Green et al., 2001; Vadlamudi et al., 2005) using EcoRI and XhoI restriction enzyme sites engineered into the primers (1049 bp). PITX2 deletion constructs have been previously described (Amendt et al., 1999). The plasmids were confirmed by DNA sequencing and transformed into BL21 cells. Protein was isolated as described (Amendt et al., 1999; Cox et al.). Tbx1 proteins were cleaved from the GST moiety using 80 units of PreScission protease (Pharmacia Biotech) per ml of Glutathione Sepharose. Cleaved proteins were stored in 10% glycerol. Protein concentration was quantitated with Bradford Reagent (Bio-Rad Laboratories, Hercules, CA). Proteins were examined by electrophoresis on denaturing SDS–polyacrylamide gels, followed by Coomassie Blue staining.

GST pull-down assays

Immobilized GST-PITX2A, GST-PITX2A homeodomain only (GST-PITX2A HD) and GST-PITX2A C173 fusion proteins were prepared as described above and suspended in binding buffer (20 mM HEPES pH 7.5, 5% glycerol, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, with or without 1% milk and 400 μg/ml of ethidium bromide). Purified bacteria expressed Tbx1 (200 ng) was added to 15 μg immobilized GST-PITX2A, GST-PITX2A HD and GST-PITX2A C173 fusion proteins or GST in a total volume of 100 μl and incubated for 30 min at 4°C. The beads were pelleted and washed 4 times with 200 μl binding buffer. The bound proteins were eluted by boiling in SDS-sample buffer and separated on a 10% SDS–polyacrylamide gel. Following SDS gel electrophoresis, the proteins were transferred to PVDF filters (Millipore), immunoblotted and detected using Tbx1 antibody (Zymed) and ECL reagents from Amersham.

Electrophoretic mobility shift assay (EMSA)

Complementary oligonucleotides containing a PITX2 binding site within the Dlx2 promoter with flanking partial BamHI ends were annealed and filled with Klenow polymerase to

generate ^{32}P-labeled probes for EMSAs as described (Green et al., 2001). Standard binding assays were performed as previously described (Amendt et al., 1999b). The bacteria expressed and purified Tbx1 protein was used in the assays. The samples were electrophoresed and visualized as described previously (Green et al., 2001).

Chromatin immunoprecipitation (ChIP) assays

The ChIP assays were performed as previously described using the ChIP Assay Kit (Upstate) with the following modifications (Amen et al., 2007; Diamond et al., 2006). LS-8 cells were fed for 24 h, harvested and plated in 60 mm dishes. Cells were cross-linked with 1% formaldehyde for 10 min at 37°C the next day. The PCR reactions were 5 min at 94°C, 1 min at 94°C, 1 min at 60°C, 1 min at 72°C X 40 cycles and 7 min at 72°C. Two primers for amplifying the Pitx2 binding site in the *p21* promoter are as follows: sense-5'GGATGTCATGTAACCTTGATGAATT3' and antisense, 5'GCTACATAGCAAGACCACCATCTCAG3'. All the PCR products were evaluated on a 2% agarose gel in 1× TBE for appropriate size and confirmed by sequencing. As controls the *p21* primers were used without chromatin, normal rabbit IgG (Santa Cruz) was used replacing the PITX2 antibody (Capra Science) to reveal non-specific immunoprecipitation of the chromatin. An additional control consisted of primers to another transcription factor binding site in another gene.

RESULTS

Tooth morphogenesis is disrupted in *Tbx1*$^{-/-}$ mouse embryos

The expression pattern of *Tbx1* during tooth development has been previously reported using in situ probes (Caton et al., 2009; Mitsiadis et al., 2008; Zoupa et al., 2006). We used the *Tbx1-lacZ* knock-in allele to examine *Tbx1* expression at later stages using whole mount *Tbx1*$^{-/+}$ mouse embryos. *Tbx1* (LacZ) expression was observed in the palate rugae and well as incisor and molar tooth germs at E14.5 and E16.5 (Fig. 1A,B). The mandible was excised from P0 *Tbx1*$^{-/+}$ mice to reveal strong *Tbx1* (LacZ) expression in the incisor posterior region (includes the cervical loop) and molar cusps (Fig. 1C). We also observed strong *Tbx1* expression in the hair follicles (data not shown).

To determine the role of Tbx1 in tooth development *Tbx1*$^{-/-}$ mutant embryos were analyzed at E14.5, 16.5 and 18.5 for defects in tooth morphogenesis, as these mice fail to survive past birth (Jerome and Papaioannou, 2001). At E14.5 there is little difference in the structure of the incisors and molars of the *Tbx1*$^{-/-}$ mice compared to wild type embryos (Fig. 1D). The *Tbx1* mutant tooth germs are slightly smaller than wild type at this stage. Thus, *Tbx1* does not appear to affect tooth development at this stage of development.

The E16.5 *Tbx1*$^{-/-}$ embryos begin to reveal defects in tooth morphogenesis as the molars are smaller, cusps are not forming and the pre-ameloblast cell layer is thin compared to wild type (Fig. 2 A–D). The mutant incisor phenotype is more severe at this stage. The incisors are approximately half the size of wild type incisors and the cervical loops are less developed (Fig. 2E,F).

A clear defect was observed in the structure of the developing molar at E18.5 in the *Tbx1*$^{-/-}$ mouse (Fig. 2H). Compared to the wild type (WT) mouse the epithelium does not form the characteristic bulges that will give rise to molar cusps (Fig. 2H). Furthermore, the epithelial layer appears thin and undifferentiated in the *Tbx1*$^{-/-}$ embryos compared to wild type embryos (Fig. 2H,J). The *Tbx1*$^{-/-}$ mutant incisors demonstrate a lack of epithelial cell proliferation and differentiation as seen by the decrease in epithelial cells in the cervical loop and a defined region of differentiated epithelial cells or pre-ameloblasts (Fig. 2L). During tooth development there is a natural developmental gradient of pre-ameloblasts, with

the least differentiated epithelial cells proliferating from the cervical loop to more differentiated cells in the regions distant from the cervical loop. Their characteristic columnar shape denotes the differentiated epithelial cells or pre-ameloblasts.

Pre-ameloblast differentiation is defective in the *Tbx1*$^{-/-}$ mice

To determine if pre-ameloblast differntiation was affected in the *Tbx1*$^{-/-}$ mice we stained for amelogenin, a marker for ameloblast differentiation and required for enamel synthesis. Because Tbx1 mutant mice die at birth we assayed for amelogenin expression at E18 in the upper incisors as they are developmentally advanced compared to molars and begin to express amelogenin at this time point. Comparable wild type and mutant sections were used and IHC staining for amelogenin revealed expression in the wild type incisors but amelogenin was not detected in the mutant incisors (Fig. 3). Thus, the *Tbx1* mutant incisors appear to have a defect in ameloblast differentiation corresponding to reduced amelogenin expression.

Tbx1 regulates epithelial cell proliferation

BrdU labeling was used to determine if decreased epithelial cell proliferation was causative for the tooth anomalies. *Tbx1*$^{-/-}$ mutant and wild type embryos were harvested at E17.5, sectioned and probed for BrdU incorporation. The E17.5 *Tbx1*$^{-/-}$ embryo molar sections revealed a decrease in BrdU positive epithelial cells compared to wild type sections (Fig. 4A–B). A similar defect in cell proliferation was observed in E17.5 *Tbx1*$^{-/-}$ mice incisor sections compared to wild type (Fig. 4C,D). To determine if a proliferation defect occurred at an early embryonic stage, E14.5 *Tbx1*$^{-/-}$ mice molar sections did not reveal a specific defect in cell proliferation compared to wild type (Fig. 4F,G). Because Tbx1 is specifically expressed in the dental epithelium, proliferation defects were not detected in the mesenchyme tissue (Fig. 4E,H).

The cyclin-dependent kinase inhibitor, p21 is up-regulated in the *Tbx1*$^{-/-}$ mouse dental epithelium

To understand the molecular mechanism of decreased epithelial cell proliferation we focused on *p21* gene expression. *p21* expression is associated with non-proliferating cells and is normally down-regulated during active cell proliferation. p21 is expressed in the primary and secondary enamel knot structures, which are transient non-proliferating epithelial cell regions or signaling centers (Thesleff et al., 2001). IHC staining revealed low p21 expression as expected throughout the dental epithelium in wild type E16.5 molars (Fig. 5A). In contrast p21 expression was increased in the E16.5 *Tbx1*$^{-/-}$ molar dental epithelium (Fig. 5B). In wild type E18.5 molars minimal p21 expression was observed in the dental epithelium (Fig. 5C). However, in the *Tbx1*$^{-/-}$ E18.5 embryos, p21 expression was further increased throughout the dental epithelium (Fig. 5D). Thus, Tbx1 appears to repress p21 expression in the normal tooth germ to allow for epithelial cell proliferation.

Tbx1 represses PITX2 transcriptional activation

To functionally test if PITX2 and/or Tbx1 regulated the p21 promoter PITX2 and/or Tbx1 were co-transfected with the p21 promoter/luciferase construct. The 2.4 kb p21 promoter was activated by PITX2A at 26-fold and Tbx1 minimally activated the p21 promoter at 4-fold (Fig. 6A). Interestingly, Tbx1 repressed PITX2 activation of the p21 promoter. Tbx1 expression vector was titrated from 0.5 μg to 3.75 μg with PITX2 co-expression at 2.5 μg. Tbx1 demonstrated a dose response in attenuating PITX2 transcriptional activation from 26-fold to 4-fold with increasing concentrations of Tbx1 plasmid (Fig. 6A). At equal concentrations of both Tbx1 and PITX2 plasmids, Tbx1 decreases PITX2 activity 4-fold, demonstrating that Tbx1 functionally attenuates PITX2 activation of the p21 promoter.

Lef-1 can regulate cell proliferation through the activation of cyclin D1. PITX2A activated the LEF-1 promoter at ~19-fold and Tbx1 does not independently regulate the LEF-1 promoter (Fig. 6B). Tbx1 repressed PITX2 transcriptional activation from 19-fold to 12-fold (Fig. 6B). PITX2A activated the 3.0 kb Pitx2c promoter at ~17-fold and Tbx1 does not regulate the Pitx2c promoter (Fig. 6C). However, Tbx1 represses PITX2A activation of its promoter from 17-fold to ~4-fold (Fig. 6C). These data reveal a combinatorial effect of Tbx1 on both PITX2 expression and transcriptional activity. Where Tbx1 can repress PITX2 activation of the Pitx2c promoter to reduce Pitx2 expression and also repress its activation of the p21 promoter during normal tooth morphogenesis. This new molecular mechanism provides a strong modulation of PITX2 transcriptional activity.

Western blot analyses of co-transfected Tbx1 and PITX2 did not reveal degradation of the Tbx1/PITX2 complex as a possible mechanism for the repressed PITX2 transcriptional activity (Fig. 6D). Furthermore, the attenuated PITX2 transcriptional activity was not due to decreased DNA binding by the Tbx1-PITX2 complex. Electrophoretic mobility shift assays demonstrate that Tbx1 has no effect on the DNA binding activity of PITX2 (Fig. 6E).

Pitx2 endogenously regulates *p21* expression

We were unable to demonstrate Tbx1 directly repressing *p21* expression through various assay systems. Because *Pitx2*, *Tbx1* and *p21* are co-expressed in the dental epithelium and enamel knot (Jernvall et al., 1998; Venugopalan et al., 2008) we asked if Pitx2 could be directly regulating p21. Analyses of the p21 promoter revealed multiple Pitx2 binding elements (Fig. 7A). ChIP assays using LS-8 cells (oral epithelial cell line) (Chen et al., 1992) revealed endogenous Pitx2 binding to the p21 promoter in vivo (Fig. 7B). The Pitx2 antibody immunoprecipitated the p21 chromatin (Fig. 7B, lane 3), and the input control confirms that the p21 primers amplify the p21 chromatin (Fig. 7B, lane 4). Rabbit IgG control did not immunoprecipitate the p21 chromatin (Fig. 7B, lane 5) and the Pitx2 antibody did not non-specifically immunoprecipitate chromatin seen by using control primers to another gene (Fig. 7B, lane 6). As a control we show that the control primers do amplify the input chromatin (Fig. 7B, lane 7). These data demonstrate direct DNA binding of endogenous Pitx2 to the p21 promoter.

To demonstrate endogenous PITX2 activation of p21 expression, PITX2C transgenic (Tg) mouse embryo fibroblasts (MEF) (Venugopalan et al., 2008) were analyzed for p21 expression. Wild type MEFs and PITX2C Tg MEFs were harvested, lysed and probed by Western blot for p21 expression. Low levels of p21 protein expression were observed in wild type MEFs, however using PITX2 over-expressing MEFs revealed increased p21 expression (Fig. 7C). As a loading control the same blot was probed for GAPDH expression (Fig. 7C). To demonstrate that Tbx1 attenuates Pitx2 transcriptional activation of p21 in LS-8 cells, these cells were transfected with PITX2A or both PITX2A and Tbx1 and p21 expression observed by Western blot. LS-8 cells endogenously express p21 (Fig. 7D, lane 1) and transfected PITX2A increased endogenous p21 expression (Fig. 7D, lane 2). However, co-transfection of Tbx1 with PITX2A revealed a decrease in endogenous p21 expression (Fig. 7D, lane 3). These data clearly demonstrate that PITX2 endogenously regulates p21 expression.

We demonstrated in figure 6 that Tbx1 attenuated PITX2 activation of the mouse Pitx2c promoter. Thus, we asked if endogenous Tbx1 expression regulated endogenous Pitx2 isoform expression. Wild type MEFs endogenously express Tbx1 (data not shown) and wild type MEFs predominantly express the Pitx2a isoform and we are unable to detect the Pitx2c isoform (Fig. 7E). However, *Tbx1* null MEFs have increased Pitx2a expression but more importantly reveal Pitx2c expression (Fig. 7E). Tbx1 appears to inhibit Pitx2a activation of the Pitx2c promoter. The Pitx2a promoter is longer and more complex with regards to DNA

transcription factor binding elements compared to the internal Pitx2c promoter. Furthermore, we have shown that the Pitx2c promoter is positively regulated by PITX2A, providing a positive feedback loop for the continuous expression of Pitx2c. Tbx1 appears to be a major regulator of Pitx2 isoform expression through its interaction with Pitx2 and regulation of Pitx2 transcriptional activities.

Tbx1 directly interacts with the PITX2 C-terminal tail

PITX2 acts as a transcriptional activator but can be repressed through its interaction with other transcription factors (Amen et al., 2008; Berry et al., 2006). Because PITX2 and Tbx1 are co-expressed during critical times of craniofacial development we asked if they physically interacted (Hjalt et al., 2000). Furthermore, a PITX2 and Tbx1 interaction would provide a mechanism for the modulation of PITX2 transcriptional activity by Tbx1. The PITX2 and Tbx1 interaction was assayed using a Tbx1 antibody to immunoprecipitate a PITX2A/Tbx1 complex in transfected CHO cells. The immunoprecipitation experiments revealed a PITX2A interaction with Tbx1 (Fig. 8A, lane 4). As controls, empty vector, PITX2A and Tbx1 transfection alone did not immunoprecipitate PITX2A. PITX2A expression is shown in transfected CHO cell lysates (input control) and also seen when co-transfected with Tbx1. Mock and Tbx1 input lanes were used as controls with the PITX2A antibody (Fig. 8A). The transfected PITX2 protein migrates slower in the gel compared to pure protein due to a myc/his tail on the transfected protein. Furthermore, CHO cells do not endogenously express Pitx2 as shown in the mock and Tbx1 transfected cell lysates.

GST pull-down assays were performed using immobilized GST-PITX2A on Sepharose beads and incubated with purified Tbx1 protein under stringent binding conditions. PITX2A full-length, PITX2 HD (homeodomain only) and the PITX2 C-terminal proteins were immobilized to map the Tbx1 interaction with PITX2A (Fig. 8B). Tbx1 bound to the full-length protein as well as to the PITX2A C173 protein, which contains only the C-terminus of PITX2 (Fig. 8C). As a control, Tbx1 did not bind to the PITX2A homeodomain. The PITX2A C-terminus contains a transcriptional activation domain and protein interaction domain present in all PITX2 isoforms (A,B,C, and D) (Amendt et al., 1999; Cox et al., 2002), which contribute to the transcriptional activities of PITX2.

Pitx2 and *Tbx1* double heterozygous mice develop an extra premolar-like tooth

To demonstrate a genetic interaction between *Pitx2* and *Tbx1*, mice heterozygous (het) for both genes were mated and the $Pitx2^{-/+}/Tbx1^{-/+}$ double het mice survived and presented with an extra tooth bud in the diastema region at P1 (Fig. 9A). At 6 weeks of age the mutant mice were analyzed for teeth phenotypes and revealed an extra functional tooth on the right side mandible next to the first molar in the diastema region (Fig. 9C). The insert shows an SEM photo of the extra tooth, without cusp formation associated with molars (Fig. 9C). X-ray analyses of the mandible from the double het identified the extra tooth as having one root structure unlike a molar (Fig. 9E). The crown of the extra tooth appears more like a canine than an incisor. These data demonstrate a dose effect of both Pitx2 and Tbx1 in regulating tooth patterning. Both genes are co-expressed in the developing dental epithelium at early time points and their combined antagonistic interactions may be key regulators in establishing a gene expression hierarchy required for normal murine dentition.

DISSCUSSION

There are many genetic mutations and syndromes that affect craniofacial and tooth development. Transcription factors play a major role in determining the temporal and spatial control and timing of tooth development. T-box factors are involved in numerous developmental processes and are essential transcription factors, which interact with other

factors to regulate gene expression. *Tbx1* is expressed in the head mesenchyme, first pharyngeal arch, and second heart field (Vitelli et al., 2002; Zhang et al., 2006). *Tbx1* demonstrates a restricted pattern of expression during craniofacial development to epithelial tissues of the tooth germs, palatal shelves, and hair follicles (Zoupa et al., 2006). *TBX1* mutations are associated with DiGeorge syndrome (DGS) or velocardiofacial syndrome and is deleted in 22q11 deletion syndrome (Yagi et al., 2003). The craniofacial anomalies in patients (e.g. hypodontia, cleft palate, facial dysmorphogenesis and ear defects) correlate well with *Tbx1* expression in mice (Scambler, 2000).

Tbx1 regulates later stages of tooth development

Previous reports using in situ hybridization assays demonstrated *Tbx1* expression starting at E11.5 in the dental placode (Caton et al., 2009; Mitsiadis et al., 2008; Zoupa et al., 2006). The *Tbx1* mice carry the lacZ knock-in allele and using LacZ staining we observe a similar expression patterns. We show that *Tbx1* is expressed in the palate rugae and palate midline. Because the dental defects do not appear until after E14.5 this suggests that Tbx1 affects the later stages of dental development. The E16.5 and E18.5 *Tbx1* null embryos reveal incisor and molar anomalies including severely regressed growth of both types of tooth germs and apparent epithelial cell proliferation defects. These data indicate a role for Tbx1 in cell proliferation through possible regulation of signaling factors, transcription factors or cell cycle control genes.

Tbx1 and signaling factors during development

There is a gradient of LacZ expression at the cap stage (E14.5) with *Tbx1* expression predominately observed in the cervical loop and enamel knot regions, with less expression in the inner enamel epithelium throughout the tooth germ (data not shown). These data suggest that *Tbx1* expression is highest in the proliferating cells of the cervical loop, with a decrease in expression as these cells differentiate into the pre-ameloblasts, which will give rise to enamel. In contrast, *Tbx1* expression was also localized to the enamel knot (EK) structures (primary and secondary knots), which are clusters of non-dividing epithelial cells (data not shown). The expression of *Tbx1* in the EK may represent its role in regulating the expression of signaling factors such as fibroblast growth factors (FGF's) that are required to stimulate proliferation of adjacent mesenchymal and epithelial cells (Thesleff et al., 2001). *Tbx1* has been shown to regulate FGF's in the pharyngeal endoderm and second heart field (Vitelli et al., 2002; Xu et al., 2004). Tbx1 may also be regulating Fgf signaling in the cervical loop. A recent report using bead implantation experiments has suggested that Fgf activates *Tbx1* expression in the dental epithelium (Mitsiadis et al., 2008). Thus, there may be a regulatory loop between *Tbx1* and *Fgf* expression during tooth development in maintaining epithelial cell proliferation. At P0 *Tbx1* expression is highest in the incisor cervical loop, a structure containing progenitor cells that allow for the continued growth of the mouse incisor. At this stage high levels of *Tbx1* expression are also observed in the molar cusps derived from the secondary enamel knots. *Tbx1* expression at later stages in the cusps themselves supports a role for Tbx1 in epithelial cell proliferation required for cusp formation.

Epithelial cell proliferation is markedly decreased in the *Tbx1*$^{-/-}$ mouse tooth germ

In the *Tbx1*$^{-/-}$ mouse the dental epithelium layer is thinner with decreased amounts of cells and BrdU labeling demonstrated a specific defect in epithelial proliferation throughout the molar epithelium and specifically in the cervical loop. Furthermore, the molar structure of the mutant mouse lacks the characteristic bulges that give rise to the molar cusps. Thus, the cusp size, which is one factor of cusp patterning, is determined solely by the epithelium (Cai et al., 2007). During molar development *Tbx1* appears to play a major role in cusp size, but not patterning. The incisors of the *Tbx1*$^{-/-}$ mutant mouse are formed but due to the early

lethality of the mice it is not possible to study their mature erupted structure. The cervical loop on the lingual side clearly revealed less progenitor cells and BrdU labeling demonstrated a specific defect in proliferation of the epithelial cells.

p21 expression is up-regulated in the *Tbx1*$^{-/-}$ mouse dental epithelium

The cyclin-dependent kinase inhibitor, p21 is involved in the differentiation of the enamel knot, where it is highly expressed during tooth development (Jernvall et al., 1998). BMP-4 can induce p21 expression and p21 is associated with apoptosis of the enamel knot, linking it to the transient nature of the enamel knot structure (Jernvall et al., 1998). p21 can also be induced by transforming growth factor-β (TGF-β) and because TGF-β is expressed in the bud stage tooth epithelium it may also be a candidate to regulate p21 expression (Bloch-Zupan et al., 1998; Datto et al., 1995). At later stages beyond the cap stage (E14.5) p21 expression is decreased in the dental epithelium and at E18.5 we detect low p21 protein expression in the epithelium. However, in the *Tbx1*$^{-/-}$ mouse we observed p21 expression throughout the dental epithelium, including the cervical loop region. Thus, Tbx1 appears to be facilitating cell proliferation through the repression of p21 expression.

Tbx1 interacts with and attenuates PITX2 transcriptional activity

We have shown that Tbx1 interacts with the C-terminal tail of PITX2 and this interaction inhibits PITX2 transcriptional activation of the p21, LEF-1 and Pitx2c promoters. Both Lef-1 and Pitx2 are expressed in the dental epithelium and the enamel knot and are also associated with cell proliferation by their ability to activate cyclin D1. However, because Tbx1 is required for cell proliferation its repression of PITX2 transcriptional activity appears to modulate a cell differentiation pathway. We have shown that PITX2 activates a variety of genes regulating dental epithelial cell differentiation (Amen et al., 2007; Cox et al., 2002; Espinoza et al., 2005; Green et al., 2001; Vadlamudi et al., 2005; Venugopalan et al., 2008). A recent study showed that β-catenin over-expression increased Pitx2 and p21 expression in the dental epithelium (Wang et al., 2009). These data suggest that Pitx2, which directly interacts with β-catenin could be regulating p21 expression. Our demonstration that PITX2 activates the p21 promoter reveals a new transcriptional mechanism for the regulation cell differentiation. During normal tooth development Tbx1 represses PITX2 transcriptional activation of the Pitx2c promoter, which decreases Pitx2 expression. Tbx1 null MEFs revealed an increase in Pitx2 expression however, Tbx1 does not directly regulate the Pitx2 promoter but interacts with the Pitx2 protein to attenuate Pitx2 transcriptional activity and the Pitx2 positive feed back loop regulating the Pitx2c promoter. Thus, decreased Pitx2 expression coupled with the ability of Tbx1 to repress PITX2 activation of p21 would significantly down-regulate *p21* expression. Tbx1 attenuation of PITX2 transcriptional activation may promote cell proliferation by decreasing p21 levels. These data reveal for the first time a role for Tbx1 in cell proliferation through its ability to indirectly regulate the *p21* gene, which is associated with cell cycle arrest. Furthermore, we demonstrate a novel molecular function for Pitx2 in its ability to activate p21, which would repress cell proliferation.

Tbx1 and Pitx2 control tooth patterning

To understand the combinatorial activities of *Tbx1* and *Pitx2* in controlling tooth morphogenesis the *Tbx1*$^{-/+}$/*Pitx2*$^{-/+}$ double heterozygous mice were analyzed for tooth anomalies. Null mutants for both genes severely affect tooth development, however *Pitx2* null mice present with tooth arrest at early stages (Lu et al., 1999). Both genes are expressed early during the tooth development program and specifically in the dental epithelium. The double het mutant presents with an extra tooth in the diastema region of the mouse mandible and the extra tooth appears similar to a canine. Interestingly, we have observed an extra tooth on either side of the mandible but not both. We speculate that the decreased dose of

both factors relieves an inhibitory mechanism by signaling factors or transcription factors to stimulate tooth formation. More experiments are required to understand the molecular mechanisms of decreased *Tbx1* and *Pitx2* expression on extra tooth formation and the type of tooth produced in the diastema region. However, these experiments demonstrate a genetic interaction between *Tbx1* and *Pitx2* in regulating tooth development.

Function of Tbx1 in the dental epithelium

We propose a mechanism for the function of Tbx1 during tooth development based on our studies and those of other investigators (Fig. 10). Tbx1 may enhance cell proliferation in the cervical loop by directly activating factors associated with cell cycle progression or through its activation of Fgf signaling in the dental epithelium. In this report we have identified a new molecular mechanism for dental epithelial cell proliferation and differentiation. PITX2 activates many genes involved in dental epithelial cell differentiation and proliferation and repression by Tbx1 may inhibit proliferation. Therefore, PITX2 activates *p21* expression suggesting a new mechanism where Tbx1 promotes cell proliferation through its ability to repress PITX2 activation of *p21* expression. These data reveal a possible molecular mechanism for hypodontia in the DGS phenotype.

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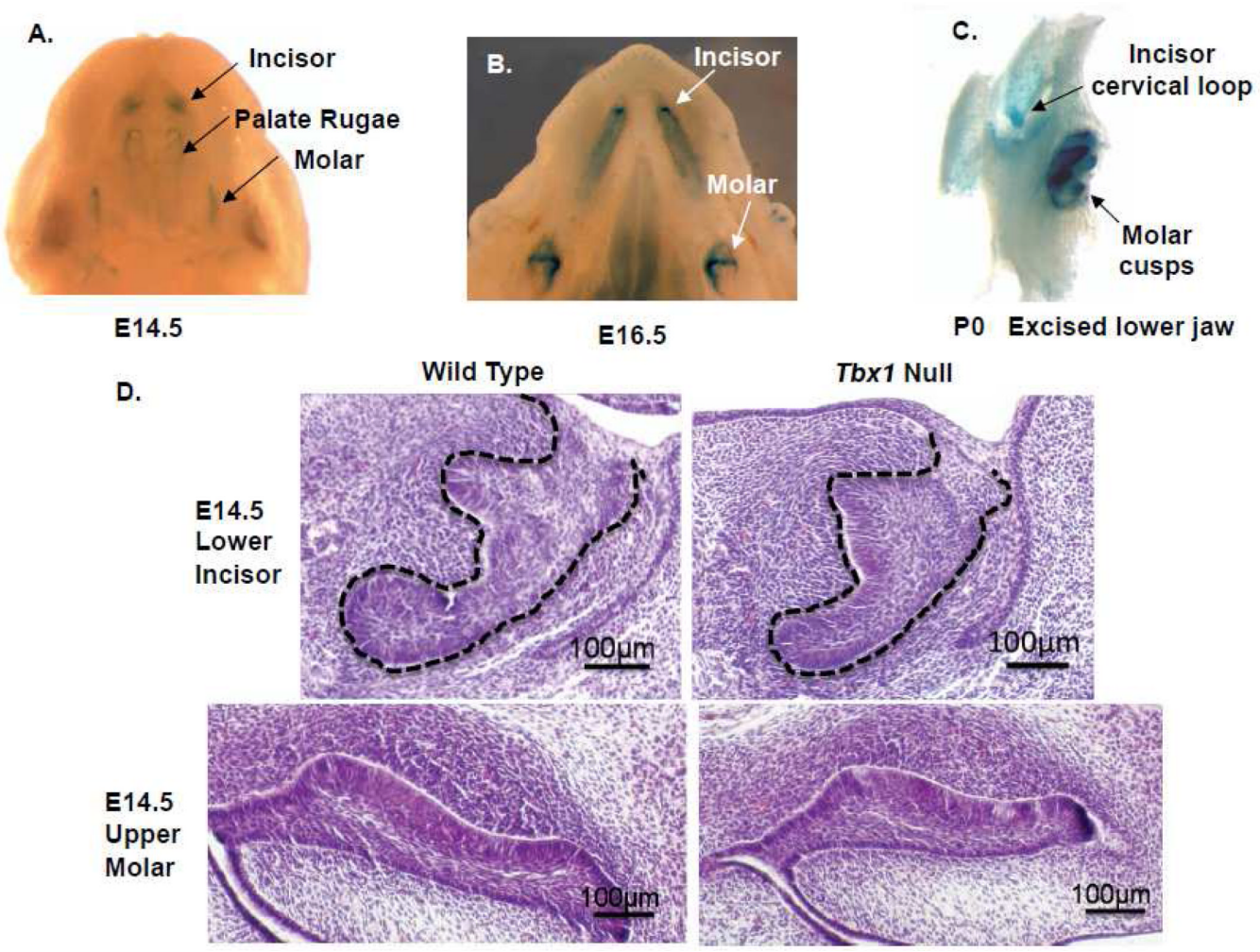

Fig. 1. Early tooth development in *Tbx1*$^{-/-}$ mouse embryos

The *Tbx1-lacZ* knock-in allele was used to confirm developmental expression of *Tbx1* during murine tooth morphogenesis. **A)** *LacZ* expression is shown in the incisors, molars and palate at E14.5 whole mount mice. **B)** E16.5 whole mount LacZ expression in the incisors, molars and palate. **C)** Excised lower jaw (mandible) of P0 (birth) *Tbx1*$^{lacZ/+}$ mice revealing strong *Tbx1* expression in the incisor cervical loop and molar cusps. **D)** Tooth development at E14.5 was compared between wild type (WT) embryo molars and incisors and *Tbx1*$^{-/-}$ null embryos. Tissues were sectioned (sagital) and stained with hematoxylin and eosin to visualize the structures. The lower incisor tooth bud is outline to show the dental epithelium.

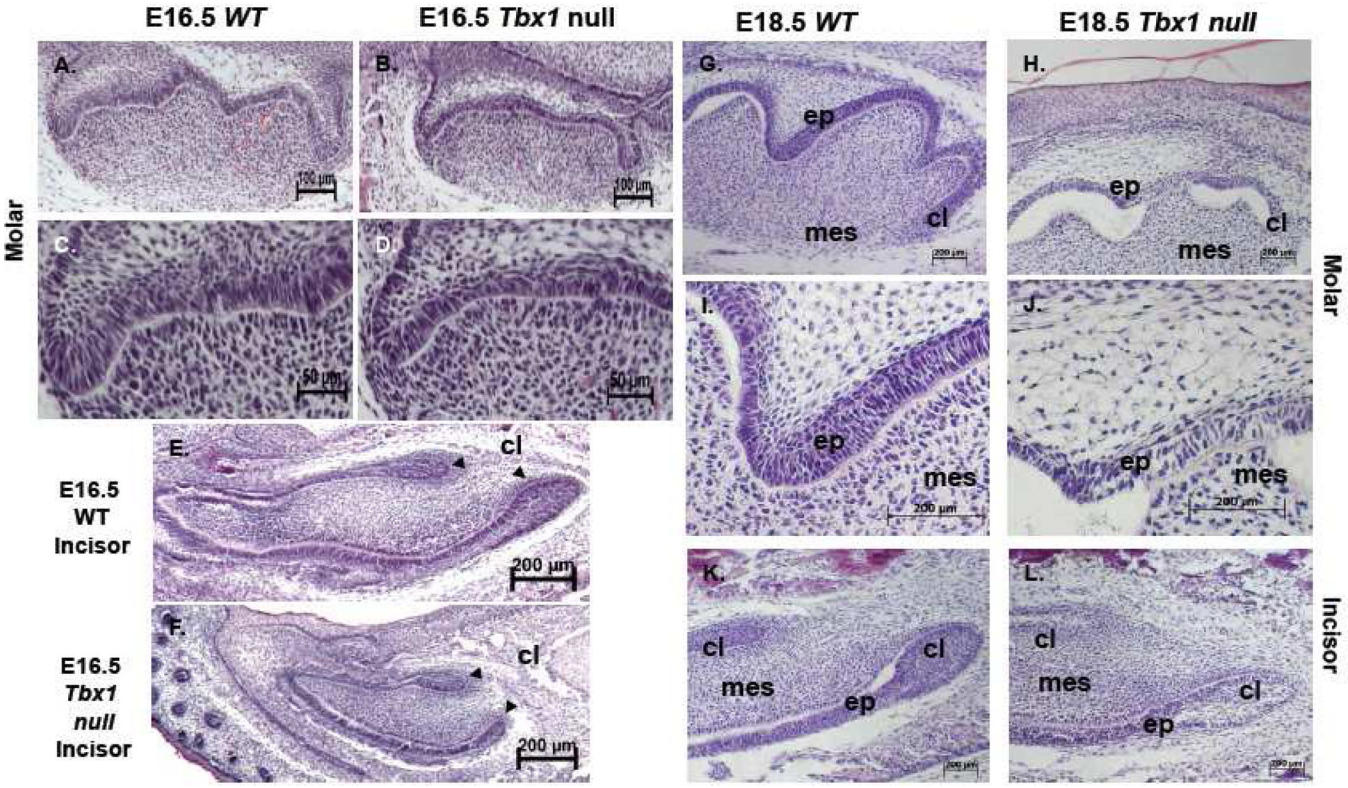

Fig. 2. Abnormal tooth development in Tbx1−/− mouse embryos at later stages

A) E16.5 WT molar tissue section (sagital) showing the characteristic shape of the molar with cusps and epithelial and mesenchyme tissues. **B)** *Tbx1*$^{-/-}$ molar tissue section revealing a defect in molar cusp formation and subsequent abnormal epithelial cell layer. **C)** Higher magnification of panel A showing the normal epithelial cells that have differentiated into pre-ameloblasts and their characteristic columnar shape. **D)** Higher magnification of panel B revealing a defect in epithelial cell differentiation to polarized pre-ameloblasts (the epithelial cell layer is not a thick or as well polarized as the control in panel C). **E)** E16.5 WT embryo incisor tissue section showing normal size cervical loops (cl). **F)** E16.5 *Tbx1*$^{-/-}$ embryo incisor and cervical loops are smaller. **G)** E18.5 WT molar with advanced molar cusp formation and morphology. **H)** E18.5 Tbx1 null molars with defective cusp formation and epithelial layer differentiation. **I)** Higher magnification of panel G showing normal pre-ameloblast polarization and morphology. **J)** Higher magnification of Panel H showing defective epithelial cell proliferation and differentiation (polarization). **K)** High magnification of the E18.5 WT lower incisor cervical loops. **L)** High magnification of E18.5 Tbx1 null lower incisor reveals decreased epithelail cells in the cervical loops and defective epithelial cell differentiation. cl, cervical loop; ep, epithelium; mes, mesenchyme; ek, enamel knot; 2nd ek, secondary enamel knot.

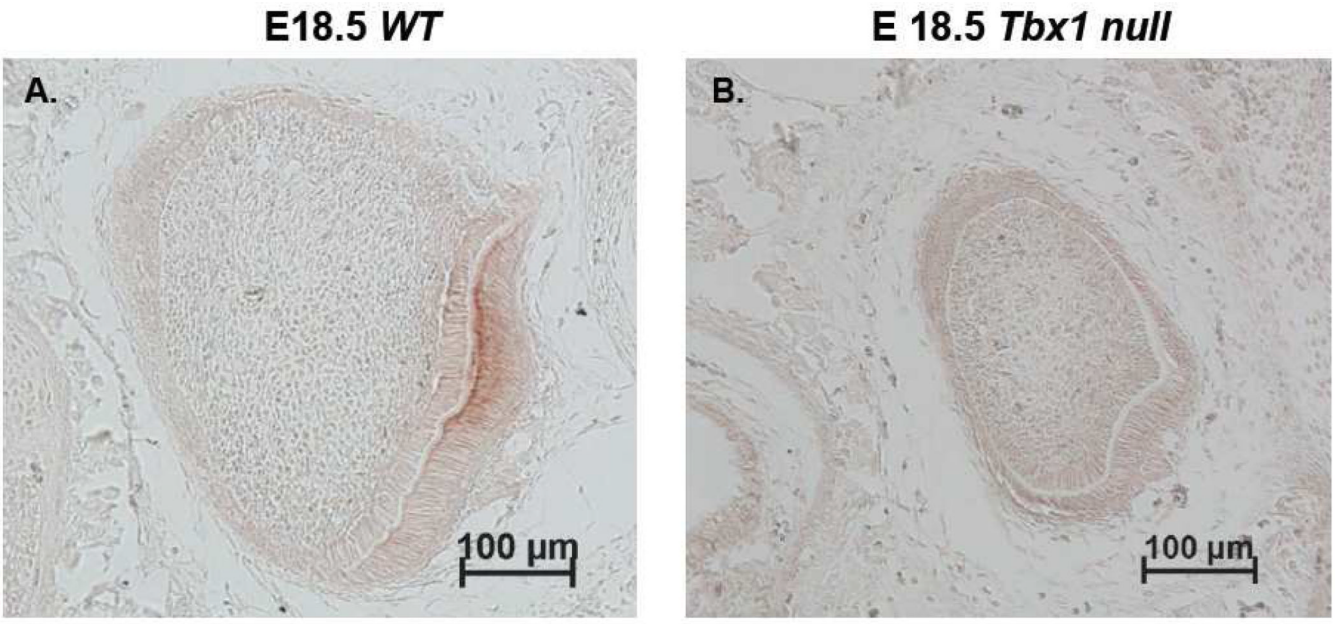

Fig. 3. Amelogenin expression in wild type and Tbx1 null mice

E18.5 Tbx1 null mutant epithelial cells do not express the ameloblast differentiation marker amelogenin. **A)** E18.5 upper incisors from WT embryos are beginning to express amelogenin from the epithelial cell layer. **B)** E18.5 Tbx1 null mutants do not express amelogenin at this stage.

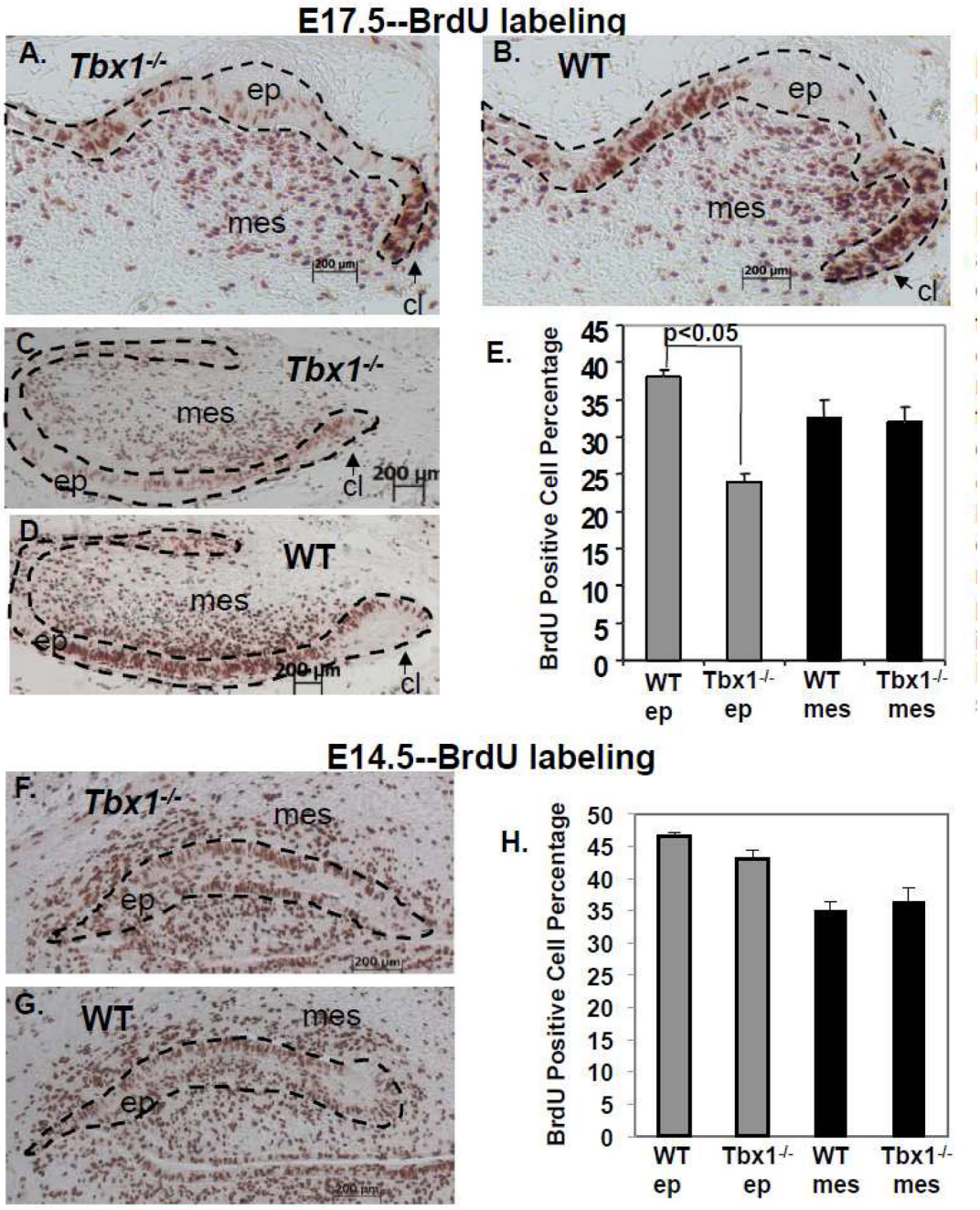

Fig. 4. Dental epithelial cell proliferation is defective in *Tbx1*$^{-/-}$ mouse embryos

WT and *Tbx1*$^{-/-}$ embryos were injected with BrdU and molar and incisor tissues harvested at E14.5 and E17.5 sectioned (sagital) and stained for BrdU. **A)** BrdU labeling of the E17.5 *Tbx1*$^{-/-}$ embryo molar tissue demonstrates a loss of epithelial cell proliferation compared to the WT molar in panel B. **C)** BrdU labeling of the E17.5 *Tbx1*$^{-/-}$ embryo incisor reveals decreased epithelial cell proliferation compared to WT incisor in panel D. **F)** BrdU labeling of the E14.5 *Tbx1*$^{-/-}$ embryo molar tissue demonstrates a minimal loss of epithelial cell proliferation compared to the WT molar in panel G. **E, H)** Quantitation of the BrdU positive cells in the molar sections, N>6.

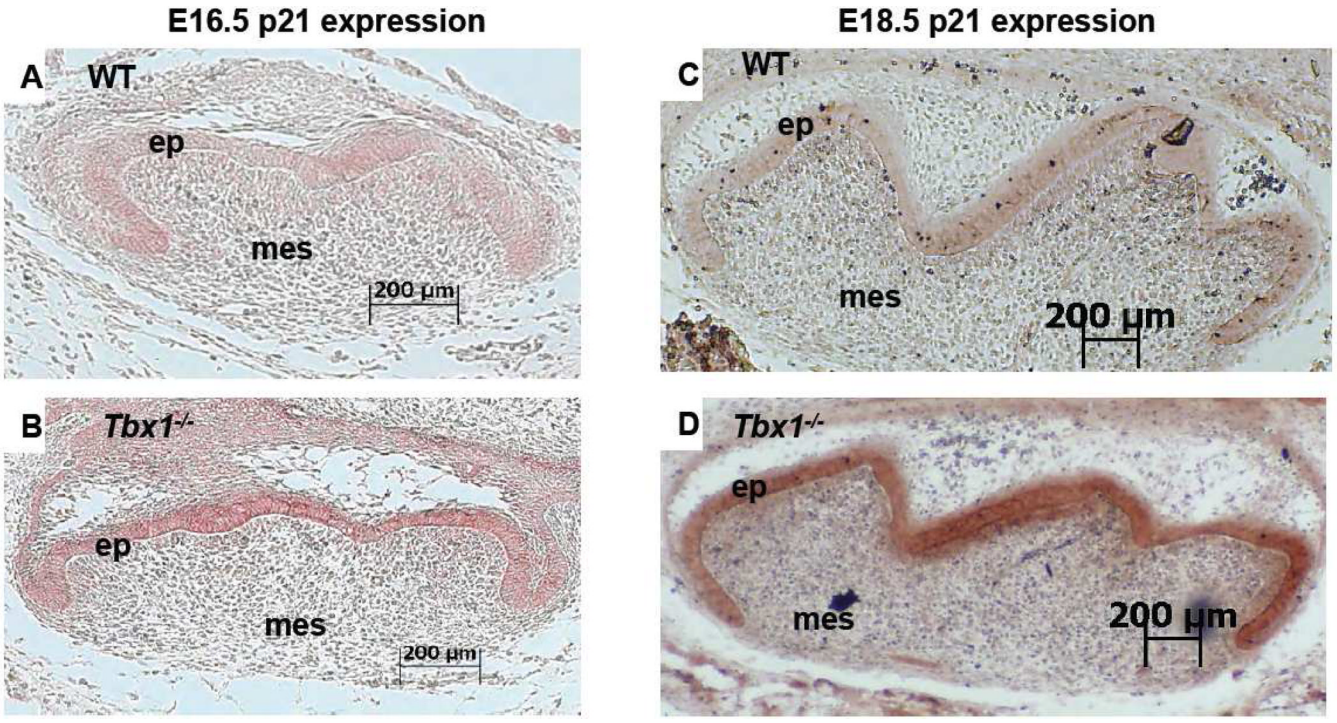

Fig. 5. Increased *p21* expression in the *Tbx1*$^{-/-}$ mouse embryo molar

E 16.5 and E18.5 mouse embryo molar sections (sagital) were incubated with p21 antibody and visualized using the AEC staining kit. **A)** Wild type (WT) E16.5 molar section showing low p21 expression in the dental epithelium. **B)** *Tbx1*$^{-/-}$ E16.5 molar sections demonstrate an increase in p21 expression throughout the dental epithelium. **C)** WT E18.5 molar section revealed low p21 expression. **D)** *Tbx1*$^{-/-}$ E18.5 molar sections demonstrate an increase in p21 expression throughout the dental epithelium compared to *Tbx1*$^{-/-}$ E16.5 molars and E18.5 wild type molars. Ep, epithelium; mes, mesenchyme. These experiments were repeated more that 3 times for each embryonic stage and genotype (N>3).

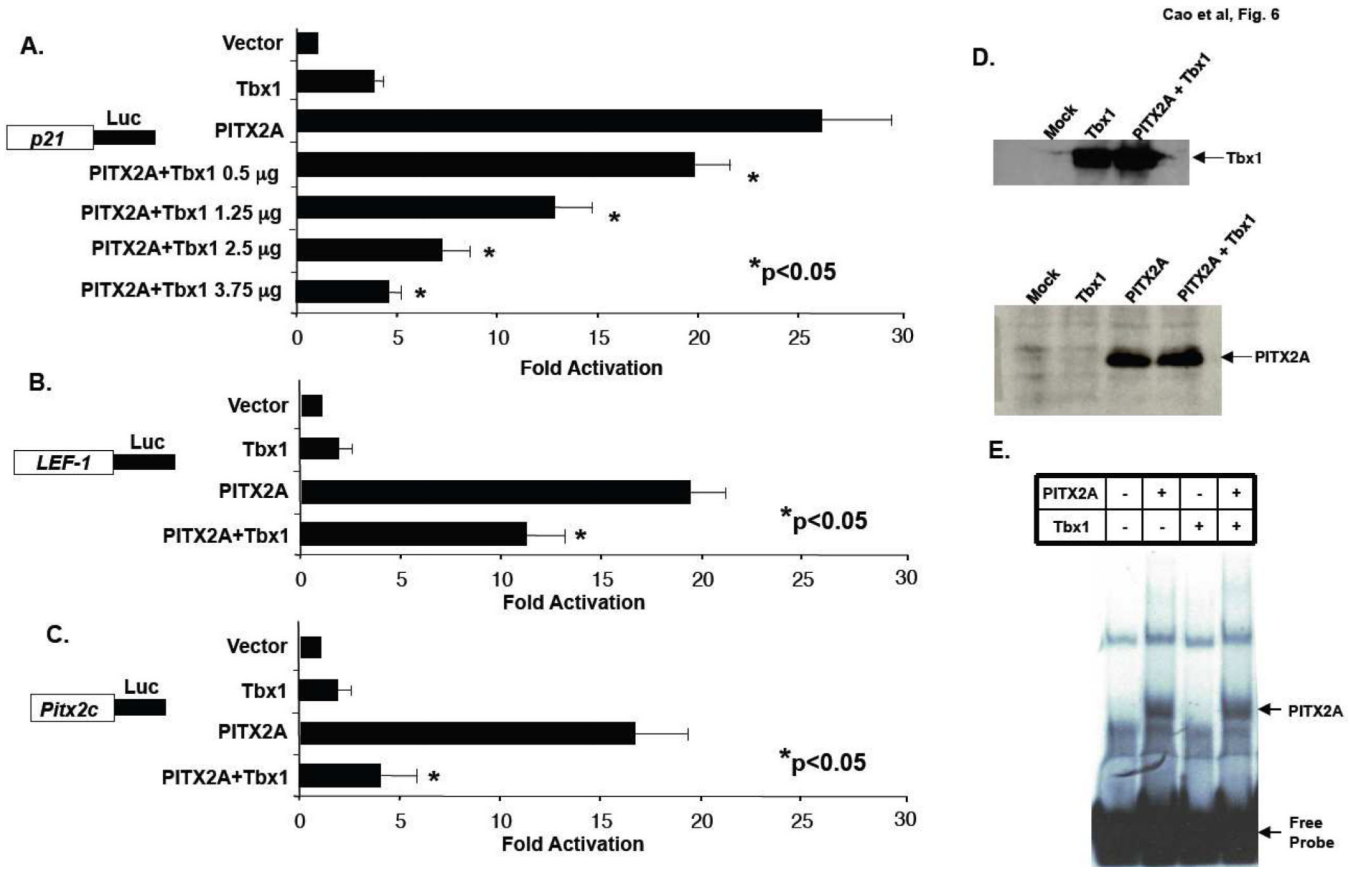

Fig. 6. Tbx1 represses PITX2 activation of the p21, LEF-1 and Pitx2c promoters

A) LS-8 oral epithelial cells were transfected with 2.5 µg of PITX2A, and indicated amounts of Tbx1 expression plasmids or a combination of both and 5 µg of the p21 promoter plasmid. All DNA was double CsCl banded for purity and cells were transfected by electroporation. To control for transfection efficiency all transfections included the SV-40 β-galactosidase reporter (0.5 µg). The activities are shown as mean-fold activation compared with the p21 promoter plasmid without expression plasmids and normalized to β-galactosidase activity (+/− S.E.M from 3 independent experiments). **B)** Transfections were performed as in panel A except that the LEF-1 promoter plasmid (Amen et al., 2007) was used instead of the p21 promoter and 2.5 µg Tbx1 and Pitx2. **C)** CHO cells were transfected with the Pitx2c promoter plasmid and the expression plasmids as in panel A. **D)** Western blot of Tbx1 transfected cell lysates demonstrating Tbx1 and PITX2 expression. **E)** PITX2 DNA binding activity is unaffected by Tbx1. PITX2 protein (80 ng) and Tbx1 protein (80 ng) were incubated with a PITX2 DNA binding element (TAATCC) as the radioactive probe. PITX2 protein bound to the DNA probe and Tbx1 protein did not bind the DNA. Tbx1 (80 ng) was added to PITX2 protein (80 ng) and did not inhibit PITX2 binding to the DNA. The EMSA experiments were analyzed in 8% native polyacrylamide gels.

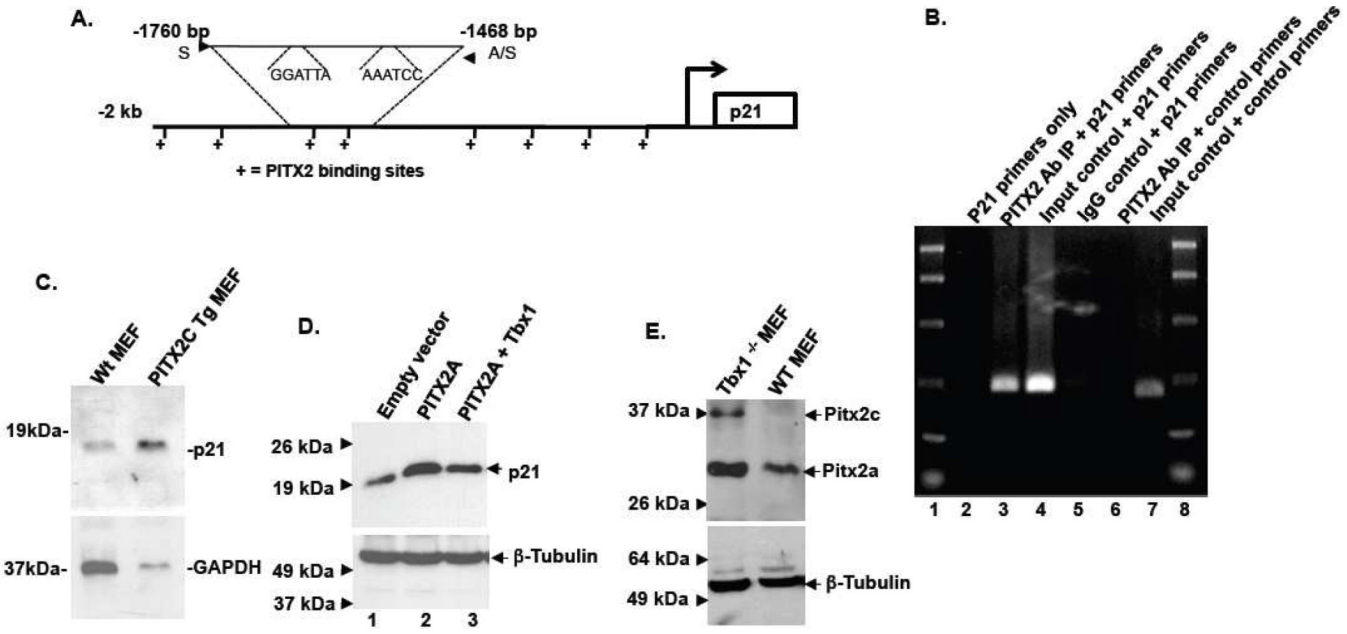

Fig. 7. Pitx2 activates p21 expression

A) Schematic of the p21 promoter indicating the Pitx2 DNA binding elements and location of primers for the ChIP assay. **B)** The ChIP assay reveals endogenous Pitx2 binding to the p21 endogenous promoter in the LS-8 oral epithelial cells. Lane 1, PCR markers; lane 2, p21 primers only with out template; lane 3, Pitx2 antibody ChIP and p21 primers showing Pitx2 binding to the p21 promoter; lane 4, input control using the p21 primers; lane 5, the IgG control ChIP; lane 6, Pitx2 antibody ChIP and control primers to another transcription factor promoter; lane 7, input control with the control primers; lane 8 PCR markers. **C)** Western blot of p21 expression in wild type MEFs and PITX2C transgenic MEFs. MEF lysates were prepared and analyzed on a Western blot probed with p21 antibody and visualized with ECL reagents (GE HealthCare). The same Western blot was probed for GAPDH expression as a loading control. **D)** LS-8 cells were transfected with empty vector (lane 1), PITX2A (Lane 2) or PITX2A and Tbx1 (Lane 3) and lysates prepared after 24 hr and Western blot probed for p21 expression. β-tubulin served as a loading control. E) Tbx1null MEFs lysates and WT MEFs lysates were cultured and lystaes probed for endogenous Pitx2 expression by Western blot. β-tubulin served as a loading control.

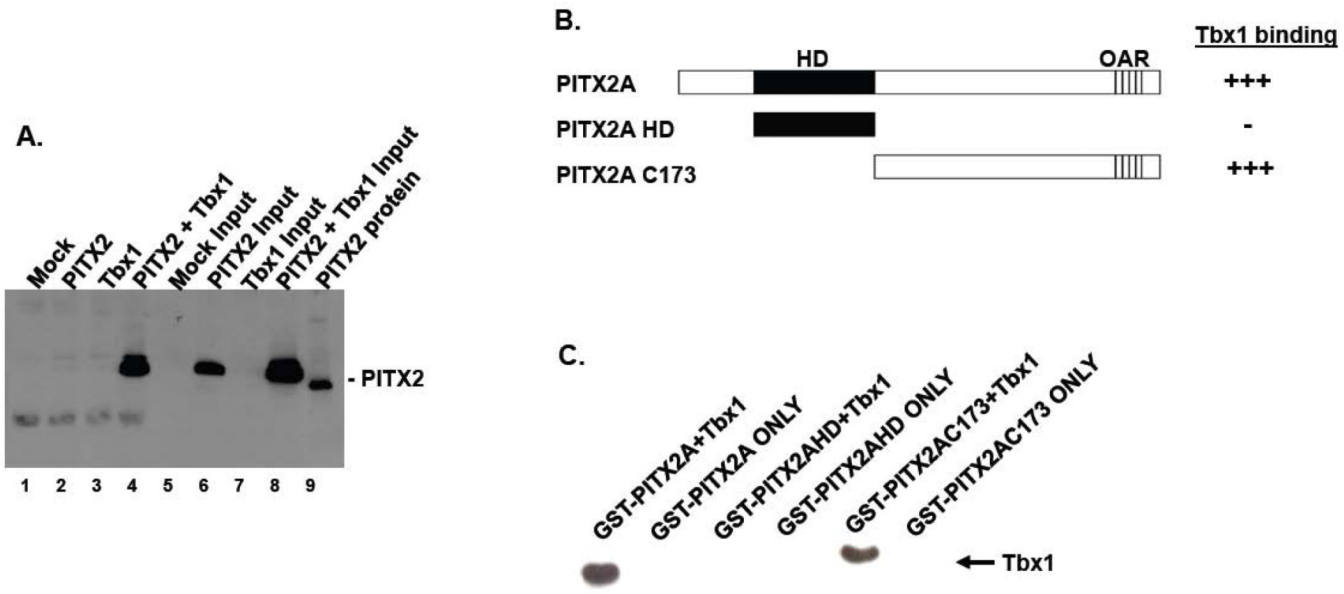

Fig. 8. Tbx1 physically interacts with the PITX2 C-terminal tail

A) Immunoprecipitation assay using the Tbx1 Ab to immunoprecipitate the Tbx1-PITX2 complex and probed on a Western blot using the PITX2 Ab. The PITX2-Tbx1 complex was immunoprecipitated from co-transfected lysates (lane 4). Transfected PITX2 input is shown in lane 6 and when co-transfected with Tbx1 (lane 8). As a control purified PITX2 protein was run on the gel (lane 9). The purified bacterial expressed PITX2 protein migrates slightly faster compared to the transfected protein due to a myc/his tag on the transfected protein. The immunoprecipitated complex was resolved on a 10% polyacrylamide gel and transferred to a PVDF filter and Western blotting was done using PITX2 antibody. **B)** Schematic of the PITX2 deletion constructs used to map the Tbx1 interaction region by GST-pull down experiments. HD, homeodomain; OAR, 14 amino acid conserved domain (protein interaction domain) **C)** GST-pull down assay using immobilized GST-PITX2 constructs and purified Tbx1 protein. Tbx1 binds to the full-length PITX2 protein and the C-terminal peptide (PITX2A C173). As a control Tbx1 did not bind to the PITX2 HD (homeodomain). The bound protein was detected by Western blot using the Tbx1 antibody.

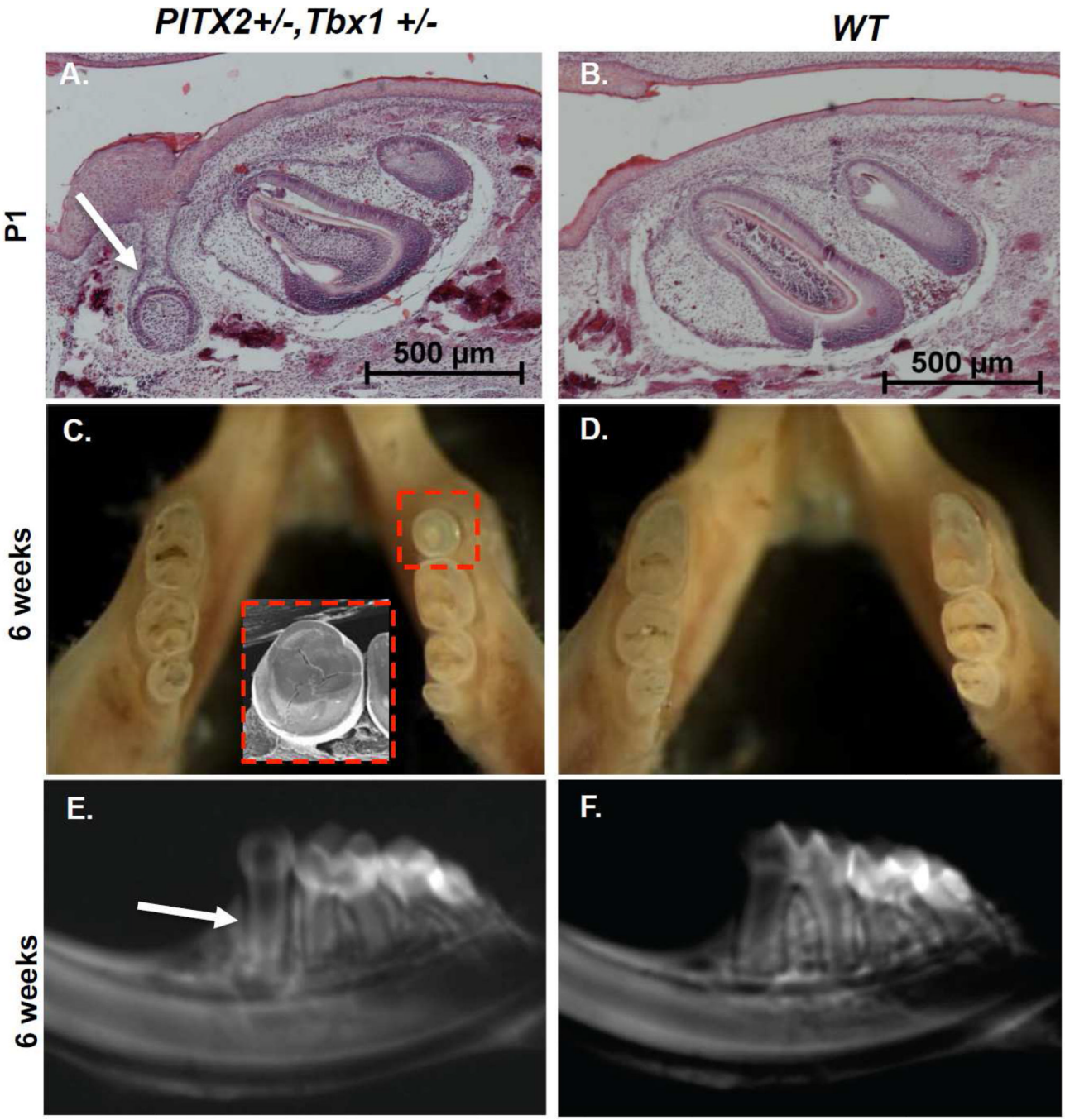

Fig. 9. *Tbx1*$^{-/+}$/*Pitx2*$^{-/+}$ double het mice development an extra canine-like tooth

The *Pitx2*$^{+/-}$ mouse crossed with a *Tbx1*$^{+/-}$ mouse results in the growth of an extra tooth in the diastema region in front of the first molar. **A)** HE staining of P1 *Pitx2*$^{+/-}$ *Tbx1*$^{+/-}$ mouse showing the early stage of a tooth structure. **B)** HE staining of P1 WT mouse. **C)** A six week old *Pitx2*$^{+/-}$ *Tbx1*$^{+/-}$ mouse with an extra tooth in front of the first molar in the lower jaw. Also shown with SEM image **D)** A six week old WT mouse. **E)** X-ray picture of the 6 week old *Pitx2*$^{+/-}$ *Tbx1*$^{+/-}$ mouse molars showing only one root on extra tooth. **F)** X-ray picture of 6 week WT mouse molars

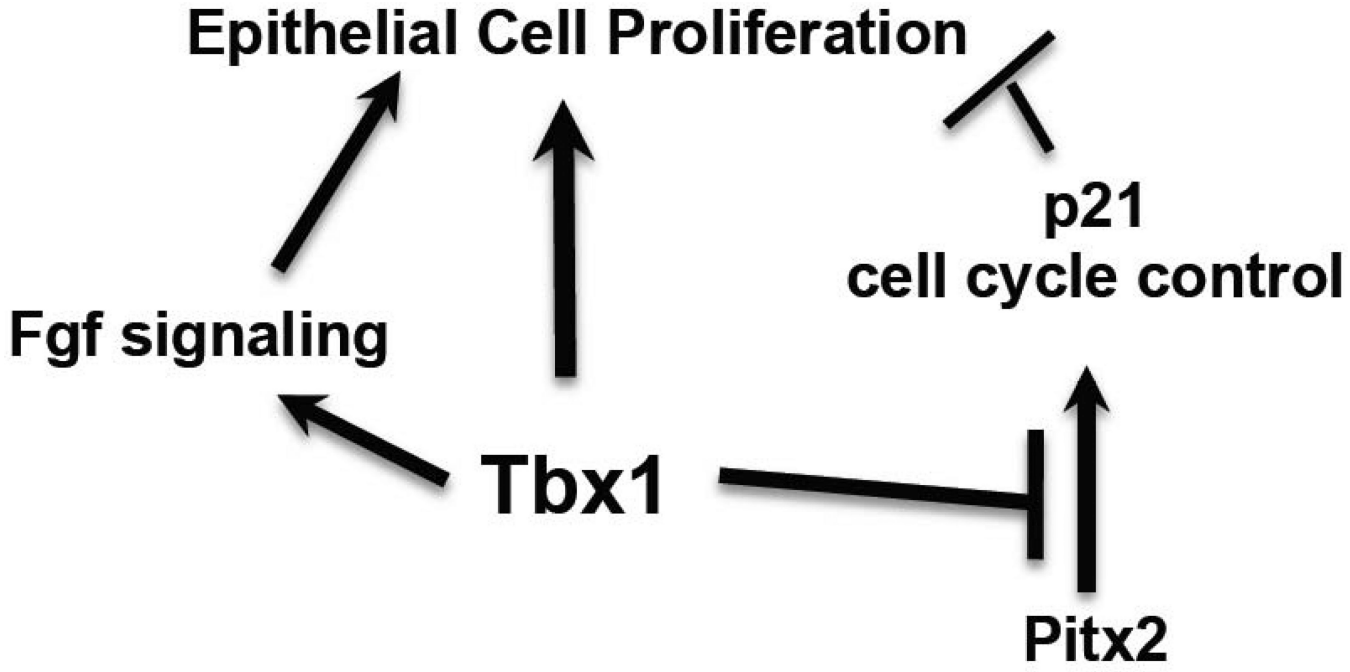

Fig. 10. Function of Tbx1 during tooth development

Tbx1 regulates cell proliferation in the cervical loop either by activating the Fgf signaling pathway or by attenuating PITX2 activation of p21 expression.