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Wnt/ β -catenin signaling directs multiple stages of tooth morphogenesis

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Abstract

Wnt/ β -catenin signaling plays key roles in tooth development, but how this pathway intersects with the complex interplay of signaling factors regulating dental morphogenesis has been unclear. We demonstrate that Wnt/ β -catenin signaling is active at multiple stages of tooth development. Mutation of β -catenin to a constitutively active form in oral epithelium causes formation of large, misshapen tooth buds and ectopic teeth, and expanded expression of signaling molecules important for tooth development. Conversely, expression of key morphogenetic regulators including *Bmp4*, *Mx1*, and *Mx2* is downregulated in embryos expressing the secreted Wnt inhibitor *Dkk1* which blocks signaling in epithelial and underlying mesenchymal cells. Similar phenotypes are observed in embryos lacking epithelial β -catenin, demonstrating a requirement for Wnt signaling within the epithelium. Inducible *Dkk1* expression after the bud stage causes formation of blunted molar cusps, downregulation of the enamel knot marker *p21*, and loss of restricted ectodermal expression, revealing requirements for Wnt activity in maintaining secondary enamel knots. These data place Wnt/ β -catenin signaling upstream of key morphogenetic signaling pathways at multiple stages of tooth development and indicate that tight regulation of this pathway is essential both for patterning tooth development in the dental lamina, and for controlling the shape of individual teeth.

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Introduction

Delineating the mechanisms by which oral epithelial cells adopt and maintain dental fates is critical for understanding developmental tooth syndromes and for designing strategies for the regeneration or repair of teeth and enamel. A key question is how the coordinated actions of broadly used signaling pathways result in the formation of a specific organ, in this case the tooth.

An early initiating signal for tooth development arises in the oral ectoderm, causing thickening of the dental lamina and its down-growth into the mesenchyme to form a tooth bud (Lumsden, 1988). The specification of tooth and intervening regions may be regulated by a competition between Fibroblast growth factor (FGF) 8, expressed in pre-tooth epithelium, and Bone morphogenetic protein (BMP) 4, expressed in intervening epithelium (Neubuser et al., 1997; St Amant et al., 2000). These factors regulate restricted expression of the homeobox transcription factor *Pitx2* that is required for tooth development beyond the bud stage (Lin et al., 1999). The Sonic hedgehog

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lamina to form tooth buds (Bei and Maas, 1998; Hardcastle et al., 1998). However, the mechanisms controlling assignment of tooth fate are incompletely understood.

At the late bud stage, the tooth bud begins to fold at its base in response to mesenchymal signals (Mina and Kollar, 1987). The enamel knot, a non-proliferating, transient epithelial structure, appears at the cap stage and is thought to regulate tooth shape (Vahtokari et al., 1996). These processes fail to occur in mice lacking the transcription factors LEF1, PITX2, MSX1, and PAX9, which are targets of the intercellular Wnt, BMP and FGF pathways (Bei and Maas, 1998; Kratochwil et al., 1996; Lin et al., 1999; Peters et al., 1998). Subsequent folding morphogenesis (the bell stage) results in the formation of multiple cusps and requires the TNF family member ectodysplasin (EDA), which signals via its receptor EDAR to activate the NF- κ B pathway (Courtney et al., 2005; Jernvall et al., 1994; Ohazama et al., 2004; Pispis et al., 1999; Schmidt-Ulrich et al., 2006). Dental papilla cells adjacent to the epithelium differentiate into odontoblasts and begin to secrete dentin (Thesleff and Harmerinta, 1981), while the epithelial cells differentiate into outer enamel epithelium, stellate reticulum, stratum intermedium, and inner enamel epithelium, lying adjacent to the papilla. The inner enamel epithelium differentiates into preameloblasts and then enamel-secreting ameloblasts (Thesleff and Harmerinta, 1981).

Wnts form a large family of secreted ligands that activate several receptor-mediated pathways (Logan and Nusse, 2004). In the Wnt/ β -catenin pathway, binding of Wnt ligands to Frizzled (FZ) receptors and LDL receptor related protein (LRP) family co-receptors causes β -catenin accumulation, nuclear translocation, and transcriptional activation by complexes of β -catenin and LEF/TCF transcription factor family members (Logan and Nusse, 2004). The Wnt/ β -catenin pathway is specifically inactivated by endogenous secreted inhibitors of the Dickkopf (DKK) family, which bind to LRP and to high-affinity receptors of the Kremen family, causing rapid internalization of Kremen–Dkk–LRP complexes and removal of LRP from the plasma membrane (Mao et al., 2002).

Activation of Wnt/ β -catenin signaling initiates the de novo formation of ectodermal appendages related to teeth, including hair follicles, feather buds, mammary placodes, and taste papillae (Chu et al., 2004; Gat et al., 1998; Hogan, 1999; Liu et al., 2007; Nornally et al., 1999; Thesleff et al., 1995). Conversely, initiation of hair follicle, mammary, and taste papilla placode development requires Wnt/ β -catenin signaling (Andl et al., 2002; Chu et al., 2004; Liu et al., 2007).

Several specific observations indicate that Wnt signaling plays key roles in tooth morphogenesis. Several *Wnt* genes are broadly expressed in oral and dental epithelium, while others are upregulated in developing teeth (Dassule and McMahon, 1998; Kratochwil et al., 2003; Sarkar and Sharpe, 1999). Loss of LEF1 causes arrested tooth development at the late bud stage, loss of expression of a direct LEF1/ β -catenin target gene, *Fgf4*,

possibly additional *Tcf* family members (Kratochwil et al., 2003; Osterwegel et al., 1993). Consistent with additional Wnt functions, constitutive ectopic expression of *Dkk1* in the oral epithelia of transgenic mouse embryos causes arrested tooth development at the lamina-early bud stage (Andl et al., 2002), and oral epithelium expressing constitutively active β -catenin develops multiple teeth following transplantation to a kidney capsule (Jarvinen et al., 2006).

Here we investigate the pattern of, and precise requirements for, Wnt pathway activation at multiple stages of tooth development, and use explant culture and in vivo loss and gain of function experiments to determine how Wnt signaling interacts with other factors important for tooth morphogenesis. Our results place Wnt upstream of other key signaling pathways at several stages of tooth development, and suggest the potential use of Wnt activation in strategies for tooth regeneration.

Materials and methods

Generation of mouse lines and genotyping

Wnt activity was monitored using TOPGAL (DasGupta and Fuchs, 1999) (Jackson Laboratories, Bar Harbor, ME, USA), *Rat-gal* (Mareni et al., 2003), and *TCF/Lef-LacZ* (Mohamed et al., 2005) Wnt reporter transgenic mice. For epidermal-specific activation of β -catenin signaling, *Cosch*^{fl/fl} mice (Ishida et al., 1999) were crossed to *K14-Cre* line 43 transgenic mice (Andl et al., 2004). For epidermal-specific deletion of β -catenin, *Cosch*^{fl/fl} mice (Ishida et al., 2001) (Jackson Laboratories) were crossed to *K14-Cre* line 43 transgenic mice (Andl et al., 2004). To assess the efficiency of *K14-Cre*-mediated recombination in oral and dental epithelia, *K14-Cre* mice were crossed with the *ROSA26* Cre reporter line (Soriano, 1999) (Jackson Laboratories). For Wnt pathway inhibition, *K5-rtTA tetO-Dkk1* mice were generated and induced by feeding with chow formulated with 1 g/kg doxycycline (BioSera, Laurel, MD, USA) as previously described (Chu et al., 2004). To monitor the efficiency of Wnt pathway activation and inhibition, *Cosch*^{fl/fl} mice and *Cosch*^{fl/fl} mice were crossed with TOPGAL mice and further crossed with *K14-Cre* mice; and *K5-rtTA tetO-Dkk1* mice were crossed with TOPGAL mice. To monitor activation of NF- κ B signaling, *K5-rtTA tetO-Dkk1* mice were crossed with the NF- κ B *LacZ* reporter transgenic mouse line (*I κ B α -crem-lacZ* (NF- κ B-GAL) (Schmidt-Ulrich et al., 1996). All experiments were performed with approved animal protocols according to the institutional guidelines established by the University of Pennsylvania IACUC committee.

Analysis of TOPGAL, *Rat-gal*, and *TCF/Lef-LacZ* expression

Heads from E11.5–E12.5 embryos were cryosectioned at 10–12 μ m followed by X-gal staining. Embryonic mandibles at E12.5–E18.5 were fixed and whole mount stained with X-gal (Firth et al., 1994), photographed and/or paraffin-embedded, sectioned and counterstained with eosin.

Histology, immunofluorescence, *BrdU* incorporation, TUNEL assays, and in situ hybridization

Tissue preparation, histology, immunofluorescence with anti- β -catenin, *BrdU* assays, TUNEL staining, and in situ hybridization with digoxigenin-labeled probes were as described previously (Andl et al., 2004, 2002; Chu et al., 2004). Sections were mounted with Vectashield containing DAPI (Vector Laboratories, Burlingame, CA, USA). *Bmp6*, *Eda*, *Lef1*, *Sfr*, *Wnt10b*, *Eda*, *Cosch1*, *Mx1*, *Mx2*, *p21*, and *Ectod1* probes were as described previously (Andl et al., 2002).

Mandible cultures and bead implantation experiments

Mandibles dissected from E11.5 TOPGAL embryos were cultured in glutamine-supplemented DMEM/F12, 10% FBS on cell culture inserts (BD Biosciences, San Jose, CA, USA) in the presence or absence of 50 nM LiCl at 37 °C, 5% CO₂ for 24 h, and stained with X-gal to assess reporter gene expression. Bead implantation and mandible culture experiments were performed according to previously described procedures (Chen et al., 1996; Valino et al., 1993). For bead implantation, heparin acrylic beads (Sigma, St Louis, MO) were incubated with 100 µg/ml recombinant human BMP4 protein (R&D Systems, Minneapolis, MN, USA) at 37 °C for 30 min. Control beads were soaked with similar concentrations of BSA under the same conditions. Some BMP4 coated beads were subsequently washed in PBS and incubated with 20 µg/ml recombinant mouse DKK1 protein (R&D Systems, Minneapolis, MN, USA) or similar concentrations of BSA at 37 °C for 1 h. Protein-soaked beads were stored at 4 °C and used within 1 week. All explants were cultured on cell culture inserts (BD Biosciences, San Jose, CA, USA) in the presence of 50 µg/ml dexamethasone, in Dulbecco's minimal essential medium with 10% FCS at 37 °C for 24 h. After culture, explants were fixed and processed for whole-mount *in situ* hybridization.

Results

Wnt/β-catenin signaling is active at multiple stages of tooth morphogenesis

To determine the spatial and temporal pattern of WNT/β-catenin pathway activation during tooth development, we

utilized mice carrying *TCF/Lef-LacZ*, *TOPGAL*, or *BAT-gal* Wnt reporter transgenes that each contain multimerized consensus LEF/TCF binding sites upstream of a minimal promoter and *lacZ* coding sequences (DasGupta and Fuchs, 1999; Maretto et al., 2003; Mohamed et al., 2005). These three independent reporter transgenic lines displayed similar expression patterns at multiple stages of tooth development. In addition, we utilized immunofluorescence for nuclear β-catenin (Andl et al., 2002) to identify cells with nuclear-localized β-catenin, an alternate readout for Wnt signaling activity. Reporter gene activity was apparent in the incisor and molar regions of the maxilla and mandible by embryonic day 11.5 (E11.5). X-gal staining of cryosectioned oral cavity revealed intense staining in developing dental epithelial placodes (Figs. 1A, B). At E12.5, reporter gene expression localized to invaginating tooth bud epithelium (Fig. 1C). Nuclear localized β-catenin was observed in epithelial and immediately underlying mesenchymal cells at this stage (Fig. 1D). At the cap stage, nuclear β-catenin and Wnt reporter gene expression localized to epithelial cells of the primary enamel knot (Figs. 1E, F). Nuclear β-catenin was also observed in a cluster of immediately underlying mesenchymal cells (Fig. 1E). The slightly different expression patterns observed for Wnt reporter genes and nuclear β-catenin at the bud-cap stages could be due to relatively greater sensitivity of nuclear β-catenin detection in mesenchymal versus epithelial

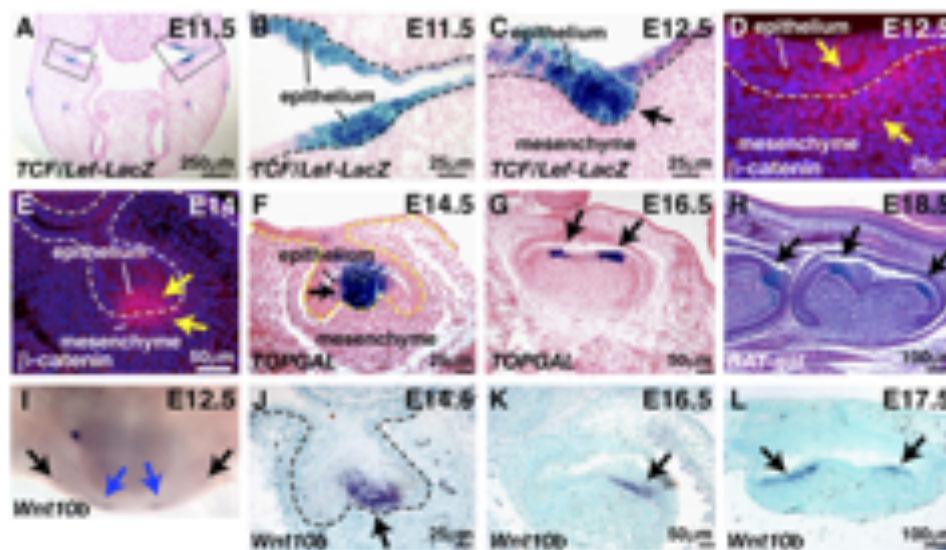


Fig. 1. Localization of Wnt reporter gene expression, β-catenin, and *Wnt10b* expression in developing tooth. Tissues were cryosectioned and then X-gal stained to reveal sites of Wnt reporter expression (blue) (A–C), sectioned prior to immunofluorescence staining for β-catenin (red) (D–E), or whole mount X-gal stained prior to paraffin sectioning (F–H). (A) Transverse section of E11.5 *TCF/Lef-LacZ* head showing reporter gene expression in molar tooth placodes (bracketed) and adjacent oral epithelium. (B) Higher magnification view of the area bracketed on the right in (A) showing *LacZ* expression in epithelial cells of the first molar. (C) Transverse section of E12.5 *TCF/Lef-LacZ* maxilla showing *LacZ* expression in molar epithelial cells. (D) Transverse section of E12.5 maxilla showing nuclear and cytoplasmic localization of β-catenin in epithelial cells and immediately underlying mesenchymal cells of the first left molar. Nuclei are counterstained with DAPI and appear blue. (E) Transverse section of E14 maxilla showing nuclear localization of β-catenin in epithelial cells and immediately underlying mesenchymal cells of the first left molar. Nuclei are counterstained with DAPI and appear blue. (F) Frontal section of E14.5 TOPGAL mandible showing reporter gene expression in the enamel knot of a first lower molar. (G) Frontal section of E16.5 TOPGAL mandible showing reporter gene expression in developing molar cusps. (H) Sagittal section of E18.5 *BAT-gal* mandible showing reporter gene expression in molar cusp cells (arrows). (I) E12.5 mandible subjected to whole mount *in situ* hybridization with digoxigenin-labeled

cells; under-reporting of mesenchymal Wnt signaling by the artificial reporter gene constructs; and/or absence in the mesenchyme of nuclear β -catenin co-factors required for activating transcription. At the early bell stage, Wnt reporter activity localized to the developing molar cusps (Fig. 1G), and by the late bell stage was present asymmetrically in the epithelial enamel knots of developing molar cusps (Fig. 1H). Notably, expression of *Wnt10b* localizes to dental placodes by E12.5 (Fig. 1I and data not shown), similar to, although slightly later than, the pattern of Wnt reporter gene activation. By the cap stage *Wnt10b*, like Wnt reporter gene expression, localizes to the primary enamel knot (Fig. 1J). *Wnt10b* expression subsequently localizes to the secondary enamel knots in molar cusp epithelium (Figs. 1K, L).

Stimulation of Wnt signaling can initiate tooth development and results in expanded expression of multiple signaling factors

To determine whether stimulation of Wnt/ β -catenin signaling is capable of initiating tooth development, we first examined the consequences of pathway stimulation *ex vivo* in cultured mandibles. *TOPGAL* transgenic mandibles were dissected at E11.5 and cultured for 24 h under conditions permissive for inductive stages of tooth development (Jowett et al., 1993), in either the presence or absence of 50 mM LiCl which inhibits GSK3- β , preventing targeting of cytoplasmic β -catenin for degradation (Hodgpeeth et al., 1997). In LiCl-treated mandibles, the sizes of clusters of *TOPGAL*-expressing cells in the regions of the incisor rudiments were significantly expanded relative to controls (Supplementary Fig. 1, white arrows) and the appearance of *TOPGAL*-expressing cell clusters in the molar regions was markedly accelerated compared with controls (Supplementary Fig. 1, red arrows) ($n=8$ control and $n=9$ LiCl-treated mandibles). In approximately 50% of LiCl-treated mandibles, clusters of blue cells with the appearance of dental placodes were formed at ectopic locations, adjacent to the incisor and molar clusters (Supplementary Fig. 1, pink arrows). Ectopic *TOPGAL*-expressing cell clusters were not observed in control mandibles (Supplementary Fig. 1). These data suggest that LiCl treatment enhances Wnt/ β -catenin pathway-mediated transcription in a region-specific fashion within the mandible, and may promote the formation of dental placodes.

To determine the consequences of Wnt pathway stimulation *in vivo*, we mutated β -catenin to a stabilized form in epithelial cells. *Cnab1*^{tsa/tst} mice, in which exon 3 of the endogenous β -catenin gene (*Cnab1*), encoding all phosphorylation target serine/threonine residues, is flanked by *loxP* sequences that are targets for Cre-mediated recombination (Harada et al., 1999), were mated to *K14-Cre* line 43 transgenic mice (Andl et al., 2004) expressing Cre recombinase under the control of a *Keratin 14* promoter (Byrne et al., 1994). *K14-Cre*-mediated recombination of a *ROS426R* Cre reporter gene (Soriano,

2007). Histological examination of the oral cavity at E14.5 revealed gross abnormalities in tooth development (Figs. 2A, B). Molar teeth developing in their normal locations were enlarged and exhibited multiple epithelial protrusions into the underlying mesenchyme. Smaller, ectopic invaginations were observed in adjacent epithelia. Similar phenotypes resulting from mutation of β -catenin to a stabilized form have also been described recently by another group (Jarvinen et al., 2006).

Strongly elevated levels of β -catenin protein were observed in the abnormal teeth and ectopic invaginations, compared with the levels in control littermate teeth (Figs. 2C, D). However, β -catenin protein levels were not uniformly elevated throughout the oral surface epithelium, suggesting either non-uniform activation of the *K14* promoter at this stage, or the existence of negative and positive feedback signaling downstream of activated β -catenin that impacts on protein levels. Interestingly, *Cnab1* mRNA was strongly upregulated in the abnormal teeth and ectopic invaginations, suggesting that stabilized β -catenin positively regulates its own expression in dental epithelial cells at the level of transcription or message stability (Figs. 2C', D').

Pitx2 expression was observed throughout the abnormal epithelial structures (Figs. 2E, F). As *Pitx2* expression is characteristic of developing teeth (Macchielli et al., 1997), but is not observed in other embryonic epithelial appendages such as hair follicle germs and mammary buds (EYC and SEM, unpublished data), this result identifies the abnormal structures as developing teeth.

Expanded Wnt/ β -catenin pathway activity, assayed by *TOPGAL* expression, was observed in the abnormal and ectopic dental structures in mutant oral cavities (Figs. 2G, H). By E17.5, multiple small, ectopic teeth could be observed on histological analysis (Figs. 2I, J). These were positive for the dental epithelial marker *Shh* (Figs. 2K, L). Similarly, expanded and ectopic expression of the enamel knot marker *Wnt10b* was observed in the mutant teeth (Figs. 2M, N). In addition, broad expression of *Left*, *Bmp4*, *Mx1*, and *Mx2* mRNAs was observed in the abnormal teeth and ectopic invaginations (Supplementary Fig. 3, and data not shown).

Broad expression of *ectodis* (also known as *Wise*), a *Bmp* and Wnt inhibitor critical for molar cusp patterning (Itasaki et al., 2003; Kassai et al., 2005; Laurikkala et al., 2003), was observed in the abnormal and ectopic mutant teeth (Figs. 2O, P) in a pattern inverse to that of *TOPGAL* and *Wnt10b*. This finding suggests that *ectodis* expression is suppressed in cells with high Wnt signaling activity but induced in adjacent cells by a secreted factor downstream of Wnt/ β -catenin. *Pitx2* expression was maintained in the mutant dental epithelium at E17.5 and E18.5 (Figs. 2Q, R and Supplementary Fig. 3).

By E18.5, dental differentiation, including the formation of ameloblasts and odontoblasts, could be observed in

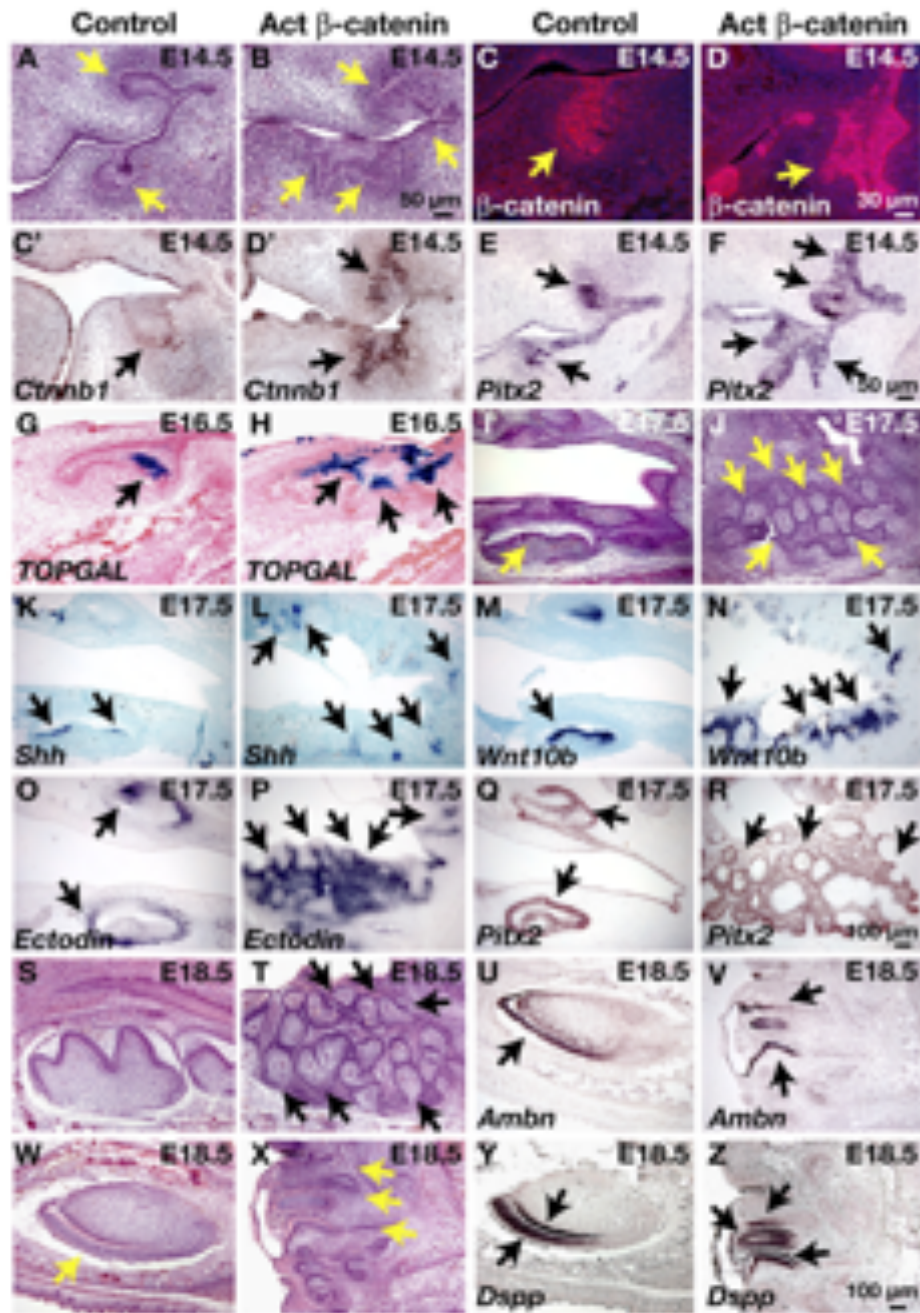


Fig. 2. Mutation of epithelial β -catenin to a stabilized form causes abnormal dental invaginations and ectopic tooth formation. Control littermate (A, C, C', E, G, I, K, M, O, Q, S, U, W, Y) and *K14-Cre Ctnnb1^{fl/fl}* (Act β -catenin)-mutant (B, D, D', F, H, J, L, N, P, R, T, V, X, Z) oral cavity from embryos at E14.5 (A–F), E16.5 (G, H), E17.5 (I–R), and E18.5 (S–Z); paraffin-embedded and sectioned frontally (A–F) or sagittally (G–Z) and subjected to hematoxylin and eosin staining (A, B, I, J, S, T, W, X); immunofluorescence with anti- β -catenin (red) (C, D); X-gal staining (G, H) (blue); or in situ hybridization with digoxigenin-labeled probe for *Ctnnb1* (C', D'), *Pitx2* (E, F, Q, R), *Shh* (K, L), *Wnt10b* (M, N), *ectodin* (O, P), *Ambn* (U, V), or *Dapp* (Y, Z) (purple or brown signals). Sections in (G and H) were taken from control and mutant embryos that also carried the TOPGAL Wnt reporter gene and were counterstained with eosin (pink). Scale bar in (B) applies to (A) and (B); scale bar in (D) applies to (C and D); scale bar in (R) applies to (C'–R); scale bar in (Z) applies to (S–Z).

sialophosphoprotein (*Dapp*) (Figs. 2U, V, Y, Z and data not shown). Developing mutant molar teeth were irregular in shape, and lacked the normal regular pattern of developing cusps (Figs.

and abnormally shaped (Supplementary Fig. 3K). These results indicate that stimulation of β -catenin signaling specifically within epithelial cells can initiate tooth development and

Wnt/ β -catenin signaling is required within the epithelium for early stages of tooth development

To determine the requirements for Wnt/ β -catenin signaling in tooth development and expression of key regulatory factors, we utilized inducible bi-transgenic *K5-rtTA tetO-Dkk1* mice, in which expression of *Dkk1*, a secreted Wnt antagonist capable of blocking the actions of Wnts that signal via LRP co-receptors and β -catenin (Zorn, 2001), can be induced by doxycycline in cells in which the *Keratin 5* promoter is active including surface and oral ectoderm and tooth buds (Chu et al., 2004; Diamond et al., 2000). Ectopic *Dkk1* expressed in surface epithelia blocks Wnt/ β -catenin pathway activity in the epithelium and in underlying mesenchymal cells (Chu et al., 2004).

To determine the efficiency of Wnt inhibition resulting from ectopic *Dkk1* expression in oral ectoderm, pregnant females carrying *K5-rtTA tetO-Dkk1 TOPGAL* and control *TOPGAL* littermate embryos were placed on oral doxycycline from the time of detection of a copulation plug and sacrificed at E11.5 or E12.5. Mandibles were dissected, subjected to whole mount X-gal staining, and sectioned. *TOPGAL* expression was reduced, but not entirely absent, in the dental region of induced *K5-rtTA tetO-Dkk1 TOPGAL* embryos at E11.5 (Figs. 3A, B). However, by E12.5, *TOPGAL* activity was absent from *K5-rtTA tetO-Dkk1 TOPGAL* dental lamina, indicating efficient Wnt inhibition by this stage (Figs. 3C–H). Immunofluorescence staining revealed that in controls, nuclear and cytoplasmic β -catenin localized to mesenchymal cells underlying the epithelium at E12.5 and was also observed in some dental epithelial cells (Fig. 3I). Nuclear and cytoplasmic localized β -catenin was not detected in *Dkk1*-expressing samples at E12.5, consistent with Wnt/ β -catenin pathway inhibition (Fig. 3J). The small number of epithelial cells displaying nuclear and cytoplasmic localized β -catenin in control molars at E12.5 despite strong expression of epithelial *TOPGAL* at this stage could be due to lower sensitivity of nuclear β -catenin detection in epithelial cells.

Histological examination at E12.5 and later stages revealed that molar tooth development was blocked at the lamina-early bud stage in *Dkk1*-expressing oral cavity (Figs. 3G, H, I, J, M–P), consistent with results from constitutive *K14-Dkk1* transgenic mice (Andl et al., 2002), while incisor tooth development was blocked at the placode stage (Figs. 3K, L). Significant differences in proliferation were not observed in *Dkk1*-expressing molars compared with controls assayed at E12.5 (Supplementary Figs. 4A, B).

Expression patterns of Wnt-reporter genes and β -catenin immunofluorescence (Fig. 1) suggested that Wnt/ β -catenin signaling activity localizes prominently in dental epithelium at the dental lamina and bud stages. To determine whether Wnt/ β -catenin signaling is required specifically within epithelial cells at these stages, we used *Keratin 14* (*K14*) promoter-driven expression of Cre recombinase to delete the cell autonomous Wnt effector gene β -catenin in dental epithelium. We crossed

Cxsb1^{fl/fl} embryos, β -catenin was deleted in a mosaic fashion in the oral and dental epithelium at E12.5 (Figs. 3Q, R). By E13.5, β -catenin was almost completely absent from dental epithelium (Figs. 3S, T). *TOPGAL* activity was markedly reduced in the dental region of *K14-Cre Cxsb1^{fl/fl} TOPGAL* compared with control *TOPGAL* embryos (Figs. 3U, V). Histological examination at E13.5 and later stages revealed that molar tooth development was blocked at the early bud stage in epithelial β -catenin depleted oral cavity (Figs. 3W', X', Y, Z), while incisor tooth development was blocked at the lamina-early bud stage (Figs. 3W, X). Significant differences in proliferation were not observed in epithelial β -catenin depleted molars compared with controls assayed at E13.5, consistent with the results of similar assays in *Dkk1*-expressing embryos (Supplementary Figs. 4C, D). These data indicate that Wnt/ β -catenin signaling is required within dental epithelial cells for tooth development beyond the lamina-early bud stage.

Wnt/ β -catenin signaling lies upstream of multiple secreted signals at early stages of tooth development

Bmp4 is expressed in the epithelium at the initiation of tooth development, and induces expression of the homeobox transcription factor genes *Msx1* and *Msx2* in the underlying mesenchyme. Lack of both *Msx1* and *Msx2* genes causes arrest at the dental lamina-early bud stage (Bei and Maas, 1998) indicating essential roles for these factors at early stages of tooth formation. Mesenchymal *Msx1* is required for expression of *Bmp4* in the mesenchyme (Chen et al., 1996). *Bmp4* then signals back to the epithelium, inducing expression of *p21* that is thought to cause cell cycle arrest and formation of the enamel knot (Jernvall et al., 1998). Consistent with this role for *Bmp* signaling in epithelial cells, deletion of epithelial *Bmp4* causes arrest of tooth development at the bud stage (Andl et al., 2004). To determine whether Wnt signaling is required for expression of *Bmp4* or *Msx* genes, we carried out whole mount and section in situ hybridization for *Bmp4*, *Msx1*, and *Msx2* on oral cavities from E12.5 embryos expressing *Dkk1* and littermate controls. *Bmp4* expression was absent and expression of *Msx1* and *Msx2* was significantly reduced in dental mesenchymal cells of *Dkk1*-expressing embryos at E12.5 (Figs. 4A, C). *Shh* signaling is required within the dental epithelium for regulation of tooth growth and shape (Dassule et al., 2000; Griffin-Linde et al., 2002). *Shh* expression was completely blocked by ectopic *Dkk1* at E12.5 (Figs. 4A, C), indicating that Wnt signaling lies upstream of *Shh* expression. Expression of the *Shh* pathway gene *Ptc2* was also absent from the dental region of *Dkk1*-expressing transgenics (Figs. 4A, C).

Left1 expression is Wnt-regulated in several developmental contexts, and the *Left1* promoter can be controlled synergistically by PITX2 and β -catenin (Vadlamudi et al., 2005). In developing teeth, *Left1* expression can also be induced by ectopic *Bmp4* (Kantochwil et al., 1996), but does not regulate its

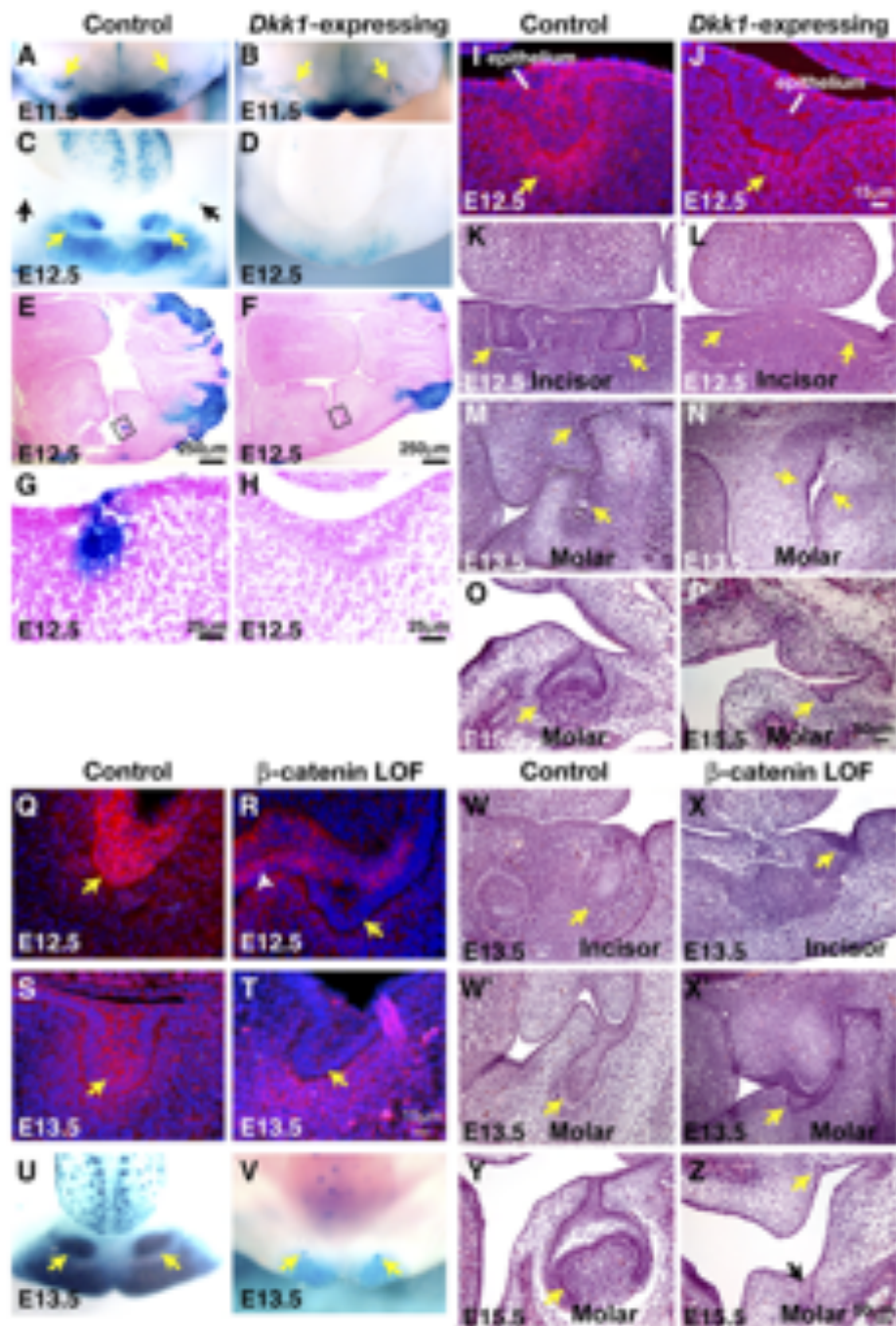
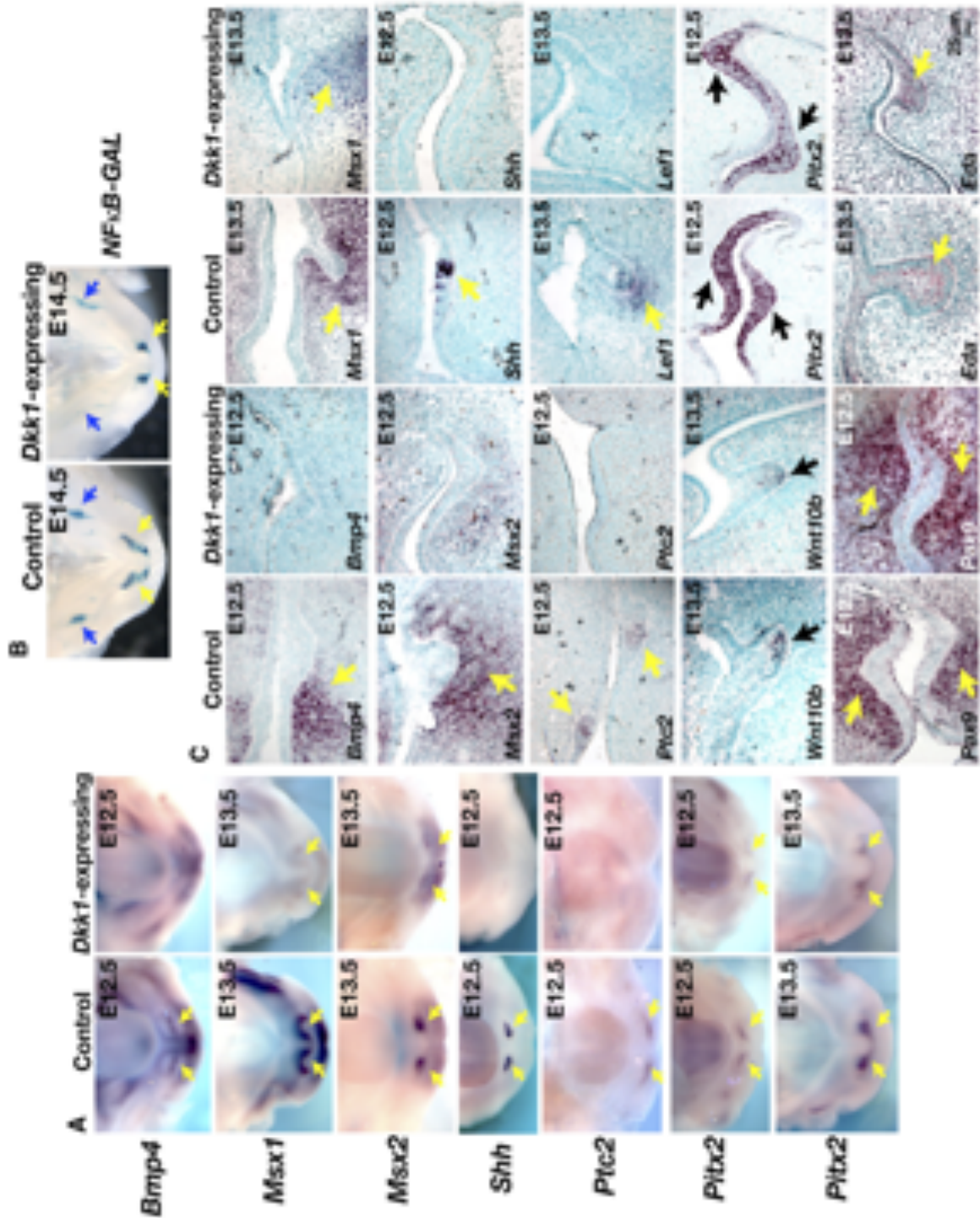


Fig. 3. Ectopic expression of *Dkk1* or epithelial deletion of β -catenin blocks Wnt pathway activity and tooth development. (A–D) X-gal stained mandible whole mounts from E11.5 (A, B) and E12.5 (C, D) littermate control TOPGAL (A, C) and *K5-rtEt wtO-Dkk1* TOPGAL (B, D) (*Dkk1*-expressing) embryos treated with doxycycline from E0.5. (E–H) Transverse sectioned X-gal stained heads from E12.5 control TOPGAL (E, G) and *K5-rtEt wtO-Dkk1* TOPGAL (F, H) (*Dkk1*-expressing) embryos. Note absence of TOPGAL staining and arrest of molar tooth development in the *Dkk1*-expressing embryo (F, H). (G and H) show higher magnification photographs of the boxed regions in (E and F). (I, J) Frontally sectioned oral cavities from E12.5 control (I) and *K5-rtEt wtO-Dkk1* (*Dkk1*-expressing) (J) embryos subjected to immunofluorescence for β -catenin (red). Sections are DAPI counterstained (blue). Note nuclear and cytoplasmic as well as membrane localization of β -catenin in dental mesenchymal and some epithelial cells in the control (I) and predominantly membrane localization in the *Dkk1*-expressing molar (J). (K–P) Frontally sectioned oral cavities from control (K, M, O) and littermate *K5-rtEt wtO-Dkk1* (*Dkk1*-expressing) (L, N, P) embryos subjected to hematoxylin and eosin staining at E12.5 (K, L), E13.5 (M, N), and E13.5 (O, P). Scale bar in (I) applies to (I and J); scale bar in (P) applies to (K–P). (Q–T) Frontally sectioned oral



Inhibition blocks expression of multiple developmental regulators. (A) Mandibles dissected from littermate control (left panels) and K3-rtTA wtO-Dkk1 (Dkk1-expressing) (right panels) embryos dosed with doxycycline at E12.5 or E13.5, and subjected to *in situ* hybridization with digoxigenin-labeled probes for *Bmp4*, *Msx1*, *Msx2*, *Shh*, *Ptc2*, and *Ptc2*. Arrows indicate positive signals (purple). (B) *Nf-κB-GAL* lines dissected from E14.5 littermate control *Nf-κB-GAL* and K3-rtTA wtO-Dkk1 *Nf-κB-GAL* (Dkk1-expressing) embryos dosed with doxycycline treated from E8.5. Note that *Nf-κB-GAL* expression in the incisor (a) and molar (blue arrows) regions persists in Dkk1-expressing mandibles. (C) Transverse sections of oral cavities from littermate control and K3-rtTA wtO-Dkk1 (Dkk1-expressing) embryos dosed with doxycycline (0.5, sacrificed at the stages indicated, and subjected to *in situ* hybridization with digoxigenin-labeled probes for *Bmp4*, *Msx1*, *Msx2*, *Shh*, *Ptc2*, *Ptc2*, and *Ptc2*. Arrows indicate *in situ* (purple/pink). Sections are counterstained with methyl green.

FGF cascade downstream of epithelial *Left1* and *Fgf4*, was downregulated in *Dkk1*-expressing dental epithelium at E13.5 (data not shown). Interestingly, *Wnt10b* expression was downregulated and more diffuse in *Dkk1*-expressing dental epithelium at E13.5 compared with littermate controls, but was not entirely absent (Fig. 4C). Expression of *Wnt10b* was lost from *Dkk1*-expressing dental epithelium by E14.5 (data not shown).

Although *Pitx2* can be regulated by Wnt/ β -catenin signaling in certain developmental contexts (Kioussi et al., 2002), we found that *Pitx2* expression was maintained in *Dkk1*-expressing dental epithelia when expression of other regulatory molecules such as *Bmp4*, *Mix* genes, and *Shh* was abrogated (Figs. 4A, C). Thus maintenance of *Pitx2* expression does not require Wnt/ β -catenin signaling in the dental lamina. We found that *Dkk1* expression also had no effect on *Pax9* expression (Fig. 4C), indicating that, like *Pitx2*, *Pax9* is regulated independently of Wnt.

Eda can promote survival of dental placodes (Mustonen et al., 2004) and has been suggested as a direct Wnt target (Dammowicz et al., 2002). EDA signals through its receptor EDAR to activate NF- κ B signaling. We used the NF- κ B *lacZ* reporter transgenic mouse line (*Ig κ conA-lacZ* (NF- κ B-GAL) (Schmidt-Ullrich et al., 1996) to determine whether Wnt inhibition blocks signaling through NF- κ B at early stages of tooth development. Pregnant females bearing *K14-rtTA tetO-Dkk* NF- κ B-GAL and control littermate embryos were doxycycline treated from the beginning of pregnancy. In X-gal stained littermate control mandibles, NF- κ B-GAL activity was clearly detectable in the incisor and molar regions at E14.5 (Fig. 4B). Similarly, despite much less extensive tooth development, clear X-gal staining was detectable in the comparable regions of *K14-rtTA tetO-Dkk* NF- κ B-GAL mandibles (Fig. 4B). Consistent with these data, *Eda* expression in dental epithelium was unaffected by ectopic *Dkk1* at E12.5 and E13.5 (Fig. 4C and data not shown). Thus expression of *Eda* and NF- κ B signaling in dental epithelial cells at early stages of tooth development is independent of Wnt/ β -catenin activity.

To determine the extent to which these alterations in gene expression reflect a requirement for Wnt signaling specifically within dental epithelial cells, we carried out whole mount and section in situ hybridization on oral tissues from *K14-Cre* *Cxsb1^{fl/fl}* and littermate control embryos with probes for

Bmp4, *Mix1*, *Mix2*, *Shh*, *Left1*, *Eda*, and *Pitx2* at E13.5, the earliest stage at which β -catenin is efficiently deleted in dental ectoderm. Expression of *Pitx2* and *Eda* was unaffected by the loss of function β -catenin mutation, in line with data from *Dkk1*-expressing embryos (Figs. 5O, P and data not shown). Depletion of epithelial β -catenin resulted in loss of *Shh* and

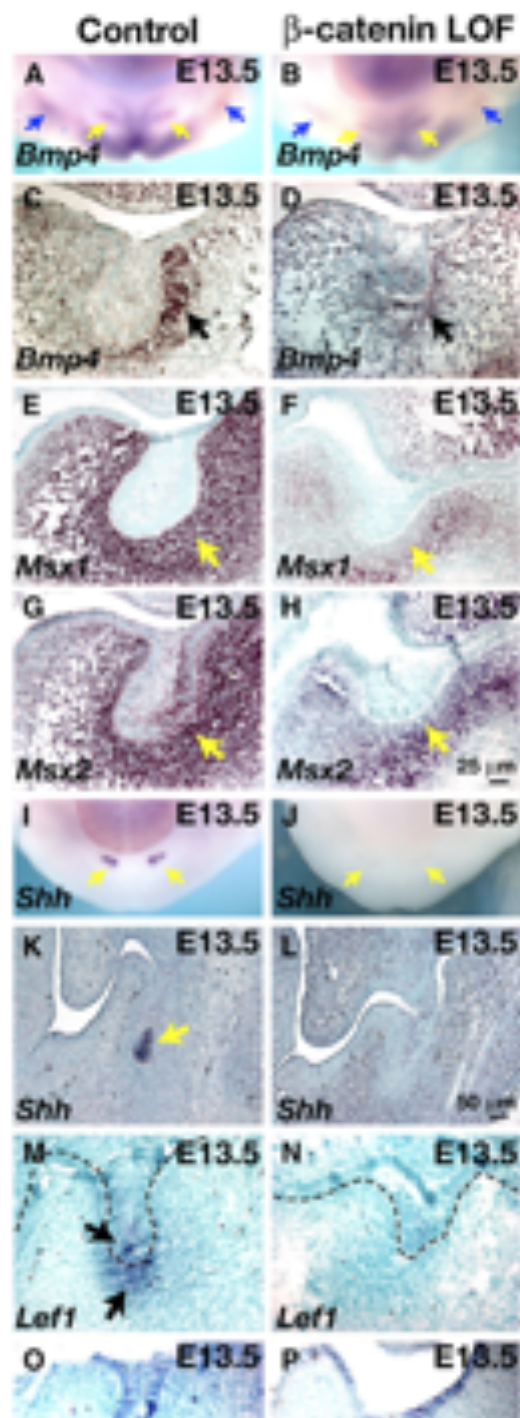


Fig. 5. Epithelial deletion of β -catenin affects expression of regulatory genes in dental epithelium and mesenchyme. (A–B) Mandibles dissected from littermate control (A) and *K14-Cre; Cxsb1^{fl/fl}* (β -catenin LOF) (B) E13.5 embryos and subjected to in situ hybridization with digoxigenin-labeled probe for *Bmp4*. (C–H) Transverse sections of oral cavities from littermate control (C, E, G) and *K14-Cre; Cxsb1^{fl/fl}* (β -catenin LOF) (D, F, H) E13.5 embryos subjected to in situ hybridization with the probes indicated. (I, J) Mandibles dissected from littermate control (I) and *K14-Cre; Cxsb1^{fl/fl}* (β -catenin LOF) (J) E13.5 embryos and subjected to in situ hybridization with digoxigenin-labeled probe for *Shh*. (K–P) Transverse sections of oral cavities from littermate control (K, M, O) and *K14-Cre; Cxsb1^{fl/fl}* (β -catenin LOF) (L, N, P) E13.5 embryos

Lef1 expression in dental epithelial cells (Figs. 5I–N). Interestingly, expression of *Lef1* was also downregulated in dental mesenchymal cells of the mutant at E13.5 (Figs. 5M, N), and mesenchymal *Bmp4* was reduced, although not entirely absent (Figs. 5A–D). Expression of *Msx1* and *Msx2* was reduced in mesenchymal cells immediately adjacent to dental epithelium of the β -catenin depleted mutant (Figs. 5E–H). As β -catenin is a cell autonomous molecule, these data suggest that epithelial β -catenin regulates mesenchymal expression of *Lef1*, *Bmp4*, *Msx1*, and *Msx2* indirectly. However, these results do not rule out the possibility that β -catenin signaling is additionally required within the mesenchyme for expression of these genes and tooth development.

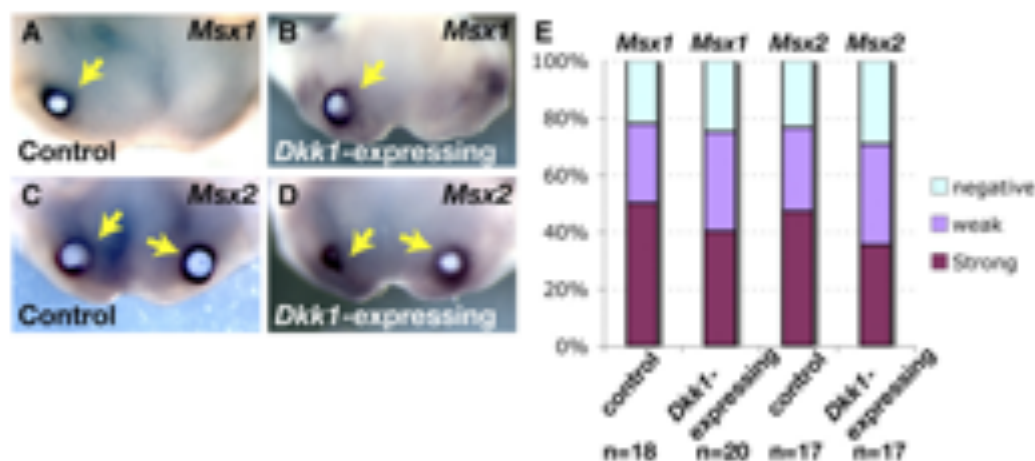
Wnt β -catenin signaling is not required for induction of *Msx1* and *Msx2* by exogenous BMP4

Bmp4 is known to regulate *Msx* gene expression in the developing tooth, and can also induce expression of *Lef1* (Kantochwil et al., 1996). Thus *Bmp4* could mediate the effects of Wnt signaling on *Lef1* and *Msx* gene expression. Alternatively, Wnt and *Bmp4* could coordinately regulate expression of *Lef1* and/or *Msx* genes. In line with the latter suggestion, LEF/TCF as well as SMAD binding sites are present in *Msx* gene promoter regions, and BMP and WNT factors synergistically activate *Msx* gene expression in human embryonic carcinoma cells and murine embryonic stem cells (Hassain et al., 2003; Wilkert et al., 2002). To distinguish among these scenarios in dental development, we asked whether *Dkk1* could block induction of *Msx* expression by exogenous BMP4. BMP4 protein coated beads were implanted into control and *Dkk1*-expressing mandibles. The tissue explants were cultured for 24 h in medium containing doxycycline to maintain *Dkk1* induction, and then subjected to *in situ* hybridization with *Msx1* and *Msx2* probes. BMP4- but not control BSA-coated beads caused robust *Msx* gene expression in immediately adjacent tissue in both control and *Dkk1*-expressing mandibles (Figs.

6A–E and data not shown). In additional experiments, beads for implantation were coated with BMP4 and BSA, or with BMP4 and recombinant DKK1. Recombinant DKK1 had no effect on the ability of BMP4 to induce *Msx* gene expression in control or *Dkk1*-expressing mandibles (data not shown). These observations indicate that *Dkk1* is not able to block the effects of exogenous BMP4, consistent with a model in which *Bmp4* functions downstream of Wnt activation to promote *Msx* gene expression, rather than acting synergistically with Wnt.

Disruption of Wnt β -catenin signaling from the early bell stage inhibits the formation of molar cusps

The TOPGAL and *BAT-gal* Wnt reporter transgenes are activated specifically in the secondary enamel knots (Fig. 1), and beads coated with the *Mfr1b1* Wnt inhibitor decrease molar tooth size in *ex vivo* experiments (Sarkar and Sharpe, 2000), suggesting involvement of Wnt β -catenin signaling in molar cusp development. To determine the requirements for Wnt β -catenin signaling in cusp development *in vivo*, *K5-rtTA terO-Dkk1* embryos and control littermates were exposed to doxycycline via treatment of the pregnant mothers starting at E16, a time point at which the molar teeth are at the late cap—early bell stages and secondary enamel knots are just beginning to form in the more advanced (first and second) molars. At these stages the *K5* promoter is active in inner and outer enamel epithelial cells and developing ameloblasts (Cascallana et al., 2005). Doxycycline treatment was continued for the remainder of pregnancy and pups were maintained on doxycycline after birth via their mothers' milk. Light microscopic analysis of mandibular molar teeth at P21 revealed that *Dkk1* induction from E16 caused development of cusps that were dramatically flattened, smaller, and irregular compared with those of control littermate molars (Fig. 7A). The second molars were more severely affected than the first molars, consistent with the later timing of second compared with first molar development.



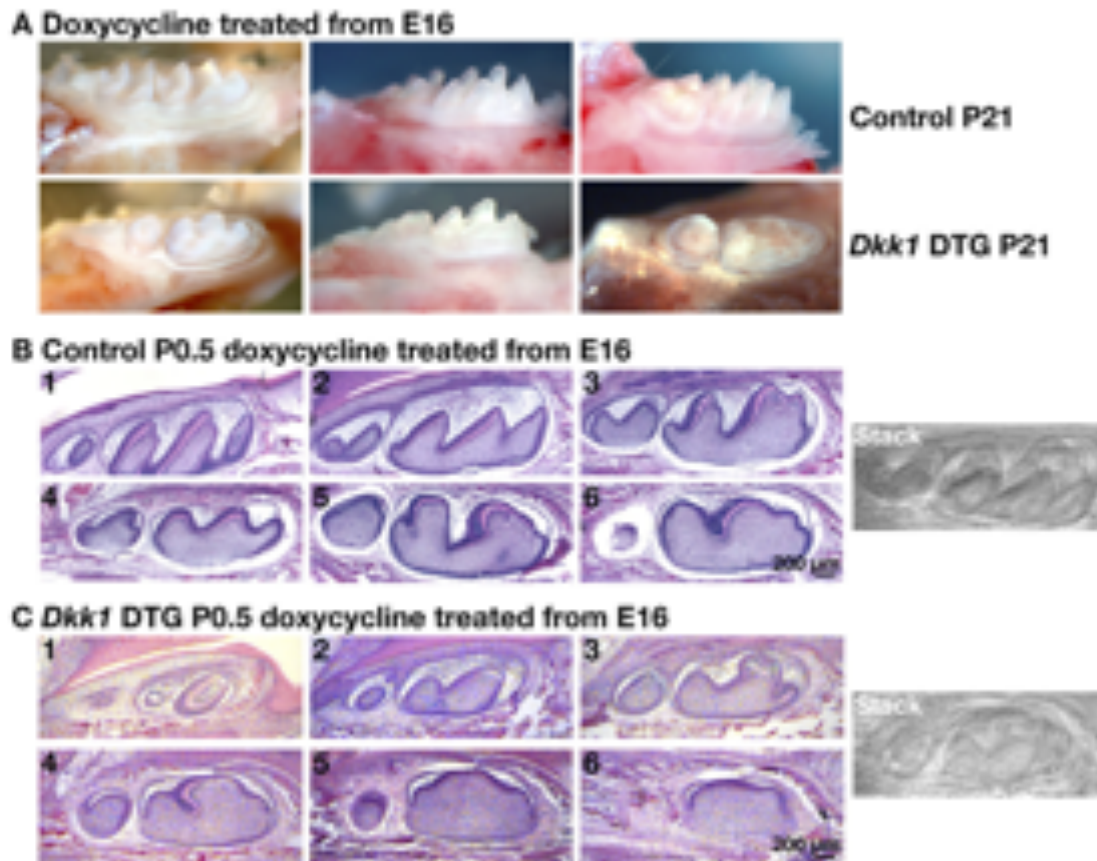


Fig. 7. *Wnt5*-catenin signaling is required for normal development of molar cusps. (A) Right mandibles dissected at P21 from control littermate and *K5-cre* *LoxP-Dkk1* (*Dkk1* DTG) mice doxycycline treated from E16. Note blunted cusp formation in *Dkk1* DTG molar teeth compared with controls. (B, C) Right mandibles from P0.5 control littermate (B) and *K5-cre* *LoxP-Dkk1* (*Dkk1* DTG) (C) mice doxycycline treated from E16.5, serially sectioned at 5 μ m in a sagittal plane from lingual to buccal side. Sections were stained with hematoxylin and eosin. The photographs shown were taken every 8 sections; each photo in each series is of a section 40 μ m from the previous photo. The images on the right represent stacked images of the serial sections, compiled using the Stack function of *ImageJ* software.

Histological analysis of serial sections showed that this phenotype was apparent in un-erupted *Dkk1*-expressing molars at birth (Figs. 7B, C). Ameloblast differentiation and enamel deposition were detected in *Dkk1*-expressing molars, but developing cusps were blunted and lacked the anterior-posterior asymmetry that was apparent in controls. These results indicate that *Wnt* inhibition from E16 represses development of molar cusps.

To determine the molecular basis for the effects of *Wnt* inhibition on molar cusp development, we examined the expression of signaling factors known to be important for cusp patterning. EDA/EDAR signaling has been shown to play a key role in cusp formation, and mice deficient in this signaling pathway display small enamel knots and decreased cusp sizes (Courtney et al., 2005; Laurikkala et al., 2001; Pispis et al., 1999). *Edar* mRNA is detected in inner enamel epithelium, stratum intermedia, and stellate reticulum at the bell stage (Tucker et al., 2000). We did not detect significant differences in the level or pattern of *Edar* expression in molars induced to

outer enamel epithelium in control E17.5 and newborn mice (Figs. 8E, F). Consistent with previous reports that *Eda* expression is *Wnt*-regulated (Laurikkala et al., 2001), we found that *Eda* expression was downregulated, although not absent, in molars induced to express *Dkk1* from E16 compared with control molars (Figs. 8E–H). Thus downregulation of EDA/EDAR signaling in *Dkk1*-expressing molars may contribute to the abnormal cusp phenotype.

Expression in developing molars of the TGF β inhibitor follistatin, a key regulator of cusp development (Wang et al., 2004), was unaffected by induced *Dkk1* (data not shown). *Bmp4* induces expression of the enamel knot marker *p27*, a cyclin-dependent kinase inhibitor (Jernvall et al., 1998), and is also implicated in ameloblast and odontoblast differentiation (Bloch-Zupan et al., 1998). *Bmp4* mRNA was detected in the dental papilla and proodontoblasts of control molars at E17.75, and in ameloblasts and odontoblasts at P0.5 (Aberg et al., 1997) (Figs. 8I, J). Expression of *Bmp4* at either stage was unaffected by *Dkk1* induction from E16 (Figs. 8K, L). By contrast, in

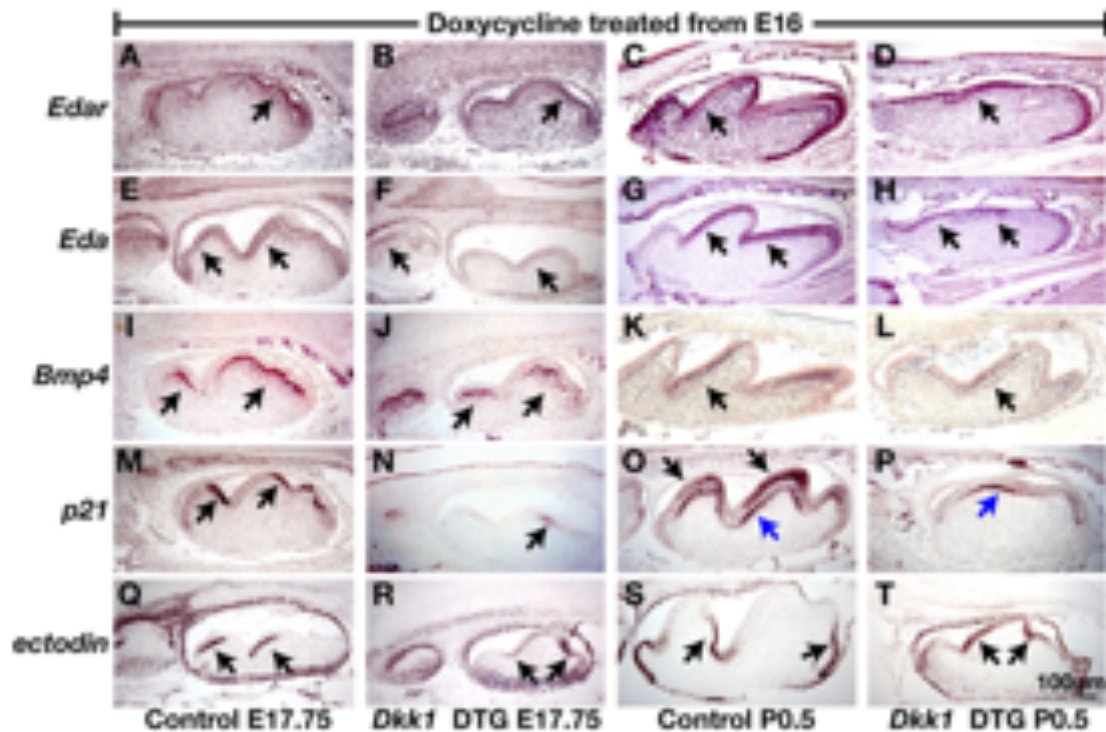


Fig. 8. *Wnt*/ β -catenin signaling is required for the maintenance of secondary enamel knots. Sagittal sections of oral cavities from control littermate (A, C, E, G, I, K, M, O, Q, S) and *K5-rtTA* *loxP-Dkk1* (*Dkk1*/DTG) (B, D, F, H, J, L, N, P, R, T) mice doxycycline treated from E16 and sacrificed at E17.75 (A, B, E, F, I, J, M, N, Q, R) or P0.5 (C, D, G, H, K, L, O, P, S, T) hybridized with digoxigenin-labeled probes for *Ectar* (A–D), *Ectd* (E–H), *Bmp4* (I–L), *p21* (M–P), and *ectodin* (Q–T). Positive signals (purple-brown) are indicated by arrows. In (D and F) black arrows indicate epithelial cells, blue arrows indicate developing odontoblasts. Scale bar in (T) applies to (A–T).

expressing teeth induced from E16, *p21* expression was downregulated in inner enamel epithelium at E17.5 and was absent from ameloblasts at P0.5. Interestingly, *Dkk1* did not affect expression of *p21* in odontoblasts, indicating that epithelial but not mesenchymal expression of *p21* is *Wnt*-dependent (Figs. 7O, P). Thus, decreased expression of *p21* in *Dkk1*-induced molars could contribute to failure of enamel knot and cusp development.

The BMP antagonist *ectodin* is expressed as a “negative image” of enamel knots and plays a key role in regulating cusp patterning (Kassai et al., 2005; Laurikkala et al., 2003). In *ectodin*-deficient mice, *p21* expression domains are expanded, molar tooth crowns are broader than normal, and buccal cusps are fused (Kassai et al., 2005). We found that in control molars the expression patterns of *p21* and *ectodin* were complementary in molar epithelial cells (Figs. 8M, O, Q, S), in agreement with previous data. In molars induced to express *Dkk1* from E16, domains expressing *ectodin* were expanded to include both sides of the developing molar cusps at E17.75, and the tips of the blunted cusps at P0.5 (Figs. 8R, T).

Discussion

regulating tooth development has been unclear. Here, using immunofluorescence detection of nuclear β -catenin, and three independent *Wnt*/ β -catenin reporter transgenic lines, we demonstrate that *Wnt*/ β -catenin signaling is active throughout tooth development. A gain of function mutation in epithelial β -catenin results in expanded expression of several key regulatory genes. Conversely, expression of these key dental regulators is disrupted when epithelial and mesenchymal *Wnt*/ β -catenin signaling is inhibited soon after tooth initiation in *Dkk1*-expressing embryos, resulting in arrested development at the early bud stage. Depletion of epithelial β -catenin produces a similar, albeit less severe, phenotype, demonstrating a requirement for epithelial *Wnt*/ β -catenin signaling at early stages of tooth development. The stronger phenotypes resulting from ectopic *Dkk1* expression compared with β -catenin depletion could be due to more efficient and earlier *Wnt* inhibition in *Dkk1*-expressing embryos, and/or to additional roles for β -catenin signaling within mesenchymal cells. We further demonstrate that inducible *Wnt* inhibition during molar cusp development results in defective cusp formation and loss of molar tooth polarity.

Arrested tooth development at the early bud stage in *Wnt*-inhibited mutants is unlikely to be due to the observed loss of

et al., 2002; Kiousi et al., 2002; Laurikkala et al., 2001), in situ hybridization signals for these mRNAs are similar in control and Wnt-inhibited epithelium at E12.5–E13.5 and NF- κ B signaling is unaffected by Wnt inhibition at this stage. Expression levels of *Pax9* are also similar in control and Wnt-inhibited dental mesenchyme. Instead, the mechanism underlying arrested development in *Dkk1*-expressing embryos appears to involve loss of expression of *Bmp4*, *Mx1*, and *Mx2*. Consistent with this model, tooth development arrests at a similar stage in *Mx1*^{-/-} *Mx2*^{-/-} mice (Bei and Maas, 1998) and Wnt-inhibited mice.

Although we observe nuclear β -catenin in dental mesenchymal cells immediately underlying the epithelium at E12.5–E14, this region only partially overlaps with the more extensive domains of *Bmp4*, *Mx1*, and *Mx2* in dental mesenchyme, suggesting that the requirement for Wnt signaling in regulation of these genes may be at least in part indirect. In support of this model, depletion of epithelial β -catenin results in decreased expression of mesenchymal *Lef1* and *Bmp4*, and reduced expression of *Mx* genes in mesenchymal cells immediately adjacent to the epithelium. Furthermore, ectopic *Dkk1* fails to block the ability of exogenous BMP4 to induce *Mx* gene expression, consistent with a mechanism in which *Bmp4* mediates *Mx* expression downstream of Wnt, rather than acting synergistically with Wnt.

Wnt/ β -catenin pathway activation in the secondary enamel knots of developing molar teeth suggested that, in addition to its roles at the lamina-early bud stage, Wnt signaling is also important for the development of molar cusps. Consistent with this hypothesis, induction of *Dkk1* expression in epithelial cells at the early bell stage resulted in blunted cusp formation. Interestingly, in contrast to the effects of Wnt inhibition at the early bud stage, *Dkk1* expression in these later stage teeth did not significantly affect expression of *Bmp4*. Expression of *Ets1*, a known regulator of cusp development, was reduced, although not eliminated, by ectopic *Dkk1*, suggesting that reduced *Ets1* may contribute to the cusp phenotype. In addition, we observed pronounced effects of Wnt inhibition on expression of the enamel knot marker *p21* and the *Bmp* and Wnt inhibitor *ectodis*. *p21* expression was markedly downregulated in the dental epithelium, although expression was observed in odontoblasts in newborn mice. Conversely, expression of *ectodis*, which is excluded from enamel knots, was expanded to include both sides and the tips of the blunted molar cusps. These results indicate that Wnt/ β -catenin signaling is necessary for maintenance of the enamel knots and plays a major role in determining tooth shape. These data further suggest that *ectodis* expression is normally suppressed in cells where the Wnt/ β -catenin signaling pathway is activated. Consistent with this, despite expanded *ectodis* expression in the β -catenin gain of function mutant, *ectodis* mRNA appears to be excluded from regions of the abnormal and ectopic teeth that exhibit expression of *TOPGAL* and *Wnt10b* (Fig. 2). As *ectodis* is

a BMP inhibitor, *ectodis* is also reported to block Wnt signaling in certain biological contexts (Itasaki et al., 2003). Thus *ectodis* and Wnt may compete to establish enamel knot and non-knot domains in the developing molar.

In summary, our data indicate that Wnt/ β -catenin signaling operates at multiple stages of tooth morphogenesis. While Wnt reporter transgene expression data and gain of function phenotypes suggest that Wnt/ β -catenin signaling can initiate the formation of dental placodes and pattern tooth development in the dental lamina, further experiments using earlier-acting promoters to drive expression of *Dkk1* or delete β -catenin in dental lamina cells will be necessary to determine whether or not Wnt/ β -catenin signaling is required for tooth initiation in vivo.

Interestingly, the effects of Wnt/ β -catenin signaling at the early bud stage appear to be mediated primarily through *Mx* and *Bmp4* gene expression, while suppression of molar cusp development does not involve *Bmp4* downregulation, indicating that different mechanisms are involved. As Wnt signaling inhibition and gain of function both result in the development of aberrantly shaped teeth, our data further indicate that Wnt activity must be tightly regulated for normal dental morphogenesis. Strategies to utilize activation of this pathway for tooth regeneration must thus include techniques for temporally and spatially limiting pathway activity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.ydbio.2007.10.016](https://doi.org/10.1016/j.ydbio.2007.10.016).

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