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RESEARCH ARTICLE

Mesenchymal Wnt/β-catenin signaling limits tooth number

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ABSTRACT

Tooth agenesis is one of the predominant developmental anomalies in humans, usually affecting the permanent dentition generated by sequential tooth formation and, in most cases, caused by mutations perturbing epithelial Wnt/β-catenin signaling. In addition, loss-of-function mutations in the Wnt feedback inhibitor *AXIN2* lead to human tooth agenesis. We have investigated the functions of Wnt/β-catenin signaling during sequential formation of molar teeth using mouse models. Continuous initiation of new teeth, which is observed after genetic activation of Wnt/β-catenin signaling in the oral epithelium, was accompanied by enhanced expression of Wnt antagonists and a downregulation of Wnt/β-catenin signaling in the dental mesenchyme. Genetic and pharmacological activation of mesenchymal Wnt/β-catenin signaling negatively regulated sequential tooth formation, an effect partly mediated by Bmp4. *Runx2*, a gene whose loss-of-function mutations result in sequential formation of supernumerary teeth in the human cleidocranial dysplasia syndrome, suppressed the expression of Wnt inhibitors *Axin2* and *Drapc1* in dental mesenchyme. Our data indicate that increased mesenchymal Wnt signaling inhibits the sequential formation of teeth, and suggest that *Axin2*/*Runx2* antagonistic interactions modulate the level of mesenchymal Wnt/β-catenin signaling, underlying the contrasting dental phenotypes caused by human *AXIN2* and *RUNX2* mutations.

KEY WORDS: Wnt/β-catenin, Axin2, Runx2, Sequential tooth formation, Hypodontia, Oligodontia, Tooth agenesis, Cleidocranial dysplasia

INTRODUCTION

Canonical Wnt/β-catenin signaling plays essential roles during tooth initiation and morphogenesis, as well as in dental cell differentiation (Liu et al., 2008; Lan et al., 2014; Balic and Thesleff, 2015). Wnts are short-range paracrine signaling molecules that regulate most developmental processes, as well as stem cell and tissue renewal (Clevers et al., 2014; Farin et al., 2016). During early tooth morphogenesis, Wnt ligands are expressed in the dental epithelium and signal predominantly within the epithelium (Sarkar and Sharpe, 1999; Liu et al., 2008). Wnt/β-catenin signaling is particularly intense in the epithelial signaling centers, including the early signaling centers, called initiation knots (IKs) (Ahtiainen et al., 2016) and the enamel knots (EKs), which regulate the budding and crown morphogenesis, respectively (Liu et al., 2008; Ahtiainen et al., 2016; Balic and Thesleff, 2015). There is also active β-catenin signaling in the mesenchyme underlying the early dental epithelium, which is necessary for normal epithelial morphogenesis (Liu et al., 2008; Fujimori et al., 2010; Balic and Thesleff, 2015) and for induction of odontogenic fate (Chen et al., 2009). However, elevated levels of Wnt/β-catenin signaling in the dental mesenchyme were shown to suppress odontogenic fate (Liu et al., 2013). Forced activation of Wnt signaling in oral epithelium results in continuous initiation of new teeth both in embryonic and adult mice (Järvinen et al., 2006; Liu et al., 2008; Wang et al., 2009), whereas mice overexpressing the Wnt inhibitor Dkk1 in dental epithelium display arrest of tooth development at the initiation stage (Andl et al., 2002). Collectively, these data strongly suggest Wnts to be the most upstream tooth induction signals.

Dental anomalies, in particular those affecting the tooth number, are among the most common developmental aberrations in humans. Predominantly, they are presented as reduced tooth number, which is known as tooth agenesis or hypodontia. In many cases, gene mutations causing the phenotype have been unraveled (Arte et al., 2013; Mues et al., 2014; Yin and Bian, 2015), but the underlying molecular mechanisms often remain unsolved. Severe tooth agenesis, or oligodontia, is frequently associated with various human syndromes, most commonly with defects in other ectodermal organs (Lefebvre and Mikkola, 2014; Plaisancié et al., 2013). Tooth agenesis is frequently caused by mutations in Wnt/β-catenin signaling pathway components, the most common gene being *WNT10A* (van den Boogaard et al., 2012; Arte et al., 2013; Plaisancié et al., 2013). Interestingly, heterozygous loss-of-function mutations in the Wnt feedback inhibitor *AXIN2* (Lustig et al., 2002; Jho et al., 2002) also leads to severe tooth agenesis (Lammi et al., 2004; Marvin et al., 2011; Wong et al., 2014; Yue et al., 2016; OMIM 608615), suggesting a negative role for Wnt signaling in tooth formation. Human *AXIN2* mutations particularly inhibited the formation of replacement teeth and posterior molars, but did not significantly affect the development of the deciduous (primary) dentition (Lammi et al., 2004). The *AXIN2* hypodontia phenotype implies that increased Wnt/β-catenin signaling may also suppress tooth formation. During tooth development, *Axin2* is expressed in the epithelial EKs and in the dental mesenchyme (Lammi et al., 2004). As the genetic activation of Wnt/β-catenin signaling in the mouse oral epithelium (Järvinen et al., 2006; Liu et al., 2008; Wang et al., 2009) induces the huge development of extra teeth, the failure of tooth formation in humans carrying *AXIN2* mutations is most likely caused by increased Wnt/β-catenin signaling in the mesenchyme.

An opposite dental phenotype is observed in cleidocranial dysplasia, a human syndrome in which heterozygous loss-of-function mutations in the transcription factor *RUNX2* cause the formation of multiple supernumerary teeth, or hyperdontia. The extra teeth develop as additional replacement teeth and posterior molars (Jensen and Kreiborg, 1990; Lee et al., 1997; Jaruga et al., 2016; OMIM 119600). During tooth development, *Runx2* function is regulated by Fgfs (Åberg et al., 2004), but studies on developing

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bone have indicated that Wnt/β-catenin signaling positively regulates Runx2 (Komori, 2011).

The human permanent dentition forms through a process called sequential tooth formation, in which a new tooth forms from the extension of dental lamina of the predecessor tooth (Juuri et al., 2013; Juuri and Balic, 2017). There are two modes of sequential tooth formation that both occur during formation of the human permanent dentition: tooth replacement, which generates the secondary teeth; and serial addition of teeth, which is how posterior molars are generated. Mice do not replace their teeth, but we have previously shown that the initiation of their posterior molars (M2 and M3) shares morphological as well as molecular characteristics with the initiation of replacement teeth in human and ferret embryos. In particular, they develop from Sox2-expressing progenitors in dental lamina (Järvinen et al., 2009; Jussila et al., 2014; Juuri et al., 2013). Thus, mouse M2 and M3 can be used as a model for studying sequential tooth formation and the molecular mechanisms of dental anomalies affecting the permanent dentition in human syndromes.

In the present work, we have examined the effects of Wnt/β-catenin signal modulation on sequential formation of molars in mouse models and in *ex vivo* cultures. We increased β-catenin expression *in vivo* by expressing a stabilized form of β-catenin, and *in vitro* by adding BIO, a GSK inhibitor, to cultured teeth *in vitro*. In addition to being an intracellular effector of Wnt signaling, β-catenin is also involved in cell adhesion by forming complexes with cadherins at cell membranes predominantly in epithelial tissues. However, the consensus in the β-catenin field seems to be that the experimental increase of cytoplasmic β-catenin mimics the effect of increased Wnt/β-catenin signaling (Fagotto, 2013; McCrea and Gottardi, 2016). We assume that the effects of increased β-catenin in the dental mesenchyme resulted from increased Wnt signaling, rather than from increased cell adhesion. This is supported by our finding that there are no obvious changes in the density of mesenchymal cells in histological sections of first molars cultured with BIO.

We demonstrate that the level of mesenchymal Wnt/β-catenin signaling is crucial for the initiation of the sequentially forming teeth: increasing Wnt/β-catenin activity in dental mesenchyme inhibited the development of posterior molars, whereas decreased mesenchymal Wnt/β-catenin was associated with their continuous development. Our results also suggest that, in humans, the congenital lack of teeth and the formation of supernumerary teeth caused by mutations in *AXIN2* and *RUNX2*, respectively, result from the modulation of Wnt/β-catenin signaling in dental mesenchyme.

RESULTS

Mesenchymal Wnt/β-catenin signaling is downregulated during continuous tooth formation in β-cat$^{\Delta ex3K14/+}$ mutants

We have previously shown that stabilization of β-catenin in the oral epithelium of mouse embryos (β-cat$^{\Delta ex3K14/+}$) results in continuous initiation of new teeth (Järvinen et al., 2006). Here, we used this mouse model to explore whether sequential tooth formation is associated with the level of mesenchymal Wnt signaling. Immunofluorescence analysis of phosphorylated β-catenin in control E16 molars showed intense β-catenin staining localized to cell surfaces in most epithelial cells (Fig. 1A). Nuclear staining was seen in the inner enamel epithelium, particularly at the sites of secondary EKs (Fig. 1A) and in the dental mesenchyme underlying the dental epithelium (asterisk in Fig. 1A). The analysis of sections of β-cat$^{\Delta ex3K14/+}$ mutant molars at E16 showed several foci of intense nuclear β-catenin staining (Fig. 1B), which is in line with our earlier demonstration of *BAT-gal* expression in these mutants (Järvinen et al., 2006). We have reported previously that these foci express several EK markers, including *Shh*, *Fgf4*, *Wnt10a* and *Edar* (Järvinen et al., 2006; Fig. 1C,D). These induced epithelial signaling centers likely represent the IKs described recently in the dental placodes at the time of tooth initiation (Ahtiainen et al., 2016) Interestingly, β-catenin staining was completely absent in the mesenchymal cells of β-cat$^{\Delta ex3K14/+}$ mutant molars (asterisk in Fig. 1B).

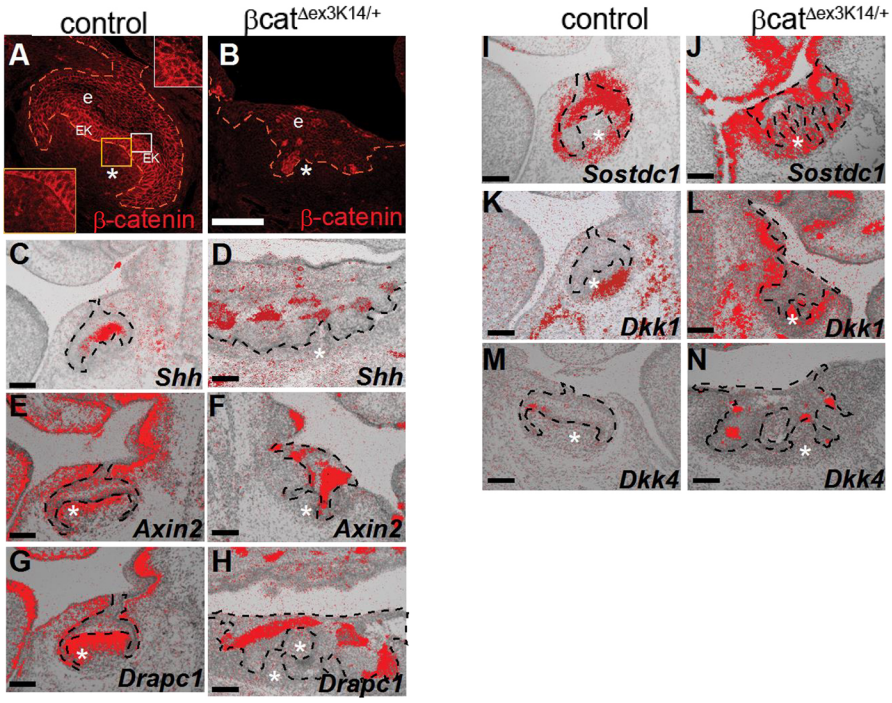

Fig. 1. Mesenchymal Wnt/β-catenin signaling is absent during continuous tooth formation in β-cat$^{\Delta ex3K14/+}$ mutants. (A,B) β-Catenin immunostaining. (C-N) *In situ* hybridization analysis of the expression of *Shh* and Wnt/β-catenin inhibitors in control and E16 β-cat$^{\Delta ex3K14/+}$ jaws. (C,D) *Shh*. (E-H) Feedback inhibitors (Wnt reporters): *Axin2* and *Drapc1*. (I-N) Inhibitors: *Sostdc1*, *Dkk1* and *Dkk4*. Scale bars: 100 µm. e, epithelium; EK, enamel knot; white asterisk indicates mesenchyme; the dashed line distinguishes the dental epithelium from the surrounding mesenchyme.

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Axin2 and *Drapc1*, targets and feedback inhibitors of Wnt/β-catenin signaling (Behrens et al., 1998; Shimomura et al., 2010), were expressed in dental papilla mesenchyme closest to the enamel epithelium in control teeth at E16, as demonstrated by *in situ* hybridization analysis (Fig. 1E,G). Expression of these genes was completely absent from the dental mesenchyme of the E16 β-cat$^{\Delta ex3K14}$ tooth germs (Fig. 1F,H), whereas in the epithelium they displayed intense and restricted expression domains corresponding to the induced epithelial signaling centers, i.e. IKs.

In situ hybridization analysis of Wnt inhibitors *Sostdc1* and *Dkk1* demonstrated their expression in the dental papilla of E16 control tooth germs, excluding its coronal (upper) part directly underlying the inner enamel epithelium, with *Sostdc1* also expressed in the mesenchyme of dental follicle surrounding the epithelial enamel organ (Fig. 1I,K). In the β-cat$^{\Delta ex3K14/+}$ mutants, the mesenchymal expression domains of these genes extended all the way to the dental epithelium, with *Sostdc1* also present in non-dental mesenchyme underlining the oral epithelium (Fig. 1J,L).

Another Wnt inhibitor, *Dkk4*, was expressed in the EKs in dental epithelium of the control teeth (Fig. 1M) and strong expression was seen in the induced IKs in the β-cat$^{\Delta ex3K14/+}$ mutants (Fig. 1N). The expression domains of β-catenin and the analyzed genes indicated that Wnt/β-catenin signaling is inhibited in β-cat$^{\Delta ex3K14/+}$ mutant mesenchyme and that this inhibition is downstream of forced β-catenin signaling in epithelium. Hence, the downregulation of mesenchymal Wnt signaling may be essential for the continuous tooth formation in the β-cat$^{\Delta ex3K14/+}$ mutants.

Stimulation of Wnt/β-catenin signaling by BIO prevents the continuous development of teeth in β-cat$^{\Delta ex3K14/+}$ mutants

We next used the *ex vivo* organ culture system to test whether increased mesenchymal Wnt/β-catenin signaling can inhibit the continuous tooth formation in the β-cat$^{\Delta ex3K14/+}$ mutant mouse. We activated Wnt/β-catenin signaling in dissected tooth rudiments by adding the GSK inhibitor BIO to culture medium, thereby preventing β-catenin degradation. The molar tooth buds were dissected from E13.5 β-cat$^{\Delta ex3K14/+}$ mutant embryos and cultured for 6 days with or without BIO and photographed daily. As controls, E13.5 tooth buds from littermate controls were cultured for 6 days without BIO (Fig. 2A,D,G).

As previously shown (Järvinen et al., 2006), new teeth were continuously initiated in the β-cat$^{\Delta ex3K14/+}$ explants during culture in the control medium (Fig. 2B,E). In the presence of BIO, β-cat$^{\Delta ex3K14/+}$ explants showed inhibition of sequential tooth initiation in a dose-dependent manner, where a lower dose (2.0 µM) prevented tooth initiation in 5/12 explants and a higher dose (10 µM) prevented tooth initiation in all four explants (4/4) (Fig. 2C,F and data not shown). Activation of Wnt/β-catenin signaling was confirmed by immunostaining of nuclear β-catenin in the mesenchymal cells in the explants cultured with BIO (3.75 µM) (Fig. 2I). The β-cat$^{\Delta ex3K14/+}$ explants cultured in the control medium demonstrated intense focal immunostaining of nuclear β-catenin in the epithelial signaling centers of the forming supernumerary teeth, whereas β-catenin staining was totally absent in the mesenchyme (Fig. 2H). Together, these data indicate that increased mesenchymal Wnt/β-catenin signaling can inhibit tooth initiation.

Forced activation of Wnt signaling in embryonic mesenchyme inhibits the formation of the posterior molars M2 and M3

We next wanted to analyze the effect of elevated mesenchymal Wnt/β-catenin signaling on sequential tooth formation *in vivo*. The development of posterior mouse molars M2 and M3 represents the process of sequential tooth formation, and resembles tooth replacement both morphologically and molecularly (Juuri et al., 2013). We generated mice in which the expression of stabilized β-catenin was directed to the embryonic mesenchyme using the *Dermo1Cre* promoter. The βcat$^{\Delta ex3Dermo/+}$ embryos, however, died prenatally before the M1 reached the bud stage and prior to the initiation of the M2 development. Therefore, we dissected the developing tooth germs from mutant and control embryos at E12.5 and cultured them *ex vivo* to analyze M1 morphogenesis and M2 formation. While both control and mutant M1 tooth germs grew in culture, neither developed further than early bell stage, and M2 failed to form in both (not shown). This was most likely due to the early developmental stage at the onset of culture.

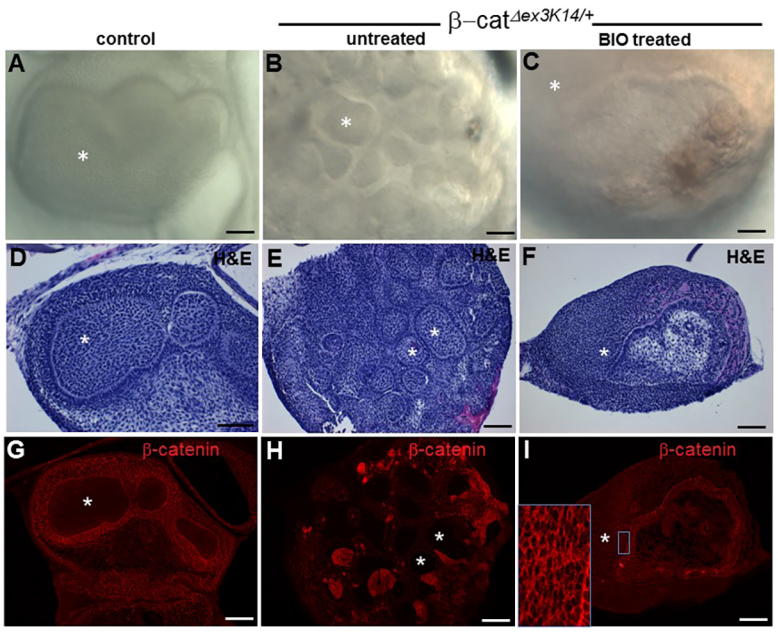

Fig. 2. Stimulation of Wnt/β-catenin signaling by BIO prevents the continuous formation of teeth in β-cat$^{\Delta ex3K14/+}$ mutants *ex vivo*. (A) Photograph of control E13.5 tooth bud cultured for 6 days. M1 and M2 are developing. (B) Multiple small teeth formed from one tooth bud of E13.5 β-cat$^{\Delta ex3K14/+}$ mutant embryo after 6 days of culture in control medium (*n*=9). (C) BIO (2.0 µM) prevented the formation of new teeth in a β-cat$^{\Delta ex3K14/+}$ mutant explant (*n*=11). (D-F) Histological sections of explants (Hematoxylin and Eosin staining). (G-I) Immunofluorescence localization of β-catenin. (G) β-Catenin is preferentially localized in epithelium, whereas no staining is seen in mesenchyme. (H) Intense focal staining of β-catenin is detected in the epithelium of the untreated β-cat$^{\Delta ex3K14/+}$ explant, but there is no staining in the mesenchyme. (I) Treatment with BIO leads to increased β-catenin staining in dental mesenchyme. Scale bars: 100 µm. White asterisk indicates mesenchyme.

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Next, we transplanted the dissected E12.5 control and βcat$^{\Delta ex3Dermo/+}$ mutant M1 tooth germs under the kidney capsule of adult nude mice and harvested the tissues after 3 weeks. The number of formed teeth varied. The control explants (n=9) formed one to three molars as follows: in one explant, all three molars; in three explants, two molars; in three explants, one molar; and in two explants, no teeth. The βcat$^{\Delta ex3Dermo/+}$ mutant explants (n=11) gave rise to one molar: in seven explants, only one molar was formed; and in four explants, no teeth formed (Fig. 3). Hence, the ability to generate more than one molar was only observed in the controls, thus suggesting that the activation of Wnt/β-catenin signaling in dental mesenchyme leads to the inhibition of sequential tooth formation.

Stimulation of Wnt/β-catenin signaling in embryonic molars by BIO inhibits the formation of M2

Embryonic lethality of βcat$^{\Delta ex3Dermo/+}$ mice impeded further analysis of forced Wnt/β-catenin signaling in the mesenchyme and its role in the sequential molar formation *in vivo*. We therefore used *ex vivo* organ cultures to examine the effect of increased Wnt/β-catenin signaling on the formation of M2 in control mice. M2 develops from the posterior extension of the M1 epithelium, also referred to as posterior dental lamina, posterior tail (tip) and continual lamina (Juuri et al., 2013; Gaete et al., 2015; Juuri and Balic, 2017). We dissected mouse embryonic M1 tooth germs at the stage when M2 is initiated (E14.5) and the continual lamina, which indicates the starting point of M2 development, is clearly visible (Fig. 4A). The explants were cultured and their development in the presence or absence of BIO was followed over the course of 6 days.

We used *Lef1*-reporter mice to facilitate the follow up of the molar development during culture. The morphogenesis of tooth crown is associated with the emergence of *Lef1*-expressing foci, i.e. primary and secondary EKs, marking crown initiation and tooth cusp formation, respectively (Fig. 4A,B). In control medium, M2 formed by day 6 in most explants (32/40) (yellow arrow in Fig. 4B). Addition of BIO inhibited the formation of M2 in a dose-dependent manner. At high concentrations of BIO (3.75 µM-15.0 µM), M2 development was inhibited in all explants (31/31), and at low concentration (2.0 µM) in most explants (29/37) (yellow arrow in Fig. 4D). M1 displayed aberrant crown morphogenesis at high concentrations of BIO, and at the low concentration (2.0 µM) the crowns were smaller than in controls, and cusps were shallow (Fig. 4D). Hence, 2.0 µM BIO was used in the following experiments.

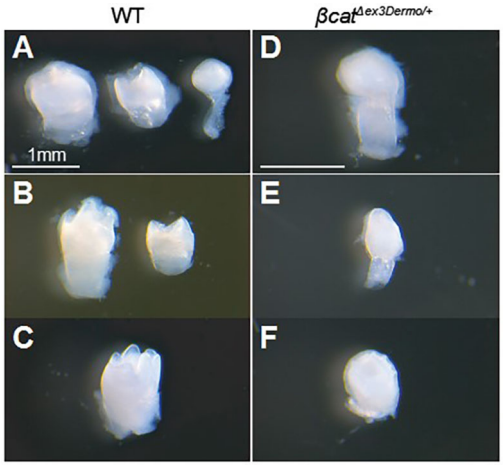

Fig. 3. Forced activation of Wnt/β-catenin signaling in embryonic mesenchyme (βcat$^{\Delta ex3Dermo/+}$) inhibits the formation of M2 and M3. (A-F) Teeth that developed from control and βcat$^{\Delta ex3Dermo/+}$ mutant molar tooth buds. Molar tooth buds were dissected from E12.5 embryonic jaws and grown under the kidney capsule of nude mice for 3 weeks. (A-C) Images of molars developed from the control tooth buds. One to three molars developed (n=9). (D-F) Images of teeth developed from the mutant explants. Only M1 developed in the mutants (n=11). Scale bars: 1 mm.

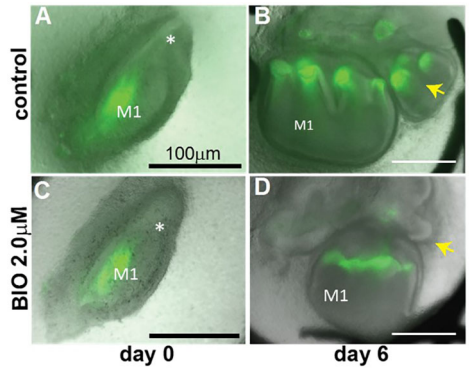

Fig. 4. Stimulation of β-catenin signaling *ex vivo* inhibits the formation of M2. (A,C) E14.5 molars (M1) from TCF/Lef:H2B-GFP mouse embryos at the onset of culture. Asterisks indicate the continual dental lamina (tail) of M1 that gives rise to M2. (B) M2 formed in control medium after 6 days of culture (arrow) (n=32). (D) BIO (2.0 µM) inhibited the formation of M2 (arrow), whereas M1 is smaller with shallower cusps (n=29). Scale bars: 100 µm.

Axin2 is intensely expressed in dental mesenchyme during initiation and morphogenesis of M2, and the expression is increased by BIO

Next, we explored the downstream effectors and molecular mechanisms associated with the arrested tooth initiation caused by increased Wnt/β-catenin signaling. *Axin2* is a target and a well-established feedback inhibitor of the Wnt/β-catenin pathway, and human *AXIN2* mutations inhibit the formation of replacement teeth and posterior molars, which form through sequential tooth formation. Therefore, we decided to first examine the role of *Axin2* in M2 initiation and its possible contribution to the phenotypes we observed. We cultured wild-type E14.5 M1 tooth germs for 2 days, separated the M1 and M2 and performed real-time quantitative PCR (RT-qPCR) on control and BIO treated M2. The inhibition of M2 development by BIO was associated with a twofold increase in *Axin2* expression (Fig. 5A).

As previously reported (Lammi et al., 2004; Lohi et al., 2010), *Axin2* was expressed in dental mesenchyme and EKs during tooth morphogenesis (Fig. 5B). In sagittal sections of the jaws during M2 initiation (E14.5) *Axin2* expression was intense in dental mesenchyme underlying the epithelium (Fig. 5B). *Lef1*, a well-known marker for Wnt/β-catenin signaling was co-expressed with *Axin2* in dental mesenchyme (Fig. 5B). At E14.5 expression of *Axin2* was detected also in the mesenchyme underlying the posterior tail of M1 (continual lamina), i.e. at the location where M2 is initiated (Fig. 5B, asterisk). At E15.5 intense *Axin2* expression was present in M1 and M2 in the dental papilla mesenchyme underlying the dental epithelium and in M2 strong mesenchymal expression surrounded the entire dental epithelium and continued posteriorly under oral epithelium all the way to the upper molars (Fig. 5B). These results indicate that there is mesenchymal Wnt/β-catenin signal activity around the developing M2 epithelium, which is most likely amplified by BIO in our experiments.

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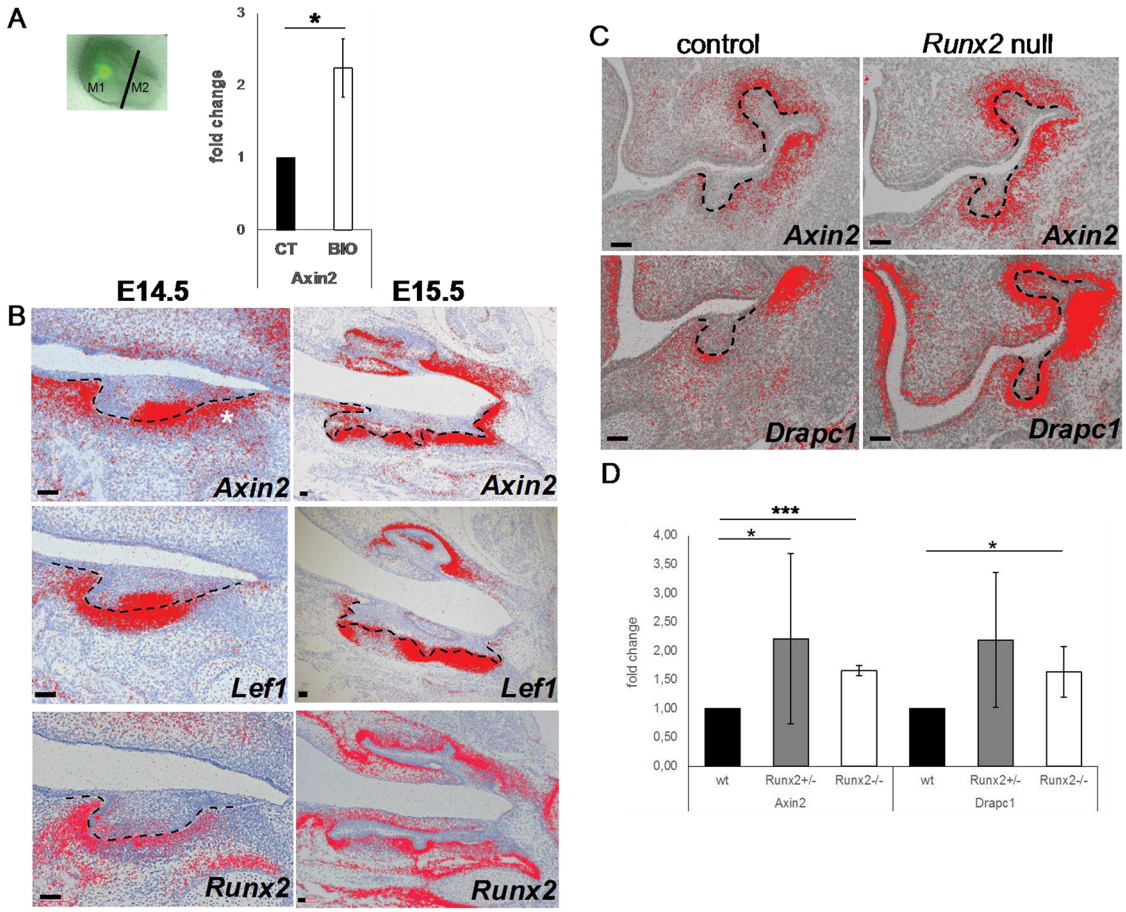

Fig. 5. *Axin2* and *Drapc1* expression is increased in *Runx2* mutant dental mesenchyme. (A) Separation of E14.5 M1 and M2 after 2 days of culture for RT-qPCR analysis. Stimulation of *Axin2* expression by BIO (2 µM) in M2 (*t*-test). (B) Expression of *Axin2*, *Lef1* and *Runx2* in sagittal sections at E14.5 and E15.5 in M1 and M2. Epithelial enamel knots express *Axin2*, but otherwise the expression is largely restricted to the mesenchyme. At E14.5, *Axin2* expression underlies the tail of M1 epithelium at the site where M2 is initiated (white asterisk). At E15.5, the mesenchymal dental papilla, the mesenchyme surrounding M1 and M2, and the mesenchyme underlying oral epithelium express *Axin2*. (C) Localization of *Axin2* and *Drapc1* in the mesenchyme of *Runx2*-null mutant and littermate control teeth at E13.5. The dashed line (B,C) distinguishes the dental epithelium from the surrounding mesenchyme. (D) *Axin2* and *Drapc1* expression is upregulated in the mesenchyme of *Runx2*-null mutant and *Runx2* heterozygote tooth germs at E13.5 (*t*-test). **P*<0.05, ****P*<0.0005. Scale bars: 100 µm.

Axin2-null mutant mice display no changes in the dentition and patterns of tooth regulatory genes

The *Axin2*-null mutant mice exhibit increased sensitivity to Wnt/β-catenin signaling and are widely used for Wnt pathway analysis. These mice display a rather mild defect in skull bone development (Yu et al., 2005). We analyzed the dentitions of adult *Axin2*-null mutant mice (n=10) under the stereomicroscope and did not detect any abnormalities when compared with littermate controls. All teeth were present and their size, shape, as well as enamel structure, appeared normal (Fig. S1). These findings indicate that, in the mouse (unlike humans), deletion of Axin2 function does not significantly affect tooth development.

We examined whether the loss of *Axin2* affected the expression of selected tooth regulatory genes by *in situ* hybridization analysis in embryonic *Axin2*-null mutant teeth. *Shh*, *Fgf4*, *Lef1*, *Fgf3*, *Dkk1*, *Runx2* and *Drapc1* were expressed in normal patterns and intensities in E13.5 and E14.5 molars (not shown). Interestingly, *Axin2*-null dental tissues were more susceptible to stimulation of Wnt/β-catenin signaling during differentiation of dentin and enamel-forming cells. When E13.5 *Axin2*-null mutant tooth explants were cultured in the presence of BIO (10 µM), the differentiation of epithelial cells to ameloblasts as well as mesenchymal cells to odontoblasts was accelerated (Fig. S2). Lack of morphological changes in the *Axin2* mutant mice suggests that the function of Axin2 may be compensated for by other Wnt antagonists or feedback inhibitors, including Drapc1.

The expression of *Axin2* and *Drapc1* colocalizes with *Runx2* and is upregulated in *Runx2*$^{-/-}$ and Runx2$^{+/-}$ dental mesenchyme

The phenotype caused by *AXIN2* mutations in the human dentition, i.e. severe tooth agenesis (oligodontia), affects preferentially sequentially forming teeth (Lammi et al., 2004) and is opposite to the phenotype resulting from *RUNX2* mutations causing sequential development of multiple supernumerary teeth in the cleidocranial dysplasia syndrome (Jensen and Kreiborg, 1990; Lee et al., 1997). Interestingly, *Axin2* has been indicated as a direct target gene of Runx2 in bone (Li et al., 2009; McGee-Lawrence et al., 2013). We compared the expression patterns of *Axin2* and *Runx2* (Fig. 5B) during M2 formation, and examined the expression of *Axin2* in *Runx2*-null mutant mice. *Runx2* showed apparent colocalization with *Axin2* in the mesenchyme surrounding M1 and M2 at E15.5 (Fig. 5B). As molar (M1) development is arrested in *Runx2* mutant embryos between E13 and E14 (before transition from bud to cap stage) (Åberg et al., 2004), we examined *Axin2* expression in *Runx2*-null mutants at E13.5. *In situ* hybridization

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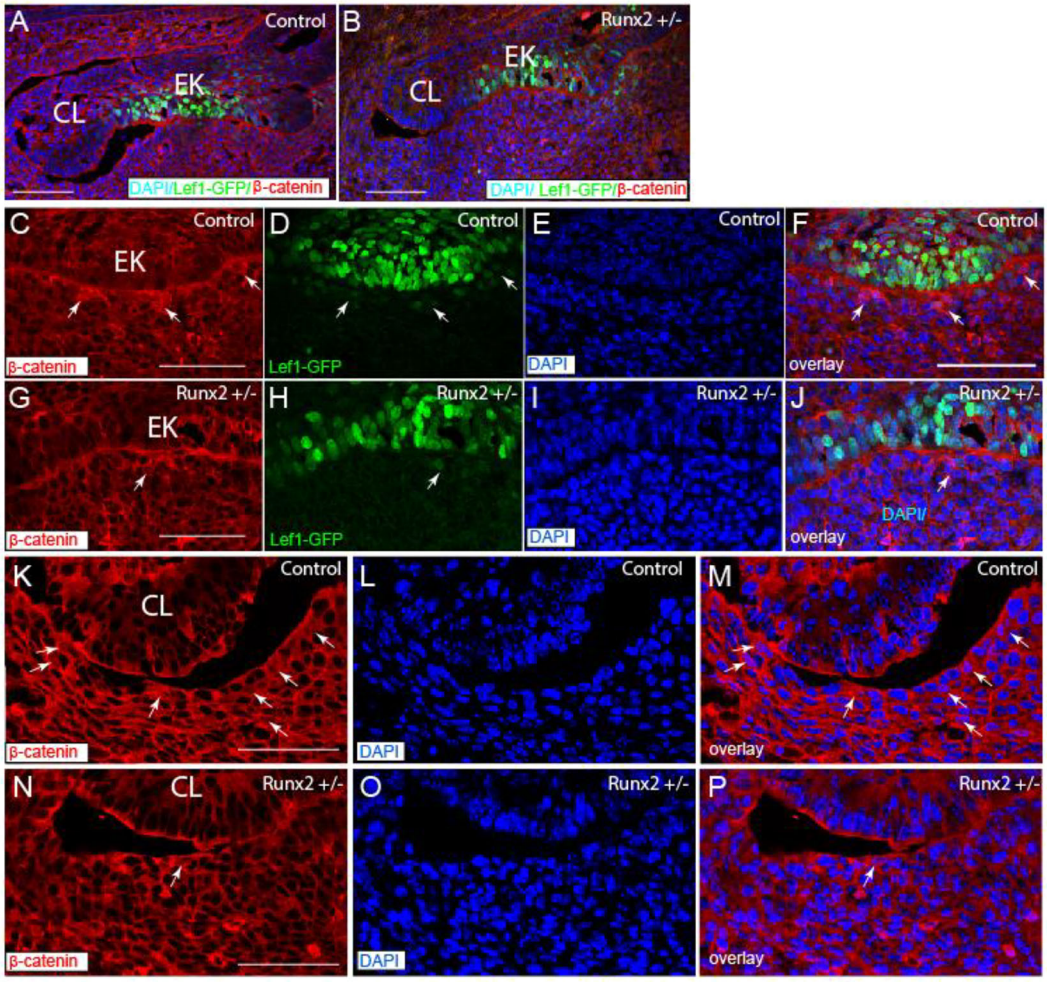

Fig. 6. Wnt/β-catenin signaling is inhibited in *Runx2* heterozygote in the mesenchyme under the enamel knot of M1 and the continual lamina forming the M2. (A,B) Lef1-GFP and nuclear β-catenin staining indicate the areas of active Wnt signaling in Runx2 control (A) and *Runx2*$^{+/-}$ (B). (C-F) Higher magnification of control tooth with active Wnt signaling in the mesenchyme under the enamel knot (arrows). (G-J) *Runx2*$^{+/-}$ with less Wnt signaling in mesenchymal cells under the enamel knot (arrow). (K-M) Under the control continual lamina, there are few cells with active Wnt signaling (arrows). (N-P) Under *Runx2*$^{+/-}$ continual lamina, there are no cells with active Wnt signaling (arrows). EK, enamel knot; CL, continual lamina. Scale bars: 100 µm.

analysis indicated a slightly higher expression of *Axin2* in the *Runx2*-null mutant teeth when compared with littermate control teeth (Fig. 5C). RT-qPCR analysis of *Axin2* expression in the E13.5 Runx2-null dental mesenchyme demonstrated that *Axin2* was expressed at significantly higher levels (P=0.0005) in the *Runx2* mutant dental mesenchyme compared with the littermate controls (Fig. 5D).

Like *Axin2*, *Drapc1* is a feedback inhibitor of β-catenin signaling and its expression pattern was similar to *Axin2* in control and *β-cat*$^{\Delta ex3K14/+}$ tooth germs (Fig. 1). Therefore, we examined whether the expression of *Drapc1* is regulated by Runx2. Indeed, both *in situ* hybridization and RT-qPCR analysis indicated increased expression of *Drapc1* in the mesenchyme of *Runx2*-null mutant tooth germs when compared with controls (P=0.035) (Fig. 5C,D).

To examine the function of Runx2 during sequential tooth formation, we analyzed Wnt/β-catenin signaling activity at the time of M2 initiation in *Runx2* heterozygotes, as the arrest of M1 morphogenesis at bud stage disables this analysis in *Runx2*-null embryos. qPCR analysis demonstrated significant increase of *Axin2* and *Drapc1* expression in the mesenchyme of *Runx2* heterozygous teeth at E14.5, suggesting increased Wnt/β-catenin activity. We next localized Wnt/β-catenin signaling activity, using Lef1-GFP reporter and nuclear β-catenin staining, in histological sections at E14.5 in *Runx2* heterozygotes and control littermates (Fig. 6). In the control teeth, cells with active Wnt signaling were detected in the mesenchyme adjacent to the EK (Fig. 6C-F), and only a few cells with active Wnt signaling were detected under the continual lamina (Fig. 6K-M). In contrast, in the heterozygote teeth the number of cells with active Wnt signaling was significantly lower (Fig. 6G-J), particularly under the continual lamina where no cells with active Wnt signaling were detected (Fig. 6N-P).

β-Catenin signaling enhances Bmp signaling and Bmp4 inhibits the formation of M2

The absence of tooth phenotype and unchanged expression of various Wnt/β-catenin pathway-associated genes in *Axin2*-null mutant mice suggests that other signaling pathways might also be involved. We decided to analyze the expression of selected genes in the BIO-treated (2 µM) tooth explants to pinpoint the signaling pathways associated with arrested M2 formation. Based on previous literature on tooth initiation, and our recent work indicating that increased mesenchymal β-catenin signaling is mediated by Fgf10 in the tooth stem cell niche, we focused on Fgf and Bmp pathways (Jussila and Thesleff, 2012; Yang et al., 2015). We examined the

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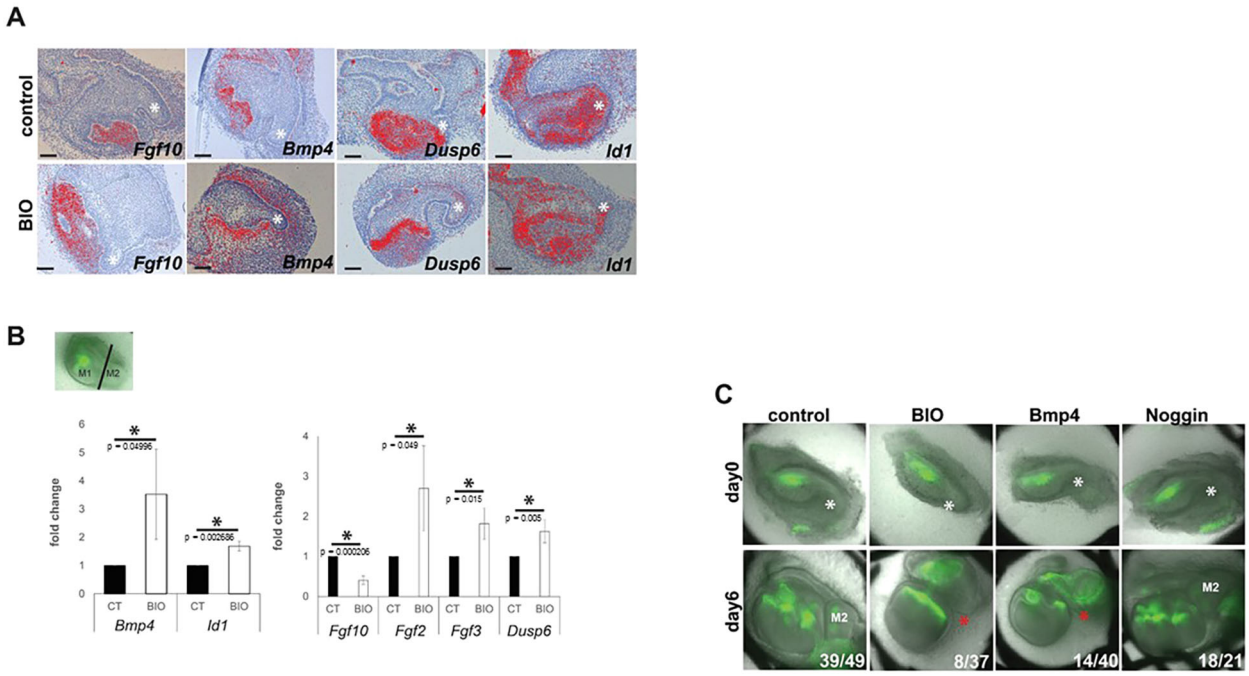

Fig. 7. Effects of BIO on Bmp and Fgf signaling, and on M2 formation. (A) *In situ* hybridization analysis of *Fgf10*, *Bmp4*, *Dusp6* and *Id1* expression in E14.5 molars after 2 days of culture with and without BIO. No major differences were detected between the control and BIO-exposed cultures in the expression patterns and intensities. Scale bars: 100 µm. (B) After 1 day of culture, the M1 and M2 were separated for RT-qPCR analysis of the effects of BIO on gene expression in M2. *Bmp4*, *Id1*, *Fgf2*, *Fgf3* and *Dusp6* expression was increased in BIO-treated M2 explants, whereas *Fgf10* expression was decreased (*t*-test). **P*<0.05. (C) E14.5 molars from *Tcf/Lef1* reporter mouse embryos were cultured with and without BIO (2.0 µM), Bmp4 (100 ng/ml) and Noggin (300 ng/ml) for 6 days to examine the development of M2. The numbers of formed M2/total number of explants are indicated in the figure. BIO and Bmp4 inhibited the formation of M2 (red asterisk), whereas Noggin had no effect. The sites of the continual dental lamina are marked with white asterisks.

BIO-treated and control explants (*Lef1*-reporter mice) after 2 days of culture for the expression of candidate ligands and targets. *In situ* hybridization analysis of E15.5 control cultures showed that *Fgf10* and *Bmp4* were expressed in the dental mesenchyme, whereas their respective targets *Dusp6* and *Id1* were expressed in the epithelium (Fig. 7A and not shown), but there was no obvious difference in the expression intensities between the BIO-treated and control explants. Therefore, we performed RT-qPCR on control and BIO-treated M2 after 2 days of culture (Fig. 7B). Increased levels of *Bmp4* and *Id1*, and downregulation of *Fgf10* expression was detected in BIO-treated M2 (Fig. 7B). In addition, *Fgf2*, *Fgf3* and the Fgf target *Dusp6* were upregulated (Fig. 7B).

These data indicated that Bmp signaling might have an agonistic role with Wnt/β-catenin signaling on the initiation of M2. To further analyze the effect of Bmp4 on M2 development, we used explant cultures of E14.5 tooth germs and treated them with recombinant Bmp4 protein (100 ng/ml). M2 formed in the majority of explants grown in control medium for 6 days (39/49), but in only 14/40 of Bmp4-treated samples (Fig. 7C). This was comparable with the frequency of M2 development in BIO-treated samples. The Bmp inhibitor Noggin (300 ng/ml) did not have a significant effect on M2 formation (18/21, Fig. 7D). When added together with BIO, Noggin did not rescue M2 formation (not shown).

DISCUSSION

Increased Wnt/β-catenin signaling in dental mesenchyme inhibits sequential tooth formation

Wnt/β-catenin signaling in the oral epithelium is generally regarded as the most upstream inducer of the initiation of tooth development (Järvinen et al., 2006; Liu et al., 2008; Wang et al., 2009). Using *in vivo* and *ex vivo* mouse models, we have now demonstrated that the stimulation of Wnt/β-catenin signaling in the dental mesenchyme has the opposite, i.e. inhibitory, effect on the initiation of sequentially developing teeth (also known as successional teeth). Genetic activation of mesenchymal Wnt/β-catenin signaling, in $\beta cat^{\Delta ex3Dermo/+}$ mice, had a negative effect on the sequential formation of mouse molars. M2 and M3 failed to develop in all $\beta cat^{\Delta ex3Dermo/+}$ tooth explants, indicating that increased mesenchymal Wnt signaling can prevent tooth initiation and that the posterior molars are more vulnerable to the effect. On the other hand, pharmacological activation of Wnt/β-catenin signaling by the GSK inhibitor BIO inhibited sequential tooth development and prevented formation of M2 in most of the cultured M1 tooth buds of control mice. In addition, BIO inhibited the initiation of new teeth in cultured M1 tooth buds of $\beta\text{-cat}^{\Delta ex3K14/+}$ embryos, which normally give rise to numerous teeth, further suggesting that these inhibitory effects were due to the stimulation of mesenchymal Wnt signaling.

The enhanced Wnt/β-catenin signaling induced by BIO in tooth explants also affected molar crown formation, detected as shallow cusps and a smaller crown, likely resulting from increased Wnt signaling in the epithelium. An epithelial effect of BIO on crown morphogenesis is also supported by the observation that similarly to the BIO-treated tooth germs, the supernumerary teeth of $\beta\text{-cat}^{\Delta ex3K14/+}$ mice are smaller than normal and have fewer cusps (Järvinen et al., 2006).

Decreased mesenchymal Wnt/β-catenin signaling is associated with stimulated sequential tooth formation and increased expression of Wnt inhibitors

The induction of continuous tooth formation by forced epithelial Wnt/β-catenin signaling in the $\beta\text{-cat}^{\Delta ex3K14/+}$ mouse (Järvinen et al.,

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2006) was associated with a dramatic downregulation of Wnt/β-catenin signaling in dental mesenchyme, as evidenced by the lack of nuclear localization of β-catenin and the striking absence of the Wnt/β-catenin reporters *Axin2* and *Drapc1*. The mechanism of this downregulation likely involves the induction of soluble epithelial proteins, which inhibit mesenchymal Wnt signaling either directly or by induction of Wnt inhibitors in the mesenchyme (Fig. 8). We have previously shown that supernumerary tooth initiation in β-cat$^{\Delta ex3K14/+}$ embryos is associated with induction of numerous epithelial signaling centers, identified by intensive expression of β-catenin and signaling molecules, including *Fgf4*, *Shh*, *Wnt10b* and *Bmp4* (Järvinen et al., 2006 and not shown). These induced signaling centers likely represent IKs, early signaling centers recently characterized in the incisor placodes (Ahtiainen et al., 2016).

We have now localized intense expression of the Wnt inhibitor *Dkk4* in the induced IKs. Dkk4 is a Wnt/β-catenin target gene (Zhang et al., 2009) and we suggest that it may diffuse to the mesenchyme to inhibit Wnt signaling in β-cat$^{\Delta ex3K14/+}$ teeth. The expanded expression domains of the Wnt inhibitors *Dkk1* and *Sostdc1* (also known as Ectodin and Wise) in the dental mesenchyme, which extended to the mesenchyme directly underlying the dental epithelium in β-cat$^{\Delta ex3K14/+}$ supernumerary teeth, further reflect increased epithelial β-catenin activity and downstream epithelial signaling from the IKs. *Dkk1* is regulated by Bmp4, Fgf4 and Shh in the tooth and jaw mesenchyme (James et al., 2006; Åberg et al., 2004; Ahn et al., 2010), and Bmp4 can induce *Sostdc1* expression in the tooth bud mesenchyme (Laurikkala et al., 2003). The Wnt inhibitors *Dkk2*, *Wif1* and *Sfrp2* have also been localized to dental mesenchyme and, interestingly, they were shown to be important for sequential tooth formation (Jia et al., 2013, 2016).

It is likely that the forced Wnt/β-catenin signaling in dental mesenchyme, both *in vivo* in βcat$^{\Delta ex3Dermo/+}$ mice and *ex vivo* after BIO administration, overrides the normal functions of the epithelium-induced Wnt/β-catenin inhibitors, thus perturbing the important fine-tuning of mesenchymal Wnt signaling induced by epithelial signals. BIO treatment seems also to affect the morphogenesis of crown epithelium of M1 by perturbing the formation and function of secondary EKs (SEKs) in the epithelium. SEKs express several signaling molecules (of all conserved pathways) and other signaling-related genes, and there is high Wnt activity in SEKs (as illustrated also by nuclear β-catenin and Lef1 in our explants). SEKs regulate the growth of the cusps and thereby also the size of tooth crown, and the small SEKs (and tooth crowns) in BIO-treated teeth indicate a negative effect of increased Wnt/β-catenin signaling on SEKs. This illustrates the complexity of the signaling networks and negative-feedback loops in EK signaling. When BIO was added to *Axin2*-null mutant teeth in culture, the differentiation of both mesenchymal odontoblasts and epithelial ameloblasts was accelerated (Fig. S2), further illustrating the multiple roles of Wnt signaling during tooth development.

Taken together, we have shown that increased mesenchymal Wnt/β-catenin signaling inhibits sequential tooth formation, while decreased mesenchymal Wnt/β-catenin signaling is associated with continuous tooth development. These results indicate that epithelial Wnt/β-catenin signaling, which initiates tooth formation and induces supernumerary tooth development, downregulates the activity of Wnt/β-catenin signaling in the mesenchyme via direct, as well as indirect, stimulation of Wnt inhibitors. We conclude that the level of mesenchymal Wnt signaling is crucial for the sequential initiation of teeth (Fig. 8).

Fine-tuning of mesenchymal Wnt/β-catenin signaling and interactions with other signaling pathways regulate tooth numbers

An 'inhibitory cascade model' has been constructed based on *ex vivo* analysis of the effects of signaling molecules on sequential mouse molar formation (Kavanagh et al., 2007). According to this model, the modulation of the balance of activators and inhibitors determines the sizes of M2 and M3. In particular, increasing the inhibition has a cumulative effect on the more posterior teeth, and the size of the earlier molar determines whether the next molar will form or not. Our results indicate that mesenchymal Wnt/β-catenin signaling is an inhibitor in this model, as increased mesenchymal Wnt/β-catenin signaling in the βcat$^{\Delta ex3Dermo/+}$ mutants did not prevent the development of M1, but inhibited the formation of M2 and M3. Although the increased β-catenin signaling induced by

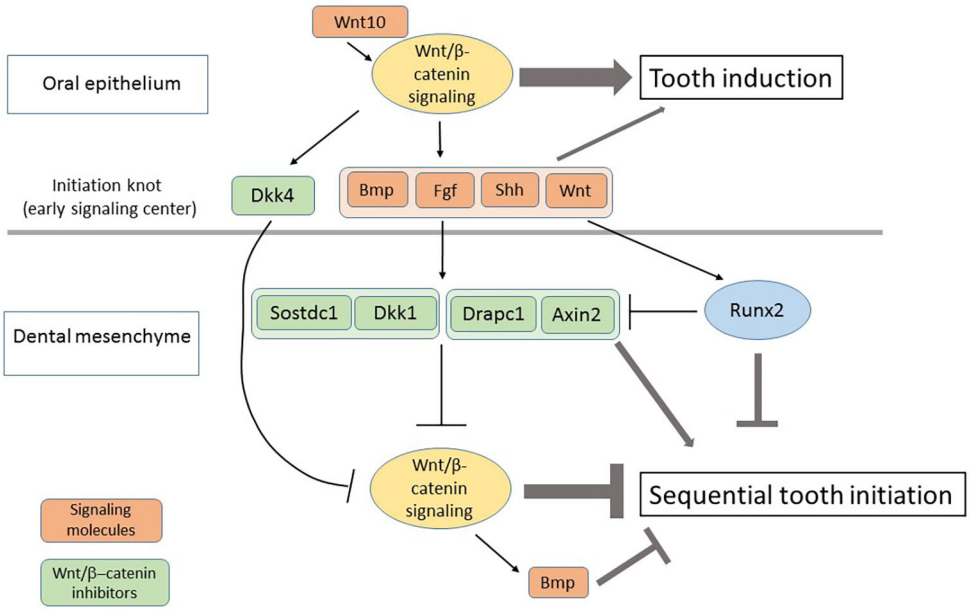

Fig. 8. Regulation of tooth numbers by Wnt/β-catenin signaling. Opposite effects of increased epithelial and mesenchymal Wnt/β-catenin signaling on tooth numbers. Suggested signaling networks regulating mesenchymal Wnt/β-catenin signaling and the proposed roles of Axin2 and Runx2 in this network, and their loss-of-function mutations in the generation of human hypodontia and supernumerary teeth, respectively. Epithelial Wnts and Wnt-induced epithelial signaling molecules induce Wnt inhibitors in the mesenchyme, thereby inhibiting the mesenchymal Wnt signaling and stimulating the initiation of sequential tooth development. The Wnt feedback inhibitor Axin2 triggers sequential tooth initiation by inhibiting Wnt/β-catenin signaling. Transcription factor Runx2 in the mesenchyme inhibits the Wnt inhibitors (including Axin2 and Drapc1) and thereby suppresses sequential tooth formation. The level of mesenchymal Wnt signaling has a key role in the initiation of the sequential teeth.

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BIO did not dramatically perturb the continued development of M1, it is likely that it affected signaling from M1 to M2. Taken together, we interpret our results as an indication of the inhibitory role of mesenchymal Wnt signaling in sequential tooth formation.

There are several mouse models in which the modulation of key signaling pathways compromises the sequential formation of molars. A classic example are the Eda-null *Tabby* mice, which lack one or more M3s in approximately half of the cases (Grüneberg, 1966). Eda is a target of Wnt/β-catenin signaling in epithelium and regulates other signaling pathways, including Shh, Fgf and Bmp in teeth (Lefebvre and Mikkola, 2014). The Eda target in the dental epithelium, *Fgf20*, has been indicated as an important activator of tooth formation in the inhibitory cascade model (Häärä et al., 2012). In addition, reducing the dose of *Pax9*, a mesenchymal transcription factor regulated by Fgf signaling (Neubüser et al., 1997), progressively reduces the number of molars: a small reduction of *Pax9* expression inhibited only M3 development, higher reduction inhibited both M3 and M2, and complete deletion of *Pax9* function prevented the formation of all three molars (Kist et al., 2005). In all these examples, the suppression of posterior molar formation results from inhibition of conserved signal pathways. However, our results indicate that stimulation of signaling pathways, in particular Wnt and Bmp, can also inhibit sequential tooth formation.

Wnt/β-catenin and Bmp signaling are intimately linked during all stages of tooth formation and they affect both epithelial and mesenchymal tissues (O'Connell et al., 2012). Crosstalk of Wnt and Bmp pathways in the dental mesenchyme is crucial for the early morphogenesis of mouse molars and their sequential formation (Jia et al., 2013). In the incisor, mesenchymal deletion of β-catenin led to the downregulation of *Bmp4* expression, which perturbed the integrity of the epithelial dental placode and led to the development of two thin incisors (Fujimori et al., 2010). We observed that the inhibition of M2 formation *ex vivo* by BIO resulted in upregulated expression of *Bmp4* in the dental mesenchyme, and of its target gene *Id1* in dental epithelium. In addition, Bmp4 prevented M2 formation similarly to BIO. However, the Bmp inhibitor Noggin did not rescue the BIO induced inhibition of M2 development, indicating that the effect of enhanced Wnt signaling was only partially mediated by Bmp.

Wnt/β-catenin appears to be a negative regulator of Fgf signaling in the dental mesenchyme. *Fgf10* expression was moderately downregulated in BIO-treated teeth, but surprisingly, the Fgf reporter *Dusp4* was upregulated. A possible explanation for this is compensation by Fgf2 and/or Fgf3, which were also upregulated by BIO. We have previously demonstrated that BIO inhibits *Fgf10* expression in the dental mesenchyme surrounding the cervical loop of the continuously growing incisor (Yang et al., 2015). This resulted in apoptosis of epithelial stem cells in the cervical loop, and in this case the effect of BIO could be rescued by Fgf10. Thus, mesenchymal Wnt/β-catenin signaling stimulates the expression of *Bmp4* and inhibits *Fgf10* expression both in incisors and molars.

Axin2, Runx2 and the sensitivity of teeth to enhanced mesenchymal Wnt/β-catenin signaling – parallels to human hypodontia and hyperdontia

In humans, heterozygous loss-of-function mutations in *AXIN2*, a direct Wnt target gene and feedback inhibitor, cause varying degrees of hypodontia (Lammi et al., 2004; Marvin et al., 2011; Wong et al., 2014; Yue et al., 2016). Our results suggest that this phenotype results from increased mesenchymal Wnt/β-catenin signaling. Similar phenotypes are also caused by mutations in the *WNT10A* gene, which constitute more than 30% of the tooth agenesis cases with known mutations (van den Boogaard et al., 2012; Arte et al., 2013; Arzoo et al., 2014; Mues et al., 2014; Yin and Bian, 2015), indicating a key role for Wnt10a in tooth development. During the initiation of mouse tooth development, the expression of *Wnt10a* and other Wnt ligands, as well as Wnt/β-catenin signaling activity, are mainly restricted to the epithelium (Dassule and McMahon, 1998; Liu et al., 2008), and stabilization of β-catenin signaling in the embryonic mouse oral epithelium leads to continuous initiation of new teeth (Järvinen et al., 2006). We demonstrate that this leads to dramatic downregulation of *Axin2* in the mesenchyme and increased initiation of teeth, suggesting that epithelial Wnt ligands function upstream of Axin2 (Figs 1F and 8).

The pattern of *Axin2* expression during sequential formation of mouse molars indicates Wnt signaling activity in the mesenchyme underlying the posterior tail of M1, or continual lamina, where M2 is initiated. We hypothesize that enhancement of β-catenin signaling in the mesenchyme at this site inhibits M2 formation. In human, the deciduous teeth are rarely missing, and the most commonly missing permanent teeth are those that develop last within a tooth class, including the third molar (M3), the second upper incisor (I2) and the second premolar (P2). These teeth are also most often affected in tooth agenesis caused by *AXIN2* and *WNT10A* mutations (Lammi et al., 2004; Arte et al., 2013; Arzoo et al., 2014; Yang et al., 2015). On the other hand, heterozygous loss-of-function mutations in *RUNX2* in human cleidocranial dysplasia syndrome stimulate sequential tooth development, resulting in excessive formation of replacement teeth and supernumerary posterior molars (Jensen and Kreiborg, 1990; Lee et al., 1997). As tooth replacement and molar addition are comparable developmental processes characterized by sequential initiation from the dental lamina associated with the previously formed tooth (Juuri et al., 2013), we hypothesize that mesenchymal β-catenin plays a similar inhibitory role in both processes.

To our knowledge, supernumerary tooth formation caused by *RUNX2* mutations has not been previously associated with Wnt signaling. We detected co-expression of *Runx2* and *Axin2* in mouse dental mesenchyme during the bud stage, as well as in association with M2 initiation, anticipating reciprocal regulatory mechanisms between these genes. Runx2 is essential for the mediation of Fgf signaling from the dental epithelium to mesenchyme at early stages of tooth development (Åberg et al., 2004). However, as arrested development at bud stage of *Runx2*-null mutant teeth was not rescued either by Fgf or Shh, we have suggested that other signaling pathways are involved (Åberg et al., 2004). We propose that Runx2 mediates Wnt signaling during the budding of the first teeth, as well as during sequential tooth formation. Previous studies have indicated a link between Runx2 and Wnt signaling during calvarial bone development and demonstrated that Runx2 regulates the repression of *Axin2*. *Axin2* was upregulated in Runx2-deficient mesenchymal cells, and Runx2 directly repressed *Axin2* (Li et al., 2009; McGee-Lawrence et al., 2013). In line with these studies, our analyses demonstrated that the expression of *Axin2* as well as the other Wnt feedback inhibitor *Drapc1* were upregulated in the *Runx2*-null mutant tooth buds in the mesenchyme surrounding the developing mouse molar.

Interestingly, both Axin2 and Runx2 dental phenotypes differ between human and mouse. Lack of any dental defects in the *Axin2*-null mice, which reportedly present normal gross phenotype with only mild calvarial bone defect (Yu et al., 2005), demonstrates a major difference between human and mouse in the sensitivity to reduced Axin2 function. It is plausible that, in mice, Axin2 function

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is compensated for by yet unknown regulatory mechanisms of Wnt/β-catenin signaling, e.g. *Drapc1*, another feedback inhibitor of Wnt/β-catenin signaling, which showed co-expression with *Axin2* in mouse tooth germs. The concurrent increase in the expression of *Axin2* and *Drapc1* in *Runx2*-null mutants supports the presence of a compensatory signaling loop between these two genes that could potentially explain the lack of tooth phenotype in *Axin2*-null mice. It can be speculated that in humans there may be less compensation by other Wnt inhibitor(s), explaining the reduction of tooth number in human *AXIN2* heterozygotes. In addition, in contrast to the human *RUNX2*$^{+/-}$ hyperdontia phenotype, *Runx2* heterozygote mice have no tooth phenotype, and *Runx2*-null mutant mice lack all teeth due to arrest of their development at the bud stage (D'Souza et al., 1999). Promotion of sequential tooth development in human *RUNX2* heterozygotes cannot be related to arrested M1 development in *Runx2*-null mice. The noted differences may result from the differences in the compositions and functions of human and mouse dentitions.

Taken together, our results indicate that mesenchymal Wnt/β-catenin signaling is a major negative regulator of sequential tooth formation, and that it limits tooth number. We propose that the continuous sequential development of supernumerary teeth caused by heterozygotic loss-of-function mutations in the human *RUNX2* gene is due to the lack of repression of *AXIN2* and other Wnt inhibitor(s), such as *DRAPC1* by RUNX2, leading to decreased mesenchymal Wnt signaling. It is also tempting to speculate that modulation of mesenchymal Wnt/β-catenin signaling by Runx2 and Wnt inhibitors, including Axin2 and Drapc1, may have played important roles in evolution when the potential for continuous tooth initiation was lost in most mammals.

Conclusions

In conclusion, our observations underline the importance of the fine-tuning of Wnt/β-catenin signaling for tooth initiation and demonstrate a link between epithelial and mesenchymal Wnt/β-catenin signaling activities in teeth (Fig. 8). These findings revealed molecular mechanisms underlying the opposite dental phenotypes of two human conditions with dental anomalies that affect the numbers of teeth. The results are potentially important for research on tooth regeneration and bioengineering. In particular the opposite consequences of increased Wnt/β-catenin signaling in epithelium versus mesenchyme are important: while high Wnt signaling in the epithelium stimulates tooth initiation, high mesenchymal Wnt signaling inhibits the initiation of sequentially forming teeth; in line with this, decreased mesenchymal Wnt signaling supports continued tooth formation.

MATERIALS AND METHODS

Animals

Generation of β-cat$^{\Delta ex3K14/+}$ mice has been described previously (Järvinen et al., 2006). *K14-cre* mice were a gift from Makoto M. Taketo (Kyoto University, Japan). *β-catenin*-flox-ex3 and *Axin2*-null mutant mice (Yu et al., 2005) were gifts from Walter Birchmeier (Max Delbruck Center, Berlin, Germany). βcat$^{\Delta ex3Dermo/+}$ embryos were generated by crossing *Dermo-cre* (*Twist2-cre*) mice with *βcat*-flox-ex3 mice (Harada et al., 1999). *Dermo-cre* mice were a gift from Christine Hartmann (Research Institute of Molecular Pathology, Vienna, Austria). *Runx2*-null mutant mice were a gift from Michael Owen (Imperial Cancer Research Fund, London, UK) (Mundlos et al., 1997; Åberg et al., 2004). Generation of *Lef1*-reporter mice (TCF/Lef:H2B-GFP) has been described elsewhere (Ferrer-Vaquer et al., 2010). Immunocompromised nude mice (Jackson Laboratory) were used in kidney capsule transplantation experiments.

Histology, immunofluorescence and radioactive *in situ* hybridization

Harvested tissues were fixed overnight in 4% paraformaldehyde, dehydrated and embedded in paraffin wax. Frontal and sagittal plane sections were cut at 5-7 µm and processed for histological analysis, immunofluorescence and radioactive *in situ* hybridization. Activated Wnt signaling was analyzed by immunofluorescence on sections that were treated with 10 mM sodium citrate buffer (pH 6.0) for antigen retrieval followed by incubation with mouse β-catenin antibody (BD Pharmingen) at 1:1000 dilution and AF568 anti-mouse secondary antibody (Invitrogen) at 1:500 dilution.

Radioactive *in situ* hybridization was performed using a standard protocol (Wilkinson and Green, 1990). [^{35}S]-UTP (PerkinElmer)-labeled RNA probes for the following genes were used to detect gene expression: *Axin2* (Lammi et al., 2004), *Drapc1* (Jukkola et al., 2004), *Dkk1* (James et al., 2006), *Sostdc1* (Laurikkala et al., 2003), *Shh* (Vaahtokari et al., 1996), *Fgf4* (Jernvall et al., 1994), *Lef1* (Travis et al., 1991), *Fgf3* (Järvinen et al., 2006), *Runx2* (James et al., 2006), *Fgf 10* (Yang et al., 2015), *Dusp6* (James et al., 2006), *Id1* (Rice et al., 2000), *Bmp4* (Vaahtokari et al., 1996) and *Dkk4* (Fliniaux et al., 2008).

Ex vivo organ culture and kidney capsule transplantation experiments

Molar teeth were dissected from E13.5 and E14.5 embryos in Dulbecco's PBS (pH 7.4) under a stereomicroscope and cultured for up to 6 days on nuclepore filters at 37°C in 5% CO_2 in a Trowell type organ culture. DMEM supplemented with 10% FCS (PAA Laboratories) was used as a basic medium. The GSK inhibitor BIO was added in concentrations ranging from 2 to 10 µM to cultures of molars of *Lef1*-reporter (TCF/Lef:H2B-GFP), β-cat$^{\Delta ex3K14/+}$ and *Axin2*-null mice. DMSO was added to control samples and the culture medium was changed every 2 days. Fluorescent and bright-field imaging under the stereomicroscope was used daily to follow and record M2 development. The effect of Bmp was studied by adding recombinant Bmp4 (100 ng/ml) and Noggin (100 and 300 ng/ml) (R&D Systems) to the cultures.

For kidney capsule transplantation, the M1 tooth buds were dissected from jaws of E12.5 βcat$^{\Delta ex3Dermo/+}$ embryos and implanted under the kidney capsule of nude mice. Implants were harvested after 3 weeks and processed for macroscopic analysis.

RNA extraction and RT-qPCR

Wild-type molar tooth germs were dissected at E14.5 and cultured *in vitro* for 2 days either with 2 µM BIO or in control medium. M1 and M2 were carefully separated, and M2 was used for RNA extraction. Developing molars were dissected from *Runx2*-null mutant and littermate controls at E13.5, and from *Runx2* heterozygotes and littermate controls at E14.5, and dental mesenchyme was separated for RNA extraction. RNA was extracted using the RNeasy Mini Kit (Qiagen) and transcribed to cDNA using Quantitec Reverse Transcription Kit (Qiagen) according to the manufacturer's guidelines. Quantitative PCR (RT-qPCR) was performed using Fast SYBR Green Master Mix (Thermo Fisher) and primers listed in Table S1. Data were normalized to *Hprt* housekeeping gene values. Results were analyzed for statistical significance using two- and one-tailed *t*-tests.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: E.J., I.T.; Methodology: E.J., J.S.-K., A.B., M.J., I.T.; Formal analysis: E.J., J.S.-K., A.B., M.J.; Investigation: E.J., J.S.-K., A.B., M.J., I.T.; Resources: I.T.; Writing - original draft: E.J., A.B., I.T.; Writing - review & editing: E.J., J.S.-K., M.J.; Visualization: E.J., A.B.; Supervision: I.T.; Project administration: I.T.; Funding acquisition: I.T.

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Supplementary information
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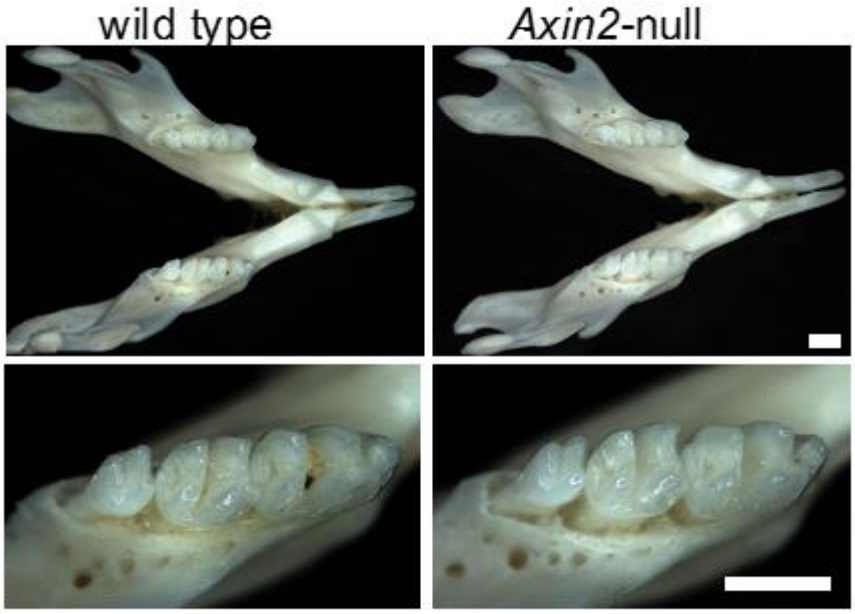
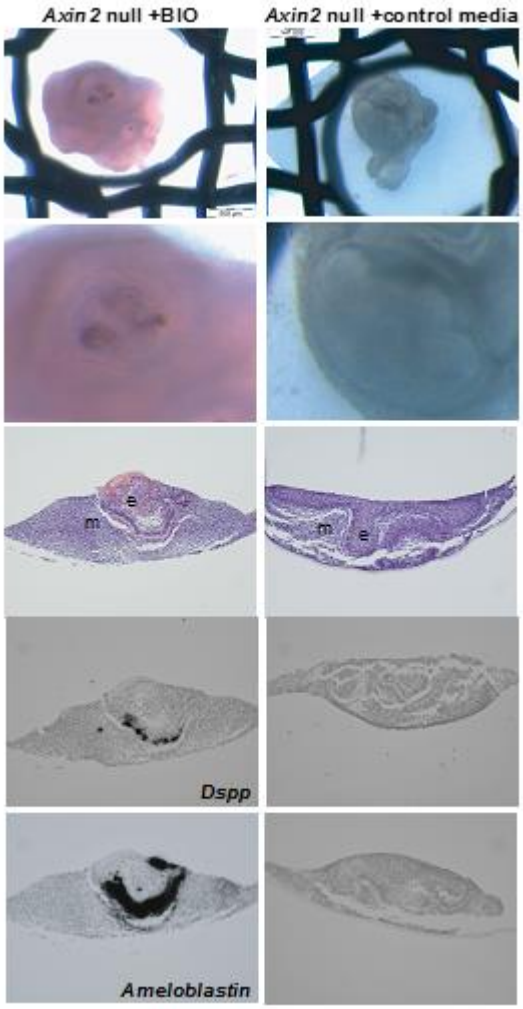


Supplementary Figure 1. *Axin2*-null mutant shows no tooth phenotype.



Supplementary Figure 2. Differentiation of dentin producing odontoblasts and enamel producing ameloblasts is accelerated by BIO (10μM) in E13.5 *Axin2*-null mutant teeth during 4 days in culture. Accelerated expression of dentin (*Dspp*) and enamel (*Ameloblastin*) in *Axin2*-null mutant. e: epithelium; m: mesenchyme.

Table S1. List of primers

Gene	Forward	Reverse
Hprt	CAGTCCCAGCGTCGTGATTA	TCGAGCAAGTCTTTCAGTCCT
Drapc1	ATGAACACCACCCTCCCATA	TCGAGCGTAGATGGTGAATG
Fgf10	CCGTACAGTGTCCTGGAGATAA	TTTCCCCTTCTTGTTCATGGCT
Runx2	TCGGAGAGGTACCAGATGGG	TGAAACTCTTGCCTCGTCCG
Axin2	ATAAGCAGCCGTTCGCGATG	TCATGTGAGCCTCCTCTCTTTTA
Id1	CTCGGAGTCTGAAGTCGGGA	GGAACACATGCCGCCTCG
Bmp4	CCTGCAGCGATCCAGTCTCT	ACTACGGAATGGCTCCATT
Fgf2	AGCGGCTCTACTGCAAGAAC	GCCGTCCATCTTCCTTCATA
Fgf3	TGCTTCGGATCACTACAACG	GGGCAGGAAGAGAGAGGACT