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ABSTRACT

Many ciliopathies have clinical features that include tooth malformations but how these defects come about is not clear. Here we show that genetic deletion of the motor protein Kif3a in dental mesenchyme results in an arrest in odontogenesis. Incisors are completely missing, and molars are enlarged in *Wnt1^{Cre+}Kif3a^{fl/fl}* embryos. Although amelogenesis and dentinogenesis initiate in the molar tooth bud, both processes terminate prematurely. We demonstrate that loss of Kif3a in dental mesenchyme results in loss of Hedgehog signaling and gain of Wnt signaling in this same tissue. The defective dental mesenchyme then aberrantly signals to the dental epithelia, which prompts an up-regulation in the Hedgehog and Wnt responses in the epithelia and leads to multiple attempts at invagination and an expanded enamel organ. Thus, the primary cilium integrates Hedgehog and Wnt signaling between dental epithelia and mesenchyme, and this cilia-dependent integration is required for proper tooth development.

KEY WORDS: Kif3a, epithelial-mesenchymal interaction, morphogenesis, odontogenesis, cell proliferation, neural crest cells.

Primary Cilia Integrate Hedgehog and Wnt Signaling during Tooth Development

INTRODUCTION

More than one-third of all birth defects involve craniofacial structures. Along with others, we have proposed that a subset of these craniofacial anomalies can be classified as ciliopathies – that is, their underlying etiology involves a disruption in the function of the primary cilium (Brugmann *et al.*, 2010b; Walczak-Sztulpa *et al.*, 2010; Khonsari *et al.*, 2013). Some ciliopathies have as secondary clinical features a disruption in the dentition (Ruiz-Perez *et al.*, 2003; Thauvin-Robinet *et al.*, 2006), and studies in mice support this observation: for example, the neural-crest-specific deletion of *Polaris* produces extra teeth (Ohazama *et al.*, 2009), and the loss of *EvC* and *EvC2* (Blair *et al.*, 2011) results in dental hypoplasia and molar fusions (Nakatomi *et al.*, 2013).

Primary cilia have been implicated in Hedgehog (Hh) (Rohatgi *et al.*, 2007) and Wnt signaling (Simons *et al.*, 2005; Gerdes *et al.*, 2007), and both pathways play critical roles in odontogenesis. For example, loss of *Sonic hedgehog* (*Shh*) inhibits the epithelial thickening that signals the onset of tooth formation, whereas gain of *Shh* function results in excessive invaginations of the dental epithelium and misshapen tooth buds (Hardcastle *et al.*, 1998). Excessive expression of β -catenin or lymphoid enhancer factor 1 (*Lef1*) in the epithelium causes the formation of supernumerary and ectopic teeth, respectively (Jarvinen *et al.*, 2006), and loss of Wnt signaling arrests tooth development (Chen *et al.*, 2009). Thus, Hh and Wnt signaling is important for odontogenesis, but how the pathways are integrated remains largely unknown.

Tooth development involves epithelial-mesenchymal interactions, and accumulating evidence indicates that the primary cilium is the centerpiece for such interactions (DeRouen and Oro, 2009). To test the role of the primary cilia in integrating Hh and Wnt signaling during odontogenesis, we generated *Wnt1^{Cre+}Kif3a^{fl/fl}* embryos, which lose the intraflagellar transport protein Kif3a specifically in dental mesenchyme.

MATERIALS & METHODS

Mouse Strains

The Stanford Committee on Animal Research approved all experimental procedures. *Wnt1^{Cre+}Kif3a^{fl/+}* were crossed with *Kif3a^{fl/fl}*, *Kif3a^{fl/fl}Axin2^{LacZ/LacZ}*, or *Kif3a^{fl/fl}Gli1^{LacZ/+}* to generate *Wnt1^{Cre+}Kif3a^{fl/fl}*, *Wnt1^{Cre+}Kif3a^{fl/fl}Axin2^{LacZ/+}*, or *Wnt1^{Cre+}Kif3a^{fl/fl}Gli1^{LacZ/+}* offspring, respectively. *Wnt1^{Cre-}* embryos served as controls. *Wnt1^{Cre+}Kif3a^{fl/+}* were crossed with *Kif3a^{fl/fl}* R26R^{LacZ/LacZ} to generate *Wnt1^{Cre+}Kif3a^{fl/fl}R26R^{LacZ/+}*, and *Wnt1^{Cre+}Kif3a^{fl/+}*

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R26R^{LacZ/+} served as controls. Timed matings were performed, and embryos were collected at indicated time points.

Sample Preparation, Processing, and Histology

Embryos were collected, fixed in 4% paraformaldehyde (PFA) at 4°C, dehydrated into ethanol, and embedded in paraffin. Eight-micron-thick coronal sections were cut. Movat's pentachrome and hematoxylin and eosin staining were performed (Kim *et al.*, 2007).

X-gal Staining

Embryos were fixed in 0.4% PFA and frozen in OCT media. Embryos were cut into 10- μ m-thick coronal sections. Sections were fixed with 0.2% glutaraldehyde and stained overnight at 37°C with 1 mg/mL Xgal solution (Invitrogen, Carlsbad, CA, USA).

Immunohistochemistry

Tissue sections were deparaffinized and blocked with 5% goat serum. Samples were incubated with primary antibodies including rabbit anti-Lef1 antibody (Cell Signaling, Beverly, MA, USA) and mouse anti-acetylated-tubulin antibody (Sigma, St. Louis, MO, USA). Sections were incubated with biotinylated secondary antibodies (Vector Labs, Burlingame, CA, USA) and then with avidin/biotinylated enzyme complex (Vector). To visualize immunoreactivity, we developed slides with 3,3'-diaminobenzidine tetrahydrochloride (Vector).

In vivo BrdU Labeling, Quantification, Statistical Analyses

Pregnant mice were injected intraperitoneally with BrdU labeling reagent (Invitrogen) and were euthanized 45 min later. BrdU labeling was detected with the BrdU Staining Kit (Invitrogen). Proliferating cells were quantified with graphic software (Adobe Photoshop CS5). The BrdU-labeling index was defined as (BrdU⁺ cells/total cells) $\times$ 100%. In the case of mesenchyme, the area of condensed mesenchymal cells surrounding the tooth buds was defined as the field of interest. Statistical analyses were performed with the Student's *t* test. Differences were considered statistically significant when *p* < .05.

In situ Hybridization

Sections were deparaffinized, rehydrated, and digested with 40 μ g/mL proteinase K. Sections were acetylated with 0.0025% acetic anhydride and hybridized with anti-DIG-labeled RNA probes. Slides were incubated with alkaline-phosphatase-conjugated anti-DIG antibody (Roche, Indianapolis, IN, USA) and developed with NBT/BCIP (Roche).

Renal Capsule Transplantations

Tooth buds from E13.5 embryos were harvested and implanted under the sub-renal capsule membrane as described in Bogden (1985). Two wk after surgery, mice were sacrificed.

Micro-CT Analysis

Transplanted tooth buds were harvested from kidneys, fixed in 4% PFA at 4°C, and processed in 70% ethanol. Micro-computed tomography (micro-CT) was carried out with MicroXCT-200 (Xradia, Pleasanton, CA, USA) with settings of 45 kV and 200 μ A. The tooth buds were scanned to produce 4.6- μ m/voxel size volumes. Sections were reconstructed with the XMReconstructor (Xradia), and Avizo Fire 7.1 software was used for morphometric quantification of dentin/enamel volumes.

RESULTS

Neural Crest Migration Does Not Depend upon an Intact Primary Cilium

We genetically deleted Kif3a in dental mesenchyme using the Wnt1^{Cre} deleter and then examined the craniofacial structures. Compared with Wnt1^{Cre}-Kif3a^{fl/+}R26R^{LacZ/+}, Wnt1^{Cre}-Kif3a^{fl/fl}R26R^{LacZ/+} showed no significant difference in the number of neural crest cells (Figs. 1A, 1B) and in the timing and direction of their migration into the pharyngeal arches (Figs. 1C, 1D; see Brugmann *et al.*, 2010a). The Wnt1^{Cre} deleter leads to *Cre* expression in the dental mesenchyme, which begins to invaginate (Fig. 1E) by embryonic day 12.5 (E12.5). Using antibodies to acetylated-tubulin, we confirmed the presence of primary cilia in controls (Fig. 1F) and then demonstrated that the number of primary cilia on mesenchymal cells was significantly reduced in Wnt1^{Cre}-Kif3a^{fl/fl} mutants (Fig. 1G).

Deletion of Kif3a Perturbs Mesenchymal Condensation

We examined tooth development starting at E12.5. Invagination of the molar dental epithelium in Wnt1^{Cre}-Kif3a^{fl/fl} mutants and Wnt1^{Cre}-Kif3a^{fl/fl} littermate controls was similar (Figs. 2A, 2B), which was expected since Kif3a was intact in the epithelium (Fig. 1). We found no evidence of incisor tooth buds, which could be related to the midline anomaly (Fig. 2C) that we previously reported (Brugmann *et al.*, 2010a).

In response to epithelial invagination, the dental mesenchyme condenses (Fig. 2A), but this response was poorly demarcated in Wnt1^{Cre}-Kif3a^{fl/fl} embryos (Fig. 2B). Compared with Wnt1^{Cre}-Kif3a^{fl/fl} controls, cell proliferation was reduced in Wnt1^{Cre}-Kif3a^{fl/fl} mesenchyme (Figs. 2D, 2E, quantified in 2F). Cell proliferation in the mutant dental epithelium, however, was equivalent to that in controls (Figs. 2E, 2F), which is in keeping with the deletion of Kif3a only in the mesenchyme. Thus, invagination of the dental epithelia proceeds normally, but mesenchymal condensation, which is dependent upon cell density (Hall and Miyake, 1995), is impeded in Wnt1^{Cre}-Kif3a^{fl/fl} embryos.

At E14.5, the enamel knots and cervical loops are evident, the dental mesenchyme condenses to generate the pulp, and alveolar bone development begins (Fig. 2G). Wnt1^{Cre}-Kif3a^{fl/fl} mutants had enamel knots but no obvious cervical loops, and their dental mesenchyme failed to condense (Fig. 2H). Alveolar bone formation, in contrast, was unaffected (Fig. 2H) despite obvious midline hypertrophy (Fig. 2I).

$Wnt1^{Cre+}Kif3a^{fl/fl}$ embryos do not survive past birth because of their midline craniofacial malformation (Brugmann *et al.*, 2010a). Therefore, we explanted E13.5 $Wnt1^{Cre+}Kif3a^{fl/fl}$ and $Wnt1^{Cre+}Kif3a^{fl/fl}$ tooth buds into the renal capsule to evaluate later stages of odontogenesis. Despite earlier disruptions in proliferation and condensation, $Wnt1^{Cre+}Kif3a^{fl/fl}$ tooth buds still produced odontoblasts that secreted mineralized primary and secondary dentin (Figs. 2J, 2K). The volume of the $Wnt1^{Cre+}Kif3a^{fl/fl}$ dentin, however, was significantly reduced (Figs. 2L, 2M; quantified in 2N). We then turned our attention to understanding the basis for this impaired dentin formation.

Kif3a Deletion in Dental Mesenchyme Causes a Loss of Hedgehog Signaling

Other groups have eliminated essential components of the primary cilia using the same $Wnt1^{Cre}$ deleter and attributed the odontogenic defects to either ectopic (Ohazama *et al.*, 2009) or reduced Hh signaling (Nakatomi *et al.*, 2013). We examined E12.5 embryos for changes in the expression of Hh target genes. *Patched* is normally expressed in dental epithelium and mesenchyme (Fig. 3A), but in $Wnt1^{Cre+}Kif3a^{fl/fl}$, *Patched* was reduced in the mesenchyme (Fig. 3B). The $Gli1^{LacZ/+}$ reporter line was used to confirm that Hh signaling is normally active in the dental epithelium and mesenchyme (Fig. 3C). However, in $Wnt1^{Cre+}Kif3a^{fl/fl}Gli1^{LacZ/+}$ embryos, *Gli1* expression was lost in the mesenchyme (Fig. 3D). Thus, both dental epithelia and mesenchyme are targets of Hh signaling, and deletion of *Kif3a* rendered the mesenchyme unresponsive to a Hh signal.

Kif3a Deletion in Dental Mesenchyme Causes a Gain in Wnt Signaling

At this point, a reduction in mesenchymal cell proliferation correlated with a reduction in Hh signaling, but primary cilia have also been implicated in the modulation of Wnt signaling. We examined E12.5 embryos and found that, relative to controls, expression of Wnt target *Lef1* was expanded in the $Wnt1^{Cre+}Kif3a^{fl/fl}$ dental mesenchyme (Figs. 3E, 3F). Recently, it was reported that replacing *Wnt1* with Cre recombinase leads to increased Wnt signaling in the brain (Lewis *et al.*, 2013). To ascertain whether Wnt signaling was elevated due to the replacement of *Wnt1* with Cre or was due to *Kif3a* deletion, we examined *Lef1* expression in the teeth of $Wnt1^{Cre+}$ and $Wnt1^{Cre-}$ embryos. We found that the replacement of *Wnt1* with Cre (*i.e.*, $Wnt1^{Cre+}Kif3a^{fl/fl}$) did not affect *Lef1* immunostaining in the dentition (Appendix Fig. 1). However, the removal of *Kif3a* in $Wnt1^{Cre+}Kif3a^{fl/fl}$ did affect *Lef1* expression (compare Appendix Figs. 1A and 1B with 1C and 1D and 1E with 1F).

We further confirmed that the *Kif3a* deletion resulted in increased Wnt signaling using $Axin2^{LacZ/+}$ reporter mice to visualize Wnt target gene *Axin2*. Compared with $Wnt1^{Cre+}Kif3a^{fl/fl}Axin2^{LacZ/+}$, the domain of *Axin2* in $Wnt1^{Cre+}Kif3a^{fl/fl}Axin2^{LacZ/+}$ mutants was significantly broader (Figs. 3G, 3H). This expanded Wnt response was also evident at later stages (Figs. 3I, 3J).

Figure 1. Primary cilia are disrupted in the neural crest-derived mesenchyme of the $Wnt1^{Cre+}Kif3a^{fl/fl}$ embryo. (A) Whole-mount Xgal staining of an E8.5 $Wnt1^{Cre+}Kif3a^{fl/fl}R26R^{LacZ/+}$ embryo. Neural crest cells (in blue) emerge from the dorsal part of the neural tube and migrate ventrally to the facial prominence and branchial arches. (B) Whole-mount Xgal staining of an E8.5 $Wnt1^{Cre+}Kif3a^{fl/fl}R26R^{LacZ/+}$ mutant. Neural crest cells of both mutants and controls show similar migration patterns. (C) Xgal staining of a section from an E8.5 $Wnt1^{Cre+}Kif3a^{fl/fl}R26R^{LacZ/+}$ control. Blue cells indicate the neural crest cells migrating into the first branchial arch. (D) The neural crest cells (in blue) of $Wnt1^{Cre+}Kif3a^{fl/fl}R26R^{LacZ/+}$ exhibit migration patterns similar to those of the $Wnt1^{Cre+}Kif3a^{fl/fl}R26R^{LacZ/+}$ control embryo. (E) Xgal staining of molar tooth from a $Wnt1^{Cre+}Kif3a^{fl/fl}R26R^{LacZ/+}$ embryo shows staining in the mesenchymal cells. (F) Acetylated α -tubulin immunofluorescence of an E12.5 $Wnt1^{Cre+}Kif3a^{fl/fl}$ control tooth section. Both the dental epithelial and mesenchymal cells have primary cilia (in red) labeled by acetylated α -tubulin, and cell nuclei are counterstained with DAPI. The dotted white lines indicate the separation between the dental epithelium (de) and dental mesenchyme (dm). (G) In the E12.5 $Wnt1^{Cre+}Kif3a^{fl/fl}$ mutant tooth bud, primary cilia (in red) are found only in the dental epithelial cells but not in the dental mesenchymal cells ($n = 3$). Cell nuclei are counterstained by DAPI. Frontonasal prominence (f), first branchial arch (I), second branchial arch (II), stellate reticulum (sr), cervical loop (cl), dental papilla (dp). Scale bars: 50 μ m (A, B), 10 μ m (C, D, F, G), 20 μ m (E).

Figure 2. Neural-crest-specific deletion of Kif3a perturbs the initial stage of odontogenesis. (A) Hematoxylin and eosin staining of an E12.5 Wnt1^{Cre}Kif3a^{fl/fl} molar tooth bud shows the invagination of the tooth epithelium (n = 10). The white dotted line outlines the developing tooth bud and separates the dental epithelium (de) from the dental mesenchyme (dm). (B) A similar invagination occurs in the Wnt1^{Cre}Kif3a^{fl/fl} mutant at E12.5 (n = 10). (C) At E12.5, Wnt1^{Cre}Kif3a^{fl/fl} embryos show a midline anomaly. (D) BrdU staining labels proliferating cells (in brown) in the tooth bud of E12.5 Wnt1^{Cre}Kif3a^{fl/fl} control and (E) Wnt1^{Cre}Kif3a^{fl/fl} embryos (n = 4). (F) Quantification of the percentage of BrdU+ cells in E12.5 molars. Wnt1^{Cre}Kif3a^{fl/fl} have reduced cell proliferation in the mesenchyme in comparison with the Wnt1^{Cre}Kif3a^{fl/fl} control, but cell proliferation in the epithelia is not perturbed in the Wnt1^{Cre}Kif3a^{fl/fl} mutant. (G) Hematoxylin and eosin staining of an E14.5 Wnt1^{Cre}Kif3a^{fl/fl} control tooth section (n = 3). Dental epithelium forms a cap-shaped structure. The arrow indicates the enamel knot. (H) E14.5 tooth bud of Wnt1^{Cre}Kif3a^{fl/fl} is misshapen (n = 3). (I) At E14.5, Wnt1^{Cre}Kif3a^{fl/fl} embryos exhibit a midline anomaly. (J) Pentachrome staining of the transplanted Wnt1^{Cre}Kif3a^{fl/fl} tooth with yellow staining indicating mineralized dentin in the control and in (K) Wnt1^{Cre}Kif3a^{fl/fl} (n = 3). Primary dentin (pd), secondary dentin (sd), odontoblasts (od). (L) Segmentation with 3D image shows dentin of the transplanted Wnt1^{Cre}Kif3a^{fl/fl} and (M) Wnt1^{Cre}Kif3a^{fl/fl} mutant tooth buds. (N) Quantification of the dentin volume of the transplanted tooth bud. Total dentin volume is greatly reduced in the Wnt1^{Cre}Kif3a^{fl/fl}. Maxillary prominence (mx), mandibular prominence (mn), frontonasal prominence (f), enamel knot (ek), alveolar bone (ab). Scale bars: 50 μ m (A, B, D, E, G, H), 5 μ m (J, K), 150 μ m (L, M), and 50 mm (C, I).

Figure 3. Loss of Kif3a perturbs the balance of Hedgehog and Wnt signaling in the dental mesenchyme. (A) *In situ* hybridization of *Patched* probe to an E12.5 $Wnt1^{Cre}Kif3a^{fl/fl}$ molar. *Patched* is expressed in both the dental epithelium and the mesenchyme ($n = 3$). (B) In the mutant, expression of *Patched* is greatly reduced in the dental mesenchyme ($n = 3$). (C) Xgal staining of tooth bud from the E12.5 $Wnt1^{Cre}Kif3a^{fl/fl}Gli1^{lacZ/+}$ control. Xgal signals (in blue) are detected in both the epithelium and mesenchyme. (D) Xgal staining of a tooth bud from the E12.5 $Wnt1^{Cre}Kif3a^{fl/fl}Gli1^{lacZ/+}$ embryo. LacZ expression is lost in the mesenchymal cells. (E) Lef1 immunostaining of an E12.5 $Wnt1^{Cre}Kif3a^{fl/fl}$ tooth bud. Lef1 is expressed in both the dental epithelium and the condensed mesenchyme ($n = 3$). (F) The Lef1 expression domain in the dental mesenchyme of the $Wnt1^{Cre}Kif3a^{fl/fl}$ is expanded when compared with that in $Wnt1^{Cre}Kif3a^{fl/fl}$ controls ($n = 3$). (G) Xgal staining of a tooth bud from an E12.5 $Wnt1^{Cre}Kif3a^{fl/fl}Axin2^{lacZ/+}$ embryo. (H) Xgal staining of a tooth bud from an E12.5 $Wnt1^{Cre}Kif3a^{fl/fl}Axin2^{lacZ/+}$ mutant. Xgal signals (in blue) are broad in the mesenchyme. (I) Xgal staining of a tooth bud from an E18.5 $Wnt1^{Cre}Kif3a^{fl/fl}Axin2^{lacZ/+}$ embryo. (J) Xgal staining of a tooth bud from an E18.5 $Wnt1^{Cre}Kif3a^{fl/fl}Axin2^{lacZ/+}$ embryo. Xgal signals (in blue) are expanded in the mesenchyme. White and black dotted lines outline the invaginating tooth buds and separate the dental epithelium (de) from the dental mesenchyme (dm). Outer enamel epithelium (oee), inner enamel epithelium (iee), dental papilla (dp), follicle (f), cervical loop (cl). Scale bars: 10 μ m (A-J).

Deletion of Kif3a Stimulates Cell Proliferation in the Dental Epithelium

Like most organs, teeth develop *via* inductive tissue interactions that take place between epithelia and mesenchyme (Thesleff *et al.*, 1995). The fact that cervical loops did not form normally in $Wnt1^{Cre}Kif3a^{fl/fl}$ embryos (Figs. 2G, 2H) made us suspect that this epithelial-mesenchymal interaction was perturbed by Kif3a deletion. We examined E16.5 tooth buds at a stage when cervical loops are well delineated and dental ectoderm has differentiated into an inner and outer enamel epithelium with an intervening stellate reticulum (Fig. 4A). Although primary cilia were intact in the epithelia (Fig. 1), the organization of the $Wnt1^{Cre}Kif3a^{fl/fl}$ enamel organ was perturbed, the stellate reticulum was grossly expanded, both inner and outer enamel epithelia were thicker, and multiple attempts at invagination were evident (Fig. 4B). Moreover, BrdU incorporation was significantly increased within the $Wnt1^{Cre}Kif3a^{fl/fl}$ enamel epithelia (Figs. 4C, 4D; quantified in 4E). These early perturbations in the

organization and proliferation of cells in the dental epithelia impeded enamel formation. When E13.5 tooth buds from $Wnt1^{Cre}Kif3a^{fl/fl}$ were explanted to the renal capsule, the teeth that developed were largely devoid of enamel (Figs. 4F–4I).

To understand the basis for the enamel defect, we examined embryos at earlier stages of development. For example, at E12.5, *Shh* is normally expressed in invaginating dental epithelia (Fig. 4J; see Hardcastle *et al.*, 1998), and at this stage no significant differences were observed between control and mutant tissues (Fig. 4K). At E14.5, *Shh* expression was limited to the enamel knot (Fig. 4L), and $Wnt1^{Cre}Kif3a^{fl/fl}$ tissues exhibited a similar expression pattern (Fig. 4M).

We also evaluated the distribution of Shh protein. As previously reported (Cobourne *et al.*, 2004), Shh transcripts are limited to epithelia, while the protein is distributed in both epithelia and mesenchyme. At both E16.5 and E18.5, Shh expression appeared equivalent in $Wnt1^{Cre}Kif3a^{fl/fl}$ and in $Wnt1^{Cre}Kif3a^{fl/fl}$ embryos (Appendix Fig. 2). This conclusion was supported by our observation that the enamel knot forms normally in

Figure 4. Imbalances of mesenchymal signaling impair enamel formation. (A) Hematoxylin and eosin staining of an E16.5 $Wnt1^{Cre}Kif3a^{fl/fl}$ control molar tooth bud. The dental epithelium forms a bell-like structure ($n = 3$). (B) The tooth bud of E16.5 $Wnt1^{Cre}Kif3a^{fl/fl}$ is misshapen, and the epithelium has many folds ($n = 3$). (C) BrdU staining labels proliferating cells in the tooth buds of E14.5 $Wnt1^{Cre}Kif3a^{fl/fl}$ and (D) $Wnt1^{Cre}Kif3a^{fl/fl}$ mutants ($n = 4$). (E) Quantification of the percentage of BrdU+ cells shows that cell proliferation is increased in the dental epithelium of a $Wnt1^{Cre}Kif3a^{fl/fl}$ mutant tooth bud. (F) Pentachrome staining of the transplanted $Wnt1^{Cre}Kif3a^{fl/fl}$ tooth bud showcases the normal tooth phenotype ($n = 3$). (G) The transplanted molar from $Wnt1^{Cre}Kif3a^{fl/fl}$ is malformed ($n = 3$). (H) The segmentation w/3D image shows dentin (in blue) and enamel (in pink) of a transplanted $Wnt1^{Cre}Kif3a^{fl/fl}$ tooth. (I) The segmentation with 3D image of the transplanted $Wnt1^{Cre}Kif3a^{fl/fl}$ tooth bud shows that the volume of the enamel is greatly reduced. (J) *In situ* hybridization of *Shh* mRNA in the E12.5 $Wnt1^{Cre}Kif3a^{fl/fl}$ tooth. *Shh* is localized in the dental epithelium ($n = 3$). (K) Expression of *Shh* occurs in the dental epithelium of the E12.5 $Wnt1^{Cre}Kif3a^{fl/fl}$ mutant ($n = 3$). (L) *In situ* hybridization of *Shh* mRNA in the E14.5 $Wnt1^{Cre}Kif3a^{fl/fl}$ tooth shows that *Shh* is localized in the enamel knot ($n = 3$). (M) Expression of *Shh* in the dental epithelium of E14.5 $Wnt1^{Cre}Kif3a^{fl/fl}$ is intact ($n = 3$). (N) *In situ* hybridization of *Patched* probe in the E18.5 $Wnt1^{Cre}Kif3a^{fl/fl}$ tooth. *Patched* is expressed in both the epithelium and mesenchyme ($n = 3$). (O) In contrast, expression of *Patched* is greatly reduced in the dental mesenchymal cells of E18.5 $Wnt1^{Cre}Kif3a^{fl/fl}$ ($n = 3$). (P) Xgal staining of a tooth bud of an E18.5 $Wnt1^{Cre}Kif3a^{fl/fl}Gli1^{lacZ/+}$ embryo. Xgal signals (in blue) are detected in both the epithelium and the mesenchyme. (Q) LacZ expression is eliminated in the dental mesenchyme of an E18.5 $Wnt1^{Cre}Kif3a^{fl/fl}Gli1^{lacZ/+}$ mutant. (R) *Lef1* immunostaining of an E14.5 $Wnt1^{Cre}Kif3a^{fl/fl}$ tooth bud. *Lef1* is detected in the mesenchyme and the enamel knot ($n = 3$). (S) Expression of *Lef1* is expanded in both the mesenchyme and epithelium of the E14.5 $Wnt1^{Cre}Kif3a^{fl/fl}$ tooth ($n = 3$). (T) In E18.5 $Wnt1^{Cre}Kif3a^{fl/fl}$ molars, *Lef1* is expressed in the mesenchyme and in the inner enamel epithelium ($n = 6$). (U) However, in the E18.5 mutant, *Lef1* is ectopically expressed in the outer enamel epithelium, which is indicated by the arrowhead ($n = 6$). White dotted lines outline the tooth buds of embryos. Dental epithelium (de), dental mesenchyme (dm), outer enamel epithelium (oe), inner enamel epithelium (ie), dental papilla (dp), follicle (f), cervical loop (cl), stellate reticulum (sr). Scale bars: 10 μ m (A-D, J-U), 20 μ m (F, G), and 150 μ m (H, I).

Wnt1^{Cre+}Kif3a^{fl/fl} (Fig. 2G). Therefore, the initial epithelial Shh signaling centers were largely intact in mutant embryos.

After receiving inductive cues from the ectoderm, the dental mesenchyme signals back to the epithelium to regulate formation of the cervical loops and secondary enamel knots (Thesleff, 2003). Analyses demonstrated that this stage of the reciprocal signaling was disturbed: Compared with controls, both *Patched* (Figs. 4N, 4O) and *Gli1* (Figs. 4P, 4Q) were over-expressed in the Wnt1^{Cre+}Kif3a^{fl/fl} dental epithelium.

This gain in Hh signaling in the mutant dental epithelia was accompanied by a simultaneous gain in Wnt signaling. In the E14.5 Wnt1^{Cre+}Kif3a^{fl/fl} tooth bud, *Lef1* expression was expanded throughout the dental epithelium (Figs. 4R, 4S); this same phenotype was observed at E18.5 (Figs. 4T, 4U). Thus, the early, aberrantly broad Wnt responsiveness in the dental mesenchyme (Fig. 3J) resulted in unrestricted Wnt signaling in the epithelium and, consequently, many failed invaginations.

DISCUSSION

In multiple developmental contexts, Wnt and Hh pathways exhibit a functional antagonism (Ahn *et al.*, 2010; Borday *et al.*, 2012). This antagonistic relationship, however, is context-dependent: At some stages of tissue patterning, Wnt and Hh pathways act synergistically (Hu *et al.*, 2005; Borello *et al.*, 2006). Our analyses demonstrate that primary cilia are essential for the integration of Wnt and Hh signaling, and that, in the absence of a functional primary cilium, Hh signaling is diminished and Wnt signaling is simultaneously increased in the dental mesenchyme (Fig. 3). Using this *in vivo* system, however, we cannot distinguish whether perturbation in one pathway preceded (and thus potentially caused) a disruption in the other. Nonetheless, we had an advantage in studying an *in vivo* system because we could observe how molecular defects in the dental mesenchyme led to perturbations in the epithelium and how these imbalances ultimately affected tissue maturation. Our stage-dependent analyses allowed us to distinguish between primary defects in the dental mesenchyme (*i.e.*, decreased cell proliferation, loss of Hh responsiveness, reduced size of condensations, and expanded Wnt signaling) and secondary defects in the epithelia. These secondary effects were clearly the result of aberrant mesenchymal signaling and not because of the loss of primary cilia in the ectodermal component of the developing tooth. The cumulative effect was profound: Morphogenesis of the entire tooth organ and tissue differentiation were disrupted.

The boundary between Hh and Wnt signaling is important for determining the position of the tooth. In Wnt1^{Cre+}Kif3a^{fl/fl} embryos, this normal antagonism is replaced by overlapping and excessive Hh and Wnt activity in the epithelium (Fig. 4). Consequently, the enamel organ is initially grossly expanded in size but ultimately produces very little enamel (Fig. 4). The initiating signal for amelogenesis originates from odontoblasts, and these odontoblasts are defective in Wnt1^{Cre+}Kif3a^{fl/fl}. Thus, the epithelial/enamel problem can be traced back to a defect in mesenchymal signaling.

In many tissues, Hh signaling has a mitogenic function (Gritli-Linde *et al.*, 2002). In keeping with this observation, we found that a loss of Hh in Wnt1^{Cre+}Kif3a^{fl/fl} dental mesenchyme

correlated with diminished proliferation (Fig. 2), and a gain in Hh signaling in the Wnt1^{Cre+}Kif3a^{fl/fl} epithelium correlated with increased cell proliferation (Fig. 4). However, there is a caveat: Wnt signaling also acts in a mitogenic fashion (Niehrs and Acebron, 2012), but we found that Wnt signaling was expanded (Fig. 3) in the less proliferative dental mesenchyme (Fig. 2). Primary cilia are thought to restrain or localize a Wnt signal (Corbit *et al.*, 2008), and the expanded Wnt signaling is in keeping with this proposed function. What, then, are the individual functions of these growth factors in the dental mesenchyme? This answer is yet to be determined, but analysis of our data demonstrates that the primary cilium is a critical integration site for their activities.

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