

Sprouty Genes Control Diastema Tooth Development via Bidirectional Antagonism of Epithelial-Mesenchymal FGF Signaling

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Summary

Unlike humans, who have a continuous row of teeth, mice have only molars and incisors separated by a toothless region called a diastema. Although tooth buds form in the embryonic diastema, they regress and do not develop into teeth. Here, we identify members of the Sprouty (*Spry*) family, which encode negative feedback regulators of fibroblast growth factor (FGF) and other receptor tyrosine kinase signaling, as genes that repress diastema tooth development. We show that different Sprouty genes are deployed in different tissue compartments—*Spry2* in epithelium and *Spry4* in mesenchyme—to prevent diastema tooth formation. We provide genetic evidence that they function to ensure that diastema tooth buds are refractory to signaling via FGF ligands that are present in the region and thus prevent these buds from engaging in the

FGF-mediated bidirectional signaling between epithelium and mesenchyme that normally sustains tooth development.

Introduction

Mammalian teeth develop as a result of signaling interactions between epithelium (derived from oral ectoderm) and mesenchyme (derived from cranial neural crest) (reviewed by Tucker and Sharpe, 2004). As these two tissues interact, the developing tooth progresses through four stages. First, the epithelium thickens to form a placode. Next, the epithelium invaginates into the underlying mesenchyme, while the prospective dental mesenchyme condenses around it, forming a tooth bud. Subsequently, the epithelium folds and extends farther into the mesenchyme, surrounding the dental mesenchyme to form a cap and then a bell stage tooth germ.

Epithelial morphogenesis and growth of the dental mesenchyme during the cap and bell stages are thought to be controlled by signals produced by the enamel knot, a morphologically distinct region of the epithelium containing densely-packed, nonproliferating cells (reviewed by Thesleff et al., 2001). Enamel knot activity is proposed to be mediated, at least in part, by FGF4 and FGF9, members of the fibroblast growth factor family of secreted signaling molecules. These proteins signal to the mesenchyme by activating the mesenchyme-specific “c” isoform of FGF receptors (FGFRs) and are thought to maintain *Fgf3* expression in the dental mesenchyme. In turn, FGF3 (and FGF10) signal to the epithelium, where they regulate cell proliferation and morphogenesis, by activating the epithelium-specific FGFR “b” isoform (see Figure 6A). This model, which is based primarily on gain-of-function studies in organ culture and gene expression analyses, has been difficult to test genetically because inactivating each of these FGF family members individually has no effect on molar development (Harada et al., 2002; X. Sun, I. Thesleff, and G.R.M., unpublished data; O.K. and G.R.M., unpublished observations). This is presumably because of functional redundancy between *Fgf4* and *Fgf9* in the epithelium and *Fgf3* and *Fgf10* in the mesenchyme. The finding that tooth development is arrested at the bud stage when the b isoform of FGFR2 is specifically deleted in mice supports this hypothesis (De Moerloose et al., 2000).

The discovery of genes that encode antagonists of FGF signaling provided new opportunities for exploring FGF function in development (reviewed by Thisse and Thisse, 2005). The *sprouty* (*spry*) gene was first identified as a negative feedback regulator of FGF-mediated tracheal branching in *Drosophila*. FGF signaling induces *D. spry* expression, and via this effect, the FGF pathway limits the range of its own signaling activity (Hacohen et al., 1998). Subsequent experiments showed that *D. spry* also regulates epidermal growth factor (EGF) receptor and other receptor tyrosine kinase (RTK) signaling pathways (Casici et al., 1999; Kramer et al., 1999; Reich et al., 1999). There are four vertebrate orthologs of *D. spry*. In mice, *Spry1*, *Spry2*, and *Spry4* are widely

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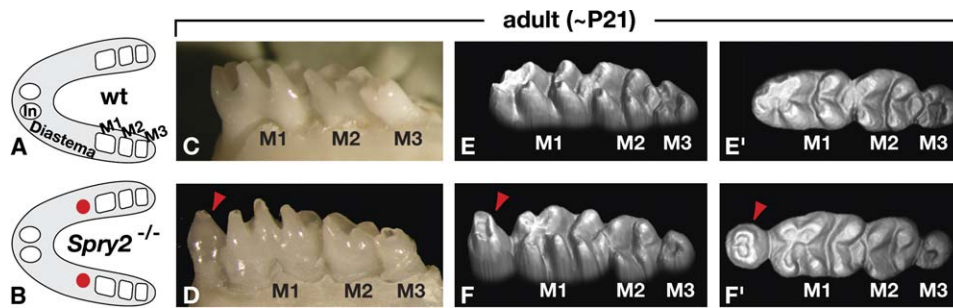


Figure 1. Loss of *Spry2* Function Causes Formation of Teeth in the Diastema

(A and B) Schematic representations of mandibles from wild-type (wt) and *Spry2*^{-/-} (null) mice at ~21 days after birth (~P21) (anterior to the left). *Spry2*-null mandibles almost always contained bilateral supernumerary teeth in the diastema (red circles), just anterior to the first molar (M1). (C and D) Side views of the molar region of wild-type and *Spry2*-null mice. Red arrowhead points to a diastema tooth. (E–F') 3D models based on laser scans of wild-type and *Spry2*-null teeth (E and F, side views; E' and F', top views of the same models). Note the altered cusps of M1 immediately posterior to the supernumerary tooth (red arrowhead) but the relatively normal cusp patterns more posteriorly in the *Spry2*-null specimen. The third molar (M3) is smaller and the cusps less visible in the *Spry2*-null sample, due to delayed eruption, which is also observed in other mutants with diastema teeth (Kangas et al., 2004) (J.J., unpublished data).

expressed in the embryo (de Maximy et al., 1999; Minowada et al., 1999; Zhang et al., 2001). Sprouty family members have been shown to function intracellularly to negatively regulate FGF and other RTK signaling through diverse biochemical mechanisms, often via effects on the RAS-MAPK pathway, although the mechanism of Sprouty function remains controversial (reviewed by Kim and Bar-Sagi, 2004 and Mason et al., 2006).

Loss-of-function analyses in mice have shown that Sprouty genes are required for normal development of many organs. For example, in *Spry1*-null mice there is excess budding of the Wolffian duct during kidney development due to hypersensitivity of the duct to GDNF, a ligand for the RET RTK (Basson et al., 2005). *Spry2* nulls have several abnormalities including a severe hearing loss due to a postnatal cell fate transformation in the auditory epithelium. This defect can be partially rescued by reducing *Fgf8* gene dosage (Shim et al., 2005), providing evidence that in this developmental context, *Spry2* affects FGF signaling.

Here, we report that although *Spry2* and *Spry4* are expressed in different tissue compartments during tooth development (epithelium and mesenchyme, respectively), loss of function of either gene results in the same phenotype, i.e., formation of teeth in a region, called the diastema, that is normally toothless. These will hereafter be referred to as “diastema teeth.” We suggest a model to explain how Sprouty genes function during odontogenesis to prevent development of diastema teeth, based on evidence that they serve to block FGF-mediated crosstalk between the epithelium and mesenchyme.

Results

Spry2-Null Mice Have Teeth in the Diastema, but Molar Morphology Is Normal

Mice have fewer teeth than most mammals, with only one incisor and three molars in each dental quadrant. The incisor and most anterior molar are separated by a toothless diastema. We found that adult mice homozygous for a null allele of *Spry2* (Shim et al., 2005) have a tooth in the diastema, just anterior to the first molar

(M1) (Figures 1A–1D). Of 55 *Spry2*^{-/-} animals examined, 92% had bilateral, 5% had unilateral, and 3% had no diastema teeth in the lower jaw. In contrast, diastema teeth were observed in the upper jaw in <5% of *Spry2*^{-/-} animals. Their *Spry2*^{+/-} littermates had normal dentition (n = 17). Thus, loss of *Spry2* function causes the formation of supernumerary teeth in the mandible but does not significantly affect the maxilla.

We found that the morphology of *Spry2*-null molar cusps was essentially normal, except that in the anterior end of M1, immediately adjacent to a diastema tooth, the cusps were further apart than in wild-type molars (Figures 1E–1F'). This resulted in shortening of M1, an effect seen in other mutant mice with diastema teeth (Kangas et al., 2004; Kassai et al., 2005), possibly because of the close packing of adjacent teeth. However, in the remainder of M1, as well as in the second and third molars (M2 and M3) of *Spry2*-null animals, there were no consistent differences in cusp morphology as compared with wild-type, although the mutant molars were generally slightly smaller and erupted slightly later than those in wild-type mice. This contrasts with what is observed in other mouse mutants with altered tooth number, in which there are extensive modifications of molar shape, such as changes in cusp position, cusp number, and formation of additional crests (Kangas et al., 2004; Kassai et al., 2005). Thus, *Spry2* is required to prevent formation of diastema teeth and does so without significantly affecting the shapes of other teeth.

Supernumerary Teeth in *Spry2*-Null Adults Result from Persistence of Embryonic Diastema Buds that Develop Active Enamel Knots

Although the diastema is toothless in wild-type adult mice, tooth primordia develop in the embryonic diastema but then undergo apoptosis and regress (Peterkova et al., 2002). To explore whether these transiently present buds abnormally persist in *Spry2*-null embryos, we compared tooth morphogenesis in wild-type and *Spry2*-null embryos by performing a 3D reconstruction analysis of serial sections through mandibular diastema and molar regions on embryonic day (E) 14.5 and E15.5 (Figures 2A–2D). Consistent with previous reports (Peterkova et al., 2002), in E14.5 wild-type mandibles,

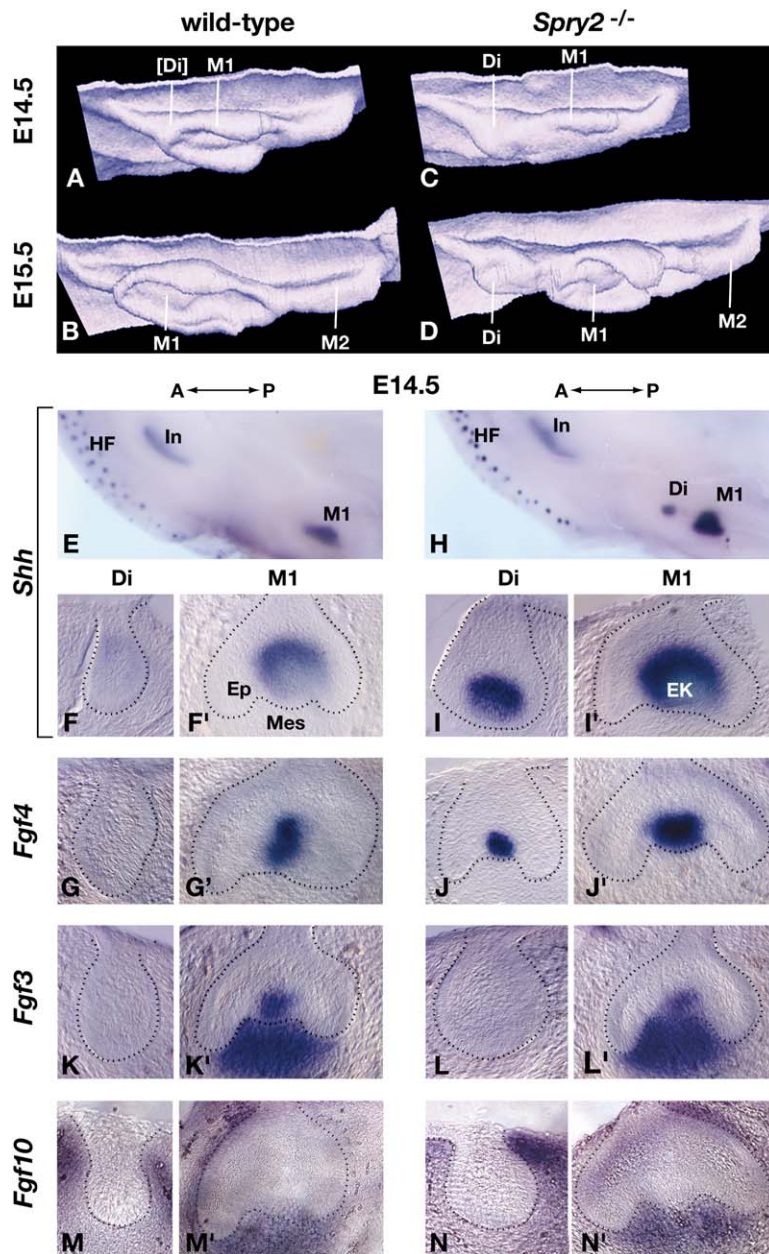


Figure 2. Loss of *Spry2* Function Results in Persistence of the Diastema Bud Enamel Knot

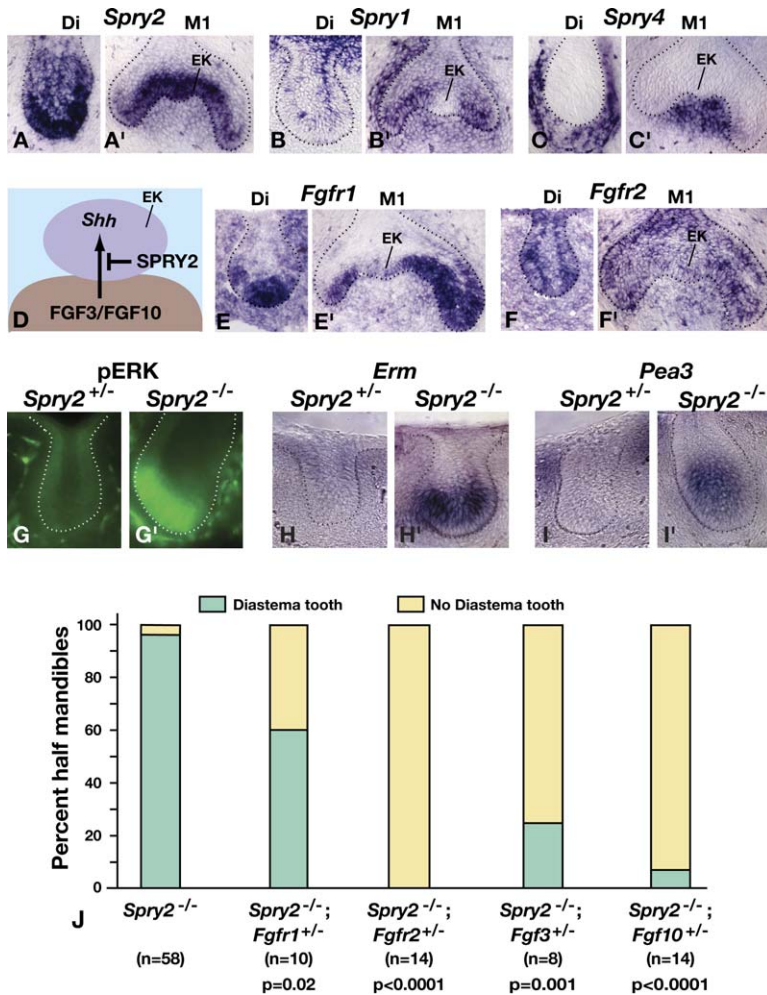
(A–D) 3D reconstructions of dental epithelium (viewed from the perspective of the mesenchyme) in wild-type and *Spry2*^{-/-} posterior mandibles at E14.5 and E15.5 (anterior is to the left). The approximate positions of the diastema (Di), first molar (M1), and second molar (M2) tooth germs are indicated. The square brackets indicate that the diastema bud in panel (A) is regressing.

(E–N') Gene expression was analyzed by in situ hybridization in whole mount followed by serial sectioning of control (wild-type or *Spry2*^{+/-}) and *Spry2*-null mandibles at the stages indicated. Examples of the whole-mount preparations are shown in panels (E) and (H). In the remaining panels, for each probe, sections from the same mandible are shown; the section on the left was taken from a more anterior region and illustrates the diastema (Di) tooth germ, and the one on the right (') illustrates the M1 tooth germ. In *Spry2*-null diastema and all M1 tooth germs, the sections pass through or are adjacent to the enamel knot. *Shh* and *Fgf4* expression is detected exclusively in the enamel knot, and *Fgf3* expression is detected in both enamel knot and dental mesenchyme. The dotted lines demarcate the boundary between epithelium and mesenchyme. Abbreviations: A, anterior; EK, enamel knot; Ep, epithelium; HF, hair follicles; In, incisor; Mes, mesenchyme; P, posterior.

we observed a thickening at the anterior end of the M1 tooth germ, which appeared to be the remnant of the regressing diastema tooth bud (Figure 2A). At this embryonic age, the M1 tooth germ was at the cap stage and contained a well-developed enamel knot (Figure 2A and data not shown). At E15.5, the diastema bud could no longer be discerned, the M1 tooth germ was at the late cap or cap-bell transition stage, and M2 was just beginning to develop (Figure 2B). In contrast, in *Spry2*-null embryos, at E14.5, the diastema bud was a robust swelling just anterior to M1 (Figure 2C), and by E15.5, it had clearly developed into a distinct tooth germ (Figure 2D). Thus, the supernumerary teeth in *Spry2*-null adults form as a result of abnormal survival and development of diastema tooth buds.

Since tooth development is dependent on the presence of a functional enamel knot, we assayed wild-

type and mutant embryonic tooth germs for the expression of two genes, *Shh* and *Fgf4*, that reflect enamel knot activity (Vahtokari et al., 1996) (Figures 2E–2J' and data not shown). In control (wild-type or *Spry2*^{+/-}) mandibles at E14.5, *Shh* and *Fgf4* expression was readily detected in sections through M1 tooth germs but not in more anterior sections, where the diastema bud remnants were localized (Figure 2E–2G'). In contrast, in *Spry2*-null embryos, *Shh* and *Fgf4* expression was readily detected in both diastema and M1 tooth germs at E14.5 and E15.5 (Figures 2H–2J' and data not shown). We also assayed for other genes that might play a role in promoting the development of diastema buds into teeth, including *Bmp4* (Neubuser et al., 1997), *Activin* (Ferguson et al., 1998), *Ectodin* (Kassai et al., 2005), and *Lef1* (Kratochwil et al., 2002), by examining their expression in whole mount at E14.5. We found no obvious



difference in the levels or domains of their expression in control versus *Spry2*-null dental epithelium or mesenchyme (not shown), suggesting that *Spry2* acts downstream of these pathways. Together, these data show that the diastema buds in *Spry2*-null mice contain a functional enamel knot, which presumably enables them to develop into teeth.

The Expression Patterns of Sprouty, FGF Receptor, and FGF Ligand Genes Suggest a Mechanism whereby Loss of *Spry2* Function Leads to Diastema Tooth Formation

To determine how loss of *Spry2* function results in enamel knot gene expression, we first analyzed the *Spry2* expression pattern by in situ hybridization. Because Sprouty proteins function intracellularly to antagonize RTK signaling (reviewed by Kim and Bar-Sagi, 2004 and Mason et al., 2006), such assays identify the primary site(s) of *Spry2* function. In wild-type diastema and M1 tooth germs at E14.5, we detected abundant *Spry2* expression throughout the epithelium adjacent to dental mesenchyme, including the M1 enamel knot. Much lower levels of *Spry2* RNA were detected throughout the rest of the dental epithelium and in the M1 dental mesenchyme (Figures 3A and 3A'). We also detected *Spry1* RNA at a low level in diastema buds and at a higher

Figure 3. Molecular and Genetic Evidence that Supernumerary Tooth Formation Results from Hypersensitivity to FGF Signaling

(A-C' and E-F') Sprouty and FGF receptor family gene expression was assayed by in situ hybridization of frontal sections of E14.5 mandibles. For each probe, sections of the diastema and M1 tooth germs were taken from the same embryo (see legend to Figure 2). Note that expression of *Fgfr1* and *Fgfr2* is detected in the enamel knot (EK).

(D) A model for the effect of SPRY2 on FGF signaling from the dental mesenchyme to the enamel knot.

(G and G') Immunolocalization of pERK in control and *Spry2* diastema buds at E14.5.

(H-I') Analysis by in situ hybridization for expression of *Erm* and *Pea3*, which are thought to be direct targets of RTK signaling, in E14.5 control and *Spry2*-null diastema buds. Assays were performed as described for gene-expression studies in Figure 2.

(J) Effects on supernumerary tooth formation of reducing dosage of the indicated genes in *Spry2*-null animals. For each animal examined, the left and right sides of the mandible were scored independently for the presence of a diastema tooth. Data on *Spry2*-null littermates from all of the crosses used to generate animals with reduced gene dosage were pooled. p values were calculated with the Fisher exact test.

level in M1 tooth germs. Expression was widespread throughout the M1 epithelium and mesenchyme, but appeared to be specifically excluded from the enamel knot (Figures 3B and 3B'). In contrast, we detected *Spry4* RNA exclusively in dental mesenchyme in both diastema and M1 tooth germs (Figures 3C and 3C'). Comparable expression patterns were observed at E12.5 and E13.5 (not shown).

Data from gene expression studies and manipulations of tooth germs in vitro have suggested that *Shh* expression in the enamel knot is induced/maintained by signaling from mesenchyme to epithelium via FGF3 and FGF10 (Kettunen et al., 2000; Kratochwil et al., 2002). Since *Spry2* is expressed in the enamel knot epithelium, this raises the possibility that the normal function of SPRY2 is to prevent *Shh* expression in the diastema bud by inhibiting FGF signaling from the mesenchyme (Figure 3D). This hypothesis relies on the assumption that FGF receptor (FGFR) genes function in the enamel knot. Although it has previously been reported that none of the FGFR genes are expressed in the enamel knot (Kettunen et al., 1998), we detected weak expression of both *Fgfr1* and *Fgfr2* in the wild-type molar enamel knot at E13.5 and E14.5 (Figures 3E' and 3F' and data not shown). More importantly, in the epithelium of the diastema bud, where the enamel knot would be

localized if the bud developed, we observed *Fgfr1* expression at a high level and *Fgfr2* at a lower level at E13.5 and E14.5 (Figures 3E and 3F and data not shown).

These data suggest a model whereby lack of SPRY2 function renders the diastema bud epithelium hypersensitive to FGF signaling from the mesenchyme, thereby maintaining *Shh* expression. In principle, either FGF3 or FGF10 could be a mesenchymal FGF that performs this function. However, we never detected *Fgf3* RNA in the diastema bud of either control or *Spry2*-null embryos at E14.5 (n = 20) or E15.5 (n = 10) (Figures 2K and 2L and data not shown), but it was detected at high level in the mesenchyme (and enamel knot) of the nearby M1 tooth germs (Figures 2K' and 2L'). *Fgf10* RNA was detected at low levels in wild-type and *Spry2*-null embryos at E13.5, E14.5, and E15.5, in a long swath in the mesenchyme of the region containing the diastema and molar tooth germs (Figures 2M–2N' and data not shown). Based on these findings, we propose that in *Spry2*-null embryos, FGFs produced in the mesenchyme are sufficient to induce and/or maintain expression of *Shh* and perhaps also other genes, such as *Fgf4*, that are required for enamel knot function, thereby enabling diastema buds to form teeth.

Loss of *Spry2* Function Causes Hypersensitivity to FGF Signaling in Diastema Bud Epithelium

One prediction that follows from the hypothesis that *Spry2* functions to inhibit FGF signaling in diastema bud epithelium is that in the absence of *Spry2* function, we should detect elevated FGF signaling. Phosphorylation of MAP kinase (ERK) is considered an indication of FGFR activation, although it also reflects signaling via other RTKs (Eswarakumar et al., 2005). Using an antibody that detects only the phosphorylated form of ERK (pERK) (Corson et al., 2003), we observed little or no staining in control and extensive staining in *Spry2*-null diastema buds at E14.5 (Figures 3G and 3G'). In addition, we detected elevated expression of *Erms* and *Pea3*, two genes considered to be direct targets of RTK signaling (O'Hagan and Hassell, 1998; Roehl and Nusslein-Volhard, 2001), in *Spry2*-null epithelium as compared with control diastema bud epithelium (Figures 3H–3I'). These data indicate that loss of *Spry2* function results in an increase in RTK signaling in diastema bud epithelium.

If it is true that loss of *Spry2* function leads to tooth development by rendering diastema bud epithelium hypersensitive to FGF signaling, then reducing dosage of the relevant FGF receptor gene(s) should eliminate the supernumerary teeth. To test this prediction, we produced *Spry2*-null animals that were wild-type for FGFR genes, or in which *Fgfr1* or *Fgfr2* gene dosage was reduced, and scored each half of the mandible (hm) for the presence or absence of a supernumerary tooth. In those *Spry2*-null animals from these crosses with wild-type FGFR gene dosage, all but one hm had a diastema tooth (Figure 3J). In contrast, heterozygosity for a null allele of *Fgfr1* (Trokovic et al., 2003) reduced the frequency of supernumerary tooth formation to 60%, and heterozygosity for a null allele of *Fgfr2* (Yu et al., 2003) reduced it to 0%. These data provide genetic evidence that diastema tooth formation in the absence of SPRY2 is due to excess signaling via FGFR1 and FGFR2. Fur-

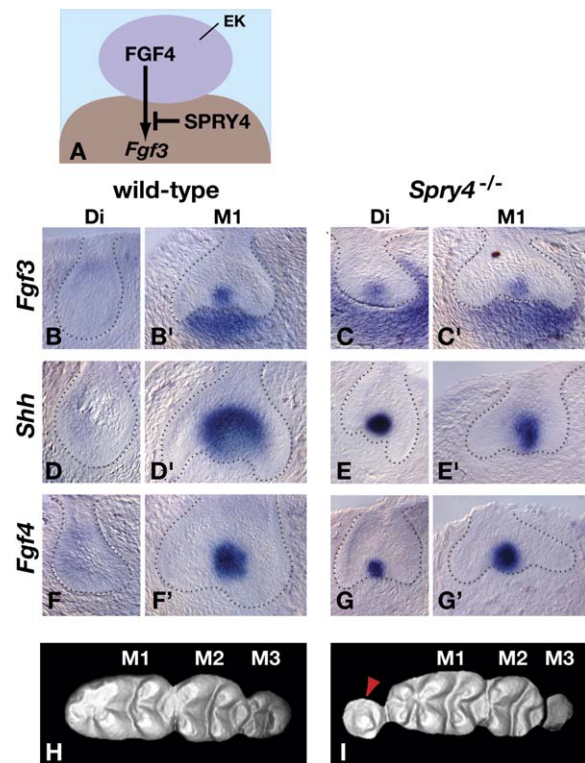


Figure 4. Loss of *Spry4* Function Results in Diastema Tooth Formation

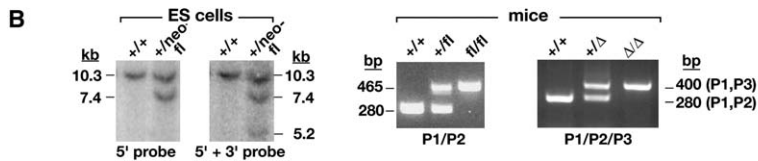
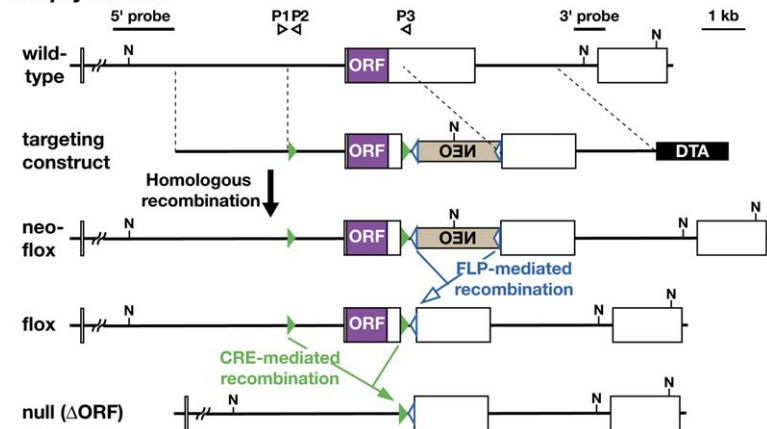
(A) A model for the effect of SPRY4 on FGF signaling from the enamel knot to the dental mesenchyme. (B–G') In situ hybridization analysis of gene expression in frontal sections of control (wild-type or *Spry4*^{+/+}) and *Spry4*-null diastema (Di) and first molar (M1) tooth germs at E14.5. The dotted lines demarcate the boundary between epithelium and mesenchyme. (H and I) Three-dimensional models based on laser confocal scans (anterior to the left) of wild-type and *Spry4*-null teeth (viewed from the top). Note the relatively normal cusp morphology in the molars posterior to the supernumerary tooth (red arrowhead) in the *Spry4*-null specimen, excluding the anterior cusp of M1. Eruption of M3 is delayed in the *Spry4*-null mouse, and the cusps are not yet visible.

thermore, we found that heterozygosity for a null allele of either *Fgf3* (Alvarez et al., 2003) or *Fgf10* (Min et al., 1998) in *Spry2*-null animals almost completely prevented diastema tooth formation (Figure 3J), indicating that FGF3 and FGF10 are ligands to which *Spry2*-null diastema buds are hypersensitive.

Loss of *Spry4* Function also Causes Teeth to Develop in the Diastema

It has been proposed that one function of FGF4 produced in the enamel knot is to induce *Fgf3* expression in the dental mesenchyme (Kettunen et al., 2000; Kratochwil et al., 2002). Surprisingly, *Fgf3* RNA was not detected in *Spry2*-null diastema buds (Figure 2L) despite the presence of a functional enamel knot expressing *Fgf4* (Figure 2J). One explanation for this observation might be that *Spry4*, which is abundantly expressed in dental mesenchyme (Figures 3C and 3C'), antagonizes the response to FGF signaling from the enamel knot and thus prevents *Fgf3* expression (Figure 4A). To explore the function of *Spry4* in diastema tooth

A *Spry4* alleles



(Rodríguez et al., 2000) to generate animals carrying the *Spry4^{flox}* conditional null allele. Mice heterozygous for *Spry4^{flox}* were mated to β -actin-cre transgenic mice (Lewandoski et al., 2000) to generate animals carrying the *Spry4^{ΔORF}*-null allele. N, *NcoI* restriction enzyme site. (B) Identification of ES cells and mice carrying *Spry4* mutant alleles. Correctly targeted ES cells carrying *Spry4^{neo-flox}* were identified by Southern blotting DNA digested with *NcoI* and by using the probes indicated. Mice heterozygous or homozygous for *Spry4^{flox}* or *Spry4^{ΔORF}* were identified by a PCR assay by using the primers indicated.

development, we produced mice carrying a *Spry4*-null allele (Figure 5), by using the same strategy that we previously employed to generate a *Spry2* mutant allelic series (Shim et al., 2005). Adults homozygous for the *Spry4*-null allele were viable and fertile.

We detected *Fgf3* RNA in diastema tooth germs in 17% of hm from *Spry4*-null animals examined at E14.5 ($n = 42$) (Figures 4B–4C'). A similar percentage of *Spry4*-null diastema tooth germs were found to express *Shh* (20% of hm; $n = 60$) and *Fgf4* (15% of hm; $n = 20$), but no *Shh* or *Fgf4* expression was detected in the diastema buds of their wild-type or *Spry4^{+/-}* littermates at E14.5 (Figures 4D–4G'). Significantly, diastema teeth were present at the same frequency in *Spry4*-null adults (16% of hm; $n = 94$) as abnormal gene expression was seen in *Spry4*-null embryos. These supernumerary teeth were almost always unilateral. As in *Spry2*-null mice, the molar cusp patterns were essentially normal in *Spry4*-null mice (Figures 4H and 4I). Thus our data suggest that SPRY4 functions to repress diastema bud development by preventing FGF signaling from maintaining *Fgf3* expression in the dental mesenchyme.

Discussion

The toothless diastema has been a feature of mouse dentition for over 50 million years. Lineages with diastemata have arisen independently several times during evolution, often as a result of a decrease in tooth number. In mice, the mechanisms for preventing tooth formation in the diastema appear to range from a block in initiation of tooth development, in the anterior region near the incisors, to suppression of tooth development beyond the bud stage, in the posterior region adjacent to the molars (Peterkova et al., 2002). Here, we demon-

Figure 5. Production of a *Spry4* Mutant Allelic Series

(A) Schematic representation of the *Spry4* wild-type allele, targeting construct, and *Spry4^{neo-flox}* allele produced by gene replacement in ES cells. A horizontal line and boxes represent *Spry4* intron and exon sequences, respectively. A purple box represents the *Spry4* open reading frame (ORF; contained within a single exon). Positive and negative selection during targeting were provided by a neomycin-resistance (NEO) expression cassette (tan box), inserted in reverse orientation to the *Spry4* gene, and a diphtheria toxin A (DTA) expression cassette (black box), respectively, each under the control of a *Pgk1* promoter. Open blue and filled green triangles represent *frt* and *loxP* sites, the recognition sites for Fip and Cre recombinases, respectively. The two pairs of dotted parallel lines demarcate the regions in which homologous recombination occurred. The positions of the probes and primers (P1–P3) used for genotyping the various alleles are indicated. Mice heterozygous for *Spry4^{neo-flox}* were produced as described in Experimental Procedures and mated to *flp* transgenic mice

strate that Sprouty genes are essential components of the molecular machinery that normally prevents embryonic diastema tooth buds from developing into teeth and that different Sprouty genes cooperate to ensure that the diastema remains toothless.

FGF Signaling in Diastema versus Molar Buds

Our data show that in the tooth buds that form in the wild-type embryonic diastema, the genetic program that normally controls progression from the bud to the cap stage is not active. Thus, we found that by E14.5, expression of *Shh*, *Fgf4*, and *Fgf3* was not detected in wild-type diastema buds (Figure 2). We made an effort to determine whether this observation reflected a lack of induction of gene expression or a failure to maintain it after it was initiated. Expression of these three genes was invariably detected by whole-mount in situ hybridization in wild-type embryos at E13.5–E14.0. In almost all cases, the signal in the prospective molar region was restricted to a single domain, presumably the M1 bud. However, occasionally we detected two adjacent but distinct domains of *Shh* expression in the molar region: a small and relatively weak anterior and a larger and stronger posterior expression domain (not shown) (Kangas et al., 2004). These results suggest that the diastema bud expresses *Shh* but only transiently and at low level. We did not detect similar dual domains of *Fgf4* or *Fgf3* expression in the wild-type molar region, suggesting that these FGF genes are never expressed in the diastema bud. However, our finding that Sprouty genes, which are known to be downstream targets of RTK signaling, are robustly expressed in diastema buds from at least E12.5 (Figure 3 and data not shown) suggests that these tooth germs have been exposed to FGF or other RTK signaling.

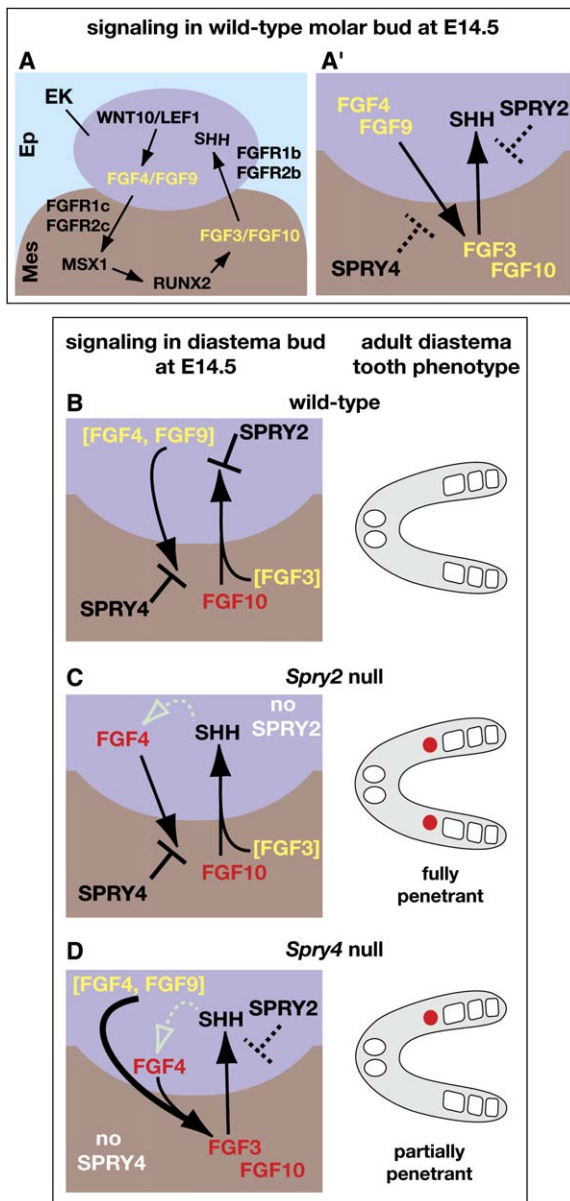


Figure 6. Model for Dual Control of Diastema Tooth Development by Sprouty Genes

Arrows indicate a stimulatory effect, and the symbol \perp indicates an inhibitory effect of one signaling molecule on the expression of another. Yellow or red lettering indicates that an FGF ligand was produced in the M1 tooth germ or diastema bud, respectively. (A) Schematic representation of FGF-dependent reciprocal signaling between the enamel knot (EK) in the epithelium (Ep) and the dental mesenchyme (Mes) and the regulatory interactions that control it in a cap-stage molar tooth germ. This model is based largely on data from gene-expression studies and manipulations of tooth germs in vitro (Kettunen et al., 2000; Kratochwil et al., 2002). (A') Condensed version of the model shown in (A) depicting *Spry2* and *Spry4* function. The dashed symbols indicate that Sprouty proteins do not prevent FGF signaling in molar tooth germs. (B) In wild-type diastema buds, FGF10 is produced in the mesenchyme, but any FGF4, FGF9, or FGF3 available was produced in the adjacent M1 tooth germ (brackets). SPRY2 in the epithelium and SPRY4 in the mesenchyme block signaling via FGF3/FGF10 and FGF4/FGF9, respectively, preventing *Shh*, *Fgf4*, and *Fgf3* expression. Consequently, the diastema bud regresses, and no supernumerary teeth form. (C) In *Spry2*-null diastema buds, hypersensitivity of the enamel knot to FGF signaling enables FGF10 produced in the diastema bud

Based on these data, we suggest that one mechanism by which diastema bud development is normally suppressed is via inhibition of FGF gene expression, including *Fgf4* in the enamel knot and *Fgf3* in the dental mesenchyme. In the developing molars, several factors are required for expression of FGF genes (Figure 6A), including the LEF1 transcription factor, whose expression is regulated by WNT10 (Kratochwil et al., 2002). BMP signaling may also be involved since the BMP and FGF signaling pathways interact during molar development, both at the time when it is initiated (Neubuser et al., 1997) and at later stages (Bei and Maas, 1998; Kassai et al., 2005). Differences in expression of one or more of these molecules between diastema and M1 buds at early stages may account for the lack of FGF gene expression in diastema buds and its abundance in M1 buds by E14.5.

A Model for Sprouty Gene Function in Suppressing Diastema Tooth Development

We show that in the absence of *Spry2* function the diastema bud persists and develops into a tooth (Figures 1 and 2), that phosphorylation of ERK and expression of RTK signaling target genes is increased in diastema bud epithelium (Figure 3), and that decreases in dosage of genes that encode FGF ligands expressed in dental mesenchyme or FGF receptors can rescue the *Spry2* loss-of-function phenotype (Figure 3). These data provide strong support for the hypothesis that loss of *Spry2* function results in the development of diastema teeth by causing increased sensitivity of diastema bud epithelium to FGF signaling. Likewise, we suggest that the reason that loss of *Spry4* function results in a similar phenotype, i.e., diastema tooth formation (Figure 4), is that it causes hypersensitivity of diastema bud mesenchyme to FGF signaling.

Interestingly, Sprouty genes are required to prevent diastema tooth development even though there is little or no FGF gene expression in wild-type diastema buds. A model for how they might function is illustrated in Figure 6. In molar tooth germs (Figure 6A'), although Sprouty genes are expressed and presumably modulate FGF signaling, they do not block the activity of either mesenchymal (FGF3 and FGF10) or epithelial (FGF4 and FGF9) FGFs. In wild-type diastema buds (Figure 6B), the only FGF gene expression we detected was that of *Fgf10*. We found no conclusive evidence that *Fgf3* or *Fgf4* are expressed in the wild-type diastema bud between E11.5 and E15.5, and we did not detect expression of *Fgf8*, *Fgf9*, or *Fgf20* at any of these stages.

mesenchyme, in conjunction with FGF3, produced in the M1 tooth germ, to induce/maintain *Shh* expression. *Fgf4* expression is also induced/maintained, possibly in response to SHH signaling (dotted open arrow). However, FGF4 cannot signal to the mesenchyme due to antagonism by SPRY4, preventing *Fgf3* expression. SHH and FGF4 produced in *Spry2*-null diastema buds enable virtually all of them to develop into supernumerary teeth (red circles). (D) In 15%–20% of *Spry4*-null diastema buds, hypersensitivity of the mesenchyme to FGF signaling, possibly FGF4/FGF9 produced in the M1 tooth germ, results in induction of *Fgf3* expression in diastema bud mesenchyme (thicker arrow). The sum total of mesenchymal FGFs is then sufficient to overcome the antagonistic effects of SPRY2, resulting in *Shh* and *Fgf4* expression, which in turn maintains *Fgf3* expression and leads to formation of a diastema tooth.

Thus, we propose that the normal function of *Spry2* is to prevent the relatively low level of signaling via FGF10 produced in diastema bud mesenchyme from inducing/maintaining *Shh* expression. However, since we found that reducing *Fgf3* gene dosage in *Spry2*-null mice prevents diastema tooth formation, *Spry2* must also normally function to prevent signaling via FGF3 from inducing/maintaining *Shh* expression. In light of our evidence that *Fgf3* is never expressed in the wild-type diastema bud, it seems likely that the source of FGF3 is the adjacent M1 tooth germ. Likewise, the normal function of *Spry4* in the mesenchyme is to prevent any epithelial FGF signals, including FGF4 and FGF9 produced in the adjacent M1 tooth germ from inducing/maintaining *Fgf3* expression. As a result of the combined activities of *Spry2* and *Spry4*, the diastema bud regresses and there are no teeth in the adult diastema.

According to our model, in the absence of Sprouty gene function, diastema bud tissues become hypersensitive to FGF signaling, and the diastema bud is sustained and develops into a tooth. In the case of the *Spry2*-null diastema bud (Figure 6C), our genetic data argue that it is both FGF10 (produced in diastema bud mesenchyme) and FGF3 (produced in the M1 tooth germ) that induces/maintains *Shh* expression. *Fgf4* expression is also induced/maintained in the *Spry2*-null enamel knot, but the factors responsible for this effect have not been identified. SHH activity is one possible candidate, by analogy to its function in the limb bud where it is required to upregulate/maintain the expression of *Fgf4*, *Fgf9*, and *Fgf17* (Sun et al., 2000). However, in vitro studies aimed at demonstrating that SHH can induce *Fgf4* expression in tooth germ epithelium have thus far been unsuccessful (J.J. and I. Thesleff, unpublished data). WNT activity might be involved since *Fgf4* is known to be a downstream target of WNT signaling in the developing molar (Kratohwil et al., 2002). However, we detected no expression of the WNT target gene *Lef1* in the diastema bud region of *Spry2*-null embryos (not shown). Interestingly, the FGF4 that is produced in the *Spry2*-null diastema buds is prevented from inducing/maintaining *Fgf3* in the mesenchyme, presumably by SPRY4 and other factors that are produced there. Despite the lack of *Fgf3* expression, the SHH and FGF4 produced in the enamel knot are sufficient to sustain development of the *Spry2*-null diastema buds, and teeth almost always form in the diastema.

In the case of the *Spry4*-null diastema bud (Figure 6D), FGF signaling from the epithelium is sufficient to induce *Fgf3* expression in mesenchyme that is hypersensitive to FGF signaling due to the loss of *Spry4* function. Because *Fgf4* and *Fgf9* are not expressed in wild-type diastema buds, we speculate that this inducing signal is FGF4/FGF9 from the nearby M1 tooth germ, or perhaps some other epithelial FGF. FGF3, in conjunction with FGF10, then overcomes the antagonistic effects of SPRY2 and induces/maintains *Shh* expression in the enamel knot. As in *Spry2*-null diastema buds, *Fgf4* expression is also induced/maintained in the enamel knot by unknown factors. This FGF4 presumably contributes to the signal that maintains *Fgf3* expression, leading to diastema tooth formation, although at low frequency (16%). The penetrance of the diastema tooth phenotype can be increased ~3-fold in *Spry4*-

null animals by heterozygosity for a *Spry1*-null allele (Basson et al., 2005), whereas heterozygosity for a *Spry2*-null allele has no effect (data not shown). Because *Spry1* is expressed in both mesenchyme and epithelium, whereas *Spry2* is expressed primarily in the epithelium, together these data suggest (but do not prove) that mesenchymal SPRY1 cooperates with SPRY4 to antagonize FGF signaling to the mesenchyme. Interestingly, *Spry1*-null adults have grossly normal dentition (not shown), indicating that *Spry1* is functionally redundant with *Spry4*, and *Spry1* function in the diastema bud is only revealed in a *Spry4*-null context.

The model we discuss here for Sprouty gene function in mouse odontogenesis is analogous to the proposed role for Sprouty genes in other developmental systems. Perhaps the most pertinent example comes from studies of *Spry1* function in kidney morphogenesis, where SPRY1 acts to ensure that the Wolffian duct anterior to the normal site of ureteric bud formation is refractory to GDNF produced at an earlier stage, thereby preventing supernumerary ureteric buds from developing (Basson et al., 2005). In that case, Sprouty genes are expressed throughout the Wolffian duct, including the region where the ureteric bud normally forms, but they do not block its development. This is remarkably similar to what is observed during mouse odontogenesis, where development of one tooth primordium—the diastema bud—is repressed by Sprouty genes, but development of the nearby molar bud is not. The molecular mechanisms that enable cells in molar tooth germs to escape the inhibitory effects of Sprouty gene expression remain to be elucidated. However, it seems likely that at least part of the reason why they do so is that molar tooth germs express higher levels of FGF genes, and perhaps other molecules that favor progression of tooth development, than do diastema buds.

Other Pathways Affecting Diastema Tooth Development

Diastema teeth similar to those observed in Sprouty mutant mice have been found in other mouse mutants. For example, they have been reported in mice carrying a hypomorphic allele of *Polaris* (Zhang et al., 2003), a gene required for assembly of cilia and for normal SHH signaling (Huangfu and Anderson, 2005; Haycraft et al., 2005). Overexpression of Ectodysplasin (*Eda*), a member of the tumor necrosis factor (TNF) family of signaling molecules, also results in formation of diastema teeth (Mustonen et al., 2003), as does inactivation of *Ectodin*, a gene that encodes an inhibitor of BMP activity (Kassai et al., 2005). The latter effect may be due to upregulation of TNF signaling as expression of *Edar*, the Ectodysplasin receptor, was found to be increased in *Ectodin*-deficient molar tooth germs (Supplemental Data in Kassai et al., 2005). As yet, no molecular analysis of the diastema buds in any of these mutants has been reported, so it is not known if there are changes in gene expression similar to those that we observed in Sprouty mutants. There are, however, some intriguing differences between the final phenotypes. In particular, in *Polaris* mutants, diastema teeth are found in both the mandible and maxilla, whereas in Sprouty mutants and in mice overexpressing *Eda*, for reasons that are not yet understood, they are found almost exclusively in

the mandible. Furthermore, overexpression of *Eda* and loss of Ectodin function primarily cause large changes in the shapes of the other teeth, particularly in cusp morphology, whereas the effects of loss of Sprouty gene function are essentially restricted to promoting development of diastema teeth. This distinction is critical because during evolution, changes in tooth number have frequently occurred without a concomitant change in the morphology of the adjacent teeth. This evolutionary evidence suggests that there must be decoupled developmental mechanisms controlling tooth number and shape. Our data identify Sprouty genes as potential mediators of evolutionary changes specifically in tooth number and implicate regulation of FGF signaling in this process.

Concluding Remarks

One of the most intriguing aspects of this study is our discovery that different members of the Sprouty gene family are deployed in different tissues to modulate the reciprocal signaling between epithelium and mesenchyme, thereby ensuring that a particular process—in this case diastema tooth formation—is prevented. Thus, *Spry2* in the epithelium, and *Spry4* in the mesenchyme, apparently perform the same function. It seems likely that similar use of complementary expression patterns of different Sprouty family members to finely tune signaling between epithelium and mesenchyme is made in other developmental contexts and that additional examples of such dual negative control of signaling between tissues will be uncovered by future studies of Sprouty and other gene families that encode antagonists of intercellular signaling.

Experimental Procedures

Mouse Lines

The targeting vector used to produce the *Spry4^{neo-flox}* allele in ES cells was constructed with ~8.6 kb of *Spry4* genomic DNA isolated from a P1 strain 129/Ola ES cell library (Genome Systems, St. Louis) and was electroporated into E14Tg2a.4 ES cells. 2/206 ES cell clones assayed by Southern blotting were found to be correctly targeted. Germline transmission of the *Spry4^{neo-flox}* allele was obtained following injection of a correctly targeted ES cell line into C57BL/6 blastocysts (performed by the Stanford University Transgenic Research Facility). Lines carrying the *Spry4^{fllox}* conditional and *Spry4^{ΔORF}*-null alleles were derived by crossing mice carrying *Spry4^{neo-flox}* to Flp- and Cre-expressing mice, as described in the legend to Figure 5. The sequences of the primers shown in Figure 5 are: P1, 5'-CAGGACTTGGGAGTGCTTCCTTAG-3'; P2, 5'-CCTCC TAGTACCTTTTGGGGAGA G -3'; P3, 5'-TACAGCAGGAATGGCT ACGGTG-3'. For both P1/P2 and P1/P3 primer combinations, standard PCR conditions were used with an annealing temperature of 57°C. The *Spry4*-null allele was maintained on a mixed genetic background.

3D Analysis of Adult Molar Shape

Selected jaw specimens were scanned with a Nextec Hawk laser scanner with 10 μm measurement sampling intervals, and each tooth row was scanned from four different directions. Tooth shapes were rendered and cusp features were examined in three dimensions as previously described (Kassai et al., 2005). Tooth crown features from 24 *Spry2*-null, 24 *Spry4*-null, and 19 wild-type mandibles were scored as characters and morphologies were compared as previously described (Kangas et al., 2004). Shape data are stored in a comparative MorphoBrowser database at <http://www.biocenter.helsinki.fi/bi/evodevo/morphobrowser/>.

Gene Expression Analysis

To stage embryos, noon of the day when a vaginal plug was detected was considered E0.5. RNA in situ hybridization was performed according to standard protocols on jaws that were fixed in 4% PFA and either embedded in OCT and cryosectioned (10 μm intervals) before hybridization (Figure 3) or hybridized in whole-mount and then sectioned on a vibratome (30 μm intervals) (Figures 2 and 4). To generate digoxigenin-labeled probes, we used plasmids containing mouse *Activin*, *Bmp4*, *Ectodin*, *Erm*, *Fgf3*, *Fgf4*, *Fgf8*, *Fgf9*, *Fgf10*, *Fgf20*, *Fgfr1*, *Fgfr2*, *Lef1*, *Pea3*, *Shh*, *Spry1*, *Spry2*, and *Spry4* sequences for in vitro transcription.

3D Reconstructions of Embryonic Tooth Germs

The contours of the mandibular dental and adjacent oral epithelium were drawn from serial frontal sections (7 μm intervals), and three-dimensional images were generated as previously described (Lesot et al., 1996).

PERK Immunohistochemistry

Mandibles were dissected in phosphate-buffered saline (PBS) and fixed overnight in 4% paraformaldehyde (PFA). After embedding in 4% agarose, serial 100 μm vibratome sections were cut. Sections were washed in Tris-buffered saline with 0.1% Triton X-100 (TBST), blocked in 3% bovine serum albumin in TBST, and incubated overnight in anti-phosphoERK antibody (Cell Signaling, #9101) diluted 1:100 in TBST. After washing, sections were incubated overnight with the anti-rabbit Alexa 488 secondary antibody (Molecular Probes) at 1:200 in TBST and then washed prior to mounting in Vectashield (Vector Laboratories). Samples were examined on a Leica fluorescent microscope.

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