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Dact1–3 mRNAs exhibit distinct expression domains during tooth development

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ABSTRACT

Wnt signaling is essential for tooth formation and Dact proteins modulate Wnt signaling by binding to the intracellular protein Dishevelled (Dvl). Comparison of the three known mouse Dact genes, *Dact1–3*, from the morphological initiation of mandibular first molar development through the onset of root formation using section *in situ* hybridization showed distinct, complementary and overlapping expression patterns for these genes. Whereas *Dact2* expression was restricted to the dental epithelium, including the enamel knot signaling centers and pre-ameloblasts, *Dact1* and *Dact3* showed developmentally regulated expression in the dental mesenchyme. Both *Dact1* and *Dact3* mRNAs were first detected in the presumptive dental mesenchyme. After being downregulated from the condensing dental mesenchyme of the bud stage tooth germ, *Dact1* was upregulated in the dental follicle mesenchyme at the cap stage and subsequently also in the dental papilla at the bell stage, where the expression persisted to the postnatal stages. In contrast, *Dact3* transcripts persisted throughout the dental mesenchyme, including the preodontoblasts, during embryogenesis before transcripts were largely downregulated from the tooth germ postnatally. Collectively, these results suggest that *Dact1* and -3 may contribute to early tooth formation by modulation of Wnt signaling pathways in the mesenchyme, including preodontoblasts, whereas *Dact2* may play important signal-modulating roles in the adjacent epithelial cells including the enamel knot signaling centers and pre-ameloblasts. Future loss-of-function studies will help elucidate whether any of these functions are redundant, particularly for *Dact1* and *Dact3*.

1. Results and discussion

The tooth, in particular the mouse first molar, is an excellent model to analyze molecular signaling mechanisms during mammalian organogenesis. Tooth formation is controlled by signaling pathways conserved across species (for reviews see Miletich and Sharpe, 2003; Thesleff, 2006; Lesot and Brook, 2009; Luukko et al., 2008). Previous studies indicated that Wnt/β-catenin signaling serves critical roles in odontogenesis. Recently, constitutive activation of the Wnt/β-catenin pathway was shown to result in continuous supernumerary tooth formation, while inhibition of this pathway interferes with tooth formation by causing arrest at early developmental stages (van Genderen et al., 1994; Andl et al., 2002; Jarvinen et al., 2006; Lammi et al., 2004; Liu et al., 2008; Wang et al., 2009). The functions of β-catenin-independent Wnt signaling pathways, including the planar cell polarity (PCP) and Wnt/Ca^{2+} pathways, remain poorly understood in the tooth. Wnt signaling is modulated by multiple mechanisms, including the intracellular Dact (also known as Dapper or Frodo) proteins. Dact proteins regulate Wnt signaling at least in part by binding to Dishevelled (Dsh/Dvl) (Suriben et al., 2009) family members, which are cytoplasmic proteins that are centrally placed in all Wnt signaling pathways (Veeman et al., 2003). Recently, *Xenopus* Dact protein (XDpr1a) was shown to inhibit Wnt/β-catenin signaling when unphosphorylated, but to promote this signaling pathway when phosphorylated in an *in vitro* assay (Teran et al., 2009).

Three Dact paralogs, *Dact1–3*, have been characterized in mouse and they show distinct expression domains during embryonic development, suggesting differential signaling functions (Fisher et al., 2006). *Dact1*$^{-/-}$ mouse embryos show posterior malformations in the spine, genitourinary and distal digestive system that result from changes in β-catenin independent signaling (Suriben et al., 2009), whereas targeted inactivation of *Dact2* has been reported to lead to accelerated re-epithelialization during cutaneous wound healing through attenuating Tgfβ signaling (Meng et al., 2008).

Epithelial expression of *Dact2* in the tooth has been reported at the cap stage (Fisher et al., 2006) whereas the expression of other Dact genes in the tooth is not known. Given the regulatory func-

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tions of Dact proteins in Wnt signaling, which is essential for tooth formation, we systemically compared mRNA expression of *Dact1–3* during initiation and epithelial morphogenesis of the mandibular first molar tooth germ from embryonic day (E) 11.5 to day 14 postnatally (PN14) using sensitive radioactive *in situ* hybridization.

Dact1 and -3 mRNAs were expressed exclusively in the mesenchyme of the molar tooth, but were differentially regulated. At the morphological onset of tooth formation, expression of both mRNAs was seen in the developing jaw mesenchyme including the presumptive dental mesenchyme (Fig. 1B1 and D1). However, *Dact1* signal appeared to be weaker in the presumptive dental mesenchyme adjacent to the epithelial thickening than in the mesenchyme surrounding this area. Two days later, at the bud stage (E13.5) differences in the expression of the *Dact1* and *Dact3* genes became evident (Fig. 1B2 and D2). While *Dact3* continued to be expressed in the condensing dental mesenchyme, *Dact1* was downregulated there. However, *Dact1* expression was observed in the jaw mesenchyme surrounding the condensing dental mesenchyme at this stage (arrows in Fig. 1B2).

Tooth-specific epithelial folding morphogenesis starts at the cap stage and continues throughout the bell stage (Kollar and Lumsden, 1979). During these stages, the mesenchymal dental follicle surrounding the dental papilla and epithelial enamel organ is visible. The dental follicle gives rise to cemento-, fibro- and osteoblasts

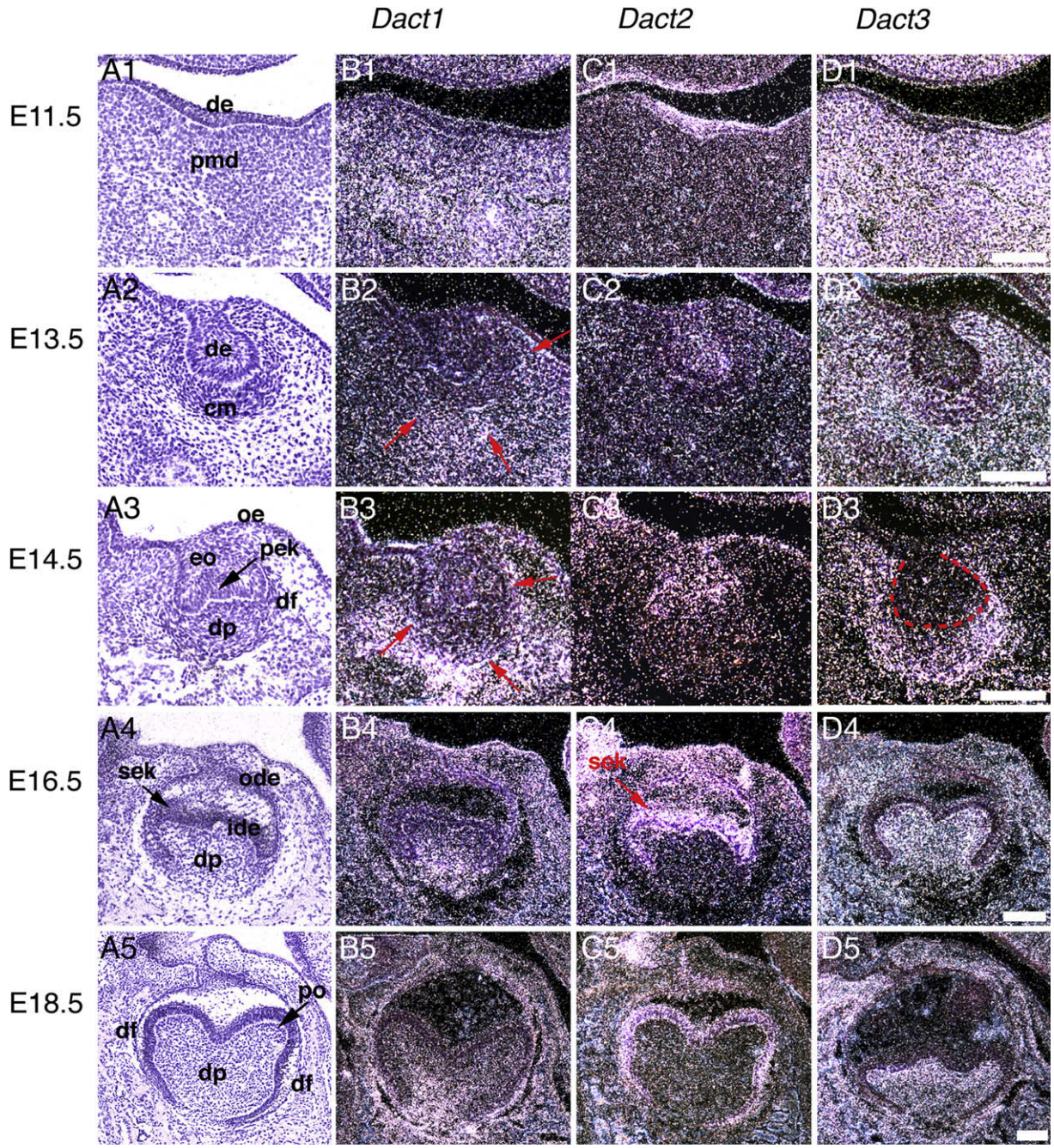

Fig. 1. Localization of *Dact1–3* mRNAs during development of the mouse tooth. Frontal sections of first mandibular molars. Arrows in B2 indicate jaw mesenchyme surrounding the epithelial bud and condensing dental mesenchyme, whereas arrows in B3 indicate the mesenchymal dental follicle. Buccal and lingual side is to the left and right, respectively. Basement membrane is marked with a dashed line in D3. Abbreviations: cm, condensing dental mesenchyme; de, dental epithelium; df, dental follicle; dp, dental papilla mesenchyme; E, embryonic age; eo, enamel organ; ide, inner dental epithelium; oe, oral epithelium; ode, outer dental epithelium; pek, primary enamel knot; pdm, presumptive dental mesenchyme; po, preodontoblasts; sek, secondary enamel knot. Scale bars: 100 μm.

forming the tooth supporting periodontium. At the cap stage (E14.5) *Dact1* expression was observed in the dental follicle and in the adjacent jaw mesenchyme (Fig. 1B3). In contrast, *Dact3* was expressed both in the dental papilla and follicle (Fig. 1D3). At this stage *Dact1* transcripts also appeared in the oral epithelium (Fig. 1B3).

At the early bell stage (E16.5) *Dact1* was upregulated in the cervical-middle part of the dental papilla mesenchyme (Fig. 1B4). In contrast, *Dact3* transcripts were present throughout the dental papilla and follicle (Fig. 1D4). One day before birth E18.5 the tooth-specific preodontoblasts are visible in the dental papilla mesenchyme adjacent to the inner dental epithelium at the most advanced cuspal areas (Lesot et al., 2001). At this stage, *Dact1* continued to be expressed in the cervical-middle part of the dental papilla as well as in the dental follicle (Fig. 1B5). This expression pattern for *Dact1* persisted after the enamel and dentin secretion and the onset of the root formation postnatally, as shown for 8-day postnatal tooth germ (Fig. 2B1 and B2). *Dact3* expression was apparent throughout the dental papilla, but the most prominent expression was detected in the coronal part of the papilla, including the preodontoblasts (Fig. 1D5). Postnatally, at PN8 little if any specific *Dact3* mRNA expression was seen in the dental pulp any longer (Fig. 2D1). No specific expression of *Dact1* or *-3* was observed in the Hertwig's epithelial root sheaths responsible for root formation (Fig. 2D2).

Dact1 and *Dact3* expression in the lower jaw incisor tooth germ correlated with that observed in the molars. At the epithelial thickening stage *Dact1* transcripts were present in the dental and jaw mesenchyme. Later, at the bud and cap stages, expression continued in the jaw mesenchyme adjacent to the dental mesenchymes as shown for the E13.5 bud stage incisor in Fig. 2B3, and subsequently expression also appeared in the dental follicle. During the bell stage, *Dact1* transcripts become upregulated in the middle part of the dental papilla and pulp (see Fig. 2B4 for a E16.5 incisors). *Dact3* expression was present in the mesenchymal tissue components during E11.5–E14.5 (Fig. 2D3). At E16.5, however, little if any expression was observed in the incisor mesenchyme (see Fig. 2D4). Postnatally, a prominent *Dact3* hybridization signal was detected in muscle tissue while little if any specific *Dact3* expression was seen in the incisor pulp (not shown).

In contrast to *Dact1* and *-3*, *Dact2* expression was restricted to the dental epithelium. At the epithelial thickening stage (E11.5),

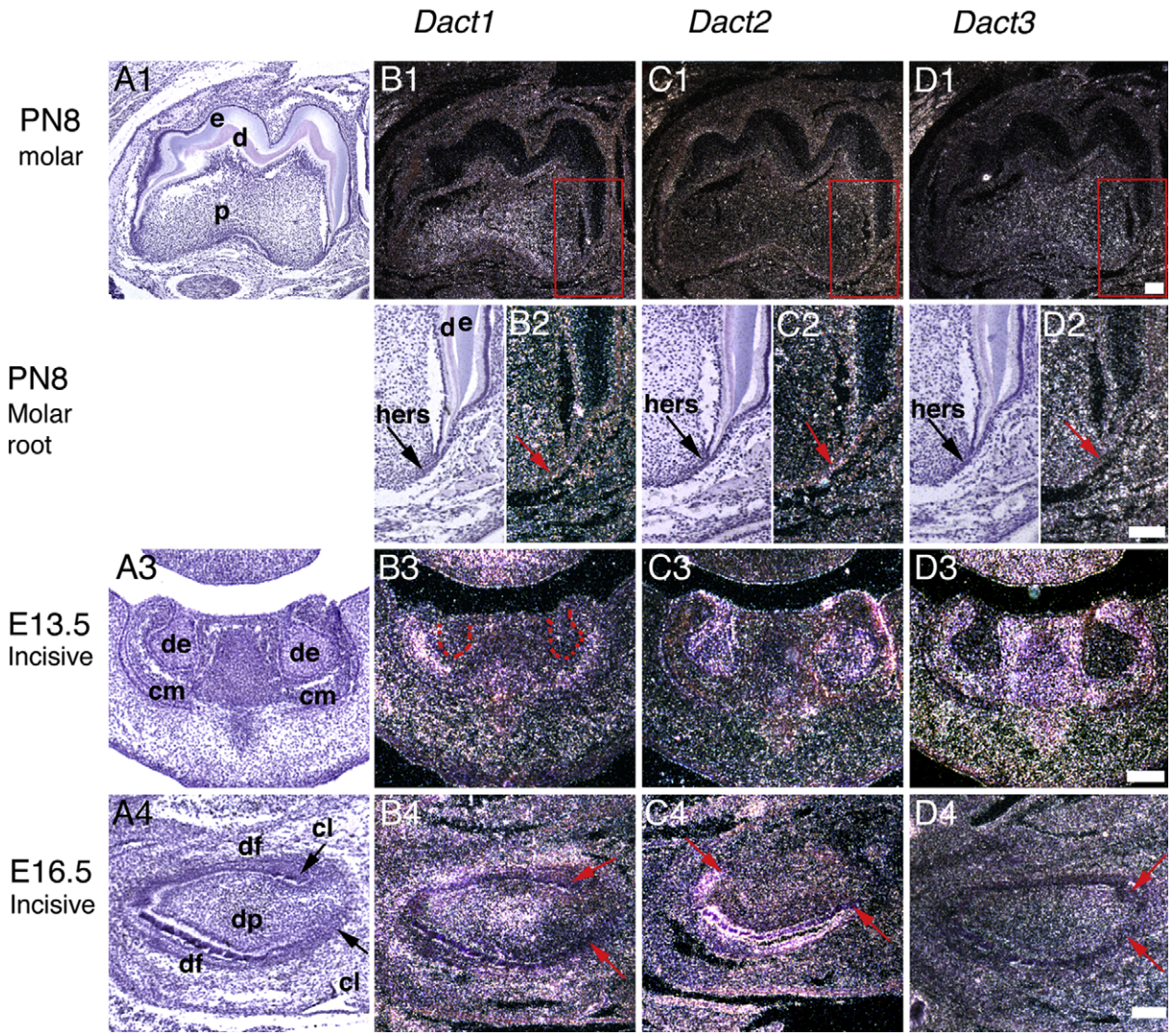

Fig. 2. Localization of *Dact1–3* mRNAs during development of the mouse tooth. Sagittal (A1–D2, A4–D4) and frontal (A3–D3) sections of the first mandibular molars (A1–D2) and mandibular incisors (A3–D4). Figures B2–D2 are higher magnifications from the areas in figures B1–D1 marked by red boxes. In frontal sections, buccal and lingual side is to the left and right, respectively. In sagittal sections rostral and dorsal side is to left and right, respectively. Basement membrane is marked with a dashed line in B3. *Dact3* does not show specific expression in tooth in D1. Abbreviations: cl, cervical loop; cm, condensing dental mesenchyme; d, dentin; de, dental epithelium; df, dental follicle; dp, dental papilla mesenchyme; e, enamel; hers, Hertwig's epithelial root sheath. Scale bars: 100 μm.

Dact2 showed prominent expression in the dental epithelium (Fig. 1C1). Expression continued in the epithelial dental bud at E13.5 and in the epithelial enamel organ of the E14.5 cap stage tooth germ. This included cells of the primary enamel knot, a signaling center that regulates tooth shape (Fig. 1C2 and C3) (Jernvall et al., 2000, 1994). Later during the bell stage, when the final shape of the tooth is established, *Dact2* expression continued in cells of the epithelial enamel organ, including the inner and outer dental epithelium, stellate reticulum, stratum intermedium and cervical loops, which later form the Hertwig's epithelial root sheath (Fig. 1C4 and C5). Molar tooth crown morphogenesis is characterized by formation of the cusps and histodifferentiation of the inner dental epithelium cells to ameloblasts, which produce enamel. Of note, *Dact2* was expressed in cells of the inner dental epithelium, which are precursors of the ameloblasts, and in the cervical loops. The secondary and tertiary enamel knots present in the bell stage molar regulate individual cusp formation and the final shape of the molar crown (Jernvall et al., 1994; Luukko et al., 2003). Of special interest is the finding that *Dact2* expression also included both the upper and lower compartments (Luukko et al., 2003) of the secondary enamel knots (SEKs), as shown for E16 molar (arrows in Fig. 1C4) and the tertiary enamel knot (TEK) (not shown). The expression of *Dact2* was downregulated from ameloblasts secreting enamel and by PN8 no specific expression of *Dact2* was seen in the tooth germ. In addition, the Hertwig's epithelial root sheaths were devoid of transcripts (Fig. 2C1 and C2).

The expression of *Dact2* in the incisors correlated with that in molars. *Dact2* transcripts were restricted to the dental epithelia including the cervical loops, as shown for E13.5 and E16.5 tooth germs (Fig. 2C3 and C4). However, at PN2 *Dact2* expression was not detected in the tooth germ (not shown).

In conclusion, our results here show that the three Dact genes exhibit distinct, overlapping and complementary expression domains during tooth development. Whereas *Dact1* and *-3* showed differentially, developmentally regulated expression in the dental mesenchyme, the expression of *Dact2* was restricted to the dental epithelium. These results suggest that regulation of Wnt signaling by Dact proteins may serve important functions in tooth initiation, crown morphogenesis and differentiation of tooth-specific cells. Moreover, the overlapping expression of *Dact1* and *-3* suggest that developmental functions of their protein products may be either redundant and/or complementary during tooth formation. Loss-of-function studies in the future will help elucidate the *in vivo* functions of the Dact proteins.

2. Experimental procedures

Animal use was approved by the Animal Welfare Committee of the Preclinical Institutes, University of Bergen. Mice (CBA & NMRI) were mated overnight and the appearance of vaginal plug was taken as day 0 of embryogenesis (E0.5). The developmental stages of the tooth germs were judged from the tissue sections according to morphological criteria. Tissue processing, section *in situ* hybridization, photomicrography and processing of the images were performed as described (Kettunen et al., 1998; Luukko et al., 1996; Kettunen et al., 2005). Plasmids containing cDNA fragments of *Dact1–3* have been described earlier in (Fisher et al., 2006). Sections were exposed for 3 weeks. No specific hybridization signal was detected in the control sections hybridized with corresponding sense probes (data not shown).

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