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Anomalous incisor morphology indicates tissue-specific roles for *Tfap2a* and *Tfap2b* in tooth development

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ABSTRACT

Mice possess two types of teeth that differ in their cusp patterns; incisors have one cusp and molars have multiple cusps. The patterning of these two types of teeth relies on fine-tuning of the reciprocal molecular signaling between dental epithelial and mesenchymal tissues during embryonic development. The AP-2 transcription factors, particularly *Tfap2a* and *Tfap2b*, are essential components of such epithelial-mesenchymal signaling interactions that coordinate craniofacial development in mice and other vertebrates, but little is known about their roles in the regulation of tooth development and shape. Here we demonstrate that incisors and molars differ in their temporal and spatial expression of *Tfap2a* and *Tfap2b*. At the bud stage, *Tfap2a* is expressed in both the epithelium and mesenchyme of the incisors and molars, but *Tfap2b* expression is restricted to the molar mesenchyme, only later appearing in the incisor epithelium. Tissue-specific deletions show that loss of the epithelial domain of *Tfap2a* and *Tfap2b* affects the number and spatial arrangement of the incisors, notably resulting in duplicated lower incisors. In contrast, deletion of these two genes in the mesenchymal domain has little effect on tooth development. Collectively these results implicate epithelial expression of *Tfap2a* and *Tfap2b* in regulating the extent of the dental lamina associated with patterning the incisors and suggest that these genes contribute to morphological differences between anterior (incisor) and posterior (molar) teeth within the mammalian dentition.

1. Introduction

Teeth arise from a series of molecular and physical interactions between epithelial and mesenchymal tissues in the embryonic oral cavity (Kollar and Baird, 1969; Lumsden, 1988; Mina and Kollar, 1987). Genetic mutations that affect these tissue interactions (*e.g., PITX2, MSX1,* and *PAX9*) can cause profound disruptions to the development of the human dentition, including loss, gain, or mis-patterning of teeth (Alappat et al., 2003; Chen et al., 1996; Dressler et al., 2010; Mostowska et al., 2003; Peters et al., 1998; Satokata and Maas, 1994). The genetic basis for tooth development has been well-studied in mice (Ahn et al., 2010; Harada et al., 2002; Harjunmaa et al., 2012; Jernvall et al., 1994; Klein et al., 2008; Pispa et al., 1999; Thesleff et al., 2001; Tummers and Thesleff, 2003), however, few studies have explicitly compared gene expression between developing incisors and molars (Hu et al., 2013; Huang et al., 2014; Laugel-Haushalter et al., 2013; Tucker et al., 1998).

Activator protein-2 (AP-2) transcription factors are known to play an essential role in craniofacial development in numerous vertebrate species, including mice, zebrafish, and chickens (Brewer et al., 2004; Brewer and Williams, 2004; de Croze et al., 2011; Hoffman et al., 2007; Knight et al., 2005; Li and Cornell, 2007; Nottoli et al., 1998; Schorle et al., 1996; Van Otterloo et al., 2018; Zhang et al., 1996). Several human genetic studies have identified dental anomalies in patients with *TFAP2A* and *TFAP2B* mutations, which cause the human syndromic disorders branchio-oculo-facial syndrome and Char syndrome, respectively (Milunsky et al., 2008; Satoda et al., 2000; Tanasubsinn et al., 2017). Although several studies have examined aspects of *Tfap2* expression in vertebrate tooth development (Laugel-Haushalter et al., 2013; Moser

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et al., 1997; Tanasubsinn et al., 2017; Uchibe et al., 2012; Wang et al., 2014), there has not been a comprehensive spatiotemporal analysis of their expression and little is known about the tissue-specific functions of AP-2 genes during dental development. To understand the roles of AP-2 genes in establishing the molecular and morphological identities of incisors and molars, here we investigate the expression dynamics and tissue-specific functions of two AP-2 paralogs, *Tfap2a* and *Tfap2b*.

We compared spatiotemporal differences in the expression of *Tfap2a* and *Tfap2b* between incisors and molars and used mouse conditional genetics to determine the tissue-specific roles of these genes in dental epithelium and mesenchyme of each tooth class. Though *Tfap2a* and *Tfap2b* are expressed in epithelial and mesenchymal tissues during tooth development, we found that epithelial-specific loss of *Tfap2a* and *Tfap2b* results in a loss or reduction of upper incisors along with a duplication of lower incisors, but deletion of these genes in the neural crest (NC)-derived mesenchyme does not perturb dental development. Despite major impacts on incisor development, molar development is essentially unaffected by epithelial loss of *Tfap2a* and *Tfap2b*. Collectively, our results identify a novel role for AP-2 family members in dental development.

2. Materials and methods

2.1. Mice

Animal experiments were conducted following the 'Guide for the Care and Use of Laboratory Animals of the National Institutes of Health' and approved by the Institutional Animal Care and Use Committees of the University of Florida or the University of Colorado – Denver. ICR (CD-1) "wild-type" laboratory mice (Envigo) were housed at the University of Florida, while *Tfap2a* and *Tfap2b* mutant lines were housed at the University of Colorado – Denver. See Supplementary Methods 5.1 for details on embryo collection and histology.

2.2. Conditional deletion of Tfap2a and Tfap2b

To generate *Tfap2* mutant embryos, we used either conditional floxed alleles or null alleles of *Tfap2a* and *Tfap2b* and two strains in which *Cre* recombinase was expressed in either the epithelium, *Crect* (Schock et al., 2017), or the neural crest (NC), *Wnt1-Cre* (Danielian et al., 1998) (Supplementary Fig. 1). Females were homozygous for the *Tfap2a* floxed conditional allele, $Tfap2a^{tm2Will/J}$ (Brewer et al., 2004) and the *Tfap2b* floxed conditional allele, $Tfap2b^{tm2Will}$ (Martino et al., 2016; Seberg et al., 2017; Van Otterloo et al., 2018). Males were heterozygous for either the epithelial or NC *Cre* allele, and the *Tfap2a* and *Tfap2b* conditional alleles (*i.e.,* $Tfap2a^{fl/wt}$;$Tfap2b^{fl/wt}$;*Wnt1-Cre* or $Tfap2a^{fl/wt}$;$Tfap2b^{fl/wt}$;*Crect)*. In the second cross, males were heterozygous for conditional null alleles of *Tfap2a* (Zhang et al., 1996) and *Tfap2b* (Martino et al., 2016; Seberg et al., 2017; Van Otterloo et al., 2018) (*i.e.,* $Tfap2a^{null/wt}$;$Tfap2b^{null/wt}$;*Wnt1-Cre* or $Tfap2a^{null/wt}$;$Tfap2b^{null/wt}$;*Crect*) and females were homozygous for the conditional alleles. See Supplementary Methods 5.2 for additional details on tissue-specific deletions and embryo genotypes. For all embryos examined, genotypes, phenotypes, and sample sizes are provided in Supplementary Table 1.

2.3. RNA in situ hybridization on cryosections

RNA probes for *in situ* hybridization (ISH) were generated for *Tfap2a, Tfap2b, Yeats4, Kctd1,* and *Ets1* (see Supplementary Methods 5.3). ISH was performed as described previously (Acloque et al., 2008) with some modifications (see Supplementary Methods 5.3). Expression patterns reported here were detected in a minimum of 3 wild-type CD-1 embryos per stage. Because the expression patterns of these genes had been documented in the head, these regions were used as positive controls (Supplementary Fig. 2A-G). Negative (sense) controls were also conducted for each gene and produced no detectable signal (Supplementary Fig. 2H-L).

2.4. Micro-CT scanning and 3-D reconstruction of Tfap2 mutant and control embryos

Mouse embryos were prepared for micro-CT (μCT) (see Supplementary Methods 5.4). and scanned in a GE v|tome|x m 240 Nano CT scanner (General Electric) at the University of Florida Nanoscale Research Facility. Tiff stacks were generated using Phoenix Datos2 software (General Electric) and VG Studio Max (Volume Graphics v3.3.4) was used for 3-D reconstructions. Length and width ratios from 3-D reconstructed first upper and lower molar crowns ($M^1/_1$) of control and mutant embryos were calculated (see Supplementary Methods 5.5 for details).

3. Results and discussion

3.1. Incisors and molars differ in temporal and spatial expression of Tfap2a and Tfap2b

To determine the spatial and temporal expression of *Tfap2a* and *Tfap2b,* we compared mRNA localization in mouse incisors and molars at the bud stage (E12.5-E13.5), the cap stage (E14.5), and the bell stage (E16.5-17). *Tfap2a* expression was detected throughout the bud and cap stages in the incisor epithelium and mesenchyme (Fig. 1A-B, G-H, M-N). *Tfap2b*, however, was first detected in the incisor epithelium at the late bud stage (Fig. 1D-E, J-K) in some (but not all) embryos, suggesting that *Tfap2b* transcripts were just beginning to accumulate at E13.5. In the molars, by contrast, both *Tfap2a* and *Tfap2b* were expressed prominently in the mesenchyme at the bud and cap stages (Fig. 1C, F, I, L, O, R), in agreement with previous reports (Tanasubsinn et al., 2017; Uchibe et al., 2012). At the cap stage, *Tfap2b* was faintly detected in the incisor epithelium (Fig. 1P, Q). At the bud and cap stages, both genes were expressed in the oral and/or surface epithelium (Fig. 1A, B, D, E, G, H, J, K), consistent with previous studies showing that at E10.5 *Tfap2a* is expressed in the NC-derived mesenchyme of the first branchial arch, and both *Tfap2a* and *Tfap2b* are expressed in the surrounding surface epithelium (Zhang and Williams, 2003; Zhao et al., 2011).

At the bell stage, in the upper incisor *Tfap2a* was expressed in the epithelium and the mesenchyme (Fig. 2A), whereas in the lower incisor, *Tfap2a* was only weakly expressed in the incisor epithelium, unlike at earlier stages when it was more prominently expressed (Fig. 1H, N). In the molars, *Tfap2a* expression was restricted to the inner enamel epithelium directly adjacent to the dental mesenchyme (Fig. 2I, M). *Tfap2b* transcripts were detected prominently in bell stage incisors within epithelial-derived ameloblasts and faintly in the mesenchyme (Fig. 2B, F); however, in the molars, *Tfap2b* expression became limited to mesenchymal cells closer to the outer regions of the tooth germ (Fig. 2J, N). Restricted expression of *Tfap2a* in the molar inner enamel epithelium suggests it may be associated with cusp formation which is facilitated by enamel knots (Cho et al., 2007; Matalova et al., 2005; Thesleff et al., 2001).

As a first step towards connecting the dynamic spatiotemporal expression of *Tfap2a* and *Tfap2b* to their potential function(s) in tooth development, we next asked whether *Tfap2a/Tfap2b* expression is associated with two modulators of AP-2 activity, *Yeats4*, which encodes an AP-2 activator protein (Ding et al., 2006), and *Kctd1* which encodes a transcriptional target of AP-2 (Marneros, 2020) or inhibitor (Ding et al., 2009). *Tfap2a, Kctd1,* and *Yeats4* exhibit similar expression patterns in the dental epithelium of the incisors and molars at bud-cap stages (E13.5 and E14.5) (Fig. 1, Supplementary Fig. 3, Supplementary Results 6.1); however, expression of *Tfap2b* is co-expressed with *Kctd1,* and *Yeats4* only at E14.5 in the incisor epithelium (Fig. 1P, Q; Supplementary Fig. 3G-H, J-K). There was limited expression of *Kctd1* and *Yeats4* in the dental mesenchyme at E14.5, where their expression domains were more similar to that of *Tfap2a* rather than *Tfap2b* (Supplementary Fig. 3I, L). At the bell stage, the incisors did not express *Kctd1*, whereas *Yeats4* was expressed in the ameloblast layer, but not the mesenchyme, similar to *Tfap2b* (Fig. 2B, C). In contrast to the incisors, both *Kctd1* and *Yeats4*

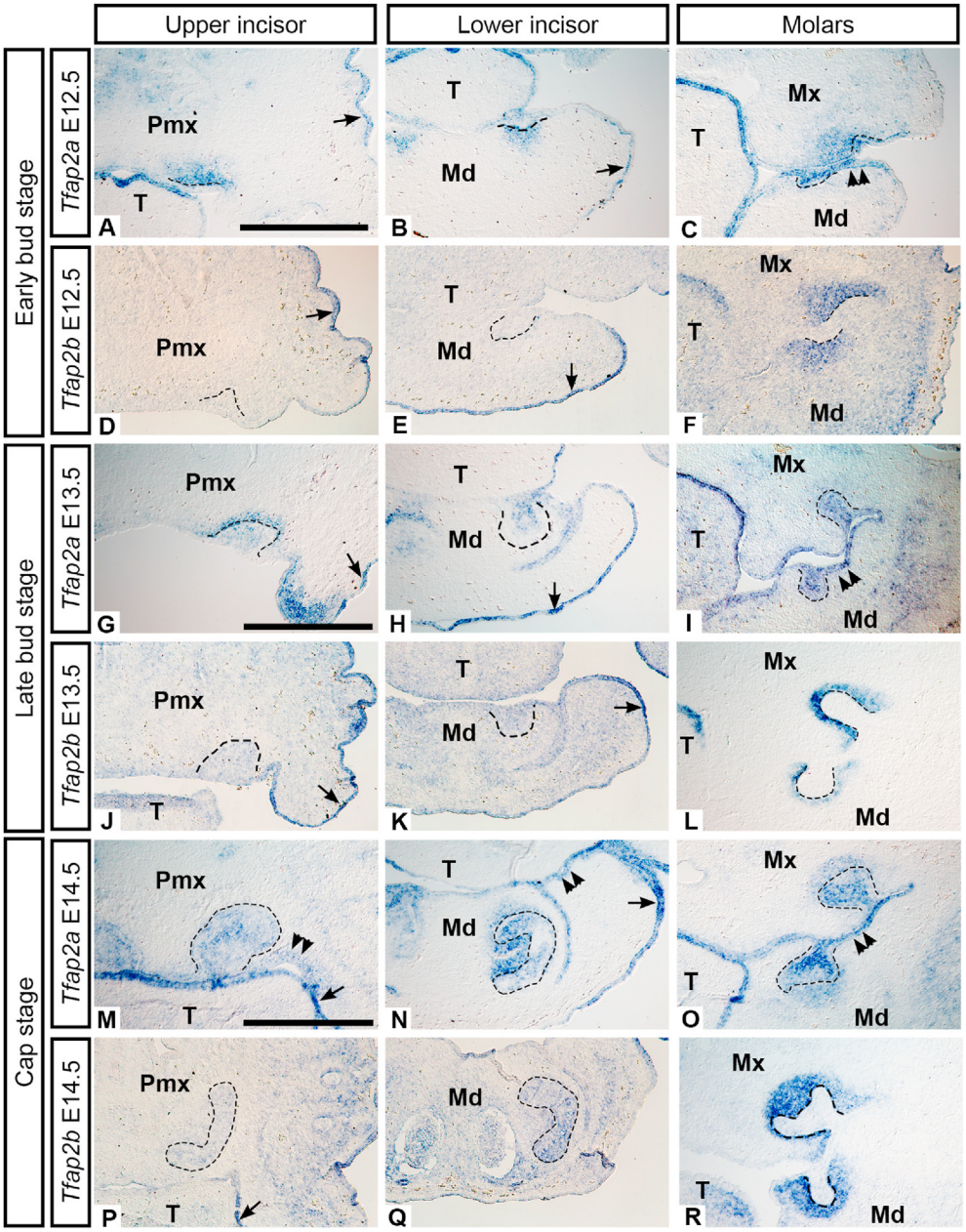

Fig. 1. Bud stage (E12.5 and 13.5) and cap stage (E14.5) mRNA expression of *Tfap2a* and *Tfap2b* in wild-type mouse embryos. Images showing mRNA transcripts detected by *in situ* hybridization on frontal cryosections through the upper incisor (left column), lower incisor (middle column), and molars (right column). The dental epithelium is outlined. There is minimal expression of *Tfap2b* in the bud stage upper and lower incisors (D-E, J-K) compared to the molar buds (F, L). Both *Tfap2a* and *Tfap2b* were detected in the surface epithelium (arrows) but only *Tfap2a* was present in the oral epithelium (M, N double arrowheads). Scale bars in A, G, M: 500 μm, all images are at the same scale. Pmx: premaxilla, Mx: maxilla, Md: mandible, T: tongue.

expression were detected to varying extents in bell stage molar epithelium in patterns more similar to *Tfap2a* (Fig. 2K, L, O, P; Supplementary Fig. 4I, J, K, L). The overlapping expression domains between *Tfap2a, Kctd1,* and *Yeats4* in the dental epithelium, and to a lesser extent in the molar mesenchyme, suggest that *Kctd1* and *Yeats4* may interact directly with *Tfap2a* to modulate the transcriptional activity of the latter in developing teeth, though this prediction remains to be tested (Fig. 2Q–T).

We also compared these patterns with *Ets1,* which, like *Tfap2a* and *Tfap2b,* is expressed in migrating NC cells, where it acts downstream of *Tfap2a* in the chick (Barembaum and Bronner, 2013). In incisors and molars, *Ets1* was not strongly expressed in mesenchyme associated with the dentition, but instead appeared to be associated with erythrocytes (Supplementary Results 6.1; Supplementary Fig. 4A-H). Altogether, these findings highlight regions of both co-expression and divergent expression domains of *Tfap2a/Tfap2b* and two regulators of AP-2 function during development of incisors and molars.

3.2. Epithelial deletion of *Tfap2a* and *Tfap2b* leads to extra incisors and misshapen teeth

The dynamic expression patterns of both *Tfap2a* and *Tfap2b* in the dental epithelium and mesenchyme suggested that these factors could play tissue-specific roles in tooth development. To test whether epithelial-specific expression of *Tfap2a* and *Tfap2b* is required for the development of properly shaped teeth, we used an epithelial-specific *Cre* recombinase allele, *Crect* (Schock et al., 2017). Mutant embryos were homozygous for *Tfap2a* and *Tfap2b* conditional alleles and heterozygous for the *Crect* transgene (*Tfap2a*$^{fl/fl}$;*Tfap2b*$^{fl/fl}$;*Crect*), resulting in deletion of *Tfap2a* and *Tfap2b* exclusively from the ectoderm, including the presumptive dental epithelium (Supplementary Fig. 1A).

The most striking difference in E18.5 embryos lacking *Tfap2a* and *Tfap2b* in the epithelium were changes in the number and/or morphology of the lower incisors (Supplementary Table 1). Control embryos at E18.5 had a single upper and single lower incisor ($I^1/_1$) on

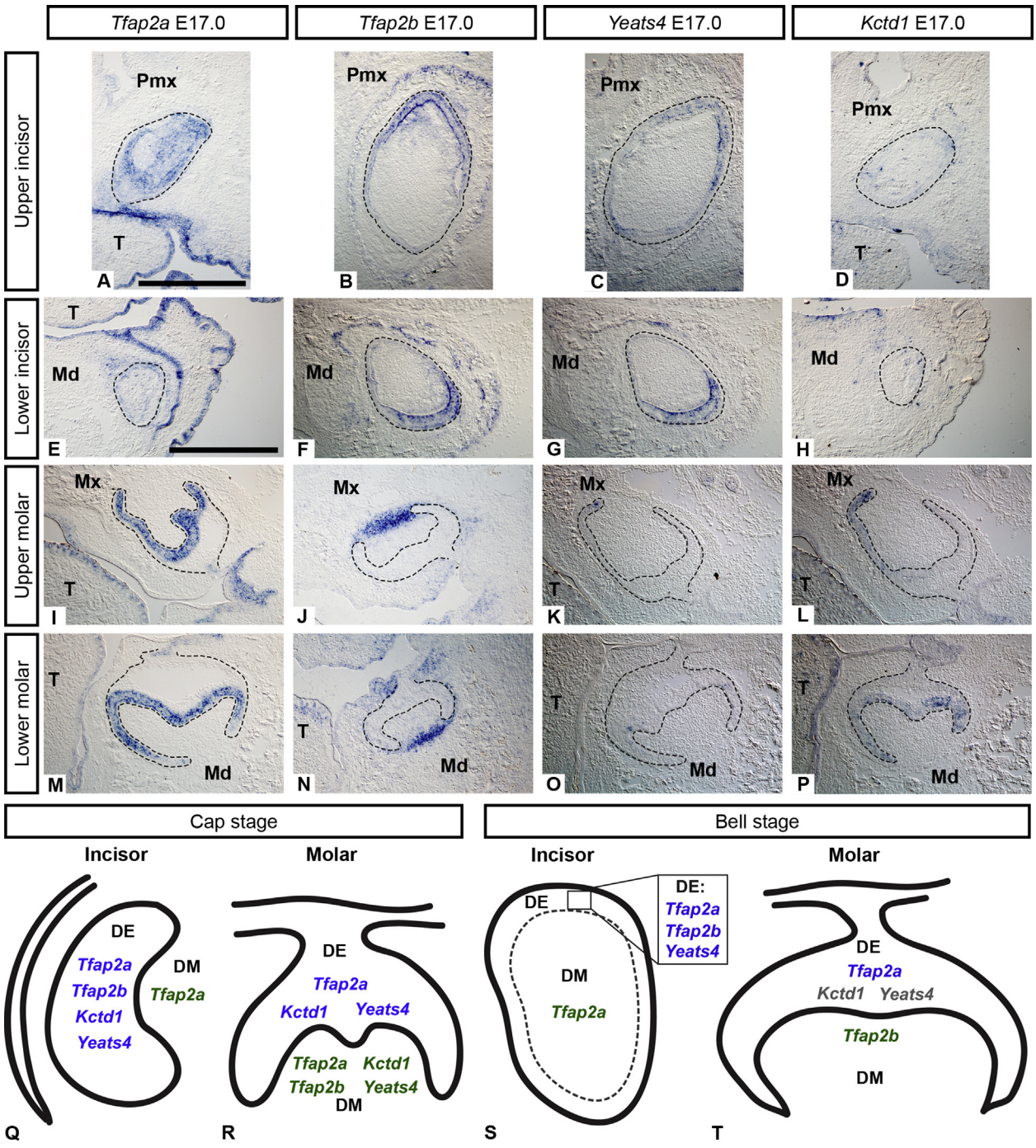

Fig. 2. Bell stage (E17.0) mRNA expression of *Tfap2a, Tfap2b, Yeats4,* and *Kctd1* (A–P) in wild-type mouse embryos. mRNA transcripts detected by *in situ* hybridization on cryosections in the frontal plane are shown in the upper incisor (A–D), lower incisor (E–H), upper molar (I–L), and lower molar (M–P). The dental epithelium is outlined. Note the spatially restricted expression of *Tfap2a* in the molars (I, M) and the overlapping expression domains of *Tfap2b* and *Yeats4* in the incisors (B, C, F, G). Scale bar in A, G: 500 μm, all images are at the same scale. Pmx: premaxilla, Mx: maxilla, Md: mandible, T: tongue. **Summary of gene expression results highlighting differences between incisors and molars at the cap (Q, R) and bell stages (S, T).** In cap stage incisors (Q), *Tfap2a*, *Tfap2b, Kctd1,* and *Yeats4* are expressed in the epithelium but only *Tfap2a* is expressed throughout the mesenchyme. In the molars (R), *Tfap2b* is expressed in the mesenchyme, while *Tfap2a, Kctd1,* and *Yeats4* transcripts are present in both the epithelium and mesenchyme. In bell stage incisors (S), the expression of each gene is similar to that of the cap stage incisors, however, *Kctd1* was not detected. In molars at the bell stage (T), *Tfap2a* was expressed in the epithelium only, *Tfap2b* expression was restricted to the mesenchyme, and minimal to no expression of *Kctd1* and *Yeats4* was detected, indicated by gray text (T). DE: dental epithelium, DM: dental mesenchyme.

each side of the jaw (Fig. 3A, C, E) whereas mutant embryos often displayed an additional bilateral lower incisor ventral to I_1 (N = 2/4 embryos) (Fig. 3B). The additional teeth appeared similar to I_1 and a complete repertoire of differentiated cell types were found in the supernumerary incisors, including enamel-forming ameloblasts and dentin-forming odontoblasts (Fig. 3B, B'). Mutants that lacked duplicated incisors had aberrantly shaped lower incisors (I_1) that exhibited ventral curvature (N = 2/4 embryos; bilaterally symmetrical) (Fig. 3G, I; Supplementary Fig. 5D, D′).

The faces of the epithelium-specific mutants were highly dysmorphic (Van Otterloo et al., unpublished observations) which made it difficult to assess the upper incisors. In some mutants, upper incisors were not

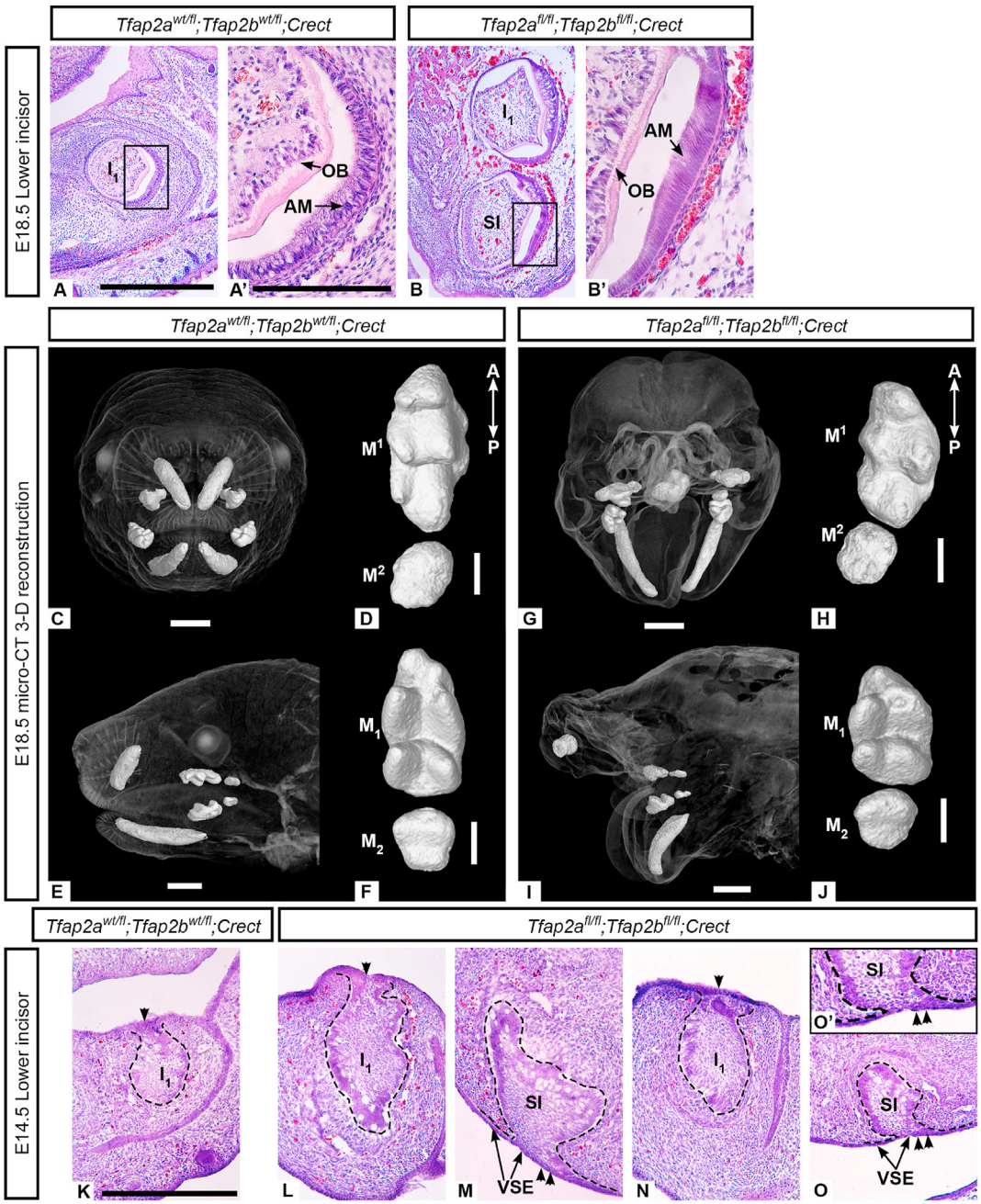

Fig. 3. ***Tfap2a*$^{fl/fl}$*;Tfap2b*$^{fl/fl}$*;Crect* mutant embryos have duplicated or ventrally curved lower incisors.** Hematoxylin and eosin (H&E) staining of bell stage lower incisors from control (A, A′) and epithelial mutant (B, B′) embryos revealed a supernumerary incisor ventral to I_1 in the mutant mandible. This additional incisor undergoes cytodifferentiation (B′) similar to I_1 in mutant (B) and control (A, A′) embryos. All mutant mandibles examined exhibited ventral curvature as seen in G and I. In the 3-D reconstructed embryo (G, I), instead of duplicated lower incisors, a single ventrally curved lower incisor was present on the right and left sides and two small right and left upper incisors were also observed. 3-D reconstructions of upper (D, H) and lower (F, J) molars show that first ($M^1/_1$) and second ($M^2/_2$) molars develop in mutants lacking epithelial *Tfap2a* and *Tfap2b* (H, J) and that the main cusps are present, though less distinct, in mutants compared with the controls (D, F). The mutant molars appear shorter along the anterior-posterior (A-P) axis than the control molars. H&E staining of frontal cryosections through cap stage lower incisors from control (K) and epithelial mutants, where I_1 and a supernumerary incisor are shown in two different individuals (L-M and N–O, respectively). I_1 is attached to the dorsal dental lamina (single arrowhead) in the control (K) and mutants (L, N). The supernumerary incisors are tethered to the ventral surface epithelium (double arrowheads, see the region of attachment shown in M, O, and enlarged in O′) and are positioned ventral and slightly posterior to I_1. Scale bars: 500 μm (A), 150 μm (A′), 1 mm (C, E, G, I), 300 μm (D, F, H, J, K). A and B, are to scale; A′ and B′ are to scale; K–O are to scale. AM: ameloblasts, OB: odontoblasts, SI: supernumerary incisor, VSE: ventral surface epithelium.

observed at E18.5 (N = 3/4 embryos) (Supplementary Fig. 6G-I), but in one embryo we observed two diminutive upper incisors (Fig. 3G, I; Supplementary Fig. 5B, B'; Supplementary Table 1). In contrast to the incisors, the first and second upper and lower molars of the mutants (Fig. 3H, J; Supplementary Fig. 7H, I, K, L) were structurally similar to those of the controls (Fig. 3D, F; Supplementary Fig. 7G, J).

In mutant embryos from a similar genetic cross in which one of the conditional alleles was null (*Tfap2a*$^{fl/null}$*;Tfap2b*$^{fl/null}$*;Crect*), we observed the same duplicated lower incisor phenotype as in the first cross (Supplementary Fig. 8D, E; Supplementary Table 1) and a single upper incisor was present (Supplementary Fig. 8B), whereas the molars appeared similar to the controls (N = 2/2) (Supplementary Fig. 8F-K).

To determine how this supernumerary lower incisor develops, we examined the histological structure of cap stage (E14.5) teeth in *Tfap2a*$^{fl/fl}$;*Tfap2b*$^{fl/fl}$;*Crect* embryos. In E14.5 control embryos, we observed bilateral I_1 (Fig. 3K), but in mutants, bilateral duplicated incisors were observed at the cap (N = 2/3; Fig. 3L–O) or bud (N = 1/3; data not shown) stage. In these mutants, I_1 appeared tethered to the dorsal dental lamina (Fig. 3L, N) as in the controls, but the duplicated (ventral) incisor was tethered to the ventral surface epithelium, a region that does not normally have the characteristics of the dental lamina (Fig. 3M, O). Upper incisors were not observed in E14.5 mutant embryos (Supplementary Fig. 6B-E) indicating that they failed to form prior to E14.5. The molars at E14.5 appeared similar to those of the controls (Supplementary Fig. 7A-F). In E14.5 "single" mutants that still contained one wild-type *Tfap2a* or *Tfap2b* allele (*Tfap2a*$^{fl/fl}$;*Tfap2b*$^{fl/wt}$;*Crect* or *Tfap2a*$^{fl/wt}$;*Tfap2b*$^{fl/fl}$;*Crect*), the dentition was similar to that of the control embryos (N = 3/3) (Supplementary Fig. 9; Supplementary Results 6.2).

TFAP2A and TFAP2B are able to form heterodimers (Ding et al., 2009; Williams and Tjian, 1991), can bind the same DNA consensus sequences (Williams and Tjian, 1991), and are capable of functioning redundantly in tissues in which they are co-expressed (Hoffman et al., 2007; Li and Cornell, 2007; Rothstein and Simoes-Costa, 2020; Seberg et al., 2017; Van Otterloo et al., 2018; Wang et al., 2008). Consistent with these observations, our analysis of *Tfap2a* and *Tfap2b* mutants shows that TFAP2A and TFAP2B cooperatively function within the craniodental ectoderm, including the dental epithelium, to regulate incisor development.

One possible explanation for the incisor duplication is that, in the absence of *Tfap2a* and *Tfap2b*, the incisor odontogenic domain is expanded ventrally, permitting a second incisor to form in the mandible. A second possibility is that there is a duplication of the odontogenic domain so that a new and separate domain is formed on the aboral surface of the mandible, thereby allowing an ectopic incisor to form. Such a

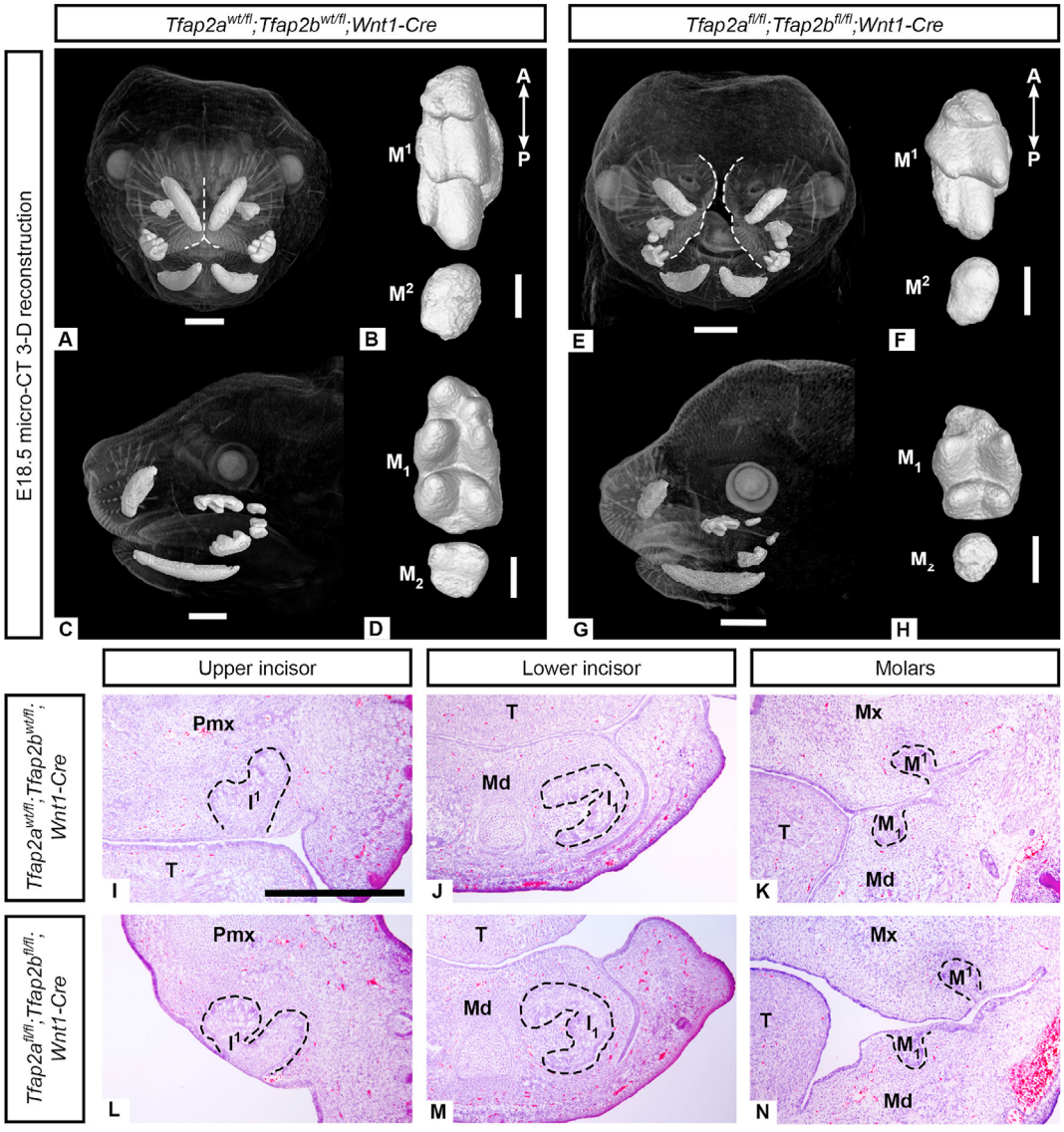

Fig. 4. Incisors and molars in *Tfap2a*$^{fl/fl}$;*Tfap2b*$^{fl/fl}$;*Wnt1-Cre* embryos lacking *Tfap2a* and *Tfap2b* expression in the NC-derived mesenchyme lack major morphological defects based on μCT (A–H) and histological (I–N) analyses. 3-D reconstructions of μCT data comparing morphology of control upper (B: $M^{1\text{-}2}$) and lower (D: $M_{1\text{-}2}$) molars with mutant upper (F: $M^{1\text{-}2}$) and lower (H: $M_{1\text{-}2}$) molars. The correct number of cusps are present but the molars appear shorter along the anterior-posterior (A–P) axis than the controls. Note the midface cleft in the mutant (E), outlined in white, compared to the control (A). H&E stained cryosections in the frontal plane (I–N) showing that mesenchyme-specific mutant incisors (L, M) and molars (N) are similar to those of the control (I–K) at the cap stage (E14.5). Due to the cleft midface and palate, in the anterior-most frontal section shown here (L) the medial aspect of the premaxilla is at an angle. Scale bars are 1 mm (A, C, E, G) and 300 μm (B, D, F, H), 500 μm (I–N). Pmx: premaxilla, Mx: maxilla, Md: mandible, T: tongue.

domain would need to be induced only at the distal end of the mandible, as the molars are not duplicated. Ventral curvature of I_1 in the mutants lacking duplicated incisors is suggestive of dorsoventral mis-patterning, however, this may be secondary to changes in mandible shape, which also curves ventrally compared to the control (Fig. 3G, I) (Van Otterloo et al., unpublished observations). In mutants in which a supernumerary cap stage lower incisor was observed at E14.5, it appeared to be connected to the ventral surface epithelium (Fig. 3M, O), raising the possibility that the surface epithelium has properties of an ectopic dental lamina. This suggests that the ectopic tooth was initiated in the ventral epithelium, but further studies will be required to ascertain how odontogenic potential is altered by the loss of ectodermal expression of these two AP-2 genes.

3.3. Mesenchymal *Tfap2a* and *Tfap2b* is dispensable for tooth development

Given that robust *Tfap2a* and *Tfap2b* expression was detected in the dental mesenchyme (Figs. 1–2), we predicted that AP-2 activity within the cranial NC-derived mesenchyme is also required for normal tooth development. To test this hypothesis, we generated mice with conditional deletions of *Tfap2a* and *Tfap2b* in the NC-derived mesenchyme using the *Wnt1-Cre* allele (Danielian et al., 1998) and floxed or null alleles of *Tfap2a* and *Tfap2b* (Van Otterloo et al., 2018) (Supplementary Fig. 1B, D). Despite an upper midfacial cleft (Fig. 4E, Supplementary Fig. 10E), mutant embryos did not possess an aberrant incisor phenotype at E18.5 (N = 3/3) (Fig. 4E, G; Supplementary Fig. 10C, D; Supplementary Table 1) or at E14.5 (N = 3/3) (Fig. 4I, J, L, M). Histological and μCT analysis showed that the molars also appeared unperturbed and were similar to controls at the cap stage (Fig. 4K, N; Supplementary Table 1) and the bell stage (Fig. 4A-H; Supplementary Fig. 10F-I). In E14.5 "single" mutants with one wild-type allele of either *Tfap2a* or *Tfap2b* in the NC-derived mesenchyme (*e.g.* $Tfap2a^{fl/fl};Tfap2b^{fl/wt};Wnt1\text{-}Cre$), we also observed the proper number of cap stage incisors and molars (N = 3/3) (Supplementary Fig. 9; Supplementary Results 6.2). In contrast to the requirement for *Tfap2a* and *Tfap2b* in the dental epithelium, these results indicate that *Tfap2a* and *Tfap2b* in the mesenchyme are not necessary, either individually or collectively, for tooth development.

3.4. Molar development is largely unaffected following loss of *Tfap2a* and *Tfap2b* in either epithelium or mesenchyme

Loss of *Tfap2a* and *Tfap2b* in either the epithelium or the mesenchyme appeared to have little effect on the molar teeth; however, 3-D reconstructions of E18.5 M crowns ($M^1/_1$) revealed that the mutant molar crowns were shorter along the anterior-posterior (mesiodistal) axis compared to the controls (Fig. 3 D, F, H, J; Fig. 4 B, D, F, H). Quantification of the upper and lower first molar crowns (ratio of molar crown length to width, see Supplementary Methods 5.5) suggested that for both crosses, anterior-posterior molar length was reduced in both the epithelium-specific and mesenchyme-specific mutants compared to the controls (Supplementary Table 4) (N = 2 teeth/individual), although, given the sample size, it was not possible to determine if mutant molar ratios were statistically different from control molar ratios.

Though deletion of *Tfap2a* and *Tfap2b* may affect molar length, the mutations had the most profound effect on the incisors. Possible explanations for the relative lack of molar defects are that AP-2 function is not required for molar development, or alternatively, that additional *Tfap2* family members, such as *Tfap2c,* which is expressed within the oral epithelium and dental mesenchyme (Chazaud et al., 1996), may compensate for the loss of *Tfap2a* and *Tfap2b*. Finally, given that *Tfap2a* homozygous null mice are so severely affected that they lack a mouth, among other ventral craniofacial structures (Zhang et al., 1996), we cannot exclude the possibility that *Tfap2a* and *Tfap2b* have an earlier role in patterning the molars, in addition to their effect on molar size.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ydbio.2020.12.017.

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Data availability

The μCT data from this study will be freely available to the public on FaceBase3 (https://www.facebase.org/) at https://doi.org/10.25550/1-YA78.

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