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LETTERS

Dual epithelial origin of vertebrate oral teeth

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The oral cavity of vertebrates is generally thought to arise as an ectodermal invagination[1,2]. Consistent with this, oral teeth are proposed to arise exclusively from ectoderm, contributing to tooth enamel epithelium, and from neural crest derived mesenchyme, contributing to dentin and pulp[3–5]. Yet in many vertebrate groups, teeth are not restricted only to the oral cavity[6–9], but extend posteriorly as pharyngeal teeth that could be derived either directly from the endodermal epithelium, or from the ectodermal epithelium that reached this location through the mouth or through the pharyngeal slits[6]. However, when the oropharyngeal membrane, which forms a sharp ecto/endodermal border[10], is broken, the fate of these cells is poorly known. Here, using transgenic axolotls with a combination of fate-mapping approaches, we present reliable evidence of oral teeth derived from both the ectoderm and endoderm and, moreover, demonstrate teeth with a mixed ecto/endodermal origin. Despite the enamel epithelia having a different embryonic source, oral teeth in the axolotl display striking developmental uniformities and are otherwise identical. This suggests a dominant role for the neural crest mesenchyme over epithelia in tooth initiation and, from an evolutionary point of view, that an essential factor in teeth evolution was the odontogenic capacity of neural crest cells, regardless of possible 'outside-in'[11] or 'inside-out'[12] influx of the epithelium.

Teeth are one of the key vertebrate innovations, but their evolutionary origins are still a matter of debate. It is widely accepted that teeth initially evolved from outer skin denticles captured in the stomodeum (the odontode theory)[11] and modified there specifically in the context of newly developed jaws ('outside-in' theory). However, as there is good evidence of teeth/denticles inside the pharyngeal regions of many fossil jawless groups[7,12], they must have evolved with a great degree of independence from the stomodeal cavity and the jaw elements. An alternative scenario reflecting these facts has been suggested, in which oral teeth arose by the progression of ancient denticles from the endodermal pharynx towards the stomodeum ('inside-out' theory)[12]. More recently, however, it was argued on the basis of fossil evidence that teeth may have evolved independently through a convergent evolution and, thus, are not homologous among jawed vertebrates[13]. A new, appealing hypothesis was then proposed, namely that the diversity and complexity of dentitions can be explained by combinatorial derivation of teeth from both external (ectodermal), and internal (pharyngeal) denticles[4].

Teeth are commonly ranked among ectodermal organs[5], although they are composite structures of dual embryonic origin. The dental mesenchyme has been shown, using a fate-mapping approach, to be derived from neural crest cells in mammals[3], urodele amphibians[14] (also this study; Supplementary Fig. 3) and fish[15], and this is generally assumed to be the case in other vertebrates as well[16]. The germ-layer origin of the epithelium, however, is far less clear. Because tooth development is most completely understood in mouse embryos[4], it is often generalized accordingly that teeth develop exclusively in the region of the oral ectoderm, which invaginates to form a stomodeum[2]. The accepted view is that the presence of teeth in any region is an indubitable criterion for the existence of the ectodermal germ layer in this region at some time of development[1]. However, in various vertebrate lineages, so-called pharyngeal teeth, or even a second set of toothed jaws, are commonly found posterior to the stomodeum in areas that are presumably lined by endoderm rather than ectodermal epithelium[6,8]. Convincing developmental evidence for an endodermal origin of teeth situated in the pharyngeal cavity is lacking, and uncertainties arise also from the fact that some structures situated within the pharyngeal cavity of bony fishes are apparently derived from the ectoderm (for example gills or opercular bones). Apart from the facts that the endoderm was suggested, on the basis of histology, to contribute to tooth formation in some lower vertebrates during the first half of the twentieth century[17,18] and that such a role has been questioned even in mammals[19,20], our understanding of the germ-layer origin of tooth epithelia is fundamentally limited by the difficulties in distinguishing between ectoderm and endoderm during critical stages of later mouth development. Hence, after the breaking of the oropharyngeal membrane, which constitutes the border between the oral (ectodermal) and pharyngeal (endodermal) epithelia, the fate of these cells is not known, owing to a lack of reliable fate-mapping studies even for model vertebrate species like mouse, chick or zebrafish.

Urodele amphibians are an interesting group for the analysis of the germ-layer origin of teeth because the presumptive border between the oral ectoderm and endoderm is substantially more anterior than in mammals[17,18,21]. To study mouth development and the germ-layer origin of dental tissues in details, we took advantage of recently developed transgenic axolotls[22]. We designed a novel experimental procedure that enables us reliably to mark the ectoderm of the entire prospective mouth area and to follow its fate during the course of development.

First we performed transplantations of four different areas of double-layered ventral epithelia using axolotl GFP-positive neurulae (Supplementary Fig 1a; GFP, green fluorescent protein) and found conclusively that for reliable marking of the ectodermal layer of the prospective mouth, it is necessary to graft both prospective oral ectoderm and transverse neural folds (in total, $n = 113$; Fig. 1a, b). Moreover, we always ascertained that these GFP-positive grafts comprised the entire mouth area, so that no GFP-negative cells could contribute to mouth formation (Supplementary Fig. 1b).

Next we used this experimental system to trace the accurate contribution of ectodermal cells to mouth and tooth formation. In the axolotl, the epidermis in the prospective mouth region initially consists of a double-layered ectoderm but subsequently becomes reduced to a single outer layer when the inner layer bends inwards over non-ectodermal mouth tissue as an 'ectodermal collar'[17,18] (Fig. 1c). This oral ectodermal lining deepens (compare Supplementary Fig. 1c, d) and during later tail-bud stages contributes to prominent buds (Fig. 1d, arrowhead). However, morphologically identical budding structures also appear in the non-ectodermal area (Fig. 1d, arrow; notice the proximity to the lower-jaw cartilage, MC). Later, still before hatching, buds are easily identified as developing teeth, which are regularly distributed in both ectodermal (Fig. 1e, arrowheads) and non-ectodermal areas (Fig. 1e,

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arrows). To confirm the identity of these structures as tooth buds, we used an antibody directed against calbindin (Sigma), a calcium-binding protein that specifically recognizes ameloblasts[23]. From sections where both GFP and immunostaining is visualized (Fig. 1f, g and Supplementary Fig. 2a, b), it is evident that the tooth primordia are developing from both ectodermal and non-ectodermal epithelia.

To substantiate our finding that in the Mexican axolotl some oral teeth develop from non-ectodermal epithelia, we invented a double-labelling approach using which cells of both oral ectoderm and foregut endoderm can be reliably marked and mapped (Fig. 2a). First, at a neurula stage, the double-layered prospective oral epithelium (from the same area as in the previous experiment) was extirpated. The exposed endodermal layer was then focally injected using the lipophilic dye DiI (Molecular Probes). Next a GFP-positive graft comprising the entire prospective oral ectoderm (as above) was transplanted orthotopically to wild-type host embryos. In this approach ($n = 91$), the entire prospective ectoderm of the oral area was marked with a green fluorescent dye and some of the foregut endodermal cells, expected to contribute to tooth buds, were labelled with a red fluorescent dye.

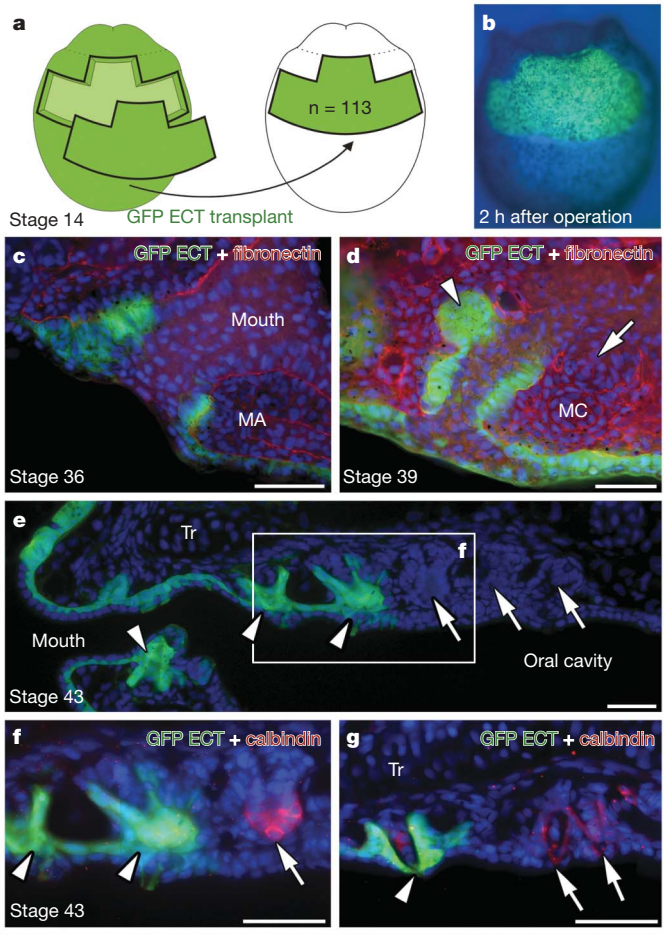

Figure 1 | Ectoderm contribution to mouth and tooth formation in the Mexican axolotl. **a**, An experimental scheme with the prospective oral ectoderm (ECT) transplanted from a GFP-positive donor to a host embryo, ventral view (END, endoderm). **b**, An embryo 2 h after operation. **c**–**g**, Paramedial sections, head to the left, showing a contribution of the oral ECT (green) to mouth and tooth formation. DAPI (blue) stains cell nuclei; fibronectin (red in **c**, **d**) marks cell and tissue borders. Initially the oral ECT (green) inflexes as a stomodeal collar (**c**). Then prominent tooth buds develop in ECT areas (arrowheads) as well as in non-ECT areas (arrows; **d**, **e**). Tooth buds, identified using anti-calbindin (red), develop within ECT areas (arrowheads) as well as non-ECT areas (arrows; **f**, **g**). Tr, trabecula; MA, mandibular arch; MC, Meckel's cartilage. **c**, **d**, Vibratome 100-μm sections; **e**–**g**, cryostat 20-μm sections. Scale bars, 50 μm.

Using this double-fate-mapping approach, we obtained strong support for our previous conclusions that the axolotl possesses oral teeth with an epithelial lining of non-ectodermal origin. Specifically, dye injected into the foregut endoderm at the neurula stage was found in oral tooth germs and later in developing teeth (Fig. 2b–d, h and Supplementary Fig. 4), as well as, notably, in the epithelium situated between GFP-positive ectodermal epithelia (Fig. 2c). Moreover, alongside the contact zone between the ecto- and endodermal oral epithelia, we found tooth germs that consistently demonstrate a mixed contribution from both ecto- and endodermal cells to their enamel epithelia (Fig. 2e–g and Supplementary Fig. 5). On the basis of our combined tracing approaches, we conclude that on the upper jaw the enamel epithelia of the premaxillary/maxillary teeth are always ectodermal, whereas the enamel epithelia of the vomero-palatal teeth are derived from the ectoderm, endoderm or

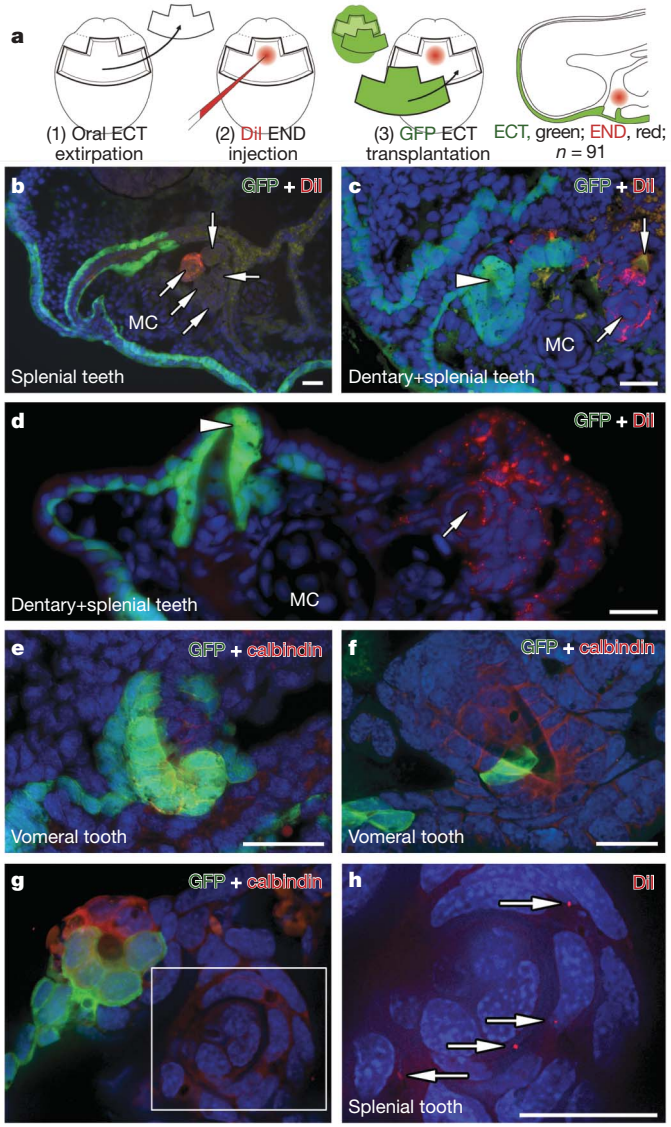

Figure 2 | Endoderm contribution to tooth formation. **a**, Sketch of double-fate-mapping experiment: following extirpation of the prospective oral ECT (1), DiI was injected into foregut END (2) and the prospective oral ECT (GFP-positive) was transplanted orthotopically (3). **b**–**h**, Paramedial (cryostat 20-μm) sections, head to the left, showing a contribution of the oral ECT (green) and mouth END (red in **b**–**d**, **h**) to tooth formation. DAPI (blue) stains cell nuclei, calbindin (red in **e**–**g**) marks tooth buds. Arrows point to END teeth; arrowheads to ECT teeth. **e**–**g**, Details of teeth of mixed origin. **h**, A confocal image, inset in **g**, showing the END (DiI, red) contribution to splenial tooth germ. Scale bars, 25 μm.

from a mixed source, according to their position (Fig. 3a). On the lower jaw, dentary teeth are basically ectodermal and splenial endodermal; however, there are teeth of mixed origin situated on the anterior parts of these fields (Fig. 3a).

Next a quantitative screening was performed in which all teeth were counted and their respective germ-layer origins determined at four different stages and based on 26 embryos from the double-labelling experiment (Fig. 4). This analysis revealed that of 1,137 teeth, 374 were derived from ectoderm, 598 from endoderm, and 155 were of mixed ecto/endodermal origin. We note that during the course of development, the proportion of ectoderm-derived teeth slightly increases as teeth located on the premaxillary and maxillary bones, which are purely ectodermal, develop very late. Thus, in the average embryo (analysed at stage 45, when the mouth opens and animals start to eat), of 82 teeth 29 were of ectoderm, 42 were of endoderm and 11 were of mixed epithelial origin (Supplementary Tables 1–4). Non-epithelial derivatives, such as tooth dentin and papillae, were derived from neural crest mesenchyme (from the trigeminal neural crest stream; Supplementary Fig. 3). All quantitative and statistical analyses were strongly significant (Supplementary Tables 1–4) and constitute robust support that our data are not biased by any technical problems.

Previous theories have identified the ectodermal border in the mouth as being central to tooth positioning[11] (Fig. 3b, upper row). However, in the Mexican axolotl, the oral ectoderm does not form a true stomodeum[17,18]. Instead, only an inner ectodermal layer bends inwards as a stomodeal collar over the dense endodermal rod, which blocks the prospective mouth at early stages of development (Fig. 3b, lower row). Because of this positioning, the collar cells develop into the basal cells, and the outer cells of the endodermal rod develop into the apical cells of the oral epithelium during the course of mouth opening (Fig. 1e and Supplementary Fig. 2a–d; summarized in Fig. 3b, lower row). The endodermal cells of the mouth, as part of the epithelial lining, are consequently found also on the outer surface of the mouth (Fig. 1e and Supplementary Fig. 2b–d, arrowheads; summarized in Fig. 3b, lower row). Thus, in the axolotl, the posterior part of the oral cavity is lined with the endodermal epithelium, whereas the anterior part is lined with an epithelium of double origin (Fig. 3b and Supplementary Fig. 2b). This provides reliable documentation of an oral endodermal epithelial lining that reaches outside the mouth, and, also, of an oral epithelium originating from two germ layers. A considerable number of reports on mouth development have been published, but, as underlined by our results, there is still a need for detailed fate-mapping approaches in studies of dynamic interactions of cells and tissues derived from different germ layers.

Progressing from recent vivid discussions on the subject[4,6,8,24], our data present reliable evidence of oral teeth of endodermal origin in vertebrates. We speculate that oral teeth of endodermal origin might form in all animals with oral endoderm, that is, in urodele and probably also lungfish species, and maybe even in some frogs[25], where the mouth develops from a structure similar to the stomodeal collar. However, as a possible interdigitation of cells from both epithelial tissue layers during mouth formation has not been fate-mapped for any vertebrate species, and some reports indicate that foregut endoderm may stretch more to

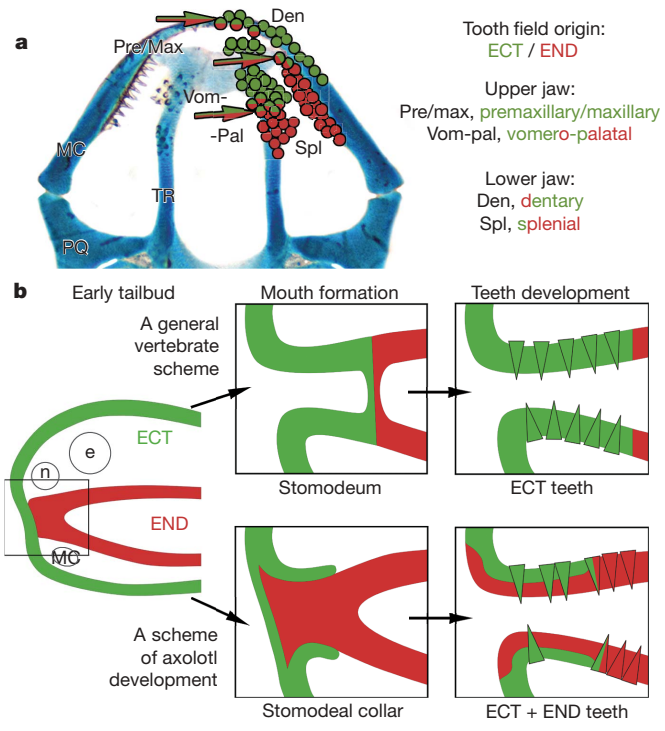

Figure 3 | Germ-layer origin and morphogenesis of teeth of the Mexican axolotl. a, A sketch of the germ-layer origin of teeth in the Mexican axolotl. ECT teeth, green; END teeth, red; teeth of mixed origin, red–green (the colouring in the key is a qualitative guide to the ratio of the components in each tooth field). Cartilages visualized using alcian blue. **b**, Comparative developmental morphogenesis of the mouth region and the germ-layer origin of teeth of vertebrate (upper row) and an axolotl (lower row) embryo. In the majority of vertebrates, the mouth develops from a stomodeum with teeth distributed in invaginated ECT. In contrast, in urodeles the mouth develops from a stomodeal collar with an oral epithelium either of a dual origin, with teeth of ECT or END, or of a mixed origin. PQ, palatoquadrate; TR, trabecula cranii; n, nose; e, eye.

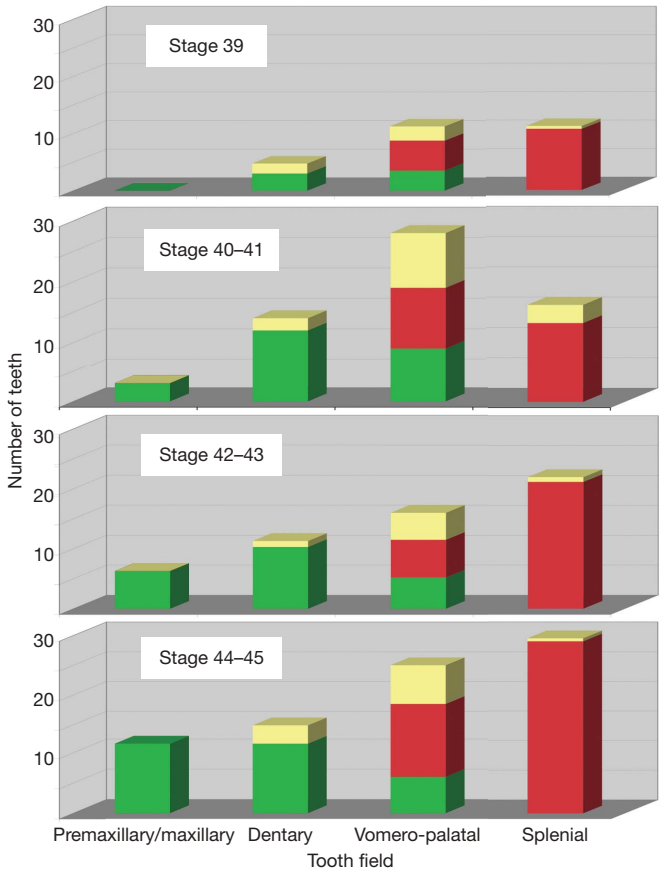

Figure 4 | A quantitative screening of teeth from different tooth fields with respect to their germ-layer origin. $n = 1{,}137$ teeth: 374 ECT (green), 598 END (red), 155 ECT–END mixture (yellow). Data based on 26 animals, visualized at four stages: four animals at stage 39, 14 animals at stage 40–41, six animals at stage 42–43, two animals at stage 44–45. For details and original data, see Supplementary Tables 1–4.

the anterior than hitherto believed[8,19–21], we speculate that oral teeth of endodermal origin might present a more common feature in vertebrate oral development than previously assumed.

Whereas the classical 'outside-in' theory implies that teeth were initially derived from the oral ectodermal layer[11], the 'inside-out' theory strongly suggests that they were derived from the endodermal layer[12], and this derivation is believed to impart differences to denticles, teeth or dentition in terms of shape and complexity[4,12,24]. However, the dual origin of enamel epithelia in otherwise morphologically identical axolotl oral tooth primordia (as regards complexity, shape, position, timing and morpho-differentiation of teeth), together with studies illustrating deep shared molecular similarities between oral (supposedly ectodermal) and pharyngeal (supposedly endodermal) teeth[15,26,27] imply that 'ectodermal' and 'endodermal' teeth do not differ essentially. It is beyond the scope of this study to identify the plesiomorphic germ-layer origin of tooth epithelium. However, our results clearly demonstrate that the germ-layer origin of epithelium into which the mesenchyme cells come into contact does not affect the final product of the odontogenic cascade. Mesenchyme cells can thus apparently interact with a host of epithelial cells, forming teeth/denticles when in the stomodeum, in the pharyngeal cavity or on the skin surface. All this suggests that the major agent of dental development is the neural crest mesenchyme rather than the epithelium, the role of which in tooth patterning[5,12,28] and even in tooth initiation[29] may be less fundamental than commonly believed. It therefore seems most likely that all teeth of extant vertebrates—or, more precisely, the developmental machinery producing them—have evolved only once, somewhere in the oropharynx, driven by a neural crest signal.

METHODS SUMMARY

Embryos. Embryos of the Mexican axolotl (*Ambystoma mexicanum*) were obtained, reared and staged as previously described[30]. GFP embryos were obtained from the Max-Planck-Institute of Molecular Cell Biology and Genetics in Dresden, Germany, and were developed in the laboratory of E. Tanaka[22].

Operations and injections. GFP ectodermal transplantations were performed as sketched in Fig. 1a ($n = 113$). At first, however, transplantations of four different areas of double-layered ventral epithelia were performed (Supplementary Fig. 1a) to define the entire ectodermal layer of the prospective mouth.

The double-labelling approach by which cells of both oral ectoderm and foregut endoderm were marked and mapped (as sketched in Fig. 2a ($n = 91$)) includes extirpation of the double-layered prospective oral epithelia (from the same area as in the previous experiment), focal injection of a CellTracker CM-DiI (Molecular Probes) into the exposed endodermal layer and, lastly, the orthotopic transplantation of a GFP-positive prospective oral ectoderm (as above) into wild-type host embryos.

Sectioning and immunostaining. Axolotl embryos were anaesthetized using MS-222 (Sigma), fixed in 4% paraformaldehyde in phosphate buffered saline and sectioned using a Vibratome 1000 sectioning system (Ted Pella) or a CM3050 cryostat (Leica). Sections were counterstained using anti-fibronectin antibody (Dako) to visualize tissue borders, with 4,6-diamidino-2-phenylindole (DAPI) to mark cell nuclei, or with anti-calbindin antibody (Sigma), which specifically recognizes ameloblasts[23].

Image acquisition. Separate fluorescence images were captured using an Olympus BX51 microscope with a SPOT RT camera, or the Olympus Cell^R IX81 with a Hamamatsu Photonics Orca camera, merged and optimized using Spot and Adobe Photoshop software.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions Tissue grafting was carried out in the laboratory of H.-H.E., who also helped with initial experiments. V.S. made transplantations and performed sectioning and image analyses as his Master's thesis under the supervision of R.C., who also designed the initial experiments and wrote the manuscript. R.C. and I.H. planned the study and interpreted the results, I.H. made statistical analyses and helped in writing the manuscript, and all authors discussed the results and commented on the manuscript.

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METHODS

Embryos. Embryos of the Mexican axolotl (*A. mexicanum*) were obtained, reared and staged as previously described[30,31]. GFP embryos were obtained from the Max-Planck-Institute of Molecular Cell Biology and Genetics in Dresden, Germany, and were spawned from a β-actin promoter-driven GFP germ-line transgenic animal that had been produced by plasmid injection[22]. Embryos were kept in tap water, and before being used for transplantations and injections, embryos were washed thoroughly with tap water and sterile Steinberg solution containing antibiotics (Antibiotic-Antimycotic, Gibco) and then decapsulated manually.

Operations and injections. GFP ectodermal transplantations were performed as sketched in Fig. 1a ($n = 113$). Operations were performed under sterile conditions using tungsten needles in an agar dish containing 1 M Steinberg solution plus antibiotics. We designed an experimental procedure that enabled us to mark the ectoderm of the entire prospective mouth area reliably and to follow its fate during the course of development. First we performed transplantations of four different areas of double-layered ventral epithelia from GFP-positive to host neurulae (Supplementary Fig. 1a; numbers of animals used for each operation are indicated there) and found conclusively that for reliable marking of the ectodermal layer of the prospective mouth it is necessary to graft both prospective oral ectoderm and a transverse neural fold (Fig. 1a, b). Using this type of transplantation, we were able to follow the fate of the entire ectodermal layer that translocates into the mouth, and, therefore, in this way all ectoderm-derived teeth became GFP-positive. We always ascertained, however, that these GFP grafts comprised the entire mouth area, so that no GFP-negative cells could contribute to mouth formation (Supplementary Fig. 1b).

Next we invented a double-labelling approach by which cells of both oral ectoderm and foregut endoderm can be reliably marked and mapped (Fig. 2a). First, at a neurula stage, double-layered prospective oral epithelia (from the same area as in the previous experiment) were extirpated. The exposed endodermal layer was then focally injected using the lipophilic dye DiI (Molecular Probes), dissolved in absolute ethanol to a concentration of 1 mg ml^{-1} and further diluted in nine parts of 10% sucrose in water just before injection. Then a graft from a GFP-positive neurula comprising the entire prospective oral ectoderm (as above) was transplanted orthotopically to wild-type host embryos. In this approach ($n = 91$), the entire prospective ectoderm of the oral area was marked green (GFP) and some of foregut endodermal cells, expected to contribute to tooth buds, were labelled red (DiI).

Neural crest transplantations. Trigeminal neural crest cells were transplanted from GFP-positive to wild-type embryos at the neurula stage as described in detail elswhere[32].

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