

Bowdoin College

## Bowdoin Digital Commons

---

Biology Faculty Publications

Faculty Scholarship and Creative Work

---

6-12-2019

# The first formed tooth serves as a signalling centre to induce the formation of the dental row in zebrafish

Yann Gibert

*University of Mississippi Medical Center*

Eric Samarut

*Centre Hospitalier de L'Universite de Montreal*

Megan K. Ellis

*Deakin School of Medicine*

William R. Jackman

*Bowdoin College*

Vincent Laudet

*Sorbonne Universite*

Follow this and additional works at: <https://digitalcommons.bowdoin.edu/biology-faculty-publications>

---

### Recommended Citation

Gibert, Yann; Samarut, Eric; Ellis, Megan K.; Jackman, William R.; and Laudet, Vincent, "The first formed tooth serves as a signalling centre to induce the formation of the dental row in zebrafish" (2019). *Biology Faculty Publications*. 121.

<https://digitalcommons.bowdoin.edu/biology-faculty-publications/121>

This Article is brought to you for free and open access by the Faculty Scholarship and Creative Work at Bowdoin Digital Commons. It has been accepted for inclusion in Biology Faculty Publications by an authorized administrator of Bowdoin Digital Commons. For more information, please contact [mdoyle@bowdoin.edu](mailto:mdoyle@bowdoin.edu), [a.sauer@bowdoin.edu](mailto:a.sauer@bowdoin.edu).

## Research



**Cite this article:** Gibert Y, Samarut E, Ellis MK, Jackman WR, Laudet V. 2019 The first formed tooth serves as a signalling centre to induce the formation of the dental row in zebrafish. *Proc. R. Soc. B* **286**: 20190401. <http://dx.doi.org/10.1098/rspb.2019.0401>

Received: 15 February 2019

Accepted: 20 May 2019

### Subject Category:

Evolution

### Subject Areas:

developmental biology, evolution

### Keywords:

tooth, initiator tooth, retinoic acid, FGF, 4 V<sup>1</sup>, dentition

### Author for correspondence:

Yann Gibert

e-mail: [ygibert@umc.edu](mailto:ygibert@umc.edu)

<sup>†</sup>These authors contributed equally to this work.

<sup>‡</sup>Present address: University of Mississippi Medical Center, Department of Cell and Molecular Biology, 2500 North State Street, Jackson, MS 39216, USA.

<sup>¶</sup>Present address: Research Center of the University of Montreal Hospital Center (CRCHUM), Department of Neurosciences, Université de Montréal, Montréal, QC, Canada.

<sup>||</sup>Present address: Sorbonne Universités, UPMC Université Paris 6, CNRS, UMR 7232, BIOM, Observatoire Océanologique de Banyuls-sur-Mer, 66650 1 Avenue du Fontaulé Banyuls-sur-Mer, France.

Electronic supplementary material is available online at <https://dx.doi.org/10.6084/m9.figshare.c.4529963>.

# The first formed tooth serves as a signalling centre to induce the formation of the dental row in zebrafish

Yann Gibert<sup>1,†,‡</sup>, Eric Samarut<sup>2,†,¶</sup>, Megan K. Ellis<sup>1</sup>, William R. Jackman<sup>3</sup> and Vincent Laudet<sup>2,||</sup>

<sup>1</sup>Metabolic Genetic Diseases Laboratory, Metabolic Research Unit, Deakin School of Medicine, 75 Pigdons Road, Waurn Ponds, Victoria 3217, Australia

<sup>2</sup>Institut de Génomique Fonctionnelle de Lyon, UMR 5242 du CNRS, Université de Lyon, Université Claude Bernard Lyon 1, Ecole Normale Supérieure de Lyon, 46 Allée d'Italie, 69364 Lyon Cedex 07, France

<sup>3</sup>Biology Department, Bowdoin College, Brunswick, ME, USA

YG, 0000-0001-8208-7223; VL, 0000-0003-4022-4175

The diversity of teeth patterns in actinopterygians is impressive with tooth rows in many locations in the oral and pharyngeal regions. The first-formed tooth has been hypothesized to serve as an initiator controlling the formation of the subsequent teeth. In zebrafish, the existence of the first tooth (named 4 V<sup>1</sup>) is puzzling as its replacement is induced before the opening of the mouth. Functionally, it has been shown that 4 V<sup>1</sup> formation requires fibroblast growth factor (FGF) and retinoic acid (RA) signalling. Here, we show that the ablation of 4 V<sup>1</sup> prevents the development of the dental row demonstrating its dependency over it. If endogenous levels of FGF and RA are restored after 4 V<sup>1</sup> ablation, embryonic dentition starts again by de novo formation of a first tooth, followed by the dental row. Similarly, induction of anterior ectopic teeth induces subsequent tooth formation, demonstrating that the initiator tooth is necessary and sufficient for dental row formation, probably via FGF ligands released by 4 V<sup>1</sup> to induce the formation of subsequent teeth. Our results show that by modifying the formation of the initiator tooth it is possible to control the formation of a dental row. This could help to explain the diversity of tooth patterns observed in actinopterygians and more broadly, how diverse traits evolved through molecular fine-tuning.

## 1. Introduction

Dentition is one of the hallmarks of vertebrates and this group harbours an impressive diversity. In particular, the *ca* 25 000 actinopterygian species display an impressive array of diversity in terms of tooth number, shape, size and localization [1]. This diversity is particularly clear for the location of teeth as they display tooth rows in many locations in the head: jaws but also on the pharyngeal arches, floor or roof of the mouth and even tongue in some species. For example, the three main developmental models for tooth development in actinopterygians, namely the zebrafish *Danio rerio*, a cypriniform; the Mexican tetra *Astyanax mexicanus*, a characiform; and the medaka *Oryzias latipes*, a beloniform, all bear teeth in different locations [1]. Therefore, actinopterygian fish represent an excellent model to understand the basis of vertebrate dentition diversity. However, little is known about the molecular mechanisms behind the evolution of such diversification.

The zebrafish like all Cyprinids, lost both oral and anterior pharyngeal teeth *ca* 65 million years ago and retain only teeth on the ventral fifth ceratobranchial arch [2]. The zebrafish pharyngeal teeth are arranged in three distinct tooth rows, a ventral (V), a mediodorsal (MD) and a dorsal (D) in adults, having five (positions 1 V–5 V), four (1MD–4MD) and two (1D and 2D) teeth, respectively [3,4]. The induction of the first formed tooth during zebrafish

development appears in the ventral row at position 4. As it is the first tooth formed at this position it is therefore named  $4 V^1$  [5] while its replacement tooth will be named  $4 V^2$  [6]. The formation of this first  $4 V^1$  tooth is dependent on retinoic acid (RA) and fibroblast growth factor (FGF) signalling [7,8] that act successively. RA signalling is required at *ca* 43 h post fertilization (hpf) while FGF signalling is necessary later at 48–49 hpf [8].

In zebrafish, the stereotyped sequential formation of teeth formed in the ventral row is well known [5]. Soon after the  $4 V^1$  tooth germ is induced, two subsequent teeth are induced almost simultaneously on each side of  $4 V^1$  along the proximo-distal axis, in positions 3 ( $3 V^1$ ) and 5 ( $5 V^1$ ) [5].

The question of the developmental patterning of the tooth row is a long standing and debated issue linked to the much controversial topic of tooth origins [9,10]. The wide diversity of situations observed in extant vertebrates, the fact that during development the patterning of the tooth row (that is, the successive appearance of teeth following skeleton growth) and the emergence of replacement teeth at each position occurred simultaneously render this question highly complex [1,11,12]. Actinopterygian fishes, with their wide diversity of tooth locations are excellent models that allow capture of most of the diversity present in non-mammalian vertebrates [5,9]. In this context it is interesting to note that unique first generation teeth from which the entire tooth row emerged have been observed in zebrafish, cichlids, sticklebacks or salmonids [4,13]. In zebrafish, pharyngeal dentition effectively starts with one single tooth on each skeletal element that bears teeth and the successive appearance of the various teeth have been meticulously described at the morphological and histological levels [4]. The extensive set of data accumulated on tooth row development in actinopterygian and other vertebrates, has allowed researchers to suggest that the first tooth acts as an ‘initiator’ tooth, also called ‘primordial tooth germ’ or ‘dental determinant’, that would be needed for the formation of the other teeth of the row [14,15]. However, this model has never, to our knowledge, been demonstrated.

In zebrafish, the existence of  $4 V^1$  is puzzling as its replacement, a second generation tooth named  $4 V^2$ , is induced before the opening of the mouth, thus showing that  $4 V^2$  induction occurs even before the first formed tooth can actually serve in mastication [4,6]. We therefore reasoned that  $4 V^1$  has been maintained during evolution because it plays an important role as an initiator of the whole dental row thus providing a strong need for its maintenance.

In this paper, we used manipulations of the RA and FGF cell-signalling pathways to investigate the role of the first formed tooth,  $4 V^1$  in the induction of the dental row. We propose a system based on the formation of a single initiator tooth, providing a novel model to explain many aspects of how the number and location of tooth rows have been diversified during vertebrate evolution.

## 2. Results

### (a) Retinoic acid and fibroblast growth factor signalling are required for 3 and $5 V^1$ induction

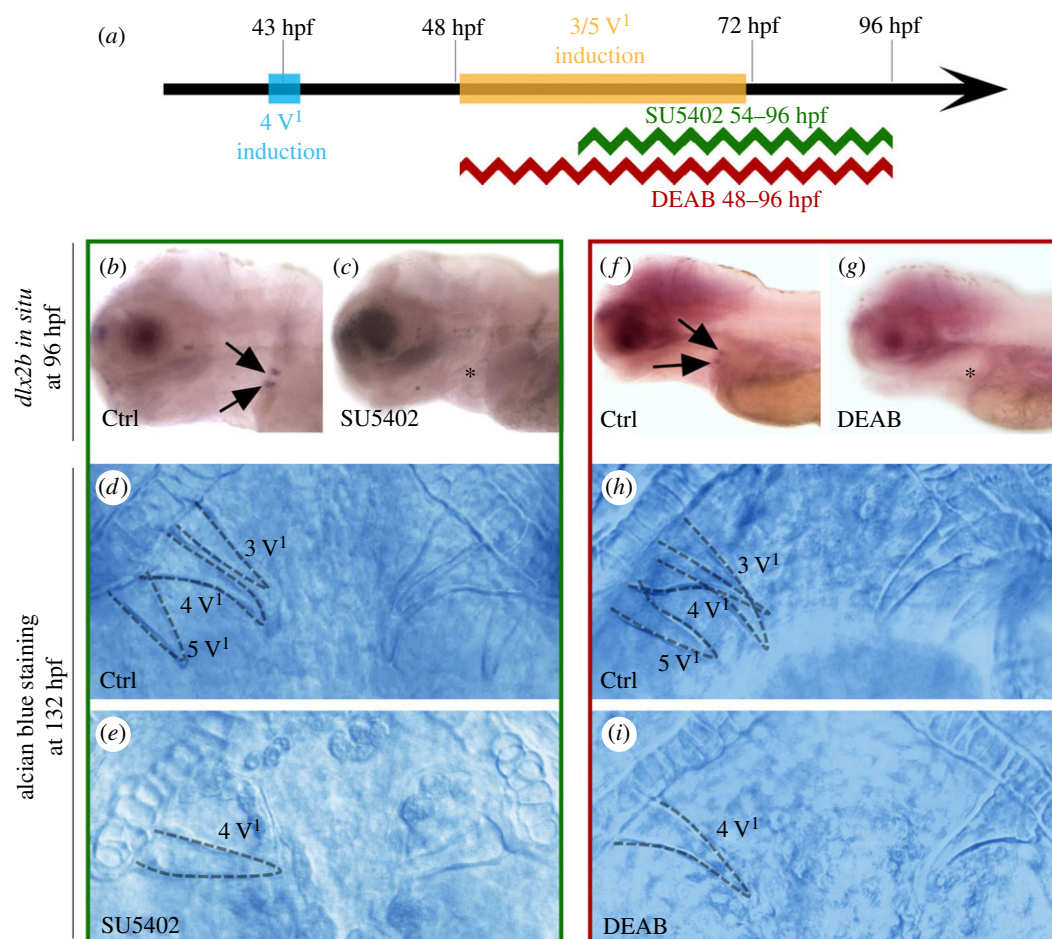
To investigate the role played by RA and FGF signalling in 3 and  $5 V^1$  induction, we took advantage of the differential

timing of  $4 V^1$  versus  $3/5 V^1$  induction. We pharmacologically blocked FGF signalling using the FGF receptor pan-inhibitor SU5402 from 54 hpf onwards (i.e. after FGF requirement for  $4 V^1$  induction which is at around 48–49 hpf [8], figure 1a) and found that 3 and  $5 V^1$  induction, as marked by *dlx2b* expression, is lost in absence of FGF signalling (figure 1b,c; 100%  $n = 30$ ). When we washed out SU5402 at 96 hpf and let the embryos develop longer at a time at which 4, 3 and  $5 V^1$  can be visualized by alcian blue staining (around 132 hpf) we observed only one pair of teeth ( $4 V^1$ ) (figure 1e; 87%  $n = 34$  we did not get 100% in this experiment because either the larvae died before observation or the observation was inconclusive) while 4, 3 and  $5 V^1$  are clearly present in a control embryo (figure 1d; 100%  $n = 39$ ). In a similar experiment, blocking RA synthesis using N,N-diethylaminobenzaldehyde (DEAB) after the induction of  $4 V^1$  prevents the formation of 3 and  $5 V^1$  (figure 1f,g; 100%  $n = 30$ ). As above, if we let the embryos develop longer after washing out the DEAB at 96 hpf, only one pair of teeth is observed at 132 hpf compared to three in controls (figure 1h,i; 84%  $n = 34$ ). These results indicate that both FGF and RA signalling are required after the induction of  $4 V^1$ , for the induction of 3 and  $5 V^1$ .

### (b) The first generation formed tooth is necessary for 3 and $5 V^1$ formation

To study the role played by  $4 V^1$  in the formation of 3 and  $5 V^1$  we pharmacologically blocked the induction of  $4 V^1$  and/or  $3/5 V^1$  using different temporal pulses of DEAB treatment as shown in figure 2a. At the time when DEAB is applied, 40 hpf at the earliest, the pharynx is already patterned and the pharyngeal arches are already specified [8,16], therefore blocking RA signalling at these late developmental stages has no influence on pharynx formation and organization in zebrafish. We visualized tooth formation by alcian blue staining at 132 (three teeth:  $3/4/5 V^1$ ) and 192 hpf (four teeth:  $2/3/4/5 V^1$ ) (figure 2a–c; 100%  $n = 30$ ). As expected when we depleted RA signalling during 4 and  $3/5 V^1$  induction (from 40 hpf onwards) no tooth is formed at 132 hpf (figure 2d; 100%  $n = 28$ ). As observed previously, only the first tooth,  $4 V^1$ , is present when RA production is blocked specifically during  $3/5 V^1$  induction (from 48 hpf onwards, figure 1i). If 3 and  $5 V^1$  are independent of  $4 V^1$  induction, when we selectively block  $4 V^1$  induction by treating with DEAB only during a short period of time (40–48 hpf) and wash out afterwards, we should expect  $3/5 V^1$  to be formed normally as RA signalling is active during their period of induction. However, this is not what we obtain, as in this condition, only one tooth is observed at 132 hpf (figure 2e; 100%  $n = 27$ ). We interpreted this tooth as being a first generation tooth because at a later stage (192 hpf) we observed three teeth on each arch in these treated larvae (100%  $n = 25$ ) resembling those of a 132 hpf-control embryo (compare figure 2f and b). A similar result is obtained if DEAB is applied later; that is when we blocked  $4 V^1$  as well as 3 and  $5 V^1$  induction (40–72 hpf, see figure 2g,h; 100%,  $n = 23$  and 78%  $n = 21$  respectively) suggesting that dental induction resumes after DEAB is washed out.

We confirmed these results by *in situ* hybridization, with *dlx3b* as a marker of developing tooth germs. We observed an absence of  $4 V^1$  and  $3/5 V^1$  induction under DEAB treatment whereas once the DEAB was removed,  $4 V^1$  and  $3/5 V^1$  were



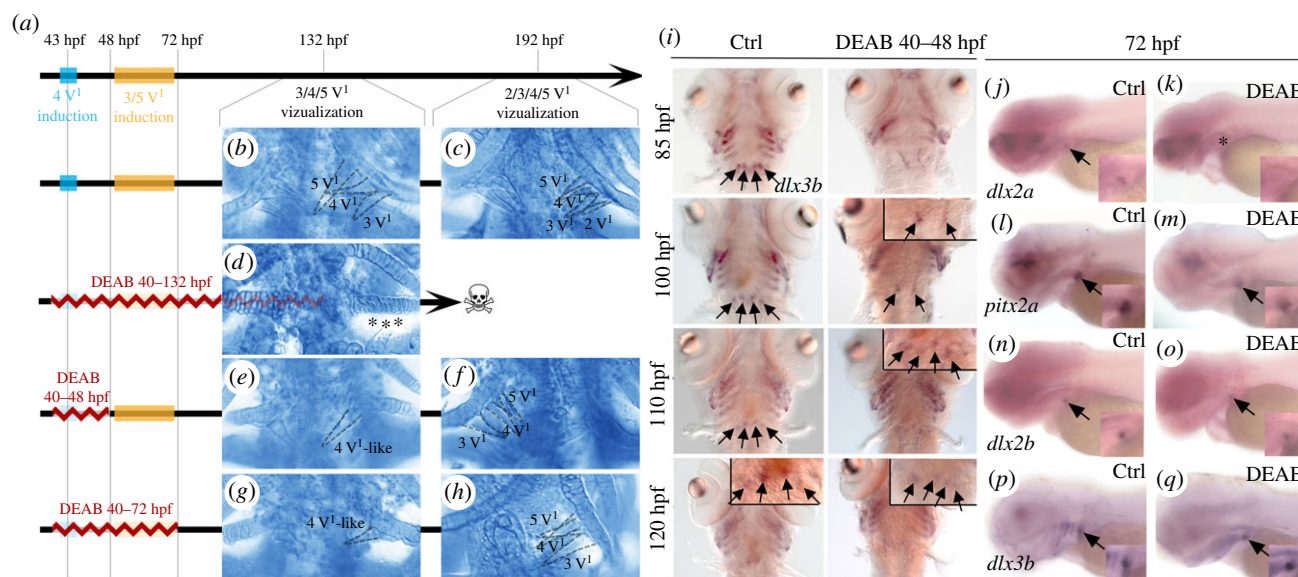
**Figure 1.** FGF and RA signalling are required for 3/5 V<sup>1</sup> formation. (a) Timeline of 4 V<sup>1</sup> and 3/5 V<sup>1</sup> induction temporal windows and DEAB or SU5402 pulse treatments. (b,c) Blocking FGF receptors using  $5 \cdot 10^{-7}$  M SU5402 from 54 to 96 hpf prevents 3/5 V<sup>1</sup> induction as visualized by a lack of *dlx2b* expression in 3/5 V<sup>1</sup> tooth germs (asterisk) compared to the control where two teeth germs are visible (arrows). (d,e) When left to develop until 132 hpf, 3 and 5 V<sup>1</sup> are not present in SU5402 treated embryos while 4 V<sup>1</sup> is fully developed, whereas three teeth are formed in a control (dashed lines). (f,g) Blocking RA synthesis using  $10^{-5}$  M DEAB from 48 to 96 hpf prevent 3/5 V<sup>1</sup> induction as visualized by a lack of *dlx2b* expression in 3/5 V<sup>1</sup> tooth germs (asterisk) compared to the control (arrows). (h,i) When left to develop until 132 hpf, 3 and 5 V<sup>1</sup> are not present in DEAB treated embryos, whereas three teeth are formed in the control (dashed lines). (Online version in colour.)

induced with a time delay corresponding to the DEAB treatment (figure 2i; 100%  $n = 30$ ). Although the four spots corresponding to 3 and 5 V<sup>1</sup> are less discrete and clearer than in the controls, nevertheless, the four 3 and 5 V<sup>1</sup> teeth are induced (figure 2i; 110 and 120 hpf). It is important to note that we obtained the same results if rather than blocking RA synthesis with DEAB we block FGF signalling with SU5402. Indeed, as for DEAB, we observed a ‘reboot’ of the dental induction sequence when the FGF inhibitor is washed out with only one pair of teeth being induced (electronic supplementary material, figure S1B) and formed (electronic supplementary material, figure S1D) at 132 hpf.

We then molecularly characterized this newly formed first tooth. We blocked the induction of 4 V<sup>1</sup> with DEAB treatment (40–48 hpf) and let the embryo develop in a DEAB free environment until fixation [8]. We then performed *in situ* hybridization using the 4 V<sup>1</sup> specific marker *dlx2a* [6,17] at different times of development (56, 60, 66, 72 and 80 hpf) but were unable to detect *dlx2a* expression in the newly formed tooth (figure 2k) suggesting that this is not a genuine 4 V<sup>1</sup>. However this newly formed tooth does express the 4 V<sup>1</sup>/4 V<sup>2</sup> specific marker *pitx2a* at 72 hpf (figure 2l,m). *pitx2a* has been reported by us and others to be solely expressed in 4 V<sup>1</sup> up until 80 hpf and not in 3/5 V<sup>1</sup> [8,18]. To confirm that *pitx2a* is not expressed in 3/5 V<sup>1</sup> we

monitored its expression in developing 3/5 V<sup>1</sup> teeth at 80, 96, 120 and 132 hpf (electronic supplementary material, figure S2). In doing so we confirmed that *pitx2a* is never detected in 3/5 V<sup>1</sup> (electronic supplementary material, figure S2A,B) but is present in 4 V<sup>2</sup> starting at 120 hpf (electronic supplementary material, figure S2C,D). Moreover, we were able to detect the expression of the 4, 3/5 V<sup>1</sup> and 4 V<sup>2</sup> specific marker *dlx2b* [6,17] at 72 hpf (figure 2o) and the 4, 3/5 V<sup>1</sup> specific markers *dlx3b* at 72 hpf (figure 2q). Therefore, this newly formed tooth does not have a 4 V<sup>2</sup> identity as it does express *dlx3b* (which is not detected in 4 V<sup>2</sup>). The two remaining options for the identity of this newly formed tooth are then a 4 V<sup>1</sup> tooth or a mix of 3/5 V<sup>1</sup> tooth. Because this tooth does express the 4 V<sup>1</sup> specific marker *pitx2a*, this tooth cannot be a mix of 3/5 V<sup>1</sup> (*pitx2a* is not detected in 3 or 5 V<sup>1</sup> see the electronic supplementary material, figure S2). That being said, this newly formed tooth is not a true 4 V<sup>1</sup> either as it does not express the 4 V<sup>1</sup> specific marker *dlx2a* (figure 2k, see table in the electronic supplementary material, figure S2E summarizing the expression data of this newly formed tooth). We therefore refer to this tooth as a 4 V<sup>1</sup>-like: it is the first formed tooth and it is present before the appearance of the second and third pair of teeth. We speculate that our DEAB treatment has shifted in time the induction of a 4 V<sup>1</sup> tooth and therefore due to a later





**Figure 2.** 4 V<sup>1</sup> is necessary for the formation of the dental tooth row. (a) Tooth induction timeline in zebrafish. The blue and yellow bars represent 4 V<sup>1</sup> and 3/5 V<sup>1</sup> induction respectively. (b,c) Alcian blue staining of control at 132 hpf and 192 hpf showing 3/4/5 V<sup>1</sup> and 2/3/4/5 V<sup>1</sup> respectively. (d) 10–5 M DEAB 40–132 hpf treated embryos with no tooth formed (asterisks). (e,g) 40–48 hpf and 40–72 hpf DEAB treated embryos fixed at 132 hpf with only 4 V<sup>1</sup> formed. (f,h) DEAB treated embryos from 40 to 48 hpf or 40 to 72 hpf fixed at 192 hpf showing three teeth: 3/4/5 V<sup>1</sup>. (i) DEAB treatment from 40 to 48 hpf blocks 4 V<sup>1</sup> induction. In controls (left) the induction of the subsequent 3 and 5 V<sup>1</sup> teeth starts at 85 hpf as shown by four spots of *dlx3b* expression and is still visible until 120 hpf (black arrows). However, in DEAB 40–48 hpf treated embryos in which 4 V<sup>1</sup> induction is blocked (right), only one pair of *dlx3b* spots is observed at 100 hpf and four spots corresponding to the 3/5 V<sup>1</sup> tooth germs are observed later from 110 hpf onwards. (l–q) inhibition on 4 V<sup>1</sup> induction by DEAB treatment from 40–48 hpf. At 72 hpf the 4 V<sup>1</sup> specific marker *dlx2a* is not detected in the 4 V<sup>1</sup>-like tooth germ (k) while the 4 V<sup>1</sup>/4 V<sup>2</sup> specific marker *pitx2a* is (m). The 4 V<sup>1</sup>, 3/5 V<sup>1</sup> and 4 V<sup>2</sup> specific marker *dlx2b* (o) and the 4 V<sup>1</sup> and 3/5 V<sup>1</sup> but not 4 V<sup>2</sup> specific marker *dlx3b* (q) are expressed in the 4 V<sup>1</sup>-like tooth germ. (j–q) inserts: high magnification images of tooth germs. (Online version in colour.)

developmental stage, *dlx2a* expression is unable to be activated explaining why this 4 V<sup>1</sup>-like tooth, induced at around 60 hpf is devoid of *dlx2a* transcripts. These results show that in addition to being RA and FGF dependant, the formation of 3 V<sup>1</sup> and 5 V<sup>1</sup> is dependent on the presence of a first formed tooth. In other words, 3 and 5 V<sup>1</sup> induction must be preceded by the formation of a first initiator tooth (4 V<sup>1</sup> in untreated control).

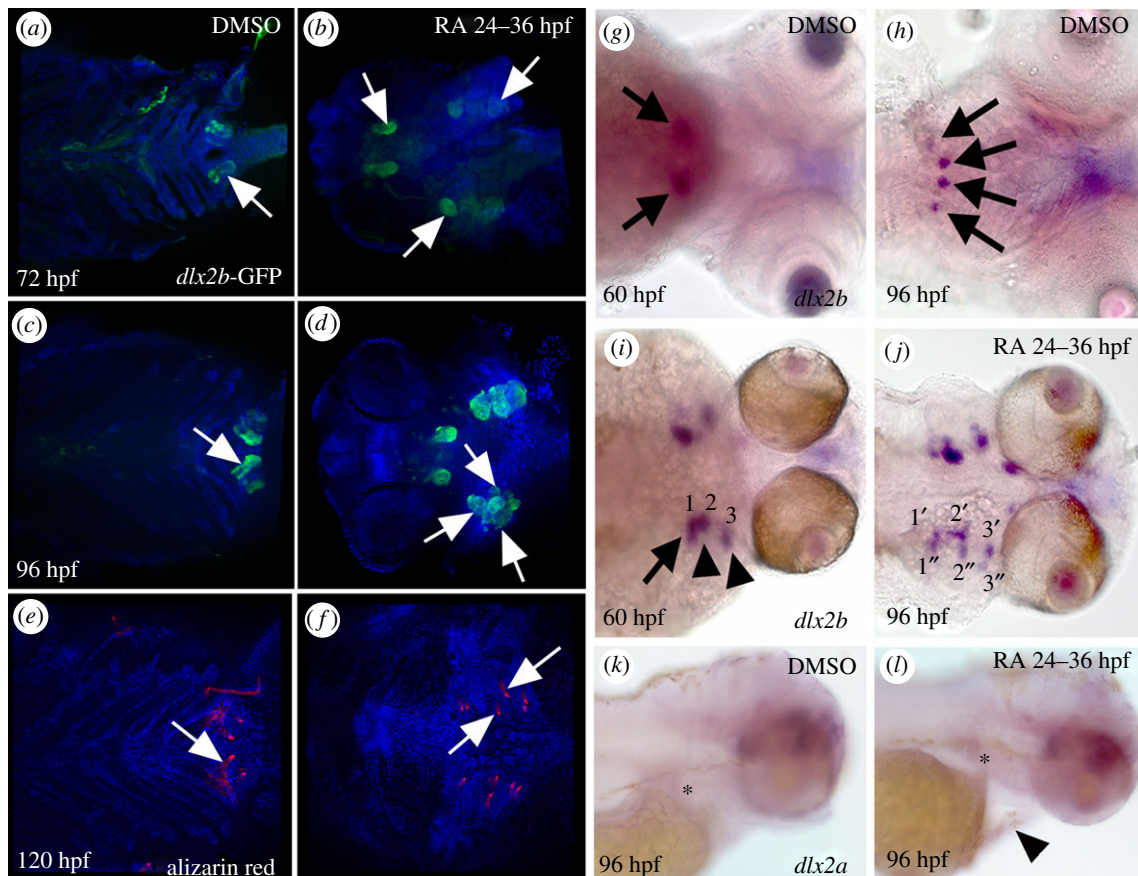
### (c) 4 V<sup>1</sup> is sufficient for 3 and 5 V<sup>1</sup> induction

Having shown that the initiator tooth is necessary for 3/5 V<sup>1</sup> induction, we wanted to test if 4 V<sup>1</sup> was sufficient to induce the whole dental row. We previously showed that exogenous RA exposure from 24 to 36 hpf can induce ectopic anterior 4 V<sup>1</sup> teeth in the pharynx [19], however in that study, the presence of later teeth (3/5 V<sup>1</sup> for example) was not assessed. To confirm that 4 V<sup>1</sup> is sufficient to induce the dental row, we investigated if these ectopic, RA-induced, 4 V<sup>1</sup> teeth were followed by 3 and 5 V<sup>1</sup> (ectopic dental rows). Using a transgenic zebrafish expressing the green fluorescent protein (eGFP) under the control of the tooth promoter *dlx2b* (Tg:*dlx2b*-eGFP; [20]) we visualized GFP expression in 4 V<sup>1</sup> at 72 hpf (figure 3a) and in 3/5 V<sup>1</sup> at 96 hpf (figure 3c). *dlx2b* expression is first detected in the early 4 V<sup>1</sup> tooth germ and stops in late tooth formation. Later on *dlx2b* is expressed in the early tooth germs of 3 and 5 V<sup>1</sup>, meaning that *dlx2b* is never expressed at the same time in the 4 V<sup>1</sup> and the 3/5 V<sup>1</sup> tooth germs [6]. After RA treatment (24–36 hpf, only, RA wash washed out at 36 hpf and the embryo left to develop with endogenous levels of RA signalling) we observed, as expected, ectopic anterior GFP staining for 4 V<sup>1</sup> at 72 hpf (arrows figure 3b). After letting these embryos develop

without exogenous RA until 96 hpf, we detected several ectopic GFP spots, in a greater number than the original spots, that corresponds to 3 and 5 V<sup>1</sup> tooth germs in each ectopic tooth row (arrows in figure 3d, 14 larvae were analysed).

To confirm that these induced ectopic 3 and 5 V<sup>1</sup> tooth germs are able to be calcified, we stained these teeth with alizarin red and detected alizarin red deposition in 3 and 5 V<sup>1</sup> teeth in the control as expected (figure 3e, *n* = 6) but also in anterior ectopic tooth rows (figure 3f, *n* = 6), demonstrating that ectopic 4 V<sup>1</sup> teeth can induce the formation of subsequent calcified 3 and 5 V<sup>1</sup> teeth. We also visualize the developing tooth germs in green superimposed to the calcified tooth in red (electronic supplementary material, figure S3).

We also confirmed the identity of these spots by *dlx2b* *in situ* hybridization that reveals the anterior ectopic 3 and 5 V<sup>1</sup> teeth (*n* = 16) (figure 3i,j). In contrast to 4 V<sup>1</sup>, these teeth do not express the 4 V<sup>1</sup> specific marker *dlx2a* (*n* = 16) which is detected in 3/5 V<sup>1</sup> neither in controls (figure 3k) nor in RA exposed embryos (figure 3l). As RA is known to posteriorize zebrafish embryos and to have a pleiotropic effect during early development [21,22] we monitored the expression of *hoxb5a*, a gene we previously reported to be upregulated after continuous exposure to RA signalling [19]. We therefore studied the expression of *hoxb5a* at the time of 3/5 V<sup>1</sup> induction (around 52 hpf) after washing out exogenous RA signalling at 36 hpf. As shown in the electronic supplementary material, figure S4, *hoxb5a* expression is similar in control and RA exposed embryos (24–36 hpf fixed at 52 hpf). This means that a change of *hoxb5a* expression or a change in arch identity cannot on its own explain the development of an ectopic dental row after RA exposure. We therefore conclude that the presence of an ectopic 4 V<sup>1</sup>, by



**Figure 3.** 4 <sup>V1</sup> is sufficient for the formation of the dental tooth row. (a,c) Control Tg:*dlx2b*-eGFP at 72 hpf and 96 hpf showing 4 <sup>V1</sup> and 3/5 <sup>V1</sup> tooth buds respectively. (b,d) RA treated embryos from 24 to 52 hpf, RA washed out at 52 hpf and the embryos were left to develop and were photographed at 72 hpf (b) and 96 hpf (d). At 72 hpf, ectopic anterior 4 <sup>V1</sup> are visible (arrows) while ectopic anterior 3 and 5 <sup>V1</sup> are detected at 96 hpf although no exogenous RA has been present since 52 hpf. (e,f) Alizarin red staining of calcified 3 and 5 <sup>V1</sup> teeth in control (e) and RA (24–36 hpf) exposed larvae (f) at 120 hpf. Ectopic 3 and 5 <sup>V1</sup> calcified teeth are clearly visible in (f, arrows). (g,j) *dlx2b* expression in the developing tooth buds. Control embryos at 60 hpf show expression of *dlx2b* in 4 <sup>V1</sup> and in 3/5 <sup>V1</sup> at 96 hpf (arrows). RA treated embryos (24–52 hpf) show ectopic 4 <sup>V1</sup> tooth germs noted 1–3 from posterior to anterior (arrowheads) and develop ectopic 3/5 <sup>V1</sup> tooth germs noted 1';1'' to 3';3'' from posterior to anterior. (k–l) Expression of *dlx2a* is not detected in control embryos and in RA exposed embryos (asterisk) the arrowhead denote the pericardial oedema induced by RA exposure in (l). DMSO, dimethyl sulfoxide. (Online version in colour.)

itself, or in combination with exogenous RA that has affected other non-tooth related tissues, is sufficient to initiate a whole dental row.

#### (d) The first formed tooth is a source of fibroblast growth factor ligand required for 3 <sup>V1</sup> and 5 <sup>V1</sup> formation

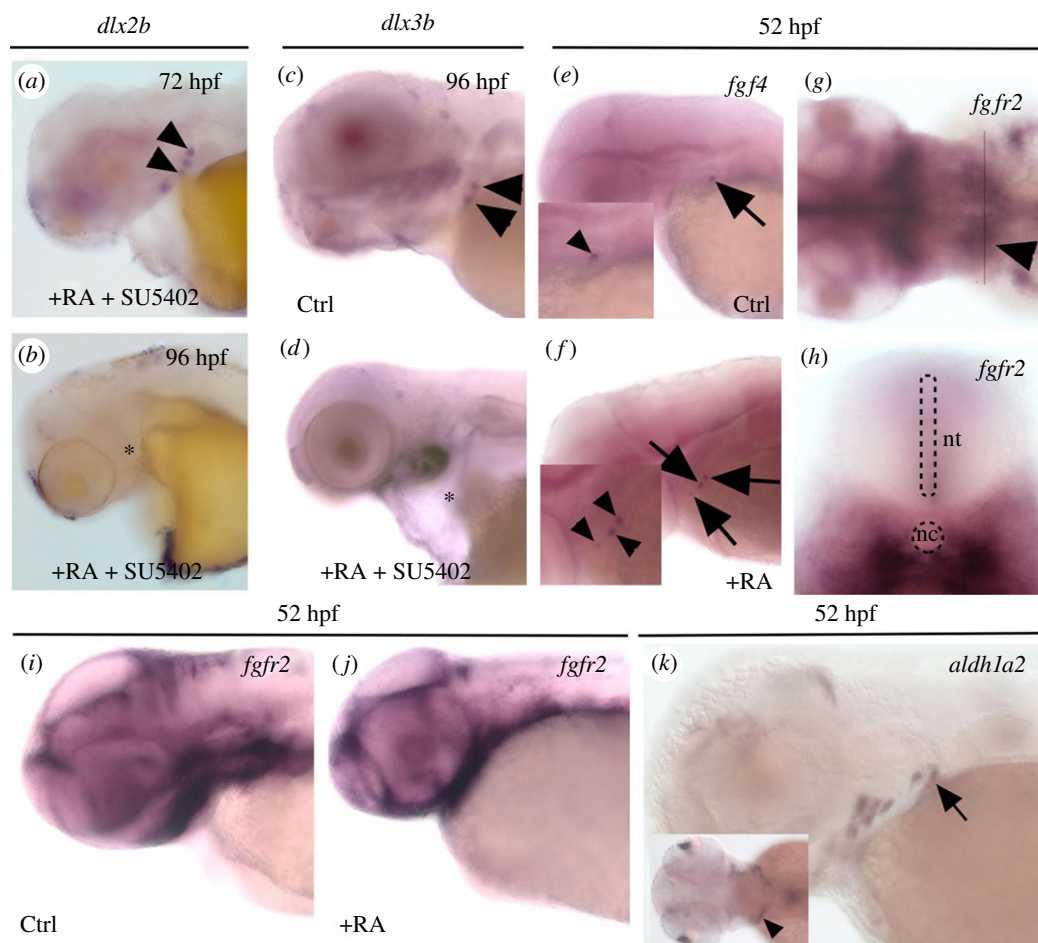
To identify the signal emanating from the initiator tooth bud that instructs the surrounding pharyngeal cells to form a tooth we induced ectopic anterior 4 <sup>V1</sup> teeth by treating embryos with RA from 24 to 36 hpf and then blocked FGF signalling in these embryos using SU5402 from 50 to 72 hpf (figure 4a–d; 80%, *n* = 30). This abolishes the formation of ectopic 3 and 5 <sup>V1</sup> teeth (figure 4b,d; 73%, *n* = 30) demonstrating that FGF signalling is also necessary to induce these teeth as it is required for normal 3 and 5 <sup>V1</sup> teeth formation. As the only ectopic structures present in the anterior pharynx after RA treatment are the supernumerary 4 <sup>V1</sup> teeth, we conclude that the 4 <sup>V1</sup> tooth germ itself probably acts as a source of FGF signals required for 3/5 <sup>V1</sup> induction.

This model implies that the initiator tooth should express at least one FGF ligand during 3/5 <sup>V1</sup> induction. In addition, the receiving tissues must be able to process FGF signalling and therefore express a FGF receptor. We therefore studied which FGF ligands are expressed in the 4 <sup>V1</sup> tooth bud at the time of 3/5 <sup>V1</sup> induction (at 52 hpf) during normal

development [5]. Out of the 32 reported FGF ligands in the zebrafish genome, only *fgf3* and *fgf4* were found to be expressed in 4 <sup>V1</sup> (www.zfin.org). At 52 hpf, we detected *fgf4* expression in the dental epithelium (figure 4e; 100% *n* = 20) as previously reported [7]. *fgf3* has also been reported to be expressed in the dental epithelium of 4 <sup>V1</sup> from 52 hpf until at least 56 hpf [7]. In accordance to our model, the anterior ectopic 4 <sup>V1</sup> teeth induced by exogenous RA exposure also express *fgf4* (figure 4f inset; 80%, *n* = 30) creating a source of FGF ligand that would normally not be present in the anterior branchial arches.

The zebrafish genome contains five FGF receptor genes (1–4 with *fgfr1* being duplicated: *fgfr1a* and *fgfr1b*) [23] of which none are known to be expressed in tooth germs. We therefore studied the expression of these five genes at the time of 3/5 <sup>V1</sup> induction and observed that *fgfr1a*, *fgfr1b* and *fgfr2* are present in a large domain of the ventral posterior pharynx at 52 hpf (figure 4g,h; 100%, *n* = 20, only *fgfr2* is shown). We noticed that RA exposure has little or no effect on *fgfr2* expression in anterior branchial arches where ectopic teeth will be located (figure 4i,j, 87%, *n* = 30). Regarding RA signalling, we previously reported that the RA producing enzyme *aldh1a2* is strongly expressed at the level of the 5th ceratobranchial arch at 43 hpf, the time of 4 <sup>V1</sup> induction, (arrow) [8]. Moreover, we detected *aldh1a2* in anterior ceratobranchial arches at the time of 3/5 <sup>V1</sup>





**Figure 4.** 4  $V^1$  is the source of FGF signalling for 3/5  $V^1$  induction. (a) After exogenous RA exposure, induction of anterior ectopic 4  $V^1$ -like teeth (arrowhead) at 72 hpf marked by *dlx2b* staining even under SU5402 treatment as long as the embryos are exposed to SU5402 after 4  $V^1$  induction at around 49 hpf [8]. (b) Same batch of embryos at 96 hpf showing that no ectopic 3 or 5  $V^1$  teeth are detected by *dlx2b* staining (asterisk). (c,d) After induction of anterior ectopic tooth germ, treatment with SU5402 abolishes *dlx3b* expression (d) indicating that unlike control (c) these embryos do not have 3/5  $V^1$  teeth (arrowheads) neither in their normal localization nor ectopically in the anterior pharynx (asterisk). (e,f) *fgf4* is expressed in the dental epithelium of ectopic anterior 4  $V^1$  under RA exposure (arrows, arrowhead in inset). (g) Expression of *fgfr2* in the ventral posterior pharynx at 52 hpf (arrowhead). (h) Transverse section of (g) (see line) at the level of the 4th ceratobranchial arch. (i,j) Expression of *fgfr2* is unchanged under RA treatment. (k) *aldh1a2* expression at 52 hpf in the last posterior ceratobranchial arch (arrow) and in anterior arches (arrowheads). (Online version in colour.)

induction (arrowheads, figure 4k; 100%,  $n = 401$ ) in untreated wild-type embryos.

We conclude from these experiments that: (i) endogenous 4  $V^1$  and ectopic anterior 4  $V^1$ -like teeth effectively provide a source of FGF ligand (*fgf4* but also *fgf3*, not shown) that is necessary for the induction of subsequent teeth; (ii) FGF receptors are present in the 5th and more anterior pharyngeal arches and are not affected by RA treatment; and (iii) endogenous RA, that is necessary for 3/5  $V^1$  development, should be present in anterior pharyngeal arches at the time of ectopic 3/5  $V^1$  induction. These data therefore identify the main actors of the signalling cascade that allow the formation of the tooth row.

### 3. Discussion

Our observations demonstrate that ectopic 4  $V^1$  teeth have all the necessary signals to induce the remaining teeth in the absence of exogenous RA signalling. The role played by FGF signalling in the cascade of events needed for tooth induction is in accordance with its function as an activator of tooth placode formation [24]. In addition Jackman *et al.*

[25], have shown using zebrafish that upregulation of FGF signalling is sufficient to produce supernumerary teeth as well as multicuspoid teeth, an activity that has also been observed in mammals [26–28]. We previously demonstrated that RA is able to control the number of teeth in a row by expanding the pharyngeal mesenchyme and therefore providing a broader domain for tooth induction [29]. In this study we link the two pathways by showing that they act in a coordinated fashion to allow the formation of the entire tooth row.

#### (a) A model of dental patterning

The dental patterns of polyphyodont vertebrates are remarkably diverse and have been extensively studied [30]. Several models have been proposed to explain the successive appearance of teeth in these animals [4]. Among these, the Zahnreihe (tooth row in German) theory proposed by Edmund in the early 1960s, is interesting to consider here [31]. In this 50 years old model, it is proposed that a signalling centre produce ‘transmitters’ that travel along the jaw to signal for the formation of the subsequent teeth. As mentioned by van der Heyden & Huysseune [4], it can be

proposed that 4  $V^1$ , the first tooth of the ventral row in zebrafish act as such a primordial signalling centre that will produce such 'transmitters' that will be responsible for the formation of the subsequent 3  $V^1$  and 5  $V^1$  teeth. We propose here that the transmitters are FGF ligands produced by 4  $V^1$  and later by any already formed tooth as a FGF source for any subsequent tooth. If our proposed hypothesis is true, ligands like *fgf4* should also be detected in every tooth before the formation of any subsequent teeth. To test this hypothesis we monitored the expression of *fgf4* in 3 and 5  $V^1$  at the time of induction of the next tooth to be formed (2  $V^1$ ). As seen in figure 5*a,b*, *fgf4* is only detected in 3  $V^1$ , which makes perfect sense as 2  $V^1$  will be induced proximally to 3  $V^1$  and no tooth is formed distally to 5  $V^1$ , therefore there is no need for *fgf4* to be expressed in 5  $V^1$  at the time of 2  $V^1$  induction. Our results obtained in zebrafish, prompt for similar studies in other contexts and in particular in species with alternate modes of dental patterning.

We further propose that RA acts mainly by creating the signalling centre that produces FGF molecules that, as demonstrated in figure 5*c*, are necessary for the formation of 3 and 5  $V^1$ .

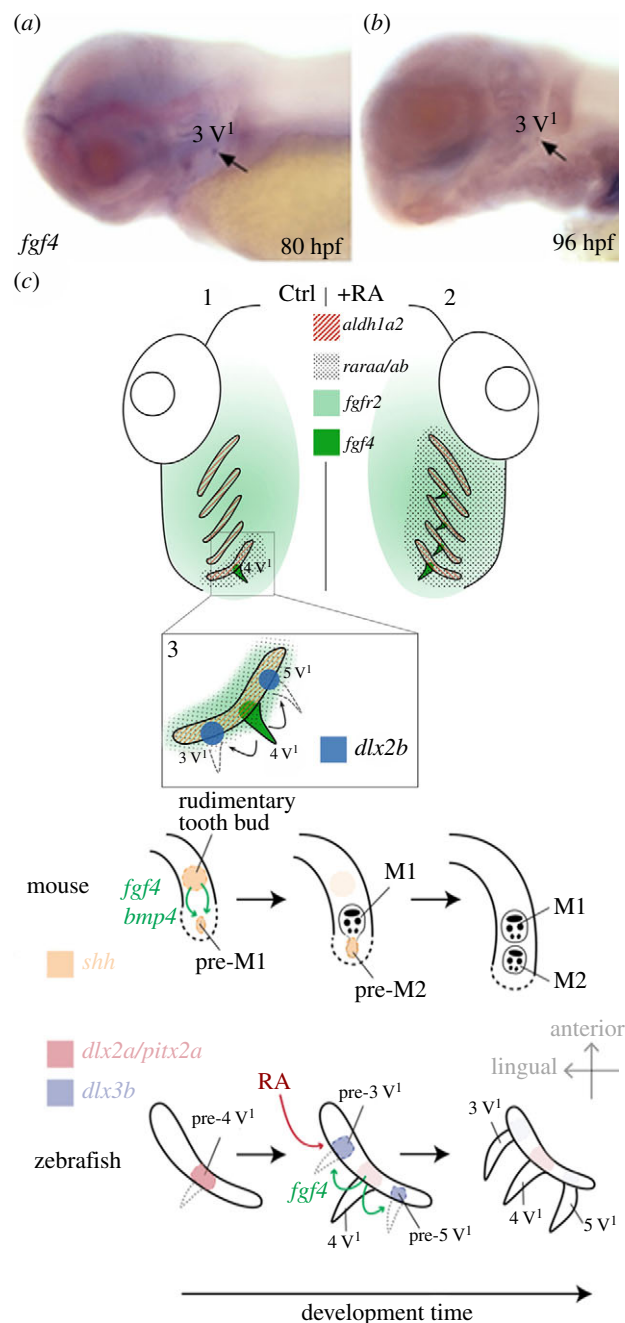
### (b) Evolution of tooth row in actinopterygian fish

The model we propose here, if extended to other species, based on *in vivo* experiments, has the potential to explain important aspects of the huge diversity of dental changes that occurred during vertebrate evolution, and in particular in actinopterygian fishes. This system is particularly plastic in terms of timing. Indeed, in other tissue or organs which are dependent of RA signalling for their formation (such as pectoral fins or the pancreas), there is a unique and precise time-window during which RA signalling must be present for organ induction [32,33]. Once the developmental time at which RA is required for their induction has passed, nothing has been found that can re-specify the structure in question. By contrast, we observed here that the time boundary of the effects on tooth-row formation is more flexible. In fact the first formed tooth can be re-induced after its normal timing of induction. It is interesting to consider how such plasticity in terms of timing might have been useful in evolution: indeed when an initiator tooth is formed in a specific place in an embryo, it will carry with it the latent ability to form the whole row.

In actinopterygian fishes, teeth are present throughout the oral and pharyngeal cavities which reflects the ecological and morphological adaptations associated with species-specific diet and feeding modes [1,34,35]. The model we propose here could explain how tooth rows can be formed once an initiator tooth is induced in new region. Such a tooth would act as a signalling centre producing FGF ligands and possibly other signalling molecules that would allow the formation of the row.

### (c) Is the concept of an initiator tooth also valid in mammals?

In mice, the three molars form sequentially, first M1, then M2 and finally M3 that erupts the latest [36]. Vestigial tooth buds develop earlier and anterior to the upper and lower first molars [37,38]. These buds were demonstrated to act as transient signalling centres that act to initiate the entire row of cheek teeth in mice [39]. This offers a striking



**Figure 5.** Model of tooth row formation. (*a,b*) *fgf4* expression is not detected prior 80 hpf or after 96 hpf in the 3  $V^1$  tooth germ and never detected in the 5  $V^1$  tooth germ. (*c*) (1, 3) In control embryos, RA production from the 5th ceratobranchial arch can activate the *raraa* and *raraab*. While FGF ligand, like *fgf4* in the dental epithelium is used by FGFR to induce 3/5  $V^1$ . *fgfr1a*, *1b* and *2* are present in the pharynx at this stage. (2) Each ectopic tooth can therefore induce whole ectopic dental rows. (*d*) Comparison of tooth row formation in mouse and zebrafish. In mouse, a wave of *fgf4* and *bmp4* signals produce from the 1st formed tooth and all rudimentary tooth buds reach the spot of pre-molar formation where tooth will erupt. In zebrafish, RA emitting from the ceratobranchial arch and *fgf4* signal form the 4  $V^1$  tooth germ worked as instructive signals to form the subsequent 3/5  $V^1$  teeth. (Online version in colour.)

parallel to the situation we observe here and suggests that despite the extreme variability of shape, size, location and number of teeth in vertebrates they all use a common system with an initiator tooth that initiates the dental row (figure 5*d*). It will be very interesting to investigate if



such an initiator tooth could in fact have a role in governing the development of entire quadrant dentition as suggested by Reif in sauropsids and actinopterygians rather than just the immediate tooth row [15]. Our model thus offers a general framework that could be challenged and from which evolutionary variations will undoubtedly be identified in the future.

## 4. Methods

### (a) Zebrafish strains

Zebrafish and their embryos were handled and staged according to standard protocols [40].

### (b) Zebrafish assays

RA, DEAB and SU5402 treatments were performed as previously described [8]. Whole mount *in situ* hybridizations were performed as described [29].

## (c) Green fluorescent protein fluorescence and alizarin red S staining

Amplification of GFP fluorescence with immunohistochemistry and alizarin red S staining of mineralized teeth was performed as in Yu *et al.* [41]. To combine the two visualization methods in a single specimen, alizarin staining was done subsequent to the antibody label.

**Ethics.** Fish and embryos were handled in accordance with the animal welfare committees of Deakin University and the Ecole Normale Supérieure de Lyon.

**Data accessibility.** This article has no additional data.

**Authors' contributions.** Y.G., E.S. and V.L. designed research; Y.G., E.S., M.K.E. and W.R.J. performed experiments; Y.G., E.S. and V.L. analysed the data; Y.G., E.S. and V.L. wrote the manuscript.

**Competing interests.** We declare we have no competing interests.

**Funding.** Y.G. is supported by the Department of Cell and Molecular Biology at the University of Mississippi Medical Center. E.S. is supported by the French Ministry of Research, funding from the ENS Lyon, V.L. is supported by the ANR program and the French Ministry of Research.

## References

- Stock DW. 2007 Zebrafish dentition in comparative context. *J. Exp. Zool. B Mol. Dev. Evol.* **308**, 523–549. (doi:10.1002/jez.b.21187)
- Patterson C. 1993 Osteichthyes: teleostei. In *The fossil record 2* (ed. MJ Benton), pp. 621–656. London, UK: Chapman & Hall.
- Wautier K, Van der heyden C, Huysseune A. 2001 A quantitative analysis of pharyngeal tooth shape in the zebrafish (*Danio rerio*, Teleostei, Cyprinidae). *Arch. Oral Biol.* **46**, 67–75. (doi:10.1016/S0003-9969(00)00091-1)
- Van der Heyden C, Huysseune A. 2000 Dynamics of tooth formation and replacement in the zebrafish (*Danio rerio*) (Teleostei, Cyprinidae). *Dev. Dyn.* **219**, 486–496. (doi:10.1002/1097-0177(2000)9999:9999<::AID-DVDY1069>3.0.CO;2-Z)
- Huysseune A, Witten PE. 2006 Developmental mechanisms underlying tooth patterning in continuously replacing osteichthyan dentitions. *J. Exp. Zool. B Mol. Dev. Evol.* **306**, 204–215. (doi:10.1002/jez.b.21091)
- Borday-Birraux V, Van der Heyden C, Debiais-Thibaud M, Verreijdt L, Stock DW, Huysseune A, Sire JY. 2006 Expression of *Dlx* genes during the development of the zebrafish pharyngeal dentition: evolutionary implications. *Evol. Dev.* **8**, 130–141. (doi:10.1111/j.1525-142X.2006.00084.x)
- Jackman WR, Draper BW, Stock DW. 2004 Fgf signaling is required for zebrafish tooth development. *Dev. Biol.* **274**, 139–157. (doi:10.1016/j.ydbio.2004.07.003)
- Gibert Y *et al.* 2010 Formation of oral and pharyngeal dentition in teleosts depends on differential recruitment of retinoic acid signaling. *FASEB J.* **24**, 3298–3309. (doi:10.1096/fj.09-147488)
- Smith MM. 2003 Vertebrate dentitions at the origin of jaws: when and how pattern evolved. *Evol. Dev.* **5**, 394–413. (doi:10.1046/j.1525-142X.2003.03047.x)
- Huysseune A, Sire JY, Witten PE. 2009 Evolutionary and developmental origins of the vertebrate dentition. *J. Anat.* **214**, 465–476. (doi:10.1111/j.1469-7580.2009.01053.x)
- Jernvall J, Thesleff I. 2012 Tooth shape formation and tooth renewal: evolving with the same signals. *Development* **139**, 3487–3497. (doi:10.1242/dev.085084)
- Tucker AS, Fraser GJ. 2014 Evolution and developmental diversity of tooth regeneration. *Semin. Cell Dev. Biol.* **25–26**, 71–80. (doi:10.1016/j.semcdb.2013.12.013)
- Berkovitz B. 1977 The order of tooth development and eruption in the rainbow trout (*Salmo gairdneri*). *J. Exp. Zool.* **201**, 221–225. (doi:10.1002/jez.1402010207)
- Osborn JW. 1973 The evolution of dentitions. The study of evolution suggests how the development of mammalian dentitions may be controlled. *Am. Sci.* **61**, 548–559.
- Reif W. 1984 Pattern regulation in shark dentitions. In *Pattern formation: a primer in developmental biology* (eds G Malacinski, S Bryant), pp. 603–621. New York, NY: Macmillan Publishing Company.
- Kopinke D, Sasine J, Swift J, Stephens WZ, Piotrowski T. 2006 Retinoic acid is required for endodermal pouch morphogenesis and not for pharyngeal endoderm specification. *Dev. Dyn.* **235**, 2695–2709. (doi:10.1002/dvdy.20905)
- Wiweger MI, Zhao Z, van Merkesteyn RJ, Roehl HH, Hogendoorn PC. 2012 HSPG-deficient zebrafish uncovers dental aspect of multiple osteochondromas. *PLoS ONE* **7**, e29734. (doi:10.1371/journal.pone.0029734)
- Ablooglu AJ, Kang J, Handin RI, Traver D, Shattil SJ. 2007 The zebrafish vitronectin receptor: characterization of integrin  $\alpha$ V and  $\beta$ 3 expression patterns in early vertebrate development. *Dev. Dyn.* **236**, 2268–2276. (doi:10.1002/dvdy.21229)
- Seritkul P, Samarut E, Lama TT, Gibert Y, Laudet V, Jackman WR. 2012 Retinoic acid expands the evolutionarily reduced dentition of zebrafish. *FASEB J.* **26**, 5014–5024. (doi:10.1096/fj.12-209304)
- Jackman WR, Stock DW. 2006 Transgenic analysis of *Dlx* regulation in fish tooth development reveals evolutionary retention of enhancer function despite organ loss. *Proc. Natl Acad. Sci. USA* **103**, 19 390–19 395. (doi:10.1073/pnas.0609575103)
- Begemann G, Marx M, Mebus K, Meyer A, Bastmeyer M. 2004 Beyond the neckless phenotype: influence of reduced retinoic acid signaling on motor neuron development in the zebrafish hindbrain. *Dev. Biol.* **271**, 119–129. (doi:10.1016/j.ydbio.2004.03.033)
- Grandel H *et al.* 2002 Retinoic acid signalling in the zebrafish embryo is necessary during pre-segmentation stages to pattern the anterior-posterior axis of the CNS and to induce a pectoral fin bud. *Development* **129**, 2851–2865.
- Rohs P, Ebert AM, Zuba A, McFarlane S. 2013 Neuronal expression of fibroblast growth factor receptors in zebrafish. *Gene Expr. Pat.* **13**, 354–361. (doi:10.1016/j.gexp.2013.06.006)
- Pispa J, Thesleff I. 2003 Mechanisms of ectodermal organogenesis. *Dev. Biol.* **262**, 195–205. (doi:10.1016/S0012-1606(03)00325-7)
- Jackman WR, Davies SH, Lyons DB, Stauder CK, Denton-Schneider BR, Jowdry A, Aigler SR, Vogel SA, Stock DW. 2013 Manipulation of Fgf and Bmp signaling in teleost fishes suggests potential

- pathways for the evolutionary origin of multicuspid teeth. *Evol. Dev.* **15**, 107–118. (doi:10.1111/ede.12021)
26. Peterkova R, Churava S, Lesot H, Rothova M, Prochazka J, Peterka M, Klein OD. 2009 Revitalization of a diastemal tooth primordium in *Spry2* null mice results from increased proliferation and decreased apoptosis. *J. Exp. Zool. B Mol. Dev. Evol.* **312B**, 292–308. (doi:10.1002/jez.b.21266)
  27. Klein OD, Minowada G, Peterkova R, Kangas A, Yu BD, Lesot H, Peterka M, Jernvall J, Martin GR. 2006 Sprouty genes control diastema tooth development via bidirectional antagonism of epithelial-mesenchymal FGF signaling. *Dev. Cell* **11**, 181–190. (doi:10.1016/j.devcel.2006.05.014)
  28. Charles C *et al.* 2011 Regulation of tooth number by fine-tuning levels of receptor-tyrosine kinase signaling. *Development* **138**, 4063–4073. (doi:10.1242/dev.069195)
  29. Gibert Y, Samarut E, Pasco-Viel E, Bernard L, Borday-Birraux V, Sadier A, Labbé C, Viriot L, Laudet V. 2015 Altered retinoic acid signalling underpins dentition evolution. *Proc. R. Soc. B* **282**, 20142764. (doi:10.1098/rspb.2014.2764)
  30. Huysseune A, Van der Heyden C, Verreijdt L, Wautier K, Van Damme N. 2002 Fish dentitions as paradigms for odontogenic questions. *Connect. Tissue Res.* **43**, 98–102. (doi:10.1080/03008200290000808)
  31. Edmund AG. 1960 Tooth replacement phenomena in the lower vertebrates. Contribution no. 51, Life Sciences Division, Royal Ontario Museum, Toronto, pp. 52–190. See <https://doi.org/10.5962/bhl.title.52196>.
  32. Stafford D, Hornbruch A, Mueller PR, Prince VE. 2004 A conserved role for retinoid signaling in vertebrate pancreas development. *Dev. Genes Evol.* **214**, 432–441. (doi:10.1007/s00427-004-0420-6)
  33. Gibert Y, Gajewski A, Meyer A, Begemann G. 2006 Induction and pre patterning of the zebrafish pectoral fin bud requires axial retinoic acid signaling. *Development* **133**, 2649–2659. (doi:10.1242/dev.02438)
  34. Stock DW. 2001 The genetic basis of modularity in the development and evolution of the vertebrate dentition. *Phil. Trans. R. Soc. Lond. B* **356**, 1633–1653. (doi:10.1098/rstb.2001.0917)
  35. Huysseune A, Sire JY. 1998 Evolution of patterns and processes in teeth and tooth-related tissues in non-mammalian vertebrates. *Eur. J. Oral Sci.* **106**(Suppl. 1), 437–481. (doi:10.1111/j.1600-0722.1998.tb02211.x)
  36. Kavanagh KD, Evans AR, Jernvall J. 2007 Predicting evolutionary patterns of mammalian teeth from development. *Nature* **449**, 427–432. (doi:10.1038/nature06153)
  37. Viriot L, Peterkova R, Peterka M, Lesot H. 2002 Evolutionary implications of the occurrence of two vestigial tooth germs during early odontogenesis in the mouse lower jaw. *Connect. Tissue Res.* **43**, 129–133. (doi:10.1080/03008200290001168)
  38. Peterkova R, Peterka M, Viriot L, Lesot H. 2000 Dentition development and budding morphogenesis. *J. Craniofac. Genet. Dev. Biol.* **20**, 158–172.
  39. Prochazka J *et al.* 2010 Patterning by heritage in mouse molar row development. *Proc. Natl Acad. Sci. USA* **107**, 15 497–15 502. (doi:10.1073/pnas.1002784107)
  40. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. 1995 Stages of embryonic development of the zebrafish. *Dev. Dyn.* **203**, 253–310. (doi:10.1002/aja.1002030302)
  41. Yu JC, Fox ZD, Crimp JL, Littleford HE, Jowdry AL, Jackman WR. 2015 Hedgehog signaling regulates dental papilla formation and tooth size during zebrafish odontogenesis. *Dev. Dyn.* **244**, 577–590. (doi:10.1002/dvdy.24258)