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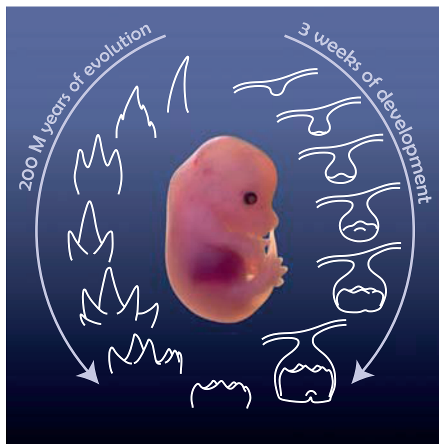
ENNI HARJUNMAA

Tinkering with Cusp Patterning: Developmental Genetic Mechanisms in Mouse Molar Development



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Tinkering with Cusp Patterning: Developmental Genetic Mechanisms in Mouse Molar Development

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Academic dissertation

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O chestnut-tree, great-rooted blossomer,
Are you the leaf, the blossom or the bole?
O body swayed to music, O brightening glance,
How can we know the dancer from the dance?
- *W.B. Yeats*

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This thesis is based on the following articles (referred to in the text by their Roman numerals) and on unpublished results. The published articles are reprinted with the permission of their copyright holders.

I Kassai Y., Munne P., Hotta Y., **Penttilä E**., Kavanagh K., Ohbayashi N., Takada S., Thesleff I., Jernvall J., Itoh N. (2005). Regulation of mammalian tooth cusp patterning by ectodin. *Science* 309, 2067-2070.

II **Harjunmaa E.,** Kallonen A., Voutilainen M., Hämäläinen K., Mikkola M.L., Jernvall J. (2012). On the difficulty of increasing dental complexity. *Nature* 483, 324-327.

III **Harjunmaa, E.,** Renvoisé, E., Jernvall, J. Ectodysplasin in molar morphogenesis: gradients in signalling and patterning. *Manuscript*.

IV Häärä, O., **Harjunmaa, E.,** Lindfors, P., Huh S-H., Fliniaux, I., Åberg, T., Mikkola, M.L., Jernvall J., Ornitz D., Thesleff I. Ectodysplasin regulates activator-inhibitor balance in murine tooth development through modulation of Fgf20 signalling. *Submitted manuscript.*

Contributions:

I The author (née Penttilä) contributed to planning the experiments and discussing the results, and performed the three-dimensional mapping of the expression pattern of *ectodin* in three dimensions, and the rendering of molar topography into three-dimensional images.

II The author contributed to planning the study, analyzing the data and writing the paper, and performed the tooth culture experiments and their quantification.

III The author contributed to planning the study, analyzing the data and writing the paper, and performed all the experiments and their quantification.

IV The author contributed to planning the experiments and discussing the results, commented on the manuscript, and performed the tooth culture experiments.

ABBREVIATIONS

Bmp	Bone morphogenetic protein, a signalling protein family
BrdU	Bromodeoxyuridine, a synthetic analogue of thymidine
Cre	Cre recombinase, a recombination enzyme
Dkk	Dickkopf homolog, a protein inhibiting Wnt signalling
Eda	Ectodysplasin, a signalling protein, in this thesis refers to the isoform Eda-A1
Edar	Eda-A1 receptor
Edaradd	Eda-A1 receptor-associated adapter protein
EdU	Ethynyldeoxyuridine, a synthetic analogue of thymidine
Fgf	Fibroblast growth factor, a signalling protein family
GFP	Green fluorescent protein
Hox	Homeobox-domain containing, a gene family
I-κB	Inhibitor of NF-κB, an enzyme complex
IKK	I-κB kinase, an enzyme complex
K14	Keratin-14 promoter, activates transcription in the developing ectoderm
Lef-1	Lymphoid enhancer-binding factor 1, a transcription factor associated with active Wnt signalling
NF-κB	Nuclear factor κB, a transcription factor
Pitx2	Paired-like homeodomain transcription factor 2
p21	A cyclin dependent kinase inhibitor, expressed in cells that do not proliferate
Smad	a protein family, homologs of *Drosophila* MAD and *C. elegans* SMA
Shh	Sonic hedgehog, a signalling protein of the Hedgehog family
TRAF	TNF associated factor, a protein family
Wnt	Wingless integrated, a signalling protein family
wt	wild type, not mutated

The names of genes and mRNAs are in *italic type*, whereas protein products are in roman type. Human recombinant proteins used in tissue cultures are in CAPITALS.

SUMMARY

Teeth display considerable morphological variability, which mammals have been able to use to their advantage. Consequently, mammal teeth provide a bountiful research subject that combines information on development, functional properties, and thanks to their durable substance, evolutionary history. This thesis work is focused on the patterning of cusps, the peaks that form the shape of the tooth crown, in the mouse.

Mouse tooth development has been studied extensively and offers a wide variety of established methods, including culture of embryonic teeth, which allows their observation and manipulation, and the mapping of gene expression patterns and protein distributions on histological sections. It has been established that teeth develop through a series of inductive interactions between the epithelium and the mesenchyme. The interactions are mediated by signalling molecules mostly belonging to the Wnt, Bmp, Fgf, and Shh families and are used similarly in the development of other organs. The growth of a tooth is controlled by epithelial signalling centres called enamel knots, each of which gives rise to a cusp. The patterning of enamel knots, and thus of cusps, can be modelled with reaction-diffusion dynamics, which suggests the patterning to be robust against interference yet capable of propagating change. As a semi-independent developmental module, teeth can vary without affecting the rest of the organism, an assumed prerequisite for evolvability. However, the use of tooth development in evolutionary studies has been hampered by a lack of mutations and manipulations causing small-scale variation.

We have explored the dynamics of cusp patterning by studying mouse mutants with altered cusp patterns and by producing cusp pattern variation in cultured molars. In addition to taking advantage of established methods, we have shown *Shh*$^{wt/GFPcre}$ reporter molars to allow real-time observation of cusp patterning in culture, derived quantified data from developing molars, and imaged their three-dimensional structure at cellular resolution with X-ray scanning.

Our results indicate that cusp patterning is controlled by feedback inhibition of enamel knot differentiation, and we identify Bmp, Activin, Eda, and Fgf20 as activators, and ectodin and Shh as inhibitors of differentiation. Each of these has slightly different functions and the correct regulation of all of these is required for normal cusp patterning. Bmp and ectodin, and Eda and Shh, seem to form feedback loops providing developmental stability. The manipulation of Eda signalling provided an opportunity to quantify development, revealing that variation increased in a linear fashion the further one deviated from the wild type level of signalling. Our results support the use of reaction-diffusion dynamics in modelling cusp patterning, but they also show that growth dynamics play an equally important role. Consequently, the evolution of crown shape can be

followed cusp by cusp, and the developmental order of enamel knot induction closely corresponds to the evolutionary order of cusp appearance. Thus the mechanisms of molar development can be assumed to restrict, or channel, variation available to selection. In agreement with this, most of the molar features we generated have counterparts in extinct or extant rodent species.

A general trend in evolution, evident also in molars, has been an increase in complexity. In the absence of experimental ways to repeat this phenomenon, its dynamics have remained elusive. In tuning Eda, Activin, and Shh signalling we found that an increase in cusp number correlated with the number of signalling pathways tuned simultaneously. Though intuitively obvious, the result had not previously been reported. Should an increase in complexity require multiple simultaneous changes in development as a rule, the overwhelming majority of reports on decreasing complexity, typically studying the effects of a single change at a time, would be explained.

In conclusion, our results provide new information on the developmental genetic mechanisms of cusp patterning, how they provide developmental stability, and what kind of evolutionary constraints they cause.

REVIEW OF THE LITERATURE

Introduction

Teeth can be considered a key innovation in vertebrate evolution, and the variability of tooth crown shape has been an asset in mammalian evolution in particular. Tooth morphology varies considerably between mammal species, in addition to which most modern mammals have dentitions consisting of different types of teeth. Teeth are a vital part of an animal's interaction with its environment and thus affect fitness directly. The tooth crown consists of an outer layer of enamel and an inner layer of dentin, both of which are mostly composed of minerals and are thus extremely hard. Teeth become preserved as fossils more easily than other, softer tissues of the body, thanks to which the approximate evolutionary history of many modern tooth forms is known. The development of embryonic teeth has been studied extensively, accumulating both information and a wide selection of established research methods. All in all, teeth provide a unique research model that combines information about functional properties, evolution, and development.

The continuing progress of the evolutionary theory

Charles Darwin's The Origin of Species in 1859 was the culmination point of a long effort by him and others to explain the current diversity and distribution of species by means other than spontaneous creation (Gould 2002; Berry 2008). The theory of evolution – in short, descent with modification – triggered the reformation of biology from a descriptive science to the empirical discipline we know today.

Explaining how evolution works remains a major goal for biology (Gould 2002; West-Eberhard 2003; Pigliucci 2007). Although the mechanics of heredity are now better understood, many aspects of the modification of existing properties and the appearance of new ones remain controversial. For example, if and how do gradual, small-scale changes between individuals (microevolution) add up to the sudden, large-scale differences observed in the fossil record and between modern species (macroevolution). One of the major leaps forward was the discovery of *Hox* genes (Lewis 1978) that regulate the patterning of the body plan in insects as well as in vertebrates (Wilkins 2002). This unexpected deep similarity of development paved the way for the merging of evolutionary and developmental biology into 'evo-devo'. It has now been established that changes in gene regulation correspond to changes in structures of the body, and that gene groups like the *Hox* genes provide 'toolkits' that can be modified for different

purposes. For example, the vertebral *Hox* patterning code has been modified for reuse in the limb.

Much has been learned of how an organism uses its genotype, the genetic makeup, to produce its phenotype, the observable characteristics. Yet, open questions remain: Although we can identify what kinds of changes in the genotype have been linked to changes in the phenotype, we cannot satisfactorily explain how ecological factors caused these changes to become accommodated into the genotype in the first place. Emergent properties of organisms, such as multiple levels of organization, affect how features can change and how these changes can be selected. The significance of the interplay between ecology and such emergent properties has recently been recognized anew and highlighted with the label 'eco-evo-devo', short for ecological evolutionary developmental biology (Gilbert 2009). The phenomena under study include, for example, the ability to buffer development against genetic and environmental variation (canalization), and the opposite ability to alter development according to environmental cues (phenotypic plasticity). Also the tendencies of development to bias variation (developmental constraints) and to become organized into semi-independently developing sub-units (modularity) are assumed to play essential roles in evolution. An additional layer of complexity is added by the notion that these phenomena facilitate evolution and are thus actively selected for and modified (evolvability).

All in all, we live in interesting times, and teeth as a research model show great promise in contributing to the next mould of the evolutionary theory (Kangas *et al.* 2004; Kavanagh *et al.* 2007; Salazar-Ciudad & Jernvall 2010).

The evolution of mouse molars

Tooth-like structures first appeared over 500 million years ago and thus outdate other famous innovations such as legs, hard-shelled eggs, and endothermy (Smith & Hall 1990). The simplest definition of a tooth is an odontode, a structure composed of an outer layer of enamel and an inner layer of dentin, or similar highly mineralized tissues, and a mesenchymal bone of attachment (Fraser *et al.* 2010). Odontodes are assumed to have originated as armour scales on the skin, and to have evolved into a dentition after the jaws appeared (Butler 1995), though odontodes in the pharynx might have contributed to the process (Fraser *et al.* 2010). The common evolutionary origin of all skin appendages, including teeth, scales, nails, hairs, horns, and feathers, is reflected in the similarity of their development in the embryo (Gurdon 1992; Thesleff *et al.* 1995; Fraser *et al.* 2010).

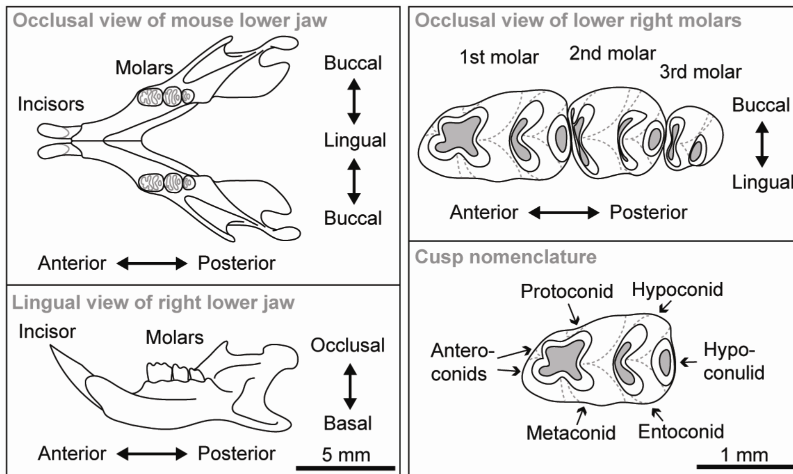


Figure 1. An illustration of the mouse dentition. Cusp nomenclature based on Osborn 1907 and Rodrigues 2010.

Teeth facilitate predatory behaviour, defence, intake of food in digestible portions, gripping, digging, and carrying. The evolution of heterodonty, meaning the diversification of the dentition into different types of teeth, takes full advantage of this versatility. Modern heterodont mammals have four different kinds of teeth; incisors, canines, premolars, and molars, often with each type of tooth being specialized to perform a certain function better than others. Although most modern mammals are heterodont, it is not a mammal-specific trait: Cetaceans, such as dolphins, have derived homodont dentitions (Déméré *et al.* 2008), while some dinosaurs, lizards, and crocodylians have had heterodont dentitions (Nyam *et al.* 2000; Buckley *et al.* 2000; Smith 2005). Also many fish, such as cichlids, have multicuspid teeth (Streelman *et al.* 2003).

The simplest teeth are one-cusped cones (Fig. 2), functionally suited for grabbing, and retained for example in modern lizards and fish, and in the incisor and canine teeth of most mammals. The early phases of cusp pattern evolution are difficult to reconstruct, due to the incompleteness of the fossil record and possible occurrences of similar evolution at different times in different lineages. However, it seems that first small wrinkles and then two additional cusps evolved to form an anterior-posterior row. In the lower teeth, the cusps are called the paracone, protocone, and metacone, from the anterior to the posterior (Osborn 1907). The occlusion was parallel, so that the upper and lower teeth acted like serrated scissors, adding a shearing function. Next the configuration of the cusps changed from linear to triangular, with the upper 'trigons' and lower 'trigonids' interlocking in occlusion. The addition of a posterior extension, the talonid, added a grinding function by enabling mortar-and-pestle action (Hiiemae

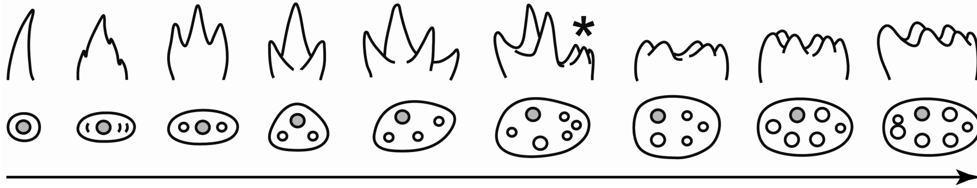


Figure 2. A schematic illustration of the presumed evolution of the lower molar from pre-mammal to modern mouse. Tilted lingual view on top, occlusal view below, with the most ancestral cusp, the protoconid, marked with grey. Anterior to the left. In the tribosphenic molar the posterior extension, i.e. the talonid (*), forms a basin for the protocone cusp of the upper molar, thus introducing a grinding function. Not to scale. Based on Osborn 1907, Weller 1968, Butler 1990, Luo et al. 2001, Rodrigues et al. 2010, and Ungar 2010.

& Crompton 1985; Luo *et al.* 2001). Efficiency was increased when the talonid part expanded to accommodate three cusps, the hypoconid, entoconid, and hypoconulid. This tribosphenic tooth form first appeared in the Jurassic era, over 150 million years ago (Fig. 2). It is generally considered a key innovation contributing to the success of mammals (Fig. 3 A) (Osborn 1907; Weller 1968; Butler 1990; Rensberger 2000). The same design seems to have evolved independently at least two times with slightly different realizations (Luo *et al.* 2001; Luo *et al.* 2007; Luo 2007).

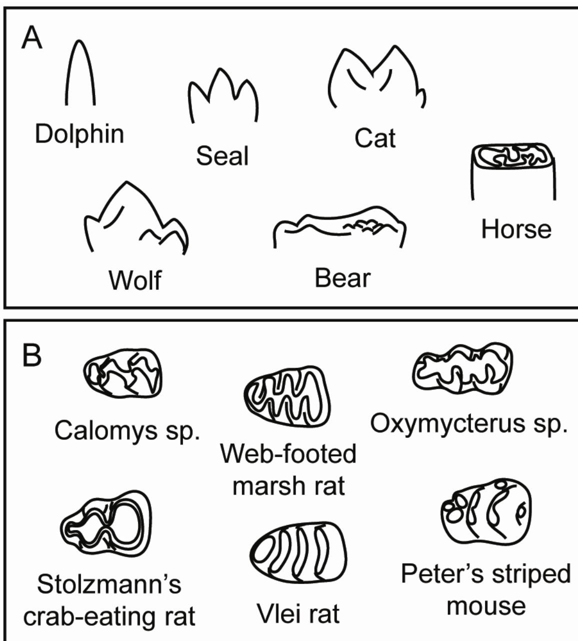


Figure 3. (A) Examples of cusp pattern diversity originating from the tribosphenic type. In the gradient from carnivores through omnivore (bear) to herbivore (horse), cusps become smaller, lower and more numerous. Illustrations of first molars adapted from Hillson 2005. Tilted lingual view, anterior to the left. Not to scale. (B) Examples of cusp pattern diversity in mouse-like rodents, in the family Muridae. Illustrations based on photographs by Alistair Evans. Occlusal view, anterior to the left. Not to scale.

The earliest undisputed rodent fossils date back to the Paleocene era, about 60 million years ago (Macdonald 2001; Ungar 2010), although molecular data indicate that the rodent family tree might have sprouted earlier (Steppan *et al.* 2004). The order Rodentia is most closely related to Lagomorpha (rabbits and hares) (Fig. 4), and more distantly to Primates, Scandentia (tree shrews), and Dermoptera (colugos). The most easily distinguishable feature of rodents is the pair of continuously growing incisors in both the upper and the lower jaw. The rest of the incisors, as well as canines and most premolars, have been lost, leaving a toothless gap through which gnawed material is expelled when the lips are drawn in to seal the throat (Macdonald 2001). Rodent molars have moved far back in the mouth, which eliminates their shearing function but allows increased pressure in grinding (Butler 1985). As in the molars of most herbivores, the toughness of plant fibres is countered with numerous, relatively small, low cusps and crests arranged in a serial array of blades, with the cutting edges of the upper and lower molars opposing each other at an angle during chewing (Hiiemae & Crompton 1985). At least in the line leading to mice (*Mus musculus*), the paraconid was lost and the anteroconid cusp appeared at its location, eventually splitting in two (Fig. 2) (Luckett 1985; Rodrigues 2010). Today, almost 2000 rodent species occupy a wide range of habitats, from the gerbils in the deserts to the beavers in the forest lakes. Although most rodents are more or less herbivorous, diet components range from tree bark to fish. Accordingly, the diversity of molar cusp patterns is impressive, even just among the family Muridae (Figs 3 B and 4) (Evans *et al.* 2007). The phylogeny of rodents remains

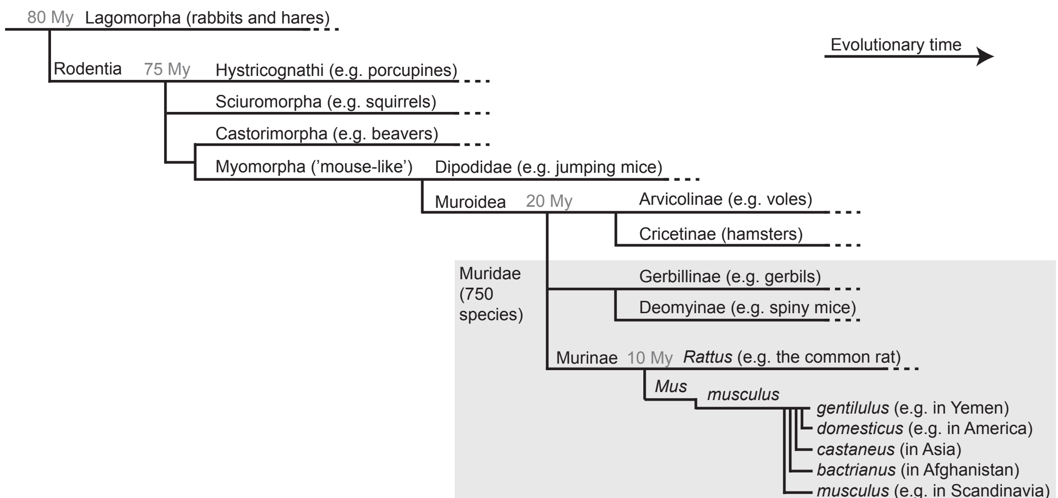


Figure 4. A possible phylogeny of the house mouse (*Mus musculus*), with only a subset of the branches shown. Branch lengths are not scaled to time. Some of the divergence time estimates, based on molecular data, are given at branching points (in grey, My = million years). Based on Ungar 2010 and references therein, Steppan *et al.* 2004, Bonhomme *et al.* 1984, and Duplantier *et al.* 2002.

complex and the details unresolved, apparently due to recent bursts of adaptive radiation leaving relatively small differences between many species (Ungar 2010). In recent textbooks, the common mouse (the house mouse, *Mus musculus*) has five subspecies. Laboratory strains, such as C57BL/6, seem to be derived from a mixture of three of these subspecies (*M. m. musculus*, *M. m. domesticus*, and *M. m. castaneus*) (Musser & Carleton 2005).

Since teeth are composed of the hardest tissue in the body, they are preserved as fossils more frequently than other structures. Of many animals, only a few teeth remain. Thus palaeontologists have long used tooth shapes and cusp patterns to identify taxa and, together with tooth wear, various properties such as age and diet. Such deductions must have a solid base in research, including developmental experimentation. For example, variation between individuals can be mistaken for variation between species, and vice versa, if the dynamics of developmental mechanisms and variation in extant populations are not considered. To clarify the matter, covariation between dental features can be identified by analyzing tooth phenotypes of transgenic mice (Kangas *et al.* 2004; Charles *et al.* 2009). In addition, developmental analysis can reveal mechanisms affecting the development, and thus the evolution of structures, such as the mouse incisor being formed by fusion of two tooth buds (Munne *et al.* 2010), and lateral inhibition controlling the relative sizes of adjacent molars (Kavanagh *et al.* 2007).

Signalling molecules in development

Communication is vital in all relationships, including those between the cells of the body. In order for all structures to achieve proper patterns, shapes, and sizes during embryogenesis, cells must continuously send each other messages carrying information on their relative position and state of differentiation. These messages, referred to in general as signalling molecules, can be secreted out of the cell and are then able to travel in the space between cells. Signalling molecules are usually protein-based when used between cells directly connected or near to each other, and lipid-based in hormonal signalling via the blood stream. Receptors are usually transmembrane proteins bound by their structure to the cell wall, with the extracellular part capable of transiently binding the signalling molecule. Interaction with the signalling molecule, referred to in this context as the ligand, triggers a conformational change in the receptor. This in turn triggers a cascade of events, called a signal-transduction pathway, inside the cell. The intended result can be any kind of change or inhibition of change in the cell state, such as cell differentiation by the activation or silencing of specific genes.

Some signalling molecules can interact with only one receptor, while others have a wide variety of receptors capable of responding to their presence, often in different ways. Still, there are only a limited number of ligands and receptors, so even though the amount and selection of the secreted signalling molecules can be changed, context-specific responses are achieved mostly by modifying the competence of the receiving cells. Competence refers to the cell's ability to respond correctly to a given signal, or in actuality to the cross-fire of several signals. It is effectuated by *e.g.* the specific amount, distribution, and structural composition of the receptors and components of the internal signal-transduction pathway present in the cell at the time the signal is received, and is therefore a product of the cell's life history. In textbooks, pattern development is usually simplified into a hierarchical series of signalling events, when in truth it rather resembles a self-organizing chaos based on reciprocal, dynamic inter-actions.

Usually, signal activation is rather straightforward, as in the case of Bone morphogenetic protein (Bmp) signalling: The ligand associates with its receptor, which phosphorylates the proteins Smad1, -5 and -8, enabling them to form a heterodimeric complex with Smad4, which is then translocated into the nucleus, where the complex can activate target genes (Nie *et al.* 2006). Sometimes signal

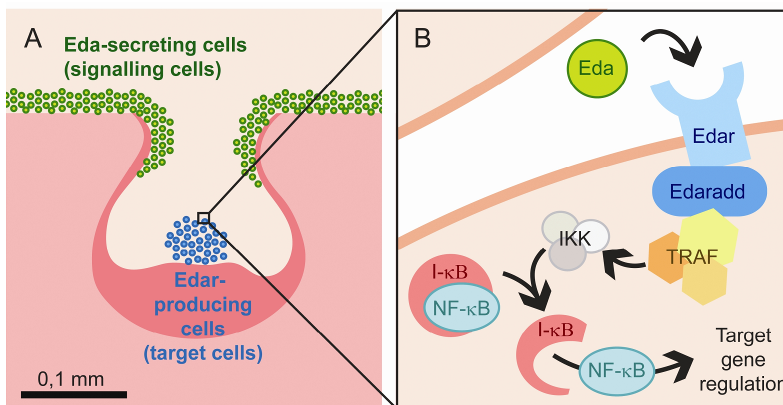


Figure 5. A schematic illustration of Eda signalling in the developing molar as an example of a diffusing signalling molecule activating a signal-transduction pathway. (A) In the developing molar of a 14 days old mouse embryo, Eda is produced only by cells in the outer dental epithelium (green), while the Eda receptor Edar is produced about 50 µm away, in the primary enamel knot cells in the middle of the tooth (based on frontal sections in Laurikkala *et al.* 2001). (B) Eda is secreted into the extracellular space, where it travels by diffusion and can bind to its receptor Edar. The binding activates Edaradd, which activates TRAF molecules, which recruits the IKK molecule complex, which phosphorylates I-κB, which releases the transcription factor NF-κB, which trans-locates into the nucleus to regulate the transcriptional activity of target genes (Mikkola 2008).

activation happens by releasing inhibition, as in Sonic hedgehog (Shh) signalling: The transmembrane receptor protein Patched keeps another trans-membrane protein, Smoothened, inactive, until the binding of the ligand Shh to Patched inhibits this inhibition, activating the signal-transduction pathway (Varjosalo & Taipale 2008). Many signalling pathways, such as Ectodysplasin (Eda), combine both activation and inhibition (Fig. 5): The ligand binding to its receptor triggers a series of activating interactions between adapter molecules, leading to the inhibition of I-κB, which allows NF-κB to regulate the activity of its target genes (Mikkola 2008).

Patterning mechanisms

Patterns in the body are realized mostly by the gradual differentiation of cells, for example from an undifferentiated embryonic cell to an epithelial cell, to an epithelial limb cell, to the next-to-last skin cell on the index finger. Differentiation is usually permanent, with only the stem cells retaining the use of their whole genetic potential, or at least part of it. Thus they are responsible for regeneration, for example in humans for the continuous production of blood cells and the periodic cycling of hairs.

In addition to cell differentiation, patterns are produced by a variety of cellular mechanisms, including migration, differential adhesion, proliferation, death, and changing the size of the cell or of its extracellular layer (Gilbert 1997). Examples of each can be given in tooth development: mesenchymal cells migrate from the neural crest to the sites of tooth initiation (Jernvall & Thesleff 2000); differential adhesion, effectuated through the differential expression of cell surface molecules, causes the segregation of dental epithelium and dental mesenchyme cells into distinct clusters (Jernvall & Thesleff 2000; Ikeda *et al.* 2009; Mammoto *et al.* 2011); carefully directed proliferation causes the dental epithelium to grow first into a bud, then a cap, and finally a bell-shape (Butler 1956; Jernvall *et al.* 1994; Jernvall *et al.* 2000); cell death, called apoptosis, removes the cells of the posterior part of the primary enamel knot, which if left undisturbed creates a crest onto the molar surface (Kangas *et al.* 2004); and finally, at the onset of tooth mineralization, epithelial cells differentiate into ameloblasts, which causes them to change their shape from round to columnar and to secrete enamel, which changes the size and shape of their surroundings (Hillson 2005).

Coding spatial information into molecule gradients

Diffusive signalling molecules can be secreted from a distinct area in order to produce a gradient which provides positional information for the nearby cells. Signalling molecules that induce different cellular responses along such a gradient are generally called morphogens. A habitually used example of morphogen action is the limb bud, where digit identity is interpreted from a gradient of Shh, secreted from the position of the future little finger (Tickle 2006). To qualify as a morphogen, a molecule must elicit at least two qualitatively different responses, though experiments reveal the number to most often fall between three and seven (Ashe & Briscoe 2006). Concentration-dependent cellular responses seem to be effectuated by thresholds in amount of signalling receptors activated, and this system can correctly interpret as small as two-fold differences in a linear gradient (Ashe & Briscoe 2006). The distance a molecule can diffuse in extracellular space varies considerably according to the biochemical properties of the molecule. For example, Activins and Bmps can travel over 10 cell diameters, while Wnts are restricted to shorter distances, and Shh can do either depending on its modification (Arias & Stewart 2002). Naturally these properties are affected by the composition of the extracellular space and the possible use of a carrier molecule. Diffusion, a process automated by the random thermal movement of molecules, is insufficient for sustaining a gradient, and so slow that additional mechanisms should be the rule (Howard *et al.* 2011). Complexity is further added by the fact that spatial information is probably never provided by a single molecular gradient alone (Ashe & Briscoe 2006).

Automated patterning

It seems that most patterns of the body are produced by dynamic, self-organizing networks emerging from the tissue-specific, pre-ordained interactions of signalling pathways. One of the most elegant examples of such self-organization is the specification of the anchor cell in the roundworm *Caenorhabditis elegans* (Arias & Stewart 2002): Initially there are two identical cells, both of which express a Notch signalling family ligand and its receptor. The binding of the ligand to the receptor results in the inhibition of the ligand expression, which is a negative feedback loop, but also in the upregulation of the receptor expression, which is a positive feedback loop. Eventually, the random events affecting cellular function cause one of the cells to exceed the other in the level of signalling, after which the feedback loops quickly cause one of the cells to express only the ligand and the other only the receptor, establishing the ligand-expressing cell as the future anchor cell.

Feedback loops are common and essential, since they provide not only a means of patterning but also developmental robustness against small random changes in cellular function and position (Wilkins 2002). For example, in one of the signalling loops governing mouse limb development, in addition to providing digit identities, Shh upregulates the expression of Gremlin1, which antagonizes Bmp4, which would otherwise have inhibited the Fibroblast growth factors (Fgfs) that are needed for promoting limb growth and Shh expression (Tickle 2006). It was recently shown that Bmp4 also directly contributes to upregulating Gremlin1, which makes the loop robust against reduction in the levels of Shh and Bmp4 signalling (Bénazet *et al.* 2009).

In 1952, Alan Turing proposed a mathematical model for explaining how complex patterns could be achieved with simple interactions of only two chemicals (Howard *et al.* 2011). In the developmental interpretation of this reaction-diffusion model (Fig. 6), two signalling molecules are initially expressed either uniformly or randomly. One of the molecules, called the activator, induces the differentiation of cells, for example black spots on an otherwise yellow skin. The activator also upregulates its own expression and the expression of the second molecule, the inhibitor, so named because it inhibits the activator from inducing differentiation. The diffusion of the two molecules is different, so that the inhibitor acts at a longer range than the activator. The combination of the positive and negative feedback loops with differences in range of action automatically causes the distribution of the activator and inhibitor to form a wave-pattern. Viewed in two dimensions, this lateral inhibition restricts

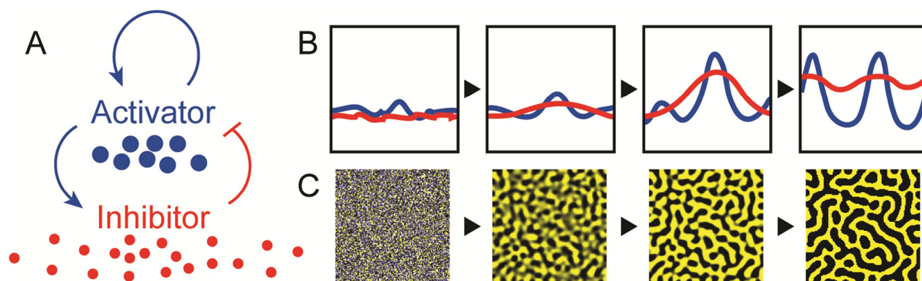


Figure 6. A schematic illustration of reaction-diffusion model dynamics. (A) The interactions of the activator (blue) and the inhibitor (red) molecules, with positive influence indicated with sharp arrows and negative influence with a blunt arrow. The inhibitor acts at a longer range than the activator, for example due to smaller size aiding diffusion. (B) Viewed as concentrations in one dimension, the activator and inhibitor start out with a random distribution, which automatically changes into a stable wave-pattern due to their interactions and differential ranges of action. The activator is now able to induce differentiation only at its concentration peaks, generated at regular intervals. (C) An example of a two-dimensional pattern generated by a reaction-diffusion model provided for free online by Kondo & Miura (2010).

differentiation into distinct spots. Since the pattern is self-organizing, it does not require previous patterning events and is robust against disturbances (Kondo & Miura 2010).

Computer simulations of reaction-diffusion mechanisms can reproduce two-dimensional biological patterns, such as the variety of complex colour patterns on vertebrate skins, by tinkering with the parameters defining the molecular interactions and ranges of action (Kondo & Miura 2010; Howard *et al.* 2011). The models used can also be expanded to include morphogens, a term also coined by Turing; this changes the induction of differentiation from a simple on/off-system to a more complex one, presumably closer to reality (Kondo & Miura 2010). Although the reaction-diffusion model is generally acknowledged as a very useful theoretical tool, recognizing activator and inhibitor molecules and characterizing their behaviour in real life has only just begun.

In addition to colour patterns, vertebrate skin can also produce hair or feather placodes in patterns reminiscent of reaction-diffusion dynamics. Wnt, Fgf and Eda signalling exhibit properties of activators, in that placodes fail to be initiated in their absence, whereas their overactivation results in the initiation of extra placodes (Mikkola 2007). Activator-inhibitor loops seem to be formed by Wnt and Dkk (Schlake & Sick 2007) and by Eda and Bmps (Mou *et al.* 2006), though their exact interactions remain to be elucidated. Of these, Eda seems especially interesting, since the receptor is initially uniformly expressed, but becomes restricted to hair placodes as they differentiate (Laurikkala *et al.* 2002).

Another intensely studied system is the cusp patterning of teeth. Information on existing signalling networks and activator-inhibitor dynamics has been successfully used to simulate the development of mouse, vole, and seal molars (Salazar-Ciudad & Jernvall 2002; Salazar-Ciudad & Jernvall 2010). Tinkering with the model parameters can replicate individual variation seen in natural populations (Salazar-Ciudad & Jernvall 2010), providing insights into which biochemical properties are likely to vary more easily.

Turing is also credited with the famous remark about zebra development: “The stripes are easy, but what about the horse part?” This serves as a reminder that in addition to biochemical patterning, also biomechanical forces are required for the shaping of organs (Howard *et al*. 2011). This requirement is well illustrated in the shaping of the mouse molar crown: Regardless of the placement of cusp initiation, mechanical forces such as water pressure in the stellate reticulum, constraint by the surrounding dental follicle, and changes in pressure caused by uneven cell proliferation probably all contribute to the folding of the tooth surface (Butler 1956; Salazar-Ciudad & Jernvall 2002; Osborn 2008; Salazar-Ciudad & Jernvall 2010).

Evolution of patterning via changes in gene regulation

Comparing gene sequences of different species has revealed that, surprisingly, many genes change very little during evolution. Even between species as diverged as the fly and the mouse, many gene sequences are strictly conserved (Davidson 2001). Since the differences between phenotypes cannot be entirely explained by the differences in gene sequences, the explanation must lie with how those sequences are used.

A gene is called active when its sequence is transcribed, *i.e.* its instructions read, in order to produce a biochemically active molecule from amino acids or RNA. The activity of genes is proximally controlled via regulatory sequences, short stretches of DNA often located physically near the gene sequence. Molecules that can bind to regulatory sequences, and interact with each other and the transcription complex, are called transcription factors (Wilkins 2002). The interactions can be weak or strong, activating or inhibiting, and the combination of many factors determines whether a gene is 'on' or 'off'. Regulatory sequences and the factors that bind them seem to commonly work in modules, so that working combinations are context-specific (Davidson 2001). Thus genetic mutations as small as changing a single nucleotide in a single binding site could potentially have any kind of phenotypic effect from none at all to inducing the development of an extra limb. Especially the capability of reaction-diffusion models to create complex patterns from simple elements implies that the evolution of complex structures might require only small changes in gene regulation. We have barely scratched the surface in the study of such evolutionary tinkering, yet examples of its potential abound, from differences in the genetic regulation of anterior-posterior body patterning between fruit flies and centipedes, to the differences in digit patterning between fish and mammals (Davidson 2001). Recently it has been discovered that also microRNAs contribute to gene regulation, and their apparent tendency to fine-tune the expression of developmental genes has sparked a growing interest in their role in the evolution of development (Ying *et al.* 2008; Wheeler *et al.* 2009).

Evolution of development via changes in gene regulation can be expected to have consequences. Though by no means exclusive to this mode of evolution, it is nevertheless worthwhile to list some of them here. First, regulatory evolution can be expected to speed up morphological change, both by facilitating small-scale tinkering and enabling radical large-scale reorganizations. Both the theory and actual examples are heavily debated (Gould 2002). Second, that development need not recapitulate evolutionary history, as it indeed does not always do (Wilkins 2002). Third, that one type of phenotypic effect should be attainable by many kinds of genotypic changes, and if the effect provides a

fitness advantage, it should evolve repeatedly in different lineages. Doubtless many conditions restrict this occurrence, called homoplasy, but, for example, in molars the hypocone cusp can be produced in computer simulations with many different kinds of parameter modifications (Salazar-Ciudad & Jernvall 2002), and it has indeed evolved independently in several lineages apparently because it adds a valuable grinding function (Butler 1985; Hunter & Jernvall 1995). Fourth, that the modularity of genetic regulation should allow modular evolution. Such 'mosaic evolution' has been documented for example in the fossil record of birds, where different traits appear and change apparently independently (Wilkins 2002). Indeed, it has even been suggested that modular evolution is the reason modular regulation has evolved in the first place (Gould 2002). Fifth, that since most regulatory sequences and transcription factors individually have but small effects, small-scale changes should be more common than large-scale ones. This is one of the paths that lead to the concept of developmental constraint, where developmental mechanisms producing a structure restrict, or in a positive view direct, the variation exposed to natural selection. Examples are not wanting, for example Osborn wrote about tooth forms in 1897 as follows: "My study of teeth in a great many phyla of Mammalia in past times has convinced me that the evolution of teeth is marked out beforehand by hereditary influences which extend back hundreds of thousands of years. These predispositions are aroused under certain exciting causes and the progress of tooth development takes a certain form converting into actuality what has hitherto been potentiality." (cited in Gould 2002).

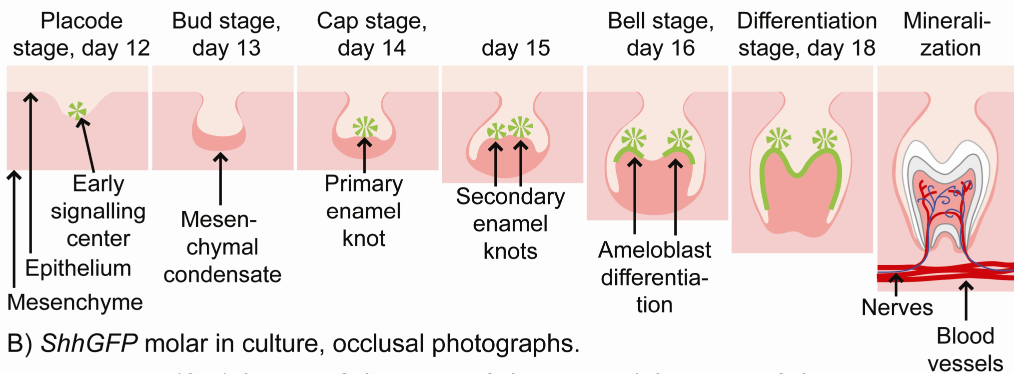
Tooth development

Mouse teeth have been intensely studied as one of the models of epithelial-mesenchymal interactions in mammal organogenesis. Signalling pathways and reciprocal interactions described in teeth seem to act similarly in the development of other organs, especially in those containing an epithelial component, and especially at the early stages of development (Gurdon 1992; Thesleff *et al.* 1995).

By embryonic day 11, the embryo has developed enough to be recognized, if not yet as a mouse, at least as a mammal. All tissues are still soft, which facilitates cell migration and tissue folding. A population of neural crest – derived mesenchymal cells has migrated from the neural tube in the midbrain region to the jaws (Imai *et al.* 1996). The sites of the future tooth buds may already have been determined by antagonistic signalling between Fgfs and Bmps (Neübuser *et al.* 1997; Peters *et al.* 1998), and also the dental marker *Pitx2* is already expressed (Mucchielli *et al.* 1997). The first tooth-specific structure to form is the epithelial thickening, called a primary epithelial band, which forms a

U-shape comprising both future incisor and molar areas (Jernvall & Thesleff 2000). At this point the epithelium has the initiative, and is able to produce tooth-specific tissues also from cells which do not normally participate in tooth formation (Mina & Kollar 1987; Lumsden 1988). The epithelium also seems to control the patterning of the primary epithelial band into the four tooth types (incisors, canines, premolars and molars), possibly via a dental homeobox code and gradients in Bmp and Fgf signalling (Sharpe 1995; Mitsiadis & Smith 2006).

The primary epithelial band and the underlying mesenchyme communicate and direct their development by reciprocal interactions (Jernvall & Thesleff 2000). Most of the signalling molecules used seem to belong to the Bmp, Wnt, and Fgf families, but also others are involved (for a full list of molecules expressed during tooth development see http://bite-it.helsinki.fi); for



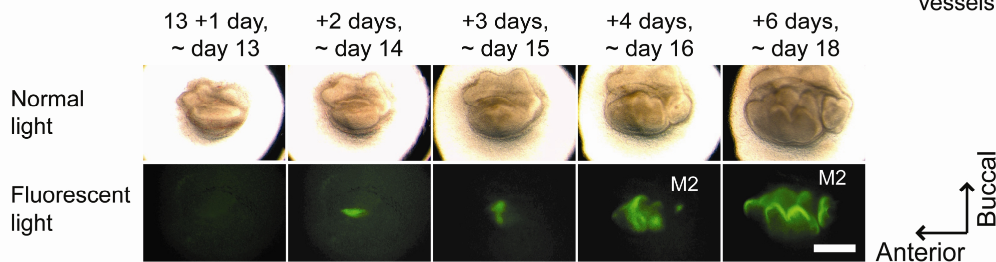


Figure 7. Mouse molar development. (A) A schematic illustration of molar development, with Green fluorescent protein (*Shh-GFP* construct) reporter activity in enamel knots and in differentiating ameloblasts in green. Based on frontal histological sections; posterior end towards the viewer, buccal to the right. Not to scale. (B) A $Shh^{wt/GFPcre}$ first lower molar in culture from embryonic day 13 onwards for six days. Photographs in the bottom row taken in fluorescent light, revealing ShhGFP reporter activity. Placing a molar into culture initially causes a 12–24 hour delay in development, causing 13 +1 day in culture to correspond roughly to day 13 *in vivo.* The second molar (M2) appears at +4 days. Proper mineralization does not take place in cultured teeth. Scale bar 0,5 mm.

example, in lower molars epithelial Fgf8 is required to induce mesenchymal *Activin-βA* expression, which in turn must induce epithelial *Follistatin* expression in order for tooth development to proceed (Ferguson *et al.* 1998). Indirect evidence places Fgf upstream of also Wnt and Bmp signalling in early tooth development (Cho *et al.* 2011; Ohazama *et al.* 2008). The epithelium grows further downwards at distinct spots, thus forming placodes, apparently one for each tooth type (Järvinen *et al.* 2008). Cells in the middle of each placode differentiate into an early signalling centre (Fig. 7). Among other molecules, they express *p21*, which contributes to preventing proliferation, and *Shh*, which contributes to inducing proliferation in the surrounding cells (Keränen *et al.* 1998; Jernvall *et al.* 1998; Dassule *et al.* 2000). The early signalling centres undergo apoptosis as the placode grows into a bud (Vaahtokari *et al.* 1996). At this point mesenchymal cells condense around the bud, and *Bmp4* expression and tooth-inducing potential shifts to the mesenchyme (Mina & Kollar 1987; Vainio *et al.* 1993).

Apparently because of a mesenchymal Bmp4 signal, cells at the tip of the epithelial bud differentiate into primary enamel knot cells, and begin to express *p21* (Jernvall *et al.* 1998). *Shh* expression is possibly induced by a Wnt – Fgf signalling loop (Kratochwil *et al.* 2002). In addition, enamel knot cells express Wnt, Bmp, and Fgf signalling factors which direct the growth of the surrounding tissue (Jernvall & Thesleff 2000). The importance of the primary enamel knot is demonstrated by the number of mouse mutants in which it does not become differentiated and tooth development fails to proceed (Jernvall & Thesleff 2000; Matzuk *et al.* 1995; Hardcastle *et al.* 1998; Andl *et al.* 2004). Due to the primary enamel knot causing uneven growth, the bud takes on a cap-like appearance (Fig. 7). The downwards growing edges are called cervical loops, and the mesenchyme they surround is called the dental papilla. The posterior part of the primary enamel knot undergoes apoptosis, while the anterior part stays on to become the first secondary enamel knot (Cho *et al.* 2007). Despite the change in name, the set of expressed molecules does not appear to change, nor does the function: the knot cells themselves do not proliferate, but they induce proliferation around them, causing the future tooth surface to fold and grow into cusps.

The lingual cervical loop grows more quickly than the buccal one. This might be due to the buccal gradient of Bmp4, which seems to be involved in tipping the balance from proliferation to differentiation (Åberg *et al.* 1997; Zhang *et al.* 2009). A condensation of epithelial cells called the enamel rope anchors the primary enamel knot onto the buccal side (Butler 1956; Cho *et al.* 2007). As the cap grows, the next secondary enamel knot becomes differentiated in the nascent lingual space. The first secondary enamel knot will give rise to the protoconid cusp, while the second will produce the metaconid cusp (Fig. 1)

(Jernvall *et al.* 2000; Cho *et al.* 2007). As the tooth grows further into the bell-stage, secondary enamel knots appear that will give rise to the anterior anteroconid cusps and the posterior talonid cusps (Gaunt 1955). Eventually, the secondary enamel knots are removed by apoptosis (Vaahtokari *et al.* 1996).

During the bell stage, the adjacent epithelial and mesenchymal cells of the crown begin to differentiate into ameloblasts and odontoblasts, respectively, beginning from the cusp tips and with the odontoblasts becoming functional first (Nanci 2008). Ameloblasts move outwards as they secrete enamel, ending up on the tooth surface. These surface cells die once the tooth erupts into the oral cavity, so injury to enamel cannot be repaired. Conversely, odontoblasts move inwards as they secrete dentin, ending up in the pulp chamber of the tooth. There reparative dentin can be produced, apparently by mesenchymal stem cells capable of differentiating into odontoblasts. Enamel and dentin are originally composed of an organic matrix, which is degraded once it becomes mineralized, *i.e.* filled with hydroxyapatite crystals formed from phosphate and calcium. It is yet unclear how exactly the mineralization is achieved.

As the tooth grows roots, the patterning of which is as distinctive as that of the cusps, mesenchymal cells differentiate into odontoblasts and epithelial and/or follicular cells differentiate into cementoblasts. Cementum, also a mineralized tissue, provides an anchor for the periodontal ligament that attaches the tooth into its socket in the jaw bone. The root tips remain open to provide access for blood vessels and nerves. So even though a tooth develops as a semi-independent module, the interaction of many tissues is still required. Tooth eruption is accomplished by apoptosis of the surface epithelium, bone resorption between the oral cavity and the tooth, and bone formation at the base of the roots (Nanci 2008). In a sense, the eruption process is never terminated in continuously growing teeth (hypselodont teeth, such as the rodent incisors and vole molars), apparently thanks to the maintenance of epithelial stem cells inside the cervical loops (Tummers & Thesleff 2009).

Variation: Within individuals and between individuals

Miscarriages and congenital defects can easily produce an impression that any departure from the ‘perfect’ development dictated by genes is deleterious. However, even identical twins are not in fact identical, as can be seen for example by observing their dental X-rays. The realization of the phenotype according to the flexible instructions of the genotype is a process open both to environmental influence and chance. Thus one genotype can give rise to a variety of phenotypes, even if the differences are very subtle, as in the case of twins, or easily visible, as in the case of temperature-regulated gender in crocodilians. This developmental plasticity and instability can be viewed as the

ultimate engine of evolution, since it produces the phenotypic variation exposed to natural selection (West-Eberhard 2003; Gilbert 2009).

Variation can be sorted into two types: within an individual and between individuals. Within an individual, variation is measured as asymmetry between the left and the right side, and is mostly produced by developmental instability, *i.e.* 'developmental noise' (Willmore & Hallgrímsson 2005; Dongen 2006; Willmore *et al.* 2007). In a way, this noise is a built-in feature stemming from developmental mechanisms; for example, the diffusion of signalling molecules is based on random thermal movement and cannot be totally controlled. Developmental stability remains a relatively unexplored field, mostly due to the technical difficulties of detecting left-right variation, which often stays below the threshold of measurement error, and also due to the difficulties of detecting the cause of the variation. In addition, most mechanisms that are assumed to promote developmental stability, for example the organization of gene regulation into networks, can just as well be assumed to promote developmental instability. Though developmental stability is clearly a fitness advantage and appears to be heritable to a low degree, it is generally assumed not to feature significantly in the evolution of other traits.

Variation between individuals is mostly caused by genetic and environmental differences. Mechanisms that buffer development against these differences are referred to as canalization, while the ability of the genome to respond adaptively to environmental differences is referred to as phenotypic plasticity. Canalization is often revealed only when it is undone (Dworkin 2005). For example, the number of vibrissae in the mouse is highly invariable, except in *Eda*-null mutants, implying the existence of a canalizing mechanism requiring Eda (Dun & Fraser 1958). On top of its obvious usefulness in constructing a viable organism, canalization is assumed to promote evolvability by hiding random mutations from the pruning force of natural selection until their combined effect overcomes a certain threshold and becomes expressed (Gilbert 2009).

Studying variation can also reveal other phenomena besides developmental instability and canalization. Gaps in a variation gradient can point to developmental constraints, meaning emergent features of development that can affect the rate and direction of evolution, while covariation among features can reveal developmental modularity, meaning the compartmentalization of development into semi-independent sub-units (Klingenberg 2005). For example, the molars form a developmental module independent enough to go through morphogenesis even when moved from the embryo into tissue culture conditions. Modularity is a common phenomenon in multicellular organisms and is assumed to facilitate evolutionary change, in part because change can be contained and is thus less likely to be detrimental to the organism as a whole, and in part because

the semi-independence of modules seems to be realized through dynamic gene regulation networks that can propagate change.

Premolar resurrection

Rodents have evolved ever-growing, self-sharpening incisors, a recipe for success as testified for by the staggering number of rodent species and their vast distribution. Like rabbits, they have lost all canines and most premolars, apparently in order to easily expel gnawed material through the toothless gap. Some rodents, for example squirrels, still retain the most posterior premolar (Fig. 8), but it has not erupted in the lower jaw of mouse-like rodents in at least 45 million years (Hillson 2005; Ungar 2010; Rodrigues *et al.* 2011).

The development of the three mouse molars begins at the site of the first molar and continues in the posterior direction. Also molar size and complexity, when changing from one molar to the next, often diminishes in the posterior direction, and loss of molars during evolution starts from the posterior end (Hillson 2005; Ungar 2010). For premolars, the opposite seems to be true, and thus the last premolar remaining is located anterior to the set of molars (Luckett 1985; Luckett *et al.* 1993; Järvinen *et al.* 2009; Ungar 2010).

In general, mammal dentitions have undergone numerous changes, including developmental arrest of teeth, and thus tooth rudiments are not uncommon. The occasional occurrences of upper canines in bovids and of lower second molars in felids have been interpreted as atavisms, resurrections of structures lost (Miles & Grigson 1990). In mice, rudimentary tooth buds are transiently present both in the incisor region and anterior to the molars (Sofaer 1969; Luckett 1985; Viriot *et al.* 2000; Peterková *et al.* 2002). These epithelial swellings weakly express some tooth markers, such as *Shh*, *Lef1* and *Bmp4* (Keränen *et al.* 1999; Klein *et al.* 2006; Prochazka *et al.* 2010) but are removed by apoptosis during bud stage (Turečkova *et al.* 1996).

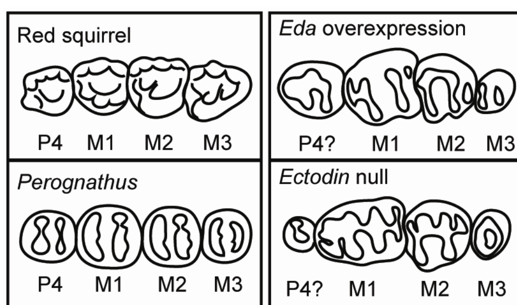


Figure 8. The left column shows the cheek teeth of two rodent species retaining the fourth premolar: the Red squirrel (adapted from Macdonald 2001) and a pocket mouse (adapted from Hillson 2005). The right column shows the cheek teeth of two mouse mutants producing an extra tooth, presumed to be the resurrected premolar. P4 = fourth premolar; M = molar. Occlusal view, anterior to the left. Not to scale.

Mouse	Mutation	Phenotype	References
Tabby (Eda-null in the text*)*	Lack of *Eda*.	Premolars uncommon in hetero-zygotes and extremely rare in full *Eda*-null. In all mutants reduced tooth size, number and cusp number; loss of third molars common; loss of incisors infrequent; reduced development of hair, nails and glands.	Grüneberg 1966; Sofaer 1969, Srivastava *et al.* 1997; Peterková *et al.* 2005; Article III.
K14-*Eda (Eda*-overexpression in the text*)*	Overexpression of *Eda* in developing ectoderm.	Premolars common; lack of enamel; slight increase of molar crown complexity; enhanced growth of hair, nails and glands.	Mustonen *et al.* 2003; Kangas *et al.* 2004.
Sprouty2-null	Lack of epithelial Fgf inhibitor *Sprouty2*.	Premolars common.	Klein *et al.* 2006.
Sprouty4-null	Lack of mesenchymal Fgf inhibitor *Sprouty4*.	Premolars infrequent.	Klein *et al.* 2006.
Lrp4 (a.k.a. *Megf7*) hypomorph	Reduction of Wnt inhibitor Lrp4.	Premolars and extra incisors common; molars have fusions, extra cusps and cusp fusions.	Ohazama *et al.* 2008.
Ectodin-null (a.k.a. *Sostdc1*-, *Wise*-, or *USAG1*-null)	Lack of Bmp inhibitor and Wnt context-dependent inhibitor/activator *ectodin*.	Premolars and extra incisors common; molars have fusions, extra cusps and cusp fusions; increased growth of mammary glands and extra whiskers.	Article I; Murashima-Suginami *et al.* 2007; Närhi *et al.* 2012.
$Ectodin^{wt/null}$; $Shh^{wt/GFPcre}$	Reduction of ectodin and Shh signalling.	Premolars common.	Ahn *et al.* 2010.
$Ctnnb1^{(ex3)fx}$; Shh^{CreERT}	Forced activation of Wnt signalling in *Shh*-expressing cells.	Premolars appear, frequency unknown.	Ahn *et al.* 2010.
$Tg737^{orpk}$ hypomorph	Reduction of polaris required in cilia, Shh signalling affected.	Premolars common; several defects in multiple organs, including extra digits and digit fusions.	Zhang *et al.* 2003: Ohazama *et al.* 2009.
Wnt1-Cre; $polaris^{flox/flox}$	Lack of polaris in dental mesenchyme, Shh signalling affected.	Premolars common; several defects in multiple organs; mice die at birth.	Ohazama *et al.* 2009.
Gas1-null	Lack of Shh inhibitor *Gas1* normally expressed in non-dental mesenchyme.	Premolars common; extra cusp lingual to the first molars.	Ohazama *et al.* 2009.

Table 1. Mouse mutants with a fully rescued premolar.

Mammals usually form two sets of teeth: First the deciduous ‘milk’ teeth, which are then replaced with the second, permanent teeth. Molars and rodent incisors are an exception to this rule, and are produced only once (Ungar 2010). In rodent species with premolars, as in mammals in general, the development of the most posterior deciduous premolar (dP4) precedes the development of the molars (Luckett 1985; Luckett 1993). The tooth rudiment anterior to the first molar in the mouse lower jaw develops slightly earlier than the molar and has therefore been identified as the remnant of dP4 (Viriot *et al.* 2002; Kangas *et al.* 2004). Some studies also report an earlier, more anterior tooth rudiment, interpreted to represent the second-to-last deciduous premolar (dP3) (Viriot *et al.* 2002; Prochazka *et al.* 2010).

The first mouse mutants to produce a mineralized extra tooth anterior to the first molar seems to have been heterozygous *Eda*-null mice (a.k.a. *Tabby*, Grüneberg 1966; Sofaer 1969). On the basis of histological sections, Sofaer identified this extra tooth as forming from a “normal anterior extension of the dental lamina” that was visible also in wild type, *i.e.* normal mice. Later it has been confirmed that the extra tooth in *Eda*-null, *Sprouty*-null, and *ectodin*-null mice develops from the rescued dP4 rudiment (Peterková *et al.* 2002; Kangas *et al.* 2004; Klein *et al.* 2006; Ahn *et al.* 2010). Probably the same holds true also for all other mouse mutants with a similar extra tooth (Table 1). It has not been investigated whether the rescued premolar remains true to its deciduous fate, and eventually falls out and is replaced by a permanent premolar, but it seems unlikely, since no such observations have yet been reported.

The premolar is often rescued in mice that overexpress *Eda*, but also sometimes in mice that lack *Eda* (Table 1). This unintuitive result might be explained by *Eda* mosaicism: In mice that lack *Eda*, full premolar rescue seems to happen mostly in heterozygous mice (Sofaer 1969; Peterková *et al.* 2005). The *Eda* gene is located in the X chromosome (Falconer 1953; Srivastava *et al.* 1997) so only females can carry two copies, and since one of them becomes randomly silenced in each cell, heterozygous females become mosaics. Presumably their developing dentition consists of patches of cells either expressing or not expressing *Eda*, rendering the rare appearance of an extra tooth plausible. Although an extra tooth has also been reported to appear extremely rarely in full *Eda*-null mice (Peterková *et al.* 2005; Article III), the criteria used were different from previous studies, and the possible contribution of additional mutations remains unconfirmed.

In addition to mosaic or increased *Eda* expression, the development of the dP4 can be rescued by increasing either Fgf or Wnt signalling (Table 1), indicating that the suppression of these signals, which promote proliferation and differentiation, has contributed to the suppression of dP4 development. The results on Shh signalling are less straightforward: Shh signalling is required

during tooth development first in the placode and later in the enamel knots (Hardcastle *et al.* 1998; Dassule *et al.* 2000). The dP4 rudimental bud expresses *Shh* in a weak, transient manner, and the upregulation and stabilization of this expression is associated with the rescue of dP4 development (Kangas *et al.* 2004: Klein *et al.* 2006). In concurrence with this, dP4 rescue has been shown to be due to increased Shh signalling in mice with reduction of polaris (Table 1). Polaris is a protein required in the formation of cellular extensions called primary cilia that play a varied role in Shh signal transduction (Haycraft *et al.* 2005; Badano *et al.* 2006; Davis *et al.* 2006; Rohatgi *et al.* 2007). Apparently conflicting with these results, dP4 development can also be rescued by diminishing Shh signalling via *Shh* heterozygosity, provided that Wnt signalling is also decreased. In addition, *Shh* heterozygosity makes dP4 rescue more frequent in mutants with decreased Bmp/Wnt inhibition (Ahn *et al.* 2010). All in all, mice have retained the last premolar rudiment, and the full development of this rudiment can be restored by a variety of mutations affecting the main signalling pathways used in tooth development (Table 1).

Importantly, the presence of the dP4 rudiment has implications for how histological data should be interpreted: At the earliest stages of mouse molar development, the cells at the tip of the tooth bud express many signalling molecules, including *Shh*. This expression has been assumed to represent the early signalling centre of the molar placode (Dassule & McMahon, 1998; Keränen *et al.* 1998). However, Prochazka *et al.* (2010) suggest that this early signalling centre represents instead the placode of the dP4. Histological sections have revealed that in many mammals, for example in squirrels, the molars do not develop from a placode of their own. Instead, the dental epithelium of the dP4 grows in the posterior direction and gives rise to the bud of the first molar, with the second and third molars sequentially budding off from the previous molar (Luckett 1985). Expression patterns during early dental development have not been studied in many mammals yet, apart from mice, but at least in the ferret only the first incisor, canine, and premolar develop from *Shh*-expressing placodes, while the following teeth, including the molars, bud off from the dental epithelium of the preceding teeth, and thus express *Shh* for the first time when their primary enamel knots become initiated (Järvinen *et al.* 2008). These results support the suggestion of Prochazka *et al.* and raise the interesting question whether the transient development of the dP4 in the mouse is in fact required for the initiation of the first molar.

In addition, it has been suggested that the dP4 rudiment becomes incorporated into the first molar and forms the most anterior cusp pair, the anteroconids (Viriot *et al.* 2000; Peterková *et al.* 2002; Prochazka *et al.* 2010). Though the regression of the premolar into a rudiment seems to temporally coincide with the appearance of the anteroconids in evolution (Viriot *et al.*

2002), there does not appear to be a causal relationship (Rodrigues *et al.* 2011; Elodie Renvoisé, personal communication). Furthermore, none of the mouse mutants with a rescued dP4 seem to have any reduction in the size of the anteroconids, indicating that though possible, the fusion of the dP4 rudiment is not required for normal first molar development.

Research on bioengineering teeth

Teeth require constant attention through life and demand professional care more frequently than any other part of the human body (Simmer & Snead 1995). Often the most convenient and aesthetically pleasing option is to replace a damaged or lost tooth with an artificial dental implant, which can involve several rounds of surgery and treatment. Admittedly, a tooth made of ceramics and steel will not develop cavities. Unfortunately, it also has its weak points: It has no nerves, so it is more likely to be overstrained and to fracture, and to damage the surrounding tissue due to its rigid attachment. Neither does the attachment adjust when the jaw grows during adolescence.

Although many vertebrates, such as sharks and crocodiles, produce new teeth throughout their lives, this ability has been suppressed in mammals. In mice, it can be reactivated by upregulation of Wnt signalling in the epithelium, which causes continuous production of small teeth (Järvinen *et al.* 2006; Wang *et al.* 2009). In humans, extra teeth develop in two dental disorders, one of which has been linked to upregulation of the Wnt pathway (Järvinen 2008). Therefore it might be possible to reactivate development of replacement teeth in humans as well.

Stem cells are studied intensely, and one application could be new therapies in dentistry (Ohazama *et al.* 2004, Bluteau *et al.* 2008, Yu *et al.* 2008). Generating a tooth from embryonic dental cells and implanting it into an adult jaw has been done in mice, proving it to be a possibility (Ikeda *et al.* 2009). If we learn enough of tooth development, it might one day be possible to use a patient's own cells to produce 'teeth to order'.

AIMS OF THE STUDY

In 1985, W. P. Luckett wrote: “Rodents (especially the mouse) are commonly studied as experimental models for early aspects of dental development, especially for investigations of pattern formation and epithelial-mesenchymal interactions [...]. These studies have provided valuable insights into tissue interactions during early phases of dental development, but they have not contributed as yet to our understanding of rodent evolution.” The aim of this study was to remove the negative from that statement by providing evolutionarily relevant information on the dynamics of cusp patterning.

By the time this study was initiated, the mouse molar had already shown great promise as a model organ for studies integrating evolution and development. For example, it had been established that the primary enamel knots initiate the teeth, while the secondary enamel knots initiate the cusps in a species-specific pattern (Jernvall *et al.* 2000), and that this patterning seems to follow reaction-diffusion dynamics, with Bmps being involved in activation, and Shh in inhibition (Salazar-Ciudad *et al.* 2002). Comparing the molars of wild type, *Eda*-null, and *Eda*-overexpressing mice had revealed that many dental characters have the potential for correlated changes, implying the existence of developmental constraints, and also that Eda and Shh signalling are important in inhibiting the development of the last premolar, which has persisted as a developmental rudiment for the last 45 million years (Kangas *et al.* 2004).

A recently discovered Bmp feedback inhibitor, *ectodin*, had been reported to be expressed in the developing molar excepting the primary enamel knot (Laurikkala *et al.* 2003). This expression pattern was unique and suggestive of a function in restricting enamel knot differentiation, which we set out to confirm. The second line of investigation was initiated by the creation of a $Shh^{wt/GFPcre}$ reporter mouse line (Harfe *et al*. 2004). Since *Shh* expression functions as a marker for enamel knot cells, the culture of $Shh^{wt/GFPcre}$ molars enabled real-time visualization of cusp pattern development. This was expected to facilitate tinkering on the cusp patterning, *i.e.* altering the activity of signalling pathways by adding either the ligand or an inhibitor onto cultured molars, hopefully thus altering the cusp pattern. We also wanted to continue exploring the effects of Eda signalling by adding different concentrations of EDA protein onto cultured *Eda*-null;$Shh^{wt/GFPcre}$ molars. In theory, this should produce a gradient of morphotypes ranging from the reduced molar morphology of the *Eda*-null mice to the ‘overdeveloped’ molar morphology of the *Eda*-overexpressing mice. Quantifying the development of these morphotypes was expected to provide insights into the role of Eda signalling, and into developmental constraints in molar development. In addition, we wanted to confirm

whether Fgf20 is upregulated by Eda signalling (Fliniaux *et al*. 2008), and to investigate the role of Fgf20 in molar development.

The methods available included growing embryonic molars in culture, studying gene expression patters with *in situ* hybridization, studying protein distributions with immunohistochemistry, and studying details on mineralized teeth by producing three-dimensional high-resolution images of the topography with regular or confocal laser scanning. During the study we also created *ectodin*-null and *Fgf20*-null mice, and began a collaboration with Keijo Hämäläinen's group in the Helsinki University Physics department in order to adapt three-dimensional X-ray scanning for molars.

The specific aims of this study were:

1. To investigate the role of ectodin in cusp patterning by studying *ectodin*-null mice.
2. To observe the details of secondary enamel knot induction by culturing the molars of *Shh*$^{wt/GFPcre}$ reporter mice, and then to alter the pattern of induction by manipulation with proteins and chemicals.
3. To quantify the effects of Eda signalling on molar development by culturing *Eda*-null;*Shh*$^{wt/GFPcre}$ molars in a gradient of EDA protein.
4. To adapt three-dimensional X-ray scanning for embryonic molars in order to image the enamel-dentin junction, and thus the future topography, of unmineralized molars.
5. To confirm Fgf20 as a target of Eda signalling and to investigate its role in molar development.

MATERIALS AND METHODS

Table 2. The mouse strains used. Crosses are not listed here, but are written in the text with a semicolon, for example *Eda*-null;*Shh*$^{wt/GFPcre}$.

Mouse strain	Used in
NMRI (wild type mouse strain)	I, II, III, IV, unpub.res.
C57BL/6 (wild type mouse strain)	III, IV
ectodin-null (in C57BL/6)	I, unpub.res.
Shh$^{wt/GFPcre}$ reporter (in NMRI)	I, II, III, unpub.res.
Eda-null (in C57BL/6 x CBA)	II, III, IV, unpub.res.
Eda-overexpression (in C57BL/6)	III, IV, unpub.res.
Eda-overexpression (in FVB)	II
Edar-overexpression (in FVB)	II
NF-κB β-galactosidase reporter (in C57BL/6)	II, III, unpub.res.
Fgf20-null (in C57BL/6 x 129Sv/J)	IV, unpub.res.

Table 3. The RNA probes used to visualize gene expression with *in situ* hybridization.

Probe	Reference	Used in
ectodin	Laurikkala *et al*. 2003	I, unpub.res.
Edar (a.k.a. *downless*)	Laurikkala *et al*. 2001	I, III, unpub.res.
Fgf3	Kettunen *et al.* 2000	IV
Fgf4	Jernvall *et al.* 1994	I
Fgf20	Ohmachi *et al.* 2000	IV
Lef-1	Travis *et al*. 1991	unpub.res.
p21	Jernvall *et al.* 1998	I
Runx2 (a.k.a. *Cbfa*)	D'Souza *et al.* 1999	IV
Shh	Bitgood & McMahon 1995	I,unpub.res.
Sprouty2	Zhang *et al.* 2001	IV
Sprouty4	Zhang *et al.* 2001	IV
Wnt10b	Sarkar & Sharpe 1999	IV

Table 4. The molecules used in molar cultures, either added to the culture media or attached to beads placed on cultures.

Molecules	Source	Used in
ACTIVIN-A	Dr. Marko Hyvönen	II, unpub.res.
BMP4	R&D Systems	I, unpub.res.
cyclopamine	Sigma-Aldrich	II, unpub.res.
EDA (Fc-EDA-A1)	Dr. Pascal Schneider	II, IV
ectodin	Prof. Nobuyuki Itoh	I
FGF4	R&D Systems	IV
FGF9	R&D Systems	IV
FGF20	R&D Systems	IV
SHH	R&D Systems	II, unpub.res.

Table 5. The methods used.

Method	Used in
Generation of mutant mouse strain.	I, IV
Culture of embryonic teeth.	I, II, III, IV, unpub.res.
Analysis of histology by hematoxylin-eosin dyed sections.	I, III, IV, unpub.res.
Analysis of gene expression pattern by *in situ* hybridization.	I, III, IV, unpub.res.
Analysis of cell proliferation by incorporation of either BrdU or EdU, and detection by immunohistochemistry.	IV, unpub.res.
Analysis of Eda signalling activity by immunohistochemistry using NF-κB β-galactosidase reporter mice.	II, unpub.res.
Confirmation of gene upregulation by luciferace assay.	IV
3D reconstruction of gene expression pattern.	I
3D reconstruction of molar crown topography from confocal laser scans.	I
3D reconstruction of molar crown topography from x-ray scans.	II
3D reconstruction of molar crown topography from laser scans.	IV
Quantifying developmental characteristics of cultured molars.	II, III, IV, unpub.res.
Quantifying phenotypic characteristics of mineralized, *in vivo* grown molars.	I, II, III, IV, unpub.res.
Quantifying complexity of molar crown topography with Orientation Patch Count.	II
Quantifying gene upregulation by RT-PCR.	IV

RESULTS AND DISCUSSION

Enamel knot patterning is controlled by feedback inhibition of differentiation (I and unpublished results)

Terminus. He who walks the boundaries. […]
Terminus is the only god to whom Jupiter must bow.
– Neil Gaiman

When discovered in mouse molars, ectodin was shown to act as a secreted feedback inhibitor of the Bmp pathway (Laurikkala et al. 2003). (This gene is now called *Sostdc1*, but is referred to as *ectodin* in this thesis since that name was used in Article I. Other synonyms are *USAG1* and *Wise*.) With *in situ* hybridisation on serial sections, made into three-dimensional images, we confirmed that *ectodin* is widely expressed in the developing molars but excluded from the primary enamel knot (Laurikkala *et al.* 2003), and found the expression to similarly skirt the secondary enamel knots (I and unpublished results). This unique expression pattern suggested a function in regulating cusp patterning.

Analysis of *ectodin*-null mutant mouse dentitions revealed extra incisors (studied further in Murashima-Suginami *et al.* 2007; Munne *et al.* 2009), rescued premolars, molar fusions and a highly altered cusp pattern including extra cusps and cusps fusing into crest-like formations (Fig. 9 A). Many of these features,

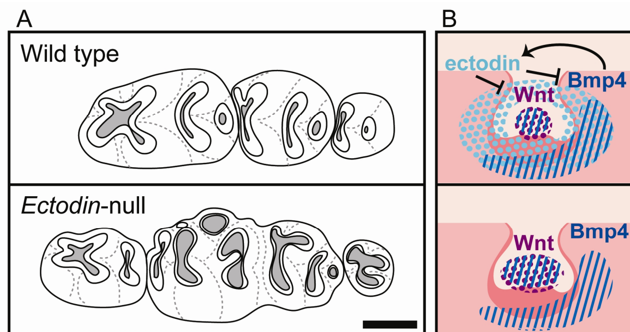


Figure 9. (A) An example of the ectodin-null cheek tooth phenotype, including a rescued premolar, fusion of the first and second molars, and an aberrant cusp pattern. Illustrations based on occlusal photographs taken by Pauliina Munne. Anterior to the left, buccal upwards. Scale bar 0.5 mm. (B) A general illustration of ectodin (blue dots), Wnt (purple dots), and Bmp4 (blue lines) expression patterns and interactions in the developing molar. Based on frontal histological sections of 14 days old mouse molars. Ectodin-null molars have enlarged enamel knot areas, presumably due to less restricted Wnt and Bmp function.

though not present in the modern mouse, appear in rodent ancestry: The common ancestor of rabbits and rodents had two incisors and at least one premolar in each jaw quadrant (Ungar 2010), and crests of variable size and orientation are common in rodents in general (Butler 1985). The ability to produce these structures has apparently been retained, perhaps as an integral feature in the development of the current dentition.

The presence of extra teeth and cusps, and tooth and cusp fusions indicated that ectodin might have a role in restricting the differentiation of both primary and secondary enamel knot cells. Indeed, *ectodin*-null molars showed expanded expression of the enamel knot markers *Shh* (unpublished result), *p21*, *Edar,* and *Fgf4*, both in primary and in secondary enamel knots. Bmp4 has been implicated in the induction of enamel knot differentiation in cichlid fish (Streelman & Albertson 2006) and in mice (Jernvall *et al.* 1998). We confirmed that in the mouse molar epithelium, BMP4 protein was able to induce the expression of the enamel knot marker *p21*. We also confirmed that BMP4 induced the expression of *ectodin* (Laurikkala *et al.* 2003), and found that ectodin protein dose-dependently antagonized the upregulation of *p21* by BMP4. Thus Bmp4 and ectodin seemed to form a negative feedback loop, which should provide developmental robustness. Indeed, treating wild type molars with BMP4 in tissue culture had negligible effect, while treating *ectodin*-null molars markedly accelerated the differentiation of secondary enamel knots, and later on the differentiation of ameloblasts and odontoblasts. From these results we concluded that ectodin delineates enamel knot differentiation by functioning as a feedback inhibitor of Bmp4. Such self-regulation had been expected but not shown previously in tooth development.

Treating $Shh^{wt/GFPcre}$ molars with BMP4 in culture revealed that also *ectodin*-wild type molars do respond to increasing levels of Bmp4 signalling, although not as strongly as *ectodin*-null molars. In cultured $Shh^{wt/GFPcre}$ molars, BMP4 caused a dose-dependent acceleration of crown differentiation (Fig. 10), which was independent of the amount of mesenchyme, and of the level of Shh responsiveness (unpublished results). However, BMP4 did not cause extra cusps, or cusp or molar fusions, like those seen in *ectodin*-null molars. Neither did BMP4 augment the *ectodin*-null cusp aberrations when added onto cultured molars (unpublished result). Therefore Bmp4 signalling alone is not sufficient for the induction of enamel knot differentiation.

In frog embryos, ectodin has been shown to act as a Wnt activator and inhibitor, context dependently, through competitive binding to the Wnt receptor LRP6 (Itasaki *et al.* 2003). Canonical Wnt/β-catening signalling is active at least in the enamel knots and in the topmost layer of the underlying mesenchyme (Liu *et al.* 2008; Lohi *et al.* 2010). Many mouse mutants with reduced Wnt signalling fail to induce the primary enamel knot and thus arrest tooth development at this

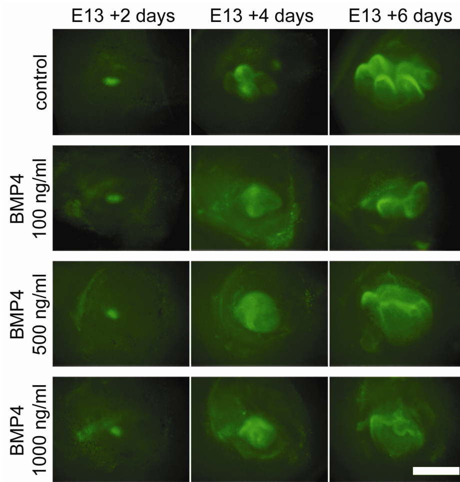


Figure 10. Adding BMP4 onto cultured molars accelerates the rates of secondary enamel knot induction and crown maturation, as seen from the activity of the Shh-GFP reporter. The effect is dose-dependent. With highest BMP4 concentrations the rate of crown maturation is accelerated more strongly than the rate of secondary enamel knot induction, leading to small teeth with reduced cusp patterns. BMP4 was kept in the culture media from 13 +1 day in culture to +3 days in culture. Anterior to the left, buccal upwards. Scale bar 0.5 mm.

point (Lin *et al.* 1999; Kratochwil *et al.* 2002; Andl *et al.* 2002; Liu *et al.* 2008). Conversely, forced activation of epithelial Wnt signalling leads to runaway induction of primary enamel knots and thus to continuous production of small, supernumerary teeth (Järvinen *et al.* 2006; Nakamura *et al.* 2008; Wang *et al.* 2009). Reduction in the amount of the Wnt inhibitor Lrp4 leads to a tooth phenotype very similar to that found in *ectodin*-null mice (Ohazama *et al.* 2008), while reduction in the amounts of the Wnt receptors Lrp5 and Lrp6 is sufficient to rescue the *ectodin*-null phenotype (Ahn *et al.* 2010). These results indicate that the main function of ectodin is to restrict Wnt target areas during enamel knot induction, while ectodin's ability to antagonize Bmp signalling might have more of a role in regulating molar size and maturation rate (Fig. 9 B). Interestingly, although reducing the amount of Lrp4 or completely removing ectodin leads to the same phenotype, these two molecules are expressed in a complementary pattern: *Lrp4* is a transmembrane protein whose expression is restricted to the enamel knots cells (Ohazama *et al.* 2008), while ectodin is expressed in nearly all other cells. However, ectodin is secreted out of the cells (Laurikkala *et al.* 2003), and can probably diffuse to the edges of the enamel knots. Thus the similarity of the Lrp4-reduced and *ectodin*-null phenotypes indicates that it is at the edges of the enamel knots where both Lrp4 and ectodin must antagonize Wnt signalling in order to ensure correct cusp patterning.

Unfortunately, the matter is far from straightforward: Decreasing either Wnt or Bmp signalling in the dental epithelium, which includes the enamel knots, leads to an early arrest of molar development (Andl *et al.* 2002; Plikus *et al.* 2005). If the main function of ectodin were to inhibit Wnt signalling, overexpression of *ectodin* in the dental epithelium could be expected to lead to

arrested tooth development. Instead, epithelial overexpression of *ectodin* only slightly reduces lower molar size (Ahn *et al.* 2010). In incisor development, ectodin has been suggested to antagonize primarily Wnts in the epithelium and Bmps in the mesenchyme (Munne *et al.* 2009). This might also be the case in molars: *Ectodin*-null mutants occasionally have tiny extra teeth on the lingual side of the molars, while the buccal side often produces extra cusps and cusp fusions (I and Ahn *et al.* 2010). During the development of wild type molars, *ectodin* expression is strongest in lingual epithelium and in buccal mesenchyme (Laurikkala *et al.* 2003 and unpublished results). This might indicate that ectodin antagonizes Wnt-mediated primary enamel knot induction in the epithelium, and Bmp-mediated secondary enamel knot induction in the mesenchyme. The matter requires more research in the future.

Also the reduction of Shh signalling can lead to molar fusions (Gritli-Linde *et al.* 2002), and to the rescue of the premolar rudiment (Ohazama *et al.* 2009). Both of these features, and the extra cusps of the *ectodin*-null phenotype, can be reproduced by treating embryos with a Shh-inhibiting antibody (Cho *et al.* 2011). In addition, reducing the expression of *Shh* in *ectodin*-null mice augments the phenotype (Ahn *et al.* 2010). Thus ectodin and Shh have similar effects in restricting enamel knot differentiation. It could thus be hypothesized that Shh upregulates *ectodin* expression, and this does seem to be the case in dental mesenchyme (Cho *et al.* 2011). However, *ectodin*-null molars remain sensitive to SHH and cyclopamine treatment (unpublished results), demonstrating that the effects of Shh are not mediated by ectodin, at least not completely. In molar fusions caused by reduced levels of ectodin or Lrp4, the fusion sites exhibit ectopic Wnt and Bmp signalling, and slightly downregulated Shh responsiveness (Ohazama *et al.* 2008). In molar fusions caused by a reduction in the level of Shh, the levels of Wnt signalling, Bmp signalling, and Shh responsiveness remain unaffected (Ohazama *et al.* 2008), which places Shh downstream of the Wnt/Bmp-ectodin loop.

Crown complexity can be increased in an additive manner (II and unpublished results)

I would not give a fig for the simplicity this side of complexity,
but I would give my right arm for the simplicity on the other side of complexity.
– Oliver Wendell Holmes

Increasing biological complexity has been a ruling trend in evolution (Carroll 2001). For example, molars have evolved from single-cusped, conical teeth to a startling variety of cusp patterns and forms (Fig. 3). This increase in crown complexity can be quantified, for example, by counting patches with dissimilar slope orientation (Evans *et al*. 2007), or simply by counting the number of cusps on the occlusal surface. Though simple to measure, the dynamics of increasing complexity have proven difficult to study experimentally: Of the 34 mouse mutants with tooth phenotypes, only five (including *ectodin*-null) show increase in the number of cusps, and even then by only one cusp on average (listed in II). In the only report on increasing cusp number in culture, a mouse molar epithelium was combined with a larger rat molar mesenchyme, resulting in three extra cusps (Cai *et al.* 2007). Whereas evolutionary increase in cusp number has

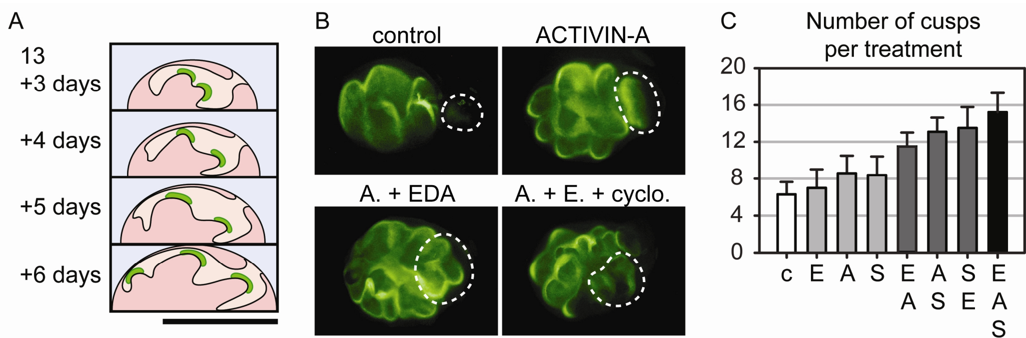


Figure 11. Crown complexity can be increased in an additive manner by tuning Activin, Eda, and Shh signalling activity. (A) An illustration of extra cusp development, based on frontal sections of ACTIVIN-A treated molars. Enamel knots (green) express *Shh, p21, Lef1*, and *Edar*, and are devoid of *ectodin*. Lingual to the left. Scale bar 0,5 mm. (B) Examples of cultured $Shh^{wt/GFPcre}$ molars treated with ACTIVIN-A protein, EDA protein, and the Shh-inhibiting chemical cyclopamine (cyclo.). Dashed line indicates second molar. Anterior to the left, lingual downwards. Scale bar 0,5 mm. (C) The number of cusps per treatment, including both the first and second molars. c = control; E = Eda signalling (increased), A = Activin signalling (increased); S = Shh signalling (decreased). Columns in greyscale gradient according to the number of signalling pathways manipulated. On average, cusp number increases by three per number of pathways. Error bars indicate standard deviation.

sometimes occurred hand in hand with increase in size, for example in the lengthening of the vole first molar, mostly changes have been in cusp density (Ungar 2010).

Experimenting on cultured molars with a variety of signalling molecules required for normal tooth development revealed that increase of Activin (a.k.a. *Inhibin*) signalling (by adding ACTIVIN-A protein) or decrease of Shh signalling (by adding cyclopamine, a plant steroid alkaloid) induced development of extra cusps (Fig. 11). The effect was dose-dependent, but high doses also deterred development, a common occurrence in tissue manipulation. Maximal increase in cusp number with minimal decrease in growth yielded 2,3 extra cusps on average.

In addition to extra cusps, decreasing Shh signalling also caused the first and second molars to fuse as soon as the second molar became induced (Fig. 11 B). Increasing Shh signalling had the opposite effect, causing decrease in the number of cusps and a delay in the initiation of the second molar. In feather patterning, Shh and Bmp2 form a feedback loop and promote proliferation and differentiation, respectively (Harris *et al.* 2002). Shh has long been suspected to tip the balance from differentiation to proliferation also in teeth (Salazar-Ciudad & Jernvall 2002): First, the lack of Shh signalling causes diffuse enamel knots, leading to small, fused molars (Dassule *et al.* 2000, Gritli-Linde *et al.* 2002). Second, Shh is the last enamel knot cell –specific gene to become expressed (Keränen 2000). Third, only the cells surrounding the enamel knot are receptive to Shh (Hardcastle *et al.* 1998; Motoyama *et al.* 1998; Carpenter *et al.* 1998). Thus its effect of promoting cell proliferation and survival (Varjosalo & Taipale 2008) is probably directed at the non-knot cells, apparently also preventing the differentiation of new knots too close to the previous ones. Confirming this conclusion also *in vivo*, injection of Shh antagonist into pregnant mice produced extra cusps and fused molars in the pups (Cho *et al.* 2011).

Shh heterozygosity has been shown to decrease the amount of Shh protein by 30% with no effect to the phenotype (Benazet *et al.* 2009). To ascertain that *Shh* heterozygosity did not affect our results, we compared wild type and $Shh^{wt/GFPcre}$ molars *in vivo* and in culture. *In vivo*, Shh heterozygosity caused only a few significant differences, which were acceptably small (max 7%, unpublished results), and in culture, Shh heterozygous and wild type molars responded identically to manipulation of Shh signalling activity (II and unpublished results).

To confirm that the extra cusps seen in our two-dimensional photographs represented true topographical features, we reconstructed the three-dimensional epithelium-mesenchyme interface using X-ray tomography. This method had been recently developed for whole embryos by Metscher (2009) and we adapted the procedure for cultured molars. Once established, the method proved quicker

and more accurate than laser confocal scanning or reconstruction from histological sections would have been. The three-dimensional resolution was 1 μm, whereas the typical cell diameter is 10 μm.

Crests develop from folding of the epithelium (Butler 1985) and can be mistaken for cusps (Tucker *et al.* 2004). *In situ* hybridization on histological sections of ACTIVIN-A treated molars confirmed the existence of extra enamel knots above the extra cusps (illustrated in Figure 11 A, unpublished result). We were unable to find changes in gene expression prior to the physical appearance of the extra cusps (expressions of 17 genes checked, unpublished results, not listed in Methods). The earliest non-morphological change detected was an increase in proliferation: Directly beneath the lingual cervical loop, mesenchymal proliferation was increased by 25%, as quantified from serial sections with BrdU staining (unpublished result). Since this ectopic proliferation seemed to increase the density of the mesenchyme, it can be hypothesized to have directed the growth of the lingual cervical loop from downwards to sidewards. Assuming reaction-diffusion type dynamics, extra enamel knots would become induced automatically once the cervical loop grew beyond the inhibition field generated by the previous enamel knots. This model has been shown to apply to the primary enamel knots of molars (Kavanagh *et al.* 2007), and is in agreement with the fusion of the first and second molars observed when Shh signalling was decreased, provided that the primary and secondary enamel knots function similarly.

Two of the mouse mutants with a modest increase in crown complexity are the transgenic strains overexpressing *Eda* (Mustonen *et al.* 2003; Kangas *et al.* 2004) or its receptor *Edar* (Pispa *et al.* 2004). We were unable to further increase the number of cusps either by crossing these two mouse lines, or by adding EDA protein onto cultured molars (II). Together with our results on Activin and Shh, this indicated that the potentials of an individual pathway are quickly exhausted, and led us to try tinkering with multiple pathways simultaneously. Surprisingly, tuning Activin, Shh, and Eda signalling in pairs exceeded the effects of similar treatments performed individually (Fig. 11 B,C). This makes intuitive sense; if you want to bake a bigger cake, adding more of just one ingredient will just result in a big pancake. As a matter of fact, tuning all three pathways together proved the most effective: Including both the first and second molar, cusp number increased from the normal 6,3 up to 15,2 on average (Figure 11 C). Neither the size nor the developmental schedule of the molars changed correspondingly, confirming that the principal effect was on the patterning process.

It remains to be tested whether the same pattern of results can be repeated using other pathways and other organs. If so, the apparent conflict between evolutionary trends and experiments would be solved: Due to practical

considerations, developmental biologists prefer to manipulate only one pathway at a time, and to concentrate their efforts on the pathways with the most dramatic phenotypic effects. A requirement for multiple simultaneous small-scale changes would not be met. Such a requirement would also generate an inherent bias against increase in complexity. This yields two testable predictions: that in extant populations individual variation should be biased onto the side of decreased complexity; and that during evolution an increase of complexity should have taken a longer time to occur than an equivalent decrease. At least body size can be decreased 10 times faster than it can be increased (Evans et al. 2012), a change which can be assumed to involve the complexity of many organs. In evolutionary literature in general, increase in complexity is acknowledged to require many changes in development, while decrease is assumed to be attainable with a single, disruptive change (McShea 2005). Also, in a recent computational model (Salazar-Ciudad & Jernvall 2010), changing individual parameters is twice more likely to decrease than to increase cusp number. An alternative explanation is that a yet to be discovered ‘master complexity gene’ is affected additively by Eda, Activin and Shh signalling.

We were unable to find a common molecular target for the three pathways manipulated, though they are certainly linked in development. Activin upregulates the expression of Edar in the primary enamel knot (Laurikkala *et al.* 2001; Laurikkala *et al.* 2002) and thus promotes the activation of the known Eda target NF-κB (unpublished results). However, the *Eda-Edar* double overexpression mice did not reproduce the phenotype achieved with ACTIVIN-A protein in culture, verifying that the Activin and Eda signalling pathways have also separate effects. Eda signalling has been shown to upregulate the expression of *Shh* in hair placodes (Schmidt-Ullrich *et al.* 2006; Pummila *et al.* 2007), but inhibiting Shh signalling did not affect NF-κB, or add to the upregulation of NF-κB by EDA and ACTIVIN-A (unpublished results). Rather than have common targets, Eda and Shh seemed to have opposite effects, in that either increase of both Eda and Activin, or decrease of Shh signalling caused molar fusion.

The lingual side of the tooth proved the most prone to produce extra cusps. A lingual bias has also been found in the rare extra cusps appearing in dogs and gorillas, and extra teeth, when not situated in the tooth row, seem to favour the lingual side (Miles & Grigson 1990). This is probably ultimately due to the lingual side giving rise to successional teeth (Leche 1895 as cited in Järvinen *et al*. 2009; Handrigan *et al*. 2010). Histologically, the forking of the lingual cervical loop, caused by our treatments, looked similar to the budding of successional teeth in ferrets and snakes (Järvinen *et al.* 2009; Buchtova *et al.* 2008). It has been shown in snakes and lizards that this budding does not require Shh signalling (Buchtova *et al*. 2008; Handrigan *et al*. 2010). Our results suggest that perhaps it is indeed the absence of Shh signalling which is required.

The growth pattern constrains the cusp pattern (III and IV)

Nothing in biology makes sense except in the light of evolution.
– Theodosius Dobzhansky, 1964
Nothing about variation makes sense except in the light of development.
– Jukka Jernvall, 2006

Developmental experiments usually involve large-scale changes, whereas subtle tinkering is assumed to account for the bulk of evolutionary change. The Eda signalling pathway offers a chance for research on small-scale changes and the variation involved: Decreasing or increasing Eda signalling is not lethal, and tooth form is altered in *Eda*-null (Grüneberg 1966) and in *Eda*-overexpression mice (Mustonen *et al.* 2003). Cultured wild type molars respond weakly to added EDA protein, while administering it to *Eda*-null molars produces a gradient of phenotypes from null to full rescue (II). And most importantly, Eda has an instructive role in the development of many epithelial appendages and has been implicated as a key factor in their evolution (Pantalacci *et al.* 2008; Barrett *et al.* 2008; Mou *et al.* 2008; Mikkola 2008; Harris *et al.* 2008).

Eda and its receptor Edar are initially uniformly expressed in the dental epithelium, but once molar development begins, Eda expression becomes restricted to the outer layer of the epithelium, closest to the oral cavity, while Edar becomes restricted to the enamel knots (Laurikkala *et al.* 2001). This expression pattern enables the treatment of dissected molars with EDA protein in culture without perturbing the gradient perceived by the receptor. Although the *Eda*-null mutant strain has been studied for a long time, in the absence of a real-time enamel knot marker, the identity of the cusps and the occurrence of possible fusions has remained unclear (Sofaer 1969; Lisi *et al.* 2001). By quantifying the development of cultured *Eda*-null;$Shh^{wt/GFPcre}$ molars treated with different concentrations of EDA, we found that the order in which the cusps were initiated was unaltered in *Eda*-null mice, and mostly corresponded to the evolutionary order of cusp appearance (Fig. 12 A) (Osborn 1907; Butler 1990; Rodrigues *et al.* 2010). Thus the cusps retained in *Eda*-null molars are the evolutionarily oldest ones, of which the cusp pairs proto- and metaconid, and hypo- and entoconid, can sometimes fuse (III).

Eda has been shown to inhibit Bmp signalling during incisor and skin development (Wang *et al.* 2004; Plikus *et al.* 2005; Mou *et al.* 2006; Pummila *et al.* 2007). Since Bmps are involved in the induction of crown maturation (I, Nie *et al.* 2006), it could be assumed that the reduced cusp pattern and molar size of *Eda*-null mice might result from premature onset of mineralization. However, our molar cultures confirmed that although tooth development is delayed by approximately 24 hours in *Eda*-null (Kangas *et al.* 2004), so is crown maturation

(Sofaer 1969; Lisi *et al.* 2001). Thus although the lack of Eda signalling did delay development, the reduced molar phenotype of the *Eda*-null mice seemed to be caused mostly by a reduction in the growth rate of the molar, and in the elongation of the molar (III). In the cultures of *Eda*-null;*Shh*$^{wt/GFPcre}$ molars, increasing the concentration of EDA restored the posterior elongation of the primary enamel knot. This elongation correlated with the rescue of the posterior elongation of the crown, *i.e.* the talonid (Fig. 12 A). The talonid is considered a key innovation in mammal evolution (Osborn 1907), and like the comparable hypocone in upper molars, has possibly been invented several times independently (Hunter & Jernvall 1995; Luo *et al.* 2001; Luo *et al.* 2007). Our results suggest that the posterior elongation of the primary enamel knot, though a transient phase, has nevertheless been important in talonid evolution.

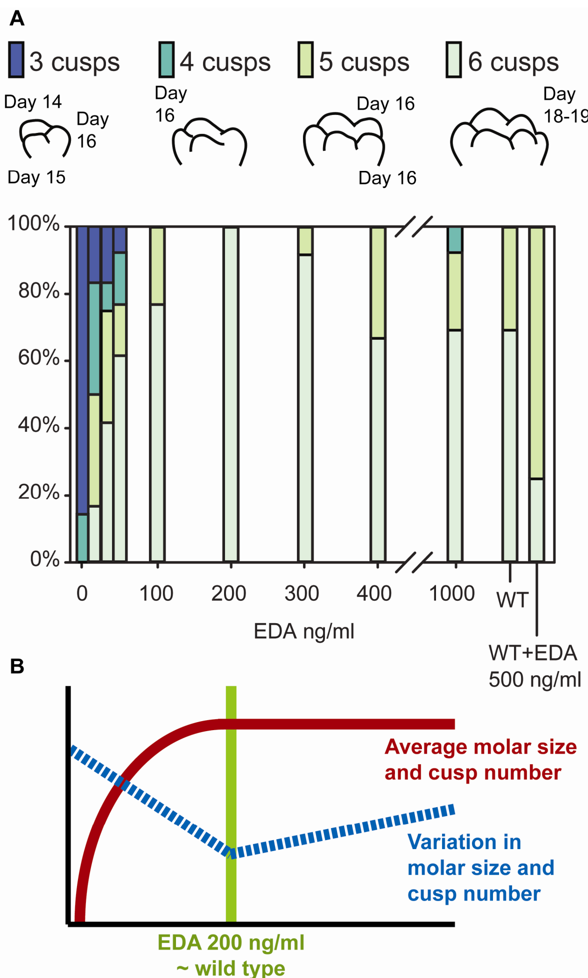


Figure 12. (A) *Eda*-null molars are reduced in size and cusp number, a phenotype which can be dose-dependently rescued in culture with EDA protein. Molars were taken into culture on day 13 of embryonic development and treated with EDA protein for the first four days. Time of cusp initiation (as seen from the appearance of the corresponding secondary enamel knot) is indicated next to each cusp in the illustrations of crown types. The order of cusp initiation resembled the order of cusp evolution. (B) The average molar size and cusp number (solid red line) responded to the increase in EDA concentration by rising exponentially until reaching a plateau after 100 ng/ml. The coefficients of variation of the same variables (dashed blue line) first decreased, then increased steadily. At EDA 200 ng/ml, variation was minimal and values resembling wild type were reached in most variables measured, indicating it to be the effective concentration *in vivo*.

The order the cusps became initiated in was not affected by the level of Eda signalling, and neither were the relative positions of the cusps. Therefore increasing the growth rates of the cultured *Eda*-null;*Shh*$^{wt/GFPcre}$ molars, by increasing the concentration of EDA, most strongly affected those features that were the last ones to develop. A comparison of the molars of adult *Eda*-null, wild type, and *Eda*-overexpressing mice revealed the same trend, implicating Eda signalling mostly in molar growth (III). Eda has previously been shown to inhibit Bmp and Wnt signalling (Mou *et al.* 2006; Pummila *et al.* 2007; Fliniaux *et al.* 2008), which could promote growth by inhibiting differentiation (I). Direct induction of proliferation in the molar is associated with Shh and Fgf signalling (Hardcastle *et al.* 1998; Dassule *et al.* 2000; Kettunen *et al.* 2000; Kratochwil *et al.* 2002). Eda has been shown to upregulate Shh in the skin (Schmidt-Ullrich *et al.* 2006; Pummila *et al.* 2007), and also Fgf20 has been implicated as a putative target gene (Fliniaux *et al.* 2008). We were able to confirm that Eda signalling upregulates *Fgf20*, which is co-expressed with Edar in the tooth placode and later in the enamel knots, and which is able to induce proliferation in the underlying mesenchyme (IV). Therefore Eda can affect growth dynamics through all of the major signalling pathways used in molar development, namely Bmp, Wnt, Shh, and Fgf signalling.

To distinguish the effects of Fgf20 from those of other Eda targets, we created *Fgf20*-null mice. Their molar size was reduced less than that of *Eda*-null mice, and instead of the talonid being most strongly affected, only the anteroconids were significantly smaller (IV). This indicates that Fgf20 contributes to the growth-promoting effect of Eda, particularly in the anteroconids. In *Eda*-null, the size reduction most severely affected the first molar, changing the relative sizes of the molars (III). Molars have been shown to impede each other's growth in a first-come, first-served style, apparently via lateral inhibition mediated by the enamel knots, countered by activation from the mesenchyme (Kavanagh *et al.* 2007). Comparing relative molar sizes of *Eda*-null, wild type, *Eda*-overexpression, and *Eda*-overexpression;*Fgf20*-null mice revealed that changing the level of Eda signalling from the wild type in either direction caused the activator-to-inhibitor ratio to increase (III). The simultaneous deletion of *Fgf20* compensated for this increase (IV). In addition, the stability of the ratio was lost in *Eda*-null mice (III). Thus Eda would seem to be needed not only for promoting molar growth in general, but also for maintaining the ratio between the activation and inhibition of enamel knot differentiation. One way to fulfil this function could be through regulation of Shh, which we have confirmed to act as a lateral inhibitor (II). Since Eda activates tooth development in general, is able to upregulate its own receptor (Mou *et al.* 2006), and is possibly able to induce inhibition via Shh, its function in cusp patterning could probably be modelled with reaction-diffusion dynamics (Fig. 6).

In concurrence with a lost activator-inhibitor balance, the lack of *Eda* increased variation between individuals, and furthermore, it increased variation within individuals to an equal level. The distinction could not be made in the cultured molars, but variation in general increased the further one deviated from EDA 200 ng/ml, which seemed to correspond to wild type levels of signalling (Fig. 12 B). Small deviations increased variation without altering the population mean phenotype. The same phenomenon, although more strongly, occurs in fruit flies when the action of Hsp90 protein is reduced, which can happen in stressful situations (Rutherford & Lindquist 1998). Hsp90 is thus assumed to mediate evolvability (Bergman & Siegal 2003), but it remains to be confirmed whether the same conclusion can be drawn for Eda. For example, it would be interesting to test whether different molar phenotypes could be selected for in the *Eda*-null and *Eda*-overexpression mouse lines.

An ancient premolar rudiment affects the modern mouse dentition (I, III, IV and unpublished results)

Omnia mutantur, nihil interit.
[Everything changes, nothing perishes.]
– Ovid, AD 8

In mice, first molar development is preceded in time and in location by the transient development of a tooth placode, presumed to be the developmental rudiment of the last premolar (Luckett 1985; Viriot *et al.* 2000; Peterková *et al.* 2002). The premolar rudiment is either removed by apoptosis (Turečkova *et al.* 1996), or it contributes to the anteroconids of the first molar (Prochazka *et al.* 2010), or both.

The development of the premolar rudiment was rescued with a high frequency in the *ectodin*-null mice (I), *Eda*-overexpression mice (Kangas *et al.* 2004) and *Eda*-overexpression;*Fgf20*-null compound mutants (IV). The earliest sign of rescue was the continued and enhanced *Shh* expression in the premolar location at embryonic day 13 (I, Kangas *et al.* 2004). Shh seems to act downstream of Bmp and Wnt, the signalling pathways ectopically activated in *ectodin*-null mice (Ahn *et al.* 2010), and also downstream of Eda signalling (Pummila *et al.* 2007). Also increased Fgf signalling can rescue the rudiment's Shh expression, and its whole development (Klein *et al.* 2006), implicating also Fgfs as regulators of Shh. Since the development of the premolar can also be rescued by the increase of Shh signalling (Ohazama *et al.* 2009), it seems likely that decreasing the expression of *Shh* has been the key factor in repressing premolar development 45 million years ago. Interestingly, the reduction of Shh signalling by *Shh* heterozygosity promotes premolar rescue in *ectodin*-null mice

(Ahn *et al.* 2010), *Eda*-overexpressing mice (III), and *Fgf20*-null mice (unpublished result). In all three mouse strains, the activation of enamel knot differentiation seems to have increased, at least in the premolar area (I; II; III), and since Shh seems to act as a lateral inhibitor of enamel knot differentiation (II), the simultaneous slight reduction of Shh signalling might further increase activation and result in the rescue of the premolar rudiment.

When a structure becomes lost during evolution, even the developmental rudiment is expected to gradually disappear due to relaxation of selection pressure and subsequent accumulation of random mutations that eventually incapacitate the developmental network. The premolar rudiment might have persisted simply because most components and interactions used in its development are probably required for the development of the incisors and molars. It has also been suggested that the premolar rudiment contributes to the anteroconids of the first molar (Prochazka *et al.* 2010). However, at least in *Eda*-overexpression mice, full rescue of the premolar reduced the size of all molars quite evenly by approximately 10% (III). The anteroconids were reduced in relative size, while the proportion occupied by the next cusp pair, the proto- and metaconid, respectively increased. Thus premolar rescue did not seem to remove material from the anteroconids selectively, but to reduce the size of all molars equally, and on the first molar to reallocate material from the anterior to the more posterior cusps.

Fgf20-null is the only mouse mutant strain in which selective reduction of anteroconids is visible (IV). *In situ* hybridization on serial sections of 14 days old *Fgf20*-null molar areas revealed the dental epithelium anterior to the first molar to be elongated and to ectopically express *Edar* and *Shh* (unpublished results). It would seem likely that this elongation corresponded to a partially rescued premolar, since the lack of *Fgf20* increased the frequency of premolar rescue in *Eda*-overexpressing mice. However, the issue remains unconfirmed, since the *in situ* results indicated that the elongation might have been situated between the premolar rudiment and the first molar.

Premolar contribution to the anteroconids is not visible during culture of wild type molars, or of mutant molars with transient premolar development, such as *Fgf20*-null or heterozygous *Follistatin*-null mutants (personal observation). When the premolar appears in culture, either transiently or permanently, the only visible effect is the temporal retardation of molar development by approximately 24 hours (Kangas *et al.* 2004 and personal observations). Thus the inhibitory cascade that regulates relative molar size (Kavanagh *et al.* 2007) seems to extend to include the premolar as well. If molars bud off from each other (Luckett 1985; Järvinen *et al.* 2008), delay in development of the first one could affect them all uniformly. Final size is regulated differently in different organs, for example in pancreas a predetermined number of progenitor cells go through a predetermined

number of proliferation rounds, and adjustment is not possible even in the case of disruptions (Stanger *et al.* 2007). Tooth size in humans correlates with the size of the tooth-supporting structures, but not with head or body size, although in mammals in general such a correlation is strong (Hillson 2005). This suggests that the development of the molars is tied to the development of the surrounding tissues. If this includes the temporal schedule, the delay in molar development, caused by premolar rescue, might shorten the growth period available, causing the size reduction. No doubt the issue will be clarified in the future.

CONCLUDING REMARKS

In the book of life, the answers are not in the back.
– Charles M. Schulz

We have established the culture of *Shh*$^{wt/GFPcre}$ reporter mouse molars as a useful model system for studying developmental patterning in an evolutionary context. For example, decreasing Eda signalling activity produced a gradient of morphotypes mapping a possible evolutionary path from the ancestral rodent molar to the reduced cusp patterns in the modern water rats, while increasing Activin signalling resulted in a third row of cusps, reminiscent of the third cusp row in modern Eurasian field mice. While such results do not necessarily pinpoint evolutionary changes in gene regulation, they nevertheless provide valuable information on the dynamics and possibilities of the current regulatory network.

All in all, our findings support the use of reaction-diffusion dynamics in describing molar cusp patterning, and show Bmp, Eda, and Activin signalling pathways to be involved in the activation of enamel knot differentiation, while ectodin and Shh are involved in the inhibition. Additionally, although the primary enamel knot appears at tooth initiation, while the secondary enamel knots appear later and give rise not to individual teeth but to cusps, both primary and secondary enamel knots express the same genes and responded similarly to all our developmental alterations, suggesting that they might be functionally analogous.

Reaction-diffusion dynamics provide self-organizing patterning via regulatory feedback loops (Fig. 6). Bmp and ectodin appear to form one such loop, and Eda and Shh might form another. Feedback loops should provide developmental stability, and we have shown how breaking a loop by removing *ectodin* or *Eda* increases variation in molar shape in general and in cusp patterning in particular (Figs 9 and 12).

It has been suggested that teeth have high evolvability as a result of modular development employing reiterative signalling events in a non-linear, dynamic manner (Roth 2005). We have found that the Eda signalling pathway might contribute to this evolvability, since deviations from the wild type level of signalling activity increase variation without initially affecting the population mean phenotype, though it remains to be tested whether the phenomenon is common to all signalling pathways.

Our results also underline that the reaction-diffusion model is not the only mechanism at work in cusp patterning; otherwise it would be as futile to trace the evolutionary origins of specific cusps as it would be to trace the ancestral stripe of the zebra. None of the manipulations we performed on

cultured molars were able to alter the order of development or the relative positions of the evolutionarily oldest cusps, the protoconid and the metaconid, in addition to which at least either the anteroconid or the talonid cusps could always be distinguished despite varying size and number. This seemed to result from the growth dynamics of the molar, especially its lengthwise elongation, and from the buccal anchorage of the primary enamel knot. As a consequence, the evolutionary history of the mouse molar is present in its development to such a degree that it can be assumed to shape the variation available to natural selection. A direct 'prehistoric' influence is provided by the premolar rudiment, the development of which can be rescued relatively easily, resulting in retardation of molar development and reduction in molar size.

We have been able to demonstrate many ways in which changes in gene regulation might have produced morphological changes during evolution. In addition, increase and decrease of complexity seem to not be equal in the number of changes required. Decrease in dental complexity is known to be achievable with a single change, while we have shown that increase in complexity, the dominant trend in evolution, requires multiple simultaneous changes. It remains to be confirmed whether our findings describe a common phenomenon, or an exception specific to tooth cusp patterning.

Our results point to many future lines of enquiry, one being how have cusp patterning mechanisms been adapted to teeth of different sizes: Although diffusion distances are long enough to be applicable to cusp patterning in the mouse, for example lion molars have cusps 15 mm apart. At the other end of the scale, the pygmy shrew has molars small enough to beg the question, can a single cell function as an enamel knot. One obvious tool to use would be X-ray scanning, which we have adapted to imaging unmineralized teeth at a cell-level resolution. The method can also be applied to studying mouse molar development in more detail: For example, increased mesenchymal proliferation beneath each enamel knot can be assumed to contribute to the growth of the cusps. Such biomechanical stress should be visible as increased cell density. Another area that could be studied with X-ray scanning is biomineralization. The details of enamel and dentin formation remain a black box. Also, we do not know how the change in density affects the ameloblasts and odontoblasts as they move outwards and inwards from the epithelium-mesenchyme interface. Imaging the dentin tubules each odontoblast cell creates should reveal whether the cells moving inwards into more confined space change their shapes, reduce their sizes, or reduce their numbers.

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If you want to go fast, travel alone.
If you want to go far, travel in company.
– An African saying

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REFERENCES

Åberg, T., Wozney, J. and Thesleff, I. (1997). Expression Patterns of Bone Morphogenetic Proteins (Bmps) in the Developing Mouse Tooth Suggest Roles in Morphogenesis and Cell Differentiation. Dev. Dyn. 210, 383-396.

Ahn, Y., Sanderson, B. W., Klein, O. D. and Krumlauf, R. (2010). Inhibition of Wnt Signaling by Wise (Sostdc1) and Negative Feedback from Shh Controls Tooth Number and Patterning. Development 137, 3221-3231.

Andl, T., Ahn, K., Kairo, A., Chu, E. Y., Wine-Lee, L., Reddy, S. T., Croft, N. J., Cebra-Thomas, J. A., Metzger, D., Chambon, P. et al. (2004). Epithelial Bmpr1a Regulates Differentiation and Proliferation in Postnatal Hair Follicles and is Essential for Tooth Development. Development 131, 2257-2268.

Andl, T., Reddy, S. T., Gaddapara, T. and Millar, S. E. (2002). WNT Signals are Required for the Initiation of Hair Follicle Development. Dev. Cell. 2, 643-653.

Arias, A. M. and Stewart, A. (2002). Molecular Principles of Animal Development, pp. 410. New York, U.S.A.: Oxford University Press.

Ashe, H. L. and Briscoe, J. (2006). The Interpretation of Morphogen Gradients. Development 133, 385-394.

Badano, J. L., Mitsuma, N., Beales, P. L. and Katsanis, N. (2006). The Ciliopathies: An Emerging Class of Human Genetic Disorders. Annu. Rev. Genomics Hum. Genet. 7, 125-148.

Barrett, R. D., Rogers, S. M. and Schluter, D. (2008). Natural Selection on a Major Armor Gene in Threespine Stickleback. Science 322, 255-257.

Benazet, J. D., Bischofberger, M., Tiecke, E., Goncalves, A., Martin, J. F., Zuniga, A., Naef, F. and Zeller, R. (2009). A Self-Regulatory System of Interlinked Signaling Feedback Loops Controls Mouse Limb Patterning. Science 323, 1050-1053.

Bergman, A. and Siegal, M. L. (2003). Evolutionary Capacitance as a General Feature of Complex Gene Networks. Nature 424, 549-552.

Berry, A. and Browne, J. (2008). The Other Beetle-Hunter. Nature 453, 1188-1190.

Bitgood, M. J., McMahon, A. P. (1995). Hedgehog and Bmp genes are coexpressed at many diverse sites of cell-cell interaction in the mouse embryo. Dev. Biol. 172, 126-138.

Bluteau, G., Luder, H. U., De Bari, C. and Mitsiadis, T. A. (2008). Stem Cells for Tooth Engineering. Eur. Cell. Mater. 16, 1-9.

Bonhomme, F., Catalan, J., Britton-Davidian, J., Chapman, V. M., Moriwaki, K., Nevo, E. and Thaler, L. (1984). Biochemical Diversity and Evolution in the Genus Mus. Biochem. Genet. 22, 275-303.

Buchtova, M., Handrigan, G. R., Tucker, A. S., Lozanoff, S., Town, L., Fu, K., Diewert, V. M., Wicking, C. and Richman, J. M. (2008). Initiation and Patterning of the Snake Dentition are Dependent on Sonic Hedgehog Signaling. Dev. Biol. 319, 132-145.

Buckley, G. A., Brochu, C. A., Krause, D. W. and Pol, D. (2000). A Pug-Nosed Crocodyliform from the Late Cretaceous of Madagascar. Nature 405, 941-944.

Butler, P. M. (1956). The Ontogeny of Molar Pattern. Biol Rev 31, 30-70.

Butler, P. M. (1985). Homologies of Molar Cusps and Crests, and their Bearing on Assessments of Rodent Phylogeny. In Evolutionary Relationships among Rodents: A Multidisciplinary Analysis (ed. W. P. Luckett and J.-L. Hartenberger), pp. 381-401. New York, U.S.A.: Plenum Press.

Butler, P. M. (1990). Early Trends in the Evolution of Tribosphenic Molars. Biol. Rev. 65, 529-552.

Butler, P. M. (1995). Ontogenetic Aspects of Dental Evolution. Int. J. Dev. Biol. 39, 25-34.

Cai, J., Cho, S. W., Kim, J. Y., Lee, M. J., Cha, Y. G. and Jung, H. S. (2007). Patterning the Size and Number of Tooth and its Cusps. Dev. Biol. 304, 499-507.

Carpenter, D., Stone, D. M., Brush, J., Ryan, A., Armanini, M., Frantz, G., Rosenthal, A. and de Sauvage, F. J. (1998). Characterization of Two Patched Receptors for the Vertebrate Hedgehog Protein Family. Proc. Natl. Acad. Sci. U. S. A. 95, 13630-13634.

Carroll, S. B. (2001). Chance and Necessity: The Evolution of Morphological Complexity and Diversity. Nature 409, 1102-1109.

Charles, C., Lazzari, V., Tafforeau, P., Schimmang, T., Tekin, M., Klein, O. and Viriot, L. (2009). Modulation of Fgf3 Dosage in Mouse and Men Mirrors Evolution of Mammalian Dentition. Proc. Natl. Acad. Sci. U. S. A. 106, 22364-22368.

Cho, S. W., Kwak, S., Woolley, T. E., Lee, M. J., Kim, E. J., Baker, R. E., Kim, H. J., Shin, J. S., Tickle, C., Maini, P. K. et al. (2011). Interactions between Shh, Sostdc1 and Wnt Signaling and a New Feedback Loop for Spatial Patterning of the Teeth. Development 138, 1807-1816.

Cho, S. W., Lee, H. A., Cai, J., Lee, M. J., Kim, J. Y., Ohshima, H. and Jung, H. S. (2007). The Primary Enamel Knot Determines the Position of the First Buccal Cusp in Developing Mice Molars. Differentiation 75, 441-451.

Dassule, H. R., Lewis, P., Bei, M., Maas, R. and McMahon, A. P. (2000). Sonic Hedgehog Regulates Growth and Morphogenesis of the Tooth. Development 127, 4775-4785.

Dassule, H. R. and McMahon, A. P. (1998). Analysis of Epithelial-Mesenchymal Interactions in the Initial Morphogenesis of the Mammalian Tooth. Dev. Biol. 202, 215-227.

Davidson, E. H. (2001). Genomic Regulatory Systems: Development and Evolution, pp. 261. San Diego, U.S.A.: Academic Press.

Davis, E. E., Brueckner, M. and Katsanis, N. (2006). The Emerging Complexity of the Vertebrate Cilium: New Functional Roles for an Ancient Organelle. Dev. Cell. 11, 9-19.

Démére, T. A., McGowen, M. R., Berta, A. and Gatesy, J. (2008). Morphological and Molecular Evidence for a Stepwise Evolutionary Transition from Teeth to Baleen in Mysticete Whales. Syst. Biol. 57, 15-37.

Dongen, S. V. (2006). Fluctuating Asymmetry and Developmental Instability in Evolutionary Biology: Past, Present and Future. J. Evol. Biol. 19, 1727-1743.

D'Souza, R. N., Åberg, T., Gaikwad, J., Cavender, A., Owen, M., Karsenty, G. and Thesleff, I. (1999). Cbfa1 is Required for Epithelial-Mesenchymal Interactions Regulating Tooth Development in Mice. Development 126, 2911-2920.

Dun, R. B. and Fraser, A. S. (1958). Selection for an Invariant Character - 'Vibrissa Number' - in the House Mouse. Nature 181, 1018-1019.

Duplantier, J. M., Orth, A., Catalan, J. and Bonhomme, F. (2002). Evidence for a Mitochondrial Lineage Originating from the Arabian Peninsula in the Madagascar House Mouse (Mus Musculus). Heredity (Edinb) 89, 154-158.

Dworkin, I. (2005). Canalization, Cryptic Variation, and Developmental Buffering: A Critical Examination and Analytical Perspective. In Variation, a Central Concept in Biology (ed. B. Hallgrímsson and B. K. Hall), pp. 131-158. U.S.A.: Elsevier Academic Press.

Evans, A. R., Jones, D., Boyer, E. G., Brown, J. H., Costa, D. P., Ernest, S. K. M., Fitzgerald, E. M. G., Fortelius M., Gittleman, J. L., Hamilton, M. J., Harding, L. E., Lintulaakso, K., Lyons, S. K., Okie, J. G., Saarinen, J. J., Sibly, R. M., Smith, F. A., Stephens, P. R., Theodor, J. M. and Uhen M. D. (2012) The maximum rate of mammal evolution. PNAS 2012 ; published ahead of print January 30, 2012, doi:10.1073/pnas.1120774109.

Evans, A. R., Wilson, G. P., Fortelius, M. and Jernvall, J. (2007). High-Level Similarity of Dentitions in Carnivorans and Rodents. Nature 445, 78-81.

Falconer, D. S. (1953). Total Sex-Linkage in the House Mouse. Z. Indukt Abstamm Vererbungsl. 85, 210-219.

Ferguson, C. A., Tucker, A. S., Christensen, L., Lau, A. L., Matzuk, M. M. and Sharpe, P. T. (1998). Activin is an Essential Early Mesenchymal Signal in Tooth Development that is Required for Patterning of the Murine Dentition. Genes Dev. 12, 2636-2649.

Fliniaux, I., Mikkola, M. L., Lefebvre, S. and Thesleff, I. (2008). Identification of dkk4 as a Target of Eda-A1/Edar Pathway Reveals an Unexpected Role of Ectodysplasin as Inhibitor of Wnt Signalling in Ectodermal Placodes. Dev. Biol. 320, 60-71.

Fraser, G. J., Cerny, R., Soukup, V., Bronner-Fraser, M. and Streelman, J. T. (2010). The Odontode Explosion: The Origin of Tooth-Like Structures in Vertebrates. Bioessays 32, 808-817.

Gaunt, W. A. (1955). The Development of the Molar Pattern of the Mouse (Mus Musculus). Acta Anat. 24, 249-268.

Gilbert, S. F. (1997). Developmental Biology, pp. 918. Massachusetts, U.S.A.: Sinauer Associates, Inc.

Gilbert, S. F. and Epel, D. (2009). Ecological Developmental Biology: Integrating Epigenetics, Medicine, and Evolution, pp. 459. Massachusetts, U.S.A.: Sinauer Associates Inc.

Gould, S. J. (2002). The Structure of Evolutionary Theory, pp. 1433. Cambridge, Massachusetts, U.S.A.: The Belknap Press of Harvard University Press.

Gritli-Linde, A., Bei, M., Maas, R., Zhang, X. M., Linde, A. and McMahon, A. P. (2002). Shh Signaling within the Dental Epithelium is Necessary for Cell Proliferation, Growth and Polarization. Development 129, 5323-5337.

Grüneberg, H. (1966). The Molars of the Tabby Mouse, and a Test of the 'Single-Active X-Chromosome' Hypothesis. J. Embryol. exp. Morph. 15, 223-244.

Gurdon, J. B. (1992). The Generation of Diversity and Pattern in Animal Development. Cell 68, 185-199.

Handrigan, G. R. and Richman, J. M. (2010). Autocrine and Paracrine Shh Signaling are Necessary for Tooth Morphogenesis, but Not Tooth Replacement in Snakes and Lizards (Squamata). Dev. Biol. 337, 171-186.

Hardcastle, Z., Mo, R., Hui, C. C. and Sharpe, P. T. (1998). The Shh Signalling Pathway in Tooth Development - Defects in Gli2 and Gli3 Mutants. Development 125, 2803-2811.

Harris, M. P., Fallon, J. F. and Prum, R. O. (2002). Shh-Bmp2 Signaling Module and the Evolutionary Origin and Diversification of Feathers. J. Exp. Zool. 294, 160-176.

Harris, M. P., Rohner, N., Schwarz, H., Perathoner, S., Konstantinidis, P. and Nusslein-Volhard, C. (2008). Zebrafish Eda and Edar Mutants Reveal Conserved and Ancestral Roles of Ectodysplasin Signaling in Vertebrates. PLoS Genet. 4, e1000206.

Haycraft, C. J., Banizs, B., Aydin-Son, Y., Zhang, Q., Michaud, E. J. and Yoder, B. K. (2005). Gli2 and Gli3 Localize to Cilia and Require the Intraflagellar Transport Protein Polaris for Processing and Function. PLoS Genet. 1, e53.

Hiiemae, K. M. and Crompton, A. W. (1985). Mastication, Food Transport, and Swallowing. In Functional Vertebrate Morphology (ed. M. Hildebrand, D. M. Bramble, K. F. Liem and D. B. Wake), pp. 263-290. Cambridge, U.S.A.: The Belknap Press of Harvard University Press.

Hillson, S. (2005). Teeth, pp. 373. Cambridge, U.K.: Cambridge University Press.

Howard, J., Grill, S. W. and Bois, J. S. (2011). Turing's Next Steps: The Mechanochemical Basis of Morphogenesis. Nat. Rev. Mol. Cell Biol. 12, 392-398.

Hunter, J. P. and Jernvall, J. (1995). The Hypocone as a Key Innovation in Mammalian Evolution. Proc. Natl. Acad. Sci. U. S. A. 92, 10718-10722.

Ikeda, E., Morita, R., Nakao, K., Ishida, K., Nakamura, T., Takano-Yamamoto, T., Ogawa, M., Mizuno, M., Kasugai, S. and Tsuji, T. (2009). Fully Functional Bioengineered Tooth Replacement as an Organ Replacement Therapy. Proc. Natl. Acad. Sci. U. S. A. 106, 13475-13480.

Imai, H., Osumi-Yamashita, N., Ninomiya, Y. and Eto, K. (1996). Contribution of Early-Emigrating Midbrain Crest Cells to the Dental Mesenchyme of Mandibular Molar Teeth in Rat Embryos. Dev. Biol. 176, 151-165.

Itasaki, N., Jones, C. M., Mercurio, S., Rowe, A., Domingos, P. M., Smith, J. C. and Krumlauf, R. (2003). Wise, a Context-Dependent Activator and Inhibitor of Wnt Signalling. Development 130, 4295-4305.

Järvinen, E., Salazar-Ciudad, I., Birchmeier, W., Taketo, M. M., Jernvall, J. and Thesleff, I. (2006). Continuous Tooth Generation in Mouse is Induced by Activated Epithelial Wnt/beta-Catenin Signaling. Proc. Natl. Acad. Sci. USA 103, 18627-18632.

Järvinen, E., Tummers, M. and Thesleff, I. (2009). The Role of the Dental Lamina in Mammalian Tooth Replacement. J. Exp. Zool. B. Mol. Dev. Evol. 312B, 281-291.

Järvinen, E., Välimäki, K., Pummila, P., Hanski, I., Thesleff, I. and Jernvall, J. (2008). The Taming of the Shrew Milk Teeth. Evolution and Development.

Jernvall, J., Åberg, T., Kettunen, P., Keränen, S. and Thesleff, I. (1998). The Life History of an Embryonic Signaling Center: BMP-4 Induces p21 and is Associated with Apoptosis in the Mouse Tooth Enamel Knot. Development 125, 161-169.

Jernvall, J., Keränen, S. V. and Thesleff, I. (2000). Evolutionary Modification of Development in Mammalian Teeth: Quantifying Gene Expression Patterns and Topography. 97, 14444-14448.

Jernvall, J., Kettunen, P., Karavanova, I., Martin, L. B. and Thesleff, I. (1994). Evidence for the Role of the Enamel Knot as a Control Center in Mammalian Tooth Cusp Formation: Non-Dividing Cells Express Growth Stimulating Fgf-4 Gene. Int. J. Dev. Biol. 38, 463-469.

Jernvall, J. and Thesleff, I. (2000). Reiterative Signaling and Patterning during Mammalian Tooth Morphogenesis. Mech. Dev. 92, 19-29.

Kangas, A. T., Evans, A. R., Thesleff, I. and Jernvall, J. (2004). Nonindependence of Mammalian Dental Characters. Nature 432, 211-214.

Kavanagh, K. D., Evans, A. R. and Jernvall, J. (2007). Predicting Evolutionary Patterns of Mammalian Teeth from Development. Nature 449, 427-4U1.

Keränen, S. V., Åberg, T., Kettunen, P., Thesleff, I. and Jernvall, J. (1998). Association of Developmental Regulatory Genes with the Development of Different Molar Tooth Shapes in Two Species of Rodents. Dev. Genes Evol. 208, 477-486.

Keränen, S. V., Kettunen, P., Åberg, T., Thesleff, I. and Jernvall, J. (1999). Gene Expression Patterns Associated with Suppression of Odontogenesis in Mouse and Vole Diastema Regions. Dev. Genes Evol. 209, 495-506.

Keränen, S. V. E. (2000). The Developmental Basis for the Evolution of Muroid Dentition: Analysis of Gene Expression Patterns and Tooth Morphogenesis in the Mouse and Sibling Vole, pp. 90. Helsinki, Finland: University of Helsinki.

Kettunen, P., Laurikkala, J., Itäranta, P., Vainio, S., Itoh, N. and Thesleff, I. (2000). Associations of FGF-3 and FGF-10 with Signaling Networks Regulating Tooth Morphogenesis. Dev. Dyn. 219, 322-332.

Klein, O. D., Minowada, G., Peterkova, R., Kangas, A., Yu, B. D., Lesot, H., Peterka, M., Jernvall, J. and Martin, G. R. (2006). Sprouty Genes Control Diastema Tooth Development Via Bidirectional Antagonism of Epithelial-Mesenchymal FGF Signaling. Dev. Cell 11, 181-190.

Klingenberg, C. H. (2005). Developmental Constraints, Modules, and Evolvability. In Variation, a Central Concept in Biology (ed. B. Hallgrímsson and B. K. Hall), pp. 219-247. Massachusetts, U.S.A.: Elsevier Academic Press.

Kondo, S. and Miura, T. (2010). Reaction-Diffusion Model as a Framework for Understanding Biological Pattern Formation. Science 329, 1616-1620.

Kratochwil, K., Galceran, J., Tontsch, S., Roth, W. and Grosschedl, R. (2002). FGF4, a Direct Target of LEF1 and Wnt Signaling, can Rescue the Arrest of Tooth Organogenesis in Lef1(-/-) Mice. Genes Dev. 16, 3173-3185.

Laurikkala, J., Kassai, Y., Pakkasjarvi, L., Thesleff, I. and Itoh, N. (2003). Identification of a Secreted BMP Antagonist, Ectodin, Integrating BMP, FGF, and SHH Signals from the Tooth Enamel Knot. Dev. Biol. 264, 91-105.

Laurikkala, J., Mikkola, M., Mustonen, T., Åberg, T., Koppinen, P., Pispa, J., Nieminen, P., Galceran, J., Grosschedl, R. and Thesleff, I. (2001). TNF Signaling Via the Ligand-Receptor Pair Ectodysplasin and Edar Controls the Function of Epithelial Signaling Centers and is Regulated by Wnt and Activin during Tooth Organogenesis. Dev. Biol. 229, 443-455.

Laurikkala, J., Pispa, J., Jung, H. S., Nieminen, P., Mikkola, M., Wang, X., Saarialho-Kere, U., Galceran, J., Grosschedl, R. and Thesleff, I. (2002). Regulation of Hair Follicle Development by the TNF Signal Ectodysplasin and its Receptor Edar. Development 129, 2541-2553.

Lewis, E. B. (1978). A Gene Complex Controlling Segmentation in Drosophila. Nature 276, 565-570.

Lin, C. R., Kioussi, C., O'Connell, S., Briata, P., Szeto, D., Liu, F., Izpisua-Belmonte, J. C. and Rosenfeld, M. G. (1999). Pitx2 Regulates Lung Asymmetry, Cardiac Positioning and Pituitary and Tooth Morphogenesis. Nature 401, 279-282.

Lisi, S., Peterkova, R., Kristenova, P., Vonesch, J. L., Peterka, M. and Lesot, H. (2001). Crown Morphology and Pattern of Odontoblast Differentiation in Lower Molars of Tabby Mice. J. Dent. Res. 80, 1980-1983.

Liu, F., Chu, E. Y., Watt, B., Zhang, Y., Gallant, N. M., Andl, T., Yang, S. H., Lu, M. M., Piccolo, S., Schmidt-Ullrich, R. et al. (2008). Wnt/beta-Catenin Signaling Directs Multiple Stages of Tooth Morphogenesis. Dev. Biol. 313, 210-224.

Lohi, M., Tucker, A. S. and Sharpe, P. T. (2010). Expression of Axin2 Indicates a Role for Canonical Wnt Signaling in Development of the Crown and Root during Pre- and Postnatal Tooth Development. Dev. Dyn. 239, 160-167.

Luckett, W. P. (1985). Superordinal and Intraordinal Affinities of Rodents: Developmental Evodence from the Dentition and Placentation. In Evolutionary Relationships among Rodents: A Multidisciplinary Analysis (ed. W. P. Luckett and J.-L. Hartenberger), pp. 227-276. New York, U.S.A.: Plenum Press.

Luckett, W. P. (1993). Ontogenetic Staging of the Mammalian Dentition, and its Value for Assessment of Homology and Heterochrony. 1, 269-282.

Lumsden, A. G. (1988). Spatial Organization of the Epithelium and the Role of Neural Crest Cells in the Initiation of the Mammalian Tooth Germ. Development 103 Suppl, 155-169.

Luo, Z. X., Cifelli, R. L. and Kielan-Jaworowska, Z. (2001). Dual Origin of Tribosphenic Mammals. Nature 409, 53-57.

Macdonald, D. (2001). The Encyclopedia of Mammals, pp. 930. London, England: The Brown Reference Group.

Mammoto, T., Mammoto, A., Torisawa, Y. S., Tat, T., Gibbs, A., Derda, R., Mannix, R., de Bruijn, M., Yung, C. W., Huh, D. et al. (2011). Mechanochemical Control of Mesenchymal Condensation and Embryonic Tooth Organ Formation. Dev. Cell. 21, 758-769.

Matzuk, M. M., Kumar, T. R. and Bradley, A. (1995). Different Phenotypes for Mice Deficient in either Activins Or Activin Receptor Type II. Nature 374, 356-360.

McShea, D. W. (2005). A Universal Generative Tendency Towards Increased Organismal Complexity. In Variation, a Central Concept in Biology (ed. B. Hallgrímsson and B. K. Hall), pp. 435-454. U.S.A.: Elsevier Academic Press.

Metscher, B. D. (2009). MicroCT for Developmental Biology: A Versatile Tool for High-Contrast 3D Imaging at Histological Resolutions. Dev. Dyn. 238, 632-640.

Mikkola, M. L. (2007). Genetic Basis of Skin Appendage Development. Semin. Cell Dev. Biol. 18, 225-236.

Mikkola, M. L. (2008). TNF Superfamily in Skin Appendage Development. Cytokine Growth Factor Rev. 19, 219-230.

Miles, A. E. W. and Grigson, C. (1990). Colyer's Variations and Diseases of the Teeth of Animals. 672.

Mina, M. and Kollar, E. J. (1987). The Induction of Odontogenesis in Non-Dental Mesenchyme Combined with Early Murine Mandibular Arch Epithelium. Arch. Oral Biol. 32, 123-127.

Mitsiadis, T. A. and Smith, M. M. (2006). How do Genes make Teeth to Order through Development? J. Exp. Zool. B. Mol. Dev. Evol. 306, 177-182.

Motoyama, J., Takabatake, T., Takeshima, K. and Hui, C. (1998). Ptch2, a Second Mouse Patched Gene is Co-Expressed with Sonic Hedgehog. Nat. Genet. 18, 104-106.

Mou, C., Jackson, B., Schneider, P., Overbeek, P. A. and Headon, D. J. (2006). Generation of the Primary Hair Follicle Pattern. Proc. Natl. Acad. Sci. U. S. A. 103, 9075-9080.

Mou, C., Thomason, H. A., Willan, P. M., Clowes, C., Harris, W. E., Drew, C. F., Dixon, J., Dixon, M. J. and Headon, D. J. (2008). Enhanced Ectodysplasin-A Receptor (EDAR) Signaling Alters Multiple Fiber Characteristics to Produce the East Asian Hair Form. Hum. Mutat. 29, 1405-1411.

Mucchielli, M. L., Mitsiadis, T. A., Raffo, S., Brunet, J. F., Proust, J. P. and Goridis, C. (1997). Mouse Otlx2/RIEG Expression in the Odontogenic Epithelium Precedes Tooth Initiation and Requires Mesenchyme-Derived Signals for its Maintenance. Dev. Biol. 189, 275-284.

Munne, P. M., Felszeghy, S., Jussila, M., Suomalainen, M., Thesleff, I. and Jernvall, J. (2010). Splitting Placodes: Effects of Bone Morphogenetic Protein and Activin on the Patterning and Identity of Mouse Incisors. Evol. Dev. 12, 383-392.

Munne, P. M., Tummers, M., Jarvinen, E., Thesleff, I. and Jernvall, J. (2009). Tinkering with the Inductive Mesenchyme: Sostdc1 Uncovers the Role of Dental Mesenchyme in Limiting Tooth Induction. Development 136, 393-402.

Murashima-Suginami, A., Takahashi, K., Kawabata, T., Sakata, T., Tsukamoto, H., Sugai, M., Yanagita, M., Shimizu, A., Sakurai, T., Slavkin, H. C. et al. (2007). Rudiment Incisors Survive and Erupt as Supernumerary Teeth as a Result of USAG-1 Abrogation. Biochem. Biophys. Res. Commun. 359, 549-555.

Musser, G. G. and Carleton, M. D. (2005). Rodentia: Myomorpha: Muroidea: Muridae. In Mammal Species of the World: A Taxonomic and Geographic Reference. (eds. D. E. Wilson and D. M. Reeder), pp. 894-1531. Baltimore, U.S.A.: Johns Hopkins University Press.

Mustonen, T., Pispa, J., Mikkola, M. L., Pummila, M., Kangas, A. T., Jaatinen, R. and Thesleff, I. (2003). Stimulation of Ectodermal Organ Development by Ectodysplasin-A1. Dev. Biol. 259, 123-136.

Nakamura, T., de Vega, S., Fukumoto, S., Jimenez, L., Unda, F. and Yamada, Y. (2008). Transcription Factor Epiprofin is Essential for Tooth Morphogenesis by Regulating Epithelial Cell Fate and Tooth Number. J. Biol. Chem. 283, 4825-4833.

Nanci, A. (2008). Ten Cate's Oral Histology: Development, Structure, and Function, pp. 432. St Louis, Missouri, U.S.A.: Mosby Elsevier.

Neubuser, A., Peters, H., Balling, R. and Martin, G. R. (1997). Antagonistic Interactions between FGF and BMP Signaling Pathways: A Mechanism for Positioning the Sites of Tooth Formation. Cell 90, 247-255.

Nie, X., Luukko, K. and Kettunen, P. (2006). BMP Signalling in Craniofacial Development. Int. J. Dev. Biol. 50, 511-521.

Nyam, R. L., Gauthier, J. A. and Chiment, J. J. (2000). The Mammal-Like Teeth of the Late Cretaceous Lizard Peneteius Aquilonius Estes 1969 (Squamata, Teiidae). Journal of Vertebrate Paleontology 20, 628-631.

Närhi, K., Tummers, M., Ahtiainen, L., Itoh, N., Thesleff, I. and Mikkola, M.L. (2012). Sostdc1 defines the size and number of skin appendage placodes. Developmental Biology 364, 149-161.

Ohazama, A., Modine, S. A. C., Miletich, I. and Sharpe, P. T. (2004). Stem-Cell-Based Tissue Engineering of Murine Teeth. Journal of Dental Research 85, 518-522.

Ohazama, A., Haycraft, C. J., Seppala, M., Blackburn, J., Ghafoor, S., Cobourne, M., Martinelli, D. C., Fan, C. M., Peterkova, R., Lesot, H. et al. (2009). Primary Cilia Regulate Shh Activity in the Control of Molar Tooth Number. Development 136, 897-903.

Ohazama, A., Johnson, E. B., Ota, M. S., Choi, H. Y., Porntaveetus, T., Oommen, S., Itoh, N., Eto, K., Gritli-Linde, A., Herz, J. et al. (2008). Lrp4 Modulates Extracellular Integration of Cell Signaling Pathways in Development. PLoS One 3, e4092.

Ohmachi, S., Watanabe, Y., Mikami, T., Kusu, N., Ibi, T., Akaike, A. and Itoh, N. (2000). FGF-20, a Novel Neurotrophic Factor, Preferentially Expressed in the Substantia Nigra Pars Compacta of Rat Brain. Biochem. Biophys. Res. Commun. 277, 355-360.

Osborn, H. F. (1907). Chapter II: First Outline of Tritubercular Evolution in Mammals. In Evolution of Mammalian Molar Teeth to and from the Triangular Type Including Collected and Revised Researches Trituberculy and New Sections on the Forms and Homologies of the Molar Teeth in the Different Orders of Mammals (ed. W. K. Gregory), pp. 36-47: New York, Macmillan.

Osborn, J. W. (2008). A Model of Growth Restraints to Explain the Development and Evolution of Tooth Shapes in Mammals. J. Theor. Biol. 255, 338-343.

Pantalacci, S., Chaumot, A., Benoit, G., Sadier, A., Delsuc, F., Douzery, E. J. and Laudet, V. (2008). Conserved Features and Evolutionary Shifts of the EDA Signaling Pathway Involved in Vertebrate Skin Appendage Development. Mol. Biol. Evol. 25, 912-928.

Peterkova, R., Lesot, H., Viriot, L. and Peterka, M. (2005). The Supernumerary Cheek Tooth in tabby/EDA Mice-a Reminiscence of the Premolar in Mouse Ancestors. Arch. Oral Biol. 50, 219-225.

Peterkova, R., Peterka, M., Viriot, L. and Lesot, H. (2002). Development of the Vestigial Tooth Primordia as Part of Mouse Odontogenesis. Connect. Tissue Res. 43, 120-128.

Peters, H., Neubuser, A., Kratochwil, K. and Balling, R. (1998). Pax9-Deficient Mice Lack Pharyngeal Pouch Derivatives and Teeth and Exhibit Craniofacial and Limb Abnormalities. Genes Dev. 12, 2735-2747.

Pigliucci, M. (2007). Do we Need an Extended Evolutionary Synthesis? Evolution 61, 2743-2749.

Pispa, J., Mustonen, T., Mikkola, M. L., Kangas, A. T., Koppinen, P., Lukinmaa, P. L., Jernvall, J. and Thesleff, I. (2004). Tooth Patterning and Enamel Formation can be Manipulated by Misexpression of TNF Receptor Edar. Dev. Dyn. 231, 432-440.

Plikus, M. V., Zeichner-David, M., Mayer, J. A., Reyna, J., Bringas, P., Thewissen, J. G., Snead, M. L., Chai, Y. and Chuong, C. M. (2005). Morphoregulation of Teeth: Modulating the Number, Size, Shape and Differentiation by Tuning Bmp Activity. Evol. Dev. 7, 440-457.

Prochazka, J., Pantalacci, S., Churava, S., Rothova, M., Lambert, A., Lesot, H., Klein, O., Peterka, M., Laudet, V. and Peterkova, R. (2010). Patterning by Heritage in Mouse Molar Row Development. Proc. Natl. Acad. Sci. U. S. A. 107, 15497-15502.

Pummila, M., Fliniaux, I., Jaatinen, R., James, M. J., Laurikkala, J., Schneider, P., Thesleff, I. and Mikkola, M. L. (2007). Ectodysplasin has a Dual Role in Ectodermal Organogenesis: Inhibition of Bmp Activity and Induction of Shh Expression. Development 134, 117-125.

Rodrigues, H. G., Marivaux, L. and Vianey-Liaud, M. (2010). Phylogeny and Systematic Revision of Eocene Cricetidae (Rodentia, Mammalia) from Central and East Asia: On the Origin of Cricetid Rodents. J. Zool. Syst. Evol. Res. 48, 259-268.

Rodrigues, H. G., Charles, C., Marivaux, L., Vianey-Liaud, M. and Viriot, L. (2011). Evolutionary and Developmental Dynamics of the Dentition in Muroidea and Dipodoidea (Rodentia, Mammalia). Evol. Dev. 13, 361-369.

Rohatgi, R. and Scott, M. P. (2007). Patching the Gaps in Hedgehog Signalling. Nat. Cell Biol. 9, 1005-1009.

Roth, V. L. (2005). Variation and Versatility in Macroevolution. In Variation, a Central Concept in Biology (ed. B. Hallgrímsson and B. K. Hall), pp. 455-474. U.S.A.: Elsevier Academic Press.

Rutherford, S. L. and Lindquist, S. (1998). Hsp90 as a Capacitor for Morphological Evolution. Nature 396, 336-342.

Salazar-Ciudad, I. and Jernvall, J. (2002). A Gene Network Model Accounting for Development and Evolution of Mammalian Teeth. Proc Natl Acad Sci U S A 99, 8116-8120.

Salazar-Ciudad, I. and Jernvall, J. (2010). A Computational Model of Teeth and the Developmental Origins of Morphological Variation. Nature 464, 583-586.

Sarkar, L. and Sharpe, P. T. (1999). Expression of Wnt Signalling Pathway Genes during Tooth Development. Mech. Dev. 85, 197-200.

Schlake, T. and Sick, S. (2007). Canonical WNT Signalling Controls Hair Follicle Spacing. Cell. Adh Migr. 1, 149-151.

Schmidt-Ullrich, R., Tobin, D. J., Lenhard, D., Schneider, P., Paus, R. and Scheidereit, C. (2006). NF-kappaB Transmits Eda A1/EdaR Signalling to Activate Shh and Cyclin D1 Expression, and Controls Post-Initiation Hair Placode Down Growth. Development 133, 1045-1057.

Sharpe, P. T. (1995). Homeobox Genes and Orofacial Development. Connect. Tissue Res. 32, 17-25.

Simmer, J. P. and Snead, M. L. (1995). Molecular Biology of the Amelogenin Gene. In Dental Enamel - Formation to Destruction (ed. C. Robinson, J. Kirkham and R. Shore), pp. 59-84. The United States of America: CRC Press, Inc.

Smith, J. B. (2005). Heterodonty in Tyrannosaurus Rex: Implicatios for the Taxonomic and Systematic Utility of Therapod Dentitions. Journal of Vertebrate Paleontology 25, 865-887.

Smith, M. M. and Hall, B. K. (1990). Development and Evolutionary Origins of Vertebrate Skeletogenic and Odontogenic Tissues. Biol. Rev. Camb. Philos. Soc. 65, 277-373.

Sofaer, J. A. (1969). Aspects of the Tabby-Crinkled-Downless Syndrome. I. the Development of Tabby Teeth. J. Embryol. exp. Morph. 22, 181-205.

Srivastava, A. K., Pispa, J., Hartung, A. J., Du, Y., Ezer, S., Jenks, T., Shimada, T., Pekkanen, M., Mikkola, M. L. et al. (1997). The Tabby Phenotype is Caused by Mutation in a Mouse Homologue of the EDA Gene that Reveals Novel Mouse and Human Exons and Encodes a Protein (Ectodysplasin-A) with Collagenous Domains. Proc. Natl. Acad. Sci. USA 94, 13069-13074.

Stanger, B. Z., Tanaka, A. J. and Melton, D. A. (2007). Organ Size is Limited by the Number of Embryonic Progenitor Cells in the Pancreas but Not the Liver. Nature 445, 886-891.

Steppan, S., Adkins, R. and Anderson, J. (2004). Phylogeny and Divergence-Date Estimates of Rapid Radiations in Muroid Rodents Based on Multiple Nuclear Genes. Syst. Biol. 53, 533-553.

Streelman, J. T. and Albertson, R. C. (2006). Evolution of Novelty in the Cichlid Dentition. J. Exp. Zool. B. Mol. Dev. Evol. 306, 216-226.

Streelman, J. T., Webb, J. F., Albertson, R. C. and Kocher, T. D. (2003). The Cusp of Evolution and Development: A Model of Cichlid Tooth Shape Diversity. Evol. Dev. 5, 600-608.

Thesleff, I., Vaahtokari, A. and Partanen, A. M. (1995). Regulation of Organogenesis. Common Molecular Mechanisms Regulating the Development of Teeth and Other Organs. Int. J. Dev. Biol. 39, 35-50.

Tickle, C. (2006). Making Digit Patterns in the Vertebrate Limb. Nat. Rev. Mol. Cell Biol. 7, 45-53.

Travis, A., Amsterdam, A., Belanger, C., Grosschedl, R. (1991). LEF-1, a gene encoding a lymphoid-specific protein with an HMG domain, regulates T-cell receptor alpha enhancer function. Genes Dev. 5, 880-894.

Tucker, A. S., Headon, D. J., Courtney, J. M., Overbeek, P. and Sharpe, P. T. (2004). The Activation Level of the TNF Family Receptor, Edar, Determines Cusp Number and Tooth Number during Tooth Development. Dev. Biol. 268, 185-194.

Tummers, M. and Thesleff, I. (2009). The Importance of Signal Pathway Modulation in all Aspects of Tooth Development. J. Exp. Zool. B. Mol. Dev. Evol. 312B, 309-319.

Tureckova, J., Lesot, H., Vonesch, J. L., Peterka, M., Peterkova, R. and Ruch, J. V. (1996). Apoptosis is Involved in the Disappearance of the Diastemal Dental Primordia in Mouse Embryo. Int. J. Dev. Biol. 40, 483-489.

Ungar, P. S. (2010). Mammal Teeth: Origin, Evolution, and Diversity, pp. 304. Baltimore, U.S.A.: The Johns Hopkins University Press.

Vaahtokari, A., Åberg, T. and Thesleff, I. (1996). Apoptosis in the Developing Tooth: Association with an Embryonic Signaling Center and Suppression by EGF and FGF-4. Development 122, 121-129.

Vainio, S., Karavanova, I., Jowett, A. and Thesleff, I. (1993). Identification of BMP-4 as a Signal Mediating Secondary Induction between Epithelial and Mesenchymal Tissues during Early Tooth Development. Cell 75, 45-58.

Varjosalo, M. and Taipale, J. (2008). Hedgehog: Functions and Mechanisms. Genes Dev. 22, 2454-2472.

Viriot, L., Lesot, H., Vonesch, J. L., Ruch, J. V., Peterka, M. and Peterkova, R. (2000). The Presence of Rudimentary Odontogenic Structures in the Mouse Embryonic Mandible Requires Reinterpretation of Developmental Control of First Lower Molar Histomorphogenesis. Int. J. Dev. Biol. 44, 233-240.

Viriot, L., Peterkova, R., Peterka, M. and 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.

Wang, X. P., O'Connell, D. J., Lund, J. J., Saadi, I., Kuraguchi, M., Turbe-Doan, A., Cavallesco, R., Kim, H., Park, P. J., Harada, H. et al. (2009). Apc Inhibition of Wnt Signaling Regulates Supernumerary Tooth Formation during Embryogenesis and Throughout Adulthood. Development 136, 1939-1949.

Wang, X. P., Suomalainen, M., Jorgez, C. J., Matzuk, M. M., Werner, S. and Thesleff, I. (2004). Follistatin Regulates Enamel Patterning in Mouse Incisors by Asymmetrically Inhibiting BMP Signaling and Ameloblast Differentiation. Dev. Cell 7, 719-730.

Weller, J. M. (1968). Evolution of Mammalian Teeth. Journal of Paleontology 42, 268-290.

West-Eberhard, M. J. (2003). Developmental Plasticity and Evolution, pp. 794. New York, U.S.A.: Oxford University Press.

Wheeler, B. M., Heimberg, A. M., Moy, V. N., Sperling, E. A., Holstein, T. W., Heber, S. and Peterson, K. J. (2009). The Deep Evolution of Metazoan microRNAs. Evol. Dev. 11, 50-68.

Wilkins, A. (2002a). Evolution and Embryology: A Brief History of a Complex Pas De Deux. In The Evolution of Developmental Pathways., pp. 3-34. Sunderland: Sinauer Associates Inc.

Wilkins, A. S. (2002b). The Evolution of Developmental Pathways, pp. 603. Massachusetts, U.S.A.: Sinauer Associates, Inc.

Willmore, K. E. and Hallgrímsson, B. (2005). Within Individual Variation: Developmental Noise Versus Developmental Stability. In Variation, a Central Concept in Biology (ed. B. Hallgrímsson and B. K. Hall), pp. 191-218. Massachusetts, U.S.A.: Elsevier Academic Press.

Willmore, K. E., Young, N. M. and Richtsmeier, J. T. (2007). Phenotypic Variability: Its Components, Measurement and Underlying Developmental Processes. Evol. Biol. 33, 99-120.

Yaguchi, Y., Yu, T., Ahmed, M. U., Berry, M., Mason, I. and Basson, M. A. (2009). Fibroblast Growth Factor (FGF) Gene Expression in the Developing Cerebellum Suggests Multiple Roles for FGF Signaling during Cerebellar Morphogenesis and Development. Dev. Dyn. 238, 2058-2072.

Ying, S. Y., Chang, D. C. and Lin, S. L. (2008). The microRNA (miRNA): Overview of the RNA Genes that Modulate Gene Function. Mol. Biotechnol. 38, 257-268.

Yu, J., Shi, J. and Jin, Y. (2008). Current Approaches and Challenges in Making a Bio-Tooth. Tissue Eng. Part B. Rev. 14, 307-319.

Zhang, S., Lin, Y., Itaranta, P., Yagi, A. and Vainio, S. (2001). Expression of Sprouty Genes 1, 2 and 4 during Mouse Organogenesis. Mech. Dev. 109, 367-370.

Zhang, Q., Murcia, N. S., Chittenden, L. R., Richards, W. G., Michaud, E. J., Woychik, R. P. and Yoder, B. K. (2003). Loss of the Tg737 Protein Results in Skeletal Patterning Defects. Dev. Dyn. 227, 78-90.

Zhang, Z., Lan, Y., Chai, Y. and Jiang, R. (2009). Antagonistic Actions of Msx1 and Osr2 Pattern Mammalian Teeth into a Single Row. Science 323, 1232-1234.