

SYMPOSIUM

The Role of Core and Variable Gene Regulatory Network Modules in Tooth Development and Evolution

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Synopsis Among the developmental processes that have been proposed to influence the direction of evolution, the modular organization of developmental gene regulatory networks (GRNs) has shown particular promise. In theory, GRNs have core modules comprised of essential, conserved circuits of genes, and sub-modules of downstream, secondary circuits of genes that are more susceptible to variation. While this idea has received considerable interest as of late, the field of evo-devo lacks the experimental systems needed to rigorously evaluate this hypothesis. Here, we introduce an experimental system, the vertebrate tooth, that has great potential as a model for testing this hypothesis. Tooth development and its associated GRN have been well studied and modeled in both model and non-model organisms. We propose that the existence of modules within the tooth GRN explains both the conservation of developmental mechanisms and the extraordinary diversity of teeth among vertebrates. Based on experimental data, we hypothesize that there is a conserved core module of genes that is absolutely necessary to ensure tooth or cusp initiation and development. In regard to tooth shape variation between species, we suggest that more relaxed sub-modules activated at later steps of tooth development, for example, during the morphogenesis of the tooth and its cusps, control the different axes of tooth morphological variation.

Introduction

Why do some morphologies repeatedly evolve, while others, although theoretically possible, do not? In the 19th century, scientists proposed that this phenomenon could result from developmental processes biasing the evolution of phenotypes (Smith et al. 1985). The idea of developmental biases or constraints was revisited by Raup (1966), who proposed that biases imposed by the mechanisms of growth and development were responsible, at least in part, for his finding that only a small proportion of all possible snail shapes are realized in nature. This idea was also explored more recently by researchers in the field of genetics. For example, Schluter (1996) proposed that adaptive morphological differentiation tends to occur preferentially in certain directions

along “lines of least resistance” (Schluter 1996). However, while the concept of developmental bias has existed for over a hundred years, the prevalence and significance of developmental bias in phenotypic evolution remain unresolved, in part due to a historical lack of relevant experimental data (reviewed in Uller et al. 2018; Brakefield 2006; Hendrikse et al. 2007).

In the past 20 years, advances in the study of developmental mechanisms and their ability to generate variation have begun to fill this critical knowledge gap. As one example of these recent, fine-scale studies, explorations of the mechanisms controlling limb development suggest that development constrains the phalangeal variation observed among vertebrates by limiting morphologies within

a continuum (Kavanagh et al. 2013). Additionally, developmental mechanisms have been shown to constrain organ positioning in the cephalic horns of scarab beetles (Busey et al. 2016). Moreover, study of development has generated insights into its role in the generation of phenotypic diversity in bird beaks; morphological variation in the bird beak has been linked to the structure of the gene network that controls beak development in Darwin's finches. Among Darwin's finches, species that eat hard seeds tend to have shorter, broader beaks, while finches that pick seeds out of cactus fruits tend to have longer, more pointed beaks. Researchers have found that development of the beak in Darwin's finches is decomposable into two modules: one that controls depth and width and is regulated by the BMP pathway through *BMP4*, and one that controls length and is regulated by the calmodulin pathway (Abzhanov et al. 2004, 2006; Mallarino et al. 2011). This finding suggests that there is a simple mechanism for independent evolutionary changes in beak length and width within Darwin's finches. Interestingly, while closely related finch genera display a morphological diversity similar to that of Darwin's finches, research suggests that the pathways generating that diversity are distinct from those observed in Darwin's finches (Mallarino et al. 2012). This appears to be true for more distantly related bird groups as well (Cheng et al. 2017). Taken together, these findings suggest that developmental processes might have the potential to bias the generation of form within groups, and they might themselves be highly evolvable.

Among the developmental processes that have been proposed to bias the evolution of phenotypes, the modular organization of developmental gene regulatory networks (GRNs) is a good candidate (Davidson 2010; Hinman and Jarvela 2014). GRNs provide a map of the interactions between the transcription factors (TFs; see Box 1), enhancers, and target genes (Erwin and Davidson 2009) that control the development of organisms. As such, GRNs can be represented as networks or circuits with beginnings and ends and various possible topologies. Using theoretical and experimental approaches, researchers have started to investigate the inherent properties of GRNs and the impact of these properties on the evolution of form (Davidson and Levine 2008; Davidson 2010). From these studies, two properties of particular interest to the topic of developmental bias have emerged. First, GRNs are hierarchical, meaning that the portion of the GRN that controls the initial stage of development of an organism or an organ (generally its induction) precede other parts of the network, which in turn

control more specific functions such as morphogenesis and cell differentiation (Erwin and Davidson 2009). Second, GRNs are modular, meaning that they can be decomposed into sub-circuits or modules, which are defined for this article as semi-autonomous units responsible for the development of a phase or a part of an organism or organ (Davidson and Erwin 2006; Davidson and Levine 2008; Erwin and Davidson 2009). Building on the hierarchical and modular architecture of GRNs, we can further predict that organ GRNs have two types of modules (Box 1): a core module, also called a kernel, comprised of a primary circuit of genes that are essential for correct initiation of the organ in question; and sub-modules of downstream and peripheral (i.e., at the periphery of the GRN, spatio-temporally) circuits of genes that control the phenotype of parts of the organ (e.g., morphogenesis or cell differentiation; Lipson et al. 2002; Erwin and Davidson 2009; Clune et al. 2013; Kouvaris et al. 2017; Uller et al. 2018).

Evidence in support of the hierarchical and modular nature of GRNs comes from studies in several systems. For example, research has shown that the GRN that controls the dorsal-ventral patterning of *Drosophila* embryos composed of several sub-circuits, each of which controls the specification of a single tissue type (e.g., mesoderm, ventral neurogenic ectoderm, and dorsal neurogenic ectoderm; reviewed in Levine and Davidson 2005; Ochoa-Espinosa et al. 2005). Similarly, studies suggest that the GRN that controls endoderm and mesoderm specification in the sea urchin is partitioned into modules that control distinct developmental processes, such as the specification of most larval cell types (Peter and Davidson 2010). However, while findings to date are consistent with GRN structure being inherently hierarchical and modular, research in a wider range of organisms and organs is needed to further test this hypothesis.

A hierarchical and modular GRN structure has been proposed to contribute to the non-uniform distribution of shapes across morphospaces (Espinosa-Soto and Wagner 2010; Andreas Wagner 2011; Uller et al. 2018). GRN modules have been described for different organs in different organisms (Raff 2007; Reno et al. 2008; Zeller et al. 2009; Lacquaniti et al. 2013) and the biasing impact of this modular GRN structure on evolutionary change has been theorized (reviewed in Uller et al. 2018). However, there remains little direct, experimentally-based evidence of how the modular structure of GRNs impacts evolutionary change and is modified in response to a fluctuating environment over evolutionary time.

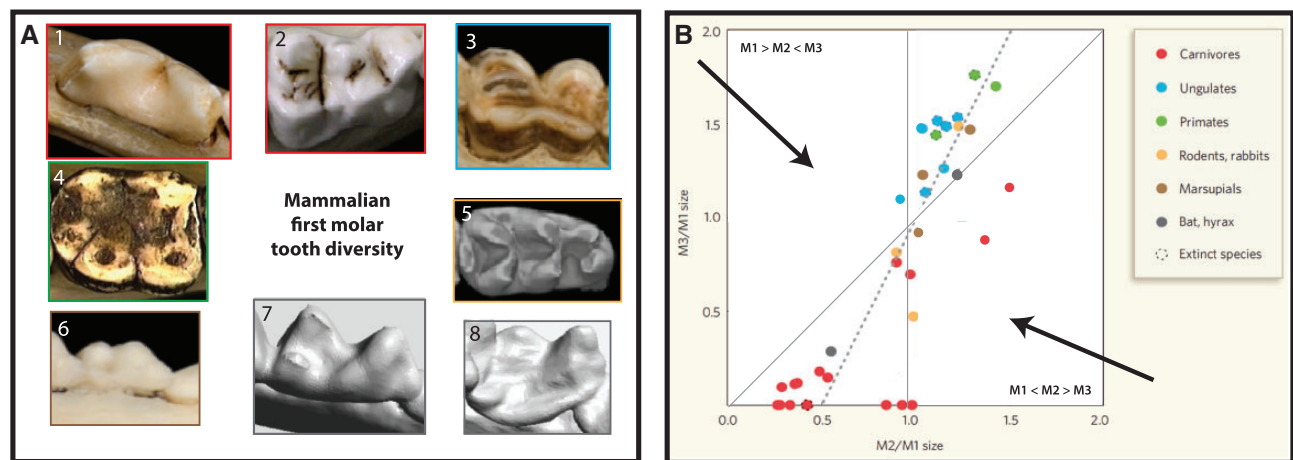


Fig. 1 Molar diversity in mammals and molar morphospace. **(A)** Mammalian molars exhibit an extraordinary diversity in terms of size, cusp number, and overall morphology. First molar of species from left to right, top to bottom: 1, cheetah (Phil Myers, ADW); 2, giant panda (Phil Myers, ADW); 3, big horn sheep (Phil Myers, ADW); 4, chimpanzee (Phil Myers, ADW); 5, mouse (Charles et al. 2009b); 6, water opossum (Phil Myers, ADW); 7, Waterhouse's leaf-nosed bat and; 8, tent making bat. ADW, Animal Diversity Web—Museum of Zoology of Michigan-Ann Arbor. **(B)** Molar inhibitory cascade morphospace. This schematic illustrates that not all possible molar proportions are realized in mammals: some regions of the morphospace (emphasized by black arrows) are not occupied. The uneven distribution of realized morphologies is thought to be due to developmental mechanisms (inhibitory cascades) controlling tooth proportions during molar development. Adapted from (Polly 2007). M1, first molar; M2, second molar; M3, third molar.

Vertebrate teeth: A model system for studying relationships between GRN structure and evolution

Teeth are serially homologous structures (i.e., repeating elements or organs within a single organism that share a large proportion of their genetic architecture and developmental pathways). Teeth are also functionally-important anatomical elements of the jaw that vary tremendously in number, shape, and location across mammals and vertebrates (Fig. 1A) (Stock 2001; Tucker and Sharpe 2004; Ungar 2010; Jernvall and Thesleff 2012). As teeth physically interface with food items during biting and mastication, their shape and number are thought to be under strong selection to match the demands imposed by the material properties of foods and the feeding behaviors used by vertebrates (Evans et al. 2007; Ungar 2010). Consistent with this, the evolution of teeth and their shape shows tight links with diet in many vertebrate groups (Evans et al. 2007; Ungar 2010, 2015). Beyond extrinsic factors, research in rodents suggests that teeth may also exhibit a differential evolvability along developmental lines of least resistance (Fig. 1B) (Renaud et al. 2006, 2011; Kavanagh et al. 2007). Furthermore, the genes that comprise the GRN that controls tooth development have been well described in model organisms, including mammals. Findings of this research suggest that the GRN controlling at least the initiation of tooth development is extremely conserved among mammals and potentially among all vertebrates (Jernvall and Thesleff 2012; Rasch et al. 2016). As

a result, vertebrate teeth represent an ideal model system with which to study how the inherent architecture of GRNs can facilitate variation while ensuring that essential processes (e.g., organ formation) are preserved. In this article, we use mammalian teeth as a model system to illustrate how applying the GRN module framework to study of teeth can advance understanding of the ways in which development biases morphological evolution and facilitates the generation of variation. In addition, we discuss how the modular structure of GRNs might facilitate the reiteration of developmental units during development.

Teeth develop through successive signaling centers that act as key checkpoints for morphogenesis

During development, teeth initially form through interactions between two embryonic tissues: the dental epithelium and the underlying mesenchyme (Tucker and Sharpe 2004). These interactions cause a thickening of dental epithelium which then invaginates into the dental mesenchyme to form a tooth bud (Fig. 2A). After invagination, the epithelium wraps around the underlying mesenchyme and the tooth progresses into cap and bell stages of development that phenocopy adult tooth form (Fig. 2A). Like tooth initiation, tooth progression through the bud, cap, and bell stages is also regulated by cross-talk between the epithelium and mesenchyme. This cross-talk takes place in a series of signaling centers that express a set of genes in a pattern conserved

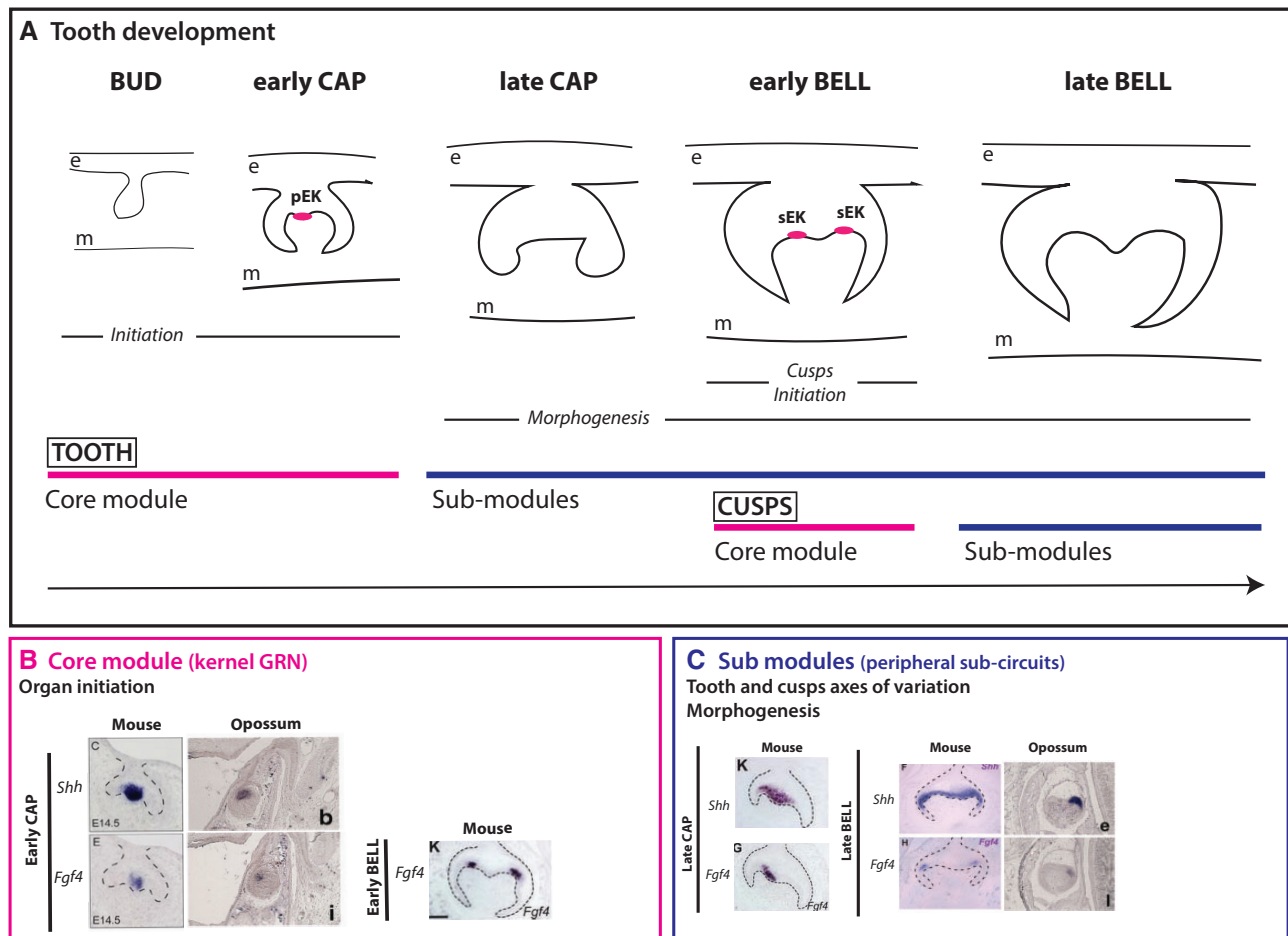


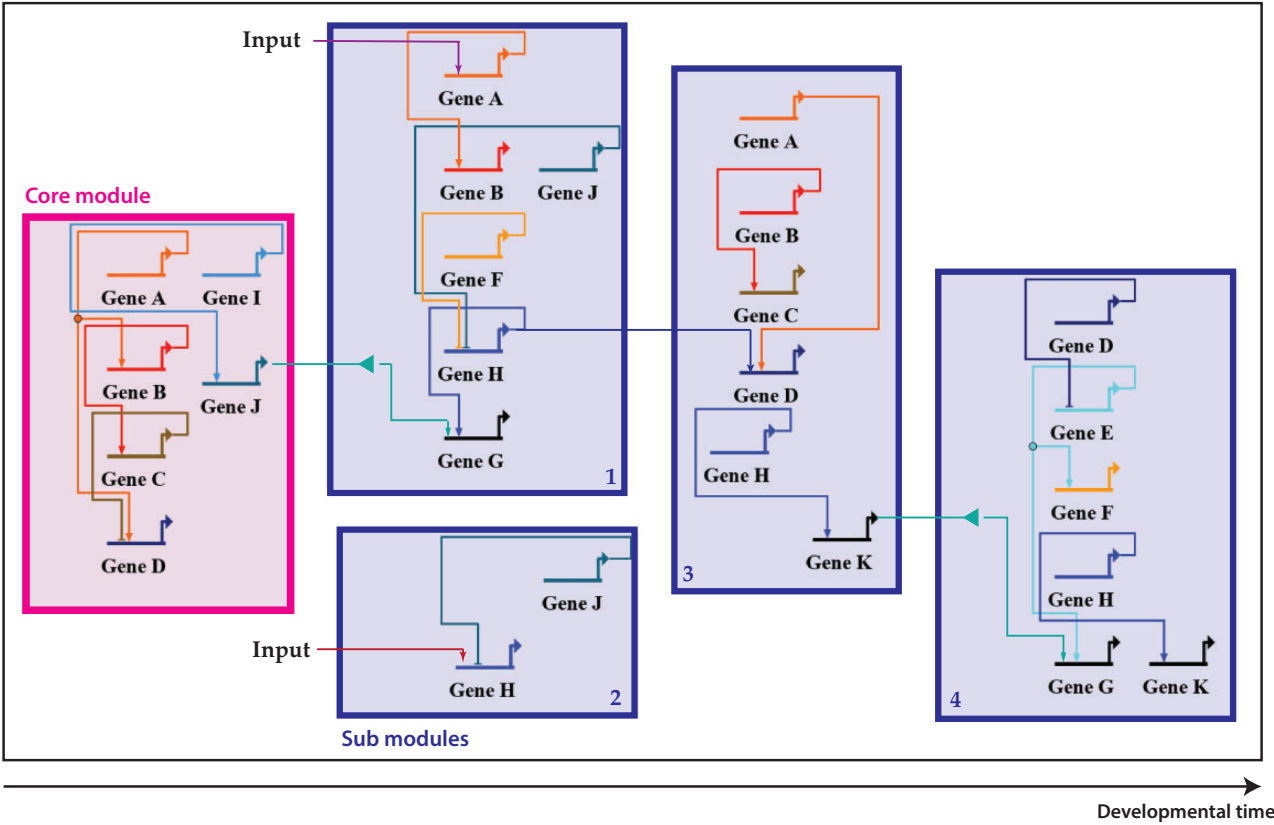
Fig. 2 Tooth development, spatiotemporal context of GRN modules activation, and gene expression patterns. **(A)** Tooth development involves two tissues: the epithelium and the neural-crest-derived mesenchyme. In mammals, the different stages of tooth development are called: bud, cap, and bell. Tooth development starts with the invagination of the epithelium into the underlying mesenchyme, followed by the induction of a first signaling center that is necessary to initiate tooth development. In multicusp teeth, signaling centers are reactivated for the formation of each cusp. These signaling centers are transient structures that express signaling molecules and are thought to regulate tooth morphogenesis. In mammals, signaling centers are called pEK (bud/cap stages) and sEKs (bell stage). In the framework presented here, we propose that the GRN that controls tooth development is partitioned into modules that are active at different stages of tooth development: the core module is active at early stages of signaling center formation (pEK and sEKs in mammals) and the sub-modules are active at later stages of tooth or cusp development. **(B)** Core module gene expression pattern. The GRN of the core module is characterized by genes with a conserved expression pattern in signaling centers, with many of them exhibiting a restricted expression pattern to signaling centers. **(C)** Sub-modules gene expression pattern. Expression patterns of the genes expressed when the sub-modules are active vary between species and can be linked to the direction of variation. *In situ* hybridization: mouse early cap from Liu et al. (2014), opossum early cap from Moustakas et al. (2011), late cap and early bell in mouse from Nakatomi et al. (2013).

among vertebrates (Fig. 2B; Jernvall and Thesleff 2012). In teeth with one cusp (i.e., monocuspid), a single signaling center likely regulates tooth development through the bud, cap, and bell stages. In contrast, development of the multicusp teeth of mammals is regulated by multiple signaling centers. In mammals, the first of these forms during the bud stage of development is called the primary Enamel Knot (pEK; Fig. 2). Additional signaling centers form later at the tips of future tooth cusps and are called secondary EKs (sEKs; Fig. 2; Jernvall and Thesleff

2000; Ungar 2015). Mammalian EKs are characterized by restricted expression patterns of key genes and distinct patterns of apoptosis (Lesot et al. 1996; Vaahtokari et al. 1996; Jernvall et al. 1998).

EKs are crucial to induction of the tooth as a whole and, in multicusp teeth, induction of each tooth's individual cusps. Consistent with this, disruption of pEKs or sEKs leads to developmental arrest of whole teeth or individual cusps, respectively (Fig. 3B). In contrast, perturbation of tooth development after pEK or sEK induction (Fig. 3B) alters

A Theoretical GRN



B Theoretical GRN Example of a gene shared by different modules

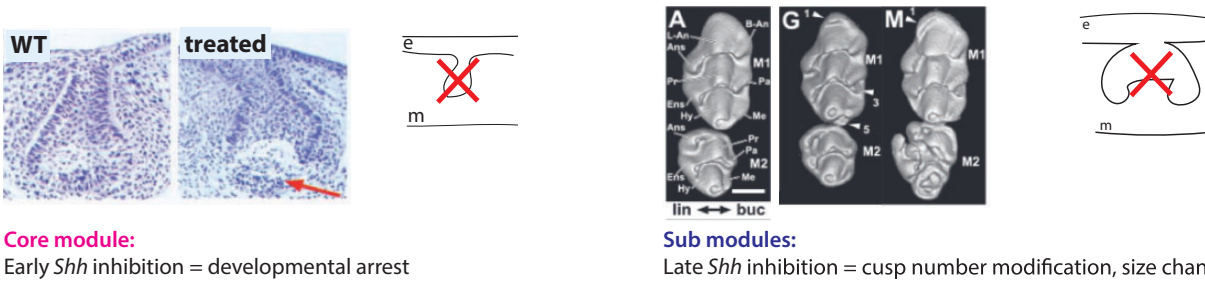


Fig. 3 GRN, core, and submodules. (A) Representation of a theoretical GRN. This GRN is both hierarchical and modular; and can be decomposed into circuits or modules: a core module, also called kernel, is comprised of an essential, primary circuit of genes that ensure the correct initiation the organ (here, the tooth and its cusps); and sub-modules of downstream, secondary peripheral circuits of genes are responsible for more peripheral functions during morphogenesis (here, the shape of the tooth and its cusps). In this theoretical example, the GRN is composed by 10 genes involved in different modules (one core and four sub modules). The core module is responsible for organ development initiation and each sub-module (1–4) is responsible for a given biological function or controls the development of a given trait (e.g., sub-module 1 could control tooth length). As many pathways and genes are pleiotropic and active during the development of a given organ (here, the tooth), many are shared between modules. However, each module circuit is unique as the regulatory loops inside these modules are specific to it. For example, Gene D is involved in the core and sub-module 3 and 4 but involved in different regulatory loops. As a result, disrupting gene D function in the core and sub-modules 3 and 4 will have different consequences (see below). (B) Example of a gene shared by different modules and functional consequences of disruptions. *Shh* is known to be involved both in tooth initiation and morphogenesis. Because of this, we hypothesize that *Shh* is involved in at least two different regulatory loops responsible for two different functions. As a disruption of *Shh* at an early stage leads to developmental arrest (Cobourne et al. 2001), we predict that *Shh* is part of the core module. Because late *Shh* inhibition leads to cusp number modification and tooth size change (Kim et al. 2019), we propose that *Shh* is also involved in sub-modules controlling these tooth traits through a different regulatory loop.

the morphology of teeth or cusps, respectively, but does not impact their presence (reviewed in [Catón and Tucker 2009](#); [Jernvall and Thesleff 2012](#)). We, therefore, hypothesize that there are phases of tooth development that are critical (i.e., when EKs are formed; pre- and early cap for pEK and early bell stages for sEKs) and phase that are peripheral (i.e., after EK formation; late cap for pEKs, and late bell for sEKs). We further speculate that the critical, early phases are indispensable for tooth and cusp formation and are highly conserved across species, while the peripheral later phases are more sensitive to evolutionary change and thus more variable across species. In this framework, these phases could represent modules of a tooth GRN, with the critical phase representing a core module of tooth development that controls presence/absence of structures and peripheral phases representing sub-modules that control distinct aspects of those structure's shapes.

A core gene network controls tooth initiation and development and is conserved across vertebrates

The genes that underlie the early phases of tooth development have been intensively studied in model and, over the last 10 years, non-model organisms ([Fraser et al. 2009, 2020](#); [Moustakas et al. 2011](#); [Jernvall and Thesleff 2012](#); [Rasch et al. 2016](#); [Landova Sulcova et al. 2020](#); [Sadier et al. 2020](#)). As a result, researchers have identified many of the developmental pathways that are required to make a mouse molar and its cusps: (TGF β [BMP], Fgf, HH, Wnt, Eda, and Notch; [Thesleff and Jernvall 1997](#); [Tucker and Sharpe 1999, 2004](#); [Jernvall et al. 2000](#); [Tucker et al. 2000, 2004](#); [Pispa et al. 2003](#); [Catón and Tucker 2009](#); [Jernvall and Thesleff 2012](#); [Jussila and Thesleff 2012](#); [O'Connell et al. 2012](#)). When these pathways are genetically disrupted in mouse at early stages, this generally results in arrest of tooth development at early stages, or significant changes in tooth shape. For example, manipulation of *Shh* ([Dassule et al. 2000](#)) or *Bmp4* ([Jia et al. 2013](#)) signaling at early stages of tooth development leads to a total arrest of tooth development before the cap stage, and manipulation of *Eda* or *Edar* signaling at early stages disrupts the correct establishment of the EKs ([Fig. 4](#)) ([Tucker et al. 2000](#)).

Comparative studies of transcriptomes have provided additional insights into the core developmental program controlling tooth development within a species. In particular, comparisons of the transcriptomes of the upper and lower molars ([Pantalacci et al. 2017](#)) and upper and lower molars and incisors ([Laugel-Haushalter et al. 2013](#)) in mice have

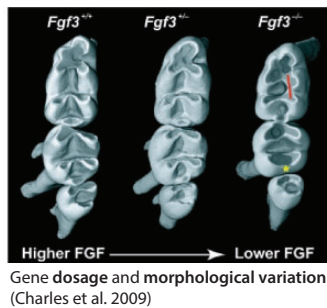
identified some key regulatory interactions with similar roles in the development of different tooth types. Other studies suggest that the core module could contain a Wnt-BMP feedback loop, and the peripheral pEK module could contain a Wnt-Shh-Sostdc1 negative feedback loop ([Cho et al. 2011](#); and see [O'Connell et al. 2012](#)). However, while some signaling pathways have been implicated in tooth developmental modules, the TFs and their controlling enhancers that regulate tooth development remain more elusive.

The core module for tooth development may be conserved across vertebrates. Studies have revealed remarkable conservation of the genes involved, and their expression patterns, at early stages of tooth development (e.g., bud to cap stages, [Fig. 2](#)) across vertebrate groups: (1) in mice and other rodents (e.g., vole; [Keränen et al. 1999](#); [Laffont et al. 2009](#)), the pEK has been shown to express a large set of genes (e.g., *Shh*, *Fgf4*, *Edar*, *Edaradd*, *Spry2*, *Wnt10a*, *p21*, *Pitx2*, and *Lef1*) in a spatially-restricted fashion during tooth induction (see <http://bite-it.helsinki.fi/>); (2) in opossum ([Moustakas et al. 2011](#)), the pEK has been shown to express *Fgf3*, *Fgf10*, *Fgf4*, *Shh*, *Spry2*, *Spry4*, (3) while no true EKs were traditionally thought to be formed in lizards and snakes, tooth signaling centers in these groups express a set of genes similar to those expressed in mammalian EKs (*Wnt6*, *Wnt7a*, *Bmp4*, *Shh*, and *Edar*; [Buchtová et al. 2008](#); [Handrigan and Richman 2010, 2011](#); [Richman and Handrigan 2011](#); [Landova Sulcova et al. 2020](#)), and a recent study revealed “EK-like” structures complete with apoptosis in the veiled chameleon as well as in crocodiles and geckos ([Landova Sulcova et al. 2020](#)); (4) a similar set of genes is conserved in bony fishes (zebrafish), medaka ([Fraser et al. 2004, 2006, 2009](#)), catshark ([Debiais-Thibaud et al. 2015](#); [Rasch et al. 2016](#)), and cichlids ([Hulsey et al. 2016](#)). Using existing and new transcriptomic data, this last study by [Hulsey et al. \(2016\)](#) found strong support for the conservation of tooth gene expression across vertebrates.

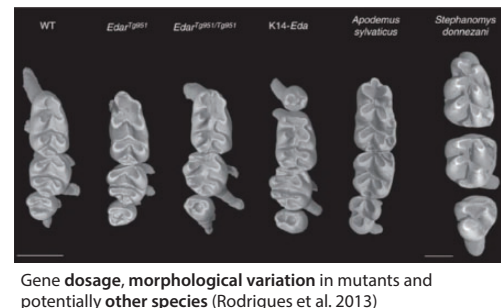
Taken together, these and other studies support the existence of a core module of genes within the tooth GRN at early stages of tooth development (bud and early cap stage) that is absolutely necessary for correct tooth development (i.e., disruption leads to developmental arrest or severely impacts shape), which has been conserved over 450 million years of vertebrate dental evolution. The genes comprising this core module include, but are likely not limited to, *B-catenin*, *Bmp4*, *Fgf3*, *Fgf10*, *Lef1*, *Pitx1*, *Pitx2*, *Shh*, and *Sox2* ([Rasch et al. 2016](#); see [Box 1](#)). Using computer science terminology, this core module

Linking sub-modules and phenotypic variation

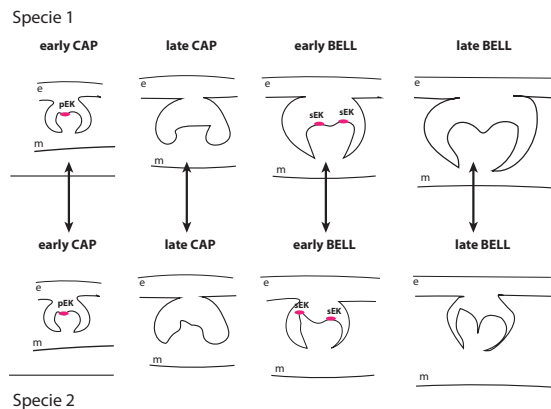
A Genetically modified mice



B Genetically modified mice, cross-species comparisons



C Transcriptomic comparison between species



D In vitro manipulations

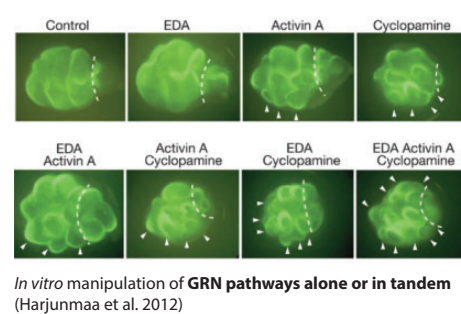


Fig. 4 Identifying the regulatory loops of modules. Identifying regulatory loops of each module require integrative approaches using morphometrics, transcriptomics, *in vitro* and genetic manipulation, species comparison, and modeling. **(A)** Genetically modified mice—genetically modified mice are useful tools to identify the role of a gene in the initiation and morphogenesis of an organ, and thus help resolve the genotype–phenotype map. In our framework, we hypothesize that dosage variation of genes implicated in the sub-modules could lead to variation in tooth morphology (e.g., width, length, or cusp number). For example, variation in FGF dosage in the *fgf3* mutant leads to variation in tooth size and cusp number (Charles et al. 2009a). **(B)** Genetically modified mice and cross-species comparison. Cross-species comparison between species and with genetically modified mice can help identifying the role of particular pathways in the development of particular morphologies seen in nature. For example, variation in *Eda* signaling through mutation (*Edar* mutants) or overexpression (*K14-Eda*) leads to variation in tooth and cusp number and size, leading to a stephanodont-like tooth morphology (Rodrigues et al. 2013). **(C)** Transcriptomic comparison between species. Transcriptomic is crucial to identify changes between the regulatory states of two or more different species. Comparison of transcriptomes of similar developmental stages between species exhibiting different tooth morphology will help identifying the regulatory loops of each tooth developmental modules. **(D)** *In vitro* manipulations—teeth can be cultured *in vitro* fairly late in their developmental processes. Because of this, it is possible to manipulate the pathways that control their development and test hypothesis regarding their implication in core and/or sub-modules. For example, the modulation of some pathways dosage *in vitro*, alone or in tandem, has been shown to increase the number of cusps and overall dental complexity (Harjunmaa et al. 2012). This suggests a circuit between these genes controls cusp number (see also Kim et al. 2019).

represents a “kernel” for the tooth development program (Fig. 3a, box 1). Additional investigation across diverse species is needed to generate a comprehensive understanding of the genes involved in this putative core module and their interactions.

Peripheral sub-modules of the tooth GRN shape tooth variation along distinct axes

The existence of a conserved, core developmental module across vertebrates poses a conundrum: If molar shape is controlled by such a robust, conserved

developmental network, then how did the great diversity in vertebrate tooth shape arise? Answering this question requires, in part, thorough comparative and quantitative analyses of tooth morphology across clades to identify: the major axes of morphological variation, and how homologous features of teeth (e.g., cusps) changed over evolutionary time and with changes in the tooth GRN. However, homologous traits are not always easily identifiable across the widely divergent tooth morphologies seen across vertebrates, rendering some current approaches (e.g., geometric morphometrics) very difficult to apply.

As a potential solution, researchers have begun to investigate this question using mouse assays, genetic manipulations, and computational *in silico* approaches. Results of these studies suggest that modifications in the expression levels of genes from the primary molar developmental signaling pathways are sufficient to modify molar shape along some axes of variation, without compromising the initial formation of the tooth and its cusps. Pathways whose perturbation modifies shape along single axes of variation, without disturbing other aspects of shape, could represent peripheral sub-modules of the tooth GRN. One such pathway is *Eda*. Disruption of *Eda* or *Edar* levels in mice modifies tooth size and crown shape in a dose-dependent manner (Fig. 4). *Eda* and *Edar* loss of function mice exhibit a trend toward a reduction of tooth size and cusp number, as well as a disorganization of tooth patterning (Tucker et al. 2000; Charles et al. 2009b). Genetically modified mice that overexpress *Edar* have misshapen molars, with phenotypes ranging from fewer cusps to more cusps, and sizes ranging from shorter/narrower to wider, both depending on the dosage (Fig. 4; Pispas et al. 2004; Tucker et al. 2004). In addition, people with the EDAR 370 A variant, which induces a higher signaling activity of the *Eda* pathway *in vitro* (Bryk et al. 2008; Mou et al. 2008), exhibit a higher prevalence of additional cusps on their molars as well as a double shoveling phenotype in their upper incisors (Park et al. 2012). Together, these results suggest that the *Eda* pathway regulates tooth traits including cusp number and tooth size and/or width.

Other genetically modified mice, for example, those misexpressing *Fgf20*, *Barx1*, *Lrp4*, *Fgf3* (Fig. 4), *Sostdc1*, or *Wnt10a*, also exhibit changes in the patterning, number, or size of teeth (Yamashiro et al. 2007; Charles et al. 2009a; Cho et al. 2011; Miletich et al. 2011; Häärä et al. 2012; Ahn et al. 2017). Within teeth, cusp number has also been shown to be regulated by *Sostdc1*-mediated cross-talk between Wnt and Shh signaling (Kim et

al. 2019) downstream of EK formation. Single and combined modification of the HH, Activin, and *Eda* pathways during mouse development can alter molar size and complexity (Harjunmaa et al. 2012, Fig. 4). In addition, variation in the temporal, spatial, and functional differences in tooth signaling center activities has been shown to be linked to variation of tooth length in the upper molars of mice (Hayden et al. 2020).

Comparative studies across rodent strains and species also provide support for the existence of peripheral, sub-modules of the tooth GRN that control the variation of specific morphological traits. For example, *in silico* manipulation of single or multiple signaling pathways within a computational model of mouse molar development (ToothMaker: Salazar-Ciudad and Jernvall 2010; Salazar-Ciudad 2012; Harjunmaa et al. 2014) can generate morphologies that mirror those observed across living and extinct rodents (Harjunmaa et al. 2014). *In vivo* comparisons of two strains of mice also have linked variation in the timing, localization, and level of EK or pre-EK gene expression to variation in tooth length within and among murine species (Hayden et al. 2020). These comparative studies suggest that the balance of activator and inhibitor levels and their regulators (e.g., *Edar*, BMP4, Activin, and/or *Shh* pathways and their regulation) within the tooth GRN are critical to shaping the variation within and among rodent species (Laugel-Haushalter et al. 2013 and Fig. 4).

In the hierarchical and modular framework proposed in our article, each of the pathways or circuits that control an aspect of tooth shape would represent a sub-module, that is, a sub-circuit of one or multiple pathways and their regulators, associated with one given trait (see examples above). Intriguingly, the comparative studies discussed here demonstrate that simple tweaking of existing GRN sub-modules within a single species can generate a pattern of morphological variation that mirrors that observed among species. At least for rodent teeth, this finding is consistent with the evolution of morphology occurring along “developmental lines of least resistance.” However, additional comparative research in model and non-model vertebrates is necessary to fully characterize the sub-modules, their regulatory loops, and their impact on morphological variation within and among species.

Evolutionary implications of core and sub-modules in the tooth GRN

A major goal of modern research on tooth diversity is to assemble a comprehensive picture of GRNs

regulating tooth shape. This goal is complicated, in part, by the fact that many pathways with roles in tooth development are pleiotropic and expressed at multiple developmental stages (see Fig. 3A for an example of a theoretical GRN), and likely has roles in multiple GRN modules. For example, *Edar* is not only expressed in EKs but also more diffusely at other stages of tooth development (Tucker et al. 2000; Sadier et al. 2019). Similarly, *Shh* is expressed at different stages of tooth development and is both a critical gene for tooth initiation (*Shh* KO leads to the arrest of tooth development at early stages, Fig. 3B) and subsequent morphogenesis (Dassule et al. 2000). However, while many genes and pathways may have pleiotropic roles in tooth development, the circuit of their interactions with other genes and pathways within a given developmental stage is often unique from that in other stages (see Box 1 and Fig. 3A). Therefore, the topology of gene interactions (i.e., the interaction map between developmental genes, TFs, and CREs) can be used to identify GRN sub-circuits that represent modules and regulate specific traits (see Box 1 and Fig. 3A; see Peter and Davidson 2009, “4.1 Subcircuits and their biological ‘jobs’”) (Fig. 4). Given this, modules are often most easily identified when a combination of techniques are used to resolve genes’ roles in tooth development and their interactions with other genes (e.g., genetic manipulation in animals, experimental manipulation *in vitro*, comparative expression assays, TF/CRE interaction assays, computational modeling).

Previous results suggest that the HH, Activin, and *Eda* pathways and some of their regulators together form a sub-module that controls the number of cusps and therefore, to some degree, dental complexity (Fig. 4; Harjunmaa et al. 2014). Analysis of *Eda* pathway variants further suggests that the *Eda* pathway, at least, is also implicated in regulating overall tooth size and tooth width (see above). These findings, and others, support the hypothesis that individual pathways can regulate different dental traits depending on the spatiotemporal context in which they are deployed. Thus, a given pathway could be part of multiple sub-modules, including, for example, the core module and sub-modules that are active at different stages during development (Fig. 3). From an evolutionary perspective, the incorporation of the same pathway in multiple modules could help explain instances in which some traits (e.g., tooth length and width) co-vary.

The existence of a hierarchical and modular structure for tooth GRNs can also help explain the repeated, independent evolution of similar tooth traits in multiple lineages. As an example of this, the molars of transgenic mice overexpressing *Eda* and

Edar display a stephanodont-like phenotype, which is characterized by longitudinal crests (Mustonen et al. 2003; Kangas et al. 2004; Tucker et al. 2004; Harjunmaa et al. 2012). Researchers have proposed that a similar modulation of *Edar* signaling drove the independent evolution of stephanodonty in the *Apodemus* and *Stephanomys* rodent lineages (Rodrigues et al. 2013; Fig. 4). This example illustrates how, in theory, the simple tweaking of a single, potential tooth sub-module could drive the repeated evolution of a complex phenotype such as stephanodonty. In a more complex example, the regulatory circuit that links *Shh*, *Wnt*, and *Sostdc1* (Cho et al. 2011) has been proposed to serve as a key coordinator of dental evolution in Murinae by regulating the number and distance among cusps, and their shape (Kim et al. 2019). Molars of *Shh*-activity-suppressed-*Sostdc1* null mice exhibit extra cusps and overall shorter distance among cusps, a morphology that is rarely seen in the genus *Mus* but has been documented in other murine genera. Thus, the *Shh*, *Wnt*, and *Sostdc1* regulatory circuit could be part of a module that controls differences in molar morphology across species via changes in cusp number and spacing (Kim et al. 2019).

Reiteration of the GRN core and sub-modules during vertebrate tooth development and evolution

An important feature of mammalian tooth development is the reuse of the same signals for the formation of the tooth itself (during the bud and early cap—pEK stage) and its cusps (during the early bell—sEK stage; Fig. 2). As outlined in the examples presented in this article, mutations that affect tooth number generally also affect cusp numbers. This suggests that the same mechanisms are required for tooth and cusp induction (Harjunmaa et al. 2012; Kim et al. 2019) and that the part of the tooth GRN that is active for the formation of a tooth is reiterated for the formation of the cusps (Jernvall and Thesleff 2000; Cho et al. 2007; Kim et al. 2019; Fig. 3). Applying the definition of GRN modules presented here, we suggest that, in mammals, cusp formation involves reiteration of the core and sub-modules, with the core module being responsible for the formation of each cusp at earlier developmental stages and the sub-modules being responsible for determining the shape of cusps at later developmental stages. Modification of the sub-modules over evolutionary time would explain differences in cusp shape among species (Figs. 2 and 4).

While the mechanisms behind the morphogenesis of multicuspid teeth (e.g., sEKs, etc.) are well understood in mammals, the situation in other vertebrates

Box 1 Definitions and putative list of core module genes

Developmental GRN: Circuits of genes and TFs that govern the development of an anatomical element (e.g., a molar) or an organism.

GRN module: A sub-circuit of the GRN (comprising genes and their TFs and regulatory interactions). The output of a module executes a given developmental function (e.g., initiate a tooth; controlling the height of a cusp), see Fig. 3A.

Core module: Module of the GRN that is essential for the induction of the development of an anatomical element (e.g., setting up the initial domain).

Putative core module genes list: *B-catenin*, *Bmp4*, *Fgf3*, *Fgf10*, *Lef1*, *Pitx1*, *Pitx2*, *Shh*, and *Sox2* (as identified in Rasch et al. 2016). Given their central role in tooth signaling centers and tooth patterning, and their potentially conserved evolutionary role, other possible candidates could include but are not restricted to *Eda*, *Edar* and *Edaradd*, *Sostdc1*.

Sub-module: Module at the periphery of the GRN that has secondary functions in the development of an anatomical element (e.g., patterning one part of the element or controlling one axis of variation), downstream of the core module.

is less clear. In particular, the existence in other vertebrates of structures homologous to mammalian EKs is debated, in particular, in other vertebrates with multicuspid teeth. While EKs are thought to be a mammalian innovation (Weeks et al. 2013), cell division, histology, and gene expression data suggest that “EK-like” structures might also participate in the formation of cusps in non-mammalian species with multicuspid teeth. In catsharks (Rasch et al. 2016), a signaling center that expresses *Bmp4*, *Fgfs 3-10*, *Ptc2*, and *Shh* and bears non-dividing cells have been observed at the apex of each cusp in multicuspid teeth, a finding that suggests homology with EKs. In cichlid fishes, EK-like structures have also been observed (in particular, regarding the restricted expression patterns of signaling center genes, see Fraser et al. 2008, 2013) and are thought to arise through mechanisms resembling those seen in mammals (Streelman et al. 2003). In squamates, the existence of “EK-like” structures is more equivocal. In the ball python, bearded dragon, leopard gecko, and alligator, the same set of genes is expressed during tooth or cusp induction, but their expression does not follow the spatially-restricted pattern that characterizes mammalian EKs (Handrigan and Richman 2011; Richman and Handrigan 2011; Weeks et al. 2013), suggesting that reptiles do not possess structures homologous to mammalian EKs. However, more recent studies in anole and chameleon have revealed that their tooth cusps are formed via folding of the inner epithelium, a process that is reminiscent of the manner in which mammalian cusps are formed (Zahradnick et al. 2014). Further, “EK-like” structures complete with EK-like apoptotic and molecular signatures have been characterized in the veiled chameleon (Landova Sulcova et al. 2020). Altogether, previous work suggests that EK-like structures for cusp formation may be conserved across vertebrates, with extreme specialization in mammals, and might have

been lost in some lineages (e.g., some squamates). Studies of such structures and their characteristics (e.g., expression patterns, apoptosis) in a wider range of taxa are needed to confirm this idea. If EK-like structures are widely present across vertebrates, this would provide an important line of evidence that the core and sub-modules are active in a reiterative manner for the formation of multicuspid teeth in vertebrates.

Conclusion

Investigations of the role of GRN modules in shaping the pattern of morphological evolution have remained largely theoretical (reviewed in Schlosser and Wagner 2004; Klingenberg 2010, 2014; Deline et al. 2018; Uller et al. 2018). Given the current knowledge of vertebrate tooth development, we propose that this organ provides an excellent system with which to test this hypothesis from developmental and evolutionary points of view. Investigating the factors that underlie tooth development and evolution from a modular perspective holds great potential to fill many important knowledge gaps. First, it could help explain why patterns of expression of tooth GRN genes are restricted and highly conserved at early stages of tooth development (such as bud and early cap), and less constrained and more variable at later stages (cap/late cap). Second, this framework could explain why tooth morphology at bud/early cap stages is generally more conserved among species, whereas tooth morphology at the cap stage—when adult tooth shape starts to establish—varies highly among species. Ultimately, a full understanding of tooth GRN components, their links, and spatial and temporal variation will contribute to defining the intrinsic factors and mechanisms that underlie the high morphological diversity of vertebrate dentitions.

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