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Enhancer pleiotropy and cell proliferation dynamics of tooth regeneration in threespine stickleback fish

By

Alyssa Joy Rowley

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Molecular and Cell Biology

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Craig T. Miller, Chair

Professor Richard Harland

Professor Hernan Garcia

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Abstract

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The greatest symphony is found in the composition of life. A large-scale consonance of sounds, an arrangement of different shapes and sizes. Within the symphony are many foundational players. Populations make up each movement and follow their own structure and format. Proteins compose the melody that confer this structure and this melody must harmonize the notes of DNA and RNA to avoid dissonance. Darwin diligently studied each measure within a movement to try and piece together their origins. His theory of natural selection realized how the harmonizing of consonance notes of a melody will be favored, survived, and thrive while the dissension of sound will be phased out and cease to exist. Mendel listened and observed how these notes are sung and inherited from one generation to the next and how the variation in alleles lead to different chord progressions. Evolved differences among organisms serve as an arrangement: a musical reconceptualization of a previously composed work. It may differ from the original work by means of reharmonization, melodic paraphrasing, orchestration, development or regeneration of the formal structure. The resulting organism is similar but also different in many ways. Almost a century later, Huxley reprised these ideas into the Modern Synthesis, the changes in chord progression, or allele frequency, within a movement, or population. Not much later Watson, Crick, and Franklin identified the notes that make up genes and alleles as DNA. This dissertation aims to describes just a few of the instruments responsible for regulating how and when these melodies are played.

All living things have certain characteristics in common: we have cellular organization, the ability to reproduce, grow, develop, respond and adapt to our environment. Chapter One will provide a brief overview of the field of evolutionary biology past and present. It will review the origins of teeth, why they make an excellent model system for studying evolution and the relationships they share with other regenerating epithelial appendages in morphology and genetic regulation. It will describe how small changes in the notes of non-coding regions of DNA can lead to large phenotypic differences and defend why these regions are the source for driving

evolution and melodic diversity among living things. It will also explain why the threespine stickleback fish, *Gasterosteus aculeatus,* is the model organism of choice to gain a better understanding of the greater symphony. Chapter Two will play the instruments of genetic regulation, pleiotropy, and transcriptional enhancers. We investigated the pleiotropy of an intronic enhancer of the stickleback *Bmp6* gene, previously shown to regulate evolved changes in tooth number and tooth regeneration. Transgenic analysis and site-directed mutagenesis experiments both deleting and scrambling highly conserved notes in the binding site for a predicted Foxc1 transcription factor revealed that this binding site is required for enhancer activity in both teeth and fins throughout embryonic, larval, and adult development. Collectively these data support a model where the melody of this enhancer depends on the very specific notes of this binding site in order to be played in more than one tissue from the embryo to the adult. Finally, Chapter Three will reveal arranged evolutionary differences on a cellular measure, assessing proliferation dynamics and cell cycling kinetics. Cell division is a vital process for long-term renewal of organs but the tempo of proliferation rates of slow cycling cells can vary greatly based on cell type, tissue, sex, age, health and organism. Exploiting the natural variation between marine and freshwater stickleback populations and the dynamic variations of slow cycling, label-retaining cells (LRCs), we reveal evolved reductions in LRCs in the adult pharyngeal dentitions of a high-toothed, freshwater population relative to an ancestral low-toothed marine population. These findings are some of the first to demonstrate the evolved differences in tempo of proliferating cells which give rise to evolved regeneration differences between populations. Together these completed works are a score in the greater musical work of the symphony of life and aims to amplify the sound of evolution and developmental biology.

“It takes a fearless, unflinching love and deep humility to accept the universe as it is. The most effective way… to accomplish that… is the scientific method, which over time winnows out deception. It can't give you absolute truth because science is a permanent revolution, always subject to revision, but it can give you successive approximations of reality.”

― Ann Druyan

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Chapter 1: Introduction

"There is grandeur in this view of life… from so simple a beginning endless forms most beautiful and most wonderful have been, and are being evolved."
– Charles Darwin, On the Origin of Species (1859)

1.1 The beginning

The observations our species have made of the natural world have been profound, inspiring, and fostered our desire to understand the origins of life. We are fortunate to indulge ourselves with the diversity of life our planet has to offer. The complexity of flora and fauna has inspired art, love, philosophy, and science. Charles Darwin's theory of Natural Selection describes how specific traits considered favorable in a given environment can increase that organism's ability to survive and reproduce (Darwin, 1859). Gregor Mendel's research in pea plants unveiled basic patterns of inheritance, in which heritable traits can be passed from parents to offspring (Mendel, 1865). Combing these theories with population genetics, Julian Huxley published the Modern Synthesis which emphasized the genetic basis of evolution and proposed a new definition of evolution as "changes in allele frequencies within populations" (Huxley, 1942). However, with great diversity, also comes great homology and we have come to learn that many traits derive from a common evolutionary origin. Not only do these traits hold historical continuity in their morphological similarities, but also genetic continuity in which regulatory circuitry was inherited from a common ancestor (Shubin et al., 2009). Existing genes can be redeployed into new places and at new times over development to generate new traits (Li et al., 2015; Shubin et al., 2009). Investigations into the evolutionary origin of epithelial appendage regeneration within and between species, must take into consideration both the morphological similarities and conserved genetic programming that serve to develop, maintain, and regenerate an organ.

1.2 On the origins of teeth, and other epithelial appendages

"The skin and the appendages of hair, feathers, hoofs, horns, and teeth, are homologous over the whole body… and… are apparently connected."
- Charles Darwin, The Variations of Animals and Plants Under Domestication (1875)

A tooth is not made of bone but of a hard, calcified structure derived from multiple tissues of varying density that originate from the embryonic ectoderm or endoderm germ layer and the neural crest. For more than 30 years, teeth have served as an excellent model for studying evolution and organogenesis. Given the structural integrity, hardness, and preservability, teeth have contributed greatly to the vertebrate fossil record. The earliest fossil dentition dates back almost 400 million years ago in the most phylogenetically basal jawed vertebrates with teeth, called acanthothoracids (Vaškaninová et al., 2020). There are two classic theories that have been debated over the evolutionary origin of teeth 1) the 'inside-out' model where patterned odontodes evolved independently from jaws from endoderm inside pharyngeal denticles, and migrated outward (Smith & Coates, 1998) and 2) the 'outside-in' model which skin denticles evolved first and odontodes evolved at the same time as, or after, jaws by merging inside the oral cavity to form teeth (Reif, 1982).

The birth of a tooth is regulated by reciprocal epithelial-mesenchymal interactions, with the epithelium arising from the ectoderm and mesenchyme from the neural crest (Soukup et al., 2008). The development and formation of a tooth shares many morphological features with other ectodermal organs, such as hair, scales, and feathers. These featured stages include the initial epithelial invagination, called bud and cap stages, followed by various lengths of bell stage which contribute to specific cusp patterns and overall growth of the tooth. Finally, mesenchymal odontoblasts differentiate to form dentin and epithelial ameloblasts secrete enamel or enameloid to create a hard, functional tooth (Leche, 1895). Some animals, like rodents, are monophyodont and develop only one set of teeth, while others like humans, are diphyodonts and develop two successive sets of teeth known as deciduous and permanent. The ancestral dental phenotype of jawed vertebrates is polyphyodonty, or continuous tooth replacement, a trait retained in sharks, fish, and reptiles. The threespine stickleback fish, *Gasterosteus aculeatus*, retains the ancestral vertebrate condition of polyphyodonty and is our model organism of interest for studying evolved tooth gain and regeneration.

In addition to the similarities that teeth, hair, scales, and feathers, share in histogenesis, there is increasing evidence for homology within genetic regulatory networks between all epithelial appendages (inner and outer ornamental epithelial-derived organs which regenerate). For example, regenerating teeth in zebrafish and sticklebacks, which are around 250 million years diverged (Near et al., 2012), have been shown to express a conserved battery of nine genes, which are also dynamically expressed in regenerating hair follicles (Square et al., 2021). In mice, these nine genes are also expressed in hair follicle stem cells (HFSCs) and play important roles in regulating hair regeneration (Kandyba et al., 2013). These findings, along with many other examples, support an overarching principle in which cells and tissues use a conserved network to communicate during development and that a similar conserved network may coordinate the regeneration of different organs throughout time. The similarities in histogenesis, gene expression, and development suggest that hair and teeth may regenerate using a conserved genetic regulatory network that could be broadly applied to regeneration of other ectodermal organs. This dissertation investigates the similarities and differences that teeth share within and between species to gain a better understanding of the origin and evolution of teeth.

1.3 Threespine sticklebacks as a model system

At the end of the last glacial maximum, coastal areas flooded and were left exposed, allowing ancestral marine sticklebacks to colonize freshwater lakes and streams across the northern hemisphere. These colonization events led to the adaptive radiation of the threespine stickleback (*Gasterosteus aculeatus*) and created a rich diversity of phenotypes that has fostered the study of morphological evolution (Bell & Foster, 1994). During mating, marine sticklebacks travel to freshwater environments repeatedly and independently to spawn and establish new resident populations (Jones et al., 2012; McKinnon & Rundle, 2002). The settlement of stickleback populations in novel environments with new prey and predators fueled the evolution of morphological traits, including reduced armor plating (Colosimo, 2005; Cresko et al., 2004; Indjeian et al., 2016), body shape (Albert et al., 2007; Reid & Peichel, 2010; Walker & Bell, 2000), pelvic spines (Chan et al., 2010), pigmentation (Miller et al., 2007), and craniofacial bones associated with feeding, like branchial bone length (Erickson et al., 2014) and gill raker number (Glazer et al., 2014). Recent studies identified a near twofold increase in ventral pharyngeal tooth number in derived freshwater benthic fish, which are adapted to live on the

bottom of a lake, compared to anadromous marine fish, which migrate up rivers from the sea to spawn (Cleves et al., 2014). The pharyngeal teeth are a crucial part of the branchial skeleton and are found in the throat. Sticklebacks have two dorsal pharyngeal tooth plates (DTP) and one ventral pharyngeal tooth plate (VTP). While the oral jaw is typically used for catching and gripping in fish, the pharyngeal teeth are thought to carry out mastication (Wainwright, 2005). Many of these evolved traits are due to changes affecting non-coding sequence (Jones et al., 2012). Differences in regulatory regions between marine and freshwater fish were identified in genes associated with reduction in armor plating (Colosimo, 2005; Indjeian et al., 2016), pelvic spines (Chan et al., 2010), and pigmentation (Miller et al., 2007), as well as increases in branchial bone length (Erickson et al., 2014) and pharyngeal tooth number (Cleves et al., 2014, 2018). These findings support a strong case for the study of non-coding regulatory elements in evolutionary biology.

In the 1950s, Niko Tinbergen established the threespine stickleback as a model system in ethology for use in behavioral studies (Tinbergen, 1952). In addition to the plethora of diverse morphological traits, sticklebacks offer distinct advantages for evolutionary and developmental genetic studies, such as large clutch size, compact and sequenced genome, and efficient transgenic and genome editing methods. Adult marine and freshwater fish are easy to maintain in captivity and can produce a range of 100-300 externally fertilized embryos per mating that hatch in approximately 10 days. A high quality assembly of the ~450Mb stickleback genome has been sequenced in 21 populations (Jones et al., 2012) and improved by a high-resolution linkage map (Glazer et al., 2015) and quantitative trait loci (QTL) mapping (Albert et al., 2007; Miller et al., 2007). The large size of the stickleback embryo allows for transgenesis via microinjection (Kingsley et al., 2004), an avenue for the application of genome editing tools such as Tol2, CRISPR/Cas9, and BACs (Erickson et al., 2015; Erickson et al., 2016; Hart & Miller, 2017; Wucherpfennig et al., 2019). Together, these qualities have made the threespine stickleback an excellent resource for studying evolution and development and understanding genetics through functional manipulation.

1.4 Evolved tooth gain

Teeth have long served as a classic model system of organogenesis since tooth development and regeneration are orchestrated by reciprocal epithelia-mesenchymal interactions which are repeated throughout the adult life of most non-mammalian vertebrates (Jernvall & Thesleff, 2012; Pispa & Thesleff, 2003). Teeth also serve as one of the distinct morphological differences between marine and freshwater stickleback populations. A benthic freshwater population from Paxton Lake, Canada was shown to have nearly two-fold higher tooth number compared to their ancestral marine population from Rabbit Slough, Alaska (Cleves et al., 2014). The increase in tooth number was associated with new teeth developing on the edges of the tooth plate. However, a different freshwater population found in Cerrito Creek, California had an evolved increase in tooth number linked with increased tooth regeneration rates (Ellis et al., 2015) but a completely different set of QTL regulating tooth number. These data support two convergently evolved developmental genetic mechanisms underlying tooth number in sticklebacks.

Interested in understanding the relationship between genotype and phenotype, genome-wide quantitative trait locus (QTL) mapping found the largest effect QTL controlling stickleback tooth gain in the chromosome XXI QTL cluster which explains almost one third of the variance in pharyngeal tooth number. Deeper examination with fine mapping identified a regulatory allele

in an enhancer in the fourth intron of *Bone morphogenetic protein 6* (*Bmp6*), a gene previously found to have a cis-regulatory change in dental tissue that is highly associated with evolved increases in tooth number (Cleves et al., 2014). *In situ* hybridization data shows *Bmp6* is dynamically expressed with spatiotemporal pattern differences in the two populations with marine *Bmp6* mostly localized to the mesenchyme and freshwater *Bmp6* mostly distributed throughout the inner dental epithelium in late bell stages during tooth development (Ellis et al., 2016; Stepaniak et al., 2021). Quantification of allele specific expression ratios from tooth plates from an F1 hybrid cross of high-toothed PAXB and low-toothed RABS revealed a 1.4 fold decrease in *Bmp6* expression in the freshwater compared to the marine allele, in adult but not juvenile fish (> 20 mm standard length fish) (Cleves et al., 2014). BMPs play a critical role in activating and inhibiting mammalian tooth development (Åberg et al., 1997; Bei & Maas, 1998; Chen et al., 1996; Neubüser et al., 1997; Zhao et al., 2000). The PAXB freshwater *cis*-regulatory down-regulation in late, not early, stages of tooth development suggested that *Bmp6* may inhibit tooth development or replacement (Cleves et al., 2014). To better understand the role *Bmp6* plays in evolved tooth number differences, it is important to investigate the genetic regulation of *Bmp6* expression. Chapter Two identifies a key transcription factor binding site in the intronic *Bmp6* enhancer which is required for normal *Bmp6* enhancer activity.

1.5 Cis-regulatory changes and enhancer pleiotropy

The structure of an organ determines function. This structure is defined by proteins regulated by genes controlling cellular processes during development. The central dogma of molecular biology as described in 1958 was an explanation of the flow of genetic information within a biological system: DNA is transcribed as RNA and RNA is translated to make protein (Crick, 1958). In 1983, Walter Gehring and his collaborators, discovered a group of homeotic genes, the homeobox, that control the pattern of body formation during early embryonic development in *Drosophila,* directly linking genotype (DNA) to phenotype (expressed trait). These homeotic genes are not only present in arthropods and their ancestors, but also in vertebrates like humans (Gehring & Hiromi, 1986). Mutations in these genes lead to severe phenotypic deformities since they are essential for controlling early development over time and space (Nüsslein-volhard & Wieschaus, 1980; Sabarís et al., 2019). Considering the high degree of protein homology between species, like the coding sequences of chimpanzees and humans, a mutation in coding regions would likely lead to deleterious phenotypes in the developing organism (King & Wilson, 1975). This suggested that the vast differences between the evolution and development of various species must lie in the non-coding, regulatory regions of genes which dictate when and where the protein is expressed. The differences in gene expression pattern during development correlate with morphological diversity among species and several studies support the idea that evolution in regulatory, not coding, regions are the basis of morphological differences (Carroll, 1995; King & Wilson, 1975). Gene expression variation can occur via cis- or trans-regulation. Cis-regulatory elements (CREs) regulate the transcription of nearby genes and include non-coding regions like promoters, enhancers, and silencers (Jacob & Monod, 1961; Stanojevic, Small, & Levine, 1991). Whereas trans-regulatory elements (TREs) are DNA sequences encoding upstream regulators (transcription factors, small RNAs, and enzymes) that modify or regulate the expression of distant genes by combining with their target sequences (Cowles et al., 2002; Pai & Gilad, 2014; Prud'homme et al., 2007). CREs control dynamic expression patterns of genes during early development and therefore are considered to be a central region in which structural and functional changes give rise to morphological evolution (Furlong & Levine, 2018).

Proteins responsible for pattern regulation were found to have several CREs containing binding sites for multiple transcription factors which control gene expression in different tissues at different stages of development (spatial-temporal regulation) (Carroll, 1995).

Many genes are integral to canonical signaling pathways (e.g. Wnt, Bmp, Fgf) which have been repeatedly coopted for regulating expression in different cell types for different structures throughout a developing organism (Pires-daSilva & Sommer, 2003). What differentiates these tissues, despite having a deployment of similar genes, is how these genes are expressed in time and space. Pleiotropy occurs when a gene influences more than one phenotypic trait. A pleiotropic gene is involved in regulating multiple phenotypes, and a pleiotropic enhancer regulates the expression of a gene in two or more tissues. An enhancer region that is pleiotropic can be regulated by a) transcription factors binding to the same site (site pleiotropy) or b) transcription factors interacting with different binding sites within a defined enhancer region. The specificity of this type of regulation allows the same gene to be expressed or repressed in different locations at different times for tissue-specific patterning and development (Pennacchio et al., 2013). Enhancer evolution of cis-regulatory regions can happen rapidly, maintain similar functions, alter developmental patterning, and instigate morphological diversity (Fisher et al., 2006; Rebeiz & Tsiantis, 2017). Chapter 2 dissects the pleiotropic effects of a *cis*-regulatory enhancer of *Bmp6* and analyzes differences in tissue specific expression.

1.6 BMP signaling & shared genetic networks

Previous studies have revealed shared genetic regulatory networks responsible for formation of teeth and hair. For example, mutations in *Ectodysplasin* or the gene encoding its receptor *Edar* in humans, fish, mice, and dogs disrupt tooth and hair development (Harris et al., 2008; Headon et al., 2001; Pispa et al., 2008; Sadier et al., 2014; Waluk et al., 2016). In mice, *Bmp6* levels are elevated in adult HFSCs and BMP signaling is thought to counteract activating Wnt signaling that drives HFSCs to lose quiescence and regenerate the next hair follicle (Blanpain et al., 2004; Kobielak et al., 2007). Essential mesenchyme and epithelium interactions for tooth development are regulated by FGF and BMP signaling (Bei et al., 2000; Mandler & Neubüser, 2001; Vainio et al., 1993; Wang et al., 2012). In fish, a *cis*-regulatory allele of *Bmp6* underlies evolved tooth number and regeneration in freshwater compared to marine populations (Cleves et al., 2014) and comparative RNA sequencing of dental tissue from loss-of-function, *Bmp6* mutant sticklebacks revealed significant downregulation of genes expressed in mouse hair follicle stem cells (HFSCs) in a BMP-dependent fashion (Cleves et al., 2018). Interestingly, the fourth intron of *Bmp6* contained a pleiotropic enhancer sufficient to drive expression in developing fins and teeth and further fine mapping revealed a predicted Foxc1 transcription factor binding site (Berry et al., 2008; Cleves et al., 2018). In mice, *Foxc1* regulates hair regeneration such that a conditional knockout produces an increase in hair regeneration rates and has been shown to directly regulate *Bmp6* (Wang et al., 2016). Investigations into the similarities of gene regulatory networks in fish teeth and mammalian hair may shed light on a broader role that BMPs play in regulating epithelial appendage regeneration and how regulatory changes influence the evolution of morphological differences between similar organs.

1.7 FOX genes

Fox transcription factors are important for the formation of tissues from all three germ layers and several developmental processes during organogenesis (Tümer & Bach-Holm, 2009). *Foxc1* has been found in both protostomes and deuterostomes and is one of the few *Fox* genes that has been maintained since the time of early bilaterians and most likely evolved as a distinct gene before the Cambrian radiation of bilaterians (Mazet et al., 2006). FOXC1 is proposed to have a key role in skeletal, cardiovascular, renal, ocular, and cerebral morphogenesis (Hannenhalli & Kaestner, 2009; Lehmann et al., 2003). Nonfunctional mutations resulting in *Foxc1*- null alleles, cause early death when homozygous in mice with a wide range of defects including, skeletal, ocular, cardiovascular, cerebellar, as well as hydrocephalus (Kume et al., 2001; Rice et al., 2003; Seo & Kume, 2006).

In humans, mutations in *FOXC1* are associated with Axenfeld-Rieger syndrome (ARS), a rare autosomal dominant disorder that encompasses a selection of congential malformations characterized by the anterior segment dysgenesis of the eye. The most common defects include point mutations but chromosome rearrangements in the *FOXC1* gene and of the chromosome region 6p25 also lead to ARS (Chanda et al., 2008; Gould et al., 2004). Patients with mutations in the 6p25 chromosomal region frequently present malformations also seen in the mouse model, including ocular, craniofacial, skeletal, cardiac, and renal defects as well as hydrocephalus (DeScipio et al., 2005; Gould et al., 2004; Le Caignec et al., 2005). With some presenting systemic malformations such as mild tooth abnormalities (microdontia, hypodontia, oligodontia and adontia) (Lin et al., 1999). Similarly, deletions or duplications spanning the *FOXC1* gene lead to the most common human cerebellar malformation, Dandy-Walker syndrome, and contribute to cerebellar vermis hypoplasia (CVH), megacisterna magna (MCM) (Aldinger et al., 2009). *FOXC1* is dosage sensitive and changes in concentration of functional protein or inactivation through haploinsufficiency have shown disease-causative mechanisms. Studies have also revealed that duplication of *FOXC1* contributes to the development of ARS, by overcoming the repressive activity of PITX2 (Berry et al., 2006).

Given the importance of *Fox* genes in development and gene regulation, they are likely to be a family of genes that are heavily conserved in their coding region, genomic location, and *cis*-regulatory elements, since a change in any of these could result in deleterious effects on the organism. The predicted Foxc1 TFBS found in sticklebacks is one of the most conserved sites in the intron four enhancer region of *Bmp6*. Given its high sequence conservation, it is possible that this pleiotropic binding site may function as a proto-enhancer that has been constrained throughout evolution. Previous studies suggest that proto-enhancers serve as a nucleation point on which more TFBSs can evolve and enhancer cores can form, diversifying function, regulation, and expression pattern (Emera et al., 2016). Teleosts underwent a whole genome duplication (Amores et al., 1998), resulting in two co-orthologs for *Foxc1* in sticklebacks: *Foxc1a* and *Foxc1b.* The different co-orthologs could be supporting distinct roles in regulating the genetic networks necessary for various stages of tooth development. In general, paralogous transcription factors that share a similar DNA recognition motif can diversify and have divergent expression domains, such that one paralog is expressed at a high level in a particular cellular context over another (L. N. Singh & Hannenhalli, 2008). Chapter 2 reveals the differential expression patterns of *Foxc1* genes in stickleback dental epithelia and mesenchyme and a pleiotropic binding site required for driving the intron four *Bmp6* enhancer in teeth and fins.

Future investigations on the mechanistic roles of *Foxc1a* and *Foxc1b* could elucidate the roles these transcription factors play in regulating development, tooth regeneration, and BMP signaling.

1.8 Stem cells and slow-cycling kinetics

The ability of a developing organism to have specialized organs which grow, maintain homeostasis, and regenerate after injury depends on stem cells. Stem cells are the raw, unspecified source for long-term renewal of the body and are essential for living organisms. Considering the vital role these cells play in regeneration and normal physiological processes, it has long been proposed that these cells would be few, preserved in a defined niche, and only proliferate when necessary. The first postulation of a very small number of cells, and possibly just one cell, giving rise to colonies of viable cells dates back almost 50 years ago (Till & McCulloch, 1961). However, more recent studies on the proliferation rates of slow cycling cells have found turnover rates vary greatly among different tissues. In mice, the proliferation rate of mammary epithelial cells was 2.9% per day while vascular smooth muscle cells divided drastically slower, with a half-life between 270-400 days (Neese et al., 2002). The lifespan of a cell in humans also varies across cell type and tissue, with gut epithelia lasting 3-5 days and cardiomyocytes and neurons lasting for years. A recent study found that the total turnover rate of the human body is 330 ± 20 billion cells (80 ± 20 grams) per day, of which approximately 86% of cells are blood cells from bone marrow and 14% of the remaining cells are gastrointestinal and gut cells (Sender & Milo, 2021).

The stem cells supporting these populations of cells must proportionally have varied cycling kinetics to replenish the lost or damaged supply of cells. Cell turnover rates can vary based on cell type, tissue, sex, age, health and organism. The presence of 'slow-cycling' label-retaining cells (LRCs) has already been established in the dentition of multiple polyphyodont species, including leopard geckos (Handrigan et al., 2010), alligators (P. Wu et al., 2013), and sharks (Martin et al., 2016). However, other studies in bichir and salmon have exposed the apparent lack of slow-cycling LRCs in oral teeth (Vandenplas et al., 2014, 2016). While sticklebacks retain the ancestral gnathostome characteristic of continuous tooth replacement and differences in tooth number and regeneration rates have previously been described, little is known about the cell cycling kinetics of regenerating teeth. What role does cell turnover rate play in tooth regeneration differences between marine and freshwater populations? Chapter 3 investigates the cell proliferation rates that vary based on evolved differences, environment, and population.

1.9 References

Abduweli, D., Baba, O., Tabata, M. J., Higuchi, K., Mitani, H., & Takano, Y. (2014). Tooth replacement and putative odontogenic stem cell niches in pharyngeal dentition of medaka (*Oryzias latipes*). *Microscopy*, *63*(2), 141–153. https://doi.org/10.1093/jmicro/dft085

Åberg, T., Wozney, J., & Thesleff, I. (1997). Expression patterns of bone morphogenetic proteins (Bmps) in the developing mouse tooth suggest roles in morphogenesis and cell differentiation. *Developmental Dynamics*, *210*(4), 383–396. https://doi.org/10.1002/(SICI)1097-0177(199712)210:4<383::AID-AJA3>3.0.CO;2-C

Albert, A. Y. K., Sawaya, S., Vines, T. H., Knecht, A. K., Miller, C. T., Summers, B. R., … Schluter, D. (2007). The genetics of adaptive shape shift in stickleback: pleiotropy and effect size. *Evolution*, *0*(0), 071115145922005-??? https://doi.org/10.1111/j.1558-5646.2007.00259.x

Aldinger, K. A., Lehmann, O. J., Hudgins, L., Chizhikov, V. V., Bassuk, A. G., Ades, L. C., … Millen, K. J. (2009). FOXC1 is required for normal cerebellar development and is a major contributor to chromosome 6p25.3 Dandy-Walker malformation. *Nature Genetics*, *41*(9), 1037–1042. https://doi.org/10.1038/ng.422

Amores, A., Force, A., Yan, Y. L., Joly, L., Amemiya, C., Fritz, A., … Postlethwait, J. H. (1998). Zebrafish hox clusters and vertebrate genome evolution. *Science*, *282*(5394), 1711–1714. https://doi.org/10.1126/science.282.5394.1711

Arnold, K., Sarkar, A., Yram, M. A., Polo, J. M., Bronson, R., Sengupta, S., … Hochedlinger, K. (2011). Sox2+ Adult Stem and Progenitor Cells Are Important for Tissue Regeneration and Survival of Mice. *Cell Stem Cell*, *9*(4), 317–329. https://doi.org/10.1016/J.STEM.2011.09.001

Bei, M, Kratochwil, K., & Maas, R. L. (2000). BMP4 rescues a non-cell-autonomous function of Msx1 in tooth development. *Development (Cambridge, England)*, *127*(21), 4711–4718. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11023873

Bei, Marianna, & Maas, R. (1998). FGFs and BMP4 induce both Msx1-independent and Msx1-dependent signaling pathways in early tooth development. *Development*, *125*(21), 4325–4333. https://doi.org/10.1242/dev.125.21.4325

Bell, M. A., & Foster, S. A. (1994). The Evolutionary Biology of the Threespine Stickleback. *Oxford University Press*, *64*(3), 418. https://doi.org/10.2307/5902

Berry, F. B., Lines, M. A., Oas, J. M., Footz, T., Underhill, D. A., Gage, P. J., & Walter, M. A. (2006). Functional interactions between FOXC1 and PITX2 underlie the sensitivity to FOXC1 gene dose in Axenfeld-Rieger syndrome and anterior segment dysgenesis. *Human Molecular Genetics*, *15*(6), 905–919. https://doi.org/10.1093/hmg/ddl008

Berry, F. B., Skarie, J. M., Mirzayans, F., Fortin, Y., Hudson, T. J., Raymond, V., … Walter, M. A. (2008). FOXC1 is required for cell viability and resistance to oxidative stress in the eye through the transcriptional regulation of FOXO1A. *Human Molecular Genetics*, *17*(4), 490–505. https://doi.org/10.1093/hmg/ddm326

Blanpain, C., Lowry, W. E., Geoghegan, A., Polak, L., & Fuchs, E. (2004). Self-renewal, multipotency, and the existence of two cell populations within an epithelial stem cell niche. *Cell*, *118*(5), 635–648. https://doi.org/10.1016/j.cell.2004.08.012

Bloomquist, R. F., Fowler, T. E., An, Z., Yu, T. Y., Abdilleh, K., Fraser, G. J., … Streelman, J. T. (2019). Developmental plasticity of epithelial stem cells in tooth and taste bud renewal.

Proceedings of the National Academy of Sciences of the United States of America, *116*(36), 17858–17866. https://doi.org/10.1073/pnas.1821202116

Boffelli, D., Nobrega, M. A., & Rubin, E. M. (2004, June). Comparative genomics at the vertebrate extremes. *Nature Reviews Genetics*. https://doi.org/10.1038/nrg1350

Bonferroni, C. E. (1936). Teoria statistica delle classi e calcolo delle probabilità. *Pubblicazioni Del R Istituto Superiore Di Scienze Economiche e Commerciali Di Firenze*.

Carroll, S. B. (1995). Homeotic genes and the evolution of arthropods and chordates. *Nature*, *376*(6540), 479–485. https://doi.org/10.1038/376479a0

Chan, Y. F., Marks, M. E., Jones, F. C., Villarreal, G., Shapiro, M. D., Brady, S. D., … Kingsley, D. M. (2010). Adaptive evolution of pelvic reduction in sticklebacks by recurrent deletion of a pitxl enhancer. *Science*, *327*(5963), 302–305. https://doi.org/10.1126/science.1182213

Chanda, B., Asai-Coakwell, M., Ye, M., Mungall, A. J., Barrow, M., Dobyns, W. B., … Lehmann, O. J. (2008). A novel mechanistic spectrum underlies glaucoma-associated chromosome 6p25 copy number variation. *Human Molecular Genetics*, *17*(22), 3446–3458. https://doi.org/10.1093/hmg/ddn238

Chen, Y. P., Bei, M., Woo, I., Satokata, I., & Maas, R. (1996). Msx1 controls inductive signaling in mammalian tooth morphogenesis. *Development*, *122*(10), 3035–3044. https://doi.org/10.1242/dev.122.10.3035

Cirillo, L. A., Lin, F. R., Cuesta, I., Friedman, D., Jarnik, M., & Zaret, K. S. (2002). Opening of compacted chromatin by early developmental transcription factors HNF3 (FoxA) and GATA-4. *Molecular Cell*, *9*(2), 279–289. https://doi.org/10.1016/S1097-2765(02)00459-8

Clavel, C., Grisanti, L., Zemla, R., Rezza, A., Barros, R., Sennett, R., … Rendl, M. (2012). Sox2 in the dermal papilla niche controls hair growth by fine-tuning BMP signaling in differentiating hair shaft progenitors. *Developmental Cell*, *23*(5), 981–994. https://doi.org/10.1016/j.devcel.2012.10.013

Cleves, P. A., Ellis, N. A., Jimenez, M. T., Nunez, S. M., Schluter, D., Kingsley, D. M., & Miller, C. T. (2014). Evolved tooth gain in sticklebacks is associated with a cis-regulatory allele of Bmp6. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(38), 13912–13917. https://doi.org/10.1073/pnas.1407567111

Cleves, P. A., Hart, J. C., Agoglia, R. M., Jimenez, M. T., Erickson, P. A., Gai, L., & Miller, C. T. (2018). An intronic enhancer of Bmp6 underlies evolved tooth gain in sticklebacks. *PLOS Genetics*, *14*(6), e1007449. https://doi.org/10.1371/journal.pgen.1007449

Colosimo, P. F. (2005). Widespread Parallel Evolution in Sticklebacks by Repeated Fixation of Ectodysplasin Alleles. *Science*, *307*(5717), 1928–1933. https://doi.org/10.1126/science.1107239

Cotsarelis, G., Sun, T.-T., & Lavker, R. M. (1990). Label-retaining cells reside in the bulge area of pilosebaceous unit: Implications for follicular stem cells, hair cycle, and skin carcinogenesis. *Cell*, *61*(7), 1329–1337. https://doi.org/10.1016/0092-8674(90)90696-C

Cowles, C. R., Hirschhorn, J. N., Altshuler, D., & Lander, E. S. (2002). Detection of regulatory variation in mouse genes. *Nature Genetics*, *32*(3), 432–437. https://doi.org/10.1038/ng992

Cresko, W. A., Amores, A., Wilson, C., Murphy, J., Currey, M., Phillips, P., … Postlethwait, J. H. (2004). Parallel genetic basis for repeated evolution of armor loss in Alaskan threespine stickleback populations. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(16), 6050–6055. https://doi.org/10.1073/pnas.0308479101

Crick, F. (1958). On Protein Synthesis. *Symposia of the Society for Experimental Biology, Number XII: The Biological Replication of Macromolecules. Cambridge University Press.*, 138–163.

Cuny, G. D., Yu, P. B., Laha, J. K., Xing, X., Liu, J. F., Lai, C. S., … Peterson, R. T. (2008). Structure-activity relationship study of bone morphogenetic protein (BMP) signaling inhibitors. *Bioorganic and Medicinal Chemistry Letters*, *18*(15), 4388–4392. https://doi.org/10.1016/j.bmcl.2008.06.052

Darwin, C. (1859). On the Origins of Species by Means of Natural Selection, or the Preservation of Favoured Races in the Struggle for Life.

DeScipio, C., Schneider, L., Young, T. L., Wasserman, N., Yaeger, D., Lu, F., … Krantz, I. D. (2005). Subtelomeric deletions of chromosome 6p: Molecular and cytogenetic characterization of three new cases with phenotypic overlap with Ritscher?Schinzel (3C) syndrome. *American Journal of Medical Genetics Part A*, *134A*(1), 3–11. https://doi.org/10.1002/ajmg.a.30573

Ellis, N. A., Donde, N. N., & Miller, C. T. (2016). Early development and replacement of the stickleback dentition. *Journal of Morphology*, *277*(8), 1072–1083. https://doi.org/10.1002/jmor.20557

Ellis, N. A., Glazer, A. M., Donde, N. N., Cleves, P. A., Agoglia, R. M., & Miller, C. T. (2015). Distinct developmental and genetic mechanisms underlie convergently evolved tooth gain in sticklebacks. *Development*, (June), 2442–2451. https://doi.org/10.1242/dev.124248

Emera, D., Yin, J., Reilly, S. K., Gockley, J., & Noonan, J. P. (2016). Origin and evolution of developmental enhancers in the mammalian neocortex. *Proceedings of the National Academy of Sciences of the United States of America*, *113*(19), E2617–E2626. https://doi.org/10.1073/pnas.1603718113

Erickson, P. A., Cleves, P. A., Ellis, N. A., Schwalbach, K. T., Hart, J. C., & Miller, C. T. (2015). A 190 base pair, TGFB responsive tooth and fin enhancer is required for stickleback Bmp6 expression. *Developmental Biology*, *401*(2), 310–323.

https://doi.org/10.1016/j.ydbio.2015.02.006

Erickson, P. A., Ellis, N. A., & Miller, C. T. (2016). Microinjection for Transgenesis and Genome Editing in Threespine Sticklebacks. *Journal of Visualized Experiments*, (111), e54055–e54055. https://doi.org/10.3791/54055

Erickson, P. A., Glazer, A. M., Cleves, P. A., Smith, A. S., & Miller, C. T. (2014). Two developmentally temporal quantitative trait loci underlie convergent evolution of increased branchial bone length in sticklebacks. *Proceedings of the Royal Society B: Biological Sciences*, *281*(1788). https://doi.org/10.1098/rspb.2014.0822

Farh, K. K. H., Marson, A., Zhu, J., Kleinewietfeld, M., Housley, W. J., Beik, S., … Bernstein, B. E. (2015). Genetic and epigenetic fine mapping of causal autoimmune disease variants. *Nature*, *518*(7539), 337–343. https://doi.org/10.1038/nature13835

Fish, A., Chen, L., & Capra, J. A. (2017). Gene regulatory enhancers with evolutionarily conserved activity aremore pleiotropic than those with species-specific activity. *Genome Biology and Evolution*, *9*(10), 2615–2625. https://doi.org/10.1093/gbe/evx194

Fisher, S., Grice, E. A., Vinton, R. M., Bessling, S. L., & McCallion, A. S. (2006). Conservation of RET regulatory function from human to zebrafish without sequence similarity. *Science*, *312*(5771), 276–279. https://doi.org/10.1126/science.1124070

Fraser, G. J., Bloomquist, R. F., & Streelman, J. T. (2013). Common developmental pathways link tooth shape to regeneration. *Developmental Biology*, *377*(2), 399–414. https://doi.org/10.1016/J.YDBIO.2013.02.007

Frazer, K. A., Pachter, L., Poliakov, A., Rubin, E. M., & Dubchak, I. (2004). VISTA: Computational tools for comparative genomics. *Nucleic Acids Research*, *32*(WEB SERVER ISS.), W273–W279. https://doi.org/10.1093/nar/gkh458

Furlong, E. E. M., & Levine, M. (2018, September 28). Developmental enhancers and chromosome topology. *Science*. American Association for the Advancement of Science. https://doi.org/10.1126/science.aau0320

Gehring, W. J., & Hiromi, Y. (1986). Homeotic Genes and the Homeobox. *Annual Review of Genetics*, *20*(1), 147–173. https://doi.org/10.1146/annurev.ge.20.120186.001051

Glazer, A. M., Cleves, P. A., Erickson, P. A., Lam, A. Y., & Miller, C. T. (2014). Parallel developmental genetic features underlie stickleback gill raker evolution. *EvoDevo*, *5*(1), 1–16. https://doi.org/10.1186/2041-9139-5-19

Glazer, A. M., Killingbeck, E. E., Mitros, T., Rokhsar, D. S., & Miller, C. T. (2015). Genome assembly improvement and mapping convergently evolved skeletal traits in sticklebacks with genotyping-by-sequencing. *G3: Genes, Genomes, Genetics*, *5*(7), 1463–1472. https://doi.org/10.1534/g3.115.017905

Gould, D. B., Jaafar, M. S., Addison, M. K., Munier, F., Ritch, R., MacDonald, I. M., & Walter, M. A. (2004). Phenotypic and molecular assessment of seven patients with 6p25 deletion syndrome: Relevance to ocular dysgenesis and hearing impairment. *BMC Medical Genetics*, *5*(1), 17. https://doi.org/10.1186/1471-2350-5-17

Handrigan, G. R., Leung, K. J., & Richman, J. M. (2010). Identification of putative dental epithelial stem cells in a lizard with life-long tooth replacement. *Development*, *137*(21), 3545–3549. https://doi.org/10.1242/dev.052415

Handrigan, Gregory R., Leung, K. J., & Richman, J. M. (2010). Identification of putative dental epithelial stem cells in a lizard with life-long tooth replacement. *Development*, *137*(21). Retrieved from http://dev.biologists.org/content/137/21/3545

Handrigan, Gregory R., & Richman, J. M. (2010). A network of Wnt, hedgehog and BMP signaling pathways regulates tooth replacement in snakes. *Developmental Biology*, *348*(1), 130–141. https://doi.org/10.1016/J.YDBIO.2010.09.003

Hannenhalli, S., & Kaestner, K. H. (2009, April). The evolution of Fox genes and their role in development and disease. *Nature Reviews Genetics*. NIH Public Access. https://doi.org/10.1038/nrg2523

Harada, H., Kettunen, P., Jung, H. S., Mustonen, T., Wang, Y. A., & Thesleff, I. (1999). Localization of putative stem cells in dental epithelium and their association with Notch and FGF signaling. *Journal of Cell Biology*, *147*(1), 105–120. https://doi.org/10.1083/jcb.147.1.105

Harris, M. P., Rohner, N., Schwarz, H., Perathoner, S., Konstantinidis, P., & Nüsslein-Volhard, C. (2008). Zebrafish eda and edar Mutants Reveal Conserved and Ancestral Roles of Ectodysplasin Signaling in Vertebrates. *PLoS Genetics*, *4*(10), e1000206. https://doi.org/10.1371/journal.pgen.1000206

Hart, J. C., & Miller, C. T. (2017). Sequence-Based Mapping and Genome Editing Reveal Mutations in SticklebackHps5Cause Oculocutaneous Albinism and thecasperPhenotype. *G3 (Bethesda, Md.)*, *7*(9), 3123–3131. https://doi.org/10.1534/g3.117.1125

Headon, D. J., Emmal, S. A., Ferguson, B. M., Tucker, A. S., Justice, M. J., Sharpe, P. T., … Overbeek, P. A. (2001). Gene defect in ectodermal dysplasia implicates a death domain adapter in development. *Nature*, *414*(6866), 913–916. https://doi.org/10.1038/414913a

Huang, X., Zhong, L., Hendriks, J., Post, J. N., & Karperien, M. (2018). The effects of the WNT-signaling modulators BIO and PKF118-310 on the chondrogenic differentiation of human mesenchymal stem cells. *International Journal of Molecular Sciences*, *19*(2). https://doi.org/10.3390/ijms19020561

Huxley, J. (1942). Evolution, the Modern Synthesis. *G. Allen and Unwin Limited.*

Indjeian, V. B., Kingman, G. A., Jones, F. C., Guenther, C. A., Grimwood, J., Schmutz, J., … Kingsley, D. M. (2016). Evolving New Skeletal Traits by cis-Regulatory Changes in Bone Morphogenetic Proteins. *Cell*, *164*(1–2), 45–56. https://doi.org/10.1016/j.cell.2015.12.007

Infante, C. R., Mihala, A. G., Park, S., Wang, J. S., Johnson, K. K., Lauderdale, J. D., & Menke, D. B. (2015). Shared Enhancer Activity in the Limbs and Phallus and Functional Divergence of a Limb-Genital cis-Regulatory Element in Snakes. *Developmental Cell*, *35*(1), 107–119. https://doi.org/10.1016/j.devcel.2015.09.003

Jackman, W. R., & Stock, D. W. (2006). Transgenic analysis of Dlx regulation in fish tooth development reveals evolutionary retention of enhancer function despite organ loss. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(51), 19390–19395. https://doi.org/10.1073/pnas.0609575103

Jacob, F., & Monod, J. (1961, June 1). Genetic regulatory mechanisms in the synthesis of proteins. *Journal of Molecular Biology*. Academic Press. https://doi.org/10.1016/S0022-2836(61)80072-7

Jernvall, J., & Thesleff, I. (2012). Tooth shape formation and tooth renewal: evolving with the same signals. *Development*, *139*(19), 3487–3497. https://doi.org/10.1242/dev.085084

Jia, S., Zhou, J., Gao, Y., Baek, J.-A., Martin, J. F., Lan, Y., … Sucov, H. M. (2013). Roles of Bmp4 during tooth morphogenesis and sequential tooth formation. *Development (Cambridge, England)*, *140*(2), 423–432. https://doi.org/10.1242/dev.081927

Jones, F. C., Grabherr, M. G., Chan, Y. F., Russell, P., Mauceli, E., Johnson, J., … Kingsley, D. M. (2012). The genomic basis of adaptive evolution in threespine sticklebacks. *Nature*, *484*(7392), 55–61. https://doi.org/10.1038/nature10944

Juuri, E., Jussila, M., Seidel, K., Holmes, S., Wu, P., Richman, J., … Thesleff, I. (2013). Sox2 marks epithelial competence to generate teeth in mammals and reptiles. *Development (Cambridge, England)*, *140*(7), 1424–1432. https://doi.org/10.1242/dev.089599

Juuri, E., Saito, K., Ahtiainen, L., Seidel, K., Tummers, M., Hochedlinger, K., … Michon, F. (2012). Sox2+ stem cells contribute to all epithelial lineages of the tooth via Sfrp5+ progenitors. *Developmental Cell*, *23*(2), 317–328. https://doi.org/10.1016/j.devcel.2012.05.012

Kandyba, E., Leung, Y., Chen, Y.-B., Widelitz, R., Chuong, C.-M., & Kobielak, K. (2013). Competitive balance of intrabulge BMP/Wnt signaling reveals a robust gene network ruling stem cell homeostasis and cyclic activation. *Proceedings of the National Academy of Sciences*, *110*(4), 1351–1356. https://doi.org/10.1073/pnas.1121312110

Kawakami, K., & Shima, A. (1999). Identification of the Tol2 transposase of the medaka fish Oryzias latipes that catalyzes excision of a nonautonomous Tol2 element in zebrafish Danio rerio. *Gene*, *240*(1), 239–244. https://doi.org/10.1016/S0378-1119(99)00444-8

Kimmel, C. B., Miller, C. T., Kruze, G., Ullmann, B., BreMiller, R. A., Larison, K. D., & Snyder, H. C. (1998). The Shaping of Pharyngeal Cartilages during Early Development of the Zebrafish. *Developmental Biology*, *203*(2), 245–263. https://doi.org/10.1006/DBIO.1998.9016

Kimmel, C. B., Miller, C. T., & Moens, C. B. (2001). Specification and Morphogenesis of the Zebrafish Larval Head Skeleton. *Developmental Biology*, *233*(2), 239–257. https://doi.org/10.1006/DBIO.2001.0201

King, M. C., & Wilson, A. C. (1975). Evolution at two levels in humans and chimpanzees. *Science*, *188*(4184), 107–116. https://doi.org/10.1126/science.1090005

Kingsley, D. M., Zhu, B., Osoegawa, K., De Jong, P. J., Schein, J., Marra, M., … Myers, R. (2004). New genomic tools for molecular studies of evolutionary change in threespine sticklebacks. In *Behaviour* (Vol. 141, pp. 1331–1344). Brill. https://doi.org/10.1163/1568539042948150

Kobielak, K., Stokes, N., de la Cruz, J., Polak, L., & Fuchs, E. (2007). Loss of a quiescent niche but not follicle stem cells in the absence of bone morphogenetic protein signaling. *Proceedings of the National Academy of Sciences*, *104*(24), 10063–10068. https://doi.org/10.1073/pnas.0703004104

Kume, T., Jiang, H., Topczewska, J. M., & Hogan, B. L. M. (2001). The murine winged helix transcription factors, Foxc1 and Foxc2, are both required for cardiovascular development and somitogenesis. *Genes and Development*, *15*(18), 2470–2482. https://doi.org/10.1101/gad.907301

Kume, Tsutomu, Deng, K. Y., Winfrey, V., Gould, D. B., Walter, M. A., & Hogan, B. L. M. (1998). The forkhead/winged helix gene Mf1 is disrupted in the pleiotropic mouse mutation congenital hydrocephalus. *Cell*, *93*(6), 985–996. https://doi.org/10.1016/S0092-8674(00)81204-0

Le Caignec, C., De Mas, P., Vincent, M.-C., Bocéno, M., Bourrouillou, G., Rival, J.-M., & David, A. (2005). Subtelomeric 6p deletion: Clinical, FISH, and array CGH characterization of two cases. *American Journal of Medical Genetics Part A*, *132A*(2), 175–180. https://doi.org/10.1002/ajmg.a.30409

Leche, W. (1895). Zür Entwicklungsgeschichte des Zahnsystems des Säugethiere, zugleich ein Beitrag zur Stammesgeschichte dieser Thiergruppe. I. Ontogenie. Zoologica 6, 1-160. (n.d.).

Lee, C. S., Friedman, J. R., Fulmer, J. T., & Kaestner, K. H. (2005). The initiation of liver development is dependent on Foxa transcription factors. *Nature*, *435*(7044), 944–947. https://doi.org/10.1038/nature03649

Lehmann, O. J., Sowden, J. C., Carlsson, P., Jordan, T., & Bhattacharya, S. S. (2003, June 1). Fox's in development and disease. *Trends in Genetics*. Elsevier Ltd.

https://doi.org/10.1016/S0168-9525(03)00111-2

Levine, M., Cattoglio, C., & Tjian, R. (2014, March 27). Looping back to leap forward: Transcription enters a new era. *Cell*. Elsevier B.V. https://doi.org/10.1016/j.cell.2014.02.009

Li, A., Lai, Y. C., Figueroa, S., Yang, T., Widelitz, R. B., Kobielak, K., … Chuong, C. M. (2015, August 1). Deciphering principles of morphogenesis from temporal and spatial patterns on the integument. *Developmental Dynamics*. John Wiley and Sons Inc. https://doi.org/10.1002/dvdy.24281

Lin, C. R., Kloussi, C., O'Connell, S., Briata, P., Szeto, D., Liu, F., … Rosenfeld, M. G. (1999). Pitx2 regulates lung asymmetry, cardiac positioning and pituitary and tooth morphogenesis. *Nature*, *401*(6750), 279–282. https://doi.org/10.1038/45803

Lukoseviciute, M., Gavriouchkina, D., Williams, R. M., Hochgreb-Hagele, T., Senanayake, U., Chong-Morrison, V., … Sauka-Spengler, T. (2018). From Pioneer to Repressor: Bimodal foxd3 Activity Dynamically Remodels Neural Crest Regulatory Landscape In Vivo. *Developmental Cell*, *47*(5), 608-628.e6. https://doi.org/10.1016/j.devcel.2018.11.009

Mandler, M., & Neubüser, A. (2001). FGF signaling is necessary for the specification of the odontogenic mesenchyme. *Developmental Biology*, *240*(2), 548–559. https://doi.org/10.1006/dbio.2001.0490

Martin, K. J., Rasch, L. J., Cooper, R. L., Metscher, B. D., Johanson, Z., & Fraser, G. J. (2016). Sox2+ progenitors in sharks link taste development with the evolution of regenerative teeth from denticles. *Proceedings of the National Academy of Sciences*, *113*(51), 14769–14774. https://doi.org/10.1073/pnas.1612354113

Maurano, M. T., Humbert, R., Rynes, E., Thurman, R. E., Haugen, E., Wang, H., … Stamatoyannopoulos, J. A. (2012). Systematic localization of common disease-associated variation in regulatory DNA. *Science*, *337*(6099), 1190–1195. https://doi.org/10.1126/science.1222794

Mazet, F., Amemiya, C. T., & Shimeld, S. M. (2006, May 9). An ancient Fox gene cluster in bilaterian animals. *Current Biology*. Elsevier. https://doi.org/10.1016/j.cub.2006.03.088

McKinnon, J. S., & Rundle, H. D. (2002, October 1). Speciation in nature: The threespine stickleback model systems. *Trends in Ecology and Evolution*. Elsevier Current Trends. https://doi.org/10.1016/S0169-5347(02)02579-X

Mendel, G. (1865). Versuche ber Pflanzen-HyBriden. *Verh Naturforsch Ver Brnn, 4(347)*.

Miller, C. T., Beleza, S., Pollen, A. A., Schluter, D., Kittles, R. A., Shriver, M. D., & Kingsley, D. M. M. (2007). cis-Regulatory Changes in Kit Ligand Expression and Parallel Evolution of Pigmentation in Sticklebacks and Humans. *Cell*, *131*(6), 1179–1189.

https://doi.org/10.1016/j.cell.2007.10.055

Miller, C. T., Glazer, A. M., Summers, B. R., Blackman, B. K., Norman, A. R., Shapiro, M. D., … Kingsley, D. M. (2014). Modular skeletal evolution in sticklebacks is controlled by additive and clustered quantitative trait loci. *Genetics*, *197*(1), 405–420. https://doi.org/10.1534/genetics.114.162420

Miyazono, K., Maeda, S., & Imamura, T. (2005). BMP receptor signaling: Transcriptional targets, regulation of signals, and signaling cross-talk. *Cytokine and Growth Factor Reviews*, *16*(3 SPEC. ISS.), 251–263. https://doi.org/10.1016/j.cytogfr.2005.01.009

Near, T. J., Eytan, R. I., Dornburg, A., Kuhn, K. L., Moore, J. A., Davis, M. P., … Smith, W. L. (2012). Resolution of ray-finned fish phylogeny and timing of diversification. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(34), 13698–13703. https://doi.org/10.1073/pnas.1206625109

Neese, R. A., Misell, L. M., Turner, S., Chu, A., Kim, J., Cesar, D., … Hellerstein, M. K. (2002). Measurement in vivo of proliferation rates of slow turnover cells by 2H2O labeling of the deoxyribose moiety of DNA. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(24), 15345–15350. https://doi.org/10.1073/pnas.232551499

Neubüser, A., Peters, H., Balling, R., & Martin, G. R. (1997). Antagonistic interactions between FGF and BMP signaling pathways: A mechanism for positioning the sites of tooth formation. *Cell*, *90*(2), 247–255. https://doi.org/10.1016/S0092-8674(00)80333-5

Nüsslein-volhard, C., & Wieschaus, E. (1980). Mutations affecting segment number and polarity in drosophila. *Nature*, *287*(5785), 795–801. https://doi.org/10.1038/287795a0

O'brown, N. M., Summers, B. R., Jones, F. C., Brady, S. D., & Kingsley, D. M. (2015). A recurrent regulatory change underlying altered expression and Wnt response of the stickleback armor plates gene EDA. *ELife*, *2015*(4). https://doi.org/10.7554/eLife.05290

Pai, A. A., & Gilad, Y. (2014, December 1). Comparative studies of gene regulatory mechanisms. *Current Opinion in Genetics and Development*. Elsevier Ltd. https://doi.org/10.1016/j.gde.2014.08.010

Pennacchio, L. A., Bickmore, W., Dean, A., Nobrega, M. A., & Bejerano, G. (2013, April 18). Enhancers: Five essential questions. *Nature Reviews Genetics*. Nature Publishing Group. https://doi.org/10.1038/nrg3458

Pires-daSilva, A., & Sommer, R. J. (2003, January 1). The evolution of signalling pathways in animal development. *Nature Reviews Genetics*. Nature Publishing Group. https://doi.org/10.1038/nrg977

Pispa, J., Pummila, M., Barker, P. A., Thesleff, I., & Mikkola, M. L. (2008). Edar and Troy signalling pathways act redundantly to regulate initiation of hair follicle development.

Human Molecular Genetics, *17*(21), 3380–3391. https://doi.org/10.1093/hmg/ddn232

Pispa, J., & Thesleff, I. (2003). Mechanisms of ectodermal organogenesis. *Developmental Biology*, *262*(2), 195–205. https://doi.org/10.1016/S0012-1606(03)00325-7

Plikus, M. V., Baker, R. E., Chen, C. C., Fare, C., De La Cruz, D., Andl, T., … Chuong, C. M. (2011). Self-organizing and stochastic behaviors during the regeneration of hair stem cells. *Science*, *332*(6029), 586–589. https://doi.org/10.1126/science.1201647

Plikus, M. V., Mayer, J. A., De La Cruz, D., Baker, R. E., Maini, P. K., Maxson, R., & Chuong, C. M. (2008). Cyclic dermal BMP signalling regulates stem cell activation during hair regeneration. *Nature*, *451*(7176), 340–344. https://doi.org/10.1038/nature06457

Preger-Ben Noon, E., Sabarís, G., Ortiz, D. M., Sager, J., Liebowitz, A., Stern, D. L., & Frankel, N. (2018). Comprehensive Analysis of a cis-Regulatory Region Reveals Pleiotropy in Enhancer Function. *Cell Reports*, *22*(11), 3021–3031. https://doi.org/10.1016/j.celrep.2018.02.073

Prud'homme, B., Gompel, N., & Carroll, S. B. (2007). Emerging principles of regulatory evolution. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(SUPPL. 1), 8605–8612. https://doi.org/10.1073/pnas.0700488104

Rebeiz, M., & Tsiantis, M. (2017, August 1). Enhancer evolution and the origins of morphological novelty. *Current Opinion in Genetics and Development*. Elsevier Ltd. https://doi.org/10.1016/j.gde.2017.04.006

Reid, D. T., & Peichel, C. L. (2010). Perspectives on the Genetic Architecture of Divergence in Body Shape in Sticklebacks. *Integrative and Comparative Biology*, *50*(6), 1057–1066. https://doi.org/10.1093/icb/icq030

Reif, W.-E. (1982). Evolution of Dermal Skeleton and Dentition in Vertebrates. The Odontode-Regulation Theory. *Evolutionary Biology*, *15*.

Rice, R., Rice, D. P. C., Olsen, B. R., & Thesleff, I. (2003). Progression of calvarial bone development requires Foxc1 regulation of Msx2 and Alx4. *Developmental Biology*, *262*(1), 75–87. https://doi.org/10.1016/S0012-1606(03)00355-5

Rosenfeld, J. A., Wang, Z., Schones, D. E., Zhao, K., DeSalle, R., & Zhang, M. Q. (2009). Determination of enriched histone modifications in non-genic portions of the human genome. *BMC Genomics*, *10*(1), 143. https://doi.org/10.1186/1471-2164-10-143

Sabarís, G., Laiker, I., Preger-Ben Noon, E., & Frankel, N. (2019, June 1). Actors with Multiple Roles: Pleiotropic Enhancers and the Paradigm of Enhancer Modularity. *Trends in Genetics*. Elsevier Ltd. https://doi.org/10.1016/j.tig.2019.03.006

Sadier, A., Viriot, L., Pantalacci, S., & Laudet, V. (2014, January 1). The ectodysplasin pathway:

From diseases to adaptations. *Trends in Genetics*. Elsevier Current Trends. https://doi.org/10.1016/j.tig.2013.08.006

Sender, R., & Milo, R. (2021). The distribution of cellular turnover in the human body. *Nature Medicine*, *27*(1), 45–48. https://doi.org/10.1038/s41591-020-01182-9

Seo, S., & Kume, T. (2006). Forkhead transcription factors, Foxc1 and Foxc2, are required for the morphogenesis of the cardiac outflow tract. *Developmental Biology*, *296*(2), 421–436. https://doi.org/10.1016/j.ydbio.2006.06.012

Shubin, N., Tabin, C., & Carroll, S. (2009, February 12). Deep homology and the origins of evolutionary novelty. *Nature*. Nature Publishing Group. https://doi.org/10.1038/nature07891

Sievers, F., Wilm, A., Dineen, D., Gibson, T. J., Karplus, K., Li, W., … Higgins, D. G. (2011). Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Molecular Systems Biology*, *7*(1), 539. https://doi.org/10.1038/msb.2011.75

Singh, D., & Yi, S. V. (2021). Enhancer Pleiotropy, Gene Expression, and the Architecture of Human Enhancer–Gene Interactions. *Molecular Biology and Evolution*, *38*(9), 3898–3909. https://doi.org/10.1093/molbev/msab085

Singh, L. N., & Hannenhalli, S. (2008). Functional Diversification of Paralogous Transcription Factors via Divergence in DNA Binding Site Motif and in Expression. *PLoS ONE*, *3*(6), e2345. https://doi.org/10.1371/journal.pone.0002345

Smith, M. M., & Coates, M. I. (1998). Evolutionary origins of the vertebrate dentition: Phylogenetic patterns and developmental evolution. *European Journal of Oral Sciences*, *106*(1 SUPPL.), 482–500. https://doi.org/10.1111/j.1600-0722.1998.tb02212.x

Soukup, V., Epperlein, H.-H., Horácek, I., & Cerny, R. (2008). Dual epithelial origin of vertebrate oral teeth. *Nature*, *455*(7214), 795–798. https://doi.org/10.1038/nature07304

Square, T. A., Sundaram, S., Mackey, E. J., & Miller, C. T. (2021). Distinct tooth regeneration systems deploy a conserved battery of genes. *EvoDevo*, *12*(1), 1–17. https://doi.org/10.1186/s13227-021-00172-3

Stanojevic, D., Small, S., & Levine, M. (1991). Regulation of a segmentation stripe by overlapping activators and repressors in the Drosophila embryo. *Science*, *254*(5036), 1385–1387. https://doi.org/10.1126/science.1683715

Stepaniak, M. D., Square, T. A., & Miller, C. T. (2021). Evolved Bmp6 enhancer alleles drive spatial shifts in gene expression during tooth development in sticklebacks. *Genetics*. https://doi.org/10.1093/genetics/iyab151

Stewart, S., Gomez, A. W., Armstrong, B. E., Henner, A., & Stankunas, K. (2014). Sequential

and opposing activities of Wnt and BMP coordinate zebrafish bone regeneration. *Cell Reports*, *6*(3), 482–498. https://doi.org/10.1016/j.celrep.2014.01.010

Talbot, J. C., & Amacher, S. L. (2014). A streamlined CRISPR pipeline to reliably generate zebrafish frameshifting alleles. *Zebrafish*, *11*(6), 583–585. https://doi.org/10.1089/zeb.2014.1047

Taylor, G., Lehrer, M. S., Jensen, P. J., Sun, T. T., & Lavker, R. M. (2000). Involvement of follicular stem cells in forming not only the follicle but also the epidermis. *Cell*, *102*(4), 451–461. https://doi.org/10.1016/S0092-8674(00)00050-7

Thesleff, I. (2003). Epithelial-mesenchymal signalling regulating tooth morphogenesis. *Journal of Cell Science*, *116*(9), 1647–1648. https://doi.org/10.1242/JCS.00410

Till, J., & McCulloch, E. (1961). A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiat Res.*, *14:213-22.*

Tinbergen, N. (1952). The curious behavior of the stickleback. *Sci. Am. 187:22–26.*

Tumbar, T., Guasch, G., Greco, V., Blanpain, C., Lowry, W. E., Rendl, M., & Fuchs, R. (2004). Defining the Epithelial Stem Cell Niche in Skin. *Science*, *303*(5656), 359–363. https://doi.org/10.1126/science.1092436

Tümer, Z., & Bach-Holm, D. (2009). Axenfeld-Rieger syndrome and spectrum of PITX2 and FOXC1 mutations. *European Journal of Human Genetics : EJHG*, *17*(12), 1527–1539. https://doi.org/10.1038/ejhg.2009.93

Vainio, S., Karavanova, I., Jowett, A., & Thesleff, I. (1993). Identification of BMP-4 as a signal mediating secondary induction between epithelial and mesenchymal tissues during early tooth development. *Cell*, *75*(1), 45–58. https://doi.org/10.1016/S0092-8674(05)80083-2

Vandenplas, S., Willems, M., & Huysseune, A. (2012). Dual BrdU-PCNA immunodetection of proliferative cells in dental and orofacial tissues of teleosts. *Journal of Applied Ichthyology*, *28*(3), 336–340. https://doi.org/10.1111/j.1439-0426.2012.02003.x

Vandenplas, Sam, De Clercq, A., & Huysseune, A. (2014). Tooth replacement without a dental lamina: The search for epithelial stem cells in *Polypterus senegalus*. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*, *322*(5), 281–293. https://doi.org/10.1002/jez.b.22577

Vandenplas, Sam, Vandeghinste, R., Boutet, A., Mazan, S., & Huysseune, A. (2016). Slow cycling cells in the continuous dental lamina of Scyliorhinus canicula: New evidence for stem cells in sharks. *Developmental Biology*, *413*(1), 39–49. https://doi.org/10.1016/j.ydbio.2016.03.004

Vandenplas, Sam, Willems, M., Witten, P. E., Hansen, T., Fjelldal, P. G., & Huysseune, A.

(2016). Epithelial Label-Retaining Cells Are Absent during Tooth Cycling in Salmo salar and Polypterus senegalus. *PLOS ONE*, *11*(4), e0152870. https://doi.org/10.1371/journal.pone.0152870

Vaškaninová, V., Chen, D., Tafforeau, P., Johanson, Z., Ekrt, B., Blom, H., & Ahlberg, P. E. (2020). Marginal dentition and multiple dermal jawbones as the ancestral condition of jawed vertebrates. *Science*, *369*(6500), 211–216. https://doi.org/10.1126/science.aaz9431

Wainwright, P. C. (2005, January 1). Functional Morphology of the Pharyngeal Jaw Apparatus. *Fish Physiology*. Academic Press. https://doi.org/10.1016/S1546-5098(05)23003-0

Walker, J. A., & Bell, M. A. (2000). Net evolutionary trajectories of body shape evolution within a microgeographic radiation of threespine sticklebacks (Gasterosteus aculeatus) . *Journal of Zoology*, *252*(3), 293–302. https://doi.org/10.1111/j.1469-7998.2000.tb00624.x

Waluk, D. P., Zur, G., Kaufmann, R., Welle, M. M., Jagannathan, V., Drögemüller, C., … Galichet, A. (2016). A splice defect in the EDA gene in dogs with an X-linked hypohidrotic ectodermal dysplasia (XLHED) phenotype. *G3: Genes, Genomes, Genetics*, *6*(9), 2949–2954. https://doi.org/10.1534/g3.116.033225

Wang, L., Siegenthaler, J. A., Dowell, R. D., & Yi, R. (2016). Foxc1 reinforces quiescence in self-renewing hair follicle stem cells. *Science (New York, N.Y.)*, *351*(6273), 613–617. https://doi.org/10.1126/science.aad5440

Wang, X.-P., Suomalainen, M., Felszeghy, S., Zelarayan, L. C., Alonso, M. T., Plikus, M. V, … Thesleff, I. (2007). An Integrated Gene Regulatory Network Controls Stem Cell Proliferation in Teeth. *PLoS Biology*, *5*(6), e159. https://doi.org/10.1371/journal.pbio.0050159

Wang, Y., Li, L., Zheng, Y., Yuan, G., Yang, G., He, F., & Chen, Y. (2012). BMP activity is required for tooth development from the Lamina to Bud Stage. *Journal of Dental Research*, *91*(7), 690–695. https://doi.org/10.1177/0022034512448660

Wilson, A., Laurenti, E., Oser, G., van der Wath, R. C., Blanco-Bose, W., Jaworski, M., … Trumpp, A. (2008). Hematopoietic Stem Cells Reversibly Switch from Dormancy to Self-Renewal during Homeostasis and Repair. *Cell*, *135*(6), 1118–1129. https://doi.org/10.1016/j.cell.2008.10.048

Wu, P., Wu, X., Jiang, T.-X., Elsey, R. M., Temple, B. L., Divers, S. J., … Chuong, C.-M. (2013). Specialized stem cell niche enables repetitive renewal of alligator teeth. *Proceedings of the National Academy of Sciences*, *110*(22), E2009–E2018. https://doi.org/10.1073/pnas.1213202110

Wu, Z., Wang, F., Fan, Z., Wu, T., He, J., Wang, J., … Wang, S. (2019). Whole-Tooth Regeneration by Allogeneic Cell Reassociation in Pig Jawbone. *Tissue Engineering Part A*, *25*(17–18), 1202–1212. https://doi.org/10.1089/ten.tea.2018.0243

Wucherpfennig, J. I., Miller, C. T., & Kingsley, D. M. (2019). Efficient CRISPR-Cas9 editing of major evolutionary loci in sticklebacks. *Evolutionary Ecology Research*, *20*(1), 107–132. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/34899072

Xu, P., Balczerski, B., Ciozda, A., Louie, K., Oralova, V., Huysseune, A., & Gage Crump, J. (2018). Fox proteins are modular competency factors for facial cartilage and tooth specification. *Development (Cambridge)*, *145*(12). https://doi.org/10.1242/dev.165498

Xu, P., Yu, H. V., Tseng, K. C., Flath, M., Fabian, P., Segil, N., & Crump, J. G. (2021). Foxc1 establishes 1 enhancer accessibility for craniofacial cartilage differentiation. *ELife*, *10*, 1–50. https://doi.org/10.7554/eLife.63595

Zhao, X., Zhang, Z., Song, Y., Zhang, X., Zhang, Y., Hu, Y., … Chen, Y. P. (2000). Transgenically ectopic expression of Bmp4 to the Msx1 mutant dental mesenchyme restores downstream gene expression but represses Shh and Bmp2 in the enamel knot of wild type tooth germ. *Mechanisms of Development*, *99*(1–2), 29–38. https://doi.org/10.1016/S0925-4773(00)00467-6

Chapter 2: Site pleiotropy of a stickleback tooth and fin enhancer

Alyssa J. Rowley, Tyler A. Square, and Craig T. Miller

2.1 Abstract

Development and regeneration are orchestrated by gene regulatory networks that operate in part through transcriptional enhancers. Although many enhancers are pleiotropic and are active in multiple tissues, little is known about whether enhancer pleiotropy is due to 1) site pleiotropy, in which individual transcription factor binding sites (TFBS) are required for activity in multiple tissues, or 2) multiple distinct sites that regulate expression in different tissues. Here, we investigated the pleiotropy of an intronic enhancer of the stickleback *Bone morphogenetic protein 6* (*Bmp6*) gene. This enhancer was previously shown to regulate evolved changes in tooth number and tooth regeneration, and is highly pleiotropic, with robust activity in both fins and teeth throughout embryonic, larval, and adult life. We tested the hypothesis that the pleiotropy of this enhancer is due to site pleiotropy of an evolutionarily conserved predicted Foxc1 TFBS. Transgenic analysis and site-directed mutagenesis experiments both deleting and scrambling this predicted Foxc1 TFBS revealed that the binding site is required for enhancer activity in both teeth and fins throughout embryonic, larval, and adult development. Collectively these data support a model where the pleiotropy of this *Bmp6* enhancer is due to site pleiotropy and this putative binding site is required for enhancer activity in multiple anatomical sites from the embryo to the adult.

Keywords: stickleback, enhancer pleiotropy, site pleiotropy

2.2 Introduction

Eukaryotic gene expression is governed by enhancers, non-coding cis-regulatory elements that positively regulate transcription. Enhancers bind transcription factors that promote cell-type-specific gene expression programs throughout development and drive transcription in response to stimuli (Levine et al., 2014). Decades of genetic studies have revealed that most human disease phenotypes (Farh et al., 2015; Maurano et al., 2012), and much of natural phenotypic variation in animals are associated with sequence changes in non-coding DNA (Rebeiz & Tsiantis, 2017). These findings suggest that enhancers play a critical role in both evolution and disease, thereby motivating further study of basic enhancer biology.

Many enhancers are known to function in multiple distinct tissues, suggesting that these elements are pleiotropic. A study on chromatin from a subset of human tissues found that at least 1% of identified *cis*-regulatory elements were active in at least two spatially distinct domains (Singh & Yi, 2021). In vivo studies in fish and mice have additionally identified enhancer sequences that drive gene expression in more than one tissue type (Cleves et al., 2018; Erickson

et al., 2015; Jackman & Stock, 2006; Jumlongras et al., 2012; Stepaniak et al., 2021). Two different described mechanisms of enhancer pleiotropy are (1) tightly clustered, but different, transcription factor binding sites (TFBS) that drive expression in different tissues and (2) site pleiotropy, where a single TFBS drives expression in multiple tissues (Preger-Ben Noon et al., 2018). These two mechanisms of enhancer pleiotropy have different implications for evolution; site pleiotropy would constrain evolution to preserve the integrity of critical regulatory sequences, as a single mutation in the TFBS could have a significant impact on gene expression in multiple tissues (Boffelli et al., 2004; Fish et al., 2017; Infante et al., 2015; Sabarís et al., 2019). In contrast, tightly clustered TFBS could allow for more modular evolution of gene expression in different tissues or at different developmental stages.

Threespine stickleback fish (*Gasterosteus aculeatus*) are a powerful system to study enhancer biology, as abundant natural variation throughout their adaptive radiation occurs largely due to non-coding variation, pointing to the importance of enhancers in phenotypic evolution (Jones et al., 2012). For example, changes in tooth number have evolved repeatedly in sticklebacks, with derived freshwater populations having increases in tooth number and tooth regeneration rates relative to ancestral marine populations (Cleves et al., 2014; Ellis et al., 2015). Quantitative trait loci mapping revealed a large effect locus that contains the gene *Bone Morphogenetic Protein 6* (*Bmp6*), which is dynamically expressed in both epithelial and mesenchymal cells within developing teeth (Cleves et al., 2014). Further high-resolution genetic mapping of this large effect locus linked the evolved phenotypic effects to an enhancer in the fourth intron of *Bmp6* (Cleves et al., 2018). This intronic enhancer is highly pleiotropic and drives expression in all developing and regenerating teeth, as well as the distal edges of the pectoral, median, and caudal fins (Cleves et al., 2018). Comparing the marine and freshwater versions of this intronic enhancer in doubly transgenic lines revealed evolved spatial shifts in enhancer activity in both tooth epithelium and mesenchyme, consistent with an evolved change in enhancer activity driving phenotypic evolution of tooth number (Stepaniak et al., 2021). In addition, although both enhancers drive strong expression in the distal edges of embryonic fins, only the freshwater enhancer was detected in fin ray joints in pectoral and caudal fins (Stepaniak et al., 2021). However, whether the enhancer utilizes common inputs for tooth and fin activity, and which sites are required for this activity are unknown.

Previous studies have found that fish teeth and mammalian hair share many aspects of early development: both are derived from placodes (Pispa & Thesleff, 2003), continuously regenerate in adults, are regulated by BMP signaling during replacement (Jia et al., 2013; Vainio et al., 1993; Wang et al., 2012), and express similar batteries of genes during regeneration (Square et al., 2021). In mice, BMP signaling has been proposed as a major regulator of maintaining stem cell quiescence. Conditional ablation of the *Bmpr1a* gene in skin epithelium activated the stem cell niche, causing quiescent hair follicle stem cells to proliferate (Kandyba et al., 2013; Kobielak et al., 2007). Further, Foxc1 regulates *Bmp6* expression in regenerating hair. Foxc1 binds to a regulatory region adjacent to *Bmp6* in mice and inhibits hair regeneration, as conditional knockout of *Foxc1* in skin results in accelerated hair regeneration (Wang et al., 2016). Moreover, Foxc1 was proposed to be a candidate regulator of the *Bmp6* intron 4 enhancer, as a putative Foxc1 TFBS was identified within this enhancer (Cleves et al., 2018). Given that this enhancer is expressed in regenerating teeth, has been linked to evolved changes in tooth regeneration, and the similarities between hair and tooth regeneration, a parsimonious model is

that BMP signaling negatively regulates mammalian hair and fish tooth regeneration using homologous gene regulatory networks. Here we test the hypotheses that (1) a predicted Foxc1 TFBS is required for *Bmp6* enhancer activity in developing and regenerating teeth and (2) this predicted Foxc1 TFBS has site pleiotropy and is required for enhancer activity in teeth and fins.

2.3 Methods

Animal husbandry
All animal work was approved by UC Berkeley IACUC protocol AUP-2015–01-7117. Sticklebacks were raised as previously described (Cleves et al., 2014; Square et al., 2021). All experiments used lab-reared marine (Rabbit Slough, Alaska) sticklebacks.

Generation of transgenic GFP enhancer stickleback lines
Tol2 plasmid transgenesis was performed using pT2HE plasmid backbone as previously described (Erickson et al., 2016; O'brown et al., 2015). To generate GFP reporter constructs, PCR-based site-directed mutagenesis was done on plasmids containing the ~1300 bp Paxton benthic (PAXB) high-tooth-associated intron four enhancer allele (Cleves et al., 2018; Stepaniak et al., 2021). Primers for site-directed mutagenesis were designed using the Quickchange tool and PCR was carried out according to Erickson et al. (Erickson et al., 2015) https://www.agilent.com/store/primerDesignProgram.jsp. Primer sequences used for the deleted were CTAATTACCCCGACGAGGTCGGGTGGGGAG and CTCCCCACCCGACCTCGTCGGGGTAATTAG and for the scrambled CCCCACCCGACCTTTTGAATACCGTCGGGGTAATTA and TAATTACCCCGACGGTATTCAAAAGGTCGGGTGGGG. Transposase messenger RNA was synthesized as described previously (Kawakami & Shima, 1999). Stickleback embryos at the one-cell stage were microinjected as described (Erickson et al., 2016). Two stable transgenic lines were generated with each of the intact, scrambled, and deleted reporter constructs.

Imaging fluorescent transgenic sticklebacks
Transgenic GFP reporter lines were imaged using a Leica M165FC stereoscope with a DFC340 FX camera. Transgenic embryos were imaged live. Transgenic juvenile and adult fish were fixed in 4% paraformaldehyde for 4 hours for 9-19 mm standard length (SL) or overnight (>20mm SL). Teeth were imaged using Montage z-stack projections on a Leica M165FC dissecting microscope with a GFP2 filter.

Bioinformatics
The following genome assemblies were used to compare *Bmp6* sequences: stickleback *Gasterosteus aculeatus*, "Gac": Broad/gasAcu, medaka *Oryzias latipes*, "Ola": NIG/UT MEDAKA1/oryLat2, zebrafish *Danio rerio*, "Dre": GRCz10/danRer10, gar *Lepisosteus oculatus* "Loc": LepOcu1 (GCA_000242695.1). A ~25 kb window centered on stickleback *Bmp6* was aligned to orthologous sequences from these three other species using mVISTA and LAGAN at https://genome.lbl.gov/vista/index.shtml (Frazer et al., 2004) using default visualization parameters of 100 bp windows showing minimum y-axis of 50% and 70% sequence identity to color windows as conserved. The entire fourth intron sequence from all four species was aligned as above, and the first 5.5 kb of the ~6.5 kb intron shown in Figure 2.2C. Sequences orthologous to the minimally sufficient ~500 bp *Bmp6* intron 4 enhancer (Cleves et al., 2018) were aligned using Clustal Omega at https://www.ebi.ac.uk/Tools/msa/clustalo/ (Sievers et al., 2011) and

default parameters. The resulting text alignment was opened in MS Word and conserved bases in all four species highlighted manually.

In situ hybridizations on sections

Sample preparation, sectioning, *in situ* hybridization, and riboprobe synthesis were carried out as previously described (Square et al., 2021). Riboprobes were designed as described previously (Ellis et al., 2016) against *Foxc1a* and *Foxc1b*. Riboprobe template plasmids were created by PCR cloning gene fragments from genomic DNA using primers to *Foxc1a*: 5'-GCCGctcgagGGGACAGGTCTAGCCACTTG-3'; 5'-GCCGtctagaACGGCGATA-TACACGTTCCT-'3 and *Foxc1b*: 5'-GCCGctcgagCCTGCCCGACTATTGCATCA-3'; 5'-GCCGactagtAGACGGCAC-TTTATTAAACAAACA-3'.

2.4 Results

Previous research mapped evolved increases in tooth number in freshwater sticklebacks to an enhancer located in the fourth intron of *Bmp6* (Cleves et al., 2014, 2018). This enhancer drives robust expression in all developing pharyngeal and oral teeth, as well as in the distal margins of the pectoral and median fins (Fig. 2.1A,B) (Cleves et al., 2018; Stepaniak et al., 2021). The sequence of this intronic enhancer is evolutionarily conserved in teleosts, with clear homology detectable in medaka and zebrafish, as well as to gar, an outgroup to teleosts (Fig. 2.1C,D). Comparison of enhancer sequences within fish and outgroups suggests different levels of constraint within this enhancer, possibly representing functionally required TFBSs. Of those regions with 100% sequence conservation across the aforementioned fish species, one includes a nine base pair motif TTTGTTTAC that perfectly matches the binding site consensus previously reported for human FOXC1 (Fig. 2.1D, Fig. S2.1) (Berry et al., 2008).

Teleosts underwent a whole genome duplication (Amores et al., 1998), resulting in pairs of co-orthologs of many teleost genes relative to outgroups. In sticklebacks, both *Foxc1a* and *Foxc1b* have been maintained. Expression of both *Foxc1* co-orthologs was detected in developing adult teeth, with *Foxc1a* largely restricted to the dental mesenchyme (Fig. 2.1E), and *Foxc1b* distributed throughout the inner and outer dental epithelium (Fig. 2.1F). Thus, *Foxc1* gene expression overlaps with activity of the *Bmp6* intronic enhancer in teeth (Stepaniak et al., 2021).

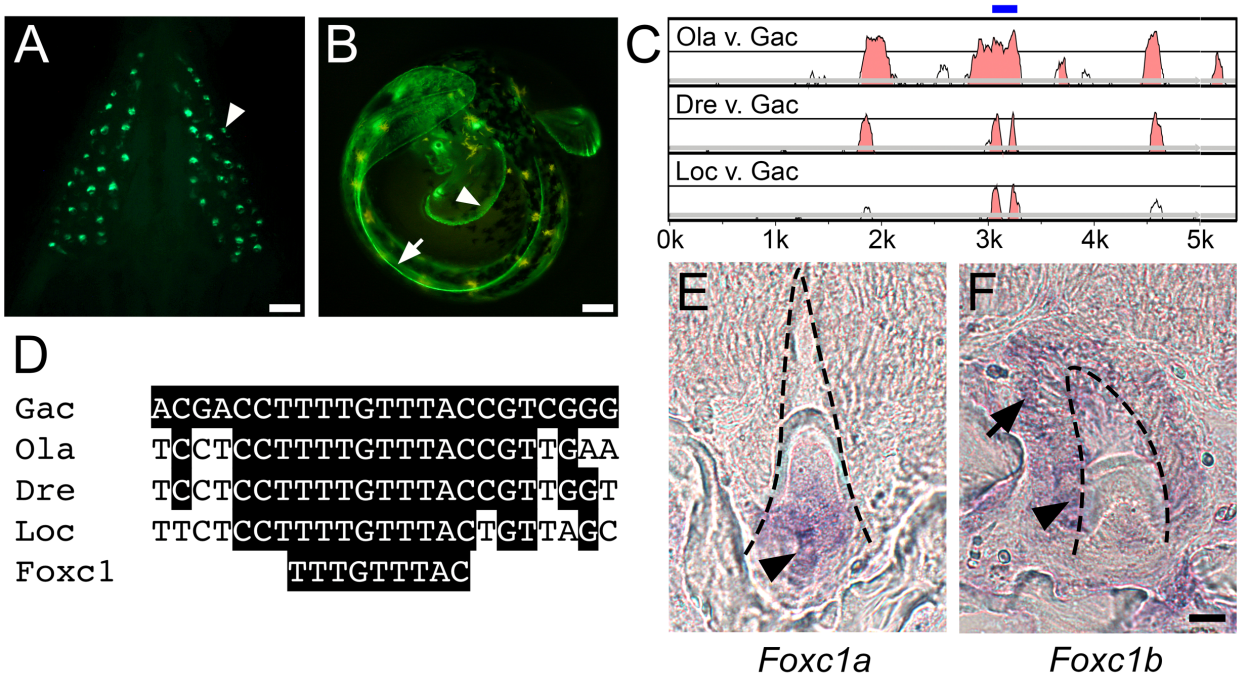


Figure 2. 1 A pleiotropic tooth and fin enhancer includes an evolutionarily conserved predicted Foxc1 binding site and Foxc1 genes are expressed in teeth.

eGFP reporter gene driven by a 1.3 kb *Bmp6* intronic enhancer (Square et al., 2021; Stepaniak et al., 2021) reveals expression in (A) ventral pharyngeal teeth (arrowhead) of juvenile stickleback fish and (B) distal edge of pectoral (arrowhead) and median (arrow) fin fold of unhatched 7dpf developing stickleback embryo. (C) mVISTA LAGAN sequence alignment showing central region of stickleback Bmp6 intron four is conserved to gar. Intron four of stickleback *Bmp6* (x-axis) is aligned to medaka (top), zebrafish (middle) and gar (bottom). Graph shows a sliding window of 100 base pairs, with y-axis starting at 50% identity and windows shaded at a threshold of 70% nucleotide identity. Blue bar above highlights central island of conservation (see Figure S2.1). Gac = *Gasterosteus aculeatus* (stickleback), Ola = *Oryzias latipes* (medaka), Dre = *Danio rerio* (zebrafish), Loc = *Lepisosteus oculatus* (gar). (D) Predicted Foxc1 binding site TTTGTTTAC (Berry et al., 2008) within central island of conservation is conserved from sticklebacks to gar. In situ hybridization of *Foxc1a* (E) and *Foxc1b* (F) expression on sections of adult stickleback pharyngeal teeth. *Foxc1a* expression was detected in dental mesenchyme (black arrowhead), while *Foxc1b* expression was detected in the inner (black arrowhead) and outer dental epithelium (black arrow). Black dotted lines demarcate the outline of the developing mineralized tooth. Scale bar = 10 µm.

To test the hypothesis that the predicted Foxc1 TFBS is required for enhancer activity, we introduced mutations in a *Bmp6* reporter construct previously described (Cleves et al., 2018; Stepaniak et al., 2021). The unaltered construct drove GFP expression in several tissues including the pectoral and median fins, and oral and pharyngeal teeth. Site directed mutagenesis was used to make two mutant enhancer variants: (1) a scrambled enhancer, where two thymidine nucleotides in the predicted Foxc1 TFBS were mutated to adenine, and (2) a deleted enhancer, where the entire nine base pair Foxc1 predicted TFBS was deleted (Fig. 2.2A). We generated two stable transgenic lines each for three different enhancer transgenes: intact wild-type, scrambled, and deleted (Fig. 2.2A). For all three genotypes, we imaged transgenic larvae, juveniles, and adults to test for possible effects on enhancer activity in different tissues. All scrambled and deleted transgenes dramatically reduced GFP expression in the pectoral and median fins of the developing embryo (Fig. 2.2B).

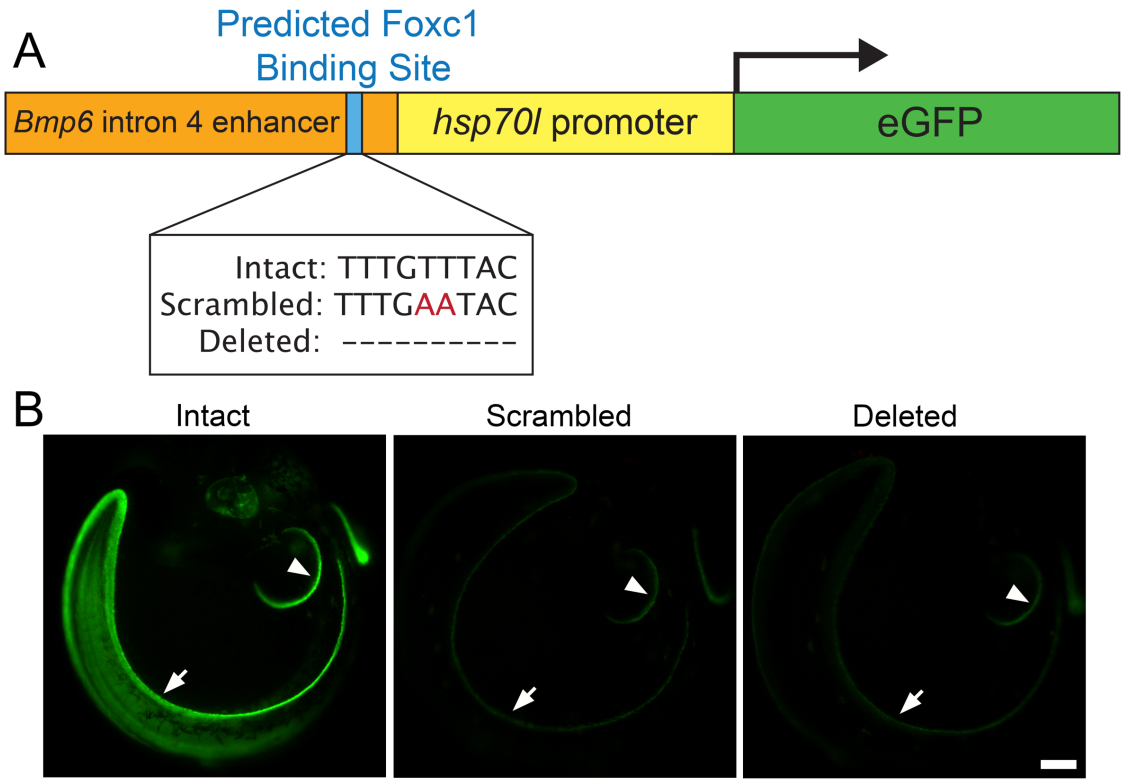


Figure 2. 2 Predicted Foxc1 binding site is required for embryonic enhancer activity.

(A) Schematic of eGFP reporter construct with *Bmp6* intronic enhancer with intact, scrambled, and deleted sequences for the predicted Foxc1 binding site upstream of the *hsp70l* promoter driving eGFP. (B) eGFP reporter expression pattern driven by the *Bmp6* intronic enhancer with intact, scrambled, or deleted sequence for the Foxc1 binding site in 7 dpf (stage 22, (Swarup, 1958)) stickleback embryos. Strong expression was seen in the distal edge of the developing pectoral fin (arrowhead) and median fin (arrow) for the enhancer with an intact sequence of the predicted Foxc1 binding site (left). Severely reduced eGFP expression in all domains of enhancer activity was detected for enhancers with the scrambled (middle) or deleted (right) sequences of the predicted Foxc1 binding site. Scale bar = 100 μm.

While the stable transgenic reporter lines containing the intact binding site drove robust expression in every detectable tooth in the pharyngeal and oral jaw, neither the scrambled nor deleted reporter lines drove detectable GFP expression in pharyngeal teeth (Fig. 2.3). As a control, GFP lens expression driven by the *hsp70l* promoter (Erickson et al., 2015) was comparably bright in the intact, scrambled, and deleted transgenic lines (Fig. 2.3A). Within developing primary oral and pharyngeal teeth, transgene GFP expression was observed in the dental epithelium and mesenchyme for the intact enhancer, but in neither epithelium nor mesenchyme for the scrambled or deleted enhancer in pharyngeal teeth (Fig. 2.3). GFP expression was also seen in the epithelium and mesenchyme of adult teeth for the intact enhancer. Faint GFP expression was detected in some oral teeth in the premaxilla at adult stages for the scrambled enhancer (Fig 3C). Overall, adult tooth expression was largely abolished in the scrambled and deleted enhancer reporter lines, suggesting that the predicted Foxc1 TFBS is required for enhancer activity in replacement as well as primary teeth (Fig. 2.3).

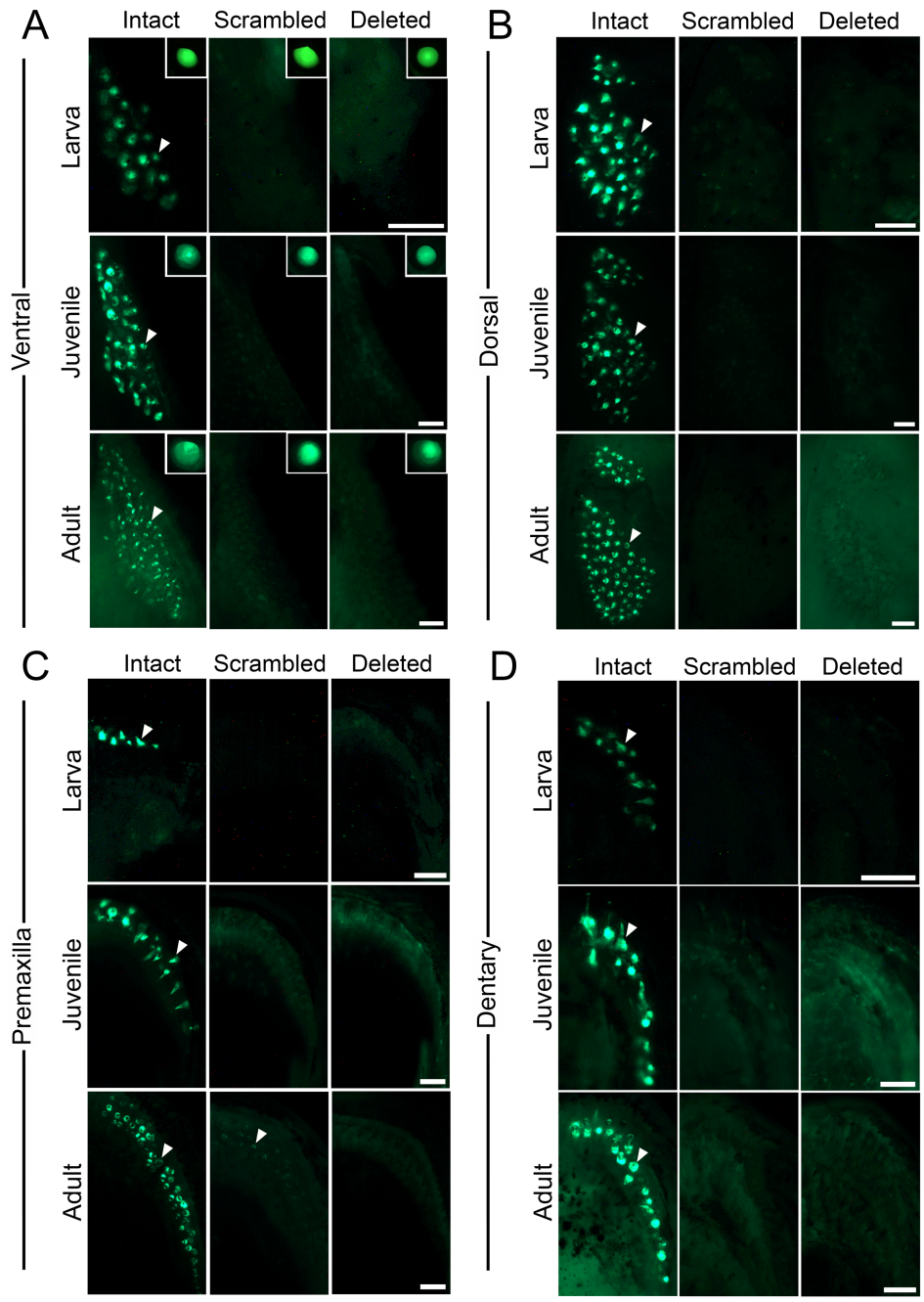


Figure 2. 3 Predicted Foxc1 binding site is required for pharyngeal and oral tooth expression in larva, juveniles, adults.

eGFP reporter gene expression in ventral (A,C) and dorsal (B,D) pharyngeal (A,B) and oral (C,D) jaws in larva (7-11mm SL), juvenile (17-21mm SL), and adult (37-55mm SL) sticklebacks. GFP expression was detected in the epithelium and mesenchyme of developing teeth (arrowheads) in larva, juvenile and adult stages with the intact sequence of the predicted Foxc1 binding site (left columns). No expression was detected in any developing teeth of larva, juvenile and adults with the scrambled or deleted sequence of the predicted Foxc1 binding site (middle and right columns, respectively), except for faint expression in some adult premaxilla teeth (arrow in adult premaxilla panel in C). (A insets) Lens of eye with eGFP expression as an internal control driven by the *hsp70l* promoter used in the transgenic construct. Scale bars = 100 µm (larva and juvenile), 250 µm (adult).

Additionally, we found that the predicted Foxc1 TFBS is required for enhancer function in fins at all developmental time points examined, including in the intersegmental joints of the juvenile and adult pectoral and caudal fins. All fin domains were nearly abolished in both the scrambled and deleted reporter lines at all developmental stages (Fig. 2.4). GFP expression was detected in rare intersegmental joints of the pectoral fins in juveniles for the scrambled enhancer, and in the base of the pectoral fin rays for both the scrambled and deleted construct (Fig. 2.4A). Some GFP expression persisted in the caudal peduncle for the deleted enhancer (Fig. 2.4B). In general, all sites of fin expression, like tooth expression, were severely reduced in both the scrambled and deleted enhancer lines relative to the intact enhancer lines. These data support a crucial role for this single putative Foxc1 binding site in positively regulating both tooth and fin expression across development from embryos to adults.

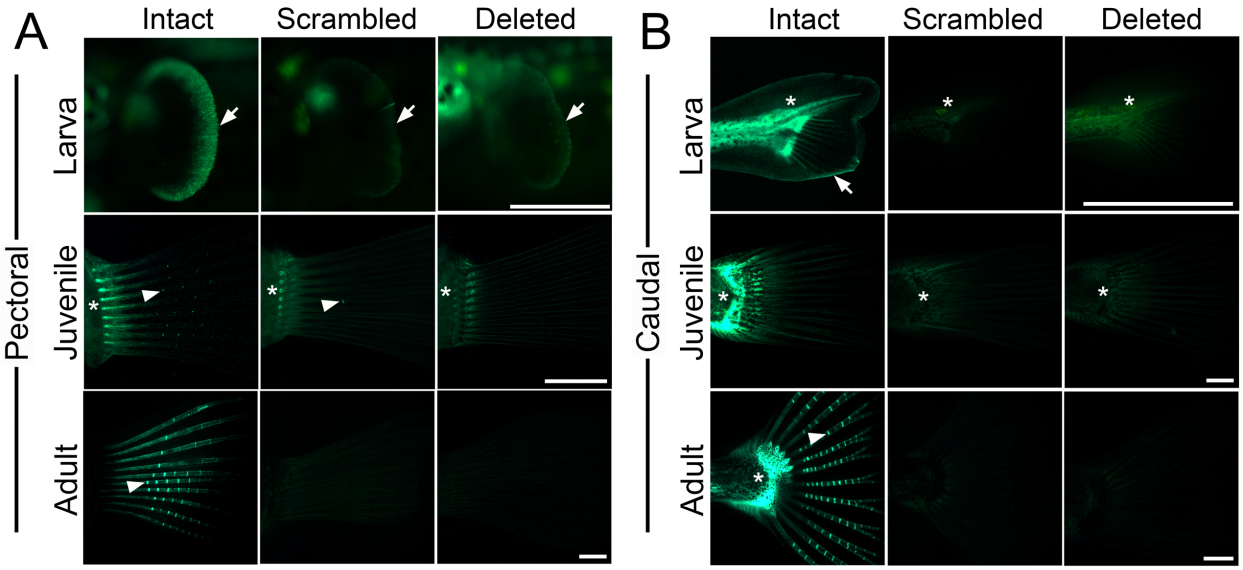


Figure 2. 4 Predicted Foxc1 binding site is required for fin expression in larva, juveniles, and adults.

eGFP reporter gene expression in the left pectoral fin (A) and caudal fin (B) of larva, juveniles and adults. (A) Expression was detected in the distal edges of the pectoral fin (arrows) in larva, the base of the pectoral fin rays (asterisk), and the intersegmental joints (arrowheads) of juveniles and adults with the intact sequence of the predicted Foxc1 binding site (left column). Severely reduced expression was seen in the distal edges of the pectoral fin in larva and the intersegmental joints of juveniles and adults with the scrambled or deleted sequence of the predicted Foxc1 binding site (middle and right columns, respectively), while some expression at the base of the fin rays remained. (B) eGFP reporter expression in the caudal fin. In larva, expression was observed in the distal edge of the median fin fold (arrow) and caudal peduncle (asterisk) with the intact enhancer, but was severely reduced with the scrambled and deleted enhancers. Some peduncle expression remained with the deleted enhancer (right). In juveniles and adults, expression was detected in the peduncle (asterisk) and the intersegmental joints of the caudal fin (arrowheads) for the intact enhancer, but not for the scrambled or deleted enhancer. Scale bars = 500 µm (larva), 1 mm (juvenile), 2 mm (adult).

2.5 Discussion

A short, conserved, pleiotropic binding site is required for enhancer activity in teeth and fins

Here we show a predicted Foxc1 TFBS is required for *Bmp6* intron four enhancer activity in teeth and fins at all stages examined of stickleback development. Altering the predicted Foxc1

TFBS by scrambling two nucleotides or deleting nine nucleotides via site-directed mutagenesis severely downregulated enhancer activity in both teeth and fins, suggesting that this predicted TFBS is critical for enhancer function in multiple expression domains. This site pleiotropy is reminiscent of a previously described enhancer 5' to the stickleback *Bmp6* gene, where a conserved predicted SMAD3 binding site is required for enhancer activity in teeth and fins (Erickson et al., 2015). While both marine and freshwater intron four *Bmp6* enhancers drive strong expression in the distal edges of embryonic fins, only the freshwater enhancer was detected in intersegmental joints in pectoral and caudal fins (Stepaniak et al., 2021). The intact enhancer tested here was the freshwater allele and drove robust fin ray joints as previously reported. Both early distal fin fold and later fin joint expression domains driven by the intact enhancer were absent in the scrambled and deleted Foxc1 TFBS reporter lines.

Enhancers have displayed both site pleiotropy and tightly linked binding sites driving expression in different tissues at different developmental stages. A study investigating the *shavenbaby* enhancers in *Drosophila* found that while one enhancer used the same TFBS to regulate embryonic and pupal expression (site pleiotropy), another enhancer required distinct tightly linked binding sites for expression in different tissues (Preger-Ben Noon et al., 2018). While we hypothesize that this predicted TFBS studied here is bound by Foxc1, it may serve as a binding site for other Fox or non-Fox transcription factors with similar binding site affinity. It is possible that in generating scrambled or deleted TFBSs, one or more new TFBS were generated. However, it would be unlikely that both deleted and scrambled nucleotides would create the same TFBS, given the different primary sequence of these two alleles. Once stickleback Foxc1 antibodies are available, biochemical experiments could test whether stickleback Foxc1a and/or Foxc1b binds this predicted TFBS. Genetic experiments, crossing this enhancer into *Foxc1a* and *Foxc1b* single and double mutants could directly test whether either or both of these duplicate *Foxc1* genes regulate this pleiotropic enhancer.

Roles for Foxc1 in BMP regulation

Previous studies have revealed *Foxc1* as a molecular switch for regulating regeneration in mouse hair follicles. As a transcription factor, Foxc1 activates BMP signaling in self-renewing hair follicle stem cells (HFSCs) (Wang et al., 2016). The conditional knockout of *Foxc1* in mouse results in reduced quiescence in HFSCs and an increase in hair regeneration rates. RNA sequencing (RNA-Seq) and quantitative polymerase chain reaction (qPCR) revealed that genes associated with quiescence of HFSCs, like *Bmp6*, were down-regulated. The direct regulation of *Bmp6* by Foxc1 was supported by chromatin immunoprecipitation PCR (Wang et al., 2016). Here we suggest Foxc1 regulates the BMP pathway for tooth, and possibly fin, development and regeneration through this TFBS in *Bmp6*. Future experiments analyzing the phenotypic effects of *Foxc1a* and *Foxc1b* single and double mutants could shed light on the role that these sticklebacks *Foxc1* co-orthologs play in maintaining quiescence and organ development and regeneration through the BMP pathway.

Foxc1 in organogenesis and chromatin remodeling

Previous studies of enhancer evolution have suggested that enhancers are born as "proto-enhancers" with an initial low-information content but containing a TFBS that serves as a

nucleation point around which other TFBSs then evolve (Emera et al., 2016). The winged-helix structure of the forkhead DNA-binding domain, which is highly conserved across all members of the Fox family, resembles the structure of the linker histone H1, a key modulator of chromatin structure. Because of its structure and chromatin binding interactions, FoxA proteins have been proposed to act as pioneer transcription factors, opening compacted chromatin to allow the binding of other transcription factors (Cirillo et al., 2002; Lee et al., 2005). Foxd3 has been shown to act as a pioneer factor by re-arranging the chromatin landscape and opening *cis*-regulatory elements to maintain multipotency in the early neural crest lineage (Lukoseviciute et al., 2018). Together, these studies suggest Fox family transcription factors promote chromatin accessibility within promoter and enhancer regions.

The loss of Foxc1 co-orthologs (*foxc1a* and *foxc1b*) in zebrafish results in severe reductions of upper facial cartilages as well as missing trabecular cartilage of the neurocranium. The zebrafish *foxc1a*$^{-/-}$ mutants died by 7 dpf, and displayed facial cartilage defects and *foxc1b*$^{-/-}$ mutants displayed a truncation in the symplectic cartilage (Xu et al., 2018). In double homozygous *foxc1a*; *foxc1b* mutants, severe reductions of upper facial cartilages occurred, suggesting that Foxc1a and Foxc1b act redundantly in upper facial cartilage development (Xu et al., 2018). In zebrafish embryos, doubly homozygous *foxc1a* and *foxc1b* mutations, the cartilaginous skeleton did not form properly, likely because many of the regulatory sequences in cartilage regulatory genes were not accessible (Xu et al., 2021). These results support a model in which zebrafish Foxc1 proteins serve as pioneer transcription factors to open chromatin, making it accessible for other transcription factors to bind and regulate genomic regions responsible for cartilage development. It is possible that stickleback Foxc1 proteins may serve similar roles by opening chromatin regions specific to tooth and fin enhancers so other transcription factors can regulate development or regeneration. Future experiments analyzing the genetic effects of *Foxc1a* and *Foxc1b* single and double null-mutants could elucidate the role of the Foxc1 co-orthologs in sticklebacks, and test whether they serve as pioneer transcription factors to promote chromatin accessibility.

2.6 Supplemental Figures

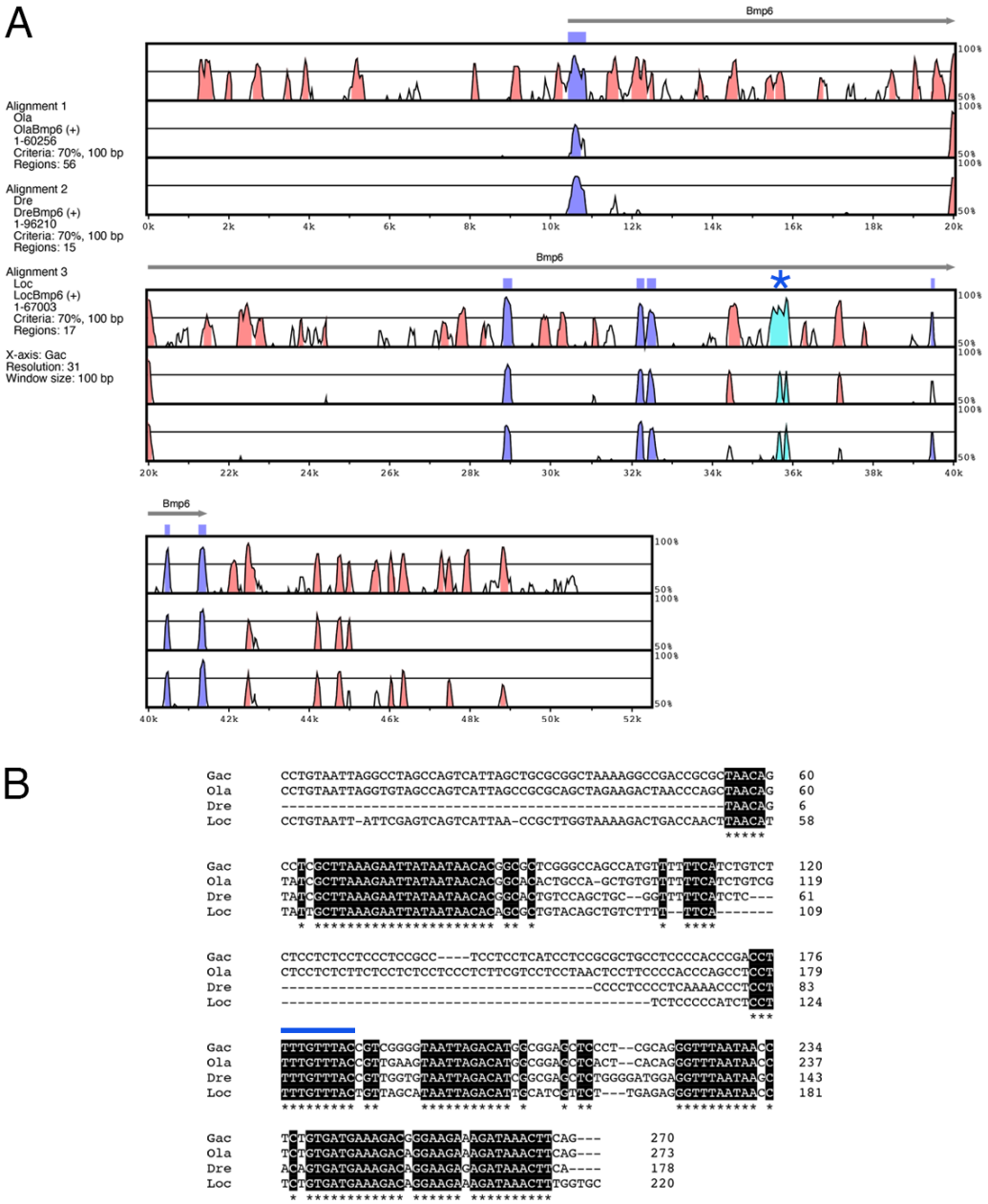


Figure S2. 1 Evolutionary conservation of a predicted Foxc1 binding site within the fourth intron of Bmp6.

(A) mVISTA LAGAN alignment of stickleback *Bmp6* locus to medaka, zebrafish, and gar genome sequences. Gac = *Gasterosteus aculeatus* (stickleback), Ola = *Oryzias latipes* (medaka), Dre = *Danio rerio* (zebrafish), Loc = *Lepisosteus oculatus* (gar). The x-axis shows ~25 kb centered on stickleback *Bmp6* aligned to medaka (Ola, top), zebrafish (Dre, middle) and gar (Loc, bottom). Y-axis starts at 50% nucleotide identity and sliding windows of 100 bp are colored at a 70% nucleotide identity threshold. *Bmp6* exons are annotated as purple regions. The minimally sufficient ~500 base pair intron 4 enhancer (Cleves et al., 2018) is evolutionarily conserved, annotated in light blue, and marked with a blue asterisk. (B) Zooming in on this conserved region reveals that a predicted Foxc1 binding site (TTTGTTTAC) (Berry et al., 2008) (marked by blue line above) is perfectly conserved in medaka, zebrafish, and gar.

2.7 Acknowledgements

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2.9 Appendix

CRISPR/Cas9 Genome Editing Foxc1 A & Foxc1B

Genome editing reagents were designed as previously described (Talbot & Amacher, 2014). Cas9 protein was obtained from UC Berkeley QB3 MacroLab. Guide RNAs (gRNAs) were designed using ZiFiT Targeter (http://zifit.partners.org/ZiFiT/) and Broad Institute CRISPick (https://portals.broadinstitute.org/gppx/crispick/public). DNA oligos were ordered from IDT, and gRNA templates were created with T7 promoters by PCR using Phusion polymerase. Resulting PCR products were gel purified, 100 ng of the resulting elution was used as input to the MAXIscript T7 kit (Ambion), and guide RNA quality verified by running 0.5 µl of the reaction in a 50% formamide buffer on a Tris-acetate-EDTA (TAE) gel. Resulting gRNAs were precipitated using 8M lithium chloride, incubated at -80°C following addition of 75 µl 100% ethanol, and centrifuged at 15,000 rpm for 1 hour at 4°C. Following a wash with 200 µL of 75% ethanol and an additional centrifugation at 15,000 rpm for 10 minutes at 4°C, RNAs were resuspended in 20 µl of DEPC treated water. Stickleback embryos at the one cell stage were microinjected as described (Erickson et al., 2016) with some modifications. First, the concentration of gRNA in the 0.2M KCl injection mixture was increased to 200 ng/µl, 3.33uM of Cas9 protein was used in place of Cas9 mRNA, and 0.025% phenol red was used as a tracking dye. Embryos were monitored for viability prior to hatching. F_0 injected mosaics were used to create stable F_1 heterozygous mutants, which were incrossed to produce F_2s. DNA from uninjected, wild-type injected, and gRNA mosaic injected embryos was purified at 4-6dpf and adults >20mm SL, and mutatant alleles in *Foxc1a* and *Foxc1b* were validated using Sanger sequencing of PCR products as described below.

Table 2.1 CRISPR-Cas9 gRNA target sequences and genotyping primers

Catalog #	Name	Target	Sequence (5' → 3')
AJB014	Foxc1a_sgRNA3 target	Foxc1a	GCGTAATACGACTCACTATAAAGGATATGGT CAAACCCCCGTTTTAGAGCTAGAAATAGC
AJB015	Foxc1a_sgRNA4 target	Foxc1a	GCGTAATACGACTCACTATAGTACCCCGCCA GCATGGCCCGTTTTAGAGCTAGAAATAGC
AJB016	Foxc1a_Fwd1 genotyping	Foxc1a	CTCGCTACTCCGTCTCCAG
AJB017	Foxc1a_Fwd2 sequencing	Foxc1a	GTCGTGCCCTACATCAGCAG
AJB019	Foxc1a_Rev2 genotyping	Foxc1a	TCTCTTTCACGACGTCCTTCT
AJB020	Foxc1b_sgRNA2 target	Foxc1b	GCGTAATACGACTCACTATAGCACAATCTGT CTCTCAACGGTTTTAGAGCTAGAAATAGC

AJB021	Foxc1b_sgRNA3 target	Foxc1b	GCGTAATACGACTCACTATACATGGCAATCC AGAATTCAGGTTTTAGAGCTAGAAATAGC
AJB024	Foxc1b_Fwd1 genotyping	Foxc1b	TGGATGATGGAGCACGCTTT
AJB025	Foxc1b_Fwd2 sequencing	Foxc1b	GAAACAGATTGGGAGCACGC
AJB027	Foxc1b_Rev2 genotyping	Foxc1b	GGATGTTTGGTTGGGGGAGT

Genotyping

For DNA purification in sticklebacks >20mm SL, DNA was extracted from caudal fin tissue or sperm and digested for 12+ hours at 55°C in 600 µl of tail digestion buffer (10mM Tris pH 8.0, 100mM NaCl, 10mM EDTA, 0.05% SDS, 2.5 µl Proteinase K (Ambion AM2546)). Following addition of 600µL of 1:1 phenol:chloroform solution and an aqueous extraction, DNA was precipitated with the addition of 1ml 100% ethanol, centrifuged, washed with 75% ethanol, and resuspended in water. For DNA purification in stickleback embryos, DNA was extracted from the embryo by dissociating embryos using a polypropylene pellet pestle, and digesting cells at 94°C for 20 minutes in 50uL of Zebrafish lysis buffer (ZLB)(10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.3 % Tween-20, 0.3% NP-40). Proteinase K was added to the samples at 2.5 uL of 20 mg/ml and incubated for 55°C for 60 minutes and then 94°C for 20 minutes to inactivate the proteinase. The supernatant of this liquid was then used as template for PCR. For sequencing, DNA was amplified by PCR using Phusion polymerase and primers listed above. The PCR products were PCR purified and Sanger sequenced at UC Berkeley DNA Sequencing Facility.

Alizarin Calcein skeletal staining and visualization

Alizarin live vital dye bone staining was performed as described in (Ellis et al., 2015) by pulsing fish with 100 µg/ml Alizarin Red S buffered with 1 mM HEPES (pH 7.0) in tank water for 18 hours in the dark (Kimmel et al., 1998, 2001) . After replacing Alizarin with fresh stickleback water, fish were returned to tanks for 14 days, and then chased with 50 µg/ml Calcein buffered with 1 mM sodium phosphate, dibasic, anhydrous (pH 8.0) in tank water for 12 hours in the dark and the replaced with fresh stickleback water. Sticklebacks were euthanized with MS222 (Western Chemical, Inc., FisherScientific NC0342409) and fixed by immersion in 4% paraformaldehyde (PFA) in 1xPBS (Sigma P6148) at 4°C overnight (4hrs for fry). Standard length (tip of snout to caudal peduncle) and/or total length (tip of snout to most posterior tip of tail) were measured with digital calipers following PFA fixation. Branchial skeletons were dissected, cleared, and mounted as previously described (Ellis et al., 2016; Miller et al., 2014). Representative tooth plate z-stack projections were imaged on a Zeiss 700 confocal microscope.

Quantification of Tooth Regeneration Rates in sticklebacks

We quantified tooth number and the rate of tooth replacement in adult fish using the Alizarin-Calcein pulse-chase method as described above to mark new tooth formation. By pulsing first with Alizarin, waiting 2 weeks, then chasing with Calcein, mineralizing teeth are marked at two points in time with red (Alizarin) and green (Calcein) fluorescence, respectively, allowing the visualization of new teeth that formed between the two dye soaks (Calcein-positive, Alizarin-negative teeth). Dorsal and ventral pharyngeal tooth number was quantified using z-stack projections taken on a Zeiss LSM700 confocal microscope. For both ventral and dorsal tooth counts, total tooth number equals the sum of the left and right sides (of ventral and dorsal

pharyngeal teeth, respectively). To account for any total difference in tooth number between wild type and *Foxc1a* or *Foxc1b* mutant groups, we divided the number of new teeth by the total tooth number to quantify a normalized rate of tooth gain.

Results

We used CRISPR-Cas9 to disrupt endogenous *Foxc1a* and *Foxc1b* transcription factors *in vivo* and found that standard length is reduced in heterozygous mutants of the +13nt allele (P = 1.55e-07) and -4nt allele (P = 0.00091). Future experiments investigating differences in tooth number and regeneration rates will elucidate which of these transcription factors are required for enhancer activity and whether shared regulatory networks control fin and tooth development.

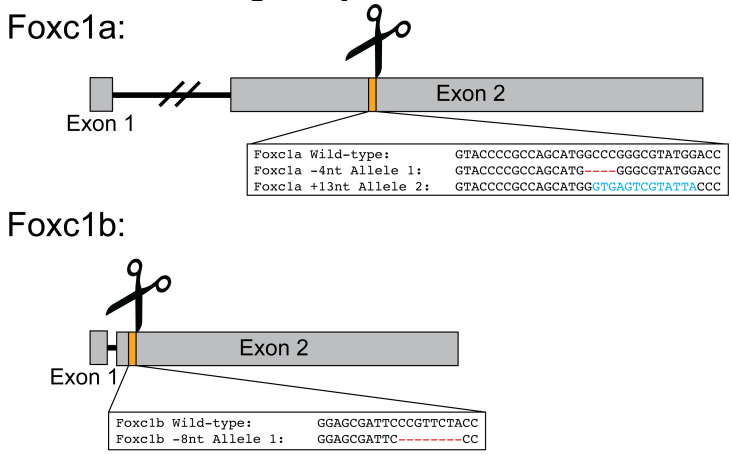


Figure S2. 2 Graphic representation of CRISPR-Cas9 editing of Foxc1a and Foxc1b and their respective alleles.

Guide RNAs targeting exon two of both *Foxc1a* and *Foxc1b* at the endogenous locus were injected into 1-cell stage embryos generating two alleles for *Foxc1a,* -4nt deletion and +13nt insertion, and one allele for *Foxc1b,* -8nt deletion.

2.10 References

Abduweli, D., Baba, O., Tabata, M. J., Higuchi, K., Mitani, H., & Takano, Y. (2014). Tooth replacement and putative odontogenic stem cell niches in pharyngeal dentition of medaka (*Oryzias latipes*). *Microscopy*, *63*(2), 141–153. https://doi.org/10.1093/jmicro/dft085

Åberg, T., Wozney, J., & Thesleff, I. (1997). Expression patterns of bone morphogenetic proteins (Bmps) in the developing mouse tooth suggest roles in morphogenesis and cell differentiation. *Developmental Dynamics*, *210*(4), 383–396. https://doi.org/10.1002/(SICI)1097-0177(199712)210:4<383::AID-AJA3>3.0.CO;2-C

Albert, A. Y. K., Sawaya, S., Vines, T. H., Knecht, A. K., Miller, C. T., Summers, B. R., … Schluter, D. (2007). The genetics of adaptive shape shift in stickleback: pleiotropy and effect size. *Evolution*, *0*(0), 071115145922005-??? https://doi.org/10.1111/j.1558-5646.2007.00259.x

Aldinger, K. A., Lehmann, O. J., Hudgins, L., Chizhikov, V. V., Bassuk, A. G., Ades, L. C., … Millen, K. J. (2009). FOXC1 is required for normal cerebellar development and is a major contributor to chromosome 6p25.3 Dandy-Walker malformation. *Nature Genetics*, *41*(9), 1037–1042. https://doi.org/10.1038/ng.422

Amores, A., Force, A., Yan, Y. L., Joly, L., Amemiya, C., Fritz, A., … Postlethwait, J. H. (1998). Zebrafish hox clusters and vertebrate genome evolution. *Science*, *282*(5394), 1711–1714. https://doi.org/10.1126/science.282.5394.1711

Arnold, K., Sarkar, A., Yram, M. A., Polo, J. M., Bronson, R., Sengupta, S., … Hochedlinger, K. (2011). Sox2+ Adult Stem and Progenitor Cells Are Important for Tissue Regeneration and Survival of Mice. *Cell Stem Cell*, *9*(4), 317–329. https://doi.org/10.1016/J.STEM.2011.09.001

Bei, M, Kratochwil, K., & Maas, R. L. (2000). BMP4 rescues a non-cell-autonomous function of Msx1 in tooth development. *Development (Cambridge, England)*, *127*(21), 4711–4718. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11023873

Bei, Marianna, & Maas, R. (1998). FGFs and BMP4 induce both Msx1-independent and Msx1-dependent signaling pathways in early tooth development. *Development*, *125*(21), 4325–4333. https://doi.org/10.1242/dev.125.21.4325

Bell, M. A., & Foster, S. A. (1994). The Evolutionary Biology of the Threespine Stickleback. *Oxford University Press*, *64*(3), 418. https://doi.org/10.2307/5902

Berry, F. B., Lines, M. A., Oas, J. M., Footz, T., Underhill, D. A., Gage, P. J., & Walter, M. A. (2006). Functional interactions between FOXC1 and PITX2 underlie the sensitivity to FOXC1 gene dose in Axenfeld-Rieger syndrome and anterior segment dysgenesis. *Human Molecular Genetics*, *15*(6), 905–919. https://doi.org/10.1093/hmg/ddl008

Berry, F. B., Skarie, J. M., Mirzayans, F., Fortin, Y., Hudson, T. J., Raymond, V., … Walter, M. A. (2008). FOXC1 is required for cell viability and resistance to oxidative stress in the eye through the transcriptional regulation of FOXO1A. *Human Molecular Genetics*, *17*(4), 490–505. https://doi.org/10.1093/hmg/ddm326

Blanpain, C., Lowry, W. E., Geoghegan, A., Polak, L., & Fuchs, E. (2004). Self-renewal, multipotency, and the existence of two cell populations within an epithelial stem cell niche. *Cell*, *118*(5), 635–648. https://doi.org/10.1016/j.cell.2004.08.012

Bloomquist, R. F., Fowler, T. E., An, Z., Yu, T. Y., Abdilleh, K., Fraser, G. J., … Streelman, J. T. (2019). Developmental plasticity of epithelial stem cells in tooth and taste bud renewal. *Proceedings of the National Academy of Sciences of the United States of America*, *116*(36), 17858–17866. https://doi.org/10.1073/pnas.1821202116

Boffelli, D., Nobrega, M. A., & Rubin, E. M. (2004, June). Comparative genomics at the vertebrate extremes. *Nature Reviews Genetics*. https://doi.org/10.1038/nrg1350

Bonferroni, C. E. (1936). Teoria statistica delle classi e calcolo delle probabilità. *Pubblicazioni Del R Istituto Superiore Di Scienze Economiche e Commerciali Di Firenze*.

Carroll, S. B. (1995). Homeotic genes and the evolution of arthropods and chordates. *Nature*, *376*(6540), 479–485. https://doi.org/10.1038/376479a0

Chan, Y. F., Marks, M. E., Jones, F. C., Villarreal, G., Shapiro, M. D., Brady, S. D., … Kingsley, D. M. (2010). Adaptive evolution of pelvic reduction in sticklebacks by recurrent deletion of a pitxl enhancer. *Science*, *327*(5963), 302–305. https://doi.org/10.1126/science.1182213

Chanda, B., Asai-Coakwell, M., Ye, M., Mungall, A. J., Barrow, M., Dobyns, W. B., … Lehmann, O. J. (2008). A novel mechanistic spectrum underlies glaucoma-associated

chromosome 6p25 copy number variation. *Human Molecular Genetics*, *17*(22), 3446–3458. https://doi.org/10.1093/hmg/ddn238

Chen, Y. P., Bei, M., Woo, I., Satokata, I., & Maas, R. (1996). Msx1 controls inductive signaling in mammalian tooth morphogenesis. *Development*, *122*(10), 3035–3044. https://doi.org/10.1242/dev.122.10.3035

Cirillo, L. A., Lin, F. R., Cuesta, I., Friedman, D., Jarnik, M., & Zaret, K. S. (2002). Opening of compacted chromatin by early developmental transcription factors HNF3 (FoxA) and GATA-4. *Molecular Cell*, *9*(2), 279–289. https://doi.org/10.1016/S1097-2765(02)00459-8

Clavel, C., Grisanti, L., Zemla, R., Rezza, A., Barros, R., Sennett, R., … Rendl, M. (2012). Sox2 in the dermal papilla niche controls hair growth by fine-tuning BMP signaling in differentiating hair shaft progenitors. *Developmental Cell*, *23*(5), 981–994. https://doi.org/10.1016/j.devcel.2012.10.013

Cleves, P. A., Ellis, N. A., Jimenez, M. T., Nunez, S. M., Schluter, D., Kingsley, D. M., & Miller, C. T. (2014). Evolved tooth gain in sticklebacks is associated with a cis-regulatory allele of Bmp6. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(38), 13912–13917. https://doi.org/10.1073/pnas.1407567111

Cleves, P. A., Hart, J. C., Agoglia, R. M., Jimenez, M. T., Erickson, P. A., Gai, L., & Miller, C. T. (2018). An intronic enhancer of Bmp6 underlies evolved tooth gain in sticklebacks. *PLOS Genetics*, *14*(6), e1007449. https://doi.org/10.1371/journal.pgen.1007449

Colosimo, P. F. (2005). Widespread Parallel Evolution in Sticklebacks by Repeated Fixation of Ectodysplasin Alleles. *Science*, *307*(5717), 1928–1933. https://doi.org/10.1126/science.1107239

Cotsarelis, G., Sun, T.-T., & Lavker, R. M. (1990). Label-retaining cells reside in the bulge area of pilosebaceous unit: Implications for follicular stem cells, hair cycle, and skin carcinogenesis. *Cell*, *61*(7), 1329–1337. https://doi.org/10.1016/0092-8674(90)90696-C

Cowles, C. R., Hirschhorn, J. N., Altshuler, D., & Lander, E. S. (2002). Detection of regulatory variation in mouse genes. *Nature Genetics*, *32*(3), 432–437. https://doi.org/10.1038/ng992

Cresko, W. A., Amores, A., Wilson, C., Murphy, J., Currey, M., Phillips, P., … Postlethwait, J. H. (2004). Parallel genetic basis for repeated evolution of armor loss in Alaskan threespine stickleback populations. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(16), 6050–6055. https://doi.org/10.1073/pnas.0308479101

Crick, F. (1958). On Protein Synthesis. *Symposia of the Society for Experimental Biology, Number XII: The Biological Replication of Macromolecules. Cambridge University Press.*, 138–163.

Cuny, G. D., Yu, P. B., Laha, J. K., Xing, X., Liu, J. F., Lai, C. S., … Peterson, R. T. (2008). Structure-activity relationship study of bone morphogenetic protein (BMP) signaling inhibitors. *Bioorganic and Medicinal Chemistry Letters*, *18*(15), 4388–4392. https://doi.org/10.1016/j.bmcl.2008.06.052

Darwin, C. (1859). On the Origins of Species by Means of Natural Selection, or the Preservation of Favoured Races in the Struggle for Life.

DeScipio, C., Schneider, L., Young, T. L., Wasserman, N., Yaeger, D., Lu, F., … Krantz, I. D.

(2005). Subtelomeric deletions of chromosome 6p: Molecular and cytogenetic characterization of three new cases with phenotypic overlap with Ritscher?Schinzel (3C) syndrome. *American Journal of Medical Genetics Part A*, *134A*(1), 3–11. https://doi.org/10.1002/ajmg.a.30573

Ellis, N. A., Donde, N. N., & Miller, C. T. (2016). Early development and replacement of the stickleback dentition. *Journal of Morphology*, *277*(8), 1072–1083. https://doi.org/10.1002/jmor.20557

Ellis, N. A., Glazer, A. M., Donde, N. N., Cleves, P. A., Agoglia, R. M., & Miller, C. T. (2015). Distinct developmental and genetic mechanisms underlie convergently evolved tooth gain in sticklebacks. *Development*, (June), 2442–2451. https://doi.org/10.1242/dev.124248

Emera, D., Yin, J., Reilly, S. K., Gockley, J., & Noonan, J. P. (2016). Origin and evolution of developmental enhancers in the mammalian neocortex. *Proceedings of the National Academy of Sciences of the United States of America*, *113*(19), E2617–E2626. https://doi.org/10.1073/pnas.1603718113

Erickson, P. A., Cleves, P. A., Ellis, N. A., Schwalbach, K. T., Hart, J. C., & Miller, C. T. (2015). A 190 base pair, TGFB responsive tooth and fin enhancer is required for stickleback Bmp6 expression. *Developmental Biology*, *401*(2), 310–323. https://doi.org/10.1016/j.ydbio.2015.02.006

Erickson, P. A., Ellis, N. A., & Miller, C. T. (2016). Microinjection for Transgenesis and Genome Editing in Threespine Sticklebacks. *Journal of Visualized Experiments*, (111), e54055–e54055. https://doi.org/10.3791/54055

Erickson, P. A., Glazer, A. M., Cleves, P. A., Smith, A. S., & Miller, C. T. (2014). Two developmentally temporal quantitative trait loci underlie convergent evolution of increased branchial bone length in sticklebacks. *Proceedings of the Royal Society B: Biological Sciences*, *281*(1788). https://doi.org/10.1098/rspb.2014.0822

Farh, K. K. H., Marson, A., Zhu, J., Kleinewietfeld, M., Housley, W. J., Beik, S., … Bernstein, B. E. (2015). Genetic and epigenetic fine mapping of causal autoimmune disease variants. *Nature*, *518*(7539), 337–343. https://doi.org/10.1038/nature13835

Fish, A., Chen, L., & Capra, J. A. (2017). Gene regulatory enhancers with evolutionarily conserved activity aremore pleiotropic than those with species-specific activity. *Genome Biology and Evolution*, *9*(10), 2615–2625. https://doi.org/10.1093/gbe/evx194

Fisher, S., Grice, E. A., Vinton, R. M., Bessling, S. L., & McCallion, A. S. (2006). Conservation of RET regulatory function from human to zebrafish without sequence similarity. *Science*, *312*(5771), 276–279. https://doi.org/10.1126/science.1124070

Fraser, G. J., Bloomquist, R. F., & Streelman, J. T. (2013). Common developmental pathways link tooth shape to regeneration. *Developmental Biology*, *377*(2), 399–414. https://doi.org/10.1016/J.YDBIO.2013.02.007

Frazer, K. A., Pachter, L., Poliakov, A., Rubin, E. M., & Dubchak, I. (2004). VISTA: Computational tools for comparative genomics. *Nucleic Acids Research*, *32*(WEB SERVER ISS.), W273–W279. https://doi.org/10.1093/nar/gkh458

Furlong, E. E. M., & Levine, M. (2018, September 28). Developmental enhancers and

chromosome topology. *Science*. American Association for the Advancement of Science. https://doi.org/10.1126/science.aau0320

Gehring, W. J., & Hiromi, Y. (1986). Homeotic Genes and the Homeobox. *Annual Review of Genetics*, *20*(1), 147–173. https://doi.org/10.1146/annurev.ge.20.120186.001051

Glazer, A. M., Cleves, P. A., Erickson, P. A., Lam, A. Y., & Miller, C. T. (2014). Parallel developmental genetic features underlie stickleback gill raker evolution. *EvoDevo*, *5*(1), 1–16. https://doi.org/10.1186/2041-9139-5-19

Glazer, A. M., Killingbeck, E. E., Mitros, T., Rokhsar, D. S., & Miller, C. T. (2015). Genome assembly improvement and mapping convergently evolved skeletal traits in sticklebacks with genotyping-by-sequencing. *G3: Genes, Genomes, Genetics*, *5*(7), 1463–1472. https://doi.org/10.1534/g3.115.017905

Gould, D. B., Jaafar, M. S., Addison, M. K., Munier, F., Ritch, R., MacDonald, I. M., & Walter, M. A. (2004). Phenotypic and molecular assessment of seven patients with 6p25 deletion syndrome: Relevance to ocular dysgenesis and hearing impairment. *BMC Medical Genetics*, *5*(1), 17. https://doi.org/10.1186/1471-2350-5-17

Handrigan, G. R., Leung, K. J., & Richman, J. M. (2010). Identification of putative dental epithelial stem cells in a lizard with life-long tooth replacement. *Development*, *137*(21), 3545–3549. https://doi.org/10.1242/dev.052415

Handrigan, Gregory R., Leung, K. J., & Richman, J. M. (2010). Identification of putative dental epithelial stem cells in a lizard with life-long tooth replacement. *Development*, *137*(21). Retrieved from http://dev.biologists.org/content/137/21/3545

Handrigan, Gregory R., & Richman, J. M. (2010). A network of Wnt, hedgehog and BMP signaling pathways regulates tooth replacement in snakes. *Developmental Biology*, *348*(1), 130–141. https://doi.org/10.1016/J.YDBIO.2010.09.003

Hannenhalli, S., & Kaestner, K. H. (2009, April). The evolution of Fox genes and their role in development and disease. *Nature Reviews Genetics*. NIH Public Access. https://doi.org/10.1038/nrg2523

Harada, H., Kettunen, P., Jung, H. S., Mustonen, T., Wang, Y. A., & Thesleff, I. (1999). Localization of putative stem cells in dental epithelium and their association with Notch and FGF signaling. *Journal of Cell Biology*, *147*(1), 105–120. https://doi.org/10.1083/jcb.147.1.105

Harris, M. P., Rohner, N., Schwarz, H., Perathoner, S., Konstantinidis, P., & Nüsslein-Volhard, C. (2008). Zebrafish eda and edar Mutants Reveal Conserved and Ancestral Roles of Ectodysplasin Signaling in Vertebrates. *PLoS Genetics*, *4*(10), e1000206. https://doi.org/10.1371/journal.pgen.1000206

Hart, J. C., & Miller, C. T. (2017). Sequence-Based Mapping and Genome Editing Reveal Mutations in SticklebackHps5Cause Oculocutaneous Albinism and thecasperPhenotype. *G3 (Bethesda, Md.)*, *7*(9), 3123–3131. https://doi.org/10.1534/g3.117.1125

Headon, D. J., Emmal, S. A., Ferguson, B. M., Tucker, A. S., Justice, M. J., Sharpe, P. T., … Overbeek, P. A. (2001). Gene defect in ectodermal dysplasia implicates a death domain adapter in development. *Nature*, *414*(6866), 913–916. https://doi.org/10.1038/414913a

Huang, X., Zhong, L., Hendriks, J., Post, J. N., & Karperien, M. (2018). The effects of the WNT-signaling modulators BIO and PKF118-310 on the chondrogenic differentiation of human mesenchymal stem cells. *International Journal of Molecular Sciences*, *19*(2). https://doi.org/10.3390/ijms19020561

Huxley, J. (1942). Evolution, the Modern Synthesis. *G. Allen and Unwin Limited.*

Indjeian, V. B., Kingman, G. A., Jones, F. C., Guenther, C. A., Grimwood, J., Schmutz, J., … Kingsley, D. M. (2016). Evolving New Skeletal Traits by cis-Regulatory Changes in Bone Morphogenetic Proteins. *Cell*, *164*(1–2), 45–56. https://doi.org/10.1016/j.cell.2015.12.007

Infante, C. R., Mihala, A. G., Park, S., Wang, J. S., Johnson, K. K., Lauderdale, J. D., & Menke, D. B. (2015). Shared Enhancer Activity in the Limbs and Phallus and Functional Divergence of a Limb-Genital cis-Regulatory Element in Snakes. *Developmental Cell*, *35*(1), 107–119. https://doi.org/10.1016/j.devcel.2015.09.003

Jackman, W. R., & Stock, D. W. (2006). Transgenic analysis of Dlx regulation in fish tooth development reveals evolutionary retention of enhancer function despite organ loss. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(51), 19390–19395. https://doi.org/10.1073/pnas.0609575103

Jacob, F., & Monod, J. (1961, June 1). Genetic regulatory mechanisms in the synthesis of proteins. *Journal of Molecular Biology*. Academic Press. https://doi.org/10.1016/S0022-2836(61)80072-7

Jernvall, J., & Thesleff, I. (2012). Tooth shape formation and tooth renewal: evolving with the same signals. *Development*, *139*(19), 3487–3497. https://doi.org/10.1242/dev.085084

Jia, S., Zhou, J., Gao, Y., Baek, J.-A., Martin, J. F., Lan, Y., … Sucov, H. M. (2013). Roles of Bmp4 during tooth morphogenesis and sequential tooth formation. *Development (Cambridge, England)*, *140*(2), 423–432. https://doi.org/10.1242/dev.081927

Jones, F. C., Grabherr, M. G., Chan, Y. F., Russell, P., Mauceli, E., Johnson, J., … Kingsley, D. M. (2012). The genomic basis of adaptive evolution in threespine sticklebacks. *Nature*, *484*(7392), 55–61. https://doi.org/10.1038/nature10944

Juuri, E., Jussila, M., Seidel, K., Holmes, S., Wu, P., Richman, J., … Thesleff, I. (2013). Sox2 marks epithelial competence to generate teeth in mammals and reptiles. *Development (Cambridge, England)*, *140*(7), 1424–1432. https://doi.org/10.1242/dev.089599

Juuri, E., Saito, K., Ahtiainen, L., Seidel, K., Tummers, M., Hochedlinger, K., … Michon, F. (2012). Sox2+ stem cells contribute to all epithelial lineages of the tooth via Sfrp5+ progenitors. *Developmental Cell*, *23*(2), 317–328. https://doi.org/10.1016/j.devcel.2012.05.012

Kandyba, E., Leung, Y., Chen, Y.-B., Widelitz, R., Chuong, C.-M., & Kobielak, K. (2013). Competitive balance of intrabulge BMP/Wnt signaling reveals a robust gene network ruling stem cell homeostasis and cyclic activation. *Proceedings of the National Academy of Sciences*, *110*(4), 1351–1356. https://doi.org/10.1073/pnas.1121312110

Kawakami, K., & Shima, A. (1999). Identification of the Tol2 transposase of the medaka fish Oryzias latipes that catalyzes excision of a nonautonomous Tol2 element in zebrafish Danio rerio. *Gene*, *240*(1), 239–244. https://doi.org/10.1016/S0378-1119(99)00444-8

Kimmel, C. B., Miller, C. T., Kruze, G., Ullmann, B., BreMiller, R. A., Larison, K. D., & Snyder, H. C. (1998). The Shaping of Pharyngeal Cartilages during Early Development of the Zebrafish. *Developmental Biology*, *203*(2), 245–263. https://doi.org/10.1006/DBIO.1998.9016

Kimmel, C. B., Miller, C. T., & Moens, C. B. (2001). Specification and Morphogenesis of the Zebrafish Larval Head Skeleton. *Developmental Biology*, *233*(2), 239–257. https://doi.org/10.1006/DBIO.2001.0201

King, M. C., & Wilson, A. C. (1975). Evolution at two levels in humans and chimpanzees. *Science*, *188*(4184), 107–116. https://doi.org/10.1126/science.1090005

Kingsley, D. M., Zhu, B., Osoegawa, K., De Jong, P. J., Schein, J., Marra, M., … Myers, R. (2004). New genomic tools for molecular studies of evolutionary change in threespine sticklebacks. In *Behaviour* (Vol. 141, pp. 1331–1344). Brill. https://doi.org/10.1163/1568539042948150

Kobielak, K., Stokes, N., de la Cruz, J., Polak, L., & Fuchs, E. (2007). Loss of a quiescent niche but not follicle stem cells in the absence of bone morphogenetic protein signaling. *Proceedings of the National Academy of Sciences*, *104*(24), 10063–10068. https://doi.org/10.1073/pnas.0703004104

Kume, T., Jiang, H., Topczewska, J. M., & Hogan, B. L. M. (2001). The murine winged helix transcription factors, Foxc1 and Foxc2, are both required for cardiovascular development and somitogenesis. *Genes and Development*, *15*(18), 2470–2482. https://doi.org/10.1101/gad.907301

Kume, Tsutomu, Deng, K. Y., Winfrey, V., Gould, D. B., Walter, M. A., & Hogan, B. L. M. (1998). The forkhead/winged helix gene Mf1 is disrupted in the pleiotropic mouse mutation congenital hydrocephalus. *Cell*, *93*(6), 985–996. https://doi.org/10.1016/S0092-8674(00)81204-0

Le Caignec, C., De Mas, P., Vincent, M.-C., Bocéno, M., Bourrouillou, G., Rival, J.-M., & David, A. (2005). Subtelomeric 6p deletion: Clinical, FISH, and array CGH characterization of two cases. *American Journal of Medical Genetics Part A*, *132A*(2), 175–180. https://doi.org/10.1002/ajmg.a.30409

Leche, W. (1895). Zür Entwicklungsgeschichte des Zahnsystems des Säugethiere, zugleich ein Beitrag zur Stammesgeschichte dieser Thiergruppe. I. Ontogenie. Zoologica 6, 1-160. (n.d.).

Lee, C. S., Friedman, J. R., Fulmer, J. T., & Kaestner, K. H. (2005). The initiation of liver development is dependent on Foxa transcription factors. *Nature*, *435*(7044), 944–947. https://doi.org/10.1038/nature03649

Lehmann, O. J., Sowden, J. C., Carlsson, P., Jordan, T., & Bhattacharya, S. S. (2003, June 1). Fox's in development and disease. *Trends in Genetics*. Elsevier Ltd. https://doi.org/10.1016/S0168-9525(03)00111-2

Levine, M., Cattoglio, C., & Tjian, R. (2014, March 27). Looping back to leap forward: Transcription enters a new era. *Cell*. Elsevier B.V. https://doi.org/10.1016/j.cell.2014.02.009

Li, A., Lai, Y. C., Figueroa, S., Yang, T., Widelitz, R. B., Kobielak, K., … Chuong, C. M. (2015,

August 1). Deciphering principles of morphogenesis from temporal and spatial patterns on the integument. *Developmental Dynamics*. John Wiley and Sons Inc. https://doi.org/10.1002/dvdy.24281

Lin, C. R., Kioussi, C., O'Connell, S., Briata, P., Szeto, D., Liu, F., … Rosenfeld, M. G. (1999). Pitx2 regulates lung asymmetry, cardiac positioning and pituitary and tooth morphogenesis. *Nature*, *401*(6750), 279–282. https://doi.org/10.1038/45803

Lukoseviciute, M., Gavriouchkina, D., Williams, R. M., Hochgreb-Hagele, T., Senanayake, U., Chong-Morrison, V., … Sauka-Spengler, T. (2018). From Pioneer to Repressor: Bimodal foxd3 Activity Dynamically Remodels Neural Crest Regulatory Landscape In Vivo. *Developmental Cell*, *47*(5), 608-628.e6. https://doi.org/10.1016/j.devcel.2018.11.009

Mandler, M., & Neubüser, A. (2001). FGF signaling is necessary for the specification of the odontogenic mesenchyme. *Developmental Biology*, *240*(2), 548–559. https://doi.org/10.1006/dbio.2001.0490

Martin, K. J., Rasch, L. J., Cooper, R. L., Metscher, B. D., Johanson, Z., & Fraser, G. J. (2016). Sox2+ progenitors in sharks link taste development with the evolution of regenerative teeth from denticles. *Proceedings of the National Academy of Sciences*, *113*(51), 14769–14774. https://doi.org/10.1073/pnas.1612354113

Maurano, M. T., Humbert, R., Rynes, E., Thurman, R. E., Haugen, E., Wang, H., … Stamatoyannopoulos, J. A. (2012). Systematic localization of common disease-associated variation in regulatory DNA. *Science*, *337*(6099), 1190–1195. https://doi.org/10.1126/science.1222794

Mazet, F., Amemiya, C. T., & Shimeld, S. M. (2006, May 9). An ancient Fox gene cluster in bilaterian animals. *Current Biology*. Elsevier. https://doi.org/10.1016/j.cub.2006.03.088

McKinnon, J. S., & Rundle, H. D. (2002, October 1). Speciation in nature: The threespine stickleback model systems. *Trends in Ecology and Evolution*. Elsevier Current Trends. https://doi.org/10.1016/S0169-5347(02)02579-X

Mendel, G. (1865). Versuche ber Pflanzen-HyBriden. *Verh Naturforsch Ver Brnn, 4(347)*.

Miller, C. T., Beleza, S., Pollen, A. A., Schluter, D., Kittles, R. A., Shriver, M. D., & Kingsley, D. M. M. (2007). cis-Regulatory Changes in Kit Ligand Expression and Parallel Evolution of Pigmentation in Sticklebacks and Humans. *Cell*, *131*(6), 1179–1189. https://doi.org/10.1016/j.cell.2007.10.055

Miller, C. T., Glazer, A. M., Summers, B. R., Blackman, B. K., Norman, A. R., Shapiro, M. D., … Kingsley, D. M. (2014). Modular skeletal evolution in sticklebacks is controlled by additive and clustered quantitative trait loci. *Genetics*, *197*(1), 405–420. https://doi.org/10.1534/genetics.114.162420

Miyazono, K., Maeda, S., & Imamura, T. (2005). BMP receptor signaling: Transcriptional targets, regulation of signals, and signaling cross-talk. *Cytokine and Growth Factor Reviews*, *16*(3 SPEC. ISS.), 251–263. https://doi.org/10.1016/j.cytogfr.2005.01.009

Near, T. J., Eytan, R. I., Dornburg, A., Kuhn, K. L., Moore, J. A., Davis, M. P., … Smith, W. L. (2012). Resolution of ray-finned fish phylogeny and timing of diversification. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(34), 13698–

13703. https://doi.org/10.1073/pnas.1206625109

Neese, R. A., Misell, L. M., Turner, S., Chu, A., Kim, J., Cesar, D., … Hellerstein, M. K. (2002). Measurement in vivo of proliferation rates of slow turnover cells by 2H2O labeling of the deoxyribose moiety of DNA. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(24), 15345–15350. https://doi.org/10.1073/pnas.232551499

Neubüser, A., Peters, H., Balling, R., & Martin, G. R. (1997). Antagonistic interactions between FGF and BMP signaling pathways: A mechanism for positioning the sites of tooth formation. *Cell*, *90*(2), 247–255. https://doi.org/10.1016/S0092-8674(00)80333-5

Nüsslein-volhard, C., & Wieschaus, E. (1980). Mutations affecting segment number and polarity in drosophila. *Nature*, *287*(5785), 795–801. https://doi.org/10.1038/287795a0

O'brown, N. M., Summers, B. R., Jones, F. C., Brady, S. D., & Kingsley, D. M. (2015). A recurrent regulatory change underlying altered expression and Wnt response of the stickleback armor plates gene EDA. *ELife*, *2015*(4). https://doi.org/10.7554/eLife.05290

Pai, A. A., & Gilad, Y. (2014, December 1). Comparative studies of gene regulatory mechanisms. *Current Opinion in Genetics and Development*. Elsevier Ltd. https://doi.org/10.1016/j.gde.2014.08.010

Pennacchio, L. A., Bickmore, W., Dean, A., Nobrega, M. A., & Bejerano, G. (2013, April 18). Enhancers: Five essential questions. *Nature Reviews Genetics*. Nature Publishing Group. https://doi.org/10.1038/nrg3458

Pires-daSilva, A., & Sommer, R. J. (2003, January 1). The evolution of signalling pathways in animal development. *Nature Reviews Genetics*. Nature Publishing Group. https://doi.org/10.1038/nrg977

Pispa, J., Pummila, M., Barker, P. A., Thesleff, I., & Mikkola, M. L. (2008). Edar and Troy signalling pathways act redundantly to regulate initiation of hair follicle development. *Human Molecular Genetics*, *17*(21), 3380–3391. https://doi.org/10.1093/hmg/ddn232

Pispa, J., & Thesleff, I. (2003). Mechanisms of ectodermal organogenesis. *Developmental Biology*, *262*(2), 195–205. https://doi.org/10.1016/S0012-1606(03)00325-7

Plikus, M. V., Baker, R. E., Chen, C. C., Fare, C., De La Cruz, D., Andl, T., … Chuong, C. M. (2011). Self-organizing and stochastic behaviors during the regeneration of hair stem cells. *Science*, *332*(6029), 586–589. https://doi.org/10.1126/science.1201647

Plikus, M. V., Mayer, J. A., De La Cruz, D., Baker, R. E., Maini, P. K., Maxson, R., & Chuong, C. M. (2008). Cyclic dermal BMP signalling regulates stem cell activation during hair regeneration. *Nature*, *451*(7176), 340–344. https://doi.org/10.1038/nature06457

Preger-Ben Noon, E., Sabarís, G., Ortiz, D. M., Sager, J., Liebowitz, A., Stern, D. L., & Frankel, N. (2018). Comprehensive Analysis of a cis-Regulatory Region Reveals Pleiotropy in Enhancer Function. *Cell Reports*, *22*(11), 3021–3031. https://doi.org/10.1016/j.celrep.2018.02.073

Prud'homme, B., Gompel, N., & Carroll, S. B. (2007). Emerging principles of regulatory evolution. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(SUPPL. 1), 8605–8612. https://doi.org/10.1073/pnas.0700488104

Rebeiz, M., & Tsiantis, M. (2017, August 1). Enhancer evolution and the origins of morphological novelty. *Current Opinion in Genetics and Development*. Elsevier Ltd. https://doi.org/10.1016/j.gde.2017.04.006

Reid, D. T., & Peichel, C. L. (2010). Perspectives on the Genetic Architecture of Divergence in Body Shape in Sticklebacks. *Integrative and Comparative Biology*, *50*(6), 1057–1066. https://doi.org/10.1093/icb/icq030

Reif, W.-E. (1982). Evolution of Dermal Skeleton and Dentition in Vertebrates. The Odontode-Regulation Theory. *Evolutionary Biology*, *15*.

Rice, R., Rice, D. P. C., Olsen, B. R., & Thesleff, I. (2003). Progression of calvarial bone development requires Foxc1 regulation of Msx2 and Alx4. *Developmental Biology*, *262*(1), 75–87. https://doi.org/10.1016/S0012-1606(03)00355-5

Rosenfeld, J. A., Wang, Z., Schones, D. E., Zhao, K., DeSalle, R., & Zhang, M. Q. (2009). Determination of enriched histone modifications in non-genic portions of the human genome. *BMC Genomics*, *10*(1), 143. https://doi.org/10.1186/1471-2164-10-143

Sabarís, G., Laiker, I., Preger-Ben Noon, E., & Frankel, N. (2019, June 1). Actors with Multiple Roles: Pleiotropic Enhancers and the Paradigm of Enhancer Modularity. *Trends in Genetics*. Elsevier Ltd. https://doi.org/10.1016/j.tig.2019.03.006

Sadier, A., Viriot, L., Pantalacci, S., & Laudet, V. (2014, January 1). The ectodysplasin pathway: From diseases to adaptations. *Trends in Genetics*. Elsevier Current Trends. https://doi.org/10.1016/j.tig.2013.08.006

Sender, R., & Milo, R. (2021). The distribution of cellular turnover in the human body. *Nature Medicine*, *27*(1), 45–48. https://doi.org/10.1038/s41591-020-01182-9

Seo, S., & Kume, T. (2006). Forkhead transcription factors, Foxc1 and Foxc2, are required for the morphogenesis of the cardiac outflow tract. *Developmental Biology*, *296*(2), 421–436. https://doi.org/10.1016/j.ydbio.2006.06.012

Shubin, N., Tabin, C., & Carroll, S. (2009, February 12). Deep homology and the origins of evolutionary novelty. *Nature*. Nature Publishing Group. https://doi.org/10.1038/nature07891

Sievers, F., Wilm, A., Dineen, D., Gibson, T. J., Karplus, K., Li, W., … Higgins, D. G. (2011). Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Molecular Systems Biology*, *7*(1), 539. https://doi.org/10.1038/msb.2011.75

Singh, D., & Yi, S. V. (2021). Enhancer Pleiotropy, Gene Expression, and the Architecture of Human Enhancer–Gene Interactions. *Molecular Biology and Evolution*, *38*(9), 3898–3909. https://doi.org/10.1093/molbev/msab085

Singh, L. N., & Hannenhalli, S. (2008). Functional Diversification of Paralogous Transcription Factors via Divergence in DNA Binding Site Motif and in Expression. *PLoS ONE*, *3*(6), e2345. https://doi.org/10.1371/journal.pone.0002345

Smith, M. M., & Coates, M. I. (1998). Evolutionary origins of the vertebrate dentition: Phylogenetic patterns and developmental evolution. *European Journal of Oral Sciences*, *106*(1 SUPPL.), 482–500. https://doi.org/10.1111/j.1600-0722.1998.tb02212.x

Soukup, V., Epperlein, H.-H., Horácek, I., & Cerny, R. (2008). Dual epithelial origin of vertebrate oral teeth. *Nature*, *455*(7214), 795–798. https://doi.org/10.1038/nature07304

Square, T. A., Sundaram, S., Mackey, E. J., & Miller, C. T. (2021). Distinct tooth regeneration systems deploy a conserved battery of genes. *EvoDevo*, *12*(1), 1–17. https://doi.org/10.1186/s13227-021-00172-3

Stanojevic, D., Small, S., & Levine, M. (1991). Regulation of a segmentation stripe by overlapping activators and repressors in the Drosophila embryo. *Science*, *254*(5036), 1385–1387. https://doi.org/10.1126/science.1683715

Stepaniak, M. D., Square, T. A., & Miller, C. T. (2021). Evolved Bmp6 enhancer alleles drive spatial shifts in gene expression during tooth development in sticklebacks. *Genetics*. https://doi.org/10.1093/genetics/iyab151

Stewart, S., Gomez, A. W., Armstrong, B. E., Henner, A., & Stankunas, K. (2014). Sequential and opposing activities of Wnt and BMP coordinate zebrafish bone regeneration. *Cell Reports*, *6*(3), 482–498. https://doi.org/10.1016/j.celrep.2014.01.010

Talbot, J. C., & Amacher, S. L. (2014). A streamlined CRISPR pipeline to reliably generate zebrafish frameshifting alleles. *Zebrafish*, *11*(6), 583–585. https://doi.org/10.1089/zeb.2014.1047

Taylor, G., Lehrer, M. S., Jensen, P. J., Sun, T. T., & Lavker, R. M. (2000). Involvement of follicular stem cells in forming not only the follicle but also the epidermis. *Cell*, *102*(4), 451–461. https://doi.org/10.1016/S0092-8674(00)00050-7

Thesleff, I. (2003). Epithelial-mesenchymal signalling regulating tooth morphogenesis. *Journal of Cell Science*, *116*(9), 1647–1648. https://doi.org/10.1242/JCS.00410

Till, J., & McCulloch, E. (1961). A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiat Res.*, *14:213-22.*

Tinbergen, N. (1952). The curious behavior of the stickleback. *Sci. Am. 187:22–26.*

Tumbar, T., Guasch, G., Greco, V., Blanpain, C., Lowry, W. E., Rendl, M., & Fuchs, R. (2004). Defining the Epithelial Stem Cell Niche in Skin. *Science*, *303*(5656), 359–363. https://doi.org/10.1126/science.1092436

Tümer, Z., & Bach-Holm, D. (2009). Axenfeld-Rieger syndrome and spectrum of PITX2 and FOXC1 mutations. *European Journal of Human Genetics : EJHG*, *17*(12), 1527–1539. https://doi.org/10.1038/ejhg.2009.93

Vainio, S., Karavanova, I., Jowett, A., & Thesleff, I. (1993). Identification of BMP-4 as a signal mediating secondary induction between epithelial and mesenchymal tissues during early tooth development. *Cell*, *75*(1), 45–58. https://doi.org/10.1016/S0092-8674(05)80083-2

Vandenplas, S., Willems, M., & Huysseune, A. (2012). Dual BrdU-PCNA immunodetection of proliferative cells in dental and orofacial tissues of teleosts. *Journal of Applied Ichthyology*, *28*(3), 336–340. https://doi.org/10.1111/j.1439-0426.2012.02003.x

Vandenplas, Sam, De Clercq, A., & Huysseune, A. (2014). Tooth replacement without a dental lamina: The search for epithelial stem cells in *Polypterus senegalus*. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*, *322*(5), 281–293.

https://doi.org/10.1002/jez.b.22577

Vandenplas, Sam, Vandeghinste, R., Boutet, A., Mazan, S., & Huysseune, A. (2016). Slow cycling cells in the continuous dental lamina of Scyliorhinus canicula: New evidence for stem cells in sharks. *Developmental Biology*, *413*(1), 39–49. https://doi.org/10.1016/j.ydbio.2016.03.004

Vandenplas, Sam, Willems, M., Witten, P. E., Hansen, T., Fjelldal, P. G., & Huysseune, A. (2016). Epithelial Label-Retaining Cells Are Absent during Tooth Cycling in Salmo salar and Polypterus senegalus. *PLOS ONE*, *11*(4), e0152870. https://doi.org/10.1371/journal.pone.0152870

Vaškaninová, V., Chen, D., Tafforeau, P., Johanson, Z., Ekrt, B., Blom, H., & Ahlberg, P. E. (2020). Marginal dentition and multiple dermal jawbones as the ancestral condition of jawed vertebrates. *Science*, *369*(6500), 211–216. https://doi.org/10.1126/science.aaz9431

Wainwright, P. C. (2005, January 1). Functional Morphology of the Pharyngeal Jaw Apparatus. *Fish Physiology*. Academic Press. https://doi.org/10.1016/S1546-5098(05)23003-0

Walker, J. A., & Bell, M. A. (2000). Net evolutionary trajectories of body shape evolution within a microgeographic radiation of threespine sticklebacks (Gasterosteus aculeatus) . *Journal of Zoology*, *252*(3), 293–302. https://doi.org/10.1111/j.1469-7998.2000.tb00624.x

Waluk, D. P., Zur, G., Kaufmann, R., Welle, M. M., Jagannathan, V., Drögemüller, C., … Galichet, A. (2016). A splice defect in the EDA gene in dogs with an X-linked hypohidrotic ectodermal dysplasia (XLHED) phenotype. *G3: Genes, Genomes, Genetics*, *6*(9), 2949–2954. https://doi.org/10.1534/g3.116.033225

Wang, L., Siegenthaler, J. A., Dowell, R. D., & Yi, R. (2016). Foxc1 reinforces quiescence in self-renewing hair follicle stem cells. *Science (New York, N.Y.)*, *351*(6273), 613–617. https://doi.org/10.1126/science.aad5440

Wang, X.-P., Suomalainen, M., Felszeghy, S., Zelarayan, L. C., Alonso, M. T., Plikus, M. V, … Thesleff, I. (2007). An Integrated Gene Regulatory Network Controls Stem Cell Proliferation in Teeth. *PLoS Biology*, *5*(6), e159. https://doi.org/10.1371/journal.pbio.0050159

Wang, Y., Li, L., Zheng, Y., Yuan, G., Yang, G., He, F., & Chen, Y. (2012). BMP activity is required for tooth development from the Lamina to Bud Stage. *Journal of Dental Research*, *91*(7), 690–695. https://doi.org/10.1177/0022034512448660

Wilson, A., Laurenti, E., Oser, G., van der Wath, R. C., Blanco-Bose, W., Jaworski, M., … Trumpp, A. (2008). Hematopoietic Stem Cells Reversibly Switch from Dormancy to Self-Renewal during Homeostasis and Repair. *Cell*, *135*(6), 1118–1129. https://doi.org/10.1016/j.cell.2008.10.048

Wu, P., Wu, X., Jiang, T.-X., Elsey, R. M., Temple, B. L., Divers, S. J., … Chuong, C.-M. (2013). Specialized stem cell niche enables repetitive renewal of alligator teeth. *Proceedings of the National Academy of Sciences*, *110*(22), E2009–E2018. https://doi.org/10.1073/pnas.1213202110

Wu, Z., Wang, F., Fan, Z., Wu, T., He, J., Wang, J., … Wang, S. (2019). Whole-Tooth Regeneration by Allogeneic Cell Reassociation in Pig Jawbone. *Tissue Engineering Part A*,

25(17–18), 1202–1212. https://doi.org/10.1089/ten.tea.2018.0243

Wucherpfennig, J. I., Miller, C. T., & Kingsley, D. M. (2019). Efficient CRISPR-Cas9 editing of major evolutionary loci in sticklebacks. *Evolutionary Ecology Research*, *20*(1), 107–132. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/34899072

Xu, P., Balczerski, B., Ciozda, A., Louie, K., Oralova, V., Huysseune, A., & Gage Crump, J. (2018). Fox proteins are modular competency factors for facial cartilage and tooth specification. *Development (Cambridge)*, *145*(12). https://doi.org/10.1242/dev.165498

Xu, P., Yu, H. V., Tseng, K. C., Flath, M., Fabian, P., Segil, N., & Crump, J. G. (2021). Foxc1 establishes 1 enhancer accessibility for craniofacial cartilage differentiation. *ELife*, *10*, 1–50. https://doi.org/10.7554/eLife.63595

Zhao, X., Zhang, Z., Song, Y., Zhang, X., Zhang, Y., Hu, Y., … Chen, Y. P. (2000). Transgenically ectopic expression of Bmp4 to the Msx1 mutant dental mesenchyme restores downstream gene expression but represses Shh and Bmp2 in the enamel knot of wild type tooth germ. *Mechanisms of Development*, *99*(1–2), 29–38. https://doi.org/10.1016/S0925-4773(00)00467-6

Chapter 3: Differential cell proliferation dynamics during tooth development and replacement between marine and freshwater sticklebacks

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3.1 Abstract

Regeneration is a vital process for long-term renewal of organs and often depends on few, slow-cycling cells from which new, specified cells are derived. However, the proliferation rates of slow cycling cells can vary greatly based on cell type, tissue, sex, age, health and organism. Stickleback fish retain the ancestral dental phenotype of continuous tooth replacement. Relative to marine ancestral fish, derived freshwater fish populations have evolved increases in tooth number and tooth regeneration rates, both of which occur late in development. Exploiting the natural variation between marine and freshwater stickleback populations and the dynamic variability of slow cycling, label-retaining cells (LRCs), we revealed evolved reductions in LRCs in the adult pharyngeal dentitions of a high-toothed, freshwater population relative to an ancestral low-toothed marine population. While we observed a dense band of LRCs in the inner dental epithelium, there was not a definitive localization of 'slow-cycling' cells adjacent to the tooth germs after long chase times. The number of LRCs found in the dental epithelium and mesenchyme across all stages of tooth development were correlated within individual fish. Proliferation in mesenchymal cell populations in late, erupted stages of tooth development was negatively regulated by Wnt signaling. The freshwater reduction of LRCs suggests evolved increases in cell proliferation contribute to increased tooth number and regeneration rates. These findings represent one of the first demonstrated examples of evolved differences of cell cycling kinetics that are associated with robust evolved phenotypic differences in regeneration rates.

3.2 Introduction

Teeth have long served as a classic model system of organogenesis. Tooth development and regeneration are orchestrated by reciprocal epithelial-mesenchymal interactions. These interactions repeat throughout the adult life of most non-mammalian vertebrates leading to continual tooth regeneration, or polyphyodonty (Jernvall & Thesleff, 2012). Previous studies have found that fish teeth and mammalian hair share many aspects of development. First, on a morphological level, both teeth and hair are derived from thickened placode epithelium and underlying condensed mesenchyme (Pispa & Thesleff, 2003; Thesleff, 2003). Second, on a molecular level, both teeth and hair are regulated by common factors including WNT, BMP, and FGF signaling (Jia et al., 2013; Plikus et al., 2008; Vainio et al., 1993; Wang et al., 2012). Third, both teeth and hair continuously regenerate in adults and deploy similar batteries of gene expression (Harada et al., 1999; Plikus et al., 2011; Square et al., 2021; Wang et al., 2007).

Previous research investigating renewal of the mouse hair follicle used a pulse-chase strategy whereby retention of tritiated thymidine revealed a population of epithelial cells with exceedingly slow cell cycling times near the base of the follicle, now known as bulge stem cells

or hair follicle stem cells (HFSCs) (Cotsarelis et al., 1990). These label-retaining cells (LRCs) in the mammalian hair follicle epithelial bulge have been shown to be stem cells that drive hair regeneration (Taylor et al., 2000).

Teleost fish retain the ancestral gnathostome characteristic of continuous tooth replacement. BrdU pulse-chase labeling experiments have more recently identified slow-cycling LRCs in teeth in a variety of polyphyodont organisms, including putative tooth stem cells (TSCs) in the oral jaws of leopard geckos (Handrigan et al., 2010), alligators (Wu et al., 2013), and sharks (Martin et al., 2016) as well as the pharyngeal jaw of medaka (Abduweli et al., 2014). In these examples, slow-cycling LRCs were found in the dental epithelium near regenerating teeth. Data from geckos, alligators, and sharks have also revealed that some genes that are expressed in mammalian HFSC niches are also expressed in TSCs (Handrigan et al., 2010; Martin et al., 2016; Wu et al., 2013). These data support the hypothesis that TSCs are involved in tooth replacement in polyphyodonts. However, similar BrdU pulse-chase labeling experiments performed in the oral jaw of salmon and bichir did not identify clear populations of LRCs after long chase time points (Vandenplas et al., 2016). Given the variation observed in the medaka compared to salmon and bichir and that no current examples exist in organisms without tooth families (teeth that share a dental lamina and regenerate in a continuous conveyor belt-like manner), it remains unclear how common LRCs are in teleost fish and whether variation in the presence, placement or cycling of these cells may change on short evolutionary time scales to facilitate adaptive changes in tooth number.

Natural variation in tooth number and tooth regeneration rates in stickleback fish populations has provided a powerful system to study heritable changes in dental patterning following shifts in environment and diet. Multiple derived freshwater populations have convergently evolved increases in tooth number relative to ancestral marine populations (Cleves et al., 2014; Ellis et al., 2015). Two independent freshwater stickleback populations with convergently evolved tooth gain both display an increase in tooth number late in development associated with increased rates of tooth regeneration in adults (Ellis et al., 2015). Genetic mapping of the evolved tooth gain phenotype in one of these high-toothed freshwater populations identified an enhancer in the fourth intron of *Bone morphogenetic protein 6* (*Bmp6*), a gene previously found to have a *cis*-regulatory change in dental tissue that is associated with evolved increases in tooth number (Cleves et al., 2014, 2018). In situ hybridization data showed *Bmp6* is dynamically expressed in the dental epithelium and mesenchyme tissue layers of fish teeth during tooth development (Cleves et al., 2014). RNA sequencing of dental tissue from loss-of-function, *Bmp6* homozygous mutant sticklebacks revealed significant downregulation of genes expressed in mouse HFSCs in a BMP-dependent fashion (Cleves et al., 2018). In mice, *Bmp6* has been proposed to be a promoter of stem cell quiescence in HFSCs (Kandyba et al., 2013) and is sufficient upon viral overexpression to inhibit hair regeneration (Wu et al., 2019). Additionally, self-renewal of quiescent HFSCs during mouse hair regeneration is dependent on the Foxc1 transcription factor which binds to a regulatory region adjacent to *Bmp6* and regulates *Bmp6* gene expression (Wang et al., 2016). Together these data support the parsimonious hypothesis that BMP signaling plays conserved roles in negatively regulating regeneration of homologous epithelial appendages including mammalian hair and fish teeth.

BMP signaling is transduced by the effector proteins SMAD1, SMAD5, and SMAD8, which are phosphorylated by BMP type I receptors upon BMP ligand binding, and then translocate to the nucleus to regulate the transcription of genes (Miyazono et al., 2005). LDN-193189 (LDN) is a potent inhibitor of the bone morphogenetic (BMP) pathway, inhibiting BMP type I receptors ALK1, ALK2, ALK3, and ALK6 (Cuny et al., 2008). To test the hypothesis that BMP signaling regulates cell proliferation, we soaked low-toothed marine fish in LDN to knockdown BMP signaling. If BMP is required for the maintenance of slow cycling cells, we would expect to see a loss of quiescence, increase in proliferation rates, activation of slow cycling cells, and overall reduction in LRCs compared to the DMSO control. We also treated fish with BIO, an agonist of WNT signaling, that we hypothesized would also promote tooth regeneration and activate quiescent cells to differentiate into replacement teeth. Here we tested the hypotheses that (1) the pharyngeal dentitions from a low-toothed marine population have differences in dental cell proliferation dynamics (i.e. has more slower cycling cells (LRCs) than a high-toothed freshwater population (2) cell proliferation dynamics are regulated by BMP signaling and (3) cell proliferation dynamics are regulated by Wnt signaling.

3.3 Methods

Animal husbandry

All animal work was approved on UC Berkeley IACUC protocol AUP-2015–01-7117. Sticklebacks were raised in 110-L aquaria in brackish water (3.5 g/L Instant Ocean salt, 0.0217 g/L sodium bicarbonate, hereafter called "stickleback water") at 18°C in 8 hours of light per day until adults, and 16 hours of light per day for breeding adults. Fry with standard length (SL) less than ~10 mm were fed a diet of live artemia, with frozen daphnia added as fish reached ~10 mm SL. Adults and late juveniles with SL greater than ~20 mm were fed a combination of frozen bloodworms and *Mysis* shrimp.

BrdU pulse-chase

Newly hatched fry (SL ~6mm, 10dpf) and late juvenile sticklebacks (SL ~25mm) derived from marine (Rabbit Slough [RABS], Alaska) and freshwater (Cerrito Creek [CERC], California) were immersed in 5-bromo-2'-deoxyuridine (BrdU; 5mM) (Sigma-Aldrich, St Louis, MO, USA) in stickleback water daily for 5 days (pulse). Fish were then transferred to stickleback water and grown under regular conditions for 0, 1, 2, and 4 weeks (chase) prior to euthanasia. Sticklebacks were euthanized with 0.04% MS222 (Western Chemical 200-226) and fixed by immersion in 4% paraformaldehyde (PFA) (Sigma P6148) at 4°C overnight (or 4 hrs for fry). Standard length (tip of snout to caudal peduncle) and/or total length (tip of snout to most posterior tip of caudal fin) were measured with digital calipers following PFA fixation. Branchial skeletons were dissected as previously described (Ellis et al., 2016; Miller et al., 2014).

Sectioning

Dissected branchial skeletons were decalcified for 5–7 days in 20% ethylenediaminetetraacetic acid (EDTA, pH 8.0) at room temperature on a tube rotator. Once decalcified, samples were washed 2 × 5 min in 1xPBS, then stepped into 100% EtOH via 10 min washes in 25, 50, 75, and 100% EtOH in RNAse-free DEPC-treated water. Samples were stepped into 100% Hemo-De

with 10 min washes in 50/50 and 25/75 EtOH/Hemo-De then in 100% Hemo-De at room temperature, washed for 30 min in 50/50 Hemo-De/paraffin (Paraplast X-tra, Fisher) at 65°C, then incubated overnight at 65°C in 100% paraffin. Samples were embedded in plastic molds with 100% paraffin (heated to 65°C), mounted, sectioned in the transverse plane with a Microm HM 340 E microtome. Sections were captured on Superfrost™ Plus Microscope Slides and stored at room temperature prior to analysis. To prepare slides for histology, slides were dewaxed (10 min incubation at 65°C, left to cool, submerged for 2 x 8 min in 100% Hemo-De, 8 min 100% EtOH, 8 min 80% EtOH/water, 8 min 100% DEPC treated water).

Immunohistochemistry

Dewaxed paraffin slides were subjected to heat-mediated antigen retrieval in 0.01 M sodium citrate (pH= 6.0) for 20 min before blocking and subsequent antibody labeling. Primary antibody labeling with mouse G3G4 (AntiBrdU, DSHB), and either rabbit anti-Sox2 (Abcam ab97959), rabbit anti-PCNA (Abcam ab92552), or rabbit Anti-Histone H3 (acetyl K27) (Abcam ab4729) was incubated at room temperature on a tube rotator for 2 hours. Secondary antibody incubation with goat anti-Mouse IgG, Alexa Fluor 568 (Invitrogen A11019) and goat anti-Rabbit IgG, Alexa Fluor 488 (Invitrogen A11070) and incubated at room temperature for 3 hours. All antibodies were diluted 1:500. Slides were counterstained with 1 µg/mL DAPI (Invitrogen D1306) and mounted in ProLong™ Gold Antifade Mountant (ThermoFisher P36930). Imaging was carried out on a Zeiss LSM700 confocal microscope and micrographs subsequently visualized with ImageJ software.

Statistical and graphical analyses of cell proliferation counts

Percent BrdU saturation was determined by dividing the number of BrdU+ cells by the total number of DAPI labeled cells in epithelial and mesenchymal cells surrounding the tooth germ. Statistical analyses of BrdU-positive cells were based on one transverse section of each tooth germ from each tooth stage defined as follows: bud (pre-mineralized stages including bud and cap stage), bell (early to late bell stage with mineralized tooth), and erupted (functional stage)(Ellis et al., 2015). Results were plotted and analyzed using a generalized linear model (GLM) for BrdU saturation. For assessing differences between epithelial and mesenchymal cells an association test for paired samples was performed based on Pearson's product moment correlation coefficient. An analysis of variance (ANOVA) model was used to determine differences in BrdU retention of marine fish treated with DMSO, BIO, or LDN. The resulting *P* values from GLM and ANOVA tests were corrected for multiple hypothesis testing using the Bonferroni method (Bonferroni, 1936). *P* values were considered significant when below a threshold of $P < 0.008$, or below the Bonferroni-corrected *P* values. All statistical analyses were performed using available CRAN packages on RStudio software. To summarize graphically the spatial locations of BrdU+ cell, at least one representative tooth germ (using the germs closest to the median BrdU+ cell count, if two germs were equally distant from median, the higher count image was used) from 5 fish had all of their BrdU+ cells plotted onto a cartoon tooth germ at bud, bell, and erupted stages, with the exceptions of 0 week bud (4 fish) and 4 week erupted (6 fish) for the VTP, 1 week bud (4 fish) and 2 week erupted (6 fish) for the DTP. To summarize the spatial locations of BrdU+ cells in the drug treated marine fish, at least two representative tooth germs (using the germs closest to the median BrdU+ cell count) from 5 fish had all of their

BrdU+ cells plotted onto a cartoon tooth germ at bud, bell, and erupted stages, with the exceptions of 0 week bud & bell (6 fish) and 1 week bud & erupted (6 fish).

Small molecule treatment

A stock solution of LDN-193189 (Sigma-Aldrich, hereafter called "LDN") was rehydrated to 10 mM in dimethyl sulfoxide (DMSO) and stock solution of small molecule glycogen synthase kinase-3 inhibitor 6-bromoindirubin-3-oxim (BIO) was rehydrated to 20 mM in DMSO. After day 5 of the BrdU pulse, RABS sticklebacks (SL >20mm) were immersed in stickleback water with either 20 µM LDN or 10 µM BIO dissolved in 1% DMSO, or 1% DMSO only. Fish were raised in treatment for 4 days, and then transferred to stickleback water for 10 more days to complete a 14 day BrdU chase before euthanasia.

3.4 Results

The first stickleback tooth replacement occurs at approximately 21 dpf (Ellis et al., 2016) and the divergence in tooth number between marine and freshwater populations begins around 25mm standard length (SL) (Cleves et al., 2014). Given the difference between tooth number and tooth regeneration rates at these late stages of stickleback development, we hypothesized that cell proliferation dynamics are different between marine and freshwater fish. To test this hypothesis, cell cycling rate was quantified through loss of label retention in a BrdU pulse-chase experiment (Fig. 3.1) comparing high-toothed fish from Cerrito Creek, California (CERC, hereafter called "freshwater") to ancestral low-toothed marine fish from Rabbit Slough, Alaska (RABS, hereafter called "marine"). Pulse-chased fish were then embedded, sectioned transversely, and then stained for BrdU immunoreactivity.

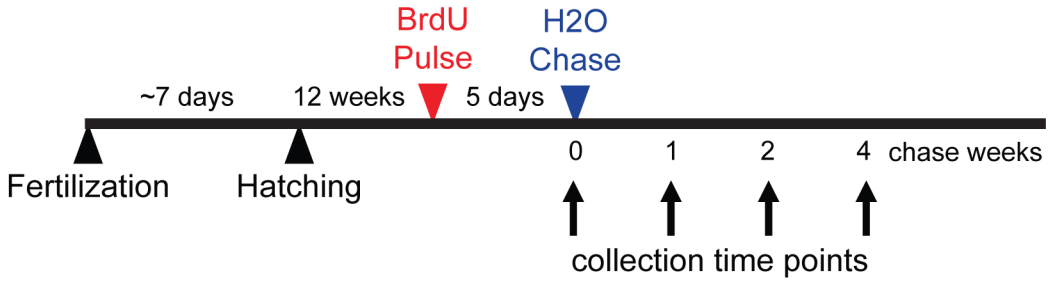


Figure 3.1 Schematic of BrdU pulse-chase experiment.

Young adult sticklebacks were immersed in 5mM BrdU approximately 12 weeks post-hatching and pulsed for 5 days followed by chase times of 0, 1, 2, and 4 weeks.

In pharyngeal teeth with no chase time (0 week column) between the initial BrdU pulse, most epithelial and mesenchymal cells had incorporated the BrdU during the pulse time period in both marine and freshwater fish, showing tooth germ cells in both populations are highly mitotic. The bud stage for both populations was the rarest to identify compared to bell and erupted since this stage is short lived for a developing tooth germ. The 0 week bud stage had the highest proportion of BrdU incorporation (Fig. 3.2A-A') followed by 0 week bell stage (Fig. 3.2E-E'). Fewer BrdU labeled cells are seen during the erupted stage of tooth development. BrdU label was still retained but at a lower proportion by 1 week chase with the most obvious decrease in

distribution between the 1 week to 2 week chase in both marine and freshwater populations (Fig. 3.2; 0, 1, and 2 weeks). By week 4 most, if not all, LRCs were absent in the cell layers surrounding the tooth germ (Fig. 3.2; 4 weeks) but present in other tissues, such as the pharyngeal luminal epithelium and deeper jaw mesenchyme. Considering that most tooth germs had cycled and depleted the BrdU after 4 weeks chase, we did not expect to see a significant difference between populations at this time point.

In comparing BrdU label-retention between marine and freshwater populations, we observed the marine population steadily retained more BrdU+ cells than their freshwater counterparts in the ventral, as well as, dorsal pharyngeal tooth plates (Fig. 3.2M-M', Fig. S3.1). The earlier stages of tooth development did not reveal significant differences in BrdU saturation in either bud and bell epithelium ($P = 0.97$ and 0.0089, respectively) or bud and bell mesenchyme ($P = 0.55$ and 0.015, respectively, GLM (see methods)) (Fig. 3.2M-M', bud and bell). However, the erupted stage of tooth development revealed a significant difference in BrdU saturation with the marine population retaining more label than the freshwater population in both epithelial and mesenchymal cells ($P = 0.0029$ and 0.00016, respectively, GLM) (Fig. 3.2M-M', erupted). In evaluating cell type differences, we found the epithelial and mesenchymal cells are individually correlated and distributions between the cell types are not significant in either population ($P = 0.32$).

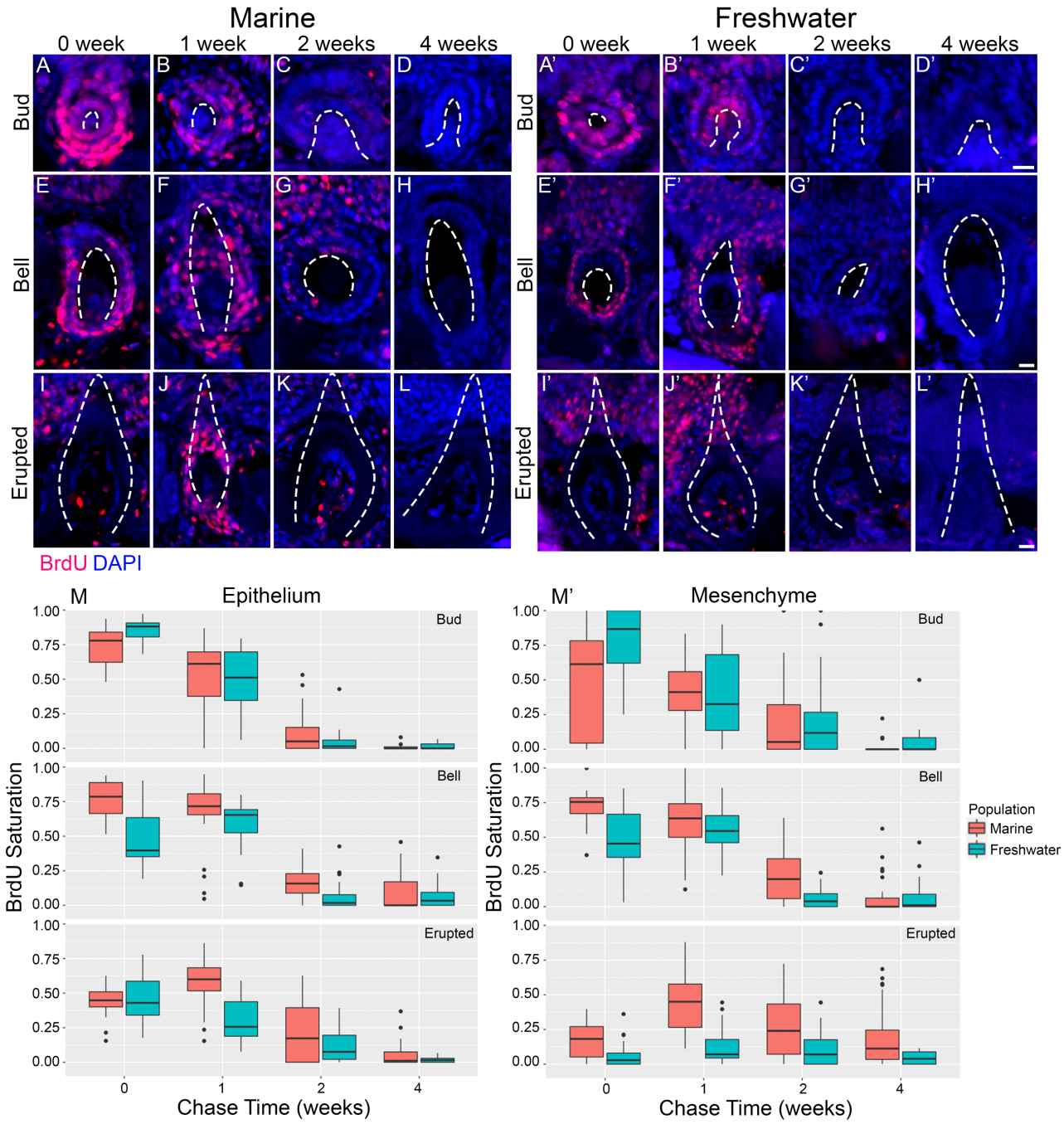


Figure 3.2 Evolved reductions in LRC number in freshwater compared to marine ventral pharyngeal teeth.

Confocal images of transverse paraffin sections from marine (A-L) and freshwater (A'-L') ventral pharyngeal tooth germs at bud, bell, and erupted stages (see Methods) of development (y-axis) and at varying chase time points (x-axis). White dotted lines demarcate the outline of the tooth germ, separating epithelium from mesenchyme. Scale bar = 10 µm. Boxplots (M-M') show overall average BrdU saturation of the dental epithelium and mesenchyme between freshwater (blue) and marine (pink) populations in ventral tooth germs at bud, bell, and erupted stages of development (y-axis) and at 0, 1, 2, and 4 week chase time points (x-axis). Marine showed significantly greater BrdU retention compared to Freshwater in the erupted stage in both epithelial ($P = 0.0029$, GLM) and the mesenchymal ($P = 0.00016$) cells. However, differences at early stages of development showed a smaller effect in

the epithelium bud and bell ($P = 0.97$ and 0.0089, respectively) and mesenchyme bud and bell ($P = 0.55$ and 0.015, respectively).

Past studies revealed LRCs and putative tooth and hair stem cells are found immediately adjacent to the regenerating organ (Handrigan et al., 2010; Martin et al., 2016; Wu et al., 2013). To test whether a similar niche of LRCs concentrated in a specific layer and location relative to the preceding tooth germ, we visualized the spatial location of LRCs as follows. For every tooth stage and time point quantified, we identified an equal number of the most median tooth germs to serve as an average representation of LRC distribution in each marine and freshwater fish. At least one representative tooth germ from 5 fish had all of their BrdU+ cells plotted onto a cartoon tooth germ at bud, bell, and erupted stages, with the exceptions of 0 week bud (4 fish) and 4 week erupted (6 fish). Within these germs, we show every BrdU+ cells as a yellow dot on an idealized cartoon of each tooth stage. Qualitatively, this summary figure revealed a concentration of BrdU+ cells near the basal layer of the pharyngeal epithelium and deep mesenchyme of the dental papilla (Fig. 3.3). By the 4 week chase, there appeared to be a greater supply of LRCs in the inner dental epithelium (IDE) compared to the outer dental epithelium (ODE) at bell stage, suggesting that that ODE proliferates more than the IDE (Fig. 3.3). As an independent way to test whether the ODE proliferates more than the IDE, we assayed immunostaining of proliferating cell nuclear antigen (PCNA), which showed a greater concentration of proliferating cells in the ODE compared to IDE (Fig. S3.4). Detailed positional information represented here is limited, since we averaged the inner most cross section of tooth germs and consolidated left-right, medial-distal, and anterior-posterior orientations. Based on the illustrated graphic generated we did not identify a definitive 'niche' in which LRCs reside and distribution of BrdU+ cells between freshwater and marine populations does not overall appear to be qualitatively different.

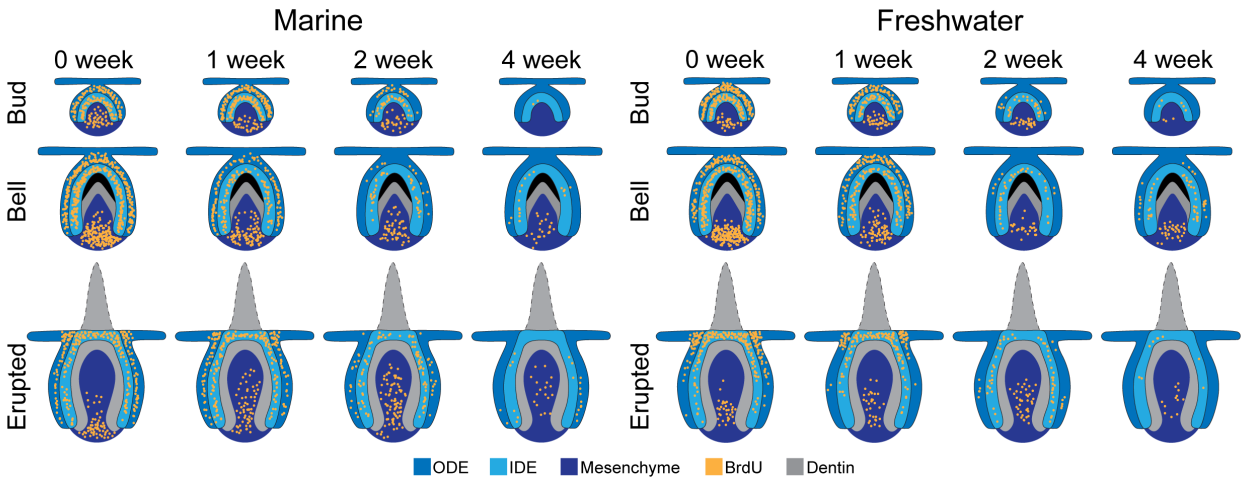


Figure 3.3 Distribution of LRCs in ventral pharyngeal teeth.

Summary of spatial distribution of LRCs in marine and freshwater ventral tooth germs at bud, bell, and erupted stages of development (y-axis) and at varying chase time points (x-axis). LRCs remain concentrated near pharyngeal epithelium and deep mesenchyme in both marine and freshwater tooth germs.

Previous research conducted in zebrafish found that inhibition of the BMP pathway via BMPR inhibitor LDN193189 (Cuny et al., 2008) reduced bone formation and mature lepidotrichia including the joints between ray segments and failed to produce mineralized bone (Stewart et al., 2014). Conversely, Activated WNTβ-catenin signaling is well-known to stimulate cell proliferation and the small molecule glycogen synthase kinase-3 inhibitor 6-bromoindirubin-3-oxim (BIO) has been demonstrated to increase WNT signaling (Huang et al., 2018). Considering the roles that BMP/WNT pathway play in hair and tooth regeneration, we were interested in testing what the effects if any of both a BMP-inhibitor, LDN, and a WNT-activator, BIO, would have on BrdU label retention and cell proliferation at the 2 week chase time point (Fig. 3.4) in the low-toothed marine population. If the BMP pathway regulates stickleback tooth regeneration by maintaining cell quiescence, then we would expect to see a decrease in BrdU label retention as a function of increased cell proliferation.

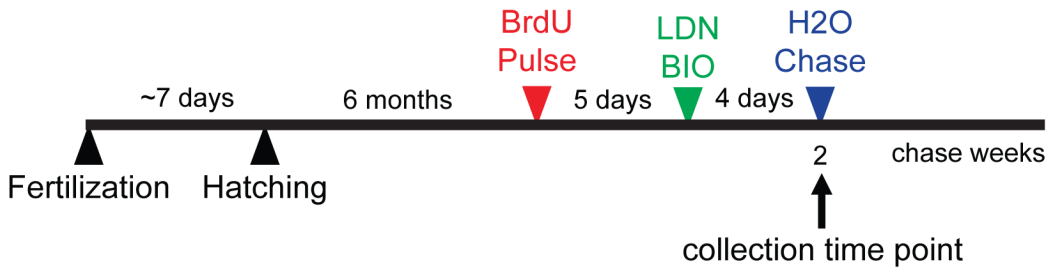


Figure 3.4 Schematic of BrdU pulse-chase timeline after treatment with LDN, BIO, or DMSO.

Adult sticklebacks were immersed in 5mM BrdU approximately 6 months post-hatching and pulsed for 5 days followed by treatment in BIO, DMSO, or LDN for 4 days and collected at the 2 week chase time point.

The graphic reveals no obvious differences in cell distribution in BIO and LDN compared to DMSO control group. However, BrdU+ cells were more commonly seen in the IDE compared to the ODE in all groups (Fig. 3.5A). While the BrdU saturation in the epithelium was similar across bud, bell, and erupted stages of tooth development ($P = 0.26, 0.21, 0.45$, respectively) and early stages of development in bud and bell mesenchymal cells ($P = 0.14$ and 0.092, respectively), there was a significance difference in BrdU retention for the erupted teeth of BIO treated fish compared to DMSO and LDN ($P = 0.0075$) (Fig. 3.5B). BrdU saturation was also significant in dorsal pharyngeal teeth for the BIO treated fish compared to DMSO and LDN in mesenchymal cells of bud and erupted teeth ($P = 0.0042$ and 0.0048, respectively) (Fig. S3.3). Future experiments manipulating the WNT and BMP pathways will shed light on how these two pathways regulate cell proliferation during tooth replacement in different populations.

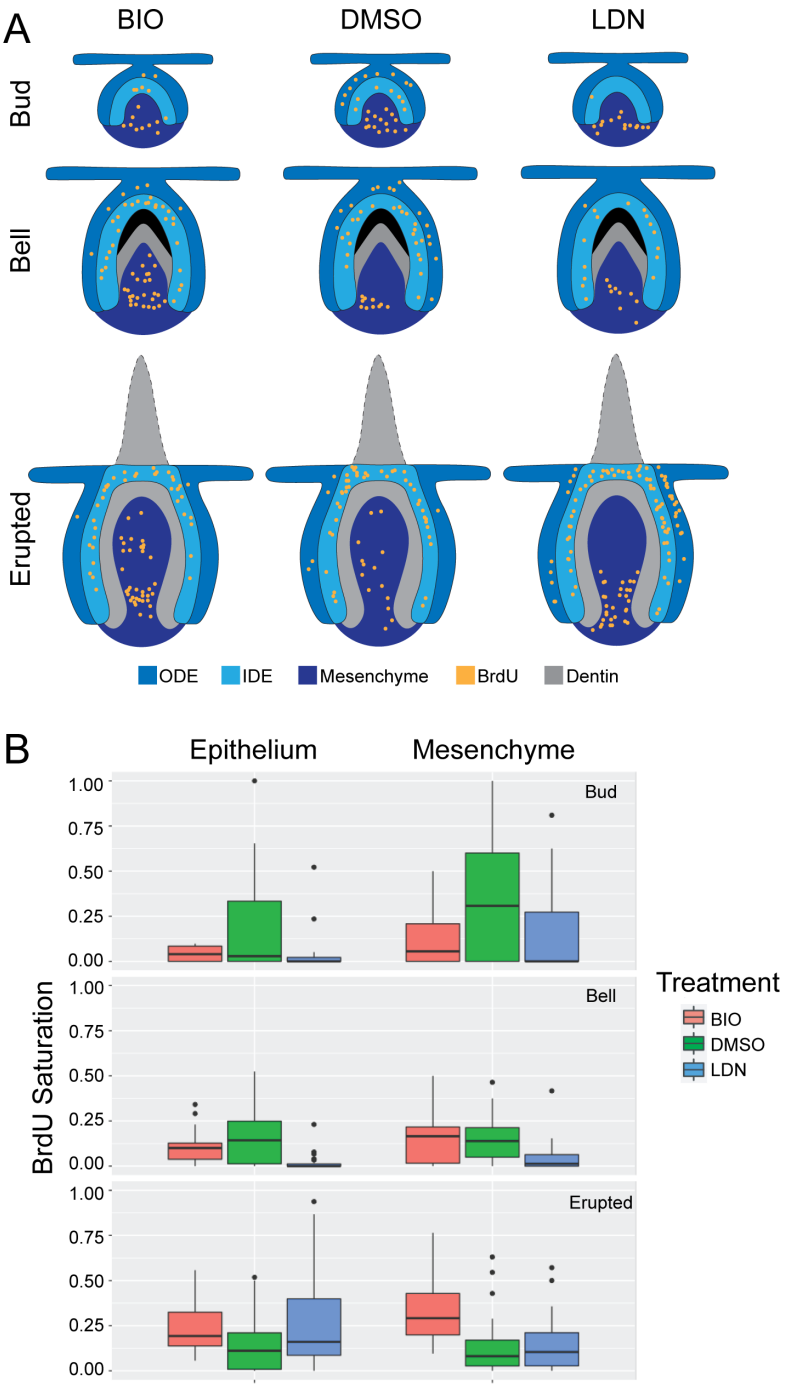


Figure 3.5 Distribution and proportion LRCs in marine ventral pharyngeal teeth after drug treatment.

(A) Graphic summary of spatial distribution of LRCs in marine ventral tooth germs at bud, bell, and erupted stages of development (y-axis) with various drug treatment: BIO, DMSO, or LDN (x-axis). (B) Boxplots describe overall BrdU saturation (y-axis) of the dental epithelium and mesenchyme between BIO (pink), DMSO (green), and LDN (blue) treated marine fish in ventral tooth germs at bud, bell, and erupted stages of development. BrdU saturation was significant for the BIO treated fish compared to DMSO and LDN in mesenchymal cells of erupted teeth (P = 0.0075). No significant differences were observed across bud, bell, and erupted stages of tooth development in epithelial cells (P = 0.26, 0.21, and 0.45, respectively) or early stages of development in bud and bell mesenchymal cells (P = 0.136 and 0.092, respectively).

3.5 Discussion

Differential saturation of label retaining cells between marine and freshwater fish

Cell proliferation is an essential characteristic of living organisms and a necessary process for development and regeneration. A BrdU incorporation assay can be used to measure cell proliferation since label that was incorporated into the DNA during active cell proliferation is progressively lost after each cell division. This method has been used to observe cell proliferation in regenerating teeth of geckos, alligators, and sharks and identify slow-cycling, tooth stem cells (TSC) niches (Handrigan et al., 2010; Martin et al., 2016; Wu et al., 2013) or apparent lack thereof in salmon and bichir (Vandenplas et al., 2014, 2016). The presence and extent of label-retaining cells during tooth replacement remains controversial and poorly understood.

Here we evaluated cell proliferation dynamics of the stickleback pharyngeal dentition by quantifying rates of BrdU incorporation and label retention, where high rates of BrdU incorporation and subsequent depletion correlate with high cell proliferation rates and high saturation of BrdU retention relate to low cell proliferation rates. The evidence presented here supports the hypothesis that the underlying cause of evolved increases in tooth number and tooth gain rate in freshwater fish can be attributed to increases in proliferation rates at the cellular level. In observing the earliest developmental stage of tooth development, the bud and bell stage, we saw that there is no significant difference in BrdU saturation between the marine and freshwater populations in either cell epithelial or mesenchymal cells. However, the latest, erupted stage of tooth development, revealed significant differences between marine and freshwater BrdU saturation in both epithelial and mesenchymal cell types. Overall, the marine population consistently retained the BrdU label more in both dental epithelia and mesenchyme compared to freshwater populations, supporting the hypothesis that freshwater sticklebacks have increases in tooth number due to evolved increases in cell proliferation and cycling turnover rate compared to marine counterparts and these proliferation differences occur late, but not early in tooth development. The marine-freshwater difference in cell proliferation dynamics is consistent with freshwater having higher rates of tooth regeneration possibly stemming from this increase in cell proliferation rates. Similarly, marine populations having slower tooth regeneration rates could reflect this ancestral population having fewer cells actively dividing and giving rise to new teeth.

Considering how others identified ‘slow-cycling’ LRCs, and putative stem cell niches, in or near regenerating tooth organs (Handrigan et al., 2010; Martin et al., 2016; Wu et al., 2013), we were interested in the distribution of LRCs surrounding the regenerating tooth germ in stickleback pharyngeal teeth. The successional dental lamina (SDL) is often described as the histologically distinct location in which many species, including most mammals, reptiles, and amphibians, derive replacement tooth epithelium (Reif, 1982). However, the use of the SDL is not common among all species, including ray-finned fishes like sticklebacks, medaka, salmon, and bichir (Abduweli et al., 2014; Vandenplas et al., 2014; Square et al., 2021). Despite the lack of a histologically distinct SDL, sticklebacks still regulate tooth regeneration in a similar fashion to their SDL counterpart, zebrafish. A set of nine genes (*Bmpr1a*, *Bmp6*, *CD34*, *Gli1*, *Igfbp5a*, *Lgr4*, *Lgr6*, *Nfatc1*, and *Pitx2*) related to expression or function in tooth or hair regeneration were identified in the basalmost endodermal cell layer, which is described as the “successional

dental epithelium" (SDE) and is the region responsible for generating replacement tooth germs (Square et al., 2021).

Unlike these proposed epithelial progenitor cell populations for tooth replacement, less is known about sources of dental mesenchyme for replacement teeth in polyphyodonts. Thus, we were interested in the distribution of slow-cycling cells in both epithelial and mesenchymal cell populations. We hypothesized that if a stem cell niche exists it would derive from the previously described SDE. While we observed a pocket of high density of LRCs in an extension of the SDE, the IDE, there was not an obvious definitive localization of 'slow-cycling' cells adjacent to the tooth germ after the 4 week chase time, as has been described in other models of tooth regeneration (Vandenplas et al., 2014, 2016). However, the cycling time and rate of cell turn-over between species, populations, and stage of development may vary. The small numbers of LRCs reported here might represent true quiescent stem cells that mediate tooth replacement. Future fate mapping data will be needed to address clonal relationships between epithelial and mesenchymal cells during tooth replacement.

Stem cells are often classified as slow-cycling and only active when a deployment of new cells is needed for regeneration. Measuring cell proliferation as a function of BrdU incorporation can be used to identify slow-cycling cells. However, 'slow-cycling' is a broad characteristic that may not apply to all types of stem cells. The BrdU+ label retention is a common, but not essential characteristic of stem cell niches. It is possible that fish like sticklebacks, salmon, and bichir have different classifications of stem cells: long-term tooth stem cells that respond to injury or growth, which could be the slowest cycling, or short-term tooth stem cells that maintain homeostasis, which cycle more frequently. The LRCs that we observed could be transit amplifying cells that were actively dividing during the pulse, then stopped proliferating but maintained their position next to the tooth with marine having greater retention than freshwater in the late stage tooth development. Conversely, failure to mark highly quiescent cells may be a function of them only dividing very rarely, such that only the transiently amplifying daughter cells incorporate the label during the initial pulse time period.

BrdU is widely incorporated throughout the pharyngeal and dental epithelium and mesenchyme during the pulse period, but saturation is never complete. It is possible that the unlabeled cells in the initial pulse period are the slowest cycling cells which is why they did not incorporate BrdU. An example of rare-cycling cells has previously been described in long-term hematopoietic stem cells (LT-HSCs). The LT-HSCs cycle as few as five times per the lifetime of a mouse and remain dormant unless stimulated by injury or induces for growth (Wilson et al., 2008). We propose that the resting phase of regenerating tooth cells is longer in low-toothed marine fish than high-toothed freshwater fish which explains the differences in tooth number and regeneration rates. However, future experiments using a more sensitive assay like H2B-GFP transgene inducible pulse-chase (Tumbar et al., 2004) would more precisely label cells and identify true LRCs and future cell lineage tracing experiments could solidify the identification of a tooth stem cell niche.

The roles of BMP/WNT pathways on maintaining and regulating cell proliferation

In addition to measuring relative rates of proliferation between marine and freshwater stickleback populations, we were interested in determining the effects that two different pharmacological compounds would have on cell proliferation rates in the low-toothed marine population. Past research on mammalian hair regeneration has proposed a model in which stem cell activity is balanced by the reciprocal antagonistic competition, between BMP and WNT signaling (Kandyba et al., 2013). This cross talk between BMP and Wnt is necessary for maintaining homeostasis, such that activator (WNT) and inhibitor (BMP) competitively regulate activities in bulge HFSCs (Kandyba et al., 2013). Additional work conducted on regenerating zebrafish caudal fins, uncovered how BMP signaling promotes osteoblast differentiation by activating expression and countering WNT activity. This evidence supports the competitive activities of WNT and BMP in regulating growth, regeneration, and differentiation during bone regeneration (Stewart et al., 2014). Furthermore, treatment of cichlids with the small molecule, BMP-inhibitor, LDN, caused a reduction in tooth density and the tooth field became artificially populated with tastebuds (Bloomquist et al., 2019).

Considering the important roles that BMP/WNT pathways play in development and hair and tooth regeneration, we were interested in testing the effects of both a BMP-inhibitor, LDN, and a WNT-activator, BIO, on BrdU label retention in ancestral, low-toothed, marine stickleback populations. However, while we expected the inhibition of BMP via LDN to increase cell proliferation and decrease BrdU saturation, there was no significant difference detected across all stages of tooth development in both epithelial and mesenchymal cell types. Additional studies with the BMP-pathway could reveal how the cycling rate and number of LRCs are regulated by BMP signaling and *Bmp6*, which have already been linked to promoting the number of slow-cycling stem cells in the mouse hair follicle.

Activated WNTβ-catenin signaling is well-known to stimulate cell proliferation and the small molecule glycogen synthase kinase-3 inhibitor 6-bromoindirubin-3-oxim (BIO) has been demonstrated to increase WNT signaling. When the WNT signaling pathway was stimulated using the GSK3b inhibitor BIO in gecko dental explants it caused the dental lamina to thicken possibly due to an increase in cell proliferation (Handrigan et al., 2010). In snakes, it has been suggested that WNT activity is repressed by Hedgehog signaling during stages of tooth replacement, by way of negatively regulating BMP levels in the dental mesenchyme (Handrigan & Richman, 2010). Activating the Wnt pathway, revealed a significant difference in BrdU saturation in mesenchymal cells of fish treated with BIO compared to DMSO and LDN in late but not early stages of tooth development. Future experiments will determine if inhibition of the WNT pathway leads to cell quiescence instead of cell proliferation in regenerating tooth germs.

3.6 Supplemental Results

Immunohistochemistry of cell proliferation markers

We were interested in observing differences in cell proliferation dynamics looking at various cell proliferation markers including: PCNA, H3, and Sox2 (Fig. S3.4). Proliferating Cell Nuclear Antigen (PCNA) is a stain that has been commonly used to describe mitotic activity in the teeth of alligator (Wu et al., 2013), salmon and zebrafish (Vandenplas et al., 2012), shark (Martin et

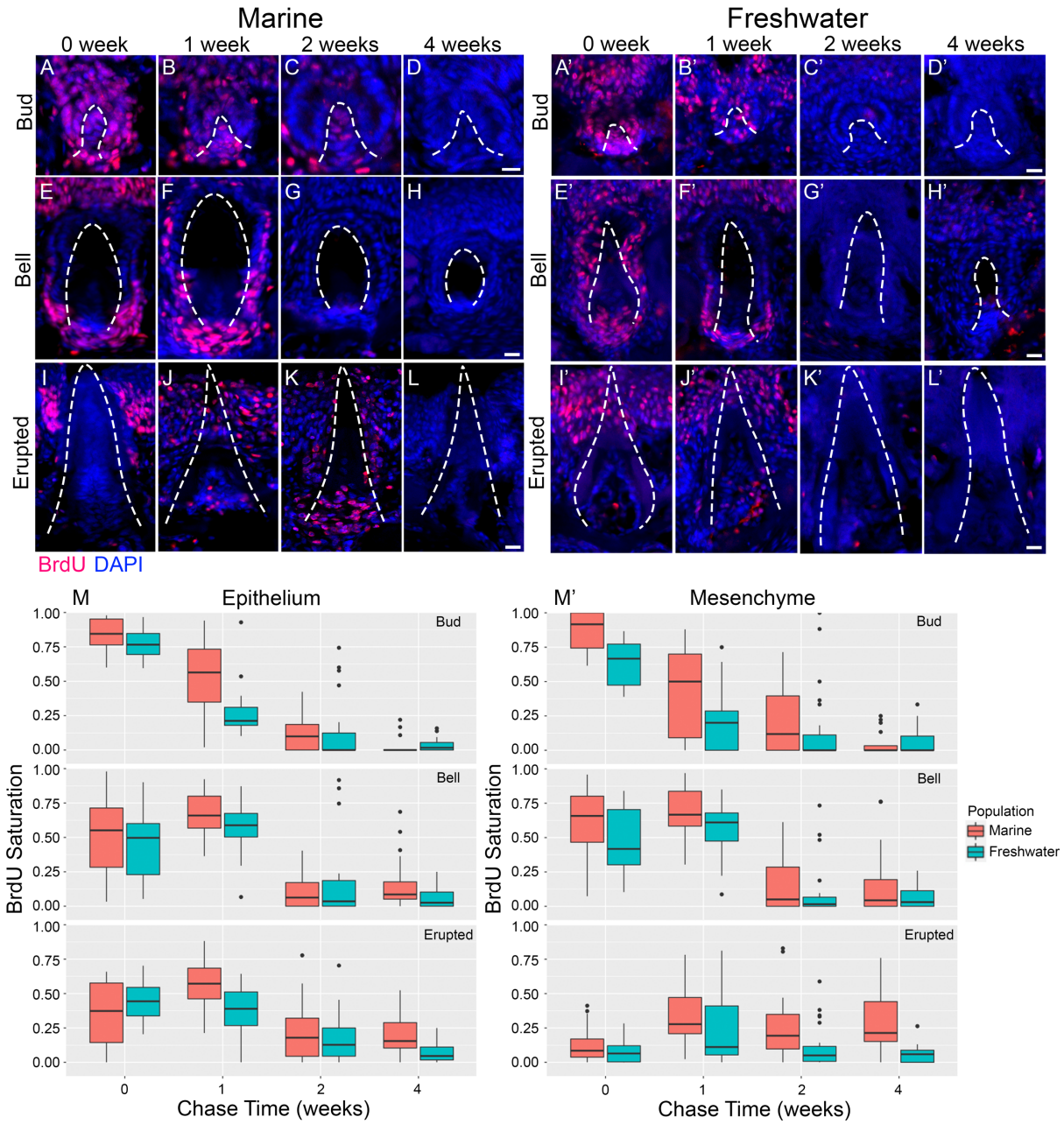
al., 2016) and cichlid (Fraser et al., 2013). We observed that PCNA is widely expressed during the bud stage of tooth development then was present more in the outer dental epithelium (ODE) than the inner dental epithelium (IDE). This difference suggests proliferating cells are more active in the ODE compared to IDE. Histones are nuclear proteins responsible for the nucleosome structure of chromosomes. Modification of histones can alter chromatin structure leading to shifts in cellular processes and transcriptional regulation. The acetylation of Histone H3 appears to control histone deposition, chromatin assembly and active transcription, whereas the phosphorylation of H3 has been linked to cell proliferation (Rosenfeld et al., 2009)**.** We used an anti-H3 antibody to detect H3 in regenerating tooth germs and found that unlike PCNA, which is concentrated in the ODE, H3 is distributed throughout both the IDE, ODE, and dental mesenchyme.

Previous research found that putative stem cells in medaka and shark dentitions expressed the epithelial progenitor and stem cell marker *Sox2* (Abduweli et al., 2014; Martin et al., 2016). *Sox2* has also been demonstrated to play a role in regulating adult epithelial stem cells required for tooth ameloblast regeneration in mammals and reptiles (Arnold et al., 2011; Juuri et al., 2013, 2012). Furthermore, *Sox2* has been identified as a key regulator of hair growth that controls progenitor migration via BMP-mediated mesenchymal-epithelial crosstalk (Clavel et al., 2012). We hypothesized that a subset of epithelial Sox2+ LRCs near regenerating teeth could indicate the presence of a TSC niche. However, instead of dental epithelium expression, we saw that Sox2 immunoreactivity was largely restricted to the dental mesenchyme, contrary to its expression in other model systems. There were no significant differences in PCNA or Sox2 distribution noticed between freshwater and marine stickleback populations.

BrdU pulse-chase in larvae developing teeth

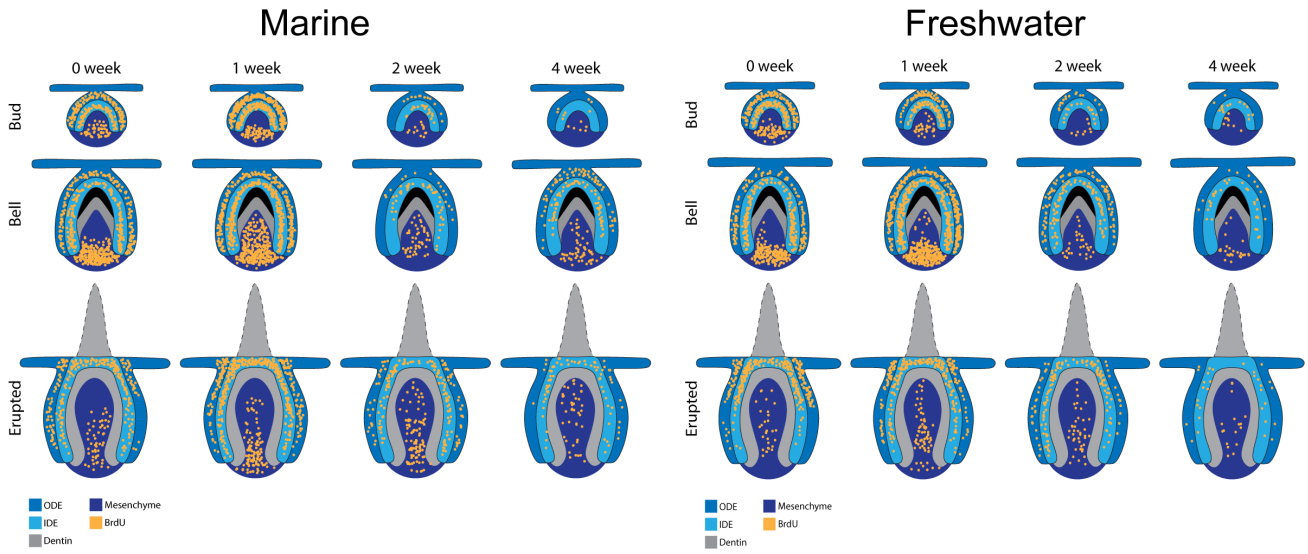
We were also interested in evaluating the differences in BrdU retention in marine and freshwater populations during the earliest stages of larvae development (Fig. S3.5 and 6). Newly hatched sticklebacks were immediately immersed in 5mM BrdU and pulsed for 5 days before being sacrificed at the 0, 1, 2, and 4 week chase time points. BrdU was broadly distributed throughout the epithelium and mesenchyme for both populations. However, the larvae lost their BrdU label at a greater rate compared to adult fish for both populations. The rapid loss of label may be explained from young fish cells proliferating faster during early development. Once the fish reaches a more mature stage of development the cells are likely not dividing as quickly and therefore retain more label than the developing larvae. Overall, not enough data were collected to observe any significant differences between marine and freshwater larvae.

3.7 Supplemental Figures



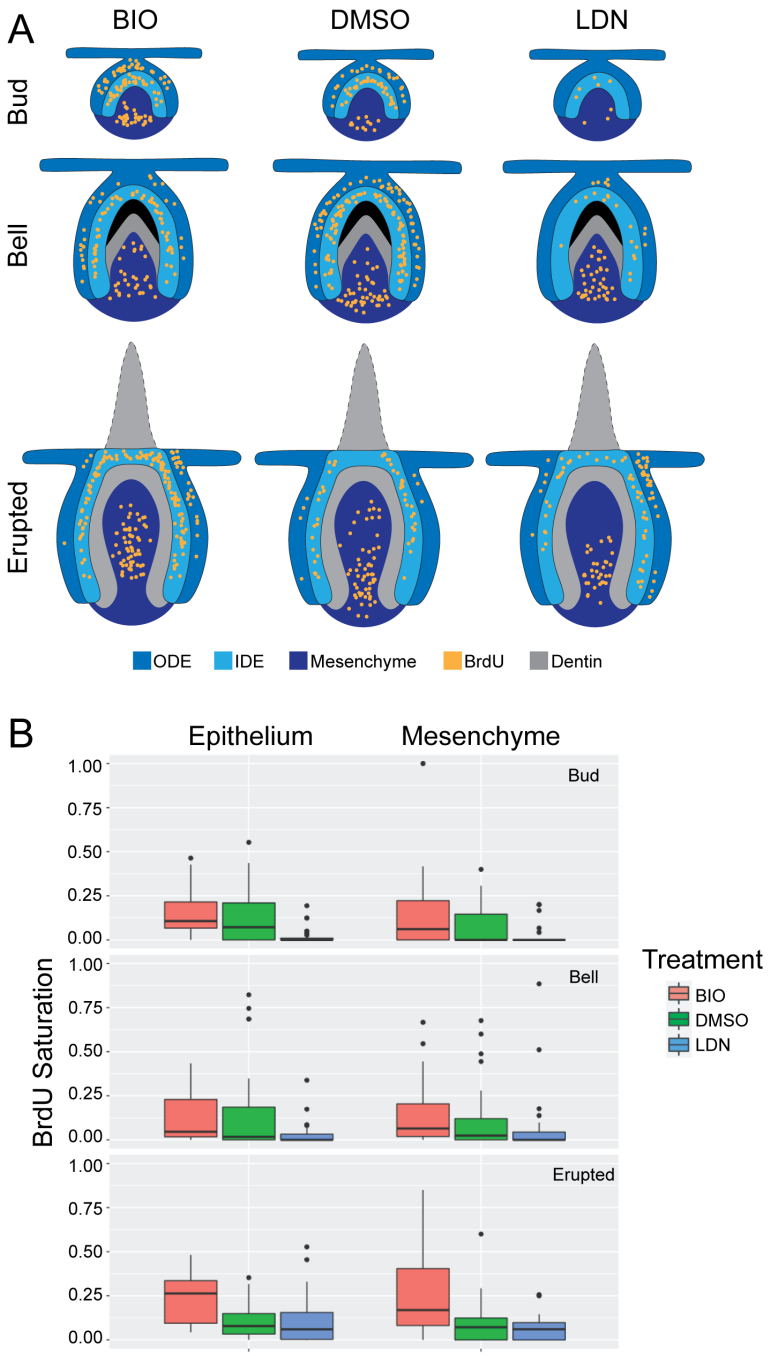
Supplemental Figure 3.1 Proportion of LRCs in marine compared to freshwater dorsal pharyngeal teeth.

Confocal images of transverse paraffin sections from marine (A-L) and freshwater (A'-L') dorsal tooth germs at bud, bell, and erupted stages of development (y-axis) and at varying chase time points (x-axis). White dotted lines demarcate the outline of the tooth germ, separating epithelium from mesenchyme. Scale bar = 10 µm. Boxplots (M-M') show overall BrdU saturation of the dental epithelium and mesenchyme between freshwater (blue) and marine (pink) populations in dorsal tooth germs at bud, bell, and erupted stages of development (y-axis) and at 0, 1, 2, and 4 week chase time points (x-axis). Marine showed significantly greater BrdU retention compared to Freshwater in the bell and erupted stage of mesenchymal cells ($P = 0.052$ and $P = 0.00065$, respectively). However, differences in the earliest stages of tooth development showed a smaller effect in the mesenchyme bud ($P = 0.063$) and all stages of epithelium bud, bell, and erupted ($P = 0.23$, 0.15, and 0.26, respectively).



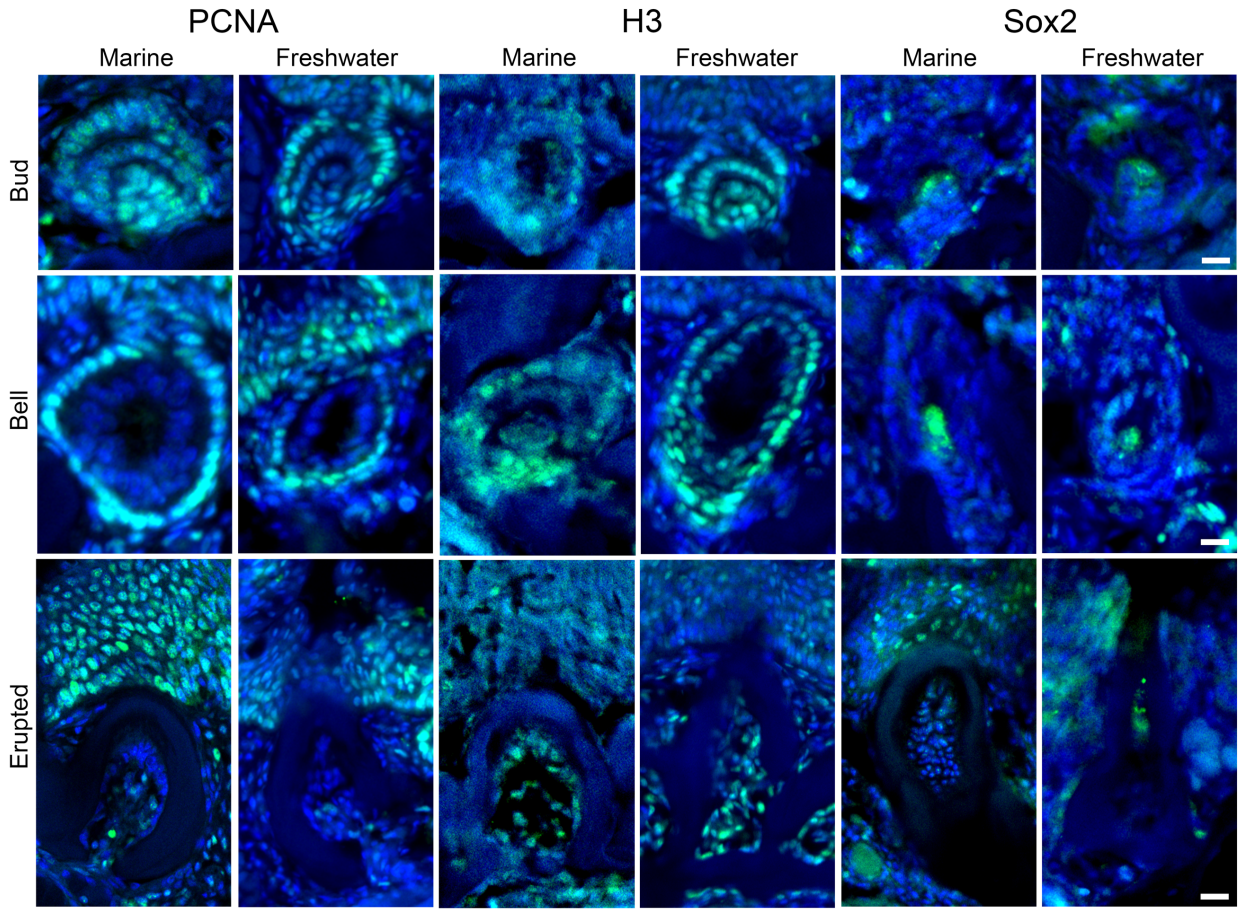
Supplemental Figure 3.2 Evolved reductions in LRC number in freshwater compared to marine dorsal pharyngeal teeth.

Summary of spatial distribution of LRCs in marine and freshwater dorsal pharyngeal tooth germs at bud, bell, and erupted stages of development (y-axis) and at varying chase time points (x-axis). LRCs remain concentrated near pharyngeal epithelium and deep mesenchyme in both marine and freshwater tooth germs.



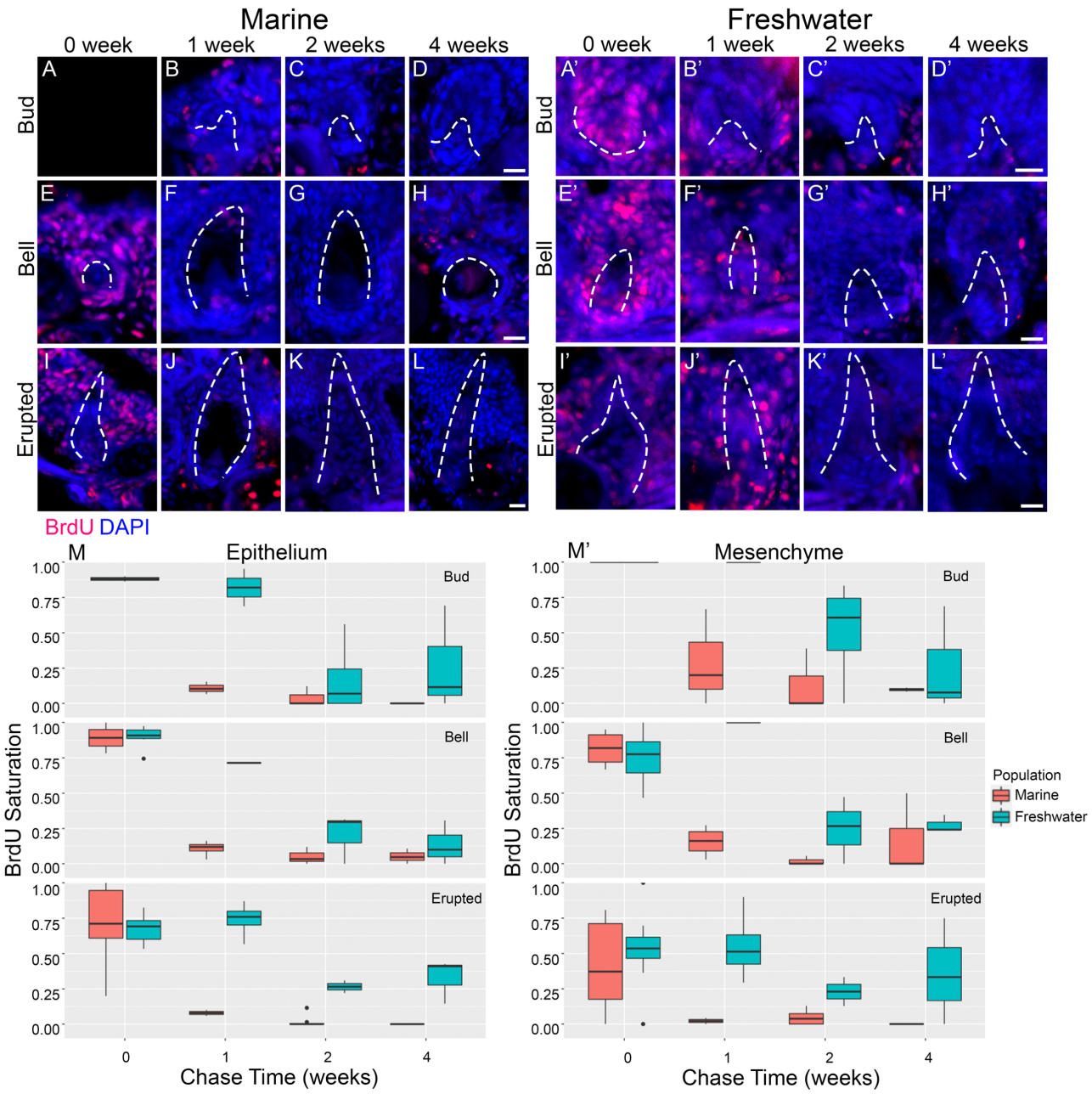
Supplemental Figure 3.3 Distribution and proportion of LRCs in marine dorsal pharyngeal teeth after drug treatment.

(A) Graphic summary of spatial distribution of BrdU-positive cells in marine dorsal tooth germs at bud, bell, and erupted stages of development (y-axis) with various drug treatment: BIO, DMSO, or LDN (x-axis). (B) Boxplots describe overall BrdU saturation (y-axis) of the dental epithelium and mesenchyme between BIO (pink), DMSO (green), and LDN (blue) treated marine fish in dorsal tooth germs at bud, bell, and erupted stages of development. BrdU saturation was significant for the BIO treated fish compared to DMSO and LDN in mesenchymal cells of bud and erupted teeth ($P = 0.0042$ and 0.0048, respectively). No significant differences were observed across bud, bell, and erupted stages of tooth development in epithelial cells ($P = 0.029$, 0.28, and 0.16, respectively) or mesenchymal cells of bell stage ($P = 0.63$).



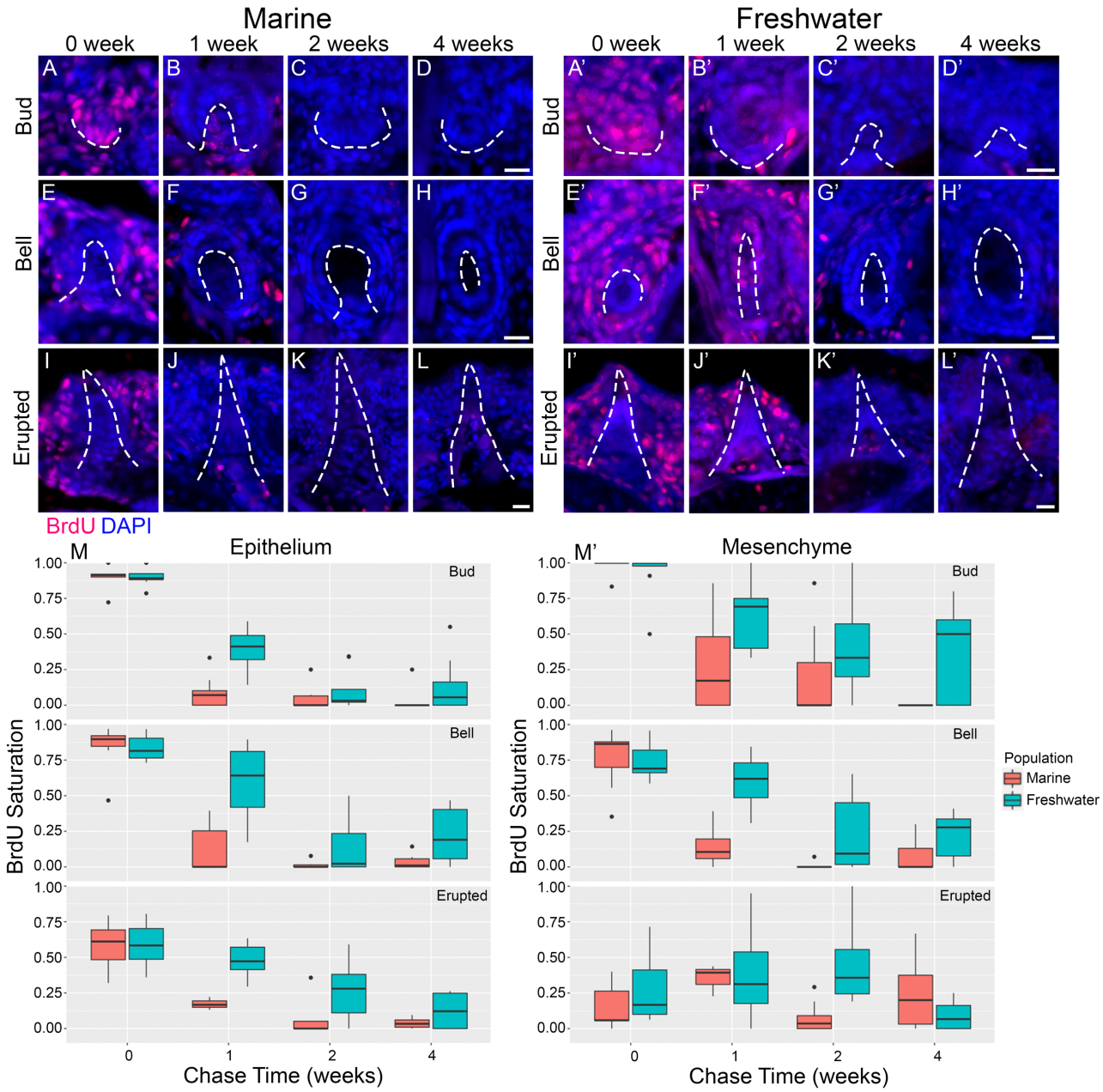
Supplemental Figure 3.4 IHC of PCNA, H3 and Sox2 in marine ventral tooth plate.

Confocal images of paraffin sections from marine ventral pharyngeal tooth germs at bud, bell, and erupted stages of development (y-axis) stained for immunoreactivity for PCNA, H3, and Sox2. White dotted lines demarcate the outline of the tooth germ, separating epithelium from mesenchyme. Scale bar = 10 μm.



Supplemental Figure 3.5 Proportion of LRCs in larvae marine compared to freshwater ventral pharyngeal teeth.

Confocal images of transverse paraffin sections from larvae marine (A-L) and freshwater (A'-L') ventral tooth germs at bud, bell, and erupted stages of development (y-axis) and at varying chase time points (x-axis). White dotted lines demarcate the outline of the tooth germ, separating epithelium from mesenchyme. Scale bar = 10 µm. Boxplots (M-M') show overall BrdU saturation of the dental epithelium and mesenchyme between freshwater (blue) and marine (pink) populations in ventral tooth germs at bud, bell, and erupted stages of development (y-axis) and at 0, 1, 2, and 4 week chase time points (x-axis). No statistical analysis performed.



Supplemental Figure 3.6 Proportion of LRCs in larvae marine compared to freshwater dorsal pharyngeal teeth.

Confocal images of transverse paraffin sections from larvae marine (A-L) and freshwater (A'-L') dorsal tooth germs at bud, bell, and erupted stages of development (y-axis) and at varying chase time points (x-axis). White dotted lines demarcate the outline of the tooth germ, separating epithelium from mesenchyme. Scale bar = 10 µm. Boxplots (M-M') show overall BrdU saturation of the dental epithelium and mesenchyme between freshwater (blue) and marine (pink) populations in dorsal tooth germs at bud, bell, and erupted stages of development (y-axis) and at 0, 1, 2, and 4 week chase time points (x-axis). No statistical analysis performed.

3.8 Acknowledgements

We would like to thank Tyler Square for assistance in sectioning and staining, Sophie Archambeault for advice on coding and RStudio, Naama Weksler for feedback on data, and Maya Samuels-Fair from the UC Berkeley Data Lab for consultations on statistical analyses.

3.9 References

Abduweli, D., Baba, O., Tabata, M. J., Higuchi, K., Mitani, H., & Takano, Y. (2014). Tooth replacement and putative odontogenic stem cell niches in pharyngeal dentition of medaka (*Oryzias latipes*). *Microscopy*, *63*(2), 141–153. https://doi.org/10.1093/jmicro/dft085

Åberg, T., Wozney, J., & Thesleff, I. (1997). Expression patterns of bone morphogenetic proteins (Bmps) in the developing mouse tooth suggest roles in morphogenesis and cell differentiation. *Developmental Dynamics*, *210*(4), 383–396. https://doi.org/10.1002/(SICI)1097-0177(199712)210:4<383::AID-AJA3>3.0.CO;2-C

Albert, A. Y. K., Sawaya, S., Vines, T. H., Knecht, A. K., Miller, C. T., Summers, B. R., … Schluter, D. (2007). The genetics of adaptive shape shift in stickleback: pleiotropy and effect size. *Evolution*, *0*(0), 071115145922005-??? https://doi.org/10.1111/j.1558-5646.2007.00259.x

Aldinger, K. A., Lehmann, O. J., Hudgins, L., Chizhikov, V. V., Bassuk, A. G., Ades, L. C., … Millen, K. J. (2009). FOXC1 is required for normal cerebellar development and is a major contributor to chromosome 6p25.3 Dandy-Walker malformation. *Nature Genetics*, *41*(9), 1037–1042. https://doi.org/10.1038/ng.422

Amores, A., Force, A., Yan, Y. L., Joly, L., Amemiya, C., Fritz, A., … Postlethwait, J. H. (1998). Zebrafish hox clusters and vertebrate genome evolution. *Science*, *282*(5394), 1711–1714. https://doi.org/10.1126/science.282.5394.1711

Arnold, K., Sarkar, A., Yram, M. A., Polo, J. M., Bronson, R., Sengupta, S., … Hochedlinger, K. (2011). Sox2+ Adult Stem and Progenitor Cells Are Important for Tissue Regeneration and Survival of Mice. *Cell Stem Cell*, *9*(4), 317–329. https://doi.org/10.1016/J.STEM.2011.09.001

Bei, M, Kratochwil, K., & Maas, R. L. (2000). BMP4 rescues a non-cell-autonomous function of Msx1 in tooth development. *Development (Cambridge, England)*, *127*(21), 4711–4718. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11023873

Bei, Marianna, & Maas, R. (1998). FGFs and BMP4 induce both Msx1-independent and Msx1-dependent signaling pathways in early tooth development. *Development*, *125*(21), 4325–4333. https://doi.org/10.1242/dev.125.21.4325

Bell, M. A., & Foster, S. A. (1994). The Evolutionary Biology of the Threespine Stickleback. *Oxford University Press*, *64*(3), 418. https://doi.org/10.2307/5902

Berry, F. B., Lines, M. A., Oas, J. M., Footz, T., Underhill, D. A., Gage, P. J., & Walter, M. A. (2006). Functional interactions between FOXC1 and PITX2 underlie the sensitivity to FOXC1 gene dose in Axenfeld-Rieger syndrome and anterior segment dysgenesis. *Human Molecular Genetics*, *15*(6), 905–919. https://doi.org/10.1093/hmg/ddl008

Berry, F. B., Skarie, J. M., Mirzayans, F., Fortin, Y., Hudson, T. J., Raymond, V., … Walter, M. A. (2008). FOXC1 is required for cell viability and resistance to oxidative stress in the eye through the transcriptional regulation of FOXO1A. *Human Molecular Genetics*, *17*(4), 490–505. https://doi.org/10.1093/hmg/ddm326

Blanpain, C., Lowry, W. E., Geoghegan, A., Polak, L., & Fuchs, E. (2004). Self-renewal, multipotency, and the existence of two cell populations within an epithelial stem cell niche. *Cell*, *118*(5), 635–648. https://doi.org/10.1016/j.cell.2004.08.012

Bloomquist, R. F., Fowler, T. E., An, Z., Yu, T. Y., Abdilleh, K., Fraser, G. J., … Streelman, J. T. (2019). Developmental plasticity of epithelial stem cells in tooth and taste bud renewal. *Proceedings of the National Academy of Sciences of the United States of America*, *116*(36), 17858–17866. https://doi.org/10.1073/pnas.1821202116

Boffelli, D., Nobrega, M. A., & Rubin, E. M. (2004, June). Comparative genomics at the vertebrate extremes. *Nature Reviews Genetics*. https://doi.org/10.1038/nrg1350

Bonferroni, C. E. (1936). Teoria statistica delle classi e calcolo delle probabilità. *Pubblicazioni Del R Istituto Superiore Di Scienze Economiche e Commerciali Di Firenze*.

Carroll, S. B. (1995). Homeotic genes and the evolution of arthropods and chordates. *Nature*, *376*(6540), 479–485. https://doi.org/10.1038/376479a0

Chan, Y. F., Marks, M. E., Jones, F. C., Villarreal, G., Shapiro, M. D., Brady, S. D., … Kingsley, D. M. (2010). Adaptive evolution of pelvic reduction in sticklebacks by recurrent deletion of a pitxl enhancer. *Science*, *327*(5963), 302–305. https://doi.org/10.1126/science.1182213

Chanda, B., Asai-Coakwell, M., Ye, M., Mungall, A. J., Barrow, M., Dobyns, W. B., … Lehmann, O. J. (2008). A novel mechanistic spectrum underlies glaucoma-associated chromosome 6p25 copy number variation. *Human Molecular Genetics*, *17*(22), 3446–3458. https://doi.org/10.1093/hmg/ddn238

Chen, Y. P., Bei, M., Woo, I., Satokata, I., & Maas, R. (1996). Msx1 controls inductive signaling in mammalian tooth morphogenesis. *Development*, *122*(10), 3035–3044. https://doi.org/10.1242/dev.122.10.3035

Cirillo, L. A., Lin, F. R., Cuesta, I., Friedman, D., Jarnik, M., & Zaret, K. S. (2002). Opening of compacted chromatin by early developmental transcription factors HNF3 (FoxA) and GATA-4. *Molecular Cell*, *9*(2), 279–289. https://doi.org/10.1016/S1097-2765(02)00459-8

Clavel, C., Grisanti, L., Zemla, R., Rezza, A., Barros, R., Sennett, R., … Rendl, M. (2012). Sox2

in the dermal papilla niche controls hair growth by fine-tuning BMP signaling in differentiating hair shaft progenitors. *Developmental Cell*, *23*(5), 981–994. https://doi.org/10.1016/j.devcel.2012.10.013

Cleves, P. A., Ellis, N. A., Jimenez, M. T., Nunez, S. M., Schluter, D., Kingsley, D. M., & Miller, C. T. (2014). Evolved tooth gain in sticklebacks is associated with a cis-regulatory allele of Bmp6. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(38), 13912–13917. https://doi.org/10.1073/pnas.1407567111

Cleves, P. A., Hart, J. C., Agoglia, R. M., Jimenez, M. T., Erickson, P. A., Gai, L., & Miller, C. T. (2018). An intronic enhancer of Bmp6 underlies evolved tooth gain in sticklebacks. *PLOS Genetics*, *14*(6), e1007449. https://doi.org/10.1371/journal.pgen.1007449

Colosimo, P. F. (2005). Widespread Parallel Evolution in Sticklebacks by Repeated Fixation of Ectodysplasin Alleles. *Science*, *307*(5717), 1928–1933. https://doi.org/10.1126/science.1107239

Cotsarelis, G., Sun, T.-T., & Lavker, R. M. (1990). Label-retaining cells reside in the bulge area of pilosebaceous unit: Implications for follicular stem cells, hair cycle, and skin carcinogenesis. *Cell*, *61*(7), 1329–1337. https://doi.org/10.1016/0092-8674(90)90696-C

Cowles, C. R., Hirschhorn, J. N., Altshuler, D., & Lander, E. S. (2002). Detection of regulatory variation in mouse genes. *Nature Genetics*, *32*(3), 432–437. https://doi.org/10.1038/ng992

Cresko, W. A., Amores, A., Wilson, C., Murphy, J., Currey, M., Phillips, P., … Postlethwait, J. H. (2004). Parallel genetic basis for repeated evolution of armor loss in Alaskan threespine stickleback populations. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(16), 6050–6055. https://doi.org/10.1073/pnas.0308479101

Crick, F. (1958). On Protein Synthesis. *Symposia of the Society for Experimental Biology, Number XII: The Biological Replication of Macromolecules. Cambridge University Press.*, 138–163.

Cuny, G. D., Yu, P. B., Laha, J. K., Xing, X., Liu, J. F., Lai, C. S., … Peterson, R. T. (2008). Structure-activity relationship study of bone morphogenetic protein (BMP) signaling inhibitors. *Bioorganic and Medicinal Chemistry Letters*, *18*(15), 4388–4392. https://doi.org/10.1016/j.bmcl.2008.06.052

Darwin, C. (1859). On the Origins of Species by Means of Natural Selection, or the Preservation of Favoured Races in the Struggle for Life.

DeScipio, C., Schneider, L., Young, T. L., Wasserman, N., Yaeger, D., Lu, F., … Krantz, I. D. (2005). Subtelomeric deletions of chromosome 6p: Molecular and cytogenetic characterization of three new cases with phenotypic overlap with Ritscher?Schinzel (3C) syndrome. *American Journal of Medical Genetics Part A*, *134A*(1), 3–11. https://doi.org/10.1002/ajmg.a.30573

Ellis, N. A., Donde, N. N., & Miller, C. T. (2016). Early development and replacement of the stickleback dentition. *Journal of Morphology*, *277*(8), 1072–1083. https://doi.org/10.1002/jmor.20557

Ellis, N. A., Glazer, A. M., Donde, N. N., Cleves, P. A., Agoglia, R. M., & Miller, C. T. (2015). Distinct developmental and genetic mechanisms underlie convergently evolved tooth gain in sticklebacks. *Development*, (June), 2442–2451. https://doi.org/10.1242/dev.124248

Emera, D., Yin, J., Reilly, S. K., Gockley, J., & Noonan, J. P. (2016). Origin and evolution of developmental enhancers in the mammalian neocortex. *Proceedings of the National Academy of Sciences of the United States of America*, *113*(19), E2617–E2626. https://doi.org/10.1073/pnas.1603718113

Erickson, P. A., Cleves, P. A., Ellis, N. A., Schwalbach, K. T., Hart, J. C., & Miller, C. T. (2015). A 190 base pair, TGFB responsive tooth and fin enhancer is required for stickleback Bmp6 expression. *Developmental Biology*, *401*(2), 310–323. https://doi.org/10.1016/j.ydbio.2015.02.006

Erickson, P. A., Ellis, N. A., & Miller, C. T. (2016). Microinjection for Transgenesis and Genome Editing in Threespine Sticklebacks. *Journal of Visualized Experiments*, (111), e54055–e54055. https://doi.org/10.3791/54055

Erickson, P. A., Glazer, A. M., Cleves, P. A., Smith, A. S., & Miller, C. T. (2014). Two developmentally temporal quantitative trait loci underlie convergent evolution of increased branchial bone length in sticklebacks. *Proceedings of the Royal Society B: Biological Sciences*, *281*(1788). https://doi.org/10.1098/rspb.2014.0822

Farh, K. K. H., Marson, A., Zhu, J., Kleinewietfeld, M., Housley, W. J., Beik, S., … Bernstein, B. E. (2015). Genetic and epigenetic fine mapping of causal autoimmune disease variants. *Nature*, *518*(7539), 337–343. https://doi.org/10.1038/nature13835

Fish, A., Chen, L., & Capra, J. A. (2017). Gene regulatory enhancers with evolutionarily conserved activity aremore pleiotropic than those with species-specific activity. *Genome Biology and Evolution*, *9*(10), 2615–2625. https://doi.org/10.1093/gbe/evx194

Fisher, S., Grice, E. A., Vinton, R. M., Bessling, S. L., & McCallion, A. S. (2006). Conservation of RET regulatory function from human to zebrafish without sequence similarity. *Science*, *312*(5771), 276–279. https://doi.org/10.1126/science.1124070

Fraser, G. J., Bloomquist, R. F., & Streelman, J. T. (2013). Common developmental pathways link tooth shape to regeneration. *Developmental Biology*, *377*(2), 399–414. https://doi.org/10.1016/J.YDBIO.2013.02.007

Frazer, K. A., Pachter, L., Poliakov, A., Rubin, E. M., & Dubchak, I. (2004). VISTA: Computational tools for comparative genomics. *Nucleic Acids Research*, *32*(WEB SERVER ISS.), W273–W279. https://doi.org/10.1093/nar/gkh458

Furlong, E. E. M., & Levine, M. (2018, September 28). Developmental enhancers and chromosome topology. *Science*. American Association for the Advancement of Science. https://doi.org/10.1126/science.aau0320

Gehring, W. J., & Hiromi, Y. (1986). Homeotic Genes and the Homeobox. *Annual Review of Genetics*, *20*(1), 147–173. https://doi.org/10.1146/annurev.ge.20.120186.001051

Glazer, A. M., Cleves, P. A., Erickson, P. A., Lam, A. Y., & Miller, C. T. (2014). Parallel developmental genetic features underlie stickleback gill raker evolution. *EvoDevo*, *5*(1), 1–16. https://doi.org/10.1186/2041-9139-5-19

Glazer, A. M., Killingbeck, E. E., Mitros, T., Rokhsar, D. S., & Miller, C. T. (2015). Genome assembly improvement and mapping convergently evolved skeletal traits in sticklebacks with genotyping-by-sequencing. *G3: Genes, Genomes, Genetics*, *5*(7), 1463–1472. https://doi.org/10.1534/g3.115.017905

Gould, D. B., Jaafar, M. S., Addison, M. K., Munier, F., Ritch, R., MacDonald, I. M., & Walter, M. A. (2004). Phenotypic and molecular assessment of seven patients with 6p25 deletion syndrome: Relevance to ocular dysgenesis and hearing impairment. *BMC Medical Genetics*, *5*(1), 17. https://doi.org/10.1186/1471-2350-5-17

Handrigan, G. R., Leung, K. J., & Richman, J. M. (2010). Identification of putative dental epithelial stem cells in a lizard with life-long tooth replacement. *Development*, *137*(21), 3545–3549. https://doi.org/10.1242/dev.052415

Handrigan, Gregory R., Leung, K. J., & Richman, J. M. (2010). Identification of putative dental epithelial stem cells in a lizard with life-long tooth replacement. *Development*, *137*(21). Retrieved from http://dev.biologists.org/content/137/21/3545

Handrigan, Gregory R., & Richman, J. M. (2010). A network of Wnt, hedgehog and BMP signaling pathways regulates tooth replacement in snakes. *Developmental Biology*, *348*(1), 130–141. https://doi.org/10.1016/J.YDBIO.2010.09.003

Hannenhalli, S., & Kaestner, K. H. (2009, April). The evolution of Fox genes and their role in development and disease. *Nature Reviews Genetics*. NIH Public Access. https://doi.org/10.1038/nrg2523

Harada, H., Kettunen, P., Jung, H. S., Mustonen, T., Wang, Y. A., & Thesleff, I. (1999). Localization of putative stem cells in dental epithelium and their association with Notch and FGF signaling. *Journal of Cell Biology*, *147*(1), 105–120. https://doi.org/10.1083/jcb.147.1.105

Harris, M. P., Rohner, N., Schwarz, H., Perathoner, S., Konstantinidis, P., & Nüsslein-Volhard, C. (2008). Zebrafish eda and edar Mutants Reveal Conserved and Ancestral Roles of Ectodysplasin Signaling in Vertebrates. *PLoS Genetics*, *4*(10), e1000206. https://doi.org/10.1371/journal.pgen.1000206

Hart, J. C., & Miller, C. T. (2017). Sequence-Based Mapping and Genome Editing Reveal Mutations in SticklebackHps5Cause Oculocutaneous Albinism and thecasperPhenotype. *G3 (Bethesda, Md.)*, *7*(9), 3123–3131. https://doi.org/10.1534/g3.117.1125

Headon, D. J., Emmal, S. A., Ferguson, B. M., Tucker, A. S., Justice, M. J., Sharpe, P. T., … Overbeek, P. A. (2001). Gene defect in ectodermal dysplasia implicates a death domain adapter in development. *Nature*, *414*(6866), 913–916. https://doi.org/10.1038/414913a

Huang, X., Zhong, L., Hendriks, J., Post, J. N., & Karperien, M. (2018). The effects of the WNT-signaling modulators BIO and PKF118-310 on the chondrogenic differentiation of human mesenchymal stem cells. *International Journal of Molecular Sciences*, *19*(2). https://doi.org/10.3390/ijms19020561

Huxley, J. (1942). Evolution, the Modern Synthesis. *G. Allen and Unwin Limited.*

Indjeian, V. B., Kingman, G. A., Jones, F. C., Guenther, C. A., Grimwood, J., Schmutz, J., … Kingsley, D. M. (2016). Evolving New Skeletal Traits by cis-Regulatory Changes in Bone Morphogenetic Proteins. *Cell*, *164*(1–2), 45–56. https://doi.org/10.1016/j.cell.2015.12.007

Infante, C. R., Mihala, A. G., Park, S., Wang, J. S., Johnson, K. K., Lauderdale, J. D., & Menke, D. B. (2015). Shared Enhancer Activity in the Limbs and Phallus and Functional Divergence of a Limb-Genital cis-Regulatory Element in Snakes. *Developmental Cell*, *35*(1), 107–119. https://doi.org/10.1016/j.devcel.2015.09.003

Jackman, W. R., & Stock, D. W. (2006). Transgenic analysis of Dlx regulation in fish tooth development reveals evolutionary retention of enhancer function despite organ loss. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(51), 19390–19395. https://doi.org/10.1073/pnas.0609575103

Jacob, F., & Monod, J. (1961, June 1). Genetic regulatory mechanisms in the synthesis of proteins. *Journal of Molecular Biology*. Academic Press. https://doi.org/10.1016/S0022-2836(61)80072-7

Jernvall, J., & Thesleff, I. (2012). Tooth shape formation and tooth renewal: evolving with the same signals. *Development*, *139*(19), 3487–3497. https://doi.org/10.1242/dev.085084

Jia, S., Zhou, J., Gao, Y., Baek, J.-A., Martin, J. F., Lan, Y., … Sucov, H. M. (2013). Roles of Bmp4 during tooth morphogenesis and sequential tooth formation. *Development (Cambridge, England)*, *140*(2), 423–432. https://doi.org/10.1242/dev.081927

Jones, F. C., Grabherr, M. G., Chan, Y. F., Russell, P., Mauceli, E., Johnson, J., … Kingsley, D. M. (2012). The genomic basis of adaptive evolution in threespine sticklebacks. *Nature*, *484*(7392), 55–61. https://doi.org/10.1038/nature10944

Juuri, E., Jussila, M., Seidel, K., Holmes, S., Wu, P., Richman, J., … Thesleff, I. (2013). Sox2 marks epithelial competence to generate teeth in mammals and reptiles. *Development*

(Cambridge, England), *140*(7), 1424–1432. https://doi.org/10.1242/dev.089599

Juuri, E., Saito, K., Ahtiainen, L., Seidel, K., Tummers, M., Hochedlinger, K., … Michon, F. (2012). Sox2+ stem cells contribute to all epithelial lineages of the tooth via Sfrp5+ progenitors. *Developmental Cell*, *23*(2), 317–328. https://doi.org/10.1016/j.devcel.2012.05.012

Kandyba, E., Leung, Y., Chen, Y.-B., Widelitz, R., Chuong, C.-M., & Kobielak, K. (2013). Competitive balance of intrabulge BMP/Wnt signaling reveals a robust gene network ruling stem cell homeostasis and cyclic activation. *Proceedings of the National Academy of Sciences*, *110*(4), 1351–1356. https://doi.org/10.1073/pnas.1121312110

Kawakami, K., & Shima, A. (1999). Identification of the Tol2 transposase of the medaka fish Oryzias latipes that catalyzes excision of a nonautonomous Tol2 element in zebrafish Danio rerio. *Gene*, *240*(1), 239–244. https://doi.org/10.1016/S0378-1119(99)00444-8

Kimmel, C. B., Miller, C. T., Kruze, G., Ullmann, B., BreMiller, R. A., Larison, K. D., & Snyder, H. C. (1998). The Shaping of Pharyngeal Cartilages during Early Development of the Zebrafish. *Developmental Biology*, *203*(2), 245–263. https://doi.org/10.1006/DBIO.1998.9016

Kimmel, C. B., Miller, C. T., & Moens, C. B. (2001). Specification and Morphogenesis of the Zebrafish Larval Head Skeleton. *Developmental Biology*, *233*(2), 239–257. https://doi.org/10.1006/DBIO.2001.0201

King, M. C., & Wilson, A. C. (1975). Evolution at two levels in humans and chimpanzees. *Science*, *188*(4184), 107–116. https://doi.org/10.1126/science.1090005

Kingsley, D. M., Zhu, B., Osoegawa, K., De Jong, P. J., Schein, J., Marra, M., … Myers, R. (2004). New genomic tools for molecular studies of evolutionary change in threespine sticklebacks. In *Behaviour* (Vol. 141, pp. 1331–1344). Brill. https://doi.org/10.1163/1568539042948150

Kobielak, K., Stokes, N., de la Cruz, J., Polak, L., & Fuchs, E. (2007). Loss of a quiescent niche but not follicle stem cells in the absence of bone morphogenetic protein signaling. *Proceedings of the National Academy of Sciences*, *104*(24), 10063–10068. https://doi.org/10.1073/pnas.0703004104

Kume, T., Jiang, H., Topczewska, J. M., & Hogan, B. L. M. (2001). The murine winged helix transcription factors, Foxc1 and Foxc2, are both required for cardiovascular development and somitogenesis. *Genes and Development*, *15*(18), 2470–2482. https://doi.org/10.1101/gad.907301

Kume, Tsutomu, Deng, K. Y., Winfrey, V., Gould, D. B., Walter, M. A., & Hogan, B. L. M. (1998). The forkhead/winged helix gene Mf1 is disrupted in the pleiotropic mouse mutation congenital hydrocephalus. *Cell*, *93*(6), 985–996. https://doi.org/10.1016/S0092-

8674(00)81204-0

Le Caignec, C., De Mas, P., Vincent, M.-C., Bocéno, M., Bourrouillou, G., Rival, J.-M., & David, A. (2005). Subtelomeric 6p deletion: Clinical, FISH, and array CGH characterization of two cases. *American Journal of Medical Genetics Part A*, *132A*(2), 175–180. https://doi.org/10.1002/ajmg.a.30409

Leche, W. (1895). Zür Entwicklungsgeschichte des Zahnsystems des Säugethiere, zugleich ein Beitrag zur Stammesgeschichte dieser Thiergruppe. I. Ontogenie. Zoologica 6, 1-160. (n.d.).

Lee, C. S., Friedman, J. R., Fulmer, J. T., & Kaestner, K. H. (2005). The initiation of liver development is dependent on Foxa transcription factors. *Nature*, *435*(7044), 944–947. https://doi.org/10.1038/nature03649

Lehmann, O. J., Sowden, J. C., Carlsson, P., Jordan, T., & Bhattacharya, S. S. (2003, June 1). Fox's in development and disease. *Trends in Genetics*. Elsevier Ltd. https://doi.org/10.1016/S0168-9525(03)00111-2

Levine, M., Cattoglio, C., & Tjian, R. (2014, March 27). Looping back to leap forward: Transcription enters a new era. *Cell*. Elsevier B.V. https://doi.org/10.1016/j.cell.2014.02.009

Li, A., Lai, Y. C., Figueroa, S., Yang, T., Widelitz, R. B., Kobielak, K., … Chuong, C. M. (2015, August 1). Deciphering principles of morphogenesis from temporal and spatial patterns on the integument. *Developmental Dynamics*. John Wiley and Sons Inc. https://doi.org/10.1002/dvdy.24281

Lin, C. R., Kloussi, C., O'Connell, S., Briata, P., Szeto, D., Liu, F., … Rosenfeld, M. G. (1999). Pitx2 regulates lung asymmetry, cardiac positioning and pituitary and tooth morphogenesis. *Nature*, *401*(6750), 279–282. https://doi.org/10.1038/45803

Lukoseviciute, M., Gavriouchkina, D., Williams, R. M., Hochgreb-Hagele, T., Senanayake, U., Chong-Morrison, V., … Sauka-Spengler, T. (2018). From Pioneer to Repressor: Bimodal foxd3 Activity Dynamically Remodels Neural Crest Regulatory Landscape In Vivo. *Developmental Cell*, *47*(5), 608-628.e6. https://doi.org/10.1016/j.devcel.2018.11.009

Mandler, M., & Neubüser, A. (2001). FGF signaling is necessary for the specification of the odontogenic mesenchyme. *Developmental Biology*, *240*(2), 548–559. https://doi.org/10.1006/dbio.2001.0490

Martin, K. J., Rasch, L. J., Cooper, R. L., Metscher, B. D., Johanson, Z., & Fraser, G. J. (2016). Sox2+ progenitors in sharks link taste development with the evolution of regenerative teeth from denticles. *Proceedings of the National Academy of Sciences*, *113*(51), 14769–14774. https://doi.org/10.1073/pnas.1612354113

Maurano, M. T., Humbert, R., Rynes, E., Thurman, R. E., Haugen, E., Wang, H., …

Stamatoyannopoulos, J. A. (2012). Systematic localization of common disease-associated variation in regulatory DNA. *Science*, *337*(6099), 1190–1195. https://doi.org/10.1126/science.1222794

Mazet, F., Amemiya, C. T., & Shimeld, S. M. (2006, May 9). An ancient Fox gene cluster in bilaterian animals. *Current Biology*. Elsevier. https://doi.org/10.1016/j.cub.2006.03.088

McKinnon, J. S., & Rundle, H. D. (2002, October 1). Speciation in nature: The threespine stickleback model systems. *Trends in Ecology and Evolution*. Elsevier Current Trends. https://doi.org/10.1016/S0169-5347(02)02579-X

Mendel, G. (1865). Versuche ber Pflanzen-HyBriden. *Verh Naturforsch Ver Brnn, 4(347)*.

Miller, C. T., Beleza, S., Pollen, A. A., Schluter, D., Kittles, R. A., Shriver, M. D., & Kingsley, D. M. M. (2007). cis-Regulatory Changes in Kit Ligand Expression and Parallel Evolution of Pigmentation in Sticklebacks and Humans. *Cell*, *131*(6), 1179–1189. https://doi.org/10.1016/j.cell.2007.10.055

Miller, C. T., Glazer, A. M., Summers, B. R., Blackman, B. K., Norman, A. R., Shapiro, M. D., … Kingsley, D. M. (2014). Modular skeletal evolution in sticklebacks is controlled by additive and clustered quantitative trait loci. *Genetics*, *197*(1), 405–420. https://doi.org/10.1534/genetics.114.162420

Miyazono, K., Maeda, S., & Imamura, T. (2005). BMP receptor signaling: Transcriptional targets, regulation of signals, and signaling cross-talk. *Cytokine and Growth Factor Reviews*, *16*(3 SPEC. ISS.), 251–263. https://doi.org/10.1016/j.cytogfr.2005.01.009

Near, T. J., Eytan, R. I., Dornburg, A., Kuhn, K. L., Moore, J. A., Davis, M. P., … Smith, W. L. (2012). Resolution of ray-finned fish phylogeny and timing of diversification. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(34), 13698–13703. https://doi.org/10.1073/pnas.1206625109

Neese, R. A., Misell, L. M., Turner, S., Chu, A., Kim, J., Cesar, D., … Hellerstein, M. K. (2002). Measurement in vivo of proliferation rates of slow turnover cells by 2H2O labeling of the deoxyribose moiety of DNA. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(24), 15345–15350. https://doi.org/10.1073/pnas.232551499

Neubüser, A., Peters, H., Balling, R., & Martin, G. R. (1997). Antagonistic interactions between FGF and BMP signaling pathways: A mechanism for positioning the sites of tooth formation. *Cell*, *90*(2), 247–255. https://doi.org/10.1016/S0092-8674(00)80333-5

Nüsslein-volhard, C., & Wieschaus, E. (1980). Mutations affecting segment number and polarity in drosophila. *Nature*, *287*(5785), 795–801. https://doi.org/10.1038/287795a0

O'brown, N. M., Summers, B. R., Jones, F. C., Brady, S. D., & Kingsley, D. M. (2015). A recurrent regulatory change underlying altered expression and Wnt response of the

stickleback armor plates gene EDA. *ELife*, *2015*(4). https://doi.org/10.7554/eLife.05290

Pai, A. A., & Gilad, Y. (2014, December 1). Comparative studies of gene regulatory mechanisms. *Current Opinion in Genetics and Development*. Elsevier Ltd. https://doi.org/10.1016/j.gde.2014.08.010

Pennacchio, L. A., Bickmore, W., Dean, A., Nobrega, M. A., & Bejerano, G. (2013, April 18). Enhancers: Five essential questions. *Nature Reviews Genetics*. Nature Publishing Group. https://doi.org/10.1038/nrg3458

Pires-daSilva, A., & Sommer, R. J. (2003, January 1). The evolution of signalling pathways in animal development. *Nature Reviews Genetics*. Nature Publishing Group. https://doi.org/10.1038/nrg977

Pispa, J., Pummila, M., Barker, P. A., Thesleff, I., & Mikkola, M. L. (2008). Edar and Troy signalling pathways act redundantly to regulate initiation of hair follicle development. *Human Molecular Genetics*, *17*(21), 3380–3391. https://doi.org/10.1093/hmg/ddn232

Pispa, J., & Thesleff, I. (2003). Mechanisms of ectodermal organogenesis. *Developmental Biology*, *262*(2), 195–205. https://doi.org/10.1016/S0012-1606(03)00325-7

Plikus, M. V., Baker, R. E., Chen, C. C., Fare, C., De La Cruz, D., Andl, T., … Chuong, C. M. (2011). Self-organizing and stochastic behaviors during the regeneration of hair stem cells. *Science*, *332*(6029), 586–589. https://doi.org/10.1126/science.1201647

Plikus, M. V., Mayer, J. A., De La Cruz, D., Baker, R. E., Maini, P. K., Maxson, R., & Chuong, C. M. (2008). Cyclic dermal BMP signalling regulates stem cell activation during hair regeneration. *Nature*, *451*(7176), 340–344. https://doi.org/10.1038/nature06457

Preger-Ben Noon, E., Sabarís, G., Ortiz, D. M., Sager, J., Liebowitz, A., Stern, D. L., & Frankel, N. (2018). Comprehensive Analysis of a cis-Regulatory Region Reveals Pleiotropy in Enhancer Function. *Cell Reports*, *22*(11), 3021–3031. https://doi.org/10.1016/j.celrep.2018.02.073

Prud'homme, B., Gompel, N., & Carroll, S. B. (2007). Emerging principles of regulatory evolution. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(SUPPL. 1), 8605–8612. https://doi.org/10.1073/pnas.0700488104

Rebeiz, M., & Tsiantis, M. (2017, August 1). Enhancer evolution and the origins of morphological novelty. *Current Opinion in Genetics and Development*. Elsevier Ltd. https://doi.org/10.1016/j.gde.2017.04.006

Reid, D. T., & Peichel, C. L. (2010). Perspectives on the Genetic Architecture of Divergence in Body Shape in Sticklebacks. *Integrative and Comparative Biology*, *50*(6), 1057–1066. https://doi.org/10.1093/icb/icq030

Reif, W.-E. (1982). Evolution of Dermal Skeleton and Dentition in Vertebrates. The Odontode-Regulation Theory. *Evolutionary Biology*, *15*.

Rice, R., Rice, D. P. C., Olsen, B. R., & Thesleff, I. (2003). Progression of calvarial bone development requires Foxc1 regulation of Msx2 and Alx4. *Developmental Biology*, *262*(1), 75–87. https://doi.org/10.1016/S0012-1606(03)00355-5

Rosenfeld, J. A., Wang, Z., Schones, D. E., Zhao, K., DeSalle, R., & Zhang, M. Q. (2009). Determination of enriched histone modifications in non-genic portions of the human genome. *BMC Genomics*, *10*(1), 143. https://doi.org/10.1186/1471-2164-10-143

Sabarís, G., Laiker, I., Preger-Ben Noon, E., & Frankel, N. (2019, June 1). Actors with Multiple Roles: Pleiotropic Enhancers and the Paradigm of Enhancer Modularity. *Trends in Genetics*. Elsevier Ltd. https://doi.org/10.1016/j.tig.2019.03.006

Sadier, A., Viriot, L., Pantalacci, S., & Laudet, V. (2014, January 1). The ectodysplasin pathway: From diseases to adaptations. *Trends in Genetics*. Elsevier Current Trends. https://doi.org/10.1016/j.tig.2013.08.006

Sender, R., & Milo, R. (2021). The distribution of cellular turnover in the human body. *Nature Medicine*, *27*(1), 45–48. https://doi.org/10.1038/s41591-020-01182-9

Seo, S., & Kume, T. (2006). Forkhead transcription factors, Foxc1 and Foxc2, are required for the morphogenesis of the cardiac outflow tract. *Developmental Biology*, *296*(2), 421–436. https://doi.org/10.1016/j.ydbio.2006.06.012

Shubin, N., Tabin, C., & Carroll, S. (2009, February 12). Deep homology and the origins of evolutionary novelty. *Nature*. Nature Publishing Group. https://doi.org/10.1038/nature07891

Sievers, F., Wilm, A., Dineen, D., Gibson, T. J., Karplus, K., Li, W., … Higgins, D. G. (2011). Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Molecular Systems Biology*, *7*(1), 539. https://doi.org/10.1038/msb.2011.75

Singh, D., & Yi, S. V. (2021). Enhancer Pleiotropy, Gene Expression, and the Architecture of Human Enhancer–Gene Interactions. *Molecular Biology and Evolution*, *38*(9), 3898–3909. https://doi.org/10.1093/molbev/msab085

Singh, L. N., & Hannenhalli, S. (2008). Functional Diversification of Paralogous Transcription Factors via Divergence in DNA Binding Site Motif and in Expression. *PLoS ONE*, *3*(6), e2345. https://doi.org/10.1371/journal.pone.0002345

Smith, M. M., & Coates, M. I. (1998). Evolutionary origins of the vertebrate dentition: Phylogenetic patterns and developmental evolution. *European Journal of Oral Sciences*, *106*(1 SUPPL.), 482–500. https://doi.org/10.1111/j.1600-0722.1998.tb02212.x

Soukup, V., Epperlein, H.-H., Horácek, I., & Cerny, R. (2008). Dual epithelial origin of vertebrate oral teeth. *Nature*, *455*(7214), 795–798. https://doi.org/10.1038/nature07304

Square, T. A., Sundaram, S., Mackey, E. J., & Miller, C. T. (2021). Distinct tooth regeneration systems deploy a conserved battery of genes. *EvoDevo*, *12*(1), 1–17. https://doi.org/10.1186/s13227-021-00172-3

Stanojevic, D., Small, S., & Levine, M. (1991). Regulation of a segmentation stripe by overlapping activators and repressors in the Drosophila embryo. *Science*, *254*(5036), 1385–1387. https://doi.org/10.1126/science.1683715

Stepaniak, M. D., Square, T. A., & Miller, C. T. (2021). Evolved Bmp6 enhancer alleles drive spatial shifts in gene expression during tooth development in sticklebacks. *Genetics*. https://doi.org/10.1093/genetics/iyab151

Stewart, S., Gomez, A. W., Armstrong, B. E., Henner, A., & Stankunas, K. (2014). Sequential and opposing activities of Wnt and BMP coordinate zebrafish bone regeneration. *Cell Reports*, *6*(3), 482–498. https://doi.org/10.1016/j.celrep.2014.01.010

Talbot, J. C., & Amacher, S. L. (2014). A streamlined CRISPR pipeline to reliably generate zebrafish frameshifting alleles. *Zebrafish*, *11*(6), 583–585. https://doi.org/10.1089/zeb.2014.1047

Taylor, G., Lehrer, M. S., Jensen, P. J., Sun, T. T., & Lavker, R. M. (2000). Involvement of follicular stem cells in forming not only the follicle but also the epidermis. *Cell*, *102*(4), 451–461. https://doi.org/10.1016/S0092-8674(00)00050-7

Thesleff, I. (2003). Epithelial-mesenchymal signalling regulating tooth morphogenesis. *Journal of Cell Science*, *116*(9), 1647–1648. https://doi.org/10.1242/JCS.00410

Till, J., & McCulloch, E. (1961). A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiat Res.*, *14:213-22.*

Tinbergen, N. (1952). The curious behavior of the stickleback. *Sci. Am. 187:22–26.*

Tumbar, T., Guasch, G., Greco, V., Blanpain, C., Lowry, W. E., Rendl, M., & Fuchs, R. (2004). Defining the Epithelial Stem Cell Niche in Skin. *Science*, *303*(5656), 359–363. https://doi.org/10.1126/science.1092436

Tümer, Z., & Bach-Holm, D. (2009). Axenfeld-Rieger syndrome and spectrum of PITX2 and FOXC1 mutations. *European Journal of Human Genetics : EJHG*, *17*(12), 1527–1539. https://doi.org/10.1038/ejhg.2009.93

Vainio, S., Karavanova, I., Jowett, A., & Thesleff, I. (1993). Identification of BMP-4 as a signal mediating secondary induction between epithelial and mesenchymal tissues during early tooth development. *Cell*, *75*(1), 45–58. https://doi.org/10.1016/S0092-8674(05)80083-2

Vandenplas, S., Willems, M., & Huysseune, A. (2012). Dual BrdU-PCNA immunodetection of proliferative cells in dental and orofacial tissues of teleosts. *Journal of Applied Ichthyology*, *28*(3), 336–340. https://doi.org/10.1111/j.1439-0426.2012.02003.x

Vandenplas, Sam, De Clercq, A., & Huysseune, A. (2014). Tooth replacement without a dental lamina: The search for epithelial stem cells in *Polypterus senegalus*. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*, *322*(5), 281–293. https://doi.org/10.1002/jez.b.22577

Vandenplas, Sam, Vandeghinste, R., Boutet, A., Mazan, S., & Huysseune, A. (2016). Slow cycling cells in the continuous dental lamina of Scyliorhinus canicula: New evidence for stem cells in sharks. *Developmental Biology*, *413*(1), 39–49. https://doi.org/10.1016/j.ydbio.2016.03.004

Vandenplas, Sam, Willems, M., Witten, P. E., Hansen, T., Fjelldal, P. G., & Huysseune, A. (2016). Epithelial Label-Retaining Cells Are Absent during Tooth Cycling in Salmo salar and Polypterus senegalus. *PLOS ONE*, *11*(4), e0152870. https://doi.org/10.1371/journal.pone.0152870

Vaškaninová, V., Chen, D., Tafforeau, P., Johanson, Z., Ekrt, B., Blom, H., & Ahlberg, P. E. (2020). Marginal dentition and multiple dermal jawbones as the ancestral condition of jawed vertebrates. *Science*, *369*(6500), 211–216. https://doi.org/10.1126/science.aaz9431

Wainwright, P. C. (2005, January 1). Functional Morphology of the Pharyngeal Jaw Apparatus. *Fish Physiology*. Academic Press. https://doi.org/10.1016/S1546-5098(05)23003-0

Walker, J. A., & Bell, M. A. (2000). Net evolutionary trajectories of body shape evolution within a microgeographic radiation of threespine sticklebacks (Gasterosteus aculeatus) . *Journal of Zoology*, *252*(3), 293–302. https://doi.org/10.1111/j.1469-7998.2000.tb00624.x

Waluk, D. P., Zur, G., Kaufmann, R., Welle, M. M., Jagannathan, V., Drögemüller, C., … Galichet, A. (2016). A splice defect in the EDA gene in dogs with an X-linked hypohidrotic ectodermal dysplasia (XLHED) phenotype. *G3: Genes, Genomes, Genetics*, *6*(9), 2949–2954. https://doi.org/10.1534/g3.116.033225

Wang, L., Siegenthaler, J. A., Dowell, R. D., & Yi, R. (2016). Foxc1 reinforces quiescence in self-renewing hair follicle stem cells. *Science (New York, N.Y.)*, *351*(6273), 613–617. https://doi.org/10.1126/science.aad5440

Wang, X.-P., Suomalainen, M., Felszeghy, S., Zelarayan, L. C., Alonso, M. T., Plikus, M. V, … Thesleff, I. (2007). An Integrated Gene Regulatory Network Controls Stem Cell Proliferation in Teeth. *PLoS Biology*, *5*(6), e159. https://doi.org/10.1371/journal.pbio.0050159

Wang, Y., Li, L., Zheng, Y., Yuan, G., Yang, G., He, F., & Chen, Y. (2012). BMP activity is required for tooth development from the Lamina to Bud Stage. *Journal of Dental Research*,

91(7), 690–695. https://doi.org/10.1177/0022034512448660

Wilson, A., Laurenti, E., Oser, G., van der Wath, R. C., Blanco-Bose, W., Jaworski, M., … Trumpp, A. (2008). Hematopoietic Stem Cells Reversibly Switch from Dormancy to Self-Renewal during Homeostasis and Repair. *Cell*, *135*(6), 1118–1129. https://doi.org/10.1016/j.cell.2008.10.048

Wu, P., Wu, X., Jiang, T.-X., Elsey, R. M., Temple, B. L., Divers, S. J., … Chuong, C.-M. (2013). Specialized stem cell niche enables repetitive renewal of alligator teeth. *Proceedings of the National Academy of Sciences*, *110*(22), E2009–E2018. https://doi.org/10.1073/pnas.1213202110

Wu, Z., Wang, F., Fan, Z., Wu, T., He, J., Wang, J., … Wang, S. (2019). Whole-Tooth Regeneration by Allogeneic Cell Reassociation in Pig Jawbone. *Tissue Engineering Part A*, *25*(17–18), 1202–1212. https://doi.org/10.1089/ten.tea.2018.0243

Wucherpfennig, J. I., Miller, C. T., & Kingsley, D. M. (2019). Efficient CRISPR-Cas9 editing of major evolutionary loci in sticklebacks. *Evolutionary Ecology Research*, *20*(1), 107–132. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/34899072

Xu, P., Balczerski, B., Ciozda, A., Louie, K., Oralova, V., Huysseune, A., & Gage Crump, J. (2018). Fox proteins are modular competency factors for facial cartilage and tooth specification. *Development (Cambridge)*, *145*(12). https://doi.org/10.1242/dev.165498

Xu, P., Yu, H. V., Tseng, K. C., Flath, M., Fabian, P., Segil, N., & Crump, J. G. (2021). Foxc1 establishes 1 enhancer accessibility for craniofacial cartilage differentiation. *ELife*, *10*, 1–50. https://doi.org/10.7554/eLife.63595

Zhao, X., Zhang, Z., Song, Y., Zhang, X., Zhang, Y., Hu, Y., … Chen, Y. P. (2000). Transgenically ectopic expression of Bmp4 to the Msx1 mutant dental mesenchyme restores downstream gene expression but represses Shh and Bmp2 in the enamel knot of wild type tooth germ. *Mechanisms of Development*, *99*(1–2), 29–38. https://doi.org/10.1016/S0925-4773(00)00467-6

“All the world’s a stage, And all the men and women merely players; They have their exits and their entrances; And one man in his time plays many parts”

― William Shakespeare, *As you like it* (1599)