DEVELOPMENTAL PLASTICITY OF STEM CELLS IN TEETH AND TASTE BUD RENEWAL

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LIST OF ABBREVIATIONS

AJ *Aulonocara jacobfreibergeii*

BMP Bone Morphogenic Protein

BrdU Bromodeoxyuridine

CA *Cynotilapia afra*

CB Copadichromis borleyi

cM Centi-morgan

Cyc Cyclopamine

DPF Days Post Fertilization

DL Dental Lamina

DMSO Dimethyl Sulfoxide

ESC Epithelial Stem Cell

FGF Fibroblast Growth Factor

Hh Hedgehog

H&E Hematoxylin and Eosin

IDE Inner Dental Epithelium

IHC Immunohistochemsitry

ISH In Situ Hybridization

LDN LDN-193189

LF *Labeotropheus fuelleborni*

LiCl Lithium Chloride

LM Lake Malawi

LOD Logarithm of the odds

MC *Mchenga conophorus*

mm Millimeter

MPM Multi-Photon Microscopy

MQM Multiple QTL model

MTOM Many-to-one-mapping

MZ *Metriaclima zebra*

NBF Neutral Buffered Formalin

NLO Non-Linear Optics

OB Odontogenic Band

ODE Outer Dental Epithelium

O/N Overnight

PCNA Proliferating Cell Nuclear Antigen

PC *Petrotilapia Chitimba*

PVE Percent variance explained (estimated)

QTL Quantitative trait locus

RNA-Seq RNA-Sequencing

RT Replacement Tooth

SC Stem Cell

SD Standard deviation

SL Succesional Lamina

Sl Standard length

SNP Single nucleotide polymorphism

SR Stellate Reticulum

TB Taste Bud

WNT Wingless

SUMMARY

Science and medicine have progressed in unfathomable ways over the past century. Paradoxically, as our result of our advancements in medicine we live in a progressively aging society where the majority of people will have multiple morbidities associated with senescence. The World Health Organization estimates that nearly 100% of the population will suffer dental maladies, which left untreated compound with age.

We hope to gain new biomedical insight applicable to the advancing field of dental regenerative therapeutics. This dissertation reveals new dental biology through studying the embryology, genetics and evolution of teeth across patterning, morphogenesis and regeneration. We exploit an innovative model, the Lake Malawi cichlid fishes, to study these processes in a natural system. Malawi cichlids have rapidly evolved diverse species-specific dentitions, ranging from hundreds to thousands of teeth that represent a rainbow of shapes and sizes, yet Malawi cichlid species has nearly identical genomes, offering us a powerful genetic system. Furthermore, unlike classic vertebrate models in embryology such as zebrafish, chicken or mice, cichlids have oral teeth and the ability to replace each tooth constantly throughout their lifetimes.

In the first study, we break-down the process of whole de-novo tooth replacement in cichlids. We then explore the re-deployment of initiating gene

pathways later in the morphogenesis of each replacement tooth (RT). In the second study we investigate the co-patterning of two placode derived oral organs, teeth and taste buds (TBs), and uncover new genes that may modulate their number and size. We subsequently discover a bipotency of progenitor tissue to form both organs and a later plasticity to trans-fate it through coordination of a Wnt-BMP- Hh genetic hierarchy. In the last study, we explore the stem cells that are responsible for the phenomenon of lifelong cichlid tooth replacement. We describe a common epithelium connected to TBs and rich in stem cells, with a newly discovered stem cell niche at the tip of the RT. We uncover the transcriptomes of both organs, and through differential gene expression informed manipulations, coerce dental cells to display TB characteristics. We hypothesize that TB stem cells may be used in dental therapeutics.

CHAPTER 1

INTRODUCTION

1.1: Significance

Roughly 25% of the human population is born with congenital defects of the dentition arising from genetic aberrations (Fleischmannova et al., 2008). While this figure is significant, a far greater number of human beings will suffer from dental disease arising from dental decay and selective tooth loss. In contrast to most vertebrates, who continuously replace their dentitions throughout ontogeny, humans have evolved to possess only two sets of teeth: a primary dentition and a successional dentition that must serve throughout adulthood. Although the human successional replacement tooth (RT) possesses limited regenerative potential in the cellular pulp chamber and periodontal ligament, injury to the adult tooth is largely irreversible. The fields of restorative and prosthodontic dentistry seek to address these congenital and adult dental defects. For the large part, this has been accomplished by employing synthetic materials such as plastics and ceramics. To better restore a healthy dentition, the field of regenerative dentistry alternatively looks to cell-based dental repair.

Regenerative dentistry is a promising field aimed at replacing damaged oral structures with live tissues. One of the most exciting therapies of

regenerative dentistry lies in the possibility of replacing agenic and lost teeth through biomedical engineering. By transplanting cells or cell scaffolds into oral tissues, researchers aim to culture new teeth (Oshima et al., 2011)to achieve ideal dental function and aesthetic where it was once lost. To wield this technology, we need to understand how those tissues were formed in the first place. Conventional model organisms studied in developmental biology, such as the chicken, frog, and zebrafish, lack teeth on their oral jaws. While the capability of replacing the dentition is limited to one generation in humans, the mouse never replaces its single set of teeth. Scientists have learned a great deal from studying the mouse dentition. However, if scientists are to best address the mechanisms underlying tooth replacement in order to regenerate missing teeth, they must turn their research to a model with a naturally replacing dentition.

Tooth replacement requires cells that both have some degree of potency and can obtain dental competence. The cells that coordinate this process must be either stem cells or cells in a more limited progenitor state. There has been keen interest in identifying the stem-cell niche(s) involved in tooth development. This is because these cells hold the most promise for regenerating lost dental tissues from a cultured germ layer. The most widely accepted data on mouse incisor suggest that the stem cells responsible for the constant renewal of rodent enamel reside in an intermediate germ layer known as the stellate reticulum (Harada et al., 2002; Wang et al., 2007). It has also been proposed that this same layer, albeit at the tip of the tooth rather than at the apices or cervical loops

as seen in the mouse, likely holds stem cells that help differentiate a RT germ in the gecko (Handrigan et al., 2010).

The most promising therapeutics for addressing morbidities that arise from congenital, traumatic, or infectious pathology lie in the field of regenerative medicine. As we turn to cell-based therapies for repair of missing teeth and damaged oral tissues, we must look to developmental biology to understand how nature grew these tissues in the first place. What we know about tooth development or odontogenesis has been almost exclusively derived from the mouse. While we have learned a great deal from this system, in contrast to humans and most other vertebrates, the mouse lacks a successional or replacement set of teeth. We know relatively little about how to replace the dentition. To discover how nature perfected the growth of a tooth in an adult organism, we turn to the de-novo continuously replacing dentition of Lake Malawi cichlid fishes. As we move into an era where the replacement of missing or damaged tissues has become not only a promising therapy, but a viable possibility, it is imperative that we appreciate how those tissues were formed in the first place. With a more global health impact, we seek to identify the epithelial and mesenchymal stem cells responsible for the natural replacement of an adult organ. These findings will advance our understanding of dental stem cells so that we may one day design therapies based on endogenous mechanisms of repair.

1.2: Malawi cichlids

We employ Lake Malawi cichlids as models of dental regeneration. Cichlid teeth exhibit complex shapes in tightly conserved patterns. Cichlid species may possess only a few unicuspid teeth, many bicuspid and tricuspid teeth together, or even over a thousand tricuspid teeth on their oral jaws (Fraser et al. 2008). In addition, Malawi cichlids constantly replace their teeth throughout ontogeny, enabling us to learn more about how teeth are regenerated in a natural system.

Lake Malawi cichlids represent one of the greatest examples of adaptive radiation. Rapid evolution was driven by extrinsic ecological factors such as lake depth and levels of solar radiation combined with intrinsic factors like behavioral sexual selection (Wagner et al., 2012) and a genetic background replete with gene duplications and non-coding element divergence (Brawand et al., 2014). The auspicious amalgamation of extrinsic factors inherent to Lake Malawi and intrinsic factors inherent to cichlid biology permitted what is largely considered the highest known incidence of vertebrate evolution, with over 500 species (Turner et al., 2001) evolving in less than 2 million years (Kocher, 2004; Meyer, 1993). Owing to this rapid rate of evolution, high phenotypic variability across Malawi cichlid species derives from genomes that are said to be interspecifically less divergent than that of laboratory strains of *Danio Rerio* when comparing nucleotide diversity (Watterson's $\theta w = 0.26\%$ for cichlids compared to $\theta w = 0.48\%$ for zebrafish) (Loh et al., 2008).

Malawi cichlids therefore serve as a paradigmatic animal for studying evolutionary developmental biology. Classical model in embryology are often studied by first describing the natural state of the organism (i.e., a phenotype corresponding to gene expression), and then by performing a functional assay such as a transgenic or chemical mutagenesis to verify a hypothesized embryologic process. As an alternative approach, researchers have turned to powerful models like the three-spined stickleback (*Gasterosteus aculeatus*) and *Astyanax mexicanus* to compare differences between naturally occurring vertebrate species. Genome-wide linkage maps generated from crosses between larger benthic and smaller limnetic species of stickleback have revealed portions of chromosomes that map to traits such as body size and armor plate number (Peichel et al., 2001). Furthermore, positional cloning and fine-mapping between low-armored lake species and the ancestral heavily armored pelagic species reveal a migration into the lake and selection on the ancestral allele of the gene locus coding for Ectodysplasin (Eda) for a derived Eda allele, believed to have resulted from standing genetic variation (Barrett et al., 2008; Colosimo et al., 2005). In the case of Astyanax mexicanus, hypothesized roles of Hedgehog (Hh) signaling in eye evolutionary development have been established by comparing expression differences of Hh mediators in a normal surface morph and a blind cave morph of the single species, which were then recapitulated by Hh factor overexpression (Yamamoto et al., 2004).

The cichlid model has thus become increasingly exploited to answer new questions in evolutionary biology and biomedicine. Cichlids occupy a rainbow of

phenotypic extremes and intermediates (Figure A.1). In their short evolutionary history, cichlids have divergently selected for brains specialized for forebrain function or brains specialized for midbrain function (Sylvester et al., 2010) and dentitions with 50 to over 1000 teeth (Fraser et al., 2009). They have convergently evolved extreme phenotypes like hump heads and trident shaped teeth across different species within a lake as well as in sister African Great Lakes(Fryer and Iles, 1972). With cichlids to occupy a spectrum of morphs, scientists can study a myriad of “natural mutants” for a given phenotype. By hybridizing a species with broad, short jaws used for scraping algae (*Labeotropheus fuelleborni, LF*) to a species with longer more gracile jaws used for generalized feeding (*Metriaclima zebra, MZ*), researchers have scored craniofacial morphometrics and utilized Quantitative Trait Loci mapping (QTL) to predict differential expression of *bmp4* (Albertson et al., 2005) and *ptch1* (Hu and Albertson, 2014) linked to jaw morphology and function. This same cross has been further fine mapped to a region of the genome controlling jaw length containing the gene *lbh*, which was functionally demonstrated in both zebrafish and frog to control migration of cranial neural crest cells (Powder et al., 2014). Even extreme hybridizations are possible from the most distant open water dwelling Utaka with rock-dwelling Mbuna (Figure A.2).

Figure 1.1. Composite of Malawi cichlid diversity in laboratory species. This figure demonstrates the range of phenotypes represented in the animals used in this work.

Beyond mapping experiments and forward genetics approaches, methods for studying cichlid biology are rapidly being developed. Like other lower vertebrates, cichlids have immense regenerative potential and have been used to understand stem cell-mediated renewal, like how tooth acquire shape upon replacement (Fraser et al., 2013). Variation in cichlid brains and underlying neurogenesis are being studied to understand complex behaviors and neuronal disease (Fernald and Maruska, 2012; Korzan et al., 2014; Sylvester et al., 2013). A detailed staging system for the a species from the Cichlidae Family, Nile tilapia *Oreochromis niloticus,* has recently been described (Fujimura and Okada, 2007) and we have charted the ontogeny of Malawi cihlids (figure 1.1). Furthermore, transgenic cichlids (Farlora et al., 2009; Fujimura and Kocher, 2011; Juntti et al.,

2013) and culture systems (Gardell et al., 2014; Mack and Tiedemann, 2013) have been created and more are on the way. With five published cichlid genomes published and annotated (Brawand et al., 2014), the cichlid is emerging as a premier model for studying evolution and development.

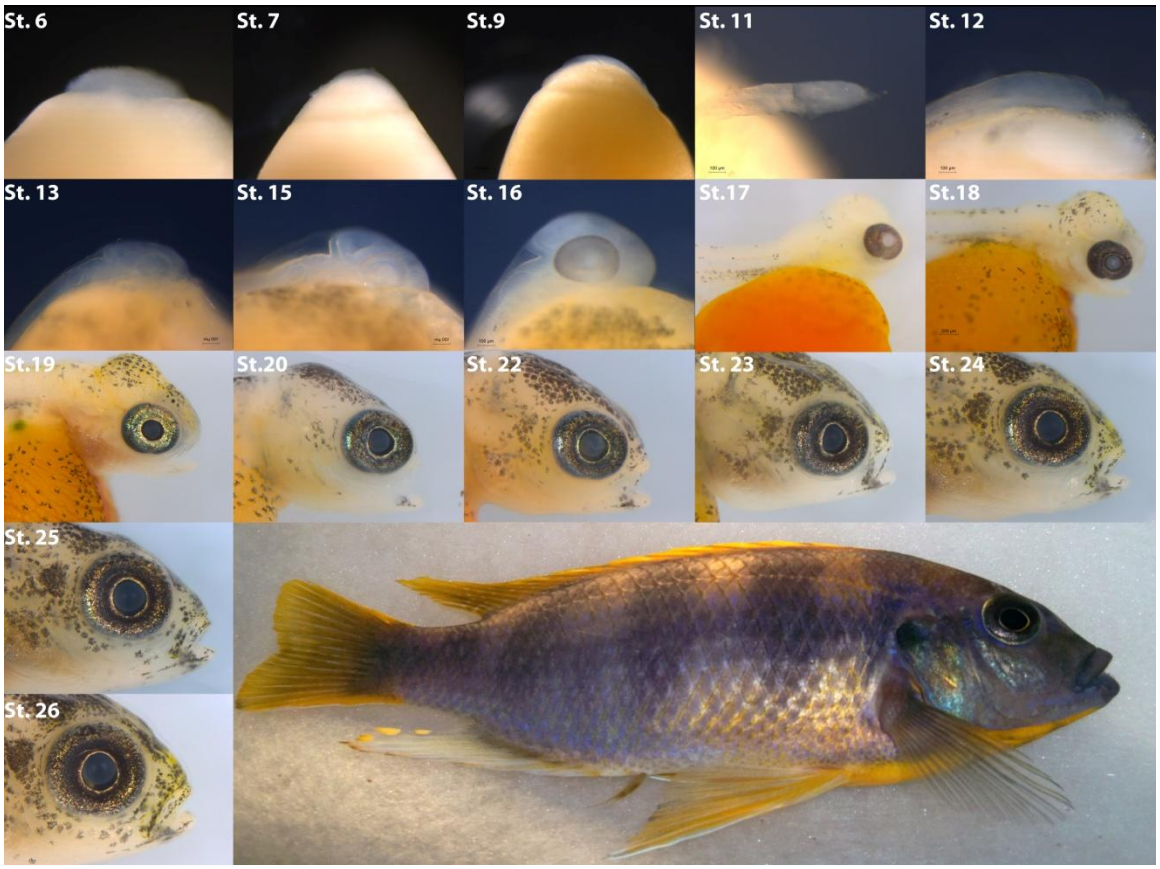

Figure 1.2. Malawi cichlid ontogenetic staging series.

1.3: Approach

In Chapter 2 of this dissertation we uncover the basic properties of cichlid tooth regeneration. In doing so, we highlight the relationship between gene

pathways that initiate and facilitate tooth replacement and are later redeployed to aid in morphogenesis, giving cichlid teeth their distinctive and diverse tooth shapes. The genetic coupling of these processes was hypothesized in a review shortly before the publication of this chapter (Jernvall and Thesleff, 2012). Mammals are unique in the sense that they develop complex shaped teeth in the primary dentitions. Cichlids and other fishes with shaped teeth, in contrast, have simple conical primary teeth and only exhibit complex shapes in successional teeth. We charted odontgenesis, from the first to subsequent generations of cichlid RT, and employed H&E staining and PCNA immunohistochemistry (IHC) to break replacement tooth formation into 3 stages: initiation, encompassing placode or succesional lamina stages of tooth formation; differentiation, encompassing cap and bell stages;, and secretion, encompassing late bell and secretion stages where complex shape is constructed. Next, we explored expression patterns of BMPs, Hhs, Wnts, FGFs, and the Notch pathway across these three stages, describing those factors recycled from initiation stages to secretion stages. Armed with a spatial and temporal map of RT gene expression, we manipulated all five pathways with chemical antagonists, noting which pathway treatments affected replacement, which affected shape, and which affected both. This chapter provided insight to the phenomenon of whole-vertebrate tooth replacement, a relatively undescribed process, and how complex-tooth shape can be generated as a result of it.

In Chapter 3, we delved into the co-patterning of two oral organs, teeth and taste buds (TBs). Both oral organs are present in numbers of hundreds

to thousands and are tightly patterned in shared space of the cichlid jaw. We noted that the numbers of teeth and TBs anecdotally were positively correlated across species with divergent dentitions. We examined the initiation of these two organs with ISH and found that both were derived from the same embryonic tissue, previously considered the “odontogentic” or dental band. We then crossed two species with low and high numbers of each organ, respectively, and again performed an intercross in order to generate a QTL map of tooth and taste bud densities, defined as tooth number over area of jaw. F_2 species exhibited a strong positive correlation of tooth and TB densities, suggesting genetic linkage. We identified several genetic candidates that may help co-pattern these organs. Two of which, *bmper* and *sfrp5,* new to the literature on patterning of both organs, were differentially expressed in the jaws of cichlids with different dental patterns as well as in both organs in mice. We then functionally tested the roles of BMP, Hh, and Wnt family members (important to forming both organs and host pathways to our genetic candidates in other animal models) in determining the oral pattern through chemical inhibition of each pathway. We identified a genetic hierarchy, wherein Wnts are positively correlated to the density of both teeth and taste buds and lie upstream of both BMPs and Hhs. The latter two pathways demonstrated positive roles on the promotion of tooth formation and were restrictive to TBs, where antagonism of these pathways led to the generation of ectopic TBs at the expense of teeth. This study uncovered a bi-potency of embryonic oral lamina to form both teeth and TB as well as a plasticity to trans-fate epithelium from tooth to TB. These boundaries are defined by Wnt, BMP,

and Hh pathways and two new genetic candidates, *bmper* and *sfrp5*, may modulate them, a vestigial hierarchy on placode patterning exhibited across vertebrates redolent of a common ancestral organ.

In the final study of this dissertation, Chapter 4, we investigate intriguing findings from the Chapters 2 and 3 related to the stem cell mediated renewal of cichlid RT. In chapter 2, we note that all RT examined histologically develop in the presence of a labial ball of cells, identified later as TBs. In Chapter 3, we demonstrate the shared embryonic origin of both teeth and TBs. Here we find through ISH that adult stem cell markers are prominent throughout RT and TB epithelium and that the two organs maintain a connection through the succesional dental lamina. Through cell cycling pulse-chase experiments and double labeling with immunohistochemistry for adult stem markers, we pinpoint stem cell niches in the oral organ renewal of cichlids and highlight a new population of stem cells at the tip of cichlid replacement teeth, a population not described in the literature. We then perform RNA-sequencing and note differential expression between RT and TB bearing oral epithelium, with BMPs being biased to RT, a finding we confirm with ISH. Lastly, we down-regulate BMPs in-vivo with small molecule chemical treatments, resulting in cells within the RT that display TB characteristics. We conclude from the culmination of these chapters that TB stem cells have clinical potential for use in the bioengineering of cell based tooth repair. Taken together, our work in cichlids uncovers a plethora of new developmental, genetic, and evolutionary data on the generation of teeth.

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CHAPTER 2

COMMON DEVELOPMENTAL PATHWAYS LINK TOOTH SHAPE TO REGENERATION

2.1: Abstract

In many non-mammalian vertebrates, adult dentitions result from cyclical rounds of tooth regeneration wherein simple unicuspid teeth are replaced by more complex forms. Therefore and by contrast to mammalian models, the numerical majority of vertebrate teeth develop shape during the process of replacement. Here, we exploit the dental diversity of Lake Malawi cichlid fishes to ask how vertebrates generally replace their dentition and in turn how this process acts to influence resulting tooth morphologies. First, we used immunohistochemistry to chart organogenesis of continually replacing cichlid teeth and discovered an epithelial down-growth that initiates the replacement cycle via a labial proliferation bias. Next, we identified sets of co-expressed genes from common pathways active during *de novo*, lifelong tooth replacement and tooth morphogenesis. Of note, we found two distinct epithelial cell populations, expressing markers of dental competence and cell potency, which may be responsible for tooth regeneration. Related gene sets were simultaneously active in putative signaling centers associated with the differentiation of replacement teeth with complex shapes. Finally, we manipulated

targeted pathways (BMP, FGF, Hh, Notch, Wnt/β-catenin) in vivo with small molecules and demonstrated dose-dependent effects on both tooth replacement and tooth shape. Our data suggest that the processes of tooth regeneration and tooth shape morphogenesis are integrated via a common set of molecular signals. This linkage has subsequently been lost or decoupled in mammalian dentitions where complex tooth shapes develop in first generation dentitions that lack the capacity for lifelong replacement. Our dissection of the molecular mechanics of vertebrate tooth replacement coupled to complex shape pinpoints aspects of odontogenesis that might be re-evolved in the lab to solve problems in regenerative dentistry.

2.2: Introduction

Vertebrate animals differ in their capacity to renew and regenerate body parts. Various lineages have retained or evolved the ability to regenerate nervous systems (Kizil et al., 2011 and Kroehne et al., 2011), limbs (Kragl et al., 2009 and Nacu and Tanaka, 2011), fins (Jaźwińska et al., 2007 and Singh et al., 2012) and tails (Echeverri and Tanaka, 2002 and Lin and Slack, 2008), internal organs like the heart (Wang et al., 2011), as well as iterative elements like hairs, scales, taste buds and teeth (Chang et al., 2009, Harada et al., 1999, Plikus et al., 2011, Plikus et al., 2008 and Wang et al., 2007). Developmental biologists are captivated by regeneration because the process may recycle well-known mechanisms of embryonic patterning and likely involves the deployment of stem

cells in post-embryonic tissues. Precisely because humans (and more generally mammals) lose regenerative capacity with age, keen biomedical interest revolves around natural instances of regeneration and renewal from stem cells as exemplars for cellular reprogramming (Christen et al., 2010).

In many examples of animal regeneration, the trigger or impetus is external and unpredictable. A lizard can re-grow a tail after escaping a predator; such an interaction may be probable, but not necessary over an individual's lifetime. By contrast, predictable programs characterize other cases of regeneration, like the shedding of hair, teeth, scales and feathers. For instance, adult cichlid fishes replace each tooth in the oral jaw approximately every 30–100 days (Tuisku and Hildebrand, 1994). When programmed regeneration is coupled with the functional requirement to maintain a particular organization of elements (feathers for flight, scales for swimming, teeth for mastication), the developmental phenomena of patterning, morphogenesis and renewal, often studied independently, must be deeply integrated across space and time. In most systems, biologists do not understand how this integration is achieved.

Vertebrate dentitions represent a seemingly apposite system in which to decipher how individual organs (teeth) develop complex shapes and inter-unit patterns, while simultaneously exhibiting programmed regeneration. Many vertebrates possess teeth in multiple rows on multiple elements of the oral jaw and pharyngeal region that are continuously replaced throughout life. In ray-finned fishes, dipnoans and urodeles, first generation teeth are small and simple unicuspids lacking blood vessels and nerves; therefore the numerical majority of

vertebrate teeth develop shape and increased complexity (e.g., size, curvature, cusps) through replacement (Sire et al., 2002). Teeth likely arose in jawless vertebrates more than half a billion years ago (Fraser et al., 2009; Smith, 2003; Smith and Coates, 1998 and Smith and Coates, 2000)—there is thus a long evolutionary record and broad phylogenetic distribution to bolster our understanding of how patterned dentitions are likewise regenerated.

The fact that we know very little about the coupled patterning, morphogenesis and regeneration of vertebrate dentitions can be partly explained by the peculiar biology of teeth in the mouse model. The mouse dentition is comprised of one incisor and three molars, in a single row, on each left and right quadrant of the upper and lower jaws. Incisors are separated in space from molars by a toothless diastema, and the early patterning of the incisor and molar domains is well understood (Tucker and Sharpe, 2004). Molars develop complex three-dimensional shape while incisors generally do not (Jernvall et al., 2000). Incisors exhibit self-renewal via continuous deposition of enamel on the labial surface, supported by a stem cell niche biased to the labial cervical loop (Harada et al., 1999 and Wang et al., 2007); molars lack this potential. Thus, mouse molars are models of complex morphogenesis. Classic studies have demonstrated how molars develop under the influence of well-known signaling pathways (e.g., BMP, FGF, Hh, Wnt and Eda) and downstream transcription factors (i.e., Pitx, Pax, Dlx, Barx, Msx) (Åberg et al., 1997, Bei and Maas, 1998, Chen et al., 1996, Dassule et al., 2000, Jernvall et al., 1994, Peters et al., 1998, Sarkar and Sharpe, 1999, Sharpe, 1995 and Thesleff and Sharpe, 1997). Recent

reports point to genes that may couple these developmental pathways in the molar field (Ahn et al., 2010 and Cho et al., 2011). By contrast, incisors are models of stem-based continuous growth, with context-dependent function of common pathways (BMP, FGF, Wnt) as well as novel roles for additional factors (Notch (Felszeghy et al., 2010); Follistatin (Wang et al., 2007)). Notably, neither molars nor incisors are replaced over mouse ontogeny.

Because mice do not replace their teeth, a set of new models for dental regeneration has emerged. This includes the shrew (Järvinen et al., 2008), ferret (Järvinen et al., 2009), zebrafish (Huysseune, 2006) and a cadre of reptiles (Handrigan et al., 2010). Taken together, studies suggest that tooth replacement requires (i) an epithelial connection between the functional tooth and its successor, known as successional lamina (SL), which is borne from (ii) putative dental/epithelial stem cells capable of forming a replacement tooth *de novo*. One limitation of these new replacement models is that the dentitions in question are relatively simple: few teeth total, often in a single row, with each tooth generally conical or spatulate in shape.

In this report, we ask how complex dentitions are shaped as they are replaced, using cichlid fishes from Lake Malawi, East Africa. The main advantage of this system is the sheer dental diversity among closely related species (Fraser et al., 2008 and Streelman et al., 2003). Most cichlids endemic to Lake Malawi have evolved from a common ancestor in the last 500,000 years; their genomes are highly similar (e.g., less nucleotide diversity than observed in lab strains of zebrafish) and species share genetic polymorphism (Loh et al., 2008). Against

this backdrop of genomic similarity, dental patterns and shapes vary considerably. For example, *Cynotilapia afra*, a rock-dwelling planktivore, possesses a small number of large, widely spaced, conical teeth in 2–3 rows while species of the algal-brushing rock-dweller genus *Petrotilapia* exhibit hundreds of small, tightly packed tricuspid teeth in 10–15 rows. The particular tooth pattern, that is the size and spacing of teeth as well as the extent of the tooth field in the jaw, is set with the initiation of the first generation dentition, prior to the development of tooth shapes (Fraser et al., 2008). As in other cichlids, these first generation teeth are small conical unicuspids and are not innervated (Huysseune and Sire, 1997). Complex shape and innervation are thus the phenomenological consequence of tooth replacement in cichlids, with adult shapes developing during multiple, early rounds of replacement (Streelman et al., 2003) into patterns set during initiation (Fraser et al., 2008).

We used a combination of immunohistochemistry and in situ hybridization, at multiple stages of development, to identify cell populations, putative signaling centers and molecular pathways involved in cichlid tooth replacement and morphogenesis. Armed with this information, we employed a set of small molecules to manipulate these pathways in vivo, documenting effects of treatment on both shape and replacement. The key finding from this study is that the processes of tooth morphogenesis and replacement are linked by common pathways that likely control the balance between growth, proliferation and differentiation as cusps form on tooth tips and as new dental organs initiate development from their predecessors. We suggest that this coupling of

morphogenesis and lifelong regeneration is the ancestral vertebrate condition, largely lost or decoupled in the mammalian dentition. Our integrative understanding of continuous tooth replacement from nature may pinpoint features of the process to be re-evolved by bioengineers.

2.3: Materials and methods

2.3.1: Fish husbandry

Species of Lake Malawi cichlids used in this analysis include: Aulonocara jacobfreibergi [AJ], Cynotilapia afra [CA], Labeotropheus fuelleborni [LF], Mchenga conophoros [MC], Metriaclima zebra [MZ], Petrotilapia chitimba [PC] Petrotilapia tridentiger [PT] and Pseudotropheus lombardoi (PL). These species were chosen to represent diversity in feeding behavior, adult tooth shape and ontogeny of tooth replacement (Table S1). Adult cichlids were maintained in re-circulating aquarium systems at 28 °C (GIT). Fertilized embryos were removed from the mouths of brooding females and staged in days post-fertilization (dpf) according to a developmental series from the Nile Tilapia (Fujimura and Okada, 2007). Embryos/fry were raised to desired stages for chemical treatment or anesthetized with MS-222 for fixation in 4% paraformaldehyde followed by dehydration into MeOH.

2.3.2: Immunohistochemistry

Embryos were fixed in 10% NBF for 24 h, dehydrated through ethanol, cleared with butanol and embedded in paraffin. Embryos were sectioned at 10 μm and H&E stained using a Leica Autostainer XL. For proliferation assays, cichlid fry undergoing active dental replacement were incubated in 5-Bromo-2-deoxy-uridine (BrdU) for periods of 6–8 h for nucleic acid incorporation. Fry were immediately anesthetized (MS-222), fixed, and paraffin sectioned at 10 μm. We then applied the 5-Bromo-2-deoxy-uridine Labeling and Detection Kit II (Roche) according to manufacturer's specifications (secondary antibody conjugated with AP activated NBT/BCIP, Roche). Similarly, PCNA staining was carried out on paraffin sections of wild type embryos according to manufacturer instructions (PCNA staining kit, Invitrogen), with DAB color reaction.

2.3.3: In-situ hybridization

Digoxigenin-labeled antisense riboprobes were prepared using partial cichlid genome assemblies (Loh et al., 2008) as well as recently assembled tilapia and MZ genomes (https://www.broadinstitute.org/ftp/pub/assemblies/fish). DNA sequence diversity across the Lake Malawi assemblage is 0.28%; less than reported values for laboratory strains of zebrafish. cDNA sequences for probe design have been deposited in GenBank (accession numbers KC633829—KC633848). ISH was performed according to previously published protocols (Fraser et al., 2008 and Fraser et al., 2009). Embryos were re-hydrated from MeOH and ISH was carried out in whole-mount. Digoxigenin-labeled antisense

riboprobes were generated using the Riboprobe System Sp6/T7 kit (Promega). AP-conjugated anti-dig antibodies were visualized at the end of color reaction (NBT/BCIP; Roche) using light microscopy. Embryos were embedded in chick albumin cross-fixed with 2.5% gluteraldehyde and post-fixed with 4% PFA. A Leica Microsystems VT1000 vibratome was used to cut sections at 15–25 μm. Histological sections were then mounted with glycerin and imaged at 10–63× using a Leica DM2500 compound microscope.

2.2.4: Treatment with small molecules

Stock solutions were prepared for each chemical treatment experiment using Dimethyl Sulfoxide (DMSO, MP Biomedicals) or water as a solvent. Stock solutions were as follows: 5 mM Cyclopamine (LC Laboratories) in DMSO, 10 μm DAPT (Tocris) in DMSO, 10 μm Dorsomorphin (Sigma-Alrich) in DMSO, 5 mM LiCl (Alexis Biochem) in H_2O, and 50 μm SU5402 (see acknowledgments) in DMSO. Cichlids were raised to appropriate stages for treatment and embryos from single broods were split into small molecule and solvent control groups. All treatments were designed to evaluate perturbations to complete, fully shaped adult first-row dentitions; because species differ in the number of replacement generations (and hence time) until adult first-row tooth shape is established (Table S1), the onset of treatment varied by species accordingly (e.g., as early as 40 dpf in LF). Treatment doses varied across chemicals to produce dental phenotypes without gross anodontia or fatality. All chemical and control experiments were performed in Erlenmeyer flasks at 28 °C in an oscillating platform culture incubator (Barnstead Lab-Line Max 4000). After treatment, fry

were washed extensively with fresh fish water and raised for 14 days prior to sacrifice, fixation, clearing and staining.

2.2.5: Clearing and staining

Fry previously fixed in PFA were washed with DEPC-H_2O for thirty minutes. Specimens were then placed into a 1% trypsin solution for one hour. After protein digest, calcified tissues were stained using Alizarin red S solution (1 g/50 mL KOH). Staining averaged 30 min, with larger specimen requiring a longer stain time. Once the tips of the pelvic fins stained red, fry were moved to a 2% KOH solution for a period of 24 h. Cleared and stained fishes were then graded into 100% glycerin, with thymol as a biocide.

2.4: Results

2.4.1: One-for-one replacement of cichlid teeth

We explored the histological events surrounding cichlid tooth replacement using both standard staining methods (i.e., hemotoxalin and eosin), as well as antibodies to proliferating cell nuclear antigen (PCNA) and incorporated bromodeoxyuridine (5-bromo-2′-deoxyuridine, *BrdU*). We present data for the oral dentition only, but general observations hold for oral and pharyngeal jaws, both of which house teeth in cichlids (Fraser et al., 2009). Throughout, we divide our description into three stages of replacement tooth development: (1) initiation, (2) cellular differentiation, and (3) secretion.

Cichlids exhibit intramedullary (inside the jawbone; i.e., intraosseous (Trapani, 2001)) replacement, similar to humans but distinct from other animals like reptiles (Handrigan et al., 2010), zebrafish (*Danio rerio*) (Huysseune, 2006) and rainbow trout (*Oncorhynchus mykiss*) (Fraser et al., 2006) where replacement germs develop in an extramedullary location. Cichlids replace teeth in one-for-one fashion like some other bony fishes (Bemis et al., 2005, Fraser et al., 2006, Kerr, 1960 and Motta, 1984); each functional tooth serves as a placeholder, as well as a supply of epithelial cells, for subsequent replacement by a single successor tooth—a process repeated over ontogeny. This is different from the many-for-one replacement system as observed in sharks (Fraser and Smith, 2011 and Smith et al., 2009), and pufferfish (Fraser et al., 2012), where many replacements form in advance of function for each tooth family.

Labial epithelial cells associated with developing first generation teeth form each successional lamina (SL, Fig. 1A–C), and together with contributions from labial oral epithelium, initiate the continued supply of tooth replacements. Using *BrdU* and PCNA, we found that the primary stage of SL invagination is marked by high rates of proliferation (Fig. 1B, C). When the lamina extends further below the existing primary tooth, proliferation continues and the lamina interacts with the receptive neural crest-derived mesenchyme to begin the process of replacement tooth organ development.

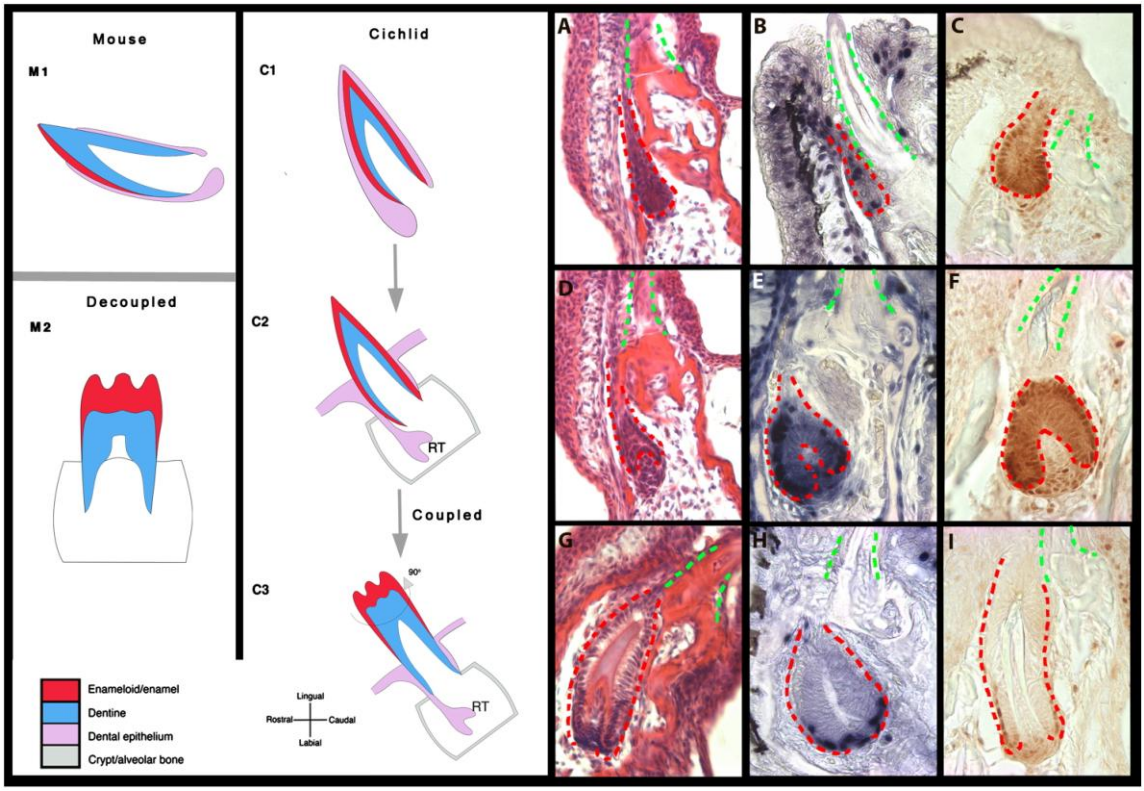

Figure 2.1. Dynamics of shape and (self-) renewal in mouse and cichlid dentitions. In the mouse, the processes of self-renewal in incisors (M1) and complex shape morphogenesis in molars (M2) are decoupled in space and time. By contrast, cichlid replacement teeth form complex shapes as they regenerate (C1–C3). Cichlid tooth replacement can be broken into three stages: initiation (A–C), cellular differentiation (D–F) and secretion (G–I). We illustrate these stages by H&E histology (A, D, G); BrdU (B, E, H) and PCNA (C, F, I) immunohistochemistry. First generation teeth are outlined in green and replacement dental epithelium in red. These are paraffin sections in sagittal plane at 10 μm thickness, imaged at 63× magnification. (A, D, G)—*Metriaclima zebra*; (B, C, E, F, H, I)—*Labeotropheus fuelleborni.*

As the epithelial SL interacts with the underlying mesenchyme, the bone surrounding and attached to the predecessor tooth begins to remodel and encases the newly initiated replacement organ (Fig. 1D–F) in a crypt that will house it throughout maturation. We observe that the oral epithelium and SL remain connected to the developing replacement tooth by a thin epithelial stream of cells, until eruption of the replacement (Fig. 1D–I). The bone forms around this ‘connector’ cell stream (the gubernacular cord (Avery and Steele, 2000)) leaving small pores called gubernacular canals, observed across vertebrates from fishes to humans (Avery and Steele, 2000 and Huysseune, 2000). The SL, through the gubernacular canal, maintains a link to oral epithelia and continuity between the extramedullary epithelium and the intramedullary (crypt) mesenchyme.

The replacement tooth germ transitions to stages of cellular differentiation as the mesenchyme condenses into the dental papilla (Fig. 1D, F). The epithelium contorts into a dental bud, followed by an inward folding of the epithelium to form the cap stage tooth—the first stage of the tooth-shaping process. This epithelial folding leads to the formation of three cell layers: the inner dental epithelium (IDE), outer dental epithelium (ODE) (Fraser et al., 2008), and an intermediate layer of cells between the IDE and ODE (Fig. S1), putatively analogous to the stellate reticulum of mammalian teeth (Huysseune and Thesleff, 2004 and Wang et al., 2007). Epithelial and mesenchymal cells differentiate at the cap to bell transition and form enameloid-secreting ameloblasts from the IDE and dentine-secreting odontoblasts from the dental papilla. As the replacement

tooth transitions from bud to cap and from cap to bell stages, we note three main sites of cell proliferation: at the tip of the developing replacement tooth, and in both cervical loops. These areas have similarly been identified in the gecko as regions of proliferation for hard tissue-secreting (enameloid and dentin) cells (Handrigan et al., 2010).

During terminal stages of cichlid replacement tooth development, the ameloblasts and odontoblasts secrete their respective hard tissue matrices. The ameloblasts continue to elongate as columnar cells and the bony crypt is remodeled to accommodate the growing tooth. As the eruption process initiates, we find that the lamina stream (gubernacular cord) that connects the oral epithelium with the successional tooth begins to break down (Fig. 1G–I). *BrdU* and PCNA analyses during hard tissue secretion highlight a slight labial bias in proliferation at the cervical loops, presumably giving rise to additional enameloid-secreting cells on the labial surface of the tooth (Fig. 1E, F, I). This asymmetry provides evidence of spatial differences in hard tissue deposition during the formation of cichlid replacement teeth.

2.4.2 Gene co-expression domains direct de novo tooth replacement

Cichlid one-for-one tooth replacement is initiated as an epithelial invagination, labial to the predecessor tooth (Fig. 1). We sought to understand the molecular pathways that might guide this process. Because little is known about tooth replacement in vertebrates, we focused on pathways involved in the patterning and regeneration of hairs and feathers, as well as the development of

mouse molars and incisors. A priori, *de novo* tooth replacement must combine factors providing dental competence to the epithelium and associated mesenchyme, coupled with signals of cell potency.

pitx2 is one of the earliest markers of dental-competent epithelium (Fraser et al., 2008). Consistent with expectation, *pitx2* expression is observed within the extended SL, throughout the epithelial downgrowth (Fig. 2A). The reciprocal neural crest-derived ectomesenchyme condenses and expresses a set of genes, including transcription factors such as *runx2* (Fig. 2B), and signaling molecules from the Wnt and FGF pathways (e.g., *wnt10a*, *fgf10*, Fig. 2C, D). Genes of the BMP pathway are also recruited to the replacement tooth. The invaginating SL expresses *bmp4* (Fig. 2E) and *bmp2* (not shown, see Fig. 3A), as does the reciprocal condensing ectomesenchyme; this expression is maintained throughout the process of lamina extension and proliferation. We note a second mesenchymal domain of *bmp4* expression, labial to the first generation tooth and the SL downgrowth (blue arrowhead in Fig. 2E). The two BMP domains are separated in space by cells expressing *osr2*, a transcription factor that represses BMP expression in mouse dental mesenchyme (Zhang et al., 2009) (Fig. 2F). *Osr2*-null mice exhibit expansion of lingual BMP expression and ultimately form lingual supernumerary teeth. It is possible that *osr2* acts similarly here, to properly position the first cycle of dental replacement through restriction of odontogenic BMP.

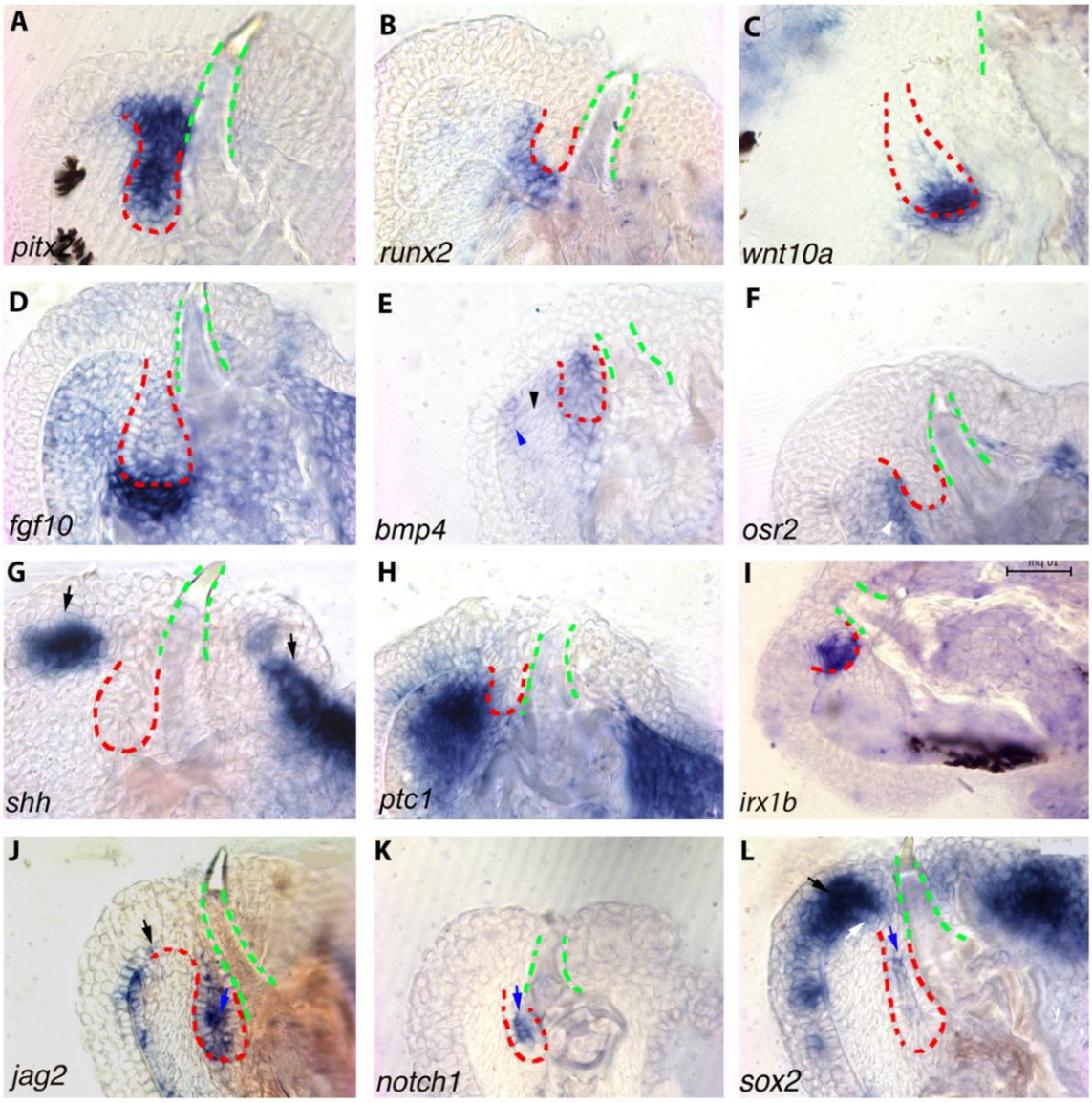

Figure 2.2. Replacement teeth recruit markers of dental competence and cell potency. The epithelial successional lamina (SL) expresses dental-commissioning *pitx2* (A) in close proximity to condensing mesenchyme marked by *runx2* (B), *wnt10a* (C), and *fgf10* (D). *bmp4* is active throughout epithelium and mesenchyme of the replacement tooth (E) and in a second region of labial mesenchyme (blue arrowhead) separated from the tooth germ by cells expressing the BMP inhibitor *osr2* (F). *shh* (G) is not expressed in the invaginating SL, but is active in the oral epithelium both lingual to the replacement tooth (presumed dental lamina for lingual rows) and oral epithelium labial to the replacement germ (black arrows). *ptc1* expression (H) is observed in the mesenchyme subjacent to *shh*-expressing oral epithelium. *irx1b*, a putative regulator of Hh signal, is expressed in the aboral-most region of the [Hh-negative] invagination (I). The invaginating SL contains an intermediate layer,

between inner and outer dental epithelium (see Fig. S1), expressing *jag2* (J), *notch1* (K), and *sox2* (L). *jag2* and *sox2* are also expressed in a continuous ribbon of epithelium labial to the replacement organ (arrows). First generation teeth are outlined in green and replacement dental epithelium in red. These are vibratome sections in sagittal plane at 15 μm thickness, imaged at 63× magnification. Labial is oriented to the left and oral toward the top of the page. Fishes used in this panel are ~15 dpf. (A, B, D, E, F, G, H, K, J, L)—*Metriaclima zebra*, (C)—*Labeotropheus fuelleborni*, (I)—*Aulonocara jacobfreibergi.*

BMPs interact with the Hedgehog (Hh) pathway during the patterning of many organ systems, including teeth (Handrigan et al., 2010 and Zhang et al., 2000); we thus examined expression of Hh ligands and receptors in the cichlid replacement program. Hedgehog is essential for tooth development in the mouse (Cobourne et al., 2004, Cobourne and Sharpe, 2004 and Dassule et al., 2000), initiates the first generation dentition in cichlids (Fraser et al., 2008), but does not play a role in the initiation of the replacement dentition in the trout (Fraser et al., 2006), nor in squamates (Handrigan and Richman, 2010). Here, we did not observe activity of the extracellular ligand *shh* or its receptor *ptc1* (Fig. 2G, H) until later replacement tooth morphogenesis (Fig. 3 and Fig. 5). Molecules from the Iroquois homeobox family have been documented in mouse tooth development (Ferguson et al., 2001), but their roles in the process are not understood. *irx1b* is known to respond to Wnt signals and restrict *shh* in the embryonic forebrain (Scholpp et al., 2007, Scholpp et al., 2006 and Sylvester et al., 2010). Here, we observe *irx1b* expression in the aboral-most epithelium of the extending SL (Fig. 2I), in close proximity to *wnt10a* (Fig. 2C). A putative function for *irx1b* in replacement tooth initiation is thus the regulation of Hh signal in the early SL downgrowth.

Although *shh* and *ptc1* expression are absent from the early replacement SL, they are nonetheless active in regions relevant to tooth development (Fig. 2G, H). The epithelium lingual to the outer row of erupted teeth strongly expresses *shh*, and as expected, the underlying mesenchyme maintains strong expression of the receptor *ptc1*. Cichlids continue to add posterior (lingual) rows of teeth throughout ontogeny and this lingual Hh signaling domain, in conjunction with *pitx2* and BMP, may provide the potential for the initiation of first generation teeth in lingual rows (Fraser et al., 2008). Notably, there is a second region of *shh*-expressing epithelium and corresponding *ptc1*-expressing mesenchyme labial to the SL (Fig. 2G, H), corresponding to the labial domain of *bmp4* noted above (Fig. 2E). This labial domain of BMP and Hh co-expression is maintained during subsequent stages of replacement tooth development (Fig. 3). As no teeth form labial to the first generation dentition, we explored this cell population as a putative source of dental potency for replacement.

To do so, we first examined expression of Notch signaling family members. The Notch pathway is involved in the patterning of teeth (Mitsiadis et al., 2010, Mitsiadis et al., 1998 and Mitsiadis et al., 2005), the stem niche of mouse incisors (Harada et al., 1999), and the general regulation of stem cells (Androutsellis-Theotokis et al., 2006). The Notch ligand *jag2* is expressed in both (i) the Hh- and BMP-positive cells labial to the SL (Fig. 2J, black arrow) and (ii) the intermediate cells of the SL (Fig. 2J, blue arrow). These intermediate cells, between IDE and ODE, will give rise to stellate reticulum-like cells in the differentiated replacement tooth (Fig. 1D–F; Fig. S1). The receptor *notch1* is

expressed in the intermediate cells, but not labial to the SL (Fig. 2K). However, the receptors *notch2* and *notch3* are active in both locations during subsequent developmental stages and rounds of replacement (RFB, unpublished). Next, we examined activity of the stem cell transcription factor *sox2* in the replacing cichlid dentition. *sox2* is expressed in both the labial domain (as well as lingual to the first tooth row) and within the intermediate cells of the SL (Fig. 2L). Taken together, these patterns of gene co-expression give an mRNA signature to two distinct populations of cells (i) labial and superficial to the replacement organ and (ii) within the intermediate cells of the SL, one or both of which may enable and maintain continuous *de novo* dental replacement. Notably, in reptiles (Handrigan et al., 2010), stem-like cells are arranged superficially along the non-tooth forming outer layer of the dental lamina while in zebrafish (Huysseune, 2006 and Huysseune and Thesleff, 2004), intermediate cells between IDE and ODE are suggested to exhibit stem-like properties.

2.4.3 Gene expression is evolutionarily conserved in replacement tooth differentiation

Cichlid replacement teeth undergo development within a bony crypt constantly remodeled to accommodate jaw growth and dental renewal. The replacement dental organ differentiates in a series analogous to the bud, cap and bell stages of mammalian teeth, while remaining connected to oral epithelium by a cord of cells through the gubernacular canal (Fig. 1). We know very little about the molecules accompanying the morphogenesis of replacement teeth in any organism. We thus examined the expression of genes from five signaling

pathways involved in the development and regeneration of vertebrate organs: BMP, FGF, Hh, Notch, and Wnt/*β-catenin*.

bmp2 and *bmp4* are expressed in both the epithelium and mesenchyme as the developing tooth transitions from cap to bell stages (Fig. 3A, B). We observe two centers of expression: one in dental mesenchyme (black arrowhead) and one at the tip of developing teeth (yellow arrowheads). While BMPs are expressed at the oral-most cap and bell stage epithelium, we note a general absence of activity in the lateral and aboral-most epithelium. Interestingly, we find the BMP antagonist, *sostdc* (*ectodin*; *wise* (Laurikkala et al., 2003)), expressed in the epithelium where *bmp2* and *bmp4* are not (Fig. 3C). *sostdc* expression is strongest in the intermediate cells between the ODE and IDE of the cap to bell-stage replacement tooth, and less strong at the cusp tip or mesenchymal papilla (3C, yellow arrow).

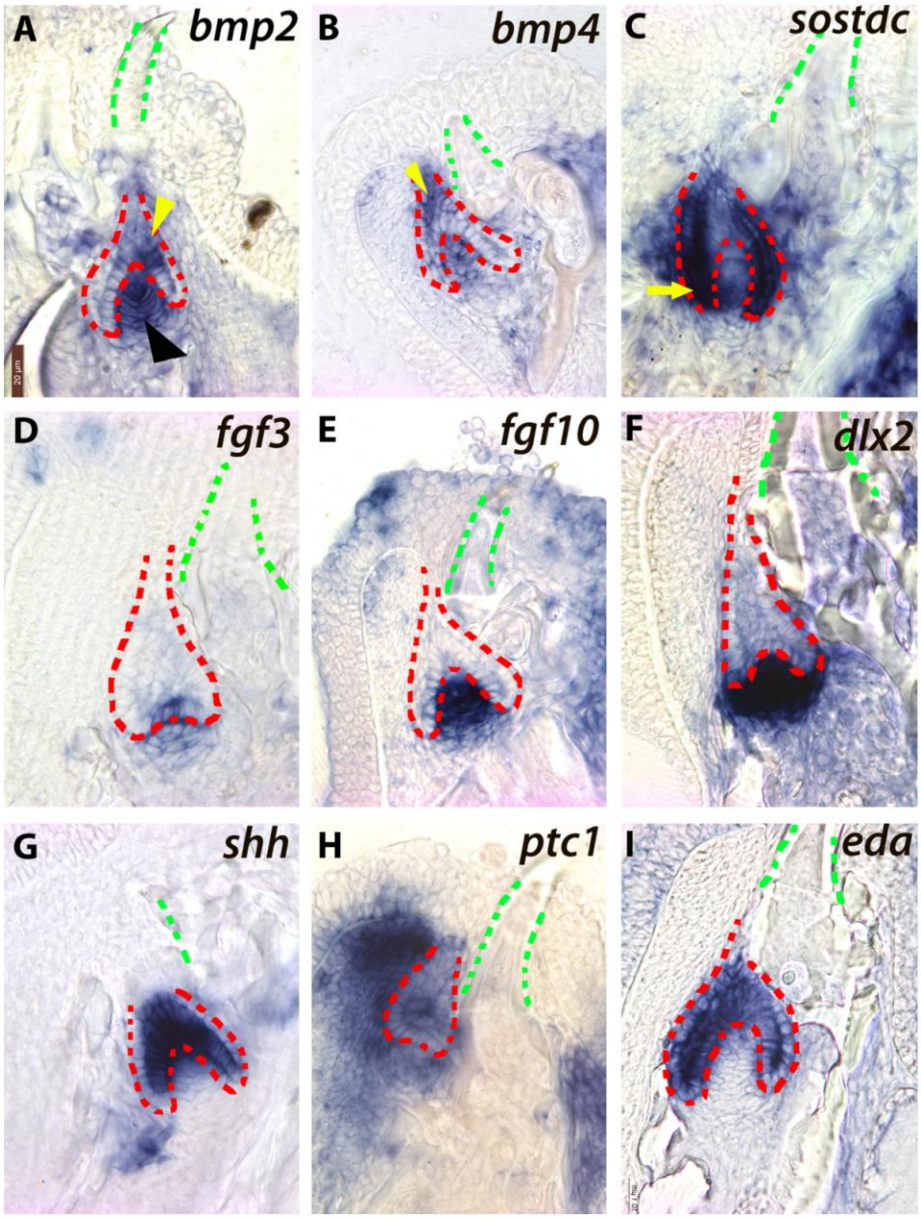

Figure 2.3. Expression of genes from the BMP (A–C), FGF (D–F), and Hh pathways (G–I) during the differentiation stage of cichlid replacement tooth development. First generation teeth are outlined in green and replacement dental epithelium in red. Three expression domains are highlighted at this stage; (i) the tooth tip (A, B; yellow arrowhead), the condensing papilla (A; black arrowhead), and the cervical loops (C; yellow arrow). These are vibratome sections in sagittal plane at 15 μm thickness, imaged at 63× magnification. Labial is oriented to the left and oral toward the top of the page. Fishes used in this panel are ~15–30 dpf. (A, B, D, F, G, H, I)—*Metriaclima zebra*, (C)—*Labeotropheus fuelleborni*, (E)—*Petrotilapia chitimba*.

sostdc influences tooth development by integrating BMP, FGF, Hh and Wnt pathways (Ahn et al., 2010, Cho et al., 2011, Kassai et al., 2005 and Laurikkala et al., 2003). As cichlid replacement teeth initiate differentiation, fgf3 and fgf10 are expressed in dental mesenchyme, and fgf3 is expressed transiently in the aboral epithelium (Fig. 3D, E). FGF signals induce dlx2 expression in the zebrafish pharyngeal dentition (Jackman et al., 2004 and Stock et al., 2006). Similarly here, dlx2 is co-expressed with fgf10 in the papilla throughout the bud to bell stage transition (Fig. 3F). shh transcripts, on the other hand, initiate expression in the invaginated SL only after the ectomesenchyme condenses, and the bud stage begins (Figs. 2G; 3G). shh becomes concentrated briefly from the cap to bell stage at the tooth tip, but later is localized to the epithelium analogous to mammalian cervical loops (Fig. 5G). As in the snake (Handrigan and Richman, 2010), the cichlid replacement tooth expresses the receptor ptc1 in both epithelium and mesenchyme of the differentiating tooth, implying that the mode of action of Hh signaling is both autocrine and paracrine (Fig. 3H). eda, a ligand in the ectodysplasin pathway is thought to induce Hh activity in hair (Pummila et al., 2007), feathers (Houghton et al., 2005), salivary glands (Häärä et al., 2011), and teeth (Laurikkala et al., 2001). Here, we observe its expression in the epithelium of replacement teeth (Fig. 3I). Expression of eda in the epithelium of cichlid replacement dental organs is notable because it is restricted to the mesenchyme during initiation of first generation teeth (Fraser et al., 2008).

In mouse incisors, FGF signaling from the mesenchyme maintains Notch activity in cervical loop epithelium (Harada et al., 1999) and presumptive stem cells in the stellate reticulum (Harada et al., 2002). Consistent with our observations from the initiation of dental replacement, jag2 and notch1 are expressed in localized cells of the epithelium and at the tooth tip (Fig. 4A, B, yellow arrow in B). Because we observed FGF signal in dental mesenchyme and Notch activity in the epithelium, we evaluated whether the stem cell marker sox2 was expressed at this stage. sox2 expression is maintained in the epithelium labial to the replacement tooth organ and is also observed in discrete epithelial cells favoring the labial side of the tooth (Fig. 4C).

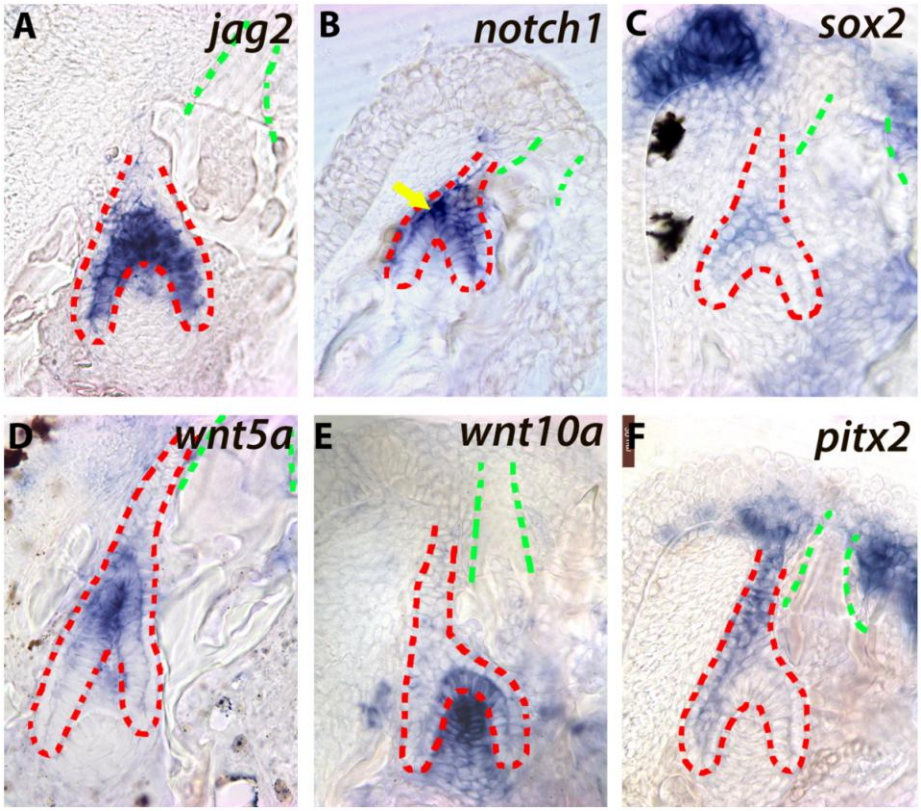

Figure 2.4. Expression of genes from the Notch (A–C) and Wnt pathways (D–F) during the differentiation stage of cichlid replacement tooth development. First generation teeth are outlined in green and replacement dental epithelium in red. Genes expressed in the intermediate cell layer include *notch1* (B; yellow arrow) and *sox2* (C) at differentiation stage. These are vibratome sections in sagittal plane at 15 µm thickness, imaged at 63x magnification. Labial is oriented to the left and oral toward the top of the page. Fishes used in this panel are ~15–30 dpf. (A, E, F)—*Metriaclima zebra*, (B)—*Mchenga conophoros*, (C)—*Petrotilapia chitimba*, (D)—*Labeotropheus fuelleborni.*

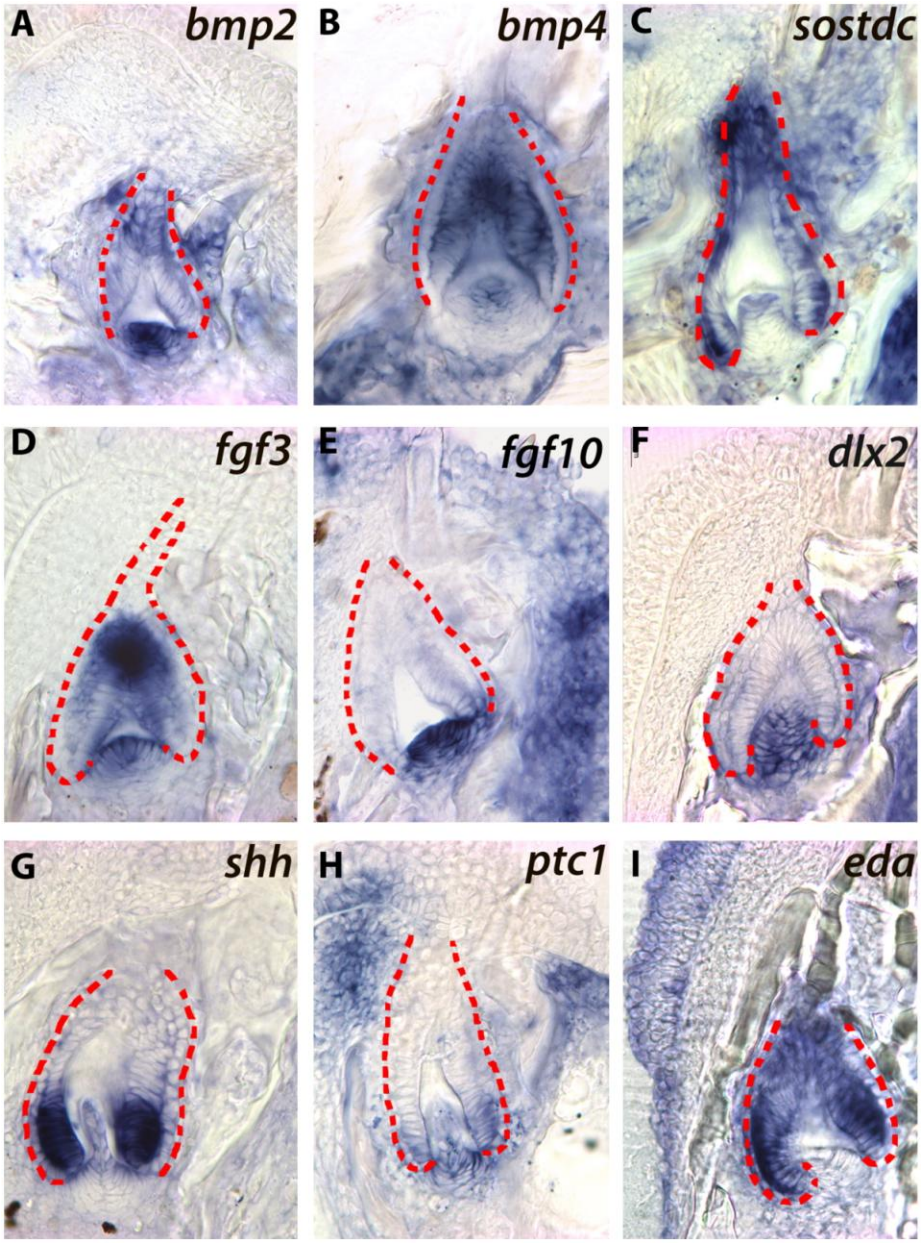

Figure 2.5. Expression of genes from the BMP (A–C), FGF (D–F), and Hh pathways (G–I) during the secretion stage of cichlid replacement tooth development. Replacement outer dental epithelium is outlined in red. These are vibratome sections in sagittal plane at 15 μm thickness, imaged at 63x magnification. Labial is oriented to the left and oral toward the top of the page. Fishes used in this panel are ~15–30 dpf. (A, D, F, G, I)—*Metriaclima zebra*, (B, H)—*Cynotilapia afra*, (C)—*Labeotropheus fuelleborni*, (E)—*Petrotilapia chitimba*.

The Wnt/*β-catenin* pathway is similarly active in differentiating replacement teeth. We observe *lef1* and *β-catenin* expression throughout the dental epithelium during the bud to cap transition (Fig. S2A, B). *wnt5a* (Fig. 4D) exhibits a local focus of expression at presumptive tooth tips; in the mouse, this gene is active in dental epithelium (including enamel knots) as well as in mesenchyme (Cai et al., 2011). *wnt10a* continues to be expressed in dental mesenchyme (Fig. 4E). In the mouse dentition, *Pitx2* and *β-catenin* directly interact to regulate *Lef1* (Vadlamudi et al., 2005). Here, *pitx2* marks a distinct set of labial epithelial cells that connect the oral epithelium to the replacement tooth (Fig. 4F). Overall, the molecular events that choreograph the progression from dental bud to bell stage tooth development are highly conserved between single generation mouse teeth and the continuously replacing cichlid dentition. Conservation of the genetic toolkit for individual tooth differentiation is particularly notable in this context of continuous one-for-one dental replacement.

2.4.4 Gene expression domains sharpen during secretion stage

By secretion stage, the replacement organ has begun to deposit hard tissues and nears eruption (Fig. 1G–I). We observe the same set of pathways active in replacement teeth at this stage, although expression domains for certain molecules have shifted with tooth maturation. *bmp4* and *bmp2* are expressed in the replacement organ at the tooth tip and the dental papilla (Fig. 5A, B) but are nearly absent from the putative cervical loops; *sostdc* is expressed in these cervical loops as well as a region far oral to the *bmp4*-positive tooth tip (Fig. 5C). This complimentary pattern of expression between signal and antagonist is also

observed for members of the FGF pathway. *fgf3* is expressed in the epithelium, including the tooth tip, and the dental papilla (Fig. 5D), but is absent from the aboral-most cervical loops; *fgf10* is strongly expressed in the papilla (Fig. 5E); the receptor *fgfr2*, which transduces FGF signal in teeth (Parsa et al., 2010), is active throughout (Fig. S3A). The FGF inhibitor *spry4* (Boran et al., 2009 and Charles et al., 2011) is concentrated along the cervical loop epithelium, also expressed in the dental papilla (Fig. S3B).

Ligands and receptors of the Hh pathway are dynamically expressed through the sequence of replacement tooth development. *shh* is initially absent from the invaginating SL (Fig. 2G), then is active throughout the bud to bell stage epithelium (Fig. 3). At mature stages of replacement tooth morphogenesis, *shh* is strongly localized to the basal cervical loops (Fig. 5G). The receptors *ptc1* (epithelium and mesenchyme, Figs. 2H, 3H) and *ptc2* (mesenchyme only), as well as the Hh activator *eda*, are likewise confined to cervical loop epithelium and contact mesenchyme (Fig. 5H, I; Fig. S3C). These aboral domains of activity for the Hh pathway are similar to observations from mouse incisors, where Hh is required for the differentiation of ameloblasts from dental stem cells within the stellate reticulum (Parsa et al., 2010). In contrast to the changing patterns of activity exhibited by Hh, molecules in the Notch pathway are consistently expressed across the stages of replacement tooth development, localized to the intermediate stellate reticulum-like cells between IDE and ODE, as well as at the tooth tips (Fig. 6A, B).

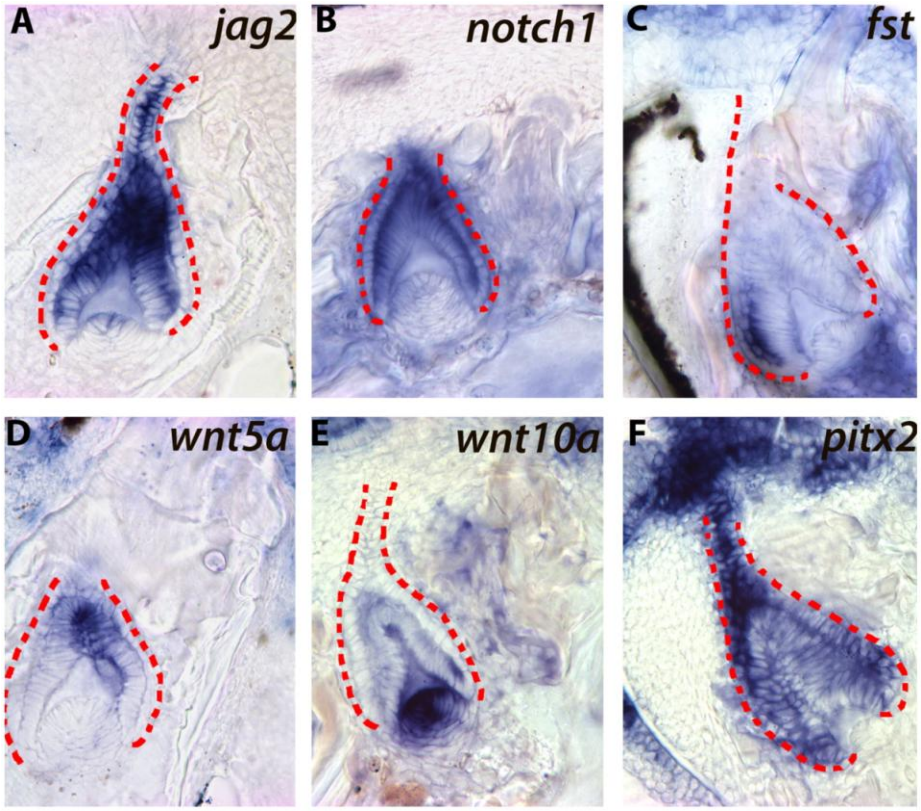

Figure 2.6. Expression of genes from the Notch (A–C) and Wnt pathways (D–F) during the secretion stage of cichlid replacement tooth development. Replacement outer dental epithelium is outlined in red. These are vibratome sections in sagittal plane at 15 μm thickness, imaged at 63x magnification. Labial is oriented to the left and oral toward the top of the page. Fishes used in this panel are ~15–30 dpf. (A, F)—*Metriaclima zebra*, (B)—*Cynotilapia afra*, (C)—*Petrotilapia chitimba*, (D, E)—*Labeotropheus fuelleborni*.

We note an intriguing and strong bias in the expression of *follistatin* (*fst*) during the final stage of replacement tooth development. *Fst* is expressed with a lingual bias in mouse incisors where, by antagonizing BMP activity, it contributes to the reduction in enamel-secreting ameloblasts on the lingual surface (Wang et al., 2004). Cichlids, however, express *fst* with a labial bias overlapping Notch-

expressing intermediate cells (Fig. 6C). Such biases might contribute to asymmetries in cell proliferation and differentiation to give cichlid teeth their characteristic slight lingual curvature (Fig. 1), but the molecular mechanism would then be distinct from that explaining the same curve of mouse incisors.

wnt5a and *wnt10a* continue to be expressed at the tooth tip (Fig. 6D) and the dental papilla (Fig. 6E), as in earlier stages and *pitx2* maintains expression linking the dental epithelium of the replacement organ to the oral epithelium (Fig. 6F). *lef1* expression spans the epithelium and mesenchyme of the replacement tooth unit (Fig. S3D). Notably, *axin2*, an effector of Wnt/β-catenin signaling, is active in dental mesenchyme, as well as dental epithelium including the cervical loops and the intermediate cells between IDE and ODE (Fig. S3E). This is in contrast to mouse incisors, where *Axin2* is only weakly expressed in cervical loop epithelium and is absent from the stellate reticulum (Suomalainen and Thesleff, 2010). Irx family members *irx1b* (not shown) and *irx2* also localize to the cervical loop domains during late replacement tooth morphogenesis (Fig. S3F).

The data presented here in conjunction with previous reports of cichlid tooth initiation (Fraser et al., 2008 and Fraser et al., 2009) demonstrate that five signaling pathways (BMP, FGF, Hh, Notch, Wnt/β-catenin) are sequentially active in specific cell populations (the SL as an extension of oral epithelium, the tooth tip, the dental papilla, and intermediate cells of the cervical loops) during the process of replacement tooth development. It is likely that the signaling pathways we highlight (i) set the precise dental pattern (the size and spacing of teeth, (Fraser et al., 2008)), (ii) requisition one-for-one replacement, which

maintains that pattern, and (iii) build the tooth organ for every new generation. The spatial domains of pathway gene co- and complementary expression are thus presumed to confine odontogenesis to precise locations and to accurately reset the process of tooth development for regeneration.

2.4.5: Treatment with small molecules affects replacement and shape

Cichlids replace (shaped) teeth in one-for-one fashion, using a set of common signaling pathways throughout (Fig. 2, Fig. 3, Fig. 4, Fig. 5 and Fig. 6). The spatial proximity of patterned tooth families and the temporal continuity of morphogenesis plus regeneration suggest to us that these processes are deeply integrated. One prediction, then, is that manipulation of key pathways should result in altered replacement and shape phenotypes. To test this prediction, we exposed cichlid individuals to temporally precise, non-lethal doses of small molecules known to agonize or antagonize the BMP, FGF, Hh, Notch and Wnt/β-catenin pathways.

A typical treatment experiment involved (i) culture of replicate cichlid juveniles at the appropriate stage (e.g., 40–100 dpf) in fish water with the small molecule or delivery control (1% DMSO) for 24 h, (ii) a 14-day recovery period under standard conditions and (iii) finally, sacrifice for phenotypic analysis (see Materials and methods). There are two major advantages of this approach in the cichlid system. First, treatment over such a brief temporal window will affect only those teeth at sensitive stages of development; there are thus ‘control’ individuals that did not receive small molecule baths, as well as ‘control’ teeth in the jaws of

experimental animals. Second, non-lethal in vivo treatment, subsequent growth in fish water and post-sacrifice clearing and staining with Alizarin red allows us to examine the presence or absence of replacement teeth in the jaw's bony crypt, as well as additional effects of treatment (i.e., jaw hypertrophy) that might influence tooth development. A general caveat holds for all of these experiments; effects differ depending upon the duration and concentration of the dose applied.

Manipulation of two of the five signaling pathways did not affect cichlid tooth replacement. Treatment with the Hh antagonist cyclopamine strongly disrupts the patterning of the cichlid dentition when administered during the initiation of first generation teeth (Fraser et al., 2008) but does not interfere with dental replacement in snakes and lizards (Handrigan and Richman, 2010). Our results are similar here upon treatment at tooth replacement stages; cyclopamine (25 μm) affects the shaping and morphogenesis of teeth (below), but does not abrogate the replacement process (Table 1). This is consistent with our gene expression data (Fig. 2, Fig. 3 and Fig. 5) wherein Hh molecules are not active in the replacement tooth until differentiation begins. Treatment with the FGF antagonist SU5402 does not interfere with the production of replacement teeth per se (we observe replacement tooth development deep to the predecessor), but seems to interfere in some animals with the process of functional tooth shedding, thus an indirect effect on replacement (Table 1). In some tooth positions in treated animals, we noted that functional teeth were elongated and that bony crypt morphology was disrupted, perhaps the result of jaw hypertrophy (data not shown).

Table 2.1. Concentrations and phenotypes of chemical treatments. Effective non-lethal doses of small molecules are arranged in rows. **1A**: Numbers of affected and treated cichlid broods and species used; MZ, *Metriaclima zebra*; LF, *Labeotropheus fuelleborni*; PL, *Pseudotropheus lombardoi*; PT, *Petrotilapia tridentiger*. **1B**: Phenotypes are recorded with respect to numbers of teeth; mean and standard deviations (SD) of both affected and total teeth are presented. Phenotypes are reported as having an effect on shape (*S*) and/or replacement (*R*). Higher concentrations elicited multiple phenotypes in a dose dependent manner. NB, unaffected individuals developed complete dentitions with no patterning defects despite being exposed to the same chemicals at the same time as their affected siblings. A single animal may have more than one phenotype.

Chemical	Concentration	Affect/Treat—Brood	Affect/Treat—Individual	Species	Mean tooth no. affected/individual	Mean tooth no. Total/individual	% Affected phenotype
DAPT	100 µM	5/6	6/11	LF; MZ; PT	2.5 (SD 1.77)	10.0 (SD 2.80)	R/S–35/65 %
DAPT	75 µM	5/5	9/10	LF; MZ; PT	4.75 (SD 2.42)	9.0 (SD 2.49)	R/S–22/78 %
DAPT	50 µM	3/3	9/9	LF; MZ	6.25 (SD 2.05)	9.67 (SD 1.30)	S–100%
DAPT	40 µM	2/2	3/3	MZ	4.5 (SD 1.38)	9.5 (SD 0.84)	S–100%
Dorsomorphin	1.0 mM	1/1	3/3	PL	4.0 (SD 2.94)	10.5 (SD 4.04)	R/S–21/79 %
Dorsomorphin	0.5 mM	6/6	12/14	PL	4.07 (SD 1.62)	10.07 (SD 2.12)	R/S–3/97%
Cyclopamine	25 µM	4/5	7/9	LF; MZ; PT	4.44 (SD 2.04)	9.28 (SD 2.19)	S–100%
LiCl	250 µM	5/5	10/10	LF; MZ	3.15 (SD 1.07)	8.69 (SD 1.97)	R/S–10/90 %
LiCl	500 µM	2/2	4/4	LF	5.0 (SD 0.89)	8.33 (SD 0.52)	R/S–37/63 %
SU5402	50 µM	2/2	5/5	MZ	3.5 (SD	8.57 (SD	R/S–

Chemical	Concentration	Affect/Treat—Brood	Affect/Treat—Individual	Species	Mean tooth no. affected/individual	Mean tooth no. Total/individual	% Affected phenotype
					1.51)	2.82)	14/86 %

Small molecules targeting any of three pathways, BMP, Notch and Wnt/β-catenin, affected the process of cichlid tooth replacement. Treatment with the BMP antagonist dorsomorphin (BML-275), at 1 mM concentration, results in tooth positions that do not undergo natural replacement on both upper and lower jaws (Fig. 7A–C; Table 1). Furthermore, there is no evidence of replacement teeth (at any stage of development) in the underlying bony crypt of affected positions. Dorsomorphin exposure thus has a major effect on the replacement dentition and uniquely (among our treatments) perturbs adjacent tooth positions. We also observed a replacement phenotype after DAPT exposure (100 μM), which inhibits the Notch signaling pathway. Treatment with DAPT produces a number of tooth positions that lack replacements (at any developmental stage) across multiple tooth rows (Fig. 7D–F). Notably, this manipulation differs from treatment of the BMP pathway in that the disrupted tooth families tend not to be nearest neighbors, and are mirrored across the jaw symphysis. Lastly, treatment with LiCl, an agonist of Wnt/β-catenin signaling, has only modest effects on tooth replacement at low concentration (0.25 mM; Table 1), but results in cusp and replacement phenotypes at higher (0.5 mM) concentration (Fig. 7G–I and below).

This regeneration phenotype is intriguing because it is not a complete knockout of the replacement tooth unit. Rather, treatment appears to affect the rate and/or timing of replacement cycles, such that the phasing of tooth replacement in even vs. odd positions and across the symphysis is disrupted, compared to control. Together, these experiments demonstrate that the BMP, Notch and Wnt/β-catenin pathways are necessary for the proper initiation, rate and/or timing of continuous tooth replacement cycles in cichlid fishes. Given the expression of molecules from these pathways at early stages (Fig. 2, Fig. 3 and Fig. 4), our treatments have likely affected the invagination or potency of the epithelial SL and/or the responsive mesenchyme that facilitates dental replacement. We have yet to conduct molecular analysis of treated morphants; therefore effects from individual small molecules might be the indirect result of interactions between BMP, Notch and Wnt/β-catenin pathways, well known from other systems (Mitsiadis et al., 2010, Mustonen et al., 2002 and Plikus et al., 2008).

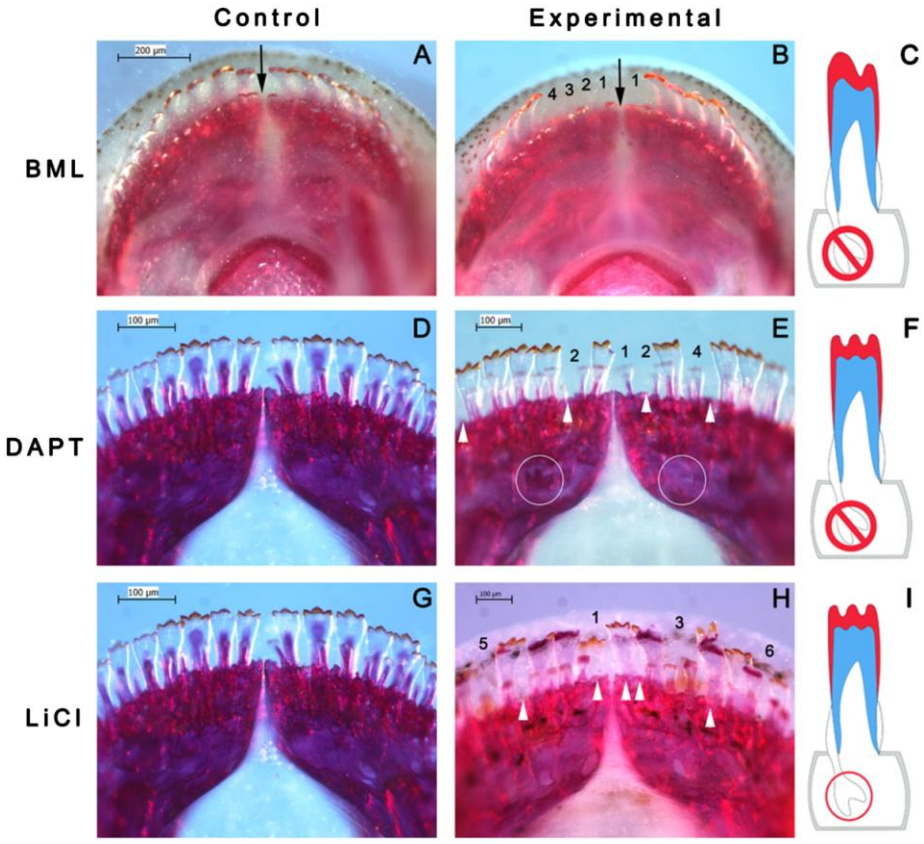

Figure 2.7. Small molecules targeting the BMP, Notch and Wnt/β-catenin pathways modulate tooth regeneration. We present dorsal views of Alizarin red stained upper (A–B) and lower (D–E, G–H) oral jaws from a variety of Malawi cichlid species. All individuals received small molecule treatments or vehicle controls for 24 h, followed by 14 days of recovery in fish water prior to sacrifice and analysis. (A–B) *Pseudotropheus lombardoi*, 100 dpf at the start of treatment. (D–E) and (G–H) *Labeotropheus fuelleborni*, 40 dpf at the start of treatment. A, D and G are vehicle controls and show the normal tooth formula. In (B), after treatment with the BMP pathway inhibitor BML275 (dorsomorphin, 1 mM), teeth from positions 1–4 of the first row, right quadrant and position 1, left quadrant, are not replaced; black arrow indicates the symphysis of the upper jaw. E, after treatment with the Notch pathway inhibitor DAPT (100 µM), teeth from positions 1, 2, and 4 of the first row (right quadrant) and tooth positions 2 and 7 (left quadrant) are not replaced. White circles show bony crypt space deep to functional teeth, with a replacement tooth present in the left circle and absent at right. (H) after treatment with the Wnt/β-catenin pathway agonist, LiCl (0.5 mM), teeth in multiple positions are delayed in eruption (red circle in I) and/or are out of phase in the replacement cycle (white arrowheads). Red circle in (I) refers to the white arrowhead positions (in H) showing functional positions without teeth; however tooth replacements are present in the crypts—hence a delay to the replacement process rather than a loss of tooth positions. Colors in schematics (C, F, and I): red=enameloid; blue=dentine; gray=bony crypt.

Notably, manipulation of all five signaling pathways for brief durations, and typically lower concentrations, produced *tooth shape* phenotypes (Fig. 8; Table 1). For instance, treatment with 0.5 mM dorsomorphin (BMP antagonist) results in transformation of outer row bicuspid to tricuspid teeth in *Pseudotropheus lombardoi* (Fig. 8C,D). Similarly, treatment with 50 μM SU5402 (antagonist of FGF signaling) results in triscuspid teeth in bicuspid first-row positions of *Metriaclima zebra* (Fig. 8E,F). Thus, inhibition of both BMP and FGF pathways affects bicuspid teeth in the same way—through the addition of a medial cusp. Treatment with cyclopamine (Hh antagonist) for only 6 h has a dramatic effect on tooth morphogenesis in *Petrotilapia tridentiger* (Fig. 8G,H), a species with an exclusively tricuspid dentition. Inhibition of Hh signaling interferes with natural cusp formation, resulting in teeth with no cusps, unevenly patterned enameloid, and even an unusual four-cusp phenotype (not shown). The variation in shape phenotypes after cyclopamine treatment matches the dynamic patterns of Hh gene expression during replacement tooth development (Fig. 2, Fig. 3 and Fig. 5). Treatment with DAPT, a Notch antagonist, impacts cusp development in *Labeotropheus fuelleborni*, a species with tricuspid teeth (Fig. 8I, J). We observe mineralization defects in both the dentine and enameloid of lateral and central tooth cusps, implying that Notch signaling is essential for correct hard-tissue biogenesis and cusp formation. Similar mineralization defects are observed in fishes treated with the Wnt/β-catenin agonist LiCl (Fig. 8K, L). Small-molecule treatment effects on replacement as well as shape (i) are reproducible in

replicate individuals and experiments across multiple broods of different species (Table 1) and (ii) are often matched in upper and lower jaws and on each side of the symphysis. Such replacement phenotypes and shape transformations have not been observed in healthy fishes from natural populations (Streelman et al., 2007). Our in vivo manipulations demonstrate an essential role for these five signaling pathways in the proper morphogenesis and shaping of cichlid teeth. Particularly exciting are treatments of the BMP and FGF pathways that transform tooth type from bicuspid to tricuspid, mimicking ecologically relevant differences among closely related species (Fraser et al., 2008, Streelman and Albertson, 2006 and Streelman et al., 2003). Taken together, these experiments (Fig. 7 and Fig. 8) provide evidence for a model linking tooth morphogenesis to tooth replacement through the function of key signaling pathways.

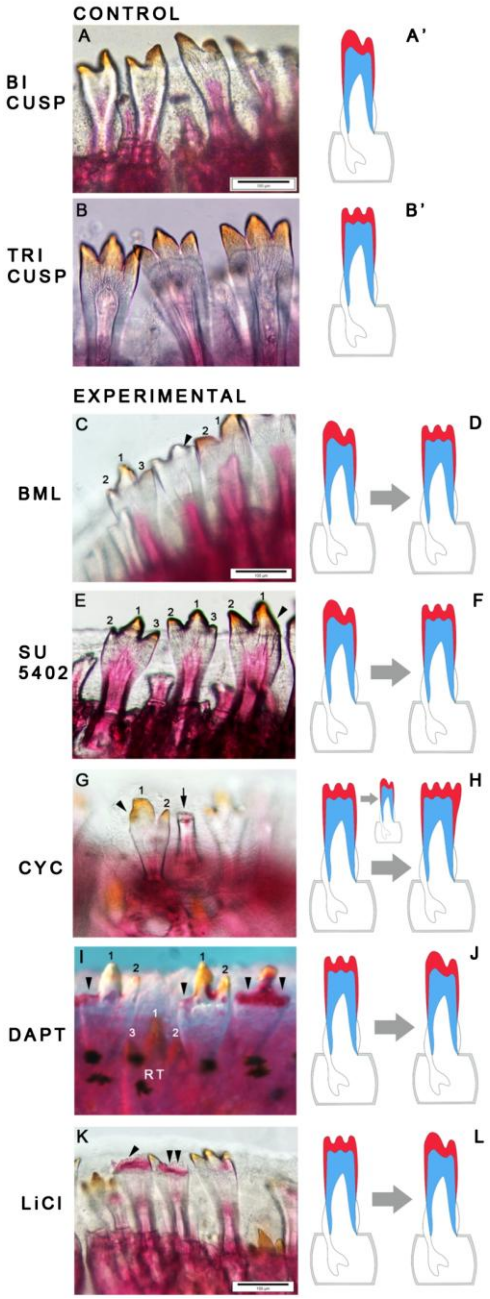

Figure 2.8.Small molecules targeting five signaling pathways modulate tooth shape. Control bicuspid (A–A′; *Metriaclima zebra*) and tricuspid (B–B′; *Labeotropheus fuelleborni*) dentitions are shown above the solid black line and small molecule treated dentitions are indicated below. All individuals received small molecule treatments or vehicle controls for 24 h, followed by 14 days of recovery in fish water prior to sacrifice and analysis. (C) BML275 treatment, (dorsomorphin, BMP inhibitor, 0.5 mM) results in transformation of bicuspid to tricuspid teeth (*Metriaclima zebra*). (E) Similarly, treatment with the FGF inhibitor SU5402 (50 μM) transforms teeth from bicuspid to tricuspid (*Metriaclima zebra*). In each of these cases, 'control' bicuspid teeth are present next to those sensitive

to the temporal window of small molecule application. (G) Treatment with cyclopamine (Hh antagonist, 25 μM) elicits numerous effects on shape, primarily through variation in enameloid patterning (arrows and arrowheads; *Petrotilapia tridentiger*). (I) Inhibition of the Notch pathway with DAPT (25 μM) affects cusp development and mineralization (arrowheads; *Labeotropheus fuelleborni*). (K) Treatment with the Wnt/β-catenin agonist LiCl (0.25 mM and 0.5 mM) results in mineralization defects with increasing dose (*Labeotropheus fuelleborni*). Colors in schematics (D, F, H, J, L): red=enameloid; blue=dentine; gray=bony crypt. Mineralization defects are inferred in treated individuals when the dentine (alizarin red stained) and/or the enameloid (yellow-orange color from Fe deposition) are abnormal, compared to controls.

2.5: Discussion

2.5.1 The cichlid dentition integrates tooth replacement and shape

The 'homeobox code' for the mammalian dentition posits that tooth shape is the product of linear position along the jaw margin (Sharpe, 1995 and Tucker and Sharpe, 2004). Mouse teeth represent the extreme condition of this general model, wherein only incisors and molars develop under distinct gradients of BMP-Msx and FGF-Barx1, respectively. In the mouse (and more generally the mammalian) dentition, molars undergo complex morphogenesis and develop cusps, which are absent and perhaps suppressed (Ohazama et al., 2010) from incisors. Mouse incisors, by contrast, exhibit enamel renewal via a labial stem cell niche. Thus for the mouse model, the phenomena of (i) complex cusp development and (ii) stem-based (self-)renewal are decoupled in space and in time (Fig. 1). Many other vertebrate dentitions do not follow such binary rules. For instance, cichlid teeth are shaped through rounds of replacement (all first generation teeth are conical) such that tricuspid teeth may replace unicuspid

teeth in the same jaw position (Streelman et al., 2003). Once adult tooth shapes are present, tooth replacement continues through ontogeny, maintaining shape and pattern fidelity. Tooth shape therefore is not correlated with position within a row and teeth with complex shapes undergo regeneration. The key finding from this study is that common signaling pathways are active and essential during the coupled phenomena of replacement and morphogenesis of cichlid dentitions, forcing us to think differently about the integration of these processes in odontogenesis (Jernvall and Thesleff, 2012). Our data inspire a model implicating specific signaling pathways (BMP, FGF, Hh, Notch, Wnt/β-catenin) in the process of cichlid tooth regeneration, explicitly coupled to tooth morphogenesis and shape. Genes from these families are sequentially co- and complimentarily expressed in spatial domains throughout tooth development and replacement. Our model posits a mechanistic connection between replacement and shape, as these signaling pathways likely mediate proliferation and differentiation at both the tooth tips and in presumed stem cell populations for renewal (Fig. 9).

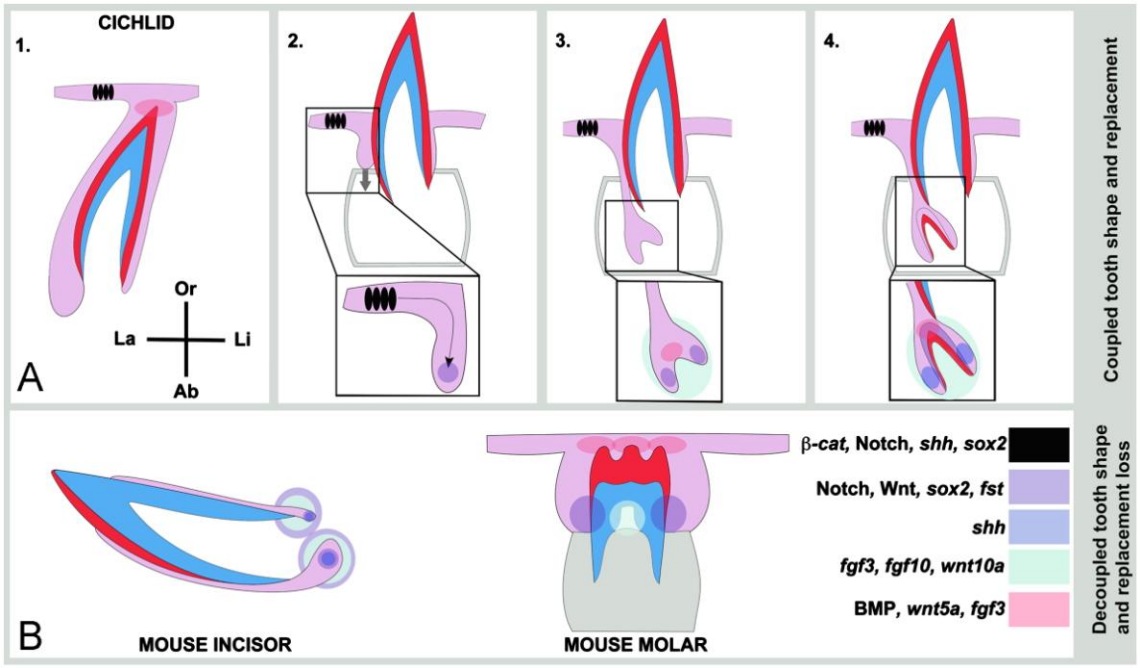

Figure 2.9. Cichlid teeth integrate tooth shape and lifelong regeneration, a linkage lost in mammals. Cichlid dental organs simultaneously coordinate shape morphogenesis and regeneration developmental programs, in the same tooth position (also Fig. 1 schematic). This is achieved via tight control of gene co-expression in zones of differentiation and zones of renewal. We identify cell populations and putative signaling centers that may regulate cichlid tooth shape and regeneration (A1–4), including (i) a population of epithelial cells (black) labial to the predecessor tooth superficial to the sucessional lamina of the replacement organ (gray arrow), (ii) cells of the intermediate layer between IDE and ODE (purple), (iii) cervical loop regions (blue), (iv) dental papilla (green) and (v) putative enameloid knots at the tips of teeth (pink). These cellular domains and putative signaling centers are color-coded based on empirical measure of gene co-expression (Fig. 2, Fig. 3, Fig. 4, Fig. 5, Fig. 6 and Fig. 7). In B, we show comparable gene activity in mouse incisors (Felszeghy et al., 2010, Harada et al., 1999, Harada et al., 2002, Jernvall and Thesleff, 2012, Wang et al., 2007 and Wang et al., 2004) capable of self-renewal and mouse molars (Jernvall et al., 2000, Jernvall et al., 1994, Kettunen et al., 2000 and Zhang et al., 2012) that develop complex 3D shapes. Throughout, tooth tissue colors are as in Fig. 1.

Cichlid teeth retain the capacity for lifelong *de novo* renewal. Our data suggest that at least two cellular domains may be important for tooth replacement. The first is similar in location to that housing putative stem-like cells in the zebrafish (Handrigan et al., 2010, Huysseune, 2006 and Huysseune and Thesleff, 2004). These cells are found in an intermediate layer between IDE and ODE that may be analogous to the stellate reticulum of mouse incisor cervical loops. These intermediate cells in cichlids co-express a common set of markers throughout replacement tooth development: *sostdc*, an inhibitor of BMP expression; Notch pathway ligands and receptors; *β-catenin*, and the stem transcription factor *sox2*. Molecules active at discrete, later stages of tooth replacement in the intermediate cells include *spry4*, an inhibitor of FGF signaling, *shh*, *fst* and *axin2*. A second population of cells that may contribute stem potential to tooth replacement in cichlids is the labial oral epithelium, superficial to each invaginating SL. This epithelium co-expresses *shh*, Notch pathway ligands and receptors, *β-catenin* and *sox2* throughout the stages of replacement tooth development; as noted, this labial epithelial domain is matched with mesenchymal expression of *ptc1* and *bmp4*. In reptiles, stem-like cells are located superficially, along the non-tooth forming regions of the dental lamina (Handrigan et al., 2010). It is notable that the pathways (BMP, Notch and Wnt/β-catenin) active throughout cichlid tooth development in both of these domains are those where small molecule manipulation elicit the strongest tooth replacement phenotypes (Fig. 7, Table 1). Our immunohistochemical, gene expression and small molecule experiments do not definitively prove that either of these cell

populations contains dental stem cells. Yet, based on gene co-expression signatures and the anatomical position of these cellular domains with respect to regenerating dental organs in other species, it is tempting to speculate that each of these populations contributes to and/or regulates the stem niche for cichlid tooth replacement (Fig. 9).

Cichlid teeth are shaped as they are replaced. First generation teeth are conical; generally, the first shaped replacement teeth have sharper cusps and more rapid replacement cycles than those that follow (Streelman et al., 2003). Once adult tooth shape is reached, teeth continue to be replaced with shape fidelity, roughly every 30–100 days. This means that the molecular signals that determine tooth shape do so later in the life of an individual cichlid than say, in the life of an individual mouse, whose first and only set of molars are shaped during embryogenesis. Two aspects of tooth shape are relevant given the diversity among cichlid species and the data we report here. The first is the degree of lingual curvature of the tooth, taken to the extreme in some algal brushing species that exhibit a near 90° angle between the long and flexible tooth ‘stalk’ and the multicuspid ‘brush’ at the tip (Fryer and Illes, 1972). We note from our histological data that cichlid replacement teeth begin as a downward extension of the SL on the labial side of the functional tooth, and that a labial bias in cell proliferation persists into hard tissue secreting stages (Fig. 1). Both the labial and lingual surfaces of cichlid teeth are covered with enameloid, but a slight bias in the production and proliferation of ameloblasts on the labial side might be facilitated by slight differences in molecular signaling in the labial vs.

lingual cervical loops. BMPs and FGFs seem to be largely absent from both cervical loop locations while the Hh, Notch and Wnt/β-catenin pathways are active. We observe a striking labial bias in the expression of *fst* that might contribute to different rates of ameloblast production and/or proliferation on the labial surface; this is a prime focus of future research because the bias is opposite that observed in mouse incisors (Wang et al., 2007).

The second relevant aspect of tooth shape is the number of cusps on each tooth, which in cichlids ranges from one to three with dramatic variation in the relative size and pattering of individual cusps (Fryer and Illes, 1972 and Streelman et al., 2003). Our data from ISH and small molecule treatments illustrate that (i) genes from all five pathways studied are active in putative signaling centers associated with cusp morphogenesis and (ii) manipulation of these pathways, individually, is sufficient to modulate shape. Strikingly, we observe a suite of molecules (*bmp2/4*, *fgf3*, *shh*, Notch ligands and receptors, *wnt5a*) active at the tooth tip in expression foci with similarity to mammal enamel knots (Jernvall et al., 1994). We suggest then that fishes (and perhaps all vertebrates with complex cusp shapes) possess primitive enamel knot-like signaling centers that function to control cusp number, sharpness and size.

One-for-one replacement of a complex dentition requires simultaneous activation of molecular programs for morphogenesis and regeneration within the same tooth. Our analysis has focused on specific cell populations and putative signaling centers that co- and sequentially express stem and dental markers

because it is likely that the spatio-temporal complementarity of gene activity is what allows a dental organ to tune proliferation, growth and differentiation all at once. In this sense, the coordination of these processes may depend as much on excluding molecular signals from a specific domain at a specific time as it does on the integration of signaling. Our data may be particularly useful in understanding how this segregation of gene expression is regulated in space and time. For instance, *shh* expression is absent from the initial downgrowth of the SL; this observation is consistent with data from other bony fishes and reptiles (Fraser et al., 2006 and Handrigan et al., 2010). We observe the expression of *irx1b*, a known mediator of Wnt signaling, antagonist of *shh* in the embryonic forebrain (Houweling et al., 2001, Scholpp et al., 2007, Scholpp et al., 2006 and Sylvester et al., 2010), and antagonist of *Bmp4* at gastrulation (Gomez-Skarmeta et al., 2001), in the aboral-most epithelium of the SL invagination. The *irx1/2* genes are later active in the cervical loop regions, this time co-expressed with Hh molecules and complementarily expressed with *bmp2/4*. Irx molecules have been noted in mouse teeth, but function is unknown (Ferguson et al., 2001). Our data suggest that these transcription factors may couple signals from the BMP, Hh and Wnt pathways and may be important negative regulators of Hh in the early SL. Similarly, *osr2* is expressed with a complementary pattern to *bmp4* at the initiation of primary replacement and may facilitate delineation of odontogenic cell populations across the jaw. At later stages of replacement tooth development, genes from the BMP and FGF pathway are invariably confined to activity in the dental papilla and the putative

enameloid-knot signaling centers at the tooth tip, and in particular are largely absent from the cervical loops and intermediate cells between IDE and ODE. We observe antagonists in each of these pathways (*sostdc*, *spry4*) expressed precisely in those cells where BMPs and FGFs are absent. Taken together, our data suggest that cichlid dental organs integrate shape and replacement by sometimes combining and other times segregating differentiation signals (BMPs, FGFs, Hh) from renewal and regeneration signals (Notch, Wnt, *sox2*), with temporal and spatial precision. It is likely that these interactions, necessary to pattern regenerating dentitions with complex shapes, prefigure the molecular programs found within multicuspid molars and self-renewing incisors (Fig. 9) (Jernvall and Thesleff, 2012). We speculate that the difference between organisms with lifelong regeneration of complex dentitions (i.e., cichlids, reptiles) and those without (e.g., mammals) lies in the continued maintenance and repeated activation of stem-like cells in positions superficial to successional lamina (Handrigan et al., 2010).

2.5.2 Stem cells and programmed evolvability of patterned elements

The majority of patterned dentitions in the long evolutionary history of vertebrates have been capable of continuous replacement (Huysseune and Thesleff, 2004), and yet we do not understand for any dentition how the processes of patterning, morphogenesis and regeneration are integrated in space and time. From first principles, we see that the replacement of dentitions *de novo* in a one-for-one fashion, while maintaining shape fidelity of individual units and inter-unit pattern, requires (i) signals of dental competence to specify

tooth vs. non-tooth, (ii) a morphogenesis program that can be recruited again and again within a tooth family, (iii) signals of renewal that can provide cell potency, and (iv) a clock mechanism to coordinate timing. Our study addresses the first three of these *a priori* requirements. Cichlid teeth carry out largely conserved morphogenesis programs coupled to regeneration via the co- and complementary expression of key signaling molecules (Fig. 9). Notable among these signals of cell potency is the transcription factor *sox2* that, among other functions, acts to maintain the undifferentiated stem state in embryonic and adult stem cells (Avilion et al., 2003) and specifically marks stem cells of the mouse incisor (Juuri et al., 2012). We observe *sox2* expression in two cellular domains that may mark the location of stem-like for cichlid tooth replacement. One of these domains shares anatomical features with dental stellate reticulum-like cells in other vertebrates. The second domain, labial to tooth rows, is particularly interesting because it may shuttle cells to the developing tooth unit (Fig. 9). A recent report of SOX2 anophthalmia syndrome in humans documented multiple dental phenotypes including supernumerary impacted teeth and the persistence of deciduous teeth (Numakura et al., 2010). It is likely then that sox2/Sox2/SOX2 plays (and has played) a central role in tooth replacement across vertebrates.

It is not clear what factors contributed to evolutionary modifications of the dentition in mammals, including the reduction in tooth number, tooth rows and lifelong replacement cycles. What is clear, however, is that this latter contingency has constrained the plasticity of mammalian teeth over an individual's ontogeny (particularly so for molars) and has limited the developmental window available

for evolutionary tinkering over a lineage's phylogeny. The recently noted ‘difficulty of increasing dental complexity’ in mammals (Harjunmaa et al., 2012) may be a direct result of this constraint. By contrast, we suggest that the phenotypic plasticity and dramatic shape diversity observed in cichlid teeth is facilitated by the potential for evolvability afforded by lifelong replacement. It is particularly this feature of cichlid teeth, the simultaneous integration of morphogenesis and regeneration programs, which might galvanize bio-inspired advancement in the field of regenerative dentistry.

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CHAPTER 3

CO-EVOLUTIONARY PATTERNING OF TEETH AND TASTE BUDS

3.1: Abstract

Teeth and taste buds are iteratively patterned structures that line the oro-pharynx of vertebrates. Biologists do not fully understand how teeth and taste buds develop from undifferentiated epithelium, or how variation in organ density is regulated. These organs are typically studied independently because of their separate anatomical location in mammals: teeth on the jaw margin and taste buds on the tongue. Yet, in many aquatic animals like bony fishes, teeth and taste buds are co-localized one next to the other. Using genome mapping in cichlid fishes, we identified shared loci controlling a positive correlation between tooth and taste bud densities. Genome intervals contained candidate genes expressed in tooth and taste bud fields. *sfrp5* and *bmper*, notable for roles in Wnt and BMP signaling, were differentially expressed across cichlid species with divergent tooth and taste bud density, and were active in the development of both organs in mice. Synexpression analysis and chemical manipulation of Wnt, BMP and Hh pathways suggest that a common cichlid oral lamina is competent to form teeth or taste buds. Wnt signaling couples tooth and taste bud density while BMP and Hh mediate distinct organ identity. Synthesizing data from fish and mouse, we suggest that the Wnt-BMP-Hh regulatory hierarchy that

configures teeth and taste buds on mammalian jaws and tongues may be an evolutionary remnant inherited from ancestors wherein these organs were co-patterned from common epithelium.

3.2: Introduction

Hughes and Chuong (Hughes and Chuong, 2003) called the mammalian oral cavity a “mouthful of epithelial-mesenchymal interactions” because the same precursor epithelium must ultimately differentiate to form teeth in one row on the oral jaw margin, taste buds and filiform papillae on the tongue, and salivary glands in precise locations. Developmental biologists have worked for decades to understand how this oral epithelium is properly fated to form such a diversity of structures. Although progress has been made to discover the molecules and pathways responsible for individual organ identity (Tucker and Sharpe, 2004), we know much less about the genetics and development of tooth and taste bud patterning (i.e., the spacing, organization and density of organs), and almost nothing about adaptive variation in dental and taste bud density across populations and species.

Because placode-derived structures such as teeth and taste buds tend to be located in different regions of the integument or the gastrointestinal tract to engage a particular function, they are studied independently. However, commonalities are apparent in the patterning of these appendages. Hair, feathers, glands, teeth, taste buds, and intestinal crypt villi all form under the

direction of epithelial and mesenchymal interactions resulting in placode induction. Similar reaction-diffusion models have been proposed to control the size and spacing of manifold placode-derived organs (Kondo and Miura, 2010) such as hair (Sick et al., 2006), feathers (Mou et al., 2011) and teeth (Cho et al., 2011). Activators and inhibitors in the bone morphogenetic protein (BMP), fibroblast growth factor (FGF), Hedgehog (Hh), and Wingless (Wnt) pathways execute the pattern. Genetic abnormalities in these pathways often lead to syndromic human diseases, such as Gardner's syndrome, Gorlin syndrome and ectodermal dysplasia, which affect multiple appendages (Mikkola and Millar, 2006). Feedback loops have been independently identified wherein Wnt drives the initiation of dental (Ahn et al., 2010) or taste (Iwatsuki et al., 2007) placodes. Wnt/β-catenin acts upstream of BMP and Hh signaling ((Liu et al., 2008; Liu et al., 2007)), which in turn may regulate Wnt and organ identity (5-10).

Mammals typically possess a single row of teeth on the oral jaw and a distinct taste papillae-bearing tongue and posterior palate. By contrast, many vertebrate dentitions are found in multiple rows on multiple bony or cartilaginous structures throughout the oro-pharynx. Taste buds often co-localize with teeth at these sites as both organs are regenerated, with pattern fidelity, throughout the lifetime of an individual. Teeth and taste buds may share an evolutionary origin and deep molecular homology (Fraser et al., 2010). There is tremendous variation in tooth and taste bud numbers among vertebrates. Among closely related species, this variation likely has ecological relevance (Yamamoto et al., 2009). For instance in Lake Malawi cichlids, planktivores typically possess a

small number of widely spaced teeth (Fraser et al., 2008) with reduced taste bud counts on the oral jaws. Alternatively, the oral jaws of algivores are packed with 100s-1000s of teeth and taste buds at high density. We sought to understand the genetic and developmental underpinnings of co-variation in tooth and taste bud density in Lake Malawi cichlids; in particular (i) how these distinct organs are patterned from a shared oral epithelium, (ii) how diversity in organ density is achieved across closely related species, and (iii) which molecular pathways and genomic regions control patterning. Working from insights in the cichlid system, we explored the activity of novel candidate genes for tooth and taste bud patterning in the mouse model.

3.3: RESULTS

3.3.1: Common regions of the cichlid genome control the positive correlation between tooth and taste bud density

The positive phenotypic correlation between tooth and taste bud density observed across Malawi cichlid species could be controlled by: (i) pleiotropy, wherein common genetic intervals control both tooth and taste bud density and/or (ii) epigenetics (in the general sense), wherein the density of one of these structures constrains or determines the density of the other. To explore the genetic basis of tooth and taste bud densities, we assessed the correlation in F_2 fishes from an intercross between two rock-dwelling Lake Malawi cichlid species,

Cynotilapia afra (CA), a planktivore with few teeth and taste buds and *Pseudotropheus elongatus* (PE), an algivore with many of both. Tooth and taste bud densities each ranged >4-fold and were positively correlated across F_2 (r^2 = 0.43, Figure S1). To identify the genome intervals controlling tooth and taste bud density, we employed a QTL mapping framework, using fully informative RAD-Tag single nucleotide polymorphisms (SNPs), as previously described for other phenotypes (Parnell et al., 2012).

Briefly, we genotyped informative SNPs in the F_2 and constructed a genetic linkage map. We joined 370 loci in 22 linkage groups (Malawi cichlids have 22 haploid chromosomes (Poletto et al., 2012). The data set exhibits nearly complete genotypes across 382 F_2 (0.4% missing data). The linkage map was translated to genome assemblies of tilapia, *Oreochromis niloticus* (an East African river cichlid) and *Metriaclima zebra* (another Lake Malawi rock-dwelling cichlid) (Brawand et al., 2014). We used the assembled linkage map to determine QTL location and mode of effects for lower jaw tooth and taste bud densities in 263 F_2 animals with complete phenotypic data (see Methods). We integrated single-QTL scans using standard and composite interval mapping with two-dimensional scans to identify pairwise (epistatic) QTL interactions and built multiple QTL models (MQM) incorporating QTL interactions, as well as phenotypic sex as a covariate. Marker quality and size of the data set give us suitable power to detect epistasis and sex-specific effects (Parnell et al., 2012).

The final model for tooth density (logarithm of odds, LOD=32.10) incorporated eight loci and three epistatic effects, accounting for over 43% of the

phenotypic variance in this trait (Phenotypic Variance Explained, PVE=43.12; see Supplemental Table 1, Figure 3.1). The largest effect was seen at position 5.8 cM on chromosome 11, explaining an estimated 15.47% of the phenotypic variance. Another QTL (PVE<8%) was found on chromosome 11 at a location 10 cM distal (11@16.0) to the first. Three other large effect loci were each associated with ~8% of the variance in tooth density, cumulatively accounting for a large proportion of the PVE for this trait. One of these loci (17@34.8) was identically identified as a determiner of taste bud density (Supp. Table 1). Four other loci and one epistatic interaction were included in the full MQM for taste bud density (LOD=13.77, PVE=21.49), and all loci in the taste bud model shared chromosomes and LOD peaks with QTL for tooth density (chromosomes 3, 14, 19, 20; Supp. Table 1, Figure 1). We did not detect a significant effect of phenotypic sex on either density measure. Overall, these genome mapping data suggest that pleiotropy and/or genetic linkage contribute to the observed positive correlation between tooth and taste bud densities in Malawi cichlids.

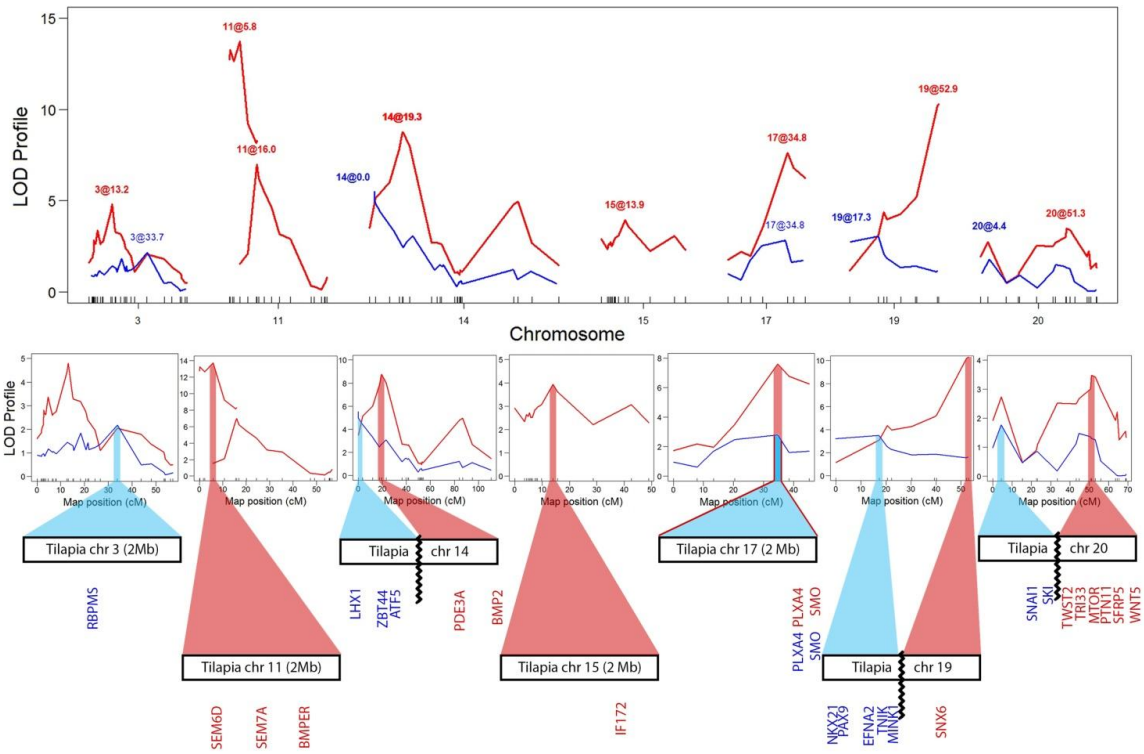

Figure 3.1. QTL profile for significant tooth (red) and TB (blue) genetic effects, with chromosome position plotted against LOD score. Best-scoring SNP markers from MQM models were located in cichlid genomes and all annotated genes 1 Megabase (Mb) on either side were identified. Bottom plot, candidate genes for tooth and taste bud density are indicated along expanded 2Mb portion of x-axis approximately positioned from the center of the peak for tooth (red) and taste bud (blue). Note shared QTL on chromosome 17.

Using annotated cichlid genomes (Brawand et al., 2014), we manually curated all predicted genes within one megabase in both directions (2Mbp total) around the highest LOD-scoring SNPs from MQM models. We highlight positional candidate genes based on either (i) published interactions in placode-derived organ development or (ii) known roles in BMP, FGF, Hh, or Wnt pathways. Notable among these positional candidates is the gene *smo*, a well-known mediator of Hh signaling, in close proximity to 17@34.8 -- a QTL for both

tooth and taste bud density; the gene *bmper*, a modulator of BMP activity, near the largest-effect QTL for tooth density (11@5.8); *bmp2*, well studied in tooth development, near a tooth density QTL (14@19.3); genes *sfrp5* and *wnt5a*, putative effectors of Wnt/β-catenin signaling near the 20@51.3 tooth density QTL (with marginal LOD signal for taste bud density); and *pax9*, known to regulate mouse tongue papillae (Jonker et al., 2004), near a taste bud density QTL (19@17.3).

3.3.2 Cichlid tooth and taste bud fields are specified from a common lamina

Because cichlid teeth and taste buds are co-localized on the jaw, we sought to understand the developmental ontogeny of each. In cichlids and other vertebrates, the earliest markers of the dental epithelium, or dental lamina, are *shh* and *pitx2* (Fraser et al., 2008). Similarly, presumptive taste bud epithelium is known to express *sox2* and *shh* at the earliest stages (Hall et al., 1999; Okubo et al., 2006). We used *in situ* hybridization (ISH) at 5 days post fertilization (5dpf) when the oral jaws first become apparent, to chart the spatial activity of these markers (Figure 2). The earliest oral lamina expresses *pitx2* (a marker of dental epithelium), *shh* (a marker of dental and taste epithelium), and *sox2* (a marker of taste epithelium) with near-overlapping patterns. A day later (6dpf), we observe the first *pitx2*+, *shh*+ dental placodes and the initiation of *calb2*+ (a marker of mature taste buds with more distinct expression than *sox2*) taste buds forming lingual to them (Figure S2, S3). Subsequently (7dpf), a labial band of taste buds

appears flanking the newly forming first row of teeth. Throughout, the epithelial dental field is circumscribed by the odd-skipped transcription factor *osr2* expressed in mesenchyme; *osr2* constrains tooth rows in mice (Zhang et al., 2009). Successive rows of teeth will be added between lingual rows of taste buds as the animal matures (Figure S4). Therefore, each tooth row is flanked by labial and lingual bands of taste buds.

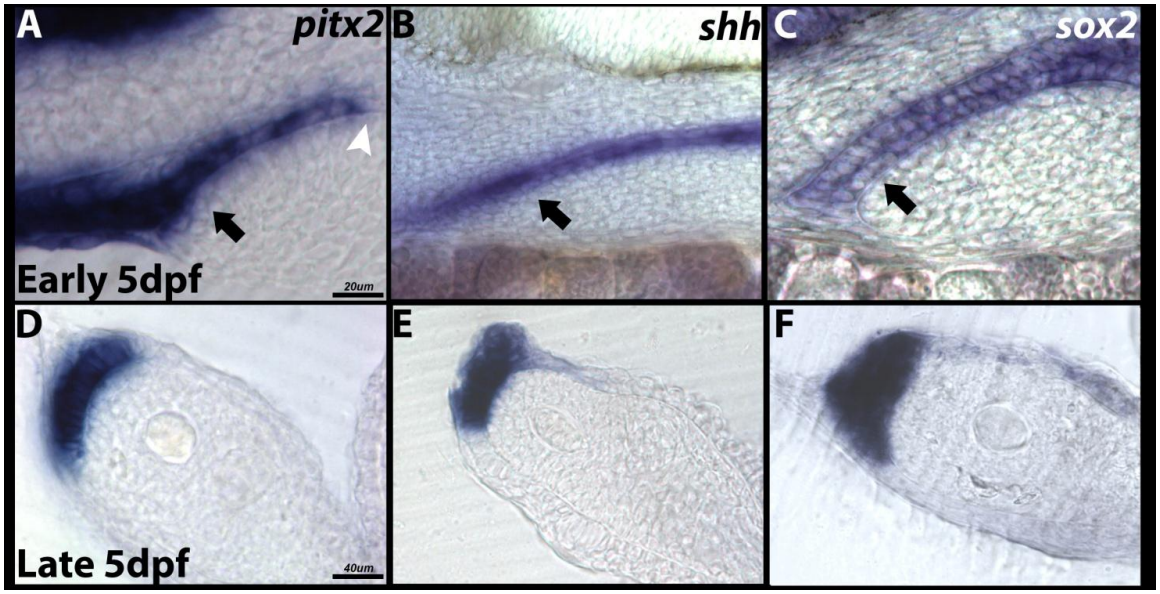

Figure 3.2. ISH of earliest markers of teeth and taste buds. ISH of progenitor dental marker *pitx2* (A,D), progenitor taste marker *sox2* (C,F) and early marker of both, *shh* (B,E). Sagittal section at initial stages of jaw formation, early 5dpf (20X; scale=20µm) and late 5dpf (40X; scale=40µm). Black arrows show shared 1st arch lamina, white arrowhead shows reduced *pitx2* in posterior pharynx. Rostral is to the left of page, ventral to the bottom. 18µm thickness sections.

We focused on the stage of initial tooth and taste bud condensation (6dpf) because this is the first point during which common epithelium becomes

differentiated as either *pitx2*+ tooth or *calb2*+ taste buds. We therefore assayed the spatial expression by ISH of a number of placodal markers as well as genes in the BMP, FGF, Hh and Wnt pathways, in both whole mount and histological section (Figure S5; S6; Supp. Table 2). FGF signal plays known roles in the patterning of teeth and taste buds in the mouse (Neubüser et al., 1997; Petersen et al., 2011) and in the zebrafish pharynx (Jackman et al., 2004; Kapsimali et al., 2011), but function in the oral jaws of teleost fishes is less clear. Here, we observed *fgf10* in condensed dental mesenchyme, *fgf7* in the forming velum lingual to teeth/TBs, and no activity of *fgf8* in the oral organ field (see also (Fraser et al., 2008)). We detected *fgfr1* and *spry4* in the oral epithelium, consistent with the position of initial tooth and taste buds (Figure S6), suggesting that FGF signal is transduced in these placodes. Wnt ligands *wnt7b* and *wnt10a* delineate and mark the tooth placodes respectively; *β-catenin* is expressed broadly across the tooth and taste epithelium. *shh* marks both the initial tooth placodes as well as the lingual taste bud field; the Hh receptor *ptc1* is broadly expressed. *sox2* is expressed in epithelium corresponding to lingual and labial taste bud fields, flanking the *pitx2*+ tooth row. The BMP receptor *bmpr2b* is expressed diffusely in the epithelium of tooth placodes and the lingual taste field, and *bmp4* is strongly activated in both dental epithelium and mesenchyme.

Notably, numerous BMP antagonists are active in the epithelium or mesenchyme marked for lingual and labial taste bud fields; this includes *ectodin* (*wise, sostdc*), *osr2* and *fst*. Similarly, the Pitx2 repressor *tbx1* (Cao et al., 2010) is strongly expressed lingual to the initial tooth row. Taken together, these data

from gene expression suggest roles for the Hh, Wnt and BMP pathways in co-patterning of cichlid teeth and taste buds and furthermore that BMP signaling promotes the differentiation of teeth from a common epithelial field, while multiple BMP and *pitx2* antagonists are protective of *sox2*+, *calb2*+ taste domains.

3.3.3: Positional candidate genes are expressed in teeth and taste buds of fishes and mice

We examined the expression of positional candidate genes from QTL analysis (above) during the placode condensation stage (Figure S4 and S6, inset box). *smo*, a co-receptor in the Hh pathway, near a coincident QTL for tooth and taste bud density, was expressed generally throughout the jaw epithelium, except in the primary dental placodes. As observed in the mouse (Du et al., 2012), activity of this transcript was not detectable at later stages. *bmp2* and *wnt5a*, near separate tooth density QTL, are expressed in dental epithelium and mesenchyme, or in the mesenchyme flanking dental placodes, respectively. *pax9*, near a QTL for taste bud density, was active throughout the mesenchyme subjacent to both tooth and lingual taste bud fields. *sfrp5* and *bmper* are two of our most interesting positional candidate genes, because they have not been heavily studied in oral placode development. Both are expressed in mesenchyme, in complementary anti-tooth patterns. *sfrp5* has been described as a Wnt inhibitor in several systems (Li et al., 2008; Suzuki et al., 2004), an integrator of Wnt-BMP signaling in the zebrafish gut (Stuckenholz et al., 2013),

and a regulator of mouse incisor renewal (Juuri et al., 2012). *bmper* has been reported as both a positive and negative mediator of BMP signaling in other organs of the mouse, frog and fly ((Ikeya et al., 2006), (Moser et al., 2003), (Kelley et al., 2009)), and as an antagonist of BMP in mouse incisor ameloblasts (Cao et al., 2013). We assayed expression of these candidates in species with divergent densities of teeth/taste buds --- C. *afra*, a species with few teeth and taste buds and *L. fuelleborni*, a species with many of both (Figure S7). *bmper* is expressed diffusely in bands flanking the tooth field; expression is stronger in *C. afra*. Notably, *sfrp5* is expressed from the midline in a gradient that encompasses much of the tooth and taste bud fields of both species; expression is strongest and broadest in *C. afra*. These expression differences between species are consistent with antagonistic roles for each gene in BMP (*bmper*) and Wnt (*sfrp5*) signaling, at this stage.

Since these genes have not been well studied in mammal tooth and taste bud development, we assessed gene expression by ISH in teeth on mouse jaws and taste buds on mouse tongues (Figure 3). *Bmper* expression, observed at the molar bud stage (E12.5) was restricted to buccal mesenchyme, a pattern similar to that of *Bmp4* (Figure 3A) (Vainio et al., 1993). By E15.5, expression can be seen surrounding the cap stage tooth bud in mesenchyme, in areas corresponding to the sites of osteogenesis (Figure 3A'). The highest expression level was still observed in buccal mesenchyme, adjacent to the dental epithelium. *Bmper* expression in the developing tongue was restricted to the deeper mesenchyme in the areas where musculature develops (Figure 3 B, B', C, C').

Sfrp5 was also observed predominantly in buccal molar tooth mesenchyme at the bud stage, with some expression in the epithelium at the tip of the bud (Figure 3D). At E15.5, mesenchymal expression of *Sfrp5* is largely undetectable but highly restricted expression was located in the epithelium of the cervical loop with a greater level in the buccal aspect (Figure 3D'). In the tongue, *Sfrp5* expression was weak and diffuse except for a small area corresponding to the single circumvallate papilla that also expresses *Wnt10b* (Figure 3E) (Iwatsuki et al., 2007). By E15.5, punctate expression in the deep tongue mesenchyme was observed (Figure 3E') with localized expression on the dorsal and lateral epithelial surfaces corresponding to (Figure 3F'). In addition *Sfrp5* was expressed in the incisor cervical loop (Figure 3E').

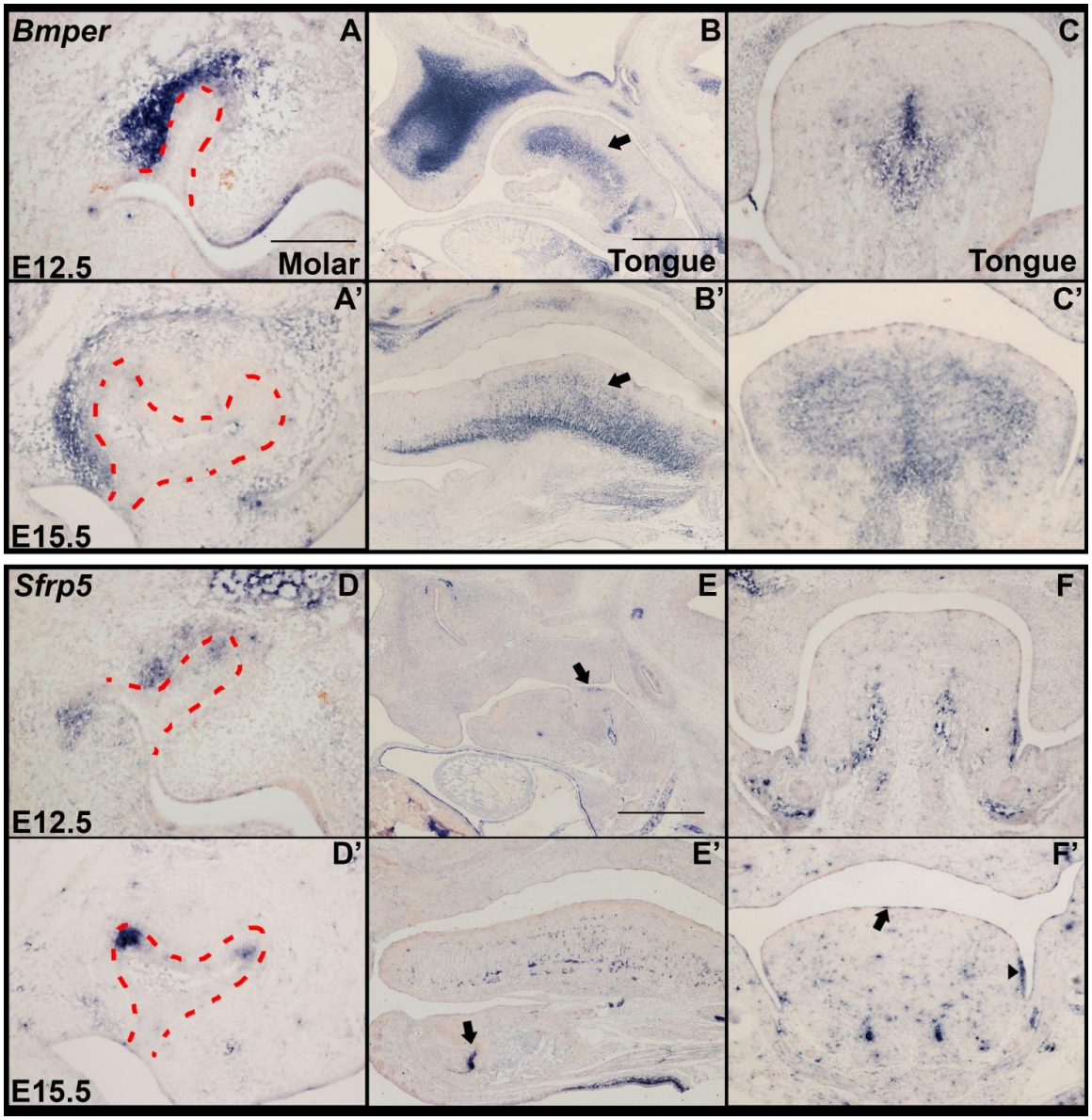

Figure 3.3 Expression of candidates *Bmper* and *Sfrp5* in mouse teeth and tongues. *Bmper* and *Sfrp5* expression, as shown in frontal section of bud stage molar teeth at E12.5 (A, D) and cap stage teeth at E15.5 (A', D') (20x; Scale=200μm). Gene expression in tongue as observed in sagittal (B, B', E, E') and frontal (C, C', F, F') sections at E12.5 and E15.5, respectively (10x; Scale=400μm).

3.3.4: Manipulation of signaling reveals epithelial plasticity via a complex logic of placode specification

QTL and gene expression data implicate the Wnt, BMP and Hh pathways in the regulation of tooth and taste bud densities, as well as the patterning of these organs from a common oral epithelium. To test the precise role of these pathways, we employed small molecule antagonists to modulate signaling during the placode condensation stage. We applied LDN-193189 (LDN), an inhibitor of the BMP pathway; endo IWR-1 (IWR), an antagonist of the Wnt pathway; and cyclopamine (cyc), an antagonist of the Hh pathway, in fishwater, at 6dpf for 24 hours. Split broods received small molecules or vehicle control. Following chemical or sham treatment, a subset of embryos was washed, returned to fishwater and allowed to develop until sacrifice at 14dpf, for quantification of tooth and taste bud densities. A second subset of embryos was sacrificed immediately after treatment, followed by ISH to examine the effects of pathway manipulation on gene expression.

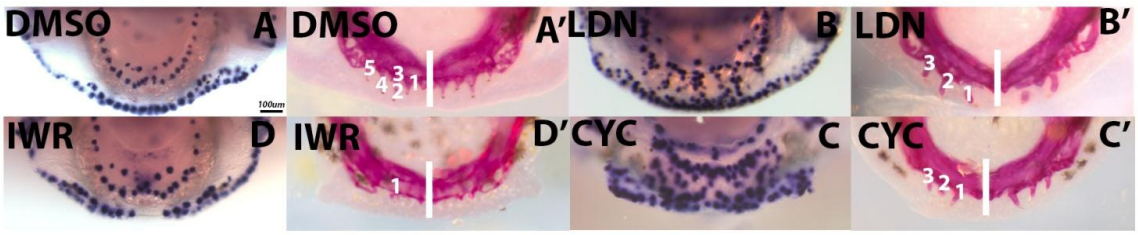

Figure 3.4. Effects of chemical treatment on tooth and taste bud density. *calb2* ISH was used to score TB density (A-D) and cleared and stained jaws were used to score tooth density (A'-D'). Midline of cleared and stained fish marked by white line, teeth in each half of dentary marked by white numerals. Statistics in S6. Dorsal views of dentaries, labial to bottom of page, scale=100µm.

Treatment with IWR significantly reduced the density of teeth and taste buds, compared to control siblings (Figure 4; quantified in Figure S9; P<0.0001). Abrogated or reduced expression levels of *sox2*, *pitx2*, BMP- and Hh-pathway members likely mediate this effect on both oral organs, observed after 24 hours of treatment (Figure S8). The only gene whose expression increased or expanded after IWR treatment was the putative Wnt antagonist, *sfrp5*. Notably, we raised a small number of IWR-treated animals to 1 month and noted a lasting reduction in the density of teeth and taste buds (Figure S10). LDN treatment (knockdown of BMP signaling) resulted in a striking increase in taste bud density at the expense of tooth density (Figure 4; quantified in Figure S9; P<0.0001). In LDN treated animals, taste papillae invaded the tooth field and occupied inter-dental spaces, suggesting the breakdown of developmental boundaries. It is possible that *calb2*+ cells migrated to occupy the normally obligate tooth field, or that the oral epithelium holds inherent plasticity at this juncture. Observations of gene expression 24 hours after treatment support the latter notion (Figure 5). LDN treatment resulted in reduced expression of dental placode transcription factors *pitx2* and *lef1*, and dramatic expansion of *sox2* and *shh* into the tooth field. *calb2*+ taste papilla development was accelerated and the putative BMP antagonists *osr2* and *bmper* showed up-regulation and expanded spatial domains, as observed for *Osr2* in mouse conditional *Bmp4* knockouts (Jia et al., 2013). Similar to knockdown of BMP signaling, Hh antagonism via temporary cyclopamine treatment reduced the density of teeth and increased the density of taste buds, compared to controls (Figure 4; quantified in Figure S9; P<0.0001).

This appears to be mediated by an increase in both lingual and labial taste bud fields coincident with a smaller dental domain. After just 24 hours, cyclopamine almost completely abolished expression of the *ptch1* receptor (Figure 5). As in LDN treatment, cyclopamine exposure yielded an expansion of TB markers *calb2* and *sox2* at the expense of the *pitx2*+ tooth field. Expression of the transcription factor *pax9* was reduced; we noted negligible effects on *bmp2* expression.

Finally, to assay if ectopic taste buds in taste and dental fields after LDN and cyclopamine treatment held potential for functional activity, we employed double whole-mount immunohistochemistry against; 1) Acetylated-Tubulin (red, Figure 6), a marker of innervation (LeClair and Topczewski, 2010), and 2) Calretinin/Calb2 (green) and visualized fluorescence using nonlinear optics (NLO) in conjunction with multiphoton microscopy. Taste buds that invade the tooth field after LDN treatment were well innervated, while following cyclopamine treatment their counterparts were not innervated (arrows). Recent study in adult mice shows that mis-expression of Shh is sufficient to induce ectopic taste placode development in lingual regions of the tongue, surprisingly independent of innervation (Castillo et al., 2014). Those data, in conjunction with ours, highlight differing roles of Hh and innervation in embryonic development versus adult maintenance of taste placodes (Barlow et al., 1996).

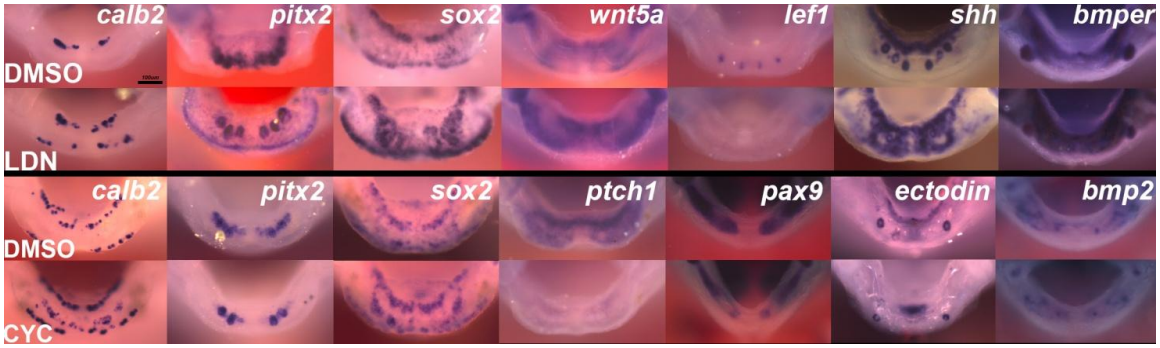

Figure 3.5. Changes in gene expression following treatment. ISH of genes in cichlid dentary following 24h treatment with LDN or CYC initiated at 6dpf and immediate sacrifice. Dorsal views, labial to bottom of page, scale=100µm.

To sum up, our treatment data demonstrate that when teeth and taste buds are patterned from common epithelium, Wnt signaling exerts a positive influence on the densities of both organs, while BMP and Hh signaling both promote, or reinforce, the development of teeth. Assessment of gene expression after treatment suggests that the Wnt pathway acts upstream of BMP and Hh as teeth and taste buds initiate development. Notably, the epithelial plasticity (or bi-potency) revealed from both LDN and cyclopamine treatments may be a common feature of organ systems like teeth and taste buds that function together, as observed for liver and pancreas (Xu et al., 2011), and hearts and lungs (Peng et al., 2013).

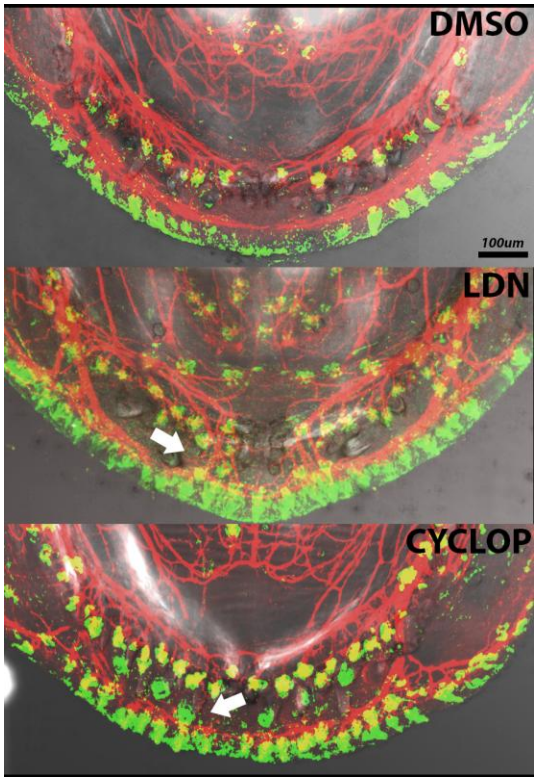

Figure 3.6. Effects of treatment at protein level. Dorsal views of dentary after whole-mount IHC of TB marker Calb2 (green) and nerve marker Acytelated Tubulin (red) following LDN or Cyclopamine treatment. 3-D rendering of 150µm optical sections overlay to bright-field image at 10X. White arrows indicate ectopic TB. Labial to bottom of page, scale=100µm.

3.4: DISCUSSION

3.4.1: Deep ancestry of the Wnt-BMP-Hh regulatory hierarchy in oral placode development

In many vertebrates, teeth and taste buds are co-localized in the oro-pharyngeal cavity and function together as potential food items are assessed, acquired and then (in some cases) masticated. In Malawi cichlids, the number and density of teeth and taste buds varies widely (Fraser et al., 2008) and the

positive phenotypic correlation between tooth and taste bud densities makes adaptive sense. Planktivores typically assess food/prey using acute vision and swallow it whole – they tend to have reduced tooth and taste bud densities. By contrast, algivores use taste and smell to make food choices and then employ flexible or shearing teeth to comb or nip from the substrate – they generally possess many teeth and many taste buds.

We showed here that the phenotypic correlation between tooth and taste bud densities is, at least partly, explained by genetic variants in common regions of the cichlid genome. We identified positional candidate genes in the BMP, Hh and Wnt pathways whose gene expression in fishes and mouse suggest conserved roles in the patterning of teeth and taste buds, regardless of whether these organs are co-localized from common epithelium (fish), or located in spatially restricted parts of the mouth (mouse). Using QTL and gene expression data as a lead, we manipulated BMP, Hh and Wnt pathways and demonstrated that Wnt signaling couples the density of teeth and taste buds, while BMP and Hh signaling promote the development of teeth at the expense of taste buds, at these early stages.

Taken together, these observations are notable for two reasons. Firstly, manipulation of Wnt signaling in cichlid morphants provides a positive correlation between tooth and taste bud densities, similar to the correlation we observe across natural species and in the F_2 of our intercross. Modulation of Wnt signaling early in the development of cichlid teeth and taste buds is sufficient to phenocopy natural differences among cichlid species (Figure S10). Second, our

small molecule treatment experiments, followed by assays of gene expression, suggest that Wnt acts upstream of BMP and Hh in the co-patterning of tooth and taste bud fields from common epithelium. Therefore, the function of these pathways, as well as their relative position in a regulatory hierarchy, is strikingly consistent between fishes and mammals, given the noteworthy spatial difference in organ distribution (Figure 7). In cichlids, Wnt signal promotes the initiation of both organs, as well as BMP and Hh from a bi-potent epithelium. As tooth and taste placodes mature, distinct fields develop wherein taste buds are patterned initially in a proximal or lingual zone to the tooth field and are recruited by Wnt signals, but repressed by BMP and Hh elsewhere. In contrast all three pathways synergistically support the maturation of tooth placodes distally. Finally, neural crest derived mesenchyme is induced to the placodes under a BMP+ tooth environment expressing *bmp2,4* and a BMP- taste environment, likely restricted by repressors like *osr2* and possibly *bmper*. It is likely that factors expressed by maturing tooth and taste buds are in turn restrictive to one another and help delineate the respective fields (Figure 7).

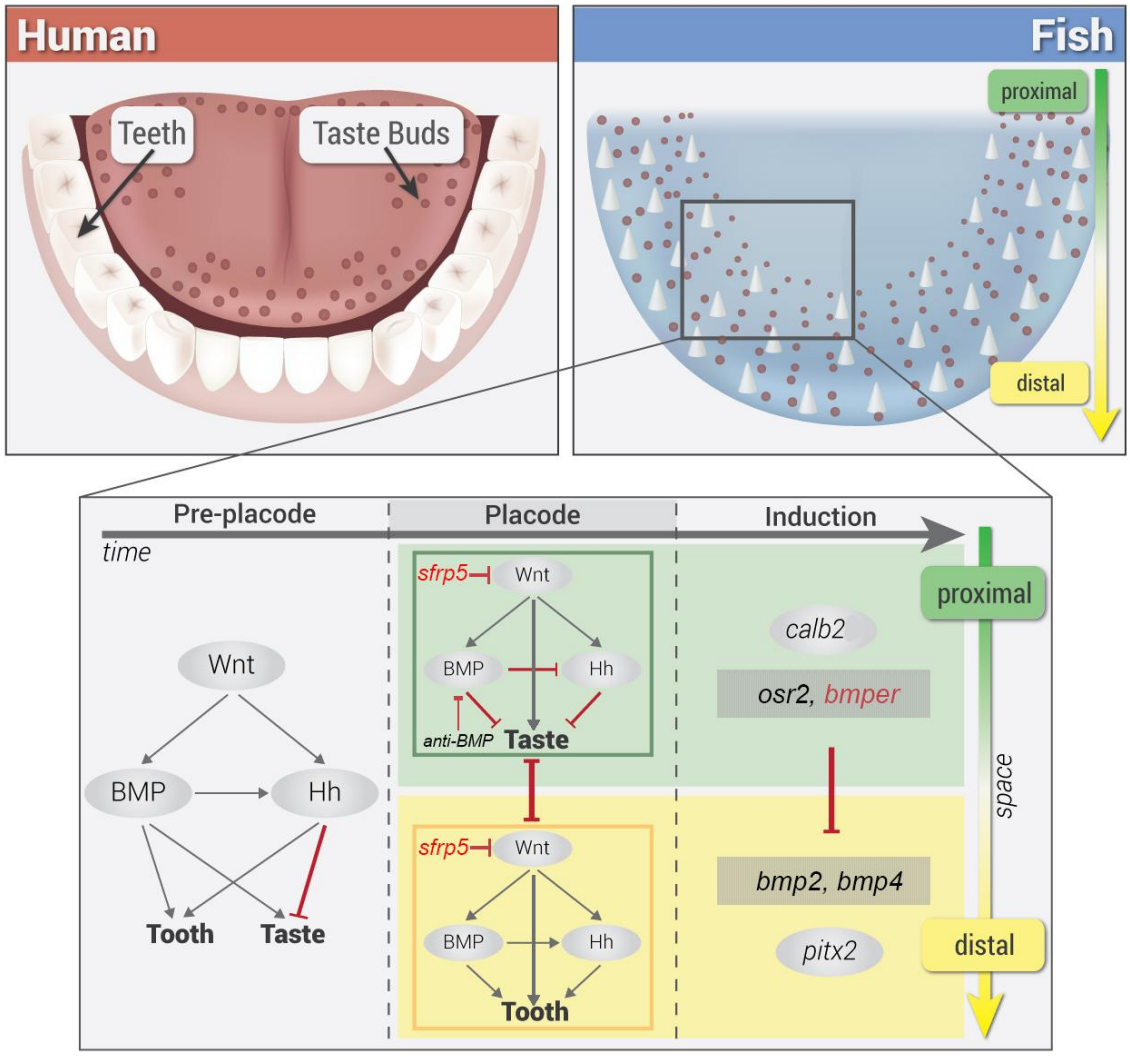

Figure 3.7. A model of evolutionary conserved patterning networks for oral organs. Humans possess TB on the tongue and teeth on the dental arch, while these organs are co-patterned in cichlids and other fishes. Inferred roles of Wnt-BMP-Hh interactions and effects on tooth and TB patterning in cichlids represented before placode condensation, during placode formation, and during induction of mesenchyme to both organs, from proximal to distal of horizontal plane. Genomic candidates are highlighted in red text. Pre-placode stage represents the model of placode recruitment from a bi-potent epithelium. These interactions are consistent with what has been reported in the mouse for taste bud [9, 11, 69, 70] and tooth [8, 21, 37, 46, 48, 71, 72] patterning networks, when studied independently. Based on cichlid expression and treatment data, Wnts drive the formation of taste placodes proximally and tooth placodes distally, while BMPs and Hhs are inhibitory of taste bud differentiation and permissive for tooth germs. Anti-BMPs, such as *osr2*, may reduce BMP activity in the taste field to promote taste bud formation and *sfrp5*, expressed in both fields, may repress Wnt signaling. At the point of mesenchyme induction, *pitx2* is expressed in tooth

placodes and the underlying mesenchyme expresses *bmp2/4*. Likewise, taste buds express *calb2* and anti-BMPs *osr2* and *bmper* are expressed in lingual mesenchyme.

In the mouse, Wnt/β-catenin signal is one of the earliest markers of dental placodes on the jaw margin and taste placodes on the tongue, acting upstream of BMP and Hh in both organs (Liu et al., 2008; Liu et al., 2007). Up-regulation of Wnt signaling in the mouse dentition, via constitutive activation of β-catenin or genetic ablation of Apc, leads to extra teeth (Järvinen et al., 2006; Wang et al., 2009). Similarly, culture of mouse tongues with LiCl, an agonist of Wnt/β-catenin, and the activating β-catenin mutation, both increase taste papillae number and size (Iwatsuki et al., 2007; Liu et al., 2007). Hedgehog signaling is necessary for proper development of teeth in fishes and mammals (Dassule et al., 2000; Fraser et al., 2008) and ectopic activity in the mouse diastema leads to extra teeth (Ohazama et al., 2009). Culture of mouse tongues with 5E1, an antibody against Shh leads to more papillae; culture with purified Shh reduces papillae number (Iwatsuki et al., 2007). Numerous reports using gene targeting have shown that ablating function of BMP antagonists [Ectodin (Sostdc1, Wise; (Kassai et al., 2005)); Noggin (Plikus et al., 2005); Osr2 (Zhang et al., 2009)] increases tooth number. Likewise, ectopic expression of the BMP antagonist Follistatin, using the K14 promoter, reduces tooth number (Wang et al., 2004). BMP data for mammalian taste papillae are complicated and stage-specific. A variety of BMPs are expressed in mammalian tongue papillae with considerable variation across stages (Kawasaki et al., 2012). Zhou and colleagues (Zhou et al., 2006) report

that culture of rat tongues with purified BMPs or the antagonist Noggin at E13 results in increased numbers of taste papillae, but that treatment at E14 with BMPs decreases papillae while Noggin treatment increases them. Interestingly, genetic ablation of Follistatin increases BMP signal in the mouse tongue and gives rise to ectopic posterior papillae that express *Sox2* and *Foxa2*, but which appear to invaginate rather than evaginate like typical taste buds (Beites et al., 2009). Overall, teeth and taste buds share gene synexpression and a deep molecular homology (Fraser et al., 2010).Our work here implies that the Wnt-BMP-Hh regulatory hierarchy patterning these organs is conserved, despite release from the constraint of spatial co-localization in mammals (and other vertebrates). The conservation of regulatory interactions is a well-known tenet of development for homologous structures (e.g., the heart, (Davidson and Erwin, 2006)) and core regulatory circuits are sometimes used in different organs, such as the case of Pax-Dach-Eya-Six in eye and muscle development (Heanue et al., 1999). We highlight a special case of conservation in evolutionary development, wherein the function and interaction of signaling in independent regulatory networks (to make teeth on the mammalian jaw margin and taste buds on the tongue) may be evolutionary remnants of a single gene circuit that evolved long ago to co-pattern these organs from common oral epithelium.

3. 5: Materials and Methods

3.5.1: Cichlid husbandry

Species of Lake Malawi cichlids used in this analysis include: *Tramitochromis intermedius* [TI], *Cynotilapia afra* [CA], *Labeotropheus fuelleborni* [LF], *Metriaclima zebra* [MZ], *Petrotilapia chitimba* [PC] and *Pseudotropheus elongatus* (PE). These species were chosen to represent diversity in tooth and taste papillae densities. Adult cichlids were maintained in re-circulating aquarium systems at 28°C (GIT). Fertilized embryos were removed from the mouths of brooding females and staged in days post-fertilization (dpf) according to Nile Tilapia developmental series (Fujimura and Okada, 2007). Embryos were raised to desired stages for chemical treatment or euthanized with MS-222 for fixation in 4% paraformaldehyde followed by dehydration into MeOH.

3.5.2: Mouse husbandry

Outbred CD1 mice were obtained from Charles River. Noon of the day when a vaginal plug was detected was designated as E0.5. Time-mated embryos were collected at E12.5, E13.5 and E15.5. All animal procedures were approved by the UK Home Office.

3.5.3: Tooth and taste bud phenotyping in cichlid adult F_2

Following euthanasia (IACUC standard protocols) measurements of standard length (nearest mm) were taken on each individual and the dentary was dissected out in 70% EtOH. Jaws were rehydrated in reverse osmosis purified

water (60 seconds) and immersed in toluidine blue for 60 seconds to visualize taste buds (Su et al., 2013). The dentary was photographed at 7 – 16X on a Leica MZ16 dissecting microscope with a scale bar merged to each photo. Taste buds (found in the shared epithelial field, see below) were counted in ImageJ (Schneider et al., 2012) using the cell counter plugin. Jaws were then cleared with KOH and glycerin using standard protocols (Dingerkus and Uhler, 1977) to allow accurate counts of all teeth (erupted and replacement teeth) as well as measurement of the tooth/taste field. Each dentary was then photographed again as described above, without staining. Tooth counts were made using ImageJ (Schneider et al., 2012). The tooth/taste bud field was quantified by calibrating the scale bar in ImageJ and creating a polygon extending around the entire field; this delineation included the area in which teeth and taste buds were quantified above. Total tooth and taste bud counts were divided by total shared tooth/taste bud field area to account for any potential differences in jaw size due to allometry.

3.5.4: QTL mapping of cichlid tooth and taste bud densities

We used RAD-tag SNPs to map tooth and taste bud densities in F_2 animals, as described previously for other phenotypes (Parnell et al., 2012; Streelman et al., 2003a), with one important addition. From genome-anchored linkage maps of *Oreochromis niloticus* and *Metriaclima zebra* (Brawand et al., 2014), we handpicked an additional set of SNPs predicted to fill gaps in linkage group coverage. This second group of SNPs was genotyped in the same F_2 population using the Fluidigm Dynamic Array (Parnell et al., 2012). This resulted

in a fully informative set of SNP markers covering the cichlid genome, with nearly complete genotypic data across F_2 (0.4% missing data). A genetic linkage map was constructed with SNP marker genotype data using JoinMap® 3.0 software, as described previously (Parnell et al., 2012). The map was created using Kosambi's mapping function, a LOD threshold of 1.0, a recombination threshold of 0.4, a jump threshold of 6.0, and a ripple function with no fixed order of loci. A LOD threshold of 4.0 was used to join 370 loci in 22 linkage groups with a total map size of 1381 cM and average marker distance 4.38 ± 1.85 cM with an average of 16.8 markers per linkage group. To facilitate comparison to other genetic maps in Malawi (Albertson and Kocher, 2005; Albertson et al., 2005; Parnell and Streelman, 2012; Streelman et al., 2003b) as well as tilapiine cichlids (Lee et al., 2005), our linkage group names represent consensus cichlid chromosomes.

The linkage map was used to determine genomic locations for tooth and taste bud densities in the F_2 population using the R/qtl package (Broman and Sen, 2009). We used an iterative approach by scanning for single QTL with standard and composite interval mapping (CIM), followed by two-dimensional scans to (i) identify QTL x QTL interactions (i.e., epistasis) and (ii) detect additional QTL. Finally, using results of the previous steps we built multiple QTL models (MQM) incorporating QTL interactions and covariates (i.e., sex). In the MQM process, we used a forward-backward selection algorithm to add and remove QTL based on overall model effects and the effects of single QTL as they were removed from the model. Genotype-phenotype associations are scored

using the logarithm of the odds (LOD) which represents the $\log_{10}$ likelihood ratio comparing the hypothesis of a QTL at a marker location to the null hypothesis of no QTL ($LOD=(n/2)\log_{10}(RSS_0/RSS_1)$; RSS=residual sum of squares (Broman and Sen, 2009). The variance in a phenotype is assigned to each significant QTL (or covariate) and reported as percent variance explained (PVE) in the analysis output. The total variance accounted for by QTL is a proxy for the heritability of a trait and is calculated as $1 - 10^{-(2/n)LOD}$ (Broman and Sen, 2009). Significance thresholds for LOD scores were estimated using 1000 permutations of phenotypes relative to genotypes to build a distribution of maximum genome-wide LOD scores. From this distribution, the 95th percentile LOD score was calculated to serve as a threshold for significant QTL associations (Broman and Sen, 2009).

To identify positional candidate genes, we manually curated all predicted genes within 1 Mb up- and downstream from the highest LOD-scoring SNPs from MQM models, using annotated cichlid genomes (Brawand et al., 2014). Candidates were selected based on published interactions in placode-derived organ development or a known relationship to BMP, Hh, Wnt, or FGF signaling pathways.

3.5.5: Cichlid in situ hybridization

Digoxigenin-labeled antisense riboprobes were prepared using partial cichlid genome assemblies (Loh et al., 2008) as well as recently assembled tilapia and MZ genomes (Brawand et al., 2014). DNA sequence diversity across

the Lake Malawi assemblage is 0.28%; less than reported values for laboratory strains of zebrafish. cDNA sequences for probe design have been deposited in GenBank (accession numbers XXXX-XXXX). ISH was performed according to previously published protocols (Fraser et al., 2008; Fraser et al., 2013). Embryos were re-hydrated from MeOH and ISH was carried out in whole-mount. Digoxigenin-labeled antisense riboprobes were generated using the Riboprobe System Sp6/T7 kit (Promega). AP-conjugated anti-dig antibodies were visualized at the end of color reaction (NBT/BCIP; Roche) using Leica Mz16 dissecting microscope. Embryos were embedded in chick albumin cross-fixed with 2.5% gluteraldehyde and post-fixed with 4% PFA. A Leica Microsystems VT1000 vibratome was used to cut sections at 15-25µm. Histological sections were then mounted with glycerine and imaged at 10-63x using a Leica DM2500 compound microscope.

3.5.6: Mouse histology and in situ hybridization

Embryos were dissected in ice-cold PBS, fixed in 4% PFA overnight at 4^{o}C, before embedding in paraffin wax. Serial sections of the embryo were obtained at 10mm and dried overnight at 42^{o}C. ISH was carried out using standard methods (Wilkinson et al., 1989). Digoxigenin-labeled antisense probes for *Sfrp5* (IMAGE ID 1395864) and *Bmper* (IMAGE ID 3483063) were used. After completion of the color reaction, sections were counterstained with nuclear fast red, dehydrated and mounted with DPX.

3.5.7: Treatment of cichlid embryos with small molecules

Stock solutions were prepared for each chemical treatment experiment using Dimethyl Sulfoxide (DMSO, MP Biomedicals). Stock solutions were as follows: 10µm LDN-193189 (Enzo) in DMSO, 50mM endo IWR-1 (Enzo) in DMSO, and 16mM Cyclopamine (LC Laboratories) in DMSO. Cichlids were raised to 6 dpf and embryos from single broods were split into small molecule and solvent control groups. All chemical and control experiments were performed in Erlenmeyer flasks at 28°C in an oscillating platform culture incubator (Barnstead Lab-Line Max 4000). Treatments were performed at 1.5µM LDN, 3.75µM IWR, and 2.5µM Cyclopmamine. After 24 hours treatment in the small molecule dilution, fry were sacrificed immediately. ISH was carried out on experimental animals to understand effects of treatment on gene expression.

Alternatively, embryos were washed extensively with fresh fish water and raised to 14dpf for sacrifice to understand effects of treatment on organ densities. Embryos were fixed and were either cleared and stained to assay effects of treatment on tooth density or ISH for the taste marker *calb2* was performed to assay effects of treatment on density of taste papillae. Tooth and taste densities were measured in different animals because the clearing/staining process and ISH damaged the tissues in combination. Post treatment and staining, dentaries were photographed using a Leica MZ16 dissecting scope. Teeth (C&S) or taste buds (*calb2* ISH) were counted manually using ImageJ software, and jaw size was calculated using the measure function which converted pixel area of scale bar to millimeters. Total tooth and taste bud counts were divided by jaw area to

account for possible size differences between specimens. All measurements for TB controls were pooled because experiments were conducted in the same species (PC) while those for tooth density were analyzed separately because experiments were conducted in different species (IWR-PC, LDN/CYC-MZ). Data were organized into box plots for each treatment set using JMP 11.0 software. Mann-Whitney U non-parametric tests were performed to test the null hypothesis of equal organ densities between treatment and control.

3.5.8: Cichlid whole-mount immunohistochemistry (IHC) and NLO microscopy

For IHC, embryos were sacrificed and fixed in 10% NBF for 24 hours at RT. Antigen retrieval was performed by washing 3X10min. PBS, placing in 2β-mercaptonol for 1 hr, washing in PBS and incubating at 70°C 150mM Tris-HCL for 1hr. Embryos were incubated in blocking solution (3% goat serum, 1% bovine serum, 0.1% Triton 100X) for 3hrs RT, followed by 48hr incubation in a 1:1000 dilution of rabbit anti-calretinin [Millipore] and mouse IgG anti-acetylated tubulin [Sigma] at 4°C in blocking solution. Embryos were then washed 6X1hour at RT in PBS and incubated 24 hours at 4°C in 1:400 alexa-fluor 488 goat anti-rabbit IgG [Molecular Probes] and alexa-fluor 568 goat anti-mouse IgG [Molecular Probes]. Unbound secondary antibody was removed by washing 48 hours in PBS and specimen were stored in a 50:50 glycerin:Vectashield mixture for imaging. Deep tissue whole-mount fluorescence was imaged by mounting embryos on glass depression slides and scanning with nonlinear optics (NLO) using a Zeiss 710

system coupled with multiphoton microscopy. Conjugated antibodies were excited with a Coherent Chameleon Ti:Sapphire laser at 780nm and scanned at their respective wavelengths.

3. 6 Acknowledgements

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CHAPTER 4

PLASTICITY OF STEM CELLS IN TOOTH AND TASTE BUD RENEWAL

4.1 Abstract

We use the continuously replacing dentition of Lake Malawi cichlid fishes to understand de-novo tooth replacement in adult vertebrates. In this system, each tooth is replaced in a one-for-one fashion every ~50 days. Here, we explore the source of epithelial stem cells for tooth replacement. We first characterized spatial expression of putative stem cell factors, including *bmi1*, *celsr1*, *igfbp5*, *hopx1*, *lgr4/6*, *sox2* and Wnt/Hh activity, in replacement teeth (RT). We noted that RT shared epithelium with adjacent taste buds (TBs) and that both organs co-expressed putative stem factors in subsets of cells. This is intriguing because both organs regenerate continuously in cichlids. We identified putative stem cells in RT and TBs after double IHC experiments with markers Bmi1, Gli3, Sox2 or Trp63, and BrdU after 100 days of chase. In the early patterning of cichlid teeth and taste buds, BMP signaling appears to promote tooth development at the expense of TBs and knockdown of BMP reveals developmental plasticity between these organs that share a common epithelium. We dissected replacement tooth germs and TB bearing oral epithelium for RNA extraction, on which we performed next generation RNA-seq. Transcriptome analysis reveled differential up-regulation of BMP family members in RT compared to TB oral epithelium. Similarly at replacement stages, we observed *bmp2/4* expressed in RT but outside of TBs using ISH. Morphants bathed in the BMP antagonist LDN

exhibited RT with abrogated *shh* expression in the inner dental epithelium (IDE) and the ectopic expression of *calb2* (a TB marker) in these very cells. Our data suggest that RT epithelium retains inherent plasticity to form non-dental cell types. These findings may reveal an endogenous supply of oral stem cells with promising potential in bioengineering and dental therapeutics.

4.2 Introduction

Roughly 25% of the human population is born with congenital defects of the dentition arising from genetic aberrations (Fleischmannova et al., 2008).While this figure is significant, according to the World Health Organization a far greater number, approaching 100 percent, of all human beings will suffer from dental disease throughout their lifetimes arising from dental disease and selective tooth loss. Most vertebrates, including the majority of fishes and reptiles, are defined as polyphydonts by their prowess to replace teeth many times over and usually throughout ontogeny. In contrast, most mammals including humans are diphyodont and have evolved to possess only two sets of teeth: a primary dentition and a successional adult dentition that must serve throughout adulthood. Further still, evolution has progressed towards specialized cases of a reductive dentition in monophyodonts like most rodents, favoring the capacity to renew damaged tissues over the capacity for whole tooth-replacement. Although the human “adult” or successional replacement tooth (RT) possesses limited regenerative potential in the cellular pulp chamber and periodontal ligament,

injury to the adult tooth is largely irreversible. The fields of restorative and prosthodontic dentistry have traditionally sought to address dental morbidities by employing synthetic tissue substitutes forged from alloys, plastics, and ceramics. On the forefront of regenerative medicine the emerging field of regenerative dentistry alternatively looks to cell-based dental repair aimed at replacing damaged oral structures with live tissues. By transplanting cells or cell scaffolds into oral tissues, biomedical engineers have cultured teeth and transplanted them in mice to achieve ideal dental function and aesthetic where it was once lost (Kim et al., 2010; Oshima et al., 2011).

A caveat of regenerative medicine is the prerequisite of an adequate reservoir of cells to compose a desired tissue. Because stem cells (SCs) possess the ability to both self renew and generate vast numbers of several cell types, they underlie most biological regenerative processes. In the instance of therapeutic research SCs are typically either harvested from sources such as embryonic and animal tissues or generated from somatic cells induced to carry out stem-like functions. While each holds immense clinical potential, these types of cells are often muddied with hurdles to practical applications arising from immunogenicity, ethical dilemmas, tumorigenicity and degrees of potency. Consequently it stands to reason that a clinically idyllic SC would be both endogenous to the patient and similar in native potential to generate the desired tissue. In the pursuit of tooth regeneration, the top cellular candidates for the task would be dental SCs or their nearest neighbor.

While at the turn of the century we knew relatively nothing about SCs that mediate tooth renewal and replacement, we have now gleaned a great deal about de-novo dental regeneration from a host of models. The mouse compensates for its monophyodont dentition by renewing damaged enamel asymmetrically on the labial surface of its incisors, which bear the brunt of primary mastication. At the base of each incisor lies, known as the cervical loop (CL), a stem-cell niche that has become a powerful model for understanding SC biology and the generation new of adult tissues in-vivo. Initially the epithelial stem cell (ESC) niche was the focus of this work. Mechanistically, a histologically apparent group of mesnmchymal-like epithelial cells, called stellate reticulum (SR), lie sandwiched in between an inner (IEE) and outer (OEE) enamel epithelium. A subset of these cells from within the SR serve as stem/progenitor cells to the epithelium, differentiating into transit amplifying (TA) cells that will multiply to form pre-ameloblasts and ultimately enamel-secreting ameloblasts along the iee (Harada et al., 2002; Wang et al., 2007). Several factors common to ESCs have been identified within this stem-niche that referee labial enamel renewal in mice. For instance, the polycomb group gene *Bmi1*, well-known for its participation in neural (Molofsky et al., 2003) and hematopoietic (Park et al., 2003) self-renewal, is now known to contribute to incisor SC renewal through repression of *Ink4a*/*Arf* and *Hox* genes (Biehs et al., 2013). *Sox2* has been used as a marker of the putative ESC niche in a cadre of mammals and reptiles (Juuri et al., 2013), cichlid fishes (Fraser et al., 2013) and sharks (Tucker and Fraser, 2014) and genetic fate mapping experiments have demonstrated that *Sox2*-

positive SCs contribute to all lineages of the dental epithelium (Juuri et al., 2012). It stands to reason, however, that if mesenchymally derived tissue, (ie dentin), is to be regenerated then progenitors must exist for this tissue type as well. Recently, nerve-associated SHH protein ligand has been demonstrated to activate *Gli1*-postive mesenchymal stem cells (MSCs) in mouse incisors, where dennervation experiments lead to a disruption of the MSC niche and defects in the generation of odontoblast and pulpal derivatives (Kaukua et al., 2014; Zhao et al., 2014).

These genetic and cellular interactions are not unique to SCs belonging to rodent incisors. In fact, many were first recognized in other placode derived organs such as hair, feathers, taste buds and scales, all of which self-renewal across ontogeny, show similarities in development and anatomy, and likely share a common ancestor (Dhouailly, 2009; Sharpe, 2001). For instance, nerve associated *Shh*- activation of *Gli1* responsive MSC's was first demonstrated in mouse hair follicles (Brownell et al., 2011) and evidence of the same pattern has been reported in taste buds (Castillo et al., 2014; Liu et al., 2013). *Lgr5* was shown as markers of a population of SC's in intestinal crypt-villi (Barker et al., 2007), later shown to be distinct from *Bmi1* positive SCs (Yan et al., 2012), while *Lgr6* from the hair follicle was demonstrated to generate all lineages of the dermis (Snippert et al., 2010). Congruently, *lgr5* is co-expressed with pulse-chase labeled BrdU-positive putative SCs of teeth that undergo of whole replacement in the gecko (Handrigan et al., 2010), as well as in the mouse incisor ESC niche (Suomalainen and Thesleff, 2010).

We set out to understand the phenomenon of vertebrate whole-tooth replacement in Lake Malawi cichlids, fishes that have the ability to replace each of their teeth with fidelity approximately every 50 days(Hildebrand et al., 1995). This is quite a task for animals that occupy a rainbow of dental formulas, with hundreds to thousands of teeth depending on the ecological demands of the particular Malawi cichlid species. In a previous study where we explored the phenomenon of cichlid RT formation with respect to tooth shape. We noted that each RT maintained a physical connection, through a ribbon of epithelium known as the succesional dental lamina, to a superficial ball of “onion-shaped” cells labial to the functional tooth (FT) and that expressed proliferative factors such as *β-cat* and *sox2* throughout each RT germ’s maturation (Fraser et al., 2013). Closer examination of this population through histology revealed that this sub-set of cells was actually a taste bud (TB). In another study we described the co-patterning of cichlid oral organs (Bloomquist et al., in submission), specifically teeth and taste buds, and we came across three intriguing findings: First, we noted that in cichlids teeth and TBs arise from common epithelium embryonically and that the dental lamina is really an “oral lamina” with bi-potency to form either tissue. Next we found that while many factors are shared across oral tissues, certain genes such as bone morphogenetic proteins (BMPs) were expressed in the presumptive dental field and flanked by BMP antagonists such as *osr2* and *bmper* in the presumptive taste field. Finally, we discovered that while teeth and TB placodes are patterned in distinct fields, chemical manipulation of gene

function, such as BMP antagonism, demonstrated the plasticity of embryonic oral epithelium by forming TBs in the dental field.

Given the described ancestral and embryonic homology between teeth and what is perhaps their closest cousin spatially and developmentally, the TB, we set out to uncover the SCs in these two organs responsible for their constant renwal in such close proximity. Furthermore, we wanted to understand why RT and TB have such an intimate connection maintained, by the dental lamina, and why this connection was sustained for much of the RT germ's incubation. We first used in-situ hybridization (ISH) for a host of adult SC markers to localize putative stem cell populations in each organ, ne of which is new to what is currently understood about tooth renewal. Next, we verified the "stemness" of these populations through pulse-chase analyses coupled with immunohistochemistry (IHC) against SC factors. We then extracted tissues and perfomed RNA-seq, and through independent analysis with ISH highlighted BMP factors to be differentially expressed in RT and not TB. Finally, we utilized a chemical antagonist of BMPs to alter the characteristics of TA cells within the RT germ, which after treatment favored TB expression profiles to that of normal RT. Our study highlights the homology of these two organs in lower vertebrates, not just in their proximity and structure, but in their prowess to renew and the cells that their stem populations can generate; and, incriminates taste bud SCs, a reservoir of SCs that can be endogenously harvested from most patients, as top-candidates for dental therapeutics.

4.3 Materials and Methods

4.3.1: Fish Husbandry

Adult Malawi cichlids were housed in re-circulating aquarium systems at 28°C (GIT) for embryo production. Species of Lake Malawi cichlids include *Labeotropheus fuelleborni* [LF], *Metriaclima zebra* [MZ], *Petrotilapia chitimba* [PC] and were selected based on embryo availability with a preference toward MZ, owing to their genome assemblage (Brawand et al., 2014) and partial albinism morph which permitted better imaging of histological stain. Fertilized embryos were harvested from mouth brooding females and staged in days post-fertilization (dpf) according to Nile Tilapia developmental series (Fujimura and Okada, 2007). Embryos were raised to desired stages for ISH, pulse-chase experiments, or chemical treatment and euthanized with buffered MS-222 for fixation in either 4% paraformaldehyde or 10% neutral buffered formalin.

4.3.2: In situ hybridization

Primers for target probe sequence were designed using the published and annotated genomes of tilapia species *Oreochromis niloticus* (Brawand et al., 2014) and the aligned genome of Malawi cichlid *Metriaclima Zebra* from the University of Maryland Cichlid Blast Server Tool. It has been reported that genomic sequence diversity across the Lake Malawi assemblage is 0.28%, less than reported values for laboratory strains of zebrafish (Loh et al., 2008), and riboprobes were reactive across Malawi cichlid species. Target sequences were transformed and cloned, and sequences were deposited in GenBank (accession

numbers XXXX-XXXX). Riboprobes were synthesized and labeled with Digoxigenin (Roche) using the Promega System Sp6/T7. In-situ hybridization was performed using previously published methods in whole-mount (Fraser et al., 2008) and visualized using a AP conjugated anti-digoxigenin antibody (Roche) to activate a NBT/BCIP (Roche) blue color reaction. Specimen were embedded in chick albumin and cross-fixed with 2.5% gluteraldehyde followed by a post-fixed with 4% PFA. Histological sections were cut at 18-20μm using a Leica Microsystems VT1000 vibratome and then mounted with glycerine for imaging using a Leica DM2500 compound microscope with 20-40x objectives.

4.3.3: BrdU labeling

5-bromo-2′-deoxyuridine pulse-chase experiments were carried out to label slow-cycling cells, a property of stem cells. Specimens reared to 4dpf were bathed in a 2% solution of BrdU in-vivo labeling reagent (Invitrogen 00-0103)in 200mL of fish room water at 28^{O}C in an erlenymer flask. Daily 1mL aliquots of BrdU solution were added for a total labeling period of 1 week to complete the “pulse” period. Embryos were rinsed 2X and then moved to fresh water at 28^{O}C in a re-circulating aquarium system (GIT). Embryos were sacrificed over 20 day periods up until a period of 100 days “chase”. This period was verified by BrdU immunohistochemistry as the chase time-point where only discreet populations of slow-cycling cells were labeled.

4.3.3: Immunohistochemistry

Embryos were sacrificed as described and fixed in 10% NBF at RT at 4^{O}C. Embryo were then rinsed in PBS and decalcified for a period of 48-72 hrs in a mild acid (0.1M EDTA) at RT before being processed through a graded series of EtOH (25%, 50%, 75%, 100%, 100%) and 2 washes in xylene. Embryos were washed in xylene for 3 hours and incubated 60^{O}C /embedded in paraffin for sectioning on a Thermo Scientific *Microm* HM355S microtome at 5µm. Slides were dried for 24 hours at 42^{O}C and rehydrated through xylene and a graded series of EtOH for incubation in blocking solution (3% goat serum, 1% bovine serum, 0.1% Triton 100X) for 1hr at RT. Slides were then incubated O/N in a 1:100 dilution of anti-rabbit primary antibody (rabbit ant-Gli3 [Genetex GTX26050], rabbit ant-Sox2 [Genetex GTX124477], rabbit ant-Trp63 [Genetex GTX124660] rabbit ant-Bmi1 [Neuromics RA25083]) in conjunction with mouse IgG2a anti-BrDU [GE Healthcare RPN202]) and the provided blocking solution containing nuclease enzyme at 4^{O}C. Slides were then rinsed 2 x 1h in PBS and incubated in secondary antibodies at 1:400 HRP conjugated goat anti-rabbit IgG [Molecular Probes] and alexa-fluor 568 goat anti-mouse IgG2a [Molecular Probes] in blocking solution at RT. Unbound secondary antibody was removed by washing 2 x 1h in PBS and the HRP signal was amplified using a 488-tyramide chemistry signal amplification kit (Molecular Probes). Slides were again rinsed 2x 1h and mounted with a 50:50 glycerin:Vectashield mixture for imaging using a Zeiss 710 confocal imaging system.

4.3.5: Chemical Treatment

A 10µm stock solution of LDN-193189 (Enzo) was prepared for each chemical treatment experiment using Dimethyl Sulfoxide (DMSO, MP Biomedicals). All chemical and control experiments were performed in Erlenmeyer flasks at 28°C in an oscillating platform culture incubator (Barnstead Lab-Line Max 4000). For changes in gene expression assayed by ISH, cichlids were raised to 20 dpf and embryos from single broods were split into a small molecule treatment and a solvent control group .Treatments were performed at 4µM LDN in 200mL fish H_20. After 48 hours treatment in the small molecule dilution, fry were sacrificed immediately and fixed in 4%PFA. ISH was then carried out to assay effects of treatment on gene expression. For RNA-Seq analysis, adult MZ males with a standard length of approximately 2.5" were treated under the same concentrations and conditions as above in 1L of H_20 for a period of 48 hours, alongside control animals in water containing equivalent volumes DMSO. Animals were sacrificed in buffered MS-222 and immediately dissected for RNA extraction.

4.3.6: RNA extraction and sequencing

Animals were sacrificed and immediately dissected for RNA extraction. A ribbon approximately 1mm x 10mm of epithelium was removed labial to the outer row of teeth from the dentary of experimental animals using a #12 scalpel blade. The extra-osseous soft tissue was removed from the entire jaw to reduce the risk of TB containing epithelium carryover. The bone was then shaved down using a

scalpel to expose the bony crypts and intraosseous RT were extracted with fine forceps. Extracted tissue was quickly placed in RNA*later* RNA Stabilization Reagent (Qiagen). Tissues were frozen in liquid nitrogen, homogenized using a mortar and pestle and placed in trizol. Following standard chloroform extraction RNeasy mini columns (Qiagen) were utilized to purify RNA for storage at -80^{O}C. Total RNA was quantified using Qubit (Molecular probes) and quality analyzed using the Agilent 2100 Bioanalyzer System for RNA library preparation. RNA input was normalized to 1µg and libraries were prepared using the TruSeq Stranded mRNA Sample Prep Kit (Illumina- Kit A). Libraries were again quantified, quality assessed, and normalized for sequencing on the HiSeq 2500 Illumina Sequencing System.

4.3.7: RNA-Seq analysis

Raw sequence reads from RT and TB samples were quality controlled using the NGS QC Toolkit (Patel and Jain, 2012). Raw reads with an average PHRED quality score below 20 were filtered out. The remaining reads were further trimmed of low-quality bases at the 3' end. Quality controlled reads for each sample were aligned to the *Metriaclima zebra* genome v1.1 (Brawand et al., 2014) using TopHat v2.0.9 (Kim et al., 2013). The resulting TopHat2 output bam files were sorted and converted to sam files using samtools v0.19 (Li et al., 2009). Sorted sam files were used as input for the HTSeq-count v0.6.1 program to obtain fragment counts for each locus (Anders et al., 2014).

Fragment counts were scale-normalized across all samples using the calcNormFactors function in the edgeR package v3.6.8 (Robinson et al., 2010). Multidimensional scaling (MDS) plots were also produced via the edgeR package v.3.6.8 to determine the relative consistency among the replicates and samples. Scale-normalized fragment counts were converted into $\log_2$ counts per million reads mapped (cpm) with precision weights using voom and fit to a linear model using the limma package v3.20.9 (Law et al., 2014; Smyth, 2005). Pairwise contrasts were constructed between RT and TB samples. After correcting for multiple comparisons using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995), genes were considered differentially expressed between RT and TB samples if they exhibited both an adjusted P-value < 0.05 and a fold change > 2.

4.4 Results and Discussion

4.4.1: ISH of stem markers reveal a highly potent oral epithelium

We began our study with aim of identifying the location of putative stem cell niches in both renewing TBs and succesional teeth through ISH. Three distinct stages of cichlid replacement tooth maturation are known: initiation, which encompasses placode or succesional dental lamina stages of odontogenesis; differentiation, which encompasses cap and bell stages; and secretion, which encompasses late bell and mineralization stages (Fraser et al., 2013). We chose to focus our study on the latter because RT spend the longest

time in this stage and secretion is the most consistent histologically across different aged cichlids, allowing us to study the RT unit at the same stage for all experiments presented in this work. We first used *pitx2*, a marker of dental tissues in cichlids (Fraser et al., 2008) and in mice (Lin et al., 1999) to identify those cells belonging to the RT germ, but noted expression along the succesional dental lamina, along basement membrane cells deep in the oral epithelium, both labial and lingual to the taste bud unit (figure 4.1, TB in orange, RT in red, FT in green). As we have done before in cichlids (Bloomquist et al, unpublished) and has been done in trout (Díaz-Regueira et al., 2005), we used *calretnin* (*calb2*) to mark TB, which more specifically was expressed in the elongated taste bud proper intragemmal cells and the support perigemmal cells that surround it. We attempted to further characterize delineate the taste unit using *foxa2*, a marker of the endoderm and taste buds in mice (Luo et al., 2009) and in zebrafish (Kapsimali et al., 2011), but to our surprise *foxa2* not only marked TBs, but was strongly expressed in all RT cells and across ectodermally derived outer oral epithelium associated with these two organs, a pattern that became increasingly apparent with most of the factors that we investigated. The last prop of the stage we studied before turning to the actors in stemness was *trp63*, a p53 transcription factor family member and a marker of proliferation and mitotic activity. Trp63-defecient mice embryos exhibit both anodontia and a thin degenerate tongue epithelial layer (Mills et al., 1999) and a hypothesized bipotential progenitor layer shared between both filliform and fungiform taste papilla is marked by Trp63 in mice (Okubo et al., 2009). We find expression of

trp63 to mirror expression of the progenitor layer in mice, being strongly expressed in the cells basal surrounding the intragemmal TB cells continuing across the oral epithelium overlying the dental lamina and in the CLs of the RT epithelium.

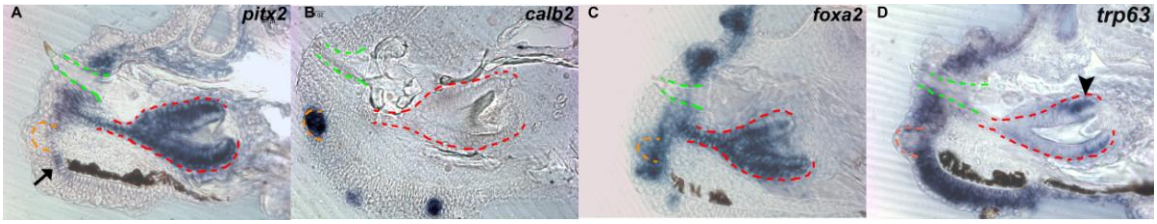

Figure 4.1. ISH identification of tissue in RT-TB unit. Expression of *pitx2* labial to (black arrow, A) and lingual TB outlined in orange, and expressed in RT in red, which is labial to FT in green. *calb2* expressed in TB (B), *foxa2* across all epithelium (C), and *trp63* in CLs (Black arrowhead, D) expression.

We then turned our focus to markers of adult stem cells. Perhaps one of the most studied of these factors in both taste bud development, stemness, and more recently in tooth regeneration is *sox2*. While Sox2 is a well known TB marker, important for both the generation of TBs as well as maintenance of its stem cell populations, it has more recently been implicated in dental ESCs of both mice (Juuri et al., 2012) and other vertebrates (Juuri et al., 2013). We found *sox2* expression in TB's, as well as RT and across epithelium associated with the two structures (Figure 4,2). *Bmi1*, a polycomb gene required for adult stem cells

in a host of organs (Molofsky et al., 2003), is essential for incisor renewal through the repression of *Ink4a/Arf* and *Hox* genes (Biehs et al., 2013), while in TBs it appears that a population of *Bmi1* positive SCs renew keratinized epithelial cells distinct from SCs that renew TB cells themselves. We observed expression of *bmi1* distinct from that of *sox2*, diffusely across TB and surrounding epithelium and in RT epithelium. The differences between *sox2* and *bmi1* expression come as no surprise. In the mouse intestine, *Bmi1* marks a population of stem cells that is relatively quiescent and activated in response to injury, while another distinct population of *Lgr5* positive stem cells is more active and is responsible for regular renewal of the crypt unit (Yan et al., 2012), and further each population responds differently to tissue perturbations such as apoptosis (Zhu et al., 2013). While no homolog of *Lgr5* exists in teleosts, the ortholog *lgr4* was expressed in cichlid RT and TBs more similarly to that of *sox2*, although more restricted to the basal layers of epithelium in and around the taste unit. *igfbp5*, shown coincident in expression to *lgr5* in gecko RT dental lamina (Handrigan et al., 2010), indeed co-labels the basal epithelial cells associated with the regenerating RT-TB unit. Meanwhile *Hopx,* which has been used to label SCs in intestine (Takeda et al., 2011) and hair follicles (Takeda et al., 2013), has not yet been described in teeth or TBs but is expressed in both the *sox2/lgr4/igfbp5* positive and *bmi1* positive populations. To complement the renewal of the epithelium, we investigated the role of MSCs in cichlid oral organ renewal. It is now well established that nerve mediated Hh responsive MSCs derived from glia in mouse incisors express *Gli1* coincident with other stem labels, and upon denervation incisors fail to produce

significant mesenchymal cell progeny (Kaukua et al., 2014) . We found *gli1/3* expressed in the epithelium and papillary mesenchyme of the RT and mesenchyme subadjacent to the SC marker-positive basal epithelial cells. In contrast, the other typical MSC markers, *celsr1* and *sox10*, the former of which has not been described in teeth, were oddly expressed in the epithelium of RT, the mesenchyme sub-adjacent to epithelium to the TB and dental lamina, and outside of the dental papilla itself but in crypt mesenchyme foci near the CLs (figure 4.2). Taken together, our ISH data imply a rich band of stemness for both epithelium and mesenchyme, containing distinct subtypes of SCs within and connected by a dental lamina to comprise a unit labial to the FT with robust regenerative potential.

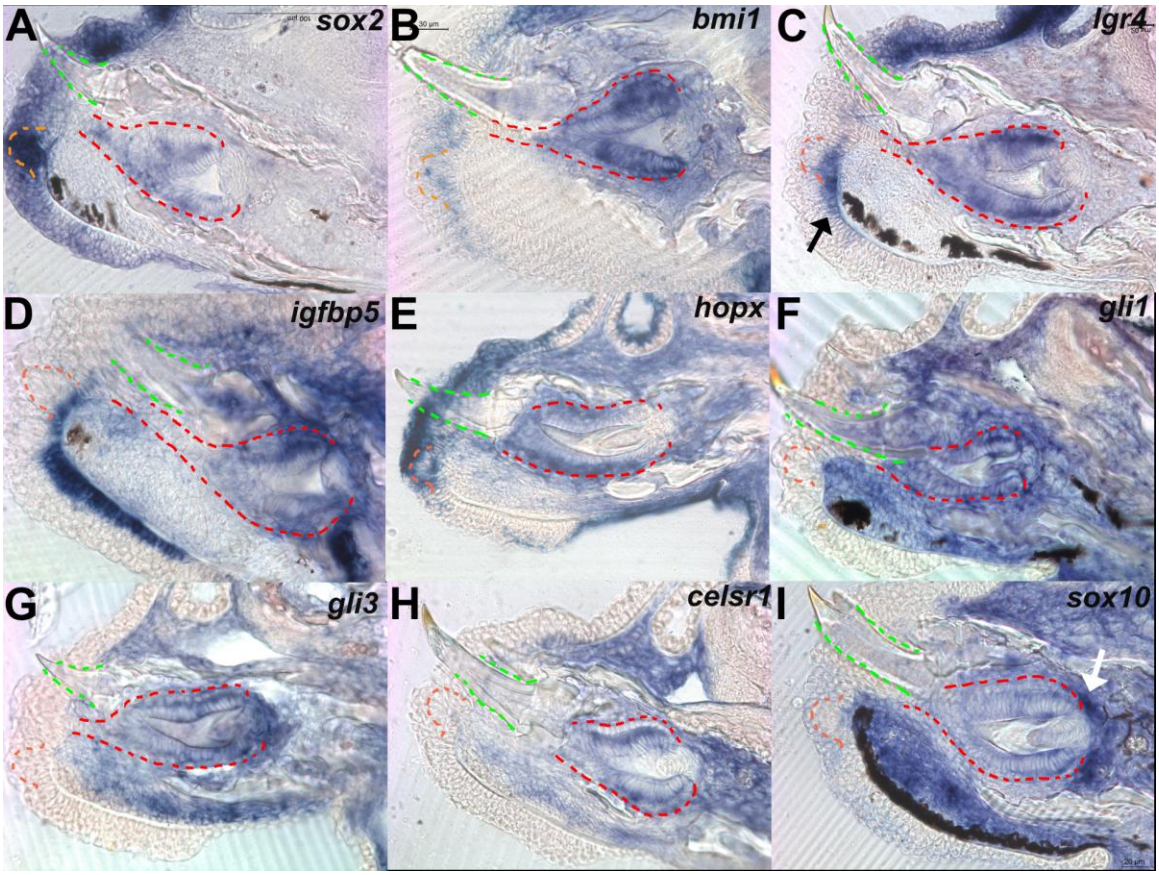

Figure 4.2. ISH of adult stem markers, Basal epithelial cell layer marked by lgr4 (black arrow, C) and CL mesenchyme of crypt outside of dental papilla (white arrow, I)

4.4.2: Double labeling with stem markers and pulse chase identifies stem cell niches across a potent oral epithelium

While we highlighted a host of adult SC-associated transcripts in the RT-TB unit, it is a not sufficient to infer that these cells are indeed SCs by gene expression alone. To validate the SC properties of these tissues and to more precisely identify SC niche environments, we conducted the first known pulse-chase experiments in cichlid fishes, one of the primary experiments done to first identify the stem cells the mouse incisor (Harada et al., 1999). By exposing animals to the synthetic nucleoside, 5-bromo-2′-deoxyuridine (BrdU), it is incorporated into newly created cells and once removed, only those cells that are slow cycling or non-dividing, a property of stem cells, will be label retaining cells (LRC). We bathed cichlid fry in a solution containing the BrdU at pharyngula stage for a period of 1 week, and then sacrificed sequentially until LRCs were identified. The high levels of exposure to BrdU early resulted in almost all cells being labeled, including potential SC populations, at a period of 40 days-chase, but by 100 days-chase discreet cell populations were apparent (figure 4.3). By 100 days-chase, there were a dense number of LRCs across all epithelium labial to the FT and within the RT. There were a far lower of LRC detectable in the mesenchyme, mostly associated in a band approximate to the epithelium. Because ISH revealed distinct subsets of adult stem markers within a tissue, we used double labeling with immunohistochemistry (IHC) to better characterize stem niches amongst the oral regenerate RT-TB unit. In accordance with mRNA expression, Trp63 protein was detected in TB support cells and co-expressed

with LRC across the basal cells of oral epithelium. In the RT, Trp63 was detected in those outer dental epithelial (ODE) cells, analogous to mammalian OEE, associated with the CL. In contrast to the basal co-labeling of Trp63, Sox2 and Gli3 were found co-expressed with almost all LRC associated with both organs, and Gli3 was co-expressed with mesenchymal LRC as well. While Bmi1 protein co-labeled a smaller subset of LRCs, its domain in the epithelium of both organs was largely in the more superficial cells, many of which were negative for label retention, and far less of those basal LRC were co-labeled than that of the other three markers. Taken together, Trp63 and Bmi1 occupied distinct and contrasting regions of LRCs. Co-labeling of Sox2 and Gli3 with LRC confirmed the presence of a SC rich regenerate unit epithelium and within it three distinct ESC niches became apparent: those associated with the base of the taste bud analogous to murine TB SCs (Okubo et al., 2006), those associated with the incisor CL (Juuri et al., 2012; Kaukua et al., 2014), and a new SC niche not described in the dental literature, occupying most cells at the tip of the maturing RT. It is likely the first description of this niche because very little to date has been published on the stem cell populations involved in whole tooth replacement in a one-for-one replacement system. We conclude that the RT-TB regenerate unit is rich in subtypes of stem cells in order to host lifelong renewal.

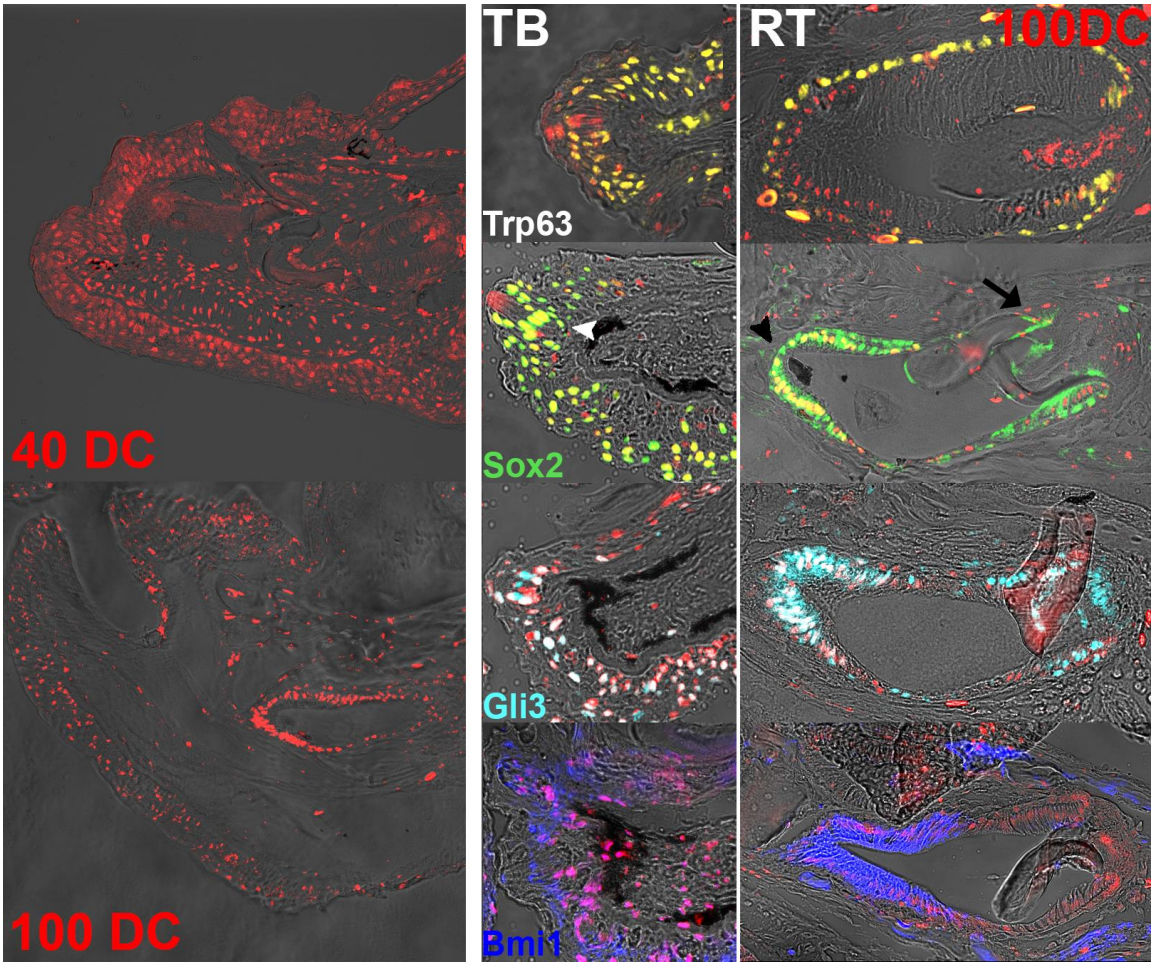

Figure 4.3. Double label for LRC and IHC of adult stem markers. LRC after 40DC labels almost all cells, 100DC labels more distinct cells in epithelium. Three distinct SC-rich niches identified, one in TB (white arrowhead), one at the tip of the RT (black arrowhead), and one in the CLs (black arrow).

4.4.3: Unique transcriptome environments across SC abundant oral tissues confers organ identity

Given the high number of stem cells we identified in cichlid oral tissues it does not astonish that cichlids and most other teleosts have outstanding regenerative capacity, with the ability to renew each tooth and TB throughout ontogeny. With their close proximity, common embryonic tissues (Bloomquist et al., unpublished), shared epithelium through the dental lamina, common patterns of markers for stemness and LRCs, and likely ancestry (Fraser et al., 2010), we asked what influenced these highly homologous stem cells to confer organ identity in their renewal. We addressed this question first through an unbiased approach of whole transcriptome RNA –sequencing. We dissected out a band of epithelium, just labial to the outer row of adult cichlid FT, which contained a high density of TBs (Bloomquist et al. unpublished). We then removed the periosteum surrounding the dental bony crypts and isolated RT germs at secretion stages that were easily identified through their hypermineralized acrodin cap (figure 4.4). We pooled TB bearing epithelial tissues and RT germs for each animal, extracted RNA, prepared RNA-libraries and performed RNA-seq on the Illumina 2500 platform.

Raw sequence reads were quality controlled and reads with PHRED score < 20 were filtered out. Overall, sequence reads were generally of high quality with only about less than 5% on average of reads having PHRED score < 20. High quality reads were aligned to the *M. zebra* genome v1.1 and on average, across all samples, over 95% of reads mapped to the reference genome.

Fragment counts across all samples were obtained, normalized and fit to a linear model to determine differential expression between the tissue types. Genes were considered significantly differentially expressed between RT and TB tissues if they exhibited both a two-fold expression difference or greater and an adjusted P-value < 0.05. Using this criterion, we found 2,527 genes were differentially expressed between the tissue types (Figure 4.4). Of those, 1623 were up-regulated in RT tissues while 905 were up-regulated in TB tissues. Significant RT biased genes included *bmp4, bmb2b, smad3, smad6, msxa, msxc, krt18, krt80, dmp1, odam, wnt9b, wnt3, axin2, pdgfra, snail1a, jag2, igf2, igfbp5, p21, irx1, irx6, MMPs, nrp1, nrp2, notch1, lrp5, lrp8, fgf14, ptch1, itga2, itgbl1 (other integrins), dlx6, per2, sparc*, solute carriers. Genes biased in TB tissue included: *krt15, neurod4, lrrn1, advillin, foxp4, per3, tspan8, cntn4, grb10, atoh1, grid2, kcnq4, cx30.9, nkx2, cntnap2, wnt4b, cntn2, sox14, sox7, itgb4, lrp2, jag1, sox13, fgf7, nkx6.1, osr1,* and solute carriers. Differentially expressed genes between RT and TB samples are represented in a heatmap in Figure 4. An analysis of functional enrichment of gene ontology (GO) categories revealed genes with functions related to development (Supplemental file 1).

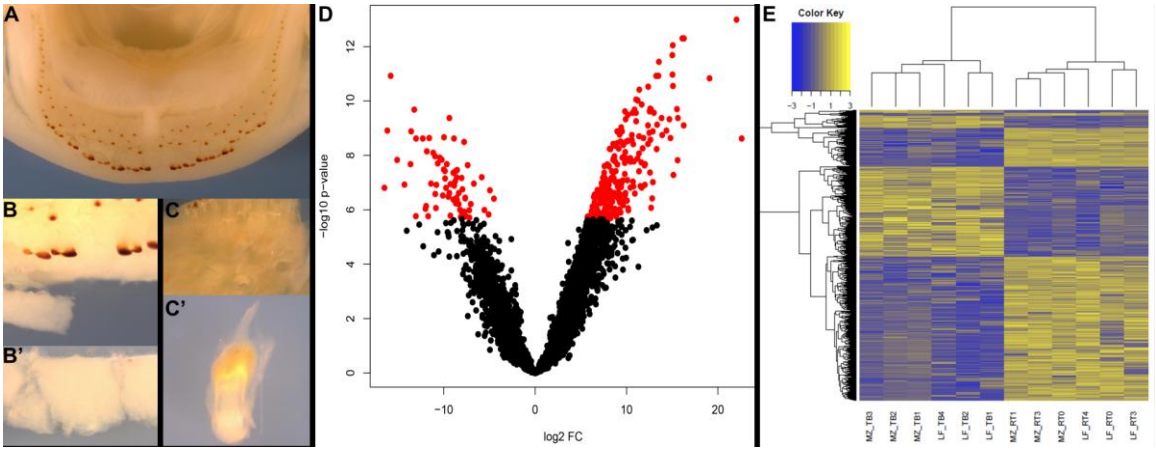

Figure 4.4. RNA-seg of RT and TB bearing epithelium. Dorsal view of cichlid jaw (A), dissected by removal of TB bearing epithelium (B') just labial to the outer row of teeth (B) and subsequent removal of extra-crypt tissue (C) to isolate RT germs (C') for RNA extraction. Volcano plot of all differentially expressed genes, y-axis log of p-value with those significantly d-expressed in red and not d-expressed in black, X-axis is log fold change of d-expression left of 0 value are TB biased and right of are RT biased (D). Heat map of those differentially expressed genes show clustering based on sample type and fold genes represented by color key (E) TB right 6 samples, RT left 6.

Major developmental pathways important for tooth development in the mouse include Bone Morphogenetic Protein (BMP), Fibroblast Growth Factor (FGF), Hedgehog (Hh), Notch, and Wingless (Wnt) in patterning (Cobourne and Sharpe, 2010) and renewal (Seidel et al., 2010; Wang et al., 2007), all of which are expressed in cichlid RT (Fraser et al., 2013). We found BMP genes biased to RT on our list and the BMP pathway was the most significantly biased developmental pathway on our GO analysis. It has long been established that BMPs are critical to the proper formation of teeth (Vainio et al., 1993) and more recently odd-skipped related protein 2 (*Osr2*), through BMP inhibition, has been proven to prevent the lingual formation of ectopic teeth by restricting the dental

competent field of mice (Zhang et al., 2009). While BMPs are expressed in lingual epithelial cells of mouse tongues, they are described as having switching roles from a brief period of activating to highly inhibitory to taste buds in cultured rat tongues (Zhou et al., 2006) and as negative regulators of taste formation through Wnt inhibition in mice (Beites et al., 2009). We have recently demonstrated that BMP inhibition results in ectopic TB formation in dental field progenitor epithelium. We further examined the dichotomy of pathway genes through ISH and, as predicted by our transcriptome profiling, found that *β-catenin*, *fgf10*, *jag2*, and *shh* were expressed in both organs, but *bmp2* and *bmp4* were sharply expressed in RT epithelium and mesenchyme but excluded from all tissues in and around the TB (figure 4.5). We hypothesized that from the common environment of SC-rich oral tissues, BMPs were a differentiation pathway that helped confer organ identity from neighboring oral SCs.

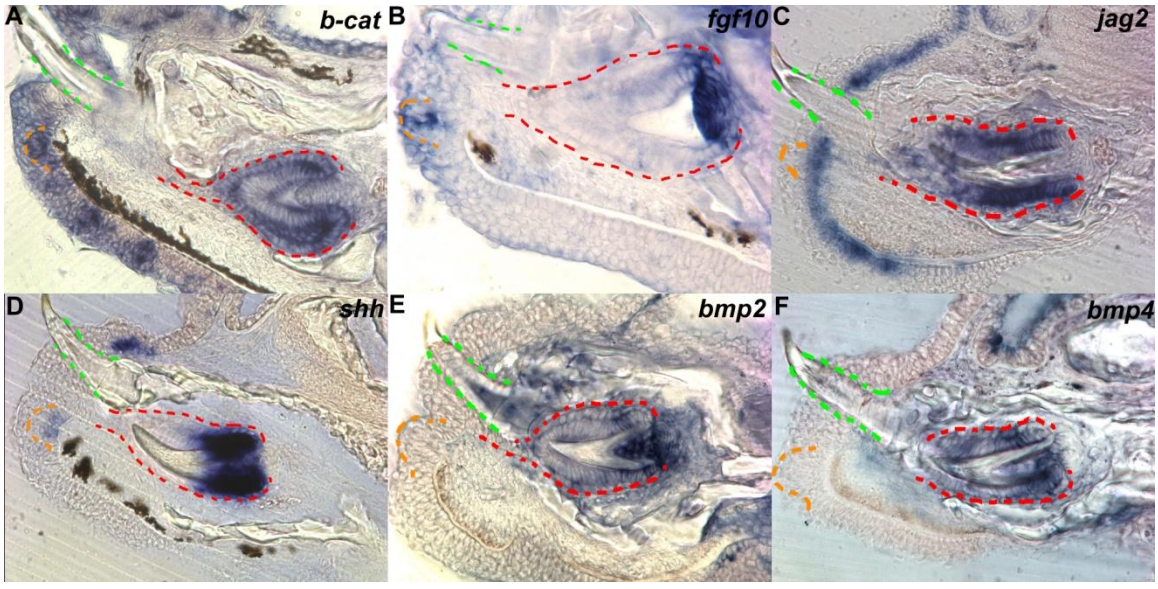

Figure 4.5 Pathway expression in RT and TB. *bmp2/4* bias to RT (E,F).

4.4.4: Antagonism of the BMP pathway blurs the line between RT and TB

The environment in which a stem cell resides can sharply influence either its quiescence, its self-renewal, or what progeny it can differentiate into it. A striking example of this is in the case of cell fate determination of the rodent incisor stem cell niche, where conditional deletion of transcriptional co-activator, Mediator 1 (Med1) resulted in hair growth; another organ with adult SC based renewal, from within the incisor (Yoshizaki et al., 2014). We've shown before that BMP antagonism through chemical inhibition abrogates cichlid tooth replacement (Fraser et al., 2013). Armed with our knowledge of cichlid RT and TB transcriptomes and renewal properties, we set to characterize this phenotype at a deeper level. We bathed juvenile cichlids in the small molecule inhibitor of BMPs, LDN, for a period of 48 hours, long enough to affect the cellular differentiation of SCs from within cichlid RT at secretion stages, and then sacrificed to characterize treated RT at the cellular and mRNA level. Shh regulates pre-ameloblasts in the rodent incisor that are making the transition from SC derived transit amplifying cells to enamel secreting ameloblasts (Seidel et al., 2010), and *shh* was expressed in the analogous IDE of cichlid teeth (figure 4.6). Upon exposure to LDN, *shh* expression was completely undetectable and RT appeared shorter and malformed, particularly in the CLs. We then explored expression of our TB marker, *calb2*, and found compared to control animals where *calb2* was specific to TB and excluded from the entire RT germ, LDN treated animals showed expression of *calb2* in CL epithelium in regions where *shh* expression was reduced (figure 4.6). At earlier initiation and differentiation

stages of RT odontogenesis, ectopic RT calb2 expression appeared in rounded elongate TB like cells (Appendix D.1). We were excited to find that BMP antagonism in-vivo across the entire animal resulted in a loss of dental characteristics in RT in exchange for TB like characteristics.

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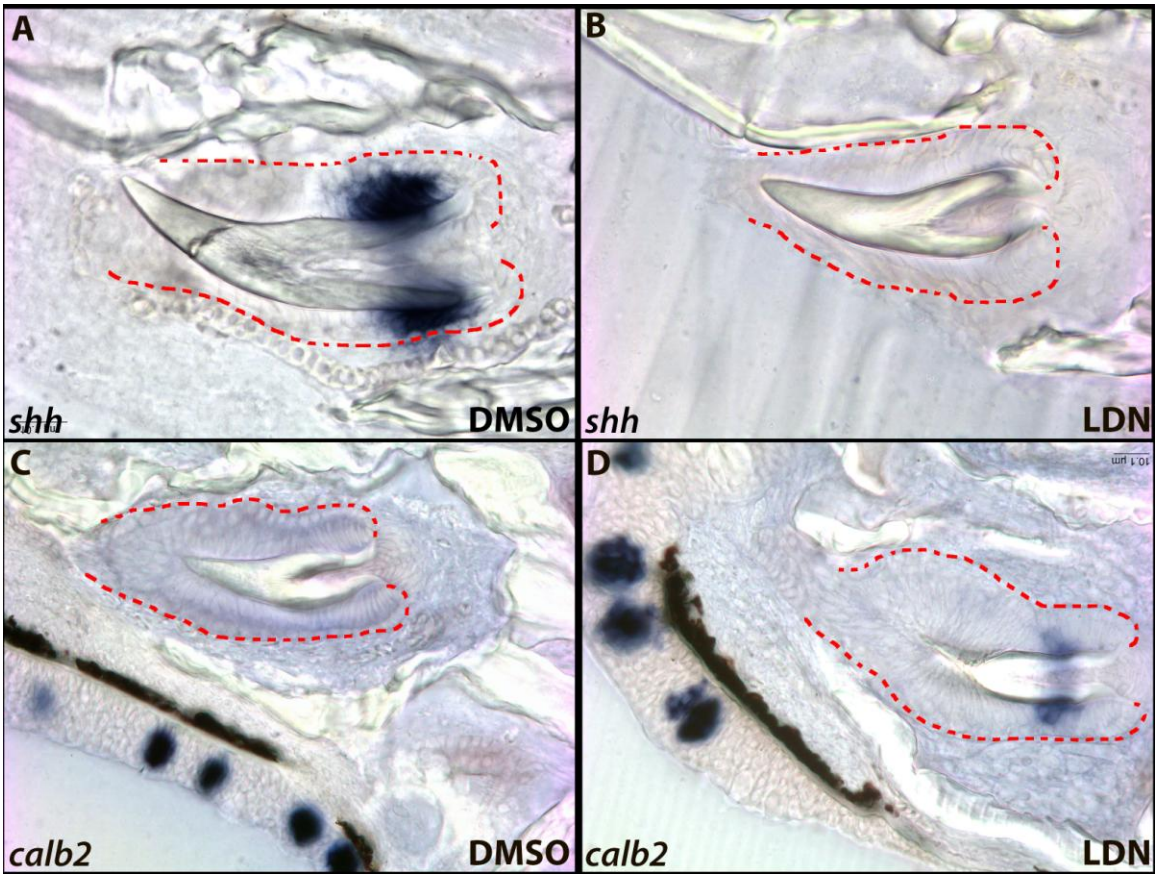

Figure 4.6. Effect of LDN on RT SC differentiation. Solvent control RT express *shh* (A) and not *calb2* (C) in CL epithelium, but LDN treated RT express *calb2* (D) and not *shh* (B).

4.5 Conclusions

In this study we employ Lake Malawi cichlids, animals with immense regenerative capabilities, to uncover new biology in the process of vertebrate whole tooth replacement. As tetrapods and mammals innovate through evolution to outcompete and execute highly specialized tasks, their tissues have become increasingly complex. With this increase in complexity comes an evolutionary tradeoff, away from the ability to regenerate and towards wound repair or scarring. A paradigm of this tradeoff is in the highly specialized dentitions belonging to mammals and rodents, which have evolved to possess limited replacement capacity (Koussoulakou et al., 2009), and where teeth reside in environments isolated from SC containing taste bud and salivary tissues. In many fishes and reptiles, including cichlids, both teeth and TBs populate the first to the most posterior pharyngeal arches. As opposed to the distinct TB and incisor models of the mouse, from this crowded environment we found that RT formed juxtaposed to TBs and that dental lamina of the successional tooth maintained a connection to the TB containing oral epithelium labial to the FT throughout RT maturation (figure 4.7). ISH revealed that adult stem cell markers were rampant across this epithelium and that with exception of BMPs, few factors were unique to either organ. Double label experiments with pulse chase in combination with IHC for adult SC markers highlighted Bmi1/Sox2/Gli3 positive LRCs and Trp63/Sox2/Gli3 positive LRCs superficially and basally respectively, with a SC niche surrounding the TB, in the RT CLs, and a newly identified niche at the tip of the RT (figure 4.7). Finally, we demonstrated that the unique BMP

biased transcriptome of RT could be manipulated, through BMP inhibition, to reduce the dental characteristic of CL epithelium and confer TB attributes, a phenotype reminiscent of hair generated from transfating through genetic conditional deletions in rodent incisors (Yoshizaki et al., 2014). We propose that cichlid RT SCs are highly similar to those of their nearest neighbor, the TB, and that this evolutionary remnant may perhaps be exploited in medicine by utilizing human endogenous TB SCs for dental regenerative therapies.

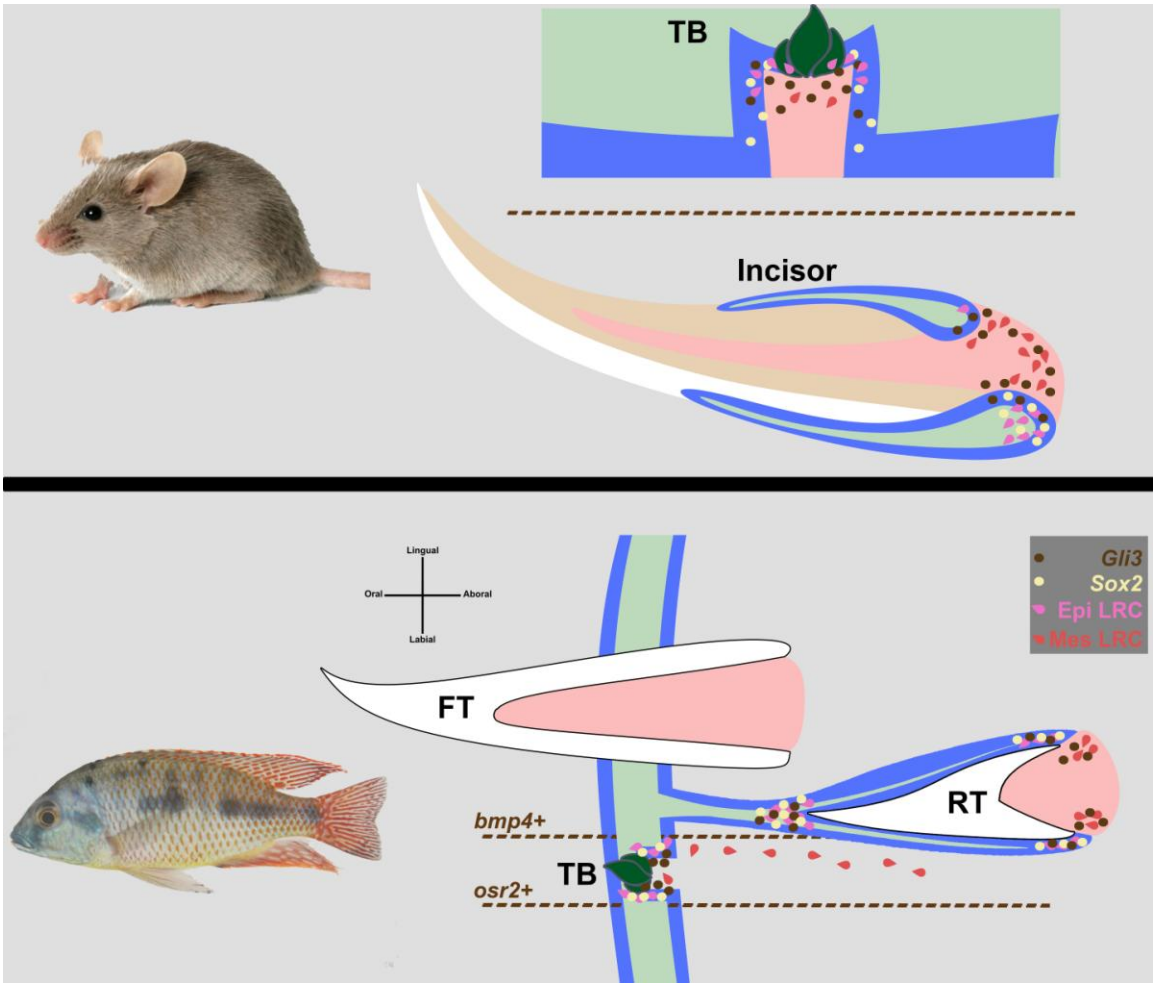

Figure 4.7. Model of mouse and cichlid renewal. Mouse incisor CL epithelium (green SR, blue iee/oee) rich with LRC, Gli1, and Sox2 while mesenchyme (pink) rich with Gli1 and LRC. Same patterns are reported for TB basal epithelium and mesenchyme. In cichlids, three niches are rich in SC at TB, tip of tooth, and CL with same epithelial and mesenchymal zones as seen in rodent inscisors for LRC/Gli3/Sox2. RT renew in *bmp2/4*+ environment while TB do so in BMP repressor environment.

4.6 Acknowledgements

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CHAPTER 5

CONCLUSIONS

The goal of this dissertation was to uncover new insight into the phenomenon of odontogenesis and natural whole-vertebrate tooth replacement. By building on what is known about mouse and other animal dentitions and drawing parallels to ancestrally linked placode derived organs, such as hair and feather, we have learned a great deal in regards to the patterning, regeneration and morphogenesis of teeth. We have done so using an animal model ideally suited for evolutionary and genetic approaches, the Lake Malawi cichlid.

In the first study, we set out to understand the most basic mechanisms of cichlid tooth replacement, an intricate, well timed and robust process. We then sought to understand how this splendidly orchestrated progression accounted for replacement tooth morphogenesis with fidelity, cichlids being one of the few examples of in nature with both species dependent complex tooth shapes and continuous dental regeneration. We dissected tooth replacement into three key stages: initiation, differentiation, and secretion. We discovered that highly proliferative oral epithelium generates RT in a one-for-one fashion, labially or rostrally to the erupted row of functional teeth, and that a dental lamina maintained its contact to the outer epithelial environment throughout RT odontogenesis in close association to a cluster of cells we later discovered to be taste buds. We then charted expression of major developmental pathways, (BMP, FGF, Hh, Notch, Wnt/β-catenin), noting the high degrees of homology to

tooth formation and renewal in comparison to the studied mouse dentition, and highlighting the fact that BMP, FGF, Notch and Wnt pathways are redeployed for both initiation and morphogenesis of the RT, while Hh only plays a part after RT initiation. Through chemical treatment we found that all five pathways were essential for proper shaping of teeth, and that BMPs, Notches and Wnts were needed for proper initiation of the tooth replacement process.

In the next study, we asked how lower vertebrates, such as fishes, coordinated the patterning of a multitude of placode derived organs, such as teeth and TBs, across the oral and pharyngeal cavities, a process that takes place in regionally delineated tissues such as the tongue and alveolar arch of mammals. We first studied the embryonic origin of cichlid teeth and TBs at the pharyngula to the establishment of placodes and we were shocked to find no distinction between the earliest dental tissue, the odontogenic band or dental lamina, and the earliest TB tissues. We re-considered this tissue an oral lamina competent or bi-potent to form either organ. We then employed QTL mapping to understand the genetic loci and candidate genes within that control the patterning of both organs between a species with high density of both organs and a species with low densities of both organs. We generated F_2 populations and found that there was a strong positive correlation between the densities of teeth and TBs, suggesting genetic linkage between pattern effectors of both. We identified QTL and candidate genes in our analysis and highlighted two of which, *bmper* and *sfrp5*, that were spatially differentially expressed between the two species and that were both expressed in mouse tooth germs. Neither of these genes has

been demonstrated in the patterning of either organ. We then pushed our model of the co-patterning of these genes further by showing BMP, Hh, and Wnt pathway expression in the embryonic tissue of each and through chemical antagonism we generated taste placodes in the place of tooth placodes, a demonstration of the plasticity between these two organs. Through an analysis of phenotypes and changes in gene expression following chemical antagonism experiments, we generated a model wherein teeth and taste buds are both positively regulated by Wnts while BMPs and Hhs coordinated the fating of oral tissue between one organ or the other.

In our final study, we addressed questions that emerged as we revealed more about tooth patterning and replacement in our system. It became clear to us from the first work that cichlid teeth renewed with an association to the TB, but we wanted to know if this relationship was more than an anecdote of spatial constraints. Furthermore, in the second study we discovered that both teeth and taste buds are embryonically derived from a common bipotent oral lamina and that at the onset of placode formation the tissue is plastic to the response of BMP and Hh signaling, still capable of forming either organ. Given the capacity of both teeth and TB to renew throughout ontogeny and the likely shared ancestor to both, we wanted to know where the stem cells resided within this TB-RT unit and how they are coaxed to generate their respective organs. Through histology and ISH we found that RT always initiated and matured in the presence of TBs and maintained a connection through the successional dental lamina. We discovered that stem factors were rampant across the entire oral epithelium labial to the FT,

including that of the RT, and that stem markers were expressed with common patterns between RT and TBs. Through pulse-chase and double labeling with IHC for adult stem cell markers, we localized stem cells across this highly prolific unit where $Gli3^{+}Sox2^{+}$ co-labeled with most LRCs and Bmi1+ LRC superficially versus Trp63+ LRC deep delineated distinct stem environments. Furthermore, three concentrations of SCs or stem niches emerged: one at the base of the TB analogous to that of the mouse, one at the CL of RT analogous to that of incisor CL, and a newly uncovered population at the tip of the RT, likely unknown to biologists because of the lack of published data on animals that undergo one-for-one continuous tooth replacement. Uncovering the high degree of stemness in the shared environment of RT and TB, we sequenced transcriptomes using RNA-Seq to identify unique gene-ontologies of either tissue and found that BMPs, which we later verified with ISH, were differentially biased in their expression to RT. We concluded our study by antagonizing BMPs in entire live animals through chemical inhibition and found that in BMP deficiency, RT lost cellular identity of untreated RT cells and gained characteristics of TB cells, insinuating a conversion of SC fates from RT like to TB like within the tooth germ. We hope to progress in our findings by removing the potent epithelium from the renewal unit and have strong preliminary evidence that ablation of TB-bearing epithelium ceases tooth replacement and leads to total edntulism. Our colleagues are also addressing our findings by studying renewing mouse taste buds, and through genetic deletions of BMP repressor genes, have evidence of conversion to dental characteristics in the mouse tongue.

From a biological perspective these finding are quite intriguing and from a biomedical insight these findings are exciting. At the most basic level, we now understand a great deal more about how teeth are uniquely patterned and replaced, even in the context of complex tooth morphologies, across species. At a more optimistic level we now have demonstrated good evidence that teeth and their nearest neighbors, the ever-renewing TBs, possess embryonically and ancestrally agnate stem cell populations. Perhaps the spatial distinctions of these two organs in higher vertebrates contributed to the reduction in stemness and subsequent renewal capacity of higher vertebrates. It stands to reason, therefore, that TB SCs have promising potential in the field of regenerative dentistry.

5.1 Publications

The following is a list of publications in chronological order, either pertaining to or resulting in this body of work, as well as other scientific contributions made during my PhD candidature. *denotes equal contributions.

1. Bloomquist RF**,** Fowler TE, Streelman JT. “"Developmental plasticity of stem cells in tooth and taste bud renewal”. In preparation.
2. Bloomquist RF***,** Fowler TE*, Sylvester JB, Streelman JT. “"A compendium of developmental gene expression in Lake Malawi cichlid fishes”. In preparation.
3. Bloomquist RF, Parnell NF, Phillips KA, FowlerTE, Yu T, Sharpe P, Streelman JT. “Co-evolutionary patterning of teeth and taste buds”. In Review, PNAS.
4. Brawand D, Catherine Wagner C, Li Y, Bloomquist RF (author position 22/75), Di Palma F. “The genomic substrate for adaptive radiation: genomes of five African cichlid fish”. Nature, 2014, 7518.
5. Fraser GJ*****, Bloomquist RF*****, Streelman JT. “Common developmental pathways link tooth shape to regeneration”. Developmental Biology, 2013, 377(2).
6. Nadler JH, Mercer AJ, Culler M, Ledford KA, Bloomquist RF, Lin A. “Structures and Function of Remora Adhesion”. MRS proceedings, 2012.
7. Fraser, G.J., C.D. Hulsey, R.F. Bloomquist, K. Uyesugi, N.R. Manley and J.T. Streelman. “An ancient gene network is co-opted for teeth on old and new jaws.” PloS Biology 2009, 7(2).
8.
9. Fraser, G.J., R.F. Bloomquist and J.T. Streelman. “A periodic pattern generator for dental diversity.” BMC Biology, 2008, 6, 32.

APPENDIX A

SUPPLEMNTAL MATERIALS FOR CHAPTER 1

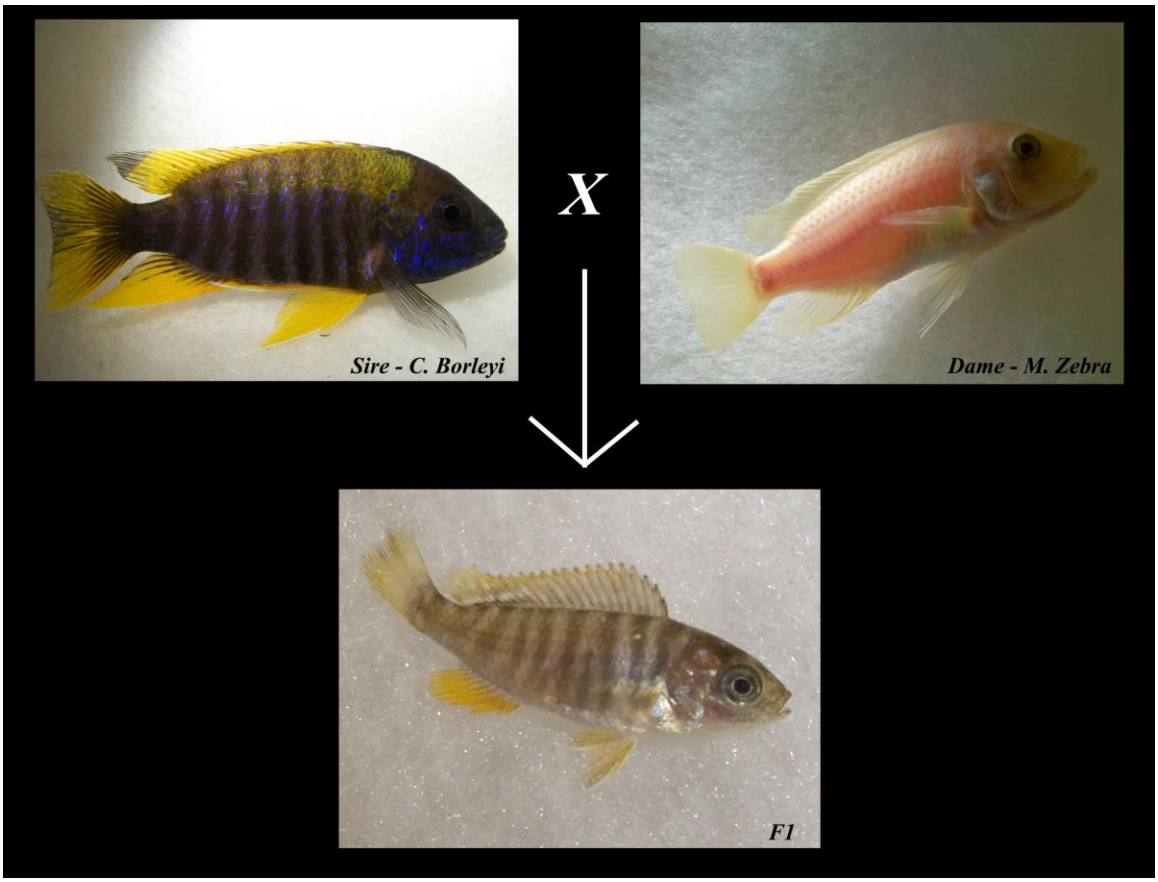

Figure A.1. Mbuna and Utaka generated hybrid. Example of several Mbuna crossed to Utaka hybrids generated in-vitro in the laboratory demonstrate genetic similarities

APPENDIX B

SUPPLEMNTAL MATERIALS FOR CHAPTER 2

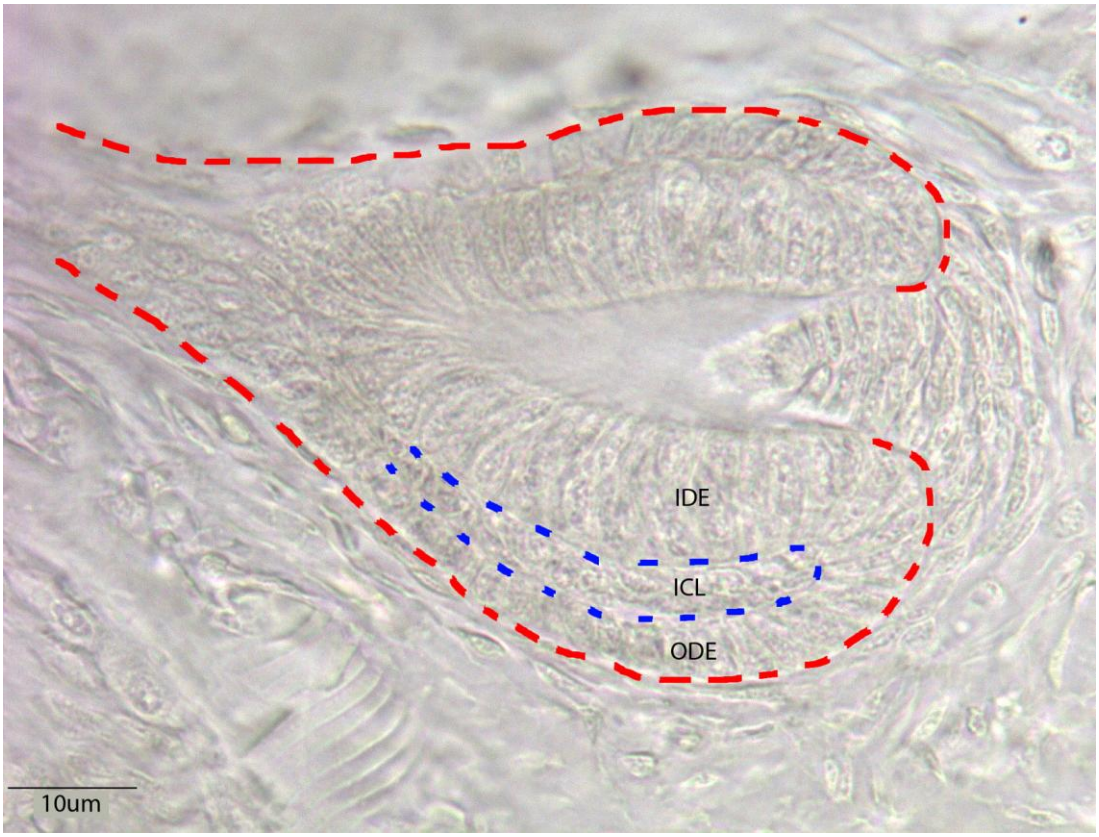

Figure B.1. Close-up image of a cichlid replacement tooth showing the intermediate cell layer (ICL) between inner- (IDE) and outer dental epithelium (ODE). Sagittal section (15μm) of a replacement tooth organ from *Labeotropheus fuelleborni* (45dpf); dental epithelium is outlined in red, the labial ICL in blue, imaged at 63x.

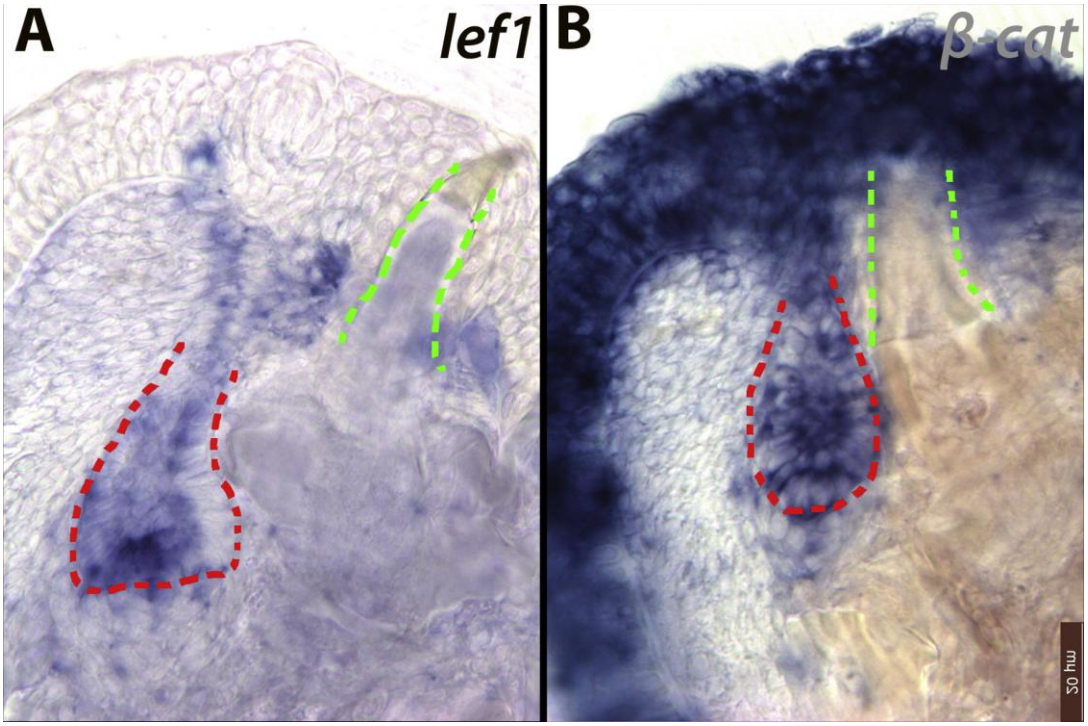

Figure B.2. Expression of additional genes from the Wnt/ß-catenin pathway during cellular differentiation stages of cichlid tooth replacement. First generation teeth are outlined in green and replacement dental epithelium in red. These are vibratome sections in sagittal plane at 15μm thickness, imaged at 63x magnification. Labial is oriented to the left and oral toward the top of the page.

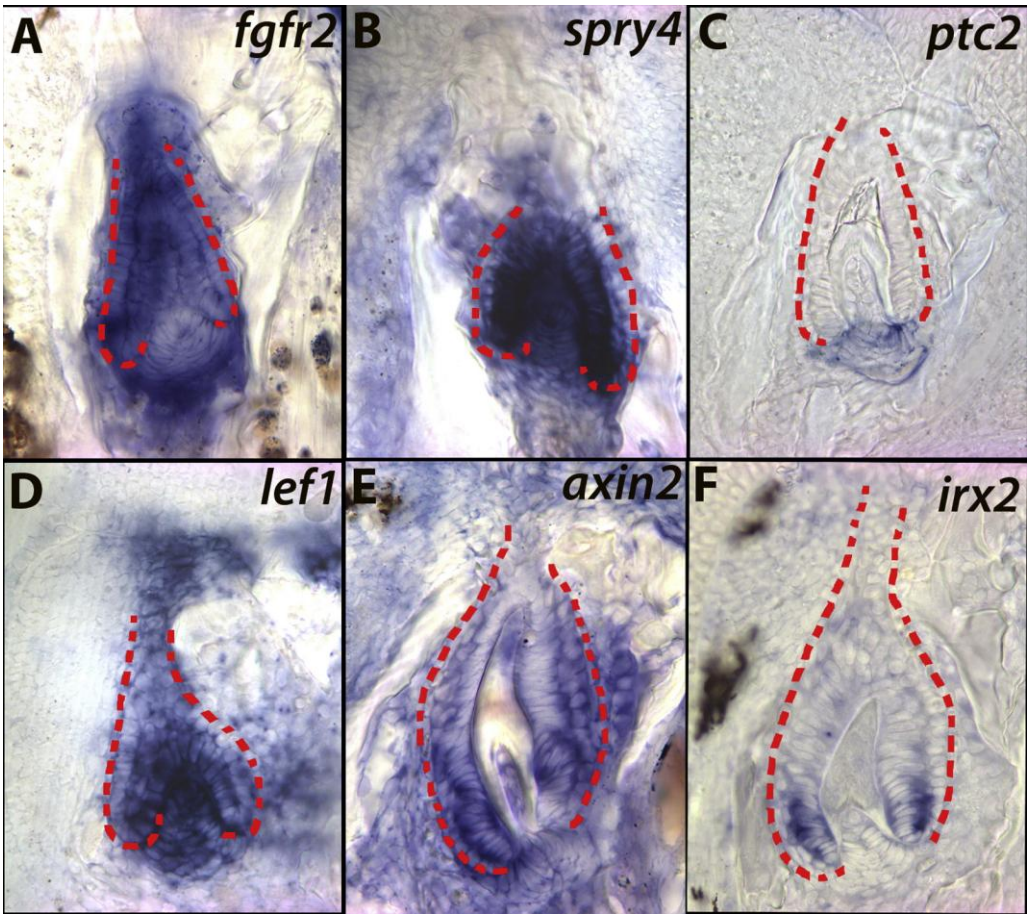

Figure B.3. Expression of additional genes from the FGF, Hh and Wnt pathways during the secretion stage of cichlid tooth replacement. These are vibratome sections in sagittal plane at 15μm thickness, imaged at 63x magnification. Labial is oriented to the left and oral toward the top of the page. Fishes used in this panel are ~15-30dpf.

APPENDIX C

SUPPLEMNTAL MATERIALS FOR CHAPTER 3

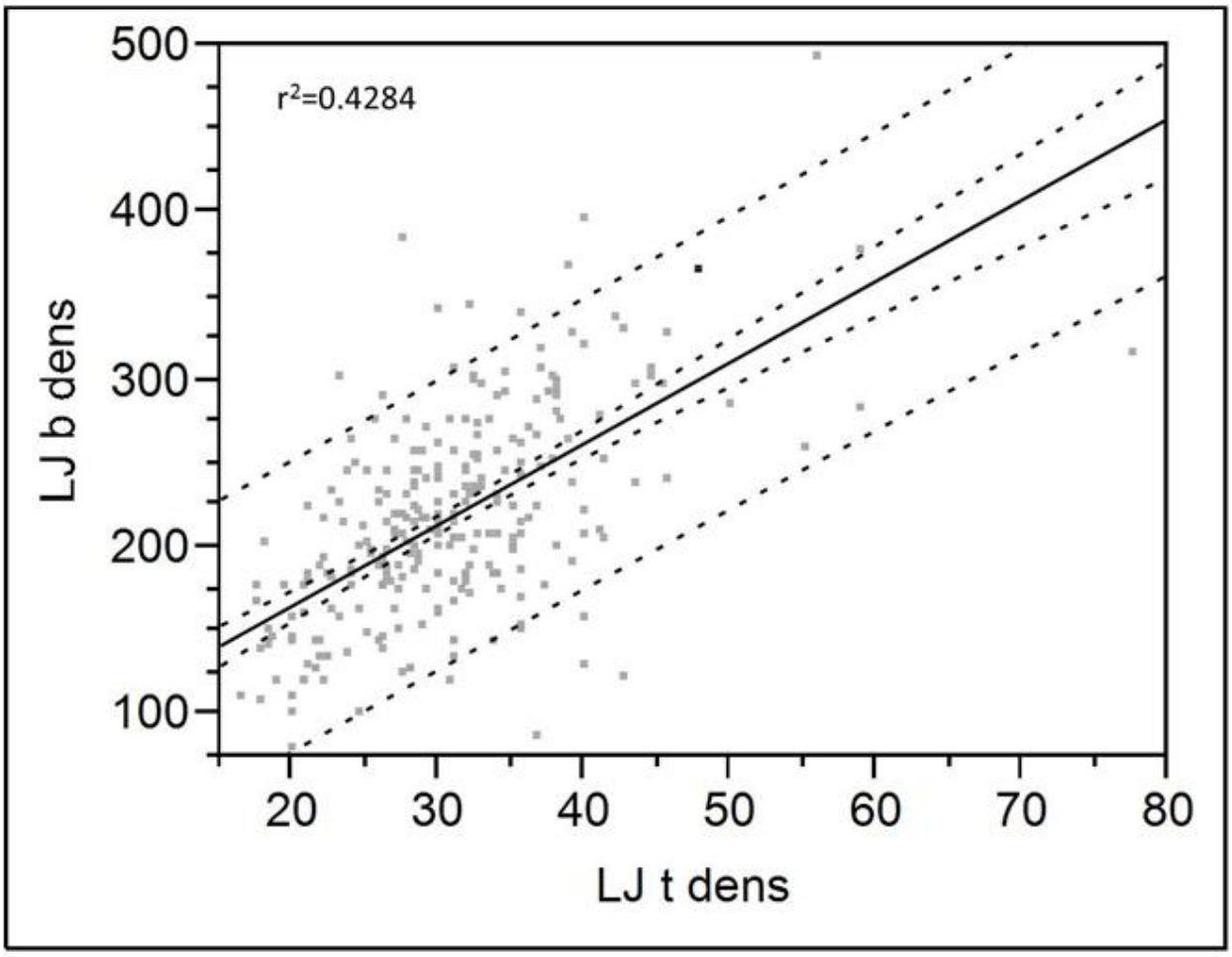

Figure C.1. Genetic linkage suggested by positive correlation of tooth and taste bud densities. R^2=0.4284, y-axis #tb/mm^2, x-axis # teeth/mm^2.

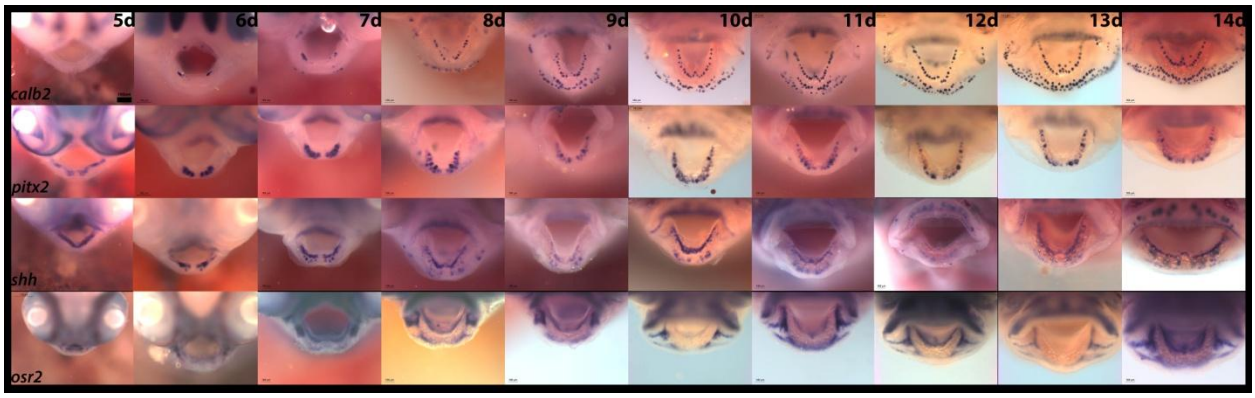

Figure C. 2. Ontogeny of *Metriaclima zebra* jaw development. ISH for markers of taste (*calb2*), tooth (*pitx2*), both (*shh*), and BMP antagonist (*osr2*) across juvenile ontogeny starting with dentary formation at 5dpf in *Metriaclima zebra*. Dorsal views, labial to bottom of page, scale=100µm.

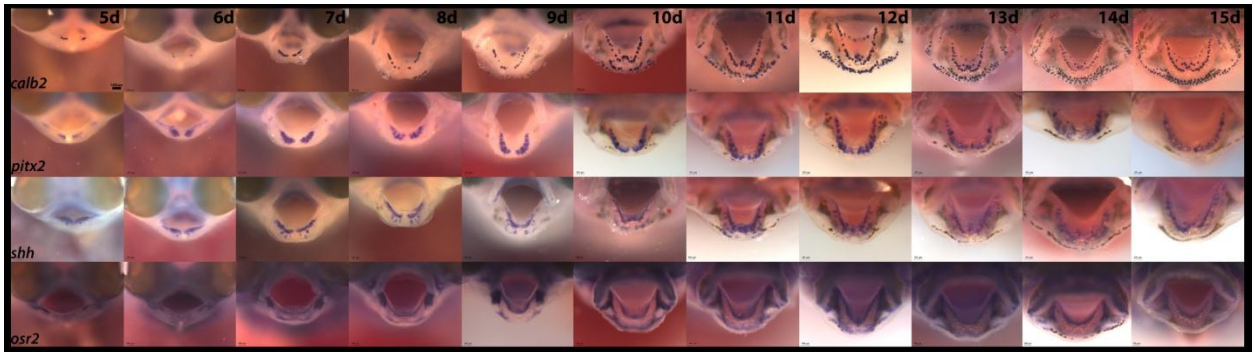

Figure C.3. Ontogeny of *Petrotilapia chitimba* jaw development. ISH for markers of taste (*calb2*), tooth (*pitx2*), both (*shh*), and BMP repressor (*osr2*) across juvenile ontogeny starting with dentary formation at 5dpf in *Petrotilapia chitimba*. Dorsal views, labial to bottom of page, scale=100µm.

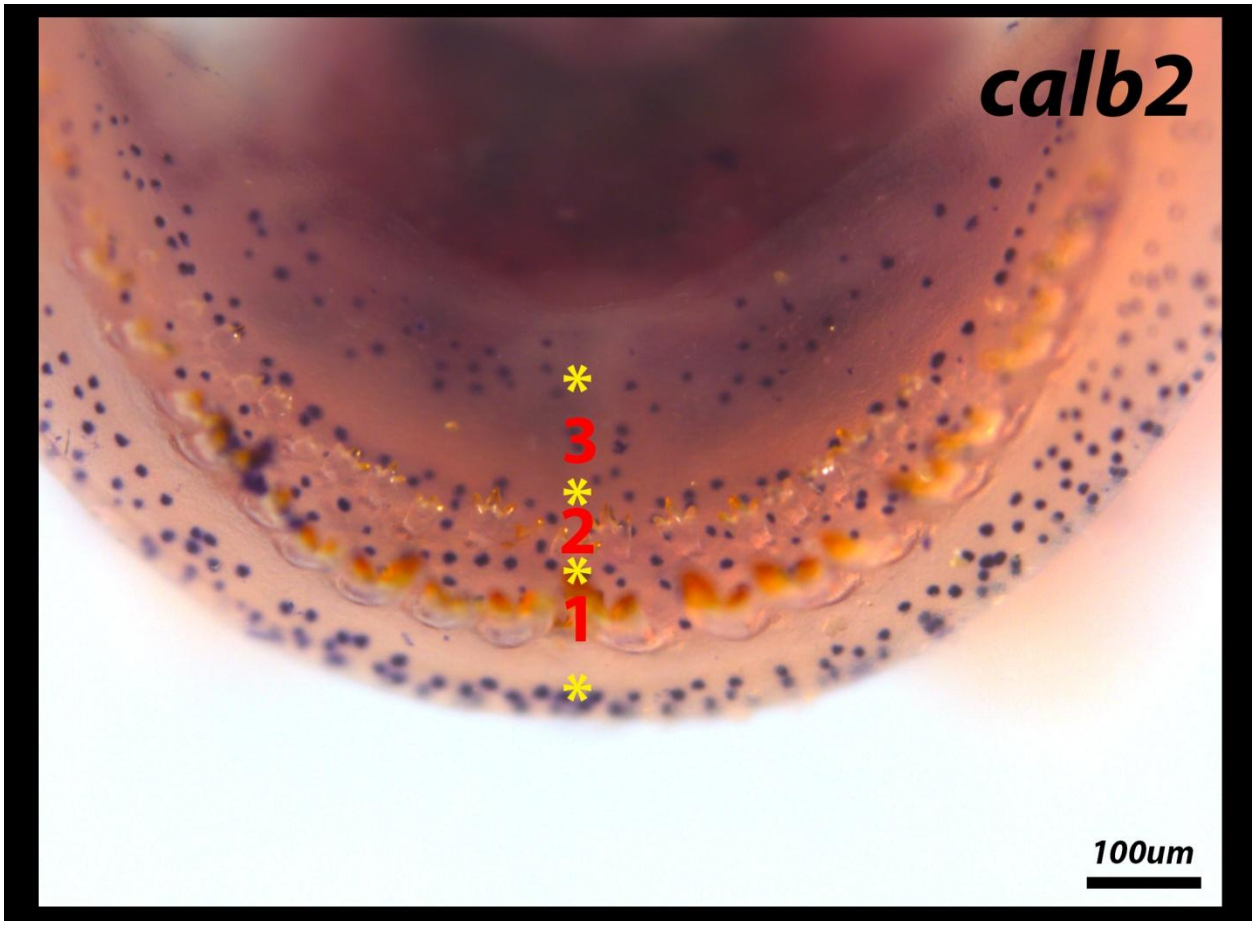

Figure C.4. ISH of *calb2* in cichlid lower jaw at 4 months. Yellow asterisk marks rows of expression (TB) while rows of teeth are marked by red numerals. Note row 3 not yet erupted. Dorsal view, labial to bottom of page, scale=100µm.

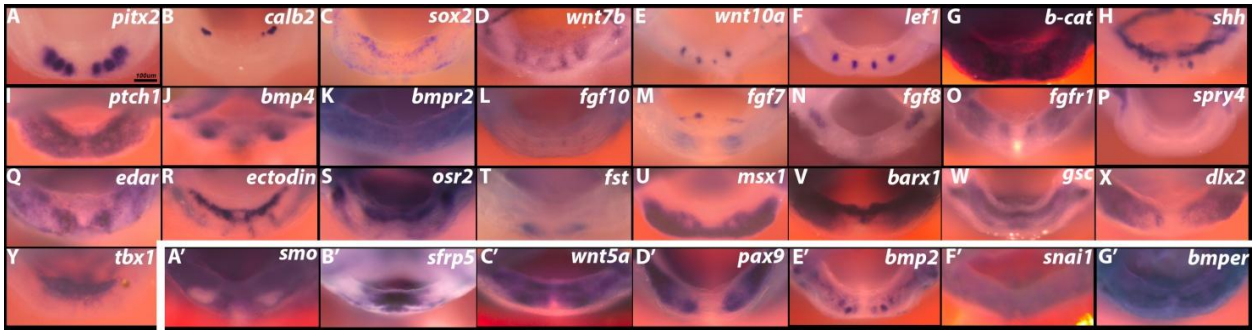

Figure C.5. Expression of patterning genes in cichlid dentaries. Dorsal views of dentaries (lower jaw) at 6dpf after ISH for *pitx2* (A), which marks the tooth field while *calb2* (B) and *sox2* (C) mark the taste field. Factors in the Wnt pathway (D-G), Hh pathway (H,I), BMP pathway (J,K), FGF pathway (L-P) and *edar* (Q) are expressed across the jaw in discrete patterns. Putative repressors (R-T) and transcription factors (U-Y) likely serve as intermediaries in these pathways. White lines delineate expression of candidate genes (A'-G') from QTL mapping. Labial to the bottom of page, Scale=100µm.

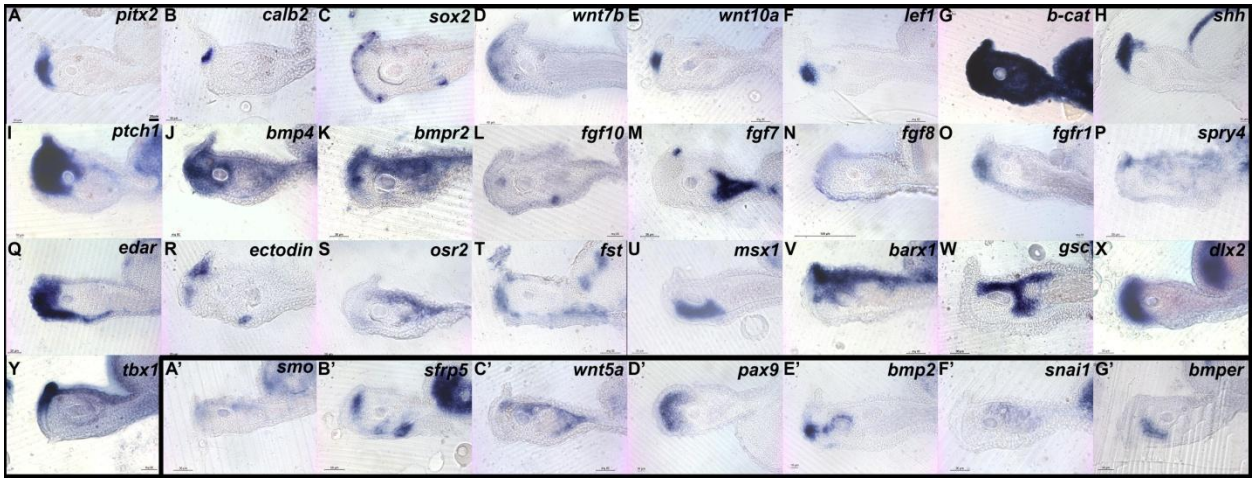

Figure C.6. ISH of cichlid dentary in sagittal histological section for markers in main Figure 3.2. Black lines bound candidate genes (A'-G'). Labial to bottom of page, rostral to left. 18µm thickness, Scale=20µm.

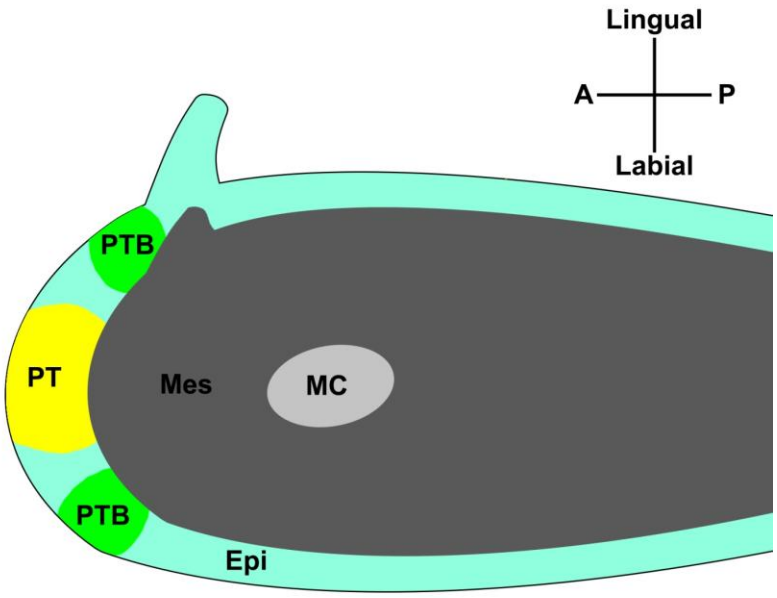

Figure C.6a. Diagram of histological sections presented in Figure B.5. Presumptive TB (PTB) field marked in green and presumptive tooth (PT) field in yellow. Epithelium in blue, mesenchyme in dark grey, and Meckel's cartilage in light grey.

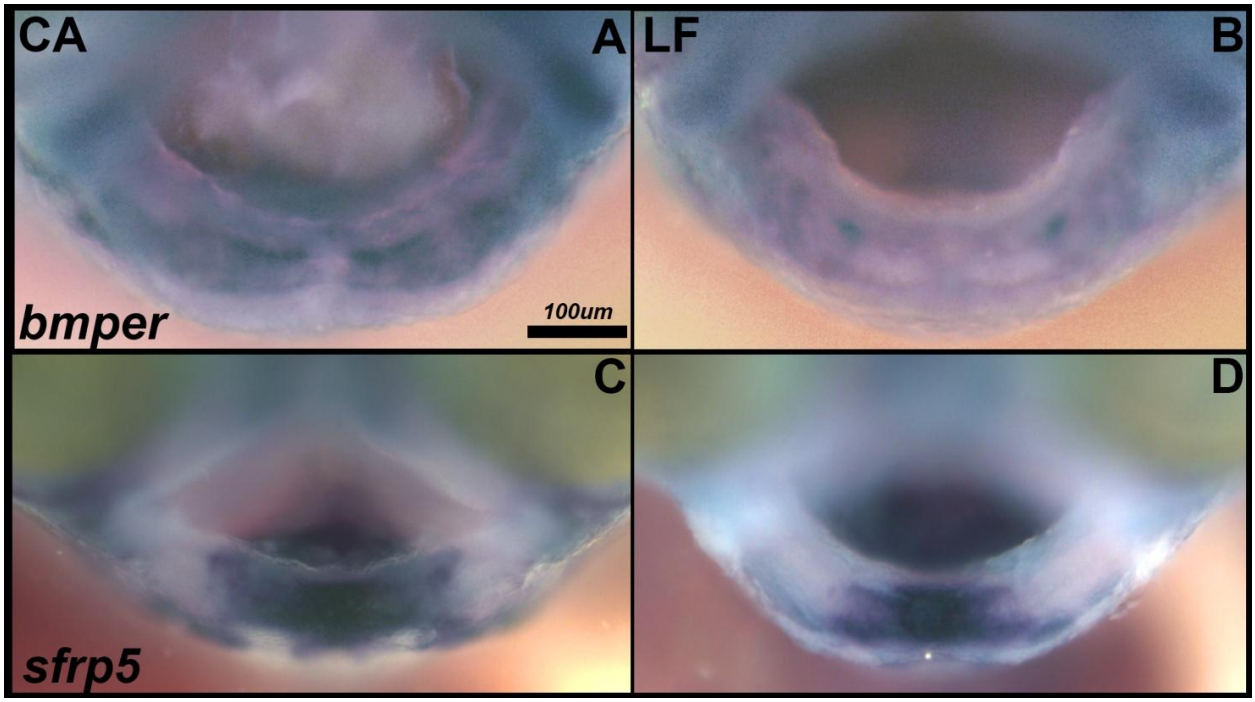

Figure C.7. ISH of candidates between species. *bmper* (A,B) and *sfrp5* (C,D) at patterning stage (5dpf), across species that exhibit (i) many adult teeth and TBs, *Labeotropheus fuelleborni* (LF) or (ii) fewer of each organ, in the adult phenotype*, Cynotilapia afra* (CA). Dorsal views of dentaries, labial to bottom of page, Scale=100µm

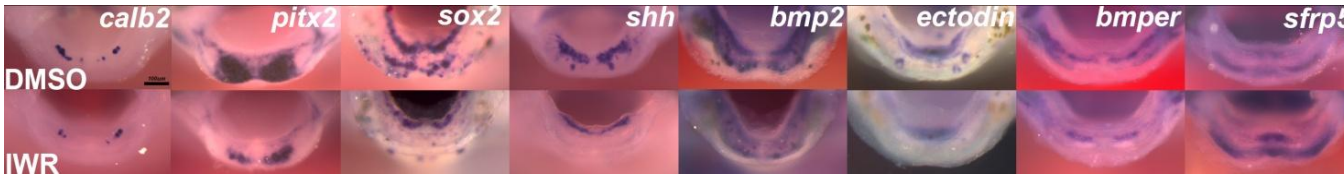

Figure C.8. ISH of genes in cichlid dentary following 24h treatment with IWR initiated at 6dpf and immediate sacrifice. Dorsal views, labial to bottom of page, scale=100µm

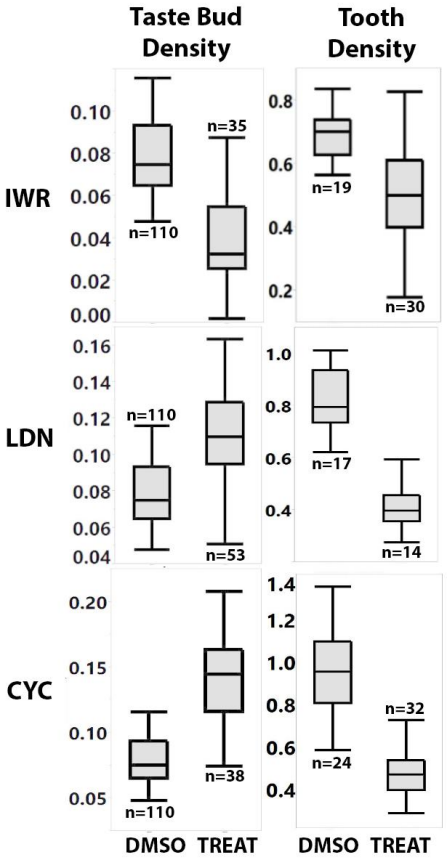

Figure C.9. Box plots summarize statistical analysis of treatments in Figure 3.6 when plotted next to control (DMSO) of TB (# of TB/10μm^2) and teeth (# of teeth/100μm^2). All treatments significant with $p<0.0001$. n= numbers of animals used.

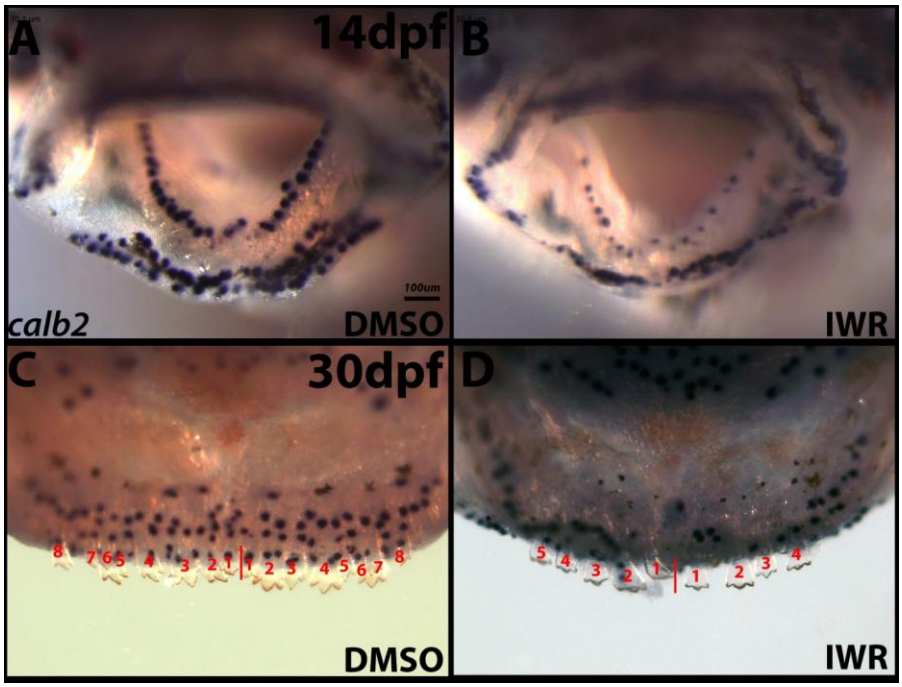

Figure C.10. Lasting effect after 24 hour treatment at 6dpf assayed at 14dpf by *calb2* ISH (A,B). Broodmates from same treatment sacrificed at 30dpf (C,D; ~3wk after IWR removal), shows bilateral reduction in tooth (red numerals) and TB number. Dorsal views, labial to bottom of page, scale=100μm.

Table C.1. Quantitative trait loci for tooth and TB density, including degrees of freedom (df), logarithm of odds ratio (LOD), percentage of variance explained (PVE), P-Value.

Model	df	LOD	PVE	P-Value
Tooth (full)	28	32.1	43.12	2.2E-16
11@5.8 (46509)	10	13.68	15.47	0.00
19@52.9 (50229)	6	10.23	11.21	0.00
14@19.3 (19640)	6	8.73	9.43	0.00
17@34.8 (104101340)	6	7.62	8.15	0.00
11@16.0 (26374)	6	6.97	7.41	0.00
3@13.2 (51426)	2	4.77	4.98	0.00
15@13.9 (44647)	2	3.95	4.09	0.00
20@51.3 (44246)	2	3.48	3.59	0.00
11@5.8:19@52.9	4	8.71	9.41	0.00
11@16.0:14@19.3	4	5.45	5.72	0.00
11@5.8:17@34.8	4	4.16	4.31	0.00
Taste (full)	14	13.77	21.49	7.26E-08
14@0.0 (47999)	6	5.42	7.85	0.00
19@17.3 (30635)	6	3.51	5	0.02
17@34.8 (104101340)	2	2.81	3.97	0.00
20@4.4 (31653)	2	1.77	2.48	0.02
3@33.7 (54663)	2	2.12	2.99	0.01
14@0.0:19@17.3	4	2.24	3.15	0.04

Table C.2. Tissue distribution of genes assayed by ISH in Figure 3, S5. Mesenchyme in blue, epithelium in purple. Genes below black line are candidates from mapping analysis.

Gene	Mes	Epi
pitx2	-	+
calb2	-	+
wnt7b	+	+
wnt10a	-	+
lef1	+	+
bcat	+	+
shh	-	+
ptch1	+	+
bmp4	+	+
bmpr2	+	+
fgf10	+	+
fgf7	-	+
fgf8	-	-
fgfr1	+	+
spry4	-	+
edar	+	+
ectodin	+	+
osr2	+	-
fst	+	+
sox2	-	+
msx1	+	-
barx1	+	+
gsc	+	-
dlx2	+	-
tbx1	-	+
smo	+	+
sfrp5	+	+
wnt5a	+	-
pax9	+	-
bmp2	+	+
snai1	+	-
bmper	+	-

APPENDIX D

SUPPLEMNTAL MATERIALS FOR CHAPTER 4

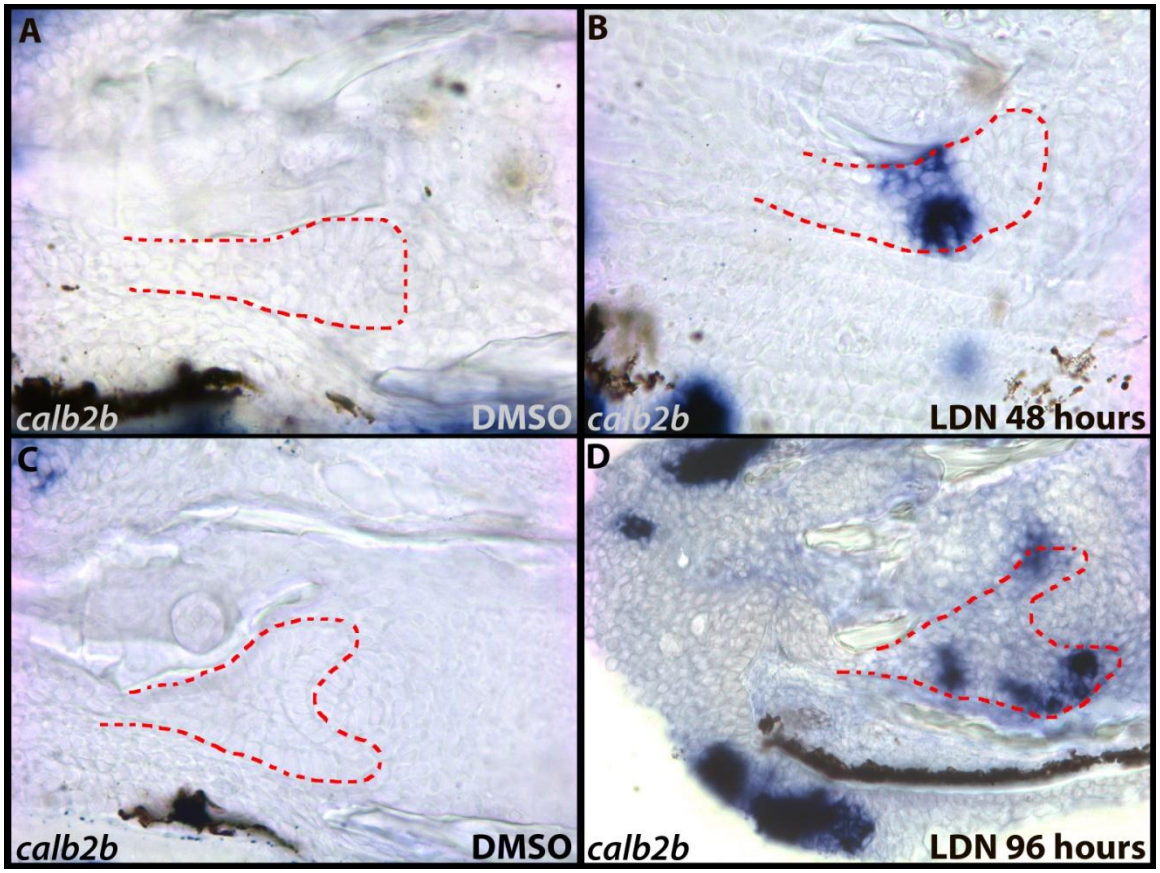

Figure D.1. Effect of treatment on initiation and differentiation stages of RT maturation. Cells express *calb2* in elongate blebs within the RT germs and the effects are more severe with longer 96 hour treatments (D).