Regulatory Mechanisms Underlying Regeneration of the Adult Zebrafish Exoskeleton

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Abstract

The fin exoskeleton of the zebrafish is comprised of lepidotrichia (or fin rays) and actinotrichia. In uncut fins, the fin rays span the entire length of the fin and the actinotrichia are found at the distal tips of each of the fin rays. Both of these fin exoskeletal components are capable of regenerating following amputation or injury. The regulation of the regeneration of these exoskeletal components is the central topic of this thesis and is explored in two different projects. The first project focuses on zebrafish fin ray regeneration during which bone segments are periodically added at the distal tips of each fin ray, each segment being separated by a joint. Joint formation involves the expression of a unique set of genes: *hoxa13a, evx1*, and *pthlha*. The alternation between bone segment formation and joint formation during fin ray regeneration seems to closely correlate with positional outgrowth during regeneration. We investigated whether or not the calcineurin and retinoic acid (RA) signalling pathways, both of which may be potential regulators of positional outgrowth, are involved in regulating joint formation. FK506-induced calcineurin inhibition and RA treatments each resulted in the suppression of joint marker expression. In RA-treated fins, bone deposition occurs in the joints as a result of joint cells being induced to differentiate into osteoblasts. These results suggest that the calcineurin and RA pathways may provide the positional information that regulates joint and bone segment formation. The second project focuses on the regulation of actinotrichia formation during adult fin regeneration. Throughout the early to intermediate stages of fin ray regeneration, actinotrichia fibers are found deep to the regenerating hemirays. As regeneration progresses, these actinotrichia fibers become gradually restricted to the distal domain of the fin regenerate. Actinotrichia contain structural proteins known as actinodin. There are four *actinodin* genes in zebrafish, *actinodin1-4*. We studied the comparative activity of the *cis*-acting regulatory elements of *actinodin1* during fin regeneration. We have previously identified tissue-specific *cis*-acting regulatory elements in a 2kb genomic region upstream of the first exon, termed 2P, that drive reporter expression
in the fin fold ectoderm and mesenchyme during embryonic development. Within 2P is a 150bp region, named \textit{epi}, which contains an ectodermal/epithelial enhancer. Using \textit{in silico} analysis, we have identified four main clusters of transcription factor binding sites within \textit{epi}, termed \textit{epi1-4}. Using a reporter transgenic approach, we have identified \textit{epi3} as a site containing an early mesenchymal-specific repressor and an epithelial-specific enhancer. We have also shown that the first exon and intron of \textit{actinodin1} contains a general transcriptional enhancer in adulthood and an alternative promoter. Overall, these results suggest that there is a difference between the regulation of \textit{actinodin1} during embryonic development and that of adult fin regeneration.
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<table>
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<th>Description</th>
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<tbody>
<tr>
<td>4-OHT</td>
<td>4-hydroxy-tamoxifen</td>
</tr>
<tr>
<td>AER</td>
<td>Apical Ectodermal Ridge</td>
</tr>
<tr>
<td>And/and</td>
<td>Actinodin/actinodin (Protein &amp; gene, respectively; structural proteins of actinotrichia)</td>
</tr>
<tr>
<td>BEL</td>
<td>Basal epithelial layer</td>
</tr>
<tr>
<td>bglap</td>
<td>bone γ-carboxyglutamic acid-containing protein</td>
</tr>
<tr>
<td>bp</td>
<td>base pair(s)</td>
</tr>
<tr>
<td>cmcl2</td>
<td>cardiac myosin light chain type 2</td>
</tr>
<tr>
<td>col10a1a</td>
<td>collagen 10a1a</td>
</tr>
<tr>
<td>cx43</td>
<td>connexin43 (gene coding for a gap junction protein)</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>dlx5a</td>
<td>distal-less homeobox 5a (gene coding for a transcription factor)</td>
</tr>
<tr>
<td>dpa</td>
<td>Days post amputation</td>
</tr>
<tr>
<td>dpt</td>
<td>Days post start of treatment</td>
</tr>
<tr>
<td>eGFP</td>
<td>Enhanced green fluorescent protein</td>
</tr>
<tr>
<td>evx1</td>
<td>even skipped homeobox 1 (gene coding for a transcription factor)</td>
</tr>
<tr>
<td>FF</td>
<td>Fin fold</td>
</tr>
<tr>
<td>Fgf</td>
<td>Fibroblast Growth Factor</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescence in situ hybridization</td>
</tr>
<tr>
<td>FK506</td>
<td>Tacrolimus</td>
</tr>
<tr>
<td>FKBP</td>
<td>FK506-binding protein</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>hox</td>
<td>homeobox</td>
</tr>
<tr>
<td>hoxa13a</td>
<td>homeobox A13a (gene coding for a transcription factor)</td>
</tr>
<tr>
<td>hpa</td>
<td>Hours post amputation</td>
</tr>
<tr>
<td>hpf</td>
<td>Hours post fertilization</td>
</tr>
<tr>
<td>HS4</td>
<td>Hypersensitivity site 4 (from chicken)</td>
</tr>
<tr>
<td>ihhA</td>
<td>indian hedgehog a (gene coding for a signalling molecule)</td>
</tr>
<tr>
<td>Ins</td>
<td>Insulator</td>
</tr>
<tr>
<td>kb</td>
<td>kilo base pair(s)</td>
</tr>
<tr>
<td>lef1</td>
<td>lymphoid enhancer-binding factor 1 (gene coding for a transcription factor)</td>
</tr>
<tr>
<td>MFF</td>
<td>median fin fold</td>
</tr>
<tr>
<td>msxe</td>
<td>muscle segment homeobox e</td>
</tr>
<tr>
<td>oct4</td>
<td>octamer-binding transcription factor 4</td>
</tr>
<tr>
<td>TCF</td>
<td>T-Cell-specific Transcription Factor</td>
</tr>
<tr>
<td>Tg</td>
<td>Transgenic line</td>
</tr>
<tr>
<td>TRANSFAC</td>
<td>Transcription factor identification software</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
</tr>
<tr>
<td>ptthla</td>
<td>parathyroid hormone-related protein a (gene coding for a signalling molecule)</td>
</tr>
<tr>
<td>RA</td>
<td>Retinoic Acid</td>
</tr>
<tr>
<td>runx2a/b</td>
<td>runt-related transcription factor 2a or 2b</td>
</tr>
<tr>
<td>sp7</td>
<td>osterix (gene coding for a transcription factor)</td>
</tr>
<tr>
<td>Wnt/wnt</td>
<td>Wingless-related integration site/wingless-related integration site (Protein &amp; gene respectively; signalling molecule)</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
<tr>
<td>ZFIN</td>
<td>Zebrafish Information Network</td>
</tr>
</tbody>
</table>
Genetic Nomenclature in Zebrafish

1117-1+EI 5’ end of epi region towards 3’ end of first intron of actinodin1
2P 2 kilobase pair region upstream of first exon (including a promoter) of actinodin1
2PΔepi The 2P region of actinodin1 with the omission of the epi fragment that drives mesenchymal specific expression
2PΔepi+EI The 2P region of actinodin1 with the omission of the epi fragment attached to the first intron and exon of actinodin1
2P+EI Regulatory region including 2P and the first exon and intron of actinodin1
E first exon of actinodin1
EI Regulatory region including the first exon and intron of actinodin1
epi A 150 base pair region located within the 2P region of actinodin1 driving ectodermal/epithelial- specific expression in the fins
I first intron of actinodin1
m-inta11 Mouse intron of Homeobox A11 gene; contains an enhancer bound by Hoxa13
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Chapter 1. General Introduction

1.1. Epimorphic Regeneration

Regeneration is the process by which damaged or lost structures are perfectly or near-perfectly replaced, which is a remarkable phenomenon that has been documented since 1900 by Thomas Hunt Morgan. In contrast, repair is a healing process that entails inflammation, fibrosis and the formation of a permanent collagen-rich connective tissue known as a scar. In mammals, including humans, regenerative capacity is limited, which makes it difficult for them to recover from injuries, such as those to the heart and brain. Most mammalian organs heal by scar formation. In invertebrates and in some vertebrates that lay their eggs in water, regenerative capacity is much higher. Teleost fish, which is an infraclass of the Actinopterygii or bony ray-finned fish class, are examples of anamniotes that are capable of appendage regeneration. The process by which appendage regeneration occurs in anamniotes involves the formation of the blastema, which is a highly proliferative mass of dedifferentiated cells that serves as a key intermediate during regeneration. Regenerative capability that entails blastema formation is more specifically known as epimorphic regeneration (Morgan, 1901; Akimenko et al., 2003; Poss et al., 2003). The study of the regenerative mechanisms in species that have high regenerative capacity can bring about great advancements in human regenerative medicine.

1.2. Zebrafish as a Model Organism for the Study of Regeneration

Zebrafish, Danio rerio, are teleost fish that have the ability to regenerate all of their fin types, along with several other organs, following amputation or injury. They possess two sets of paired fins, which are named the pectoral and pelvic fins, as well as three unpaired fins, which are named the dorsal, caudal and anal fins. Zebrafish serve as a very convenient model organism for the study of development and regeneration because they are amenable to standard molecular and genetic
manipulations. Zebrafish researchers have access to well-established procedures for mutagenesis screening, a complete and high-quality sequence of the zebrafish genome, and well-developed transgenesis and knockout techniques. Zebrafish also have a relatively short generation time, they can be housed and maintained in large numbers, and they can be studied using various reagents and technologies. The zebrafish caudal fin is primarily used to assay regeneration due to its accessibility, simpler anatomy, short regeneration time and symmetry. The symmetrical shape of the caudal fin allows one of the two lobes to be used as a control while the other can undergo experimentation (Akimenko et al., 2003; Poss et al., 2003; Pfefferli and Jazwinska, 2015; Wehner and Weidinger, 2015). The majority of zebrafish fin regeneration studies have been done using the caudal fin; therefore, aside from its functional role in locomotion, it is a well-established model for the study of regeneration.

1.3. Overview of of the Zebrafish Caudal Fin Anatomy

All of the zebrafish fins possess a skeletal structure that consists of an endoskeleton and an exoskeleton. The endoskeleton is composed of endochondral bones, which have a cartilaginous origin and are found deeper within the body. The exoskeleton is located in the external part of the fin and consists of two main components: (1) lepidotrichia, which are dermal or intramembranous bones that form via the direct mineralization of the bone matrix, and (2) actinotrichia, which are unmineralized, rigid bundles of fibres of elastoidin (Figure 1.1). Intramembranous ossification differs from endochondral ossification in that the latter requires a cartilage intermediate. The exoskeleton is connected to the endoskeleton via ligaments.

The structural integrity of the adult zebrafish fin is maintained by the lepidotrichia, also known as bony fin rays (see Figure 1.1 for fin ray anatomy). These fin rays are important for providing structure to the fin and for protecting the softer tissues located within. The fin rays run along the proximal-
distal axis of the fin and are separated by soft interray tissue (Akimenko et al., 2003). The caudal fin, in particular, consists of 16-18 fin rays. The entirety of each fin ray consists of two segmented, biconcave-shaped hemirays facing one another that shelter various types of mesenchymal tissue ("mesenchymal" describes loose tissue organization). The bone segments of the fin rays are separated by joints and are joined together by collagenous ligaments. Each fin ray, with the exception of the lateral fin rays is capable of successive bifurcations during fin outgrowth. Each hemiray is lined with layers of bone-forming cells known as osteoblasts.

**Figure 1.1. Zebrafish Exoskeletal Elements.** (A) Bright field image of whole caudal fin. Red arrows point to fin rays and lateral rays. (B) Schematic representation of fin ray skeleton. Red arrows and brackets indicate individual bone segments and joints, the bifurcation point, the sister rays, the actinotrichia and the hemirays that constitutes the fin ray skeleton. (C) Transverse cross-section of a single fin ray showing tissues enclosed within the hemirays and within the interrays (interray = region located in between two fin rays). f: fin ray; l: lateral ray; sg: bone segment; sr: sister ray; j: joint; b: bifurcation point; hm: hemiray. Scale bar = 200μm.
Enclosed within the hemirays and interray tissue (interray = region located in between two fin rays) are several other types of mesenchymal tissues, which include blood vessels, fibroblasts, pigments cells and nerve tissue. The actinotrichia are found at the distal tips of each fin ray and are maintained by actinotrichia-forming cells, which are located within the mesenchymal compartment deep to the osteoblasts (reviewed in: Akimenko et al., 2003; Poss et al., 2003; Pfefferli and Jazwinska, 2015).

1.4. Overview of Zebrafish Fin Regeneration

The process of fin regeneration can be summarized into three main events (reviewed in: Akimenko et al., 2003; Poss et al., 2003; Pfefferli and Jazwinska, 2015; Wehner and Weidinger, 2015):

(1) **Formation of the wound epithelium (Figure 1.2, 1.3).** Within one to three hours of injury, epithelial cells of the amputated or injured fin rearrange and migrate to form a single layer of epithelial cells that serves to cover the wound. Over the next 12 to 18 hours, the epithelium accumulates additional layers of epithelial tissue, which together constitutes the wound epithelium. This process appears to occur via cell migration, and not proliferation.

(2) **Formation of the blastema (Figure 1.2, 1.3).** Mature cells that are located a few segments proximal to the amputation plane will dedifferentiate and migrate distally to become part of a large mass of dedifferentiated mesenchymal cells, which are collectively known as the blastema. The blastema consists of two distinct populations, one of which is the proximal blastema, which is highly proliferative. The other population of blastemal cells is known as the distal blastema which consists of slow-cycling cells which are believed to provide the progenitors that will become part of the proximal blastema. As the name implies, the proximal blastema is located in the more proximal regions of the fin with respect to the distal blastema. The blastema is fully established at approximately 72 hours post amputation (hpa) in a housing environment of 28°C.
(3) **Regenerate outgrowth (Figure 1.2, 1.3).** Several tissue domains can be distinguished in the zebrafish fin regenerate. Cells of the blastema eventually enter a region just proximal to the blastema, known as the differentiation zone, and differentiate into more specific cell types to reform the lost fin. The differentiation zone and the blastema are segregated by opposing mechanisms which either stimulate cell proliferation within the blastema or cause cells entering the differentiation zone to lose their proliferative state and to differentiate into more specific cell types. The regenerative program relies on signaling interactions between the wound epithelium and underlying mesenchyme (Figure 1.3). The deepest layer of the epithelium lining the regenerate is known as the basal epithelial layer (BEL). The BEL contains important signaling centres that also mediate differentiation. The mesenchymal cells that participate in the cellular signaling interactions with the BEL make up the blastema as well as the differentiating cells exiting the blastema (Figure 1.3).

![Figure 1.2. Schematic representation of a longitudinal section of a 4 dpa (days post amputation) fin regenerate, stained with DAPI.](image-url)

The wound epidermis is located at the very tip of the regenerate. The entire regenerate is lined with several epithelial layers. The location of the blastema is delineated by the yellow dotted circle. The basal epithelial layer is the deepest epithelial layer of all epithelial layers that emerges throughout regeneration. The hemirays are the linear regions devoid of cells (blue dotted lines) and are lined with layers of osteoblasts. Actinotrichia-forming cells are found deep to the osteoblasts and adjacent to another thin space where longitudinally organized actinotrichia fibers span the proximal-distal axis of the regenerate (orange dotted lines). The amputation plane is delineated by the dotted red line. **we:** wound epithelium; **e:** epithelium; **b:** blastema; **bel:** basal epithelial layer; **h:** hemiray; **ob:** osteoblasts; **af:** actinotrichia-forming cells; **a:** actinotrichia. Scale bar = 50μm.
Figure 1.3. Simplified schematic of the cellular compartments of the fin regenerate. Once formed, the blastema matures into a highly organized structure that can be divided into several different tissue domains with distinct proliferative potential and gene expression.
1.5. Overview of Zebrafish Fin Ray Regeneration

Following amputation, mature osteoblasts that are located within a few segments proximal to the amputation plane dedifferentiate, proliferate and migrate towards the wound site (Figure 1.4B). Despite their dedifferentiation, osteoblasts remain fate-restricted, as previously indicated by lineage-tracing studies (Knopf et al., 2011; Stewart and Stankunas, 2012). In the nascent blastema, osteoblast-derived mesenchymal cells, which eventually become pre-osteoblasts, form a subpopulation within the lateral regions of the blastema underneath the wound epithelium (Figure 1.4C). After several rounds of division, osteoblast-derived mesenchymal cells exit the blastema, lose their proliferative state and redifferentiate into mature osteoblasts (Stewart et al., 2014; Knopf et al., 2011) (Figure 1.4D). In the distal part of the regenerate, osteoblasts secrete bone matrix, which is released between the basement membrane of the epithelium and the osteoblasts. In the more proximal regions of the regenerate, osteoblasts surround the developing hemiray. During regenerative outgrowth, osteoblasts are periodically interrupted by fin ray joints (Figure 1.5). Fin ray segmentation involves the periodic formation of joints at the distal tips of the outgrowing fin ray; the formation of new joints depends on the differentiation of joint cells, which flank the forming joint. The molecular mechanisms involved in joint formation between dermal bones remains largely unknown compared to what is known about the formation of joints of endochondral bones.
Figure 1.4. Osteoblast dedifferentiation and redifferentiation during fin regeneration. Schematic representations of longitudinal sections of an (A) intact fin and of a (B-D) regenerating fin. Legend indicates different cell types at different stages of differentiation. Grey lines represent hemirays, which are flanked by mature osteoblasts on the inner and outer sides. Red arrow in B indicate direction of cell migration.

Figure 1.5. 7dpa regenerate of reporter transgenic line of zebrafish in which mature osteoblasts fluoresce red. mCherry expression in mature osteoblasts is periodically interrupted at the level of the joints (yellow arrowheads). Scale bar = 100μm.
1.6. Actinotrichia

Aside from the lepidotrichia, the actinotrichia are also part of the zebrafish fin exoskeleton. As previously described, actinotrichia are unmineralized fibres that form brush-like bundles at the distal tips of the fin rays (Figure 1.1). Their functional roles in the adult intact fins remain unknown (Becerra et al., 1996; Montes et al., 1982; Duran et al., 2011). Actinotrichia are the first exoskeletal elements to form during embryonic development and adult fin regeneration.

1.6.1. Actinotrichia Role and Formation in Fin Development

The zebrafish pectoral fins originate from an embryonic structure known as the fin bud, which is an outgrowth of the lateral plate mesoderm. Zebrafish embryos possess fin buds, which arise 24 hours post fertilization (hpf) (Kimmel et al., 1995). The fin bud contains important signalling centres that include the apical ectodermal ridge (AER), which patterns the proximal-distal axis. The AER is a thickened layer of ectoderm that rims the distal edge of the fin bud and promotes cell proliferation and outgrowth of the underlying mesenchyme (Grandel and Schulte-Merker, 1998; Grandel et al., 2000; Kawakami et al., 2003). In fin development, the AER eventually folds on itself to form a structure known as the fin fold (FF), which will continue to extend distally. The FF consists of two sheets of ectodermal cells that are separated by two rows of actinotrichia fibres (Figure 1.6) (Duran et al., 2011; Géraudie and Meunier, 1980; Grandel and Schulte-Merker, 1998). Here, in the subepidermal space of the FF, the actinotrichia not only helps to maintain the structure of the fin fold, but also serve as a scaffold for the migration of mesenchymal cells into the FF (Wood and Thorogood, 1984). As fin development progresses, the lepidotrichia eventually emerge and form in the proximal-to-distal direction, while actinotrichia fibers become progressively restricted to the distal part of the fin (Géraudie and Meunier, 1980; Géraudie, 1984).
1.6.2. Acinotrichia in Fin regeneration

As of now, the role of actinotrichia in the adult fin has yet to be determined. However, it has been suggested that, similarly to what occurs in embryonic development, the actinotrichia have vital importance in maintaining structure, and in cell migration and signaling in adult fin regeneration. For example, in fin ray regeneration, they have been suggested to indirectly regulate the commitment and differentiation of osteoblasts (Wehner et al., 2014). Actinotrichia are organized in longitudinal bundles within the subepithelial space of the distal-most regions of the regenerating fin, where they are found between the epithelium and underlying mesenchymal cells of the blastema (Figure 1.7B). They, additionally, occupy the mesenchymal compartment deep to the osteoblasts in the more proximal regions of the regenerate (Figure 1.7A). As regeneration progresses, the actinotrichia degrade in the more proximal regions and become restricted to the distal tips of the lepidotrichia (Duran et al., 2011; Konig et al., 2017); the fate of the actinotrichia-forming cells during this distalizing degradation of the actinotrichia remains unknown. Aside from providing mechanical support at the distal-most tissues of the fin regenerate, the actinotrichia may potentially act as a substrate for the migration of blastemal cells (Knopf et al., 2011; van den Boogaart et al., 2012; Konig et al., 2017).
1.6.3. The actinodin family and the cis-acting regulation of actinodin1

Actinotrichia fibers are composed of collagenous and non-collagenous components (Duran et al., 2011; Géraudie and Meunier, 1980; Mari-Beffa et al., 1989). The non-collagenous components are known as the Actinodin proteins, which are tyrosine-rich proteins. These proteins are encoded by the actinodin (and) gene family, which consists of four actinodin genes, and1-4 (Zhang et al., 2010). During fin regeneration, the formation of actinotrichia correlates spatially and temporally with and1 expression. And1 expression occurs in actinotrichia-forming cells which are composed of a portion of the BEL and a subset of mesenchymal cells deep to the osteoblasts (Zhang et al., 2010; Duran et al., 2011; König et al., 2017).

The and1 gene contains five exons, (of which the first exon is untranslated), and four introns. The cis-acting regulatory elements of actinodin1 have been characterized in developing zebrafish embryos (this will be discussed in more detail in chapter 3). It was found that a 2-kb region located upstream of the first exon of actinodin1 contains several tissue-specific cis-acting regulatory elements as well as a promoter located approximately 200bp upstream of the first exon (Figure 1.8). This 2-kb region is termed 2P. As found in reporter transgenesis experiments, 2P is sufficient to drive reporter

Figure 1.7. Actinotrichia fibers in 4dpa fin regenerate. Immuno staining for And1, labeling actinotrichia fibers (in green). (A) Longitudinal section showing actinotrichia in the mesenchymal compartment in the more proximal regions of a 4dpa fin regenerate. (B) Tranverse section showing actinotrichia in the subepithelial space within the distal region of a 4dpa fin regenerate. Red lines delineate subepithelial space (beneath basal epithelial layer). Left-most panel shows a brightfield image of part of a 4dpa fin regenerate. Fin rays are indicated. Locations from where sections were obtained are delineated by yellow dotted lines. a: actinotrichia; se: subepithelial space; f: fin ray. Scale bars = 50μm. (Jing Zhang, unpublished).
expression in the ectoderm and underlying mesenchyme of the pectoral and median fin folds of zebrafish embryos (Lalonde et al., 2016), where endogenous *actinodin1* expression also occurs (Zhang et al., 2010). The characterization of the *cis*-acting regulatory elements within 2P involves expression analysis of several reporter transgenic lines, each of which have DNA fragments, originating from 2P, of varying lengths driving *egfp* expression. These analyses have mainly been done in zebrafish embryos.

Figure 1.8. Activity of 2P *cis*-acting regulatory elements of *actinodin1* in reporter transgenic zebrafish 3dpf embryos. (A) Bright field image of a 3dpf embryo showing the yolk sac and the median fin fold seen in the yellow dotted box. The pectoral fin fold is difficult to see in a brightfield image and is therefore indicated in panel C by the yellow arrow. (B) Schematic representation of 2P upstream of the first exon of *actinodin1*. (C) Reporter transgenic line in which 2P drives eGFP expression in 3dpf zebrafish larvae and schematic of reporter construct. Expression occurs in ectoderm and fin fold mesenchyme of the median and pectoral fin fold (yellow arrow). Ectodermal- and fin fold mesenchymal-specific expression can be separately seen in the following transgenic lines: (C’) Reporter transgenic line showing only ectodermal-specific reporter expression. (C”) Reporter transgenic line showing only fin fold mesenchymal-specific reporter expression. y: yolk sac; mff: median fin fold. The green fluorescence seen in the yolk sac is autofluorescence. Note: The regulatory elements used to generate the reporter transgenic lines shown in panels C’ and C” will be discussed in greater detail in chapter 3. Scale bar: A-C, C’, C” = 200μm.
1.7. General Summary of Objectives

The main objectives of the studies discussed in this thesis are centered on further understanding the regulation of exoskeleton formation during adult zebrafish fin regeneration. The topics that will be explored are the formation of the fin ray joints and the molecular regulation of *actinodin1*.

Objective (1): Lepidotrichia segmentation during fin ray regeneration occurs via the alternation between bone segment and joint formation at specific intervals during outgrowth; the molecular mechanisms that underlie this alternation have yet to be understood. Moreover, studies done on the formation of joints that articulate dermal bones, such as the zebrafish fin ray skeleton, are limited compared to those done on joints that articulate endochondral bones. In chapter two of this thesis, candidate molecular pathways that govern the formation of fin ray joints will be investigated. We hypothesize that the calcineurin and retinoic acid signaling pathways have a role in joint formation (to be discussed in more detail in chapter 2). The main objective of this chapter is to observe the effects of inhibiting calcineurin signalling or enhancing retinoic acid signalling on the formation of joints during fin regeneration.

Objective (2): Reporter transgenic lines that visualize actinotrichia-forming cells in embryos have been well established through the identification of *cis*-acting regulatory elements that are required for full recapitulation of *actinodin1* expression in zebrafish embryos. As for adult zebrafish, the activity of these *cis*-acting regulatory elements has yet to be characterized. We hypothesize that there is a difference in the regulation of the *actinodin1* gene between embryonic development and adulthood. The main objective of chapter three of this thesis is to perform a comparative analysis on the expression patterns of various *actinodin1* reporter lines in which eGFP is driven by various DNA fragments that are believed to contain candidate enhancer and repressor sequences; these analyses are
done in order to identify DNA fragments containing *cis*-acting regulatory elements of *actinodin1* that are active during adult fin regeneration.

Objective (3): The characterization of the *actinodin1* regulatory elements would allow us to complete the third objective (to be discussed in Appendix A): to determine the fate of actinotrichia-forming cells throughout the regeneration program. The last objective would be accomplished through the use of lineage-tracing experiments that would allow us to permanently label *actinodin1*-expressing cells and to follow them throughout regeneration; this would allow us to further study the dynamics of actinotrichia-forming cells in zebrafish fin regeneration. The only way to fully establish the transgenic lines necessary for these lineage-tracing experiments would be with the use of the *cis*-acting regulatory elements of *actinodin1* to drive adequate transgene expression in adulthood and adult fin regeneration.
Chapter 2. Effects of FK506 and Retinoic Acid on Joint Formation

The results discussed in this chapter have been published in Development as of June 11, 2018 in a subsection of the following article:


Contributions to results discussed in this chapter:

Hue-Eileen Phan performed all drug treatments, staining and epifluorescence imaging.

Hue-Eileen and Jing Zhang equally contributed all data obtained from in situ hybridization experiments, TUNEL assays and confocal imaging and processing.

Jing Zhang performed all Zns5 immunostaining experiments.

All images discussed in section 2.3 have been modified from McMillan, Zhang et al. (2018).
2.1. Background Information

The skeleton of the zebrafish fin rays form via intramembranous ossification during development and adult fin regeneration (Akimenko et al., 2003; Poss et al., 2003). Following amputation, mature osteoblasts within the stump dedifferentiate, migrate and populate the lateral regions of the blastema. Osteoblast-derived blastemal cells remain fate-restricted and, therefore, redifferentiate into osteoblasts as they exit the blastema (Figure 1.4) (Knopf et al., 2011; Stewart and Stankunas, 2012). Throughout fin ray outgrowth, bone formation is periodically interrupted by joint formation (Figure 1.5), the mechanisms of which have yet to be understood.

2.1.1. Osteoblast Redifferentiation Pathway

The process of redifferentiation (Figure 2.1) begins with cells located within the lateral regions of the blastema that become pre-osteoblasts upon the expression of runt-related transcription factors 2a and 2b (runx2a and runx2b) (Knopf et al., 2011; Smith et al., 2006). As runx2a/b(+) pre-osteoblasts enter the differentiation zone and come into contact with the basal epithelial layer, they commit to the osteoblast fate by expressing an early bone marker known as osterix (sp7) as well as indian hedgehog a (ihha), both of which are robustly expressed in osteoblasts and redifferentiating osteoblasts throughout regeneration (Stewart et al., 2014; Sousa et al., 2011; Nakashima et al., 2002; Avaron et al., 2006). BMP signaling, from a subset of cells of the basal epithelial layer (BEL), is believed to promote sp7 expression (Stewart et al., 2014). During the intermediate stages of maturation, maturing osteoblasts express collagen, type X, alpha (col10a1a) (Avaron et al., 2006), which codes for one of the components of bone matrix. As they further mature and reach the differentiating zone, they express late differentiation markers such as osteocalcin, or bone γ-carboxyglutamic acid-containing protein (bglap) (Gavaia et al., 2006; Sousa et al., 2011) and downregulate runx2a/b and sp7 (Knopf et al., 2011).
2.1.2. Cellular and Molecular Mechanisms of Joint formation

The fin rays of uncut fins consist of bone segments that are linked together by joints. The mature joints are flanked by joint cells (Pacifici et al., 2006). At the level of the joint, the ends of consecutive bone segments are connected by collagenous ligaments, the composition of which remains largely unknown. As a matter of fact, compared to what is known about joints that articulate endochondral bone, much less is known about the physical structure and composition of joints that articulate dermal bone.

During fin ray regeneration, segments of dermal bone are formed via the periodic addition of joints at the distal end of each fin ray (Johnson and Bennett, 1999; Iovine and Johnson 2000). Joints that are in the process of forming are flanked by joint forming cells. Recent results obtained in our lab have characterized three different stages of joint cell maturation: presumptive joint cells, joint-forming cells, and mature joint cells (McMillan, Zhang et al., 2018). Joint formation (Figure 2.2) begins with the condensation of a discrete band of cells located in the presumptive joint regions at the distal part of the regenerating fin ray (Pacifici et al., 2006). This cluster of cells is named the presumptive joint cells, which then reorganizes into two distinct rows which then separate, forming a physical joint (Sims et al., 2009). Mature joint cells surround the mature joint (McMillan, Zhang et al., 2018).

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**Figure 2.1. Stages of osteoblast differentiation.**

<table>
<thead>
<tr>
<th>Mesenchymal cell</th>
<th>Pre-osteoblasts</th>
<th>Maturing osteoblasts</th>
<th>Mature osteoblasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{Runx2a/2b}$</td>
<td>$sp7$ &amp; $ihha$</td>
<td>$\text{col10a1a}$</td>
<td>$\text{bglap}$</td>
</tr>
</tbody>
</table>

**Commitment** → **Bone matrix secretion**
Figure 2.2. Steps involved in fin ray joint formation.
Schematic representation of regenerating hemiray (gray) showing joint cells (green) at different stages of maturation.

The molecular mechanisms that govern joint formation remain largely unknown; however, it is known that there is a set of genes that are uniquely expressed during joint formation. The *even-skipped homeobox 1* (*evx1*) transcription factor has been established as an important joint marker. *Evx1* is necessary for joint formation as *evx/-* zebrafish mutants lack fin ray joints (Schulte et al., 2011). In addition, recent studies in our lab have identified the *parathyroid hormone-related peptide 1* (*pthlha*) as another potential joint marker (McMillan, Zhang et al., 2018). *Pthlha* is co-expressed downstream of *evx1* at the level of the joints and is absent in *evx1/-* mutants, suggesting that it is acting downstream of *evx1* (McMillan, Zhang et al., 2018). Lastly, our lab has identified *hoxa13a* as a potential joint marker due to its expression at the level of the joints. In a transgenic line of zebrafish, *Tg(m-inta11:eGFP)*, which reports Hoxa13a activity, eGFP fluorescence is observed in joints cells. This observation prompted us to further investigate *hoxa13a* as a potential joint marker. Double fluorescence *in situ* hybridization experiments showed that *hoxa13a* is co-expressed in presumptive joint regions with *evx1* and *pthlha*; however, *hoxa13a* expression continues to persist in *evx1/-* mutants, which raises the question of whether *hoxa13a* is expressed upstream or in parallel with *evx1* and if *hoxa13a* has any direct role in joint formation (McMillan, Zhang et al., 2018).

Recent data obtained in our lab have shown that joint cells and osteoblasts originate from a common cell precursor (McMillan, Zhang et al., 2018). Both joint cells and osteoblasts are positive for Zns5 (Figure 2.3A-C), which is a pan-osteoblast marker, and for *runx2a* and *runx2b* (Figure 2.3D-D’’), which are pre-osteoblast markers (McMillan, Zhang et al., 2018). It was shown that when a new joint...
is forming, these runx2a/b+ pre-osteoblasts cells upregulate hoxa13a expression and subsequently activate evx1, then pthlha (Figure 2.4) (McMillan, Zhang et al., 2018). In contrast, in the absence of new joint formation, the runx2a/b+ pre-osteoblasts will activate sp7 expression and will commit to the osteoblast lineage (Figure 2.4).

Figure 2.3. Osteoblasts and joint cells emerge from a common cell precursor. (A) DAPI staining on longitudinal cryosections of a 4dpa fin regenerate. (B) Continuous staining for pan-osteoblast marker, Zns5, in osteoblasts and joint cells (yellow arrowhead). (C) DAPI (nuclei) staining on the same view as panel B. Joint cell clusters are indicated by dotted circle. Double fluorescence in situ hybridization (FISH) on DAPI-stained longitudinal cryosections of 4dpa fin regenerate for (D) runx2b and (D’) runx2a, which are both continuously expressed within osteoblasts and joint cells. Joint cell clusters are indicated by yellow arrows. (D”) Merge. b: blastema; e: epithelium; o: osteoblasts. Scale bars: A, D-D” = 50μm, B, C = 10μm. Zns5 staining was performed by Jing Zhang.

2.1.3. Regulation of Positional Outgrowth in Zebrafish Fin Regeneration

Size and form are achieved and maintained through the appropriate coordination of isometric and allometric growth. Isometric growth refers to growth that occurs in proportion to body size, while allometric growth refers to disproportionate growth. Zebrafish fins initially grow allometrically in relation to the body size during early juvenile development (Goldsmith et al., 2006). Once the adult
fin shape is reached, growth switches from allometric to isometric (Goldsmith et al., 2003; Iovine and Johnson, 2000). Partial loss of the fin results in the regeneration of the missing tissue to the original adult size by inducing allometric growth, which progresses to an isometric growth rate as the regenerating tissues approach their original dimensions (Kujawski et al., 2014; Lee et al., 2005). The rate of allometric regenerative outgrowth correlates with the location of injury along the proximal-distal axis (Lee et al., 2005).

A previous study performed by Kujawski et al. (2014) suggested that the calcineurin pathway may act as a molecular switch between isometric and allometric growth. Calcineurin is a serine/threonine phosphatase that initiates its pathway by binding to the FK506-binding protein (FKBP) (Liu et al., 1991). The role and mechanism of the calcineurin pathway in fin regeneration is unknown; however, studies have shown that the FK506 drug can inhibit the calcineurin pathway by blocking FKBP. FK506-treated fin regenerates exhibit enhanced fin outgrowth as a result of enhanced cell proliferation within the blastema, which indicates that the calcineurin pathway may act as a negative regulator of allometric growth (Kujawski et al., 2014). FK506 is also an immunosuppressant, however, treatments with other immunosuppressants such as rapamycin and celastrol did not induce enhanced fin outgrowth. This result suggests that the observed enhanced fin outgrowth occurs independently of the immunosuppressing effects of FK506. Interestingly, in situ hybridization results showed that the retinoic acid-synthesizing enzyme (*raldh2*) and the retinoic acid nuclear receptor, *crabp2b*, were upregulated in FK506-treated regenerates, suggesting that the calcineurin pathway regulates positional outgrowth through the inhibition of the retinoic acid (RA) signaling pathway (Kujawski et al., 2014).
2.1.4. Retinoic Acid Signaling in Regeneration and in FK506-treated fins

RA signaling is essential for blastemal cell proliferation and survival (Blum and Begemann, 2012). RA itself is a small hydrophobic molecule of which the signaling pathway is directly regulated by the RA-degrading enzyme, cyp26, and the rate-limiting RA synthesizing enzyme, aldhl2 (rahdh2) (Blum and Begemann, 2012). Cyp26 reduces proliferation of both blastemal and epithelial cells. ALDH1a2-mediated RA synthesis occurs in the distal non-proliferative blastema (Blum and Begemann, 2012; Mathew et al., 2009). Because RA is a small molecule, it is likely highly diffusible, which allows it to regulate proliferation in blastemal regions that are more proximal from its original site of synthesis (Wehner and Weidinger, 2015). RA signaling, Wingless-related integration site (Wnt)/β-catenin and Fibroblast Growth Factor (FGF) signaling have been shown to positively regulate one another to promote blastema survival (Stoick-Cooper et al., 2007; Blum and Begemann, 2012) (Figure 2.4). In conjunction to this positive regulation is the non-canonical Wnt signaling pathway that inhibits blastemal cell proliferation (Stoick-Cooper et al., 2007; Lee et al., 2005; Lee et al., 2009); the non-canonical Wnt signaling pathway is positively regulated by FGF and Wnt/β-catenin signaling and negatively regulated by RA signaling (Blum and Begemann, 2012) (Figure 2.4).

In addition, many studies have shown that the RA signaling pathway regulates positional outgrowth in regenerating amphibian appendages by proximalizing positional information (i.e. by causing cells to adopt a proximal identity). Ectopic RA has been shown to reprogram the positional identity of cells in regenerating axolotl and newt limbs to a proximal identity (Noji et al., 1991; Wanek et al., 1991; Bryant and Gardiner, 1992; McCusker et al., 2013; Brockes, 1997; Crawford and Stocum, 1988; Maden, 1982). In newt limb regeneration, excess RA proximalizes the distal blastema and the extent of proximalization of the regenerated limb increases with the dose of RA (Brockes, 1997; Crawford and Stocum, 1988; Maden, 1982). Thus, it is possible that the enhanced fin outgrowth phenotype
exhibited by FK506-treated fins may be due to RA-induced proximalization of positional information, (which means that the tissues of the fin adopt a more proximal identity).

2.1.5. Relationship between Positional Outgrowth and Joint Formation

As mentioned before, *evx1* is expressed in a segmental pattern at the level of the joints and, as inferred from the lack of dermoskeletal joints observed in null mutants, is required for joint formation (Borday et al., 2001; Schulte et al., 2011; Sims et al., 2009). Mutations in *cx43* (short fin, sof<sup>bl23</sup>) and *kcnk5b* (another long fin, alf<sup>dvy86</sup>) alter *evx1* expression (Sims et al., 2009): the sof<sup>bl23</sup> mutants possess decreased fin lengths and shorter fin ray segments (Hoptak-Solga et al., 2008; Iovine et al., 2005; Ton and Iovine, 2013), while the alf<sup>dvy86</sup> mutants possess increased fin lengths and longer or inconsistent fin rays segments (Hoptak-Solga et al., 2008; Perathoner et al., 2014; Ton and Iovine, 2013). With these data taken together, it is possible that joint formation and positional outgrowth are interrelated.

Figure 2.4. Summary of aforementioned pathways involved in fin outgrowth and osteoblast differentiation.
2.1.6. Objective 1

Little is known about the mechanisms of the formation of joints that articulate dermal bone. Previous studies on zebrafish joint mutants suggest a relationship between positional outgrowth, bone segment formation and joint formation (Sims et al., 2009; Iovine and Johnson, 2000; Iovine et al., 2005; Hoptak-Solga et al., 2008; Schulte et al., 2011); this suggests that this positional information, which has yet to be characterized, may be important in regulating the alternation between bone segment and joint formation. Furthermore, calcineurin inhibition, using FK506 treatments, alters fin outgrowth, perhaps through the upregulation of RA signalling (Kujawski et al., 2014). Based on the results of these studies, we hypothesize that the calcineurin pathway, through the inhibition of RA signaling, may be important in regulating joint formation (i.e. the calcineurin pathway may be important in determining whether a pre-osteoblast will differentiate into an osteoblast or become a joint cell). The main objective of the present study is to investigate the effects of FK506-induced inhibition of calcineurin and RA treatments on joint formation and maintenance during adult fin regeneration and in intact fins, respectively.
2.2. Materials and Methods

2.2.1. Zebrafish Husbandry

All fish used in the experiments were maintained at 28°C with a photoperiod of 14 hours of light and 10 hours of darkness. Fish were fed regularly (Westerfield, 2007). The bglap regulatory fragment was acquired from Dr Christoph Winkler and subcloned in a Tol2 vector to make the bglap:mCherry construct which was then used to create a new transgenic line. Tg(m-inta11:eGFP) line was previously described (Kherdjemil et al., 2016). All experiments were performed according to the CCAC guidelines.

2.2.2. Fin Amputations

Zebrafish were anesthetized by immersion in system water containing 0.17 mg/ml tricaine (Westerfield, 2007). Caudal fins were amputated two segments proximal from the first branch point of the lepidotrichia; referred as standard cut. Fish were then returned to fresh system water to recover.

2.2.3. Live Imaging

Adult fish were anesthetized and placed on a 1% agarose plate with the caudal fins spread out naturally. The plate was placed under a Leica MZ FLIII dissection microscope and images were taken using an AxioCam HSM digital camera and AxioVision AC software (Carl Zeiss). For live confocal imaging, fish were anesthetized and immersed in 0.17 mg/ml tricaine in a petri dish. The caudal fins were flattened to the bottom of the petri dish with a slide hold-down (Warner Instruments 64-0248) and imaged with a water-immersion objective equipped on Nikon A1RsiMP Confocal. All images were processed using ImageJ (NIH).

2.2.4. Double Fluorescence in situ Hybridizations (FISH) on Sections
Double FISH on longitudinal cryosections of adult fin regenerates was adapted from protocols that were previously described (Welten et al., 2006); Perkin-Elmer Manufacturers protocols of TSA Cyanine 3 and Fluorescein system, Perkin Elmer Cat# NEL753001KT and TSA Cyanine 5 system, Perkin Elmer Cat# NEL745001KT. Fin regenerates were fixed and sectioned as described in these protocols. Permeabilization, hybridization and post-hybridization washes on sections were also performed as described in these protocols. After washing with TBST (20 mM NaCl, 150 mM Tris Base, 0.05% Tween) on day two, slides were washed with 2% H$_2$O$_2$ in TNT (0.1 M Tris-HCl pH 7.5; 0.15 M NaCl; 0.5% Tween20) for 10 min and then washed 4x5 min in TNT, blocked for 4 hours in TBSTB (TNT with 0.5% Perkin-Elmer blocking powder), and incubated overnight in anti-DIG-POD (1:500) (Roche) in TBSTB at 4°C. Slides were then washed in TNT (6 x 20 min), stained with Tyr-Cy3 (1:100) in amplification diluent (Perkin-Elmer) for 10 min, and washed in TNT (3 x 5 min). Slides were then washed with 2% H$_2$O$_2$ in TNT for 30 min to eliminate the peroxidase of anti-DIG and washed and blocked as the previous day. Slides were then incubated overnight with anti-DNP-POD (1:500) (Perkin-Elmer) at 4°C. Slides were then washed in TNT (6x20 min), stained with Tyr-Fluorescein (1:100) in amplification diluent (Perkin-Elmer) for 10 min, and washed in TNT (3 x 5 min). Slides were then incubated in DAPI stock solution (5 mg/ml) diluted to 1:10000 with TNT (1 x 5 min), washed in TNT (3 x 5 minutes), washed briefly with water, and mounted with AquaPolymount.

2.2.5. Probe Synthesis

Digoxigenin-labelled (DIG) antisense RNA probes were made from the cDNA plasmid templates and RNA polymerases specified in Table 2.1. 10 μg of plasmid DNA was linearized by enzymatic digestion using the proper restriction enzyme for 2 hours at 37°C. The linearized plasmid was then collected and purified using a GE Healthcare IllustraTM purification kit. The transcription reaction
was prepared as follow: 1 μL of the linearized template DNA was mixed with 2 μL of NTP labeling mix (10 mM ATP, CTP, GTP, 6.5 mM UTP and 3.5 mM DIG-11-UTP) (Roche), 2 μL of 10X transcription buffer (Roche), 0.5 μL of RNase Inhibitor (Fermentas), 2 μL of RNA polymerase (Roche) and 12.5 μL of DEPC treated water. The mix was subsequently incubated for 2 hours at 37°C. The polymerase reaction was stopped, the RNA probe was isolated and purified using a SigmaSpin Post-Reaction Clean-Up Columns (Sigma-Aldrich) and stored at -80°C, as specified in Thisse & Thisse, (2008).

<table>
<thead>
<tr>
<th>Probe Name</th>
<th>Plasmid Vector</th>
<th>Restriction Enzyme</th>
<th>RNA Polymerase</th>
</tr>
</thead>
<tbody>
<tr>
<td>hoxa13a</td>
<td>pGEM-T</td>
<td>SmaI/XmaI</td>
<td>Sp6</td>
</tr>
<tr>
<td>evx1</td>
<td>pCS2+</td>
<td>BamHI</td>
<td>T3</td>
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<tr>
<td>pthlha</td>
<td>pDrive</td>
<td>XhoI</td>
<td>T7</td>
</tr>
<tr>
<td>sp7</td>
<td>pCRII-TOPO</td>
<td>HindIII</td>
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<td>pBK-CMV</td>
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<tr>
<td>ihha</td>
<td>pDrive</td>
<td>SnaBI</td>
<td>Sp6</td>
</tr>
</tbody>
</table>

Table 2.1. Antisense RNA probes for double fluorescence in situ hybridization experiments.

2.2.6. FK506 (Tacrolimus) Treatments

FK506 (Tacrolimus) (Sigma F4679) was dissolved in ethanol and added to system water at 0.1 μg/L; this concentration was chosen based on published data (Kujawski et al., 2014). Fish were treated with FK506 for two days prior to fin amputation to ensure the chemical had time to take effect. Following 2 days of treatment (dot), fins were amputated and allowed to regenerate for 4 days. Every two days, the water was changed with fresh drug. Zebrafish were fed and kept in glass tanks throughout treatments. Control tanks contained either the same percentage of ethanol or system water alone. Each experiment was performed in triplicate (4 fish/tank/experiment).
2.2.7. Retinoic Acid Treatments

RA treatment is adapted from (Jeradi and Hammerschmidt, 2016). 0.5-10 µM of RA were tested and 1 µM is the concentration that induced phenotype without causing mortality. A stock of RA (Sigma R2625) was prepared by dissolving RA powder in DMSO to obtain a final concentration of 10 mM. This stock solution is diluted to 1 mM with ethanol and further diluted to 1 µM with system water. Zebrafish were kept in this solution in 2 L plastic tanks (4 fish/tank/experiment) at 28.5°C in the dark with an air bubbler throughout treatments. RA solution was changed every two days during the period of the experiments. Control groups were left to swim in the 0.001% EtOH and 0.0001% DMSO or system water alone.

2.2.8. In vivo Alizarin Red and Calcein Staining

The alizarin red staining solution was prepared by dissolving alizarin red (Sigma A-5533) directly into fish water to obtain a final concentration of 100 mg/L. The solution was also supplemented with 1 mM Hepes (Roth). Calcein solution was prepared by dissolving calcein powder (Sigma-Aldrich) in fish water to obtain a final concentration of 100 mg/L. The pH for each staining solution was adjusted to 6.5. Fish were left to swim in the dark in Alizarin Red solution or calcein staining solution at 28.5°C for 1 hour. Subsequent to staining, fish were washed 3 times for 5 minutes in fish water. Alizarin red staining was performed on wildtype fish prior to RA treatments and imaging. Calcein staining was performed following treatments and prior to imaging.
2.3. Results

2.3.1. Inhibition of the calcineurin pathway disrupts joint formation and inhibits joint marker expression.

FK506 treatments were done in order to assess the effects of inhibiting calcineurin activity on joint formation during fin regeneration. FK506 treatments were carried out two days prior to amputation in order to ensure that the drug is effective at the time of amputation. Treatments ended four days following amputation. At 4dpa, fin regenerates normally have two or three joints that are in the process of forming and that can be readily observed under the microscope; thus, 4dpa regenerates were collected and analysed. Before FK506-treated 4dpa fin regenerates were collected and fixed, they were observed in vivo. It was noted that FK506-treated 4dpa fin regenerates completely lack joints (Figure 2.5). Based on these results, we wondered if this would be correlated with an uninterrupted, longitudinal expression of bone markers along the fin regenerate and an absence of joint markers. In order to visualize the mRNA expression patterns of bone markers (sp7, col10a1a and ihha) with respect to those of the joint markers (hoxa13a, evx1 and pthlha), double fluorescence in situ hybridization (FISH) experiments were carried out such that the expression analysis of a bone marker was paired with that of a joint marker. In 4dpa regenerates, under normal conditions, bone markers are expressed within the layers of osteoblasts in a discontinuous fashion (Figure 2.6 A’-A”, B’-B”, C’-C”). The gaps of bone marker expression occur at the level of the presumptive joint regions where the joint markers are expressed. (It is important to note that col10a1a expression occurs in two distinct cell layers. The discontinuous expression of col10a1a expression occurs within the deeper of the two layers.) In FK506-treated 4dpa regenerates, there is an absence of joint marker expression and continuous expression of the bone markers (Figure 2.6A-C). The lack of joints in FK506-treated fins suggests that the calcineurin pathway may play an important role in prompting pre-osteoblasts to become joint cells at specific positions along the fin regenerate by
promoting joint marker expression while preventing osteoblast differentiation from occurring within presumptive joint regions.

**Figure 2.5. FK506-treated fins lack joints.** (A) Fish were treated with FK506 starting from two days prior to amputation. The treatment was performed for six days. 0dpa/2dpt (dpt=days post start of treatment), fins were amputated and treatments continued for 4 days. (B, B’), 4dpa/6dpt, no joints form in the regenerates of FK506 treated fish. Fish in (C, C’) ethanol and (D, D’) water controls form joints normally in the fin regenerate (n=16 per group). Panels B, C and D show close-up bright field images on whole fins shown in B’, C’ and D’. Red arrows indicate location of joints (C, C’, D, D’). n = 4 x 4 (4 fish/replicate x 4 replicates). Scale Bars: Panels B, C, D = 200μm, B’, C’ and D’ = 200μm.

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Figure 2.6. FK506 treatments inhibit joint-related gene expression. Double FISH for (A-A’’) *hoxa13a* and *sp7*, (B-B’’) *evx1* and *col10a1a* and (C-C’’) *pthlha* and *ihha* on 4dpa longitudinal sections of (A-C) FK506-treated fins, (A’-C’) EtOH controls and (A’’-C’’) water controls. Numbers in each panel are numbers of sections with the expression pattern shown in a given panel out of the total number of observed sections. Yellow arrows indicate concentrated regions of joint-related marker expression. Scale bar = 50μm
2.3.2. Retinoic acid (RA) treatments suppress joint marker expression and induce ectopic bone deposition in mature joints.

As previously mentioned, FK506-treated regenerates exhibit an upregulation of RA signalling, suggesting that the calcineurin pathway may regulate positional outgrowth through the counter-regulation of the RA signalling pathway (Kujawski et al., 2014). We wondered if an exposure to RA would favour osteoblast differentiation and inhibit joint cell differentiation because FK506-treated regenerates have a higher bone-to-joint ratio than normal fin regenerates.

In the first experimental setup of RA treatments, we performed a pulse RA treatment on 3dpa regenerates for 24 hours. At 1day post start of treatment (dpt)/4dpa, fin regenerates were collected, fixed and longitudinally sectioned for in situ hybridizations for hoxa13a, evx1 and pthlha. 1dpt/4dpa RA-treated regenerates exhibit a loss of all three joint-related genes (Figure 2.7). In the second treatment setup, we performed RA treatments on 7dpa regenerates and continued the treatment for six days (Figure 2.8A). We could not assess the effect of RA treatment on the formation of the new bone segments and needed to allow the fin to grow for seven days before we commenced treatment because RA treatments impair fin outgrowth. As a result, our analysis was restricted to the effect of RA treatment on joint maintenance, which would not be directly affected by impaired fin outgrowth. Prior to treatment, fish were stained in vivo with Alizarin Red in order to mark all ossified bones present before treatment in red. At 6dpt/13dpa, fish were stained in vivo with calcein to label all calcified bone present after treatment in green (Figure 2.8A). Live confocal imaging was used to closely observe the joints. 7dpa fin regenerates allowed us to observe joints at different stages of maturation. In order to compare joints that are at similar maturation stages, the first joint proximal to the first bifurcation point on the second most dorsal fin ray was consistently observed. New bone deposition at the level of the joints, seen only via calcein staining, only occurred in RA-treated fish (Figure 2.8B-B”, C-C”, D-D”). Overall, overexposure to RA results in ossification of the joints.
Figure 2.7 Pulse retinoic acid treatment leads to a loss of expression of joint-related genes. (A) RA treatments were performed on 3dpa regenerates for one day. In situ hybridization for (B, B’, B”) hoxa13a, (C, C’, C”) evx1, and (D, D’, D”) pthlha on longitudinal cryosections of 4dpa/1dpt regenerates. (B, C, D) Expression of joint-related markers is lost in joint cells following RA treatment. Expression remains in joint cells of (B’, C’, D’) ethanol and (B”, C”, D”) water controls. Numbers in each panel indicate the number of sections that show the expression pattern shown in a given panel out of the number of sections analyzed. Yellow arrows indicate concentrated expression of joint-related genes. Scale bar shown in A =100μm.
Figure 2.8. Retinoic acid treatments lead to bone matrix deposition within joint spaces. (A) Retinoic acid treatments were performed on 7dpa fin regenerates for 6 days. Alizarin red staining was performed prior to treatment at 7dpa. Calcein staining was performed after treatment at 6dpt/13dpa. The first joint proximal to the first bifurcation point was observed using confocal microscopy. (B, B’, B”) Alizarin red alone showed no difference between joints of RA treated and control fins. (C) Calcein staining indicates new bone matrix being deposited within joint spaces following RA treatment. (C’, C”) no new bone is observed in ethanol and water controls. (D, D’, D”) Merged images of alizarin and calcein staining. n = 4 x 3 (4 fish/replicate x 3 replicates). Scale Bar in A=10μm.
2.3.3. Mature osteoblasts surround the fin ray joints of RA-treated fins

In order to understand why bone deposition was occurring at the level of the joints in 6dpt/13dpa RA-treated fish, we performed RA treatments on 7dpa fins of Tg(bglap:mCherry), a transgenic line in which mature osteoblasts fluoresce red as mCherry expression is driven by the regulatory region of bglap, for six days (Figure 2.9A). In 7dpa and 13dpa fin regenerates, under normal conditions, mCherry expression occurs discontinuously along the length of the fin rays and is absent at the level of the joints (Figure 2.9B-B”, C’-C”). In 6dpt/13dpa RA-treated fish, the gaps in mCherry expression seem to narrow and sometimes completely close, indicating that mature osteoblasts are present at the presumptive joint regions and are, thus, responsible for the bone deposits in the joints (Figure 2.9C). Whether the appearance of osteoblasts at the joint regions is occurring as a result of joint cells differentiating into osteoblasts or as a result of a migration of osteoblasts towards the joint regions has yet to be determined. In order to examine the possibility of joint cell death, we performed TUNEL assays on longitudinal sections of 1dpt/4dpa RA-treated fin regenerates in order to assess apoptotic cell death at the level of the joints. We found that there is no cell death occurring at the presumptive joint regions (Figure 2.10).

2.3.4. Joint cells are able to differentiate into osteoblasts

As previously described, RA treatments induce fin ray joint ossification as a result of the presence of osteoblasts at the level of the joint. Although it seems that high levels of RA signalling favours osteoblast differentiation, we cannot conclude its direct role in joint formation due to its various functions in positional outgrowth during regeneration. In addition to this uncertainty, it was not clear whether the presence of osteoblasts at the level of the joints following RA treatments was due to (1) the migration of adjacent osteoblasts towards the joint region or (2) the differentiation of joint cells into osteoblasts. This raised the question of whether or not joint cells could differentiate into osteoblasts. In order to address this question, we performed RA treatments on the intact fins of a double transgenic line named Tg(m-inta11:eGFP; bglap:mCherry). In this transgenic line, the joint cells fluoresce eGFP and the osteoblasts
fluoresce mCherry. The $Tg(m\text{-inta}11:eGFP)$ construct reports Hoxa13a activity using the mouse $Hoxa11$ intron ($m\text{-inta}11$). $M\text{-inta}11$ was found to contain an intronic enhancer that triggers reporter expression in the zebrafish pectoral fin ray mesenchyme during embryonic and early larval development (Kherdjemil et al., 2016). Interestingly, adult $Tg(m\text{-inta}11:eGFP)$ fish were found to have reporter expression within joint cells. For this reason, we investigated the potential role of $hoxa13a$ in joint formation and used the zebrafish $m\text{-inta}11$ reporter lines to observe joint cells (McMillan, Zhang et al., 2018).

If joint cells are able to differentiate into osteoblasts, RA treatments on $Tg(m\text{-inta}11:eGFP; bglap:mCherry)$ uncut fins would yield joint cell-derived osteoblasts that exhibit eGFP and mCherry colocalization at the level of the joint. It is important to note that although RA treatments would suppress $hoxa13a$ expression, as observed in RA-treated 1dpt/4dpa fin regenerates (Figure 2.7B-B”), the half-life of eGFP is approximately 24 hours (Thomas et al., 2012); therefore, at about 24 hours after $hoxa13a$ is significantly downregulated, joint cells will still be identifiable because of the persistence of eGFP fluorescence that can still be detected via confocal microscopy. At 3dpt, we detected mCherry expression in joint cells, which also exhibit weak, but detectable eGFP expression (Figure 2.11B-D). Furthermore, eGFP-expressing osteoblasts exhibit a spherical shape in contrast to the flattened shape of mature osteoblasts that surround the bone segments; this difference in cell shape indicates that osteoblasts at the level of the joints were derived from the joint cells, which are typically spherical in shape (Figure 2.11B).

In order to ensure that the yellow fluorescence observed at the level of the joints was occurring within one cell layer and is not the result of an overlap of two different cell layers individually expressing either mCherry and eGFP, we have narrowed the number of optical sections subject to Z-stacking such that the total optical thickness of the resulting Z-stack is less than the thickness of a cell. We, additionally, performed a 3-D rendering to visualize different planes of the joint cell-derived osteoblasts in order to further ensure that the yellow composite fluorescence occurs within one cell layer (Figure 2.11B’-B’’’).
Indeed, the co-localization of mCherry and eGFP is observed within one cell layer at the level of the joints, suggesting that RA treatments can induce joint cells to differentiate into osteoblasts.

**Figure 2.9. Mature osteoblasts surround the fin ray joints of RA-treated fins.** (A) Tg(bglap:mCherry) fish at 7dpa were treated with RA for six days and imaged. (B-B") At 0dpt/7dpa (Before RA treatment), mature osteoblasts are not observed in joint regions of fin regenerates (white brackets). (C-C") At 6dpt/13dpa, a closure in gap of *mCherry* expression is observed in RA-treated fish compared to ethanol and water controls. Brackets indicate gap in *mCherry* expression. Asterisk indicates an almost complete closure in gaps of mCherry expression at the joint level. n= 4 x 3 (4fish/replicate x 3 replicates). Scale bar=100μm.
Figure 2.10. Apoptotic cell death occurs in the blastema, the epithelium and/or at the amputation plane, but not in joints cells of RA-treated fins. TUNEL assay on longitudinal cryosections of 3dpa (A-C) RA-treated fins (A’-C’) ethanol controls and (A’’-C’’) water controls. (A-A’’) Sections showing cell death occurring in the blastema, epithelium and at the amputation plane. (B-B’’) Sections showing cell death at amputation and in epithelium only. (C-C’’) Sections showing no notable cell death. Numbers indicate number of sections with observed cell death pattern. Scale bar = 50μm.
Figure 2.11. (See next page)
Figure 2.11. RA treatments can induce joint cells to differentiate into osteoblasts. (A) RA treatments were performed on the intact fins of Tg(m-inta11:eGFP; bglap:mCherry) for three days and imaged. (B-D) Confocal image of joints of 3dpt RA-treated intact fins showing eGFP-expressing joint cells and mCherry-expressing mature osteoblasts. (B) eGFP-expressing joint cells begin to express mCherry (white arrowheads), while eGFP and mCherry expression domains remain separate in (C) ethanol controls and (D) water controls indicating that eGFP-positive joint cells do not express mCherry. It is important to note that Panel B is only comprised of a Z-stack of seven optical sections, which makes up a thickness of 4.5μm. This is to illustrate that the yellow composite fluorescence is found within one cell layer. Panel (B’) shows one optical section from the image in panel B, which is set as the XY plane. (B”-B”’) Orthogonal view through the fin ray showing one optical section where co-localization of eGFP and mCherry occurs within joint cells (pink arrows) in the YZ plane and XZ plane, respectively. n = 35/44 joints exhibited mCherry and eGFP co-localization in 6 fish. Scale bars: Panels A, B, C = 25μm, panels B’, B”, B’’’=10μm.
2.4. Discussion

In this study, FK506-induced calcineurin inhibition and RA treatments were performed in order to investigate the role of calcineurin and RA signaling in joint formation. We observed that FK506 treatments result in the absence of joints with a loss of hoxa13a, evx1 and pthlha expression, suggesting that the calcineurin pathway may have an important role in the early stages of joint formation. We also observed that RA treatments suppress joint marker expression and cause joint ossification. Furthermore, RA treatments on intact fins of Tg(m-inta11:eGFP; bglap:mCherry) revealed that joint ossification may be due to joint cells becoming osteoblasts, which was indicated by eGFP and mCherry colocalization in joint cell-derived osteoblasts. Nevertheless, this result does not rule out the possibility of osteoblasts migrating toward the joint region; the presence of osteoblasts in the joints may be due to both osteoblast migration and joint cells becoming osteoblasts. The only way for us to confirm if osteoblasts migrate towards the joint region upon RA treatments is to perform lineage-tracing experiments that would permanently label the osteoblasts and allow us to track them throughout RA treatments.

2.4.1. Hoxa13a may be important for joint formation

Hoxa13a was originally suspected to be a potential joint marker because of its observed activity at the level of the joint. Furthermore, hoxa13a is co-localized with evx1 (McMillan, Zhang et al., 2018), which codes for a transcription factor necessary for the formation of fin ray joints (Schulte et al., 2011); however, unlike pthlha, its expression still persists in evx1 mutants, which evokes the question of whether or not hoxa13a is a joint marker (McMillan, Zhang et al., 2018). Several studies done on joints of endochondral bone in mice suggest the importance of Hoxa13 in phalanx segmentation. In the developing autopod in mice, Hoxa13 localizes to discrete domains within the interdigits and interarticular regions, where it is believed to provide positional boundaries that regulate phalanx segmentation (Stadler et al., 2001; Fromental-Ramain et al., 1996; Knosp et al., 2004). Hoxa13+/− and
Hoxa13+/− mutants exhibit several abnormal autopodal phenotypes, which include the fusion of phalangeal segments. Moreover, Hoxa13+/− mutants exhibit a marked decrease in Gdf5, which is normally expressed in a segmental pattern along the developing phalanges; the expression of Gdf5 is necessary for joint fate specification of joint progenitors (Huang et al., 2015; Koyama et al., 2008; Storm et al., 1999; Archer et al., 2003; Pitsillides and Ashhurst, 2008). Our results pertaining to the calcineurin and RA experiments in zebrafish suggest that hoxa13a may be involved in the formation of joints that articulate the dermal bones of fin rays. Firstly, FK506 treatments result in the loss of expression of hoxa13a, evx1 and pthlha, which strongly correlates with the lack of joint formation. In place of joints forming, bone is formed continuously along the fin ray, which is concomitantly accompanied with continuous expression of osteoblast differentiation markers (sp7, ihha, col10a1a) along the cells that line the hemirays. Secondly, RA treatments on intact fins of Tg(m-inta11:eGFP; bglap:mCherry) result in a loss of eGFP expression that seems to correlate with the joint cells becoming osteoblasts. The loss of hoxa13a in FK506-treated fins and in RA-treated fins along with the loss of joint cells suggest that hoxa13a may be essential for joint cell identity.

Overall, our results suggest that hoxa13a has an important role in fin ray joint formation and positioning. Furthermore, in conjunction with studies done on loss-of-function mouse mutants, our results seem to suggest that the zebrafish hoxa13a and mouse Hoxa13 may have similar roles in joint formation, which reveals mechanistic parallels between joint formation in endochondral and dermal bones. This being said, additional experiments will need to be performed in order to confirm the role of hoxa13a in joint formation. Future experiments include the creation of a transgenic line that would allow us to determine whether or not increasing levels of hoxa13a along the regenerating fin rays can induce ectopic joint formation.
2.4.2. Calcineurin and Retinoic Acid signaling may be potential regulators of joint formation

A previously published mathematical model on fin ray patterning suggested that the rate of fin ray outgrowth and joint spacing is regulated by opposing longitudinal morphogen gradients. These gradients involve three unknown morphogens, termed X, G and S. X is transported distally and activates the distal production of a mitogen known as G. S is an inhibitor of joint formation. Low levels of G and S trigger joint formation, and joint formation activates the production of S. In short, the spacing of the joints within the fin rays depends on how far these opposing morphogens stretch along the regenerating fin ray from the previously formed joint; this is dependent on the levels of expression of each of the three morphogens (Rolland-Lagan et al., 2012). Cx43 is a gap junction protein that allows passage of signaling molecules between cells. Studies on sof mutants, which have shorter fin ray segments and defective cx43 functioning, suggest that the transportation of morphogens along the proximal-distal axis is important for mediating fin ray outgrowth and joint patterning (Rolland-Lagan et al., 2012; Iovine et al., 2005; Iovine and Johnson, 2000). Moreover, cx43 expression is upregulated in FK506-treated regenerates (Dardis et al., 2017). These studies, altogether, seem to suggest that the calcineurin pathway, (which is inhibited by FK506), may be involved in maintaining the aforementioned morphogen gradients, perhaps through the control of cx43.

From our experiments, we find that inhibiting the calcineurin pathway disrupts joint formation and suppresses the expression of joint markers, which include evx1. In the study performed by Dardis et al. (2017), FK506 treatments result in the absence of evx1 as well as in the upregulation of cx43. Similarly, increased expression of cx43 in alfphy8 mutants results in long segments and, consequently, fewer joints along the fin rays (Sims et al., 2009). These findings suggest that the calcineurin pathway regulates the early stages of joint formation, perhaps, by controlling the levels of cx43, which, in turn, affects the aforementioned morphogen gradients involved in joint patterning. In addition to these
FK506 treatments, we performed RA treatments, which were found to induce joint cells to become osteoblasts in the intact fins. Previous studies have shown that ectopic RA can reprogram the positional identity of cells in developing mouse and regenerating axolotl and newt limbs to a proximal identity (Noji et al., 1991; Wanek et al., 1991; Bryant and Gardiner, 1992; McCusker et al., 2013; Brockes, 1997; Crawford and Stocum, 1988; Maden, 1982). The fact that joint cells become osteoblasts in RA-treated fins may be the result of a RA-induced change in positional identity. Overall, the maintenance of joint cell identity may rely on the homeostatic balance between distalizing and proximalizing positional information along the fin, which becomes disrupted by excess RA.

2.4.3. Joint cells can differentiate into osteoblasts

Recent experiments performed in our lab suggested that the joint cells and osteoblasts originate from a common cell precursor known as runx2a- and runx2b-positive pre-osteoblasts (McMillan, Zhang et al., 2018). RA experiments on the intact fins Tg(m-inta11:eGFP; bglap:mCherry) have shown that mature joint cells are able to become osteoblasts. This being said, it is not known whether joint cells are cells that emerge after divergence from the osteoblast lineage or cells of the osteoblast lineage that arrested at a less differentiated state; therefore, it is not certain whether or not the RA-induction of joint cells to become osteoblasts exemplifies transdifferentiation (the process in which a cell differentiates into another cell type that results from a differentiation pathway separate from the first) or further differentiation. There are several arguments that favour either. Joint cells may be considered a differentiated cell type because they express evx1 and pthlha, which are not expressed in osteoblasts. Furthermore, the expression of runx2a and runx2b, which are markers present in both the presumptive joint cells and pre-osteoblasts during regeneration, do not persist in the joint cells of the intact fins (Knopf et al., 2011). On the other hand, it is possible that joint cells are cells that share the same differentiation pathway as osteoblasts, but that are arrested at a less differentiated state. In zebrafish, some of the endochondral bones in the craniofacial endoskeleton are articulated by hyoid joints.
During development, hyoid joint formation depends on the arrest of chondrocyte maturation (at the level of the articular region) and on the repression of cartilage matrix genes (Askary et al., 2015). The inability to maintain chondrocytes in an immature state results in the expression of cartilage matrix genes within the developing articular region and in hyoid joint fusions (Askary et al., 2015). Altogether, it is possible that \textit{hoxa13a}, \textit{evx1} and \textit{pthlha} may act to repress osteoblast differentiation (McMillan, Zhang et al., 2018). The absence of \textit{col10a1a} or \textit{bglap} in these cells renders them unable to deposit bone matrix, which results in the formation of joint spaces (McMillan, Zhang et al., 2018). Moreover, the absence of the joint markers may result in continued differentiation down the osteoblast pathway, which would lead to bone matrix deposition (McMillan, Zhang et al., 2018).

In the context of RA-induced differentiation of joint cells into osteoblasts, the repression of \textit{hoxa13a}, \textit{evx1} and \textit{pthlha} expression by RA treatments results in the removal of their arresting effects, allowing joint cells to further differentiate into osteoblasts.

The fact that RA treatments were able to induce joint cells to become osteoblasts reveals a level of positional plasticity that was not known of joint cells before. Positional plasticity refers to the ability of a cell to change its positional identity (i.e. identity that depends on its location relative to the rest of organ) (Wyngaarden and Hopyan, 2008; McCusker and Gardiner, 2014; McCusker et al., 2015). This property was found to be inherent in undifferentiated cells and is believed to vary based on the epigenetic profile of a cell (Rinn et al., 2006; Stewart et al., 2009; McCusker et al., 2013; McCusker et al., 2015; Sosnik et al., 2017). In the regenerating limb of axolotls, mature cells exhibit higher chromatin condensation, while the chromatin in regenerative-participating cells, such as the blastemal cells, de-compacts as they contribute to the regenerating organ (Sosnik et al., 2017). This finding correlates with additional studies that show that epigenetic reprogramming, which involves the expression of de-methylases, is necessary for regeneration in zebrafish (Stewart et al., 2009; Katsuyama and Paro, 2011). Altogether, the ability of a cell to reprogram its positional identity is
likely related to a more “open” epigenetic state, which is conferred by less chromatin compaction at the level of genes, (which may include *Hox* genes), related to positional reprogramming (Sosnik et al., 2017; McCusker et al., 2014). Interestingly, joint cells in the intact fins of zebrafish express *hoxa13a*. In zebrafish fin regeneration, *hoxa13a* expression is high in regenerates, but significantly lower in the intact fins or stump (Figure 2.12). However, *hoxa13a* still persists in the joint cells of intact fins and its expression is inhibited by excess RA, a chemical that is known to reprogram positional identity; this may explain the subsequent change in cell identity from joint cells to osteoblasts. Altogether, joint cells may have a relatively “opened” epigenetic state, particularly at the *hoxa13a* locus, which renders them susceptible to having their identity changed upon exposure to excess RA. Future experiments could include individually isolating joint cells and osteoblasts and determining whether or not chromatin compaction is higher in osteoblasts compared to joint cells. This could be accomplished using image processing techniques that produce high resolution images of chromatin. The extent of chromatin compaction could be calculated using Sobel Edge Detection algorithms explained in Sosnik et al. (2017).

![Figure 2.12. Hoxa13a expression in Tg(M-inta11:mCherry) in a 4dpa fin regenerate and stump. Hoxa13a expression is bright along the proximal-distal axis of the fin regenerate. In the stump, hoxa13a expression is only noticeable in the joints. This expression pattern was observed in over 20 fish. Scale bar = 200μm.](image)

The following schematic illustrates our findings in the context of what has currently been researched on fin ray regeneration (Figure 2.13).
Figure 2.13. Signaling pathways that may be involved in fin ray regeneration. FGF, Wnt/β-catenin and retinoic acid positively regulate one another in the distal blastema. FGF signaling and Hh signaling regulate one another through unknown mechanisms. Bmp2b, which may be downstream of Hh, promotes sp7-mediated commitment of runx2a/b-expressing pre-osteoblasts into osteoblast pathway. Pre-osteoblasts expressing evx1 become joint cells and subsequently express pthlha, the latter of which is presumed to inhibit bone marker expression. Calcineurin may act to promote hoxa13a expression perhaps by inhibiting retinoic acid signalling. It is not known whether calcineurin directly promotes hoxa13a and where it initiates its pathway (indicated by white background). Meanwhile, retinoic acid inhibits hoxa13a expression and may participate in a feedback loop with calcineurin in order to regulate joint positioning and, consequently, segment length. It is likely that retinoic acid exerts its effects from the distal blastema (indicated by the yellow background). Question marks show that the indicated interactions have yet to be confirmed.

Chapter 3. Differential actinodin1 expression in zebrafish embryonic development and adult fin regeneration
Chapter 3 is a manuscript in preparation.

Authors’ Contributions:

Hue-Eileen Phan (co-first author) wrote the majority of the manuscript, performed regenerative time course analyses, some of the immunos, the developmental time course analyses for Tg(2P\Delta epi1-4:eGFP), Tg(2P\Delta epi+EI:eGFP), Tg(2P\Delta epi3:eGFP), Tg(1117-1+EI:eGFP), cloned the Tg(2P\Delta epi+EI:eGFP) construct and screened transgenic lines for Tg(2P\Delta epi+EI:eGFP), Tg(1117-1+EI:eGFP) and Tg(2P\Delta epi1-2:eGFP).

Marissa Northorp (co-first author) performed all in situ hybridization experiments, most of the immunos, the developmental time course analyses for Tg(2P\Delta 1:eGFP), Tg(Epi+\beta-globin:eGFP), Tg(2P\Delta epi:eGFP) and Tg(2P+EI:eGFP), performed preliminary regenerative time course analyses and screened for Tg(1117-1+EI:eGFP) transgenic lines.

Robert Lalonde (third author) cloned the Tg(2P\Delta d1:eGFP), Tg(2P+EI:eGFP), Tg(2P\Delta epi:eGFP), Tg(Epi+\beta-globin:eGFP), Tg(2P\Delta epi1-4:eGFP), and Tg(1117-1+EI:eGFP), injected all constructs, screened most of the transgenic lines, and performed preliminary larval analyses on all transgenic lines except Tg(1117-1+EI:eGFP) and Tg(2P\Delta epi+EI:eGFP).

Hombline Poullain (third author) cloned the Tg(shh aRC+EI:eGFP) and Tg(EI:eGFP) constructs, and performed preliminary analysis on primary injected fish.

Vishal Saxena (acknowledgements) injected the Tg(2P\Delta epi+EI:eGFP) construct.

Dr. Marie-Andrée Akimenko conceived the project and directed the study. Robert Lalonde, Marissa Northorp and Dr. Marie-Andrée Akimenko provided critical analysis on the manuscript.
3.1. Abstract

Zebrafish fin regeneration involves the formation of unmineralized exoskeletal elements known as the actinotrichia. These actinotrichia fibers are found within the mesenchymal compartment of the fin regenerate where they provide structural support and serve as substrates for cell migration. In the adult intact fins, actinotrichia are restricted to the distal domain of the fin. The actinodin gene family codes for structural proteins that are crucial for actinotrichia formation. There are four actinodin genes in zebrafish: actinodin1-4. We have previously identified tissue-specific cis-acting regulatory elements in a 2kb genomic region upstream of the first exon of actinodin1, termed 2P, that drive reporter expression in the fin fold ectoderm and mesenchyme during embryonic development. Within 2P is a 150bp region, named epi, which contains an ectodermal enhancer. In the present study, we sought to further characterize the activity of these regulatory sequences in adult fin regeneration. Using a reporter transgenic approach, we have shown that a site within the epi region, termed epi3, contains an early mesenchymal-specific repressor and an epithelial-specific enhancer. We have also shown that the first exon and intron of actinodin1 contains a general transcriptional enhancer and an alternative promoter that are necessary for the persistence of reporter expression reminiscent of actinodin1 during adulthood. In this way, we have generated a reporter transgenic line that fully recapitulates actinodin1 expression. Overall, we have identified additional cis-acting regulatory elements of actinodin1 as well as their functions during adult fin regeneration.
3.2. Introduction

The zebrafish fin, like all teleost fins, is supported by two types of exoskeletal elements: lepidotrichia and actinotrichia. The lepidotrichia are calcified bones and extend along the entire length of the fin. The actinotrichia are unmineralized collagenous fibrils that form brush-like bundles at the distal tips of the lepidotrichia (Becerra et al., 1996; Montes et al., 1982; Durán et al., 2011). The zebrafish pectoral fins originate from an early embryonic structure known as the fin bud, which is a small protrusion of the lateral plate mesoderm, covered by the ectoderm. The distal region of the fin bud is rimmed by a thickened layer of ectodermal cells, collectively known as the apical ectodermal ridge that eventually folds on itself to form a more flattened structure known as the fin fold, which will extend distally (Grandel and Schulte-Merker, 1998; Yano et al., 2012). The median fin fold (MFF) is an embryonic structure that extends from the dorsal end of the embryo at the 8th somite stage; this structure will extend dorsally, caudally and ventrally to eventually form the dorsal, caudal and anal fins, respectively. The fin fold consists of two sheets of ectodermal cells separated by fibres of actinotrichia (Durán et al., 2011; Géraudie, 1977; Géraudie and Meunier, 1980; Grandel and Schulte-Merker, 1998). The actinotrichia are the first fin exoskeletal elements to form during development and are believed to maintain the structure of the early fin fold before the emergence of the lepidotrichia (Wood and Thorogood, 1984; van den Boogaart et al., 2012). During development, they serve as a scaffold for the migration of the mesenchymal cells into the fin fold (Wood and Thorogood, 1984; Grandel & Schulte-Merker, 1998; Géraudie and Meunier, 1980; Dane and Tucker, 1985). As the MFF undergoes resorption during the late larval stages, actinotrichia fibres become progressively restricted to the distal region of the developing fins as lepidotrichia formation occurs in the proximal-to-distal direction (Géraudie and Meunier, 1980; Géraudie, 1984).
Following fin amputation or injury, the actinotrichia are also the first exoskeletal elements to form; they form thick longitudinal bundles in the distal regions of the regenerate whose distal ends occupy the subepidermal space while the proximal ends are located in the mesenchymal compartment deep to the osteoblasts (Durán et al., 2011; Konig et al., 2017). As regeneration progresses, the actinotrichia degrade in the more proximal regions and become restricted to the distal tips of the lepidotrichia (Marí-Beffa et al., 1989). It has also been suggested that, aside from providing mechanical support at the distal-most tissues of the fin regenerate, the actinotrichia may also act as a substrate for the migration of mesenchymal cells that emerge from the blastema (Konig et al., 2017).

Actinotrichia are composed of elastoidin, which consist of collagenous and non collagenous proteins that confer a combination of rigidity and flexibility to the fin fold (Durán et al., 2011; Géraudie and Meunier, 1980; Marí-Beffa et al., 1989). The collagenous components are made up of type I and type II collagens (Durán et al., 2011). The non collagenous components are known as the actinodin proteins, which are tyrosine-rich proteins encoded by the actinodin (and) gene family (Zhang et al., 2010). The latter consists of four actinodin genes, actinodin1-4. During embryonic development, the expression of actindoin1 (and1) and and2 share the same spatial and temporal profile as actinotrichia formation (Zhang et al., 2010); their expression occurs, first, in the ectoderm as the fin fold is forming, then in the mesenchymal cells that are invading the fin fold and that are found beneath the ectoderm (Lalonde et al., 2016). During adult fin regeneration, and1 is expressed in the basal epidermal layer at the more distal regions of the fin regenerate and in a subset of mesenchymal cells located deep to the osteoblasts within the regenerating fin rays (Zhang et al., 2010; Durán et al., 2011; Konig et al., 2017).

Cis-acting regulatory elements of the and1 gene that are active during fin development were characterized by Lalonde et al. (2016) using several and1 reporter transgenic lines of zebrafish. The 2P region of approximately 2kb is located 1941bp upstream of the first non-coding exon (the first bp
of the first exon is denoted +1) of andl and contains important regulatory elements that drive reporter expression that is reminiscent of andl expression in the fin fold. In Tg(2Pandl:eGFP), the 2P region drives reporter expression within the migrating mesenchymal cells and ectodermal cells of the pectoral and MFF. Within the 2P region is a 150bp fragment (positions -1117 and -975), termed epi, that contains a 22bp region known as epi3, which drives reporter expression within the ectoderm of the median and pectoral fin folds. In Tg(epi+βG:eGFP), the epi region, with a beta-globin minimal promoter, is sufficient to drive reporter expression in the fin fold ectoderm. The removal of epi or epi3 from 2P in Tg(2PΔepi:eGFP) or Tg(2PΔepi3:eGFP), respectively, results in expression solely in the migrating mesenchyme and absence of expression in the overlying ectoderm (Lalonde et al., 2016).

As of now, there have been no direct studies done on the regulation of actinotrichia formation during fin regeneration. There are only limited studies that have shown that there is disruption of actinotrichia formation following pharmacologic inhibition of Sonic Hedgehog signaling, Bone Morphogenetic Protein receptors and Histone deacetylase 1 during regenerative outgrowth (Armstrong et al., 2017; Pfefferli et al., 2014; Quint et al., 2002; Thorimbert et al., 2015); however, whether or not these pathways directly act on the gene expression of actinotrichia components was not the main focus of these studies. It has also been shown that pulse-inhibition of either TGFβ/Activin-βA or FGF signaling results in the disruption of Actinodin deposition during regeneration. However, whether or not TGFβ/Activin-βA or FGF signaling directly regulates andl expression remains uncertain (Konig et al., 2017). In the present study, we sought to characterize some of the key regulatory elements that govern the expression of actinotrichia components. In doing so, we have identified potential transcription factor binding sites that could provide us with leads on potential signalling pathways that regulate actinotrichia formation during fin regeneration. As of now, the functions of the cis-acting
regulatory elements of *actinodin1* have only been explored in embryonic and early larval fin development (Lalonde et al., 2016).

In this study, we further studied the activity of the *cis*-acting regulatory elements of *actinodin1* in adult fin regeneration. At adulthood, it was remarked that the 2P regulatory elements alone were not sufficient to drive reporter expression, indicating that the full recapitulation of *and1* expression at all developmental time points require additional regulatory elements. We examined the expression patterns of the four aforementioned transgenic lines as well as additional transgenic lines in adulthood and fin regeneration via *epi* fluorescence microscopy. It was observed that the inclusion of the first exon and intron of *and1* can significantly increase reporter expression in adulthood, allowing us to generate several transgenic lines that report tissue-specific *and1* during adulthood and regeneration. We also identified a cluster of putative binding sites for a repressor and an enhancer that control the dynamics of the actinotrichia in specific regions of the regenerate. Altogether, in addition to having created a transgenic line that fully depicts endogenous *and1* expression from the embryonic stage to adulthood, we have characterized additional regulatory elements that are essential for controlling actinotrichia formation during adulthood and regeneration.
3.3. Materials and Methods

3.3.1. Zebrafish Husbandry

All fish used in the experiments were maintained at 28°C with a photoperiod of 14 hours of light and 10 hours of darkness. Fish were fed regularly (Westerfield, 2007). Animal care and all experiments were performed according to the CCAC guidelines.

3.3.2. Fin Amputations

Zebrafish were anesthetized by immersion in system water containing 0.17mg/ml tricaine (Westerfield, 2007). Caudal fins were amputated two segments proximal from the first branch point of the lepidotrichia; referred as standard cut. Fish were then returned to fresh system water to recover.

3.3.3. Live Imaging

Adult fish were anesthetized and placed on a 1% agarose plate with the caudal fins spread out. Zebrafish embryos, larvae and juveniles were anesthetized in E3 embryo medium containing 0.1 mg/ml tricaine. The plate was placed under a Leica MZ FLIII dissection microscope and images were taken using the AxioCam HSM digital camera and AxioVision AC software (Carl Zeiss). For live confocal imaging, fish were anesthetized and immersed in 0.17 mg/ml tricaine in a petri dish. The caudal fins were flattened to the bottom of the petri dish with a slide hold-down (Warner Instruments 64-0248) and imaged with a water-immersion objective equipped on Nikon A1RsiMP Confocal. All images were processed using ImageJ (NIH).
3.3.4. Cryosectioning

Fin samples were fixed in 4% (w/v) paraformaldehyde (PFA) solution for 2 hours at room temperature or overnight at 4°C. After fixation, samples were washed twice for 5 minutes in 1X PBS (phosphate buffer saline: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na$_2$HPO$_4$, 1.47 mM KH$_2$PO$_4$, adjusted to a final pH of 7.4) and samples were either directly processed for sectioning or dehydrated in 100% methanol for storage at -20°C. If stored at -20°C in fresh methanol, frozen samples were then sequentially rehydrated incrementally in PBS solutions and washed twice for 5 minutes in 1X PBST solutions (1X PBS, with 0.1% tween-20-from 20% stock). Samples were then embedded in 1.5% agar and 5% sucrose in 1X PBS solution. Agar blocks were placed in 30% sucrose in 1X PBS solution at 4°C overnight. The blocks were then embedded in optimal cutting temperature compound (OCT) embedding matrix using a standard freezing protocol. Sections were cut 18-22 μm thick, transferred onto Superfrost/Plus slides and allowed to dry for at least 30 minutes before being stored at -20°C (protocol modified by Zhang, J. from Smith et al., 2006).

3.3.5. In situ Hybridizations

In situ hybridizations (ISH) on longitudinal and transverse cryosections of at least 3 adult fin regenerates per probe were performed as previously described (Smith et al., 2006) with modifications. Briefly, fin samples were fixed with 4% PFA overnight at 4°C and cryo-sectioned to 20 μm. Sections were stored at -20°C until use. On day one of ISH, slides were thawed at 60°C for one hour. Sections were permeabilized with 0.3% Triton-X in PBS for 15 min and then with 5μg/ml proteinase K for 15 min at room temperature. Sections were post-fixed with 4% PFA in PBS to prevent detaching from slides, and acetylated with 1.25% triethanolamine and 0.3% acetic anhydride. Each slide was then covered with 500 μl hybridization buffer (1x Salt solution [1x Salt: 0.2 M NaCl, 10 mM Tris HCl, 5 mM NaH$_2$PO$_4$, 5 mM Na$_2$HPO$_4$, 1 mM Tris-base, 5 mM EDTA], 50% deionized formamide, 10%
dextran sulphate, 1 mg/ml yeast tRNA, and 1x Denhardt’s) containing approximately 1ng/µl RNA probe and hybridized overnight at 70°C. On day two, slides were washed 2x30 min with 1x SSC, 50% formamide, and 0.1% Tween-20 and then 2x30 min with TBST (20mM NaCl, 150mM Tris Base, 0.05% Tween). Slides were then blocked with 10% calf serum in TBST and incubated with anti-DIG at 1:2000 overnight at 4°C. On day three, slides were first washed 6x20 min with TBST and then stained in coplin jar containing 40 ml NTMT (100 mM NaCl, 100 mM Tris HCl pH 9.5, 50 mM MgCl₂, and 0.1% Tween-20) with 225 μg/ml NBT and 175 μg/ml BCIP. The staining was performed at 37°C to accelerate reaction. After staining, slides were washed with water and mounted for observation.

3.3.6. Double Fluorescence in situ Hybridizations (FISH) on Sections

Double FISH on longitudinal and transverse cryosections was adapted from protocols that were previously described (Welten et al., 2006); Perkin-Elmer Manufacturers protocols of TSA Cyanine 3 and Fluorescein system, Perkin Elmer Cat# NEL753001KT and TSA Cyanine 5 system, Perkin Elmer Cat# NEL745001KT). Fin regenerates were fixed and sectioned as described in these protocols. Permeabilization, hybridization and post-hybridization washes on sections were also performed as described in these protocols. After washing with TBST on day two, slides were washed with 2% H₂O₂ in TNT (0.1 M Tris-HCl pH 7.5; 0.15 M NaCl; 0.5% Tween20) for 10 min and then washed 4x5 min in TNT, blocked for 4 hours in TBSTB (TNT with 0.5% Perkin-Elmer blocking powder), and incubated overnight in anti-DIG-POD (1:500) (Roche) in TBSTB at 4°C. Slides were then washed in TNT (6x20 min), stained with Tyr-Cy3 (1:100) in amplification diluent (Perkin-Elmer) for 10 min, and washed in TNT (3 x 5 min). Slides were then washed with 2% H₂O₂ in TNT for 30 min to eliminate the peroxidase of anti-DIG and washed and blocked as the previous day. Slides were then incubated overnight with anti-DNP-POD (1:500) (Perkin-Elmer) at 4°C. Slides were then washed in TNT (6x20 min), stained
with Tyr-Fluorescein (1:100) in amplification diluent (Perkin-Elmer) for 10 min, and washed in TNT (3 x 5 min). Slides were then incubated in DAPI stock solution (5 mg/ml) diluted to 1:10000 with TNT (1 x 5 min), washed in TNT (3 x 5 minutes), washed briefly with water, and mounted with AquaPolymount.

3.3.7. Probe Synthesis

Digoxigenin-labelled (DIG) antisense RNA probes were made from the cDNA plasmid templates and RNA polymerases specified in Table 3.1. 10 μg of plasmid DNA was linearized by enzymatic digestion using the proper restriction enzyme for 2 hours at 37°C. The linearized plasmid was then collected and purified using a GE Healthcare IllustraTM purification kit. The transcription reaction was prepared as follow: 1 μL of the linearized template DNA was mixed with 2 μL of NTP labeling mix (10 mM ATP, CTP, GTP, 6.5 mM UTP and 3.5 mM DIG-11-UTP) (Roche), 2 μL of 10X transcription buffer (Roche), 0.5 μL of RNase Inhibitor (Fermentas), 2 μL of RNA polymerase (Roche) and 12.5 μL of DEPC treated water. The mix was subsequently incubated for 2 hours at 37°C. The polymerase reaction was stopped, the RNA probe was isolated and purified using a SigmaSpin Post-Reaction Clean-Up Columns (Sigma-Aldrich) and stored at -80°C, as specified in Thisse & Thisse, (2008).

<table>
<thead>
<tr>
<th>Probe Name</th>
<th>Plasmid Vector</th>
<th>Restriction Enzyme</th>
<th>RNA Polymerase</th>
</tr>
</thead>
<tbody>
<tr>
<td>and1</td>
<td>pBK-CMV</td>
<td>SalI</td>
<td>T7</td>
</tr>
<tr>
<td>eGFP</td>
<td>pDrive</td>
<td>BamHI</td>
<td>Sp6</td>
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</tbody>
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Table 3.1. Antisense RNA probes for (double fluorescence) in situ hybridization experiments

3.3.8. Immunohistochemistry

Immunohistochemistry on longitudinal and transverse cryosections was adapted as previously described by Smith et al., 2006. Zns5 immunohistochemistry was adapted from a protocol that was previously described (Smith et al., 2006). Longitudinal cryosections of 4dpa fin regenerates were
incubated with Zns5 (ZFIN), rabbit anti-And1/2 protein (Life Technologies), or rabbit anti-green fluorescent protein (Life Technologies) antibodies at 1:200. Fluorescently labeled secondary antibodies Alexa Fluor 594 goat anti-mouse IgG (H+L) or Alexa Fluor 488 goat and anti-rabbit IgG (Invitrogen, A11001) were used at 1:500. Slides were counterstained with DAPI and mounted.

3.3.9. Cloning of the 1117-1+EI:eGFP construct

The cloning and subcloning of the 1117-1+EI:eGFP region were performed following the standard cloning procedures of Sambrook & Russell (2001). The genomic sequence was amplified using the Epi forward primer (5’-GCTAGCCTTTCCGAAAACCCCAGAC-3’) and the intron reverse primer (5’-GGCGGATCCCTTGGATGAAATTAA-3’), and cloned in a pDrive cloning vector (Qiagen). The 1117-1+EI region, consisting of 4992 bp in total, was then subsequently subcloned into a modified pEGFP-N1 cloning vector via Nhel and BamHI restriction sites. The CMV regulatory region was removed from the original pEGFP-N1 cloning vector and replaced by a Tol2 (left) arm between the AseI and Nhel restriction sites and another Tol2 (right) arm was inserted at the AflII restriction site.

3.3.10. Cloning of the 2PΔepi+EI:eGFP construct

The 1941-1117 fragment from the 2P region and the region continuous from the 3’ end of epi to the end of the first intron (positions -1117 to +638) were sequentially subcloned into the pEGFP-N1 tol2 cloning vector, (obtained as previously described in section 2.2.6.). These fragments were cloned into the Nhel, KpnI and AgeI restriction sites of pEGFP-N1 tol2. The 1941-1117 fragment was amplified using the Nhel-1941-2Pand1 FW (5’-GCTAGCGGTGAATTACAGCTTTAAGAC-3’) and 1117-Epi-and1-KpnI Rev (5’-GAGCTCAAATGTGGAAACATCTGGAAA-3’) primers. The region continuous from the 3’ end of epi to the end of the first intron was amplified using the KpnI-967-(After) Epi-and1 FW (5’-GAGCTCCGTTAACATAAAGCACAGATG-3’) and (+)-681-intron-
and1-AgeI Rev (5’-GTCGACCTTGGATGAAATTAATTACGCTT-3’) primers. Each of these fragments were amplified from the 2P+E1:eGFP construct from Lalonde et al., (2016).

3.3.11. Cloning of the E1:eGFP and shha+E1:eGFP constructs

The first exon and intron of and1 (termed E1) were amplified using the FW Exon1 (5’-AACAGTGGGTCAGTCGGG-3’) and Rev Intron1 (5’-GGCGGATCCITTGGATGAAATTAA-3’) and cloned into pDrive. E1 was digested with the EcoRI restriction enzyme and subcloned into the pEGFP-N1 to2 cloning vector. The shha arC fragment was digested from the shh arC+200-1 construct from Lalonde et al., (2016) using the NheI and EcoRI restriction enzymes and was subcloned into the E1:eGFP construct.

3.3.12. Microinjections

Reporter constructs (final concentration of 100 ng/mL) are co-injected with transposase RNA (final concentration of 50 ng/mL) mixed with distilled water and 0.5% phenol red in one cell-stage zebrafish embryos.
3.4. Results

3.4.1. Differential regulation of \textit{andl} throughout development and adulthood

A time course analysis of GFP expression starting at 2 days post fertilization (dpf) and ending at 90 dpf was performed on several \textit{andl} reporter transgenic lines described in Lalonde \textit{et al.} (2016), to characterize the activity of the regulatory elements throughout fin development. The specific starting and ending time points of the time course were chosen according to the stages at which zebrafish are deemed as larvae and as sexually mature adults, respectively (Singleman \textit{et al.}, 2014). Fish were regularly anaesthetized throughout the time course analysis and imaged.

The \textit{Tg(2Pandl:eGFP)} construct (Fig. 3.1A) utilizes the 2P\textit{andl} regulatory elements (located at positions -1941 to +1) to drive eGFP expression within the fin fold ectoderm and mesenchymal tissue of the developing larvae (Fig. 3.1B); five lines for this construct have been generated, (~25 fish per line were analyzed). The \textit{Tg(2P+EI:eGFP)} construct (Fig. 3.1A) includes the first exon and intron of \textit{andl}, which are collectively referred to as the \textit{EI} region (located at positions +1 to +638). At 3 dpf, reporter expression in both \textit{Tg(2Pand1:eGFP)} and \textit{Tg(2P+EI:eGFP)} lines occurs within the median fin fold (MFF) ectoderm and migrating mesenchymal cells (Fig. 3.1B, C). The \textit{Tg(epi+βG:eGFP)} construct (Fig.1A) utilizes the \textit{epi} region (located at positions -1117 to -967) and a minimal beta-globin (βG) promoter, which, together, drive reporter expression specifically within the MFF ectoderm in 3dpf larvae (Fig. 3.1D) (Lalonde \textit{et al.}, 2016). Lastly, the \textit{Tg(2PΔepi:eGFP)} construct (Fig. 3.1A) utilizes the 2P region excluding the \textit{epi} region to drive reporter expression specifically within the MFF mesenchymal tissue of 3dpf larvae (Fig. 3.1E). Two transgenic lines (~25 fish per line were analyzed) were generated for the last three aforementioned constructs.

As zebrafish reach 30dpf, the lepidotrichia emerge, allowing the MFF to transition from a rounded, blunt shape to a bi-lobed structure that will become the caudal fin (Singleman \textit{et al.}, 2014).
reporter expression is brightly observed in the ectoderm that lines the edges of the developing tail fin, and in the fin rays (Fig. 3.1G). As for Tg(2PΔepi:eGFP), reporter expression is solely observed in the fin rays (Fig. 3.1I). In Tg(2Pand1:eGFP), reporter expression is present within the fin rays and ectodermal tissue, but is comparably fainter than that of Tg(2P+EI:eGFP) and Tg(2PΔepi:eGFP) (Fig. 3.1F, G, I). As for Tg(epi+βG:eGFP), reporter expression is no longer detectable at 30dpf and throughout the remainder of development to adulthood (Fig. 3.1H, L, P).

As zebrafish reach 60dpf, they are considered to have transitioned from the larval to juvenile stage. At the juvenile stage, the MFF has fully undergone resorption and developed into the dorsal, ventral and caudal fins (Singleman et al., 2014). At 60dpf, reporter expression is absent in Tg(2Pand1:eGFP) (Fig. 3.1J), while Tg(2P+EI:eGFP) and Tg(2PΔepi:eGFP) lines continue to have reporter expression at the distal region of the caudal fin (Fig. 3.1K, M). In Tg(2P+EI:eGFP), reporter expression is strong in the interray tissue and in the fin rays (Fig. 3.1K). As for, Tg(2PΔepi:eGFP), its expression still occurs only in the fin rays to levels that are detectable by epifluorescence microscopy; however its expression is not as strong as that of Tg(2P+EI:eGFP) (Fig. 3.1K, M). These expression patterns of all and1 reporter lines observed at 60dpf are similar to those at >90dpf (Fig. 3.1N-Q).

The absence of reporter expression in Tg(2Pand1:eGFP) and Tg(epi+βG:eGFP) reporter lines in contrast to the presence of reporter expression observed in Tg(2PΔepi:eGFP) lines throughout development to adulthood suggests that the epi region may contain a repressor that is active during adulthood. Furthermore, the presence of strong reporter expression in Tg(2P+EI:eGFP) lines, despite the inclusion of this potential epi repressor, suggests that the EI region may contain enhancers required for the maintenance of reporter expression throughout development. Overall, the variation in reporter expression seen throughout development in the aforementioned transgenic lines suggests that the cis-acting regulation of and1 differs between embryonic development and later development.
towards adulthood. The details regarding these changes in *andl* regulation will be discussed in upcoming sections.

3.4.2. Reporter expression of *Tg(2P+EI:eGFP)* recapitulates endogenous *andl* expression

3.4.2.1 Reporter expression in *Tg(2P+EI:eGFP)* throughout development

During the transition from the larval stage to the juvenile stage, reporter expression in *Tg(2P+EI:eGFP)* occurs in the interray tissue and along the proximal-distal axis of the fin rays (Fig. 3.2A-C). As development progresses towards adulthood, reporter expression gradually regresses to the more distal regions of the growing caudal fin (Fig. 3.2 B-D). At adulthood, reporter expression is fully maintained at the distal tips of the fin rays as well as along the distal epithelial tissue that lines the entire fin (Fig. 3.2E-E'). To determine the specific location of eGFP(+) cells, double immunostaining on cryosections was performed for eGFP and Zns5, the latter of which is a pan-osteoblast marker (Johnson and Weston, 1995). Reporter eGFP, in transverse cryosections through a single fin ray, is localized within the layers of cells deep to the osteoblast layers that surround the hemirays (Fig. 3.2F, G, H-H').

To determine if the distal restriction of reporter expression in *Tg(2P+EI:eGFP)* corresponds to a distal restriction in endogenous *andl* expression and actinotrichia formation during the early juvenile stage, *in situ* hybridization for *eGFP* and *andl* and immunostaining using an antibody against And1, an important component of the actinotrichia, was performed on transverse cryosections obtained from the distal and proximal (midway down the fin) regions of the developing caudal fin at 40dpf. Both *eGFP* and *andl* mRNA are absent in the sections obtained from the proximal region of the caudal fin (Fig. 3.3A-A', B-B'). Conversely, *eGFP* and *andl* are present in the fin ray mesenchymal tissue in sections obtained from distal region of the caudal fin (Fig. 3.3C-C', D-D'). Similarly, immunostaining for And1, which allow us to visualize the actinotrichia, show that the actinotrichia are absent in the
more proximal regions of the caudal fin and present in the distal regions of the caudal fin (Fig. 3.3E-E', F-F'). In order to confirm that eGFP and and1 colocalize, double fluorescence in situ hybridization (FISH) experiments were performed on proximally and distally located transverse sections with eGFP and and1 probes. Indeed, the expression patterns of eGFP and and1 co-localize within the fin ray mesenchymal tissue of the developing caudal fin of fish reaching the juvenile stage (Fig. 3.3G-G', H-H').

3.4.2.2 Reporter expression in Tg(2P+EI:eGFP) throughout adult fin regeneration

During the early stages of adult fin regeneration, at 2dpa, reporter expression is first observed in the interray tissue of the regenerate (Fig. 3.4A). At 3dpa, reporter expression within the fin rays begins to appear (Fig. 3.4B). At 4dpa to 7dpa, reporter expression is brightly observed along the proximal-distal axis in fin rays, while interray-specific reporter expression remains within the distal regions of the regenerate (Fig. 3.4C-D). After ~7-9dpa, reporter expression within the fin rays and interray tissue gradually distalizes (Fig. 3.4E, F). To determine the specific cell layers in which eGFP(+) cells occur in the fin rays and interray tissue, immunostaining for eGFP was performed on consecutive transverse cryosections of 4dpa regenerates (Fig. 3.4G-K). In the distal-most region of the regenerate, eGFP expression only occurs in the basal epithelial layers of the interrays (Fig. 3.4G). As the sections progress more proximally to approach the region where eGFP can be observed in the fin rays, eGFP expression is present in distinct layers of cells of the interrays and the fin rays (Fig. 3.4H). In the interrays, eGFP is only found in the basal epithelial layer (Fig. 3.4H-K). In the middle region of the fin rays (Fig. 3.4H-K), eGFP is only present in the mesenchymal tissue layers. It is only in the lateral parts of the hemiray that are closer to the interrays where eGFP is present in both the basal epithelial layer and mesenchymal tissue (Fig. 3.4H-K).

In order to compare endogenous and1 expression with Tg(2P+EI:eGFP) during adult fin regeneration, double FISH experiments for and1 and eGFP were performed on consecutive
longitudinal cryosections of 3dpa fin regenerates. At 3dpa, andl and eGFP expression co-localize in the fin ray mesenchyme and in the basal epithelial layer along the proximal-distal axis of the regenerate (Fig. 3.4L-L’). Overall, the observed co-localization of eGFP and endogenous andl expression throughout development and in adult fin regeneration confirms that the Tg(2P+El:eGFP) line can be used to report endogenous andl expression.

3.4.3. The epi3 site within the epi region contains an interray basal epithelial-specific enhancer and early mesenchymal-specific repressor in adult regeneration

The absence of reporter expression in intact fins of Tg(2Pandl:eGFP) in contrast to the presence of fin ray-specific expression in Tg(2PΔepi:eGFP) during adulthood suggested that the epi region may contain a repressor sequence (Fig. 3.1N, Q). To further analyze the activity of this potential repressor sequence, we performed a comparative time course analysis of reporter expression in Tg(2P+El:eGFP), Tg(2Pandl:eGFP), and Tg(2PΔepi:eGFP) during fin regeneration. The Tg(epi+βG:eGFP) line lacks reporter expression throughout regeneration (Supp. Fig. 3.1A-F) and was, therefore, excluded from comparative analyses.

As previously shown in the proximal regions of fin regenerates, reporter expression occurs in the basal epithelial layer of the interrays and in the mesenchymal cells of the fin rays (Fig. 3.4I-K). This tissue-specific expression was confirmed for all transgenic lines (data not shown). At 2dpa, Tg(2Pandl:eGFP) and (Tg(2P+El:eGFP) reporter expression is first observed in the regenerative interray tissue (Fig. 3.5B, C). In contrast, Tg(2PΔepi:eGFP) is the only line among the three to exhibit reporter expression within the fin ray regenerative tissue (Fig. 3.5D). At 3dpa, fin ray mesenchymal-specific reporter expression is detectable in Tg(2Pandl:eGFP) and Tg(2P+El:eGFP), but is fainter than that of Tg(2PΔepi:eGFP) (Fig. 3.5F-H). It is only at 4dpa when fin ray mesenchymal-specific expression in Tg(2Pandl:eGFP) and Tg(2P+El:eGFP) is comparable to that of Tg(2PΔepi:EGFP) (Fig. 3.5J-L). At 7dpa, all four transgenic reporter lines have fin ray mesenchymal-specific reporter
expression, although that of Tg(2Pand1:eGFP) is consistently fainter or patchier than that of Tg(2P+El:eGFP) and Tg(2PΔepi:eGFP) (Fig. 3.5N-P). In summary, reporter expression was faintly observed in Tg(2P+El:eGFP) and Tg(2Pand1:eGFP) within the fin ray mesenchymal tissue up to 4dpa, while, Tg(2PΔepi:eGFP) displayed strong reporter expression in the same region at the same time points. Thus, the removal of epí in Tg(2PΔepi:eGFP) resulted in notable fin ray mesenchymal-specific reporter expression during the early stages of regeneration, suggesting that epí contains an early fin ray mesenchymal-specific repressor. It was also noted that, in adulthood and regeneration, the Tg(2PΔepi:eGFP) line also completely lacked interray basal epithelial-specific reporter expression (Fig. 3.1Q, Fig. 3.5D, H, L, P), suggesting that the previously identified embryonic/early larval ectodermal enhancer within the epí region (Lalonde et al., 2016) also functions to enhance and1 expression within the basal epithelial layer of the adult fin regenerate.

TRANSFAC is a curated database of transcription factors as well as their DNA binding sequences that can be used to identify transcription factor binding sites within a sequence of interest. TRANSFAC analysis of the epí region, allowed identification of four clusters of putative binding sites; these four sites were termed epí1-4. These sites were originally identified and analyzed for their potential role during embryogenesis. Four transgenic reporter lines, in which each of the four epí sites of the constructs was deleted or substituted via site-directed mutagenesis, were generated; three mutations consisted of deletions (epí1-3) from the 2P region and one consisted of a 2bp substitution (epí4) (Lalonde et al., 2016). These same transgenic lines were also analysed in order to characterize their potential activity in adult fin regeneration. A time course analysis of the four distinct transgenic lines was performed in order to identify which of the epí sites may act as a mesenchymal-specific repressor during adult fin regeneration. Of these four transgenic lines, Tg(2PΔepi3:eGFP) showed reporter expression that was identical to that of Tg(2PΔepi:eGFP) throughout regeneration in that there is only fin ray mesenchymal-specific expression and a complete absence of interray basal
epithelial-specific expression (Fig. 3.5D-E, H-I, L-M, P-Q). Therefore, the epi3 site probably contains putative binding sites for an early mesenchymal-specific repressor. In addition, the complete absence of interray-specific expression in Tg(2PΔepi3:eGFP) throughout regeneration suggests that the epi region also contains an interray basal epithelial-specific enhancer.

3.4.4. The EI region contains an alternative promoter for andl expression during adulthood

Compared with Tg(2Pand1:eGFP), the Tg(2P+EI:eGFP) line has very strong reporter in adult intact fins and during adult fin regeneration; this suggests that the EI region contains enhancers or an additional promoter required for adequate transgene expression during adulthood. Transgenesis in zebrafish was used to determine whether or not the EI region contains an alternative andl promoter. The EI fragment was cloned into an eGFP reporter construct (Fig. 3.6A). The EI:eGFP construct was capable of transiently driving reporter expression in arbitrary regions of developing embryos. 76.7% of injected embryos (n=60, where n equals total number of viable injected embryos) exhibited reporter expression within the median fin fold or the dorsal region of the embryo (Fig. 3.6B-E). The presence of eGFP expression in primary injected embryos suggests the presence of a functional promoter within the EI region. Moreover, there seems to be no specific tissue in which reporter expression is consistently observed in all eGFP-positive embryos, which suggests that the EI region does not contain any tissue-specific enhancers. Moreover, the EI region was cloned into an eGFP reporter construct containing a sonic hedgehog a (shha) arC enhancer element (Fig. 3.6A) and zebrafish transgenic lines were generated with this construct. In the presence of a promoter, the arC enhancer can drive weak reporter expression in the floor plate and notochord regions of zebrafish larvae (Muller et al., 1999). The Tg(shh arC+EI:eGFP) embryos consistently exhibit faint, but detectable reporter expression within the floor plate and notochord at 1dpf (Fig. 3.6F-F”), which further reinforces the notion of an alternative promoter within the EI region. Taking into account the disappearance of both fin interray epithelial- and mesenchymal-specific reporter expression in Tg(2Pand1:eGFP) compared
with the $Tg(2P+EI:eGFP)$, it is possible that this alternative promoter may be required for both epithelial- and mesenchymal-specific $andl$ expression during adulthood.

### 3.4.5. The $EI$ region may contain a general transcriptional enhancer

When comparing the expression patterns of the $Tg(2Pand1:eGFP)$ and $Tg(2P+EI:eGFP)$ lines during the embryonic stage, strong reporter expression in the mesenchymal and ectodermal cells of the MFF (Fig. 3.1B, C) was observed. However, in adulthood, this reporter expression only strongly persisted in $Tg(2P+EI:eGFP)$ (Fig. 3.1K, O) and nearly disappears in $Tg(2Pand1:eGFP)$ (Fig. 3.1J, N); this suggests that the inclusion of $EI$ significantly increases reporter expression. As for fin regeneration, reporter expression in $Tg(2Pand1:eGFP)$ can be observed in both the interray basal epithelial layer and fin ray mesenchymal tissue (Fig. 3.5B, F, J, N); however, its expression is fainter and patchier than the reporter expression observed in $Tg(2P+EI:eGFP)$ (Fig. 3.5C, G, K, O). This difference in strength of reporter expression between $Tg(2Pand1:eGFP)$ and $Tg(2P+EI:eGFP)$ suggests that the enhancer functions in the maintenance of $andl$ expression throughout development and adulthood, and in boosting $andl$ expression throughout regeneration.

To test whether or not the inclusion of $EI$ could enhance interray basal epithelial-specific reporter expression driven by the $epi$ region, the region continuous from the beginning of $epi$ to the end of the first intron (positions -1117 to +638) was cloned into an $eGFP$ construct, yielding a construct named $1117-1+EI:eGFP$ (Fig. 3.7A). Notably, the region spanning from positions -967, (3’ end of $epi$), to +1 does not drive significant transgene expression on its own during embryonic development (Lalonde et al., 2016). At 3dpf, ectodermal-specific reporter expression was present in the MFF (Fig. 3.7B). In adulthood, two out of three $Tg(1117-1+EI:eGFP)$ lines ($n = 5-6$ fish per line) had strong reporter expression within the interray tissue at the distal regions of the intact fin (Fig. 3.7C). During regeneration, strong reporter expression within the interray tissue was also observed (Fig. 3.7D, Supp. Fig. 3.2). This interray-specific reporter expression mimics that of the $Tg(2P+EI:eGFP)$ line, where it
also remains confined within the distal regions of the regenerate (Supp. Fig. 3.2G-L). To confirm that this boost in expression is specifically occurring within the basal epithelial layer of the interray, transverse cryosections of 4dpa regenerates of Tg(1117-1+EI:eGFP) were obtained and subjected to immunostaining for eGFP. Transgene expression was specifically located in the basal epithelial layer of the interray tissue and absent in the middle regions of the hemirays (Supp. Fig. 3.3A-F). Taken together, we were able to successfully create a transgenic line in which reporter expression is specific to the basal epithelial layer and is maintained throughout adulthood, while showing that the EI region of andl contains a strong general transcriptional enhancer that is required for adequate reporter expression to be observed in adulthood and fin regeneration.

We, additionally, created a Tg(2P∆epi+EI:eGFP) transgenic line (Fig. 3.7A), in which the 2P+EI region excluding the epi region drive reporter expression; we generated three lines. At 3dpf, reporter expression is observed specifically in the mesenchymal tissue of the MFF (Fig. 3.7E). In adulthood, reporter expression within the fin ray mesenchymal tissue was noticeably and consistently bright in all three Tg(2P∆epi+EI:eGFP) lines (n= 10-15 fish per line) within the intact fins and within 4dpa fin regenerates (Fig. 3.7F, G). Although the Tg(2P∆epi:eGFP) line still has reporter expression at the distal tips of the fin rays, its expression tends to be weaker and patchier than that of Tg(2P+EI:eGFP) and is not consistently present in all of the fin rays of all fish that have surpassed the 90dpf time point (Supp. Fig. 3.4). This suggests that the removal of the epi repressor is not sufficient to maintain strong and consistent reporter expression throughout adulthood. It appears that the inclusion of EI is still required in order to fully recapitulate andl expression in the fin ray mesenchymal tissue of adult intact fins.
Figure 3.1. Differential regulation of and1 during larval development and adulthood. (A) Schematic representations of the constructs of various transgenic lines. Dotted line indicates that mapping is not to scale. In vivo expression of four and1 reporter lines at 3dpf (B-E), 30dpf (F-I), 60dpf (J-M) and 90dpf (N-Q). (B, F, J, N) Tg(2Pand1:eGFP) (n=5) expression fades after 30dpf. (C, G, K, O) Tg(2P+EI:eGFP) (n=2) expression persists after 30dpf. (D, H, L, P) Tg(epi+βG:eGFP) (n=2) expression is absent at 30dpf and onward; and (E, I, M, Q) Tg(2PΔepi:eGFP) (N=2) expression...
persists. n = # of lines, ~25 fish/line. Scale bars: B-E = 100μm; F-I = 200μm; J-M = 200μm; N-Q = 200μm.

**Figure 3.2.** Reporter expression in the developing caudal fin of Tg(2P+EI:eGFP). (A) Reporter expression of Tg(2P+EI:eGFP) (25 fish were analysed) occurs within the ectodermal tissue and along the proximal-distal axis of the developing caudal fin. It is notably brighter as it approaches the distal region at 30dpf. (B) Reporter expression occurs along the proximal-distal axis in the fin rays and is beginning to approach the distal edge of the fin where ectodermal-specific expression is present at 40dpf. (C, D) At 50dpf and 60dpf, eGFP expression in the fin rays distalizes and is present in epithelial tissue located at the very distal regions of the fin. (E, E’) Reporter expression only occurs at the distal tips of the fin rays and at the distal edge of the interrays (pink arrowheads). (E’) close-up on panel E. (F) Transverse sections from a 30dpf caudal fin were obtained in the region indicated by the yellow dotted line. (G) Immunostaining showing eGFP expression (white arrowheads) localized within hemirays (h; delineated by yellow line) (n=6). Immunostaining for Zns5, which is a pan-osteoblast marker, labelling osteoblasts surrounding the hemirays (h; yellow arrowheads) and eGFP (white
arrowheads) in Tg(2P+El:eGFP) on transverse cryosections of 30dpf larvae (n=6). (H) eGFP is localized in layers of cells deep and adjacent to osteoblast layers lining hemirays (h). (H’) Merge on bright field. n = # of fish from which sections were obtained. Scale bars: A-E, E’ = 200μm, F = 100μm and G, H-H’ = 50μm.
Figure 3.3. *Tg(2P+EI:eGFP)* recapitulates endogenous *and1* expression in developing caudal fin at juvenile stage. All experiments were performed on transverse cryosections of middle region (A-A’, B-B’, E, G) and distal region (C, D, F, H) of developing caudal fin of 40dpf juvenile fish. *In situ* hybridization (n=8) for *eGFP* of *Tg(2P+EI:eGFP)* (A-A’, C, C’) and endogenous *and1* expression (B-B’, D, D’). The fin rays and the interrays are indicated by brackets. (A’-D’) Higher magnification of a single fin ray from panels A-D, respectively. (E, F) Immunohistochemistry (n=9) for *And1*. (E’, F’) Merge with DAPI staining, which stains cell nuclei. (G, H) Double fluorescence *in situ* hybridization (n=12) for *and1*. (G’, H’) *eGFP* of *Tg(2P+EI:eGFP)* merged with DAPI staining. n = # of fish of which fins were sectioned and on which given experiment was performed. e: epithelium; fr: fin ray; ir: interray; m: mesenchyme. Melanocytes are the dark spots indicated by an asterisk. Scale bars: Panel A, B, C, D = 20µm, A’, B’, C’, D’ = 10µm, E-E’, F-F’ = 20µm, G-G’, H-H’=10µm
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**Annotations:**
- fr: 
- ir: 
- bel: 
- m: 
- e:
Figure 3.4. Time course analysis of Tg(2P+EI:eGFP) throughout regeneration. (A-F) In vivo time course analysis of eGFP reporter expression during fin regeneration in Tg(2P+EI:eGFP) (n=12). (A-B) At 2dpa and 3dpa, reporter expression is brightly observed only in interray tissue of Tg(2P+EI:eGFP). (C-D) At 4dpa and 7dpa, fin ray-specific reporter expression occurs along the proximal-distal axis and is as equally bright as that of interray expression in Tg(2P+EI:eGFP). Yellow arrowheads indicate location of fin ray tissue. (E-F) Fin ray-specific reporter expression becomes distally restricted in Tg(2P+EI:eGFP). (G-K) Immunostaining for eGFP in Tg(2P+EI:eGFP) on consecutive transverse cryosections of 4dpa fin regenerate. (G) In the most distal region of regenerate, reporter expression faintly occurs in the basal epithelial layer (pink arrowheads) surrounding hemirays. The fin ray and interray are indicated by white brackets. (H-K) As sections progress proximally, reporter expression within basal epithelial layer (pink arrowheads) is restricted towards interray region. Reporter expression within the mesenchymal tissue is indicated by the yellow arrowheads. (I-K) Reporter expression within basal epithelial layer is absent in middle regions of hemirays and overlaps with mesenchymal-specific expression in regions of hemirays that are closer to interrays (indicated by dotted yellow box in Panel K). Pink stars indicate autofluorescence from blood vessels. (L) Double fluorescence in situ hybridization (n=8) for and1 and eGFP of Tg(2P+EI:eGFP). (L’) Merge with DAPI staining of cell nuclei on longitudinal sections of 3dpa fin regenerate. Note that the longitudinal section shown in L-L’ was obtained from the region indicated by the yellow dotted box in panel K where and1 and eGFP reporter expression occur in the mesenchyme and basal epithelial layer. fr: fin ray; ir: interray; m: mesenchyme; bel: basal epithelial layer; e: epithelium. n = # of fish of which fins were sectioned and on which given experiment was performed. Scale bars: A=100μm, B-F= 200μm, G-L, L’ = 50μm.
Figure 3.5. The epi3 site within the epi region is a mesenchymal-specific repressor that is active early in regeneration. In vivo time course analysis of reporter expression of Tg(2Pand1:eGFP) (n=7), Tg(2P+E1:eGFP) (n=12), Tg(2PΔepi:eGFP) (n=11), and Tg(2PΔepi3:eGFP) (n=13) during fin regeneration. (B-E) At 2dpa, Tg(2Pand1:eGFP) and Tg(2P+E1:eGFP) only have interray epithelial-specific expression while Tg(2PΔepi:eGFP), and Tg(2PΔepi3:eGFP) have fin ray mesenchymal-specific expression. (F, G) At 3dpa, Tg(2Pand1:eGFP) and Tg(2P+E1:eGFP) have bright interray epithelial-specific expression and faint fin ray mesenchymal-specific expression. (H, I, L, M) As for Tg(2PΔepi:eGFP) and Tg(2PΔepi3:eGFP), strong fin ray mesenchymal-specific expression is observed. (J-M, N-Q) At 4dpa and 7dpa, mesenchymal-specific expression in Tg(2Pand1:eGFP) is faint and patchy, while that of Tg(2P+E1:eGFP) is just as bright as Tg(2PΔepi:eGFP) and Tg(2PΔepi3:eGFP). Time course analysis was done on 1 line for each construct; n=# of fish/ line. Pink stars in B and E indicate autofluorescence from white pigment cells.
and blood vessels. Yellow arrowheads indicate fin ray regenerative tissue. Amputation plane is delineated by yellow dotted line. Scale bars: B-E = 100μm; F-I = 200μm; J-M = 200μm; N-Q = 200μm

Figure 3.6. *EI* contains an alternative promoter. (B-E) 1dpf *Tg(EI:eGFP)* primary injected embryos showing *eGFP* expression at different regions of the body (indicated by pink arrows): (D) median fin fold (lateral view) or the dorsal region of the embryo from (C) dorsal and (E) lateral view (n=20/95, N=total # of viable injected embryos). (F-F") 1dpf *Tg(shha arC+EI:eGFP)* F1 generation embryos showing *eGFP* expression in the notochord of a portion of the median fin fold (n= 15 fish, 1 line). (F”) Higher magnification on panel F showing floor plate in merged GFP and bright field
images. (F") close-up image obtained from GFP filter only. fp: floor plate. fp: floor plate; y: yolk sac. Scale bars: B-D =50μm, E-E"=25μm.

**Figure 3.7. EI contains a general transcriptional booster.** *In vivo* time course analysis of reporter expression of Tg(1117-1+EI:eGFP) (N=3; n=35) and Tg(2PΔepi+EI:eGFP) (N=3; n=31), at 3dpf (B, E), in intact fins at 90dpf (C, F), and at 4dpa (D, G) during fin regeneration. (B-D) Tg(1117-1+EI:eGFP) expression only occurs in the ectodermal/basal epithelial tissue; (E-G) Tg(2PΔepi+EI: eGFP), only mesenchymal tissue. fr: fin ray. N=number of lines found; n= total number of fish analysed. Scale bars: B, E = 25μm, C, F = 200μm and D, G = 200μm.
Supplementary Figure 3.1. Time course analysis of eGFP in *Tg(epi+βG:eGFP)* throughout regeneration. (A-F) Regenerative time course analysis of *in vivo* reporter expression of *Tg(epi+βG:eGFP)* (n=25 fish/line, 2 lines), indicating a complete absence of reporter expression throughout regeneration. All scale bars= 200μm.
Supplementary Figure 3.2. Time course analysis of Tg(1117-1+EI:eGFP) and Tg(2P+EI:eGFP) throughout regeneration. In vivo time course analysis of reporter expression of Tg(2P+EI:eGFP) (n=12) (A-F) and Tg(1117-1+EI:eGFP) (n=5) (n=25 fish/line, 2 lines) (G-L) during fin regeneration. (A-B, G-H) At 2dpa and 3dpa, reporter expression is brightly observed only in interray tissue of Tg(2P+EI:eGFP) and Tg(1117-1+EI:eGFP). (C-D) At 4dpa and 7dpa, fin ray mesenchymal-specific reporter expression occurs along proximal-distal axis and is as equally bright as that of interray-specific expression in Tg(2P+EI:eGFP). (E-F) Fin ray-specific reporter expression begins to distally restrict in Tg(2P+EI:eGFP). (I-J) Reporter expression only occurs in interray of Tg(1117-1+EI:eGFP) and always remains confined within distal region of regenerate. (K-L) reporter expression occurs in interray and distal edge of fin in Tg(1117-1+EI:eGFP). Scale bar: A, G=100μm, B, H= 200μm, C, I=200μm, D, J= 200μm, E, K=200μm, F, L=200μm. (Images in panels A-F have been reused from Figure 3.4. for better comparison of eGFP expression.)
Supplementary Figure 3.3. Transverse sections of 4dpa of Tg(1117-1+E2A::eGFP).

most proximal 4dpa Transverse Sections most distal

Tg(1117-1+E2A::eGFP), Reporter
expression of (A) $Tg(1117-1+EI:eGFP)$ ($n=2$) in vivo at 4dpa. Immunostaining for eGFP in (B-F) $Tg(1117-1+EI:eGFP)$ on consecutive transverse cryosections. (H) In the most distal region of regenerate, reporter expression occurs in the basal epithelial layer surrounding hemirays. As sections progress proximally, (C-F) reporter expression within basal epithelial layer restricts towards interray region. (E, F) Reporter expression within basal epithelial layer of the interray and the fin ray region closer to the interray (indicated by dotted yellow box). It is absent in middle regions of hemirays. Basal epithelial layer-specific expression is indicated by yellow arrows. Pink star indicates autofluorescence from blood vessels. Scale bars: A= 200μm, B-F = 50μm. n = # of fish from which sections were obtained.
Supplementary Figure 3.4. The EI region is necessary to enhance fin ray mesenchymal-specific expression even without the epi repressor. (A) Distal fin ray mesenchymal-specific expression does not occur in all fin rays. Red arrows indicate presence of fin ray-specific expression while asterisk indicates absence of expression. Reporter expression is bright and consistent in all fin rays in (B) Tg(2PΔepi:EI:eGFP), and in (C, D) two different Tg(2PΔepi+EI:eGFP) lines. Scale bar = 200μm.
3.5. Discussion

Time course analysis of several andl reporter lines throughout development revealed that there is a change in andl regulation as the zebrafish reaches adulthood. During larval development, the Tg(2Pand1:eGFP) and the Tg(2P+Ei:eGFP) lines exhibit strong reporter expression in the migrating mesenchymal cells of the MFF and in the overlying ectoderm. As for the Tg(epi+βG:eGFP) and Tg(2PΔepi:eGFP) lines, reporter expression is observed in only the ectoderm or fin fold mesenchymal tissue, respectively. As the zebrafish transition towards the juvenile stage, reporter expression in Tg(2Pand1:eGFP) and in Tg(epi+βG:eGFP) disappears, while that of Tg(2P+Ei:EGFP) and Tg(2PΔepi:eGFP) remains at the distal region of the growing caudal fin. This stage-dependent disappearance of reporter expression in Tg(2Pand1:eGFP) and Tg(epi+βG:eGFP) in contrast to the persistence of that of Tg(2PΔepi:eGFP) lines suggests the existence of a repressor that is located within the epi region and active during adulthood. As we further investigated the role of this potential repressor, we found that a site, termed epi3, functions to suppress mesenchymal-specific expression during the early stages of fin regeneration and to enhance basal epithelial-specific expression. Furthermore, we, additionally, remarked that reporter expression in the Tg(2P+Ei:eGFP) line is still strongly maintained in adulthood. This observation suggests that (1) the Tg(2P+Ei:EGFP) line can fully report andl expression throughout development and adulthood, and (2) the Ei region may contain enhancers and an alternate promoter that are essential to the maintenance of andl expression throughout development (Figure 3.8).

3.5.1. The Tg(2P+Ei:eGFP) line reports andl expression in adulthood and regeneration

During the intermediate stages of larval development (30dpf), reporter expression in the Tg(2P+Ei:eGFP) line is strong in the ectoderm and along the proximal-distal axis in the mesenchymal tissue enclosed within the fin rays. This observation may reflect the presence of
actinotrichia along the developing caudal fin that provide mechanical support and that serve as a scaffold for mesenchymal cell migration during morphogenesis (Duran et al., 2011; Wood and Thorogood, 1984; Zhang et al., 2010). As development progresses towards adulthood, reporter expression gradually distalizes until it remains restricted at the distal tips of the fin rays and distal epithelial tissue lining the adult fin. In adults, actinotrichia are located at the distal tip of each lepidotrichia in the intact fin (Becerra et al., 1983). Furthermore, they have also been observed to remain distally restricted at the tip of each fin ray throughout fin development and regeneration (Grandel & Schulte-Merker, 1998; Marí-Beffa et al., 1989). Overall, the spatial and temporal dynamics of $Tg(2P+EI:eGFP)$ reporter expression throughout development matches that of actinotrichia formation. Moreover, endogenous and1 expression spatially and temporally correlates with actinotrichia formation (Zhang et al., 2010), which further suggests that the $Tg(2P+EI:eGFP)$ line recapitulates endogenous and1 expression throughout development.

In the early to intermediate stages of regeneration (3dpa-7dpa), $Tg(2P+EI:eGFP)$ reporter expression colocalizes with endogenous and1 expression within the basal epithelial layer of the interray tissue and the mesenchymal tissue located deep to the osteoblasts layers. The expression pattern of $Tg(2P+EI:eGFP)$ correlates with the presence of actinotrichia, which are found to be displaced by the osteoblasts into the mesenchymal compartment (Konig et al., 2017). Interestingly, $Tg(2P+EI:eGFP)$ expression occurs along the proximal-distal axis within the fin rays. Similarly, actinotrichia fibers are also found along the proximal-distal axis possibly because of their role in cell migration as suggested by recent findings that show the cytoplasm of blastemal cells and differentiating cells engulfing nearby actinotrichia fibers (Konig et al., 2017; Zhang, unpublished). The similarity between the expression patterns of $Tg(2P+EI:eGFP)$ and endogenous and1 expression suggests that the $Tg(2P+EI:eGFP)$ line can recapitulate endogenous and1 expression throughout regeneration.
3.5.2. The EI region can be used to drive tissue-specific expression in adulthood

During larval development, the expression patterns between $Tg(2Pand1:eGFP)$ and $Tg(2P+EI:eGFP)$, $Tg(\text{epi}+\beta G:eGFP)$ and $Tg(1117-1+EI:eGFP)$, and $Tg(2P\Delta epi:eGFP)$ and $Tg(2P\Delta epi+EI:eGFP)$ within the MFF are identical to one another (Table 3.2). The notable difference in expression strength between each of these three pairs during adulthood is believed to be associated with the inclusion or absence of the first intron and exon of $\text{and}1$, which are collectively, termed EI. Because these differences in strength of reporter expression occur as the zebrafish reaches adulthood, it is likely that the regulatory elements within EI are only activated during adulthood.

As development progresses to adulthood, reporter expression in the $Tg(2Pand1:eGFP)$ and $Tg(\text{epi}+\beta G:eGFP)$ lines disappeared, which was problematic for the analysis of $\text{and}1$ expression during adult fin regeneration. The inclusion of EI in $Tg(2P+EI:eGFP)$, $Tg(1117-1+EI:eGFP)$ and $Tg(2P\Delta epi+EI:eGFP)$ lines was found to significantly boost tissue-specific expression in adult zebrafish, suggesting that adequate $\text{and}1$ expression relies on a potential enhancer situated within the EI region. The inclusion of EI allowed us to successfully generate a transgenic line, $Tg(1117-1+EI:eGFP)$, that specifically reports $\text{and}1$ expression within the basal epithelial layer during adult fin regeneration. In addition to this observed boost in $\text{and}1$ reporter lines, the EI region can be paired with a weak enhancer, such as the $\text{shha aRC}$ enhancer, to drive reporter expression specifically within the notochord. Therefore, the inclusion of EI not only allowed us to generate $\text{and}1$ reporter lines that can be used for adulthood analyses, but also to generate reporter lines using other enhancers. Furthermore, EI alone was found to non-specifically drive reporter expression in primary injected embryos, suggesting that the EI region may also contain a promoter and that the enhancer that lies within is not tissue-specific. There are several findings that support the notion of intron-mediated enhancement. For example, the inclusion of an intron of certain genes in reporter constructs significantly increased transgene expression levels in mice, *Drosophila*, plants and zebrafish (Choi et
It was suggested that the inclusion of the first intron in reporter constructs can individually enhance each step of the dogma from transcription to translation and inhibit mechanisms that suppress these steps (Niu & Yang, 2011; Le Hir et al., 2003; Rose, 2004). Other studies proposed that the inclusion of an intron in reporter constructs may oppose epigenetic silencing to which some foreign DNA elements in zebrafish are susceptible (Horstick et al., 2015; Stuart et al., 1988; Goll et al., 2009). There are also studies that support the possibility of EI containing an alternative promoter that is necessary for driving sufficient andl expression in adults. Previously, it has been shown that alternative promoters can play an important role in boosting mRNA expression levels and/or conferring increased mRNA stability. Alternative promoters were, additionally, shown to increase translational efficiency and provide differential tissue-specific expression in yeast, zebrafish and humans (Kolondra et al., 2015; Wang et al., 2005; Chen et al., 2010; Shang et al., 2011; Regadas et al. 2013). Altogether, the EI region is likely to contain a strong general transcriptional enhancer and an alternative promoter that renders it a useful molecular tool for boosting transgene expression in adulthood.

<table>
<thead>
<tr>
<th></th>
<th>Expression during Embryonic Development</th>
<th>Adult Intact Fins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tg(2P:EGFP)</td>
<td>ectoderm + mesenchyme</td>
<td>None</td>
</tr>
<tr>
<td>Tg(2P+EI:EGFP)</td>
<td>ectoderm + mesenchyme</td>
<td>Basal epithelial layer in interray + fin ray mesenchyme</td>
</tr>
<tr>
<td>Tg(Epi + βG:EGFP)</td>
<td>ectoderm</td>
<td>None</td>
</tr>
<tr>
<td>Tg(1117-1+EI:eGFP)</td>
<td>ectoderm</td>
<td>Basal epithelial layer of interray</td>
</tr>
<tr>
<td>Tg(2PΔepi :EGFP)</td>
<td>mesenchyme</td>
<td>Fin ray mesenchyme (not in all fin rays)</td>
</tr>
<tr>
<td>Tg(2PΔepi +EI:EGFP)</td>
<td>mesenchyme</td>
<td>Fin ray mesenchyme (bright and in all fin rays)</td>
</tr>
<tr>
<td>1st Exon + Intron (EI)</td>
<td>General transcriptional enhancer (in adulthood) + alternative promoter</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.2. Change in expression of several andl reporter lines towards adulthood.
3.5.3. Candidate transcription factors associated with the epi3 element and EI region in regulating the dynamics of actinotrichia during regeneration

The epi region was discovered to likely contain a repressor that functions in suppressing mesenchymal-specific expression during the early stages of regeneration. Among Tg(2P+EI:eGFP), Tg(2Pand1:eGFP), Tg(1117-1+EI:eGFP), Tg(2PΔepi:eGFP), and Tg(2PΔepi3:EGFP), the last two of the five exhibited strong reporter expression within the fin ray mesenchymal tissue between 2dpa and 4dpa. It was only after 4dpa, when fin ray mesenchymal-specific reporter expression was comparably bright to what was observed in the interray tissue in Tg(2P+EI:eGFP), Tg(1117-1+EI:eGFP) and Tg(2Pand1:eGFP). In 2dpa fin regenerates, actinotrichia are first seen in the regenerative tissue located above the interray tissue of the stump before it is formed in mass bundles along the regenerate (Konig et al., 2017); this correlates with the emergence of Tg(2P+EI:eGFP) reporter expression in the interrays before fin ray-specific expression. This delay in actinotrichia formation in the regenerative fin ray tissue during the early stages of regeneration may be mediated by the epi3 repressor sequence. The epi3 site contains putative binding sites for TCF-4 (Lalonde et al., 2016). It was noted that TCF-4 as well as other TCF proteins, such as TCF3a and TCF3b, seem to have complementary expression patterns to mesenchymal-specific Tg(2P+EI:eGFP) and basal epithelial-specific Tg(1117-1+EI:eGFP) reporter expression throughout regeneration (Wehner et al., 2014). This complementary expression pattern strongly suggests that these transcription factors may act on epi3 in order to inhibit andl expression in specific regions of the outgrowing regenerate. In addition to its fin ray mesenchymal-specific repressor effect, epi3 was also found to be an important basal epithelial-specific enhancer, similarly to how it is an important ectodermal enhancer during embryonic and early larval development (Lalonde et al., 2016). Potential factors that may enhance basal epithelial-specific andl expression include TCF1 and LEF1, both of which also belong to the TCF/LEF family and are strongly expressed in the basal epithelial layer in the distal and distal-most
regions of the regenerate (MacDonald et al., 2009; Wehner et al., 2014). This overlap in expression with $Tg(1117-1+EI:eGFP)$ reporter expression, suggest that TCF1 and LEF1 may enhance basal epithelial-specific and1 expression throughout regeneration (Wehner et al., 2014).

TCF proteins are generally downstream effectors of Wnt/β-catenin signaling (MacDonald et al., 2009; Wehner et al., 2014). The activity of this pathway in and1-expressing cells is believed to directly and indirectly mediate cell proliferation within the blastema and osteoblast differentiation (Long, 2012; Stewart et al., 2014; Wehner et al., 2014). However, because of the various indirect functions of Wnt/β-catenin signaling (Wehner et al., 2014), it is not certain whether or not the potential repressor effects of TCF proteins on and1 expression can be associated with a negative effect of Wnt/β-catenin signaling on and1 expression.

The EI region is believed to contain a general transcriptional enhancer that is necessary for adequate and1 expression throughout adulthood and fin regeneration. TRANSFAC analysis revealed several putative binding sites within the EI region, some of which for the $oct4$, $dlx5$, $msxe$, $lef1$ and $hox$ genes (Stewart et al., 2009; Schebesta et al., 2006; Akimenko et al., 1995; Wehner et al., 2014; Thummel et al., 2007). The expression pattern of $oct4$ in zebrafish fin regeneration has yet to be characterized; however, stemming from the fact that this gene is documented to have an indispensible role in regulating cell pluripotency in stem cells, it is possible that $oct4$ may be reactivated in regenerative tissues that express and1 (Loh et al., 2006; Stewart et al., 2009). The $dlx5$ genes include $dlx5a$ which is expressed within the basal wound epidermis and likely overlaps with the and1-expressing cells within the basal epithelial layer that lines the distal-most regions of the regenerate (Schebesta et al., 2006); therefore, $dlx5a$ is a likely candidate for an activator of and1 in the basal epithelial layer. The $msxe$ gene belongs to the $msx$ homeobox gene family, which were found to be highly expressed in the blastema, hence the likelihood of $msxe$ being a candidate for an activator of and1 in the mesenchyme (Akimenko et al., 1995). As previously described, $lef1$ is expressed in the basal
epithelial layer within the distal-most region of the regenerate and may either be an activator of basal epithelial layer-specific and1 expression via the epi3 site or via the EI region (Wehner et al., 2014). Lastly, the hox genes are important regulators of embryonic patterning. More specifically, the 5’hoxA/D genes are heavily involved in proximal-distal patterning in fin/limb development (Ahn and Ho, 2008; 2012; Yano and Tamura, 2013). The Hox genes have been shown to be expressed in regenerative tissues in planarians, Xenopus, urodele amphibians and zebrafish (Bayascas et al. 1997; Christen et al., 2003; Nicolas et al., 2003; Géraudie and Borday Birraux, 2003). Unfortunately, the expression patterns of most hox genes have yet to be characterized in the zebrafish fin regenerate. As of now, it has been discovered that hoxa13a, hoxa13b, hoxc13a and hoxc13b genes are highly upregulated in the blastema, basal epithelial layer and/or osteoblasts in the regenerate (McMillan, Zhang et al., 2018; Thummel et al., 2007; Géraudie and Borday, 2003), suggesting that hox genes may also be potential activators of and1 expression.

3.6. Conclusion

While actinotrichia are absent in mammals, they play a crucial role in cell migration, cell differentiation and structural support in zebrafish during fin development and regeneration. Therefore, understanding the dynamics and distribution of actinotrichia-forming cells is indispensable to the study of zebrafish fin regeneration. In the present study, we have characterized additional cis-acting regulatory elements of actinodin1 that are active during adult fin regeneration. The identification of these regulatory elements has allowed us to generate a transgenic line that fully recapitulates actinodin1 expression and to further explore potential molecular pathways that may govern the dynamics of actinotrichia formation during adult fin regeneration. Finally, the discovery of an adult-specific, general transcriptional enhancer has provided us with an additional molecular tool that may be useful for the conception of transgenic lines that can be studied during adulthood.
Figure 3.8. Regulation of actinodin1 during embryonic and early larval development, and adult fin regeneration. (A) During early larval development, the ectodermal-specific enhancer binds to the epi region, while mesenchymal-specific enhancers bind to 2P regions outside of epi. These enhancers, together, promote and1 expression in the ectoderm and migrating mesenchymal cells of the fin fold. (B) During the early stages of regeneration and in the distal-most regions of the regenerating fin, fin ray mesenchymal-specific actinodin1 expression is inhibited, perhaps through the binding of a strong repressor within the epi region. In parallel, strong actinodin1 expression occurs within the basal epithelial layer due to the binding of an epithelial-specific enhancer at the epi region and perhaps various enhancers within the EI region. The repressor effects at the level of epi appear to override intron-mediated enhancement. (C) During the intermediate stages of regeneration, within the more proximal regions of the regenerate, the absence of the epi-specific repressor and the persistence of intron-mediated enhancement allows for strong actinodin1 expression in the basal epithelial layer and in the fin ray mesenchyme.
Chapter 4. General Conclusion

The results discussed in this thesis contribute to further understanding the regenerative mechanisms involved in zebrafish fin regeneration. More specifically, our results reveal some of the cellular and molecular mechanisms underlying the regeneration of the fin exoskeletal elements: the lepidotrichia and actinotrichia.

In the study pertaining to joint formation, the calcineurin pathway was found to be an important regulator of early joint formation as its inhibition resulted in the suppression of joint marker expression and disruption of joint formation. We also found that retinoic acid treatments caused fin ray joints to ossify by inducing joint cells to differentiate into osteoblasts. Furthermore, prior to calcineurin and retinoic acid treatments, it was hypothesized that \textit{hoxa13a} might be involved in joint formation due to its expression in joint cells and its co-localization with the known joint marker being \textit{evx1}, although this presumption had yet to be confirmed. Our results show that the loss of joint identity, which can be induced via calcineurin inhibition or retinoic acid treatments, appears to be linked with a loss of \textit{hoxa13a} expression, suggesting that \textit{hoxa13a} expression is important for maintaining joint cell identity. It is possible that the calcineurin and retinoic acid signalling pathways interact with one another to regulate \textit{hoxa13a} expression and, thus, the fate of pre-osteoblasts. Altogether, our results revealed novel mechanisms that are important for the formation of zebrafish fin ray joints and that could have parallel functions in other model organisms. Our data may have useful ramifications for future research on prospective regenerative therapies for degenerative joint diseases such as osteoarthritis.

In the second study, we discover that the first exon and intron of the \textit{actinodin1} gene contains a general transcriptional enhancer that is active during adulthood and of which the inclusion is necessary for full recapitulation of \textit{actinodin1} expression. This discovery allowed us to generate
transgenic lines that reported tissue-layer specific \textit{actinodin1} expression. Using these reporter transgenic lines, we were able to follow actinotrichia-forming cells \textit{in vivo} during fin regeneration. The differences in the expression patterns observed in these transgenic lines also allowed us to identify \textit{epi3} as an important site containing an early mesenchymal-specific repressor and a basal epithelial-specific enhancer; its timing in regulation correlated with the formation of the actinotrichia during regeneration, as explored in detail in previous studies. The identification of these additional regulatory elements, which appear to be active during adulthood, provided us with more insight on potential molecular pathways that may be involved in regulating the dynamics of the actinotrichia. Moreover, the characterization of the aforementioned general transcriptional enhancer provided us with a larger repertoire of molecular tools that may be useful for transgenic analysis during adulthood; this is especially given that there is a need of cell/tissue-specific regulatory elements in order to generate transgenic lines for adulthood analysis. Lastly, the characterization of these regulatory elements may have its implications in human molecular medicine. Past projects in our laboratory include the development a human skin disease model in zebrafish that targets the basal epithelial layer using the enhancer elements within the \textit{epi} region.

Future directions include further exploration of the role of \textit{hoxa13a} in joint positioning. We are in the process of creating a transgenic line that will allow us to increase \textit{hoxa13a} expression to induce ectopic joint formation. We are also in the process of characterizing the transcriptional profile of osteoblasts and joint cells using RNAseq analysis in order to determine additional molecular markers associated with joint formation. As for the project pertaining to the regulation of \textit{actinodin1}, the characterization of adulthood-specific \textit{actinodin1 cis}-acting regulatory elements would allow us to further explore the cellular mechanisms of actinotrichia formation during regeneration. We are in the process of generating a \textit{Cre-loxP}-based system that would allow us to perform lineage-tracing experiments on actinotrichia-forming cells (see Appendix A).
References


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Appendix A. Fate of *actinodin1*-expressing cells throughout development and regeneration

### A1. Background and Objectives

During fin regeneration, osteoblast differentiation begins with blastemal cells that are derived via the dedifferentiation of mature osteoblasts situated a few segments proximal to the amputation plane (Knopf et al., 2011). As for actinotrichia-forming cells, which can be reported using the *Tg(2P+EI:eGFP)* line, their origin during fin regeneration is unknown. Fin amputation, which is normally done two segments proximal to the amputation plane, results in the complete removal of the actinotrichia, which are distally restricted in intact fins. Moreover, no mature actinotrichia-producing cells would be present near the amputation plane. Previous experiments done in our lab have shown that the *Tg(2P+EI:eGFP)* transgenic line recapitulates *and1* expression (see chapter 3). In the intact fins, reporter expression remains restricted at the distal tips of the fin rays (Figure A1.A). Conversely, during the early to intermediate stages of regeneration, reporter expression is observed along the proximal-distal axis of the fin regenerate (Figure A1.B). We are not sure whether the *and1*-expressing cells along the proximal-distal axis migrate distally or stop expressing *and1* in the more proximal regions of the regenerate; both options would consequently result in the distal restriction of *and1* expression in the regenerate. The main objective of this project is to determine the fate of *and1*-expressing cells during regeneration through cell lineage-tracing analysis. If the distalization of *and1* is the result of a gradual cessation of *and1* expression within the more proximal regions of the outgrowing regenerate, it is possible that, following amputation, the mesenchymal cells within the stump reactivate *and1* expression and contribute to the pool of *and1*-expressing cells during fin regeneration.
Figure A1. Tg(2P+EI:eGFP) expression in adult intact fins and in 7dpa fin regenerates. (A) Reporter expression is distally restricted in intact fins. However, (B) reporter expression occurs along the proximal-distal axis in 7dpa fin regenerates. The amputation plane is delineated by the red line. Scale bar = 200µm.

**A2. Current Progress and Future Directions**

We plan to use a genetic approach to conditionally and permanently label and1-expressing cells to track them during fin regeneration. This approach is based on the use of two transgenic lines (Mosimann et al., 2011): Tg(ubi:switch), which is already available, and Tg(2P+I:Cre-Ert2-Ins-eGFP:cmlc2), which I am currently creating (Figure A2.A). In the Tg(2P+I:Cre-Ert2-Ins-eGFP:cmlc2) line, the expression of 4-hydroxy-tamoxifen-inducible Cre-ER\textsuperscript{T2} is driven by the 2P+EI regulatory elements of and1, which have been shown to drive strong expression in and1-expressing cells during adulthood. Originally from the P1 bacteriophage, Cre is an enzyme that acts as a site-specific recombinase that recognizes loxP sites, which are short DNA consensus sequences that have directionality (Hamilton and Abremski, 1984). The most common use of Cre recombinase is to excise or delete a DNA sequence flanked by loxP sites that are located on the same strand of DNA and that are in the same orientation (Nagy, 2000). It is important to note that Cre-mediated recombination is a permanent deletion of the floxed DNA; therefore, all daughter cells of cells that have been subjected
to Cre-mediated recombination will inherit this gene deletion. The location of Cre recombinase is controlled through the use of a modified estrogen receptor ligand binding domain (ER\textsuperscript{T2}), which is fused with Cre and engineered to only respond to 4-hydroxy-tamoxifen (4-OHT) (Feil et al. 1997; reviewed in Cox et al., 2012). In the absence of 4-OHT, Heat-shock protein 90 (Hsp90) binds to the modified estrogen receptors; this keeps Cre enzymes sequestered in the cytosol (Feil et al. 1996; Hayashi and McMahon 2002; Reviewed in Cox et al., 2012). Upon exposure to 4-OHT, 4-OHT binds to the ER\textsuperscript{T2} domain and Hsp90 is released, enabling the translocation of the Cre-ER\textsuperscript{T2} fusion protein to the nucleus where it catalyzes a recombination event between two \textit{loxP} sites (Feil et al. 1996; Hayashi and McMahon 2002; Reviewed in Cox et al., 2012). In many cases only a single estrogen receptor moiety is used. The \textit{cmlc2:eGFP} fragment of this construct serves as a screening tool, in which eGFP expression is driven by the heart-specific \textit{cmlc2} promoter (Mosimann et al., 2011). The \textit{cmlc2:eGFP} fragment has been placed in reverse transcriptional orientation. Moreover, an insulator sequence (Ins), obtained from the chicken hypersensitivity site 4 (HS4) (Bessa et al., 2009; Zhu et al., 2007), has been placed in between the 2\textit{P+I}:\textit{Cre-}E\textit{rt2} fragment and the \textit{cmlc2:eGFP} fragment. This insulator was included in the construct in order to block any suppressive effects on \textit{Cre-}E\textit{rt2} expression that may be associated with the inclusion of \textit{cmlc2}.

Once the \textit{Tg(2\textit{P+I}:\textit{Cre-}E\textit{rt2}-\textit{Ins}-eGFP:}c\textit{mlc2)} is obtained, it will be crossed with \textit{Tg(ubi:loxp-GFP-loxp-mCherry)}, (also known as \textit{Tg(ubi:switch)}), a reporter line that employs the \textit{ubiquitin} promoter (\textit{ubi}) to ubiquitously express GFP; following Cre-recombination, the excision of the floxed GFP would allow for \textit{ubi} to permanently drive mCherry expression (Mosimann et al., 2011). To summarize, in the double transgenic fish \textit{Tg(2\textit{P+I}:\textit{Cre-}E\textit{rt2}-\textit{Ins}-eGFP:}c\textit{mlc2}; \textit{ubi:switch}), and \textit{1l}-expressing cells express the Cre-ER\textsuperscript{T2} fusion protein and all cells at all time points express GFP (Figure A2.A). Upon exposure to 4-OHT, Cre-ER\textsuperscript{T2} enters the nucleus (Figure A2.B-D); \textit{gfp} and all
DNA flanked by loxP sites are excised and all andI-expressing cells fluoresce mCherry (Figure A2.E).

The *Tg(2P+I:Cre-Ert2-Ins-eGFP:cmIc2)* construct has been injected and fish are being raised. Unfortunately, it will take a few more months before primary injected fish can be screened and tested. Nevertheless, once the line has been obtained, tamoxifen treatments will be performed in order to test switching. If the line is functional, 4-OHT treatments will be performed during the intermediate stages of regeneration (about 7dpa) and mCherry-expressing cells will be followed.
Figure A2. Tamoxifen-induced Cre recombination in \textit{and1} expressing cells in \textit{Tg(2P+I:Cre-Ert2-Ins-eGFP:cmlc2)}. 
A3. Methods and Materials


The cloning and subcloning of the 2P+EI:cre-ert2-Ins-eGFP:cmlc2 construct were performed following the standard cloning procedures of Sambrook & Russell (2001). The cmlc2:eGFP fragment was subcloned from pDrive into the Tg(2P+EI:eGFP) construct from Lalonde et al. (2016) via the NotI and BamHI sites. This fragment was amplified using the NotI cmlc2 FW (5’-GCGGCCGCAAAGCTTAAATCAGTTGTGTTAAAT-3’) and cmlc2 SV40 REV (5’-GGATCCCTTGTTTATTGCAGGTAAATTTGG-3’) primers from the Tg(cmlc2:eGFP) construct from Kwan et al. (2007). We called this recombinant plasmid Tg(2P+EL_eGFP:cmlc2) tol2.

The chicken HS4 Insulator fragment was amplified from the pBT268_(pCA-ATG-tTA2-iiTRE-tfTii) plasmid (Addgene) using the KpnI_SV40 polyA FW (5’-GGTACCTGTTGTTGTTAATCT-3’) and Insulator REV (5’-GTGGTAAACTCGAATCAGATCT-3’) primers. This fragment was subcloned from pDrive to Tg(2P+EI_eGFP:cmlc2) tol2 plasmid via the KpnI and BamHI sites. This recombinant plasmid was named Tg(2P+EI_Ins_eGFP:cmlc2)tol2.

The Cre-Er\textsuperscript{t2} gene was subcloned from pDrive into the Tg(2P+EI_Ins_eGFP:cmlc2)tol2 plasmid via the KpnI sites. The Cre-Er\textsuperscript{t2} gene was amplified using the KpnI-Cre FW (5’-GGTACCTGTTGTTGTTAATCT-3’) primer and Cre-Ert2 REV (5’-CTGCAGGTCTCAGGATCTTTCATAA-3’) primer from the Cre-Ert2 sequence in Mosimann et al. (2011).

A3.2. Microinjections

Reporter constructs (final concentration of 100ng/mL) are co-injected with transposase RNA (final concentration of 50ng/mL) mixed with distilled water and 0.5% phenol red in one cell-stage zebrafish embryos. (Microinjections were performed by Vishal Saxena.)