Characterizing Tissue-Specific *actinodin1* Reporter Expression in *Danio rerio* Fins Throughout Development and Regeneration

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Abstract

The exoskeleton of the fins comprises fin rays and actinotrichia; the latter are small unmineralized fibrils found at the distal margin of fin rays. Actinotrichia play a role in the growth and structure of the fins during fin development and regeneration. Our lab has previously identified the *actinodin* (*and*) gene family, which codes for structural proteins in actinotrichia. Interestingly, the loss of this gene family has been proposed to be involved in the loss of fin rays, an important step in the fin-to-limb transition during evolution. Furthermore, the *and* genes are expressed in the epithelial cells and in the migrating mesenchymal cells of the zebrafish embryonic pectoral and median fin fold. The presence of tissue-specific *cis*-acting regulatory elements were found within the 2 kilobase pair genomic region (2P) located upstream of *and1*'s first untranslated exon by performing analyses of the expression of a fluorescent reporter (EGFP) placed under the control of fragments of various lengths originating from the 2P genomic fragment in zebrafish transgenic lines. Using these various *and1* reporter lines, tissue-specific *and1* expression was previously characterized during the embryonic stage of zebrafish development. However, these transgenic reporter lines were not analyzed throughout important fin morphogenesis events occurring during fin development, such as the initial formation of lepidotrichia and the resorption of the median fin fold, and throughout fin regeneration as well.

This study mainly enabled us to characterize in great details *and1* expression throughout fin development and regeneration using the various tissue-specific *and1* reporter lines by performing time course analyses. In doing so, we were able to demonstrate that these reporter lines recapitulate endogenous *and1* expression through *in*

situ hybridization and RT-PCR experiments. Furthermore, the distinct transgene expression patterns observed during lepidotrichia formation/regeneration in the various *and1* reporter lines supports previous research that proposes *and1*-expressing cells may indirectly contribute to lepidotrichia formation not only during fin regeneration but during fin development as well. Furthermore, the characterization of the tissue-specific *and1* reporter lines throughout development allowed us to characterize specific changes in the *cis*-acting regulation of *and1* in the fins of adult fish when compared with the tissue-specific *and1* reporter expression patterns characterized during the embryonic stage. All in all, this study provides further clues on the contribution of *and1*-expressing cells throughout fin development and regeneration.

Acknowledgements

First and foremost, I would like to express my deepest gratitude to my supervisor Dr. Marie-Andrée Akimenko, who most importantly presented me with the opportunity to pursue my master's degree in her hospitable laboratory. This opportunity gave me valuable life lessons and exceptional lab experience. The expertise and guidance provided by her throughout my project were invaluable to me and allowed me to strive as a graduate student. A sincere thank you to Dr. Berman for providing us the *Tg(mpeg1:EGFP)* line.

Furthermore, I would like to thank my committee members, Dr. Ekker and Dr. Bui, who with their insightful comments and input, helped shape the path of my project.

In addition, I would like to give my sincerest thanks to my lab mentor, PhD candidate Robert Lalonde, for his unwavering support, guidance as well as his everlasting patience. His input and mastery of the *actinodin* project was essential to my success.

I would like to also thank Jing Zhang, our lab technician, for always being present and tremendously helpful with lab related matter and for dealing with my constant inquiries in an encouraging manner. Her expertise in confocal imaging allowed the production of many beautiful images seen throughout this study.

I would like to thank the rest of the Akimenko lab staff for always providing a great work atmosphere. I will always remember the outings and the great discussions we had during manageable and sometimes difficult times. I will cherish those forever.

Lastly, I would like to thank my family, significant other and friends for their constant support and unwavering willingness to help at every opportunity they had.

Funding for this research was provided from the Natural Science and Engineering Council of Canada (NSERC) to Dr. Marie-Andrée Akimenko.

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2P	2 kilobase pair region upstream of first exon (including a promoter (P))
2PΔepi	The 2P region with the omission of the Epi fragment drives mesenchymal-specific expression
2P+E	Regulatory region including 2P and the first exon of *actinodin1*
2P+I	Regulatory region including 2P, the first exon and intron of *actinodin1*
AER	Apical ectodermal ridge
AF	Apical fold
af	anal fin
Aldh1a2	Aldehyde Dehydrogenase 1 Family Member A2
And/*and*	Actinodin/*actinodin* (Protein & *gene* respectively)
AP	Alkaline phosphatase
BCIP	5-Bromo-4-Chloro-3-Indolyl Phosphate
βG	β-globin
BMP	Bone Morphogenetic Protein
bp	base pair
CCAC	Canadian Council on Animal Care
Cas	Caspase
CDX	Caudal-related homeobox
cf	caudal fin
cl	cleithrum
CMV	Cytomegalovirus
col	*collagen*
CRISPR	Clustered regularly interspaced short palindromic repeats
D	Distal section
DAPI	4',6-diamidino-2-phenylindole
dEGFP	*destabilized enhanced green fluorescent protein gene*
DEPC	Diethylpyrocarbonate
df	dorsal fin
DIG	Digoxigenin
Dlx	Distal-less-related homeobox
DNP	Dinitrophenyl
dpa	days-post-amputation
dpf	days-post-fertilization
dr	distal radials
E	First exon of *actinodin1*
E3	NaCl+KCl+CaCl+$MgSO_4$ embryo solution
ed	endoskeletal disk
EDTA	Ethylenediaminetetraacetic acid
EdU	5-ethynyl-2'-deoxyuridine
EGFP/*egfp*	Enhanced green fluorescent protein (Protein & *gene* respectively)
EI	Regulatory region including the first exon and intron of *actinodin1*
Epi	A 150 base pair region located within the 2P region driving epidermal-specific expression in the fins

Epi-EI	Regulatory region spanning the 5'end of Epi to the 3'end of the first intron of *actinodin1*
Fab	Fragment antigen binding
fc	foramen coracoideus
Fgf/*fgf*	Fibroblast growth factor/*fibroblast growth factor* (Protein & *gene* respectively)
FISH	Fluorescent *in situ* hybridization
Fli1a	Fli-1 proto-oncogene, ETS transcription factor a
FW	Forward primer
Hox/*hox*	Homeobox/*homeobox* (Protein & *gene* respectively)
hpf	hours-post-fertilization
I	First intron of *actinodin1*
IgG	Immunoglobulin G
kbp	kilobase pair
L	Lepidotrichia
M	Middle section
MMP	Matrix metalloproteinase
mpeg1	*macrophage expressed gene 1*
MTF-1	Metal Transcription Factor-1
MTZ-NTR	Metronidazole-Nitroreductase
NBT	Nitro Blue Tetrazolium
NTMT	NaCl+Tris-HCl+ $MgCl_2$+Tween-20 solution
Osr	Odd-skipped-related protein
PBS	Phosphate buffer saline
PBST	Phosphate buffer saline with Tween-20
PCR	Polymerase Chain reaction
PFA	Paraformaldehyde
PI	Propidium iodide
PK	Proteinase K
pop	postcoracoid process
pr	proximal radials
RA	Retinoic acid
RT	Reverse transcriptase
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
RV	Reverse primer
sco	scapulocoracoid
SL	Standard length
SSC	Saline sodium Citrate
T3	3,5,3'-triiodothyronine
T4	Thyroxine
TBE	Tris-HCl+Borate+Ethylenediaminetetraacetic acid solution
TBST	Tris-buffered saline with Tween-20
TBSTB	Tris-HCl+NaCl+Tween-20 solution with Perkin-Elmer blocking powder
Tbx5/*tbx5*	T-box5/*t-box5* (Protein & *gene* respectively)
TCF	Transcription Factor
Tg	Transgenic line

TNT	Tris-HCl+NaCl+Tween-20 solution
TRANSFAC	Transcription factor identification software
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
Wnt/*wnt*	Wingless-related integration site/*wingless-related integration site* (Protein & *gene* respectively)
WT	Wild Type
ZFIN	Zebrafish Information Network

Chapter 1. Introduction

1.1. Overview

Tetrapod limbs are homologous structures to the paired pectoral and pelvic fins in fish (Yano & Tamura, 2013). In fact, early fin and limb development share many similar developmental mechanisms (Mercader, 2007). Fin development studies have played a key role in uncovering the complex morphological and molecular changes that may have contributed to the transition of fins to limbs during evolution (Ahn & Ho, 2008; Zhang et al, 2010). By studying the various signalling pathways responsible for the structure of the fins, it allows us to identify the fundamental similarities and differences between fin and limb development (Hall, 2008). From these studies, paired appendage evolution is theorized to have involved a number of distinct steps, one of them being the eventual loss of the fins' exoskeleton, which includes fin rays and actinotrichia fibres (Yano & Tamura, 2013; Géraudie & Landis, 1982). The latter are unmineralized fibres that play an important role in supporting and elongating the embryonic fin bud into a fin fold during early fin development and are the first and only exoskeletal element of the embryonic fins (Wood, 1982). In adult fish, they provide support to the distal margin of intact and regenerating adult fins (Becerra et al, 1983; Kemp & Park, 1970). Interestingly, these fibres are absent in tetrapods: this supports the proposition that limb bud development may lack the elongation of the bud's distal epidermis that is required to form a fin fold in fish species. Consequently, the loss of actinotrichia is a key difference between fin and limb development (Zhang et al, 2010; Zeller, López-Ríos & Zuniga, 2009).

Actinotrichia are composed of elastoidin, which is a two-part protein complex made up of collagens and a previously unknown tyrosine-rich protein component (Durán

et al, 2011; Damodaran, Sivaraman & Dharaliker, 1956). Our lab characterized the non-collagenous protein fraction of actinotrichia to be constituted of Actinodin (And) proteins, which are essential for the structural integrity of the actinotrichia fibres. These proteins are coded by the *actinodin* (*and*) gene family and are expressed in both the ectoderm and mesenchymal cells of embryonic fins. It is important to note the distinct characteristics of zebrafish nomenclature for genes and proteins; gene names are italicized and lower case and protein names are non-italic and the first letter is upper case (ex: *and1* gene; And1 protein) (Dunn, 1999). Interestingly, the loss of the *and* gene family in tetrapods is proposed to have participated in the loss of the fins' exoskeleton during evolution (Zhang et al, 2010). Among the 4 *and* genes, my study focuses on *and1*. *Cis*-acting regulatory elements have been identified upstream of the *and1* gene, including tissue-specific enhancers by cloning various genomic regions located upstream of *and1* in reporter constructs to produce zebrafish transgenic lines. This allowed us to analyze the distinct reporter expression of these genomic regions in the ectoderm and/or mesenchymal cells of the zebrafish embryonic fin folds (Lalonde et al, 2016). These transgenic lines were well characterized in the fins of zebrafish embryos, however they were not characterized throughout development where fins undergo many morphological changes to obtain the adult morphology. Moreover, zebrafish are able to regenerate their fins, and actinotrichia distribution has been well characterized throughout fin regeneration (Gemberling et al, 2013; Marí-Beffa, Carmona & Becerra, 1989). However, the *and1* tissue-specific transgenic lines were likewise not characterized throughout fin regeneration. Therefore, this study focuses on characterizing *and1* reporter expression using the tissue-specific transgenic lines throughout fin development and regeneration.

1.2. Zebrafish

1.2.1. A relevant model organism

Zebrafish (*Danio rerio*) are small fresh-water teleosts (bony) fish that have become an attractive model organism in many fields of research. These fish are native to the South and Southeast regions of Asia, mainly in North-eastern India, Bangladesh and Nepal (Nelson, 1994; Talwar & Jhingran, 1991). Zebrafish were first used in scientific research as a genetic tool for studying vertebrate biological processes in the 1960s by George Streisinger, a fish enthusiast and professor at the University of Oregon who attempted to find a cheaper alternative vertebrate model organism to mice for genetic analyses (Grunwald & Eisen, 2002). Admittedly, zebrafish were revealed to be technically very cheap and easy to maintain in laboratory facilities compared to mice, costing only $1/1000^{th}$ of the cost of mice maintenance when performing similar studies (Goldsmith & Solari, 2003). The low maintenance costs associated with the use of zebrafish as a model organism prompted many laboratories around the world to use them. In fact, an estimated 600 labs today use the zebrafish as a model organism for their own research interests (Heath, 2013).

Zebrafish have many characteristics that make them an appealing model organism for research. First, zebrafish are highly fecund animals that produce up to 300 eggs per clutch and fertilization and development are external. Furthermore, they have a short three-month generation time (Brand, Granato & Nüsslein-Volhard, 2002; Spence et al, 2008). Thus, the use of zebrafish enables the production of many offspring and generations in a short amount of time. Since zebrafish eggs, larvae and adult fins are optically transparent, they allow scientists to study many vertebrate developmental

processes using simple microscopy (Kimmel, Warga & Schilling, 1990). Moreover, the fully sequenced zebrafish genome revealed that 70% of protein-coding genes in zebrafish have at least one human ortholog. From this percentage, 82% of the zebrafish protein-coding genes were found to be disease-related genes in humans (Howe et al, 2013). Among many applications, zebrafish-tailored genome-editing methods have been used as of late to create a few disease models by knocking-in/out some of these human disease-related orthologs in the hopes of discovering candidate drugs for treating human diseases (Hisano et al, 2015; Jao, Wente & Chen, 2013; Gilbert, Trengove & Ward, 2013).

In line with our research interests, the zebrafish was found to be a convenient model organism for other numerous reasons in addition to the ones previously stated. My research particularly focuses on the development and regeneration of the zebrafish fin. To this day, numerous researches have proven the zebrafish to be a relevant model organism for studying the molecular mechanisms responsible for proper fin development (Yano et al, 2012; Fernandez-Teran & Ros, 2008). It is also through the characterization of these developmental processes in the fins that similarities as well as disparities were noted between fin and limb development (Mercader, 2007; Yano & Tamura, 2013). Admittedly, zebrafish appear to be a pertinent model organism in evolutionary developmental studies since its genome can easily be modified. In addition, zebrafish are much more convenient to grow in captivity, and well-established molecular experiments can easily be performed using zebrafish as a model organism compared to other fish models. Therefore, zebrafish can provide molecular and genetic insight on prospective mechanisms responsible for the evolutionary transition from fish fins to tetrapod limbs (Nakamura et al, 2016). Lastly, teleosts including zebrafish have the ability to regenerate

their fins at mostly any developmental stage, which makes them perfect model organisms for regenerative studies (Gemberling, et al, 2013). By using a wide-array of molecular techniques, the zebrafish proves to be an excellent and versatile model organism for studying developmental and regenerative processes occurring throughout fin development and regeneration.

1.2.2. Morphology of the adult fins

Considering the focal point of my research concerns the fins, it is adequate to get acquainted with their structure and morphology during the adult stage. Zebrafish possess 5 types of fins: they have three unpaired medial fins which consist of the dorsal, anal and caudal fins and two sets of paired fins which consist of the pectoral and pelvic fins (Fig 1.1a) (Nüsslein-Volhard & Dahm, 2002). During the adult stage, all fins contain two types of skeleton: the endoskeleton, which are bones that are generated through endochondral ossification, and the exoskeleton, which in contrast contains bones that are created via an intramembranous ossification process (Fig 1.1b) (Grandel & Schulte-Merker, 1998; Géraudie, 1983).

Endochondral ossification involves the formation of a cartilage intermediate, which is later replaced by bone (Gilbert, 2000; Hall, 2005). This type of bone constitutes the endoskeleton of the adult fins and includes the radials bones, which are found at the proximal base of the anal, dorsal and paired fins, and the numerous proximal bones found in the caudal fin complex (Grandel & Schulte-Merker, 1998; Schultze & Arratia, 1989). These bones have an important role in connecting the very base of the fin rays by means of ligaments to the girdles (paired fins) or axial skeleton (unpaired fins) of the fish (Van Eeden et al, 1996; Hall, 2008). Furthermore, a pair of protractor and retractor muscles are

also found at the base of the fins and they control a muscle set that is attached at the base of each fin ray; each muscle set consists of a pair of erector, depressor and inclinator muscles (Schneider & Sulner, 2006). The contraction of these muscles plays an important role in controlling the precise movement of the fins during swimming.

In contrast, fin rays undergo intramembranous ossification, which involves the production of bone matrix directly on the basement membrane of the fin's epidermis without the need of a cartilage intermediate structure (Hall, 2005). Fin rays (lepidotrichia) are part of the fins' exoskeleton and are intramembranous bones (Géraudie & Landis, 1982). Lepidotrichia are calcified and branched ray structures that support the proximodistal length of adult teleost fins (Fig 1.1c). All adult fins comprise a number of lepidotrichia, which are enclosed by fin epithelium and are connected together by soft inter-ray tissue in a fan-like structure (Akimenko et al, 2003). Each lepidotrichia are composed of two concave hemirays, which shelters connective tissue, mesenchymal cells, nerves and blood vessels (Santamaría & Becerra, 1991; Montes et al, 1982; Becerra et al, 1983). The vasculature of the fin is composed of a vein-artery-vein architecture where lepidotrichia hemirays distinctively surround one artery and veins are located in the inter-ray tissue flanking the lateral sides of each lepidotrichia (Huang et al, 2003). The hemirays are composed of numerous bone segments that are separated by joints (Fig 1.1d) (Arita, 1971). Furthermore, the exoskeleton also comprises actinotrichia, which are short unmineralized collagenous fusiform spicules/fibres found at the distal margin of adult fin rays (Fig 1.1e) (Becerra et al, 1983; Géraudie & Landis, 1982).

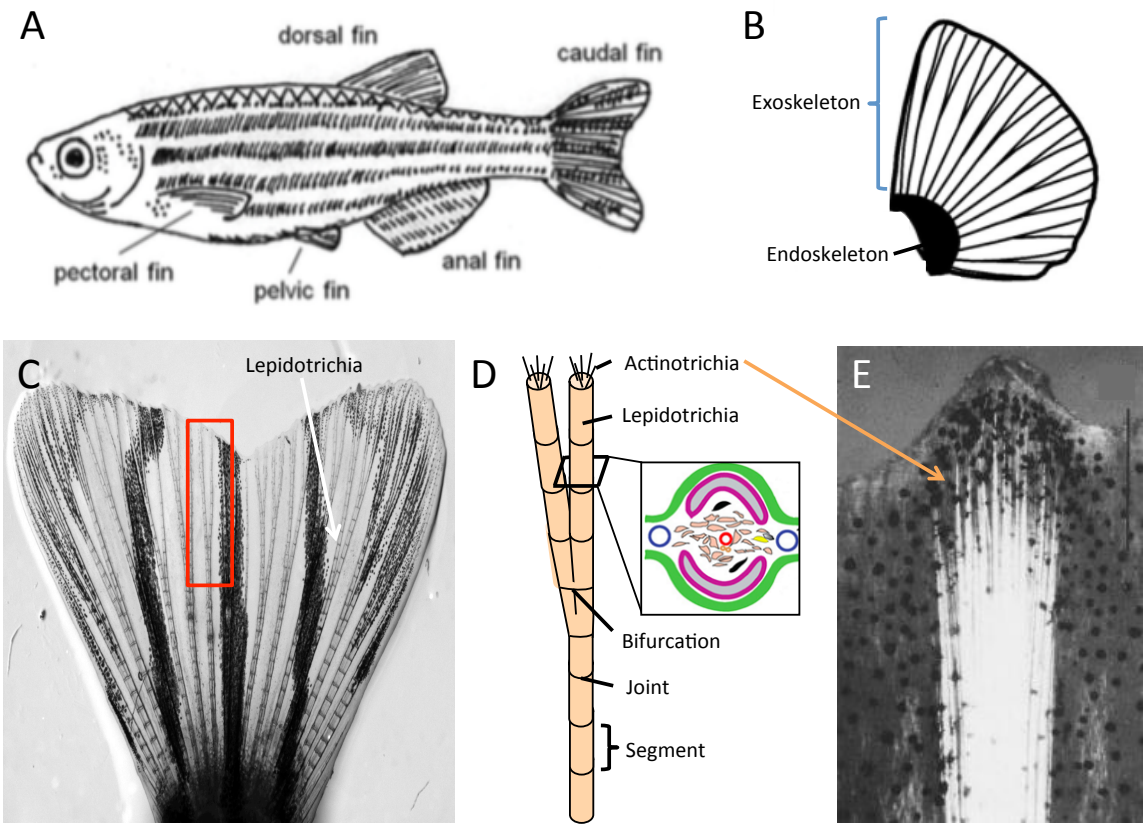


Figure 1.1. The morphology of the adult zebrafish fins. a. Zebrafish have 5 fins: three unpaired fins which consists of the dorsal, anal and caudal fins, and two paired fins which consists of the pectoral and pelvic fins. **b.** Fins are made of two types of skeletal elements: the exoskeleton and the endoskeleton. **c.** The fins contain a number of fin rays (lepidotrichia) that are separated by soft inter-ray tissue. **d.** Schematic of the morphology of the fins' exoskeleton seen in the red box of panel c. Lepidotrichia are constituted of repeated segments separated by joints and branch out by producing bifurcation points. A cross-section of a lepidotrichia shows that it is composed of two concave hemi-rays (pink structures) and surrounded by fin epithelium (green). The hemirays shelter connective tissues, nerves, mesenchymal cells and an artery (red circle). Veins are found on either sides of lepidotrichia (blue circles). **e.** Actinotrichia are found at the tip of lepidotrichia and have a brush-like appearance. Permission granted for the use of the figures taken from Jackman & Stock, 2006; Collar, Wainwright & Alfaro, 2008; Tu & Johnson, 2011; Marí-Beffa, Carmona & Becerra, 1989.

1.3. Zebrafish fin development

1.3.1. Formation of the embryonic zebrafish fins

The zebrafish embryo has two types of fins: the median fin fold, which later gives rise to the adult unpaired fins, and the pectoral fin buds, which later produce the adult pectoral paired fins (Fig 1.2a). The median fin fold and pectoral fin buds start to develop at 24 and 36 hours-post-fertilization (hpf) respectively. The pectoral fin buds form on top of the yolk sac on each side of the trunk at the level of the third somite (Kimmel et al, 1995). In fact, pectoral fin formation starts with the specification of the fin field position, which sequentially occurs first in the somites, secondly in the intermediate mesoderm and lastly in the lateral plate mesoderm of the embryo. The specification of the fin field is initiated by the synthesis of retinoic acid (RA) in the somites of zebrafish by Aldehyde Dehydrogenase 1 Family Member A2 (Aldh1a2) (Gibert et al, 2006). RA then induces the expression of *wntb2a* in the intermediate mesoderm located next to the somites, which in turn activates the expression of *t-box 5* (*tbx5*) in the lateral plate mesoderm (Ng et al, 2002; Ahn et al, 2002). Tbx5 is responsible for initiating fin bud formation and was also found to initiate forelimb formation in mice (Ahn et al, 2002; Takeuchi et al, 1999). Through a transcriptional cascade, Tbx5 activates *fibroblast growth factor 10* (*fgf10*) expression in the lateral plate mesoderm, which signals the formation of the apical ectodermal ridge (AER) (Ng et al, 2002; Yonei-Tamura et al, 1999). The AER, which is an important signalling centre for the proximodistal patterning of the fin, is the distal thickened epidermis that rims the fin bud (Fig 1.2b) (Fernandez-Teran & Ros, 2008). The AER is then maintained through the action of Fgf10, which induces the expression of

fgf8, which consequently sustains mesenchymal cell proliferation in the fin bud through a positive feedback loop with Fgf10 (Ohuchi et al, 1997).

Early fin and limb bud formation share many similar developmental processes, however the fate of the AER is a key difference between fin and limb development (Zeller, López-Ríos & Zuniga, 2009). In its mature form, the AER of mammals is composed of a thick stratified epithelium that rims the distal edge of the limb bud and persists until a fully patterned limb emerges. The AER then subsequently regresses via apoptosis and flattens into a simple epithelium (Wanek et al, 1989; Fernandez-Teran & Ros, 2008; Salas-Vidal, Valencia & Covarrubias, 2001; Guo, Loomis & Joyner, 2003). In contrast, once matured the AER of fish is composed of thickened epithelium and, in comparison to limb development, is short-lived; the AER forms an apical fold, which progressively elongates into a fin fold (Fig 1.2c) (Thorogood, 1991; Wood, 1982). The extension of the apical fold is supported by two rows of unmineralized collagenous fibres termed actinotrichia, which acts as a scaffold for the distal migration of mesenchymal cells along the fin fold (Wood, 1982; Wood & Thorogood, 1984). Interestingly, tetrapods do not have actinotrichia and it is proposed that their loss may have played a role in the fin-to-limb transition during evolution (Zhang et al, 2010).

As for the median fin fold, it initially forms along the ventral midline, posterior to the anal/cloaca region, and surrounds the tail up to the 8th somite of the dorsal midline. As development progresses, the yolk sac develops into two structures: the yolk ball and the yolk extension. The ventral portion of the median fin fold later extends anteriorly to cover the underside of the yolk extension (Kimmel et al, 1995; Abe, Ide & Tamura, 2007). The formation of the median fin fold occurs similarly to the formation of paired

fins where an AER-like structure emerges and subsequently produces a fin fold supported by actinotrichia fibres (Freitas, Zhang & Cohn, 2006). Many gene expression studies support the presence of common developmental mechanisms between the formation of paired and unpaired fins (Abe, Ide & Tamura, 2007; Freitas, Zhang & Cohn, 2006; Johanson, 2010; Yonei-Tamura et al, 2008). Studies also propose that developmental mechanisms may have been assimilated from the unpaired to paired fins during evolution (Johanson, 2010).

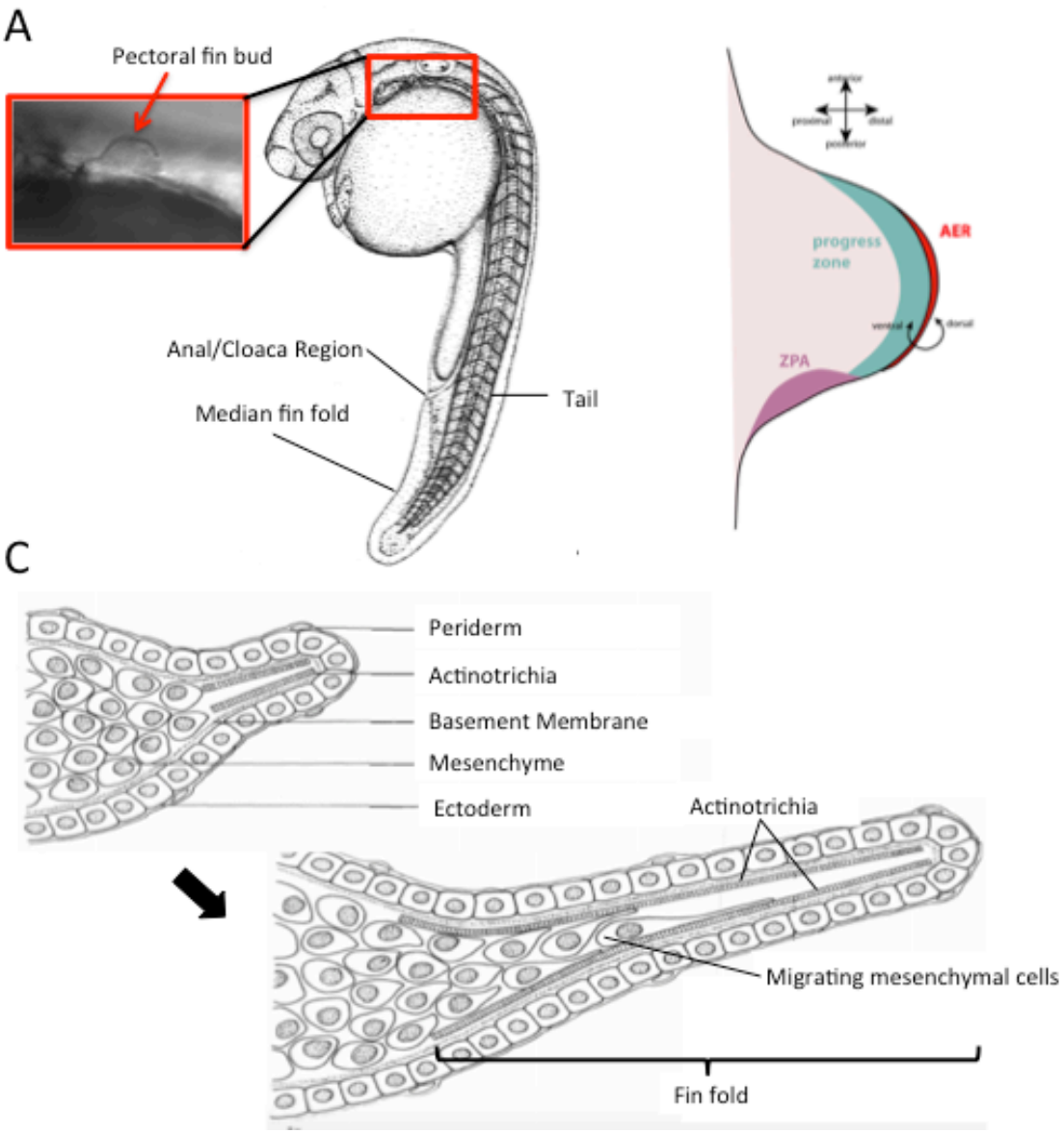


Figure 1.2. The morphology and formation of the embryonic zebrafish fin. a. Zebrafish embryos have two embryonic fins: the pectoral fins (close-up shown in the red box) and the median fin fold; the latter spans the dorsal, ventral and tip of the tail. **b.** The apical ectodermal ridge is an important organizing center during the development of the paired fins and is the distal ectoderm that rims the fin bud. **c.** Schematic showing the elongation of the AER into a fin fold during development. The tissue morphology of the fin fold is also described. AER= apical ectodermal ridge. Permission granted for the use of the figures taken from Wood & Thorogood, 1984; Kimmel et al, 1995; Lalonde, unpublished; Sisi Chen, Creative Commons.

1.3.2. Formation of endochondral and intramembranous bones in the fins

As previously mentioned, the endoskeleton results from an endochondral ossification process where a preformed cartilage matrix is progressively replaced by bone matrix (Gilbert, 2000; Hall, 2005). The endoskeleton first develops in the embryonic pectoral fin buds (36 hpf) and includes the formation of pectoral girdles and radial bones, which all emerge from the base of the pectoral fin fold (Fig 1.3a) (Grandel & Sculte-Merker, 1998). First, chondrogenic condensations appear at the centre of the pectoral fin bud during early fin development, which separates the fin bud mesenchyme into a ventral and dorsal population of muscle precursor cells (Neyt et al, 2000; Grandel & Schulte-Merker, 1998). These chondrogenic condensations are from a paraxial mesodermal origin (Neyt et al, 2000). After undergoing a number of proliferation rounds, the proximal part of the chondrogenic condensations gives rise to the girdle, which is a set of endochondral bones that connect and support the fin to the axial skeleton of the fish, and the distal part of the chondrogenic condensations gives rise to the endoskeletal disk (Fig 1.3b). At this point, the girdle and the endoskeletal disk are still connected by poorly chondrified mesenchymal cells. The most distal fin bud mesenchyme then becomes loose and migrates along actinotrichia as the apical fin fold grows (Grandel & Schulte-Merker, 1998).

Thereafter, cell proliferation concludes and the endoskeletal disk divides into 4 cartilage subdivision zones, which become the 4 proximal radials of the paired fins (Fig 1.3c-d). Furthermore, the subdivision of the endoskeletal disk also results in the separation of the girdle from the rest of the disk (Grandel & Schulte-Merker, 1998). Unlike limb development in tetrapods, the division of the endoskeletal disk of the

pectoral fin is not due to cell death, chondroclast activity or macrophage digestion (Zuzarte-Lui's & Hurle, 2002; Bronckers et al, 2000). It is rather due to the dedifferentiation of chondrocytes in the subdivision zones of the disk (Dewit, Witten & Huysseune, 2011).

As previously stated, the formation of lepidotrichia, which are part of the exoskeleton of the fin, is the result of an intramembranous ossification process that involves the production of bone matrix directly on the basement membrane of the fin's epidermis and does not involve the formation of a cartilaginous intermediate structure (Hall, 2005). Actinotrichia, which are also part of the fins' exoskeleton, are the sole skeletal elements found in the pectoral and median fin folds during embryonic fin development. As fin development progresses, actinotrichia fibres become progressively restricted distally in the fold as lepidotrichia formation occurs from a proximal-to-distal direction (Grandel & Schulte-Merker, 1998). The formation of lepidotrichia is synchronized with the cartilage subdivision of the disk (Dewit, Witten & Huysseune, 2011).

While the 4 proximal radials are forming, distal mesenchymal condensations are observed occurring at the base of the fin fold (Grandel & Schulte-Merker, 1998). The origin of the cells constituting these condensations is currently debated; some studies propose a strict mesodermal contribution while others propose an additional neural crest contribution (Lee, Thiery & Carney, 2013; Shimada et al, 2013; Smith et al, 1994; Kague et al, 2012). The mesenchymal condensations then invade the proximal end of the fin fold, which causes the basement membrane to bulge into a bracket shape and subsequently acts as a mold. Lepidotrichia formation occurs when bone matrix is

deposited in the bracket-shaped basement membrane by bone-forming cells (osteoblasts). Lepidotrichia then grow by the sequential synthesis of segments to its distal end. All the while, actinotrichia fibres gradually become spatially restricted to the distal end of the fin. Distal radials are subsequently formed from *de novo* cartilaginous condensations located at the proximal end of lepidotrichia (Fig 1.3d-f) (Grandel & Schulte-Merker, 1998). Lastly, ossification of the cartilaginous elements of the pectoral fins occurs as described in Cubbage & Mabee, (1996).

In contrast, pelvic fins are the last fins to develop during development. They first appear on the ventrolateral body wall of zebrafish (~18 days-post-fertilization (dpf)) and are positioned anterior to the anal/cloaca region, specifically in the ventral region of the ninth and tenth myotome. Although both pelvic and pectoral fins are paired structures, the development of the pelvic endoskeleton (~29 dpf) proceeds differently compared to the pectoral fins; the chondrogenic condensations that contribute to the pelvic girdles and radials directly reflect the adult endoskeletal pattern, without the need of a disk intermediate. However the sequential formation of the skeletal elements of the fins remains the same; the pelvic girdles are first to develop and three radials are subsequently formed after lepidotrichia formation (Grandel & Schulte-Merker, 1998).

The larval median fin fold gives rise to the three adult unpaired fins in fish following metamorphosis. The larval median fin fold is composed of two lobes; a major lobe, which is composed of a continuous fin fold that surrounds the dorsoventral region of the larval tail and ends on the posterior side of the anal/cloaca region, and a minor lobe, which is located ventrally along the yolk extension and ends on the anterior side of the anal/cloaca region (Fig 1.4). From these two lobes, only the major lobe gives rise to

the unpaired fins (Parichy et al, 2009). Similar to pelvic fin development, the chondrogenic condensations that are destined to form the endoskeleton of the unpaired fins directly generate individual endochondral bones in the median fin fold without the need of a disk or a girdle (Suzuki et al, 2003). The caudal fin is the second fin to develop during fin development (~14 dpf) and is distinctively characterized by the formation of the hypural complex. The formation of this complex occurs first and includes the hypurals, the parhypural and the haemal spine. This endoskeletal complex develops from chondrogenic condensations located at the ventral side of the posterior-most end of the notochord. Its function is to secure the subsequently formed lepidotrichia to the axial skeleton of the fish (Parichy et al, 2009; Bensimon-Brito et al, 2010).

Next, the anal (~15 dpf) and dorsal fins (~17 dpf) are then sequentially formed in the median fin fold. First, the proximal radials are directly formed by chondrogenic condensations located at the very base of the fin fold. After the proximal radials have been created, distal radials begin to form. Meanwhile, lepidotrichia formation occurs from an anterior-to-posterior fashion in the median fin fold. The proximal radials subsequently undergo ossification, with the exception of the distal part, which later becomes segregated and forms the medial radials between the proximal and distal radials. Lastly, complete ossification of the radials occurs (Suzuki et al, 2003).

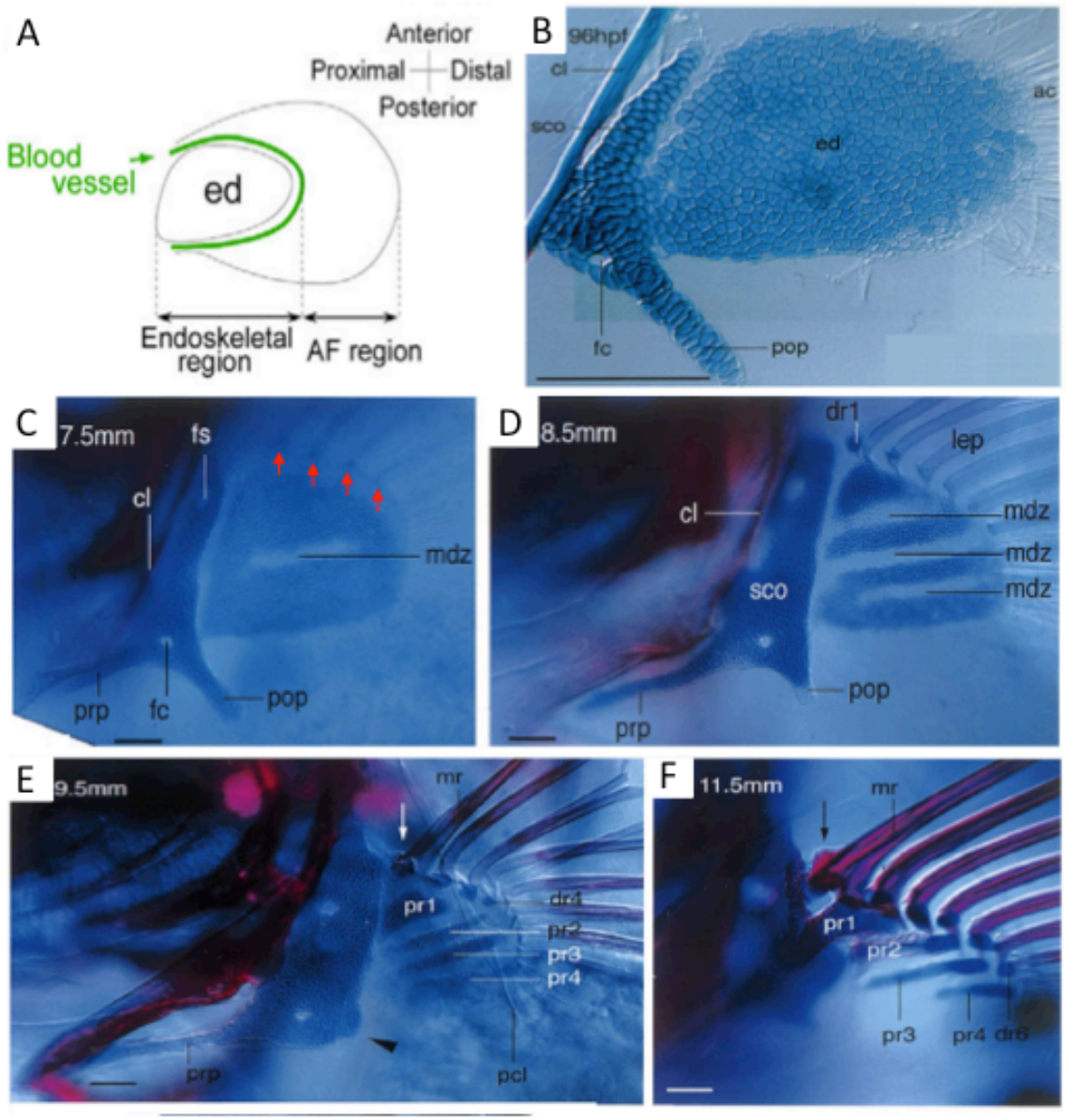


Figure 1.3. The formation of the endochondral and intramembranous bones in paired fins. a. The zebrafish paired fins consists of an endoskeletal region, where endochondral bones develop, and an apical fin fold region, where intramembranous bones develop. **b**. During fin development, there is the formation of the endoskeleton, which consists of the endoskeletal disk and the girdle (which is constituted of the cl, sco, fc and pop labelled structures). The endoskeletal disk gives rise to 4 proximal radials via a chondrogenic dedifferentiation process, seen in panels **c-e**. Meanwhile, intramembranous bones such as lepidotrichia form from mesenchymal condensations (shown by red arrows) located at the proximal end of the endoskeletal disk, see panel c. Distal radials then form from *de novo* mesenchymal condensations located at the proximal end of lepidotrichia, see panels **d-f**. ed= endoskeletal disk, AF= Apical fold, pr= proximal radials, cl= cleithrum, sco= scapulocoracoid, fc= foramen coracoideus, pop= postcoracoid process, dr= distal radials. Permission granted for the use of the figures taken from Yano et al, 2012; Grandel & Schulte-Merker, 1998.

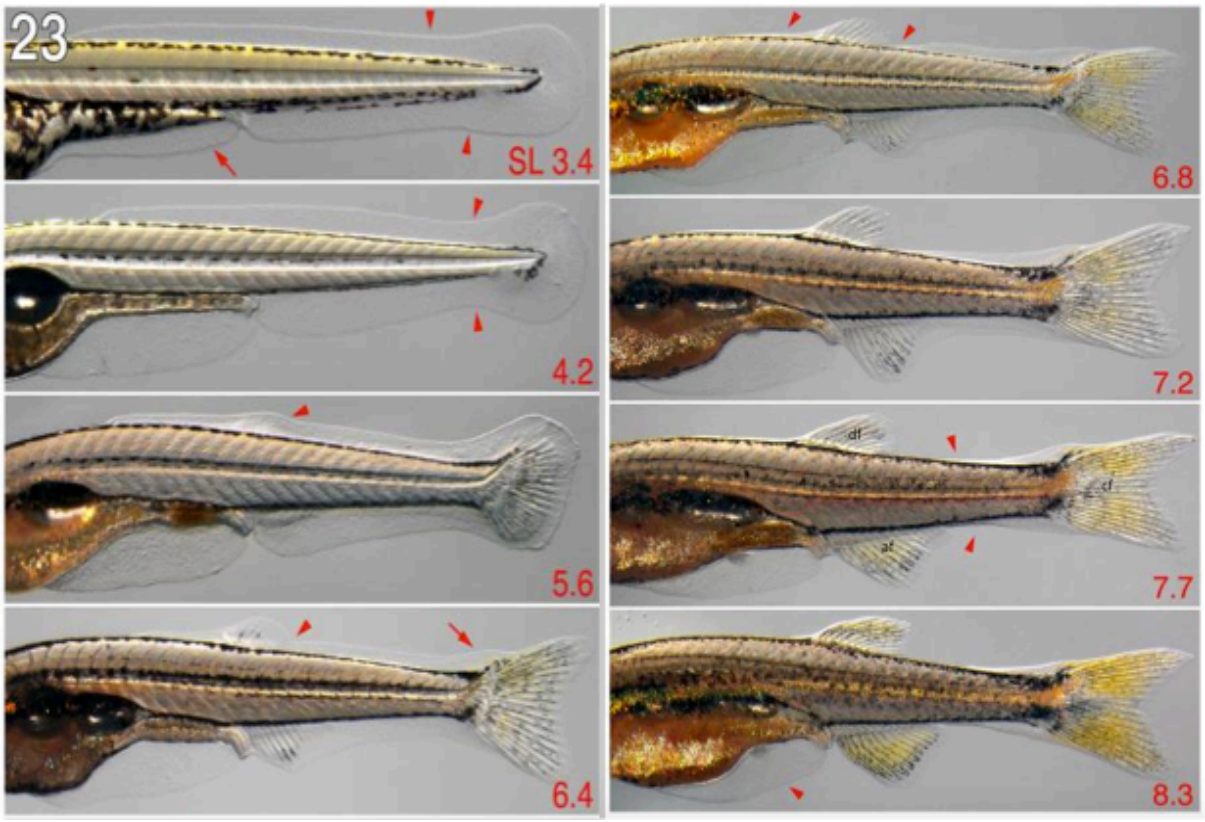


Figure 1.4. The resorption of the median fin fold. The standard lengths (SL) of the larvae are shown at the bottom right corner of the pictures. The median fin fold consists of two lobes; a major lobe (shown by red arrowheads at a SL of 3.4 mm), which is composed of a continuous fin fold that surrounds the dorsoventral region of the larval tail and ends on the posterior side of the anal/cloaca region, and a minor lobe (shown by a red arrow at a SL of 3.4 mm), which is located ventrally along the yolk sac and ends on the anterior side of the anal/cloaca region. The major lobe of the median fin fold gives rise to the three unpaired fins: the caudal fin is first to develop, the anal fin is second and the dorsal fin is the last unpaired fin to form. As skeletal elements form in the presumptive caudal, anal and dorsal areas of the median fin fold, the fin fold tissues located between them are gradually being resorbed in an anterior-to-posterior fashion (seen by the red arrowheads at a SL of 7.7 mm). The minor lobe is completely resorbed at a SL of 10.0 mm. SL= Standard length, cf= caudal fin, af= anal fin, df= dorsal fin. Permission granted for the use of the figure taken from Parichy et al, 2009.

1.3.3. The resorption of the median fin fold

As skeletal elements form in the presumptive caudal, anal and dorsal areas of the median fin fold, the fin fold tissues located between them gradually regress (Fig 1.4). Parichy et al, (2009) has morphologically described the process and refer to it as the median fin fold resorption process. Resorption of the major lobe starts at a standard length (SL) of 7.0 mm (~25 dpf) and proceeds in an anterior-to-posterior fashion. The SL measuring method entails measuring the distance from the snout to the posterior tip of the notochord and is a more accurate staging method compared to date staging since many factors can affect the rate of fish growth. As for the minor lobe, it goes through resorption much later in development and is complete at a SL of 10.0 mm (~50 dpf) (Parichy et al, 2009). From this process, the adult unpaired fins take shape: the caudal fin is first to develop, the anal fin is second and the dorsal fin is the last unpaired fin to form. Once the resorption of the median fin fold and the fin ossification is complete, the final adult morphology of the fins is achieved. The median fin fold resorption process has been extensively described morphologically, however it is still unclear what cellular processes are responsible for shaping the larval median fin fold into adult unpaired fins.

In contrast, the loss of larval-specific features has been greatly studied in amphibian models, such as the regression of the tadpole tail. Amphibian tail regression is caused by thyroid hormone-mediated apoptosis (Kerr, Harmon & Searle, 1974; Wang & Brown, 1993). Metamorphosis is initiated by the secretion of thyroid hormones into the bloodstream, such hormones include 3,5,3'-triiodothyronine (T3) and thyroxine (T4), the latter being a T3 precursor hormone (Gereben et al, 2008). Type I and II deiodinase enzymes convert T4 into T3, which is a more active hormone variant (Becker et al,

1997). In contrast, type III deiodinase enzyme inactivates T3 (St Germain et al, 1994). These deiodinase enzymes are expressed differentially in the tadpole, where structures that undergo metamorphosis express type I and II deiodinases, and the tissues that do not undergo metamorphosis express type III deiodinase (Becker et al, 1997; Huang, Marsh-Armstrong & Brown, 1999). T3 then binds to thyroid hormone receptors, which then act as a transcription factor that binds to thyroid response elements in the genome. This subsequently activates the transcription of pro-apoptotic and anti-apoptotic genes (Wang & Brown, 1993; Helbing et al, 2003). This massive deletion of the various tissues of the tail via cell death also involves macrophages, which removes auto- and heterolyzed cells through phagocytosis in dying tissues (Kinoshita, Sasaki & Watanabe, 1985). Lastly, a cell death/proliferation balance is achieved during amphibian metamorphosis where there is a loss of larval-specific structures via apoptosis and the acquisition of adult features via maintained cell proliferation (Ishizuya-Oka, 2011). Previously, Brown (1997) has found that the inhibition of thyroid hormones in zebrafish prevented the larva-to-juvenile transition from happening, which is exactly when the median fin fold resorption process occurs. However, the effects of thyroid hormones have not been appropriately researched specifically during the median fin fold resorption process in zebrafish.

1.4. Fin regeneration

As previously stated, zebrafish can regenerate multiple organs following injury including their fins, which are composed of multiple types of tissues such as epithelium, fibroblasts, bones, blood vessels, actinotrichia, etc. (Gemberling, et al, 2013). It does so through an epimorphic regenerative process, which implicates the formation of a blastema; mature differentiated cells dedifferentiate into a mass of undifferentiated cells

(blastema), proliferate and then redifferentiate during regeneration to reconstitute the lost fin (Hall, 2008; Santamaría & Becerra, 1991). Zebrafish caudal fin regeneration occurs in three distinct steps as described by Pfefferli & Jaźwińska, (2015): there is first the formation of a wound epidermis, the formation of a blastema and lastly, regenerative outgrowth of the fin (Fig 1.5a). After fin amputation, a layer of epithelial cells covers the wound at 1 day-post-amputation (dpa) and is termed the wound epidermis. It was shown to be essential for regeneration to occur (Goss, 1991). In fact, the wound epidermis secretes many factors needed for controlling the subsequent formation of the blastema (Laforest et al, 1998; Poss, Shen & Keating, 2000; Lee et al, 2009; Quint et al, 2002).

At 2 dpa, the blastema forms through the accumulation of dedifferentiated proliferative cells in the mesenchyme of the fin regenerate (Hall, 2008; Stewart & Stankunas, 2012). Although many studies support that fin regeneration is a lineage-restricted process where undifferentiated cells contributing to the regeneration of the fin re-differentiate into their original cell types, Singh, Holdway & Poss, (2012) have demonstrated trans-differentiation may be occurring as well during fin regeneration (Tu & Johnson, 2011; Stewart & Stankunas, 2012). Trans-differentiation is the process where a differentiated cell type converts into another (Shen, Burke & Tosh, 2004).

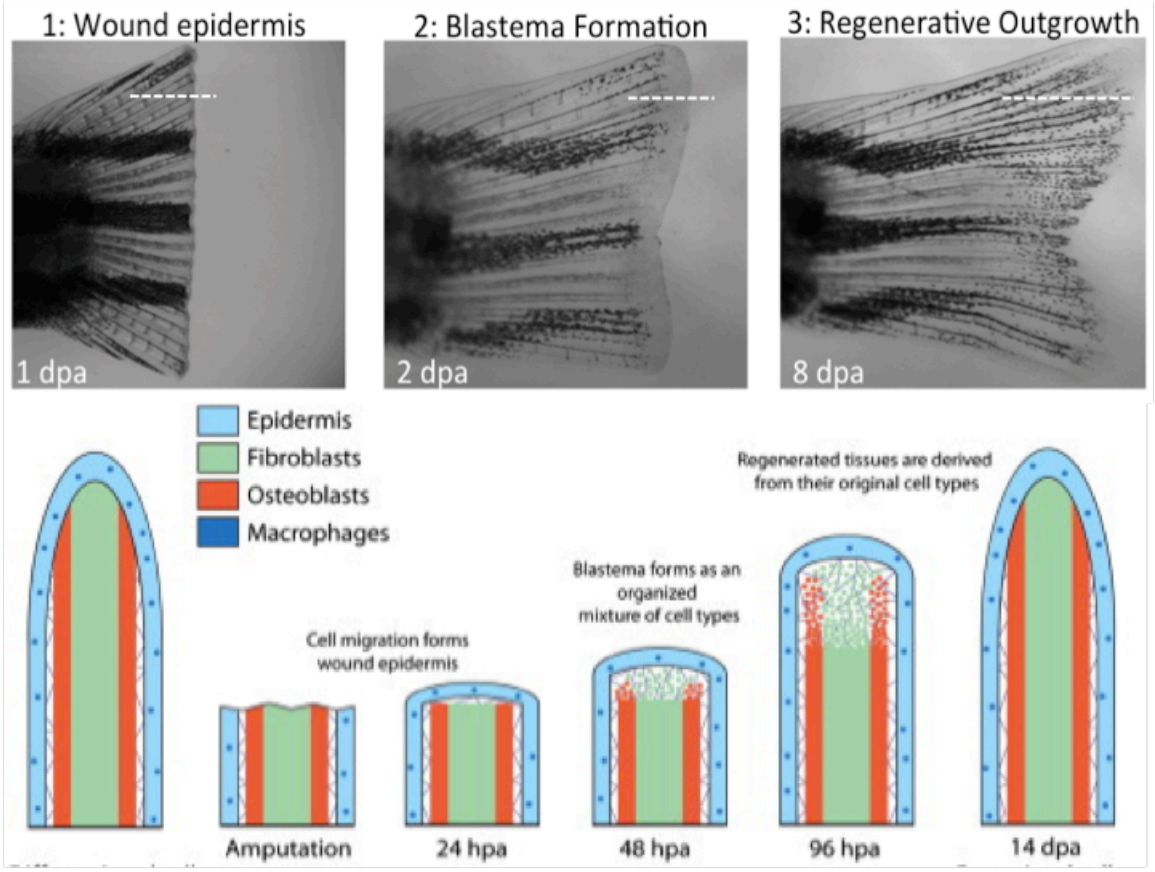
The blastema is composed of two distinct zones according to their proliferation rate: a non-proliferating distal blastema and a proliferating proximal blastema (Fig 1.5b) (Nechiporuk & Keating, 2002). It has been proposed that the distal blastema acts as an upstream organizer that regulates the patterning of the fin regenerate while the proximal blastema's task is to maintain high cell proliferation and guide redifferentiation (Wehner et al, 2014; Grotek, Wehner & Weidinger, 2013; Münch González-Rajal & de la Pompa,

2013). Furthermore, the fin regenerate is constituted of repeated blastema units located at the distal tip of each lepidotrichia stumps (Stewart & Stankunas, 2012). Interestingly, following amputation the actinotrichia fibres and presumably the actinotrichia-forming cell population are completely removed. Yet, they are the first skeletal elements to be visible in the fin regenerate (2 dpa); they form beside the basal epidermal layer and the mesenchyme of the fin regenerate (Fig 1.5b) (Kemp & Park, 1970). In contrast, regenerating lepidotrichia are only visible at 3 dpa in the fin regenerate despite having an available cell population located at their stumps (Knopf et al, 2011).

The regenerative outgrowth phase of the regeneration process then re-establishes the lost structures and tissues of the adult fin within 2-3 weeks time (Poss, Keating & Nechiporuk, 2003; Azevedo et al, 2011; Pfefferli & Jaźwińska, 2015). During this stage, the blastema maintains its distal position as newly regenerated tissues emerge from the proximal end. As for bone regeneration, mature osteoblasts located at the stumps of amputated lepidotrichia dedifferentiate and induce bone progenitor markers following the downregulation of intermediate and late bone differentiation markers. These dedifferentiated osteoblasts then proliferate and migrate towards the amputation plane to be part of the lateral blastema (Knopf et al, 2011). Redifferentiation of pre-osteoblasts into mature osteoblasts occurs in the lateral regions of the blastema in a proximal-to-distal direction and involves many signalling pathways such as BMP, RA, Wnt/β-catenin and Fgf signalling pathways to mention a few (Smith et al, 2006; Blum & Begemann, 2012; Wehner et al, 2014; Poss et al, 2000). As for actinotrichia, similarly to what was characterized in the intact adult fins, actinotrichia fibres remain distally restricted in the fin regenerate. Actinotrichia are composed of a protein complex termed elastoidin.

Radioautographic studies revealed the presence of an elastoidin turn-over, where actinotrichia are distally secreted and proximally reabsorbed in a continuous way, which allows actinotrichia to remain distal in the fin regenerate (Marí-Beffa, Carmona & Becerra, 1989).

A


1: Wound epidermis
2: Blastema Formation
3: Regenerative Outgrowth
1 dpa
2 dpa
8 dpa
Epidermis
Fibroblasts
Osteoblasts
Macrophages
Cell migration forms wound epidermis
Blastema forms as an organized mixture of cell types
Regenerated tissues are derived from their original cell types
Amputation
24 hpa
48 hpa
96 hpa
14 dpa

B

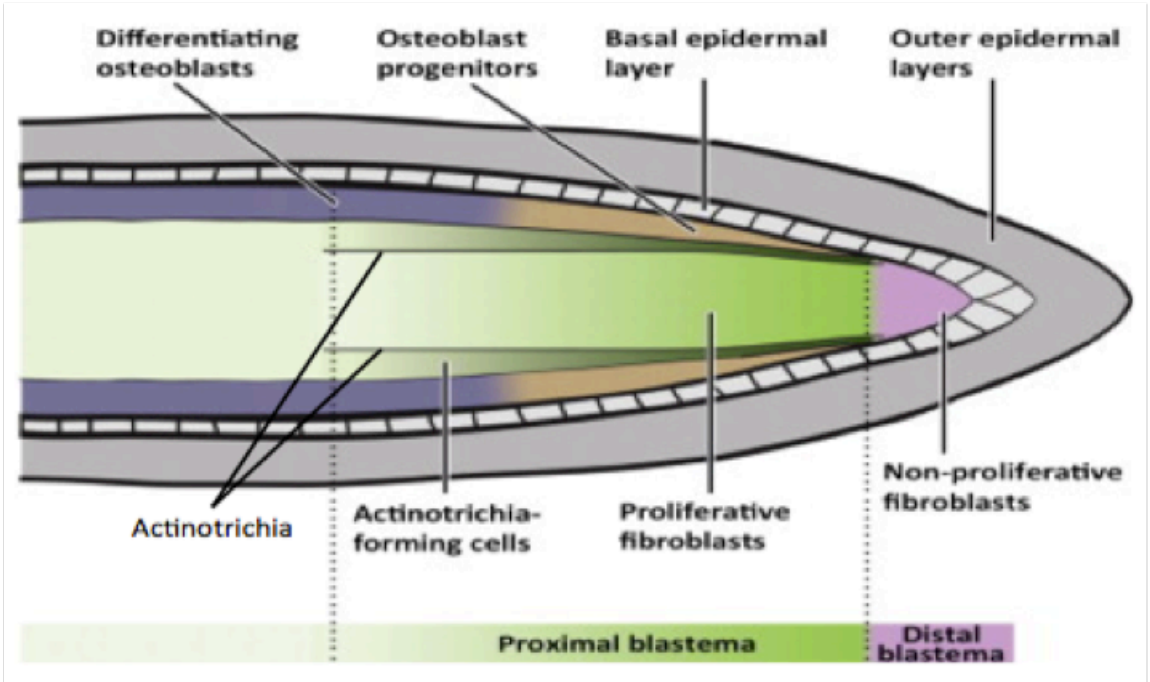

Differentiating osteoblasts
Osteoblast progenitors
Basal epidermal layer
Outer epidermal layers
Actinotrichia
Actinotrichia-forming cells
Proliferative fibroblasts
Non-proliferative fibroblasts
Proximal blastema
Distal blastema

Figure 1.5. Regenerative steps and morphology of the fin regenerate throughout fin regeneration. a. There are three distinct steps during fin regeneration: after amputation, the wound is covered by epidermis at 1 day post-amputation (dpa), next there is the formation of a blastema at 2 dpa, which is a mass of proliferative undifferentiated cells, and then regenerative outgrowth occurs where the lost part of the fin is reconstituted. **b.** Schematic of the tissue morphology of the fin regenerate. The blastema is constituted of two essential regions: the proximal proliferative blastema and the distal non-proliferative blastema. Permission granted for the use of the figures taken from Stewart & Stankunas, 2012; Wehner & Weidinger, 2015.

1.5. Actinotrichia

1.5.1. Origin and composition

My research focuses on the actinotrichia fibres observed in zebrafish fins throughout their development and regeneration. As previously described, actinotrichia are small tapered fusiform spicules found at the distal margin of adult fins of ray-finned fishes (Actinopterygii) and lobe-finned fishes (Sarcopterygii) (Becerra et al, 1983; Géraudie, 1985). Previous research suggests actinotrichia are homologous to ceratotrichia, which are skeletal elements found in the fins of cartilaginous fishes (Chondrichthyes) (Goodrich, 1904; Géraudie & Meunier, 1980). Upon their discovery, Krükenberg observed that ceratotrichia were made of a protein that had very similar characteristics to elastin and collagen and consequently, termed the protein elastoidin (Krükenberg, 1885). Biochemical analyses of elastoidin later revealed it to be a two-part protein complex constituted of unspecified collagen and an unknown tyrosine-rich component (Gross & Dumsha, 1958; Damodaran, Sivaraman & Dharaliker, 1956). Like ceratotrichia, actinotrichia found in teleost fish are also composed of elastoidin and are recognized by an anti-serum directed against selachian (Chondrichthyes) ceratotrichia (Garrault, 1936; Santamaría, Santos Ruiz & Becerra, 1996). Consequently, there is a clear homology between ceratotrichia from cartilaginous fish (Chondrichthyes) and actinotrichia from bony ray-finned fish (Teleosteii).

As previously stated, elastoidin was actually found to be a protein complex constituted of a collagen fraction and a tyrosine-rich protein component; tyrosine-rich segments increase the structural strength of proteins (Gross & Dumsha, 1958; Damodaran, Sivaraman & Dharaliker, 1956; McGaughey et al, 1998). The collagenous

properties of actinotrichia have been substantially investigated in the past through diffraction studies, chemical analyses and electron microscopy (Champetier & Fauré-Frémiet, 1937; Piez & Gross, 1959; McGavin & Pyper, 1964). However, it is only in 2011 that actinotrichia were characterized to be specifically composed of collagen I and a novel form of collagen II, whose gene is only found in the genome of fish and was also found to be the only type II collagen expressed in the fins (Durán et al, 2011). As for the unknown tyrosine-rich protein component of actinotrichia, it was only in 2010 our lab found they were coded by the *actinodin* (*and*) gene family and had an important role in the structural support of the fibre itself (Zhang et al, 2010). All in all, actinotrichia are composed of two types of collagens (type I and II) and tyrosine-rich proteins called Actinodins (And).

In the past, research proposed that the formation of actinotrichia in the subepidermal space of the pseudo apical ridge was initiated by the ectoderm (epithelium) since during the early formation of the apical fold, the first rudiment of actinotrichia fibres were observed developing closely to the basal epithelium of the pseudo apical ridge when it was devoid of mesenchymal cells (Géraudie, 1977; Géraudie & Landis, 1982). The lengthening and further growth of actinotrichia was hypothesized to be later induced by the mesenchymal cells invading the fold (Géraudie & Landis, 1982). The ectodermal origin of actinotrichia was further supported by a recent study that shows, by using tissue-specific *and1* zebrafish reporter lines, that *and1* expression was activated in ectodermal cell populations prior to mesenchymal cells in the pseudo apical ridge of the pectoral fin bud (Lalonde et al, 2016). Therefore, research strongly suggests actinotrichia

formation is initiated by the ectoderm and then mesenchymal cells, along with the ectoderm, are responsible for their lengthening and maintenance.

1.5.2. The role of actinotrichia fibres

The role of actinotrichia has been previously well characterized during early fin development. Actinotrichia are fibres whose size was found to vary: its width ranges between 1-5 μm and its length ranges between 300-500 μm when studied in an assortment of adult teleost fish (Becerra et al, 1983). During the embryonic and larval stages of teleost fish, there are ventral and dorsal rows of actinotrichia found along the proximodistal axis of the pectoral and median fin folds, which provide structural support and maintain the integrity of the fin folds throughout early fin development (Bouvet 1974; Dane & Tucker, 1985). As they support the developing fin fold, actinotrichia also act as a scaffold for the distal migration of mesenchymal cells, which may contain progenitor cells needed for the subsequent formation of lepidotrichia, along the fin fold (Wood, 1982; Zhang et al, 2010). These progenitor mesenchymal cells directionally migrate in the fold via contact guidance by using the topography of actinotrichia fibres and filopodia to distally migrate along the fin fold (Wood, 1988; Wood & Thorogood, 1984).

In contrast, little is known about the function of actinotrichia in the fins of adult fish. As fin development progresses, the supporting skeletal role of actinotrichia is gradually and almost completely replaced by lepidotrichia (Géraudie, 1984). Nevertheless, actinotrichia still remain present in adult fins however only at their distal margin: they lie between the hemirays of the most distal bone segment, occasionally reaching to the second bone segment, and seldom make contact with lepidotrichia

(Becerra et al, 1983; Géraudie & Landis, 1982). Furthermore, the presence of actinotrichia has also been characterized in the inter-ray tissue of adult fins (Zhang, unpublished). Thus, they appear to play a role in supporting the very distal edge of the fins. Furthermore, past research suggested actinotrichia to have a potential role in inducing osteoblast differentiation since lepidotrichia were observed to grow towards actinotrichia throughout fin regeneration (Santamaría & Becerra, 1991). Recent studies are just starting to uncover the molecular relation between the formation of lepidotrichia and actinotrichia during fin regeneration: Wehner et al, (2014) discovered that actinotrichia-forming cells possess a Wnt/β-catenin activity, which was shown to indirectly regulate the commitment and differentiation of pre-osteoblasts in the fin regenerate. However, previous studies have not specifically investigated the effects of the loss of actinotrichia-forming cells on bone development and regeneration.

1.6. Actinodin gene family

1.6.1. Discovery and protein characteristics

Our lab initially discovered the *actinodin* gene family during a screen for genes differentially expressed during fin regeneration (Fig 1.6a). From this screen, two genes by the name of *2-F11* and *2-H06* were identified to have unknown functions and were found to be specifically expressed in the fins (Padhi et al, 2004). Upon further sequence and expression analyses of these two genes in zebrafish, they were revealed to code for the previously unknown tyrosine-rich protein fraction of elastoidin, which constitutes actinotrichia; the 2-F11 and 2-H06 proteins are 8.25% and 5.71% rich in tyrosine respectively (Zhang et al, 2010). These two genes were named *actinodin1* (*2-F11: and1*) and *actinodin2* (*2-H06: and2*) by combining the terms actinotrichia and elastoidin

together. Sequence analyses of the gene products also uncovered that there is a signal peptide at their N-terminal and they contain two potential convertase cleavage sites, which are sites where enzymes can catalyze the maturation of proproteins into mature proteins. This suggests these two proteins are most likely secreted (Zhang et al, 2010).

Furthermore, the And1 and And2 proteins were characterized to contain 8-10 repeats of a nine-amino acid motif (C(N/D)PXXDPXC), where each repeat is theorized to form an individual loop. To add, the presence of two prolines and two cysteines in the repeated motif also suggests that each loop may stack on top of one another to form an elongated domain. Two additional *and* genes were found by performing additional zebrafish genome sequence database analyses: *and3* and *and*4 are shorter members of the *and* family and have fewer repeats (3 and 4 repeats respectively). Nevertheless, their sequences likewise showed the presence of a signal peptide at their N-terminal and one convertase cleavage site. Altogether, our lab has discovered the *and* gene family, which contains 4 genes in total in the genome of teleost fish and were found to code for the tyrosine-rich protein component of elastoidin, which compose actinotrichia fibres. Lastly, these proteins were found to be likely secreted by actinotrichia-forming cells (Zhang et al, 2010).

1.6.2. The role and expression patterns of the actinodin genes in the fins

The *and* gene family has been found to be essential in the formation of actinotrichia fibres. First, our lab observed that *and* expression spatially and temporally correlates to the formation of actinotrichia during fin development and regeneration; actinotrichia become visible after the onset of *and* expression in the developing and regenerating fin. During early fin development, *and* expression occurs at 24 hpf in the

developing median fin fold and at 36 hpf in the pectoral fin buds in both the ectodermal and mesenchymal cell populations of the fin folds, with the exception of cleft cells which are ectodermal cells located at the very distal end of the fold (Fig 1.6b-d). During fin regeneration, *and1* expression occurs prior to actinotrichia formation (a little before 2 dpa) in the distal basal epidermal layer and in the mesenchymal cells of the fin regenerate (Fig 1.6e-f) (Zhang et al, 2010). All in all, the expression pattern of *and1* spatially and temporally correlates with the formation of actinotrichia during fin development and regeneration.

Furthermore, gene functional analyses confirmed the importance of *and* genes in actinotrichia formation. By performing morpholino-mediated knockdown experiments against *and1* and *and2* individually during early fin development, no perceptible morphological effects were observed in the fin fold. However, the simultaneous knockdown of both genes caused actinotrichia loss and/or disorganization in the fin fold, which inherently caused the fin fold to fold unto itself. Furthermore, a lack of mesenchymal cells migration was also observable along the fin fold in And1/2 morphants. These results indicate first, that both genes have compensatory roles and that secondly, And proteins are needed for the structural support of actinotrichia and therefore, are also needed for the proper growth of the fin during development. Furthermore, by compromising mesenchymal cell migration along the fin fold, the subsequent formation of lepidotrichia may be jeopardized since these migrating cells may contain progenitor cells needed for bone formation (Zhang et al, 2010). Additional research also show that *and1*-expressing cells express *col1a1a* and *col2a1b*, which are genes that code for type I and type II collagens, which were found to compose

actinotrichia fibres. These cells also express *lysyl hydroxylase 1,* which is required in the post-translational processing of collagens (Durán et al, 2011). Therefore *and1*-expressing cells are directly responsible for the formation of actinotrichia.

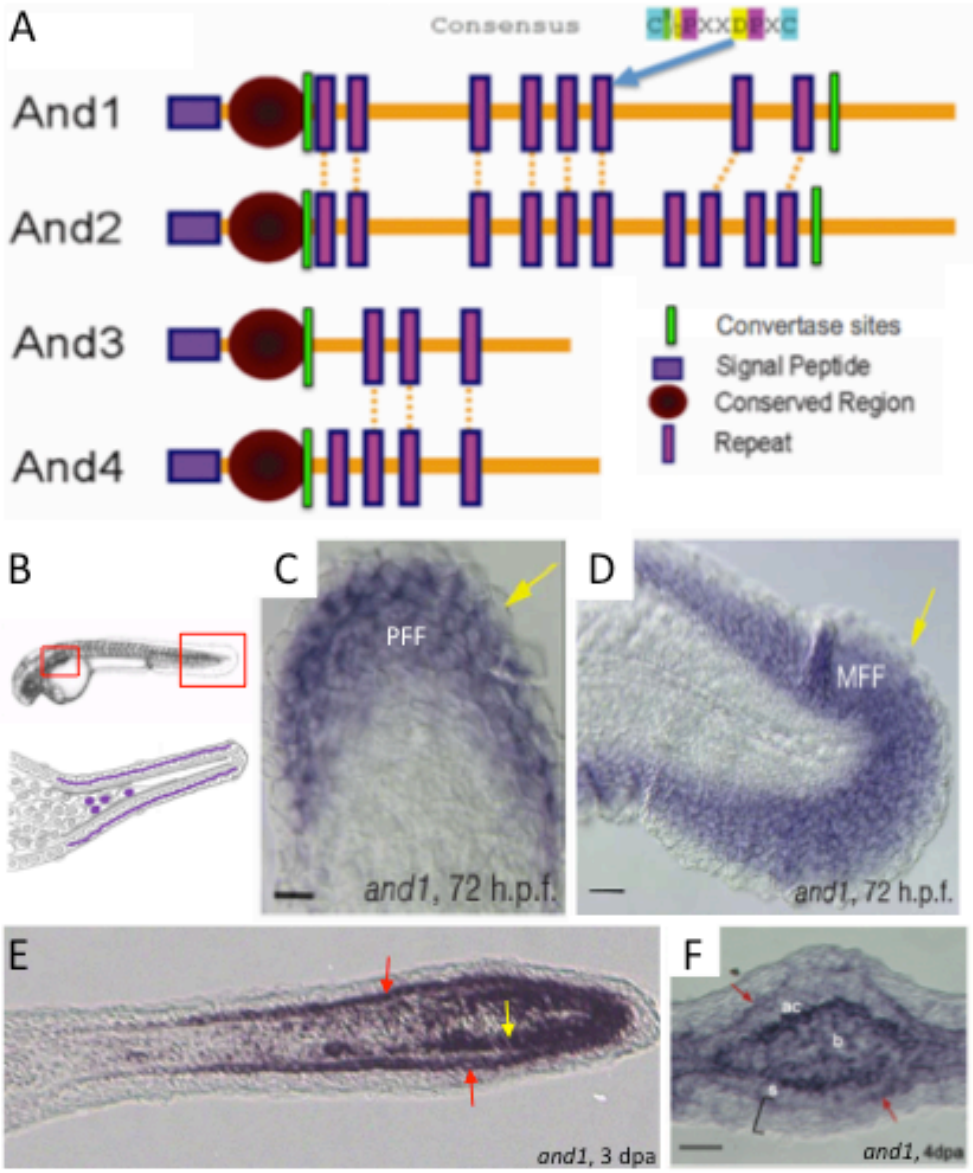


Figure 1.6. The zebrafish Actinodin proteins. a. Schematic of the structures of the Actinodin protein found in the zebrafish. **b.** Schematic of the regions where *actinodin1* (*and1*) is expressed in the zebrafish embryo; *and1* is specifically expressed in the mesenchymal and ectodermal cells of the pectoral and median fin folds of zebrafish embryos, except for the cleft cells which are the distalmost ectodermal cells of the fin (shown by yellow arrows) seen in panels **c** and **d** respectively. **e.** The *and1* gene is expressed also in the basal epidermal layer (shown by the red arrows) and mesenchyme (region shown by the yellow arrow) of the fin regenerate. **f.** A transverse view of the *and1* expression pattern in the fin regenerate. And= Actinodin, *and1*= *actinodin1*, dpa= days-post-amputation, h.p.f= hours-post-fertilization, PFF= Pectoral Fin Fold, MFF= Median Fin Fold. Permission granted for the use of the figures taken from Kimmel et al, 1995; Wood & Thorogood, 1984; Zhang et al, 2010.

1.6.3. Evolutionary significance

The *and* gene family has an important evolutionary significance in regards to the fin-to-limb transition during evolution. The fin-to-limb transition entailed many gradual morphological changes to the fins; one of them being the loss of fin rays (Yano & Tamura, 2013). Interestingly, the *and* gene family was found through genome sequence database analyses to be present in fish species, however completely absent in the genomes of tetrapods, which suggests the loss of the *and* gene family may have occurred during the transition from water to land (Zhang et al, 2010). Furthermore, actinotrichia are present in fish species and have a role in the support and extension of the AER into a fin fold (Wood, 1982). The formation of the fin fold by actinotrichia allows progenitor osteoblasts to migrate and form fin rays in the fold (Zhang et al, 2010). In contrast, actinotrichia are absent in tetrapod species and the AER of the limb bud is not supported during limb development. The AER then regresses via programmed cell death and interacts with the underlying mesenchyme, which allows the limb to form more endoskeletal elements (Kimmel et al, 2000; Guo, Loomis & Joyner, 2003; Lu et al, 2008, Benazet & Zeller, 2013). All in all, the loss of the *and* gene family is proposed to have played a role in the loss of fin rays during the fin-to-limb transition; the consequential loss of actinotrichia perhaps inhibits the distal outgrowth of the fin that is needed for subsequent fin ray formation (Zhang et al, 2010).

According to thorough sequence analyses, *and* orthologs were found to be present in many fish species. Teleosts contain 4 *and* orthologs, two long (*and1* & *and2*) and two short versions (*and3* & *and4*), due to the teleost genome duplication event that took place between 320 and 350 million years ago according to the most recent estimates (Zhang et

al, 2010; Glasauer & Neuhauss, 2014). As for cartilaginous fish, which display more ancestral characteristics than teleost fish, an *and*-like ortholog was identified in their genome (Zhang et al, 2010). Furthermore, a short and a long *and* orthologs were identified in lobe-finned fish, which share a more recent common ancestor with tetrapods than teleosts. Gene synteny comparisons have revealed important clues on establishing which *and* gene was most similar to the ancestral *and*-like gene. It seems that among the 4 *and* genes found in teleost genomes, *and1* is more representative of the ancestral long version and *and3* is more representative of the ancestral short version of the *and* genes (Ameniya et al, 2013; Moses Thesis, unpublished).

1.6.4. Cis-acting regulatory elements of and1

Past researches have been focusing distinctively on the *and1* gene for a few reasons. As previously discussed, the *and* genes are composed of a variable number of a 9 amino acid repeat motif that is proposed to grant its structural function in actinotrichia fibres. Since the *and1* gene is one of the long gene versions of the *and* gene family and naturally contains more repeats than the short versions, it is therefore presumed that the *and1* gene may play a more substantial role in the structure of actinotrichia fibres. In fact, the simultaneous knockdown of *and1* and *and2* was sufficient to considerably affect actinotrichia formation in zebrafish embryos; this result suggests that the short *and* gene versions may not play an important role in initiating the formation of actinotrichia fibres in zebrafish embryos. Furthermore, *and1* is consistently expressed in both the epithelial and mesenchymal actinotrichia-forming cell population of the fin throughout both development and regeneration unlike some of its other family members (Zhang et al, 2010). Along with the synteny and sequencing analyses that suggest *and1* is the most

representative ancestral long version of the *and* genes, these are the various reasons why studies mainly focus on the *and1* gene compared to the other paralogs.

The *and1* gene is a gene that contains 5 exons, of which the first exon is untranslated and 4 introns (Fig 1.7a). Our lab have characterized the presence of tissue-specific *cis*-acting regulatory elements in the 2 kilobase pair (kbp) genomic region located upstream of the first untranslated exon (E) of *and1* (position 1941-1 base pairs (bp)), including a promoter (P) 200 bp upstream of E. This 2 kbp genomic region was subsequently termed 2P. The 2P region drives gene expression in both the ectodermal and mesenchymal cells of the embryonic fins in zebrafish (Fig 1.7e-g). Interestingly, when used in transgenic mice, the 2P region is able to drive *lacZ* reporter gene expression in the distal ectoderm, excluding the AER cells, of mice limbs during their formation. This clearly suggests that upstream ectodermal activators are present in tetrapods and are able to recognize the zebrafish enhancer element even though *and* genes are absent in tetrapods. This also suggests that the upstream mesenchymal activators needed for *and1* expression have either been completely lost in tetrapods, are not present in the limb bud or are no longer able to recognize the zebrafish regulatory sequence (Lalonde et al, 2016).

The presence of tissue-specific *cis*-acting regulatory elements within 2P was found by performing analyses of the expression of a fluorescent reporter (EGFP) placed under the control of fragments of various lengths originating from the 2P genomic fragment in zebrafish transgenic lines. The Epi fragment, which consists of a 150 bp fragment situated at the 1117-975 bp position, was found to contain an ectodermal enhancer driving reporter expression solely in the ectodermal cells of the fin fold, with the exception of cleft cells, when paired with a human β-globin minimal promoter (βG)

(Fig 1.7k-m). Furthermore, mesenchymal specific expression occurred when removing the Epi region from the 2P fragment (Fig 1.7h-j). Further reporter gene expression analyses showed that individually, the 967-1P and 1941-1117 regions, which flank the Epi region on either side, showed either no or very minimal mesenchymal transgene expression. This suggests that there are mesenchymal enhancers present in the fragments flanking the Epi fragment, that are needed simultaneously to efficiently drive *and1* expression in mesenchymal cells of the fin folds. Together, the 967-1P and 1941-1117 fragments, which contain mesenchymal-specific enhancers, were termed the 2PΔepi region. Both tissue-specific reporter lines were found to recapitulate endogenous *and1* expression in their respective tissues in the pectoral and median fin fold of zebrafish embryos (Lalonde et al, 2016). For a complete list and detailed schematic of the many reporter constructs mentioned in this thesis, see appendix A.

Transcription factor candidates have been identified within the ectodermal and mesenchymal-specific fragments using a transcription factor identification software (TRANSFAC) and by performing site-directed mutagenesis on the identified clusters of putative sites. Transgenic zebrafish were then generated from the mutated plasmid constructs to look for a decrease in tissue-specific transgene expression. From these experiments, the removal of a 23 bp (1023-1002P position) region located within the Epi fragment was observed to considerably reduce ectodermal-specific transgene expression. This 23 bp region contains a cluster of putative binding sites for transcription factors such as members from the TCF family and MTF-1. These transcription factors are potentially responsible for the activation of ectodermal *and1* expression. In contrast, the TRANSFAC software allowed the identification of multiple Hoxa13 and Hoxa11

putative binding sites within the mesenchymal-specific 2PΔepi region (Lalonde et al, 2016). According to previously reported expression analyses, the *hoxa13a*, *hoxa13b* and *hoxa11b* genes are expressed in the mesenchyme of the embryonic fins (Ahn & Ho, 2008). All in all, the Hoxa13 and Hoxa11 transcription factors are good candidates for the mesenchymal activation of *and1* expression (Lalonde et al, 2016).

It is worth noting that in the past, the 2P+I region, which includes the 2P region with the addition of the first untranslated exon (E) and the first intron (I) of *and1* (Fig 1.7b-d), as well as the 2P+E, which includes the 2P and E regions (data not shown), drove an identical reporter gene expression profile to the *Tg(2P:EGFP)* lines during embryogenesis and early larval development. All in all, transgene expression has been well studied in the pectoral and median fin folds of the tissue-specific *and1* reporter lines during the embryonic stage of zebrafish. However, transgene expression and *cis*-acting regulation of the *and1* gene was never characterized in later stages of fin development where important fin morphogenesis events occur.

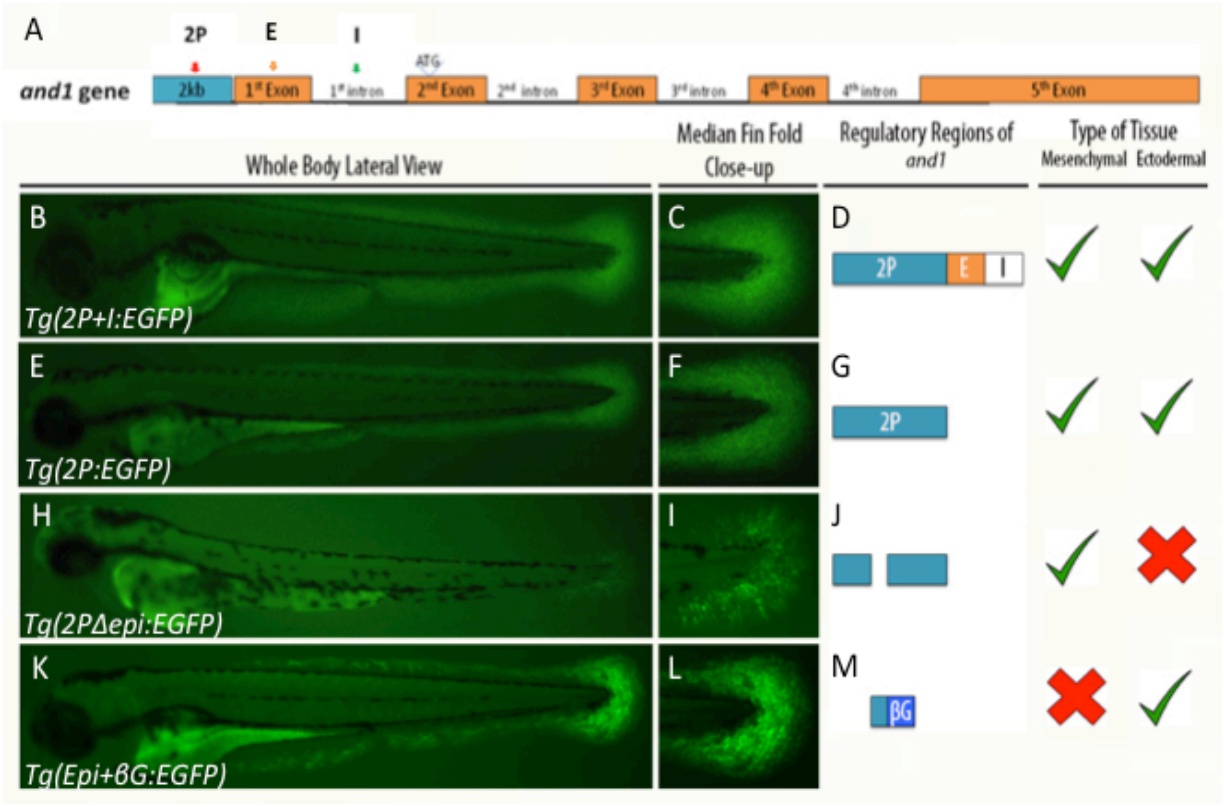


Figure 1.7. The *and1* gene and its tissue-specific *cis*-acting regulatory elements. a. The *and1* gene is a gene that contains 5 exons, of which the first exon is untranslated and 4 introns. *Cis*-acting regulatory elements have been found in the 2kbp region (2P) located upstream of the first exon, the first exon (E) and the first intron (I). **b-d**. The 2P+I region drives transgene expression in both the ectoderm and mesenchyme of the pectoral and median fin folds of zebrafish embryos. **e-g.** The 2P region produces the same expression pattern as 2P+I. **k-m.** The Epi fragment was found to contain an ectodermal enhancer driving reporter expression solely in the ectodermal cells of the fin fold, with the exception of cleft cells, when paired with a human β-globin (βG) minimal promoter. **h-j.** When the Epi fragment was removed from the 2P region, the remaining regions termed 2PΔepi were found to drive transgene expression solely in the mesenchymal cells of the fin fold. Pictures are adapted from Lalonde et al 2016 and Lalonde, unpublished.

1.7. Summary of background and objectives

Actinotrichia are small tapered fusiform fibres found in the distal margin of the adult fins and fin regenerate in zebrafish. They are also the only skeletal component of the pectoral and median fin folds during embryonic and early larval development (Becerra et al, 1983; Kemp & Park, 1970; Wood, 1982). They are composed of elastoidin, which is a protein complex composed of collagens type I and II, and tyrosine-rich Actinodin proteins that were characterized by our lab to be coded by the *actinodin* gene family (Durán et al, 2011; Zhang et al, 2010). Ands were found to play an essential role in the formation and structure of the actinotrichia fibres and consequently, was also found to play a role in the proper growth of the fin during development. Since the *and* gene family is present in the genome of fish species and completely absent in genome of tetrapods, it is hypothesized that the loss of the *and* gene family may have contributed in the fin-to-limb transition during evolution (Zhang et al, 2010). Recently, the tissue-specific *cis*-acting regulatory elements of *and1* were characterized through the creation of transgenic reporter lines; tissue-specific reporter gene expression was thoroughly characterized in the pectoral and median fin folds of zebrafish embryos (Lalonde et al, 2016). However, *and1* reporter expression was never characterized during important fin morphogenesis events that occur throughout fin development. One such event includes initial lepidotrichia formation, which involves the formation of mature fin rays within the larval fin folds and the gradual distal restriction of actinotrichia in the fold.

Another important fin morphogenesis event includes the resorption of the median fin fold, where there is the gradual regression of the median fin fold tissues located between the developing adult unpaired fins (Parichy et al, 2009). This process has been

morphologically described in zebrafish in great details, however no cellular mechanisms have been found to be responsible for shaping the larval median fin fold into the adult unpaired fins. Interestingly, the cellular mechanisms responsible for the loss of larval-specific features have been greatly studied in the amphibian models and involve cell death, macrophage digestion and a cell death/proliferation balance in larval and adult tissues respectively (Kerr, Harmon & Searle, 1974; Kinoshita, Sasaki & Watanabe, 1985; Ishizuya-Oka, 2011). Drawing inspiration from the processes present in the tadpole during metamorphosis, we believe that the median fin fold resorption process may involve one or a number of these processes during zebrafish fin development. Furthermore, since actinotrichia have an important role in the support of the embryonic and larval fin fold, we believe that there is a gradual loss of actinotrichia in the median fin fold that may account for its regression.

Moreover, the reporter gene expression patterns of these various *and1* reporter lines were not characterized throughout fin regeneration as well. All in all, the characterization of tissue-specific *and1* reporter expression throughout fin development and regeneration using these zebrafish transgenic lines may provide us with a better insight on the distribution and behaviour of *and1*-expressing cells throughout these processes. In addition, it would allow us to examine whether the *cis*-acting regulation of *and1*, as characterized during the embryonic stage, remains identical during zebrafish adulthood.

The objectives of this study are to:

1) **Characterize *and1* reporter expression throughout fin development.**

   a. Characterize *and1* reporter expression during initial lepidotrichia formation in the pectoral and median fin fold of zebrafish larvae.

   b. Characterize *and1* reporter expression in the resorbing median fin fold throughout its resorption.

   c. Investigate whether the various zebrafish *and1* reporter lines are representative of endogenous *and1* expression and actinotrichia distribution during both initial lepidotrichia formation and the resorption of the median fin fold.

   d. Determine whether the *cis*-acting regulation of *and1* differs at the adult stage.

2) **Characterize *and1* reporter expression throughout fin regeneration.**

   a. Generate an active epidermal-specific *and1* reporter line during the adult stage.

   b. Characterize *and1* reporter expression throughout the fin regeneration process.

   c. Investigate whether the various zebrafish *and1* reporter lines are representative of endogenous *and1* expression and actinotrichia distribution during the regeneration of the fin.

3) **Uncover the cellular mechanisms responsible for shaping the larval median fin fold into the three adult unpaired fins.**

   a. Examine whether the median fin fold resorption process is due to cell death.

   b. Determine whether macrophages play a role in the resorption of the median fin fold.

c. Investigate whether there is a differential cell proliferation profile along the median fin fold causing its resorption.

The *and1* gene is an important marker for labeling actinotrichia-forming cells in the developing and regenerating fins of zebrafish. Characterizing *and1* reporter expression in the various zebrafish transgenic lines may further our understanding on the distribution of actinotrichia-forming cells throughout these processes and may shed light on the *cis*-acting regulation of *and1* during the adult stage. Furthermore, these transgenic zebrafish lines may be useful tools for future mis-expression studies during specific fin development and regeneration processes.

Chapter 2 – Material & Methods

2.1. Zebrafish Care and Husbandry

All fish were bred and raised in the D' Iorio, University of Ottawa, zebrafish facility. Zebrafish (Tuebingen strain) were kept in a 28.5°C room under a photoperiod cycle of 14 hours of light proceeded by 10 hours of darkness in standard conditions as reported by Westerfield (2000). Animal care and experiments were performed according to the Canadian Council on Animal Care (CCAC) guidelines.

For microinjection purposes, breeding tanks were set up the day before embryos were to be collected, each containing a pair of females and a male. Each breeding tank comprised two stackable plastic containers where the top container was perforated at the bottom, which allowed embryos to fall through the perforations into the bottom container and enabled the eggs to be easily collected after they were laid. A plastic divider separated the male from the females. The next morning, the divider was removed within the first two hours of the light cycle to allow the fish to breed for 15 minutes. Embryos were then collected from the bottom tank by filtering the water through a fine-mesh net and then placed in a petri dish containing E3 embryo medium (5mM NaCl, 0.17mM KCl, 0.33mM $CaCl_2$, 0.33mM $MgSO_4$, 6-12 μM methylene blue), microinjected and kept in a 28.5°C incubator. For screening purposes, only one female and one male were placed per tank; this was done to correctly identify the founder fish. Furthermore, dividers were not used to control the breeding time, however, the remainder of the protocol was applied in the same fashion.

The day after zebrafish embryos were collected and/or microinjected, embryos were placed in a fine-mesh net and bleached for 10 minutes in a 0.003% NaOCl solution,

then neutralized in a 0.05% $Na_2S_2O_3$ solution (Sigma-Aldrich) for a minute and rinsed twice for 5 minutes in E3 embryo medium. The embryos were then placed in a petri dish containing E3 embryo medium and kept in a 28.5°C incubator for further uses.

2.2. Fin amputation

Adult fish were anesthetised by immersion in system water containing 0.24 mg/ml tricaine (Westerfield, 2000). Next, the fish were placed in a dry petri dish, their caudal fins were fanned out onto the plate and a standard cut was performed under a dissection microscope using a scalpel blade. A standard cut involves cutting 2 segments proximal from the first branching point of lepidotrichia. Fish were then re-animated in system water.

2.3. Whole-mount time courses analyses

Zebrafish embryos, larvae and juveniles were anesthetised in E3 embryo medium containing 0.1 mg/ml tricaine. As for adult zebrafish, they were anesthetised in system water containing 0.24 mg/ml tricaine (Westerfield, 2000). Anesthetised zebrafish were then placed on a plate filled with clear agar, their standard lengths were taken using a graduated ruler and pictures were taken using an AxioCam HRm Zeiss camera along with the complementary AxioVision 4.8.2 software under a fluorescence Leica MZ FLIII microscope. The standard length is the distance measured from snout to the posterior end of the notochord or of the caudal peduncle according to the developmental stage of the zebrafish (Parichy et al, 2009).

2.4. Cryo-sectioning

First, samples were fixed accordingly in either a 4% (w/v) paraformaldehyde (PFA) solution for 2 hours at room temperature or in a 9:1 Formaldehyde (37%): Ethanol (95%) solution overnight at 4°C. Different fixation methods are used to maintain the integrity of the targeted antigen and to conserve the cellular integrity of the tissue to maximize antibody penetration. 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_2HPO_4, 1.47 mM KH_2PO_4, adjusted to a final pH of 7.4) and samples were either directly processed for sectioning or processed later by dehydrating the samples in 100% methanol and stored in fresh methanol at -20°C. Frozen samples were then sequentially rehydrated incrementally in PBS solutions and washed thrice for 5 minutes in 1X PBST solutions (1X PBS, with 0.1% tween-20-from 20% stock). Samples were then each placed in a well from a 24-well plate and embedded by pouring a 1.5% agar and 5% sucrose in 1X PBS melted solution over the tissue. The samples were then oriented in the melted solution. Once the agar was set, the embedded samples were trimmed into blocks and put in a 30% sucrose in 1X PBS solution at 4°C overnight. The blocks were then embedded in OCT embedding matrix using a standard freezing protocol. Sections cut 20µm thick were transferred on Superfrost/Plus slides and allowed to dry for 30 minutes before slides were transferred to a box and stored at -20°C until they were required (protocol modified by Zhang, J. from Strähle et al, 1994).

2.5. Immunostaining on cryo-sections

After samples were fixed, embedded and processed for cryostat sectioning, the slides were thawed for 2 hours at room temperature and rehydrated in 1X PBS for 30

minutes. The slides were then blocked in a 0.2% Triton X-100 and 2% heat-inactivated calf serum solution in 1X PBS. A 1:200 dilution of the primary antibodies in blocking solution was applied to the slides and incubated overnight at 4°C, see table 1 for the various antibodies used for immunostaining. Afterwards, slides were washed thoroughly in 1X PBST. Next, a 1:500 dilution of the secondary antibody in 1X PBST solution was applied to the slides and incubated at room temperature for 3 hours. The slides were then thoroughly washed in 1X PBST. Slides were then counterstained with a 1:10000 dilution of DAPI and quickly rinsed in distilled water. The slides were subsequently left to dry and were mounted in aqua-poly/mount medium (Polysciences). Pictures were taken using a Zeiss LSM 510/AxioVert 200 confocal and zen2009 software. Images were then processed using ImageJ and Adobe Photoshop.

Table 1. Primary and secondary antibodies used in the various immunostaining experiments.

Primary antibodies	Secondary antibodies
Anti-And1/2 protein, rabbit*	Alexa Fluor® 488 goat, anti-rabbit IgG**
Anti-Zns-5, mouse	Alexa Fluor® 594 goat, anti-mouse IgG**
Anti-green fluorescent protein, rabbit**	Alexa Fluor® 488 goat, anti-rabbit IgG**

** custom order from Open BioSystems*
***from Life Technologies*

2.6. The making of anti-sense RNA probes

Digoxigenin-labelled (DIG) antisense RNA probes were made from the cDNA plasmid templates and RNA polymerases specified in table 2. 10µg 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 Illustra^{TM} purification kit. The transcription reaction was prepared as follow: 1µL of the linearized template DNA was mixed with 2 µL of NTP labeling mix (10mM ATP, CTP, GTP, 6.5mM UTP and 3.5mM 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 DEP-C 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 2. Antisense RNA probes employed for the various *in situ* hybridization experiments.

Probe name	Plasmid Vector	Restriction Enzyme	RNA Polymerase
and1	pBK-CMV	Sal*I*	T7
egfp	pDrive	Bam*HI*	Sp6

As for dinitrophenyl-labelled (DNP) antisense RNA probes, linearization and purification of the cDNA plasmids proceeded in the same fashion. Concerning the reaction mix, the same components were used except for the NTP labeling mix, which was replaced by 1 µL of 10mM DNP-11-UTP (PerkinElmer) and 1 µL of NTP (2mM ATP, CTP, GTP, UTP) (Roche). Furthermore, instead of using the SigmaSpin Post-Reaction Clean-up columns, the DNP-labelled antisense RNA probes were precipitated and purified using a standard LiCl RNA purification protocol.

2.7. In situ hybridization on cryo-sections

After samples were fixed, embedded and processed for cryostat sectioning, the slides destined for *in situ* hybridization experiments coupled with a proteinase K (PK) treatment were warmed for an hour at 60°C, then permeabilized for 15 minutes in a 0.3% Triton X-100/1X DEP-C PBS (pH 7.4) solution and washed twice for 5 minutes with 1X DEP-C PBS. The slides were then soaked in a 5µg/ml PK, 0.1M Tris HCl (pH 8.0) and 0.05M EDTA solution for 15 minutes for additional permeabilization and subsequently

washed with 1X DEP-C PBS. Afterwards, slides were fixed for 20 minutes in a 4% PFA solution and washed in 1X DEP-C PBS. Next, slides were incubated for 5 minutes in an acetylation mix (500µL Triethanolamine, 108µL Acetic anhydride in 40mL DEP-C water) to reduce non-specific probe binding. Slides were then washed in DEP-C water and left to dry. As for non-PK treated slides, they were left to defrost for an hour at room temperature and were directly processed for the hybridization step of the protocol.

Next, a DIG-labelled antisense RNA probe was diluted 1:200 in hybridization buffer (1X Salt, 50% deionised formamide, 10% dextran sulphate, 1mg/ml yeast tRNA, 1X Denhardt's solution and DEP-C water), denatured at 70°C, and applied to the slides. The slides were subsequently left overnight in a 70°C oven to hybridize. The next day, slides were washed twice for 30 minutes in Solution A (1X SSC, 50% Formamide, 0.1% Tween-20) at 70°C and twice for 30 minutes in a 1X TBST solution (0.14M NaCl, 2.7mM KCl, 0.025M Tris HCl, 0.1% Tween-20) at room temperature and left to block for an hour in a 10% heat-inactivated calf serum solution in 1X TBST. A 1:2000 dilution of anti-DIG AP Fab fragment antibody (Roche) was made using a 10% heat-inactivated calf serum in 1X PBS solution and was applied to the slides and incubated overnight at 4°C. The anti-DIG AP Fab fragment antibody is made from Fragment antigen-binding (Fab) fragments from polyclonal anti-DIG antibodies that are conjugated to an alkaline phosphatase (AP). The next day, the antibody solution was washed away with four 20-minute washes in 1X TBST and two 10-minute washed in NTMT (100mM NaCl, 100mM Tris HCl (pH 9.5), 50 mM $MgCl_2$, 0.1% Tween-20 in distilled water). Slides were then stained in the dark at room temperature with a staining solution (4.5µL Nitro Blue Tetrazolium (NBT) + 3.5µL 5-Bromo-4-Chloro-3-Indolyl Phosphate (BCIP)/ 1ml

NTMT) until desired contrast was achieved. The reaction is stopped with two washes in distilled water. The slides were left to dry and were mounted with aqua-poly/mount medium (Polysciences). Protocol modified by Zhang, J. from Strähle et al, (1994). Pictures were taken using the Lumen*era* Infinity 3-1 camera in combination with the Lumen*era* Infinity Capture v 6.5.4. software on a Zeiss Axioskop microscope.

2.8. Double fluorescence in situ hybridization on cryo-sections

After samples were fixed, embedded and processed for cryostat sectioning, they underwent the same hybridization step and post-hybridization washes as the previously described *in situ* hybridization on cryo-sections protocol. For double *in situ* hybridization experiments, two antisense RNA probes were used instead of one: a DIG-labelled and a DNP-labelled RNA probe. After post-hybridization washes, endogenous horseradish peroxidase was inhibited by washing the slides for 10 minutes in 2% H_2O_2 solution in 1X TBST. Slides were subsequently washed with TNT (0.1M Tris HCl pH7.5, 0.15M NaCl and 0.5% Tween-20) and blocked for 2 hours using TBSTB (TNT and 0.5% PerkinElmer blocking powder). After blocking, slides were incubated in a 1:100 polymerized horseradish peroxidase conjugated anti-DIG antibody (Roche) in TBSTB solution overnight at 4°C. Afterwards the slides were thoroughly washed in TNT and stained using a 1:100 Tyr-Cy3 solution (PerkinElmer). After washing the stain, horseradish peroxidase was inhibited and the slides were blocked again as previously described. Slides were then incubated in a 1:100 polymerized horseradish peroxidase conjugated anti-DNP antibody (PerkinElmer) in TBSTB solution overnight at 4°C. Afterwards the slides were thoroughly washed in TNT and stained using a 1:100 Tyr-fluorescein solution (PerkinElmer). Slides were then counterstained with a 1:10000 DAPI stain, left to dry

and mounted using aqua-poly/mount medium (Polysciences). Pictures were taken using a Zeiss LSM 510/AxioVert 200 confocal and zen2009 software. Images were then processed using ImageJ and Adobe Photoshop.

2.9. Reverse transcriptase polymerase chain reaction

50-100mg of zebrafish fins were collected and homogenized in TRIzol® reagent (Thermo Fisher) and total RNA isolation was performed using a standard phenol-chloroform protocol. 1000ng of the total RNA isolation was then used to produce cDNA using the Quantitect® Reverse transcription Kit (Qiagen). Polymerase chain reactions (PCR) were performed using 1/10 dilutions of the cDNA reaction mix as DNA template. 25µL volume reactions were prepared according to the GoTaq® Green Master Mix reaction (Promega) protocol using custom primers designed to target a ~200 base pair region of *and1* and a ~150 base pair region for the human *β-globin* control as specified in table 3.

Table 3. Custom primers for the RT-PCR experiments.

Primer name	Primer sequence (5' to 3')
β-globin FW	ATGGATGAGGAAATCGCTGCCCTGGTC
β-globin RV	CTCCCTGATGTCTGGGTCGTCCAAC
and1 FW	AGGTTCACACCACCACATCC
and1 RV	TGTCATCACTTCCATCGTCTCC

The amplification program started with an initial denaturation step of 2 minutes at 94°C. Next, 30 cycles of a 30-second denaturation step at 94°C, 30-second annealing step at 55°C and 30-second elongation step at 72°C were performed. A final 10-minute elongation step at 72°C finished the amplification program and PCR reaction were subsequently kept at 4°C. PCR products were then loaded on a 0.8% agarose, 0.5X TBE and 0.005% RedSafe™ (iNtRON Biotechnology, inc.) gel and ran for 30 minutes at

130V. Gel pictures were taken using the Alpha Innotech FluorChem® Q UV gel imager in combination with the AlphaView™ Q software.

2.10. Cloning of the Epi-EI:EGFP construct

The cloning and subcloning of the Epi-EI region were performed following the standard cloning procedures of Sambrook & Russell (2001). The genomic sequence was amplified using the Epi forward primer (5'-GCTAGCTTTCGGAAACCCCAGAC-3') and the intron reverse primer (5'-GGCGGATCCCTTGGATGAAATTAA-3'), and cloned in a *pDrive* cloning vector (Qiagen). The Epi-EI region, consisting of 4992 bp in total, was then subsequently subcloned into a modified *pEGFP-N1* cloning vector using the *NheI* 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 *NheI* restriction sites and another Tol2 (right) arm was inserted at the *AflII* restriction site.

2.11. Microinjection of zebrafish embryos

Collected embryos were placed in a petri dish that contained solidified 1% agarose and indented rows to prevent the embryos from moving during injection. Microinjection needles were made using borosilicate glass tubes that had an inner diameter of 0.5mm, an outer diameter of 1.0mm and 10cm long (Sutter Instruments). The needles were pulled apart using the Sutter instruments Flaming/Brown P-87 model micropipette puller. The tip of the needle was cut using a scalpel and filled with 1µl of injection solution, which contained the reporter Epi-EI:*EGFP* construct (final concentration of 100 ng/µL) and transposase RNA (final concentration of 50 ng/µL), which are needed for Tol2 gene editing, plus RNAse-free water and 0.5% phenol red.

The injection solution was injected into one-cell stage embryos using a Narishige IM300 microinjector and a Leica M27.5 microscope.

2.12. Screening transgenic zebrafish via fluorescence microscopy

First, the chorion of 2 dpf primary injected embryos was manually removed using forceps. The embryos were then anesthetised with E3 embryo medium containing 0.1mg/ml tricaine and screened for the presence of enhanced green fluorescent protein (EGFP) in the paired and unpaired fins of zebrafish embryos using a fluorescence Leica MZ FLIII microscope. Positive primary injected embryos (n=~50) were raised until sexual maturity was attained and then individually bred with wild type fish. The offspring of each primary injected fish were again screened in the same fashion to ultimately identify and raise *Tg(Epi-EI:EGFP)* lines (n=2).

2.13. Propidium Iodide Staining

Zebrafish (n=5) with a standard length spanning from 6.5 to 8.5 mm were added to an E3 embryo medium solution containing 0.02 mg/ml Propidium iodide solution (from a 1mg/ml Propidium iodide, 1mg/ml NaN_3 stock solution) (Thermo Fisher) for 5 minutes at room temperature. Larvae were then washed twice for 5 minutes in fresh E3 embryo medium, anesthetised in E3 embryo medium containing 0.1 mg/ml tricaine and pictures were taken using an AxioCam HRm Zeiss camera along with the complementary AxioVision 4.8.2 software under a fluorescence Leica MZ FLIII microscope.

2.14. Whole-mount TUNEL assays on zebrafish larvae

Zebrafish larvae (n=15) with a standard length spanning from 6.5 to 8.5 mm were euthanized via tricaine overdose and fixed for two hours at room temperature in a 4%

PFA solution. Once fixed, the larvae were washed twice for 5 minutes in 1X PBS solution and stored in 100% methanol at -20°C. After larvae were rehydrated in 1X PBST solutions, larvae samples underwent tissue permeabilization by digestion using a 10 mg/ml PK solution (Sigma-Aldrich) for 15 minutes at room temperature and washed twice for 5 minutes in 1X PBST. The larvae were then post-fixed for 20 minutes in 4% PFA and washed thoroughly in 1X PBST. Larvae samples that were either previously PK-treated or not PK-treated, were then permeabilized in a 0.1% sodium citrate in 1X PBST solution for 15 minutes at room temperature and thoroughly washed in 1X PBST. The TUNEL reaction mix from the In Situ Cell Death Kit, TMR red (Roche) was applied to the larvae and left to incubate for an hour at 37°C in darkness. Larvae were subsequently washed in 1X PBS and pictures were taken using an AxioCam HRm Zeiss camera along with the complementary AxioVision 4.8.2 software under a fluorescence Leica MZ FLIII microscope.

2.15. Whole-mount EdU assays on zebrafish larvae

Zebrafish larvae (n=29) with a standard length spanning from 6.5 to 9.0 mm were anesthetised in E3 embryo medium containing 0.1 mg/ml tricaine and placed in a foam slit belly side up in a petri dish. Larvae were then injected in the belly cavity with a 0.25 mg/g dose of EdU in Hank's solution (Life Technologies), as specified in Poleo et al (2001), using a borosilicate microinjection needle, a Narishige IM300 microinjector and a Leica M27.5 microscope. After injection, larvae were then left to swim for 18 hours after which, they were collected, euthanized via tricaine overdose and fixed in 4% PFA for two hours at room temperature. Once fixed, larvae were then washed twice in 1X PBS for 5 minutes and permeabilized in 0.5% Triton X-100 in 1X PBS three times for 20 minutes.

Larvae were then placed and incubated in a EdU detection reaction cocktail supplied with the Click-iT® EdU Alexa Fluor 488 Imaging Kit (Life Technologies) for 30 minutes at room temperature in darkness. After three 5-minute 1X PBS washes, pictures of the larvae were then taken using an AxioCam HRm Zeiss camera along with the complementary AxioVision 4.8.2 software under a fluorescence Leica MZ FLIII microscope.

Chapter 3 – Results

Objective 1: Characterization of *egfp* expression in the various transgenic *and1* reporter lines throughout fin development

3.1.1. Characterization of tissue-specific and1 reporter expression during initial lepidotrichia formation in paired and unpaired fins

A time course analysis starting at 3 dpf and ending at 90 dpf was performed on the *Tg(2P+I:EGFP)*, *Tg(2P:EGFP)*, *Tg(2PΔepi:EGFP)* and *Tg(Epi+βG:EGFP)* transgenic lines (see table 4 for the number of lines for each reporter construct) (n=~25 zebrafish/line) to characterize each of their distinctive *and1* reporter expression throughout fin development. The specific starting and ending time points of the time course were chosen according to the stages at which zebrafish begin to be considered larvae and sexually mature adults respectively as reported by the Zebrafish Information Network, ZFIN (online resource for the scientific community using the zebrafish as a model organism (https://zfin.org)). Fish from the transgenic lines were regularly anaesthetized throughout the time course, measured for their standard length (SL), which allows the evaluation of the developmental progression and their pictures were taken under a fluorescence microscope. The SL measuring method entails measuring the distance from the snout to the caudal peduncle or, since zebrafish at the larval stage do not have a caudal peduncle, to the posterior tip of the notochord (Parichy et al, 2009).

We first paid special attention to *and1* reporter expression during initial lepidotrichia formation, which is an important fin morphogenesis event that occurs during the larval stage of zebrafish development. Initial lepidotrichia formation starts at a SL of ~4.9 mm and occurs first in the developing caudal fin (Parichy et al, 2009). When larvae

reached a standard length of 5.0 mm, we observed in the *Tg(2P+I:EGFP)* lines, which drive expression in both mesenchymal and epithelial cells of the fin fold, that transgene expression was maintained throughout the median fin fold like previously observed at the embryonic stage. However, transgene expression was additionally discerned visually in the appearing lepidotrichia occurring at the proximal ventral side of the posterior-most end of the notochord in the developing caudal fin (Fig 3.1.1a-b). Concurrently, the mesenchymal-specific *Tg(2PΔepi:EGFP)* lines showed identical transgene expression in the lepidotrichia arising in the developing caudal fin (Fig 3.1.1c-d). In contrast, the epithelial-specific *Tg(Epi+βG:EGFP)* lines showed no expression in the developing lepidotrichia, but epithelial transgene expression rather became distal in the fin fold (data not shown). Thus, this suggests that mesenchymal *and1*-expressing cells correlate with the position of the initiation of lepidotrichia formation in the developing fins.

Table 4. Number of zebrafish reporter lines analyzed for each *and1* reporter construct.

Name of transgenic line	Number of lines
Tg(2P+I:EGFP)	2
Tg(2P:EGFP)	5
Tg(2PΔepi:EGFP)	2
Tg(Epi+βG:EGFP)	2

As initial lepidotrichia formation progressed, fin ray segments started to appear within the fin fold and began to elongate. Transgene expression was observed all along the proximodistal length of the developing fin rays during this process in the *Tg(2P+I:EGFP)* and *Tg(2PΔepi:EGFP)* lines (Fig 3.1.2a,b,d). In comparison, the *Tg(Epi+βG:EGFP)* lines showed transgene expression in the distal part of the developing caudal fin (Fig 3.1.2c) and as development progressed, transgene expression was additionally observed on the dorsal and ventral edges of the developing caudal fin (Fig

3.1.4g-h). To better situate the location of EGFP persisting all along the proximodistal length of developing fin rays in the *Tg(2P+I:EGFP)* and *Tg(2PΔepi:EGFP)* lines (Fig 3.1.3a-b), transverse cryo-sections of the developing caudal fin rays (n=~96) of the mesenchymal-specific lines were produced. This allowed us to observe that EGFP was located on the inner side of lepidotrichia hemi-rays, close to the location where osteoblasts are situated (Fig 3.1.3c). To better visualize the location of EGFP in relation to osteoblasts, an immunostaining for both EGFP and Zns-5, the latter being a pan-osteoblast marker, was performed on transverse cryo-sections of the developing caudal fin rays (n=~96) of the mesenchymal-specific line. This experiment revealed that EGFP did not co-localize with osteoblasts. However, EGFP was located alongside osteoblasts (Fig 3.1.3d). In other words, mesenchymal *and1*-expressing cells are located all along the proximodistal length of the developing fin rays, alongside osteoblasts, during initial lepidotrichia formation.

The presence of EGFP along the proximodistal length of developing fin rays in the *Tg(2P+I:EGFP)* and *Tg(2PΔepi:EGFP)* lines was not only observed in the developing caudal fin. In fact, EGFP was located in all of the zebrafish fins throughout development, which comprises the unpaired and paired fins (Fig 3.1.4 and 3.1.5 respectively). As a matter of fact, the expression patterns were identical in paired and unpaired fins for each transgenic reporter lines. Concerning the epithelial-specific *Tg(Epi+βG:EGFP)* lines, transgene expression remained at the distal end of the developing fins. Epithelial transgene expression was also found on the dorsal and ventral edges of the developing caudal fin, and on the anterior and posterior edges of the dorsal, anal and paired fins (Fig 3.1.4.g-h, data not shown for paired fins). Interestingly,

epithelial-specific expression in the paired and unpaired fins of *Tg(Epi+βG:EGFP)* lines gradually decreased in intensity as development progressed, to a point where no more was observed at a SL of 8.5 mm. This suggests that there may be a successive and gradual change in the epithelial *cis*-acting regulation of *and1* during development.

As for the *Tg(2P:EGFP)* lines, they had a very similar expression pattern to the epithelial-specific transgenic lines and interestingly, lacked the mesenchymal expression shown all along the proximodistal length of lepidotrichia in the *Tg(2P+I:EGFP)* and *Tg(2PΔepi:EGFP)* lines at identical developmental stages (Fig 3.1.4a-f). Likewise, transgene expression also decreased in intensity in the *Tg(2P:EGFP)* lines as development progressed, to a point where no more was observed at a SL of 9.0 mm. The fact that the *Tg(2P:EGFP)* lines displayed no more transgene expression was unexpected since the 2P region contains both the Epi and the 2PΔepi fragments, and most importantly the *Tg(2PΔepi:EGFP)* lines were shown to sustain strong mesenchymal transgene expression throughout development. This observation suggests that the Epi region may contain a mesenchymal-specific repressor.

Interestingly, the *Tg(2P+I:EGFP)* lines maintained strong epithelial and mesenchymal transgene expression throughout development. Since the inclusion of the EI region is the only difference between the 2P+I and 2P regions, we suggest that the EI region may contain enhancers (and potentially an alternate promoter) required for the maintenance of epithelial-specific expression throughout development and may also be needed to reactivate mesenchymal-specific *and1* expression in the adult fins. These suggestions are explored later in the thesis. All in all, the various *and1* reporter lines

seem to hint that the epithelial *cis*-acting regulation of *and1* differs throughout development.

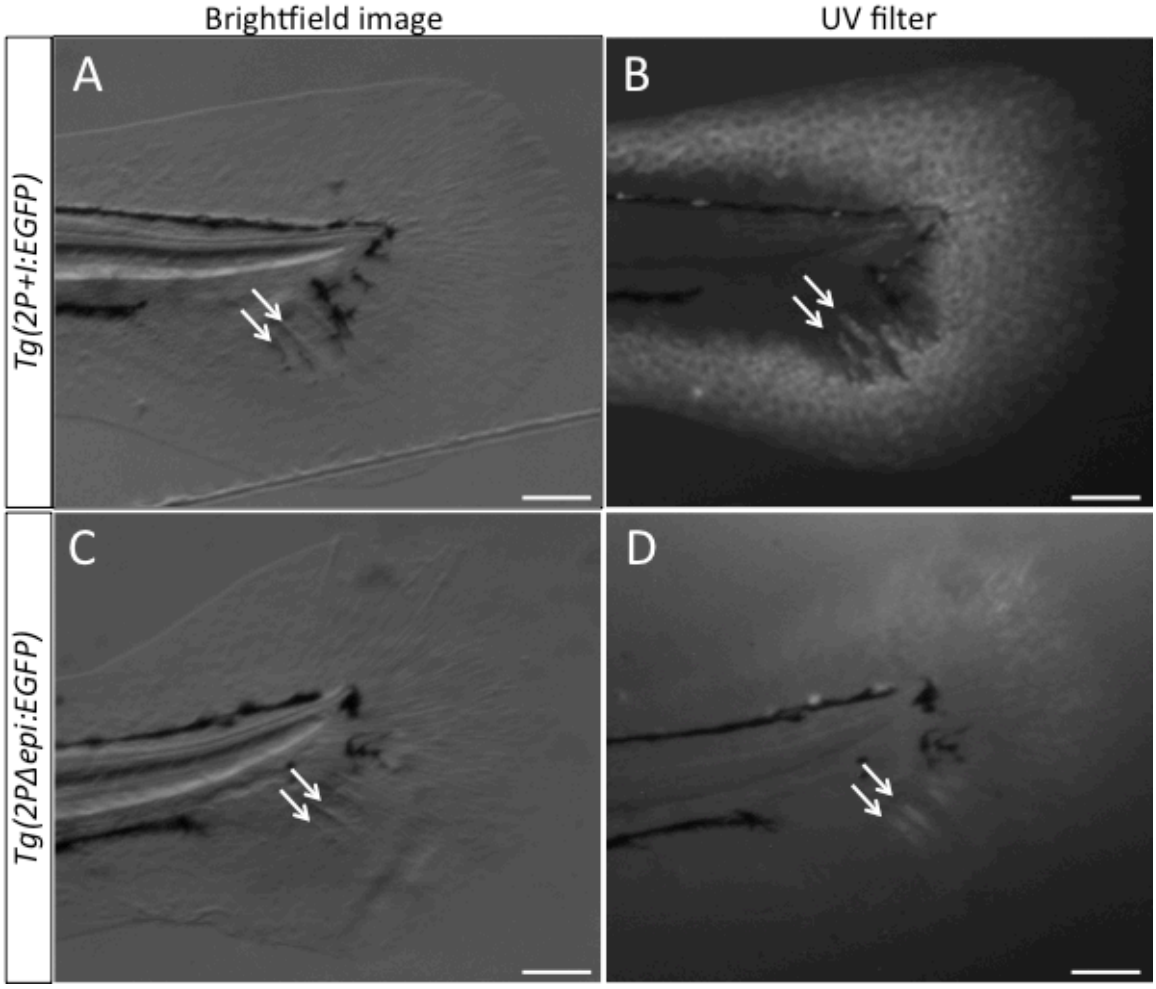


Figure 3.1.1. Reporter expressions in the appearing fin rays of the caudal fin. Developing fin rays, shown by the white arrows, appear at the ventral side of the posterior-most end of the notochord during initial lepidotrichia formation (Parichy et al, 2009). Panels **a** & **c** show the brightfield images and panels **b** & **d** show the UV filter images of the developing caudal fin of the *Tg(2P+I:EGFP)* (panels **a-b**) and *Tg(2PΔepi:EGFP)* (panels **c-d**) lines. Scale bars= 0.1 mm.

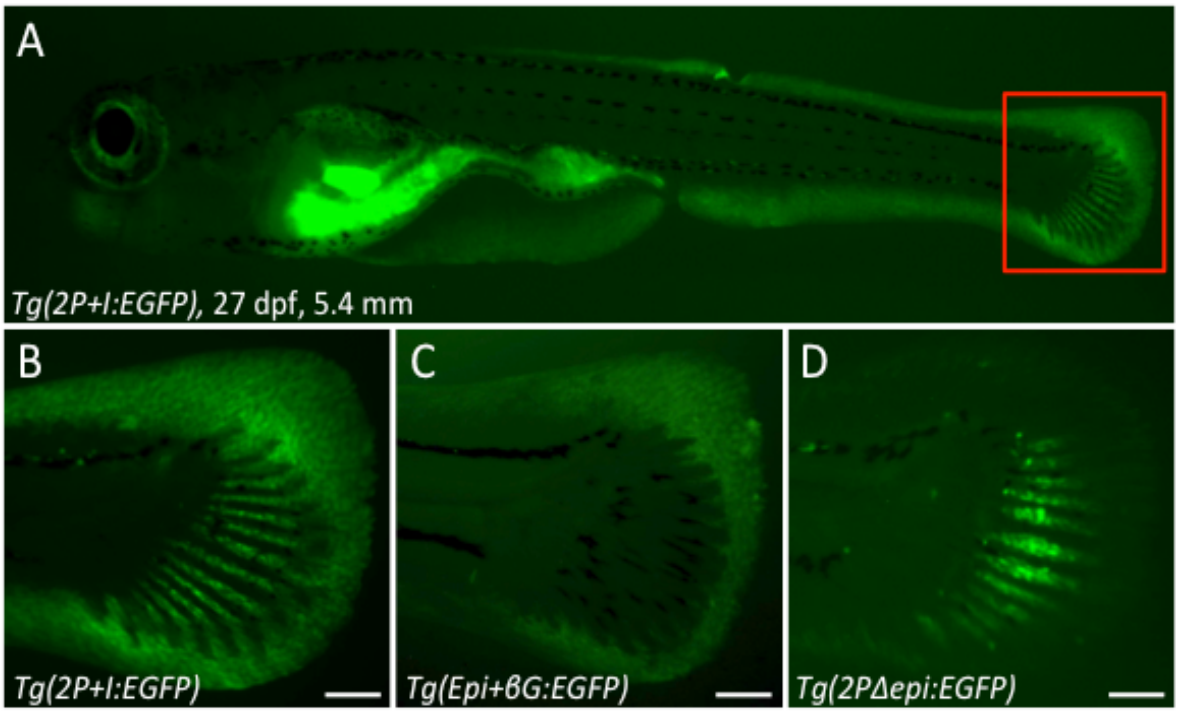


Figure 3.1.2. The *Tg(2P+I:EGFP)* lines recapitulate the reporter expression observed in the tissue-specific reporter lines during initial lepidotrichia formation. **a.** The transgene expression pattern of a 27 dpf, 5.4 mm SL *Tg(2P+I:EGFP)* larva during initial lepidotrichia formation. Autofluorescence is present in the eyes and yolk of the larva. **b.** High magnification of the developing caudal fin of the larva shown in panel a (red square). **c**. The epithelial reporter expression from the *Tg(Epi+βG:EGFP)* lines becomes distally restricted in the fin fold. **d.** The mesenchymal reporter expression from the *Tg(2PΔepi:EGFP)* lines is located all along the proximodistal length of developing fin rays. Scale bars= 0.1 mm.

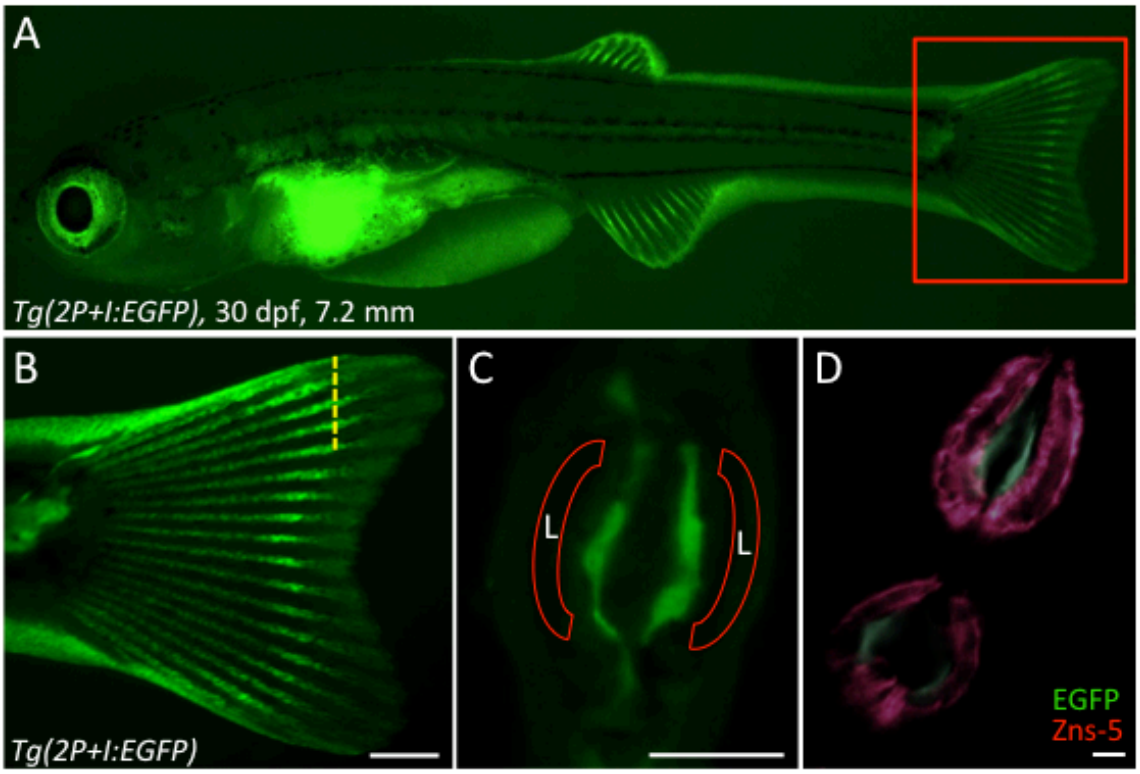


Figure 3.1.3. Mesenchymal reporter expression is specifically located on the inner side of the hemirays beside osteoblasts during lepidotrichia morphogenesis. a. Reporter gene expression pattern of a 30 dpf, 7.2 mm SL *Tg(2P+I:EGFP)* juvenile. Autofluorescence is present in the eyes and yolk area of the juvenile. **b.** High magnification of the developing caudal fin of the juvenile shown in panel a (red square). Transgene expression is located all along the proximodistal length of developing fin rays. **c.** Transverse sections of the developing caudal fin of 7.2 mm SL *Tg(2PΔepi:EGFP)* larvae (shown by yellow line in panel b) revealed that transgene expression is located on the inner side of the hemirays. **d.** Immunostaining on transverse sections of the developing caudal fin of 7.2 mm SL *Tg(2PΔepi:EGFP)* larvae show EGFP located alongside osteoblasts (Zns-5) during lepidotrichia morphogenesis. L=Lepidotrichia hemirays. Scale bars in panel b= 0.2 mm, panel c & d= 10 μm.

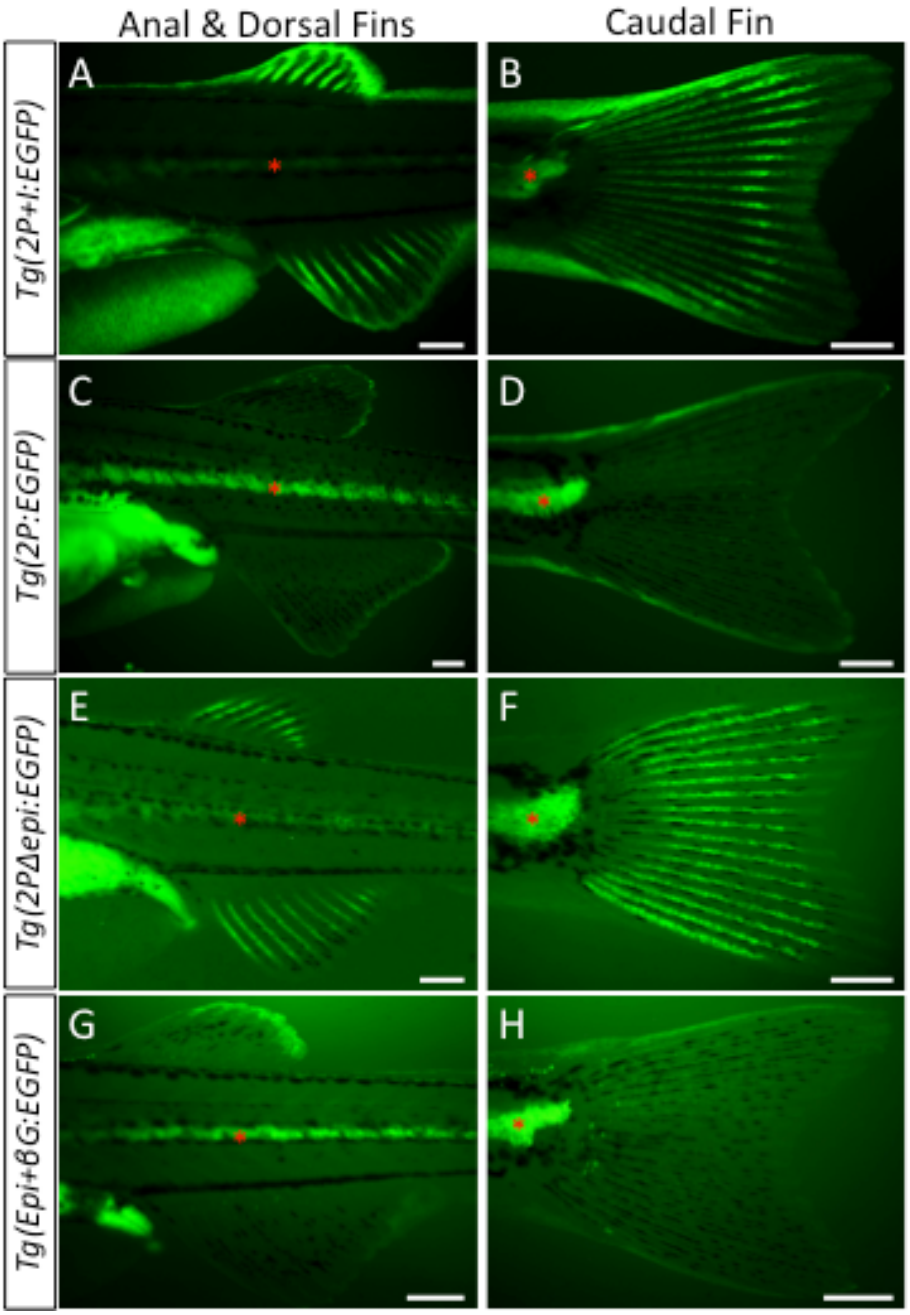


Figure 3.1.4. Reporter gene expression in the unpaired fins of the various *and1* reporter lines during the early juvenile stage. The unpaired fins consist of the anal, dorsal and caudal fins of the zebrafish. Transgene expression is observed in the dorsal and anal fins (panels **a, c, e, g**) and caudal fin (panels **b, d, f, h**) of 7.2 mm SL *Tg(2P+I:EGFP)* (panels **a-b**), *Tg(2P:EGFP)* (panels **c-d**), *Tg(2PΔepi:EGFP)* (panels **e-f**) and *Tg(Epi+βG:EGFP)* (panels **g-h**) juveniles during their formation. Mesenchymal reporter expression is present all along the proximodistal length of developing fin rays in the *Tg(2P+I:EGFP)* and *Tg(2PΔepi:EGFP)* lines. Epithelial reporter expression is found at the distal end and lateral edges of the fins. Autofluorescence is present in the lateral line. *= Lateral line. Scale bar=0.2 mm.

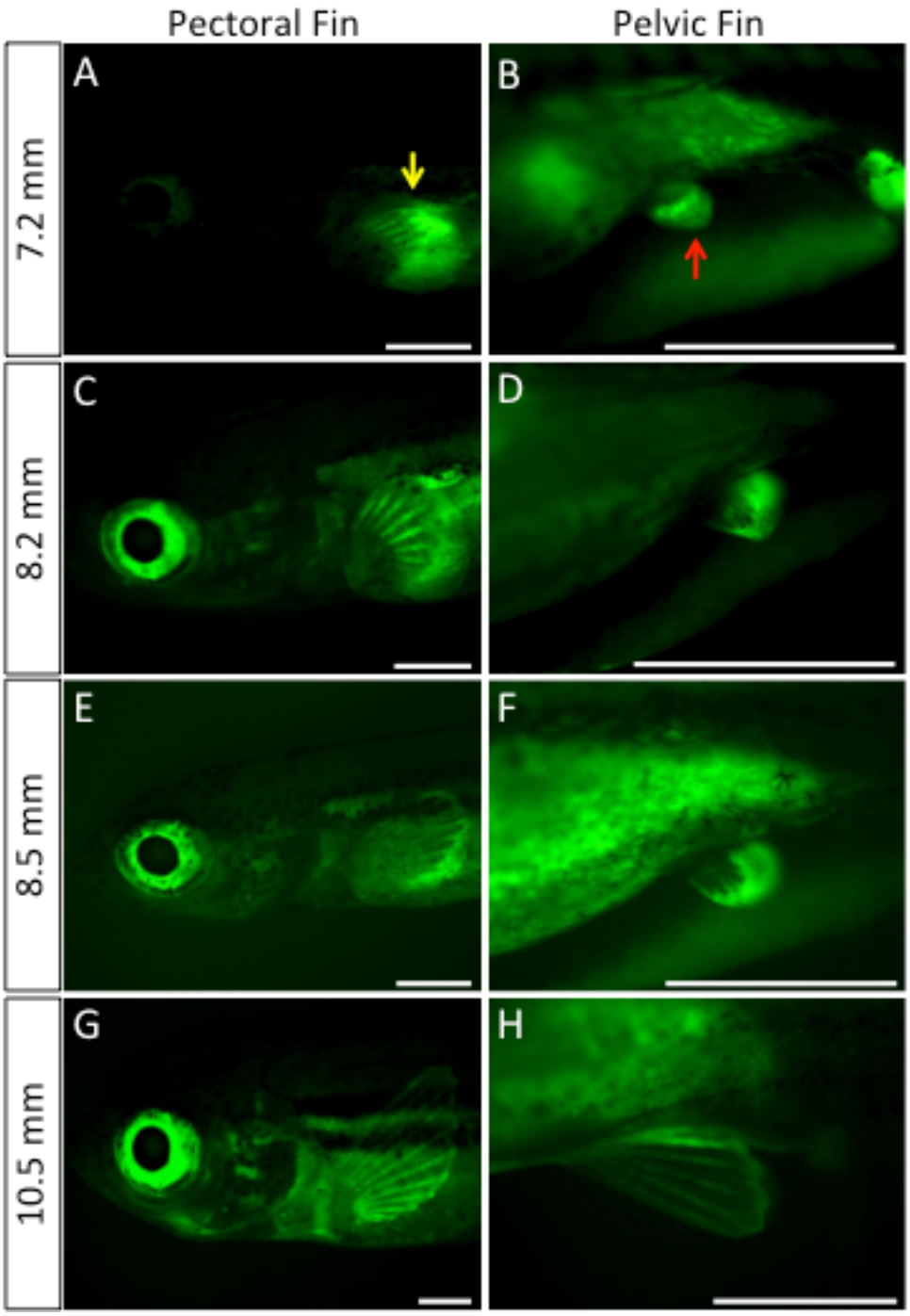


Figure 3.1.5. Reporter gene expression in the paired fins of *Tg(2P+I:EGFP)* zebrafish throughout development. The paired fins include the pectoral fins (panels **a, c, e, g**) (shown by yellow arrow) and the pelvic fins (panels **b, d, f, h**) (shown by the red arrow). The *Tg(2P+I:EGFP)* lines show transgene expression all along the proximodistal length of fin rays in the paired fins during their development and in the epithelial cells located at the distal end of the fins. Approximate SL of the zebrafish are mentioned on the left side of the pictures. Autofluorescence is present in eyes and ventral tissues. Scale bar=0.5 mm

3.1.2. Characterization of and1 reporter expression, endogenous and1 expression and actinotrichia distribution during the development of the caudal fin

Considering actinotrichia are found only at the distal tip of lepidotrichia in intact fins of adult zebrafish and presuming *and1* expression is distally restricted as well, we found it was essential to examine whether the mesenchymal reporter expression observed all along the proximodistal length of developing lepidotrichia in the *Tg(2P+I:EGFP)* and *Tg(2PΔepi:EGFP)* lines was representative of endogenous *and1* expression and actinotrichia distribution during initial lepidotrichia formation (Santamaría, Santos Ruiz & Becerra, 1996). To do so, *in situ* hybridization and immunostaining experiments were performed on transverse cryo-sections of the developing caudal fin of zebrafish larvae undergoing initial lepidotrichia formation.

Since EGFP is observed all along the proximodistal length of developing fin rays in the *Tg(2P+I:EGFP)* and *Tg(2PΔepi:EGFP)* lines, we first examined the endogenous expression pattern of *and1* in the caudal fin of zebrafish larvae. Whole-mount *in situ* hybridization experiments were performed on 40 dpf, 7.2 mm SL Wild type (WT) zebrafish juveniles (n=8) to have an overall view of endogenous *and1* expression in the developing fins. Unfortunately, this experiment was revealed to be unfeasible since the fins of the larvae were seemingly unable to withstand the proteinase K treatment, which is required for probe penetration in the tissues for this experiment. Ultimately, this experiment was unable to give us a broad view of endogenous *and1* expression in the developing fins of zebrafish (data not shown). To remedy the situation, transverse cryo-sections were generated spanning the entire proximodistal length of the caudal fin of 40 dpf, 7.2 mm WT zebrafish juveniles (n=~9) instead and *in situ* hybridization experiments

were performed on them using an antisense *and1* RNA probe. Endogenous *and1* expression was only detected in the most distal sections of the developing caudal fin (Fig 3.1.6a-b,d-e). Therefore, there is a discrepancy between the location of EGFP in the transgenic reporter lines and the expression domain of *and1* in the developing caudal fin.

Next, we investigated whether actinotrichia are similarly distributed all along the proximodistal length of developing fin rays like EGFP in the *Tg(2P+I:EGFP)* and *Tg(2PΔepi:EGFP)* lines. To do so, an immunostaining experiment using an antibody directed against a peptide sequence highly conserved between the And1 and And2 proteins (named And1/2) was performed on transverse sections spanning the developing caudal fin of 40 dpf, 7.2 mm SL WT zebrafish juveniles (n=~9) in order to visualize the location of actinotrichia. The experiment showed that actinotrichia are restricted at the distal end of the fin rays (and are approximately 480μm in length) during initial lepidotrichia formation (Fig 3.1.6c,f). This further confirms the observations of Grandel & Schulte-Merker, (1998). According to these results, there is an incongruity between the location of EGFP situated all along the proximodistal length of developing lepidotrichia in the *Tg(2P+I:EGFP)* and *Tg(2PΔepi:EGFP)* lines and the distribution of actinotrichia in the developing caudal fin.

This led us to evaluate whether the transgenic reporter lines were truly representative of endogenous *and1* expression. To do so, we analyzed the expression pattern of *egfp* from the *Tg(2P+I:EGFP)* lines to investigate whether the expression patterns of *egfp* and *and1* were similar in the developing caudal fin of zebrafish juveniles. Transverse cryo-sections were generated spanning the entire proximodistal length of the developing caudal fins of 40 dpf, 7.2 mm SL *Tg(2P+I:EGFP)* zebrafish juveniles (n=~9)

undergoing initial lepidotrichia formation. *In situ* hybridization experiments showed that *egfp* expression was only detected in the distalmost sections of the developing caudal fin, similar to *and1*'s expression pattern at this stage (Fig 3.1.7). To have a better global view of the expression patterns of both *and1* and *egfp* during initial lepidotrichia formation, we performed double fluorescence *in situ* hybridization experiments (n=12 developing caudal fins) which further confirmed that *egfp* and *and1* expression patterns co-localized and was restricted to the distal end of fin rays (Fig 3.1.8). This suggests that the 2P+I region contains enhancers required to recapitulate endogenous *and1* expression during initial lepidotrichia formation. Taken together, a discrepancy remains when the location of endogenous *and1* expression, reporter expression and actinotrichia are compared to the location of EGFP in the reporter lines during the development of the caudal fin in zebrafish larvae.

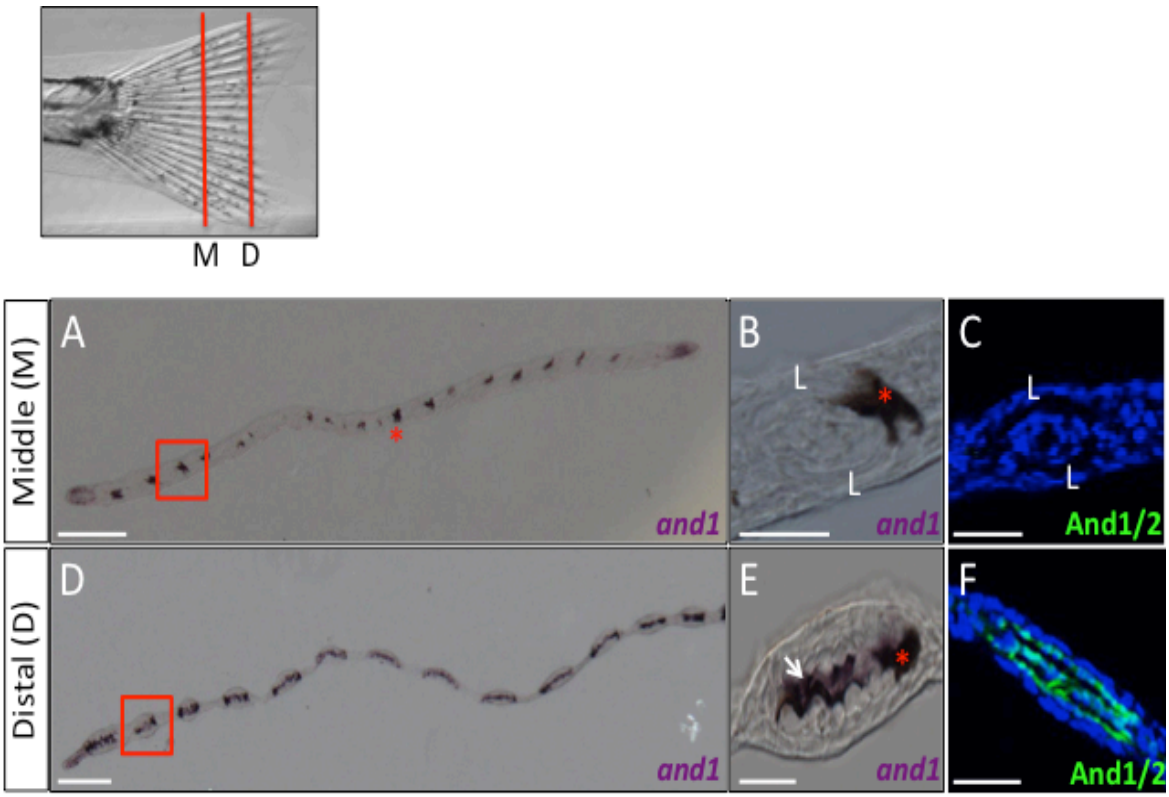


Figure 3.1.6. *and1* expression and actinotrichia are detected only in the distalmost sections of the developing caudal fin during lepidotrichia morphogenesis. Representative middle (panels **a-c**) and distal (panels **d-f**) transverse sections of the developing caudal fin of 40 dpf, 7.2 mm WT zebrafish juveniles. **a**. *In situ* hybridization experiments reveal no detectable *and1* expression in the middle sections of the developing caudal fin. **b.** High magnification of a fin ray from the middle section shown in panel a (red square). **c**. Immunostaining for And1/2 shows an absence of actinotrichia in middle sections. **d.** *In situ* hybridization experiments reveal *and1* expression in the distalmost sections of the developing caudal fin. **e.** High magnification of a fin ray from the distal section shown in panel d (red square). White arrow indicates *and1* expression. **f.** Immunostaining for And1/2 shows the presence of actinotrichia only in the distalmost sections. Images have been merged with a DAPI counterstain (panels c & f). *=Black pigment cells, L= Lepidotrichia hemirays. Scale bars in panels a & d= 100 um, panels b, c, e & f= 20 µm.

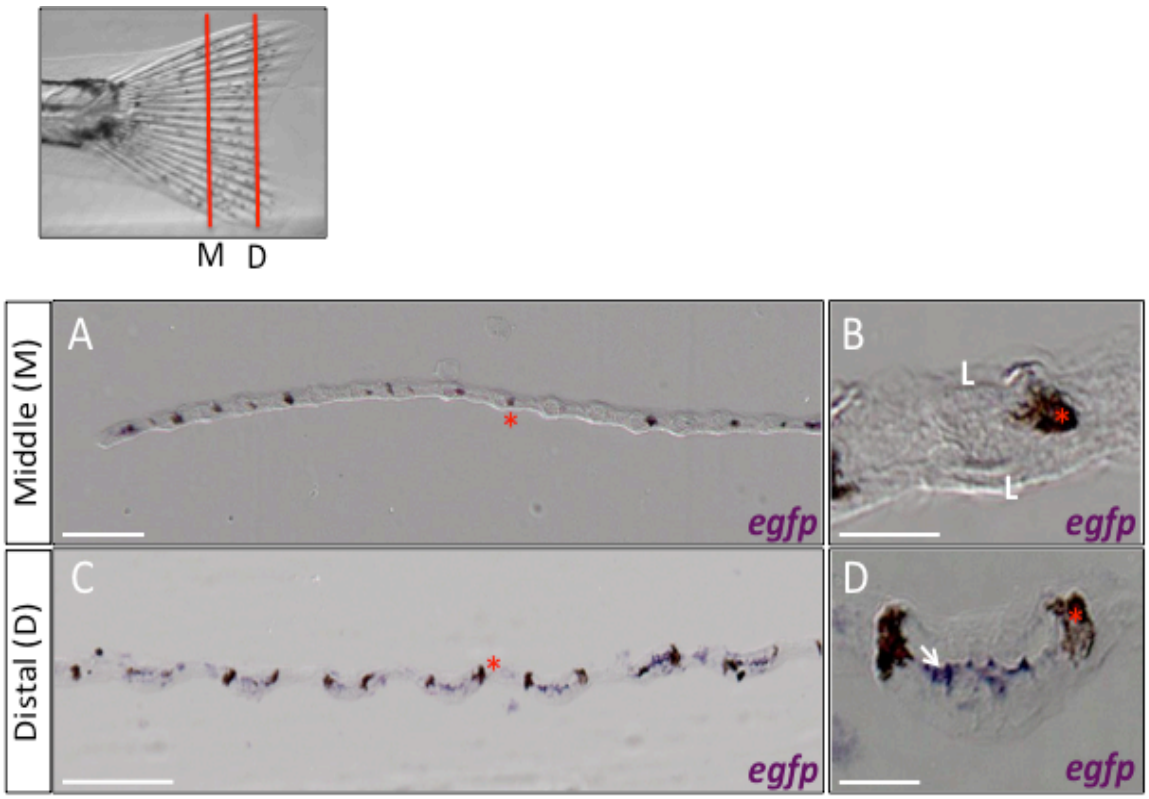


Figure 3.1.7. Reporter gene expression recapitulates endogenous *and1* expression in the developing caudal fin during lepidotrichia morphogenesis. Representative middle (panels **a-b**) and distal (panels **c-d**) transverse sections of the developing caudal fin of 40 dpf, 7.2 mm *Tg(2P+I:EGFP)* zebrafish juveniles. **a.** *In situ* hybridization experiments reveal no detectable *egfp* expression in the middle sections of the developing caudal fin. **b.** High magnification of a fin ray from the middle section. **c**. *In situ* hybridization experiments reveal *egfp* expression in the distalmost sections of the developing caudal fin. **d.** High magnification of a fin ray from the distal section. White arrow shows transgene expression. *=Black pigment cells, L= lepidotrichia. Scale bars in panel a & c= 100 μm, panels b & d= 20 μm.

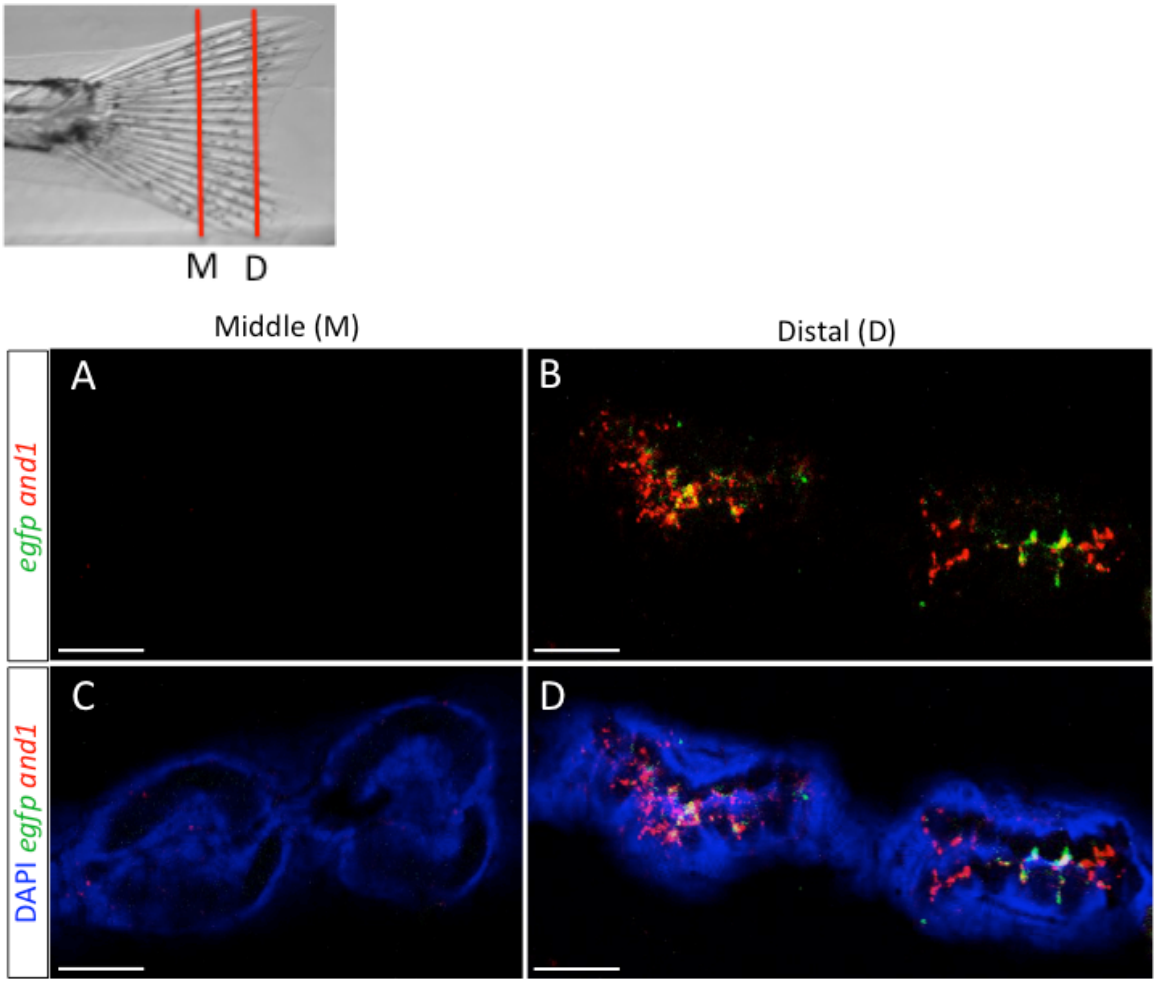


Figure 3.1.8. Reporter gene expression from the *Tg(2P+I:EGFP)* lines co-localize with endogenous *and1* expression in the developing fin rays. Representative middle (panels **a** & **c**) and distal (panels **b** & **d**) transverse sections of the developing caudal fin of 40 dpf, 7.2 mm *Tg(2P+I:EGFP)* zebrafish juveniles. **c.** Double fluorescence *in situ* hybridization experiments show an absence of *and1* and *egfp* expression in the middle sections of the developing caudal fin. **d.** *and1* and *egfp* are detected in the distalmost sections and co-localize. Images have been merged with a DAPI counterstain (panels c & d). Scale bar = 20 μm.

3.1.3. Characterization of and1 reporter expression during the median fin fold resorption

The median fin fold resorption process is the second important fin morphogenesis event that we focused on during the time course analyses. The median fin fold resorption process is defined as the process by which the median fin fold give rise to the three unpaired fins. It involves the gradual regression of the median fin fold tissues, which are located in between the newly developed fins, until the fold disappears. This process occurs in an anterior-to-posterior fashion (Parichy et al, 2009). Since actinotrichia have a structural role and supports the fin fold of zebrafish embryos and larvae, we propose that the resorption of the median fin fold during fin development may be due in part by a gradual anterior-to-posterior loss of actinotrichia along the median fin fold. Similarly, we also anticipate a loss of *and1* expression in an anterior-to-posterior trend. To examine this hypothesis, we first characterized reporter expression in the *Tg(2P+I:EGFP)* lines and compared it to endogenous *and1* expression in the median fins of zebrafish larvae undergoing resorption.

During the time course analyses of the *Tg(2P+I:EGFP)* lines, we noticed a gradual loss of transgene expression in an anterior-to-posterior direction along the resorbing median fin fold. In parallel, transgene expression simultaneously became distally restricted in the fold until it eventually vanished along with the median fin fold (Fig 3.1.9). This suggests that *and1* expression may follow a similar expression pattern during the median fin fold resorption process. To assess whether the *Tg(2P+I:EGFP)* lines were representative of endogenous *and1* expression during the median fin fold resorption process, we performed *in situ* hybridization experiments on transverse sections of the resorbing median fin fold using 8.0 mm SL WT juveniles (n=~8) (Fig 3.1.10). The

experiment confirmed that like EGFP, there is a gradual loss of *and1* expression from an anterior-to-posterior direction all the while being simultaneously distally restricted in the resorbing median fin fold.

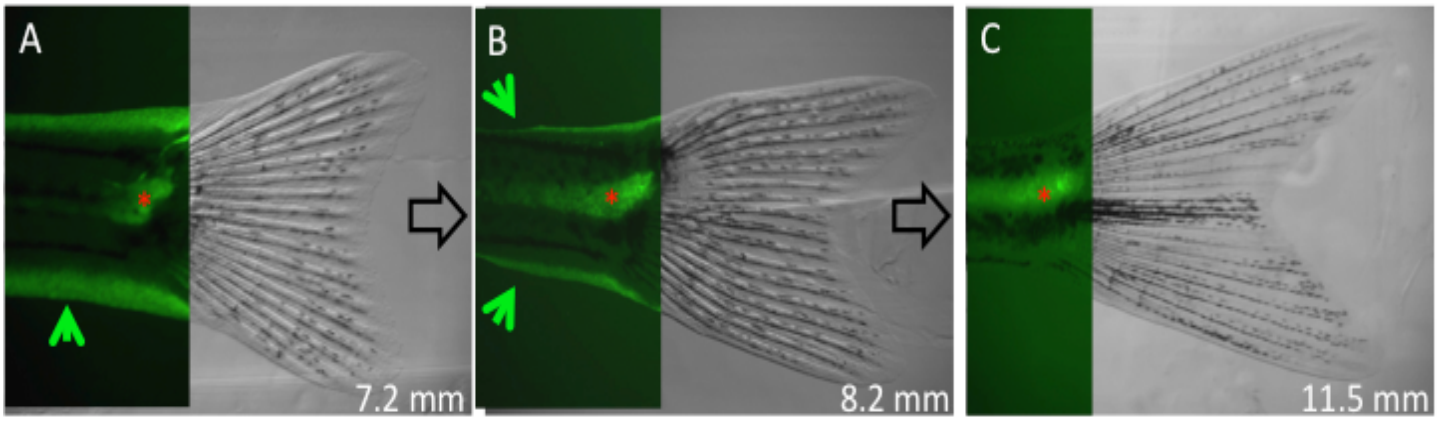


Figure 3.1.9. Reporter gene expression disappears in an anterior-to-posterior fashion while becoming gradually distally restricted in the resorbing median fin fold of *Tg(2P+I:EGFP)* juveniles. a. Transgene expression is present in the median fin fold (indicated by the green arrows) of juveniles having a SL of 7.2 mm. **b.** Transgene expression disappears in an anterior-to-posterior fashion while becoming gradually distally restricted in the resorbing median fin fold at a SL of 8.2 mm. **c.** At a SL of 11.5 mm, the median fin fold is completely resorbed and there is absence of reporter expression. Autofluorescence is present in the lateral line of the zebrafish (shown by the red asterisks).

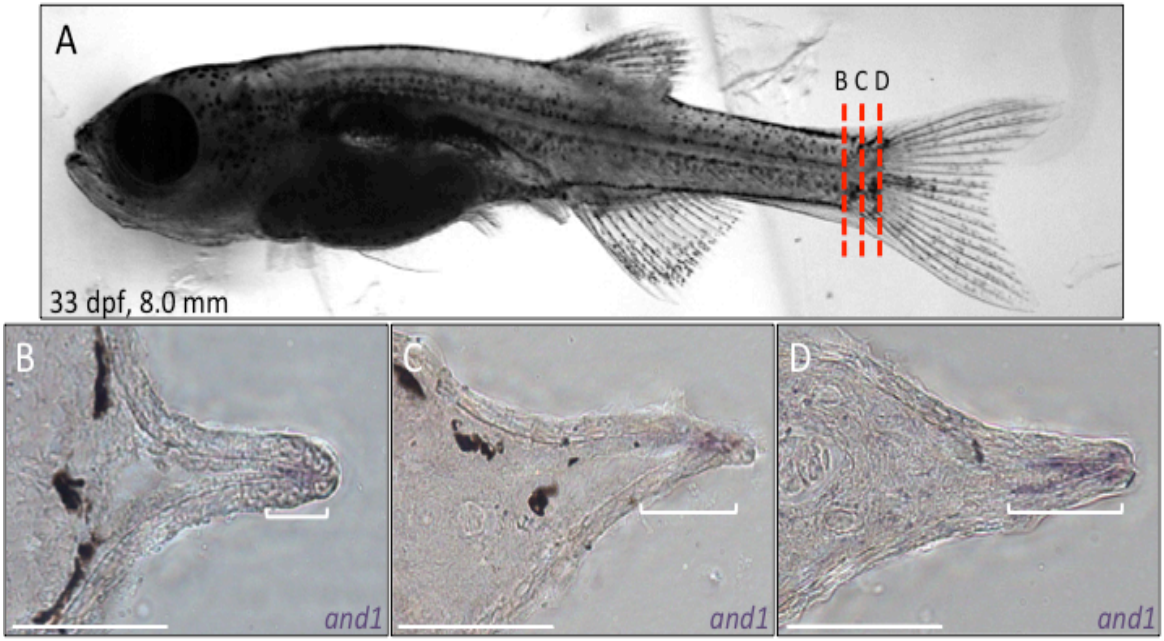


Figure 3.1.10. *and1* expression disappears in an anterior-to-posterior fashion while becoming gradually distally restricted in the resorbing median fin fold. a. View of the levels at which sections are situated in panels **b, c** and **d**. The trunk is to the left and the dorsal fin is to the right in the following panels. *In situ* hybridization experiments on transverse cryo-sections of 8.0 mm SL WT juveniles show *and1* expression along the resorbing median fin fold. White brackets show the length of the *and1* expression domain along the proximodistal axis of the median fin fold. Scale bar= 100 μm.

3.1.4. Characterization of endogenous and1 expression and actinotrichia in the median fin fold during its resorption

To further corroborate that the resorption of the median fin fold is correlated with the gradual anterior-to-posterior loss of actinotrichia, we found necessary to characterize the expression pattern of *and1* and actinotrichia distribution during median fin fold resorption through *in situ* hybridization and immunostaining experiments respectively. Since the resorption of the median fin fold is a gradual process, we analyzed actinotrichia distribution and *and1* expression during an early and a late phase. During the resorption of the median fin fold, there is a point where the median fin fold has begun receding completely in the anterior portion of the median fin fold (specifically the dorsal median fin fold located between the dorsal and caudal fin). We defined the early and the late phase as being the developmental stages prior and after this event respectively (Fig 3.1.11a compared to Fig 3.1.12a). *In situ* hybridization experiments were performed on transverse cryo-sections spanning the anteroposterior length of the resorbing median fin fold of 29 dpf, 7.0 mm SL WT larvae (n=~9) undergoing early phases of resorption. This experiment showed *and1* expression in mesenchymal cells, which are located alongside actinotrichia, and distal epithelial cells (Fig 3.1.11b-c). As for actinotrichia, their presence and distribution were assessed by performing an And1/2 immunostaining on transverse cryo-sections spanning the anteroposterior length of the resorbing median fin fold of 29 dpf, 7.0 mm WT larvae (n=~7) as well. The experiment revealed the presence of elongated and organized actinotrichia fibres supporting the median fin fold (Fig 3.1.11d-e). This shows that *and1* expression and actinotrichia are well present in the fold during the early stages of the median fin fold resorption.

As for the late phases of the median fin fold resorption, *and1* expression and actinotrichia distribution were assessed at two different locations along the anteroposterior length of the median fin fold. At a more posterior position, *and1* expression was completely absent when performing *in situ* hybridization experiments coupled with a proteinase K treatment on transverse cryo-sections spanning the anteroposterior length of the median fin fold of 38 dpf, 8.2 mm SL WT juveniles (n=~8) (Fig 3.1.12b-c). Immunostaining for And1/2 (n=~8) showed actinotrichia had receded to the tail; they seemingly lost their fibril-like structures and became agglomerated into two cluster-like structures (Fig 3.1.12d-e).

In a more anterior position, *and1* expression remained absent (n=~12) (Fig 3.1.13b-c) and actinotrichia had completely lost their structural resemblance to fibrils (n=~12) (Fig 3.1.13d-e). Instead, actinotrichia appeared fragmented and sparse at the edge of the tail. In fact, actinotrichia eventually disappeared in the tail (data not shown). We suggest that there is a gradual loss of *and1* expression along the receding median fin fold. This loss occurs from an anterior-to-posterior direction. In turn, this causes the loss of actinotrichia during the median fin fold resorption process. In other words, we think that there is a loss of actinotrichia maintenance through the consequential loss of endogenous *and1* expression along the receding median fin fold.

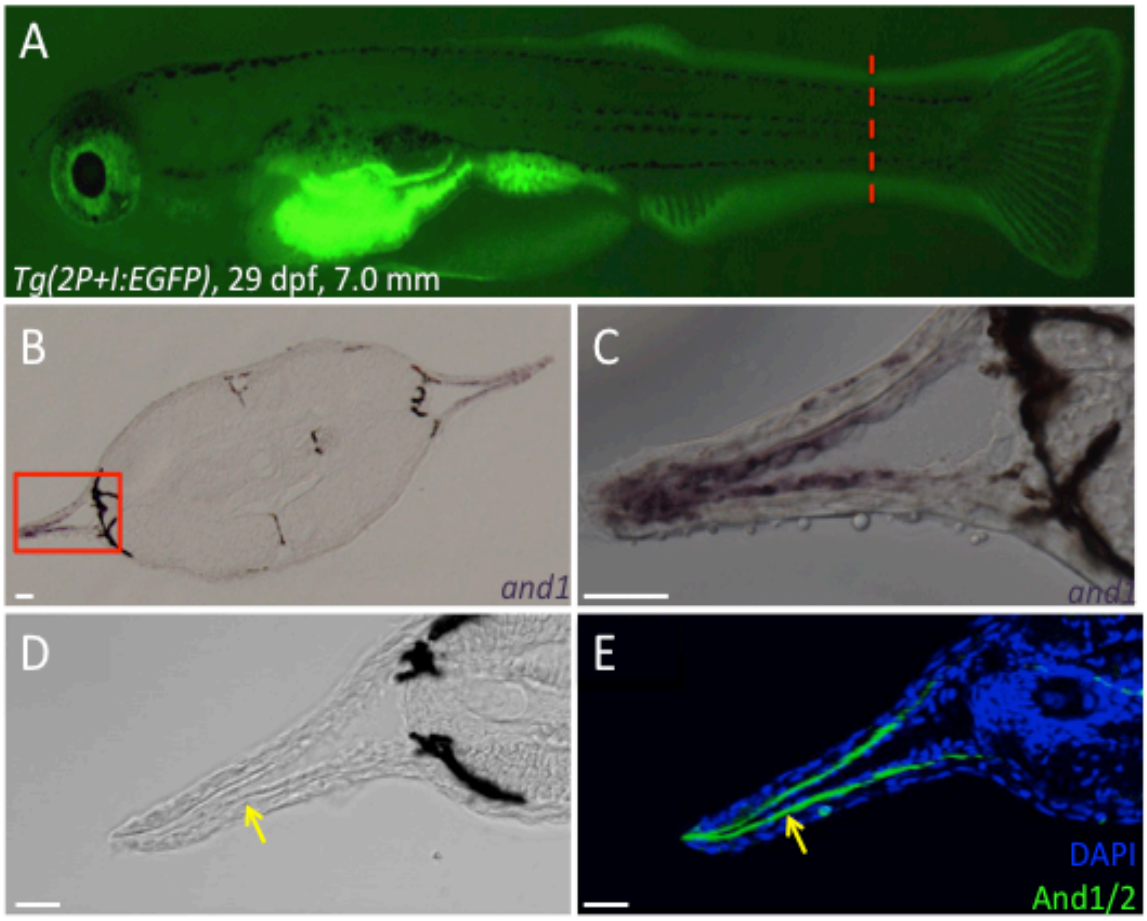


Figure 3.1.11. *and1* expression and actinotrichia fibers are well present in the median fin fold during the early phase of resorption. a. A 29 dpf, 7.0 mm SL *Tg(2P+I:EGFP)* larva during the early phase of resorption. The red dotted line shows the level at which the sections are situated in panels b-e. **b.** *and1* expression is present in both the mesenchymal and epithelial cells of the median fin fold (dorsal fin is to the left, ventral fin is to the right). **c.** High magnification of the dorsal fin shown in panel b (red square). **d**. Bright field image of actinotrichia fibers located within the ventral median fin fold. **e.** Immunostaining for And1/2 shows elongated and organized actinotrichia fibers. Image in panel e was merged with a DAPI counterstain. Yellow arrows= actinotrichia. Scale bar in panels b-e= 20 μm.

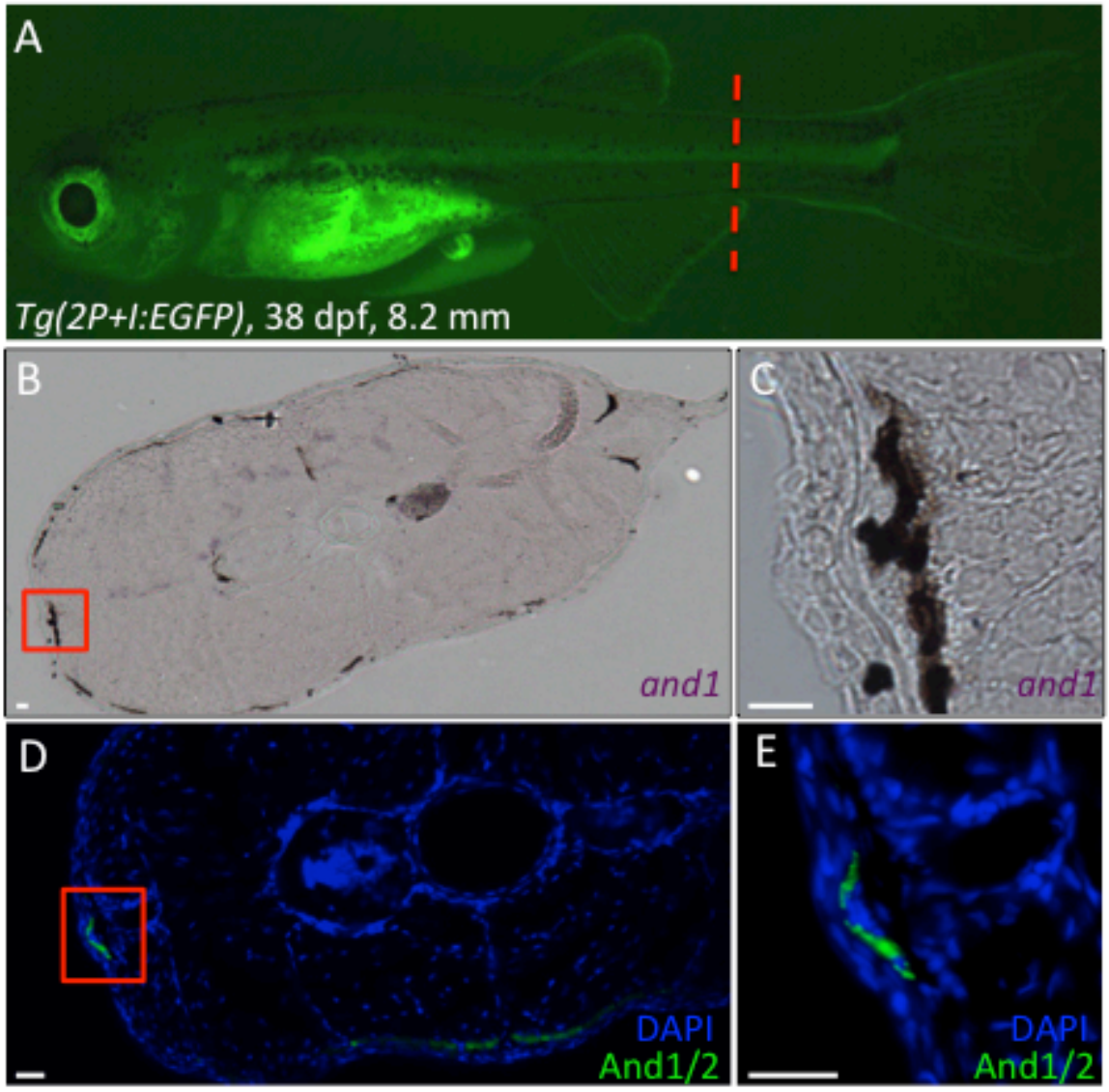


Figure 3.1.12. Posterior sections of the median fin fold during a late resorption phase show an absence of *and1* expression and actinotrichia degradation. a. A 38 dpf, 8.2 mm SL *Tg(2P+I:EGFP)* juvenile during the late phase of resorption. The red dotted line shows the level at which the sections are situated in panels b-e. **b.** *and1* expression is absent in the dorsal median fin fold. **c.** High magnification of the dorsal fin shown in panel b (red square). Dorsal fin is to the left, ventral fin is to the right. **d**. Immunostaining for And1/2 shows two agglomerated actinotrichia clusters in the dorsal median fin fold. **e.** High magnification of the dorsal median fin fold shown in panel d (red square). Images merged with a DAPI counterstain (panels d & e). Scale bars in panels b-e= 20 μm.

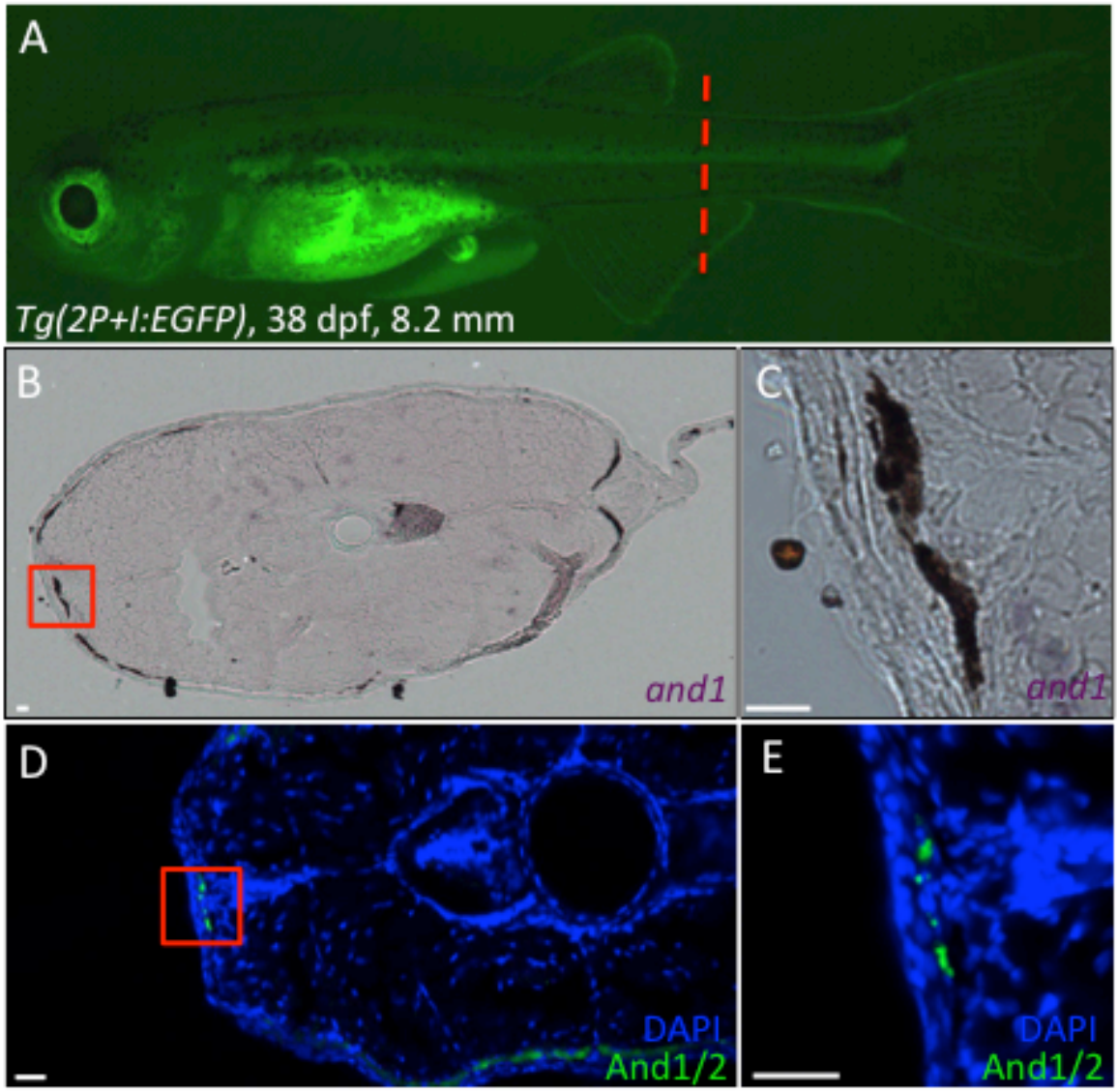


Figure 3.1.13. Anterior sections of the median fin fold during a late resorption phase show an absence of *and1* expression and further actinotrichia degradation. a. A 38 dpf, 8.2 mm SL *Tg(2P+I:EGFP)* juvenile during the late phase of resorption. The red dotted line shows the level at which the sections are situated in panels b-e. **b.** *and1* expression is absent in the dorsal median fin fold. **c.** High magnification of the dorsal fin shown in panel b (red square). Dorsal fin is to the left, ventral fin is to the right. **d**. Immunostaining for And1/2 shows fragmented and sparse actinotrichia in the dorsal median fin fold. **e.** High magnification of the dorsal median fin fold shown in panel d (red square). Images merged with a DAPI counterstain (panels d & e). Scale bar in panels b-e= 20 μm.

3.1.5. Characterization of and1 reporter expression during the adult stage

We characterized reporter expression of the various transgenic reporter lines during the late juvenile and adult stage. It is already known that actinotrichia fibres are present only at the distal end of each lepidotrichia in the intact adult zebrafish fins (Santamaría, Santos Ruiz & Becerra, 1996). Since *and1* expression is assumed to be distally restricted in the intact fins of adult zebrafish, we expected reporter expression to reflect this assumption. Additionally, we sought to observe whether the function of the tissue-specific *cis*-acting enhancer regions of *and1* characterized during the embryonic stage remains identical during the adult stage. In order to make those observations, we examined 60 and 90 dpf zebrafish, which are considered the late juvenile and adult stage respectively according to ZFIN.

When juveniles reached 60 dpf, it was clear that transgene expression was maintained in the developing fins of the *Tg(2P+I:EGFP)* and *Tg(2PΔepi:EGFP)* lines (Fig 3.1.14a,c). Moreover, transgene expression gradually became distally restricted along the fin rays and disappeared in the proximal end of the caudal fin rays. It is worthy to note that autofluorescence, which appears as a yellow colour when directly looking in the microscope, can be observed at the very base of the caudal fin rays at this stage and is not to be mistaken for reporter fluorescence: this autofluorescence was observed in WT fish as well during the time course analyses and cannot be distinguished from reporter fluorescence via camera (data not shown). Concerning the *Tg(2P:EGFP)* and *Tg(Epi+βG:EGFP)* lines, we had previously observed a gradual decrease in transgene expression throughout initial lepidotrichia formation. Evidently, transgene expression

became barely perceptible to a point where no transgene expression was observed at 60 dpf using whole-mount fluorescence microscopy (Fig 3.1.14b,d).

During the adult stage, the absence of transgene expression in the *Tg(2P:EGFP)* and *Tg(Epi+βG:EGFP)* lines was maintained (Fig 3.1.15c-d,g-h). On the other hand, transgene expression in the *Tg(2P+I:EGFP)* and *Tg(2PΔepi:EGFP)* lines was distally restricted to the tip of each fin ray, where actinotrichia are located in the adult intact fin (Fig 3.1.15a-b,e-f) (Santamaría, Santos Ruiz & Becerra, 1996). When comparing the late juvenile and adult stage analyses of these two lines, we observe that transgene expression completely becomes distalized as development progresses. This suggests that these two lines may recapitulate endogenous *and1* expression, not only during the adult stage but throughout development as well.

Furthermore, the fact that the *Tg(2P+I:EGFP)* lines have the ability to maintain epithelial expression unlike the *Tg(2P:EGFP)* and *Tg(Epi+βG:EGFP)* lines during the adult stage suggests that there is most likely a change in the *cis*-acting regulation of the *and1* gene in the fin epithelium as development progresses. However, we have to consider the possibility that the absence of fluorescence detection in the *Tg(2P:EGFP)* and *Tg(Epi+βG:EGFP)* lines during the adult stage may be due to a level of transgene expression that cannot be detected using whole-mount fluorescence microscopy.

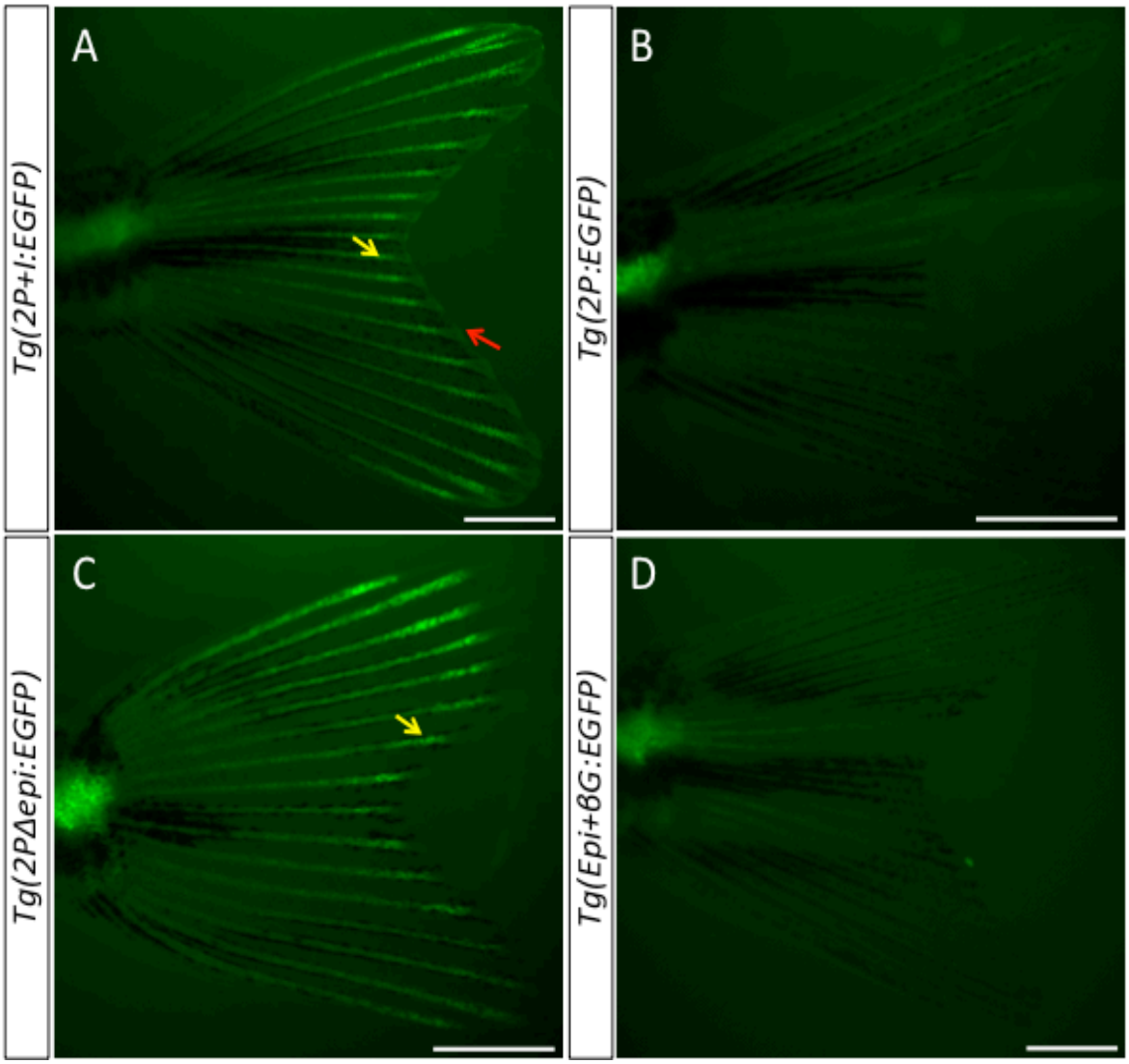


Figure 3.1.14. Reporter gene expression in the caudal fin of the various *and1* reporter lines during the late juvenile stage (60 dpf). a. Reporter gene expression is located all along the proximodistal length of fin rays (yellow arrow) and at the distal end of the fin rays (red arrow) in the *Tg(2P+I:EGFP)* lines. **b.** No reporter expression is detected in *Tg(2P:EGFP)* fins. **c**. Reporter expression is restricted all along the proximodistal length of fin rays in *Tg(2PΔepi:EGFP)* lines (yellow arrow). **d.** No reporter gene expression is detected in *Tg(Epi+βG:EGFP)* fins. Scale bars= 0.5 mm.

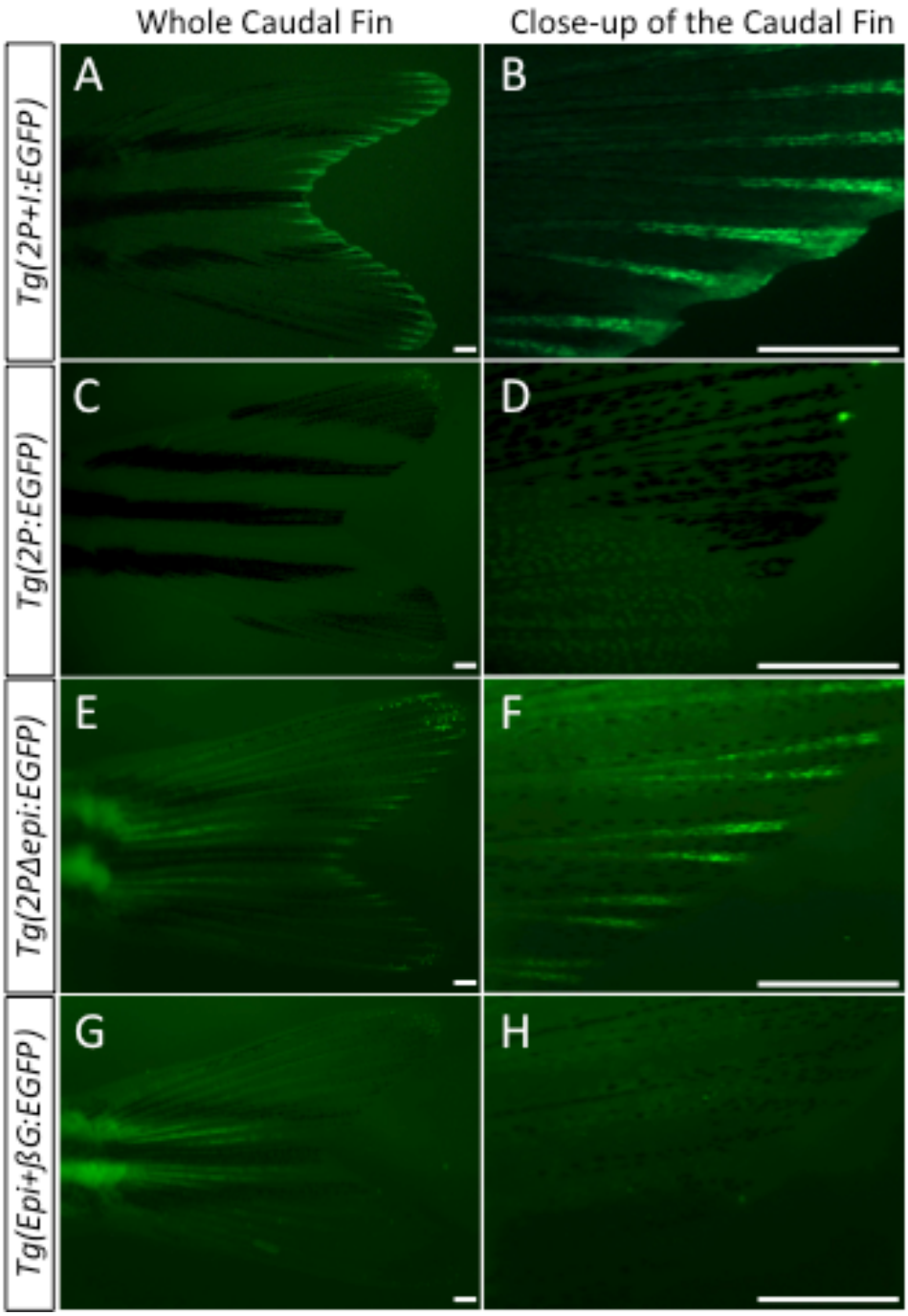


Figure 3.1.15. Reporter gene expression in the caudal fin of the various *and1* reporter lines during the adult stage (≥90 dpf). a-b. Reporter gene expression is located at the distal end of the fin rays in the *Tg(2P+I:EGFP)* lines. **c-d.** No reporter expression is detected in *Tg(2P:EGFP)* fins. **e-f**. Reporter gene expression is located at the distal end of the fin rays in *Tg(2PΔepi:EGFP)* lines. **g-h.** No reporter expression is detected in *Tg(Epi+βG:EGFP)* fins. Scale bars=0.5 mm.

Objective 2: Characterization of *egfp* expression in the various transgenic *and1* reporter lines throughout fin regeneration

3.2.1. The I region of and1 acts as a general transcriptional enhancer

In objective 1, we revealed that transgene expression was maintained in the intact fins of adult fish in *Tg(2P+I:EGFP)* and *Tg(2PΔepi:EGFP)* lines, however transgene expression disappeared in the intact fins of adult fish in *Tg(2P:EGFP)* and *Tg(Epi+βG:EGFP)* lines according to fluorescence microscopy analyses (Fig 3.1.15). Time course analyses were performed on the *Tg(2P+I:EGFP)*, *Tg(2P:EGFP)*, *Tg(2PΔepi:EGFP)* and *Tg(Epi+βG:EGFP)* transgenic lines (see table 4 for the number of lines for each reporter construct) (n=~5 zebrafish/line) to characterize each their distinctive *and1* reporter expression pattern throughout fin regeneration after performing a standard cut, which is defined as an amputation located 2 segments below the first branching point of lepidotrichia in the caudal fin. From these analyses, we were able to observe likewise that transgene expression is maintained in *Tg(2P+I:EGFP)* and *Tg(2PΔepi:EGFP)* lines and absent in the fin regenerate of *Tg(2P:EGFP)* and *Tg(Epi+βG:EGFP)* lines throughout fin regeneration (Fig 3.2.1).

Since no reporter expression was detected using fluorescence microscopy in the intact fins and fin regenerates of the *Tg(2P:EGFP)* lines and epidermal-specific *Tg(Epi+βG:EGFP)* lines, we wondered if it was due to the transgene not being expressed or due to the fact that the transgene might be so lowly expressed that fluorescence microscopy was an inadequate method to detect the presence of *egfp* expression. In order to examine this, we performed a standard cut on two transgenic lines for each constructs, collected the fin regenerates (n=10 fin regenerates/line) at 7 dpa, and did total RNA

extraction to produce cDNA via a reverse transcriptase (RT) reaction. Polymerase chain reactions (PCR) were performed using custom primers directed against a ~200bp region of the *egfp* gene. To rule out genomic DNA contamination for each sample, a PCR was performed on the DNase-treated RNA that was subsequently used for RT reactions. Furthermore, we included a negative control that consisted of water, a positive control that contained total cDNA from *Tg(2P+I:EGFP)* 7dpa regenerates and β-actin controls as reaction controls. The data was not quantified since only the presence versus absence of transgene expression was evaluated. According to the RT-PCR results, *egfp* is indeed expressed during fin regeneration in *Tg(2P:EGFP)* and *Tg(Epi+βG:EGFP)* lines, however it seems in very low levels since it cannot be detected using fluorescence microscopy (Fig 3.2.2). This also suggested that *egfp* expression may also be occurring in the intact fins of *Tg(2P:EGFP)* and *Tg(Epi+βG:EGFP)* adult fish.

Interestingly, when comparing the *Tg(2P+I:EGFP)*, *Tg(2P+E:EGFP)* and *Tg(2P:EGFP)* lines during the embryonic stage, we notice that all three drive transgene expression in mesenchymal and ectodermal cells of the fin fold (Fig 3.2.3). However, when comparing the same three transgenic lines during adult fin regeneration, transgene expression was dissimilar between them: the *Tg(2P+I:EGFP)* line displayed strong transgene expression along the regenerating fin rays in the mesenchyme and basal epidermal layer of the fin regenerate, the *Tg(2P+E:EGFP)* line presented what was seemingly distal mesenchymal transgene expression and the *Tg(2P:EGFP)* lines showed no transgene expression via fluorescence microscopy (Fig 3.2.4). This suggests the *Tg(2P:EGFP)* line may require the addition of the EI region for the transgene to be efficiently expressed in the mesenchyme and in the basal epidermal layer of the intact and

regenerating fin. In fact, when comparing the *Tg(2P+E:EGFP)* and the *Tg(2P+I:EGFP)* lines, we observe that the simple addition of the first intron of *and1* (the I region) tremendously boosts transgene expression. Therefore, we hypothesize that the I region acts as a strong general transcriptional enhancer.

To test this hypothesis we decided to generate a transgenic line in which the basal epidermal layer expression would be boosted/activated by the inclusion of the I region. In the process, it would simultaneously allow us to create an epidermal-specific *and1* reporter line in which the expression would be maintained throughout fin development and regeneration, and would be potentially useful for future experiments. The EI region was previously shown to activate transgene expression when cloned upstream of an *egfp* reporter construct, thus suggesting this region contains an alternate *and1* promoter. However the expression profile did not correspond to the expression profile of *and1*, thus suggesting this region drives non-specific expression (Poullain Thesis, unpublished). Considering this, the region spanning the 5' end of the Epi fragment to the 3' end of the first intron was cloned in an *egfp* reporter construct in collaboration with fellow PhD candidate Robert Lalonde and was named the Epi-EI*:EGFP* construct (Fig 3.2.5a). It is important note that the region spanning from the 3' end of the Epi fragment to the 5' end of E (in other words, excluding the Epi and first exon), termed 967-1P*and1:EGFP,* was previously shown to drive no significant transgene expression of its own (Lalonde et al, 2016). Thus, this suggests that any significant epidermal expression in the adult fish of *Tg(Epi-EI:EGFP)* lines would be most likely due to the addition of the I region. See appendix A for detailed schematics of the various reporter constructs mentioned in this

thesis. The Epi-EI*:EGFP* plasmid construct was then microinjected into one-cell staged zebrafish eggs to generate *Tg(Epi-EI:EGFP)* lines.

Two transgenic lines were found using whole-mount fluorescent microscopy, one showed strong transgene expression in the lateral line of adult fish, and the other displayed strong transgene expression at the distal end of each lepidotrichia of the intact fins. We suspect there are positional effects causing ectopic transgene expression in the lateral line of the adult fish in the former transgenic line. The latter transgenic line displayed strong transgene expression in the distal inter-ray tissue of the fin regenerate during fin regeneration and at the distal end of each lepidotrichia in adult intact fins (Fig 3.2.5b-c). In order to identify the cell type of the *egfp*-expressing cells, longitudinal cryo-sections of 7 dpa *Tg(Epi-EI:EGFP)* fin regenerates (n=6) were produced and imaged using confocal microscopy. Transgene expression was revealed to be specifically located in the basal epidermal layer of the fin regenerate (Fig 3.2.6). Taken together, we were able to successfully create an epidermal-specific transgenic line in which the expression is maintained throughout fin development and regeneration, all the while showing that the I region of *and1* is a strong general transcriptional enhancer that is required for the efficient expression of *and1* in the basal epidermal layer of the fin.

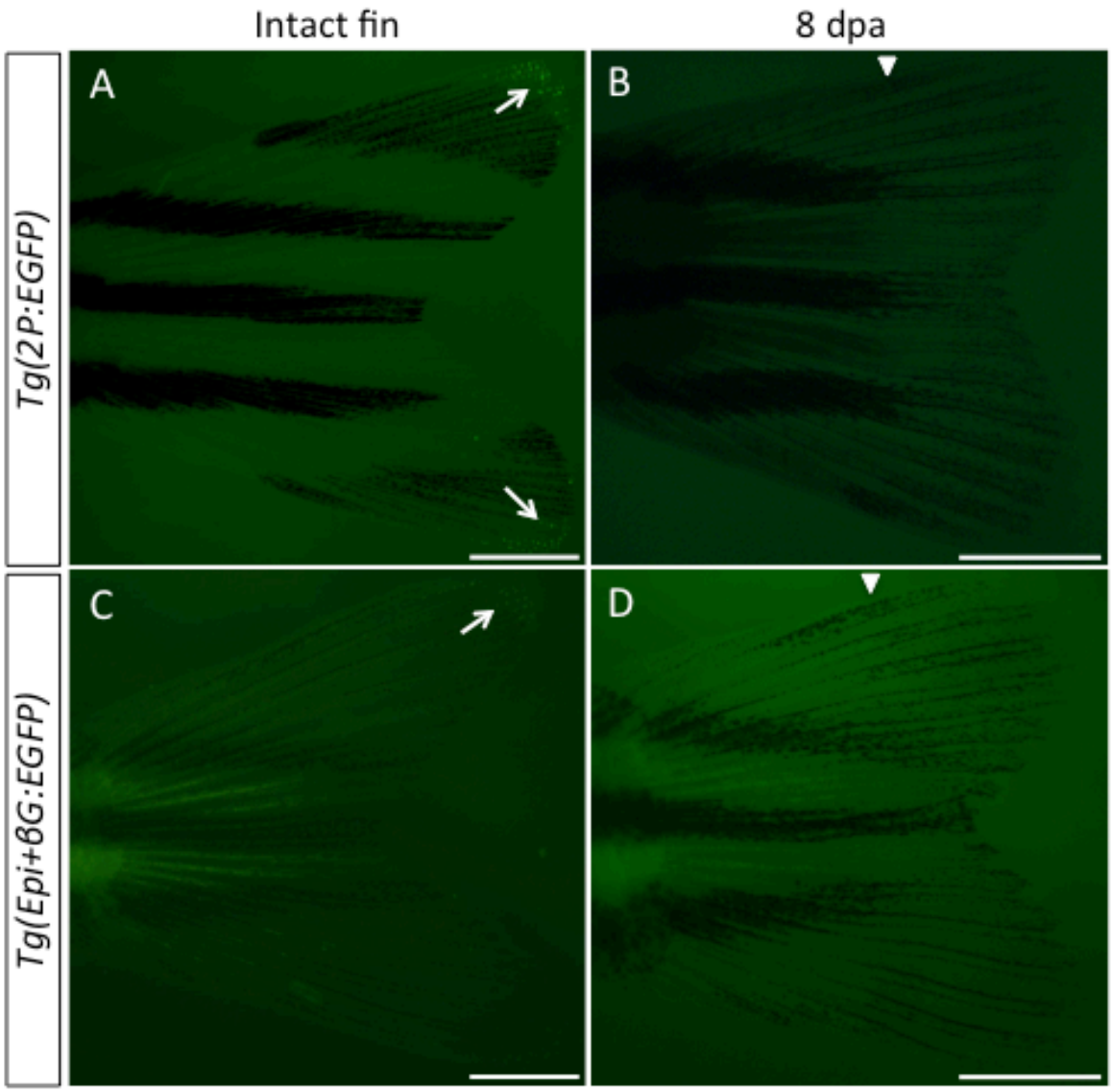


Figure 3.2.1. Absence of reporter gene expression in the intact and regenerating caudal fin of *Tg(2P:EGFP)* and *Tg(Epi+βG:EGFP)* lines using whole-mount fluorescence microscopy. Panels **a** & **c** show the intact fin and panels **b** & **d** show the 8 dpa fin regenerate of the *Tg(2P:EGFP)* line (panels **a-b**) and *Tg(Epi+βG:EGFP)* (panels **c-d**) lines. The white triangles indicate the plane of amputation. The white arrows indicate autofluorescence caused by white pigment cells. Scale bar= 2 mm.

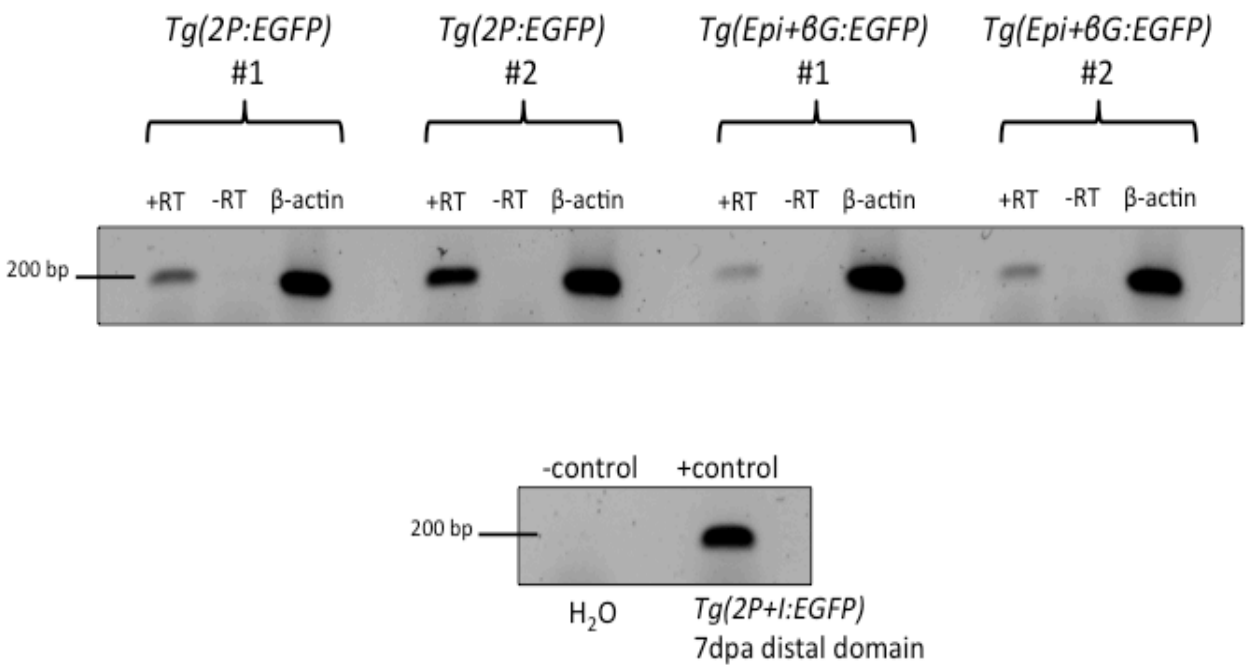


Figure 3.2.2. RT-PCR experiments show that there is low transgene expression in 7 dpa *Tg(2P:EGFP)* and *Tg(Epi+βG:EGFP)* fin regenerates. 7 dpa fin regenerates from the *Tg(2P:EGFP)* and *Tg(Epi+βG:EGFP)* lines were collected and RNA extraction was performed to produce cDNA via a reverse transcriptase reaction. Reverse transcriptase polymerase chain reactions (RT-PCR) was performed using custom primers directed against a ~200bp region of the *egfp* transgene (+RT) and compared to DNase-treated/RT-untreated total RNA (-RT) to rule out genomic DNA contamination. Furthermore, a negative control that consists of water, a positive control that contains cDNA from *Tg(2P+I:EGFP)* 7dpa regenerates and β-actin controls as reaction controls were included. The data was not quantified since we only investigated the presence versus absence of transgene expression. According to the RT-PCR results, *egfp* is indeed expressed during fin regeneration in *Tg(2P:EGFP)* and *Tg(Epi+βG:EGFP)* lines, however seemingly in very low levels since it cannot be detected using whole-mount fluorescence microscopy.

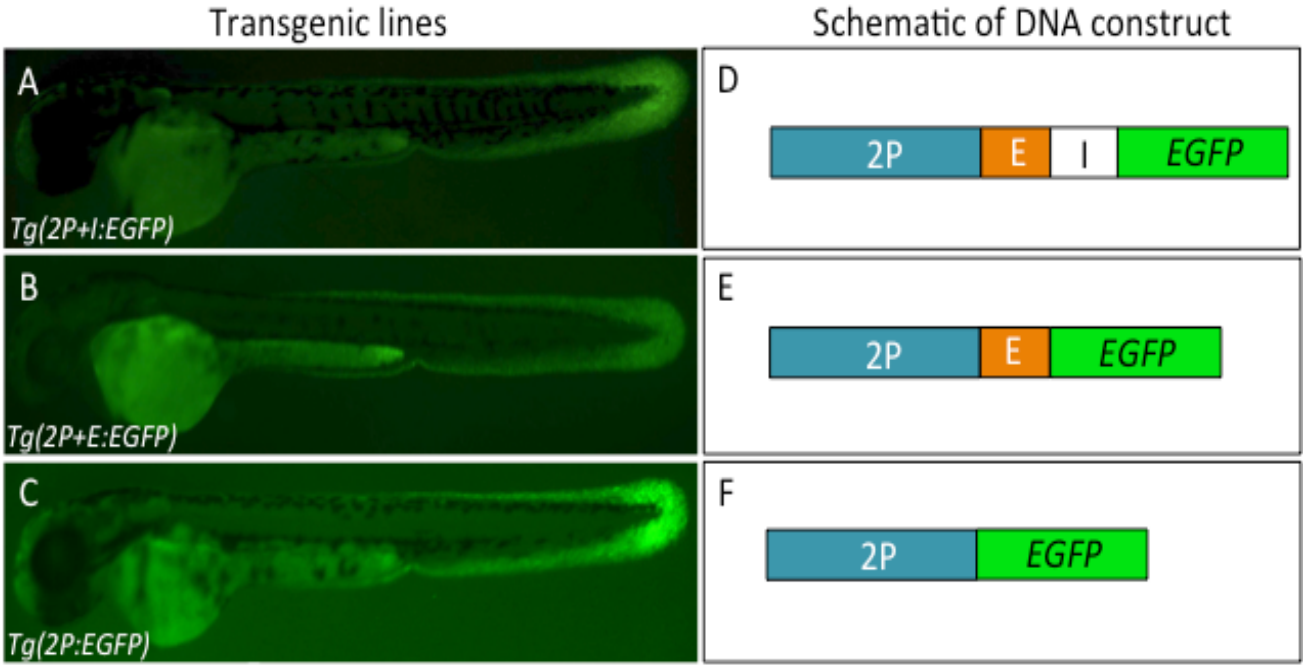


Figure 3.2.3. The *Tg(2P+I:EGFP)*, *Tg(2P+E:EGFP)* and *Tg(2P:EGFP)* lines have identical transgene expression during the embryonic stage in the mesenchyme and ectoderm of the fin fold. Panels **a-c** show lateral pictures of 3 dpf *Tg(2P+I:EGFP)* (panel **a**), *Tg(2P+E:EGFP)* (panel **b**) and *Tg(2P:EGFP)* (panel **c**) zebrafish embryos. Panels **d, e & f** show schematics of the *2P+I:EGFP, 2P+E:EGFP* and *2P:EGFP* constructs respectively. 2P stands for the 2kbp region located upstream of the first untranslated exon (E) and I stands for the first intron of *and1*. *EGFP* stands for enhanced green fluorescent protein sequence.

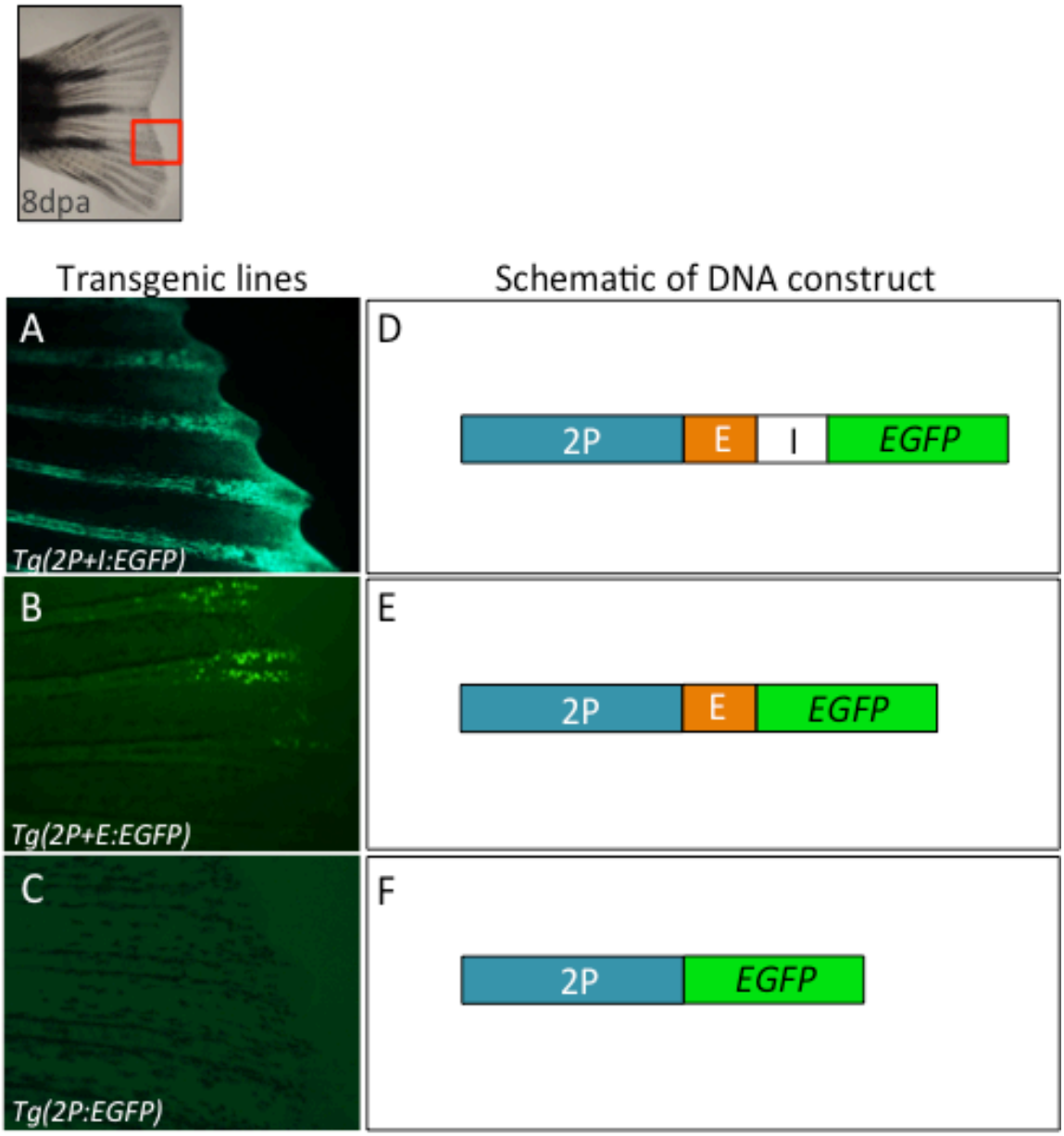


Figure 3.2.4. Transgene expression in the *Tg(2P+I:EGFP)*, *Tg(2P+E:EGFP)* and *Tg(2P:EGFP)* lines differ during the adult stage and throughout fin regeneration (8 dpa). Panels **a-c** show whole-mount pictures of the distal ends of 8 dpa *Tg(2P+I:EGFP)* (panel **a**), *Tg(2P+E:EGFP)* (panel **b**) and *Tg(2P:EGFP)* (panel **c**) fin regenerates. **a**. The *Tg(2P+I:EGFP)* line shows transgene expression all along the proximodistal length of fin rays and the distal epidermis. **b.** The *Tg(2P+E:EGFP)* line shows transgene expression distally restricted at the tip of the regenerating fin rays. **c.** The *Tg(2P:EGFP)* line shows no expression at all. Panels **d, e & f** show schematics of the 2P+I:*EGFP,* 2P+E:*EGFP* and 2P:*EGFP* constructs respectively. 2P stands for the 2kbp region located upstream of the first untranslated exon (E) and I stands for the first intron of *and1*. *EGFP* stands for enhanced green fluorescent protein sequence.

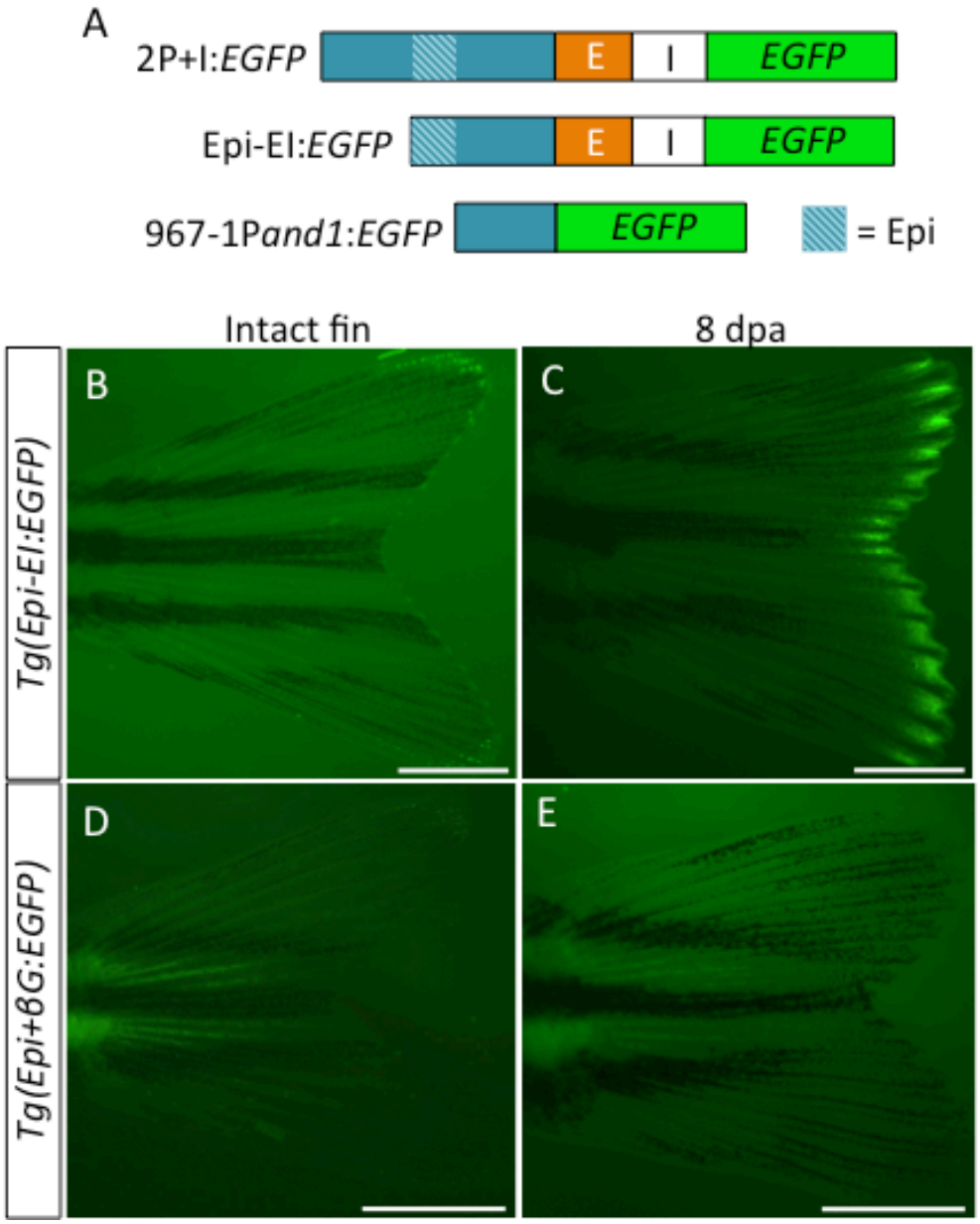


Figure 3.2.5. Reporter gene expression increased in the epidermis of the intact and fin regenerate of the *Tg(Epi-EI:EGFP)* line. a. Schematics of the 2P+I:*EGFP,* Epi-EI:*EGFP* and 967-1P*and1:EGFP* constructs. The Epi-EI:*EGFP* construct starts at the 5' end of the Epi fragment and ends at the 3' end of the first intron (I). The 2P+I:*EGFP* construct includes 2P, which is a 2kbp region located upstream of the first untranslated exon (E), E and the first intron of *and1* termed I. The 967-1P*and1:EGFP* construct begins at the 3' end of the Epi fragment and ends at the 5' end of the E. *EGFP* stands for enhanced green fluorescent protein sequence. Panels **b** & **d** show the intact fins and panels **c** & **e** show 8 dpa fin regenerates from the *Tg(Epi-EI:EGFP)* (panels **b** & **c**) and *Tg(Epi+βG:EGFP)* (panels **d** & **e**) lines. Scale bars= 2 mm.

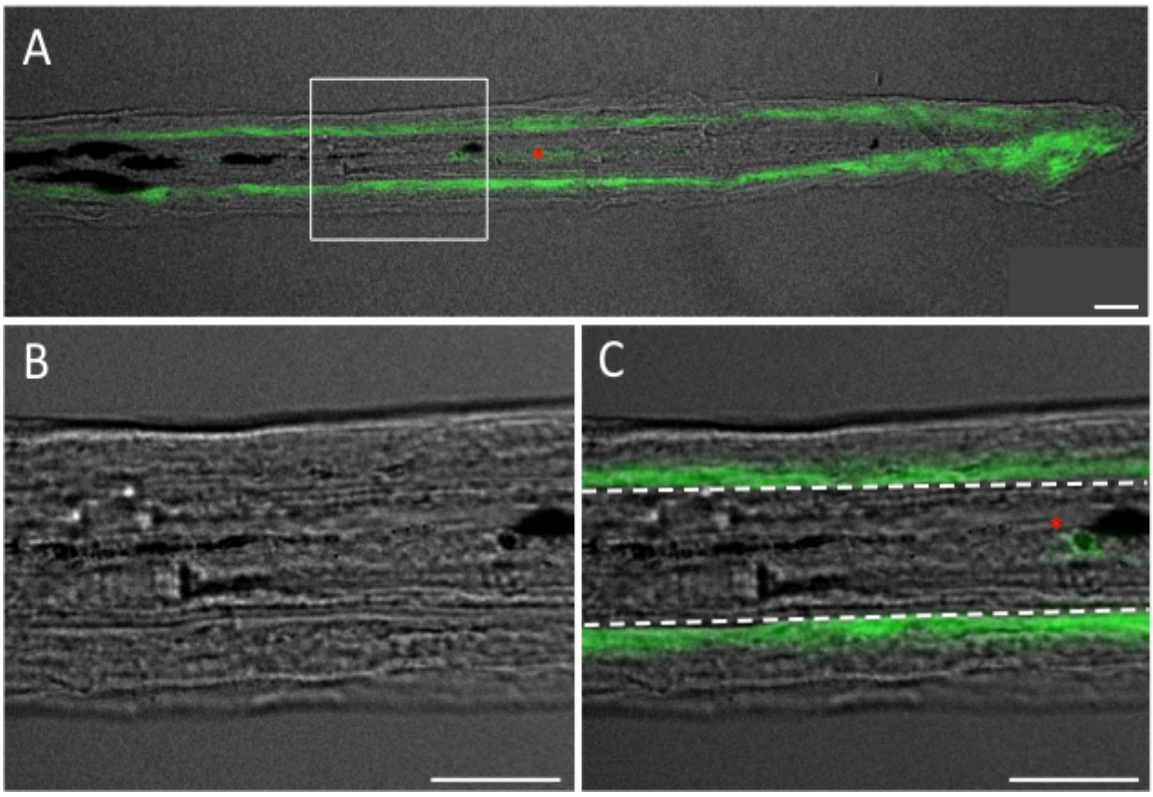


Figure 3.2.6. Reporter gene expression in the *Tg(Epi-EI:EGFP)* line is restricted to the basal epidermal layer of the fin regenerate. a. The distal end of a 7 dpa *Tg(Epi-EI:EGFP)* fin regenerate. Images from panels b & c are high magnifications of the white box seen in panel a. **b.** Brightfield high magnification image of the fin regenerate. **c.** Brightfield and green fluorescence merged high magnification image shows transgene expression is located in the basal epidermal layer of the fin regenerate. The white dotted lines indicate the limit between the basal epidermal layer and the mesenchyme of the fin regenerate. *= autofluorescence from red blood cells. Scale bar= 20 μm.

3.2.2. Characterization of tissue-specific and1 reporter expression throughout fin regeneration

Time course analyses starting at 1 dpa and ending at 14 dpa were performed on a number of the *Tg(2P+I:EGFP)*, *Tg(2P:EGFP)*, *Tg(2PΔepi:EGFP)*, *Tg(Epi+βG:EGFP)* and *Tg(Epi-EI:EGFP)* transgenic lines to characterize each their distinctive *and1* reporter expression (see table 4 for the number of lines for each reporter construct) (n=~5 zebrafish/line). *and1* expression has been previously characterized by our lab to be located in the distal basal epidermal layer and distal mesenchyme (including the blastemal cells) of the fin regenerate throughout fin regeneration (Wagh thesis, unpublished). We aimed to observe whether these transgenic lines were representative of endogenous *and1* expression throughout fin regeneration. Since the *Tg(2P:EGFP)* and *Tg(Epi+βG:EGFP)* lines showed no transgene expression throughout the regeneration process of the caudal fin, we found appropriate to only show the *Tg(2P+I:EGFP)*, *Tg(2PΔepi:EGFP)* and *Tg(Epi-EI:EGFP)* transgenic lines in the time course analyses considering that they each showed distinct expression patterns. Specific time points were chosen throughout fin regeneration to characterize transgene expression: 1 and 2 dpa time points represent wound epidermis and blastema formation respectively, the 3 dpa time point reflects the early regenerative outgrowth stage and the 7 dpa time point represents a later time point of the regenerative outgrowth stage.

As previously mentioned, endogenous *and1* expression has been characterized throughout fin regeneration to be restricted at the distal margin of the fin, including the tip of lepidotrichia and the inter-ray tissue; *and1* expression were present in both the distal mesenchymal and distal basal epidermal layer cells of the fin regenerates. *and1*

expression is first detected by *in situ* hybridization at 2 dpa, thus *and1* expression is present during the formation of the blastema but absent during the formation of the wound epidermis (Zhang et al, 2010; Wagh thesis, unpublished). We examined whether the transgenic reporter lines would reflect endogenous *and1* expression during the early stages of fin regeneration, which include the formation of the wound epidermis and the blastema. After amputation, we observed very faint transgene expression in the region corresponding to the wound epidermis of the *Tg(2P+I:EGFP)* lines and epidermal-specific *Tg(Epi-EI:EGFP)* line at 1 dpa (Fig 3.2.7a,c). At 2 dpa, strong transgene expression was observed in the inter-ray tissue of these two transgenic lines (Fig 3.2.7d,f). In contrast, transgene expression in the *Tg(2PΔepi:EGFP)* line started at 2 dpa and was located at the distal end of each lepidotrichia stumps (Fig 3.2.7b,e). There is definitely a discrepancy between the time at which endogenous *and1* expression starts according to *in situ* hybridization experiments (2dpa) versus the time at which transgene expression starts in the *Tg(2P+I:EGFP)* and *Tg(Epi-EI:EGFP)* lines (1dpa).

Similarly, another discrepancy was observed between the location where endogenous *and1* and *egfp* are expressed during the formation of the blastema. Previously, endogenous *and1* expression was predominantly observed at the tip of each lepidotrichia stumps and in the inter-ray tissue of the fin regenerate. In contrast, transgene expression was predominantly identified in the inter-ray tissue of the *Tg(2P+I:EGFP)* and *Tg(Epi-EI:EGFP)* lines, which is odd since the 2P+I includes the 2PΔepi region and the *Tg(2PΔepi:EGFP)* lines showed transgene expression only at the tip of each lepidotrichia stumps. Perhaps there are positional effects causing the *Tg(2P+I:EGFP)*

lines to not efficiently express *egfp* at the tip of each lepidotrichia stumps during blastema formation.

It is worthy to note that even though two *Tg(2P+I:EGFP)* transgenic lines similarly showed strong expression in the inter-ray tissues of the fin regenerate, they both differed in the time at which expression was first observed during early fin regeneration; one transgenic line started showing very faint expression at 1 dpa and the other started showing expression at 2 dpa. With regards to the *Tg(2PΔepi:EGFP)* lines, they seemed to accurately recapitulate previously characterized endogenous *and1* expression at the tip of each lepidotrichia stumps of the fin regenerate in a spatial and temporal manner. As for the *Tg(Epi-EI:EGFP)* line, transgene expression was first observed in the region corresponding to the wound epidermis at 1 dpa and showed strong transgene expression at 2 dpa in the inter-ray tissue of the fin regenerate, however only one transgenic line was at our possession to perform these analyses at this point. Further *and1* reporter lines need to be analyzed to confirm these results.

Seemingly, transgene expression from the *Tg(2P+I:EGFP)* and *Tg(Epi-EI:EGFP)* lines does not seem to temporally recapitulate previously characterized endogenous *and1* expression during early fin regeneration. As a reminder, *and1* expression is first detected at 2 dpa by *in situ* hybridization during blastema formation (Zhang et al, 2010). Perhaps the *in situ* hybridization method previously used to detect endogenous *and1* expression during the early stages of fin regeneration was unable to detect the possible presence of lowly expressed *and1* specifically during the formation of the wound epidermis. Therefore, *in situ* hybridization experiments need to be redone on cryo-sections of fin regenerates during the formation of the wound epidermis and need to be coupled with a

proteinase K treatment to further permeabilize the tissues and accessibility of the probe to confirm whether *and1* is expressed during the formation of the wound epidermis. Furthermore, RT-PCR experiments can also be performed in parallel on the wound epidermis of the fin regenerate to better detect the presence of low *and1* expression.

During the early stage of regenerative outgrowth (3dpa), strong transgene expression was observed in the *Tg(2P+I:EGFP)*, *Tg(2PΔepi:EGFP)* and *Tg(Epi-EI:EGFP)* lines starting from the cut site (Fig 3.2.8). Transgene expression in the mesenchymal-specific *Tg(2PΔepi:EGFP)* lines remained at the distal end of each regenerating lepidotrichia (Fig 3.2.8d) while transgene expression in the epidermal-specific *Tg(Epi-EI:EGFP)* line remained in the inter-ray tissue of the fin regenerate (Fig 3.2.8g). All the while, the reporter expression patterns from the *Tg(2PΔepi:EGFP)* and *Tg(Epi-EI:EGFP)* lines were simultaneously observed in the *Tg(2P+I:EGFP)* lines on whole-mount analyses (Fig 3.2.8a). In order to observe the specific location of EGFP and confirm the tissue specificity of the tissue-specific transgenic lines, longitudinal cryo-sections of 3 dpa fin regenerates were generated for each transgenic line (n=6 fin regenerates/line). Longitudinal 3 dpa sections of the *Tg(2PΔepi:EGFP)* lines showed EGFP limited to the mesenchyme and distal blastema (Fig 3.2.8e-f), and the *Tg(Epi-EI:EGFP)* lines showed EGFP limited to the distal basal epidermal layer of the fin regenerate (Fig 3.2.8h-i). As for the *Tg(2P+I:EGFP)* lines, they recapitulated both tissue-specific expression simultaneously in the mesenchyme, the distal blastema and the distal basal epidermal layer of the fin regenerate (Fig 3.2.8b-c). Double immunostaining experiments performed on longitudinal sections of 3 dpa *Tg(2P+I:EGFP)* fin regenerates (n=4) revealed EGFP to be located in mesenchymal cells adjacent to osteoblasts and in

the distal part of the basal epidermal layer (Fig 3.2.9). No co-localization was found between the two proteins. Taken together, this confirms that the tissue-specific lines recapitulate tissue-specific endogenous *and1* expression during early regenerative outgrowth (Fig 1.6e). Furthermore, the *Tg(2P+I:EGFP)* lines recapitulate endogenous *and1* expression at this stage.

During the later phase of regenerative outgrowth (7-8 dpa), mesenchymal transgene expression was observed all along the proximodistal length of regenerating lepidotrichia in the *Tg(2P+I:EGFP)* and *Tg(2PΔepi:EGFP)* lines on whole-mount and longitudinal 7 dpa cryo-sections (n=6 fin regenerates) (Fig 3.2.10a-f). It is worth mentioning that the *Tg(2P+I:EGFP)* lines had stronger mesenchymal transgene expression all along the length of regenerating fin rays starting from the cut site compared to the *Tg(2PΔepi:EGFP)* lines, which showed transgene expression all along the length of regenerating fin rays as well but started from a segment or two more distal of the cut site. In contrast, transgene expression located in the basal epidermal layer of the fin regenerate remained distal in the *Tg(2P+I:EGFP)* and *Tg(Epi-EI:EGFP)* lines throughout fin regeneration (Fig 3.2.10a-c,g-i). Taken together, this confirms that expression in the tissue-specific lines remain tissue-specific throughout the regeneration process. Furthermore, the *Tg(2P+I:EGFP)* lines recapitulate the expression patterns observed in both of the tissue-specific transgenic lines throughout fin regeneration.

The "trail" of EGFP located all along the proximodistal length of regenerating lepidotrichia persisted until the end of the time course (14 dpa) in the *Tg(2P+I:EGFP)* and *Tg(2PΔepi:EGFP)* lines. However, the intensity of EGFP was observed to slowly and gradually disappear in a proximal-to-distal direction along the fin rays after 14 dpa

(data not shown). We assume this proximodistal disappearance of EGFP eventually results in the distally restricted expression domains we observe in the intact fins of the various *and1* reporter lines. Nonetheless, the "trail" of EGFP located all along the length of regenerating fin rays is puzzling since our lab previously characterized *and1* expression to remain distally restricted in the fin regenerate throughout the fin regeneration process (Wagh thesis, unpublished). We can confirm whether the *Tg(2P+I:EGFP)*, *Tg(2PΔepi:EGFP)* and *Tg(Epi-EI:EGFP)* lines accurately represent endogenous *and1* expression throughout fin regeneration by examining thoroughly endogenous *and1* expression during the late phase of regenerative outgrowth.

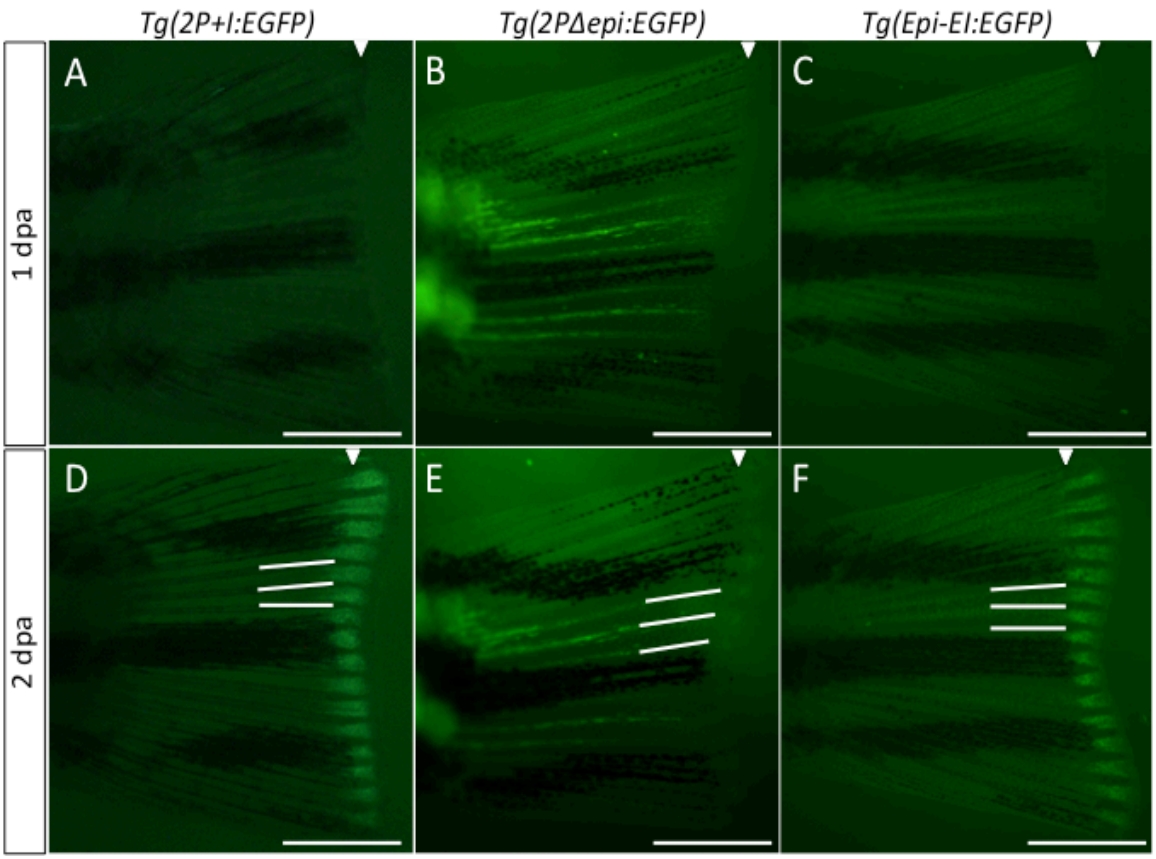


Figure 3.2.7. Reporter gene expression in the various *and1* reporter lines during wound epidermis and blastema formation in the course of fin regeneration. Panels **a-c** show 1 dpa fin regenerates during the wound epidermis formation stage and panels **d-f** show 2 dpa fin regenerates during the blastema formation stage. Panels **a** & **d** show fin regenerates of a *Tg(2P+I:EGFP)* line, panels **b** & **e** show fin regenerates of a *Tg(2PΔepi:EGFP)* line and panels **c** & **f** show fin regenerates of the *Tg(Epi-EI:EGFP)* line. White triangles show the level of amputation and white lines show the location of fin rays. Scale bar= 2 mm.

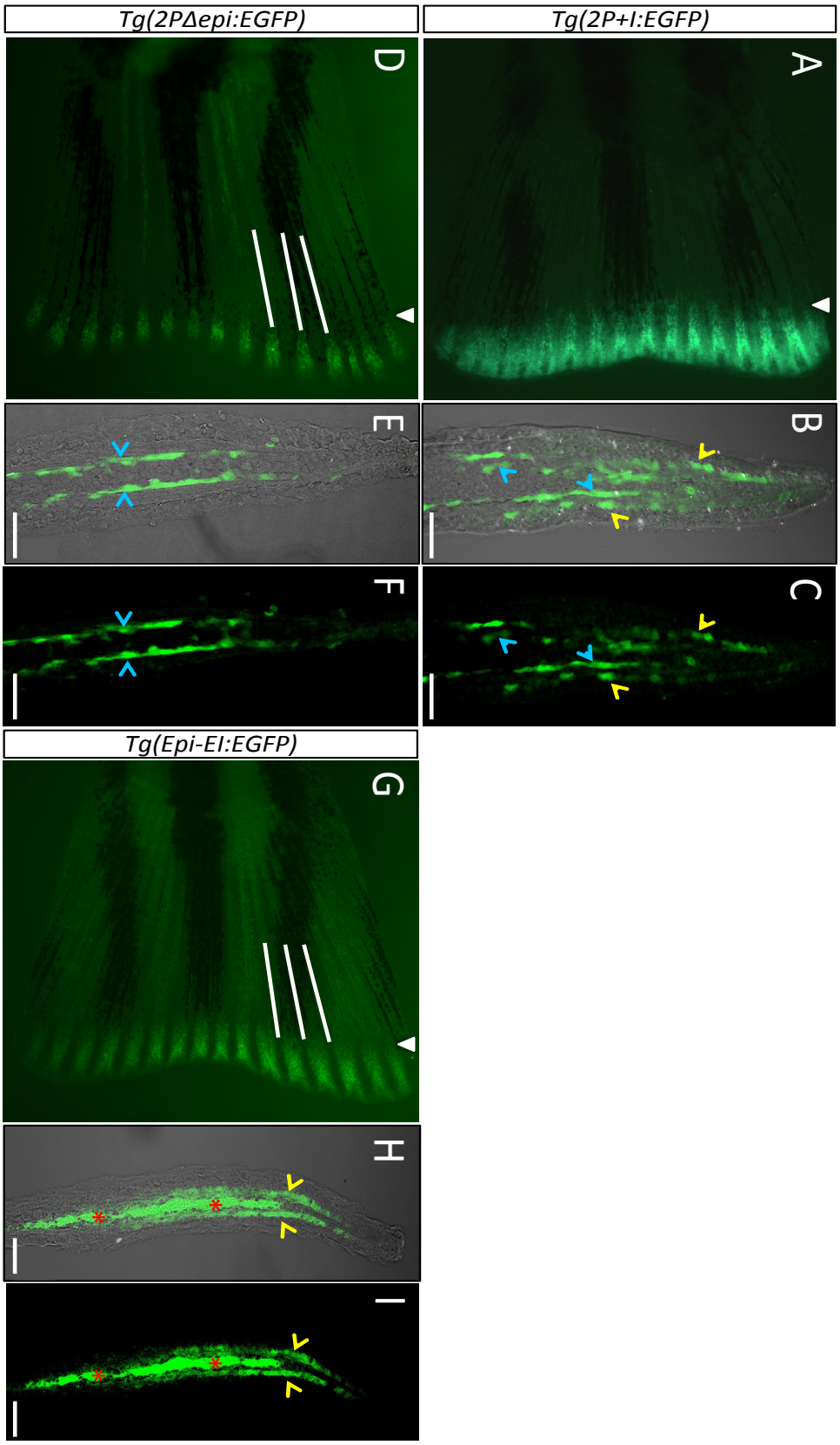

Tg(2PΔepi:EGFP)
Tg(2P+I:EGFP)
D
A
E
B
F
C
Tg(Epi-EI:EGFP)
G
H
I

Figure 3.2.8. Reporter gene expression in the various *and1* reporter lines during early regenerative outgrowth. Panels **a, d** & **g** show whole-mount pictures of 3 dpa fin regenerates, panels **c**, **f** & **i** show longitudinal sections of the fin regenerate (UV channel only) and panels **b**, **e** & **h** show the merged pictures of the sections with brightfield images of the various *and1* reporter lines. Panels **a-c** show transgene expression occurring in the mesenchyme (blue arrow heads) and the basal epidermal layer (yellow arrow heads) of *Tg(2P+I:EGFP)* fin regenerate. Panels **d-f** show transgene expression restricted in the mesenchyme of the fin regenerate of the *Tg(2PΔepi:EGFP)* line (blue arrow heads). Panels **g-i** show transgene expression restricted in the basal epidermal layer (yellow arrow heads) of the *Tg(Epi-EI:EGFP)* line. White triangles show the level of amputation and white lines show the location of fin rays. *= autofluorescence from red blood cells. Scale bar = 40 μm.

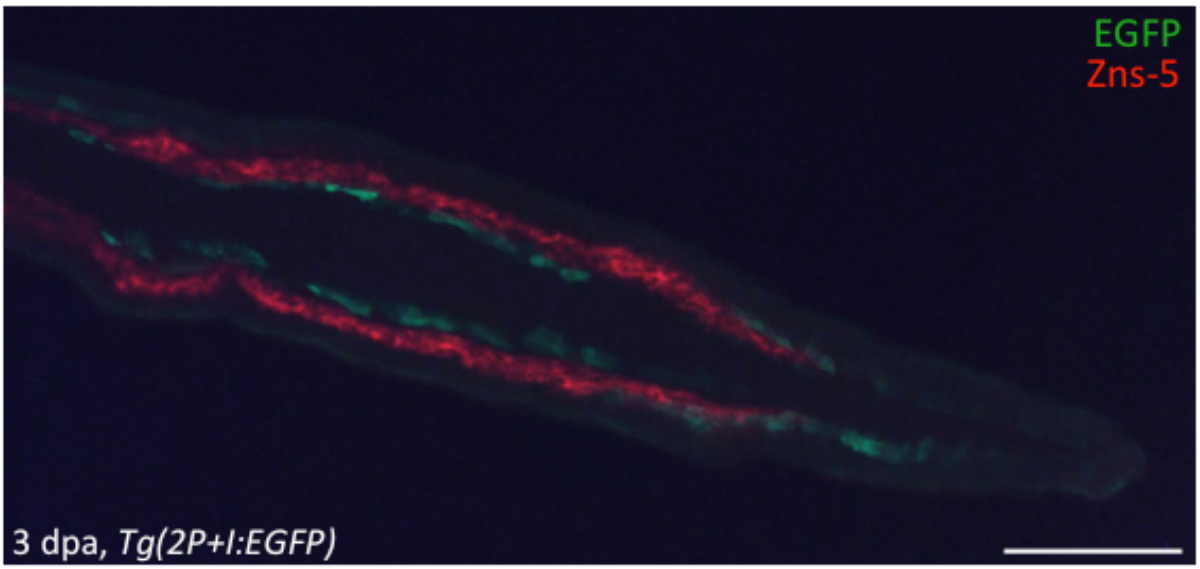


Figure 3.2.9. EGFP from the *Tg(2P+I:EGFP)* line is located beside osteoblasts during fin regeneration. Immunostaining for Zns-5 and EGFP on a 3 dpa longitudinal cryo-section of *Tg(2P+I:EGFP)* fin regenerates reveals EGFP to be located on the inner side of lepidotrichia hemi-rays and in the basal epidermal layer of the fin regenerate. The protein were located in separate domains and showed no co-localization. Scale bar= 100 μm.

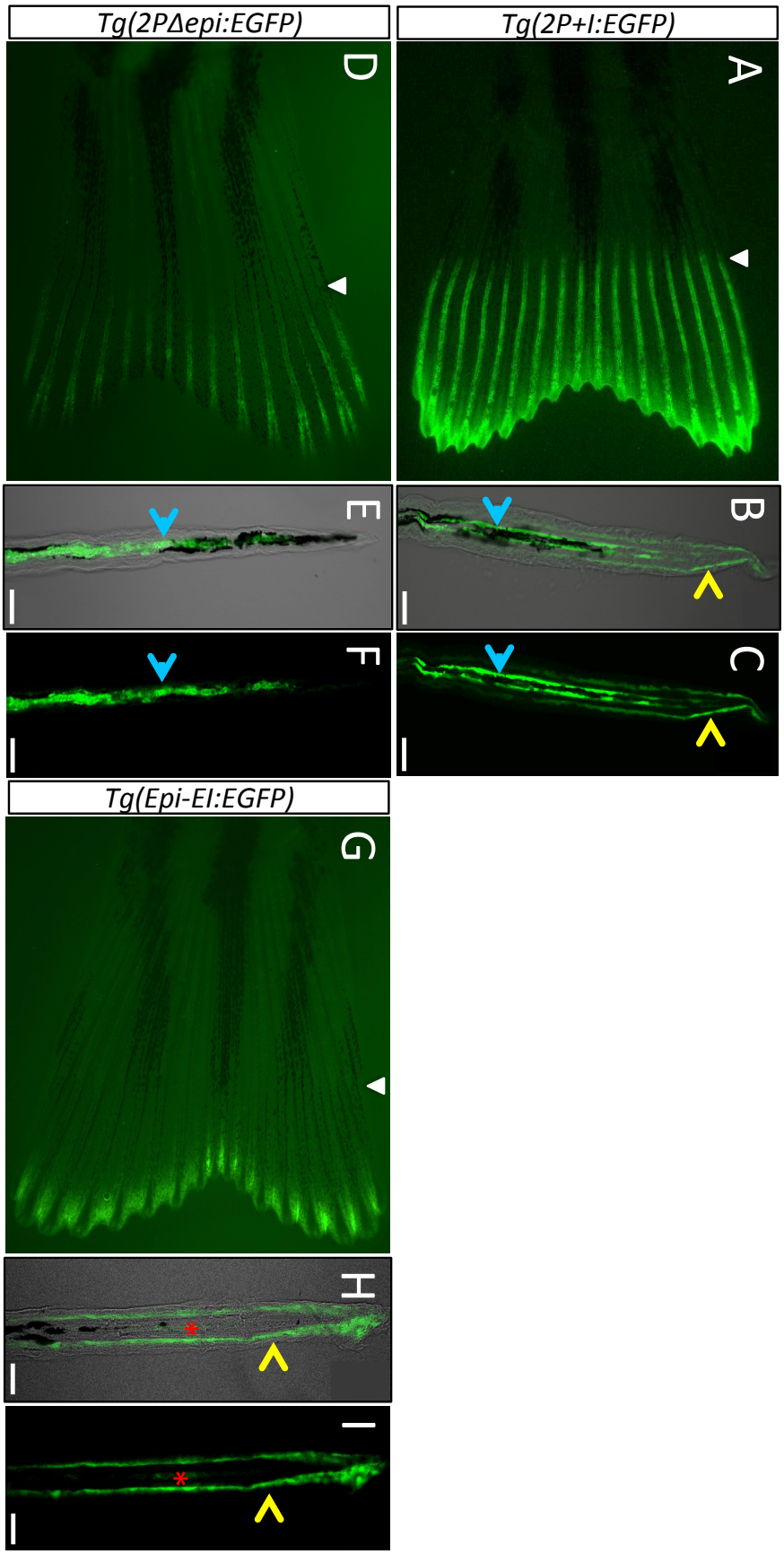

Tg(2PΔepi:EGFP)
Tg(2P+I:EGFP)
D
A
E
B
F
C
Tg(Epi-EI:EGFP)
G
H

Figure 3.2.10. Reporter gene expression in the various *and1* reporter lines during late regenerative outgrowth. Panels **a, d** & **g** show whole-mount pictures of 7 dpa fin regenerates, panels **c**, **f** & **i** show longitudinal sections of the fin regenerate (UV channel only) and panels **b**, **e** & **h** show the merged pictures of the sections with brightfield images of the various *and1* reporter lines. Panels **a-c** show transgene expression occurring in the mesenchyme (blue arrow heads) and the basal epidermal layer (yellow arrow heads) of *Tg(2P+I:EGFP)* fin regenerate. Furthermore, transgene expression occurs all along the proximodistal length of the regenerating fin rays starting from the cut site. Panels **d-f** show transgene expression restricted in the mesenchyme of the fin regenerate of the *Tg(2PΔepi:EGFP)* line (blue arrow heads). Transgene expression is located all along the proximodistal length of the regenerating fin rays starting ~2 segments distal from the cut site. Panels **g-i** show transgene expression restricted in the distal basal epidermal layer (yellow arrow heads) of the *Tg(Epi-EI:EGFP)* line. White triangles show the level of amputation and white lines show the location of fin rays. *= autofluorescence from red blood cells. Scale bar= 40 μm.

3.2.3. Characterization of actinotrichia, endogenous and1 expression and and1 reporter expression during the late phase of regenerative outgrowth

To explain the presence of EGFP all along the proximodistal length of the regenerating fin rays in the *Tg(2P+I:EGFP)* and *Tg(2PΔepi:EGFP)* lines, we found it was appropriate to first examine the location of actinotrichia in 7 dpa longitudinal cryo-sections of WT fin regenerates (n=12) by performing an And1/2 immunostaining. It revealed that actinotrichia remained distal at 7 dpa (Fig 3.2.11). In fact, actinotrichia have been previously characterized through radioautographic studies to remain distal in the fin regenerate throughout the fin regeneration process (Marí-Beffa, Carmona & Becerra, 1989). Therefore, we ruled out the possibility that actinotrichia may be located all along the proximodistal length of lepidotrichia as a potential reason as to why EGFP is present all along the length of regenerating fin rays in the *Tg(2P+I:EGFP)* and *Tg(2PΔepi:EGFP)* lines. We then focused on the possibility that endogenous *and1* expression may actually be present in the proximal region of the fin regenerate, unlike previously reported by our lab, which included whole-mount *in situ* hybridization and *in situ* hybridization on transverse cryo-sections of the fin regenerate all performed without a proteinase K treatment (Wagh thesis, unpublished).

Longitudinal cryo-sections of 7 dpa WT fin regenerates (n=6) were produced and *in situ* hybridization experiments were performed without and with PK treatments to examine whether any *and1* expression could be detected by this method in the proximal region of the fin regenerate. A proteinase K treatment further permeabilizes the tissue and increases probe accessibility. *In situ* hybridization experiments performed without PK treatment showed the majority of sections (188/192) only had *and1* expression located in

the distal region of the fin regenerate (Fig 3.2.12). However, a small number of sections (4/192) showed the presence of *and1* expression all along the proximodistal length of the regenerating fin ray (Fig 3.2.13). We next performed *in situ* hybridization experiments with a proteinase K treatment, and the majority of sections (119/125) still had *and1* expression located only in the distal region of the fin regenerate. Regardless, a small number of sections (6/125) showed *and1* expression located all along the length of the fin regenerate (Fig 3.2.14). It is likely that *and1* expression may have remained undetected in previous *in situ* hybridization experiments due to the lack proteinase K treatments at the time. These results suggest that endogenous *and1* expression may actually be expressed all along the proximodistal length of the regenerating fin rays during fin regeneration. However, *and1* may be expressed in lower levels in the proximal part of the fin regenerate.

To further confirm this, we found necessary to perform RT-PCR to detect possible low-level *and1* expression in the distal and proximal part of 7 dpa fin regenerates and intact fins of WT zebrafish as a control. The caudal fin of WT zebrafish (n=10) were amputated using a standard cut and left to regenerate for 7 days. At 7 dpa, the fin regenerates were collected and cut into proximal and distal regions as specified in Fig 3.2.15a. As for the intact fins of WT fish, the proximal and distal regions were cut similarly to the 7 dpa fin regenerates and collected as specified in Fig 3.2.15b. The central fin rays, which are very short, were removed from this analysis to avoid potential contamination of proximal samples by the distal regions containing actinotrichia (Fig 3.2.15a-b).

Total RNA extraction was performed on the distal and proximal regions of the fin regenerates and intact fins to produce cDNA via reverse transcriptase reaction. RT-PCR was performed using custom primers directed against a ~200bp region of the *and1* gene (where one primer was located on an exon-exon boundary as an extra measure against the detection of genomic DNA contamination) on the cDNA of the distal and proximal regions of the fin regenerates and intact fins. To rule out genomic DNA contamination for each sample, a PCR was performed on the DNase-treated RNA that was subsequently used for RT reactions. Furthermore, a negative control that consists of water and a β-actin control (that acts as reaction control) were included. The data was not quantified since we only needed to determine the presence versus absence of endogenous *and1* expression along the proximodistal length of the fin rays. According to the RT-PCR results, *and1* expression is located in both the distal and proximal regions of the fin regenerate (Fig 3.2.15c). In contrast, the intact fins showed the presence of *and1* expression only in the distal region of the fin ray, which reflects the observations made in the intact fins of the various *and1* reporter lines. All in all, endogenous *and1* expression is present all along the proximodistal length of regenerating fin rays at 7dpa during fin regeneration.

To further confirm that the *and1* reporter lines have the same expression pattern as *and1* during fin regeneration, we performed double fluorescence *in situ* hybridization experiments (FISH) on longitudinal 3 and 7 dpa cryo-sections of the fin regenerates (n=8) of the *Tg(2P+I:EGFP)* lines using antisense RNA probes directed against *egfp* and *and1*. The double FISH on 3 dpa longitudinal sections revealed complete co-localization of the expression domains of *egfp* and *and1* in both the mesenchyme (including the blastema cells) and the distal basal epidermal layer of the fin regenerate (Fig 3.2.16). Complete co-

localization was also similarly observed in 7 dpa longitudinal sections in both cell types as well (Fig 3.2.17). With everything considered, we confirmed that the *Tg(2P+I:EGFP)*, *Tg(2PΔepi:EGFP)* and *Tg(Epi-EI:EGFP)* lines may actually be representative of endogenous *and1* expression throughout fin regeneration.

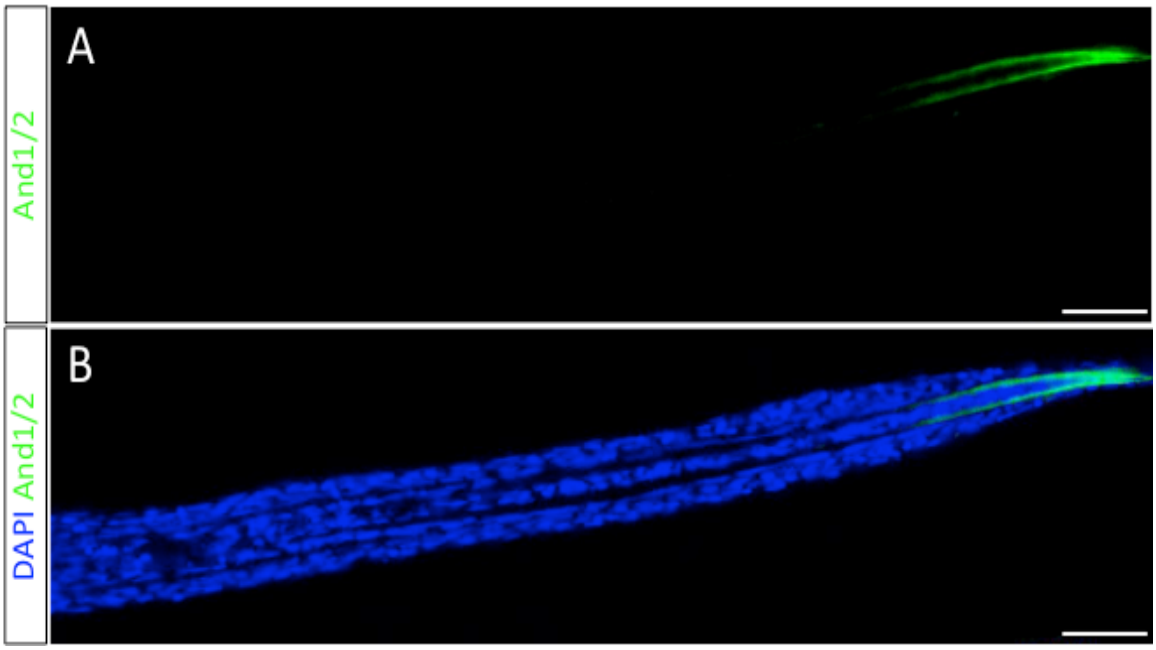


Figure 3.2.11. Actinotrichia remain distally restricted throughout fin regeneration. **a.** Immunostaining for And1/2 in 7 dpa longitudinal sections of WT fin regenerate show that actinotrichia are distally restricted throughout fin regeneration. **b.** Image merged with a DAPI counterstain. Scale bars= 40 μm.

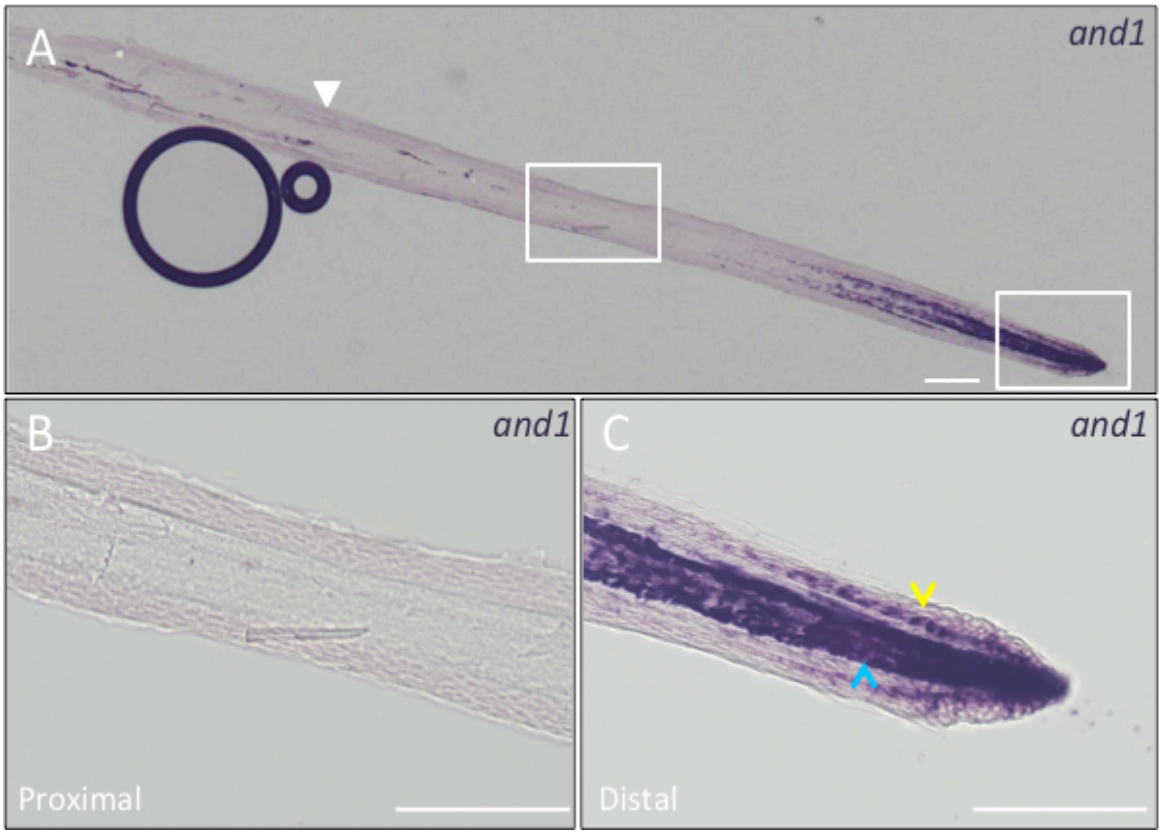


Figure 3.2.12. The majority of sections (188/192) from 7dpa WT fin regenerates showed a distally restricted *and1* expression domain when not PK-treated. a. Whole view of a longitudinal 7dpa WT sections where *and1* expression is distally restricted in the fin regenerate. The white boxes show the location at which the proximal and distal high magnifications images were taken. **b.** A high magnification of the proximal end of the fin regenerate shows no detection of the *and1* gene. **c.** A high magnification of the distal end of the fin regenerate shows *and1* expression located in both the basal epidermal layer (yellow arrow head) and mesenchyme (blue arrow head). A white triangle indicates the plane of amputation. Scale bars= 100 μm.

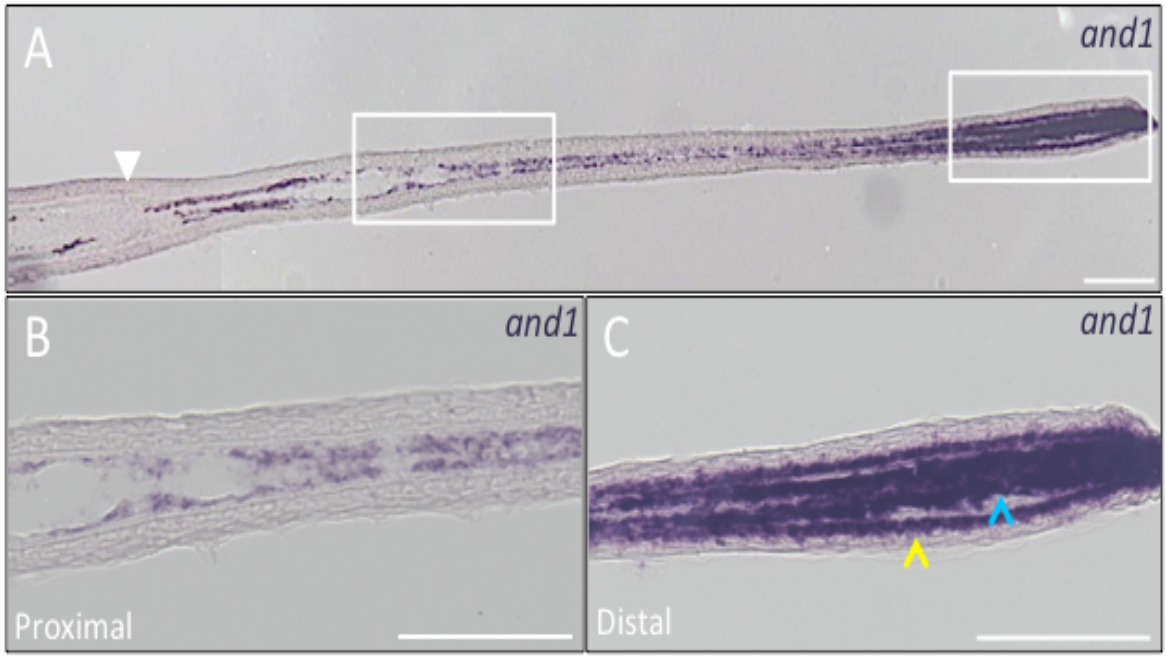


Figure 3.2.13. The minority of sections (4/192) from 7dpa WT fin regenerates showed *and1* expression all along the proximodistal length of the regenerating fin rays when not PK-treated. a. Whole view of a longitudinal 7dpa WT sections where *and1* expression is present all along the proximodistal length of the fin regenerate. The white boxes show the location at which the proximal and distal high magnifications images were taken. **b.** A high magnification of the proximal end of the fin regenerate shows the presence of *and1* expression in the mesenchyme. **c.** A high magnification of the distal end of the fin regenerate shows *and1* expression located in both the basal epidermal layer (yellow arrow head) and mesenchyme (blue arrow head). A white triangle indicates the plane of amputation. Scale bars= 100 μm.

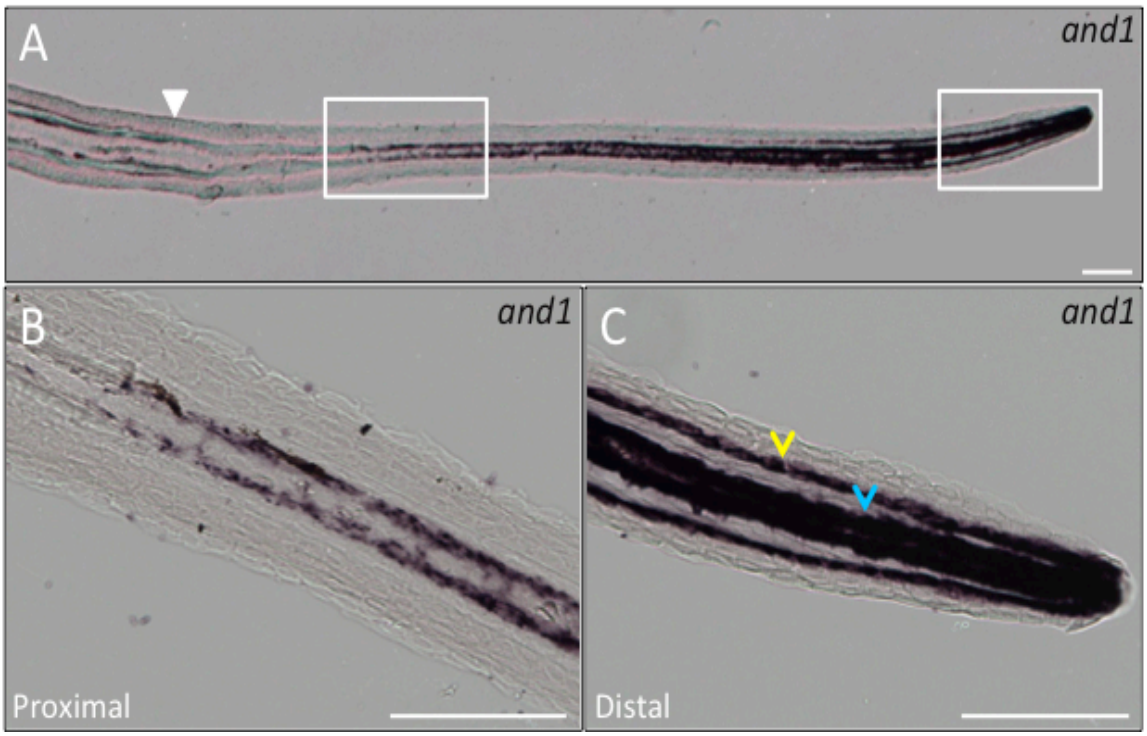


Figure 3.2.14. The minority of sections (6/125) from 7dpa WT fin regenerates showed *and1* expression all along the proximodistal length of the regenerating fin rays when PK-treated. a. Whole view of a longitudinal 7dpa WT sections where *and1* expression is present all along the proximodistal length of the fin regenerate. The white boxes show the location at which the proximal and distal high magnifications images were taken. **b.** A high magnification of the proximal end of the fin regenerate shows the presence of *and1* expression in the mesenchyme. **c.** A high magnification of the distal end of the fin regenerate shows *and1* expression located in both the basal epidermal layer (yellow arrow head) and mesenchyme (blue arrow head). A white triangle indicates the plane of amputation. Scale bars= 100 μm.

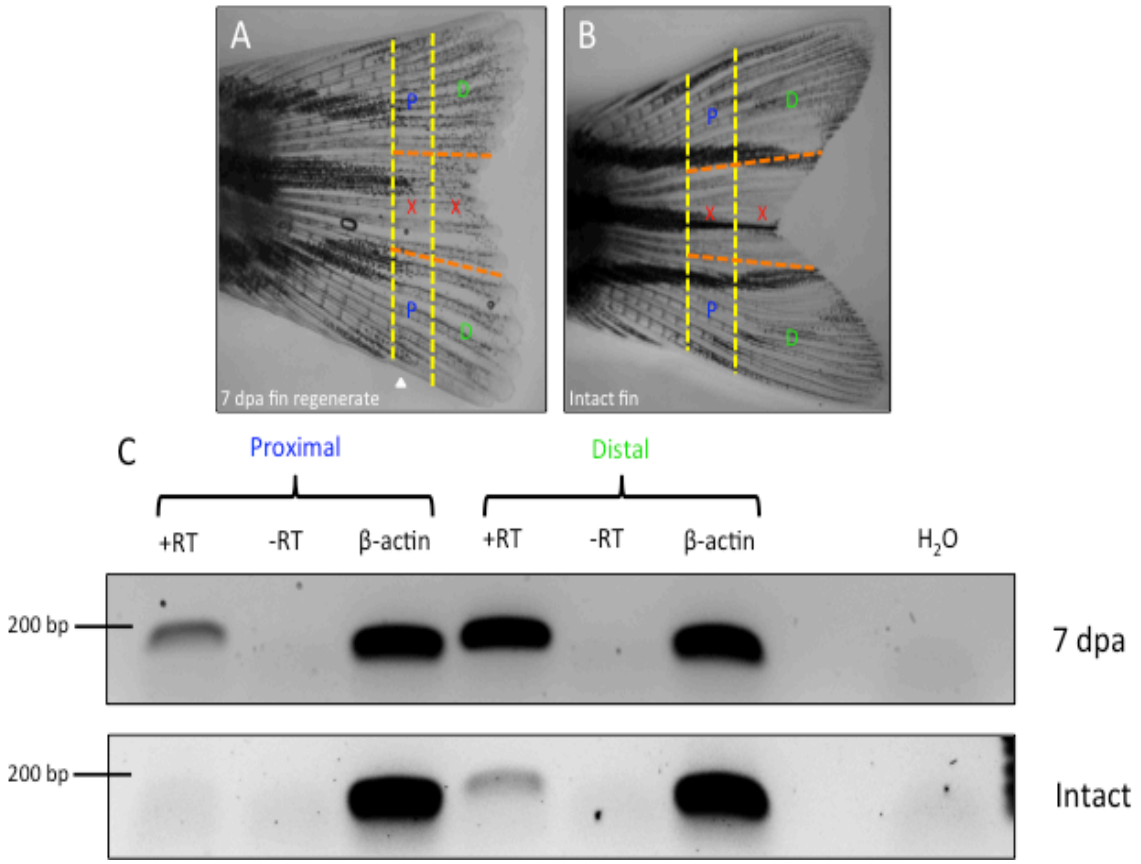


Figure 3.2.15. The *and1* gene is expressed all along the proximodistal length of regenerating fin rays during late regenerative outgrowth according to RT-PCR results. The 7 dpa fin regenerate (panel **a**) and intact caudal fin (panel **b**) of WT zebrafish were cut to produce 6 regions. **a.** For 7 dpa fin regenerate 2 cuts were made along the dorsoventral axis of the fin regenerate (indicated by yellow dotted lines) to delimit the proximal and distal region of the fin regenerate (indicated by P's and D's respectively): one located a segment below the initial standard cut (indicated by a white triangle) and one located 2 segments after the initial standard cut. Two proximodistal cuts (indicated by orange dotted lines) were made to omit the regions that contain shorter fin rays (indicated by red X's). **b**. The intact fins of WT zebrafish were cut in the same way as the 7 dpa fin regenerates, excepted the most proximal dorsoventral cut was made a segment below the first lepidotrichia bifurcation and the second dorsoventral cut was made 4 segments after the first cut. **c.** RT-PCR results for the proximal and distal regions of the 7 dpa fin regenerates and intact caudal fin of zebrafish. RT-PCR was performed using custom primers directed against a ~200bp region of the *and1* gene (+RT) and compared to DNase-treated/RT-untreated total distal or proximal RNA to rule out genomic DNA contamination (-RT). The negative controls consist of water and β-actin controls were included as reaction controls. The data was not quantified since we investigated the presence versus absence of endogenous *and1* expression along the proximodistal length of the fin rays.

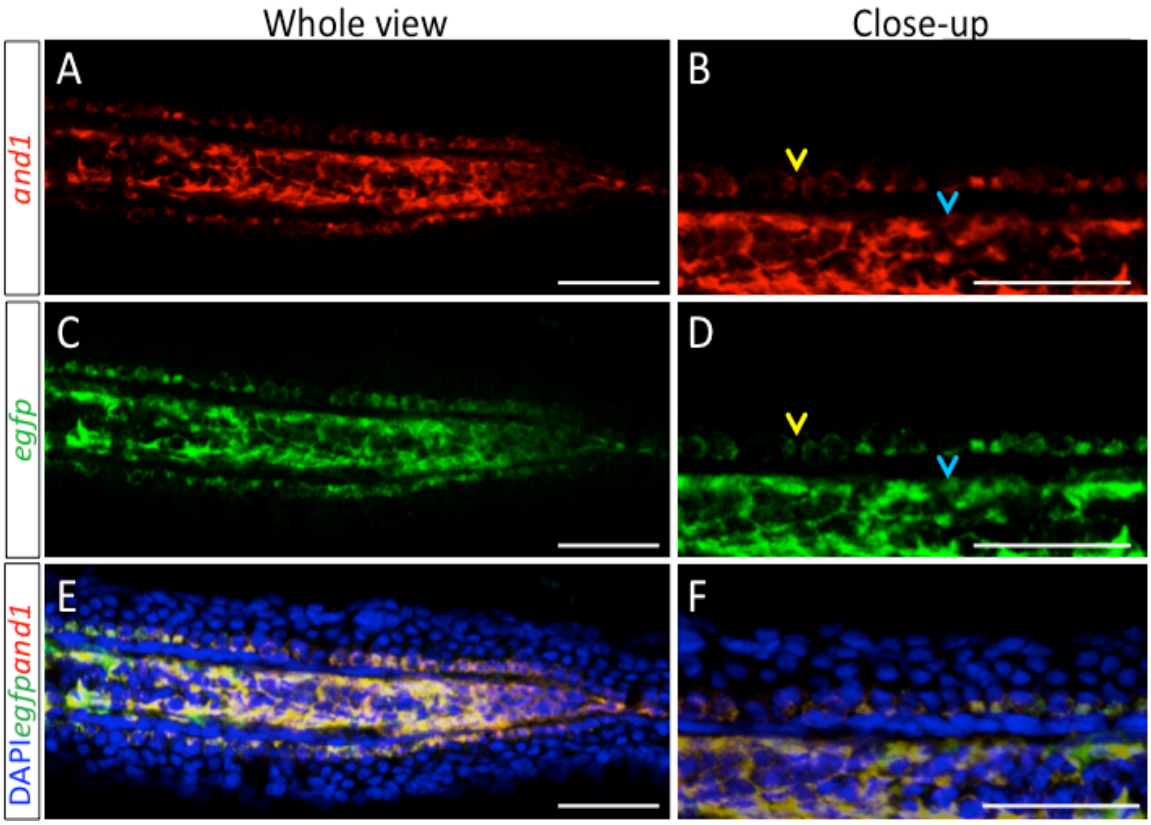


Figure 3.2.16. *and1* and *egfp* expression co-localize in the mesenchyme and basal epidermal layer of the fin regenerate during early regenerative outgrowth. Double fluorescence *in situ* hybridization was performed on 3 dpa *Tg(2P+I:EGFP)* longitudinal cryo-sections. Panels **a, c** & **e** show the whole view and panels **b, d** & **f** show high magnification views of the fin regenerate. Panels **a-b** show the *and1* expression domain and panels **c-d** show the *egfp* expression domain. Both expression domains are found in the mesenchyme (blue arrow heads) and basal epidermal layer (yellow arrow heads) of the fin regenerate. Panels **e-f** are the merged images of the *and1* and *egfp* expression domains, which show complete co-localization. Images were merged with a DAPI counterstain. Scale bars= 20 μm.

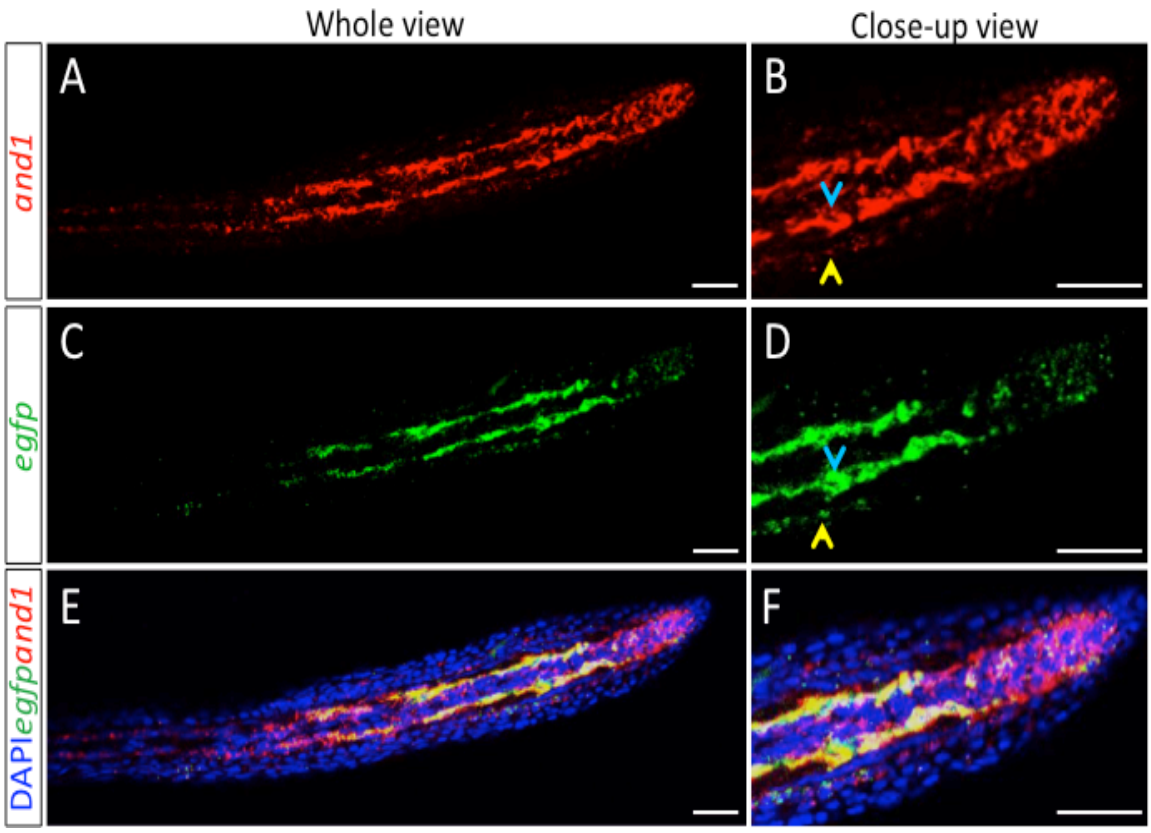


Figure 3.2.17. *and1* and *egfp* expression co-localize in the mesenchyme and basal epidermal layer of the fin regenerate during late regenerative outgrowth. Double fluorescence *in situ* hybridization was performed on 7 dpa *Tg(2P+I:EGFP)* longitudinal cryo-sections. Panels **a, c** & **e** show the whole view and panels **b, d** & **f** show high magnification views of the fin regenerate. Panels **a-b** show the *and1* expression domain and panels **c-d** show the *egfp* expression domain. Both expression domains are found in the mesenchyme (blue arrow heads) and basal epidermal layer (yellow arrow heads) of the fin regenerate. Panels **e-f** are the merged images of the *and1* and *egfp* expression domains, which show complete co-localization. Images were merged with a DAPI counterstain. Scale bars= 80 μm.

3.2.4. Presence of egfp-expressing cells around the veins of the fin regenerate

During the time course analyses of the various *and1* reporter lines throughout the regeneration process, we noticed the presence of *egfp*-expressing cells located outside of the fin rays, in the inter-ray tissue, usually starting after the third newly formed segment and extending to the end of the fin regenerate, in the *Tg(2P+I:EGFP)* and *Tg(2PΔepi:EGFP)* lines (Fig 3.2.18a-b). Upon closer look, transverse cryo-sections of the *Tg(2PΔepi:EGFP)* fin regenerates (n=8) at 7 dpa allowed us to discern that *egfp*-expressing cells are located around the veins of the fin regenerate (Fig 3.2.18c). In order to have a better view and also to confirm the location of these cells in relation to the veins, a *Tg(2P+I:EGFP)* line was crossed with a *Tg(Fli1a:DsRed)* line, where Fli1a is a tissue-specific promoter that drives gene expression in endothelial cell of the blood vessels (Liu et al, 2008), to create a *Tg(2P+I:EGFP; Fli1a:DsRed)* double transgenic line. A time course analysis of this double transgenic line throughout the regeneration process allowed us to observe that *egfp*-expressing cells found in the inter-ray tissue did not co-localize with the endothelial cells of the blood vessels. In fact, transverse cryo-sections of the fin regenerate (n=6) of this transgenic line at 7dpa confirmed that these cells were located in proximity to the veins, more specifically on the abluminal surface of the veins and spanned between the 4-6 most distal segments of the fin rays (Fig 3.2.19). The specific role of the *egfp*-expressing cells found on the abluminal surface of the veins of the fin regenerate is unknown.

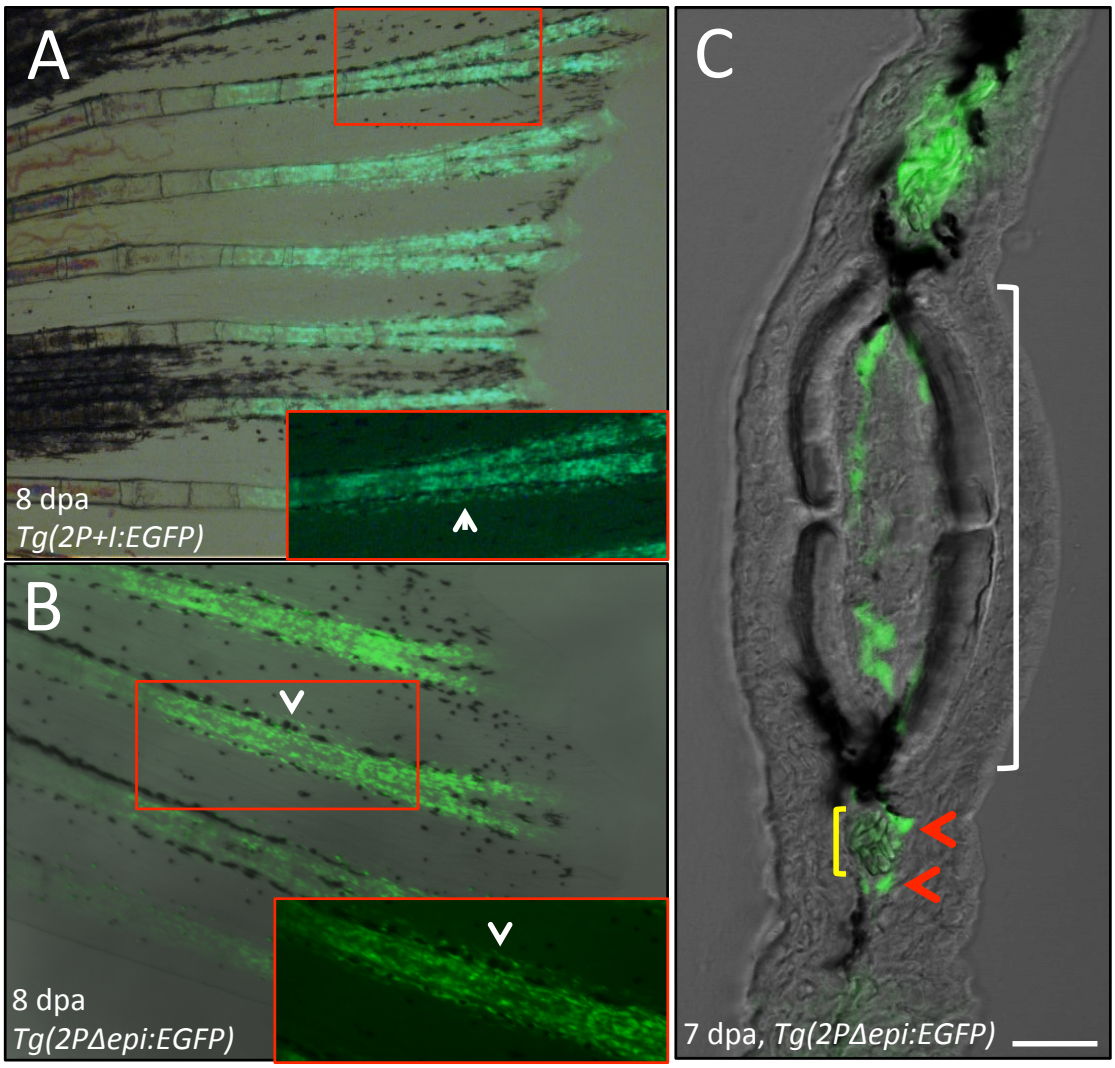


Figure 3.2.18. *egfp*-expressing cells are present in the inter-ray tissue of the *Tg(2P+I:EGFP)* and *Tg(2PΔepi:EGFP)* lines during fin regeneration. Panels **a** & **b** show *egfp*-expressing cells in the inter-ray tissue (white arrow heads) of *Tg(2P+I:EGFP)* and *Tg(2PΔepi:EGFP)* 8 dpa fin regenerates respectively. High magnification UV filter only images of the fins (location indicated by the red squares) are shown in the bottom right corner of the panels. **c.** Transverse cryo-section of 7 dpa *Tg(2PΔepi:EGFP)* fin regenerates show *egfp*-expressing cells (red arrow heads) closely located to a vein (indicated by a yellow bracket). The white bracket delimits a lepidotrichia. Scale bar= 20 μm.

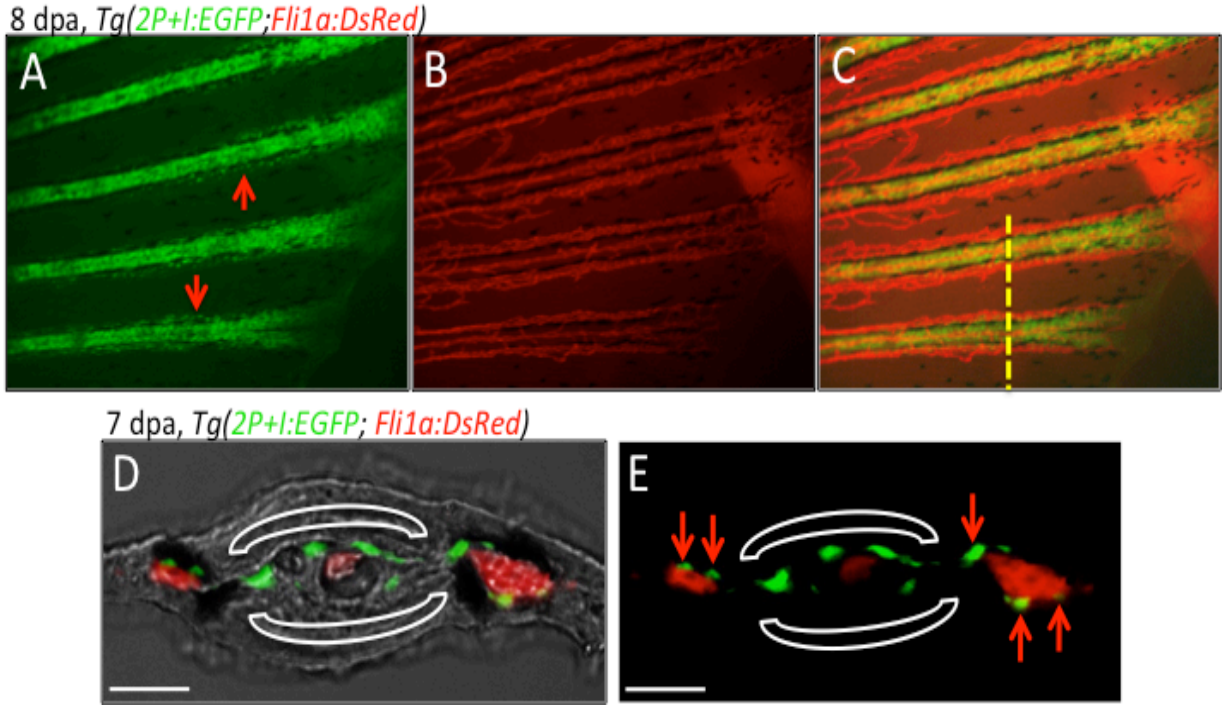


Figure 3.2.19. The *Tg(2P+I:EGFP; Fli1a:DsRed)* double transgenic line reveals *egfp*-expressing cells located on the abluminal surface of the veins in the fin regenerate. Panels **a** & **b** show the green and red fluorescence UV filters of a 8 dpa *Tg(2P+I:EGFP; Fli1a:DsRed)* fin regenerate respectively. Red arrows indicate *egfp*-expressing cells located in the inter-ray tissue of the fin regenerate. **c.** Merged image of the 8 dpa *Tg(2P+I:EGFP; Fli1a:DsRed)* fin regenerate. The yellow dotted line indicates the plane in which the following images were taken. **d.** Merged brightfield, green and red fluorescence filters image of a transverse section of 7 dpa *Tg(2P+I:EGFP; Fli1a:DsRed)* fin regenerate. Lepidotrichia hemirays are outlined in white. **e.** Merged green and red fluorescence filter image of the section. Red arrows indicate *egfp*-expressing cells located on the abluminal surface of the veins, which are labelled by red fluorescence. Scale bars= 20 μm.

Objective 3: Characterization of the cellular processes responsible of shaping the median fin fold throughout development

3.3.1. Cell death assays are inconclusive in determining whether apoptosis is responsible for the resorption of the median fin fold

The cellular mechanisms responsible for the resorption of the median fin fold during zebrafish fin development remain uncharacterized as of yet. As a reminder, the median fin fold resorption process occurs in an anterior-to-posterior direction as skeletal elements form in the presumptive caudal, anal and dorsal areas of the median fin fold; it involves the gradual resorption of the median fin fold tissues located between the developing unpaired fins (Fig 1.4) (Parichy et al, 2009). In our search to characterize the cellular mechanisms responsible for the resorption of the median fin fold, we drew inspiration from the amphibian models that undergo major tail metamorphosis throughout development. Since the loss of larva-specific features in amphibians is mainly caused by the activation of the apoptotic program via the action of the thyroid hormone in larval tissues, we found it was suitable to investigate whether apoptosis had any important role in the resorption of the median fin fold, a larva-specific structure in zebrafish (Tata, 1994). Two cell death assays were performed on whole-mount larvae undergoing different stages of the median fin fold resorption process: a propidium iodide (PI) staining experiment and a TUNEL assay.

The PI compound is unable to penetrate through intact cell membranes. For this reason, PI fluorescently labels cells whose cell membranes have been compromised by either late apoptotic processes or necrosis and it does so by intercalating into double-stranded nucleic acids (Crowley et al, 2016). We first performed PI staining on whole-

mount larvae undergoing different stages of the median fin fold resorption process (6.0 to 8.5 mm SL WT larvae) (n=5) in order to have an initial and global view of the cell death profile along the resorbing median fin fold.

PI-positive cells were detected along the distal edges of the median fin fold and the developing unpaired fins in most of the zebrafish larvae. Apoptosis has been previously studied in zebrafish embryos and likewise observed cell death only at the distal edges of the median fin fold (Cole & Ross, 2001). These PI-positive cells can be caused by the constant exposure of the fins to environmental abrasion. However, it is also possible that epithelial cells are constantly being renewed and distally migrate to eventually die and to be eliminated at the distal edge of the fin. We found that in most cases (n=4/5), excluding the distal edges of the fins, significant cell death did not occur in the resorbing median fin fold (Fig 3.3.1a-b). However in one case (n=1/5), PI-positive cells were observed in high numbers in the resorbing median fin of a 7.2 mm SL larva (Fig 3.3.1c-d). It is arguable whether these PI-positive cells are indeed late stage apoptotic cells since no PI-positive cells have been detected in the median fin fold in larvae undergoing later stages of the resorption process (8.0 and 8.5 mm SL larvae). Therefore, it is reasonable to state that the presence of PI-positive cells in the proximal area of the median fin fold of this one larva may be an anomaly rather than a genuine result. We found the PI staining experiment to be inconclusive in regards to finding whether apoptosis occurred in the resorbing median fin fold.

Since the PI staining protocol cannot differentiate between apoptotic and necrotic cells and seemed futile, we decided to proceed with *in situ* Terminal deoxynucleotidyl transferase dUTP Nick-End Labeling (TUNEL) assay instead on whole-mount larvae

undergoing different stages of the median fin fold resorption process; with and without a proteinase K treatment. The TUNEL assay relies on two components: first, the natural DNA fragmentation that occurs during late stage apoptosis and secondly, the use of the terminal deoxynucleotidyl transferase, an enzyme that adds dUTPs at the blunt ends of fragmented DNA. The TUNEL assay is able to detect the dUTPs that are added in fragmented DNA of dying cells (Garrity et al, 2003).

All larvae assayed without a PK treatment, except one, showed no TUNEL-positive cells in the resorbing median fin fold (n=14/15) (data not shown). One larva (1/15) showed TUNEL-positive cells on the edge of the median fin fold. When paired with a PK treatment to increase the penetrability of the detection compounds in the tissue, 57% of larvae assayed (n=23/40) had no TUNEL-positive cells in the resorbing median fin fold. However, 43% (n=17/40) of larvae had TUNEL-positive cells on the edges of the resorbing fin fold (Fig 3.3.2a-b) similarly to what was observed with the PI staining protocol. Only one larva had significant cluster-like TUNEL-positive cells in the resorbing median fin fold (Fig 3.3.2.c-d). Nonetheless, this result was infrequent and false-positives have been previously reported to occur when TUNEL assays are paired with a proteinase K treatment. A proteinase K treatment can produce artificial double strand breaks that may be detected through TUNEL assay (Garrity et al, 2003). All in all, TUNEL assays were inconclusive in determining whether apoptosis is responsible for the resorption of the median fin fold.

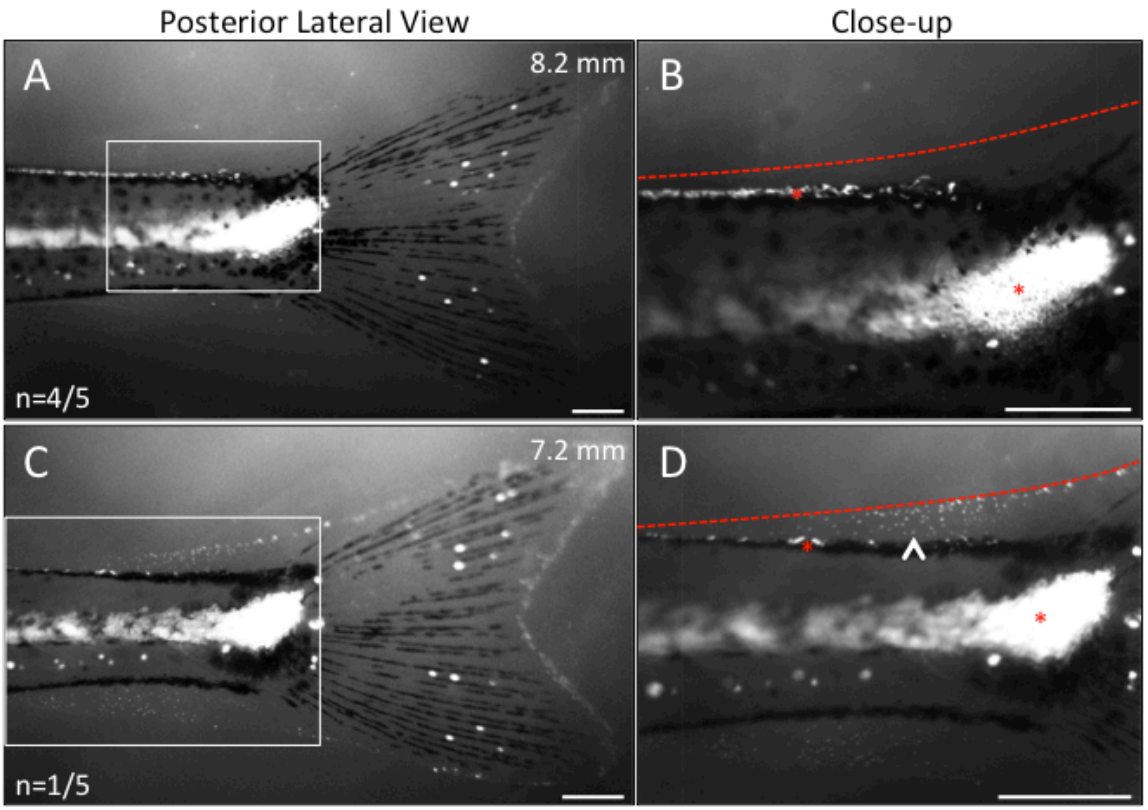


Figure 3.3.1. PI staining shows larvae do not have PI-positive cells in the resorbing median fin fold except in one instance. Panels **a** & **c** show the posterior lateral view of larvae and white boxes represent the high magnification images of the dorsal median fin fold shown in panels **b** & **d**. the SL of the displayed larvae are shown in the upper right corner of the panel and the number of larvae displaying the shown results is shown in the bottom left corner. The white arrow shows the presence of PI-positive cells all along the proximodistal length of the resorbing median fin fold. Red dotted lines delimit the dorsal resorbing median fin fold. *= Autofluorescence is present in pigment cells at the base of the median fin fold and in the lateral line. Scale bar= 200 μm.

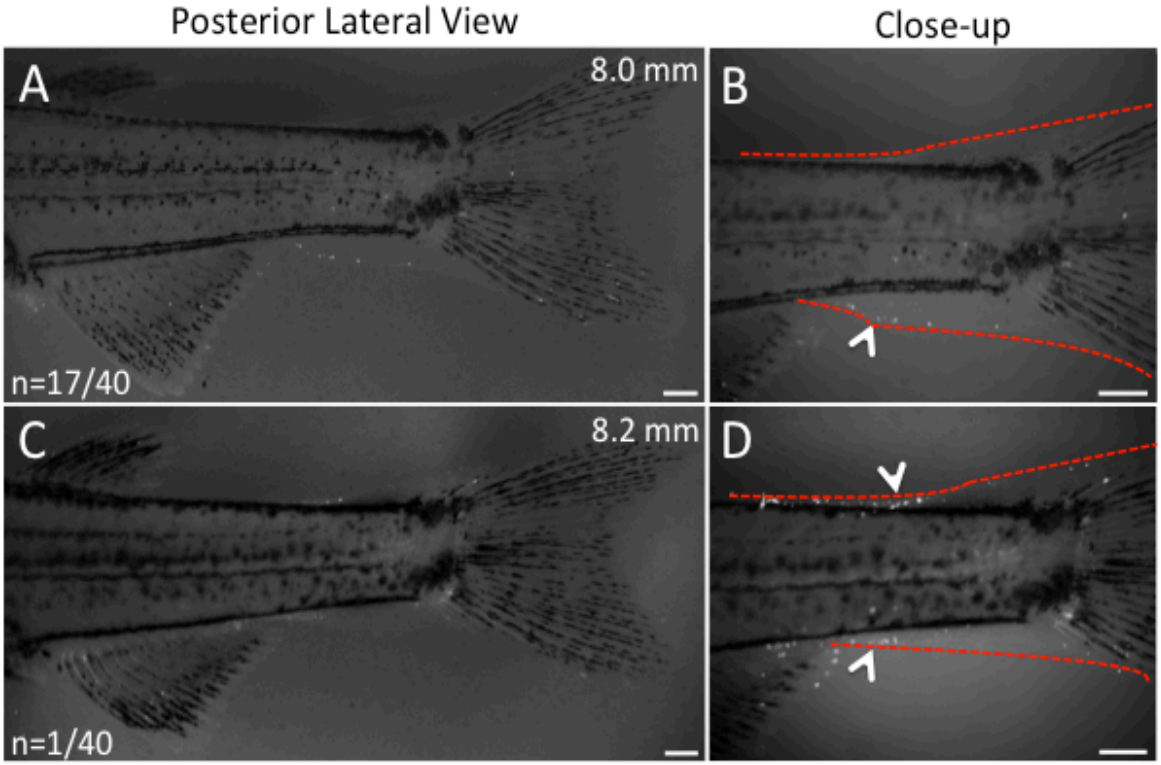


Figure 3.3.2. 43% of PK-treated larvae showed TUNEL-positive cells in the median fin fold during resorption. Panels **a** & **c** show the posterior lateral view of larvae and high magnification images of the dorsal median fin fold are shown in panels **b** & **d**. The SL of the displayed larvae are shown in the upper right corner of the panels and the number of larvae displaying the shown results is shown in the bottom left corner. White arrows show TUNEL-positive cells on the edges of the median fin fold (panels **a-b**) and in the median fin fold in clusters (panels **c-d**). Red dotted lines delimit the resorbing median fin fold. Scale bar= 200 μm.

3.3.2. The presence of macrophages in the median fin fold during its resorption suggests they may be implicated to a certain extent in the resorption of the median fin fold

Next, we wanted to examine whether macrophage digestion occurred in the resorbing median fin fold of zebrafish since it has been previously showed to be occurring following massive apoptosis in the tail of amphibians during metamorphosis (Kerr, Harmon & Searle, 1974). To do so, we acquired the *Tg(mpeg1:EGFP)* line from Dr. Berman's laboratory, which has been shown to drive transgene expression in macrophages of the zebrafish, and performed time course analysis of this transgenic line throughout the median fin fold resorption process (n=5 zebrafish) (Ellett et al, 2011). We initially observed macrophage infiltration in the median fin fold where fin ray formation was occurring in the developing unpaired fins; first in the caudal fin, then the anal fin and lastly in the dorsal fin (Fig 3.3.3). This suggests that macrophages may have a role in modeling the developing unpaired fins during initial lepidotrichia formation.

During the time course analyses of the *Tg(mpeg1:EGFP)* line, macrophages were observed along the perimeter of the tail, at the very base of the median fin fold in 6.5 mm SL larvae (Fig 3.3.4a-b). Macrophage migration was observed in the proximal half of the median fin fold, in both the ventral and dorsal sides of the median fin fold in 6.8 mm SL larvae (Fig 3.3.4c-d). As development progressed, macrophages were observed to eventually occupy all of the proximodistal length of the resorbing median fin fold in 8.5 mm SL larvae (Fig 3.3.4e-f). All in all, these analyses suggests macrophages may be implicated in the resorption of the median fin fold, however we do not know their exact role during the process.

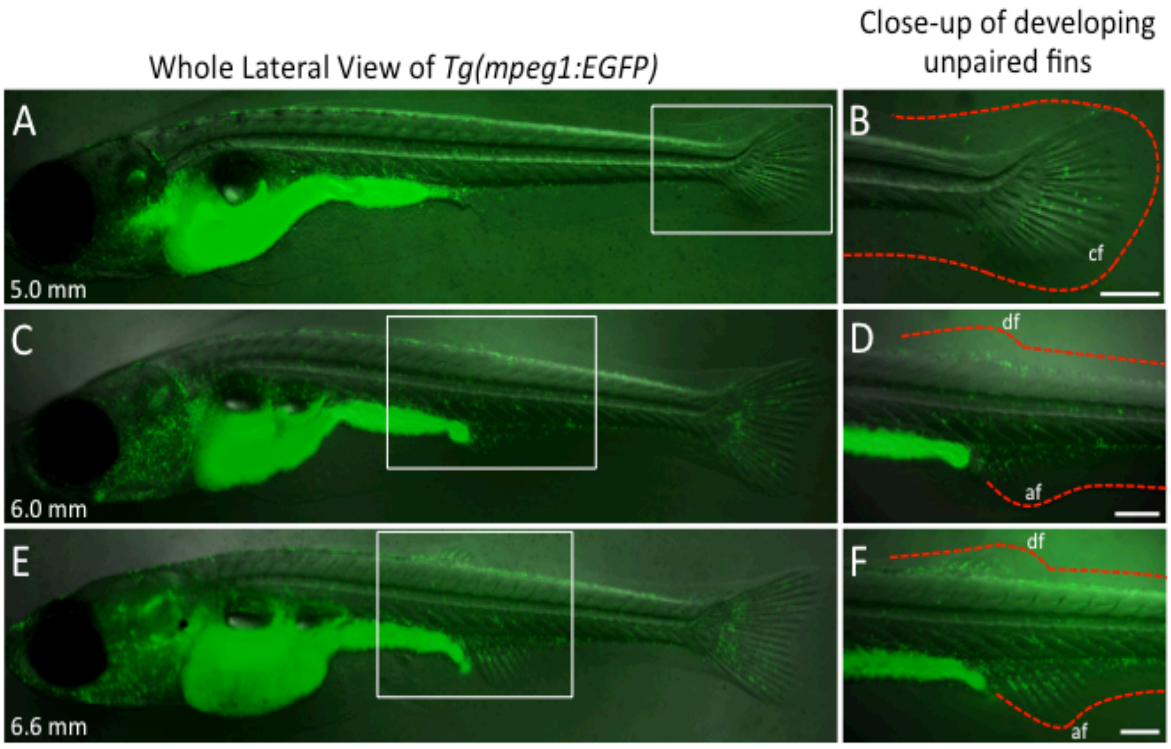


Figure 3.3.3. Macrophages infiltrate the developing unpaired fins during fin ray formation. Panels **a, c** & **e** show the whole lateral view of *Tg(mpeg1:EGFP)* larvae and the white boxes represent high magnification images of the unpaired fins shown in **panels b, d** & **f**. The SL of the displayed larvae are shown in the bottom left corner of the panels. Macrophages are first seen infiltrating the developing caudal fin (cf), then the anal fin (af) and lastly the dorsal fin (df) during lepidotrichia morphogenesis. Autofluorescence is observed in the yolk sac. Red dotted lines delimit the resorbing median fin fold. Scale bar= 200 μm.

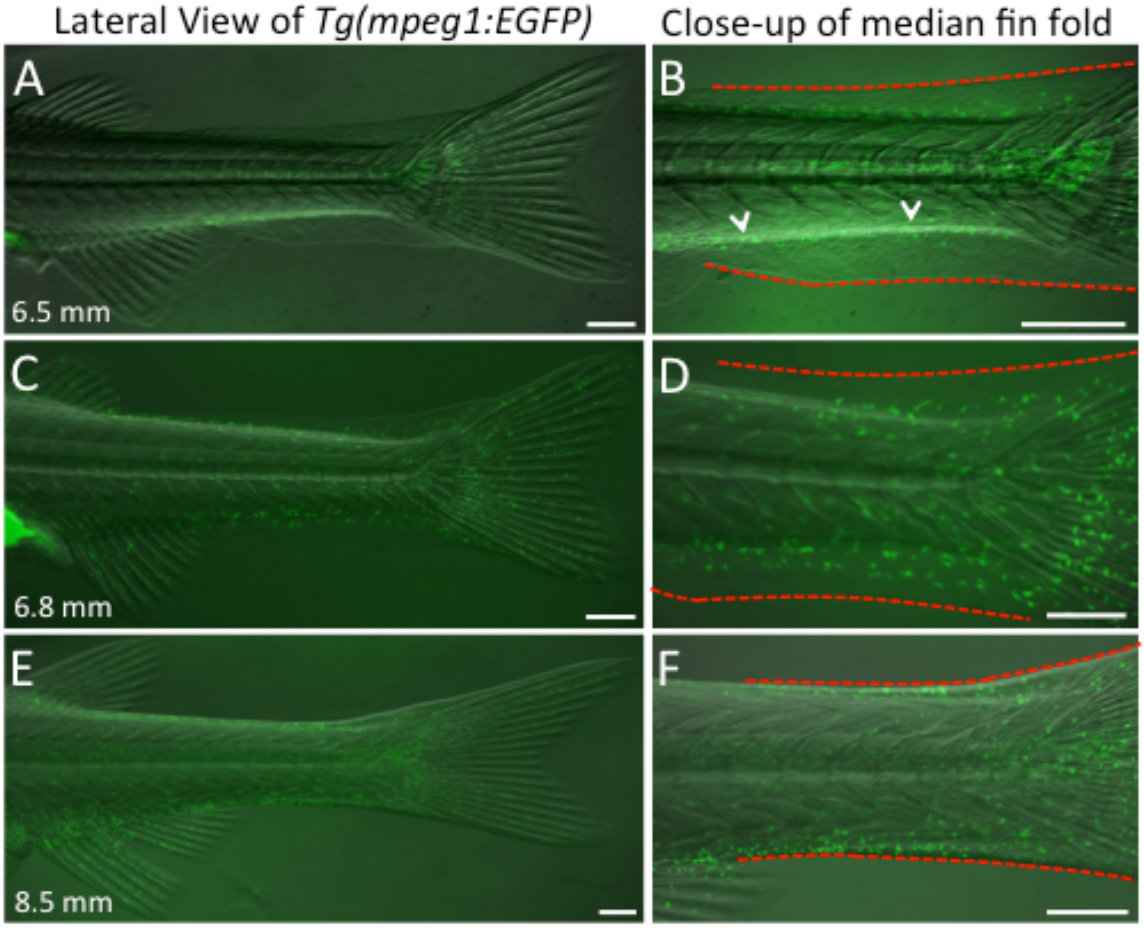


Figure 3.3.4. A time course of the *Tg(mpeg1:EGFP)* line throughout the median fin fold resorption process shows macrophage migration in the median fin fold. Panels **a, c** & **e** show the posterior lateral view of *Tg(mpeg1:EGFP)* larvae and high magnification images of the dorsal median fin fold are shown in panels **b, d** and **f**. The SL of the displayed larvae are shown in the bottom left corner of the panels. White arrows indicate the presence of macrophages at the edge of the trunk of the tail. As the time course progresses, macrophages are seen migrating in the proximal half of the median fin fold and eventually becomes present all along the proximodistal length of the receding fin fold. Red dotted lines delimit the resorbing median fin fold. Scale bar= 250 μm.

3.3.3. The presence of cell proliferation in the resorbing median fin fold invalidates the hypothesis that the resorption process may be due to a lack of cell proliferation

Lastly, we wanted to determine whether there was a differential cell proliferation profile along the median fin fold during its resorption. In other words, we investigated whether there was a lack of cell proliferation in the tissues that were being resorbed along the median fin fold compared to surrounding tissues that were either not undergoing resorption, such as the tail, or that were undergoing morphogenesis, such as the unpaired fins. To have a global view of the median fin fold's cell proliferation profile during its resorption, EdU (5-ethynyl-2′-deoxyuridine) assays were performed on whole-mount larvae undergoing different stages of the median fin fold resorption process (6.5 mm to 9.0 mm SL larvae). The EdU assay involves the injection of EdU, a thymidine analog, into the animal where it gets incorporated into newly synthesized DNA in cycling cells (Thermo Fisher, 2011). For the purpose of this study, EdU was injected in the body cavity of 6.5 to 9.0 mm SL WT larvae (n=29) undergoing different stages of the median fin fold resorption process in order to have a representative time course of the process. The larvae were then collected and fixed 18 hours post-injection and assayed.

The level of cell proliferation in the median fin fold was assessed by globally comparing the amount of positive cells in the median fin fold tissues located between the developing unpaired fins to the amount of positive cells found in the tail and the developing unpaired fins. We observed that in the majority of cases (n=20/29) there was as much, if not more cell proliferation in the median fin fold compared to the tail and the developing unpaired fins throughout the resorption process, see time course in Fig 3.3.5.

This suggests that cell proliferation does not differ in the resorbing median fin fold throughout development and does not account for its resorption.

However, we did notice strong cell proliferation domains along the median fin fold. We observed six cell proliferation domains in total; one on the anterior and one on the posterior side of the anal and dorsal fins of 6.5 mm to 7.5 mm SL WT larvae (Fig 3.3.6a-b) and two additional cell proliferation domains appeared on the dorsal and ventral sides of the developing caudal fin of 8.5 mm to 9.0 mm SL WT larvae (Fig 3.3.6c-d). The location of these cell proliferation domains on the lateral sides of the developing unpaired fins correlates to the location where there is ongoing formation of fin rays along the median fin fold. All in all, we cannot observe any lack of cell proliferation along the resorbing median fin fold compared to the domains of fin formation that would account for its resorption.

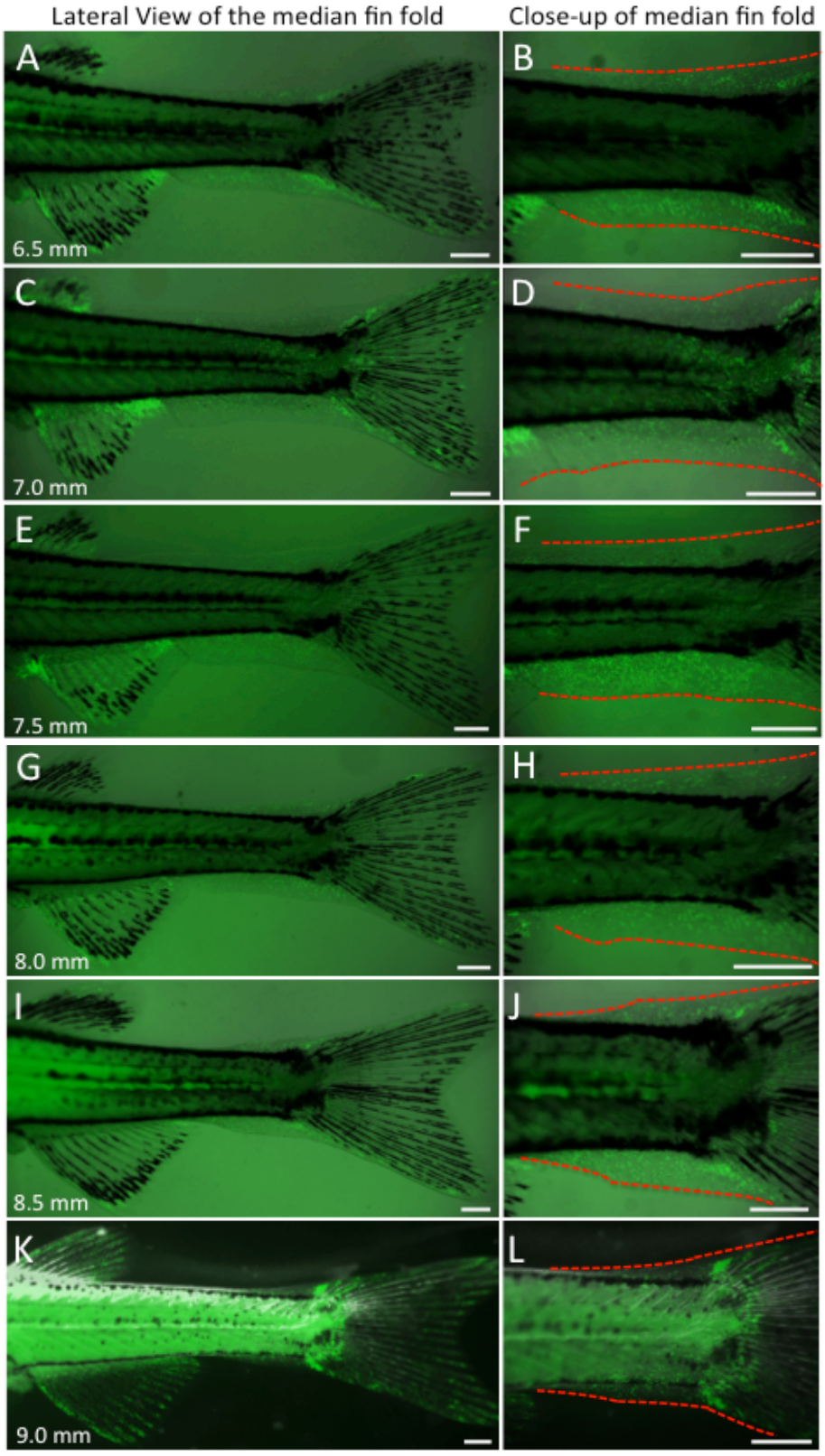

Lateral View of the median fin fold
Close-up of median fin fold
A
B
6.5 mm
C
D
7.0 mm
E
F
7.5 mm
G
H
8.0 mm
I
J
8.5 mm
K
L
9.0 mm

Figure 3.3.5. Cell proliferation is present in the median fin fold during its resorption. Panels **a, c, e, g, i** & **k** show the posterior lateral view of larvae after undergoing an EdU assay and high magnification images of the median fin fold are shown in panels **b, d, f, h, j** & **l**. The SL of the displayed larvae are shown in the bottom left corner of the panels. Cell proliferation can be seen in the median fin fold throughout its resorption. Red dotted lines delimit the resorbing median fin fold. Scale bar=250μm.

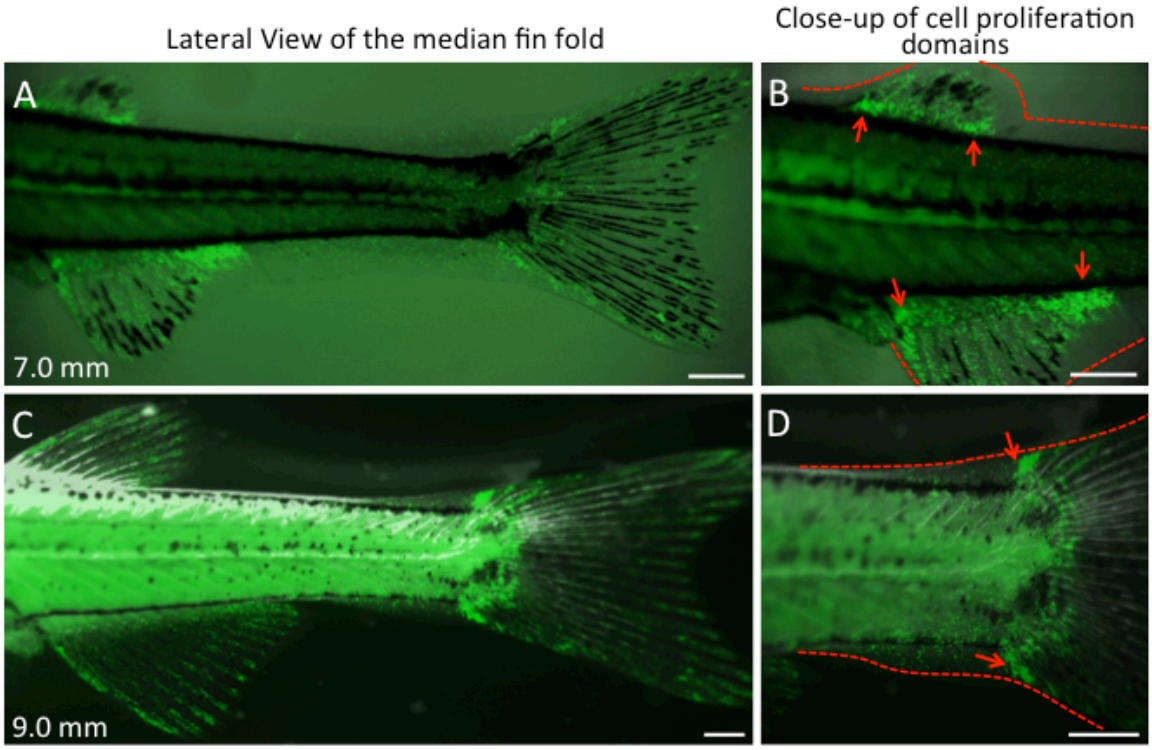


Figure 3.3.6. Cell proliferation domains are found on the lateral sides of each developing unpaired fin. Panels **a** & **c** show the posterior lateral view of larvae after undergoing an EdU assay and high magnification images of the median fin fold are shown in panels **b** ***&*** **d**. The SL of the displayed larvae are shown in the bottom left corner of the panels. Six cell proliferation domains were observed in total (red arrows); one on the anterior and one on the posterior side of the anal and dorsal fins of 6.5 mm to 7.5 mm SL larvae (panels **a-b**) and two additional cell proliferation domains appeared on the dorsal and ventral sides of the developing caudal fin of 8.5 mm to 9.0 mm SL larvae (panels **c-d**). Red dotted lines delimit the resorbing median fin fold and the developing unpaired fins. Scale bar= 250 μm.

Chapter 4. Discussion

4.1. The various and1 reporter lines recapitulate endogenous and1 expression throughout fin development and regeneration

We first characterized the tissue-specific *and1* reporter lines with the goal of determining whether they could recapitulate endogenous *and1* expression throughout fin development and regeneration. Previously, actinotrichia have been characterized to be located and to play an important role in supporting the pectoral and median fin folds of zebrafish embryos (Wood, 1982). 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 lepidotrichia throughout fin development and regeneration (Grandel & Schulte-Merker, 1998; Marí-Beffa, Carmona & Becerra, 1989).

In the same fashion, *and1* expression has been reported to be spatially and temporally correlated to the formation of actinotrichia during the embryonic stage and throughout fin regeneration (Zhang et al, 2010; Wagh Thesis, unpublished). For these reasons, we initially expected transgene expression to remain distally restricted in the fins of the various *and1* reporter lines throughout fin development and regeneration. Although, transgene expression remained distal in the epidermal-specific line during the time course analyses, mesenchymal *egfp*-expressing cells were found to be located all along the proximodistal length of developing and regenerating lepidotrichia in paired and unpaired fins; these cells were located adjacent to osteoblasts according to double immunostaining experiments.

We first hypothesized this "trail" of EGFP to be the result of the protein persisting long after the end of *and1* transcription. As a matter of fact, EGFP is a protein that has a 24 hour-long half-life (Corish & Tyler-Smith, 1999; Li et al, 1998). However, this particular expression pattern persisted for many weeks in the fins of the transgenic lines during both fin development and regeneration. Therefore EGFP's long half-life was likely not responsible for the distribution of *egfp*-expressing cells observed all along the proximodistal length of developing and regenerating fin rays. Despite this hypothesis, it would still be interesting to observe whether the same expression pattern would occur in the developing and regenerating fins of the various transgenic lines using a *destabilized enhanced green fluorescent protein* sequence (*dEGFP)*, instead of *EGFP* in the reporter constructs. This destabilized green fluorescent protein has a reduced half-life of 2 hours resulting from the fusion of a protein domain that targets EGFP for degradation (Li et al, 1998). All in all, the use of *degfp* in the *and1* reporter constructs would allow us to observe whether EGFP is present all along the proximodistal length of the fin rays.

We next hypothesized that the "trail" of EGFP was actually reflecting endogenous *and1* expression during both fin development and regeneration. Therefore, we first examined the distribution of actinotrichia throughout both processes to confirm that *and1* expression spatially and temporally correlated to actinotrichia formation (Zhang et al, 2010). The And1/2 immunostaining experiments further confirmed that actinotrichia are only located at the distal margin of fins throughout both processes (Grandel & Schulte-Merker, 1998; Marí-Beffa, Carmona & Becerra, 1989). Therefore, actinotrichia distribution does not explain the presence of EGFP all along the proximodistal length of fin rays in the various *and1* reporter lines during fin development and regeneration.

Next, we found it was adequate to compare the expression domains of *and1* and *egfp* all along the proximodistal length of developing lepidotrichia in the various *and1* reporter lines using *in situ* hybridization experiments. In doing so, we observed that the expression domains of both genes were detected only at the distal end of the fin rays and perfectly co-localized. According to these results, a discrepancy remains when the location of endogenous *and1* expression, reporter expression and actinotrichia are compared to the location of EGFP in the reporter lines during development. Intriguingly, when performing *in situ* hybridization experiments on cryo-sections of the fin regenerate, a minority of sections showed endogenous *and1* expression extending almost to the amputation site during the later stages of fin outgrowth. This expression pattern was never reported in the past. These results did put forward the idea there may actually be endogenous *and1* and reporter expression in the proximal end of developing and regenerating fin rays, however in levels so low that they generally remain undetected using the *in situ* hybridization methods.

Since the *in situ* hybridization methods were not sensitive enough to detect low levels of endogenous *and1* expression, we resorted to RT-PCR, which in comparison was previously reported to be a generally more sensitive method for detecting mRNA (Bates et al, 1997). Considering the developing fins of zebrafish larvae are minuscule and EGFP is similarly found all along the proximodistal length of fin rays in the *and1* reporter lines during fin regeneration, we decided to perform RT-PCR to detect *and1* expression using the regenerating adult caudal fin since it has more tissue and in comparison, can easily be handled. When comparing the proximal and distal regions of the fin regenerate, RT-PCR results demonstrated that *and1* was expressed all along the proximodistal length of the fin

regenerate. In contrast, RT-PCR results confirmed that *and1* is expressed only at the distal margin of intact adult fins, similarly to the reporter expression observed in the intact adult fins in the various *and1* reporter lines. Furthermore, *and1* and *egfp* expression domains co-localized throughout fin regeneration as well. Altogether, these results imply that the various *and1* reporter lines do indeed recapitulate endogenous *and1* expression throughout fin development and regeneration, and these results interestingly reveal that *and1* expression persists at a low level in cells that are located much proximal than the actinotrichia fibres.

4.2. Results further support the proposition that and1 expression may indirectly contribute to bone formation throughout fin development and regeneration

This study has clearly shown that mesenchymal reporter and *and1* expression do not spatially and temporally reflect actinotrichia distribution specifically throughout fin development and regeneration. During both processes, the expression domains of *egfp* and *and1* extend all along the proximodistal length of developing and regenerating lepidotrichia. However, the expression domains of *egfp* and *and1* do spatially and temporally reflect actinotrichia distribution in the embryonic and larval fin folds (more specifically in the areas of the fold that do not undergo bone formation) and in the intact fins of adult zebrafish. According to these observations, it puts forward the suggestion that *and1*-expressing cells may not only possess a role in the formation and maintenance of actinotrichia, but may also have a concomitant role in bone formation in the fins of zebrafish throughout fin development and regeneration.

During the time course analyses of the various *and1* reporter lines, mesenchymal-specific reporter expression was specifically observed in the appearing lepidotrichia

located at the ventral side of the posterior-most end of the notochord during early lepidotrichia morphogenesis. This expression domain was subsequently observed extending all along the proximodistal length of lepidotrichia as the lepidotrichia progressively developed distally. Eventually, mesenchymal-specific reporter expression gradually became distally restricted in the resulting intact adult fins. A similar expression pattern was observed during fin regeneration; mesenchymal-specific *and1* reporter expression occurred prior to bone formation and first temporally and spatially correlated to actinotrichia formation. However, its expression domain extended similarly all along the proximodistal length of lepidotrichia as regeneration progressed; specifically from the cut site to the distal end of the fin regenerate. Similarly to what was observed during the completion of fin development, mesenchymal-specific reporter expression gradually became distally restricted when fin regeneration was completed.

Since we observed identical expression domains throughout fin development and regeneration, the results of this study support the idea that *and1*-expressing cells may play an indirect role in bone formation during both processes in zebrafish fins. In fact, the regeneration process shares many resemblances to development; many genes expressed in the fins during larval development are re-expressed during fin regeneration (Avaron, Smith & Akimenko, 2006; Shi et al, 2015). Interestingly, *and1*-expressing cells were recently found to have an essential Wnt/β-catenin activity that indirectly regulates the commitment and differentiation of osteoblasts during fin regeneration (Wehner et al, 2014). Perhaps *and1*-expressing cells have an identical role during lepidotrichia morphogenesis. Altogether, from these results we propose that mesenchymal *and1*-

expressing cells may also indirectly contribute to bone formation in the fins of zebrafish throughout development and regeneration.

As previously stated, the loss of fin rays is deemed one of the many morphological changes that contributed to the fin-to-limb transition during evolution (Yano & Tamura, 2013). The loss of the *and* gene family during evolution is proposed to have structurally hindered the formation of the embryonic fin fold and thus the subsequent formation of fin rays (Zhang et al, 2010). If mesenchymal *and1*-expressing cells are later found to identically possess an indirect role in the commitment and differentiation of osteoblasts during lepidotrichia development as observed during fin regeneration, this puts forward the proposition that the loss of the *and* gene family during evolution may have played a more substantial role than previously contemplated in the loss of the fin rays during the fin-to-limb transition (Wehner et al, 2014).

Further cell lineage tracing experiments would allow us to determine whether the gradual distal restriction of *egfp*-expressing cells in the fins throughout both development and regeneration is due to a distal migration of *egfp*-expressing cells or a gradual inactivation of *egfp* in the proximal part of the fins. Such experiments include the establishment of a Cre/loxP system using zebrafish, which would allow the permanent genetic labelling of *and1*-expressing cells using the characterized tissue-specific *cis*-acting regulatory elements of *and1* (Kretzschmar & Watt, 2012; Mosimann et al, 2011). Time course analyses performed on these specific transgenic zebrafish lines during fin development and regeneration would allow us to follow any movement and better understand the possible function of permanently-labelled *and1*-expressing cells throughout these processes. Poleo et al, (2001) have shown by using a dye solution that

there is a great deal of cell migration from the proximal to the distal end of the fin regenerate throughout regeneration. On the other hand, if we perform cell lineage tracing experiment and we observe that there is the gradual proximal inactivation of *egfp* during fin development and regeneration, it would possibly legitimize the hypothesis that there is an available latent *and1*-expressing cell population located all along the proximodistal length of lepidotrichia. After all active *and1*-expressing cell population are amputated away, the putative latent *and1*-expressing cell population located all along the proximodistal lengths of lepidotrichia may be reactivated and may further contribute to the formation of actinotrichia in the blastema during fin regeneration. Regardless, cell lineage tracing experiments may be able to uncover more clues on the matter.

Furthermore, previous studies have shown the morpholino-mediated knockdown of certain genes causes actinotrichia formation/organization defects in the fins of zebrafish embryos (Heude, Shaikho & Ekker, 2014; Huang et al, 2009; Zhang et al, 2010). However, these studies did not focus on the possible effects these gene knockdowns may have played on lepidotrichia formation, which occurs later during development. Therefore, additional experiments are needed to examine whether the *and* genes are indirectly needed for lepidotrichia formation. Such experiments may include the establishment of a Metronidazole-Nitroreductase (MTZ-NTR) system in zebrafish, which is an inducible cell ablation system that could allow us to observe whether lepidotrichia formation is jeopardized when specifically ablating *and1*-expressing cells prior to the formation of lepidotrichia (Curano, Stainier & Anderson, 2008). Since mesenchymal *and1*-expressing cells are observed all along the proximodistal length of developing and regenerating lepidotrichia, we suggest producing *Tg(2PΔepi:NTR)* lines

to analyze whether there are any consequential effects when ablating these specific cells on the formation of lepidotrichia. Furthermore, zebrafish *and1/2* knock-out mutants could also be generated using the clustered regularly interspaced short palindromic repeats (CRISPR)/Caspase9 (Cas9) system. The latter is a genome-editing system that relies on double strand break DNA repair mechanisms, which introduce small deletions or insertions in a targeted gene to ultimately produce non-functional proteins (Irion, Krauss & Nüsslein-Volhard, 2014). All in all, further ablation and knockout experiments are needed to confirm the role of *and1*-expressing cells in lepidotrichia formation.

4.3. The function of the cis-acting regulation of and1 during adulthood is not entirely identical to the one previously characterized during the embryonic stage

One of the objectives of this study was to assess whether the function of the tissue-specific *cis*-acting regulatory elements of *and1* characterized during the embryonic stage remained identical throughout zebrafish fin development and regeneration. As a reminder, the 2P region consists of the 2kbp region located upstream of the first untranslated exon (termed E) of *and1* and includes a promoter (P) 200bp upstream of E. The 2P+I region consists of the 2P region, E and the first intron of *and1* (termed I). During the embryonic stage of zebrafish development, the *Tg(2P+I:EGFP)* and *Tg(2P:EGFP)* lines showed expression in both the mesenchymal and ectodermal cells of the pectoral and median fin folds (Fig 4a-b). The Epi region, which is a 150 bp region located within 2P, drove ectodermal-specific expression when paired with a minimal promoter (human β-globin promoter) in *Tg(Epi+βG:EGFP)* zebrafish embryos (Fig 4b). The 2PΔepi region, which is the 2P region with the exclusion of the Epi fragment, showed mesenchymal-specific expression in both the pectoral and median fin folds of

Tg(2PΔepi:EGFP) zebrafish embryos (Fig 4a). These transgenic lines recapitulated endogenous *and1* expression during the embryonic stage of zebrafish (Lalonde et al, 2016). However, these transgenic lines were not characterized during adulthood and fin regeneration. To do so, time course analyses were performed on these transgenic lines using fluorescence microscopy to determine whether the function of the tissue-specific *cis*-acting regulatory elements of *and1* characterized during the embryonic stage remained identical throughout fin development and regeneration.

The time course analyses mainly showed that the *Tg(2P+I:EGFP)* and *Tg(2PΔepi:EGFP)* lines maintained transgene expression throughout fin development and regeneration; the 2P+I region retained transgene expression in both the mesenchymal and the basal epidermal layer of the fins (Fig 4c-d), and the 2PΔepi region preserved transgene expression specifically in mesenchymal cells of the fins (Fig 4c). Therefore, we conclude the *cis*-acting regulatory elements found within the 2P+I and 2PΔepi regions are enough to maintain and drive reporter expression in their respective and previously reported cell types throughout fin development and regeneration. However, we also noticed during the time course analyses that transgene expression gradually decreased in intensity in the *Tg(2P:EGFP)* and *Tg(Epi+βG:EGFP)* lines throughout fin development to a point where no more transgene expression was detected during adulthood using fluorescence microscopy (Fig 4c-d). The lack of transgene expression extended during fin regeneration as well. Using RT-PCR, we determined these transgenic lines were indeed still driving transgene expression however, in levels so low that fluorescence microscopy was not sufficiently sensitive to detect reporter expression. From these results we gather

that the 2P and Epi *cis*-acting regulatory regions, even though weak, remain functional during the adult stage.

We first hypothesized that the low levels of transgene expression in the *Tg(2P:EGFP)* and *Tg(Epi+βG:EGFP)* lines were due to position effects caused by the random integration of the constructs in the zebrafish's genome via the Tol2 transposon system. The Tol2 transposon system is used to make transgenic zebrafish and as previously stated, involves the random integration of the reporter construct in the genome by a transposase enzyme, which can result in position effects where there is variability in reporter gene expression patterns among the transgenic lines generated from a set reporter construct. Position effects may occur when genes or regulatory elements surrounding the integration site of the reporter construct directly affect/alter transgene expression (Roberts et al, 2014). To circumvent this, we analyzed 5 transgenic lines for the 2P:*EGFP* reporter construct and 2 transgenic lines for the Epi+βG:*EGFP* reporter construct to come to a consensus transgene expression pattern for each reporter construct. All transgenic lines had similar reporter gene expression throughout fin development and regeneration. Therefore, we do not think that the low levels of transgene expression in the *Tg(2P:EGFP)* and *Tg(Epi+βG:EGFP)* lines to be due to position effects. However, the analysis of additional transgenic lines for each construct may help further solidify our conclusions.

Through the analyses of multiple *Tg(2P+I:EGFP)* and *Tg(2P:EGFP)* lines and the only *Tg(2P+E:EGFP)* line, we concluded the I region of *and1* may be containing an alternative promoter and/or may be acting as a strong general transcriptional enhancer essential for the efficient expression of epidermal-specific *and1* expression in the intact

adult fins and fin regenerate. The analysis of additional *Tg(2P+E:EGFP)* lines would be needed to solidify this supposition. In order to test the latter assumption, the Epi-EI*:EGFP* construct, which included the region spanning the 5' end of the Epi fragment to the 3' end of I, was cloned by fellow PhD candidate Robert Lalonde and zebrafish transgenic lines were subsequently generated for this construct. The EI region was previously shown to activate expression when cloned upstream of an *egfp* reporter construct, thus suggesting this region contains an alternate *and1* promoter. However the expression profile did not correspond to the expression profile of *and1*, thus suggesting this region drives non-specific expression (Poullain Thesis, unpublished). Furthermore, the region spanning from the 3' end of the Epi fragment to the 5' end of E (in other words, excluding the Epi and first exon), termed 967-1P*and1:EGFP*, was previously shown to drive no significant transgene expression of its own (Lalonde et al, 2016). All in all, these analyses suggest that any significant epidermal expression in the adult fish of *Tg(Epi-EI:EGFP)* lines would be most likely due to the addition of the I region.

Two transgenic *Tg(Epi-EI:EGFP)* lines were found and analyzed; one showed strong transgene expression in the line of adult fish, and the other displayed strong transgene expression strictly in the basal epidermal layer of the intact adult fins and in the fin regenerate. For the former transgenic line, we suspected there were positional effects causing ectopic transgene expression in the lateral line of the adult fish. The lateral line is a sensory system found on the lateral sides of fish and amphibians; it detects changes in water motion and plays a role in an assortment of behaviours in zebrafish (Ghysen & Dambly-Chaudière, 2004). Since we theorized region I to contain a strong promoter and/or transcriptional enhancer, it is reasonable to presume the position at which the

construct was integrated in the genome may have caused this unexpected ectopic expression. In other words, region I may be transcriptionally boosting any gene closely located to it. As for the latter transgenic line, transgene expression was situated in the expected tissue; the basal epidermal layer of adult intact fins and fin regenerates. Therefore, we suggest region I contains an alternative promoter and/or may be containing a transcriptional enhancer essential for epidermal-specific *and1* expression during the adult stage of zebrafish development (Fig 4d). Clearly, more *Tg(Epi-EI:EGFP)* lines need to be screened and analyzed to further confirm this conclusion. In order to further support the hypothesis that region I is a strong general transcriptional enhancer/promoter, it needs to be cloned downstream of previously characterized weak *cis*-acting regulatory elements to observe whether transgene expression will significantly increase.

It was found that in the case of certain genes, the inclusion of their first intron in a reporter construct significantly increases the expression levels of integrated transgenes in mice, *Drosophila*, plants and zebrafish (Choi et al, 1991; Pfeiffer et al, 2010; Callis, Fromm & Walbot, 1987; Horstick et al, 2015). Many mechanisms have been found to contribute to, what has been previously termed, intron-mediated enhancement. Such mechanisms include but are not limited to the ones discussed here. The inclusion of the first intron in reporter constructs was shown to enhance every step of gene expression, from transcription to translation and to inhibit mechanisms that supress these steps (Niu & Yang, 2011; Le Hir, Nott & Moore, 2003; Rose, 2004). Furthermore, a higher guanine and cytosine nucleotide content found in introns has been correlated to increased transcription levels (Kudla et al, 2006). Transgene expression is susceptible to silencing in zebrafish (Stuart, McMurray & Westerfield, 1988; Goll et al, 2009). It is proposed that

the inclusion of an intron in reporter constructs may help suppress epigenetic silencing of foreign DNA elements in zebrafish (Horstick et al, 2015). Furthermore, the use of alternative promoters was shown to play an important role in boosting mRNA expression levels and was also shown to confer distinctive mRNA stability. Additionally, alternative promoters were shown to increase translational efficiency and provide differential tissue-specific expression in yeast, zebrafish and humans (Kolondra et al, 2015; Gauss et al, 2006; Wang, Guo & Floros, 2005; Chen et al, 2010; Shang et al, 2011; Regadas et al, 2013). Therefore, region I may act as a strong transcriptional enhancer/alternative promoter due to one or a combination of these mechanisms. The specific mechanisms responsible for the strong expression levels observed in the *Tg(2P+I:EGFP)* and *Tg(Epi-EI:EGFP)* lines have yet to be determined.

Unexpectedly, we observed a discrepancy in transgene expression in the *Tg(2P:EGFP)* and *Tg(2PΔepi:EGFP)* lines throughout fin development and regeneration; the transgene expression pattern from the *Tg(2P:EGFP)* lines were not similar to *Tg(2PΔepi:EGFP)*'s transgene expression pattern in the mesenchyme; in fact, as development progressed, no more transgene expression was observed in the *Tg(2P:EGFP)* lines during the adult stage and in contrast, the *Tg(2PΔepi:EGFP)* lines showed maintained expression in mesenchymal cells. This came as a surprise since the only difference between the two reporter constructs was the omission of the Epi fragment in the 2P:*EGFP* construct. As a matter of fact, the removal of the Epi fragment from the 2P region was enough to reactivate mesenchymal-specific transgene expression, thus suggesting that the Epi fragment may contain a *cis*-acting mesenchymal-specific repressor (Fig 4c). Interestingly, the Epi fragment was found to contain clusters of

putative consensus binding sites of numerous transcription factors such as smad3, CDX-2, Osr2, MTF-1, TCF-4 and multiple sites for Dlx proteins (Lalonde et al, 2016). All of these transcription factor candidates have been found to not only possess activator abilities, but repressor activity as well in mice (Vincent et al, 2009; Wang et al, 2010; Kwon et al, 2015; Dostert & Heinzel, 2004; Brantjes et al, 2001; Le et al, 2007). Therefore, site-directed mutagenesis experiments on the putative binding sites of these transcription factors within the 2P region will allow us to determine which one is likely acting as a mesenchymal-specific repressor of *and1* during the adult stage of zebrafish.

Furthermore, when comparing the *Tg(2P+I:EGFP)* and *Tg(2PΔepi:EGFP)* lines, we observe that in both lines there is strong mesenchymal expression even though the putative mesenchymal repressor is present in the 2P+I:*EGFP* construct (Fig 4c). We hypothesize this putative mesenchymal repressor situated in the Epi region may also dictate the preferential use of a promoter over another specifically in mesenchymal *and1*-expressing cells; in the *Tg(2PΔepi:EGFP)* lines, the absence of the putative mesenchymal repressor determines that the promoter characterized at the 200-1bp position (P) is preferentially employed, however in the *Tg(2P+I:EGFP)* lines where the putative mesenchymal repressor is included, it orders the use of the alternative promoter found in the EI region instead. This hypothesis would explain the reason why no mesenchymal expression has been observed in the *Tg(2P:EGFP)* lines as well; the putative alternate promoter is not included in this construct. In sum, we hypothesize the putative mesenchymal repressor found within the Epi fragment may also serve to dictate the preferential use of promoters specifically in mesenchymal *and1*-expressing cells in the adult fish.

All in all, we observe a few changes in the *cis*-acting regulation of *and1* during adulthood versus the embryonic stage. First, the I region is seemingly essential for the efficient transgene expression in the epidermis of the fins specifically during the adult stage. Furthermore, the 2P region was unable to efficiently express the transgene in either mesenchymal and epidermal cells of the fins as development progressed; the removal of the Epi fragment from the 2P region was enough to reactivate mesenchymal-specific transgene expression, thus suggesting that the Epi fragment may contain a *cis*-acting mesenchymal-specific repressor. However, the 2P+I region contains all necessary tissue-specific *cis*-acting regulatory elements for transgene expression in both mesenchymal and epidermal cells of the adult fins. The 2PΔepi region contains all necessary mesenchymal-tissue specific *cis*-acting regulatory elements to maintain transgene expression in the mesenchymal cells of the adult fin.

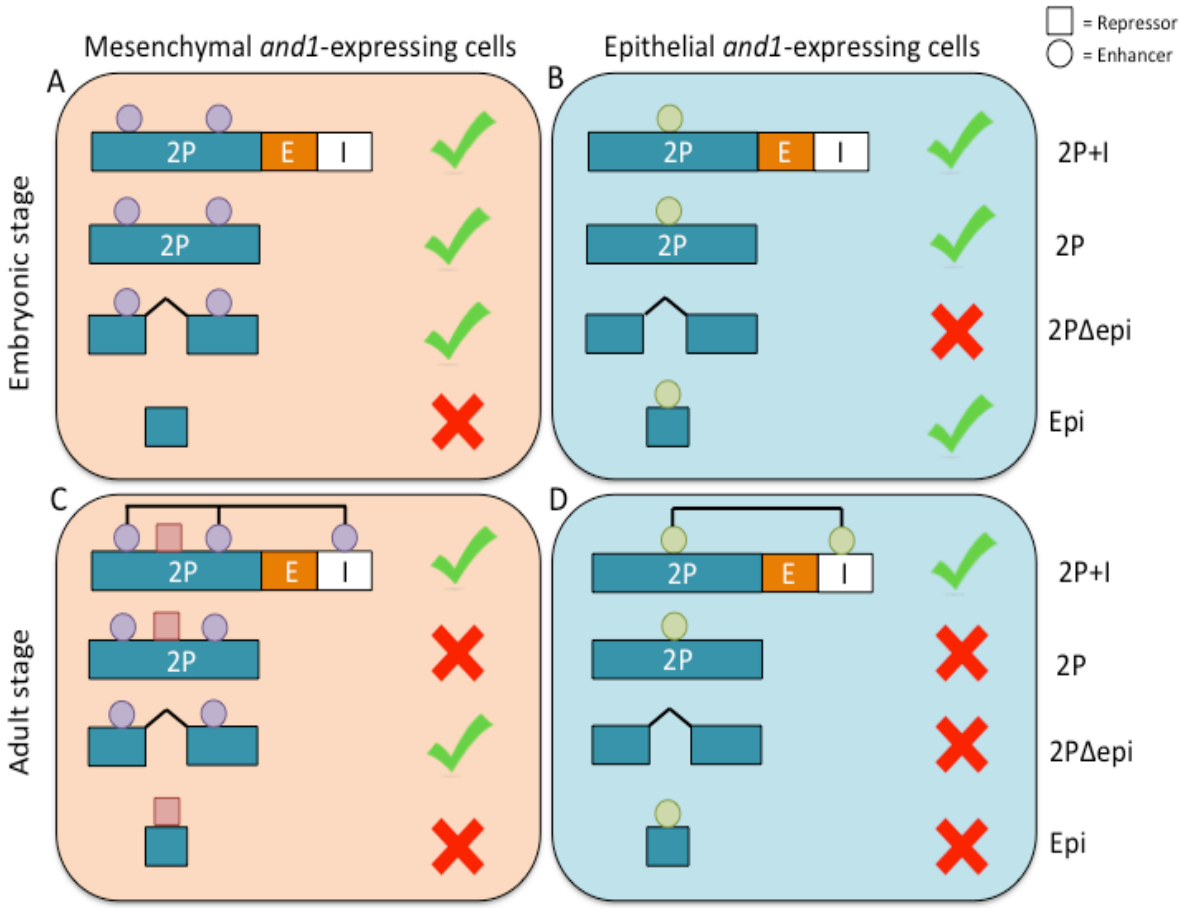


Figure 4. Schematic of the proposed *cis*-acting regulation of *and1* in mesenchymal and epithelial *and1*-expressing cells during the embryonic and adult stage of zebrafish development. During the embryonic stage of zebrafish development, the 2P *cis*-acting regulatory element alone was shown to be sufficient in terms of recapitulating *and1* expression in both the mesenchymal and ectodermal cells of the pectoral and median fin folds (the presence of reporter expression is indicated by the green checks). **a-b.** The Epi *cis*-acting regulatory element recapitulated ectodermal-specific *and1* expression when paired with a minimal promoter and when omitted from the 2P region, the remaining fragments, termed 2PΔepi, recapitulated mesenchymal-specific *and1* expression in the embryonic fins (Lalonde et al, 2016). **c-d.** However, during the adult stage of zebrafish development, the 2P *cis*-acting regulatory element was not enough to sufficiently drive reporter expression (the absence of reporter expression is indicated by the red crosses). In fact, the inclusion of the first untranslated exon (E) and the first intron (I) with the 2P element is enough to reactivate reporter expression in both mesenchymal and epithelial *and1*-expressing cells. This suggests the EI region may perhaps contain a strong transcriptional enhancer or an alternative promoter needed for the efficient expression of *and1* during later stages of zebrafish development. In fact, the EI region is required in combination with the Epi regulatory element to efficiently drive epithelial-specific reporter expression in the fins of adult zebrafish. Since the 2PΔepi regulatory elements maintains reporter expression and 2P cannot during zebrafish adulthood, we hypothesize there may be the presence of a mesenchymal-specific repressor binding

within the Epi regulatory element. In fact, the removal of the Epi fragment from the 2P regulatory region was enough to reactivate mesenchymal-specific transgene expression, thus supporting the hypothesis that the Epi fragment may contain a *cis*-acting mesenchymal-specific repressor during the adult stage of zebrafish development. Furthermore, when comparing the *Tg(2P+I:EGFP)* and *Tg(2PΔepi:EGFP)* lines, we observe that in both lines there is strong mesenchymal expression even though the putative mesenchymal repressor is present in the 2P+I:*EGFP* construct. We hypothesize the putative mesenchymal repressor situated in the Epi region may also dictate the preferential use of a promoter over another specifically in mesenchymal *and1*-expressing cells.

4.4. egfp-expressing cells are present around the veins of the fin regenerate

During fin regeneration, we noticed the presence of *egfp*-expressing cells in the inter-ray tissue of the fin regenerate of the *Tg(2P+I:EGFP)* and *Tg(2PΔepi:EGFP)* lines; more specifically on the lateral sides of the 4-6 most distal segments of regenerating lepidotrichia. Upon closer look, these cells were found to be located closely to veins of the fin regenerate. To have a better view of these cells in relation to the blood vessels, a double transgenic line was generated by crossing the *Tg(2P+I:EGFP)* line with a *Tg(Fli1a:DsRed)* line, which drives transgene expression in the endothelial cells lining the blood vessels (Liu et al, 2008). The *Tg(Fli1a:DsRed)* line reveals extremely well the vein-artery-vein layout of the vasculature of the fin; veins are located in the inter-ray tissue of the fin, more specifically on the lateral sides of each lepidotrichia, while arterial blood vessels are surrounded by the lepidotrichia hemirays (Huang et al, 2003). This allowed us to observe that the *egfp*-expressing cells located in the inter-ray tissue are actually situated on the abluminal surface of the veins, meaning the cells are located on the outer surface of the blood vessel. In contrast, *egfp*-expressing cells found within the hemirays are not particularly located close to the arterial blood vessels. All in all, the specific role of the *egfp*-expressing cells found on the outer surface of the veins remains unknown.

However, we can deduce from the location of these *egfp*-expressing cells along the proximodistal axis of lepidotrichia that they do not contribute to actinotrichia formation; the *egfp*-expressing cells present in the inter-ray tissue are located in the 4-6 most distal lepidotrichia segments whereas actinotrichia are only found along the 2 most distal segments (Marí-Beffa, Carmona & Becerra, 1989). Therefore, it is unlikely the

proximal *egfp*-expressing cells located on the abluminal surface of the veins in the inter-ray tissue of the fin contribute to actinotrichia formation.

We hypothesize that perhaps these *egfp*-expressing cells may be needed, to an unknown extent, for the regeneration of blood vessels. A previous study pertaining to collagen IX, which is coded by the *col9a1* gene in zebrafish, was found to play not only an important role in preserving the structural integrity of actinotrichia fibres but was additionally found to be responsible for regulating the formation of vascular networks during fin development and regeneration (Huang et al, 2009). It would be worth investigating whether the *egfp*-expressing cells located on the abluminal surface of blood vessels express *col9a1*; this would allow us to explore whether *and1*-expressing cells also contribute to the regulation of blood vessel formation during fin regeneration.

4.5. The cellular processes responsible for the resorption of the median fin fold have yet to be determined, however we show resorption involves the loss of actinotrichia

During zebrafish fin development, the major lobe of the median fin fold gives rise to the three unpaired fins in the adult; the dorsal, anal and caudal fins. While the skeletal elements of the adult fins form in the median fin fold, the latter undergoes resorption. This process occurs from an anterior-to-posterior fashion and involves the gradual loss of the median fin fold tissues located in between the formation of the adult unpaired fins (Parichy et al, 2009). Intriguingly, actinotrichia distribution was never assessed in the median fin during its resorption. Furthermore, no cellular processes have been identified and found responsible for this process in teleost fish. Inspired by the characterized mechanisms by which amphibians lose larval-specific features, we aimed to determine whether the presence of cell death or macrophage digestion was responsible for the

resorption of the median fin fold. We also investigated whether there was a lack of cell proliferation in the receding median fin fold that could have contributed to its resorption.

We first characterized the presence of *and1* and *egfp* expression, and actinotrichia distribution in the median fin fold undergoing either early or late stages of the resorption process. During the early stage of resorption, we noticed endogenous *and1* and reporter gene expression gradually became distal in the median fin fold as resorption began to occur from an anterior-to-posterior direction. Moreover, immunostaining for And1/2 demonstrated that actinotrichia retained their elongated fibril structure during the early stages of resorption. However during later stages of resorption, *and1* expression was undetected in the resorbed median fin fold, even when pairing the experiment with a PK treatment, which increases probe access to mRNA in tissues (Tesch, Lan & Nikolic-Paterson, 2006). In addition, during the late stages of resorption actinotrichia lost their fibril structures; they became sparse and clustered at the very proximal end of the trunk. As development progressed, they became completely absent along with the median fin fold.

From these results, we propose the loss of actinotrichia is due to the subsequent and gradual loss of *and1* expression in the median fin fold during its resorption; *and1* expression plays an important role in maintaining the structural integrity of actinotrichia. Hence, we demonstrated that actinotrichia loss correlates to the gradual resorption of the median fin fold. Since actinotrichia have an essential role in the structural support of the fin folds during the embryonic and larval stages of zebrafish development, it would be interesting to examine whether the loss of actinotrichia causes the resorption of the median fin fold. It would be worthy to investigate whether matrix metalloproteinases

(MMPs), which are extracellular matrix degradation proteins, are responsible in the breakdown of actinotrichia during the median fin fold resorption process. MMPs have been found to play essential roles in the degradation of larval tissues during tadpole metamorphosis (Visse & Nagase, 2003).

The MMP family in zebrafish includes a collagenase (MMP-13), which cleaves type I collagen, and gelatinases (MMP-2 and MMP-9), which cleave type IV collagen and denatured collagens (Wyatt et al, 2009; Hillegass et al, 2007; Nagase & Woessner, 1999). Perhaps MMPs play a role in the degradation of the collagenous actinotrichia fibres during the median fin fold resorption process. All three zebrafish MMPs candidates play a role in tissue remodelling during both zebrafish development and fin regeneration (Hillegass et al, 2007; Zhang et al, 2008; Bai et al, 2005; Zhang et al, 2003; Yoong et al, 2007; Lebert et al, 2015). Interestingly, MMP-9 and -13 are also expressed in zebrafish inflammatory cells; MMP-9 is expressed in neutrophils and eosinophils and MMP-13 is expressed in primitive macrophages and is required for macrophage migration during fin regeneration (Dahlen, Schute & Howarth, 1999; Zhang et al, 2008). This is reminiscent of the *Tg(mpeg1:EGFP)* line time course analyses where macrophage migration was observed in the resorbing median fin fold. It would be worth analyzing whether MMP-2, -9 & -13 are expressed in the median fin fold during its resorption. It would shed light on whether MMPs play a role in the degradation of actinotrichia during the resorption of the median fin fold.

The loss of larval-specific features were well characterized during amphibian metamorphosis and mainly involves thyroid hormone (T3)-induced apoptosis (Kerr, Harmon & Searle, 1974; Wang & Brown, 1993; Ishizuya-Oka, 2011). We were greatly

inspired to investigate whether cell death was involved in the resorption of the median fin fold in zebrafish using PI staining and TUNEL assays. The experiments showed that the majority of zebrafish larvae undergoing different stages of the resorption of the median fin fold showed no staining in the median fin fold. Both experiments included positive and negative controls (data not shown). Only a minority of the samples showed the presence of PI- and TUNEL-positive cells at the very distal edges of the fins, and only one larva showed PI- and TUNEL-positive cells in the resorbing median fin fold.

Since the latter result was infrequent, we hypothesized that the larvae displaying the presence of PI- and TUNEL-positive cells at the very distal edges of the fins is possibly due to environmental abrasion. However, it is also possible that epithelial cells are constantly being renewed and distally migrate to eventually die and to be eliminated at the distal edge of the fin. A similar mechanism does occur in the mammalian intestinal epithelium where cell renewal and cell death occur in distinct compartment of microvilli; cells originate at the base of microvilli, distally migrate and are terminated at the distal end of microvilli (Gordon & Hermiston, 1994; Sancho, Batlle & Clevers, 2003). As for the presence of TUNEL-positive cells in the resorbing fins of only one larva, false-positives were previously reported to occur when TUNEL assays were paired with a PK treatment (Garrity et al, 2003). All in all, cell death assays were inconclusive in determining whether apoptosis play a role in the resorption of the median fin fold; TUNEL assays should be repeated on a higher number of samples for the results to be significant. Other cell death assays, such as the Caspase 3 assay, should be considered as well to determine with greater certainty whether cell death plays a role during the resorption of the median fin fold.

Additionally, it would be interesting to investigate whether T3 plays a role in the resorption of the median fin fold in zebrafish. Type I and II deiodinase enzymes convert thyroxine (T4) into T3, which is a more active hormone variant (Becker et al, 1997). The type III deiodinase enzyme inactivates T3 (St Germain et al, 1994). These deiodinase enzymes are differentially expressed in the tadpole, where structures that undergo metamorphosis express type I and II deiodinases, and the tissues that do not undergo metamorphosis express type III deiodinase (Becker et al, 1997; Huang, Marsh-Armstrong & Brown, 1999). In zebrafish, deiodinases enzymes play an important role in embryonic development, growth, motility and pigmentation (Guo et al, 2014; Bagci et al, 2015; Walpita et al, 2009). Type II deiodinase is the main activator and type III deiodinase is the main inactivator of T3 in zebrafish. In contrast, type I deiodinase is not functional in zebrafish (Guo et al, 2014). Investigating whether type II and III deiodinases are expressed in the resorbing median fin fold would help in figuring whether thyroid hormones play a role in the resorption of the median fin fold in zebrafish.

Amphibian metamorphosis also involves macrophage digestion, which removes auto- and heterolyzed cells through phagocytosis in dying tissues (Kinoshita, Sasaki & Watanabe, 1985). As previously stated, macrophages have been analyzed in the median fin folds of zebrafish throughout the resorption process using the *Tg(mpeg1:EGFP)* line. During the embryonic stage, macrophages remained largely absent in the median fin fold (data not shown). However, macrophages started invading the developing unpaired fins during their formation. This suggests macrophages may be contributing to the morphogenesis of the fins during zebrafish development. During the early stages of the resorption process, we observed macrophages lining the base of the median fin fold and

gradually migrating halfway through the proximodistal length of the resorbing median fin fold as development progressed. In the later stages of resorption, macrophages were observed occupying all of the proximodistal length of the resorbing median fin fold. According to these observations, we do not know whether we can conclude that macrophages eventually occupy the entirety of the proximodistal length of the median fin fold or that the median fin fold eventually recedes to a point where it meets with the macrophages observed at the more proximal area of the median fin fold during earlier stages. All in all, these analyses suggests macrophages may be implicated in the resorption of the median fin fold, however we do not know their exact role during the process.

Lastly during amphibian metamorphosis, a cell death/proliferation balance is achieved where there is a loss of larval-specific structures via apoptosis and the acquisition of adult features via maintenance of cell proliferation (Ishizuya-Oka, 2011). We assessed, by performing EdU assays on zebrafish larvae undergoing different stages of the median fin fold resorption process, whether there was a difference in cell proliferation in the tissues undergoing resorption in comparison to tissues that are undergoing morphogenesis, like the unpaired fins, or none, such as the tail. Globally, there were seemingly as much, if not more, cell proliferation in the receding median fin fold compared to the unpaired fins, which undergo active morphogenesis, during the median fin fold resorption. This suggests that the amount of cell proliferation observed along the median fin fold, when compared to the domains of fin formation, does not account for its resorption. However, we did notice 6 strong cell proliferation domains along the median fin fold during its resorption; a cell proliferation domain was present on

both lateral sides of the dorsal, anal and caudal fins where fin ray formation occurred. In sum, we characterized the presence of cell proliferation domains along the median fin fold where there were active growth of lepidotrichia and globally observed no significant lack of cell proliferation in the resorbing median fin fold that would account for its resorption. All in all, we cannot state with great certainty that a differential cell proliferation profile along the resorbing median fin fold is responsible for its resorption. Further cell proliferation experiments are needed to make compelling conclusions.

All things considered, if there is still no observable cell death or there is still the presence cell proliferation in the regressing median fin fold after careful future examination, perhaps the resorption of the median fin fold is simply the result of the median fin fold giving way to provide more epidermis for the rest of the body, which undergoes a period of rapid growth. The loss of actinotrichia in the median fin fold would perhaps allow the fold to be less sturdy or cause the connections between the two epithelial layers to become more fragile. This would allow the median fin fold to recede without any cell death or changes in cell proliferation. Cell lineage tracing experiments would allow us to observe whether the cells found in the median fin fold eventually becomes part of the epidermis of the tail of zebrafish juveniles after the resorption of the median fin fold.

4.6. General Conclusion

This study mainly enabled us to characterize in great details *and1* reporter gene expression using the various tissue-specific *and1* reporter lines throughout fin development and regeneration. In doing so, we were able to demonstrate that these reporter lines recapitulated endogenous *and1* expression and further supports the

hypothesis that *and1*-expressing cells may indirectly contribute to lepidotrichia formation in the fins during development and regeneration. Furthermore, the characterization of the tissue-specific *and1* reporter lines throughout development allowed us to notice a change in the *cis*-acting regulation of *and1* specifically in the epidermal cells, which seemingly necessitate the I region to be efficiently expressed during the adult stage. Consequently, it enabled us to generate an epidermal-specific transgenic line where epidermal *and1* reporter expression is maintained throughout fin development and regeneration. Additionally, we suspect there is a repressor-binding site present in the Epi fragment that represses mesenchymal-specific transgene expression during zebrafish adulthood. Next, we have characterized for the first time that there is a loss of actinotrichia maintenance in the median fin fold during its resorption through the consequential loss of endogenous *and1* expression along the receding median fin fold. Lastly, we attempted to resolve the cellular mechanisms that caused the resorption of the median fin fold during fin development.

This study has important ramifications in the future studies of *and1*-expressing cells during both fin development and regeneration. The characterization of tissue-specific *and1* reporter expression throughout fin development and regeneration significantly furthered our understanding on the distribution of *and1*-expressing cells in the fins throughout important fin morphogenesis/regenerative events. It also advanced our knowledge on the *cis*-acting regulation of *and1* in zebrafish adults compared to the embryonic stage. However, these tissue-specific *cis*-acting regulatory elements have the potential of becoming useful tools for the mis-expression of exogenous genes in the zebrafish fins to produce zebrafish disease models for future drug screenings. For

example, the epithelial-specific *cis*-acting regulatory elements can be cloned upstream of a mutated version of a gene of interest that reflects a real life human diseased gene expressed in human epithelial cells. This construct will then drive expression of the mutated gene in epithelial cells only of the fins once transgenic fish are generated. Therefore, this results in the creation of a zebrafish disease model and allows future important research advances. In other words, the tissue-specific *cis*-acting regulatory elements of *and1* may tremendously advance our knowledge not only on the contribution of *and1* in relation to other molecular processes during both fin development and regeneration, but may be applied in other fields of study such as the understanding and treatment of human diseases.

Secondly, this study is also significant for some aspects of paired appendage evolution. The pectoral fins have undergone many profound morphological changes during the fin-to-limb transition throughout evolution, one of them being the loss of fin rays. This study further supports the hypothesis that *and1*-expressing cells indirectly regulate lepidotrichia formation during not only fin regeneration, which has been previously investigated, but also throughout fin development. All in all, this study suggests the loss of the *and* gene family may have had a stronger impact on the loss of fin rays than previously considered during the fin-to-limb transition.

Lastly, during the formation of the unpaired fins we have shown that the gradual degradation of actinotrichia in the median fin fold correlates to the median fin fold resorption. Perhaps the gradual breakdown of actinotrichia causes the resorption of the median fin fold. Although the cellular mechanisms responsible for shaping the median fin

fold into the unpaired fins remain unknown, actinotrichia may play an essential role in the resorption of the median fin fold.

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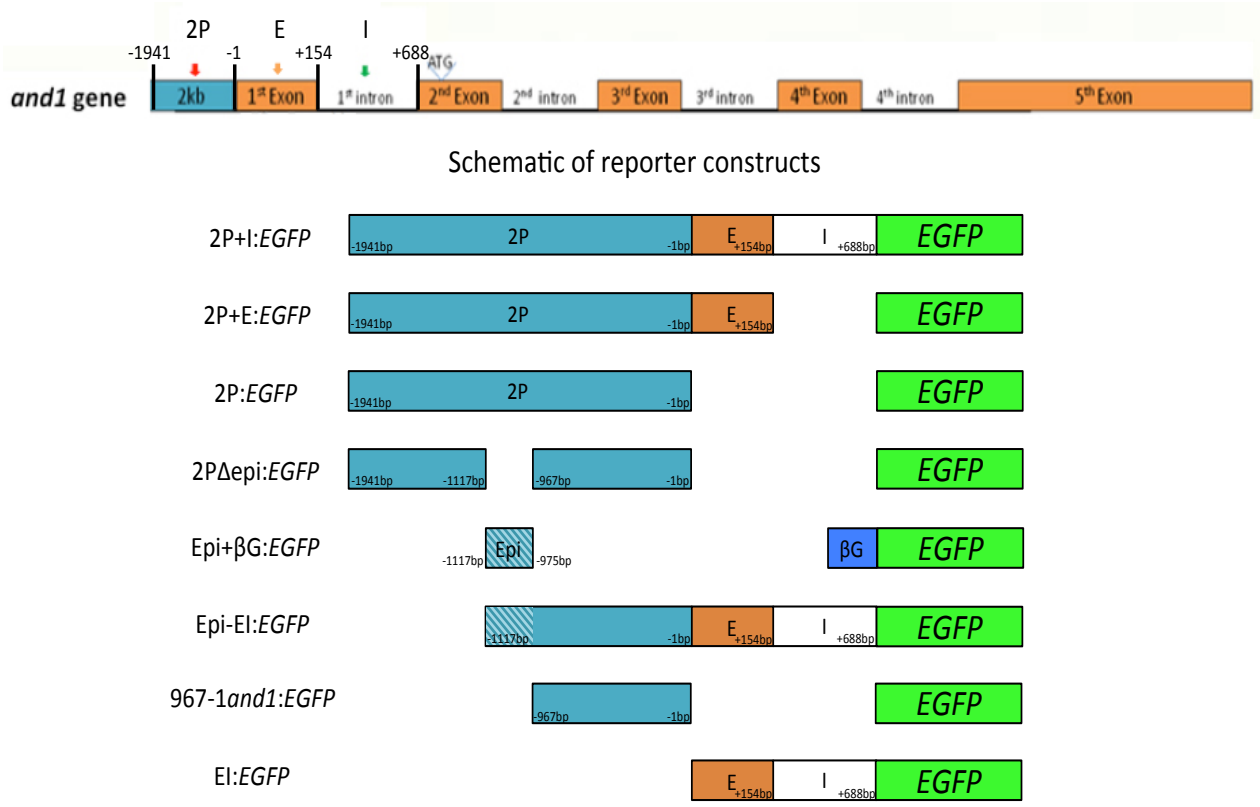
Appendix A



List of reporter constructs mentioned throughout the thesis dissertation.