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Molecular and Cellular Dissection of Zebrafish Larvae Tail Regeneration

PhD thesis by

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Abstract

Regeneration is the ability of organisms to restore their structures in form and function. While it is present in the complete animal kingdom, humans retain the capacity to restore some tissues and organs, but this capacity is limited in comparison with that of other vertebrates such as the zebrafish. Further, the growth of regenerative diseases and the lack of therapies to fully restore damaged organs and limbs highlight the importance of the study of regeneration.

To further the understanding of the regeneration process, this study focuses on molecular and cellular dissection of the tail regeneration of zebrafish larvae. The molecular dissection found that the molecular signalling Hedgehog regulates regeneration through the Wnt and Fibroblast Growth Factor (FGF) developmental pathways as follows. The regeneration marker genes *msxc*, *dlx5a* and *raldh2* are regulated by Hedgehog (Hh) through Wnt signalling, while cell proliferation is regulated by Hh through FGF signalling. Further, the role of Hh during regeneration is unique and different to its role during normal tail development, where it is not essential. Finally, it was found that the muscle differentiation marker *myod* is expressed sequentially after the regeneration marker genes *raldh2* and *dlx5a* during the late stage of tail restoration.

For the cellular dissection cell lineage tracings during regeneration were performed using several Cre expressing lines with tissue specific promoters, where Cre recombination is controlled by 4OH Tamoxifen administration. The lineage tracing analysis showed that both blood vessels and periderm cells participate during tail restoration and that both cell types remain lineage committed.

Altogether the results presented in this thesis show that the zebrafish larva tail is a suitable model to study both molecular and cellular aspects of regeneration. The knowledge generated during this PhD research can contribute to set the basis for the development of clinical therapies to assist human regeneration.

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Chapter 1

General Introduction

1.1 Regeneration

Regeneration is defined as the reconstruction of lost tissues or body parts (Poss 2010). The regeneration process can take place at a histological, cellular and physiological level, where even regeneration of entire limbs and organs can be accomplished (Carlson 2007). Complex multicellular organisms can perform regeneration from the moment of their creation. Thanks to the regeneration process, multicellular organisms can manage their unique sizes and physiological homeostasis that help them to reach greater longevity (Kawakami 2010).

Regeneration is observed in plants, algae, fungus and animals (Carlson 2007). The regeneration of plants shares features observed in animal regeneration (Birnbaum et al. 2008). For example, stem cells participate in animal tissue regeneration(Poss 2010) and a similar type of cell, called pericycle cells, have been described to participate in the regeneration of the plant shoot (under cytokinin estimulation) and roots (Atta et al. 2009). Another mechanism through which animals regenerate is the formation of a blastema (Wang et al. 2012). Similarly with plants a mass of cells called a callus can be formed where regeneration takes place (Nabors 2004). Finally the end of the meristem in a plant's roots has what is known as the quiescent centre, which is composed of stem cells that can replace damaged tissue (Sena et al. 2010). This structure resembles the apical epithelial cap found during salamander limb regeneration. (Christensen et al. 2000). However, it is important to mention that in many cases during plant regeneration, the new regenerated structure will not be the continuation of the excised tissue, but rather new tissue that resembles the particular excised tissue (Sugimoto et al. 2010), while in animals the new structure will be regrowth from the wound area with a similar size and shape to the pre-existing tissue (Kawakami 2010). Another difference observed between animal and plant regeneration is that cell de-differentiation has been documented to happen during animal regeneration. However cell de-

differentiation is not very well understood in plant regeneration. It is argued that instead of de-differentiation, trans-differentiation takes place in order to form the callus structure (Sugimoto et al. 2011).

I will address aspects of animal regeneration in the present introduction, with emphasis on the zebrafish.

1.1.1 Types of regeneration

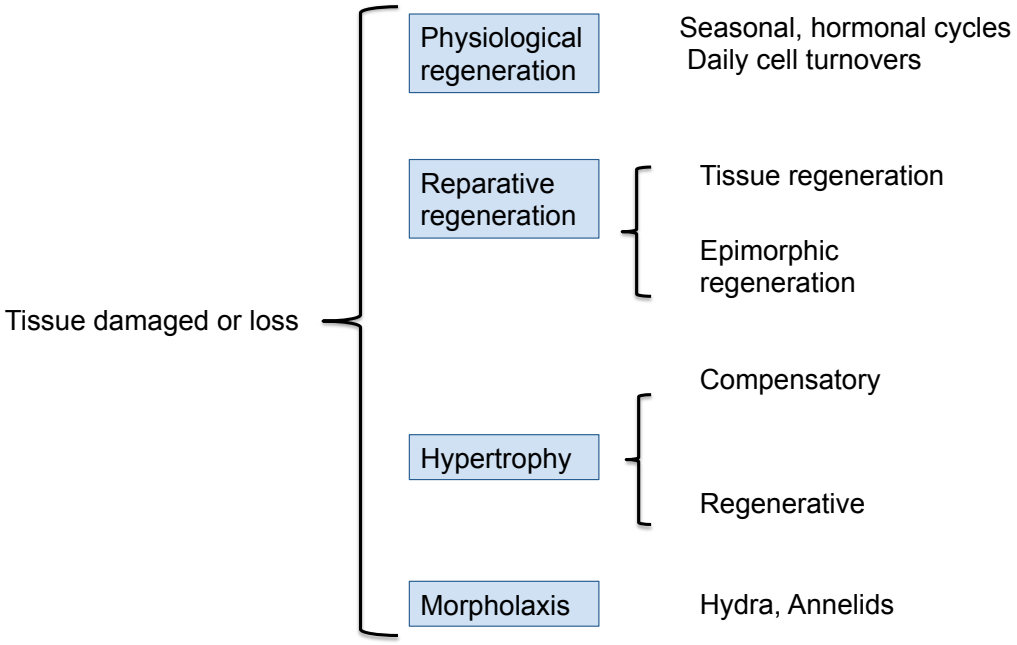

Figure 1-1: The four main types of regeneration within the animal kingdom. Physiological regeneration occurs at times when the tissue deteriorates due to everyday activities and functions, such as skin shedding, so tissue replacement is necessary. Examples include deer antlers; bone remodeling; periderms replacement, etc.. Reparative regeneration where there are two types: 1) Tissue regeneration involving reconstruction of the structure by cell division, where the formation of the blastema is not observed; and 2) Epimorphic regeneration, which is similar to tissue regeneration but involves the formation of the blastema, for example, limb or fin regeneration in salamanders and Teleosts fish. Hypertrophy also comes in two forms: compensatory and regenerative hypertrophy. Compensatory regeneration is observed in paired organs; when one of them is absent the remaining organ increase in size. Regenerative hypertrophy is also present in internal organs, but in this case the mass of the organ is restored after damage. Morpholaxis is the restoration of shape but not of size of the damaged area of the body (Carlson 2007).

Four types of regeneration are present in the animal kingdom. (Figure 1-1) The

first regeneration type is physiological regeneration, which includes bone turnover and epithelial cell replacement. It is also present during hormonal cycles of tissue in birds and the molting periods of snakes. The second type of regeneration, hypertrophy, is a type of regeneration in paired organs such as lungs. For example, after one of the organs has been lost, the remaining one increases in size in order to compensate for the function of the lost organ. Morphallaxis is the third type of regeneration, and can be observed in both annelids and the cnidarian hydra. When one part of the organism is removed, stem cells migrate to reconstitute the shape of the lost part. The result is an organism of the same shape but smaller in size than the original organism, since cell division does not take place. The fourth type of regeneration, reparative regeneration, comes in two forms: tissue regeneration and epimorphic regeneration. Tissue regeneration involves the reconstitution of the lost/damaged tissue without the formation of blastema, but only by cellular proliferation, for example lizard tail regeneration. Epimorphic regeneration is the same as tissue regeneration but with the formation of blastema. After amputation the wound closes and a layer of cells, called wound epidermis, covers the wound. The cells surrounding the wound then proliferate and migrate towards it and form a mass of cells called the blastema. Blastema cells are thought to be undifferentiated, and are highly proliferative. During cell proliferation tissue is form in order to replace the lost tail structure. Finally these cells will re-differentiate into the tissue or tissues that will reconstruct the absent structure (Kawakami 2010;Poss et al. 2003). Epimorphic regeneration will be discussed in more detail in this thesis, since my project's aims are to understand the cellular and molecular mechanism of this process.

1.1.2 Animal models where regeneration is present

To further understand the regeneration process, I will discuss a few examples found in the animal kingdom. Certain flatworms, better known as planarians, are found in fresh water and enjoy a remarkable ability to regenerate the entire body from a small piece of tissue. After tissue excision, highly proliferative stem cells called neoblasts move to the wound area. Once near to the injury area the neoblasts form a blastema that will proliferate and differentiate to replace the lost structure (Newmark et al. 2000). In order to complete regeneration, morphallaxis is necessary to accomplish the right size and shape (Reddien et al. 2004).

Planarians such as hydras also possess a dramatic level of regeneration. Hydras can to restore a fully functional body from a small portion of tissue. It has been reported by (Shimizu et al. 1993) that a minimum of 1% of the total number of the cells that form the hydra is necessary for regeneration to happen. If a hydra is divided in two, either horizontally or vertically, each of the body parts will form a new hydra that will be the same as the original in shape but smaller in size (Tanaka et al. 2011). The reason for this is that cell migration occurs in order to

restore the lost part, and then these cells differentiate into the new structures in order to complete tissue restoration. There is no cell proliferation involved. This process is called morphallaxis (Park et al. 1970; Holstein et al. 1991).

The regeneration process in vertebrates is not as broad as in planaria and hydras, since vertebrates are unable to regenerate the full body. However, there are some vertebrates that display better regenerative properties than others. These animals are salamanders, teleost fish and frogs, which present epimorphic regeneration (Tanaka et al. 2011). Regeneration in these animals can be observed in several internal organs such as the heart, kidneys, brain, liver or even entire limbs (Gemberling et al. 2013; Tseng et al. 2008; Godwin et al. 2013). Extensive studies into the regeneration of the tail of frogs, newts, salamanders and the zebrafish have been performed. The tail has become a common structure to study, due to the variety of tissues that form it and can regenerate, such as the spinal cord, muscles, epidermal cells, fibroblast cells, bone tissue and the notochord (Tseng et al. 2008;Yoshinari et al. 2011; Schnapp et al. 2005). *Xenopus laevis* (frog) tail regenerative capacity decreases after metamorphosis, which is a limitation that teleost fish and urodels do not have (Mochii et al. 2007). Moreover, even though a salamander's appendages resemble human ones more than the fins of zebrafish, zebrafish studies present several advantages over the urodel model. The zebrafish can fully regenerate an adult tail in two weeks instead of the 60 days need for a salamander limb. Thanks to the size of the zebrafish (smaller than urodel) a greater number of animals can be used, and finally the transparency of the zebrafish makes it suitable for several molecular techniques, such as the insertion of fluorescent protein for cell tracking, in situ hybridisation detection, and immunostaining (Nishidate et al. 2007; Singh et al. 2012; Gemberling et al. 2013). For this reason the present PhD research employs the zebrafish as a model in order to study tail regeneration.

As mentioned previously, regeneration is a vital process necessary to maintain tissue homeostasis in multicellular organisms. Therefore, regeneration is also present in humans but is very limited, as evident in the human inability to fully regrow limbs after amputation, and in how after a heart or brain stroke scar tissue is formed instead of tissue repair. However, there are other tissues where regeneration can be observed, such as blood cells, skin, bone, muscle and the liver (Dinsmore 2007; Gemberling et al. 2013 Pellettieri et al. 2007). The main goal when studying the regenerative mechanisms in the previously described organisms is to develop therapies that can be applied in humans. Also, since humans retain a certain level of regeneration, it would be interesting to develop techniques that can enhance this regenerative capacity (Odelberg 2005).

As we can see, regeneration is widely present among vertebrates and invertebrates, but, while there are similarities between these organisms, there are also differences. One example is Reactive Oxygen Species (ROS) signaling, which has

been identified as essential for the initiation of regeneration in both vertebrates and invertebrates. For example, in planarians, ROS is necessary for stem cell differentiation (Pirotte et al. 2015). For *Drosophila melanogaster* (fruit fly) the role of ROS has been reported to be necessary for the recruitment of macrophage cells during wound healing (Moreira et al. 2010). The role of ROS as activator of the immune response is also found in Hydra during tissue regeneration (Wenger et al. 2014) as well as during zebrafish tail regeneration (Niethammer et al. 2009). Another common molecular pathway observed in several organisms during tissue regeneration is Wnt/β-catenin signalling (Brockes et al. 2008). An example of the level of conservation in Wnt signalling during regeneration is the activation of this molecular signaling during the early stages of the regeneration process by the Wnt3 ligand in both *Xenpus laevis* (frog) (Yokoyama et al. 2007) and Hydra (Galliot et al. 2010). In the case of Hh during the regeneration of vertebrate organisms such as *Xenopus*, zebrafish and salamanders it is being found to be necessary during cell proliferation. Finally the activation development pathways such as FGF Wnt/β-catenin Notch, transforming growth factor beta (TGFB) and HH has been described to be active in the same organisms described above but the hierarchical organization varies among them (Sanchez Alvarado et al. 2006).

After highlighting the difference and similarities in the different regenerative organisms I would suggest that regeneration could have been conserved during evolution in many species and lost in others as is the case of mammals and birds. However, it is important to consider that in animals where high regenerative capacities were kept, this capacity could have changed through evolution giving rise to cellular and molecular differences already mentioned between organisms.

1.1.3 The zebrafish as a model for the study of regeneration

As mentioned earlier in this paper, teleost fish have a striking ability to regenerate several internal organs and fins. Broussonet (1786) first described the regeneration process in the pectoral fin of the goldfish. However, it was not until some decades ago that the teleost model has been used extensively to study regeneration. Examples include medaka *(Oryzias latipes)* and zebrafish *(Danio rerio)* (Nishidate et al. 2007). Zebrafish is the model employed for the regenerative research done during this PhD project. Several characteristics made it a convenient model. For instance, the small size allows a large-scale genetics study, transparency of flesh, and easy embryonic manipulation that allows gene modification. Also, it is important to mention that, due to their small size, they have low-cost maintenance in comparison with urodels. Finally, the genomic sequencing of the zebrafish has nearly been completed, which will allow the development of different molecular and genetic methods (Nishidate et al. 2007; Mathew et al. 2009; Yoshinari et al. 2009). As mentioned earlier, zebrafish are able to regenerate internal organs, such as the heart; complete appendages, such as the caudal fin; and even simpler struc-

tures such as photoreceptor cells (Brockes et al. 2001;Iovine 2007). Advances in the research of the regenerative capacity of these structures will be described in more detail in the following section.

1.1.4 Structures of zebrafish that can regenerate and their mechanisms.

Recent research has found that heart muscle regeneration in mammals is possible when clinical techniques are applied. However, mammal regeneration is very limited in comparison to zebrafish regeneration (Kikuchi 2014). The myocard regenerative capacity of the zebrafish has been reported by Poss et al. (2002). Where one fifth of the heart tissue was removed, eventually complete regeneration was achieved 60 days post-heart damage. Furthermore, the research done by Kikuchi et al. (2010) demonstrated that heart restoration was not only done by shape and size but functionally, since the new myocardial tissue had electrical conduction. Other advances in regeneration studies have reported the new findings about zebrafish photoreceptors. The motivation for these studies comes from the understanding that zebrafish have a similar number of neural retinal cell types and one type of müller glia cell, as present in mammals. After retinal injury in the zebrafish, müller cells undergo de-differentiation. Dedifferentiation allows müller cells to change into to a less specialised state. In this way, müller cells resemble stem cells. These retinal stem cells are able to differentiate into rod and cone precursors, which will re-differentiate in order to regenerate the damaged retina (Brockerhoff et al. 2011;Qin 2010). The limitation of mammals is that, even though mammalian müller glia cells can regenerate neurons, the process is insufficient to repair a wounded retina (Goldman 2014).

Zebrafish fins also display interesting regenerative properties, and correspond to the vertebrate limbs and tail of mammals (Hinchliffe 2002). Moreover, the tail fin has been identified as the fin that regenerates the fastest. For these reasons the tail fin has served as a popular model for regeneration in recent times (Pfefferli et al. 2015). Zebrafish tail regeneration includes the reconstruction of several tissues, for instance bony rays, neural axons, mesenchymal cells, epidermis, pigment cells and blood vessels (Azevedo et al. 2011; Kawakami 2010). Therefore the regeneration of the tail allows us to study the regenerative mechanism of different tissues in one appendage. Another advantage in comparison with the heart or photoreceptors is that the tail can be manipulated more easily, since it is not an internal structure. Therefore, I found the zebrafish tail to be a suitable model to study epimorphic regeneration. Furthermore, I decided to use the larval tail over the adult tail, since this presents certain advantages which will be explained in the next section

1.1.5 The use of zebrafish larvae to study tail regeneration.

The extensive use of the adult tail for regeneration studies has generated interesting insights into different molecular and cellular mechanisms. However, the tail regeneration time and the cost to raise and maintain the fish are not optimal (Yoshinari et al. 2011). Kawakami et al. 2004). I decided to analyse the regeneration of the zebrafish larval tail fin fold. This study demonstrated that the molecular and cellular mechanisms of regeneration process in the larval fin fold are similar to those of adult tail regeneration. Epimorphic regeneration was observed with the formation of a wound epidermis and blastema, and the expression of regeneration marker genes was also detected. Finally the Fibroblast Growth Factor (FGF) pathway, as in the adult tail, regulated the regeneration of the fin fold. Furthermore, Yoshinari et al. (2009) made a genetic profiling of the genes expressed in both adult and larval fin folds. It concluded that even though the structures of larval fin folds and adult tails do not share the same origin, the same genes are expressed in both appendages during regeneration. This gives us more evidence about the suitability of using the fin fold for regeneration studies. Furthermore, the fin fold completes regeneration in 3 days instead of 2 weeks in the adult fish caudal fin; feeding is not necessary during the first 5 days (thanks to the yolk); and the small size of the younger fish enables a larger sample size analysis than with adult fish. Altogether these advantages illustrate how the larval fin fold in regeneration studies can be more productive due to low-cost maintenance and fast healing process (Yoshinari et al. 2011). With this in mind I decided to use the larva zebrafish as the model to study regeneration for this doctoral research project.

However, the larval fin fold is a simple structure made of mesenchymal cells, epidermis and collagen fibers called actinotrichia. The simplicity of the fin fold limits the amount of tissue that can be studied during regeneration. Therefore, instead of analysing the fin fold, I will use the tail of the larvae to induce regeneration. As presented in Figure 1-2, amputation will be performed in the pigment gap at the end of the tail. This amputation point will allow me to sever structures such as the notochord, blood vessels, muscle, spinal cord, epidermis and mesenchyme cells, allowing me to induce regeneration in a number of tissue types.

1.2 Molecular mechanisms involved during zebrafish tail regeneration

The study of the molecular pathways activated during regeneration can yield greater understanding of how regeneration takes place in lower vertebrates and how the molecular mechanisms differ from those of mammals. I will describe the most popular pathways already reported during zebrafish regeneration.

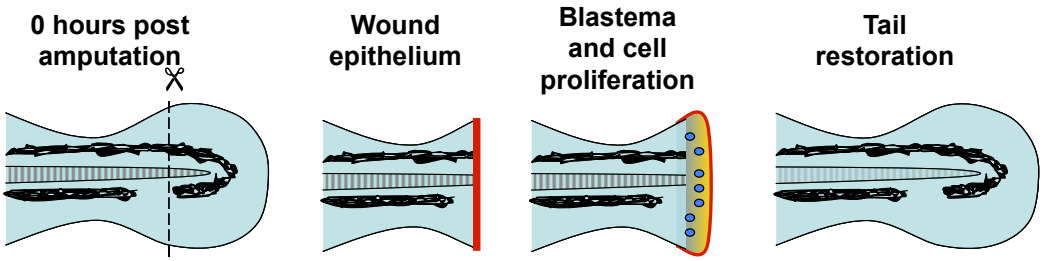

Figure 1-2: Epimorphic regeneration of a zerbrafish larva's tail. After tail amputation epithelial tissue is formed along the amputation plane of the wound. Then cell migration and proliferation take place to form the blastema structure, which in turn proliferates and gives rise to the new tissue structures that compose the regenerated tail..

1.2.1 Importance of FGF signalling during the initiation of tail regeneration and proliferation

Previous molecular dissection of the genes involved during zebrafish tail regeneration has found that the FGF pathway has a crucial role in blastema formation and proliferation Poss et al. (2000b) reports that the *fibroblast growth factor receptor 1* (*fgfr1*) expression overlaps with the expression of regeneration marker genes (*msx* genes) expressed in the blastema. Moreover, when the *fgfr1* inhibitor SU5402 is administered at the beginning of tail regeneration, the blastema structure is not formed. When FGF down-regulation occurs after the blastema is formed, regeneration is still affected as blastema cell proliferation decreases. Another FGF pathway marker gene, the *fibroblast growth factor 20* (*fgf20*) has been described as necessary for the initiation of tail regeneration. The expression of the *fgf20* gene has been reported to colocalise with the regeneration marker gene *homeobox b* (*msxb*). It is suggested, that the purpose of the *fgf20* gene is to regulate the blastema formation (Whitehead et al. 2005). The relevance of FGF signalling is not only restricted to the regulation of the blastema formation and proliferation.Lee et al. (2005) reported that FGF signalling during tail regeneration forms a gradient of expression depending on the location of the amputation site. It was observed that the FGF target genes *mkp3*, *sef* and *spry4* have a stronger expression when amputation occurred at a more proximal level in comparison to a distal amputation. The same results were found for cell proliferation when comparing proximal and distal amputation. We can conclude then that the FGF pathway is necessary to define a gradient that will indicate the position where amputation happened (proximal or distal).

1.2.2 Importance of Wnt/β-catenin signalling and RA during the blastema creation.

Another molecular pathway generating interest is Wnt/β-catenin signalling. The Wnt/β-catenin pathway is essential for tail development. Therefore, Poss et al. (2000b) investigated whether this pathway was involved in zebrafish epimorphic regeneration. Through the use of whole ISH (in situ hybridisation) the expression of different Wnt/β-catenin readouts was detected. *lef1*, a Wnt/β-catenin readout gene, was located in the wound epidermis and in epidermal cells surrounding the blastema. *wnt5* expression was found to be in the same areas as *lef1*. *wnt3* had a mild expression in the regenerated epidermis, while *wnt1* and *wnt8* expression could not observed. Further research on the role of Wnt/β-catenin signalling during tail regeneration reported a relation with FGF signalling, where Wnt/β-catenin pathways seems to be upstream (Wehner et al. 2014). The role of the *wnt5* gene seems to be a negative mediator of tail regeneration, since the overexpression of this gene reduced proliferation and therefore tail outgrowth. Finally, the *wnt8* gene was found to have mediated proliferation in a positive manner (Stoick-Cooper et al. 2007). The accumulation of evidence of different Wnt signalling markers participating in different roles during regeneration helped to set the basis of a further study. In this study, it was proposed that Wnt/β-catenin signalling controls tail epimorphic regeneration by regulating several developmental pathways. These pathways are FGF and BMP (Bone Morphogenetic Protein), which are necessary for controlling epidermal cellular arrangement, and Retinoic Acid (RA) and Hedgehog (Hh) signalling for regulating cell proliferation (Wehner et al. 2014)

As mentioned previously, RA is expressed during tail regeneration. Nonetheless, through the years of research, reports about RA have been contradictory. Geraudie et al. (1995) administered RA at different concentrations and exposure times during tail regeneration, reporting teratogenic effects. Then it was used the *long-fin mutant*, which fails to regenerate the tail in size and shape. RA was administered to the mutant but did not reverse the tail regeneration impairment. Blum et al. (2012) managed to standardise an RA concentration that avoided any negative effect such as cell death during regeneration, by injecting 1 mM RA in a low concentration of dimethyl sulfoxide (DMSO). Once stabilised, suitable RA concentration regeneration studies where done. In these studies it was found that RA is necessary during the formation of the basal epidermal layer, blastema formation and proliferation. All this regulation of regeneration by RA was suggested to happen through FGF, Wnt/β-catenin canonical signalling and Insulin-like growth factor (IGF) pathway. These results agree partially with the reports made by Wehner et al. (2014) regarding the importance of RA in the blastema proliferation. However, there is some disagreement about the pathway hierarchy, since it is suggested by Wehner et al. (2014) that Wnt/β-catenin signalling is upstream of

RA, contrary to the repots made by Blum et al. (2012). The different reports in the literature highlight the importance of further investigation into the role of RA during tail regeneration and to offer a more consistent explanation of the processes involved.

1.2.3 Hh is necessary for bone ray patterning.

Hh has been stated to be involved in adult zebrafish tail regeneration during bone patterning formation and blastema proliferation. No major roles like those of Wnt/β-catenin and FGF have been described for Hh signalling. Laforest et al. (1998) observed the expression of the *shh* (sonic hedgehog) ligand and *ptc1* Hh marker in the dermoskeleton during tail regeneration. Quint et al. (2002) also reported the expression of *shh* and *ptc1*, stating that Hh pathway is necessary during blastema cell proliferation and bone ray patterning. These findings agree with Wehner et al. (2014) who also reports the regulation of cell proliferation by Hh signalling during regeneration, where Hh is regulated by the Wnt/β-catenin pathway.

Regarding the hierarchical position in respect of the Wnt pathway during regeneration in other animals, it is reported that in both salamander and planaria regeneration Hh signalling regulates the Wnt pathway (Yazawa et al. 2009; Bhairab et al. 2012). Similar outcomes were obtained during this doctoral research and are shown later on in this report.

1.2.4 Regeneration marker genes expressed during tail regeneration

Aside from the dissection of the molecular pathways that are involved during regeneration, it is important to know the genes that are expressed during the regeneration process. Regeneration marker genes are normally up-regulated during regeneration and the expression can be observed in different regenerated tissues such as those of the heart, tail and retina (Yoshinari et al. 2011).Expression data of different genes expressed during regeneration also show that these genes take place in different parts of the blastema and wound epidermis (Nishidate et al. 2007).

For the development of this PhD project, three regeneration marker genes were used. *raldh2* is a regeneration marker expressed in the blastema. This gene has a great importance because it encodes for the *raldh2* enzyme. This enzyme, in turn, synthetises the metabolite RA that is necessary for tail regeneration (Mathew et al. 2009). *msxc* is a regeneration marker previously described by Akimenko et al. (1995) as being present during both tail regeneration and development. At 3dpf (days post-fertilization) the expression of the msxc gene is not detected by in situ hybridisation (ISH). For this reason, tail amputation took place at this stage

during my research. The *msxc* gene is suggested to maintain cell dedifferentiation in the blastema (Schebesta et al. 2006). *dlx5a* (previously *dlx4)* is expressed in the wound epidermis during regeneration and is also expressed during normal tail development (Heude et al. 2014); ISH does not detect its expression at 3 dpf. The expression of the regeneration marker genes after molecular pathway manipulation is a good indicator that allows me to understand how regeneration was affected in a genetic manner.

1.3 Molecular mechanisms involved during zebrafish tail development

1.3.1 Pathways expressed during tail bud formation

Developmental pathways such as Hh, Wnt, FGF and RA are reported to activate during tail regeneration (Schebesta et al. 2006). It was also suggested by Iovine (2007) that regeneration recapitulates ontogeny. Ontogeny is the study of the development of an organism until this reaches his final form which involved cell prolifferentaion, differentiation and patterning (Gilbert 2000). Because of the similarities in the molecular pathway activation during both tail regeneration and development, the tail development is studied by evaluating the tail regeneration. Furthermore the tail is convenient, as its amputation is easily performed. However, at the moment, to compare characteristics of normal development with other regenerative processes like morphollaxis and hypertrophy the regeneration and development have some differences. Morphollaxis in comparison to ontogeny does not present cell proliferation, while hypertrophy regeneration comprises active cell division; cell differentiation is not reported. Therefore, there are big differences that does not stick to the statement of regeneration summarises ontogeny, and the idea that regeneration reactivates the exact mechanisms involved during normal tail formation is still in debate (Iovine 2007). For this reason, I decided to study the expression of Hh, Wnt/β-catenin, FGF and RA during tail development, and compare the results with the data obtained from the molecular dissection during tail regeneration. This section will introduce the roles of the pathways already mentioned as being present during normal tail formation.

1.3.2 The importance of Hh, FGF, and Wnt signalling during tail formation

At the end of zebrafish gastrulation, an accumulation of cells forms the 'tail bud' (Kimmel et al. 1990). The tail bud gives rise to the zebrafish tail through elongation, proliferation and cell movement (which is thought to be a continuation

of gastrulation) (Kanki et al. 1997). The molecular pathway Wnt, according to Martin et al. (2012), co-ordinates the location of multipotent stem cells during the tail formation. FGF is as essential as the Wnt pathway, because the lack of both pathways leads to the absence of tail development (Griffin et al. 1995). Wnt and FGF signalling have been found to keep a cross talk during tail development. The communication of Wnt and FGF pathways regulates a molecular equilibrium in order to complete tail formation, cell differentiation and muscle formation (Stulberg et al. 2012). Finally Hh signalling is found to be necessary during somatogenesis, mainly of the slow muscle fibers, during tail development (Barresi et al. 2000).

1.4 Cellular mechanisms involved during zebrafish tail regeneration

My PhD research also involves the cell line tracing of the cells that participate during regeneration, and therefore warrants mention here alongside the molecular mechanisms.

1.4.1 Formation of wound epidermis and blastema

Epimorphic regeneration involves the formation of two essential structures: the "wound epidermis" and the "blastema" (Figure 1-2). After amputation the injury closes and epidermal cells surrounding the area move towards the wound, forming a thick layer called the wound epidermis (Poss et al. 2000b).Even though the epidermis seems to be a very simple structure, it is necessary in order for regeneration to be completed: removing this thick epidermal layer interrupts the process (Kawakami 2010). There is even a hypothesis that claims the reason why higher vertebrates as mammals do not regenerate is due to the type of wound epidermis. It is suggested by Tassava et al. (1982) that in urodels the wound epidermis regulates the blastema by keeping the cells in an undifferentiated state. For mammals, the wound epidermis does not have the same function of maintaining undifferentiated cells, and therefore regeneration never takes place; instead, scar tissue forms.

After the wound epidermis is formed, the cells close to the injury proliferate and migrate towards to wound and form an accumulation of cells called the blastema. High proliferation is observe in the blastema cell that in turn contribute to the formation of the missing strucutre. The blastema is believed to be a mass of undifferentiated cells, however, the identity of these cells is still under debate. Some hypotheses suggest that stem cells actually migrate to the wounded area in order to form the blastema, a mechanism that is observed in planarias; alternatively, it could be that mature cells surrounding the wound area migrate and then de-differentiate, enabling a higher rate of proliferation. So far none of these

explanations have been demonstrated and would require lineage-tracing studies (Kawakami 2010). Studies of cell tracking during regeneration form the second part of my PhD research. I tracked the periderm and blood vessel cells during tail regeneration and the data generated disagrees with the concept of blastema. The position of blastema (identified by the expression of the *msxc* gene) is not the same as the location of proliferation and tissue formation. The blastema appeared to be more a centre that induces de-differentiation during regeneration rather than a centre of tissue formation. I think the concept of blastema should be re-evaluated in light of the modern tools available for lineage tracing, such as the Cre-Lox system. I will describe in more detail the methods used to study different cell lineages in a different section of the introduction.

1.4.2 Dedifferentiation and transdifferentiation mechanisms

The blastema structure has generated much interest not only due to the cells that compose it, but also for the cell plasticity and destiny that the cells could have. The term dedifferentiation refers to the change of gene expression and mature states of a defined cell into a less specialised form. The level of cell differentiation can vary, since the cell can acquire different potencies, ranging from totipotency (one cell is able to differentiate into all the cells that form an organism,) to unipotency (the cell can differentiate into only in one type of cell) (Poss 2010).Previous studies regarding heart regeneration have shown that cardiac cells undergo dedifferentiation and express the *globin transcription factor 4* gene (*gata4*) which is normally expressed during heart embryonic development (Kikuchi et al. 2010). By having this undifferentiated state, cardiac cells can be more proliferative in order to restore the lost heart tissue. Another mechanism that occurs during epimorphic regeneration is transdifferentiation. Transdifferentiation is defined as the ability of a cell to change into a different cell type. An example that can be found in nature is the regeneration of newts' lenses. The newt's iris cells lose their pigment and become transparent while transforming into the cells that will regenerate the lenses (Poss 2010) (Figure 1-3).

1.4.3 Cell proliferation and tail outgrowth

After the formation of the blastema, the organism is ready to restore the missing tissue. To achieve tissue restoration blastema cells undergo cell division in order to create the tissue that will regenerate the tail. Patterning mechanisms are also essential during the outgrowth stage, due to their contribution to the regulation of size and shape of the newly regenerated structure (Poss 2010). Examples of the molecular mechanisms that regulate cell proliferation and patterning have been described by Lee et al. (2009). It is known that the *wnt5* gene acts as a negative regulator of blastema proliferation (Stoick-Cooper et al. 2007) while *shh* is reported

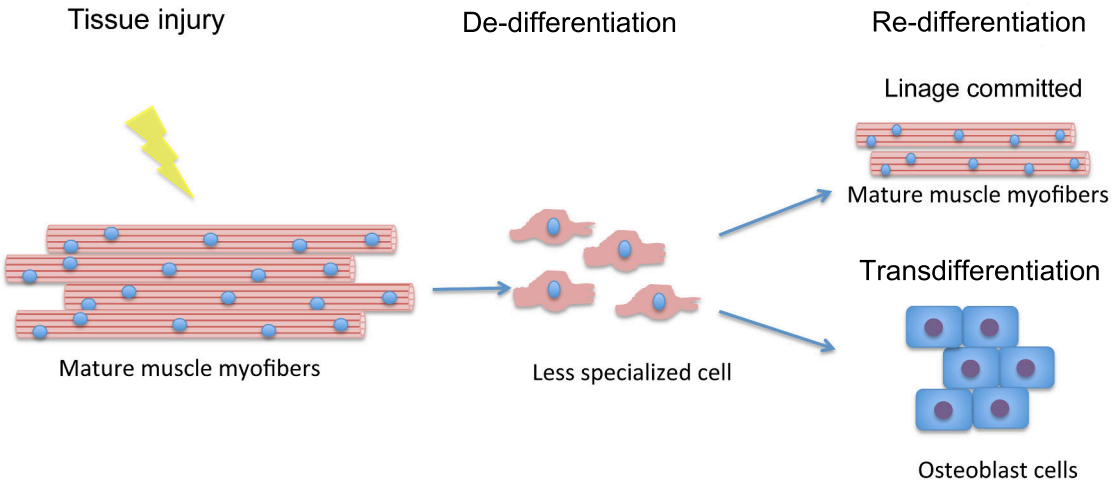

Figure 1-3: Tissue re-differentiation and trans-differentiation. After injury of the muscle fibers, the fibres fragment into individual cells that enter into a less specialised state. This process is called de-differentiation. The less specialised cells differentiate again into a more mature state, by either differentiating into de original cell type or into a different cell type, for example, osteoblast. This process is called trans-differentiation.

to regulate cell division in a positive way. FGF signalling regulates *wnt5* and *pea3* (FGF readout gene), where the expression of these gene restricts Hh signalling to the wound epidermis. Thus the interaction of FGF, Wnt and Hh pathways enables equilibrium in the level of proliferation and patterning during tail regeneration (Lee et al. 2009).

1.5 Lineage tracing methods

Even though the concept of the blastema has widely been described, its role during epimorphic regeneration, as well as the plasticity that cells can have during regeneration (de-differentiation and trans-differentiation). The origin of the cells that contribute to regeneration is still under investigation, and is a topic of much interest in both developmental biology as well as regeneration since the refutation of spontaneous generation. Scientist have been focusing on the development of lineage tracing analysis in order to understand how a whole tissue is created from pre-existing cell/cells.

1.5.1 Direct observation and cell labelling with dyes

One of the first methods of lineage tracing was done by simple direct observation. Direct observation during embryonic development of leech and *Caenorhabditis elegans* (Sulston et al. 1983; Kretzschmar et al. 2012) showed that the cells resulting from the first cell division have different fates and functions in later development.

It was also documented that the fate of the cells composing the embryo could be greatly influenced their position inside the embryo. Even though direct observation proves to be a very practical and non-invasive form of lineage tracing study, its application is limited to small organisms. Therefore, in order to analyse the embryonic development of vertebrate organisms (that tend to be larger and more complex than leeches and *C. elegants*), (Vogt 1929) labelled regions of the embryo with non-hazardous dyes. The dyes were applied at early stages of gastrulation and then the final cell type of the labelled cells was observed at late stages of development (organogenesis). It was then reported that different regions of the embryo give rise to different, which in turn form body systems with particular functions. Thanks to this technique Vogt managed to make a 'fate map' that explained the origin and fate of different regions of the amphibian embryo. One of the main disadvantages of this method is that the dyes get diluted over time.

1.5.2 Genetic markers

In order to overcome the problems associated with dye diffusion, a method that permanently labeled cells had to be developed. One method uses enzymes that, by reacting with a substrate, generate a traceable colour. Examples include β-Galactosidase and Alkaline phosphatase (Chalfie et al. 1994; Price et al. 1987; Holland et al. 1998). Another method is to use genetic markers such as fluorescence, for example GFP (Green Fluorescent Protein). The incorporation of genetic markers can be achieved by direct injection into the cell, transfection and viral infection. A good example of cell tracking using viral infection was performed by Doetsch et al. (1999), where astrocytes cell tracking was done by using the avian leukosis virus receptor, under the control of a tissue-specific promoter (in this case expressed the promoter only in astrocytes). The leukosis virus was able to bind only to the astrocytes and, once the virus was inside the cell, it could synthesise the alkaline phosphatase enzyme. The enzyme when in contact with the substrate generates a colour and allows visualisation of the cells of interest. This technique has also been applied to track the different cell lineages that participate in the tail regeneration of adult zebrafish. Tu et al. (2011) created a transgenic line that expressed GFP under the control of the EF1α (Elongation Factor 1 alpha) promoter. The construct was injected at one cell stage, expecting that it will be incorporated by only one stem cell, that in turn will give rise to only one type of tissue. Fortunately, after several genetic screenings, it was found that there were groups of fish in which GFP was only expressed in one tissue. The tissues carrying the *EF1α:GFP* construct were blood vessels, epidermis, fibroblasts, osteoblasts etc.. Regeneration was then induced by amputation, and after tail regeneration was completed it was concluded that the tissues that participated in tail regeneration remained lineage-committed. Genetic marking proved to be permanent, and the label could be inherited to the daughter cells. However, it is still necessary to

overcome other problems such as low level of genetic incorporation into the cell, along with alteration that the cell may have as a result of gene incorporation into the cell.

1.5.3 Cell and tissue transplantation

Another popular technique for cell tracing is the creation of chimeras (a single organism formed from cells of different organisms). Cells from one organism (donor) are incorporated into another (host) and this is most commonly performed during embryonic development. Differences in colour, size and cell cycle behaviour help to distinguish the donor cells from the host. These transplants can be done between organisms that belong to the same species (allotransplantation) or two different species (xenotransplantation) (Kretzschmar et al. 2012). With the use of cell and tissue transplantation techniques it has been possible to track the neural crest cells of a quail into A chicken embryo (Le Douarin 1980). It is also possible to combine cell and tissue transplantation with cell labelling using dyes or genetic markers. This technique allows for differentiation between the donor cell of the host by a marker in the cell. Gargioli et al. (2004) made a *Xenopus laevis* transgenic line, expressing GFP in the whole body under the control of the CMV (Simian Cytomegalovirus) promoter. The GFP cells were then transplanted into an organism from the same species that did not express GFP. The tracking of the GFP cells during tail regeneration generated data that suggested spinal cord and notochord cells participate during tail regeneration of the Xenopus tadpole. Cell trans-differentiation was not observed at all. The advantage of cell and/or tissue transplantation is that cell differentiation between host and donor is very obvious. However, transplantation is still an invasive technique that can affect the health of the organism and the life span of the chimera, which is normally short after birth.

1.6 Cre-lox as a tool to track cell lineages during tail regeneration

As mentioned earlier, the cell origin, fate and the level of plasticity that cells present during epimorphic regeneration is still not clear. As a second part of my PhD research I decided to perform lineage tracing of different cells that might contribute to regeneration. To this end I used the Cre-lox system where the expression of Cre is under a tissue-specific promoter. This system allowed me to label specific types of cells in a permanent manner. Furthermore, I could also control the accuracy of cell labelling by using 4-hydroxytamoxifen (4OHT). I will describe in more detail how the system works and the previous studies already performed.

1.6.1 Description of Cre -lox system

The Cre-Lox system was originally found in the bacteriophage P1, which has a linear DNA that is injected into the bacteria host. Once the linear DNA is inside the host the Cre (which causes recombination) the recombinase enzyme gets transcribed. The Cre enzyme then targets the loxP (locus of crossing over (x), Pl) located in the bacteriophage DNA, generating recombination, which results in the circularisation of the genome (Sternberg et al. 1981a; Sternberg et al. 1981b). Further applications has been given to the Cre-lox system, which is also able to cause site-specific deletion, inversions, insertion and translocations of DNA (Hans et al. 2009). Sauer (1987) was able to insert the cre-lox system into the *saccharomyces cerevisiae*, an eukaryotic organism. Later the system was implemented in other organisms such as mice and zebrafish (Hans et al. 2009; Thummel et al. 2005). The specificity of the DNA recombination is due to how the Cre molecule can recognise the lox sites which are paired DNA segment of 34 DNA bases.The orientation of the lox sites is important for either deletion (both lox sites face the same direction) or inversion (both lox sites face each other) (Sauer 1987), which can be observed in Figure 1-4. Feil et al. (1997) managed to generate a Cre-lox system where recombination can be controlled by coupling the Cre section with the ligand of the human estrogen receptor ERT2. The controlling of cell recombination is due to the Cre ERT2 enzyme is unable to enter to the cell nucleus, therefore, unable to cause any recombination. When 4OHT is administrated it functions as the ligand of the ERT2 that when binds causes configuration of the receptor, and is realised from the heat-shock proteins (hsp) that act as chaperones (Metzger et al. 1995). Cre enzyme then can enter into the nucleus of the cell and cause DNA recombination as, explained in Figure 1-5. The generation of this system that allows to control DNA recombination in specific sites is useful not only for lineage tracing but also allows the study of genes of interest.

1.6.2 Zainbow multilox system

A system to label cells with different colours within the same tissue has been developed by Livet et al. (2007). The multicolour system is called “brainbow” and has allowed the tracking of neural cells and their synaptic interactions in the brain of mice. The multicolour system consists of Cre-lox, which causes recombination in a multi-lox genetic construct. The multilox construct has two or three pairs of lox sections that are incompatible between each lox pair (see Figure 1-6)A. Each multilox has two or three fluorescent protein genes, but only one gene per construct is expressed after Cre recombination (see Figure 1-6)B. With brainbow, more than one multi-lox section is located in tandem with the same genetic sequence in the same cell. Therefore, the expression of a wide range of fluorescent protein hues along the cell population can be observed. This is the result of the combination

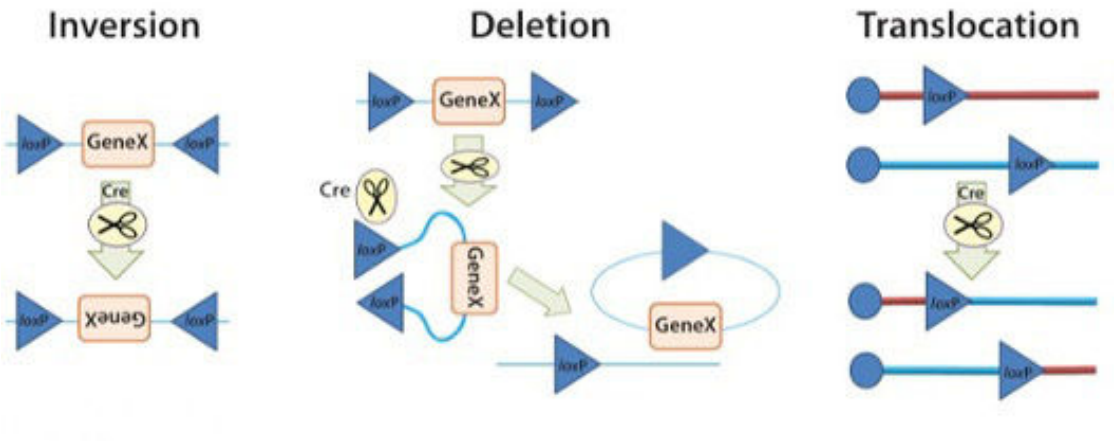

Figure 1-4: DNA inversion results as consequence of putting the lox sites in the same DNA strand facing each other. DNA deletion is happens after Cre molecules identify two lox sites in the same strand facing the same direction. DNA translocation takes place when each of the lox sites are in different DNA strands; the DNA next to the lox site is interchanged between DNA strands (Image source Haliw 2015).

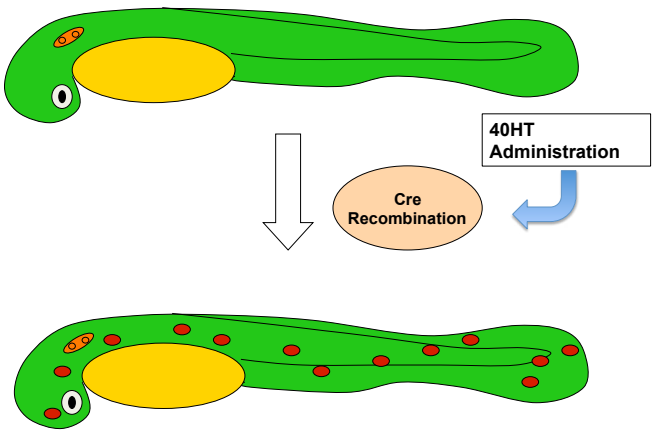

Figure 1-5: Cre recombination taking place in *krt4* expressing cells after 4OHT administration. As a result of 4OHT administration described in Figure 1-4, the larva expressing GFP fluorescent now expresses DsRed protein in the periderm cells, where Cre is driven by promoter *krt4*

of each of the fluorescent proteins from each multi-lox section within one cell (see Figure 1-6)C. This system can be applied to other models such as zebrafish for the study of tissue regeneration or normal development. The advantage of the multicolour system is the possibility to differentiate a single cell from its neighbours. This makes it possible to study the interactions.

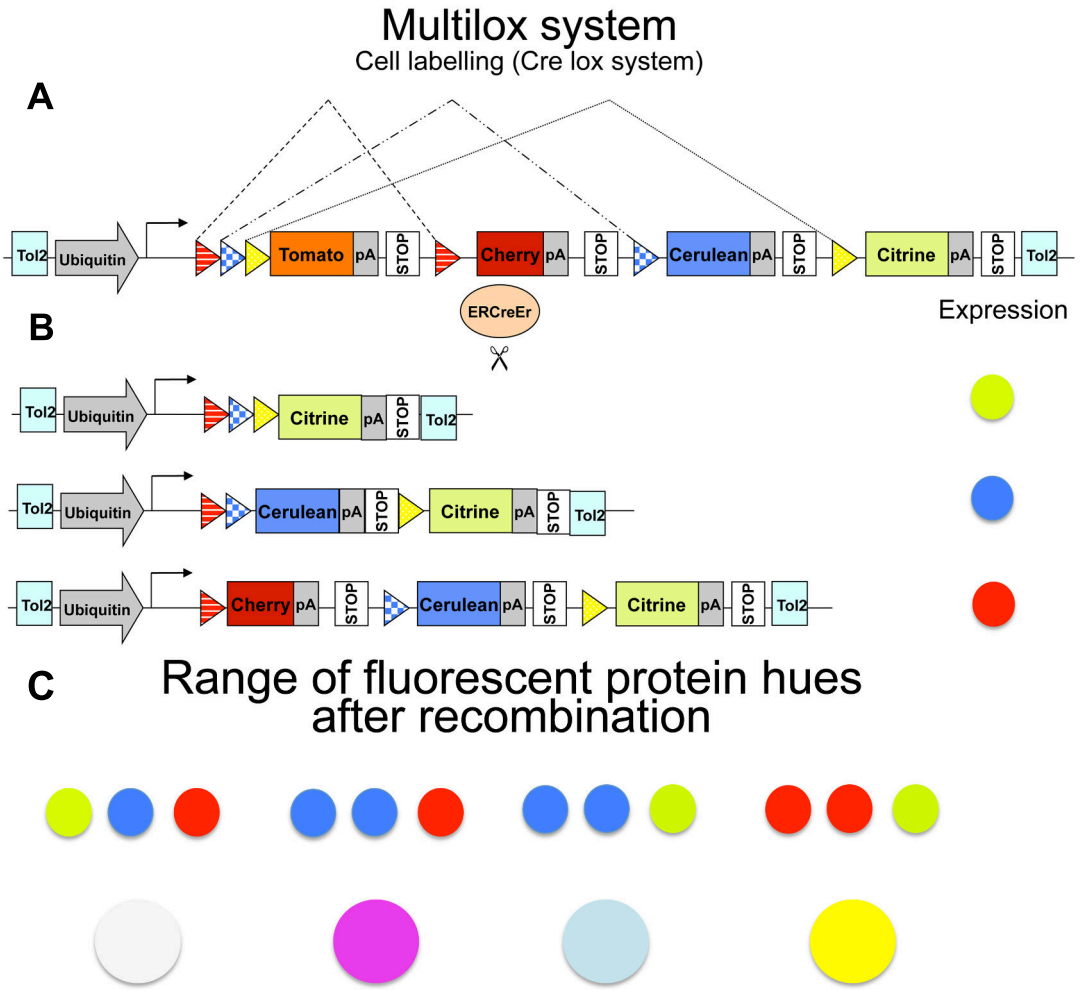

Figure 1-6: Multilox system. **A** Multilox system with three pairs of lox sites. **B** Cre recombination generates the expression of three fluorescent proteins in the same organism. **C** Besides the exression of the red, blue and green fluorescent colours, the expression two or three of them can generate different hues

The initial approach of lineage tracing analysis for the developing of the present PhD thesis was the use of the "zainbow" technique. The zainbow technique is an adaptation of the brainbow in zebrafish. The genetic construct as well as the transgenic line were successfully created. Tissue recombination and expression of three fluorescent proteins in the zainbow transgenic line were observed after the injection of Cre RNA (see Figure 1-7). Unfortunately at the moment, when crossing the zainbow line with a Cre ERT2 expressing transgenic line, recombination was not observed. Therefore, I decided to switch to a more simple version of the

lox system that consists in only one lox pair (see Figure 1-8).

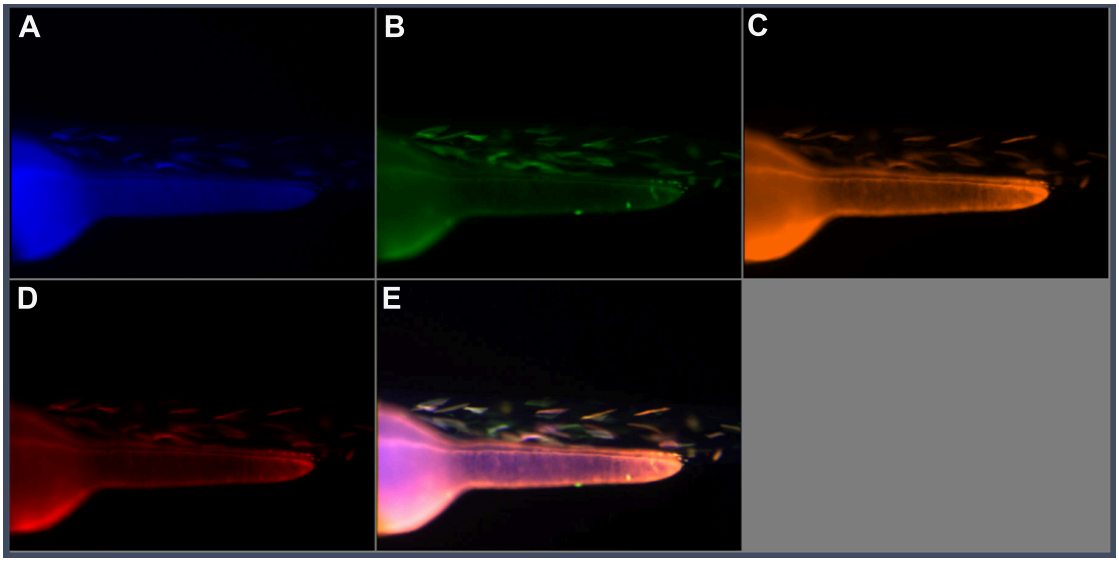

Figure 1-7: Multicolour labelled fish. Image of the zainbow line tissue recombination after Cre RNA injection. 2dpf representative embryo is depicted in the panel. **A** Cerulean fluorescent protein expressed in the muscle stripes. **B** Citrin fluorescent protein expressed in the muscle stripes. **C** Tomato fluorescent protein expressed in the muscle stripes. **D** Cherry fluorescent protein expressed in the muscle stripes. **E** Merge of the fluorescent protein expressed in the muscle stripes

Cell linage tracing in this study used the *Tg(EF1α:loxPGFPloxP-DsRed)* and *Tg(ubiquitin:loxPGFPloxP-mCherry)* transgenic lines. The transgenic fishes express the green fluorescent protein (GFP) in the whole body, since the ubiquitous genes *ubiquitin* and *EF1α* drive GFP expression. Once 4OHT is administered Cre is able to go into the nucleus and remove the GFP section from the genome, leaving only the *Discosoma Red (DsRed)* or *monomer Cherry* (*mCherry*) genes. Both of these synthesise a red fluorescent protein. The ubiquitous genes make the cell labelling permanent by driving the expression of the red fluorescent protein. Finally, specification of where tissue recombination will happen (change from GFP to red fluorescent) depends on the promoter that drives the expression of Cre.

I decided to use seven tissue-specific promoters that will drive the expression of Cre in the tissues that form the zebrafish larva tail; these promoters are described in Table 1-1

1.6.3 Previous studies using the Cre-lox system during tail regeneration

Studying the destiny of osteoblast cells during adult zebrafish tail regeneration has already been done by using a lineage tracing analysis with the Cre-lox system. Knopf et al. (2011) generated the double transgenic line *Tg(osterix:CreERT2;hsp:loxP-*

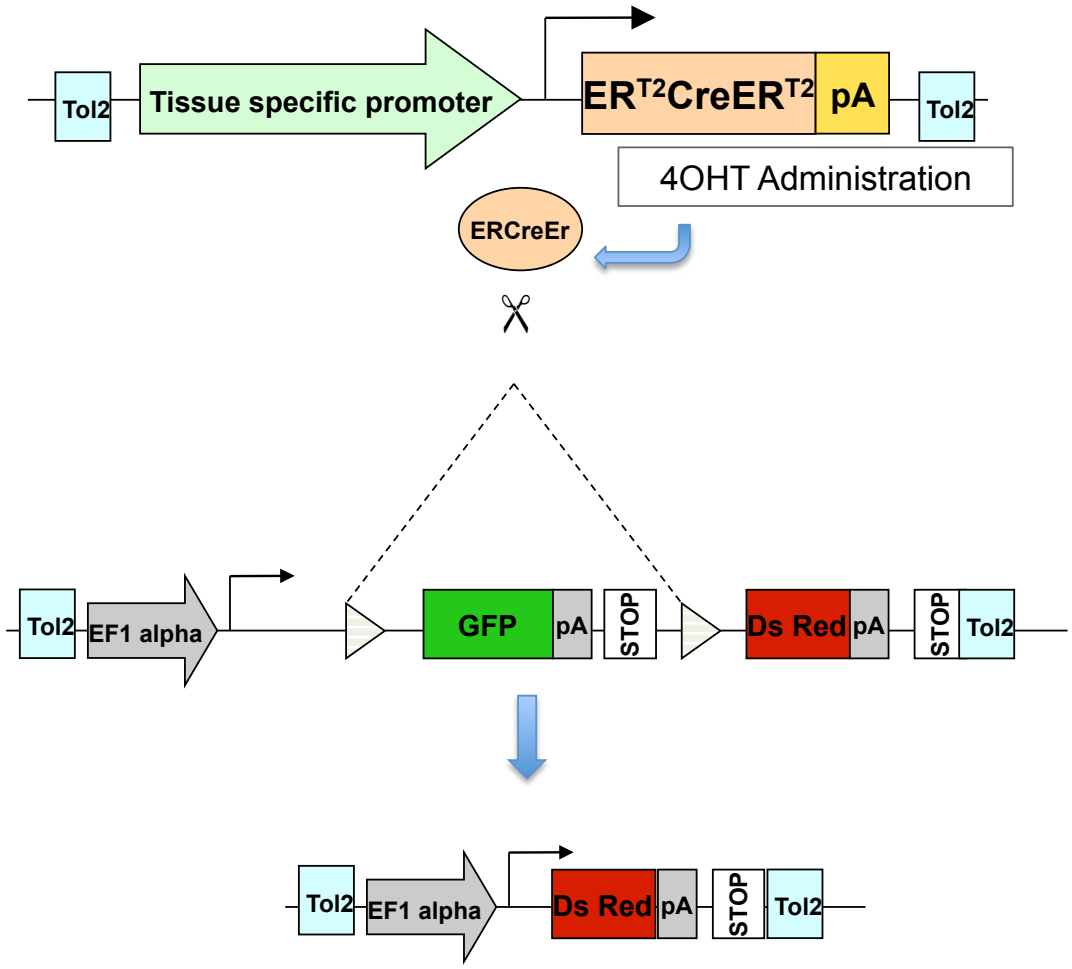

Figure 1-8: Cre expressing line driven by tissue specific promoter. Cartoon showing the Cre-lox system with only one pair of lox sequence. Recombination takes place only in the tissue where Cre is expressed, and after 4OHT administration.

Table 1-1: Tissue specific promoters

Tissue of expression	Promoter
Mature muscle	*αactin*
Periderm cells	*krt4*
Blood/Endothelium	*fl1a*
Chondrocytes and sclerotomal cells	*twist*
Osteoblasts	*osteocalcin*
Neural stem cells	*nestin*
Commited neuron cells	*huc*
Neural crest cells	*sox10*

DsRed-loxP-nls-EGFP) wheren Cre was driven by the intermediate osteoblast marker *osterix*. Once osteoblast labeling was observed (cells expressing red fluorescent colour), tail regeneration was induced by amputation.4dpa (post-amputation) green cells were found in the regenerated tissue. In order to confirm the identity of these cells, an antibody staining against the zn-5 (bone epitope) was performed. 92% of the cells were found to be osteoclasts cells. Finally, the expression of GFP was not observed in any other tissue than bone. This suggests that the formation of new bone tissue comes from pre-existing osteoblasts.

Singh et al. (2012) also decided to study the fate of the osteoblast cells by using the Cre-lox technology. For this study, genetic ablation was included. Genetic ablation allows the elimination of the desired tissue by driving Nitroreductase (Nrt) with a tissue-specific promoter. Once Nrt is in the tissue of interest, administration of metronidazole (Mtz) reacts with Nrt causeing cell death (Pisharath et al. 2009). Therefore a triple transgenic line was generated *Tg(osx:CreERT2; β-actin2:loxP-DsRed-Stop-loxP-EGFP;osx:NTR).* Recombination was done in osteoblast cells, where the fish expressed red fluorescent protein with green bones. Once cell recombination was observed Mtz was administered and the fish did not express the red fluorescent color any more, because of the death of the osteoblast cells. After this, amputation was performed and the tail was allowed to regenerate. Surprisingly, the newly regenerated tissue included the formation of bony rays, however, none of them expressed GFP. This suggested that the origin of the new cells was different from pre-existing osteoblast cells (since the osteoblast cells were eliminated). Cell flow cytometry confirmed that there were no osteoblast cells after the administration of MTZ. These results suggest that the new osteoblast cells used to be other cell types that experienced transdifferentiation.

1.6.4 Models to be tested with the use of Cre-lox system.

The outcomes presented in the two previous studies suggest the use of Cre expressing lines with tissue-specific promoters in order to increase understanding of the cell fate, plasticity and types of tissues that are involved in tail regeneration. The creation of these transgenic lines will allow me to test these models (see Figure 1-9)

1.7 Aims and objectives

For my PhD project I decided to work in parallel on two aspects of the epimorphic tail regeneration. The first involved the molecular dissection of the pathways *involved* during regeneration and the relation of these pathways with each other. The second was the creation of five Cre expressing lines with tissue-specific promoters which were used for lineage tracing analysis. I decided to start with the

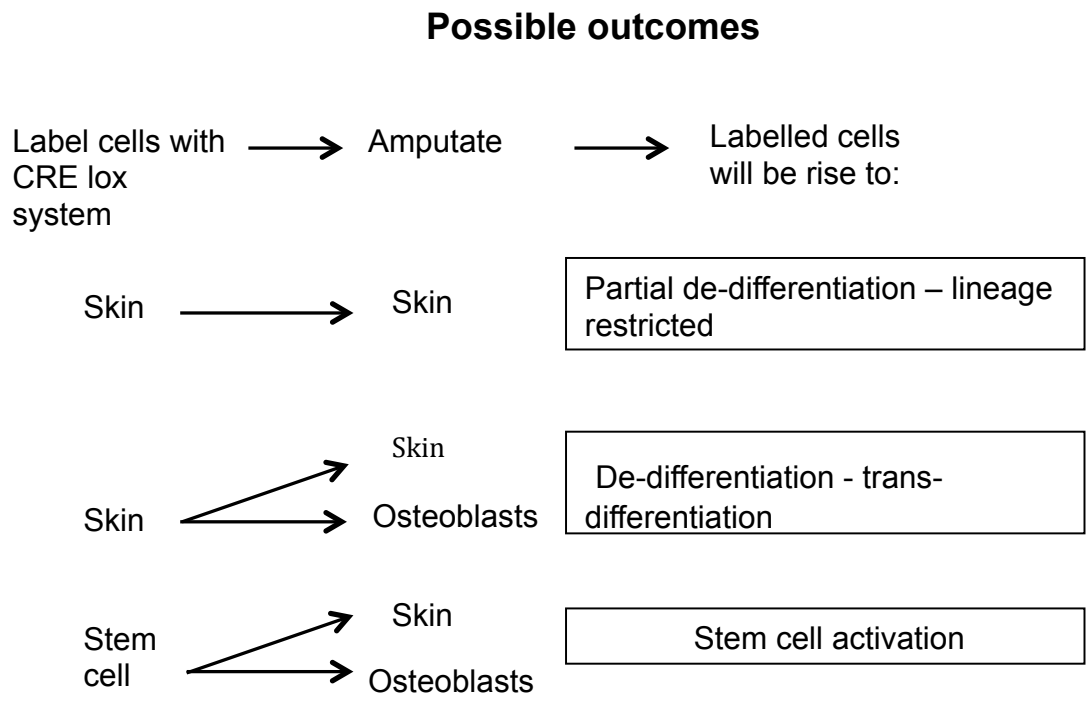

Figure 1-9: Possible outcomes expected after cell lineage tracing. During tail regeneration, cells can originate from mature cells or stem cell like. The cells can either be lineage committed or trans-differentiate into another cell type.

creation of the transgenic lines. The reason for this was that I wanted to create stable lines. Nonetheless, sexual maturation and the creation of a second generation of the transgenic line would take a year. So whilst the fish were growing and the second generation was created I could work on the molecular analysis involved in tail regeneration.

The molecular dissection project was a continuation of my Master's dissertation. My Master's research had shown that Hh signalling was regulating the regeneration marker genes in an indirect way, and that the Wnt/β-catenin pathway regulates regeneration marker genes in a more direct way. This is in contrast to the established opinion that hh signalling is only involved in bone ray patterning and proliferation (Quint et al. 2002). Furthermore Wnt/β-catenin signalling is claimed to be upstream of FGF signalling during regeneration (Stoick-Cooper et al. 2007). This research had been performed in adult fish and did not concur entirely with results of my Master's. Hh signalling had been shown to be essential for the initiation of tail regeneration and necessary for cell proliferation, while the study of FGF signalling was not very conclusive. I decided then to formulate and test the following hypothesis: Hedgehog signalling leads tail regeneration by regulating Wnt/β-catenin and FGF signalling in larval zebrafish.

The second part of my PhD project involved the creation of several transgenic lines with tissue-specific promoters that will drive Cre, with cell recombina-

tion controlled by 4OHT administration. There were a lot of advantages to Cre technology compared to the use of fluorescent proteins driven by tissue-specific promoters. The first advantage is that the labeling is permanent, which doesn't happen when using fluorescent proteins. The tissue-specific promoter is no longer active when cells start to dedifferentiate, and then the fluorescent protein is no longer observable, meaning that the cell cannot be tracked. The second advantage is that I could control when cell labeling will happen. Therefore if any new labeled cells appear in the regenerated tissue one can be certain that they came from the previous labeled cells. So this provided a more effective way to track the origin of the cell. The third advantage was that I could also study the destiny of these cells and whether any of them transdifferentiate or not. A transgenic line with a tissue-specific promoter driving a fluorescent protein would not allow me to observe this, since during trans-differentiation the promoter will no longer be active.

Once the transgenic lines were established I would make a lineage tracing analysis in order to address the following questions: Which cells participate during tail regeneration? Which is the origin of cells that participate during tail regeneration? Is there any stem cell-like participation? Therefore, for the realisation of both projects, my aims were the following:

- Study of developmental pathways involved during zebrafish tail regeneration.
  - More specifically analysis of Hh, Wnt/β catenin, FGF pathways and RA.
    - Find the relation between these pathways in order to assess their organization, two models were suggested linear hierarchy or despotic hierarchy with pathway cross talk (see Figure 1-10)
- Compare the role of developmental pathways in both tail development and regeneration.
  - Compare the mechanisms of Hh, Wnt/β catenin, FGF and RA during tail development and regeneration.
  - Assess whether the relationship between the pathways found during regeneration is the same as during tail formation.
  - Study the tissue de-differentiation stage of regeneration by assessing the expression of early tissue development marker genes.
- Create several transgenic fish lines with tissue-specific promoters as Cre drivers.
  - Create stable Cre expressing lines, where recombination can be controlled by 4OHT and spontaneous regeneration is not observed.

- Assess different characteristics of epimorphic tail regeneration using the Cre transgenic lines.
  - Observe which cell lineages participate in tail regeneration.
  - Assess whether there is stem cell-like participation in tail regeneration.
  - Assess whether cells undergo de-differentiation and/or trans-differentiation.

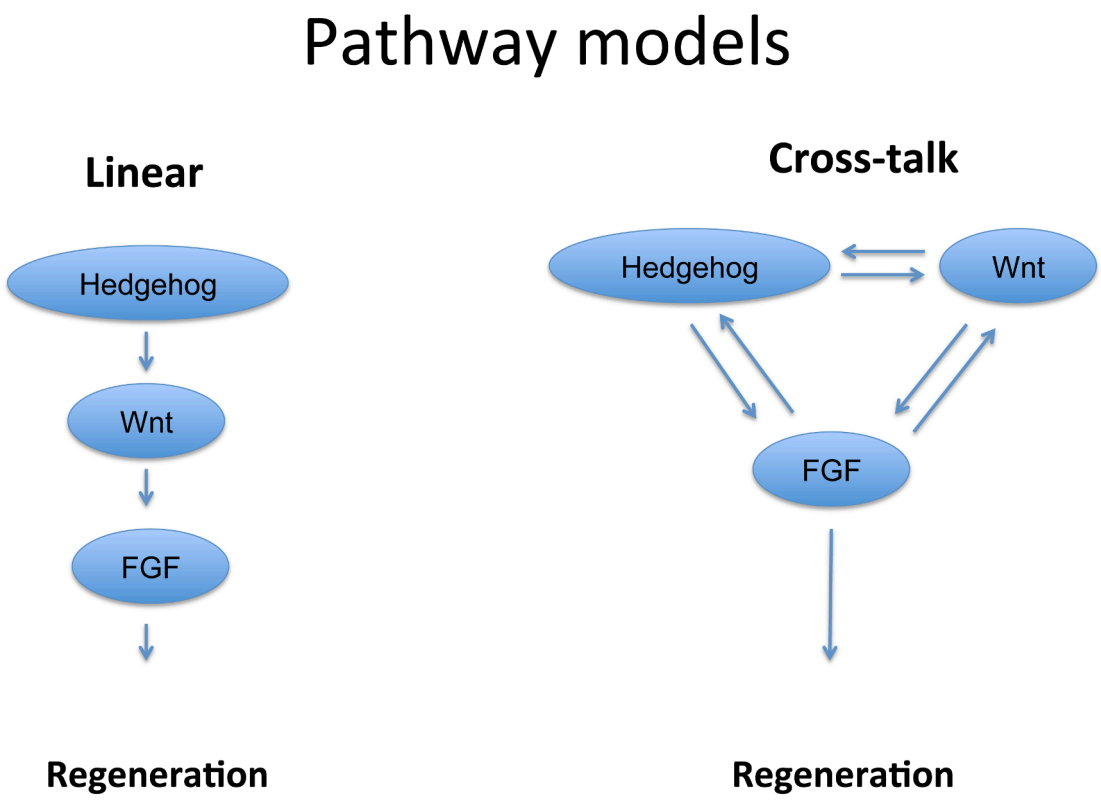

Figure 1-10: Possible models to explain the relationship between developmental molecular pathways during tail regeneration.

Chapter 2

Material and Methods

The molecular techniques and methods used during my PhD research are described in this section.

2.1 Zebrafish husbandry

Zebrafish (Danio rerio AB strain) embryos were obtained from zebrafish adults kept at an aquarium facility at Sheffield University. The embryos were staged under the dissection scope as described by Kimmel et al. (1995). Embryos were put into petri dishes (40 embryos per dish) in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4 plus 0.000001 Methylane Blue). The petri dishes were placed in incubators set to 28°C until the embryos reached the desired stage.

The regulated procedures were performed on fish 5 days post-fertilisation (dpf), or that would have become 5dpf. Next an Individual Study Plan (ISP) was elaborated and submitted to the aquarium manager for approval of the procedure.

2.2 Tail amputation, regeneration and chemical treatment

Fish tails were amputated at 3 dpf. Using a micro scalpel up to a millimetre of tissue was removed from the end of the fish tail. A piece of tape was used as a surface whilst performing the removal. The fish were put back into the E3 medium and remained there for 4 days in order to allow tail regeneration. Fish older than 5dpf were culled according to Schedule 1 of the Animals (Scientific Procedures) Act 1986, and fixed with 4% Paraformaldehyde (PFA) for image analysis.

2.2.1 Fin fold amputation and regeneration

A micro scalpel was used to remove the fin fold of the embryos. The very end of the tail pigment was used as a reference to cut the fin fold. Embryos were allowed to regenerate for 3 days or fixed at different stages of regeneration. Fish older than 5dpf were culled according to Schedule 1 of to the Animals (Scientific Procedures) Act 1986 and fixed with 4% Paraformaldehyde (PFA) for image analysis.

2.2.2 Chemical treatment

Serveral molecular pathways were activated or inhibited in order to examine their influence on the tail morphology and gene expression during tail regeneration. The chemicals listed in Table 2-1 were administered continuously from amputation until fixation. Pulse treatment involves the administration of the chemical some hours before fixation, and the exact number of hours is indicated in Table 2-2. The time of administration plus the concentration of each compound was standardised during the development of my Master's (Garcia Romero 2011) and PhD projects (both degrees were completed at "Roehl's lab"). The genetic parameters used to determine the concentration and time of administration of the chemical compounds were based on the changes in gene expression of pathway readouts of the targeted pathway and the expression of the regeneration marker genes. Gene expression was assessed by in situ hybridisation (ISH). The morphological parameters used were the overall health of the fish during the time of the compounds' administration and tail morphology. The genetic parameters were measured during fish tail regeneration, while the morphological parameters were assessed during both normal development and tail regeneration.

Table 2-1: Small molecules treatments, concentration, target molecules and pathways during continuous treatment.

Molecule	Target	Pathway	Concentration
NVP-LDE225 (Erismodegib) (Selleckchem)	Binds to Smoothened	Hedgehog (inhibition)	20 μM
Cyclopamine (Calbiochem) (Sigma)	Binds to Smoothened	Hedgehog (inhibition)	20 μM
DEAB (Sigma) 4-Diethylaminobenzaldehyde	Inhibits Aldehyde dehydrogenase	Retinoic Acid (inhibition)	50 μM
IWR-1 (Sigma)	Stabilization of Axin protein	Wnt (inhibition)	5 μM
GSK3 inhibitor (Calbiochem)	inhibits GSK3	Wnt (activation)	0.625 μM
Vehicle:Ethanol or DMSO	-	-	-
E3 medium	-	-	-

Table 2-2: Small molecules treatments, concentration, pathways and timing during pulse treatment

Molecule	**Treatment period**	**Pathway**	**Concentration**
SU5402 (Sigma)	4 hours	FGF (inhibition)	15 μM
Cyclopamine (Sigma)	8 hours	Hedgehog (inhibition)	50 μM
Retinoic Acid (RA)	4 hours	Ectopic administration	1 μM
GSK3 inhibitor (Sigma)	8 hours	Wnt (activation)	12.5 μM
DEAB (Sigma)	4 hours	RA (inhibition)	250 μM
Vehicle:Ethanol or DMSO	Depending on the chemical	-	-

Fish were put in groups of 10-20 per glass petri dish, with the chemical dissolved in the E3 medium. The list of the small molecules used during the project is listed in Tables 2-1 and 2-2.

Cyclopamine was dissolved in 100% ethanol for 2 hours at 70°C. Once dissolved it was left at -20°C overnight before administration. Aliquots were made at 10mM. Cyclopamine was stored at -20°C and heated up to 70°C for 15 minutes before administration in order to dissolve it. As the effectiveness of each batch varied, cyclopamine was tested by checking the level of tail regeneration during continuous administration at 20μM.

SU5402 was dissolved in dimethyl sulfoxide (DMSO) and stored at -80°C. Aliquots were taken at a concentration of 10 mM.

NVP-LDE225, Erismodegib was dissolved in DMSO and stored at -80°C. Aliquots were taken at a concentration of 10mM. The following procedure was used for NVP-LDE225: For a final concentration of 10μM place the chemical in a glass bottle followed by 1% of Ethanol (this will help produce a better dilution). Add the E3 medium into the bottle and vortex immediately until it is completely dissolved. Pour the contents into a petri dish and then put the embryos into the dish.

RA was dissolved in DMSO and stored at -20°C. Aliquots were taken at a concentration of 1mM.

Glycogen synthase kinase 3 (Gsk3XV-inh) was dissolved in DMSO and stored at -20°C. Aliquots were taken at a concentration of 5mM.

IWR-1 [4-(1,3,3a,4,7,7a-Hexahydro-1,3-dioxo-4,7-methano-2H-isoindol-2-yl)-N-8-quinolinyl-Benzamide] was diluted in DMSO and stored at -20°C. Aliquots were taken at a concentration of 20mM.

DEAB (Diethylaminobenzaldehyde) was prepared anew every time before ad-

ministration. The stock was prepared according to the treatment (continuous or pulse) and then diluted at a ratio of 1:1000.

2.3 Whole mount in-situ hybridization and regeneration marker detection

Antisense RNA syntesis

The DNA template for probe synthesis was done by amplifying DNA sequence inside the PCR product or plasmid, using primers in Table 2-3. The PCR program was set up with the following cycles: 35 cycles, each consisting of 45 seconds at 94 °C; 30 seconds at 58 °C; 120 seconds at 72 °C. And the reagents for the PCR amplification and transcription are described in Table 2-4, 2-5 and 2-6

Table 2-3: Probes employed to test the expression of regeneration markers and molecular pathways genes

Probe	Forward primer	Reverse primer	Poly*.	Backbone/ restriction enzyme
msxc	PBS for M13 gtaaaac gacggccagt	PBS rev M13 ggaaacag ctatgaccatg	T7	pBSK(-)/ BamHI
raldh2	M13 for gtaaaacg acggccag	M13 rev caggaa acagctatgac	T7	PCR product
dlx5a	Dlx5a R1 cttccaaatgcaa taaattacatgca	Dlx5a f2 tagtgtagactct ttggacccctat	T3	Sk- /BamHI
ptc1	M13 for gtaaaacg acggccag	M13 rev caggaa acagctatgac	T3	pBSK/ Bam HI
lef1	Lef1-for tcccagaacg tcgaataagg	lef1-rev gcttggggt tgacatcttg	T7	PCR product
wnt10b	M13 for gtaaaac gacggccag	M13 rev caggaaac agctatgac	T7	EcoRV/ pExpress-1
tcf7	PCRII for T7 agtgaat tgtaatacgactca	PCRII rev ccgccagtg tgtgtctggaattc	T7	PCR II-TOPO/BamHI
axin2	axin2-for caaccaag cacatccatcac	axin2-rev tgcgaa tgtaaggagcagtg	T3	PCR product
pea3	M13 for gtaa aacgacggccag	M13 rev cagg aaacagctatgac	T7	Sk/Not I
fgf10a	M13 for gtaa aacgacggccag	M13 rev cagga aacagctatgac	T7	pBS-SK/ Eco RI

*Poly abbreviation for Polymerase

The PCR product was cleaned using the Milipore (UFC505024) clean up columns.

The mix was incubated at 37°C for 2 hours. After this period of time 2μl of RNase-free DNAse I and 18μl was added. The mix was incubated again at 37°C for 30 minutes.

Table 2-4: DNA template synthesis from cDNA to make the PCR product

Reagent	Volume
10X PCR Buffer	2.5 μl
1:3 NTP mix	1 μl
TAQ DNA polymerase	0.5 μl
Primer For and Rev	1 μl each
cDNA	1 μl
mqwater	18 μl

Table 2-5: DNA template synthesis from DNA plasmid/PCR product

Reagent	Volume
10X PCR Buffer	2.5 μl
NTP mix	1 μl
TAQ DNA polymerase	2 μl
Primer For and Rev	1 μl each
20ng plasmid or 1μl PCR product	in 85 μl of mqwater

Table 2-6: RNA transcription reagents

Reagent	Volume
1μg	in 21μl mq water
Transcription Buffer	3 μl
RNA labelling mix DIG or Fluorescein	3 μl
RNAase Inhibitor murin	1.5 μl
T7 or T3 polymerase	1.5 μl

Transcription mix (probe) was clean through Sigmaspin™ clean-up columns (Sigma-Aldrich, S5059). Then the probe was diluted in 14μl of RNA later. Finally the 1μl of the probe was run in 1% fresh agarose gel.

The antisense RNA probes used for the ISH to assess gene expression during regeneration were chosen from previous publications and unpublished research at "Roehl Lab". The authors from the previous publications/research are stated in 2-7. The main characteristic of the chosen probes was the limitation of gene expression in wounded tissue with no expression in uninjured structures. Whole-mount in situ hybridisation was used to detect the expression of both regeneration marker genes and development pathways readouts during tail regeneration. ISH uses DIG (digoxigenin) labeled complementary Ribonucleic Acid (RNA) probe. The probe binds to the RNA of interest, which allows the seeing of the tissue and level of expression that the gene of interest has. This protocol was adapted from Thisse et al. (2008).

Fish were tail-amputated (as previously described), fixed at different time points with 4%PFA (10 embryos per tube) and kept overnight at 4°C. Methanol/-PBST series (30%, 60%, 100%) were performed the next day, after which the embryos were stored in 100% methanol at -20°C. Table 2-7 shows the probes used during my PhD project (see Table 2-9 for solutions).

In situ hybridisation, Day 1

Embryos were dehydrated by application of a methanol/PBST reverse series (100%, 60% 30%). Then the embryos were washed 5 times for 5 minutes each in PBST. For permeabilisation the embryos were incubated in Proteinase K (PK) (10μl/ml PBST) for 10 minutes at room temperature (RT). After the incubation the embryos were re-fixed by removing the PK and adding 4%PFA for 20 minutes at RT. Then embryos were rinsed 4 times for 5 minutes each. To pre-hybridisise the embryos, 500μl of Hyb+ solution was added to each tube and then the embryos were incubated at 70°C for two hours. The Hyb+ solution was replaced by 200μl of Hyb+ containing 4μl to 8μl of antisense RNA probe. Embryos were incubated overnight with the antisense probe.

In situ hybridisation, Day 2

The probe/hybridisation solution was removed (recycled) and then 1ml of pre Hyb- wash was added. Then the stringency washes were done at 70°C in 25% 2x SSCT/75% Hyb-, 50% 2x SSCT/50% Hyb-, 75% 2x SSCT/25% Hyb-, 2x SSC, 10 minutes each. The embryos were then incubated two times for 30 minutes in 0.2x SSC solution. The embryos were moved from the 70°C incubation and allowed to cool down at RT for 5 minutes. The embryos were then washed with 25% PBST/75% 0.2x SSC, 50% PBST/50% 0.2x SSC, 75% PBST/25% 0.2x SSC for 10 minutes each. Two washes were done with PBST at RT 5 minutes each. The embryos were put in blocking buffer solution for 3-4 hours at RT, then for 10

minutes each at 4°C. Finally the blocking buffer solution was replaced with fresh blocking buffer solution containing the antibody (1:10000 anti-DIG OR 1:5000 anti fluorescein) and the embryos were incubated overnight at 4°C.

In situ hybridisation, Day 3

The antibody solution was removed and the embryos were given a quick wash with PBST. The embryos were washed 6 times for 15 minutes each with PBST at RT.

The embryos were then washed 3 times for 5 minutes with the staining buffer pH 9.5. The embryos were then transferred into a 12 well plate, the staining buffer solution was removed and then fresh staining buffer was added containing staining solution, which was 3.5μl/ml (staining buffer) of BCIP and 3.5μl/ml (staining buffer) of NBT. Embryos were left at RT for the staining development from 30 minutes to 3 hours. Once satisfactory levels of staining were observed, the reaction was stopped with PBST + 1mM EDTA for 15 minutes.

Embryos were then fixed with 4% PFA for 20 minutes. To remove pink staining background the embryos were put into methanol/PBST series (30%, 60%, 100%) and left in 100% methanol for two hours. After two hours in methanol the embryos were put in reverse methanol/PBST series (100%, 60% 30%) and then washed 2 times for 5 minutes each in PBST. To bleach the pigment the embryos were put in the bleaching solution for 10 minutes at 37°C and mixed once for 10 minutes. After the bleaching the embryos were washed twice with PBST for 5 minutes and then put in glycerol series 50%glycerol/mqwater for 10 minutes and then 100% glycerol for final storage. The embryos were stored in the dark at RT for up to 5 years.

2.4 Evaluation of gene expression

In many of the experiments, clear differences were observed for the staining, representing gene expression between the control group and the experimental group (pathway manipulation or amputation). However, for embryos where the difference between staining of the experimental and the control groups was marginal, blind tests were performed to avoid a biased judgment. The blind tests involved asking another colleague if he could see differences in the staining between the control and treated group without mentioning which was the control and the treated group.

Table 2-7: Probes employed to test the expression of regeneration markers and molecular pathways genes

Probe	Regeneration markers	Molecular Pathway	Probe number	Authors
msxc	Blastema		p3	Kawakami et al. 2004*
raldh2	Blastema		p305	Mathew et al. 2009*
dlx5a	Wound epithelium		p372	Kawakami et al. 2004*
ptc1		Hh	p311	P. Jankun**
lef1		Wnt	p301	P. Jankun **
wnt10b		Wnt	p207	P. Jankun **
tcf7		Wnt	p227	P. Jankun **
axin2		Wnt	p285	P. Jankun **
pea3		FGF	p65	P. Jankun **
fgf10a		FGF	p615	P. Jankun **

*Authors of papers and/or works where these probes have been used during zebrafish larval-tail regeneration. **Manuscript in preparation

2.5 Whole mount antibody staining

To detect proteins of interest a combination of two antibodies was used. The primary antibody binds to the protein while the secondary binds to the first antibody. The secondary antibody is normally labeled (fluorescent) which enables the observation of the protein. Embryos were fixed in 4% PFA, and kept in 100% methanol (as described for whole-mount ISH).This protocol was adapted from Westerfield (2000) (see Table 2-9 for solutions).

First day of antibody staining

A reverse methanol series was applied (100%, 60%, 30%) to put the embryos back into phosphate buffered saline plus 0.1% Tween 20 (PBST). PK permeabilisation was done for 10 minutes (in order to preserve most of the tail tissue). A 4% PFA re-fix was done after PK treatment for 20 minutes. Embryos were rinsed 4 times at 5 minutes each with PBST solution. Then, the embryos were incubated for 2 hours at RT in blocking buffer solution. After the incubation period, the blocking buffer was replaced with fresh blocking buffer containing the primary antibody (see Table 2-8) for the antibodies employed). The embryos were left overnight at 4°C.

Antibody staining, Day 2

The embryos were washed 4 times at 30 minutes each with PBST at RT. The PBST was removed and the embryos were incubated for 2 hours in the blocking buffer solution. After the incubation period, the blocking buffer was replace with fresh blocking buffer containing the secondary antibody (see Table 2-8) for the antibodies employed). The embryos were left overnight at 4°C.

Antibody staining, Day 3

Embryos were washed 4 times at 30 minutes each with PBST at RT. The PBST was removed and a small amount of Vectashiel with DAPI (4',6-diamidino-2-phenylindole) just covering the embryos was added. Embryos were stored in the dark at 4°C until image analysis.

Table 2-8: List of primary and secondary antibodies used during my PhD research.

Antigen	**Antibody Concentration**	**Company**	**Secondary Ab/concentration**
Cre protein	Mouse Cre Antibody (1:300)	Millipore MAB3120	Alexa 488 Goat anti mouse (1:200)
mCherry protein	Rabbit mCherry (1:300)	Novus Biologicals NBP2-25157	Alexa 488 Goat anti rabbit (1:200) /HRP Goat anti rabbit (1:500)
Phosphorylated histon H3	Rabbit Anti PH3 (1:500)	Calbiochem Cat No.382159	Alexa 488 Goat anti rabbit (1:200)

Note: All secondary antibodies were purchased from Invitrogen

2.6 Heat Shocks transgenic lines

Heat shock consists of having an inducible heat shock promoter that controls the expression of the gene of interest. This heat shock promoter is activated under heat, stress or tissue repair (Ritossa 1962; Matz et al. 1995;Cao et al. 1999; Laplante et al. 1998). Halloran et al. (2000) cloned and used the inducible heat shock 70 gene as a promoter in the zebrafish for the first time. The heat shock transgenic fish is then used to induce the expression of the gene of interest in time and space. Incubating the heat shock transgenic embryos at 39°C for 1.5 to 2 hours activates the heat shock promoter. Normally a heat shock DNA construct carries a reporter gene that is expressed, indicating that the fish carries the heat shock promoter DNA construct. This reporter gene can be a fluorescent protein gene. The heat shock transgenic lines used in this project used the green fluorescent protein (GFP) gene as reporter gene. Therefore, after the incubation period the embryos were examined under a fluorescent scope (Zeiss Axio Zoom) where, the embryos expressing the GFP had the heat shock promoter activated.

In my studies I used the *Tg(hsp70l:dkk1b-GFP)* (simplified as *hs:dkk1-GFP*) line, provided by Dr Weidinger "Ulm University", that expresses GFP under heat shock conditions (ZFIN community 2015). The *hs:dkk1-GFP* is a transgenic line that under heat shock conditions expresses the *dkk1* gene (an antagonist of the Wnt canonical pathway). Embryos were amputated at 3dpf and then allowed to

Table 2-9: List of reagents for in-situ hybridisation (ISH) and immunohistochemestry (IHC).

Solution/Buffer	**Composition**
PBS	1 Tablet of PBS (Phosphatase-buffered saline in 200 ml mq-water)
PBST	1 PBS + 0.1 20% Tween 20 (v/v)
PK (Proteinase K) stock	For 1ml: 10mg PK in 1 ml PBS)
4%PFA (Paraformaldehyde)	For 50ml: 2g PFA in 50ml PBS
Hyb +	For 50ml: 25ml formamide, 25mg tRNA, 12.5ml 20x SSC, 250 μl 20% Tween 20, 50μl heparine stock, 460μl 1M Citric Acid and fill with mq-water up to 50ml
Hyb-	The same as Hyb+ without tRNA
20x SSC	For 0.8 Liter: 140.2g NsCl 3m 65.8g Na Citrate dehydrate 3M pH 7
Blocking buffer solution for ISH	For 50ml: 1ml sheep serum, 100mg Bovine Serum Albumin (BSA), fill it up with 50ml PBST
Staining buffer	For 50ml: 5ml tris pH 9.5, 2.5ml 1M $MgCL_2$, 1.66ml NaCl 4M, 250μl 20% Tween-20
NBT stock	For 1ml: 50mg Nitro Blue Tetrazolium in 0.7 ml of anhydrous Dimethyl-formamide + 0.3ml water. Store at 4 °C
BCIP stock	For 1ml: 50mg of 5-Bromo 4-Chloro 3 Indolyl Phosphate in 1ml anhydrous Dimethyl-formamide. Store at 4°C
Bleaching solution	10% H_2O_2, 0.5X SSC, 5% formamide (v/v)
Blocking buffer solution for IHC	PBST + 10% NBCS (New born calf serum) (v/v)

regenerate. 12 hours before fixation the amputated embryos were incubated at 39°C for heat shock for 2 hours. The embryos were fixed with 4% PFA at 48 hours post-amputation (hpa). ISH was performed in order to see how the down-regulation of Wnt signalling affects the expression of regeneration marker genes during tail regeneration.

Tg(hsp70l:fgf3) (simplified as *hs:fgf3*) (ZFIN community 2015). In the case of transgenic lines *hs:fgf3*, heat shock may lead to FGF signalling up-regulation. The reporting gene induces GFP expression in the heart of the fish that carries the *hs:fgf3* transgenic line. Previous lab members reported that down-regulation of Hh by cyclopamine affects proliferation negatively, while up-regulation of FGF increases proliferation during tail regeneration. The aim of the experimental plan was to test whether FGF signalling can increase the reduction in proliferation by cyclopamine. Figure 2-1 shows the experiment plan.

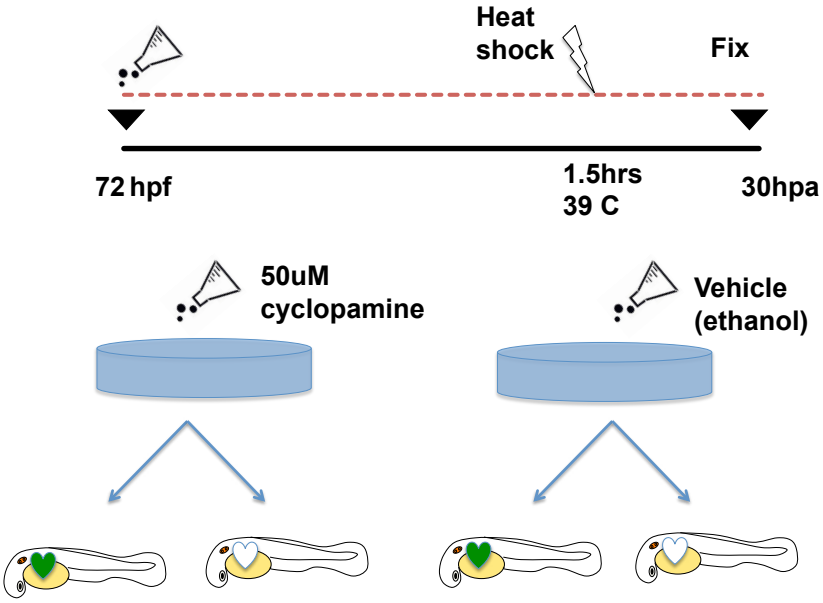

Figure 2-1: Proliferation rescue experiment. Cyclopamine (dotted line in red) is administered continuously after amputation (black line) until fish fixation. 6 hours before fixation the fish are heat shocked for a duration of 1.5 hours. The fish are separated in four groups, depending on the green expression in the heart and the treatment. There are 80 fish in total.

Fish were treated continuously with 50μM of cyclopamine, and were then incubated at 39°C for 1.5 hours at 6 hours before fixation. For the 4.5 hours between heat shock and fixation the fish were incubated at 28°C. Fish were then sorted using the Axio Zoom fluorescent scope, by selecting embryos expressing GFP in the heart. Embryos were fixed at 30hpa. Whole-mount antibody staining against phosphor-histone H3 (PH3) was performed to detect cell proliferation.

Note: Due to the lack of time, only fish treated with cyclopamine were processed for immunostaining.

2.7 16 Somite (S) and 1dpf embryo collection and molecular pathway analysis

Two adult zebrafish were put into a container and separated by a plastic divider. Next morning the divider was removed so that the fish could mate. Embryos were collected 1 hour after the removal of the divider. Using a dissection scope the two-cell stage embryos were identified and selected. Fish embryos were put to a incubator set to 28.5°C until they reached the stage of 75% epiboly stage. Embryos were dechorionated by incubating them in 5 milligrams (mg)/millilitre (ml) of pronase (diluted in E3 medium) for 3 minutes. Embryos were put into glass petri dishes, and then treated with small molecule compounds. The small molecule compounds used for the treatment were: Cyclopamine 20μM, SU5402 5μM, Gsk3XV 5μM inhibitor IWR-1 5μM and Vehicle DMSO or ethanol. Embryos were put at 28°C and the next day the embryos were fixed for tail image analysis.

For molecular analysis embryos were treated with 20μM cyclopamine, as previously described. After the treatment the embryos were put into the incubator at 22°C to slow down the development. The next day the embryos were observed under the dissection scope and fixed at the 16 somite (S) stage. Whole-mount ISH was performed to observe how cyclopamine affected the expression of retinaldehyde dehydrogenase 2 (*raldh2*) and transcription factor 7 (*tcf7*) in the tail bud.

For tail morphology analysis *hs:dkk1-GFP* embryos at 75% epiboly stage were put in the incubator at 39°C for 1.5 hours then transferred back to another incubator at 28°C. After 8 hours the embryos were sorted out for the expression of GFP and separated from the ones that lacked GFP expression. The next day the fish were fixed at 40hpf for tail image analysis.

2.8 *raldh2* expression analysis in the tail bud, under the suppression of FGF signalling and over-expression of Wnt signalling.

The expression of *raldh2* was studied during the down-regulation of FGF and up-regulation of Wnt signallings during tail bud formation and tail regeneration. Two chemical compounds were used to manipulate these pathways: SU5402 was used to down-regulate FGF while GSK-3 XV was employed to up-regulate Wnt signalling. The chemicals were administrated individually in order to manipulate one pathway per fish group, or both at the same (epistasis analysis) (see Figure3-16) To study the tail regeneration, tails were amputated at 3dpf and fixed at 48hpa. Fish were divided in 3 groups and each group had its own control (20 fish per group). The fish were treated as follows.

For FGF suppression, fish were treated with 15μM of SU5402 4 hours before fixation. For Wnt over-expression, fish were treated with 5μM of GSK-3 XV 8 hours before fixation. For epistasis analysis Wnt over-expression and FGF suppression, fish were treated as mentioned previously with both chemicals. Fish were fixed with PFA and whole-mount ISH was done for *raldh2*.

For the assessment of *raldh2* expression in tail bud formation the treatment was performed as follows. At 8hpf fish were treated with either SU5402 or GSK-3 XV inh with a concentration of 2.5μM for both chemicals. Fish were incubated at 22°C to slow down the development. The next day the fish were analysed under the dissection scope and fixed at the 16S stage. For the epistasis experiment, Wnt over-expression and FGF suppression, fish were treated as mentioned previously with both chemicals using the same concentration. Fish were fixed and whole-mount ISH for *raldh2* was performed.

2.9 Quantification of the total are of *raldh2* staining in the tail bud

raldh2 expression was analysed in the tail bud of the 16S embryo. The reason for this was to see whether the disruption of either FGF signalling or Wnt/β catening caused changes in the *raldh2* expression. The images were processed using the Image J program. The total area of the *raldh2* expression in the tail bud was measured as follows: 1.- The Background of the image was change to black colour. 2.- All the images resolution was adjusted to 300 dpi. 3.- Then I selected only the tail form the rest of the background by going to Image, Adjust, Colour Threshold. Once the window open, I selected the area of the tail with the brightness function as shown in Figure 2-2A. Then I pressed the bottom selected to chose only the tail. 4.- Now image J only recognize the tail, so to select the *raldh2* staining (blue colour) inside the tail I used again the brightness setting of the colour threshold function. However, this part can be open to subjectivity so I decided to chose a range of 80 as 160 (Figure 2-2B) (black arrows) for all the images measured, I chose this range since I considered it selected most of the *raldh2* staining, to confirm my decision I asked to a colleague Luis Medina if he agreed with me. 5.-After the *raldh2* staining of the image was selected I went to analyse then measurement. Then the area of the *raldh2* staining was given to me in square pixels. 6.- To convert square pixels into square micrometers. I first measure the distance of 100μm bar in pixels by using the line tool on image J (see Figure 2-3). Then I pressed analyse, set scale and at the top of it appears the total number of pixels of line drawn that means are the total number of pixels in 100μm. 7.- So I new that 100 μm correspond to 94 pixels, the question now is how many micrometers are in 1 pixel. So I divided 100 into 94 and gave me a number of 1.06382978723. This number is the size of a pixel in μm and to converter it into square μm I

multiplied 1.06382978723 by 1.06382978723, which is equal to 1.13173381621, this final number is the size of one square μm. 8 Finally to convert the total square pixels given by the measurements from image j into μm^2 I multiplied the area of pixels given from image J by the 1.13173381621 number.

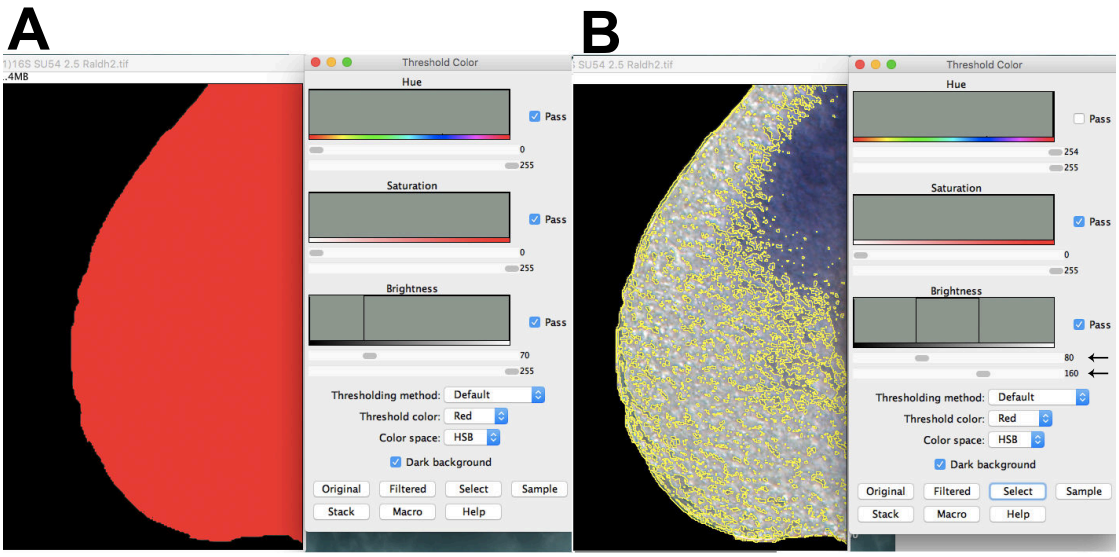

Figure 2-2: *raldh2* staining measurement. **A** The total area of the tail is selected. **B** The total area where *raldh2* is expressed is selected with the brightness settings, a scale of 80 and 160 was chose for all the pictures (black arrows).

Finally the means of each group was compared with each other group. The idea was to see whether they were equal or if there were differences amongst each other. The statistical technique used was one-way analysis of variance (ANOVA) with pairwise comparison using the Lowry (2015) website. The bar graphs were done in Microsoft Excel.

2.10 Mounting and image capture

Fish were stored in 80% glycerol and eventually mounted on a glass slide covered with a glass cover slip. DIC (differential interference contrast) light microscopy was used for images analysis. A ProgRes® camera and image capture software was used to capture the images.

hs:fgf3 transgenic line embryos processed for immune staining against phospho-histone were analysed using the Olympus confocal microscope by making a stack of optical sections (z scan).

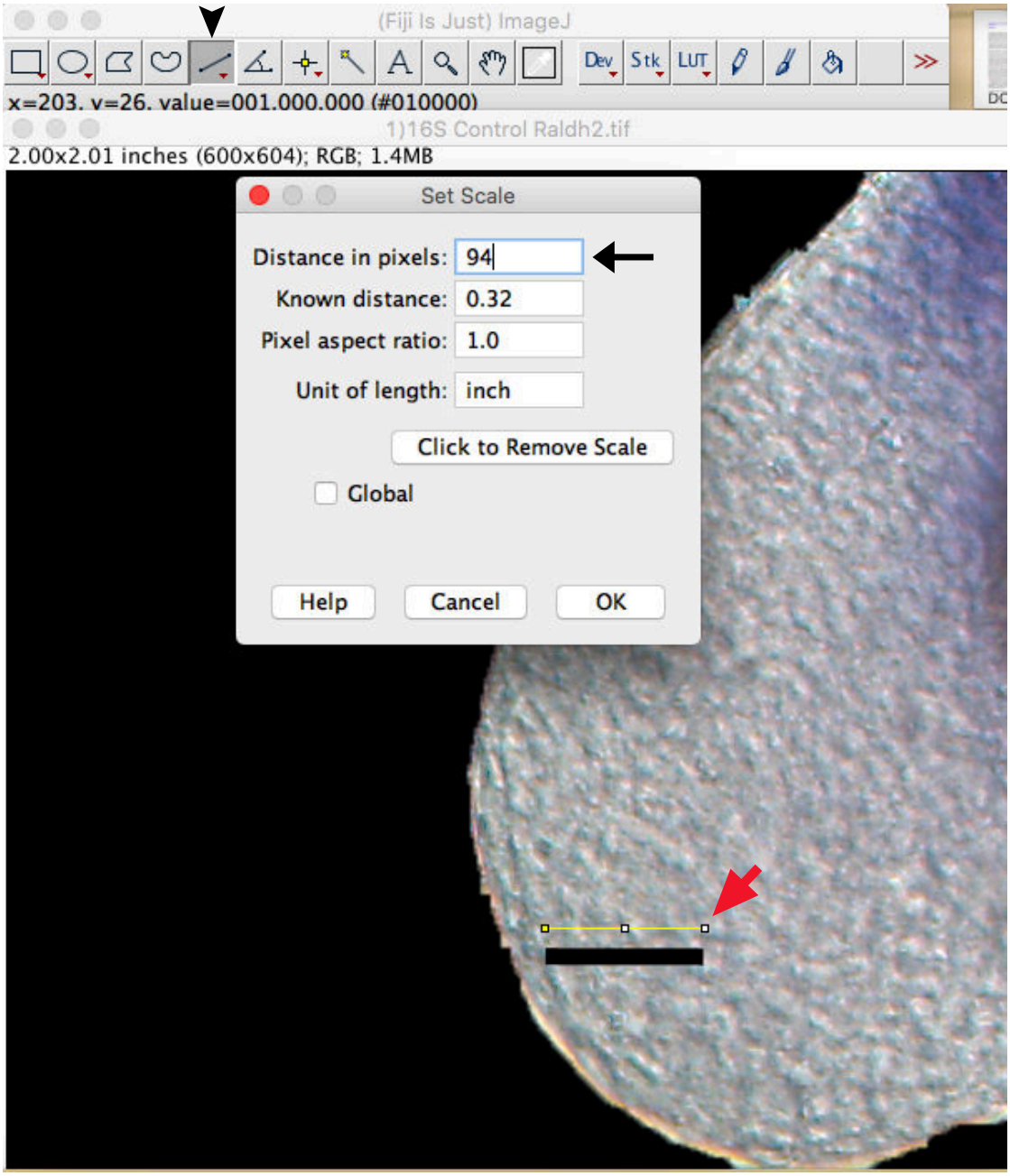

Figure 2-3: Scale calculation into μm^2. A line was draw along a bar that had a total length of 100 μm (black arrowhead and red arrow). To calculate the total number of pixel along the line I pressed analyse, then set scale and the first number showed me the total number of pixels (black arrow) in 100 μm.

2.11 Restriction digestion DNA

DNA restriction digestion is a molecular biology technique used to cut DNA at a defined site. DNA excision is done using restriction enzymes (Hartl et al. 2005). This technique can be used for DNA sub cloning by cutting the desired piece of DNA and also to verify if we have the right plasmid size by checking the restriction through DNA digestion.

The restriction enzymes were purchased from New England Biolabs inc (NEB) and were used depending on the DNA plasmid.

The digestion mix included 1 microliter (μl) of DNA, 2μl of buffer (depending on the enzyme), 2μl of BSA (bovine serum albumin) (depending on the enzyme), 1μl of restriction enzyme (it could be more that one enzyme) and mq water enough to make a final volume of 20μl.

The DNA digestion mix was put into an Eppendorf Tube and incubated in a metal rack at 37°C for 2 hours.

2.12 DNA electrophoresis, gel cutting and DNA gel extraction

In order to observe the size of a plasmid, DNA strand or RNA strand, it is necessary to put the DNA into agar gel and perform electrophoresis. 1% of agar plus 1 drop of ethidium bromide (EtBr) (the gel had several wells in it) was prepared. The gel was put into the electrophoresis unit, which already contained 1 X TBE (Tris, Borate, EDTA) buffer plus 1 drop of EtBr. The gel wells were loaded with 16μl of the DNA digestion enzyme mix plus 4μl of loading dye. A DNA ladder was also loaded into the agar gel in order to serve as a comparison against the DNA bands. The electrophoresis unit was run at 90 volts (V) for 30 minutes. The DNA band already in the gel was checked continuously under ultraviolet (UV) light. Once the bands were properly separated a picture was taken. The size of the bands was compared with the DNA ladder in order to see if we had the right plasmid.

To achieve DNA extraction from the gel without damage, the DNA bands were first cut from the rest of the gel under a UV transmission microscope illuminating at a wavelength of 302nm. Once the gel piece was cut, it was deposited into an Eppendorf Tube and weighed. The gel extraction procedure was performed according to the QIAGEN (2012a).

2.13 DNA dephosphorylation, ligation and subcloning

The transfer of a DNA section from one plasmid to another is called subcloning (Hartl et al. 2005) and requires dephosphorylation and ligation. DNA dephosphorylation is the removal of the DNA phosphate sides by the phosphatase enzyme. Dephosphorylation should avoid plasmid self ligation, which generates bacterial colonies containing the wrong DNA plasmid. For DNA dephosphorylation the shrimp alkaline phosphatase (rSAP) enzyme was used from NEB. The phosphatase reaction had a total volume of 20μl, and comprised 2μl of rSAP reaction buffer, 1μl of rSAP and mq water. The reaction was incubated at 37°C for 30 minutes and then stopped by incubating at 65°C for 5 minutes.

DNA ligation involves putting together two DNA strands, forming a phosphodiester bond between the 5' end and the 3' end. The enzyme used for this reaction was the T4 DNA Ligase from NEB. The protocol was as described in https://www.protocols.io/view/Ligation-Protocol-WITH-T4-DNA-Ligase-M0202-imss4v. Once the ligation was finished, the restriction digestion of the DNA confirmed the size of the new plasmid.

2.14 Bacterial transformation

Bacterial transformation is a technique where the bacterium incorporates a portion of DNA into its genome. This DNA gets replicated when the bacteria proliferates (Sambrook et al. 2001). The protocol employed for bacteria transformation is as follows: New 10-β competent *E.coli* cells were thawed on ice for 10 minutes. 25μl was taken from the tube containing the *E.coli* and the rest was put back at -80°C. 1-2μl of the plasmid of interest was added to 25μl of 10- beta cells and gently flicked 4 times. The mixture was put on ice for 30 minutes. Then the mix was put in a hot water bath at 42°C for 30 seconds to induce heat shock. The tube containing the cells was put back on ice for 5 minutes.

After the tube containing the cells was moved to a RT environment and 450μl of super optimal broth (SOC) was added to the tube. The mixture was placed in a shaker at 250 revolutions per minute (rpm) at 37°C for 1 hour so that cells could proliferate.

In the meantime agar plates containing antibiotics (Ampicillin, Kanamycin or Streptomycin) were warmed up to 37°C (3 plates per construct to test). The cells mix with the SOC medium was distributed across the three plates. Each plate had different volumes: 300μl, 90μl and 10μl.

The liquid containing the cells was poured onto the plates and spread close to

the Bunsen burner. Once the liquid was dry and properly spread on the surface of the agar, the plates were incubated upside-down at 37°C overnight.

2.15 DNA amplification and extraction by plasmid preparation

Plasmid preparation is a method used to extract and purify DNA. I used two types of DNA extraction: plasmid miniprep and midiprep (the latter method extracts larger amounts of DNA at a higher level of purification). For plasmid miniprep a small piece of a bacteria colony was rubbed with a toothpick and put into a 12ml Falcon tube. 10 colonies were picked in total. 2ml of lysogeny broth (LB) with the desired antibiotic was added to each tube, then the tubes were put into a shaker at 250rpm and 37°C overnight.

The next day the LB was put into an Eppendorf Tube and spinned down for 3 minutes at 80,000rpm. The supernatant was removed from the tube and the bacteria cells already at the bottom of the tube were re-suspended by adding 200μl of P1 buffer. The tube was kept on ice and re-suspension was achieved by pipetting up and down. 200μl of P2 buffer was added at RT. The tube was shaken 3 times and left to rest for 3 minutes. Then 200μl of P3 buffer was added, the tube was shaken again 3 times and then was spun down for 5 minutes at 250,000rpm. Meanwhile 600μl of isopropanol was added into individual Eppendorf Tubes. Once the spinning finished, the 600μl of the bacterial prep mix was pipetted into the isopropanol tube and shaken 3 times. The isopropanol prep was left at -20°C for 30 minutes and then spun down for 30 minutes. All the supernatant was removed and then the pellet was washed with 700μl of PE buffer. The pellet was left to dry for some minutes until no smell of ethanol was detected. Finally, 50μl of buffer was added, the tube was left to stand for 5 minutes and then it was stored at -20°C.

For plasmid midiprep a small amount of cells was taken from the agar plates with a toothpick and put into a 12ml Falcon tube with 2ml of LB medium. This start culture was left to shake for 6 hours at 37°C at 250rpm. Plasmid amplification and purification were done using the Midiprep kit and according to the QIAGEN (2012b) with some modifications: Step 8 from QIAGEN (2012b) was substituted with the filtering of the sample using tissue paper as a filter. At Step 13, the sample was divided into 8 Eppendorf Tubes and then 700μl of isopropanol was added. Tube centrifugation was done at 15,000g for 30 minutes at 4°C in the Sigma microfuge 1-14.

2.16 Creation of the *cryaa Venus*

The first part of designing the Cre-expressing lines was to make the genetic vector. The function of the vector is to transport the external DNA into the cell genome. I decided to design the *pDestTol2cryaa:Venus* vector (Crystallin Alpha A gene, venus is a variant of the yellow fluorescent protein). The insulin:cre-mCherry-cryaa:Venus construct was digested with *Apa I* in order to take the *cryaa:Venus* section (Figure 2-4I. At the same time the *pBSK II* was linearised with *Apa I* enzyme (Figure 2-4II. The *cryaa:Venus* and the *pBSK II* were fused by ligation. The idea behind putting together these two DNA pieces (and creating the *pBSK II-Cryaa:Venus* plasmid) was to take the restriction sites *Bamh I* and *Acc651* from the *pBSK II.* (Figure 2-4III). Enzyme digestion was performed again using BamH I and *Acc651* in the *pBSK II cryaa:Venus* plasmid (Figure 2-5I). At the same time another plasmid was digested, the *pDestTol2CG2* construct with *BgIII* and *Acc651* (Figure 2-5II). Both digested constructs were observed in an electrophoresis gel, where the smallest band from the *pBSK II cryaa:Venus* (the *cryaa:venus* section) was taken and ligated with the biggest band of the *pDestTol2CG2*. The ligation of these DNA sections created the Tol2cryaa:Venus (Figure 2-5III). The bacterial transformation took place using the ccdB cells that grew in agar plates containing Ampicillin (50mg/ml) and Chloramphenicol (34mg/ml ethanol). To confirm that I had gotten the right DNA construct I digested it with Pvu II, which gave me three bands as observed in Figure 5-1. Finally the vector was used to create the Cre-expressing construct and then it was injected into one-cell zebrafish embryos.

The *cryaa:Venus* vector was used as the standard vector for all my Cre-expressing transgenic lines. The creation of this vector was made using deoxyribonucleic acid (DNA) subcloning in which several steps are involved and are described in detail in this section.

Note: The insulin:cre-mCherry-cryaa:Venus was acquired from the plasmid depository Addgene website: www.addgene.org. This construct drives the expression of Cre, which is fused to mCherry under the control of the insulin promoter. The reporter gene is *cryaa:Venus*.

2.17 Synthesis of the Cre-expressing construct with the use of Gateway kit

Five constructs with tissue specific promoters expressing Cre were made using the Gateway kit (Kwan et al. 2007). The Gateway kit uses the *att* system that is found in the lambda phage (Hartley et al. 2000). A genetic contruct is made up of three entry clones (p5a: Tissue specific promoter, pMe: ERT2CreERT2/ CreERT2 &

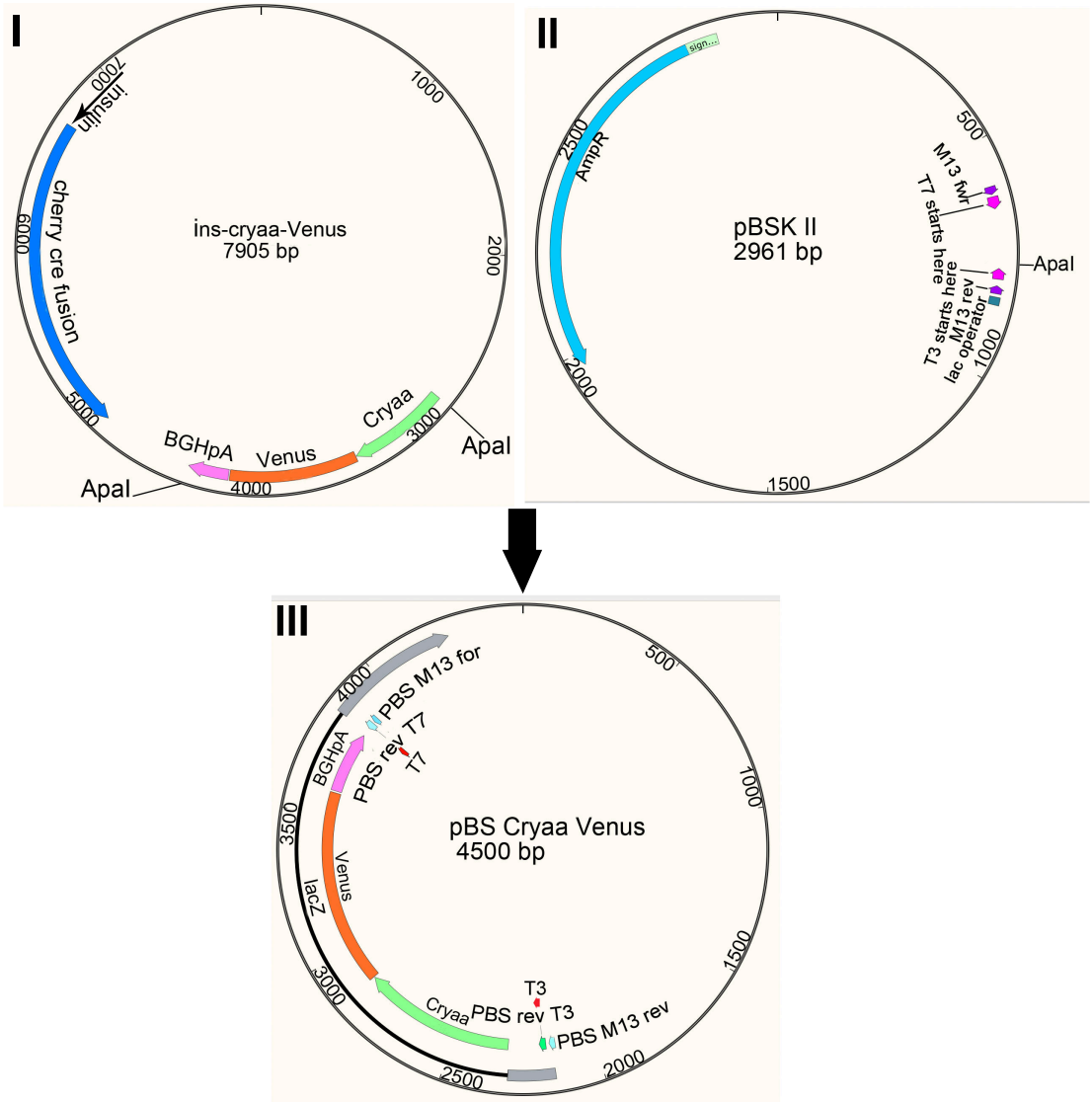

Figure 2-4: PBSKII-Cryaa:venus. **I** The *crya: venus* section is taken from the *insulin:cryaa Venus* construct with the enzyme ApaI. **II** The pBSKII construct is linearised with ApaI enzyme. **III** The *cryaa:Venus* section is ligated with the linearised pBSK II construct to create the *pBSK cryaa:Venus* construct.

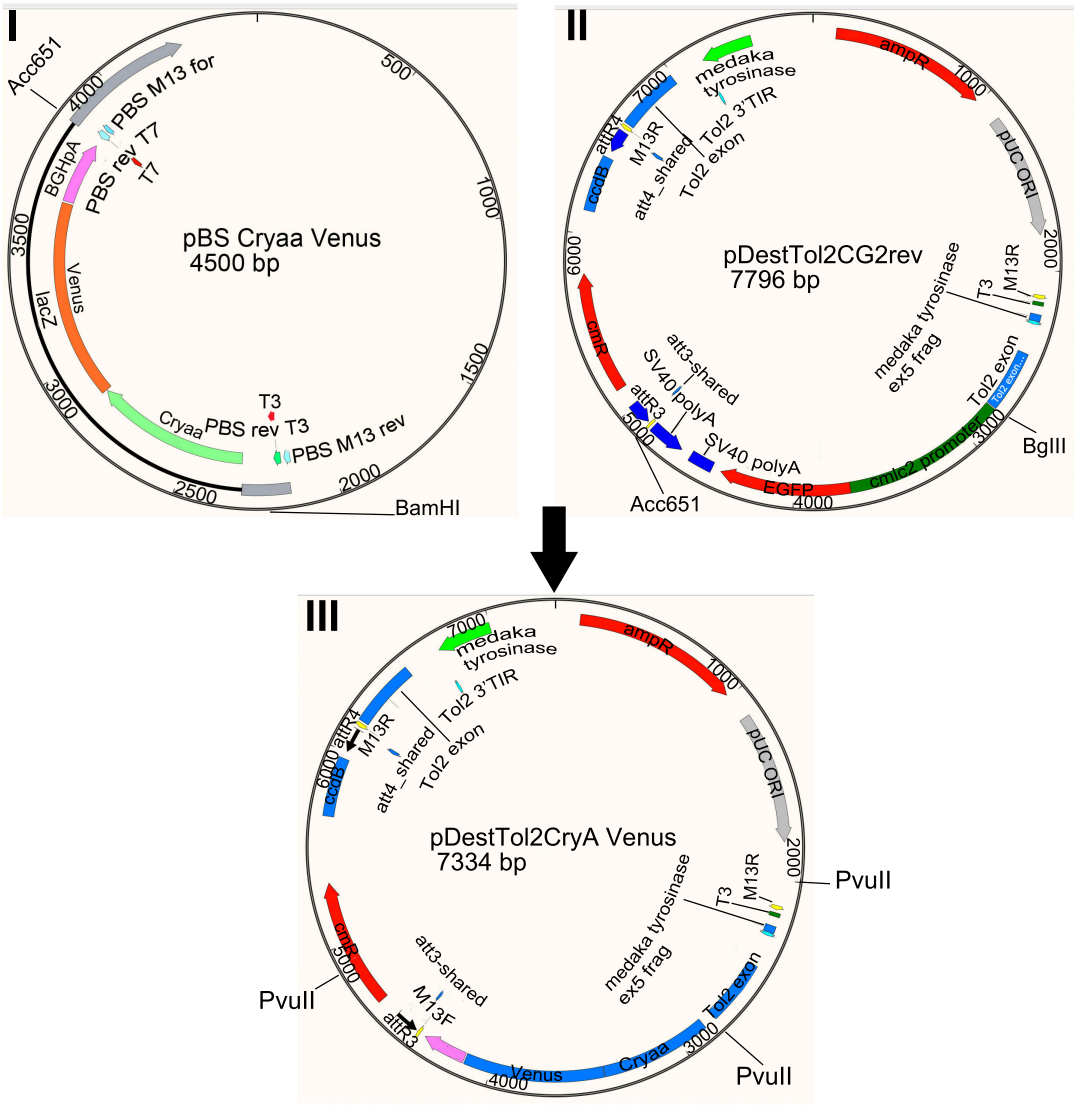

Figure 2-5: pDestTol2 cryaa:Venus.I The *cryaa:Venus* section is taken by digestion with the Acc651 enzyme and BamHI enzyme from the *pBS cryaa:Venus* construct. **II** The *cmcl2:EGFP* is taken from the *pDestTol2cg2rev* by digestion with the enzyme Acc651 and BgIII. **III** The *cryaa:Venus* section is fused into the pDestTol2 section by ligation (without the cmcl2:EGFP section) to create the *pDestTol2 cryaa:Venus* vector.

p3e: polyA) flanked with the att sites and the vector (*cryaa:Venus*).

After the LR recombination reaction the Cre-expressing construct with tissue specific promoter is generated (see Figure 2-6). Table 1-1 shows the different Cre-expressing constructs created with the Gateway kit.

According to the Gateway kit, the construct is made by mixing $10fmol$ of each entry clones, and $20fmol$ of the vector together with enough 1 X TE buffer to create a $10\mu l$ reaction.

$10fmol$ of one component corresponds to $10 \cdot \mathrm{N} \cdot 0.000660$ nanogramms (ng) of DNA (ng_{DNA}), where N is the number of base pairs of the component. This equation can be easily derived from the invitrogen manual (Invirogen):

$$
\begin{aligned}
ng_{\mathrm{DNA}} &= x \cdot \mathrm{N} \cdot \frac{660\ fg}{fmol}\frac{1\ ng}{10^6 fg} \\
ng_{\mathrm{DNA}} &= x \cdot \mathrm{N} \cdot \frac{660\ ng}{10^6 fmol} \\
ng_{\mathrm{DNA}} &= x \cdot \mathrm{N} \cdot 0.000660 \cdot \frac{ng}{fmol}
\end{aligned}
$$

Where x is the number of femtomoles ($fmol$) for each entry clone or vector.

To save cost on reagents we only created a final volume of $5\mu l$ and only half of the reagents were used.

For the entry clone the units of the final concentration were in nanograms (ng)/μl. This concentration obtained by the formula mentioned above was multiplied by 2 and then the following process was performed: 5μl from each entry clone was taken, p5a: tissue specific promoter, pMe: ERT2CreERT2/ CreERT2 gene and p3e polyA (diluted in TE buffer) and mixed with another 5μl of TE (Tris, EDTA) buffer, giving a final volume of 20μl. From this 20μl, 2μl was taken and mixed with 1μl of the clonase enzyme plus 1μl of the destination vector *cryaa:Venus* and 1μl of the TE buffer. The mix was put into a polymerase chain reaction (PCR) machine at 25°C for 16 hours. The reaction was stopped, and 1μl of PK was added to the mix, which was kept at 37°C for 10 minutes.

This process was followed by bacterial transformation, plasmid preparation and DNA restriction digestion.

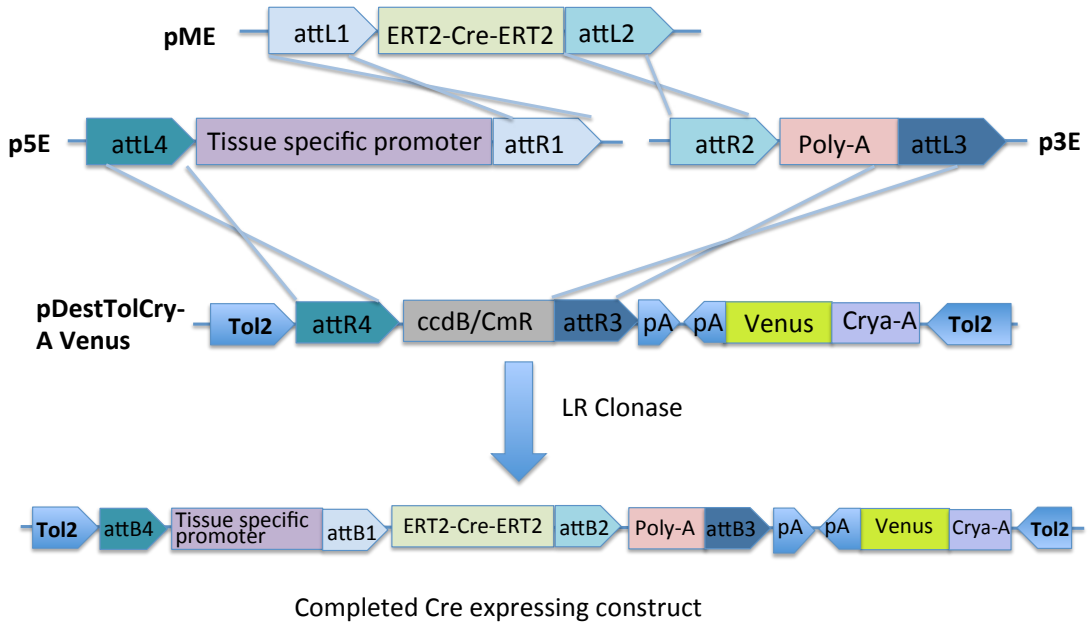

Figure 2-6: Gateway strategy for the generation of the Cre expressing lines with tissue specific promoters. The Gateway cloning strategy makes possible the rapid incorporation of the tissue specific promoter, ERcreER/erCRE gene, polyA gene and the Crya Venus vector using the LR recombination reaction.

2.18 Assessment of the genetic incorporation of the construct by DNA injection in one-cell stage embryos

Once the entire tissue-specific Cre-expressing construct was created and purified, cell injection took place. In order to increase the rate of gene incorporation, the Tol2 system was used. Therefore 20ng/μl of transposase RNA was mixed with 20μg/μl of the desired DNA construct + 0.5μl of phenol red. The DNA+RNA mix was loaded into a glass needle (approximately 3μl), and then the needle was plugged into a micro injector. One-cell embryos were collected from Sheffield University aquarium and put in a row along the edge of a glass slide. Embryos were put under a dissecting scope for a better observation during the injection. Embryos were injected with a DNA volume that corresponds to a quarter of the cell's total area.

After DNA injection the embryos were put into a petri dish containing E3 medium and were incubated at 28°C for 3 days. During that period dead embryos were removed and the E3 medium regularly replaced by clean E3 medium. At 3dpf embryos were analysed under the Axio Zoom fluorescent scope for the expression of GFP colour in the eyes. The GFP was an indicator that the fish was carrying the construct of interest. Embryos expressing GFP in the eyes were selected and put together into petri dishes (50 embryos approximately) with E3 medium. At 5dpf embryos were sent to be raised to adulthood (3 months) in the Sheffield University aquarium.

2.19 Screening of transgenic zebrafish by identification positive founders (F0)

Once the injected zebrafish reached the age of 3 months a screening was made to identify the positive F0 founders. The fish were pair mated into an overlapped container, one female and one male per container. One of the fish had to be wild type and the other DNA injected. The next day the DNA injected fish that had laid eggs were put into individual glass containers and they were given a code name, while the wild type were put back into the aquarium tank). All the eggs were collected and put into petri dishes with E3 medium and named according to the zebrafish progenitor code name. The embryos were put into a 28°C incubator for 3 days. After 3 days the embryos were analysed under the fluorescent scope and selected by those expressing GFP in the eyes. All the embryos expressing GFP green eyes were put together into a petri dish (no more than 50) and sent to be raised. The adults that managed to lay embryos expressing GFP in the eyes

were kept as the F0 generation.

2.20 Raising of the transgenic progeny of positive F1 and F2 founders into adulthood

The offspring from the F0 fish generation were classified as the F1 generation. Once they were 3 months old a screening was made in order to identify F1 positive founders carrying the Cre-expressing transgene. The selection process was carried out using the same process as described above for the F0 founders. The offspring of the F1 founders expressing GFP were sent to be raised. Finally the same process was repeated again, creating the second generation (F2) of Cre-expressing founders.

2.21 Double transgenic fish husbandry by crossing the Cre expressing fish with the Lox expressing line

Once the Cre-expressing line's stability was confirmed by obtaining a second generation (F2) of transgenic fish, the transgenic fish were grouped in numbers of 40 per tank. The Cre-expressing line fish were crossed with the transgenic fish carrying *EF1a:loxPEGFPloxP-DsRed*. The offspring were used to test Cre recombination in the tissue of interest.

2.22 4OH tamoxifen administration in the double transgenic fish and live imaging of the tissue recombination

4-Hydrotamoxifen (4OHT) (sigma) powder was diluted in 100% ethanol for 2 hours at 70°C. Aliquots were taken at 10mM and 4OHT was stored at -80°C up to 6 months. For 4OHT administration one 10mM aliquot was warmed up in a 70°C water bath for 15 minutes. In the meantime double transgenic embryos were analysed under the Axio Zoom fluorescent scope in order to choose the embryos that were 8hpf and expressing GFP (indicator of Lox genome). The embryos were dechorionated by adding pronase with a concentration of 5mg/ml diluted in E3 medium for up to 3 minutes. Embryos were rinsed 4 times in E3 medium and put in glass petri dishes containing 6ml of E3. 4OHT was added to a final concentration of 10μM. 4OHT treated embryos were put in the dark and incubated

at 28°C overnight. In order to test possible Cre leakiness (Cre recombination in the absence of 4OHT) another group of embryos was treated with ethanol. The next day the embryos were rinsed 4 times with E3 medium, and left at 28°C until they were 3 days old. At 3-4dpf embryos were put under the fluorescence scope to observe tissue recombination. The recombined tissue should express the DsRed fluorescent protein. The fish were imaged using the software ZEN pro.

2.23 Selection of embryos that present tissue recombination in the tail and live imaging during tail regeneration

4OHT administration was performed as described previously.1μM 4OHT was administered in order to have a few recombined cells in the tail. Once recombination was observed and a picture of the fish was taken, amputation was performed. A second picture was taken immediately after amputation. Fish were put into petri dishes with E3 medium for recovery. One picture per day was taken over the 3 following days, in order to track recombined cells during tail regeneration.

2.24 Double antibody staining for mCherry and Phospho histone H3

This antibody protocol was performed over 5 days. During the first 3 days I used the Anti-Ph3 antibody with a concentration of 1:300 and the Alexa 488 as secondary with a concentration of 1:200. The third day I washed off the Alexa 488 antibody and added the anti-mCherry antibody. The fourth day I added the secondary antibody HRP 1:1000. On the fifth day I made 2 quick washes with PBST, followed by 4 washes of PBST of 25 minutes each. I gently added 100μl of CY3 tyramid solution: CY3 tyramid solution was diluted 1:100 in 1x amplification buffer Plus (Perkin Elmer, catalogue number SAT704A) followed by incubation in the dark for 30 minutes. I gently washed the embryos once with PBST and then 5 times with PBST for 15 minutes each. Finally I rinsed the embryos, adding just enough Vectashield with DAPI to cover the embryos, and then stored the embryos containing tubes in the dark at 4°C.

Chapter 3

Dissection of the pathways involved during zebrafish tail regeneration.

3.1 Introduction

The zebrafish is a popular organism for the study of tissue regeneration as it can regenerate several structures, including complete organs and tissues such as heart, spinal cord, scales, bone rays and, most popularly, the caudal fin. The caudal fin displays a type of regeneration called epimorphic regeneration, and can be described as follows. Firstly, immediately after tail amputation, the injury closes and a mass of epithelial cells cover the wound, forming the *wound epidermis*. Secondly, cell migration occurs towards the injured area to form the blastema; these cells are thought to be undifferentiated. Thirdly, the blastema cells proliferate in order to generate the tissue that will regenerate the injured area. Fourthly, the new cells re-differentiate into the different tissues that will form the caudal fin, thus completing regeneration (Kawakami et al. 2004).

Epimorphic regeneration has raised different questions in relation to the molecular mechanisms that trigger, maintain and complete tail regeneration. Developmental molecular pathways are re-expressed during tail regeneration, and different functions have been described (Odelberg 2005; Poss 2010; Nechiporuk et al. 2002).The Hh (Hedgehog) molecular pathway, for example, is necessary for bone patterning during caudal fin regeneration according to Quint et al. (2002). On the other hand, Wnt signalling is active during blastema formation, while FGF (Fibroblast Growth Factor) signalling is important during proliferation and the formation of the blastema (Stoick-Cooper et al. 2007; Stulberg et al. 2012). BMP signalling is reported to be active during tail outgrowth and bone formation (Laforest et al. 1998), while retinoic acid has also been found to be required during blastema proliferation and maintenance (Mathew et al. 2009). Besides the developmental pathways already described, there are marker genes (genes expressed during tissue

regeneration) that are expressed during epimorphic regeneration; the study of these markers is necessary for the molecular dissection of regeneration. The research into the dissection of molecular pathways during regeneration has focused mainly on the modulation of developmental pathways and how these pathways regulate the expression of regeneration marker genes, such as *dlx5a* (expressed in wound epidermis) and *msxc* and *raldh2* (expressed in blastema) (Kawakami et al. 2004; Mathew et al. 2009). Even though the previous studies have generated knowledge about the role of developmental pathways involved in regeneration, many questions still need to be answered, for example, the activation order of the different pathways, the links between each other and the regeneration marker genes.

The present chapter reports the use of larval zebrafish to study the molecular pathway interactions during tail regeneration. Amputation of the larval zebrafish tail takes place at the very end of the tail, where it is possible to find a pigment gap as observed in Figure 3-1. The reason for this cutting point is that it allows for the amputation of several tissues, such as the notochord, the blood vessels, the neurons, the muscles, and the skin. This is not possible with fin fold amputation. Tail amputation, shown in the figures of the present chapter, took place at 3 days post fertilisation and was allowed to regenerate at different time lengths. The amputation age and the tail regeneration time is shown in some figures in this way: "3dpf (2dpa)". The number outside the parenthesis indicates the fish age at which the tail was amputated, while the number inside the parenthesis indicates for how long the tail was allowed to regenerate. The time is indicated in either hours or days. Note: The number of experimental repetition or trials will be represented by the letter N, while the number of total samples will be represented by letter n.

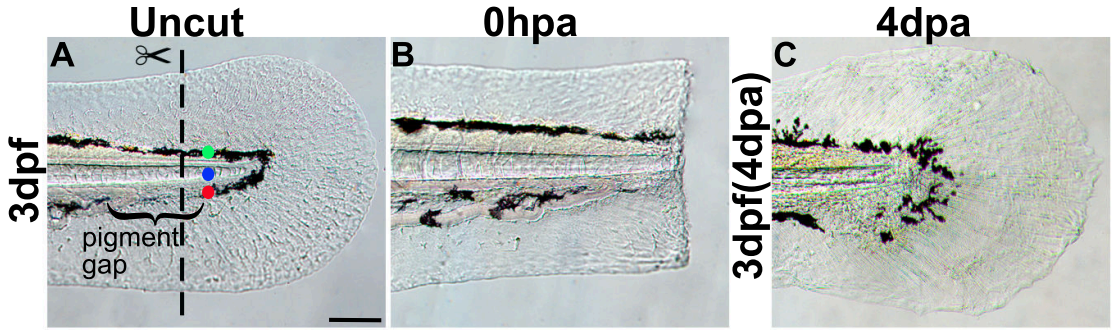

Figure 3-1: Tail amputation of the zebrafish larva. A: Tail amputation (black dashed line) is performed at the pigment gap of the tail, cutting different types of tissue including the notochord (blue dot) muscle tissue (red dot) and pigmented cells (green dot). **B:** Amputated tail. **C:** 4 days post-amputated completely regenerated tail, the embryo was 7 days old when regeneration was . The three figures shown in this panel come from 1 representative embryo, out of 10 embryos. A similar scale of regeneration was observed along the 10 amputated tails. N=20 n=10. Scale bar 100 μm.

The present research is a continuation of a study done during my Master's

degree. The results obtained during my Master's suggests a relationship between Hh signalling and Wnt during tail regeneration, where Hh signalling is upstream of Wnt during tail regeneration and Wnt regulates the regeneration marker genes *msxc*, *raldh2* and *dlx5a* (see Figure 3-2). The findings of the relationship between Hh signalling and Wnt, along with some of the data obtained from Philip Jankun's PhD reports, helped to set the basis of my research. The results section of this chapter shows figures from my Master's dissertation and P. Jankun's reports. Thus the previous investigations helped to establish the following hypothesis: *Hedgehog signalling leads to tail regeneration in larval zebrafish by regulating Wnt and FGF signalling.* In order to test this hypothesis, molecular pathways were manipulated during tail regeneration, with the use of small-molecule compounds and two transgenic lines under the control of an inducible heat-shock promoter. Whole-mount ISH was used to detect how regeneration markers were affected by the manipulation of relevant developmental pathways. Finally, immunohistochemistry was employed to analyse cell proliferation.

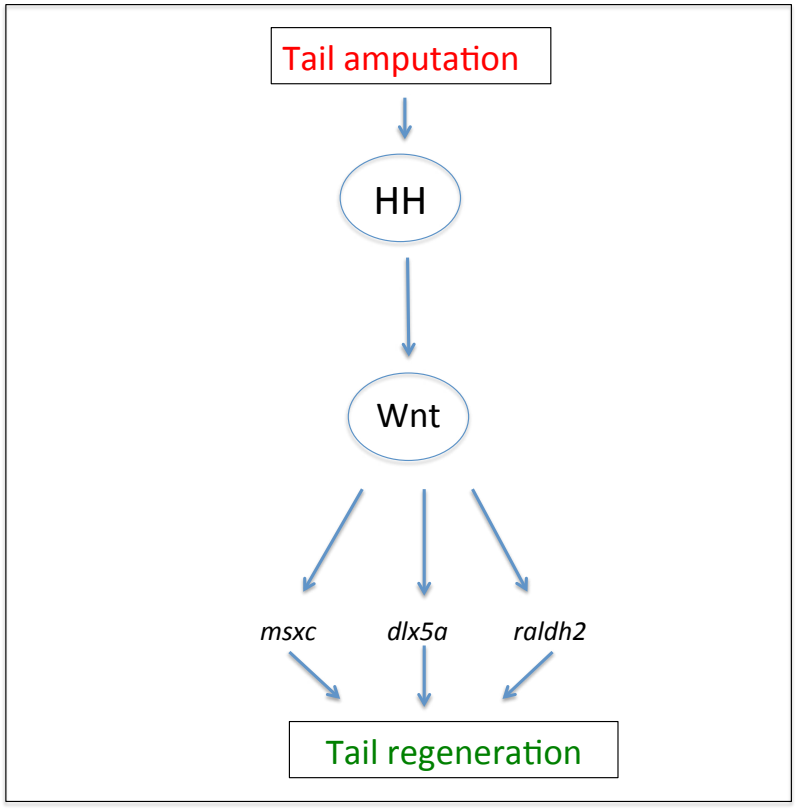

Figure 3-2: Molecular hierarchy during larval tail regeneratin. In this model Hh signalling initiates tail regeneration by activating Wnt signalling, which in turn activates regeneration marker genes.

The results presented in this chapter suggest a molecular hierarchy where Hedgehog signalling regulates Wnt signalling components and also proliferation through the *fgf3* gene. The relationship between Wnt and FGF was also studied,

by analysing the expression of *raldh2*. FGF signalling was down-regulated with the compound SU5402 during regeneration and at the same time Wnt signalling was up-regulated with the compound Gsk3XV inh. *raldh2* expression was similar to the level of expression in fish treated only with SU5402.

3.2 Hh signalling plays an important role at the beginning of tail regeneration

3.2.1 The zebrafish larval tail possesses regenerative capabilities

The start of this research is the establishment of a more appropriated tail regeneration model that allowed me to study different types of tissue regeneration. Rojas-Munoz et al. (2009) has used the tail amputation model before, but a proper characterisation of it has not been done. Therefore, I decided made a characterisation of the tail regeneration model to evaluate the morphology and genetic properties during tail regeneration. The present model was adapted from the fin fold regeneration model from Kawakami et al. (2004). The fin fold regeneration model takes place at 2 days post fertilisation (dpf) at the fin fold and completes regeneration 3 days after amputation. My model involves the amputation of the tail as described in Figure 3-1 at 3dpf, where the amputation reference point is the pigment gap. After 4 days post amputation (dpa) tail restoration can be observed. The next part was to test the expression of the regeneration marker genes *msxc*, *raldh2* and *dlx5a* by inducing regeneration through amputation, and then fix the fish at 48 hours post amputation (hpa) (This fixation time was chosen since regeneration marker genes present a high level of expression). Gene expression detection was done using whole-mount ISH. The marker genes previously mentioned are also expressed during tail development but down-regulated in larval stages (Akimenko et al. 1995). Therefore, uncut controls were also tested for the expression of these genes in order to dissect whether the expression was due to normal tail development or regeneration.

As we can observe in Figure 3-3, gene markers are only expressed during tail regeneration. These experiments were done during my Master's project and presented in this report to explain better the establishment of the zebrafish tail regeneration model.

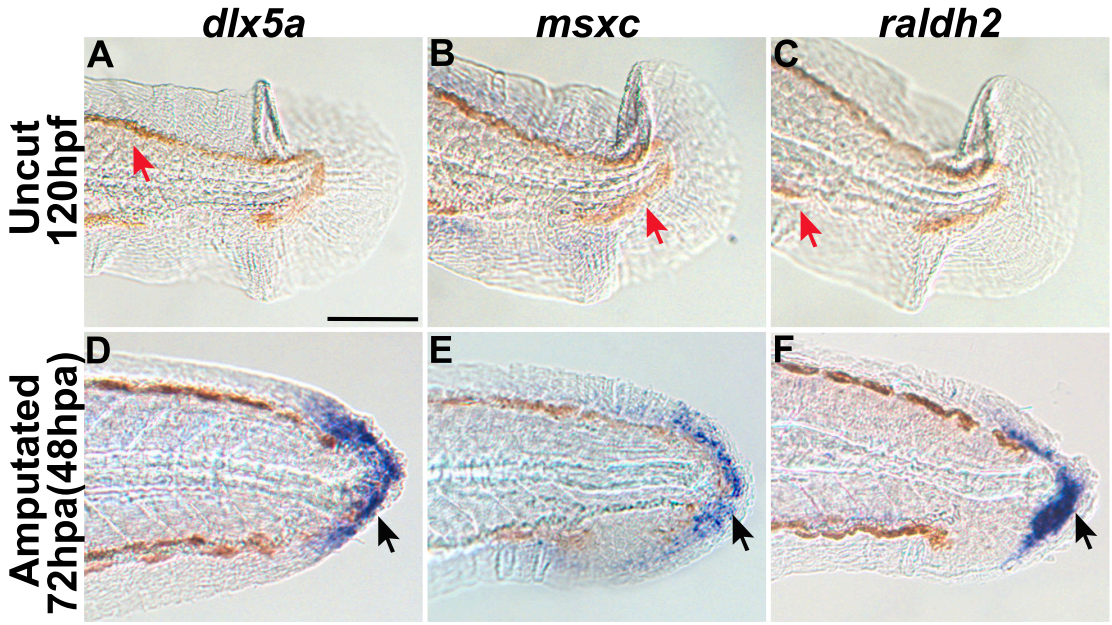

Figure 3-3: Tail amputation in the zebrafish larva. Regeneration marker genes are expressed in amputated tails but not in un-amputated tails. *dlx5*, *msxc* and *ralhd2* expression is assessed in the tails of 5-day-old. Gene expression is detected by ISH at 48 hpa (blue staining) (black arrows). **A-C:** Regeneration genes are not expressed in un-amputated tails, un cut (n=10 per gene expression). **D-F:** Regeneration markers are expressed at the end of the regenerating tail (black arrows)(n=10 per gene expression). The tail pigment is pointed with red arrows. A representative embryo was chosen for each picture. N=15. All the 10 tail amputated embryos showed regeneration marker gene expression. The embryo that had an average staining along the 10 embryos was chosen as the representative sample. Scale bar 100 μm.

3.2.2 Hedgehog signalling as an early activator during zebrafish tail regeneration

Molecular function analysis was performed in order to observe how the disruption of developmental pathways affects tail regeneration. The first part of the study was to assess the role of molecular pathways in tail regeneration by morphology. Tail amputation was executed, then the fish were continuously treated with 4 small molecules for 4 days SU5402 (FGF antagonist) 2μM, Gsk3XV inh (Wnt agonist) 0.625μM, IWR-1 (Wnt antagonist) 10μM and Cyclopamine (Hh antagonist) 20μM. These compound concentrations were standardised during my Master's degree and by previous students at Roehl's lab. The parameter used to establish the concentration were was when the compound managed to affect the pathway of interest (which was done by assessing the expression of a pathway read out gene or marker by ISH) without affecting the overall health of the larval zebrafish. After 4 days post amputation it was possible to observe that Hh and Wnt down-regulation caused the most disruption during the regeneration process. In the cyclopamine and IWR-1-treated tails it was possible to observe the amputation plane, where no tissue outgrowth took place (Figure 3-4).

The second part of the molecular functional analysis was to study the regeneration marker genes. Tails were amputated and treated continuously with the small molecular compounds already mentioned. Fish were fixed at 48hpa for whole-mount ISH to observe regeneration marker's expression (Figure 3-5). All the zebrafish embryos used for this experiment were siblings collected the same day. The fish embryos were treated with four different conditions, using different chemical compounds and processed for ISH simultaneously, except for IWR-1 treatment. As we can observe in Figure 3-5, there is a lack of expression of the regeneration marker genes where down-regulation of Hh (cyclopamine) and Wnt signalling (IWR-1) occurs. These results agree with the morphology assessment experiment that took place during my Master's degree, where down-regulation of Hh and Wnt was shown to impede tail regeneration.

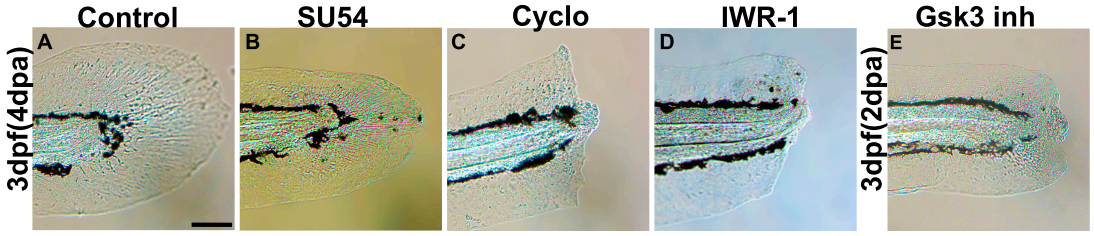

Figure 3-4: Hh and Wnt signalling is necessary for tail regeneration. The tails are 4 days post-amputation, which are continuously treated with different chemical compounds after amputation. **A:** Vehicle-treated tail (n=10). **B:** 2 μM SU5402 (n=10). **C:** 20 μM cyclopamine (n=10). **D:** 10 μM IWR-1 (n=10). **E:** 0.625 μM Gsk3XV inhibitor (n =10). N=7. Scale bar 100 μm.

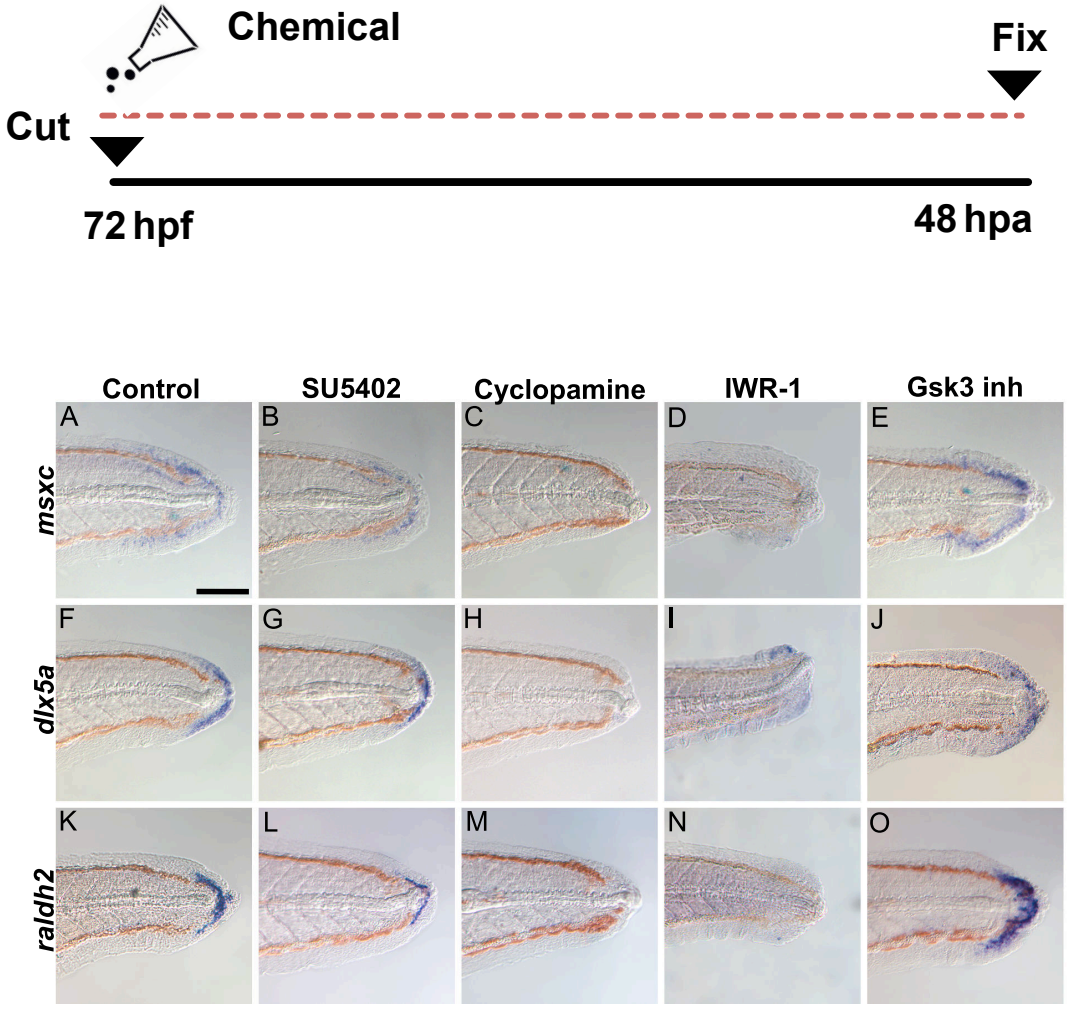

Figure 3-5: Hh and Wnt signalling is necessary for the expression of regeneration marker genes. Tails are cut at 72 hpf and allowed to regenerate for two days (black line), then fixed with PFA. Regeneration marker gene expression (*dlx5a*, *msxc* and *raldh2*) is detected by in situ hybridisation at 48 hpa. Chemical administration is done immediately after amputation (red dashed line). **A, F, K:** Vehicle-treated tails (n=10 per gene expression) . **B, G, L:** 2μM SU5402-treated tails (n=10 per gene expression). **C, H, M:** 20μM Cyclopamine-treated tails (n=10 per gene expression) **D, I, N:** 5μM IWR-1-treated tails (n=10 per gene expression) **E, J, O:** 10μM Gsk3 inh-treated tails (n=10 per gene expression). N=2. Scale bar 100μm (Adapted from Garcia Romero 2011 p.15).

It is important to mention that the molecular functional analysis experiments were performed during my master degree. However, the assessment of the down-regulation of Wnt signalling by the IWR-1 compound was incorporated later on during my PhD. Before obtaining the IWR-1 compound, Hh pathway was the most consistent in both experiments (molecular and morphology functional analysis).Therefore, the dissection of the Hh signalling was performed first, which generated significant results, which will be explained in this section.

Finally, another aspect that is important to mention is that the cyclopamine compound was the standard one to down-regulate Hh signalling during my PhD research. However I wanted to make sure that the current findings were due to cyclopamine acting towards Hh signalling and not because of stray target effects (affecting other physiological process not related to Hh signalling). In order to do this I decided to use a different Hh inhibitor called LDE225 that, like cyclopamine, is a smoothened inhibitor. A morphology and gene expression assessment during tail regeneration was performed in fish treated with 20μM LDE225 . The results showed that the tail regeneration was blocked (Figure 3-6) and expression of the regeneration markers *msxc* and *dlx5a* was down-regulated (Figure 3-7). The use of LDE225 showed similar results to the observed with cyclopamine, confirming that Hh is properly down-regulated by cyclopamine.

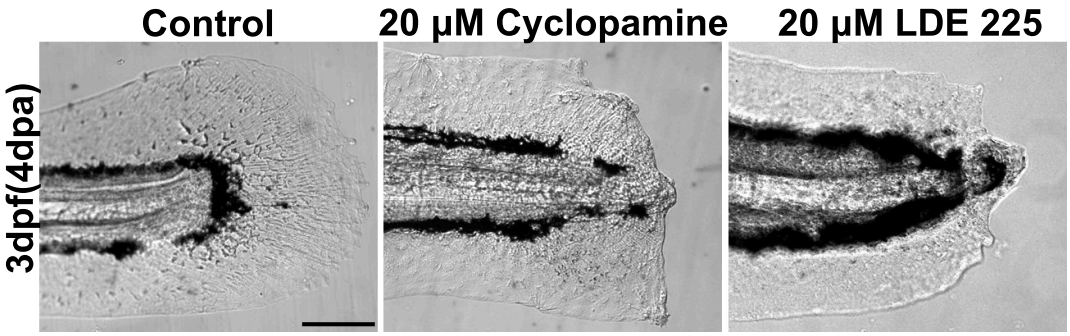

Figure 3-6: Hh down-regulation by LDE and cyclopamine suppresses tail regeneration. This shows 4 days post-amputated tails under different chemical conditions during regeneration. **A:** Control tail ethanol-treated (vehicle) (n=10). **B:** 20μM cyclopamine-treated tail (continuously) (n=10). **C:** 20μM LDE225-treated tail (continuously) (n=10).One representative embryo was chosen per group. Scale bar 100μm.

3.2.2.1 Hedgehog expression at early time points during tail regeneration

Once I identified the importance of Hedgehog signalling during zebrafish tail regeneration I decided to observe the expression of *ihh* (indian hedgehog) ligand at different time points during regeneration. My interest in *ihh* expression came from the reports from P. Jankun, which showed that*ihh* is expressed at 6 hours

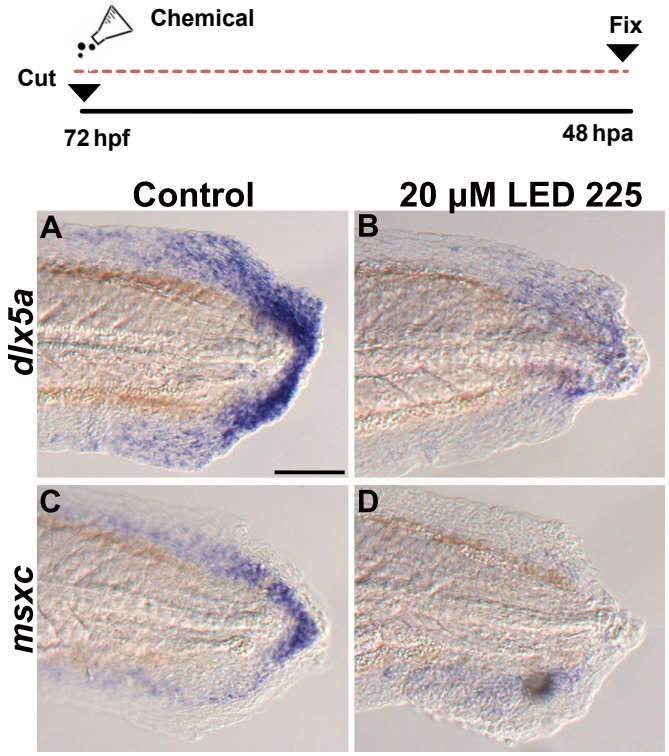

Figure 3-7: Hh down-regulation by LDE down regulates *dlx5a* and *msxc* gene expression. 2 days post-amputation tails treated with 20μM of LDE225 during regeneration, ISH is used to detect *dlx5a* and *msxc*. **A, C:** Control tails treated with DMSO (vehicle) (n=10 per gene expression). **B, D:** 20μM LDE225-treated tail (continuously)(n=10 per gene expression). One representative embryo was chosen per group. Scale bar 100μm.

post amputation. The expression of this ligand eventually decreases with time, as observed in Figure 3-8. The idea of placing this result here is to show how early Hh signalling starts and to compare it with the Wnt pathway dissection, which is described later on in this chapter.

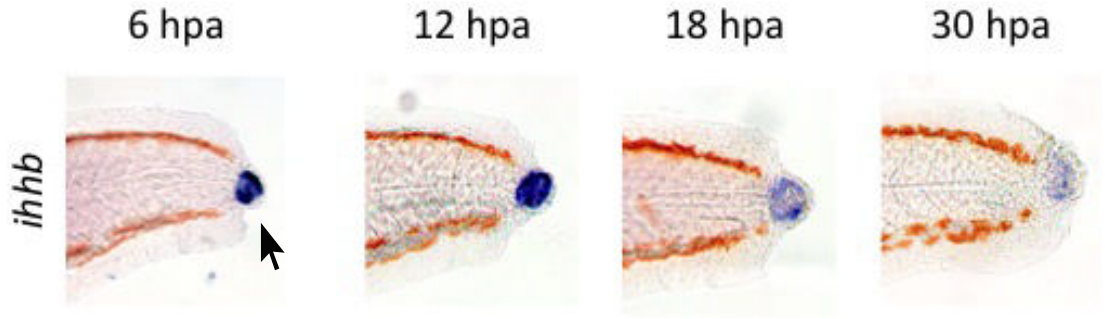

Figure 3-8: Indian hedgehog (*ihh*) is expressed in the regenerated tail. *ihh* is expressed in the bead (round protuberance that is form after tail amputation) formed after tail amputation (black arrow). Expression detected by ISH. n=10 per time point. One representative embryo was chosen per time point. (Source P. Jankun).

3.2.3 Hedgehog signalling is not dependent upon Wnt, FGF or RA pathways during tail regeneration

After testing the morphological and genetic effects of Hh signalling and observing the early expression of *ihh* during tail regeneration, I decided to test the relationship of Hedgehog to the Wnt, FGF and RA pathways. To do this I used the *patched 1* gene expression as a Hh readout. *patched* gene was first described as necessary for segmental pattern in *Drosophila* larva (Nüsslein-Volhard et al. 1980). The reason why *patched 1* is used a Hh readout is because its expression is up-regulated when Hh signalling is active (Pearse et al. 2001). I tested the expression of the *patched 1* gene under the following conditions: pulse administration of SU5402 1μM (4 hours before PFA fixation), pulse administration of Gsk3XV inhibitor 12.5μM (8 hours before fixation), and RA administration 1μM (5 hours before fixation). Wnt signalling was also down-regulated with the use of the heat-shock dkk line *hs:dkk1-GFP* (Wnt signalling is down-regulated when the transgenic fish are incubated at 39°C). The *hs:dkk1* fish were put at 39°C for 3 hours, 17 hours before fixation. Fish expressed GFP as indicator of Wnt signalling down-regulation (The GFP gene is coupled after the *dkk1* gene, so GFP is expressed when the *dkk1* gene is activated). The control group was treated with the solvent used to dilute the chemical. The fish were tail-amputated at 72hpf and fixed at 48hpa. Whole-mount ISH was performed to detect the expression of *ptc 1* during tail regeneration under four different conditions. The concentration and treatment times of the SU5402, Gsk3-inh compounds and the transgenic line *hs:dkk1-GFP* were determined based on the effectiveness of affecting the expression of the regeneration marker genes *msxc*,

dlx5 and *raldh2* during tail regeneration. RA conditions were determined by the effectiveness of affecting the expression of Wnt signalling readout *tcf7*. However, it is important to mention that the Gsk3 molecule forms part of other molecular signalling apart of Wnt, so the results observed for the Gsk3 inhibitor condition should be interpreted with care. As we can observe in Figure 3-9, the *ptc 1* gene expression was not strongly altered, in comparison with the control panel. This result suggests that Hh signalling is not dependent on Wnt, FGF and RA during tail regeneration. Still, further studies need to be done to confirm these findings, such as an epistasis analysis. Some complementary studies are presented later in this chapter.

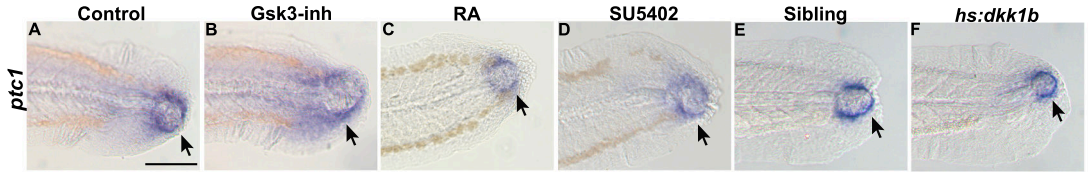

Figure 3-9: *ptc1* expression is maintained after Wnt, FGF, and RA manipulation during tail regeneration. No major differences in *ptc1* expression detected by ISH are observed in amputated fish treated with different chemical compounds. **A:** Control (n=10). **B:** Larvae treated with Gsk3XV inh at 12.5μM 8 hours before fixation (n=10). Wnt/β- Catenin pathway activator (n=10). **C:** Larvae treated with RA at 1μM from 43.5-48hpf to activate the RA ectopic administration (n=10). **D:** Larvae treated with SU5402 at 15μM 4 hours before fixation (FGF pathway inhibitor) (n=10). **E:** Larvae that were heat-shocked for 3 hours at 39°C at 17 hours before fixation; sibling of the *hs:dkk1-1b* transgenic fish (n=10). **F:** Larvae that were heat-shocked for 3 hours at 39°C at 17 hours before fixation (Wnt/β-Catenin pathway inhibition) (n=10). A representative embryo was chosen for each picture. N=2. Scale bar 100μm.

3.2.4 Fin fold and tail regeneration are regulated in a different manner by Hh signalling

Many of the studies done with larval zebrafish do not report the importance of Hh signalling during regeneration and, instead, focus more on other pathways such as Wnt, FGF, BMP and Notch. As previously mention, many of the previous published reports use the alternative larvae regeneration model where the fin fold is cut and not the tail. This fact made me wonder whether there is a difference between tail and fin fold regeneration with regard to the role of Hh signalling.

Therefore I decided to compare the down-regulation of Hh by cyclopamine treatment during tail and fin fold regeneration. As we can observe in Figure 3-10, tail amputation does not present any tissue outgrowth, while fin fold-amputated fish present certain level of regeneration but which is not complete.

Finally I made a quantitative ANOVA with pairwise comparison. As we can observe in Figure 3-11 there is a clear difference in the tail outgrowth level.

The second part of this study was the evaluation of the expression of *ptc 1* gene in both tail and fin fold regeneration.

Fish were amputated and fixed at 48hpa and processed for *ptc 1* detection. Figure 3-12 shows a clear expression of *ptc 1* in the tail-amputated fish but it is absent in the fin fold-amputated fish. These results suggest that Hh signalling acts in a different way during fin fold regeneration.

However, further studies need to be done with different Hh markers and Hh regulators, in order to explain the role of Hh in both amputation models.

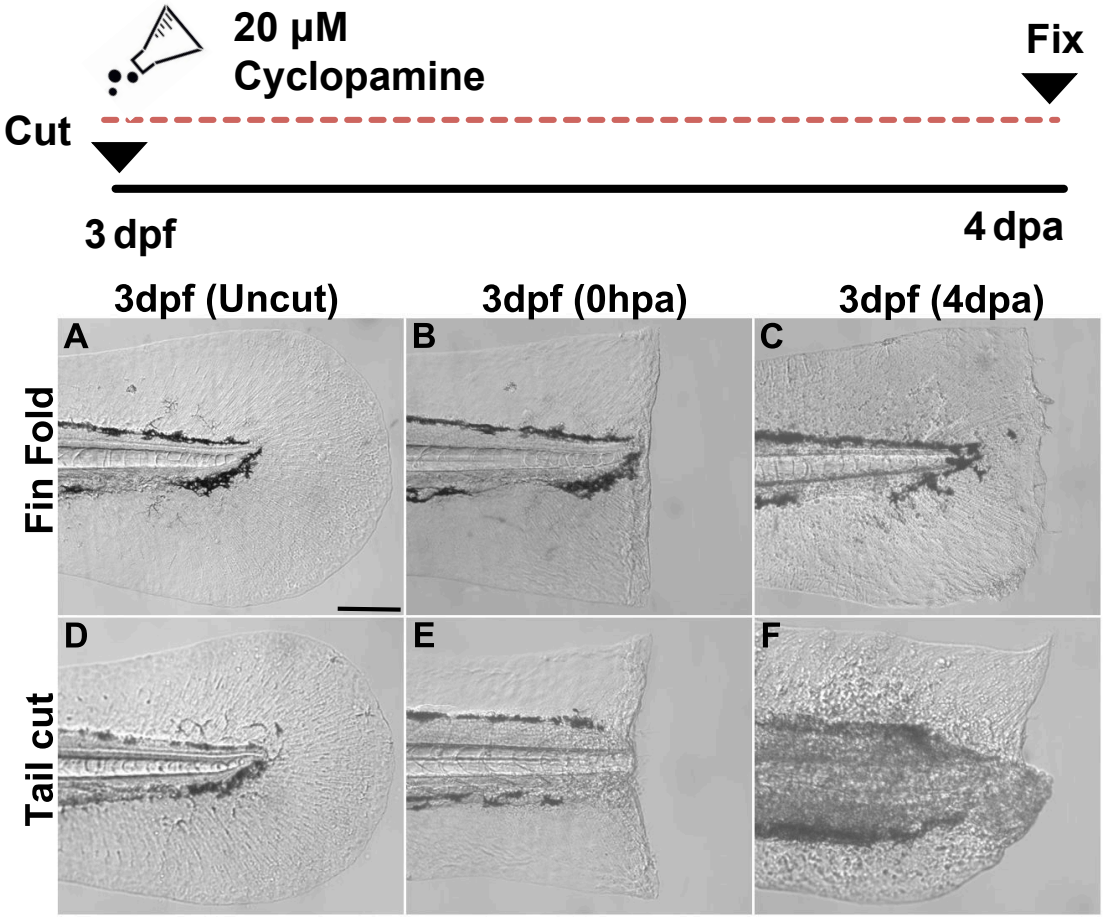

Figure 3-10: Down-regulation of Hh signalling completely suppress tail outgrowth during tail regeneration but not fin fold regeneration. A-C: Fin fold regeneration, following the 20μM cyclopamine, during the 4 days immediately after amputation, pictures showing the fin fold regeneration of the same fish (n=10 per condition). **D-F:** Tail regeneration, fish treated with 20μM cyclopamine, during the 4 days immediately after amputation, pictures showing the tail regeneration of the same fish (n=10 per condition). N=2. A representative embryo was chosen for each picture. Scale bar 100 μm.

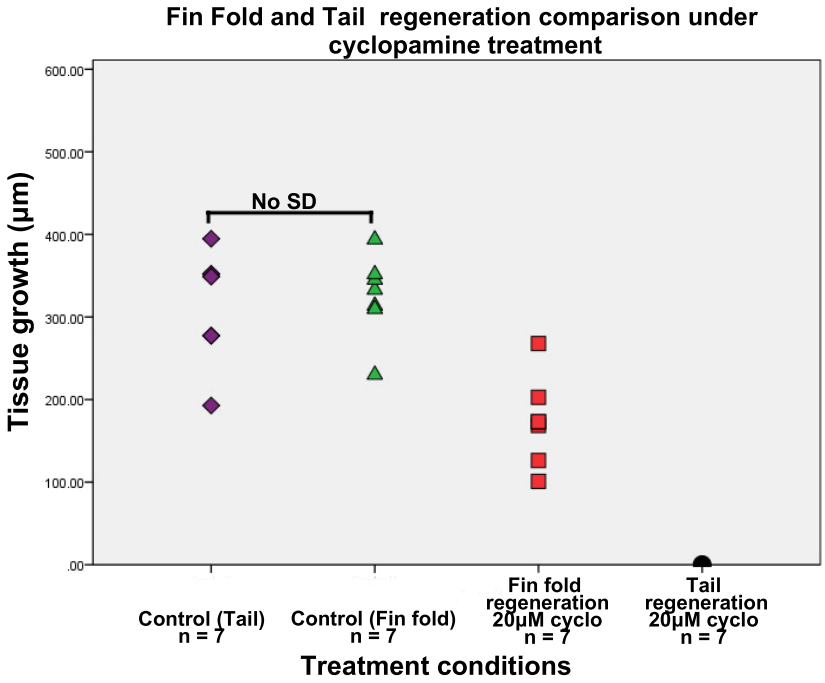

Figure 3-11: Cyclopamine-treated fin fold regenerated tails present a level of outgrowth higher than the tail amputated tails and lower than vehicle-treated tails. Fin fold cut tails treated with cyclopamine present a level of outgrowth higher than the amputated tails treated with cyclopamine, but smaller than the vehicle-treated amputated tails. Error bars represent standard error. One-way ANOVA statistics with pairwise comparisons showed a significant difference between all the conditions at $p < 0.01$, except between the two control groups (No SD: No Significant Difference).

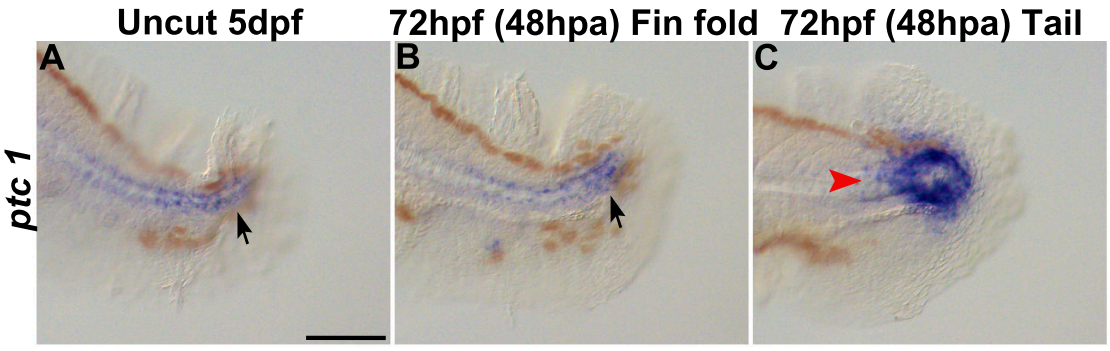

Figure 3-12: The *ptc1* gene has a stronger expression in amputated tails than in fin fold cut tails or un-cut tails. A-B: Uncut and amputated fin fold cut tails present a mild level of *ptc1* expression (black arrows) (N=10 per condition). **C:** *ptc1* is strongly expressed at the end of the regenerated tail (red arrowhead) (n=10). Gene expression detected by ISH at 48 hpa. A representative embryo was chosen for each picture. N=1. Scale bar 100 μm.

3.3 Wnt signalling is downstream of Hedgehog during zebrafish tail regeneration

3.3.1 Wnt signalling is able to regulate regeneration marker genes in a more direct way than Hedgehog signalling

The previous results, as illustrated in Figures 3-4 and 3-5, show that Wnt signalling has a crucial role during regeneration. Wnt signalling seems to be downstream of Hh during regeneration, as shown in Figures 3-9. Together these findings may indicate that Wnt signalling can have a more direct role in the regulation of the regeneration marker genes than Hh. To study further the role of Wnt signalling during regeneration, I observed the experiment that took place during my Master's course, where a series of pulse treatment were administered in tail-amputated fish. The pulse treatment consisted of the administration of the small molecular compound for a short period of time before fixation. The reason for this short period of time was to give enough time for the pathway of interest to be either up- or down-regulated, without affecting other pathways that may be downstream.

The result, shown in Figures 3-13 shows that the pulse up-regulation of Wnt signalling by the Gsk3inh XV inh compound managed to up-regulate the regeneration marker genes, while the pulse down-regulation of Hh signalling did not affect their expression. This suggests two things: 1) Other pathways are downstream of Hh signalling during regeneration, because the pulse down-regulation of Hh did not affect the regeneration markers' expression (only in continuous cyclopamine regeneration genes expression is totally down-regulated) 2) Wnt signalling seems to have a more direct role over the regulation of the regeneration markers.

In order to confirm these findings, during my PhD I decided to down-regulate Wnt signalling with the use of the *hs:dkk1-GFP* transgenic line. The *hs:dkk-GFP*-transgenic fish were amputated at 72hpf, then 36 hours after amputation I heat-shocked the fish at 39°C for 3 hours. 17 hours after heat shock I separated the fish expressing GFP (the indicator that Wnt is down-regulated) from the non-expressing GFP (siblings) and fixed them for whole-mount ISH. The ISH showed that regeneration marker genes expression was completely down-regulated in fish expressing GFP (Wnt down-regulation). This result confirms the regulation of regeneration markers by Wnt signalling during tail restoration.

3.3.1.1 Wnt signalling is expressed at a later time point than Hh signalling during tail regeneration

After assessing the importance of Wnt signalling for the expression of regeneration marker genes, I decided to assess the expression of Wnt signalling by observing the expression of the *wnt10a* ligand and *tcf7* readout at different time points during

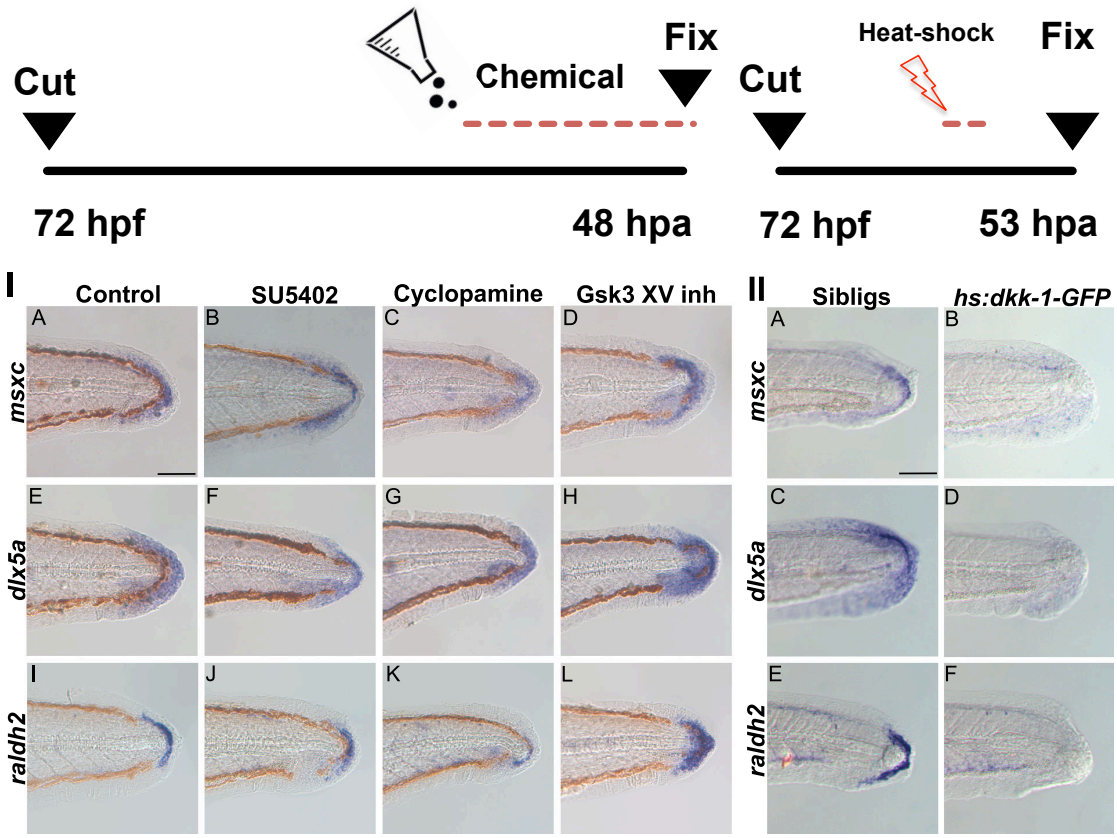

Figure 3-13: Wnt signalling has a more direct role over the expression of regeneration marker genes under pulse treatment conditions than Hh signalling. Tails are cut at 72 hpf and allowed to regenerate over 2 days (black line), then fixed with PFA. Regeneration marker genes (*dlx5a, msxc* and *raldh2*) are detected by ISH at 48 hpa and *53 hpa (for the dkk-1-GFP line).* Chemical administration is done some hours before fixation (red dashed line). **I: A, E, I** Vehicle-treated tails (n=10 per gene expression). **I: B, F, J:** 10 μM SU5402 added 4 hours before fixation (n=10 per gene expression). **I: C, G, K:** 20 μM Cyclopamine added 8 hours before fixation (n=10 per gene expression). **I: D, H, L** 10 μM Gsk3 XV inh added 6 hours before fixation (n=10 per gene expression). **II: A, C, E** siblings (n=10 per gene expression) and **II: B, D, F** *dkk-1-GFP* heat shocked for 3 hours, (n=10 per gene expression) 36 hours post amputation, the embryos were fixed at 53 hpa. A representative embryo was chosen for each picture. N=2. Scale bar 100 μm. (Adapted from Garcia Romero 2011 p.16).

tail regeneration. *wnt10a* expression has been noted to activate Wnt/β catenin signalling during development (Narita et al. 2005) and also has been reported to be a good candidate to report the activation of Wnt/β catenin during adult zebrafish tail regeneration (Stoick-Cooper et al. 2007). P. Jankun assessed the expression of *wnt10a* during tail regeneration, which was up-regulated at 18hpa. On the other hand *tcf* genes have been reported to be active during Wnt/β catenin up-regulation (Roose et al. 1999) and during adult tail regeneration (Münch et al. 2013). Therefore I decided to test *tcf7* gene expression during larval zebrafish tail regeneration to measure the activity of Wnt signalling. Figure 3-14 shows that the *tcf7* gene is also expressed during tail regeneration, where the earliest expression point was at 18hpa.

The expression of the Wnt ligand and readout took place at a later stage in comparison with the *ihh* ligand (Figure 3-8).

These results suggest that Hh precedes the activation of Wnt signalling during tail regeneration.

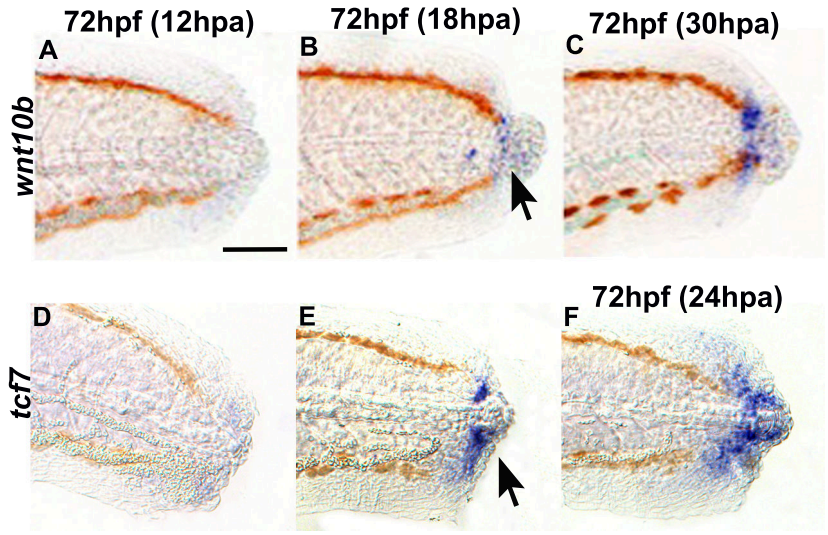

Figure 3-14: *tcf7* and *wnt10b* are expressed at 18 hpa in the regenerating tail. *tcf7* and *wnt10b* (Wnt readouts) expression is observed at the end of the amputated tail from 18 hpa onwards, (black arrow) gene expression detected by ISH (*wnt10* in situs and image analysis were done by P. Jankun). n=10 per time point. One representative embryo was chosen per time point. Scale bar 100 μm.

3.3.2 Hh signalling is required for Wnt activation during tail regeneration

The lack of regulation in the expression of *ptc1* by Wnt down-regulation and up-regulation, along with the later expression of the Wnt readouts in comparison with *ihh* (6hpa), support the statement about Hh being upstream of Wnt signalling. In order to further assess this statement I decided to test whether or not Hh

played a direct role in controlling Wnt signalling. I performed tail amputation and then continuously treated the fish with 20μM cyclopamine for the duration of 48 hours. Then, to evaluate the activity of Wnt signalling under the conditions already mentioned, I decided to see the expression of the *tcf7* Wnt readout. I also performed a short cyclopamine treatment (pulse treatment) at 100 μM for 8 hours before fixation (fixation was done at 48hpa) to see the expression of *lef1* Wnt (Filali et al. 2002). The expression of *tcf7* and *lef1* was analysed using ISH. As a control marker to measure Hh activity I analysed the expression of *ptc1* under similar pulse and continuous treatment conditions. The idea was to show that cyclopamine was targeting Hh signalling. The treated fish showed a lack of the expression of both Wnt markers, which is shown in Figure 3-15. The current result suggests a direct role of Hh signalling in Wnt expression.

Note: The reason why there are two types of treatments (short and pulse) is because the short was done at the very beginning of the PhD, while the second (continuous) was done at the very end, so I did not have time to do the pulse but still wanted to show that the expression was regulated by Hh.

The outcomes from Figure 3-15 lead to the question of whether Hh signalling is necessary for the maintenance of Wnt signalling during tail regeneration, or if it is just necessary for its activation.

To answer this question I propose a further experiment to perform a time course for the expression of *ptc1*, *lef1* and *tcf7* genes during tail regeneration. Here two possible outcomes are expected. The first is that *ptc1* is expressed temporarily at the beginning of tail regeneration, and with *lef1* and *tcf7* expressed sequentially after. This outcome would suggest that Hh signalling is necessary for the activation of Wnt signalling. To further assess this I would apply cyclopamine during the period where *ptc1* is expressed and see how the down-regulation of Hh signalling affects *lef1* and *tcf7* expression. The second outcome is that *lef1* and *lef7* are expressed after *ptc1* expression during regeneration, but the expression *ptc1* is maintained at the same time as *lef1* and *tcf7* during the regeneration process. This outcome would suggest that Hh is necessary for the maintenance of Wnt signalling during regeneration. To determine whether the interactions between Hh signalling and Wnt signalling are direct or indirect I would suggest the use of the ChiP (Chromatin immunoprecipitation) assay. ChiP is used to detect the interaction between DNA and proteins, which could be a transcription factor binding to a specific DNA sequence (Bailey et al. 2013). I would then induce regeneration through tail amputation and then fix the fish at different time points. Then I would use the ChiP assay to detect whether any Hh transcription factor binds to the DNA sequence of any Wnt genes to initiate its transcription during tail regeneration.

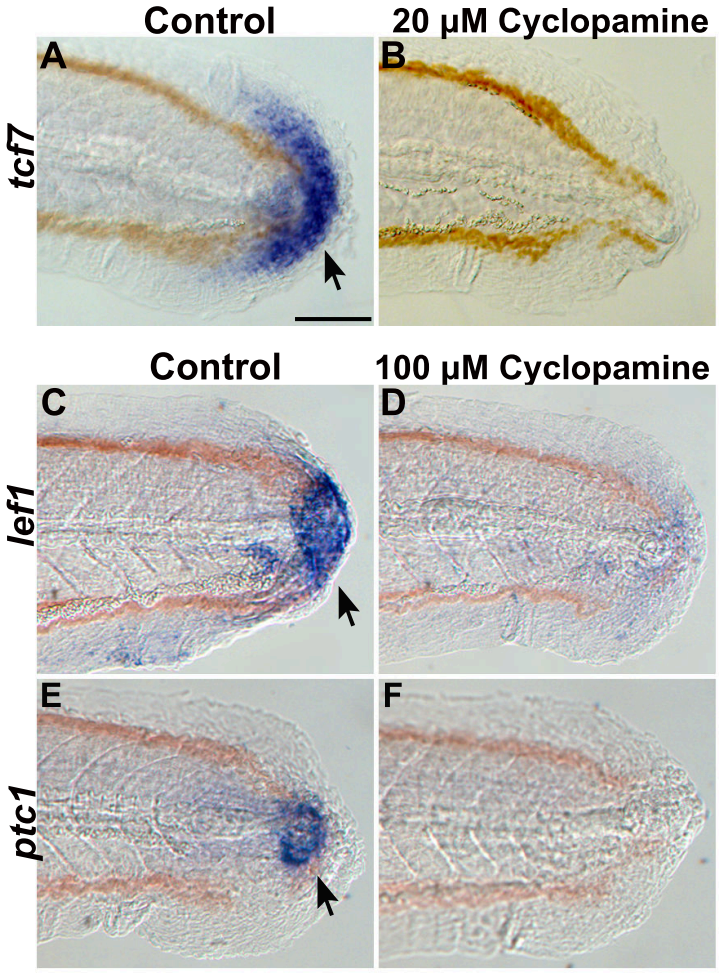

Figure 3-15: Hh signalling has a direct role over the Wnt pathway during tail regeneration. A, C and **E:** Ethanol-treated tails expression of *tcf7*, *lef1* and *ptc1*, (black arrows) gene expression detected by ISH (n=10 per gene expression). **B, D** and **F:** Cyclopamine-treated tails. *tcf7* expressing tails continuously treated with 20 μM of cyclopamine from 0 hpa to 48 hpa (n=10 per gene expression). *lef1* and *ptc1* expressing tails treated with 100 μM 8 hours before fixation. Fish were amputated at 72 hpf and fixed at 48 hpa. N=2. One representative embryo was chosen for each picture. Scale bar 100 μm.

3.3.3 Wnt signalling is epistatic of Hh signalling during the expression of regeneration marker genes

Since my previous outcome suggest that Hh signalling is upstream of Wnt signalling during larval zebrafish tail regeneration, I suggest that the down-regulation of regeneration marker genes *msxc*, *dlx5* and *raldh2* by continuous administration of cyclopamine 3-5 can be restored by Wnt signalling up-regulation. Therefore my hypothesis is that Wnt signalling is epistatic of Hh signalling during larval zebrafish tail regeneration.

To test this hypothesis I amputated the fish at 3dpf and divided them in 3 groups. The first group was treated continuously with 0.5% ethanol (Control 1), the second group was treated continuously with 50μM of cyclopamine (Control 2) and the third group was also treated continuously with 50μM cyclopamine then washed 8 hours before fixation, when a second chemical was added (the Gsk3XV inh) in order to up-regulate Wnt/β catenin signalling. The reason for this was to see whether Wnt up-regulation was able to increase (rescue) the loss of the regeneration marker genes expression by continuous cyclopamine administration. ISH was performed on fish fixed at 48 hours to detect the regeneration marker genes *msxc*, *raldh2* and *dlx5a*. As we can observe in Figure 3-16, cyclopamine-treated fish lack the expression of the regeneration marker genes while the cyclopamine- and Gsk3XV inh-treated group showed the expression of the three markers. The increase in the expression of regeneration markers genes by up-regulation of Wnt signalling is the final assessment of this chapter, and suggests Hh signalling is upstream of Wnt signalling during larval tail regeneration. Finally, even though the results are consistent with my hypothesis, they do not rule out the possibility of Hh and Wnt signalling acting in a parallel fashion. A complementary experiment to test whether Hh signalling is epistatic of Wnt signalling could be very informative regarding the relation between Hh and Wnt signalling during tail regeneration.

3.4 FGF signalling is downstream of Hh and Wnt signalling during zebrafish tail regeneration

3.4.1 FGF signalling readouts are down regulated under a continuous suppression of Hh signalling during tail regeneration

Several studies done with tail regeneration both in adult and larvae zebrafish report a role for FGF signalling in modulating different areas of regeneration, from blastema formation to cell proliferation. In order to test the hypothesis *Hedgehog signalling leads tail regeneration by regulating Wnt and FGF signalling*

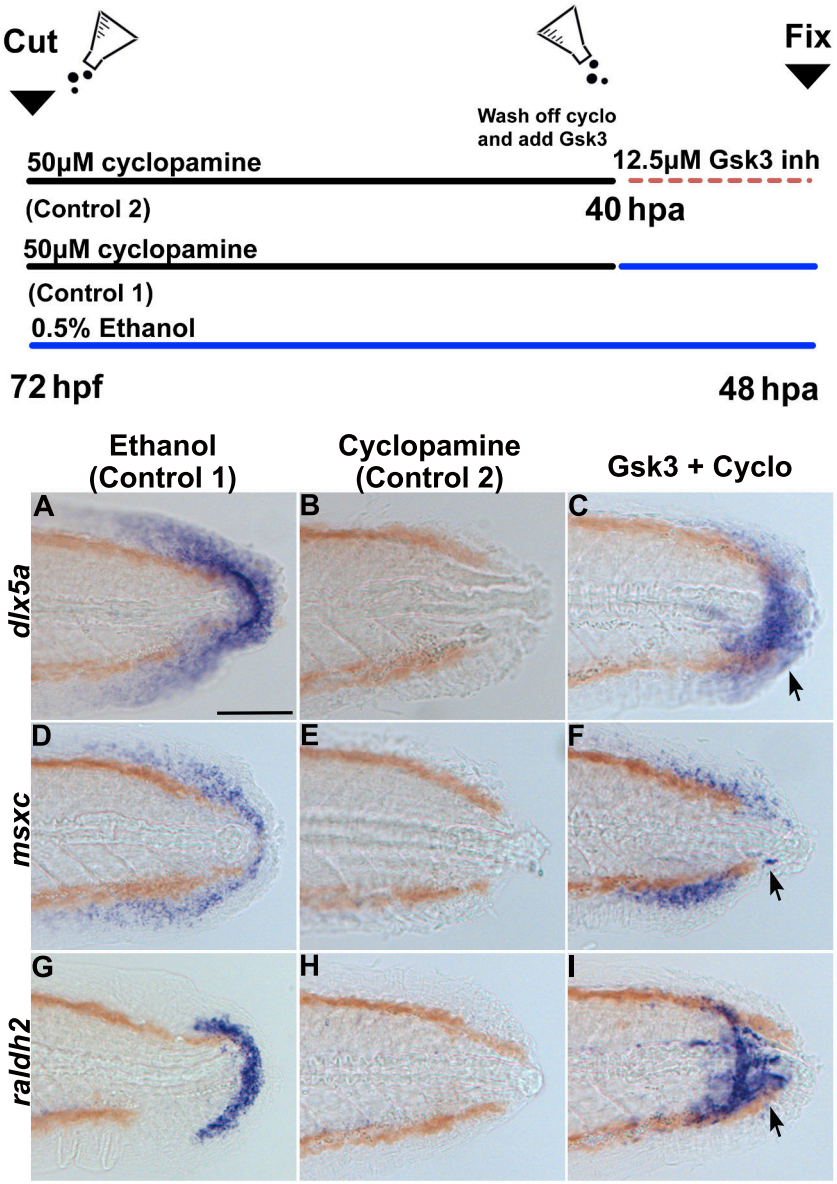

Figure 3-16: Wnt signalling is epistatic of Hh pathway during tail regeneration. Regeneration marker genes (*dlx5a, msxc* and *raldh2*) expression is detected by ISH at 48 hpa. **B, E** and **H:** Larvae treated with 50 μM cyclopamine and then incubated in E3 medium for 8 hours until fixation (n=10 per gene expression). C, F and **I:** Larvae treated during 0-40 hpa with 50 μM cyclopamine and then incubated in 12.5 μM Gsk3XV for 8 hours before fixation (n=10 per gene expression). Regeneration marker genes expression is observed (black arrows). **A, D** and **G:** Vehicle-treated (control) (n=10 per gene expression). 0/10 larvae showed expression in cyclopamine-treated embryos and 10/10 showed expression in cyclo/Gsk3XV-treated embryos. N=2. One representative embryo was chosen per picture. Scale bar 100 μm.

in larval zebrafish, I decided to test the relation between Hh and FGF by down-regulating Hh signalling and then observing the expression of FGF readouts. I therefore induced regeneration by performing zebrafish tail amputation, and then continuously treated the fish with 20μM of cyclopamine for 48hpa. Finally the fish were processed for ISH to see the expression of *pea3* and *fgf10a* (FGF readout and ligand).

As we can observe in Figure 3-17, there is a loss in the expression of *fgf10a* in cyclopamine-treated fish, and a pronounced down-regulation of the *pea3* marker. These results, along with the outcome presented in Figure 3-9 (where down-regulation of FGF signalling by the SU5402 compound does not strongly affect the expression of the Hh readout gene *ptc1*) suggest that Hh signalling positively regulates FGF signalling during tail regeneration.

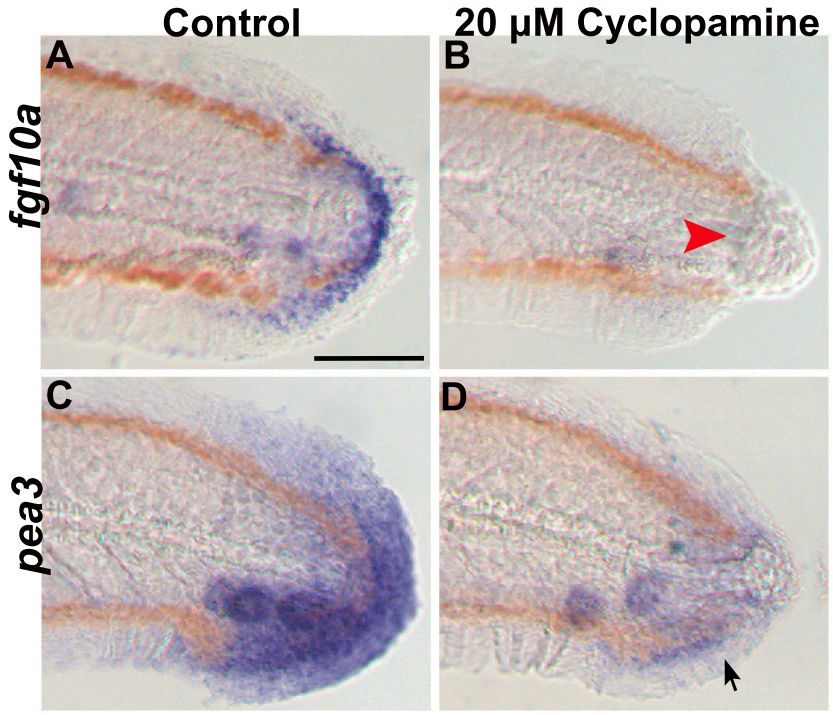

Figure 3-17: Hh signalling regulates the expression of *pea3* and *fgf10a* during tail regeneration. Tails are amputated and fixed at 48 hpa. The expression of *fgf10a* and *pea3* is detected by ISH . **A** and **C:** Control (n=12 per gene expression). **B** and **D:** Continuously cyclopamine-treated fish with 20 μM (n=12 per gene expression). *fgf10a* expression is totally suppressed, *pea3* expression is down-regulated. One representative embryo was chosen per picture. N=1. Scale bar 100 μm.

3.4.2 Wnt signalling regulates FGF readouts during zebrafish tail regeneration

After I had tested the relation between Hh and FGF, it was time to make a similar analysis to observe the relationship between Wnt and FGF signalling during tail

regeneration. To evaluate the activity of FGF signalling I used the *fgf10a* (FGF signalling ligand) and the *pea3* (FGF signalling readout) (McCarroll et al. 2013). The reason I chose these genes is that *pea3* has been reported to be up-regulated in the distal epidermal tissue during adult fin regeneration and broadly regulated by FGF (Lee et al. 2009). Regarding the choosing of the *fgf10a* gene, it has been reported by P. Jankun that there is a lower level of proliferation during tail regeneration in the *daedalus (dae)* zebrafish mutant, which has a disruption in the *fgf10* gene (Norton et al. 2005). Larvae tails were amputated at 72hpf and allowed to regenerate for 48 hours, and then Gsk3XV inh was added at 12.5μM concentration for 8 hours before fixation in order to up-regulate Wntβ catenin. To down-regulate Wnt signalling I used the transgenic line *hs:dkk1-GFP*; the embryos were heat-shocked for 3 hours at 39°C and fixed 8 hours post heat-shock at 48hpa.

fgf10a and *pea3* gene expression was assessed under the conditions already described. As we can observe in Figure 3-18 it is clear that the up-regulation of Wntβ catenin signalling by Gsk3XV inh shows up-regulate both FGF markers.

On the other hand, down-regulation of Wntβ catenin by the use of the *hs:dkk1-GFP* transgenic line shows an obvious down-regulation of *pea3*. *fgf10a* also seems to be down-regulate in the *hs:dkk1-GFP* line but the down-regulation is not as evident as *pea3*. The present outcome suggests that Wnt is upstream of FGF signalling, however, the regulation of *fgf10* gene expression by the *hs:dkk1-GFP* line was difficult to interpret, since both the sibling and *hs:dkk1-GFP* have a similar level of expression. Finally the possibility of FGF and Wntβ-catenin signalling acting in a parallel fashion has not been ruled out in this experiment. Complementary experiments are shown later in this chapter regarding the relation of FGF and Wntβ catenin during larval zebrafish tail regeneration.

3.4.3 FGF signalling is epistatic to Hh signalling during cell proliferation in zebrafish tail regeneration

The results from Figures 3-17 and 3-18 suggest that both Wnt and Hh pathways are upstream of FGF signalling during tail regeneration. The finding answers some questions and generates others. The first question I wanted to answer was whether Hh had a direct influence over FGF pathway components. To answer this question I induced tail regeneration by amputating the larval tail at 72hpf then treating the fish with 100μM cyclopamine for 8 hours before fixation. Gene expression was detected using ISH. We can observe in Figure 3-19 the down-regulation of *pea3* expression by cyclopamine. However, the changes in expression were not as evident as in the manipulation of Wnt signalling (Figure 3-18). This outcome suggests that during tail regeneration Wnt signalling regulates *pea3* expression in a more direct way than Hh signalling. On the other hand *fgf10a* was totally suppressed, indicating that Hh signalling regulates *fgf10a* in a direct way. To

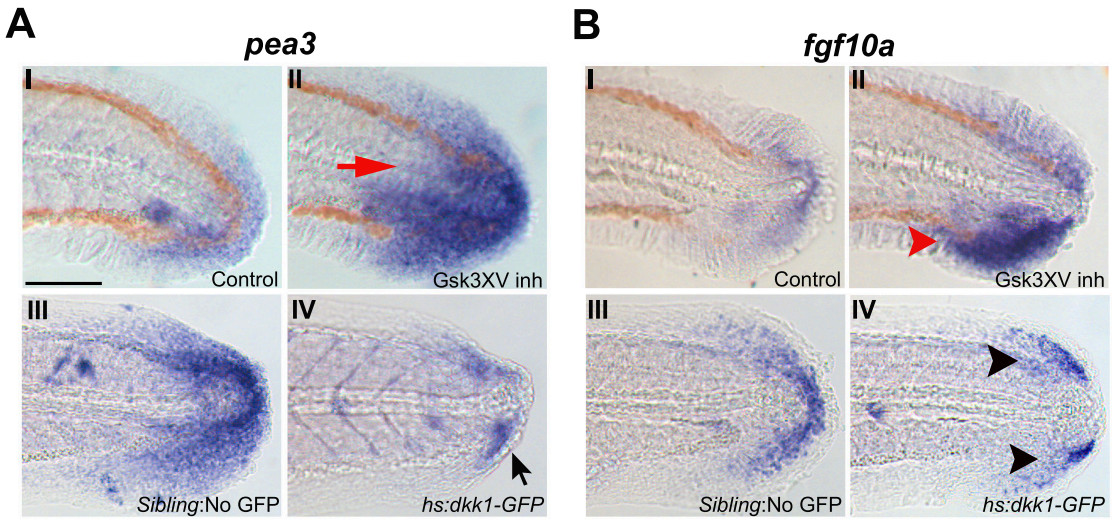

Figure 3-18: Wnt signalling regulates the expression of *pea3* and *fgf10a*. *pea3* and *fgf10a* expression is detected by ISH in fish 48 hpa. **A I, III** and **B I, III:** Control groups (n=12 per condition). **A II** and **B II:** Fish treated with 12 μM Gsk3VX 8 hours before fixation have a stronger expression of *pea3* (red arrow) and *fgf10a* (red arrowhead) than control (n=12 per condition). **A IV** and **B IV:** Fish heat shocked for 3 hours and fixed 8 hours post heat shock have a lower expression of *pea3* (black arrow) than the sibling (n=12 per condition). *fgf10a* expression is also lower that of the sibling but still presents some expression at the edges of the tail tip (black arrowheads). N=2. One representative embryo was chosen per picture. Scale bar 100 μm.

further analyse the relationship between Hh signalling and *pea3* I suggest testing different concentrations and administration points of cyclopamine.

A previous study made by P. Jankun, using the *fgf10a/Deadalus* mutant (defect in the Fgf 10 protein), showed a reduction in cell proliferation during tail regeneration. A similar finding was reported in my Master's thesis, where BrdU (Bromodeoxyuridine, used for the detection of cell proliferation) cell detection showed a decrease in cell proliferation in cyclopamine-treated fish (20μM continuously) (see Figure 3-20). It is well known that the family of fibroblast growth factors is necessary during proliferation (Lee et al. 2005). These previous reports made me think that the Hh signalling regulates cell proliferation during regeneration through FGF signalling. In order to test this idea, I decided to up-regulate FGF signalling by using the *hs:fgf3* transgenic line that, after heat-shock at 39°C, is able to up-regulate the *fgf3* gene. My test involved decreasing proliferation by continuously treating the fish with 50μM of cyclopamine (see Figure 3-20). I then heat-shocked the fish 6 hours before fixation for a period of 1.5 hours. I separated the fish expressing GFP in the hearts (GFP is the reporter gene driven by the expression of the cardiomyocyte light chain gene that is present in the transgenic fish) (*fgf3* up-regulation) from the ones that did not express it (siblings). Finally I decided to immunodetect for the phosphorylated histone H3 protein in order to observe the level of proliferation in both groups.

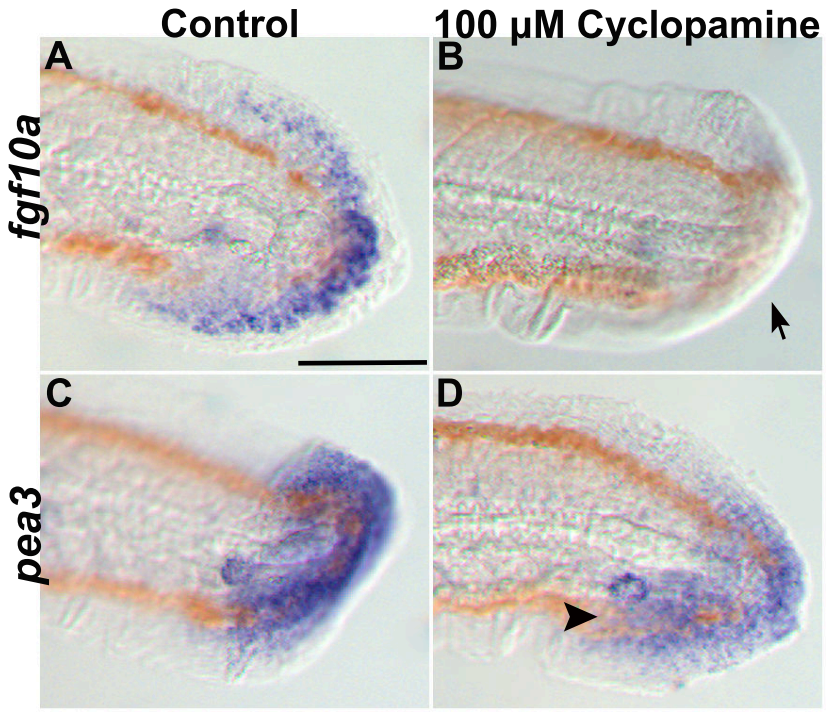

Figure 3-19: Hh signalling has a direct role in the expression of *fgf10a*. *pea3* and *fgf10a* expression is detected by ISH in fish 48 hpa. **A** and **C:** Control group (n=12 per gene expression). **B** and **D:** Fish treated with 100 μM cyclopamine 8 hours before fixation (n=12 per gene expression). A total suppression of the expression of *fgf10a* genes is observed (black arrow). *pea3* expression is down-regulated but not suppressed (black arrowhead). One representative embryo was chosen per picture. N=1. Scale bar 100 μm.

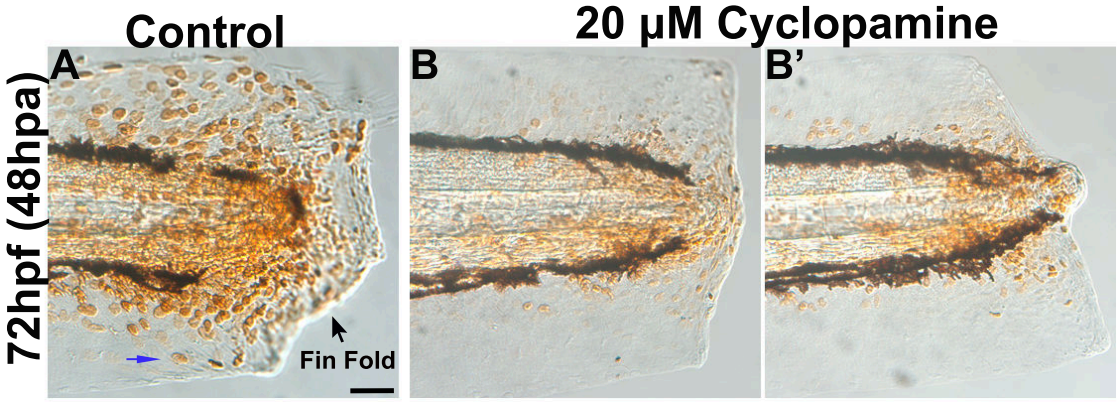

Figure 3-20: Hh signalling regulates cell proliferation during tail regeneration. Fish tails were amputated at 72hpf and were continuously treated with 1mM BrdU and fish were fixed at48 hpa. Cell proliferation is detected by immunostaining. **A:** Control treatment (Ethanol) and 1mM BrdU (n=10). Blue arrow points to a cell that incorporated BrdU. **B, B':** Fish continuously treated with 20μM cyclopamine, which results in lower cell division compared to control treatment (n=10). One representative embryo was chosen per picture. Scale bar 100 μm. (Adapted from Garcia Romero 2011 p.13).

Cell proliferation in cyclopamine-treated siblings was lower than in fish where *fgf3* was up-regulated, as observed in Figure 3-21. Cell counting was done using the Volocity program and the data was processed in Excel by P. Jankun. The Figure 3-22 shows an increase in the detection of phosphorylated histone H3 protein in fish where *fgf3* was up-regulated, in comparison with the siblings (lack of *fgf3* up-regulation).

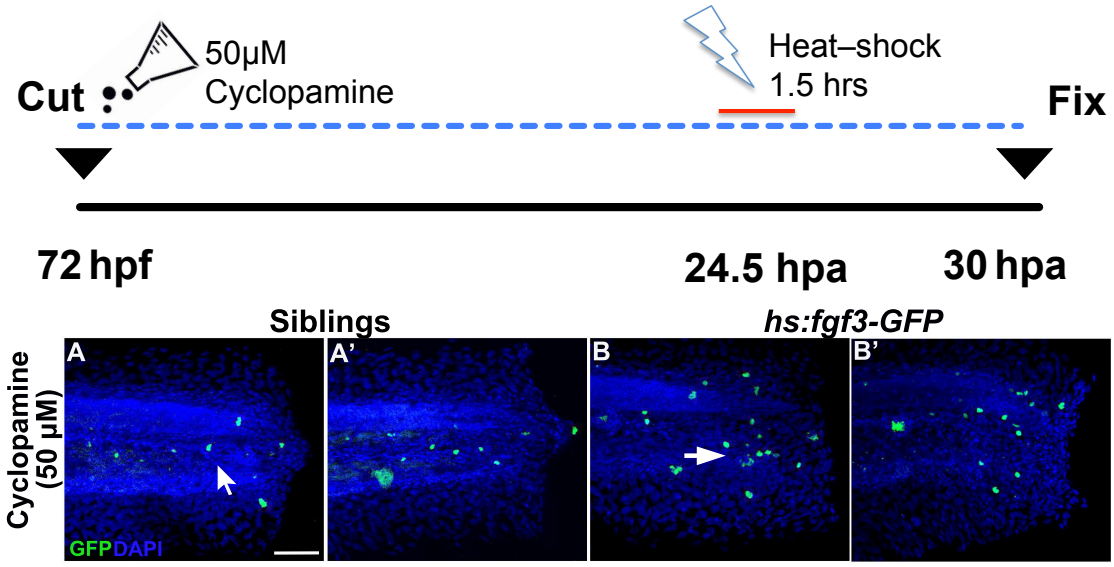

Figure 3-21: Hh signalling regulates cell proliferation through FGF signalling during tail regeneration. *hs:fgf3* transgenic fish and siblings were tail-amputated at 72hpf and continuously treated with 50μM cyclopamine. At 24.5hpf the larvae were heat-shocked for 1.5 hours and then fixed at 30hpa. Immunodetection of PH3 protein was performed to detect cell proliferation (white arrows). **A, A':** Siblings that were not *fgf3* up-regulated (n=10). **B, B':** *hs:fgf3* transgenic fish, (*fgf3* up-regulation) present a higher number of proliferative cells than siblings (n=40). N=3. Pictures taken from two representative embryos were chosen per condition. Scale bar 100μm.

This finding suggests that Hh signalling regulates cell proliferation during tail regeneration through FGF signalling. In order to further analyse this outcome it will be necessary to down-regulate FGF signalling during tail regeneration while Hh is up-regulated.

The next question was to know whether the relationship between Hh signalling and FGF signalling only affected cell proliferation or if it was also involved in regeneration gene expression. To answer this question I tested the expression of the regeneration marker gene *raldh2* where I down-regulated the expression of this gene by continuously administrating cyclopamine. I then up-regulated FGF by using the hs: *fgf3* transgenic line. The ISH detection showed that there was still a lack of *raldh2* expression even though *fgf3* was up-regulated (Figure 3-23). This final outcome suggests that Hh regulates cell proliferation through *fgf* signalling. However, the same regulation is not observed for the *ralhd2* regeneration marker gene.

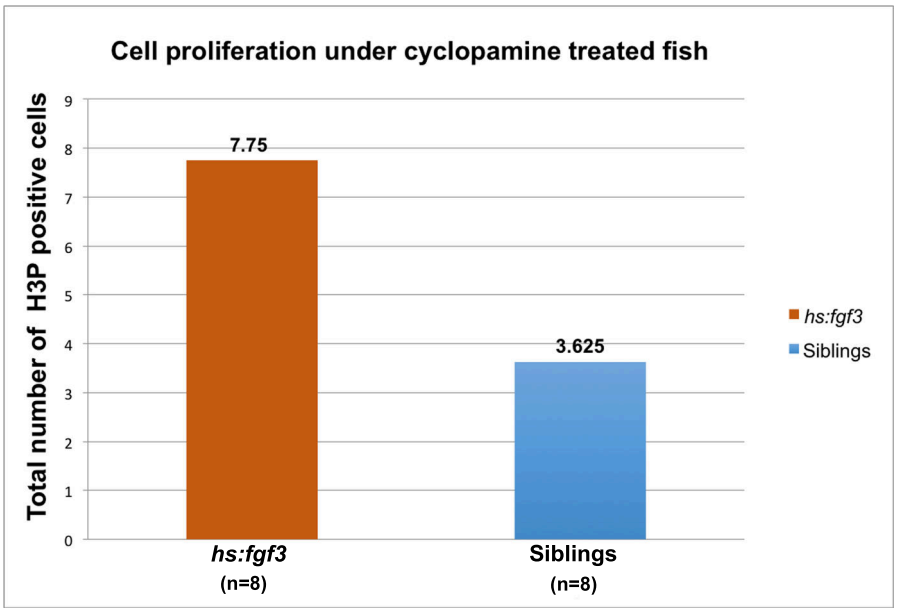

Figure 3-22: Cyclopamine-treated fish show a higher level of proliferation when *fgf3* is up-regulated. Quantification of the cells expressing PH3 is done with the Volocity software. The mean comparison (number at the top of each bar) of the total number of PH3 positive cells is done between *hs:fgf3* fish and the siblings. p-value 0.04, Un paired T-test. (quantification made by P. Jankun).

3.4.4 *raldh2* signalling down-regulation by SU5402 cannot be rescued by Wnt signalling up-regulation during tail regeneration

The previous findings helped to make a better interpretation of the relationship between Hh and Wnt with FGF signalling during regeneration. It seems that Hh regulates some components of the FGF pathway, such as *fgf10a*, while Wnt signalling is able to regulate others, such as *pea3*. In order to further analyse the relationship between Wnt and FGF pathways during tail regeneration, I decided to make an epistasis analysis and observe the expression of the *raldh2* regeneration marker gene. I induced tail amputation in a group of 40 fish by cutting their tails at 72hpf. I divided the group into 4 and each group was treated under different conditions.

The first group was the Control (only treated with DMSO). The second group was treated with SU5402 to down-regulate FGF signalling. The third group was treated with Gsk3 inh to up-regulate Wnt signalling. For the fourth group Wnt was up-regulated at the same time as FGF signalling was down-regulated by administrating both chemical compounds SU5402 and Gsk3 inh at the same time. To determine the outcome, the expression of *raldh2* from fish treated with both compounds was compared with the other three groups. For this I will suggest three possibilities. First option, if *raldh2* expression is similar to the control fish

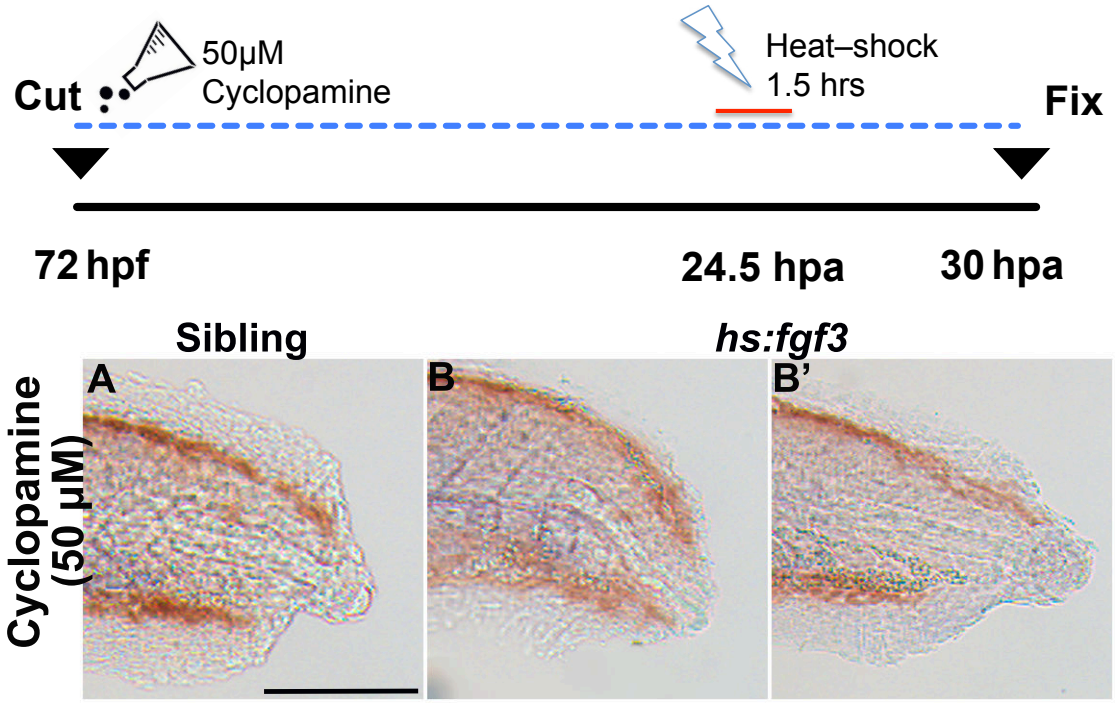

Figure 3-23: *raldh2* down-regulation by cyclopamine cannot be up-regulated by FGF signalling up regulation. Fish were amputated at 72hpf and then continuously treated with 50μM cyclopamine. Heat-shock was for 1.5 hours at 39°C, 6 hours before fixation. Fixation was at 30hpa. *raldh2* expression is detected by ISH. **A:** Sibling fish in which no *raldh2* expression is observed (n=10). *B, B': hs:fgf3* transgenic fish, no *raldh2* expression is observed (n=40). N=1. Two representative embryos were chosen per condition. Scale bar 100 μm.

raldh2 expression this would suggest that both pathways (Wnt and FGF) act in a parallel way to regulate *raldh2* expression during tail regeneration. The second option would be that *raldh2* expression from Group 4 is similar to the second group (FGF down-regulated), which would suggest that FGF acts downstream of Wnt signalling. Finally it is also possible that *raldh2* expression from the control group compared with the third group (Wnt up-regulaton) would suggest that Wnt signalling is downstream of FGF signalling to regulate *raldh2* expression during tail regeneration.

Figure 3-24 shows that both up-regulation of Wnt signalling and down-regulation of FGF altered the expression of *raldh2*, when done separately. However, when both pathways were altered at the same time the *raldh2* expression was similar to the fish treated only with SU5402 (FGF down-regulation). This result suggests that Wnt signalling cannot rescue the action of SU5402 over the expression of *raldh2* during tail regeneration, suggesting that FGF signalling is downstream of Wnt signalling during tail regeneration. In order to have a better conclusion it will be necessary to up-regulate FGF signalling and down-regulate Wnt signalling and then observe how the expression of *raldh2* during tail regeneration is affected.

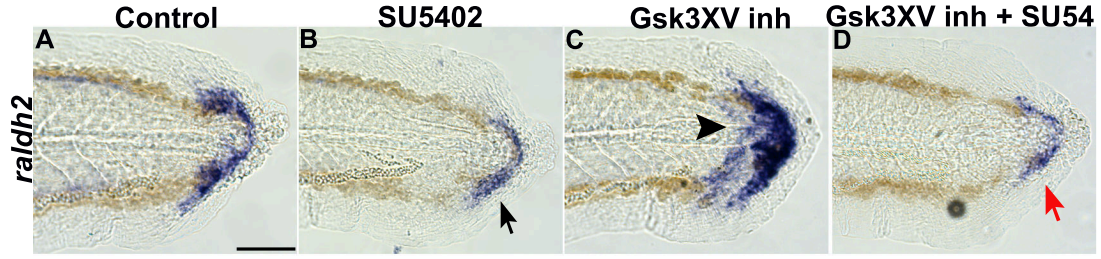

Figure 3-24: *raldh2* down-regulation by SU5402 cannot be up-regulated by up regulating Wntβ catenin signalling during tail regeneration. Tail regeneration is induced by amputation at 72 hpf, fish were fixed at 48 hpa and *raldh2* expression was detected by ISH. **A:** Control(DMSO) (n=10). **B:** 15 μM SU5402-treated fish 4 hours before fixation show lower *raldh2* expression than control (black arrow) (n=10). **C:** 5 μM Gsk3XV inh-treated fish 6 hours before fixation showed a higher level of expression than the control group (black arrow)(n=10). **D:** SU5402 and Gsk3XV inh-treated fish showed a similar level of expression as SU5402-treated fish (red arrow)(n=10).N=1. One representative embryo was chosen per picture. Scale bar 100 μm.

3.5 Discussion

Our larval zebrafish tail regeneration model displays the morphological and genetic characteristics of epimorphic regeneration. The model was established during my Master's degree where tail regeneration took 4 days (Figure 3-1). According to Laforest et al. (1998) and Heude et al. (2014)), *msxc*, *dlx5a* and *raldh2* markers are normally expressed during tail development. Nonetheless, at 3dpf, expression is no longer detected until regeneration is initiated by tail amputation. The expression

of the regeneration markers therefore is due to regeneration and not tail development (Figure 3-3). Regarding the molecular pathways involved during epimorphic regeneration, the outcomes of my Master's studies suggested that Hh signalling regulates regeneration marker genes in an indirect way (Figure 3-5) while Wnt signalling does so in a more direct way (Figure 3-13). The present chapter is the continuation of the molecular pathway dissection, the main goal of which is to study the relationship and function of Hh, Wnt, FGF and RA during larval tail regeneration.

3.5.1 Hh signalling is upstream of Wnt signalling during larva zebrafish tail regeneration.

After observing that the continuous down-regulation of Hh signalling managed to down-regulate regeneration marker genes, I decided to study this pathway by first observing the *ihh* ligand expression time course made by P. Jankun. The earliest expression reported is at 6hpa (Figure 3-8), which is earlier than Wnt readout *tcf7* and the ligand *wnt10b* where the earliest expression is observed at 18hpa (Figure 3-14). Therefore it could be that Hh expression may appear earlier than Wnt and FGF signalling. Therefore, manipulation of Wnt, FGF signallings and RA was done and then the expression of *ptc1* was observed. As we can see in Figure 3-9, the expression of *ptc1* was maintained under the manipulation of Wnt signaling, FGF and RA ectopic administration. These findings are in contrast to the absence of *ptc1* expression in the regenerated adult zebrafish tail after administration of 1μM of RA for 4 hours, reported by Laforest et al. (1998)). Wehner et al. (2014) reported a lack in *ptc2* gene expression (*ptc2* and *ptc1* are dependent upon Hh signalling with no differences in their regulations by Hh components (Lewis et al. 1999)) during adult zebrafish tail regeneration, where down-regulation of Wnt signalling was induced by heat-shocking the *hs:dkk1-GFP* line at 37°C for 1 hour.

Finally, the reports for the relationship between Hh signalling and FGF during tail regeneration in adult zebrafish made by Poss et al. (2000a)) were also different to my outcomes. Poss et al. (2000a)) down-regulated FGF signalling by administrating 17μM of SU5402 compound for 24 hourrs at 48hpa, and used *shh* (whose receptor is *ptc1* (Laforest et al. 1998) to measure Hh signalling activity. Here SU5402 treatment totally suppressed the expression of *shh*. The difference in *ptc 1/2* gene expression in adult and larval tail regeneration suggests Hh signalling is upstream of RA, Wnt and FGF signalling during larval tail regeneration, while in adult tail regeneration Hh signalling is downstream.

Wenger et al. (2014) reports that Wnt signalling is upstream of Hh during adult tail regeneration, which is different from Yazawa et al. (2009) who notes that the regulation of the anterior-posterior polarity formation during tissue planarian regeneration is due to Hh signalling acting through the Wnt pathway. To

study further the relation between Hh and Wnt signalling in larval zebrafish tail regeneration, the expression of Wnt signalling readouts *lef1* and *tcf7* was assessed during larval tail regeneration under continuous down-regulation of Hh signalling. Figure 3-15 shows that continuous down-regulation of Hh signalling leads to down-regulation of *lef1* and *tcf7*. This outcome, along with the finding shown in Figure 3-9, where *ptc1* expression is not dramatically altered by Wnt signalling manipulation, suggest that Wnt signalling is dependent on Hh during larval tail regeneration. To analyse in more detail the relationship between Hh and Wnt signalling during larval tail regeneration, an epistasis analysis was performed. The down-regulation of the regeneration marker genes by cyclopamine was rescued by a short up-regulation of Wnt signalling using the Gsk3XV inh (Figure 3-16). A very similar experiment done by Bhairab et al. (2012) involving salamander limb regeneration shows that up-regulation of Wnt signalling overrides the effects of cyclopamine. All together these results and reports suggest a model where Hh signalling governs the action of Wnt during larval zebrafish tail regeneration. These reports also show that the hierarchical relationships between Hh and Wnt signalling during tissue regeneration are different between regenerative models.

The differences between my findings and the literature made me think that either the age (larval instead of adult) or amputation area (tail instead of fin fold) may lead to the difference in the interaction of the molecular pathways. Therefore I decided to study whether Hh signalling operates in the same way as in tail regeneration, but this time with regards to fin fold regeneration. *ptc1* expression was not as obvious during fin fold regeneration as it was in the tail (Figure 3-12). In addition, down-regulation of Hh signalling did not prevent regeneration in the fin fold in the same way that it did in the tail (Figures 3-10 and 3-11). One reason for this difference is that during tail amputation the notochord is more exposed, which is a centre of Hh protein secretion (Stemple 2005). Therefore Hh signalling could have a faster action on the injured tissue during tail amputation in comparison with fin fold amputation.

3.5.2 Hedgehog and Wnt regulates some FGF markers in both indirect and direct fashion

To follow the analysis of pathway communication, Figures 3-17 and 3-19 show the relationship between Hh signalling and the expression of the FGF readout *pea3*. The continuous and pulse down-regulation of Hh signalling affects *pea3* expression, which is also sensitive to up-regulation and down-regulation of Wnt signaling, as shown in Figure 3-18. These outcomes suggest that both pathways regulate *pea3* in a parallel fashion; however, these findings disagree with Lee et al. (2009). The study by Lee in adult caudal fin regeneration shows that *pea3* (FGF readout) actually regulates *wnt5b* (Non-canonical Wnt ligand) and that FGF manages to

regulate Hh signalling but without involving the *pea3* marker. The difference in the methodology used as well as the regeneration model could explain the difference in the results.

The expression of *fgf10a* was also studied in the same manner as *pea3*, where Hh and Wnt were manipulated. The result, however, was different. As we can observe in Figure 3-19, pulse down-regulation of Hh signalling completely inhibits *fgf10a* expression, which is not observed when Wnt signalling is down-regulated by a short period of time (Figure 3-18). These findings suggest that Hh signalling may regulate *fgf10a* in a more direct way than Wnt signalling. Comparing this finding with the literature, Lee et al. (2005) suggests a model where FGF signalling regulates *shh* during blastema proliferation. This report, however, shows the opposite of my findings that suggest Hh pathway regulates FGF during regeneration. The difference may be due to the model employed, in this case adult tail regeneration.

3.5.3 Cell proliferation was rescued once *fgf3* was up-regulated

The deadelus *fgf10-/-* mutant shows a lower level of proliferation than a wild type zebrafish (Unpublished results from P. Jankun). The same result can be observed after continuous down-regulation of Hh signalling (dissertation project) (Figure 3-20), and during axolotl regeneration (Schnapp et al. 2005). These previous reports along with the discussion in the last section suggest a relationship between Hh and *fgf10a*, where Hh is upstream of *fgf10a*. To further study this pathway relationship, I tested whether the down-regulation in proliferation could be rescued by FGF up-regulation. Figure 3-21 and 3-22 depict an increase of proliferation in the *hs:fgf3*, transgenic line, under a continuous cyclopamine exposure. This relationship seems to be restricted to cell proliferation since up-regulation of *fgf3* in cyclopamine-treated fish does not rescue the expression of the regeneration marker gene *raldh2* (Figure 3-23). To reach a better interpretation of these pathways' relationship (Hh being upstream of FGF signalling) it will be necessary to up-regulate Hh signalling in tail-amputated fish where FGF is being down-regulated and observe cell proliferation as well as the expression of *raldh2*. I would expect to see that the up-regulation of Hh would not affect the down-regulation of cell proliferation by FGF down-regulation.

3.5.4 FGF signalling affects *raldh2* expression during tail regeneration in a more direct way than Wnt.

Regulation by FGF and Wnt signalling over regeneration markers has been reported in adult tail and fin fold regeneration (Kawakami et al. 2004;Wehner et al. 2014). Those findings agree with Figure 3-24, where the regeneration marker *raldh2* expression is affected by FGF down-regulation and Wnt up-regulation.

Mathew et al. (2009) suggests a pathway hierarchy where Wnt regulates RA production through FGF signalling. To tests this hypothesis an epistasis analysis was performed. Figure 3-24 shows that the up-regulation of Wnt signalling is unable to rescue the expression of *raldh2* down-regulated by SU5402 component. A complementary experiment where FGF is up-regulated while Wnt down-regulated is necessary to confirm the hypothesis.

3.6 Conclusions

The main core of my research was to analyse the relationship between Hh, Wnt and FGF developmental pathways and regeneration marker genes. Therefore, I hypothesised that *Hedgehog signalling leads tail regeneration by regulating Wnt and FGF signalling in larval zebrafish.* The data in this chapter has consistently confirmed my hypothesis. FGF and Wnt signalling seem to be downstream of Hh during regeneration, since *ptc1* expression (Hh readout) is not affected by the changes in expression of FGF and Wnt signalling (Figure 3-9). Furthermore, Wnt signalling can rescue the expression of regeneration markers affected by cyclopamine (Figure 3-16). Hh signalling seems to regulate FGF markers since changes in the expression of Hh affect *pea3* and *fgf10a* expression (FGF markers) (Figures 3-17 and 3-19). Finally Hh signalling appears to regulate cell proliferation during regeneration through *fgf3* expression (Figures 3-21 and 3-22).

The relationship between Wnt and FGF was also assessed but the results are less conclusive. Firstly, Wnt signalling seems to regulate *pea3* by changing its expression, however, changes in the expression of *fgf10a* were not always observed by Wnt signalling modification, so this last relationship was difficult to interpret (Figure 3-18). Secondly, *raldh2* regeneration marker appears to be regulated by FGF and Wnt separately since administration of SU5402 and Gsk3XV inh caused changes in the expression of *raldh2* (down-regulation and up-regulation respectively). Wnt signalling up-regulation at the same time as FGF down-regulation could not rescue the expression of *raldh2* using the SU5402 compound (Figure 3-24). Figure 3-25 shows a model that explains the molecular interaction that happened during tail regeneration. Further studies need to be done in order to understand whether there is a hierarchy or cross talk between FGF and Wnt signalling during tail regeneration.

It is important to mention that pathway activity was measured by using IHS, where a general idea could be obtained regarding the pathways involved during larval tail regeneration. However, the ISH technique is not optimal since it cannot be quantified. The use of real-time PCR (Polymerase Chain Reaction) enables the quantification of gene expression and detection of small differences between samples (Wong et al. 2005). However the equipment and reagents tend to be expensive and also an understanding and standardisation of the process is vital to

obtain reliable results. Due to the costs of this technique, it should be reserved to test an idea that has already been measured with a less expensive method such as ISH. I would also suggest the use of transgenic lines where the gene of interest (signalling readout, ligand or regeneration marker gene) drives the expression of a fluorescent protein in order to measure the activity of molecular signalling during tail regeneration. The intensity of the fluorescent protein affected during tail regeneration or pathway manipulation could be measured by using the Image J software.

The present results can help us to better understand the dynamics of the molecular pathways that occur during tail regeneration, how pathways communicate, and finally how they control the expression of regeneration markers in order to trigger regeneration. As already mentioned, tissue regeneration also exists in humans but is quite limited in comparison with zebrafish. Therefore, the zebrafish model could help us to unravel epimorphic regeneration with the aim of using this knowledge to assist human tissue regeneration in the future.

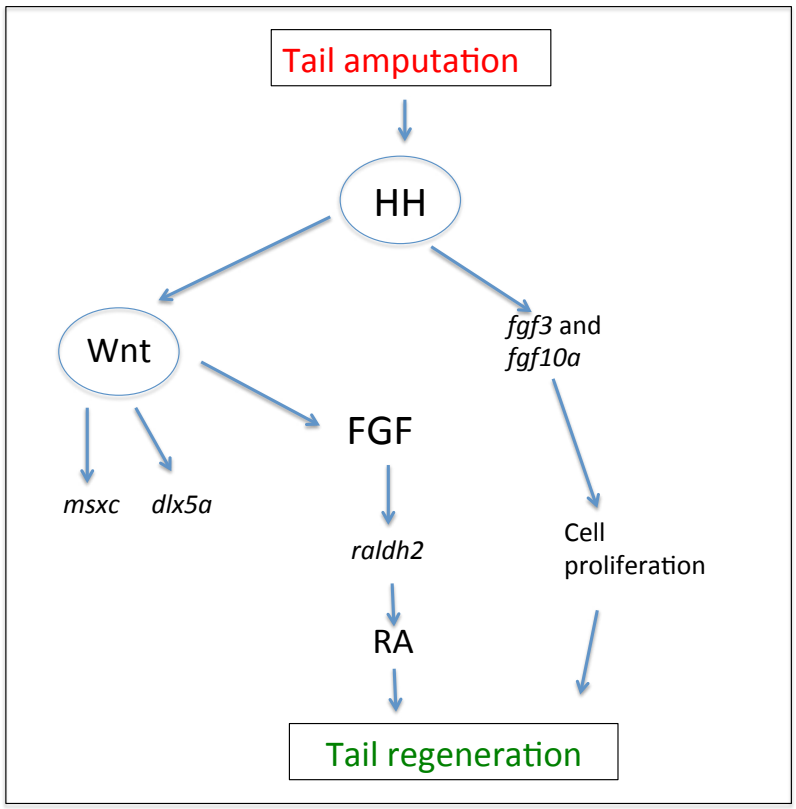

Figure 3-25: Molecular interaction during tail regeneration. In this model Hh signalling initiates tail regeneration by activating Wnt signalling, which in turn activates regeneration marker genes. Cell regeneration is also regulated by Hh through the FGF genes. Wnt signalling regulates also other FGF genes.

Chapter 4

Dissection of the pathways involved during zebrafish tail development.

4.1 Introduction

The molecular pathways that are crucially involved during zebrafish tail development have been largely described to be Hh, Wnt and FGF signalling. Hh signalling, for example, has been found to be important during anterior posterior-patterning, cell proliferation, and slow muscle fibre development of zebrafish (Schauerte et al. 1998;Barresi et al. 2000;Neumann et al. 1999). Wnt signalling has found to be important during somite formation and mesoderm maintenance; lack of this pathway therefore prevents tail formation (Pyati et al. 2006;Martin et al. 2008;Row et al. 2011). FGF signalling has been identified as necessary for anterior posterior and dorso-ventral patterning as well as axis elongation, mesoderm formation and somatogenesis. Absence of the FGF pathway truncates tail formation (Griffin et al. 1998; Fürthauer et al. 2004 and Griffin et al. 2003; Stulberg et al. 2012).

These pathways are re-expressed during tail regeneration (as described in Chapter 3). The fact that developmental pathways are expressed during regeneration suggests that tail regeneration may recapitulate tail embryonic formation. A good example of this is limb regeneration, which recapitulates *shh* expression observed during normal development of limb buds in newts (Bhairab et al. 2012). Given this example I was wondering whether these pathways communicate, and are expressed in a similar manner during tail bud formation as in tail development. I therefore formulate this hypothesis: *Hh, Wnt and FGF signalling have the same roles during both tail development and regeneration.* With this I am assuming that the lack of either Wnt or Hh signalling is enough to stop tail development (Shimizu et al. 2005), as happened during tail formation (Chapter 3), and that the regeneration molecular pathway will be similar to Figure 3-25.

Chapter 4 describes the molecular dissection of Hh, Wnt and FGF during tail

development in order to compare the molecular pathway roles versus tail regeneration. With this study I would also analyse the possible differences in both the tail regeneration and development processes. The use of the zebrafish larva model has generated question of whether tail regeneration is actually taking place because of regenerative mechanisms, or if it is just the continuation of normal development. For the realisation of the molecular analysis in zebrafish tail bud, down-regulation of the Hh, Wnt and FGF was performed before tail bud formation. The regulation of these pathways was analysed by morphology and gene expression in the tail bud.

The results shown in this chapter suggest a distinct role for Hh signalling during tail development and regeneration, regarding tail morphology and gene expression. Wnt signalling seems to have an analogous role during tail development and regeneration regarding tail morphology. Finally FGF signalling seems to have also a distinct role for the expression of the *raldh2* gene; when FGF is down-regulated *raldh2* seems to be up-regulated during tail development and down-regulated during tail regeneration.

Note: The number of experimental repetition or trials will be represented by the letter "N", while the number of total samples will be represented by letter "n".

4.2 Molecular dissection during zebrafish tail development.

4.2.1 Hedgehog has a crucial role during tail regeneration that is not observed during tail development.

As mentioned in the Section 3.2.1, questions have arisen about the use of larvae as a model for tail regeneration. The fact that the fish is still in development implies that regeneration may not actually be happening but that normal development is taking place. Akimenko et al. (1994); Akimenko et al. (1995); Kawakami et al. (2004) have shown that the regeneration marker genes *msxc* and *dlx5a* are expressed during zebrafish larval tail regeneration and early development. However, when the zebrafish embryos are aged 3 days post fertilisation (dpf) their expression can not be detected by in situ hybridisation (ISH) in the tail. However, when regeneration is induced by tail amputation, *dlx5a* and *msxc* can be detected again. Section 3.2.1 shows evidence of the re-expression of regeneration marker genes during regeneration (Figure 3-3).

However, the question of how similar tail development and regeneration are regarding the activation of developmental molecular paths remains to be answered. To answer this question I decided to analyse the role of FGF, Wnt and Hh during zebrafish tail development. The idea was to assess whether each pathway acts

in a similar way in both regeneration and tail development or whether they have independent roles during each process.

The first part of the experiment involved a morphological analysis of the Hh signalling during tail bud formation. My first approach was to use the *slow muscle omitted* mutant (*smu*), which lacks Hh signalling. The smu^{b577} mutant carries a mutation in the smu^{b577} gene that affects the zebrafish *smoothened*. *smoothened* has been described as necessary for Hh signaling (Chen et al. 2001). At 1dpf tail development was observed in the *smu* mutant, although with defective somites. However, at 2dpf the tail had a curly shape, twisted so tight that made tail amputation and imaging difficult to perform.

As an alternative to studying Hh signalling I decided to use the small molecular compound 10μM LDE 225 (smoothened inhibitor) that was reported to effectively block Hh signalling (Kelleher et al. 2010) by Dr. Stone Elworthy from "Ingham Lab". To assess zebrafish tail development during down-regulation of Hh signalling I treated one group of fish with 20μM cyclopamine, a second group with LDE 225, and the control group was treated with 0.6% ethanol. The idea was to target Hh signalling at the 14 somite stage when tail extension starts (Kimmel et al. 1995). For this reason I treated the embryos at 75% epiboly (8 hours earlier than bud stage) to give enough time for the compounds to down-regulate Hh signalling (it has been reported from a previous student at "Roehl's Lab" that 8 hours is enough time for cyclopamine to effectively down-regulate Hh signalling). Along with this treatment I down-regulated Hh signalling during tail regeneration by cutting the tail of 3dpf larvae and treating the zebrafish larvae with 20μM cyclopamine, 10μM LDE and 0.6% ethanol (control) for 4 days. The cyclopamine and LDE225 concentrations had been shown to effectively block tail regeneration, which is the reason why the same concentrations were used to assess tail development.

In Figure 4-1 we can observe 24 hours post-fertilisation embryos where Hh signalling was down-regulated. The panel shows that embryos treated with LDE 225 had fully formed and extended tails, similar to the control embryos, while the embryos treated with cyclopamine had extended tails, though the morphology does not resemble that of the control embryo (defective somites and notochord). This result suggests that the disruption of Hh signalling during development does not affect the tail development as severely as it does with tail regeneration, since the tail still manages to form and extend.

The next step was to make a molecular dissection by observing the expression of the *raldh2* regeneration marker and *tcf7* Wnt readout during Hh down-regulation. The expression of these markers was abolished by continuous cyclopamine administration (Figures 3-5 and 3-15). Figure 4-2 shows that Hh down-regulation by 20μM cyclopamine administration did not affect *raldh2* and *tcf7* expression in tail bud formation as it did with tail regeneration. The outcomes of the morphology and molecular assessment of tail bud formation under the manipulation of Hh sig-

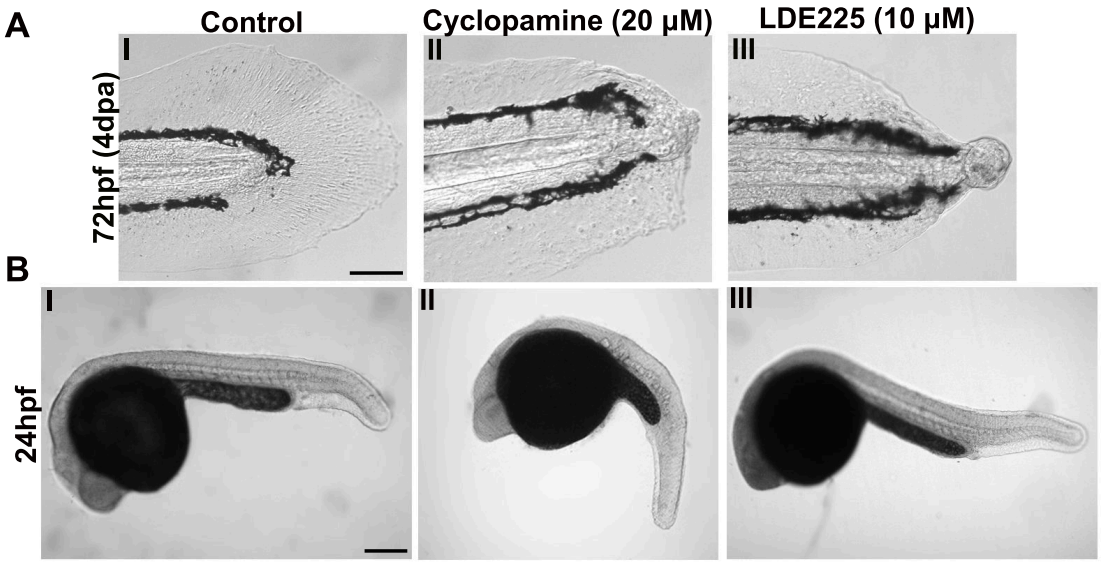

Figure 4-1: Hh signalling has a different role in tail regeneration than in tail development. Fish embryos are treated with 20 μM of cyclopamine and 10 μM of LDE and the effects are analysed by morphology. **A** (n=10) and **B I:** Vehicle-treated embryos (n=20), in which normal tail development and regeneration is observed. **A II** (n=10) and **B II:** (n=20) Cyclopamine-treated embryos (continuously), in which tail extension is observed (with some morphological defects) but not regeneration. **A III** (n=10) and **B III:** (n=20) LDE 225-treated embryos (continuously), in which normal tail development is observed but not regeneration. Number of trials N=2. One representative embryo was chosen per picture. Scale bar 100 μm.

nalling suggest that Hh signalling has a different role during tail development than it does in tail regeneration.

4.2.2 Tail development and regeneration are regulated by Wnt signalling

As described previously, Wnt signalling has a role in tail regeneration, so I decided to compare the regulation of this pathway during tail bud formation. As described in the Method section (Section 2.7) I used the *hs:dkk1-GFP* line to down-regulate Wnt signalling during tail formation. The morphological analysis shown in Figure 4-3 depicts an embryo 40hpf that did not manage to complete tail extension as the sibling did. As a point of comparison, Figure 4-3 shows an unregenerated tail where Wnt signalling was down-regulated. This result suggests that Wnt signalling has a similar role during tail development as it does during regeneration (Shimizu et al. 2005; Bajard et al. 2014). The molecular analysis is still pending in order to reach a better conclusion.

4.2.3 Canonical Wnt signalling does not mediate the transition from FGF to RA as in chicken body axis.

As mentioned in the previous section, Wnt signalling during tail bud formation still needs to be analysed at a molecular level. However, it has been shown by morphological analysis that the Wnt pathway is crucial during tail development.

Regarding the molecular role of Wnt signalling during tail formation, I would like to answer two questions: 1) What is the role of Wnt signalling in relation to regeneration markers such as *raldh2* during tail bud formation? 2) Is there any communication between Wnt signalling and FGF during tail bud formation, as there is during regeneration?

To answer the first question I decided to test the expression of *raldh2* under Wnt signalling manipulation during tail bud formation. As seen in Figure 4-4, there is not an obvious change in the expression of *raldh2* with the up-regulation of Wnt signalling. The report made by Olivera-Martinez et al. (2007)), however, mentions that during the development of chicken axis Wnt can regulate *raldh2* expression as long as FGF signalling is down-regulated. The replication of the experiment done in chickens will allow me to answer the two questions mentioned above.

To replicate Olivera-Martinez et al. (2007)'s experiment I decided to treat the fish under 4 different conditions. The first condition, Control, was DMSO 0.1%; the second was down-regulation of FGF (15μM SU5402); the third was up-regulation of Wnt/β-catenin (5μM Gsk3 inh); and the fourth was down-regulation

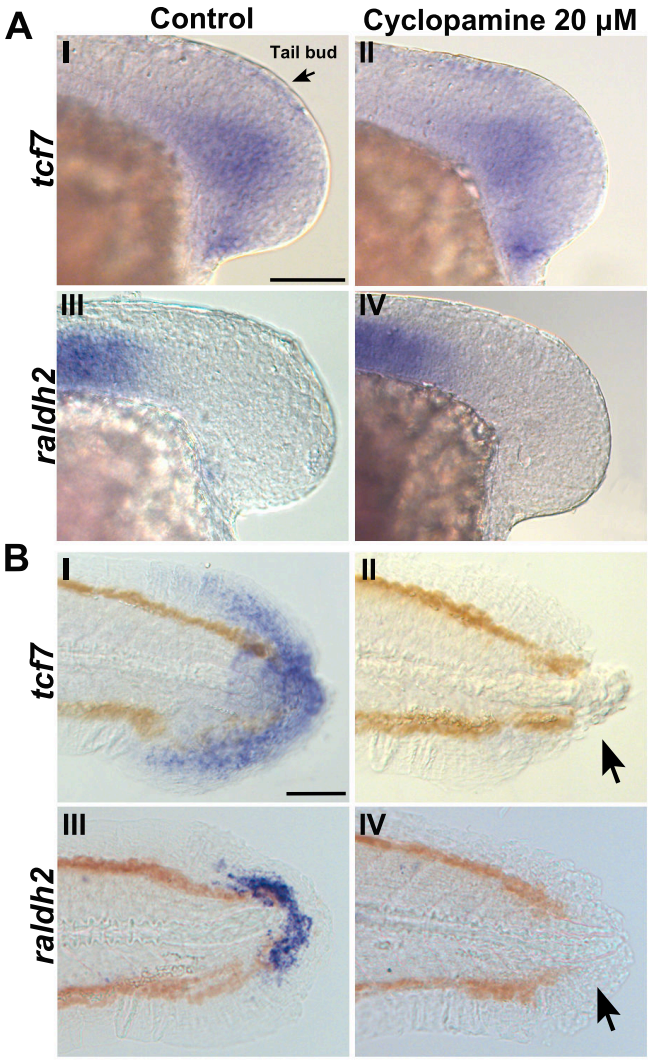

Figure 4-2: Down-regulation effects the expression of *tcf7* and *raldh2* genes during regeneration but not during tail development. Fish embryos are treated with 20 μM of cyclopamine. *tcf7* and *raldh2* expression is detected by in ISH. **A I** (n=20), **A III** (N=20),**B I** (n=20) and **B III:** (n=20) Control vehicle-treated fish present normal expression of *tcf7* and *raldh2* during normal tail development and regeneration. **A II** (n=20), **A IV** (n=20) Cyclopamine-treated fish from 75% epiboly to 16 somites present normal expression of *tcf7* and *raldh2*, when compared with controls. **B II** (n=20) and **B IV** (n=20) Fish treated with cyclopamine from 0-48hpa do not show expression of the *tcf7* and *raldh2* genes during regeneration (black arrows) when compared with controls. N=1. One representative embryo was chosen per picture. Scale bar 100 μm.

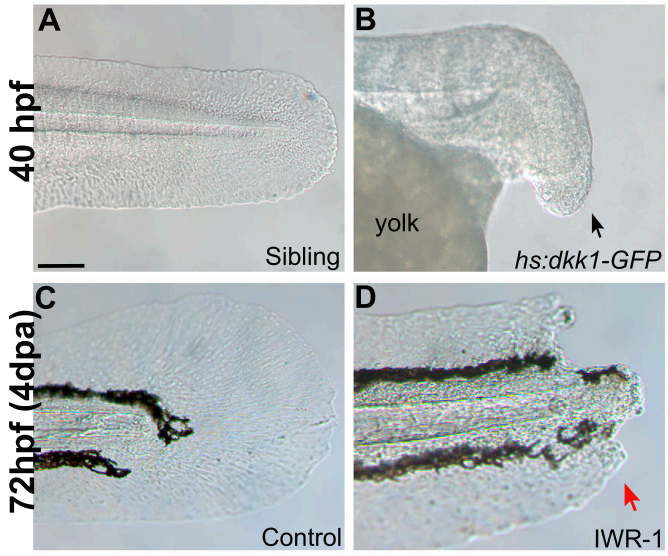

Figure 4-3: Wnt signalling has a similar role in tail regeneration and tail development. Fish embryos are treated with 10 μM of IWR-1 during regeneration for 4 days. *dkk1* embryos are heat shocked for 1 and a half hours at 39 °C from 75% epiboly and then allowed to continue with normal development until 40 hpa (fish were fixed at this stage). **A** (n=10) and **B:** (n=30) *hs:dkk1* line. Wnt down-regulated embryo did not complete tail formation (black arrow) in contrast to the control. **C** (n=10) and **D:** (n=10) Fish treated continuously with IWR-1 during regeneration did not complete tail outgrowth (red arrow), which was complete in the control. N=1. One representative embryo was chosen per picture. Scale bar 100 μm.

of FGF and up-regulation of Wnt at the same time (15μM SU5402 and 5μM Gsk3 inh) (these concentrations had previously shown to effectively manipulate FGF and Wnt pathway by modifying the expression of the pathways readouts). Fish embryos were treated at 75% epiboly to give enough time for the compounds to act on the pathway of interest, then at the 16 S (Somite) stage (tail extension starts) the embryos were fixed and ISH was performed to detect *raldh2* expression. As we can observe in Figure 4-4, there is no obvious change in the expression of *raldh2* between the four conditions.

I decided to make a more detailed image analysis, for which I measured the total area where *raldh2* is expressed in the tail bud. The measurement was done using the colour threshold option of the Image J program, and the parameters to measure the area of raldh2 expression were the same for all the pictures. In order to avoid subjectivity as much as possible I asked a colleague for his opinion of the parameters I was using to measure the area, and he agreed with my decision. Once I obtained the data I performed a one way ANOVA with pairwise comparison. The idea of the statistical analysis was to see whether there was any difference between the four molecule treatments: FGF down-regulation, Wnt up-regulation, both pathways manipulated at the same time, and the control group.

As we can observe in Figure 4-5, the p value comparison between FGF down-regulation and Control shows a difference between the samples' means. In the case of Group 4 (where FGF and Wnt manipulation were manipulated at the same time) there is not a significant difference in comparison with the control group. The present outcome suggests that down-regulation of FGF using SU5402 has an impact on the expression of *raldh2* expression (up-regulation) during tail bud formation. However, up-regulation of Wnt/β-catenin and down-regulation of FGF at the same time during tail development had no significant impact on *raldh2* expression. This method is still open to subjectivity, so I would suggest that the results are corroborated using a real-time PCR to quantify the expression of *raldh2* under the four different conditions previously described.

4.2.4 Development mechanisms are reexpressed during tail regeneration.

The final part of tail regeneration is the formation of the lost tissue (Kawakami 2010). This process involves the re-expression of early tissue markers such as *myod*. *myod* is a marker that is expressed during myogenesis (Weinberg et al. 1996) and decreases during late tail development. I decided therefore to make a regeneration time course analysis using 3dpf embryos. The reason for this was to observe the time points when *myod* is reexpressed during regeneration. As seen in Figure 4-6, *myod* expression is not existent at 24hpa, but starts to be re-expressed at 48hpa. The highest level of expression is at 72hpa. After obtaining the time

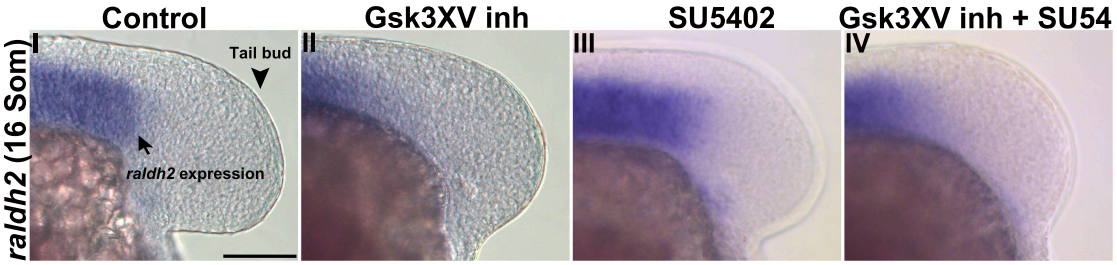

Figure 4-4: Manipulation of FGF and Wnt signalling at the same time does not affect *raldh2* expression during tail development. *raldh2* expression is detected by ISH in fish 16 somite treated with different chemicals. **I:** Control (n=20) **II:** 5 μM SU5402-treated fish from 75% epiboly to 16 somites (n=20). **III:** 5 μM Gsk3XV inh from 75% epiboly to 16 somites (n=20). **IV:** 5 μM Gsk3XV inh and 5 μM-treated fish from 75% epiboly to 16 somites (n=20). N=1. One representative embryo was chosen per condition. Scale bar 100 μm.

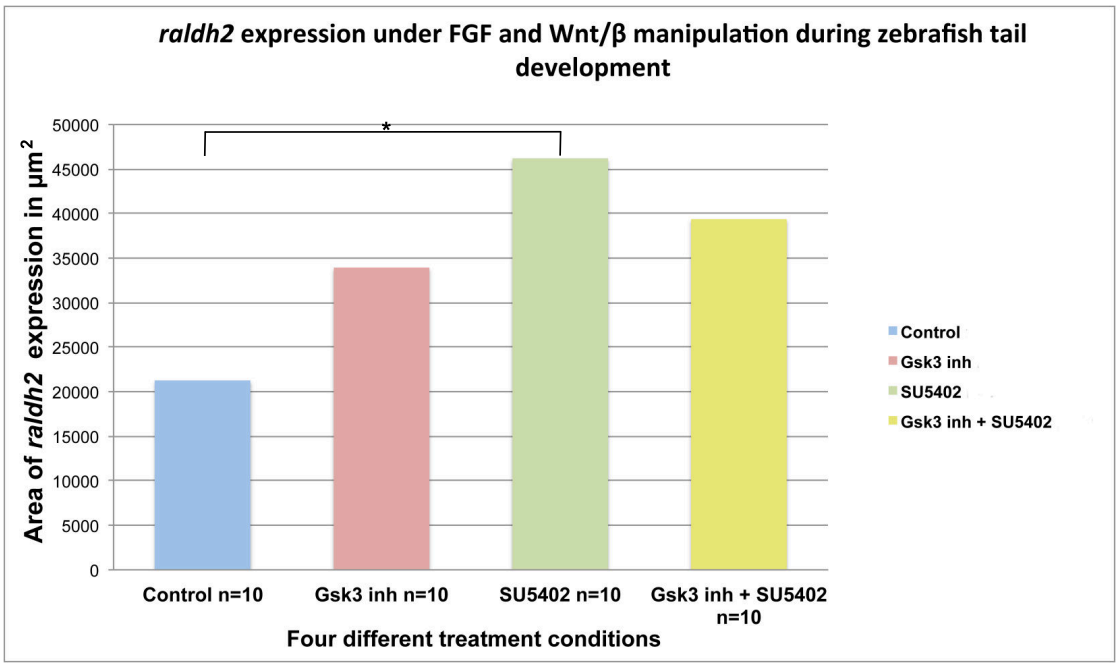

Figure 4-5: Manipulation of FGF and Wnt signalling at the same time does not affects *raldh2* expression during tail development, but FGF does. Comparison of the total area of *raldh2* expression is done between groups using the one way ANOVA test with pairwise comparison. Significant differences are found between control group and the SU5402 treated fish at $p<0.01$ (asterisk). Lowry 2015.

course expression for *myod* I decided to make a comparison with the expression of the regeneration marker genes *raldh2* and *dlx5a* using the same time points. This was to determine whether *myod* expression takes place at the same time as the regeneration marker genes. As seen in Figure 4-6, it seems that *myod* is expressed after *raldh2* and *dlx5a*. Also the highest levels of expression for *myod* seem to take place at 72hpf, a later time point than the highest level of expression of *ralhd2* and *dlx5a*, which were at 48hpa. This result suggests that regeneration marker genes *raldh2*, *dlx5a* and *myod* are expressed sequentially during tail regeneration.

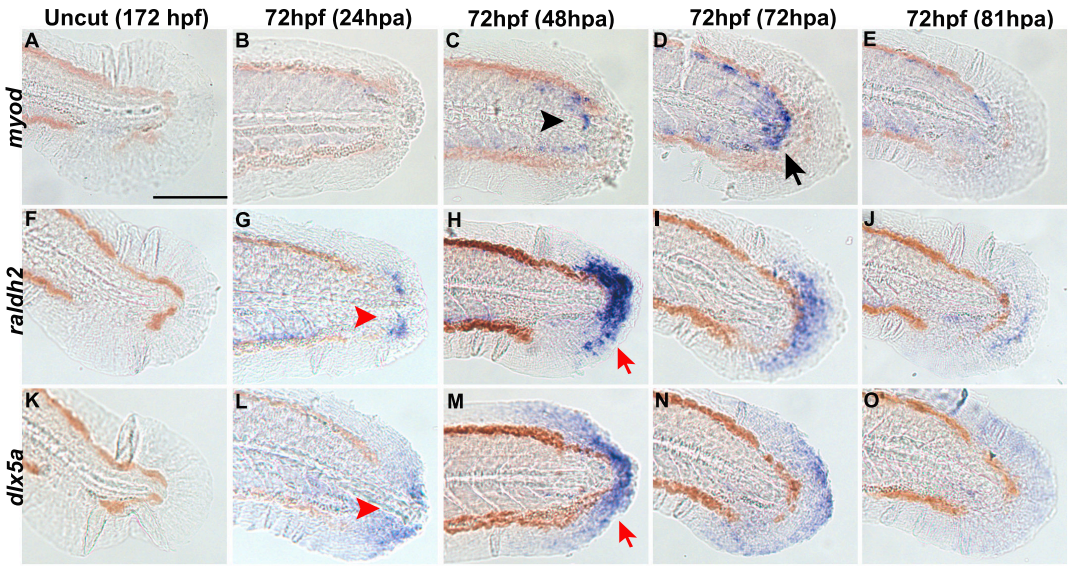

Figure 4-6: *myod* is expressed sequentially after *raldh2* and *dlx5a* expression during tail regeneration. Time course of the regeneration markers *raldh2*, *dlx5a* and the myogenic marker *myod* during tail regeneration. Expression was detected by ISH. **A-E:** *myod* expression (n=10 per time point). **C:** *myod* shows expression for the very first time at 48hpa (black arrowhead). **D:** *myod* highest level of expression at 72 hpa (black arrow). **F-J:** *raldh2* expression (n=10 per time point). **K-O:** *dlx5a* expression (n=10 per time point). **G** and **L:** *raldh2* and *dlx5a* expression is first observed at 24 hpa (red arrowheads). **H** and **M:** Highest level of *raldh2* and *dlx5a* expression is observed at 48 hpa (red arrows). N=2. Scale bar 100 μm. The amputation age and the tail regeneration time is shown "72 hpf (72 dpa)". The number outside the parenthesis indicates the age at which the tail was amputated while the number inside the parenthesis indicates for how long the tail was allowed to regenerate. The time is indicated in hours.

4.3 Discussion

4.3.1 Hedgehog signalling seems not to be crucial during tail development as during tail regeneration.

The first part of the study involves a comparison of the role of Hh signalling during both tail development and regeneration. To make this comparison I first made a

morphology assessment by down-regulating Hh signalling with cyclopamine and LDE 225 during normal tail development and regeneration. Figure 4-1 shows 24hpf embryos treated with LDE 225 with a fully developed tail, and tail-amputated fish (treated with the same concentration) that have a lack of tail outgrowth. Embryos treated with cyclopamine had a formed and extended tail that present morphological defects in comparison with the Control. The tail-amputated fish treated with the same concentration of cyclopamine did not present tail regeneration. These results agree with Barresi et al. (2000), where down-regulation of Hh signalling decreases the number of slow muscle fibres and cause partial cyclopia in 24hpf embryos. Nonetheless, tail development and extension is still observed. To complement these results I performed a molecular analysis comparison of regeneration marker genes during tail development and regeneration during Hh signalling down-regulation. As seen in Figure 4-2, the only change in expression of the regeneration marker genes is observed in the cyclopamine-treated organisms during tail regeneration. All together these results suggest that Hh signalling has a different role in tail regeneration and tail bud formation.

4.3.2 Lack of Wnt signalling is detrimental for tail formation.

The importance of Wnt signalling during tail development was assessed by morphology during embryo tail development and regeneration. Figure 4-3 shows the truncation of both tail development and tail regeneration where Wnt signalling is down-regulated. This result concurs with Thorpe et al. (2004) and Shimizu et al. (2005), in whose studies the use of morpholinos against *wnt3* and *wnt 8* was shown to suppress normal tail development. In these reports is suggested that Wnt signalling regulates *cdx1, cdx4* and *sp51* genes, which are required for posterior development.

In order to have a better interpretation of the function of Wnt signalling during tail development it is necessary to make a molecular analysis. For this molecular analysis I would propose a series of experiments. First a microarray analysis should be performed to assess which pathways are potentially downstream of Wnt/β catenin during regeneration and tail development. For the microarray analysis I will use organisms that are undergoing tail development as control and organisms undergoing tail regeneration as a test sample. I will down-regulate Wnt signalling using the *hs:dkk1-GFP* line during tail development and regeneration. The aim is to use the microarray analysis to find gene pathway components, target genes and ligands of other molecular signalling pathways that are either down-regulated or up-regulated during Wnt/β-catenin suppression during tail regeneration and development. Once I identify a group of genes that are affected in tail development and regeneration I would do an in situ to determine whether the

genes are expressed during tail development and regeneration. Finally, to corroborate these results, I would quantify the level of gene expression during both tail processes by quantitative real-time PCR.

Gilbert (2000) reports that tail development involves cell proliferation, differentiation and tissue patterning. This biological process is also found during zebrafish tail regeneration (Pfefferli et al. 2015). To assess these processes I would propose the use of the *ef1alpha:ERCreER;ubiquitin:loxP-tomato-Stop-loxP-axin2-GFP* double transgenic line that expresses the tomato fluorescent protein and after 40H tamoxifen it is possible to observe the expression of GFP (low concentration to cause mosaic recombination). GFP is an indicator of Wnt/β catenin signalling down regulation, since recombination leads to the over-expression of the *axin2* gene which is a negative regulator of the Wnt/β catenin signalling (Hart et al. 1998). I would expect the down-regulation of Wnt/β catenin to happen in a mosaic way where only few cells would present Wnt/β catenin down-regulation, indicated by the expression of the *GFP* gene. The idea is to track individual cells affected by Wnt/β catenin down-regulation and see how proliferation, cell differentiation and tissue patterning is affected and compare them with cells where Wnt/β catenin has not been down-regulated (cells expressing tomato fluorescent protein). Finally, in Figure 4-3, one can observe a reduction of tail formation in both tail regeneration and development. To assess the lack of tissue, a terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay (to detect cell apoptosis) and pHistone H3 antibody staining (to detect cell proliferation) could be done. These assays can help to see whether cell apoptosis is involved in the reduction of tissue or is actually a decrease of tail proliferation.

4.3.3 *raldh2* signalling is not regulated by Wnt signalling in the same way as chicken body axis.

Olivera-Martinez et al. (2007) reports that chicken body axis development depends on the secretion of RA. This secretion is regulated by Wnt signalling when FGF is down-regulated. I decided to replicate this experiment to see if the same pathway interaction regulates RA during body axis development in zebrafish. Figure 4-5 shows that down-regulation of FGF actually up-regulates *raldh2* expression while up-regulation of Wnt/β does not. However, the manipulation of both pathways Wnt and FGF at the same time does not show a significant difference in *raldh2* expression in comparison with the Control. A complementary experiment where Wnt is down-regulated and FGF up-regulated during *raldh2* expression is still required to better understand the relation between Wnt and FGF signalling in tail bud regeneration.

4.3.4 *myod* is expressed after regeneration marker genes during tail regeneration.

As described by Akimenko et al. (1995), the last stage during epimorphic regeneration involves tissue differentiation. I decided to study the expression of *myod* during tail regeneration, since it is described to play a major role during muscle differentiation (Zhao et al. 2004). I made a tail amputation time course in order to see at which time point *myod* is expressed, and then I compared the expression with the regeneration marker genes *dlx5* and *raldh2* time point expression. Figure 4-6 shows that *myod* marker is expressed after *dlx5a* and *raldh2*, which could be interpreted as sequentially. An explanation could come from the reports of Odelberg et al. (2000), where mouse mammalian myotubes undergo de-differentiation and *myod* expression levels are reduced when there is ectopic expression of the homeobox *msx1* gene. Once *msx1* is absent myogenic factors are re-expressed and myotubes formed again. The zebrafish homeobox gene that was tested during regeneration in this PhD thesis is *msxc*, which is expressed during tail development (Akimenko et al. 1995) and re-expressed during tail regeneration (Kawakami et al. 2004). Still, the time point testing of *msxc* gene in comparison with *myod* needs to be performed to determine the relationship between this genes.

4.4 Conclusion

The main goal of the present chapter was to test the following hypothesis: *Hh, Wnt and FGF signalling are essential during both tail development and regeneration.*

The results, however, do not support the hypothesis since differences were found during the study of the three pathways. Hedgehog signalling seems not to have the same function in tail bud formation as observed to have in tail regeneration. Zebrafish embryos under continuous cyclopamine administration still managed to develop tails (Figure 4-1). Hh signalling does not seem to be upstream of Wnt and regeneration marker genes, since the expression of *raldh2* and *tcf7* (Wnt readout) was still present even though Hh signalling was down-regulated (Figure 4-2). On the other hand, Wnt signalling's role in the bud formation seems to be more similar to its role in tail regeneration. This is because the lack of Wnt signalling manages to stop tail formation (Figure 4-3), however further molecular analysis needs to be done. FGF seems to modulate *raldh2* expression but in a different way than it does in tail regeneration. However, the relationship between FGF and Wnt seems to be more a cross-talk rather than a hierarchy (Figure 4-5), since the expression of *raldh2* in the epistasis analysis was similar to the Control. Finally, the expression of developmental tissue markers in tail regeneration was also studied in this chapter because to some extent regeneration seems to re-express embryonic regulation factors. The expression of the *myod* (myogenic marker) was studied during tail

regeneration. *myod* seems to be expressed sequentially after regeneration marker genes, which is a sign of tissue re-differentiation (Figure 4-6).

Altogether these results indicate that there is a difference in the roles of Hh Wnt and FGF during tail regeneration and development. The study provides evidence that tail regeneration in larvae is not due to a continuation of tail development but instead tail regeneration mechanisms are taking place. Finally Hh signalling mechanisms observed during tail regeneration seem to be unique for this process; while the expression of Wnt signalling could indicate a re-expression of embryonic mechanism since Wnt seems to have an analogous mechanism in tail development and regeneration that is not observed in Hh (Figure 4-7).

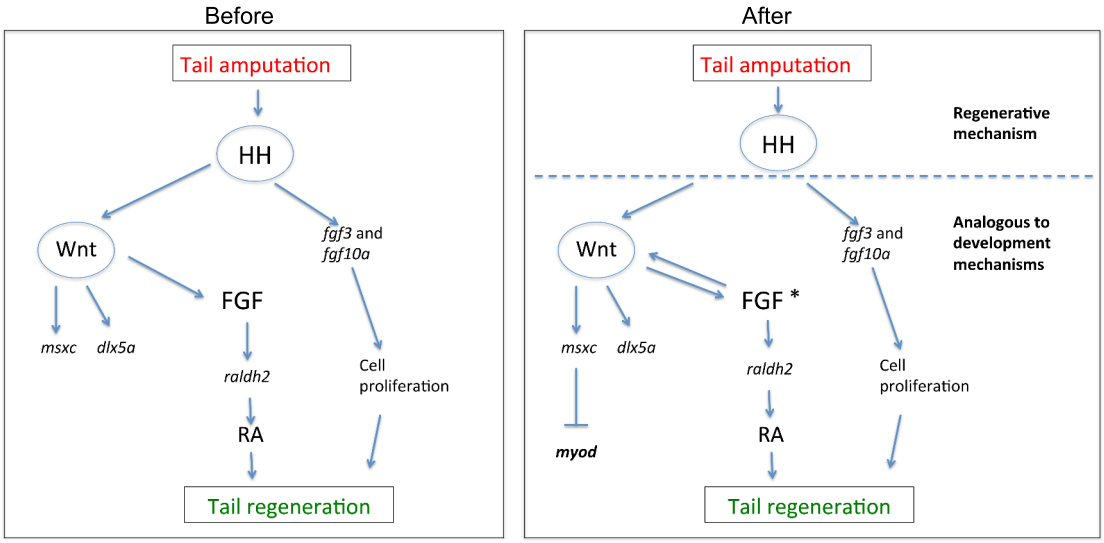

Figure 4-7: Developmental-regenerative mechanisms during tail restoration. The model on the left shows the findings before the tail development analysis, while the model on the right incorporates both the findings for the tail development and regeneration findings. The model suggest that Hh signalling has a unique mechanism during regeneration, while the pathways downstream, such as Wnt, have an analogous role to the tail developmental mechanism and also suggests a cross-talk between Wnt and FGF regarding *raldh2* expression. On the other hand, FGF signalling regulates *raldh2* but in an opposite way to when during tail regeneration (asterisk indicates that during tail development FGF down-regulation up-regulates *raldh2* expression). *myod* expression is suppressed by the regeneration marker *msxc*, which is re-expressed when *msxc* is down-regulated.

Chapter 5

Construction of fish transgenic lines with tissue-specific promoters to drive Cre expression

5.1 Introduction

Teleost organisms possess a striking ability to renew tissues and organs; zebrafish in particular have become a popular model to study tissue regeneration. Short period of regeneration, tissue transparency and well-characterised molecular techniques such as the Cre-Lox system and whole-mount in situ hybridisation (ISH) are some of the characteristics that make the zebrafish a suitable tissue/organ remodeling model. Tail regeneration is described as follows: after tail amputation, the injury immediately closes, then a structure called wound epithelium forms along the wounded area. The cells surrounding the wound migrate towards the wound epithelium in order to agglomerate, forming *the blastema*. *The blastema* is a mass of cells that is thought to be un-differentiated, and is the main characteristic of epimorphic regeneration. The blastema starts to proliferate, and then the cells will differentiate into the tissues that will reconstruct the tail, completing tail regeneration (Nishidate et al. 2007; Kizil et al. 2012).

Epimorphic regeneration has attracted the attention of researchers over the past years. The reason is that differentiated cells undergo a certain level of dedifferentiation in order to gain cell plasticity (Knopf et al. 2011). However, many questions still need to be answered regarding the origin of the cells that participate in regeneration. For example, it is not clear how the cells acquire a proliferative capacity. Are the cells able to trans-differentiate (change from one defined type cell into a different cell type) or do they remain lineage-restricted during regeneration? Also, what is the level of cell plasticity that the blastema can achieve? (Nechiporuk et al. 2002; Tu et al. 2010; Stewart et al. 2012).

Tu et al. (2010) performed a lineage-tracing analysis during regeneration with the technology called transposon-based clonal analysis. The method involves the injection of the *EF1α:GFP* construct into a one-cell embryo. This gave rise to mosaic embryos expressing GFP. Then the embryos were raised to adulthood. Adult zebrafish with GFP expressed in different tissues were identified. Once the tissue of interest was identified, in this case the fin rays, amputation was performed and it was observed that after regeneration only fin rays expressed GFP. This result suggests that the tissue in the fin rays remains lineage-restricted. This technology, however, only identifies the structure of interest, and is not tissue-specific. Therefore it is not possible to know which are the precise cells that form the fin rays and that participate in their regeneration.

A more suitable tool for the study of lineage-tracing analysis is the Cre-Lox system, where it is possible use tissue-specific promoters, depending on the tissue of interest. These promoters drive the expression of the Cre protein, which will label the tissue of interest in an irreversible manner. Also, with the help of the mutated human ligand-binding domain of the estrogen receptor (ERT2) (T2 is the name of the exon in the genome sequence of ER (Brand et al. 2002), tissue recombination can be temporally controlled by tamoxifen administration (Hans et al. 2009). Genetic recombination happens when Cre binds to the Lox sites of the genome and cause either deletion, inversion or translocation depending on the orientation of the genome (Haliw 2015). Initially, for the development of this PhD thesis, we created a multilox construct called the "zainbow", which is a multicolour system that allows the expression of three different fluorescent proteins after Cre recombination (Livet et al. 2007). The zainbow construct has three pairs of Lox sites, where each pair flanks each fluorescent protein gene (see Figure 1-6), Cre randomly binds to any of the Lox pairs, then recombination happens and only one of the three fluorescent proteins is expressed. So different cells end up expressing different fluorescent proteins, resulting in the visualisation of three different fluorescent proteins in one organism. We were able to induce recombination in the zainbow transgenic line, after injecting Cre RNA. Unfortunately, recombination was never observed after crossing the zainbow line with CreER-expressing lines, where recombination takes place after 4-Hydroxytamoxifen (4OHT) administration. For this reason I adopted a different strategy by using a Lox line with only one Lox pair, which express one fluorescent protein after Cre recombination, in order to test my Cre-expressing lines (see Figure 1-8).

The present chapter describes the design of 5 Cre-expressing lines and the test of 7 Cre-expressing lines with tissue-specific promoters. Additionally most of the lines can undergo tissue recombination where the recombination is tightly dependent on 4OHT administration.

5.2 Results

5.2.1 The *pDestTol2cryaa:venus* vector is functional in the zebrafish genome

Initially I had planned to use the *pDestTolCG2* vector from "Chien lab" (Chien lab 2016) as part of the Cre-expressing constructs. The *pDestTolCG2* has the *att* sites necessary to use the molecular tool Gateway system. The Gateway system allows a rapid integration of a vector, promoter, Cre gene and the poly A section into one DNA construct. The *pDestTol2CG2* vector also has the Tol2 transposon ends needed for the insertion of the construct into the zebrafish genome. Another feature of the pDestTol2CG vector is the ccdB section that provides negative selection in cells where the DNA construct (vector, promoter, coding sequence and 3' tag) is not present, but only the vector. The reporter gene of this vector is the EGFP, and the expression is driven by the *cardiac myosin light chain (cmcl2)* gene that is expressed in the zebrafish heart. Finally the vector has the ampicillin resistance gene that encodes β-lactamase, inactivating the ampicillin antibiotic. Therefore, after bacterial transformation, only the cells that incorporate the vector can grow in agar plates with ampicillin (Kwan et al. 2007).

However, another lab working with pDestTolCG2 reported that the *cmcl2* gene, when used as a promoter for reporter gene, had silenced the expression of the other promoter contained in the same vector. Therefore, there is a risk of *cmcl* silencing the expression of the tissue-specific promoter, which would prevent observing tissue recombination, and is therefore undesirable. I decided then to remove the *cmcl2:EGFP* section from the vector and incorporate the *cryaa:Venus* into it, creating the *pDestTol2cryaa:Venus*. The *pDestTol2cryaa:Venus* vector has all the characteristics of the pDestTolCG2 but the reporter gene is expressed in the crystalline lens of the zebrafish eye (Figure 5-1).

5.2.2 Creation of the Cre expressing lines

Five DNA constructs expressing Cre were successfully created using the Gateway cloning system (Invitrogen). The Gateway system allowed a fast incorporation of the tissue-specific promoter, ERcreER/creER gene, poly A and the DNA vector. The final Cre-expressing constructs are described in Table 5-1, where I mention the construct made by me and also made by other researchers but tested by me. Table5-2 shows the name of the tissue promoters with the source and the base pairs used from each promoter. The reason why these promoters were chosen is because they reported a satisfactory level of expression along the tissue of interest and no leakiness was observed (expression in a different tissue).

I tested the size of the Cre-expressing constructs that were synthesised by me

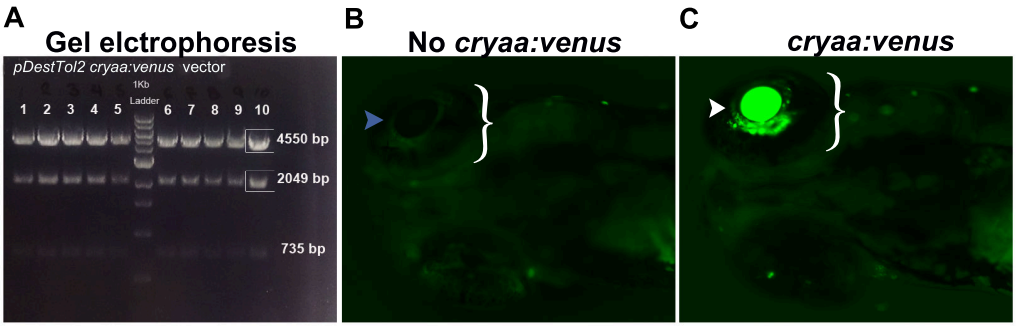

Figure 5-1: ***cryaa:Venus*** **vector making and expression in 3 dpf larva. A:** *cryaa:Venus* plasmid digested with the enzyme Pvu lI show the right band sizes (white squares). **B, C:** 3dpf embryo DNA injected at 1-cell stage shows the expression of the GFP in the crystalline lense of the eye (white arrowhead), indicating *cryaa:Venus* incorporation in the genome. Lack of DNA incorporation leads to no GFP expression (blue arrowhead). Picture taken with the GFP channel. White brackets point the eyeball

by restriction enzyme digestion, and then I ran the DNA digestion in 1% agar gel by electrophoresis. We seen in Figure 5-2 and 5-3 the DNA bands of each Cre-expressing plasmid along with the genetic map. *α actin: ERT2Cre-ins* and *twist:ERT2Cre-ins* Figure 5-3 were designed with the Cre molecule expressing only one ERT2. However, leakiness has been reported with a construct containing CreERT2 in mice where recombination takes place without 4-Hydroxytamoxifen (4OHT) administration (Kemp et al. 2004). To avoid leakiness we decided to control CreERT2 expression in a transcription level by using insulator DNA technology. Insulators are DNA sections that act as a barrier against the activation of gene expression by distant enhancers. Insulators can also prevent gene silencing due to the intrusion of chromatin condensation (Burgess-Beusse et al. 2002). Therefore, a pair of insulators were inserted flanking the DNA section that corresponds to the tissue-specific promoter and the Cre section. The insulators were kindly provided by Robert Kelsh's lab "Bath University" where the insulators used were the SINE CD insulator (more information can be found from Lunyak et al. 2007). The reason for creating constructs with CreERT2 instead of ERT2CreERT2 is because Carlos Cruz the research associated from Henry Roehl's lab had troubles seeing tissue recombination with the Cre lines expressing ERT2CreERT2. We thought that maybe 4OTH was not able to reach two ERT2 sites at the same time.

The first test to observe tissue recombination was performed by injecting the Cre-expressing construct in the one-cell of the reporter-expressing embryo (*EF1α:loxPGFPloxP-DsRed*) and then administration of 4OHT was done at 24hpf. Tissue recombination was never observed under such conditions, so I decided to adopt a different strategy by which I injected the Cre-expressing construct into a one-cell stage wild type embryo and then sent the fish to be raised to adulthood. It was once the transgenic line reached the second generation (F2) and I could

Table 5-1: Cre expressing lines

Tissue of expression	Construct	Synthesis
Blood vessels	*Tg(fli1a:ERT2CreERT2*	Montserrat Garcia
Periderm cells	*Tg(krt4:ERT2CreERT2)*	Montserrat Garcia
Muscle	*Tg(αactin:CreERT2)*	Montserrat Garcia
Chondrocytes and sclerotomal cells	*Tg(twist:CreERT2-ins)*	Montserrat Garcia
Osteoblasts	*Tg(osteocalcin: ERT2CreERT2)*	Montserrat Garcia
Osteoblasts	*Tg(osteocalcin: CreERT2)*	Luis Medina
Neural stem cells	*Tg(nestin:CreERT2)*	Dr. Carlos Cruz
Commited neuron cells	*Tg(huc:CreERT2)*	Dr. Stone Elworthy

confirm that the Cre-expressing lines were stable that I tested the line again for recombination. The test involved crossing the Cre-expressing line with the reporter line (*EF1α:loxPGFPloxP-DsRed*) and then administering 10μM 4OHT as a standard concentration to treat all the transgenic lines. With treatment of 10μM 4OHT all the Cre-expressing lines managed to recombine cells except for the *osteocalcin:ERT2CreERT2* line. Finally, after recombination was observed, I decided to grow the tissue recombined line *fli1α:ERT2CreERT2;EF1α:loxPGFPloxP-DsRed* up to 20dpf. At this stage the DsRed-expressing cells were still present, which is evidence that recombination is permanent (See Chapter 6).

5.2.3 Tissue recombination is observed in all the double transgenic lines after 4OH Tamoxifen administration

Once the five transgenic lines were proved to be stable (F2 generation), I crossed each of them with the *Tg(EF1α: loxPGFPloxP-DsRed)* or *Tg(ubiquitin:loxPGFP-loxP-mCherry)* Lox reporter lines (Table 5-3) to test tissue recombination by 4OHT administration. The pictures shown here depict tissue recombination by 4OHT and lack of recombination when 4OHT is absent. Auto fluorescence is observed in the eye, yolk and iridiophores. Regarding the Cre-expressing lines that were created by others Tg(nestin:ERT2CreERT2) and *Tg(huc:ERT2CreERT2)*, I tested them for recombination when the lines where in the F1 generation. Finally, it is important to mention that even though I observed recombination in most of the lines I tested, tissue recombination was mosaic which means it did not happen in the whole tissue.

5.2.3.1 *Tg(fli1a:ERT2CreERT2)* transgenic line

The *fli1a* promoter is expressed in the endothelia cell layer of the blood vessels (Lawson et al. 2002). Therefore this promoter allows us to track the blood vessel regeneration throughout tissue recombination. As we can see in Figure 5-4, tissue recombination was observed in the vasculature of the zebrafish embryo trunk

Table 5-2: Tissue specific promoters

Tissue specific promoter	Source	Base pairs used
fli1a	Lawson et al. 2002	Available on request Lawson et al. 2002
keratin 4 (krt4)	Gong et al. 2002	Approximately 2200 bp 5' upstream of the transcription initiation site
αactin	Higashijima et al. 1997	Approximately 3900 bp upstream from the transcription initiation site
twist	Inohaya et al. 2007	Approximately 5000 bp upstream from the transcription initiation site
ostocalcin	Inohaya et al. 2007	Approximately 3700 bp upstream from the transcription initiation site
nestin	Chen et al. 2009	Approximately 3900 bp upstream from the transcription initiation site
huc	Park et al. 2000	Approximately 2800 bp upstream from the transcription initiation site

Table 5-3: Tissue specific promoters

Lox reporter line	Recombination	Source
Tg(EF1α: loxPGFPloxP-DsRed)	From GFP to DsRed	Sinha et al. 2010
Tg(ubiquitin:loxPGFPloxP-mCherry)	From GFP to mCherry	Mosimann et al. 2011

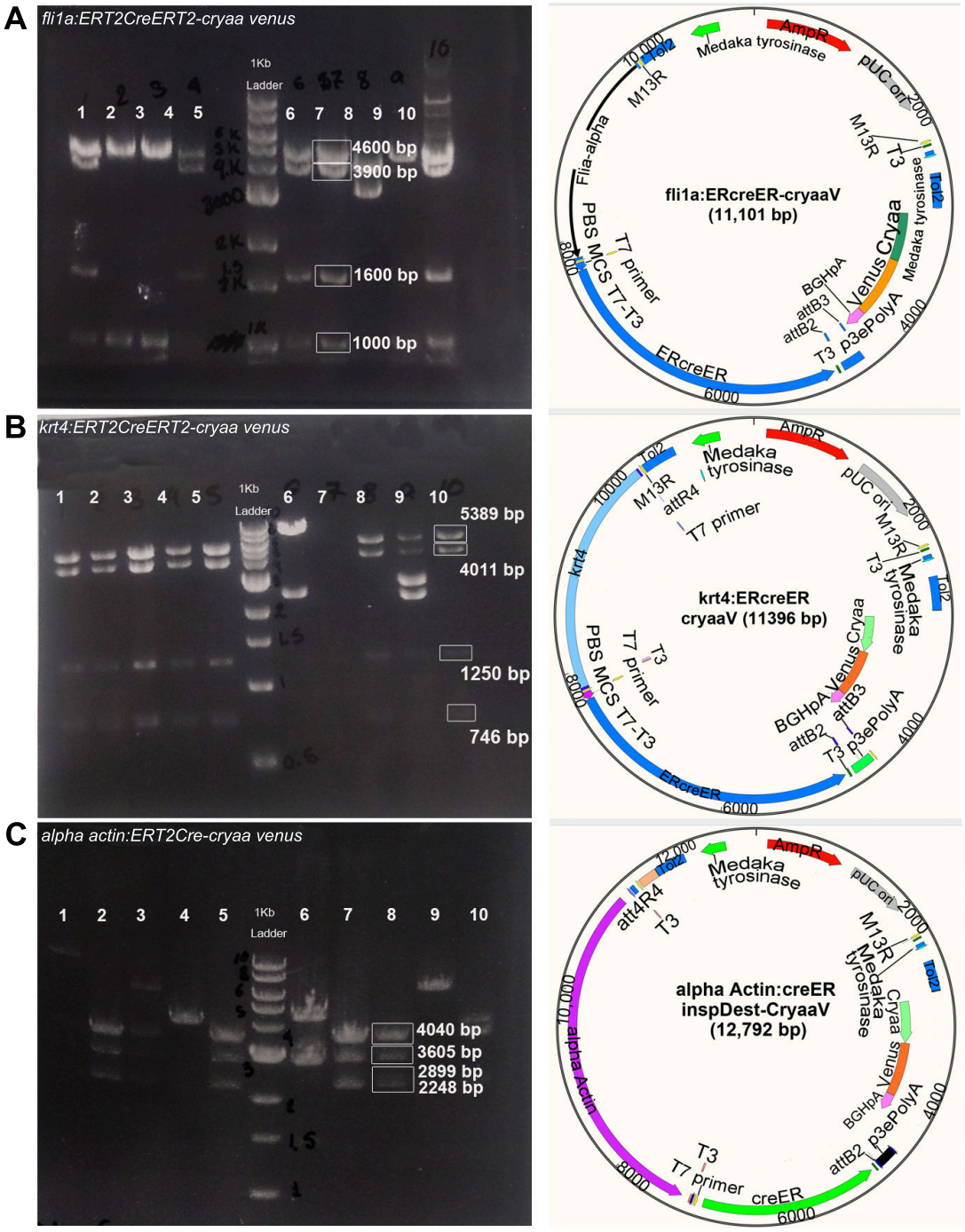

Figure 5-2: Cre expressing constructs with tissue specific promoters synthesis. Each construct is enzyme digested; the right band size is shown in the white squares of each picture. Next to the electrophoresis picture is the plasmid map of each of the Cre constructs with the size of the whole construct. **A:** *fli1a:ERT2CreERT2* construct. **B:** *krt4:ERT2CreERT2* construct. **C:** *αactin:CreERT2* construct.

Figure 5-3: Cre expressing constructs with tissue specific promoters synthesis. Each construct is enzyme digested; the right band size is shown in the white squares of each picture. Next to the electrophoresis picture is the plasmid map of each of the Cre constructs with the size of the whole construct. **D:** *osteocalcin:ERT2CreERT2* construct. **E:** *twist:CreERT2* construct.

and developing jaw in the double transgenic fish *Tg(fli1a:ERT2CreERT2;EF1α: loxPGFPloxP-DsRed)* when 4OHT is administered. Recombination of the double transgenic line is tightly regulated by 4OHT since no recombination was observed when adding ethanol (see Figure 5-4). The *fli1a* promoter has been reported to be expressed in hematopoietic stems cells during the first 24hpf; these cells could give rise to different tissues that are not blood vessels, (Lawson et al. 2002) thus compromising the specificity of the tissue-specific transgenic line. Therefore I decided to administrate 4OHT after 24hpf, in order to narrow cell recombination to blood vessels.

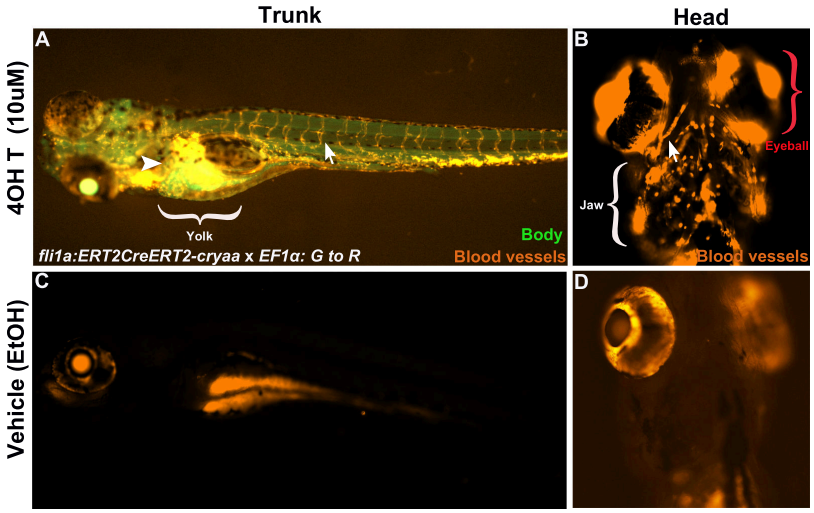

Figure 5-4: Tissue recombination in the blood vessels of the *fli1a:ERT2CreERT2* transgenic line. Fish are treated with 10 μM 4OHT and controls with the vehicle (ethanol). **A:** Tissue recombination is observed in the blood vessels along the trunk (white arrow) by expressing the DsRed fluorescent protein (n=16/20). The yolk presents auto fluorescence (white arrowhead) (pictures taken with the Tomato and GFP channel). **B:** Recombination is observed in the blood vessels in the jaw (white arrow) (pictures taken with the Tomato and GFP channel) (n=16/20). **C** and **D:** Ethanol-treated fish do not present signs of tissue recombination (n=20). 80% of double transgenic embryos show recombination (pictures taken with the Tomato channel). N=5. One representative embryo was chosen per picture.

5.2.3.2 *Tg(krt4:ERT2CreERT2)* transgenic line

The *krt4* promoter is expressed in the periderm cells of the zebrafish embryo. This epidermis cells form a layer that serves as a barrier between the zebrafish embryo and the exterior environment (Gong et al. 2002). The *Tg(krt4:ERT2CreERT2)* line crossed with *Tg(EF1α: loxPGFPloxP-DsRed)* gave rise to the double transgenic line *Tg(krt4:ERT2CreERT2;EF1α: loxPGFPloxP-DsRed)*. This double transgenic line expressed DsRed protein in several periderm cells along the body when 1μM of 4OHT was administered. No Cre leakiness was observed in double transgenic fish

that were not treated with 4OHT, as shown in Figure 5-5. This line was created to study how *krt4*-expressing cells contribute to tail regeneration and the level of cell plasticity that these cells possess (proliferation, de-differentiation and/or trans-differentiation) during regeneration.

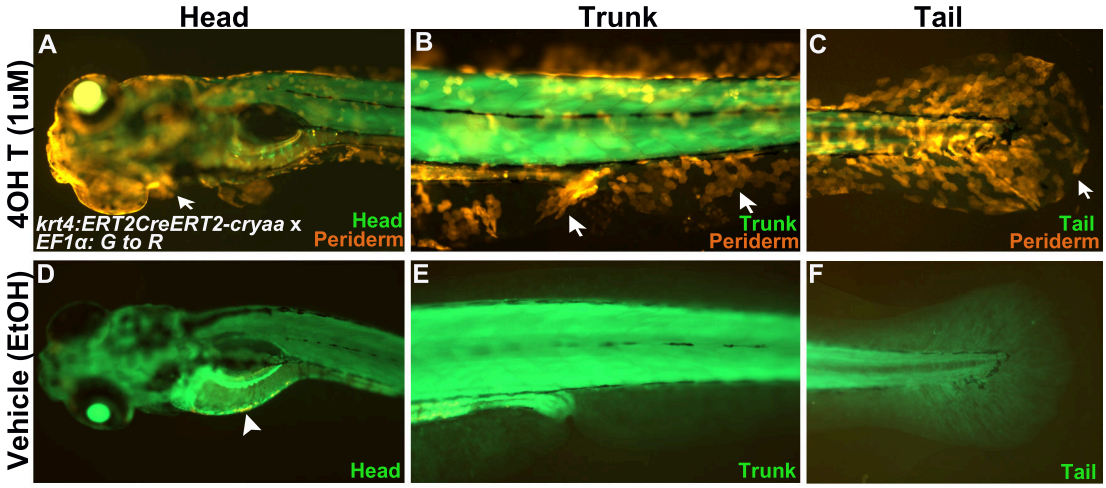

Figure 5-5: Tissue recombination in the periderm cells of the *Tg(krt4:ERT2CreERT2)* transgenic line. Fish are treated with 10 μM 4OHT to induce recombination and controls with the vehicle (ethanol). **A-C:** Treated embryos, in which tissue recombination is observed in the periderm cells of the head, trunk and tail (white arrows) by expressing DsRed fluorescent protein (pictures taken with the Tomato and GFP channel) n=(9/10). **D-F:** The control fish does not show recombination, since the expression of DsRed fluorescent protein is not detected (pictures taken with the Tomato and GFP channel) a small level of red auto fluoresce is observed in the yolk (white arrowhead)(n=10). One representative embryo was chosen per picture. 90% of double transgenic embryos had recombination.

5.2.3.3 *Tg(αactin:CreERT2-ins)* transgenic line

The αactin gene is expressed in the zebrafish muscle and has been used by Higashijima et al. (1997) to drive the expression of GFP in muscle tissue. The generation of the double transgenic line *Tg(αactin:CreERT2;EF1α: loxPGFPloxP-DsRed)* was done successfully. However, tissue recombination was observed in no more than 5 muscle strips. For this reason I decided to make a screening with the offspring from 4 different double transgenic fish *Tg(αactin:CreERT2;EF1α: loxPGFPloxP-DsRed)*. The founder tagged as 4 was treated with 10μM 4OHT and showed bigger level of recombination than 5 stripes. The recombined muscle stripes can be observed along the trunk in Figure 5-6. Robert Kelsh's lab from "Bath University" had recommended that the fish be incubated after 4OHT at different temperatures to enhance recombination. Therefore, I incubated the fish at different temperatures, but this did not show any difference in the level of recombined cells. No Cre leakiness was observed in the non-4OHT-treated fish. The

progeny of the founder 4 was sent to be raised. The creation of this transgenic line was to observe the contribution of muscle tissue during tail regeneration and the cell plasticity that such cells may present. Finally, as we can see, the recombination of the muscle stripes is mosaic. Mosaic recombination should not be an obstacle in performing a lineage-tracing analysis, since the lineage-tracing will consist of only localising and tracking the behaviour of individual muscle stripes during regeneration.

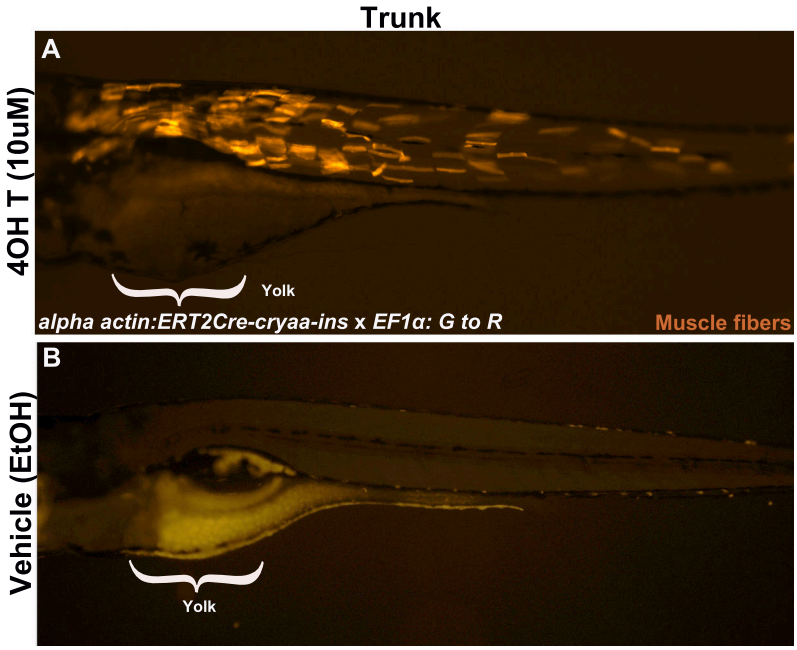

Figure 5-6: Tissue recombination in the muscle tissue of the *αactin:ERT2Cre* transgenic line. Fish are treated with 10 μM 4OHT to induce tissue recombination and controls with the vehicle (ethanol). **A:** Treated embryos, have tissue recombination by expressing the DsRed fluorescent protein in several muscle stripes (white arrow) along the trunk (white arrows) (pictures taken with the Tomato and GFP channel) (n=7/10). **B:** Control fish, where no recombination is observed (n=10). 70% of double transgenic embryos had recombination. One representative embryo was chosen per picture. (pictures taken with the Tomato and GFP channel).

5.2.3.4 *Tg(twist:CreERT2-ins)* transgenic line

The idea of using the *twist* gene from the medaka fish genome is to have a mesenchymal marker to label cells that express certain level of de-differentiation. Inohaya et al. (2007)) reports the expression in the medaka fish of the *twist* gene as a sclerotomal marker, where the sclerotome is the part of the somite that gives rise to bone tissue (Gilbert 2000). Stokes et al. (2002) reports the expression of *twist* in human chondrocytes. *Tg(twist:CreERT2;EF1α:loxPGFPloxP-DsRed)* showed tissue recombination in the jaw area after adding of 10μM 4OHT. The cells that

expressed the DsRed protein seemed have chondrocyte identity, which are cells that creates cartilage matrix (Perka et al. 2000). However, tissue recombination was restricted only to the jaw area (Figure 5-7) so further recombination assessment is pending in order to observe DsRed expression in tissue close to the tail. *twist*-expressing cells have been analysed in adult medaka during tail regeneration by in situ hybridisation (ISH) where *twist* is expressed in the blastema proliferating cells and later on remain in the un-differentiated section of the blastema (Katogi et al. 2004).

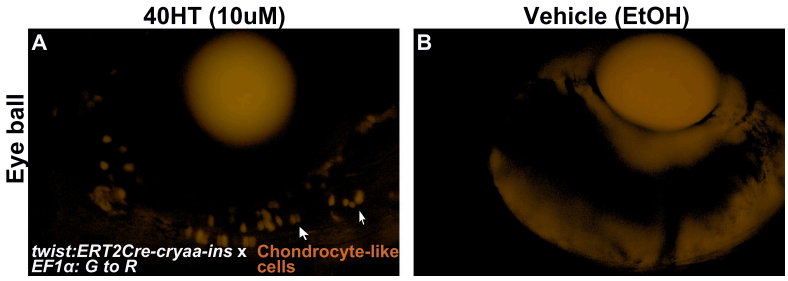

Figure 5-7: Tissue recombination in the chondrocyte-like cells of the *Twist:ERT2Cre* transgenic line. Fish are treated with 10 μM 4OHT to induce recombination and controls with the vehicle (ethanol). **A:** Treated embryos have tissue recombination by expressing DsRed fluorescent protein in the chondrocyte-like cells (white arrows)(n=4/5). **B:** Control fish do not show recombination (n=5). (pictures taken with the Tomato channel) 80% double transgenic embryos show recombination.

5.2.3.5 *Tg(nestin:ERT2CreERT2)* transgenic line

The *nestin* gene is expressed in neural stem cells, astrocytes and neuroepithelial stem cells (Chen et al. 2009). The use of this marker could allow me to see whether neural stem cells participate in tail regeneration and also to analyse their cell behaviour. Dr. Carlos Cruz prepared the plasmid construct and the generation of the transgenic line in our lab. As we seen in Figure 5-8, the double transgenic line *nestin:CreERT2;EF1α:loxPGFPloxP-DsRed* shows tissue recombination in the jaw area, in cells that seem to be chondrocytes. Similar to the Cre line driven by twist, no tissue recombination was observed in the trunk area, therefore further characterisation of this line needs to be done.

5.2.3.6 *Tg(huc:ERT2CreERT2)* transgenic line

The *huc* gene marker is expressed in differentiating neurons. (Park et al. 2000). *huc* was used as a promoter to drive the Cre expression. Dr Stone Elworthy from "Ingham Lab" prepared the construct and the generation of the transgenic line. I performed the functional test of the double transgenic line *huc:CreERT2;EF1α:loxPGFPloxP-DsRed* was done by administrating 10μM 4OHT. As seen in Figure

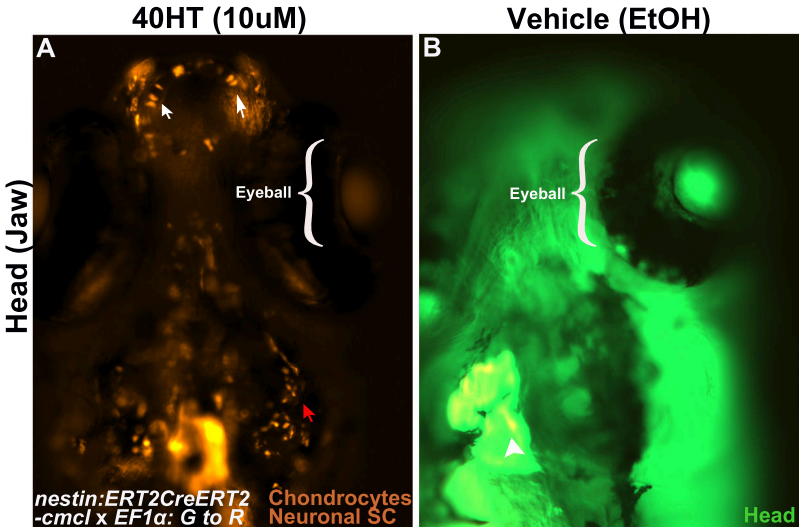

Figure 5-8: Tissue recombination in the chondrocyte like-cells and neural-like stem cells of the *nestin:ERT2CreERT2* transgenic line. Fish are treated with 10 μM 4OHT, and control fish with the vehicle (ethanol). **A:** Treated embryos, which show tissue recombination by expressing DsRed fluorescent protein in chondrocyte- like cells (white arrows) in the anterior part of the jaw, and also in neural-like stem cells (red arrow) (pictures taken with the Tomato channel)(n=7/10). **B:** Control fish, which do not show recombination, arrow head points auto fluoresce (pictures taken with the Tomato and GFP channel)(n=10). One representative embryo was chosen per picture. 70% of double transgenic embryos showed recombination.

5-9, tissue recombination is observed in neurons and axons along the spinal cord, and in the anterior part of the head. Similar tissue labelling is observed in the reports made by Park et al. (2000) where GFP driven by *huc* gene is expressed in the spinal cord, brain and body axons. Ectopic recombination (tissues recombination where the marker is not expressed) was observed in muscle, but not in all the treated embryos (approximately 10%). Finally, tissue recombination was properly regulated by 4OHT since no tissue recombination was observed in the double transgenic line when treated only with ethanol.

5.2.3.7 Tissue recombination was not observed when using the *Tg(osteocalcin:CreERT2*

The*osteocalcin* gene has reported to be expressed at 7dpf on the fifth ceratobranchial (Gavaia et al. 2006). However, a later report by Knopf et al. (2011) (supplementary information) using the *osteocalcin:GFP* transgenic line described a strong expression of GFP in the skull of the zebrafish at 22dpf. Therefore, I decided to cross the *osteocalcin:creER* with the *EF1α:loxPGFPloxP-DsRed* transgenic line and then test for recombination at 22dpf by 4OHT administration.

4OHT administration was performed at 10μM continuously for 3 days and monitored by the aquarium manager. 4OHT administration stopped at 25dpf, then the fish were analysed for possible tissue recombination at 28dpf in order to

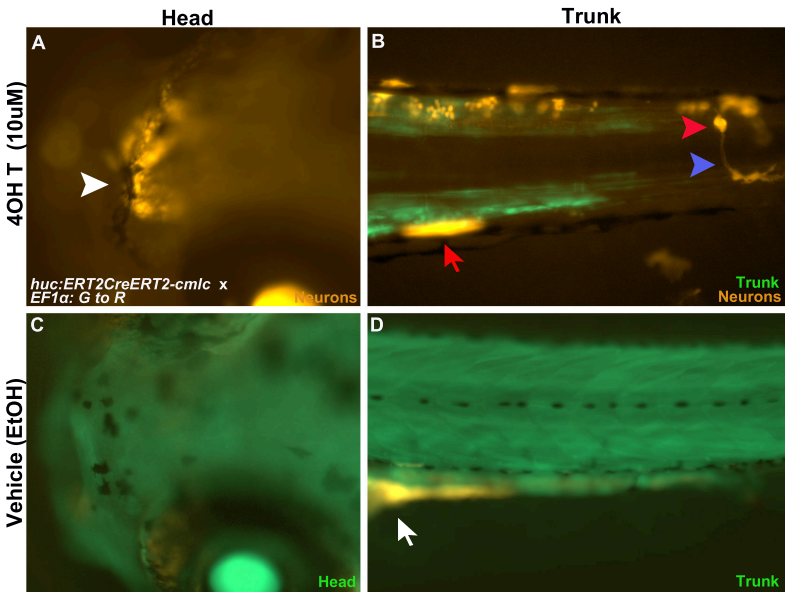

Figure 5-9: Tissue recombination in the neuron-like cells of the *huc:ERT2CreERT2* transgenic line. Fish are treated with 10 μM 4OHT, and control fish with the vehicle (ethanol). **A-B:** Treated embryos, tissue recombination is observed by expression of the DsRed fluorescent protein in neuron-like cells in the anterior part of the head (white arrowhead), neuron bodies in the trunk (red arrowhead) and axon (blue arrowhead) (pictures taken with the Tomato and GFP channel) (n=7/10). Ectopic recombination is observed in one muscle stripe (red arrow). **C-D:** Control fish, in which no recombination is observed (n=10). The yolk has auto fluoresced (white arrow) (pictures taken with the Tomato and GFP channel). One representative embryo was chosen per picture.70% of double transgenic embryos showed recombination.

Table 5-4: Cre expressing lines

Transgenic construct	Synthtesis	Test
Tg(osteocalcin: ERT2CreERT2)	Luis Medina	Montserrat Garcia Luis Medina
Tg(osteocalcin: kaede)	Monika Tomecka	Monika Tomecka

give enough time to the dsRed protein to be synthesised.

The fish did not show recombination after 6 days of the first 4OHT administration, which is normally the time frame at which I observed recombination in the other transgenic lines. We wondered if the promoter *osteocalcin* may be the problem, so we sent it for DNA sequencing and the *osteocalcin* sequence did not show any irregularity (data not shown). From this we thought that probably the *osteocalcin* promoter was not being expressed at all. I asked another lab member, Monika Tomecka, who had managed to make the *Tg(osteocacin:kaede)* line, to analyse the expression of the *osteocalcin* gene. Kaede is a green fluorescent protein that photoconverts into red fluorescent protein when hit by ultraviolet light (Ando et al. 2002). Monika Tomecka reported that the Kaede protein was expressed in the zebrafish adult bone fin rays, where *osteocalcin* is normally expressed.

This outcome indicates that *osteocalcin* as a promoter works in the zebrafish adult. Due to the time and resources required I decided to stop working with the *Tg(osteocalcin:ERT2CreERT2* line and focused more on the other Cre-expressing lines. For future analysis I would suggest the testing of whether the Lox line *EF1α:loxPGFPloxP-DsRed* is actually functional in the zebrafish adult. The contructs made by other lab members are shown in Table5-4.

5.2.4 Tissue recombination is 4OH Tamoxifen concentration dependent

The very first line to test was the double transgenic line *Tg(fli1a:ERT2CreERT2;-EF1α: loxPGFPloxP-DsRed)*. At the beginning of the 4OHT administration and recombination test, the change from GFP to DsRed (tissue recombination) in the target tissues was not observed. The problem could be permeabilisation, and for this reason the chorions of 8hpf embryos were removed and 4OHT was administered along with 0.1% of DMSO to make the cell membrane more permeable. Recombination was observed for the very first time under the condition already described in the *fli:ERT2CreERT2;EF1α:loxPGFPloxP-DsRed* line.

In order to show the importance of treating the fish under the mentioned conditions a recombination test under three conditions was done. The first condition: the chorion was removed plus 0.1%DMSO administered. The second condition:

the chorion was removed but no DMSO was administered. The third condition: the chorion was not removed and no DMSO was administered. The transgenic line used for this test was the *krt4:ERT2CreERT2;EF1α:loxPGFPloxP-DsRed* and 0.1μM of 4OHT was added to each of the three groups. As seen in Figure 5-10 there is an obvious difference between the groups where the chorion was removed to the one where the chorion was left. A bigger level of tissue recombination was observed in the groups were the chorion was removed, in which more skin cells expressed the DsRed protein. The addition of DMSO had no obvious effect on the observed number of recombinant cells. This suggests that DMSO is not that important during cell permeabilisation, but a range of different DMSO concentrations should be tested to confirm this.

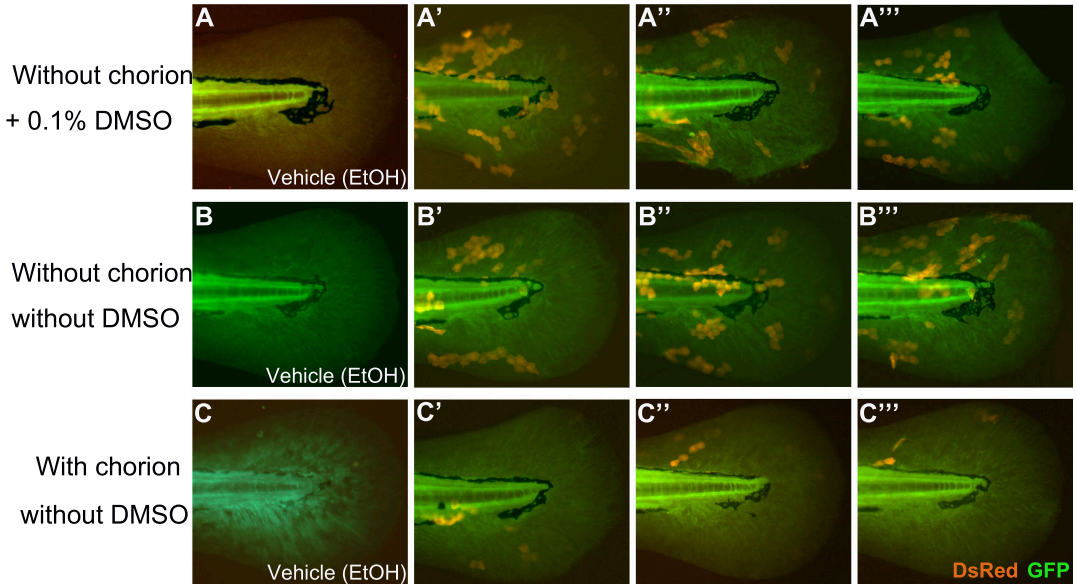

Figure 5-10: Chorion removal in the 75% epiboly stage leads to a more effective tissue recombination when treated with 4OHT. The*krt4:ERT2CreERT2;EF1α:loxPGFPloxP-DsRed* line was used for this study. Three examples per treatment are shown in the figure. **A'-B''':** The elimination of the chorion leads to a higher level of recombination than group **C'-C'''** where the chorion was intact. DMSO administration in group **A'-A'''** does not seem to affect tissue recombination. **A-C:** Control group fish, in which no recombination is observed. Fish were treated for 24 hours with either 4OHT or Ethanol. (Pictures taken with the Tomato and GFP channel)

After establishing the importance of removing the chorion I decided to test whether tissue recombination was 4OHT concentration-dependent. If so, it would be possible to control the amount of recombinant cells produced. Four groups of fish were tested and treated under different 4OHT concentrations: 0.01μM, 0.1μM, 1μM and 5μM. As seen in Figure 5-11 the level of recombination depends on the 4OHT concentration administered. Group 1, 0.01μM, shows the least tissue recombination in comparison with the other groups. Groups 2 and 3, 0.1μM and 1μM respectively, show a higher level of recombination in comparison with

Group 1. Both Groups 1 and 2 show less tissue recombination in comparison with Group 4, 5μM 4OHT. However, the difference between the tissue recombination between Group 2 and 3 was not significant. A more detailed evaluation of the cell recombination needs to be done in order to find a difference, such as cell counting with Image J for each treated fish of each group. Group 4, 5μM of 4OHT, displays the highest level of recombination; in some treated fish tissue recombination is nearly achieved in all the periderm cells that are in the tail. Finally it is important to mention that at the beginning of the recombination tests, fish were incubated with 4OHT for 24 hours and not 48 hours as had previously been done, since this period of time was enough in order to get a satisfactory level of cell recombination.

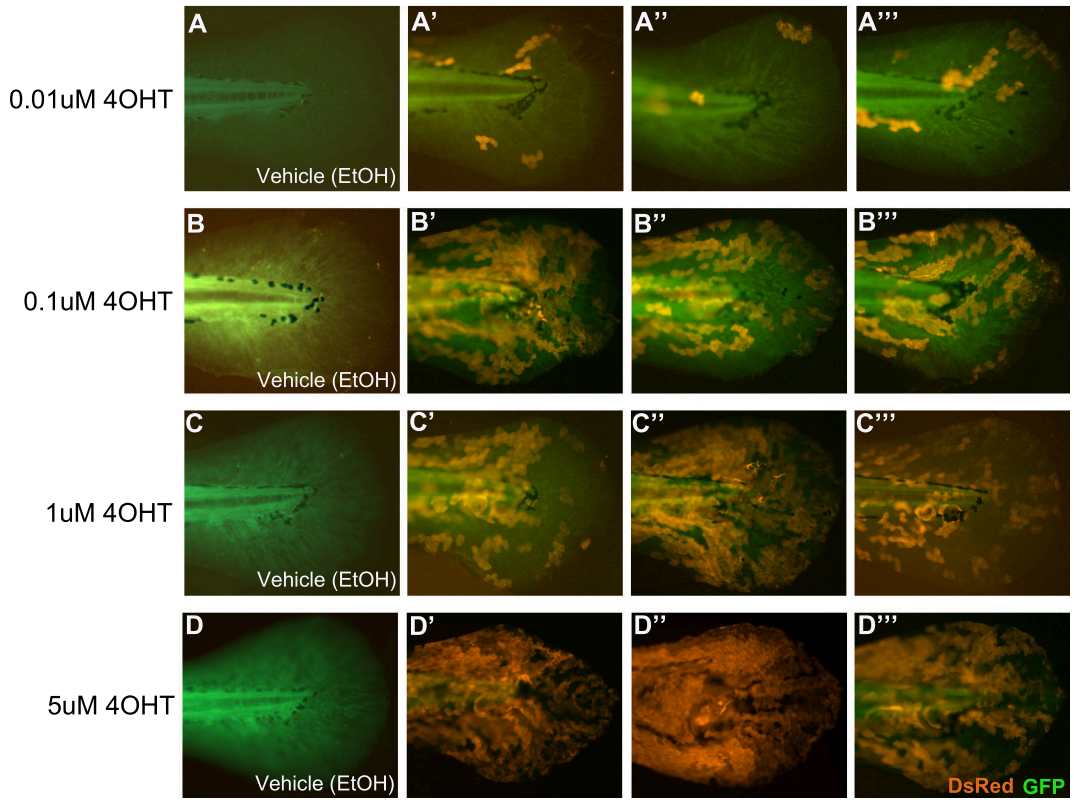

Figure 5-11: Tissue recombination is 4OHT concentration dependent. The *Krt4:ERT2CreERT2;EF1α:loxPGFPloxP-DsRed* line was used for this study. Three examples per treatment are shown in the figure. The chorion was removed in embryos at 75% epiboly stage then were treated with different concentrations of 4OHT. **A'-A"':** 0.01 μM 4OHT-treated embryos present the lowest level of recombination (n=10). **B'-B"':** 0.1 μM 4OHT-treated embryos have a higher level of recombination (n=10) than group **A. C'-C"':** 1 μM 4OHT-treated embryos have a similar level of recombination (n=10) to group **D'. D-D"':** 5 μM 4OHT-treated embryos present the highest level of recombination (n=10). **A-D:** Control groups, in which no recombination is observed. Fish were treated for 24 hours with either 4OHT or Ethanol (n=10 per condition). (Pictures taken with the Tomato and GFP channel).

The importance of this assessment is that it helped me to find a way to control

tissue recombination. Since the majority of my work involved tissue regeneration it was useful to be able to label a few individual cells and track them separately during tail regeneration or be able to label the whole tissue component in order to study it in a more general way.

5.3 Discussion

5.3.1 *Cryaa:Venus* construct was more suitable as a vector than *cmlc:EGFP* .

The first attempt to create the genetic vector was by using the cardio myosin light chain (*cmlc*) as a reporter gene, which will drive the expression of EGFP. Eventually the *cryaa:Venus* vector was used instead, since another lab reported problems with the use of *cmlc*. It was reported to us that the *cmcl* gene, when used as a promoter, had silenced the expression of the other promoter contained in the same plasmid. Therefore we wanted to avoid the risk of *cmcl* silencing the expression of the tissue-specific promoter, which would prevent observing tissue recombination.

5.3.2 Each tissue-specific Cre-expressing line, needs a specific concentration of 4OH tamoxifen in order to observe a satisfactory level of recombination in the whole body

Recombination of the *Tg(fli1α:ERT2CreERT2;EF1α:loxPGFPloxP-DsRed)* can be observed in most of the blood vessels that are present in the zebrafish larvae trunk and jaw, as we can see in Figure 5-4. However there were other areas that presented a high level of recombination as well, such as the thymus and the ventral side of the trunk. Tissue recombination in these areas could be explained by the fact that *fli1a* is not only expressed in the blood vessels' epithelia but also in hematopoietic stems cells (HSC) (Lawson et al. 2002). Chen et al. (2009) reported several HSC production centres that include the thymus and the caudal hematopoietic tissue (CHT), which is located in the ventral side of the zebrafish trunk. However at the beginning of the tissue recombination assessment I observed that muscle cells also presented DsRed expression. This observation first made me think that my promoter have certain degree of leakiness and that was misexpressed in other tissues. Another explanation for this could be that the *fli1a* marker is expressed during early muscle development. However, the closest relation between the HSC, *fli1a* marker and muscle tissue that I found has been reported was that HSC are derived from endothelial cells from somites (Nguyen et al. 2014).

fli1a:ERT2CreERT2;EF1α:loxPGFPloxP-DsRed is a suitable line to track blood vessel development/regeneration, however, it is necessary to keep in mind that recombination is not only restricted to blood vessels, but that erythrocytes, myeloid cells and cells with macrophage morphology can also be labelled.

krt4:ERT2CreERT2;EF1α:loxPGFPloxP-DsRed managed to generate a satisfactory level of cell recombination in epidermis cells of the whole body, when 1μM of 4OHT was added instead of 10μM. No ectopic recombination was observed in either 10μM or 1μM conditions. This result agrees with Gong et al. (2002) who describes that GFP in the *krt4:GFP* line is only expressed in the enveloping outer layer (EVL) cells. During embryonic development the EVL cells, after 3.7hpf, give rise to the periderm, (Kimmel et al. 1990) which in teleost larvae forms the bi-layered epidermis. Since I treated the embryos at 8hpf I can state that the recombinant cells expressing DsRed protein form the periderm and that DsRed is confined to that area only (See Figure 5-5).

Several screenings of the *Tg(αactin:ERT2CreERT2;EF1α:loxPGFPloxP-DsRed)* were done before finding a founder that presented tissue recombination in several muscle stripes along the trunk and close to the tail without ectopic recombination. Unfortunately due to time shortage, lineage-tracing analysis was not performed in this line (See Figure 5-6).

Tg(twist:ERT2CreERT2;EF1α:loxPGFPloxP-DsRed) and *Tg(nestin:ERT2CreERT2;EF1α:loxPGFPloxP-DsRed)* transgenic lines presented a good level of recombination, however, further characterisation still needs to be done in order to find founders or the right 4OHT concentration to see recombined tissues close to the tail (See Figures 5-7 and 5-8).

The Tg(huc:ERT2CreERT2;EF1α:loxPGFPloxP-DsRed) transgenic line presented a satisfactory level of recombination in neurons and axons along the trunk, however, ectopic expression was still observed. Further screening will be necessary in order to find a founder that presents more tissue-specificity recombination (See Figure 5-9).

5.3.3 *Tg(osteocalcin:ERT2CreERT2)* needs further characterisation in order to observe tissue recombination in bone tissue.

The second generation of the transgenic line *Tg(osteocalcin:ERT2CreERT2)* was obtained. However, when crossed to the *EF1α:loxPGFPloxP-DsRed* and adding 4OHT recombination was never observed. We know that the promoter sequence does not have any defects since the previous DNA sequence allow me to confirm this. Furthermore, the *osteocalcin* promoter proved to be functional in adult according to reports from the lab member Monika Tomecka, who saw the expression

of green fluorescent protein in fin rays using the *Tg(osteocacin:kaede)* line.

In the results sections I suggest that it be tested whether the *EF1α:loxPGFPloxP-DsRed* is functional in adult zebrafish. I have observed GFP expression in the adult zebrafish *EF1α:loxPGFPloxP-DsRed* transgenic line, which suggests ubiquitous expression of the *EF1α* promoter at this stage. However, Hans et al. (2009) reports that the *EF1α* ubiquitous expression decreases with age and that, for example, in the brain of the adult zebrafish *EF1α* is not expressed in the whole organ. For this reason I also suggest that tissue recombination of the *Tg(osteocalcin:ERT2CreERT2)* be tested by crossing it with a Lox line that has a different promoter that the *EF1α*.

5.3.4 Tissue permeabilisation and 4OH tamoxifen dosage administration, during tissue recombination

In order to observe tissue recombination I decided to treat the embryos with 10μM 4OHT at the earliest embryo stage reported, which is 75% epiboly (Hans et al. 2009). As we can see in Figure 5-10, recombination is higher when the chorion is removed. However, I did not observe any difference when 1%DMSO was added. This outcome suggests that permeabilisation is key in order to better deliver 4OHT into the cells. Hans et al. (2009)) with the use of the *Tg(pax2a:CreERT2; EF1α:loxPGFPloxP-DsRed* line reported that tissue recombination was dose-dependent. The same test was done using the transgenic line *Tg(krt4:ERT2CreERT2;EF1α:loxPGFPloxP-DsRed)*. Recombination was found to be 4OHT dose-dependent, as can be seen in Figure 5-11. Being able to control the level of cell recombination is important for the lineage-tracing studies during regeneration. For example, the tracking of individual cells during regeneration is necessary in order to analyse cells plasticity and migration. By increasing the concentration of 4OHT is possible to label a bigger amount of cells of a specific tissue, in order to see the behaviour of that tissue in a more broad way during regeneration or development.

5.3.5 Cre-expressing lines had mosaic recombination

Recombination was observed in most of the Cre-expressing lines with tissue-specific promoters crossed with the *EF1α:loxPGFPloxP-DsRed* Lox reporter line (except *Tg(osteocalcin:CreERT2*). However, tissue recombination was always mosaic, which means it was never observed in the whole tissue. One of the explanations for tissue recombination mosaicism could be the use of the EF1α promoter in the reporter line. Hans et al. (2009) reported that the EF1α is not expressed ubiquitously after 24hpf. Another explanation for the mosaicism could be the methods to control Cre activity to a transcriptional level by adding the insulators and the protein level by fusing the Cre with the oestrogen receptor ERT2. For instance,

Kemp et al. (2004) reported this to happen after a decrease of tissue recombination in the *Ah:CreERT2* line in comparison with the *Ah:Cre* transgenic line. In order to reduce tissue recombination mosaicism I suggest the testing of different promoters for the Lox reporter lines and complement this by screening different fish from each Cre-expressing line with a tissue-specific promoter. The screening can help to find adult fish that, when crossed with the Lox reported line, generates offspring where recombination is higher than the current data as described in the *αactin:creERT2* (Section 5.2.3.3). Finally I would also suggest the improvement of the delivery of 4OHT into the cells since, as seen in Figure 5-11, the increase of 4OHT concentration leads to a higher level of recombined cells.

5.4 Conclusion

Seven transgenic lines, driven by tissue-specific promoters, were successfully created and six of them showed tissue recombination. Tissue recombination specificity can be observed in all the lines where recombination is tightly regulated by the administration of 4OHT administration ,except the *Tg(osteocalcin:CreERT2)* line. Zebrafish embryo permeabilization by chorion removal is necessary to achieve a higher level of recombination. Tissue recombination also proved to be dose dependent, which is necessary in order to control the number of labelled cells. These Cre-expressing lines by tissue-specific promoters are an important tool for lineage-tracing analysis during both tissue regeneration and normal development. Finally, I suggest further study of the *nestin* promoter since tissue recombination was observed in chondrocyte-like cells a tissue where *nestin* is not normally expressed.

Chapter 6

Cell lineage-tracing using Cre-expressing lines 4OH Tamoxifen dependent

6.1 Introduction

One of the characteristics of epimorphic regeneration that attracts the attention of researchers in the field of regenerative medicine is the formation of the blastema. The blastema is a structure that is reported (Odelberg 2005) to arise from mature cells that undergo de-differentiation to form a mass of undifferentiated cells. Eventually these cells differentiate again into mature cells that will repopulate an amputated tail in zebrafish. Therefore, epimorphic regeneration displays interesting cellular and physiological behavior (Nechiporuk et al. 2002). Several questions regarding the cell biology during epimorphic regeneration have arisen. For instance, what is the level of cell fate plasticity during tail fin regeneration? Which cells participate during zebrafish tail regrowth? And do stem cells contribute the regeneration process?

With the use of transgenic lines, the lineage-tracing of osteoblast cells has previously been done during regeneration, where two important outcomes have been noted. The first study was done by Knopf et al. (2011) using the intermediate osteoblast marker *osterix* which drives the expression of Cre *(osterix:CreERT2)*. After amputation, osteoblast cells were tracked to reveal that during regeneration these cells de-differentiate and remain lineage restricted (Knopf et al. 2011). The second report done, by Singh et al. (2012) reveals also the possibility that transdifferentiation takes place. For this study a triple transgenic line, Tg (*osterix:CreERT2; β-actin2:LoxP-DsRed-Stop-LoxP-EGFP; osx:NTR*), was generated. The line includes the Cre-expressing line with the tissue-specific promoter *osterix*, the Loxed DNA and the nitroreductase (NTR) gene driven by *osterix*. Nitroreduc-

tase is a substance that, when in contact with Metronidazol (MTZ), causes cell ablation (Pisharath et al. 2009). Therefore cell recombination was performed, obtaining a green fish with red bone rays, and then osteoblast ablation was done by adding MTZ. In order to confirm the absence of osteoblast by using this technique, tissue disaggregation was performed, followed by flow cytometry. No osteoblast were detected in the genetic ablated transgenic fish. The tail was amputated and allowed to regenerate. After 4dpa the regenerated tail managed to form bone rays that did not express the GFP protein. This result suggests the new osteoblast originated from tissues that did not express *osterix*, which opens the possibility of cell transdifferentiation or stem cell participation. Knopf et al. (2011)'s analysis is an important report that shows evidence of osteoblast de-differentiation and lineage restriction, while Singh et al. (2012) also reports the possibility of cell transdifferentiation during tail regeneration of osteoblast. These two characteristics of epimorphic regeneration that have been in debate for many years.

Another aspect that is important to discuss is the lineage-tracing analysis during early development, since new lineage tracing techniques have been used in the past years, as fluorescent proteins driven by tissue-specific promoters or antibody staining. The analysis of lineage-tracing with these tools have shown different outcomes to the current believes, for example Slanchev et al. (2009) analysed cell adhesion of the periderm cells during early zebrafish development by using the *krt4:GFP* transgenic line. The *krt4:GFP* transgenic was used to track periderm cell adhesion and as a side outcome it was observed that periderm cells had a high proliferative rate at 24-48 hours post fertlisation (hpf) (by using BrdU antibody staining). This outcome is in contrast with the reports of Kimmel et al. (1990); Kimmel et al. (1995), where it is stated that after the mid-gastrula stage (3.5hpf approximately), periderm cells slow down cell division, and if it happens it occurs very rarely.

The previous reports regarding cell lineage-tracing emphasise the creation of Cre-expressing lines driven by tissue-specific promoters in order to explore different tissues during tail regeneration and development. Tissue-specific labeling is irreversible and permanent can make a more accessible cell analysis for the study of blastema formation and cell plasticity during tissue regeneration (Singh et al. 2012) as well as the study of early tissue development.

As mentioned in the last chapter I have already generated several transgenic lines with different promoters. With the help of these lines I made two lineage analyses during tail regeneration. The tissues studied were the blood vessels and the periderm, which is the upper layer of the larvae's epidermis (Kimmel et al. 1990). In each experiment, I first labelled the cells, then amputated the tail and studied the behaviour of the labelled cells. The outcomes of this chapter show that after tail amputation both periderm and blood vessels participate in the regeneration of the tail and both remain lineage-committed. However, due

to shortage of time it was not possible to assess the participation of other cells such as stem cells. I could not assess either if the periderm and blood vessels are able to undergo transdifferentiation in order to compensate for the lack of another tissue. The use of other Cre-expressing lines generated during my PhD could help to understand better the cellular features of epimorphic regeneration.

6.2 Results

6.2.1 Angiogenesis is the mechanism by which blood vessels develop and regenerate

After establishing that the *Tg(fli1α:ERT2CreERT2)* transgenic line was able to generate recombination when crossed with a transgenic line with Loxed genes, I decided to perform a lineage-tracing analysis. The first lineage tracing involved the normal development of blood vessels; the reason for this was to have a point of comparison for blood vessel regeneration (which I made after this analysis). At 2dpf I treated the fish with 5μM 4-hydroxytamoxifen (4OHT) for 1.5 days, in order to avoid the labelling of erythrocyte cells or other types of cells that were not blood vessels. Once I observed recombination in the blood vessels, I photographed the progression of this tissue 3 times within 16 days with the fluorescent scope. As seen in Figure 6-1, at 8 days post fertilisation (dpf) it is possible to observe a blood vessel that is growing in the direction to the ventral side of the tail (pigment gap). It was of great interest to label a blood vessel that was growing towards the pigment gap, since this site has been suggested as an important centre of cell proliferation during development and may contribute to tissue regeneration (Kawakami et al. 2004). Later on some of the cells inside the pigment gap give rise to the rays that will form the adult tail (Gavaia et al. 2006). As seen in Figure 6-1, at 14dpf the size of the blood vessel has increased and by 16dpf three branches have developed from the pre-existing blood vessel. This result shows that the normal development of this blood vessel emerges from a pre-existing one. Even though the efforts to avoid the labelling of white blood cells (it is reported that white cells express the *fli1a* gene at early stages of development) did not work. Moreover, the development of blood vessels from white-like blood cells was not observed.

After establishing that blood vessel development happens through angiogenesis by using the Cre-Lox system, I decided to make a lineage-tracing analysis during blood vessel regeneration. I treated the fish with 10μM 4OHT at 75% epiboly. At 3dpf, when blood vessel recombination was appreciated, I performed tail amputation. As seen in Figure 6-2, at 2dpa the regenerated blood vessel has reached the end of the tail, then at 3dpa the blood vessel has managed to surround the end of the tail, and finally at 5dpf the new branches have emerged from the regenerated blood vessel. The neovascularisation of the amputated blood vessel also

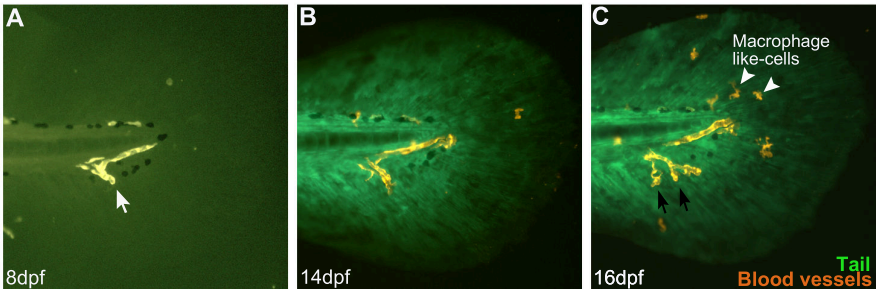

Figure 6-1: Blood vessel development tracking. Blood vessel development was tracked during 10 days. A blood vessel emerging from the pigment gap is labelled by a pulse treatment of 5 μl 4OHT treatment at 2dpf where expression of the DsRed protein is observed at 8 dpf in the blood vessel(white arrow) (**A**). Later on this blood vessel gives rise to new branches, as can be can be observed in **B** and **C** (black arrows). It is possible to observe recombinant macrophages like-cells (white arrowheads).(Pictures taken with the Tomato and GFP channel). 1 representative embryo was chosen for the whole panel. N=3. n=13.

takes place through angiogenesis, in a similar way to as observed during blood vessel development. Regeneration by angiogenesis could be used as a model where regeneration takes place from the same pre-existing tissue. It can also be used as a point of comparison in case that is found a different type of tissue regeneration, such as stem cell participation or tissue transdifferentiation (the conversion of one type of tissue into another).

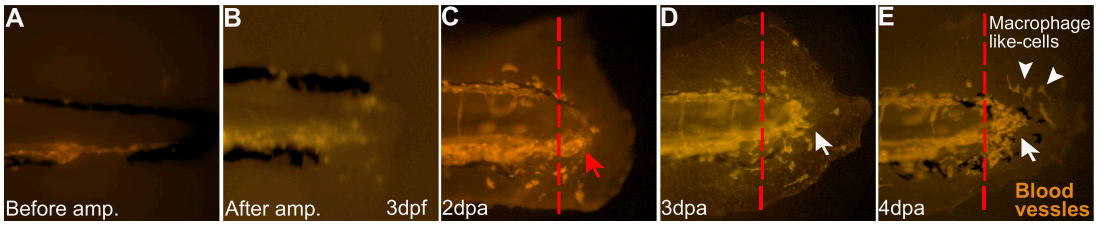

Figure 6-2: Blood vessel tracking during tail regeneration. A single fish was treated with 10 μM 4OHT at 75% epiboly stage, tail amputated at 3 dpf (panel **A** and **B**) and tracked during regeneration. During tail regeneration it is possible to observe the blood vessel regrowth at 2 dpa (red arrow in panel **C**). This blood vessel eventually gives rise to new branches of blood vessels at the tail tip, white arrows in **D-E** panels. The red dashed line indicates the plane were amputation was done. It is possible to observe recombinant macrophages like-cells (white arrowheads).(pictures taken with the Tomato channel).One representative embryo was chosen for the whole panel. n=10. N=3.

6.2.2 The regenerated tail has recombined (labelled) periderm cells

The next tissue to examine during tail regeneration was the periderm. For this study I decided to use the double transgenic line *Tg(krt4:ERT2CreERT2;Ubiquitin:LoxPGFPLoxP-mCherry)*. A single fish was treated with 1μM 4OHT at 1 day post fertilisation (dpf) for 2 days. At 3dpf I observed the expression of the mCherry fluorescent protein in the periderm cells, and at 4dpf tail amputation was performed. The 4dpf stage was suitable for analysing periderm cell behaviour since, according to Kimmel et al. (1990), the number of periderm cells after gastrulation remains the same (no cell division) until metamorphosis. As seen in Figure 6-3, at 5dpa the number of periderm cells that express the mCherry fluorescent protein and that populate the regenerated tail is greater than before tail amputation. This result suggests that periderm cells, in order to replenish the previous periderm cells, undergo cell proliferation during tail regeneration. However, the possibility of cell migration, transdifferentiation or elongation is still not excluded. Finally, in order to confirm that the mCherry-expressing cells are actually periderm cells, I would propose a double in situ hybridisation (ISH) to detect the expression of *mCherry* and *krt4*. To check the specificity I would expect to see colocalisation of the*mCherry* protein only in cells expressing *krt4*.

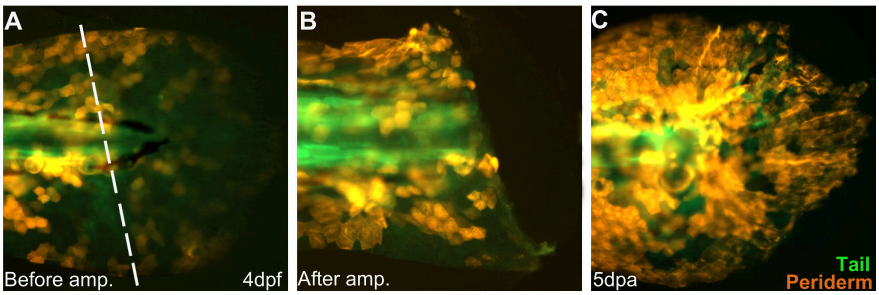

Figure 6-3: Lineage tracing of mature skin cells after tail amputation. A single fish was treated with 1 μM of 4OHT at 1 day post fertilization, tail amputated at 4 dpf (white dashed line) (**A**) and tracked during regeneration. Panel **B** shows tail amputation at 4dpf (0hpa). Fish almost completes tail regeneration at 5 dpa (**C**).(pictures taken with the Tomato channel and GFP channel).One representative embryo was chosen for the whole panel. n=10, N=2.

6.2.3 Colocalisation of the phosphorylated histone H3 protein in cells expressing mCherry suggest mitosis in periderm cells

Kimmel et al. (1990) reported that periderm cells are proliferative until the mid-gastrula stage. Once the embryo reaches gastrulation, periderm cells stop being

proliferative and spread to cover the zebrafish embryonic body. However, Figure 6-4 shows that mature periderm cells undergo proliferation during cell regeneration. In order to have a better insight into cell behaviour during tail regeneration I decided to test the level of cell proliferation during periderm regeneration by using a double antibody staining technique that allows observation of periderm cells and proliferative cells in the same sample. Embryos were treated at 75% epiboly with 1μM 4OHT for 24 hours and at 4dpf I induced tail regeneration through amputation. At 48hpa fish were fixed for immunostaining analysis to detect mCherry (expressed in the peridem cells showing recombination) and to also detect the phosphorylated histone H3 (pH3) protein, which is a cell mitosis marker (Hans et al. 2001). I also kept an un-amputated group of fish to use as control in order to compare the cell proliferation in periderm cells with periderm cells in amputated tails.

As seen in Figure 6-4, in both groups, amputated and un-amputated tails periderm cells presented nuclei with the phosphorylated histone 3 protein, which is an indication of cell proliferation. This result contrasts with Kimmel et al. (1990)'s reports, which mention the periderm cells lack of cell proliferation. Knowing that in both amputated and un-amputated tails the periderm undergoes cell division. I decided to measure the level of proliferation in each group. For this I chose to count the periderm cells with phosphorylated histone-3 protein in each group and then make a comparison between both amputated and un-amputated groups. As seen in Figure 6-5, the amount of cell division observed in periderm cells is higher in most of the tail-amputated organisms in comparison with the un-amputated ones. This result suggests that periderm cells undergo cell proliferation during normal development and that this increases once regeneration is induced. Finally it is important to mention that this was a preliminary study, the main purpose of which was to show that the detection of both mCherry and PH3 was possible. An improvement of the technique along with the use of a higher magnification lens in the confocal microscope can help to better identify the cell type to be studied.

6.3 Discussion

6.3.1 Blood vessel regeneration and development take place by angiogenesis

The results in Figures 6-1 and 6-2 show that the origin of cells during blood vessel development and regeneration are from pre-existing blood vessels. This process is called angiogenesis. This finding concurs with Huang et al. (2003)'s assertion that with the use of the *Tg(fli1a:EGFP)* line allows lineage-tracing of blood vessels during regeneration. The analysis describes that angiogenesis is the mechanism by which vascular regeneration happens in adult zebrafish tails. The

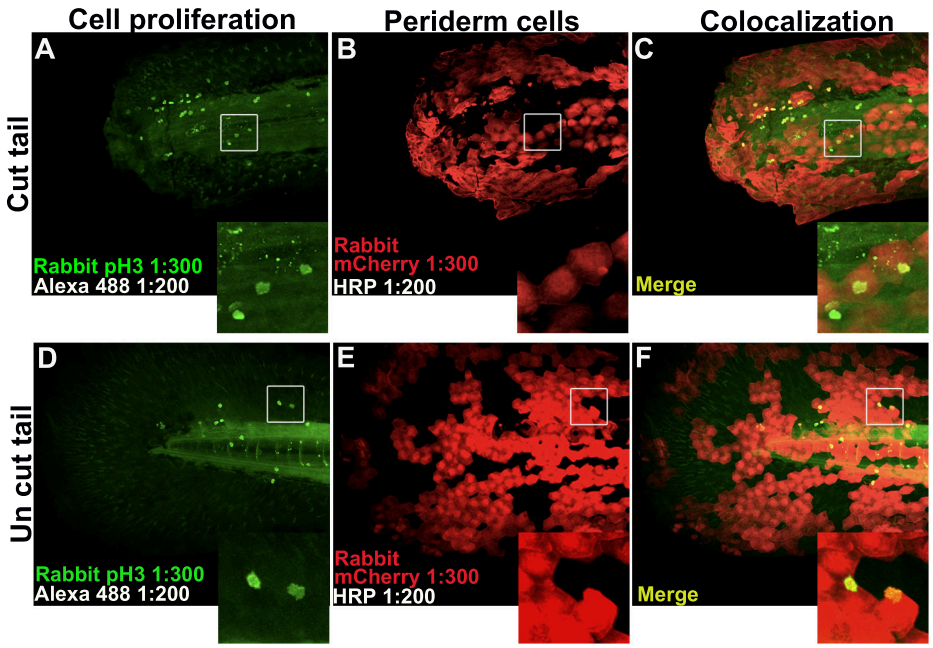

Figure 6-4: Cell proliferation in periderm cells. Embryos are treated with 1 μM 4OHT at 75%. Recombination is observed at 3 dpf and tail amputation is performed at this age. Embryos are allowed to regenerate for 48 hours and then are fixed and processed for double immunostaining against pH3 (cell division) and mCherry (tissue recombination) (secondary antibody Alexa 488 and HRP). Phosphorylated histone H3 protein (pH3) is detected in the nucleus of both groups cut and un-cut (**A** and **D**). Tissue recombination in the periderm cells is detected by the mCherry antibody (**B** and **E**). Panels **C** and **F** show an overlap of both staining. Recombinant periderm cells (mCherry) have green fluorescent nuclei (detection of pH3), which is a sign of cell division. Amplification of the stained cells is shown at the bottom right of each picture. A representative embryo was chosen from the un-cut and cut group. One representative embryo was chosen per condition. n=5 per condition. N=1. Fish imaged with the confocal Olympus microscope.

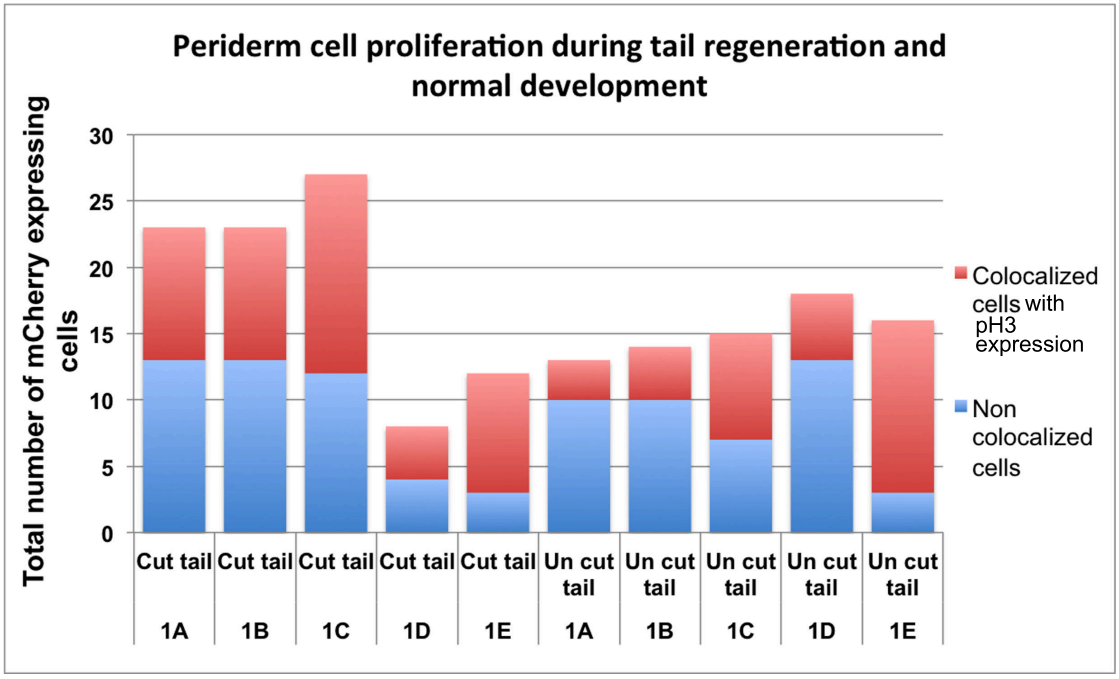

Figure 6-5: Cell division in periderm cells is higher in amputated tails than un-amputated. Stacked bar chart showing the number of proliferative peridem cells in each of the amputated and un-amputated tail fish, blue bar represent mCherry expressing cells and red bar represents mCherry expressing cells colocalized with pH3 staining. **1A cut:** Non colocalised cells: 13, colocalised cells 10. **1A uncut:** Non colocalised cells: 10, colocalised cells 3. **1B cut:** Non colocalised cells: 13, colocalised cells 10. **1B uncut:** Non colocalised cells: 10, colocalised cells 4. **1C cut:** Non colocalised cells: 12, colocalised cells 15. **1C uncut:** Non colocalised cells: 7, colocalised cells 8. **1D cut:** Non colocalised cells: 4, colocalised cells 4. **1D uncut:** Non colocalised cells: 13, colocalised cells 5. **1E cut:** Non colocalised cells: 3, colocalised cells 9. **1E uncut:** Non colocalised cells: 3, colocalised cells 13. 3 tail amputated fish out of 5 showed a higher level of proliferation than the proliferation detected the periderm cells of the un cut tails. Cell quantification was done with image J software.

report also mentions that during regeneration a web of vascular tubes forms a plexus that later on disappears into a normal vascular pattern when regeneration is completed. This observation is similar to results observed during regeneration shown in Figure 6-2. At 5dpa, at the end of the regeneration period, several vascular branches protrude, forming a small web. However my findings and the previous report do not exclude the possibility that stem cells travelling through the circulatory system could also participate and replenish the epithelium of blood vessels. Permanent and irreversible cell labelling of stem cells (with the use of the Cre-Lox system) could help us to better understand the possible participation of these cells during regeneration in general.

For the assessment of stem cells participation I would propose the use of the perivascular cells (PVC) that express the *stromal cell-derived factor* (*sdf1*) as a way to assess stem cells participation during tail regeneration. PVC cells in zebrafish have been isolated and shown characteristics of human mesenchymal stem cells (hMSC) such as differentiation in several tissues as osteocalst, chondrocyte and adiposite-like cells besides of the expression of hMSC markers (Lund et al. 2014). *sdf1* makes the study of PVC cells very attractive since this gene is also expressed in the blastema during tail regeneration (Bouzaffour et al. 2009). For the assessment of stem cell participation I would track PVC cells with the use of a Cre-expressing line driven by the *sdf1* marker. I would assess if PVC cells participate in the formation of the blastema or/and contribute to tissue formation during tail regeneration in a way independent of the blastema.

In epimorphic regeneration, after the formation of wound epidermis proliferative cells next to the wound area migrate and form the blastema, a mass of cells that is thought to be undifferentiated (Nechiporuk et al. 2002). It is thought that blastema cells later on differentiate into the tissues that will regenerate the amputated structure, although the fate of the cells that form the blastema has not been fully assessed (Kawakami 2010). Knopf et al. (2011), with the use of genetic fate mapping, observed that during adult tail regeneration mature osteoblast de-differentiate, migrate and form part of the blastema, to then differentiate again to form bone that will regenerate the fin ray. However, when I did the lineage-tracing of blood vessels during larval tail regeneration there was evidence that the only source of new blood vessels was pre-existing blood vessels and not a blastema structure. Given the current outcomes and in the light of new lineage-tracing tools it is important to analyse the function of the blastema. Besides, blastema cell origin and fate should be assessed in order to evaluate the concept of blastema. Finally it is important to assess if the blastema acts as a centre that influences cell de-differentiation in surrounding tissues during tail regeneration, if it is the case that the participation of blastema cells for tissue regeneration is minimum.

To make this analysis I will suggest the tracking of blastema cells expressing the *raldh2* marker during epimorphic regeneration by using the Cre-Lox system or

a fluorescent protein driven by this gene. *raldh2* has been described as necessary for the blastema formation and proliferation and is expressed in most of the larval tail regeneration process (Mathew et al. 2009). I suggest the inducement of tail regeneration by amputation and the tracking of *raldh2*-expressing cells, then observe if these cells contribute to blastema formation and their final fate (if they differentiate into different tissues). I would also suggest performing genetic ablation in *raldh2*-expressing cells during regeneration and evaluate if de-differentiation in surrounding tissues is affected or not. This experiment will help me to assess the classical model of the blastema that, in light of modern lineage-tracing techniques, should be evaluated for function cell composition and fate.

6.3.2 The presence of periderm recombinant cells in the new tail after amputation suggests that these new skin cells come from pre-existing ones

The use of the *Tg(krt4:ERT2CreERT2; ubiquitin: LoxPGFPLoxP-mCherry)* during regeneration allowed me study the cell behaviour of the periderm cells during normal development and regeneration (Figure 6-3). During normal development it was not expected to see proliferation in the periderm cell, as reported by Kimmel et al. (1990), who mentioned proliferation in periderm cells slow down after the mid-gastrula stage and occurs very rarely if at all. Because of this report, unamputated tails were used in my study as a negative Control with the expectation that proliferation would not be present. Nonetheless, the immunostaining to assess proliferation in the peridem cells revealed that during normal development these cells still undergo proliferation in 5 day old fish (See Figure 6-4). This results concurs with an observation made by Slanchev et al. (2009) that, during the study of cell adhesion in the zebrafish epidermis, BrdU immunostaining detection showed that periderm cells also divide. However, proliferation in both studies (Slanchev et al. 2009; Kimmel et al. 1990) was assessed in fish at the stages of 24 and 48hpf, where Kimmel et al. (1990) mentions that the final fate of periderm cells still needs to be analysed. Additional to my findings that periderm cells divide during normal development, there is evidence in my studies to show that periderm cell division increases during tail regeneration (See Figure 6-5). This result raises new questions about whether the origin of this new periderm cells is exclusively coming from pre-existing periderm cells or also basal epidermal cells. The use of the transgenic *Tg(krtt1c19e: CreERT2)* with the basal epidermal cell marker could help us to track the participation of these cells during regeneration. Also, it is important to know if lack of basal epidermal cells is detrimental for periderm regeneration and maintenance or not. This could be done by using the dominant-negative Np63 conditional mutant (p63 is a marker of the basal epidermal cells). The p63 gene is disrupted in the mutant. Both lines have reported to be used by Lee et al. (2014).

Finally, in order to complement the result obtained by the double antibody staining, it is necessary to make an image analysis with more detail, where the scan section of the periderm cells is of a size that is as small as the length of the periderm cell or even smaller. Scan sections under these parameters can help us to see whether individual cells expressing the DsRed protein are also expressing pH3. Therefore the section of the periderm cells and identification of PH3 and DsRed antibodies staining in one cell could reinforce the finding that periderm cells are undergoing proliferation.

6.4 Conclusion

The main goal of the research explained in this chapter is to assess the cellular characteristics of epimorphic regeneration. For this I suggest three outcomes. First, cells remain lineage-restricted; second, transdifferentiation occurs (mature cells convert into different cell type) and; third, there is stem cell activation (undifferentiated cells participate during tail regeneration). With the use of the Cre-expressing lines with tissue-specific promoters I managed to perform lineage-tracing for two tissues, the periderm (upper layer of the embryonic epidermis) and the blood vessels. Both outcomes showed that regeneration of these two tissues remains lineage-restricted. However, there is still possibility that transdifferentiation may happen and that stem cell participation cannot be discounted. Another interesting outcome observed with the Cre-Lox system is that periderm cells actually divide during normal development, which contradicts the current literature (Slanchev et al. 2009) (see Figure 6-4). It is important to mention that even though these tissues remain lineage-committed it does not exclude the possibility that they undergo transdifferentiation to compensate for the lack of any other tissue. Therefore the use of Cre-expressing lines with other tissue-specific promoters, for example the *pax7* marker of satellite muscle cells (Seger et al. 2011), and genetic ablation could help to analyse this possibility. A better understanding of the cell biology and plasticity during tissue regeneration can contribute to the development of new cell-based therapies for tissue regeneration in humans.

Chapter 7

General Discussion and Conclusion

7.1 General discussion and future directions

Tissue regeneration is an essential mechanism that is present throughout the animal kingdom. Regeneration helps the body to maintain tissue homeostasis, and is present to different extents depending on the organism (Kawakami 2010). The organism used to study tissue regeneration during my PhD research is the zebrafish, as it presents an interesting type of regeneration called epimorphic regeneration. Epimorphic regeneration has been widely studied in several organisms and a general understanding of the process has been described already by Tal et al. (2010). After amputation, the injury closes and a structure called wound epithelium forms along the area. Adjacent cells then migrate to the amputated plane in order to form the blastema, which, is a mass of cells that are thought to be un-differentiated. This cell mass proliferates and then differentiates in order to complete tissue restoration (Kawakami et al. 2004). The molecular pathways that govern epimorphic regeneration such as Hh (Hedgehog), Wnt, FGF (Fibroblast Growth Factor) BMP (Bone Morphogenetic Protein) and the morphogen RA are also important during early development (Yoshinari et al. 2011;Mathew et al. 2009;Poss et al. 2000b;Stoick-Cooper et al. 2007). The relation between these molecular pathways, what trigger their activation, and the order in which they are expressed, still need to be analysed. Knowing this, I decided to research the role of Hh, Wnt, FGF and RA during regeneration, in order to see whether there is a pathway hierarchy or if they act in a parallel fashion. I also compared the role of these pathways with the normal early development in zebrafish tails.

To make a molecular dissection of the regeneration process, regeneration was induced through tail amputation.Investigation into the process of regeneration with both tail bud formation and tail regeneration were performed with the help of small molecule compounds and heat shock transgenic lines. The molecular pathways previously mentioned were manipulated during tail regeneration and

bud formation. The reason was to see whether these pathways have the same role in both tail development and regeneration following an injury. The expression of the regeneration marker genes and pathway readouts was assessed with whole mount ISH.

A parallel study to the molecular dissection projection was done which entailed the dissection of cellular mechanisms during epimorphic regeneration. There are several questions regarding this process. Which cell lineages are involved? What is the level of cell plasticity? Is there stem cell participation or not? In order to answer these question I decided to design 5 transgenic lines expressing Cre, and test a total of 7 Cre lines with tissue-specific promoters, where Cre recombination is dependent on 4OH Tamoxifen administration. To investigate these questions I induced Cre recombination by administrating 4OH Tamoxifen, and when tissue recombination was observed I performed tail amputation. Finally I tracked the recombinant cells during the regeneration process. Regarding the analysis of stem cells, my original plan was to use the Cre-expressing line driven by *sox10* promoter. This line had already been synthesised and tested for recombination previously in Roehl's lab. *sox10*-expressing cells are multipotent cells occurring during neural crest development, which means that they can differentiate into several types of tissues such as melanocytes, glia cells, neurons and craniofacial cartilage (Kelsh 2006). Unfortunately due to time shortage an assessment of stem cell-like participation by using the *Sox10:ERCreER* transgenic line was never performed.

7.1.1 Developmental pathways during regeneration

Time courses following amputation were done at 6, 12, 18 and 24hpa in order to observe the stages of Hh and Wnt readouts. As we can observe in Figures 3-8 and 3-14, the ligand *ihh* is expressed 12 hours before the Wnt readouts *wnt10* and *tcf7*. These results suggest that Hh signalling appears before Wnt and that it could be upstream of Wnt. This suggestion is confirmed in Figure 3-9, which shows that Wnt signalling cannot affect the *ptc1* Hh marker gene during Wnt down-and up-regulation. On the other hand, it was observed that *tcf7* and *lef1* Wnt markers are completely down-regulated when treated with cyclopamine, as seen in Figure 3-15. Finally, the epistasis analysis presented in Figure 3-16 shows that Hh is upstream of Wnt during tail regeneration. Regeneration marker genes are expressed despite being continuously treated with cyclopamine while Wnt is up-regulated. The relationship between Hh and Wnt pathways in larvae tail regeneration contrasts with previous findings in tail regeneration of the adult zebrafish (Wehner et al. 2014). Wehner et al. (2014) showed that during *axin2* and *dkk1* up-regulation (Wnt pathway down-regulation), *ptc2* expression is absent. The expression of*ptc2* can then be observed when Hh signalling is up-regulated by SAG (smoothened agonist) administration. Nonetheless, Singh et al. (2012), reported the opposite to Wehner et al. (2014) during salamander limb regeneration by using

an activation-inhibition method. The method consisted on Hh signalling down-regulation by cyclopamine administration while Wnt signalling was activated by the BIO compound (Gsk3 inhibitor) continuously during limb regeneration for 60 days. Finally sole cyclopamine treatment in amputated salamander limb blocked regeneration. This finding concurs with the hierarchical model of Hh signalling being upstream of Wnt signalling during larval tail regeneration.

Another important aspect of the role of Hh signalling I wanted to assess was whether Hh signalling was needing during the whole tail regeneration process or just for a certain time period. To test the time for Hh to be activated during tail regeneration I down regulate Hh signalling 12 hours, before amputation by continious cyclopamine administration. Then I amputated the tail and put the fish back to cyclopamine for another 12 hours. The first experiment I did blocked regeneration unfortunately, the repetition of the experiment did not gave me the same result, even when I aimed to to treat the fish for 24 hpa with cyclopamine. Singh et al. (2012) aimed to find the same question regarding the time of Hh signalling needed for regeneration. By down regulating Hh signalling with cyclopamine at different stages during limb regeneration it was found that Hh signalling is essential during the first 14 days post-amputation. Given the results found by Singh et al. (2012) regarding the timing of Hh signalling and the hierarchical position regarding to Wnt I suggest to test again the requirements for Hh signalling during tail regeneration.The experiment I suggest is to find the period of time where the *ptc1* gene (Hh readout) initiates expression during regeneration and when is down regulated. Once the *ptc1* expression time period is known, fish will be treated with cyclopamine during the length of *ptc1* expression. I would expect that the down regulation of Hh signalling during *ptc1* expression during tail regeneration to be a reliable parameter to determine the length period where Hh needs to be active to complete tail regeneration. Regarding the relation between Hh and FGF pathways, Hh appear to regulate the *fgf10a* gene in a more direct way than Wnt signalling, as shown in Figures 3-17, 3-18 and 3-19. Additionally, Hh appears to regulate proliferation through FGF signalling, as shown in Figure 3-21. This last outcome concurs with Wehner et al. (2014), where cyclopamine administration down-regulates proliferation in adult zebrafish tail regeneration. To determine of Hh signalling has a direct role over FGF signalling during proliferation in the tail regeneration process, the use of the ChiP-sequence (chromatin immunoprecipitation) analysis.The ChiP-sequencing analysis could help to observe any DNA protein binding between Hh signalling transcription factors and FGF genes that would suggest a direct function of Hh over FGF signalling.

The relation between the Wnt and FGF pathway during regeneration has been widely studied. For adult zebrafish tail regeneration, it has been suggested that Wnt signalling directly regulates *raldh2*, independent of FGF pathway participation (Wehner et al. 2014). On the other hand Mathew et al. (2009) has shown that *raldh2* is regulated by FGF signalling during fin fold regeneration in larva

zebrafish. FGF in turn is regulated by Wnt pathway. The results shown in Figure 3-24 indicate that both Wnt and FGF can regulate *raldh2*. However, *raldh2* down-regulation by SU54 (FGF inhibitor) cannot be up-regulated by Wnt signalling. To have a better understanding about the relation between Wnt and FGF during regeneration, complementary experiments need to be done. For example, is it necessary to up-regulate FGF signalling (with the use of a transgenic line or chemical inhibitor) and down-regulate Wnt signalling during tail restoration. For this, a good candidate is the IWR-1/2 Wnt inhibitor. This could help us to know whether there is a hierarchical role between FGF and Wnt signalling, which regulates the expression of *raldh2* in tail regeneration.

To analyse the similarities and differences of the molecular mechanism in tail development and regeneration a comparison of the developmental pathways Hh, Wnt, FGF and RA was performed in both processes. Different chemical compounds and heat shock transgenic line were used to manipulate the molecular pathways of interest. It was found that Hh is not essential during tail development, as is during tail regeneration, as demonstrated in Figure 4-1 and 4-2. Nevertheless, Wnt pathway appears to be vital for both processes, as shown in Figure 4-3. The Wnt pathway evaluation was only a morphological assessment, but it was nevertheless still necessary to see how manipulation of Wnt signalling affects the expression of regeneration marker genes, Hh and FGF readouts. Finally the relationship between FGF and Wnt signalling during the modulation of *ralhd2* expression in tail bud formation appears to be different to the outcome during tail regeneration. Both FGF and Wnt regulate *raldh2* during tail development. However, up-regulation of Wnt signalling in embryos that were treated with SU54 did not cause any changes in the expression of *ralhd2* in comparison with the control (vehicle-treated) as shown in Figures 4-4 and 4-5. This suggests that FGF and Wnt operate in parallel. *raldh2* expression when Wnt signalling is down-regulated at the same time that FGF is up-regulated could confirm this result.

Late stages of regeneration were also analysed in order to evaluate the re-differentiation process. It was found that *myod* was re-expressed sequentially after *raldh2* and *dlx5* regeneration marker expression (Figure 4-6). Woloshin et al. (1995) and Odelberg et al. (2000) state that the ectopic expression of the *Msx1* gene (orthologue of the *msxc* gene) suppresses the expression of the myogenic regulatory gene *Myod* in mouse myotubes and fibroblasts. Therefore, even though there is not a time lapse of the expression of *msxc*, I had shown that *msxc* is expressed during larval tail regeneration and it could be that during later stages of zebrafish regeneration *msxc* is no longer necessary and so the expression is decreased. The decrease in *msxc* then favours *myod* gene re-expression (as during normal development) and allows muscle precursor cells to re-differentiate into mature muscle. I also analysed the neurogenesis marker *huc* by antibody staining during tail regeneration. However no expression was observed in the regenerated tail. This was an interesting outcome since my Masters research showed that after tail regenera-

tion the tail was able to move and sense the external environment. This outcome indicates that the nervous system was able to regenerate and is functional. There could be different explanations for the lack *huc*-expressing cells detection during tail regeneration. The first explanation is that the source of neuron cells is not *huc*-expressing cells but neural stem cells. The *nestin* marker has been reported to be expressed in stem cell-like cells in zebrafish (Chen et al. 2009) and could potentially be the source of regenerating neurons as observed during early neural development in mice (Yamaguchi et al. 2000). The second explanation is that differentiated neurons actually contribute to the regeneration of the spinal cord as reported by Goldshmit et al. (2012) in the adult zebrafish. However, a better cell tracking method is necessary where the *huc:ERcreER* can be used for irreversible labelling to track *huc*-expressing cells during zebrafish spinal cord regeneration.

The data shown here regarding molecular dissection during tail regeneration shows that Hh signalling governs regeneration through Wnt and FGF signalling. However, analysis of the relationship between FGF and Wnt is pending. Manipulation of Wnt and FGF during the expression of *raldh2* in tail regeneration (Chapter 3) suggested that Wnt is upstream of FGF. Up-regulation of FGF signalling and down-regulation of Wnt signalling (by either using heat shock transgenic lines or chemical compounds) during the expression of *raldh2* could further confirm the result that Wnt is upstream of FGF signalling during regeneration. Another pending assessment is that regarding whether Hh signalling has a specific time of expression that is crucial during regeneration. Further time course of the Hh marker *ptc1* can show the time where Hh is expressed during regeneration. Cyclopamine administration at the times were *ptc1* is expressed during tail regeneration might help to determine whether Hh signalling has a specific time frame of activation and how crucial this may be during regeneration.

7.1.2 Cell lineage tracing during regeneration

Seven transgenic Cre-expressing lines with tissue-specific promoters, with observable Cre recombination (Section 5.2.3), were tested. Of the seven lines five were created by me from the genetic construct and raised into the second generation. Cre recombination was tightly regulated by 4OHT (Chapter 5 Section 5.2) and tissue recombination was shown to be 4OHT concentration dependent (Section 5.2.3). Another important aspect of 4OHT administration is that permeabilisation following chorion removal was shown to generate a larger number of recombinant cells (Figure 5-10). It would be interesting to test solvents that improve permeabilisation at a cellular level to make 4OHT administration more effective, as this could increase the experimental success and cost effectiveness. Also, it is important to mention that the methods to avoid spontaneous recombination in the absence of 4OHT worked. This methods consisted of the control of Cre function on a transcription level or protein level. To control the transcription of Cre, a pair

of DNA sequences called insulators were paired next to the ERCre section. The insulator function is to avoid DNA transcription by distant enhancers (Burgess-Beusse et al. 2002). The other method to control Cre activity was to pair the Cre molecule with two oestrogen receptors (ER) instead of one as traditionally done. This further restricts the Cre molecule's ability to get inside the nucleus in the absence of 4OHT. Both methods employed to avoid Cre leakiness worked well, but on the other hand I believe that such restrictions resulted in tissue recombination mosaicism (where tissue recombination is not observed in the whole tissue). However, as I mention previously, the number of recombinant cells can also be influenced by the concentration of 4OHT administrated.

After generating the transgenic lines and confirming that recombination was dependent on 4OHT administration, cell lineage tracing was done. The cell lineage tracing was performed in two tissues. The first was of periderm cells, which form the upper layer of the embryonic epidermis (Kimmel et al. 1990), and the second was the blood vessels. The transgenic lines used for the lineage analysis were *krt4:ERT2CreERT2;ubiquitin:loxPGFPloxP-mChery* (*krt4* as periderm tissue marker) and *fli1α:ERCreER;EF1α:loxPGFPloxP dsRed* (*fli1α* as blood vessel tissue marker).

To study normal blood vessel development, a cell lineage tracing was performed. Blood vessel development was observed from 8 dpf until 16 dpf. The blood vessel lineage tracing confirmed that blood vessel development takes place by angiogenesis, as shown in Figure 6-1. This mechanism was previously reported by Risau (1997) in a 3 day old quail development. Subsequently blood vessel development was used as a control in order to compare it with blood vessel regeneration. Tail regeneration was induced by tail amputation at 3 dpf and tracked until 5 dpa. The final result showed that blood vessel regeneration takes place by angiogenesis (Figure 6-2), which means that blood vessel regeneration remains lineage committed. This result is similar to a previous lineage tracing experiment where adult amputation was done using the *fli1a:GFP* line (Huang et al. 2003). It is important to mention that, during the performance of both experiments, macrophage-like cells were labeled. However, it was not observed that this type of cells could give raise to blood vessels.

To track periderm regeneration, the same model was utilised, where the unamputated fish served as control. The number of labeled periderm cells was larger in the regenerated tail than before amputation (Figure 6-3). This result suggests that periderm cells participate in tail regeneration and most probably undergo proliferation. Cell division is an important aspect to consider, since the traditional belief is that periderm cells do not experience cell division after mid-gastrulation (Kimmel et al. 1990) or if does it is very rare. However, the report was based on fish 48 dpf where it is stated that the final fate of periderm cells in zebrafish still should be analysed. A double antibody staining, to detect the mCherry protein

expressed in the periderm cells and the phosphorylated histone 3 protein (PH3) which indicates cell division proliferation, was performed. The colocalisation of both labels indicates that that the periderm cell is undergoing cell division. The result showed that periderm cells in tails where amputation was not induced had cell division (Figure 6-4) which was unexpected. In support with this finding Slanchev et al. (2009) by using BrdU immunodetection observed the same process in a time lapse analysis in the larva zebrafish periderm cells, where it was obseved a increased in cell proliferation at 24 and 48 hpf. Cell division was also detected in periderm cells where the tail was amputated (Figure 6-4), so a cell counting was done in order to see any difference in the number of proliferative cells between amputated tails and un-amputated tails. It was observed that the total number of proliferative cells in a group of 5 fish was higher in amputated tails than in a group of 5 tail un-amputated fish (Figure 6-5). Altogether these results show that periderm cells undergo cell division after mid-gastrulation, that they participate in tail regeneration, and that they remain cell lineage committed.

The lineage-tracing analysis of both periderm cells and blood vessels generated interesting outcomes regarding the concept of blastema formation and wound epidermis. The blastema is thought to be made up of de-differentiated and highly proliferative cells, which migrated from surrounding tissues after amputation to form the blastema (Kawakami 2010). One can determine the location of the blastema by observing the expression of the *raldh2* and *msxc* regeneration marker genes (Mathew et al. 2009;Akimenko et al. 1995). However, the expression of these genes during regeneration does not match the areas where proliferation takes place, since the area of proliferation is wider than the area of blastema marker gene expression (Figures 3-20 and 3-3). Secondly the blastema is supposed to be the supplier of the tissue that will regenerate the tail, but in the case of blood vessels they regenerate by angiogenesis and not from the blastema-like structure, as shown in Figure 6-2. However, it has already been reported that the blastema is actually vascularised where blood vessels are needed for the completion of tissue regeneration (Pfefferli et al. 2015). Given the present outcome and the previous reports I think is very important to pay attention to the role of blood vessels during regeneration as I suspect that blood vessels could provide stem cells to the blastema to support regeneration. The reason for this is that the perivascular cells (PVC) which are found in the blood vessels, and express the *sdf1*, have been found to have mesenchymal stem cell characteristics (Lund et al. 2014). Besides, the *sdf1* marker is also expressed in the blastema (Yoshinari et al. 2011). Secondly, since the blood vessels vascularise the blastema, another of the function of the blood vessels could be that they supply nutrients and growth factors that support tissue regeneration. It would be interesting to assess to what extent blood vessels are required to complete tissue regeneration. It is known that when angiogenesis is suppressed regeneration is not completed in adult zebrafish tails (Bayliss et al. 2006). It would also be interesting to assess the function of blood vessels during

regeneration and re-analyse the traditional concept of blastema in the light of new reports.

Finally, periderm cells seems to have a behaviour similar to blood vessels where pre-existing periderm cells are the source of new periderm cells during tail regeneration (see Figure 6-3). Further, periderm cells do not seem to contribute to the formation of the blastema since cell proliferation was observed in a broader area than the expression of regenerative marker genes (see Figures 3-3 and 6-4). An explanation for this is that periderm cells in larval regeneration contribute solely to the formation of wound epithelium.

A different approach to confirm that both periderm and blood vessels remain lineage restricted during tail regeneration would be to perform a triple antibody staining for the PH3 protein, the Cre molecule and the mCherry protein. The reason for this is that the Cre molecule is reported to go to the nucleus after 4OHT administration (Feil et al. 1997), so colocalisation of the three proteins in the same cell will reinforce the previous findings that the recombinant cell undergoes cell division. However several attempts were done to detect the Cre molecule by using whole ISH detection and immunostaining, without success. During Cre immunodetection it was possible to observe the Cre molecule in the nucleus of the *Tg(hs:cre)* line. The in situ detection for Cre expression was observed in the transgenic lines with Cre paired to only one estrogen receptor (ERT2), *aactin:CreERT2* and *sox10:CreERT2*. Several attempts to detect the Cre RNA by ISH in transgenic lines paired with two ERT2 were done without success. Altogether this result suggests that the Cre molecule is difficult to detect by antibody staining or ISH when it is paired with two ER. An alternative detection method for Cre expression would be to develop an antisense RNA probe or antibody against the ER section only.

Pending is the use of the other transgenic lines to analyse whether mesenchymal cells, neurons, neuron precursors, stem cell-like cells, and muscle cells participate during regeneration, and the level of cell plasticity that they possess. Another interesting approach would be to assess whether the zebrafish larva is able to undergo trans-differentiation during regeneration. To investigate this a transgenic line to cause genetic ablation can be a valuable tool, in order to remove the tissue of interest. Once genetic ablation is done tail regeneration will be induced by tail amputation. Cell lineage tracing with the use of cre expressing lines with tissue specific promoters can be used to detect if any tissues is able to trans-differentiate into the ablated tissue during regeneration.

7.2 Conclusion

In this thesis I present my results on the molecular and cellular aspects of zebrafish tail regeneration and development.

The study of the molecular aspects using small molecular compounds, heat-shock conditional mutants, in situ hybridisation and immunostaining techniques yielded the following results.

In zebrafish larvae tail, regeneration is initiated by Hh signalling acting through Wnt and FGF signalling. This result gives evidence to validate my hypothesis which states: "Hedgehog signalling leads tail regeneration by regulating Wnt and FGF signalling in larval zebrafish." Furthermore, this work shows that larval zebrafish tail regeneration has a different molecular hierarchy than adult tail regeneration.

It also shows, that, as for adult tail regeneration, Hh signalling regulates cell proliferation. I anticipate that the use of CHiP assay and real-time quantitative PCR analysis will strongly validate these findings.

Furthermore, there are significant differences between the molecular hierarchy of tail regeneration and development. This demonstrates that regeneration does not just recapitulate ontogeny.

To evaluate the cellular aspects of epimorphic tail regeneration, periderm and blood vessel cells were tracked using lines with Cre expression driven by tissue specific promoters.To this end the cells were permanently and irreversibly labelled by enabling Cre recombination through the administration of 4-hydrotamoxifen.

This led to the observation that new blood vessels and periderm cells regenerate from pre-existing tissue. There was no evidence that periderm cells and blood vessels would regenerate from the blastema. Furthermore, both tissues remained lineage-committed during tail regeneration. In light of these new results it is of interest to re-evaluate the function of the blastema. For the re-evaluation of the blastema activity the Cre-expressing lines created during this thesis would be very useful.

The knowledge generated during the course of this thesis could contribute to the development of therapies enhancing the limited regenerative capacity of humans to restore organs and limbs. However, it is first necessary to further the understanding of epimorphic larval tail regeneration by investigating the mechanisms upstream of Hh signalling.

Chapter 8

List of Abbreviations

’	minutes
4OHT	4-Hydroxytamoxifen
ANOVA	one-way analysis of variance
BMP	Bone Morphogenetic protein
BSA	Bovine Serum Albumin
°C	Centigrade
ChiP-sequence	Chromatin immunoprecipitation
CHT	Caudal hematopoietic tissue
DAPI	4’,6-diamidino-2-phenylindole
DEAB	Diethylaminobenzaldehyde
DIC	Differential interference contrast
DIG	Digoxigenin
dkk1	Dickkopf
DMSO	Dimethyl sulfoxide
dpa	Days post amputation
DsRed	*Discosoma RED*
EF1alpha	Elongation Factor 1 alpha
ER	Estrogen Receptor
EVL	Enveloping outer layer
F2	Second Generation
FGF	Fibroblast Growth Factor
fgf20	*fibroblast growth factor 20*
fgfr1	*fibroblast growth factor receptor*
gata4	*globin transcription factor 4*
GFP	Green Fluorescent protein
Gsk3XV inh	Glycogen synthase kinase 3 inhibitor

Hh	Hedgehog
hpa	hours post amputation
hpf	hours post fertilization
HSC	hematopoietic stems cells
IGF	Insulin-like growth factor
ISH	In situ-hybridization
ISP	Study Plan
IWR-1	4-(1,3,3a,4,7,7a-Hexahydro-1,3-dioxo-4,7-methano-2H-isoindol-2-yl)-N-8-quinolinyl-Benzamide
LB	Lysogeny broth
mg	milligrams
ml	milliliter
mM	milimolar
mq	mili-q
msxb	homeobox b
Mtz	Metronidazol
MTZ	Metronidazol
NBCS	New Born Calf Serum
NEBinc	New England Biolabs inc
Ng	nanograms
nm	nanometers
NTR	Nitroreductase
PBST	Phosphate buffered saline plus 0.1% of tween 20
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
pH3	phosphorylated histone 3 protein
PK	Proteinase K
ptc1	patched 1
RA	Retinoic Acid
raldh2	*retinaldehyde dehydrogenase 2*
RNA	Ribonucleic Acid
Rpm	Revolution per minute
qRT-PCR	quantitative Real Time Polymerase Chain Reaction
rSAP	Shrimp Alkaline Phosphatase

RT	Room Temperature
S	Somite
SAG	Smoothened agonist
SOC	Super Optimal Broth
TBE	Tris,Borate,EDTA
tcf7	*transcritption factor 7*
TE	Tris, EDTA
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
μM	Micromolar
UV	Ultra Violet
X	Times
μl	microliter
fmol	femtomol
ng	nanogram
fg	femtogram

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