Rita Joana Soares Serrano

Licenciada em Biologia

The role of Hedgehog signaling during zebrafish larvae fin fold regeneration

Dissertação para obtenção do Grau de Mestre em Genética Molecular e Biomedicina

Orientadora: Susana Alexandra Rodrigues Pascoal, Doutora, Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa

Júri:

Presidente: Prof. Doutora Paula Maria Theriaga Mendes Bernardo Gonçalves

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Março de 2014

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

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Acknowledgements/Agradecimentos

Primeiro, gostaria de agradecer à minha orientadora, Dra. Susana Pascoal, pela disponibilidade para me ensinar durante este ano e por todo o apoio, paciência e encorajamento transmitidos que foram sem dúvida fundamentais para este trabalho. Muito Obrigada!

Gostaria, também, de agradecer à Dra. Leonor Saúde pela simpatia com que sempre me recebeu, pelos conselhos e acompanhamento ao longo deste projeto. Obrigado pela oportunidade de integrar um grupo tão dinâmico com quem pude aprender e crescer.

Aos meus colegas de laboratório: Margarida Figueira, Rita, Raquel, Margarida Pereira, Sara, Ana e Zé, um muito obrigado pelo companheirismo, pela preocupação, pela motivação e ajuda quando precisa. Ao João Pereira, um obrigado por acreditar em mim, por ser sempre motivador e estar sempre lá! Obrigado por tudo o que me ensinaste. À Rita Fior, um muito obrigado por me ter mostrado pela primeira vez o que era a Biologia do Desenvolvimento e que era este o meu caminho…

Gostaria, também, de agradecer a todas as pessoas que contribuíram para este trabalho: Aida, Lara e Sara Matos, obrigado por cuidarem dos meus peixes e por vezes também de mim. Obrigado pelos sorrisos e simpatia nos bons e maus momentos. Ao António Temudo e à Ana Nascimento, agradeço todos os ensinamentos sobre microscopia, Photoshop, e muito mais, que foram essenciais para este trabalho. Obrigado pela paciência e disponibilidade. À Andreia e Margarida Pinto, agradeço nunca terem desistido dos meus cortes histológicos…foram meses e meses de tentativas e estiveram sempre disponíveis para tentar algo diferente.

Um especial obrigado a toda a minha família por sempre ter acreditado em mim e no meu empenho, por me ter permitido vir estudar para Lisboa e seguir o meu caminho. Mami, obrigado por seres sempre compreensiva e por toda a motivação. Guga, a tia agora já pode brincar contigo!

Finalmente, um muito obrigado ao Pedro. Não há palavras suficientes para expressar o quanto significas para mim. Obrigado por sempre me apoiares e nunca me deixares cair.

“Embrace the mystery of it all…”

Abstract

Several studies have demonstrated that although the structure of the adult and larval zebrafish caudal fin is different, there are similarities at the cellular and molecular level that turn larval zebrafish fin fold a useful model to study the basic principles of regeneration. In this process, while the essential role for Hedgehog (Hh) signaling is well established in the adult zebrafish caudal fin system, its involvement in juvenile tissue regeneration is still unknown. The aim of this Master thesis was therefore to evaluate the contribution of the Hh signaling pathway to the larval zebrafish fin fold regeneration process. Accordingly, we analyzed the expression of several Hh signaling components through *in situ* hybridization. Here, we showed that several of these genes are effectively expressed in the larval regenerating fin tissue, suggesting a role for Hh signaling also during larval regeneration. However, divergence in the regulation of few Hh signaling components appears to exist between the adult and larval zebrafish fin regeneration processes. Nevertheless, similarly to adult caudal fin regeneration, when Hh signaling was blocked, by using cyclopamine, the larval fin fold regenerative outgrowth is severely impaired.

Since larval zebrafish fin fold is ciliated, and primary cilia are closely related to Hh signaling regulation in vertebrate systems, we further addressed the role of primary cilia during larval fin fold regeneration process. To this end, we used the zebrafish *iguana* mutant, in which primary cilia are not formed, to study the modulation of Hh signaling expression during larval fin fold regeneration in the absence of primary cilia. Here, we found that several genes were expressed with a delay, coincident with the delay in the mutant fin fold regeneration observed in previous work.

We show that Hh signaling in the fin fold is crucial to promote cell proliferation. When Hh signaling is blocked using cyclopamine there is a strong blockage of cell proliferation and regeneration is also blocked. Surprisingly, in *iguana* mutants where Hh signaling is impaired but not totally blocked, cell proliferation is not detected but regeneration still occurs. This raises the question about the requirement of cell proliferation in larvae fin fold regeneration. By blocking the cell cycle using aphidicolin we demonstrate that cell proliferation is not necessary for zebrafish larvae fin fold regeneration.

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Keywords: Regeneration, zebrafish larvae, Hedgehog, primary cilia, cell proliferation.

Resumo

Vários estudos têm demonstrado que, apesar da estrutura da barbatana caudal do peixe-zebra adulto e da larva ser diferente, existem semelhanças ao nível celular e molecular entre o processo regenerativo de ambos, que tornam a barbatana caudal da larva de peixe-zebra um bom modelo para estudar os princípios básicos da regeneração. Neste processo, enquanto o papel da sinalização Hedgehog (Hh) na barbatana caudal do peixe-zebra adulto está bem estabelecido, o seu envolvimento na regeneração da barbatana caudal da larva é desconhecido. O principal objetivo desta tese de Mestrado foi, assim, avaliar o contributo da via de sinalização Hh para o processo regenerativo da barbatana caudal da larva de peixe-zebra. Nesse sentido, analisámos a expressão de vários componentes da sinalização Hh através de hibridação *in situ*. Esta análise permitiu verificar que vários componentes da via Hh são efetivamente expressos no tecido regenerado da barbatana caudal da larva, sugerindo que a via Hh tem um papel importante durante este processo. No entanto, parecem existir divergências na regulação de alguns componentes da via de sinalização Hh entre os processos regenerativos do peixe-zebra adulto e da larva. Contudo, através do estudo da regeneração na ausência de sinalização Hh demonstramos, ainda, que a via Hh é essencial para regular o crescimento regenerativo da barbatana caudal de larvas de peixe-zebra tal como no adulto.

Dado que a barbatana caudal da larva de peixe-zebra é ciliada, e os cílios primários estão relacionados com a transdução do sinal da via Hh em sistemas de vertebrados, o nosso segundo objetivo foi tentar compreender o papel dos cílios primários durante o processo de regeneração da barbatana caudal de larvas de peixe-zebra. Para tal, utilizamos o mutante *iguana*, nos quais os cílios primários não se formam, para estudar a modulação da expressão da via durante este processo. Este estudo permitiu verificar que vários genes da via Hh são expressos com um atraso, que coincide com o atraso observado durante o processo de regeneração do mutante *iguana* em estudos anteriores.

Finalmente, neste trabalho é sugerido pela primeira vez que a proliferação celular não é necessariamente obrigatória para o processo regenerativo da barbatana caudal da larva de peixe-zebra.

Palavras-chave: Regeneração, larvas de peixe-zebra, Hedgehog, cílios primários, proliferação celular.

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List of Abbreviations

ACFP	Adult caudal fin primordia
BCIP	Bromo Chloro Indolyl Phosphate
BrdU	Bromodeoxyuridine
BSA	Bovine Serum Albumine
cDNA	Complementary Deoxyribonucleic Acid
dpa	days post amputation
dpf	days post fertilization
Dhh	Desert hedgehog
DIG	Digoxigenin
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
FGF	Fibroblast growth factor
Gli	Glioblastoma
GliA	Gli activator isoform
GliR	Gli repressor isoform
Hh	Hedgehog
hpa	hour post amputation
IFT	Intraflagellar Transport
igu	*iguana*
Ihh	Indian hedgehog
ISH	*In Situ* Hybridization
µm	micrometer
µM	microMolar
ml	millilitre
mM	milliMolar
NBT	Nitro Blue Tetrazolium
PBS	Phosphate Buffered Saline solution
PBST	Phosphate Buffer Saline/ % TritonX-100
PBT	Phosphate Buffer Saline/ 0.1% Tween-20
PDGF	Platelet-derived growth factor
PFA	Paraformaldehyde
Ptc1	Patched1
Ptc2	Patched2
%	Percentage
PTU	Phenyl-1-thiourea
RA	Retinoic Acid
RNA	Ribonucleic Acid
rpm	revolutions per minute
Shh	Sonic hedgehog
Smo	Smoothened
SuFu	Supressor of fused
Wnt	Wingless/Int

Chapter 1: Introduction

1.1 Regeneration in the zebrafish model

Regeneration is an event that can be found in certain organisms that permits the complete reconstitution of lost or damaged tissues and organs (Sanchez Alvarado, 2000). Depending on the context, regeneration can follow an injury, acting as a mechanism of repair, or it can be a constitutive event involved in maintaining the physiological integrity of the organism. There are two main organ/tissue regenerative strategies within different organisms: morphallaxis and epimorphic regeneration. Morphallaxis is defined as the reconstruction of the organism form by remodeling of pre-existing tissue after severe damage, such as the type of regeneration occurring in hydra (Bosch, 2007). On the other hand, epimorphic regeneration depends on cell proliferation and on the formation of a regeneration-specific structure, the blastema, which comprises proliferative cells that differentiate and lead to a complete recovery of the lost tissue. This type of regeneration is seen, for example, during amphibian limb and tail regeneration, and zebrafish (*Danio rerio*) fin regeneration (Galliot and Ghila, 2010).

Mammals have only a limited capacity to regenerate their tissues and organs during adult life. Their regeneration capacity includes the limited replacement of certain cell types in a physiological manner, which is transversal to all animals (Stoick-Cooper *et al.*, 2007). Cells of the skin, gastrointestinal epithelium, bone, skeletal muscle and hematopoietic tissue are regularly replaced upon apoptosis and aging through the activity of self-renewing stem cells. However, most adult tissues/organs, in mammals, have lost their potential for further growth and differentiation (Stoick-Cooper *et al.*, 2007). In mammals, regeneration is usually applied to processes such as liver growth after partial resection, a process that consists of compensatory increase of the mass of the organ rather than true regeneration (Michalopoulos, 2007). Consequently, damage of a tissue or organ usually produces a permanent damage from scarring to disability. Conversely, some non-mammalian vertebrate models, such as the zebrafish, retain enormous regenerative potential in tissues as diverse as cardiac muscle, retina, liver, spinal cord axons, cerebellum, and the fins (Poss *et al.*, 2003). Unlike the situation in mammals, zebrafish tissues with regenerative abilities never form scars after injury since they undergo a complete tissue reconstitution process, and do not seem to have a decline in their regenerative capabilities throughout adult life (Azevedo *et al.*, 2011; Shao *et al.*, 2011). Taken together these characteristics make zebrafish an ideal model to study the cellular and molecular processes underlying tissue regeneration that could provide the required link among species and assist the field of regenerative medicine in the development of new approaches to tissue and organ regeneration in humans.

1.1.1 Adult zebrafish fin regeneration

The zebrafish caudal fin is an established model to study regeneration that presents many advantages in comparison to other organs: is easily accessible, amputation does not compromise the survival of the fish, completely regenerates in a short time frame (2 weeks at 28ºC) and it has a relatively simple architecture composed of 18 bony rays attached to the axial skeleton by muscles (Poss *et al.*, 2003). The regeneration in the zebrafish caudal fin, termed "epimorphic regeneration", involves three stereotypic successive steps, starting from the wound healing in the first 12 hours post amputation (hpa), followed by wound epidermis and the blastema formation at 24-48hpa and regenerative outgrowth after 48hpa (Poss *et al.*, 2003). Wound healing comprises the migration of epithelial cells that cover the wound and form an epithelial cap (or wound epidermis) at the amputation site within 12hpa. Bromodeoxyuridine (BrdU) incorporation assays demonstrated that this response does not involve cell proliferation (Poleo *et al.*, 2001). From 12hpa until 48hpa, the wound epidermis becomes thicken in several layers, and completely covers the amputation site (Poleo *et al.*, 2001). This process is a critical event, since the regeneration does not proceed without it, and comprises migration events from the mesenchymal tissue underneath the wound epidermis and once again cell proliferation is not involved (Poss *et al.*, 2003). Meanwhile, cell types in the mesenchymal tissue, such as osteoblasts (the bone forming cells), undergo dedifferentiation and migrate distally (Tu and Johnson, 2011). These cells form the blastema, which is a group of undifferentiated cells, distal to the amputation plane within 48hpa. These blastema cells proliferate and re-differentiate, being responsible for replacing the lost tissue in a process called regenerative outgrowth (Poleo *et al.*, 2001; Poss *et al.*, 2003). The blastema is divided in two compartments: the proximal blastema that is composed of highly proliferative cells and the distal blastema that is composed of non-or slow-proliferative cells (Poleo *et al.*, 2001; Poss *et al.*, 2003). As the regenerative outgrowth proceeds, the blastema grows distally and cells in the proximal proliferating zone differentiate into new structures which replace the amputated part of the fin. Finally, regenerative outgrowth is completed around 14dpa (Poleo *et al.*, 2001; Poss *et al.*, 2003).

1.1.2 Molecular signaling involved in fin regeneration

In many aspects, the regenerative process is reminiscent of embryonic development. Since epimorphic regeneration yields patterned tissues with diverse cell types, it is thought that the regenerative process within the blastema recapitulates embryonic development of these missing structures. Moreover, regeneration must involve a number of signaling events to coordinate not only the blastema formation, but activate cell proliferation and differentiation in a spatio-temporal controlled manner. It may not be surprising, then, that Wnt, Fibroblast growth factor (FGF) and Hedgehog (Hh), among other signaling molecules are activated upon amputation and regulate different aspects of caudal fin regeneration (Poss *et al.*, 2000b; Quint *et al.*, 2002; Lee *et al.*, 2005).

In the adult zebrafish, FGF has been described as necessary to blastema formation and subsequent regenerative outgrowth. It has been demonstrated that inhibition of FGF signaling, through an Fgfr1 (Fibroblast Growth Factor receptor 1) inhibitor (SU5402) or a transgenic line (*hsp70:dn-fgfr1*) that expresses the dominant negative Fgfr1 protein upon heat shock, causes an aberrant regenerative epithelium and consequently impedes blastema formation (Poss *et al.*, 2000b; Lee *et al.*, 2005). In addition, a genetic zebrafish mutant study revealed that *fgf20a* (a gene of the FGF signaling) is absolutely required for the initiation and formation of the blastema (Whitehead *et al.*, 2005) and referred *fgf20a* as an initiator of regeneration. On the other hand, a proper balance of Wnt/β-catenin signaling is critical for the formation and proliferation of blastema cells that is required to complete regeneration (Kawakami *et al.*, 2006). Hh signaling has been shown to play a role not only in the caudal fin re-patterning but also in the regenerative outgrowth through the coordination of cell proliferation events (Quint *et al.*, 2002). It has been demonstrated that amputation of the caudal fin of zebrafish stimulated regeneration of the dermal skeleton and re-expression of Hh signaling pathway genes (Quint *et al.*, 2002). Moreover, disruption of the Hh signaling using cyclopamine, a steroidal compound that blocks Hh signaling (Chen *et al.*, 2002), has been shown to cause a reduction in cell proliferation within the blastema and arrest the caudal fin outgrowth (Quint *et al.*, 2002). Conversely, the ectopic expression of the ligand Shh leads to additional bone deposition, suggesting a role in proliferation and differentiation of osteoblasts (Quint *et al.*, 2002).

1.1.3 Early life stage zebrafish model

Recently, the zebrafish embryonic caudal fin ("the fin fold") of the 2 days post-fertilization (dpf) larvae has become appreciated as a new model to study the regenerative process (Kawakami *et al.*, 2004). The larval zebrafish fin fold is a simple structure, composed of mesenchymal cells with no cartilage enfolded in two epithelial layers, which is non-homologous to the adult caudal fin (Yoshinari and Kawakami, 2011). It consists of a transient structure that is replaced by the adult zebrafish caudal fin during a later larval development stage through the proliferation of a pool of cells localized in the ventral gap of the melanophores streak at the larvae fin fold (Figure 1.1). This pool of cells, termed hypural cells or adult caudal fin primordia (ACFP), is essential to the development of the adult caudal fin and is dependent on Hh signaling (Hadzhiev *et al.*, 2007).

Although an embryonic structure, the larval fin fold is capable of tissue regeneration in a process similar to that observed in the adults (Kawakami *et al.*, 2004). The larval fin fold regeneration process involves the healing of the wound around 1dpa, the formation of a blastema from 1dpa-2dpa and regenerative outgrowth from 2dpa. Within 3dpa the larvae retain the complete form and structure of the lost part of the fin fold (Kawakami *et al.*, 2004) (Figure 1.1). The wound healing occurs through the migration of epithelial cells, which surround the amputation site, within 6hpa. These epithelial cells accumulate to form a compact wound epithelium at 1dpa. Similar to adult zebrafish regeneration,

it has been shown that blastema (*msxc*, *msxe*) and regenerative epithelium (*dlx4*) markers expression occurs in the regenerating fin fold (Kawakami *et al.*, 2004). Currently, though, it is unknown whether the larval fin fold blastema has a specific function for proliferation as in the adult zebrafish caudal fin regeneration. Kawakami *et al.* (2004) reported that upon amputation of the larvae fin fold, cell proliferation occurs in a spatial restricted manner in the blastema. Actively proliferating cells denoted as blastema-like cells are detected in the area adjacent to the amputation plane from1dpa-2dpa. After the blastema formation, both the adult and larvae regenerating fins exhibit a common cell proliferation profile with the proliferation starting at the distal area. In addition, the distal-most cells do not proliferate during the late phase of repair and drastic cell proliferation occurs in the proximal region (Kawakami *et al.*, 2004). Contrasting to these data, Mateus *et al.* (2012) reported that there is a significant increase in cell proliferation in response to fin fold amputation at 11-16hpa, but cell proliferation does not appears spatially restricted to a blastema region. Instead, cell proliferation occurs in a broad area of the fin fold and not only in the most distal region of the tissue, implicating that the larval fin fold blastema is not a "classical blastema" (Mateus *et al.*, 2012). The authors propose that the difference between cell proliferation patterns might be due to the distinct protocols used in both papers to determine cell proliferation (Mateus *et al.*, 2012). Even so, it is demonstrated that, like the adult regeneration, cell proliferation is activated in the larval fin fold in response to injury (Kawakami *et al.*, 2004; Mateus *et al.*, 2012). Since the larvae fin fold presents no fully differentiated cell types, except for actinotrichia (Yoshinari and Kawakami, 2011), almost no cell differentiation occurs during larval fin fold regenerative outgrowth until the complete recovery of the amputated tissue around 3dpa.

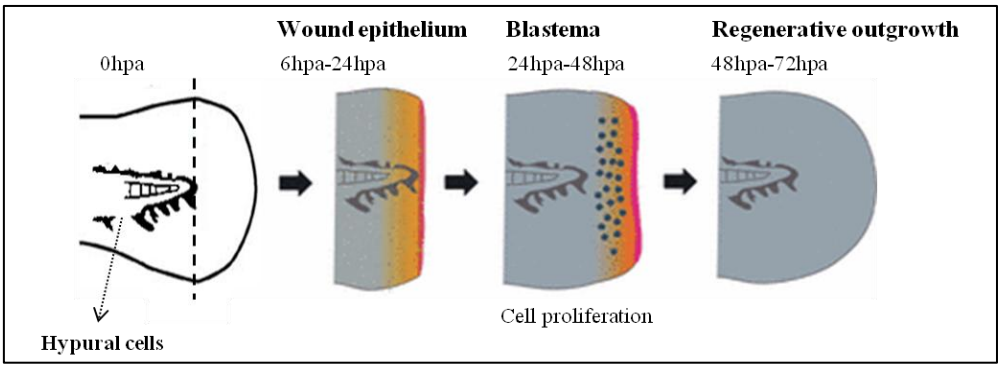

Figure 1.1 – Schematic representation of the zebrafish larvae fin fold regeneration process. After amputation of the 2dpf larva fin fold, epithelial cells surrounding the stump start to contract and seal the wound around 6 hours post amputation (hpa). These epithelial cells accumulate and form a compact wound epithelium at 24hpa. From 24hpa until 48hpa, blastema-like cells are located beneath the wound epithelium. Cell proliferation events take place in the larvae fin fold during this phase. Following regenerative outgrowth, within 72hpa the larvae retain the complete form and structure of the lost part of the fin fold (Kawakami *et al*., 2004). Adapted from Yoshinari and Kawakami (2011).

Since the caudal fin regenerative process appears to be conserved in the larval fin fold system, it presents as a powerful new way to study tissue regeneration and identify its intrinsic regulatory mechanisms. In addition, the larval fin fold has some advantages in comparison to the adult system, namely the speed of regeneration, the structural simplicity of this non-vascularized appendage and the possibility to amputate hundreds of fin folds within an hour gathering a higher experimental number. Further, zebrafish larvae do not feed for up to 5dpf, minimizing the environmental effect on the regeneration assay, and are easier to manipulate and perform chemical and molecular assays (Yoshinari and Kawakami, 2011).

It has been previously reported that, in addition to similar regenerative events between the adult and the larval zebrafish systems, several signaling pathways required during adult caudal fin regeneration are also activated during larval fin fold regeneration. This suggests that larval and adult zebrafish caudal fin regeneration also share a common molecular mechanism. Indeed, microarray studies have shown that a large number of up-regulated expression markers are coincident during adult and larval fin regeneration (Mathew *et al.*, 2009; Yoshinari *et al.*, 2009). Moreover, previous larval regeneration studies demonstrated that, similar to the adult, inhibition of Fgfr1 with SU5402 arrests larvae fin regeneration by blocking the blastema formation (Kawakami *et al.*, 2004). Also, *fgf20a* that was identified as an initiator of blastema formation in the adult regenerating fin (Whitehead *et al.*, 2005) is also highly induced in the larval fin fold tissue. When canonical Wnt signaling is blocked, the blastema and wound epithelium formation is also blocked in the larval model (Mathew *et al.*, 2008). However, the functional requirement for Hh signaling in regeneration of the larval fin fold is currently unknown.

1.2 The Hedgehog signaling pathway

The Hh signaling pathway is a well conserved signaling pathway in multicellular organisms that regulates cell growth and tissue patterning during embryonic development and adult tissue homeostasis (Ingham and McMahon, 2001). During development, it regulates the growth and patterning of tissues, such as the limb, the heart, motorneurons, muscle and bone (Ingham and McMahon, 2001). In the adult organism, Hh signaling is involved in the regulation of cell proliferation and differentiation (Ingham and McMahon, 2001), such as in regeneration of the hematopoietic tissue. Therefore, misregulation of the Hh pathway has been implicated in several congenital malformations and tumor formation (Ingham and McMahon, 2001).

1.2.1 Mechanism of vertebrate Hedgehog signaling

In vertebrates, there are mainly three *hh* genes, encoding extracellular signaling proteins of the same name. These are Desert hedgehog (Dhh), mainly involved in germ cell development (Bitgood *et al.*, 1996), Indian hedgehog (Ihh), involved in bone development (Vortkamp *et al.*, 1996), and Sonic

hedgehog (Shh) (Bumcrot *et al.*, 1995) the best characterized and widely expressed Hh protein. These Hh proteins (also called Hh ligands or Hh signals) are secreted from Hh-producing cells and transferred to adjacent cells. Once released from the cells, Hh bind to a complex of proteins on an Hh-receiving cell. This complex includes the Hh-binding protein Patched (Ptc), which has high affinity for all Hh ligands. Ptc is a 12-pass transmembrane protein that in the absence of Hh inhibits the action of the downstream signaling component Smoothened (Smo). Upon binding of Hh to Ptc, inhibition of Smo is relieved. Smo is a 7-pass transmembrane G-protein and the Hh pathway activator. Smo promotes the downstream signal transduction through interaction with the Gli (glioma-associated genes) family of zinc finger transcription factors (Fig. 1.2). The Gli transcription factors are mediators of the Hh response and have been found to function as transcriptional activators, repressors or both (Eggenschwiler and Anderson, 2007; Briscoe and Therond, 2013).

In vertebrates, there are three Gli transcription factors: Gli1, Gli2 and Gli3. All three Gli transcription factors have five highly conserved zinc finger DNA binding domains and C-terminal activation domains, while Gli2 and Gli3 also have N-terminal repressor domains (Sasaki *et al.*, 1999). Hence, Gli1 functions solely as a transcriptional activator whereas Gli2 and Gli3 can act both as activators and repressors. Their bi-functionality is determined by the presence of Hh signaling. In the absence of Hh and Smo activation, full length Gli2 and Gli3 are proteolitically processed resulting in the removal of the carboxyl-terminal activation domain. In this form (GliR), these transcription factors are translocated to the nucleus and act to repress transcription of Hh target genes. Disruption of this processing by Smo, allows full length Gli2 and Gli3 to translocate to the nucleus and act as transcriptional activators (GliA). The ratio of GliR and GliA forms varies as the concentration of Hh changes. The balance of these forms within the nucleus of a given cell ultimately determines the activation or repression of Hh target genes. Among the Hh pathway target genes are Gli1 that further enforces the Hh pathway activation, and Ptc1 that establishes a negative feedback regulation of the pathway by repressing Smo (Eggenschwiler and Anderson, 2007; Briscoe and Therond, 2013) (Fig. 1.2).

Regulation of Gli transcription factors processing and nuclear translocation has an essential role in the modulation of the Hh target gene expression. This process is mediated through a number of interacting proteins including Smo, Supressor of Fused (Sufu), Kif7 and Protein kinase A (PKA). Sufu acts as negative regulator of the Hh signaling through the formation of a complex with full length Gli proteins and promoting their processing into GliR via PKA (Merchant *et al.*, 2004). In addition, Sufu has also been shown to play a positive role on Hh signaling by maintaining sufficient full-length Gli levels in the cytoplasm required for their activation by Smo (Humke *et al.*, 2010; Tukachinsky *et al.*, 2010). Although the process is unknown, activation of Smo promotes the dissociation of Gli-Sufu complexes and leads to the formation of GliA forms (Humke *et al.*, 2010).

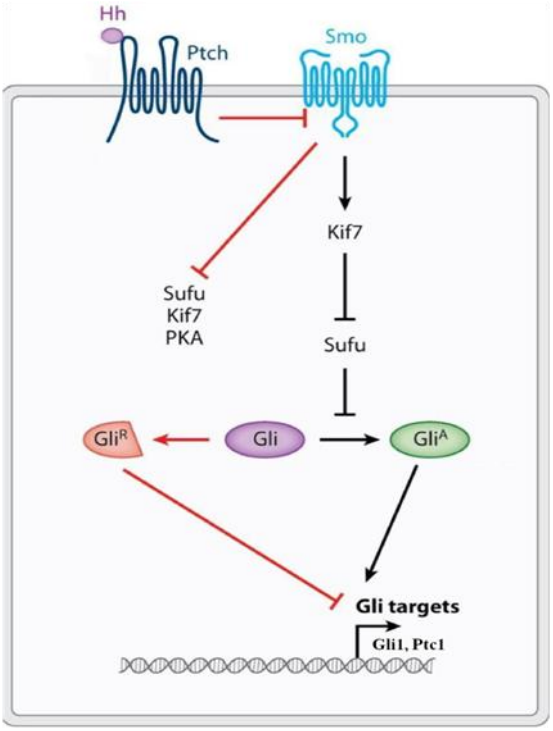

Figure 1.2 – The main components of the Hedgehog signaling pathway. Smo is the key signal transducer of the Hh pathway. In the absence of the Hh ligands, Hh receptor Ptc inhibits Smo signaling. Gli molecules are processed into repressor forms (GliR), which turn off the Hh-signaling pathway. In the presence of Hh, Ptc is unable to inhibit Smo. Smo undergoes conformational changes and promotes dissociation of Sufu-Gli complexes. Gli proteins are processed to active forms (GliA), which will activate transcription of Hh target genes, including Ptc1 and Gli1. SuFu: Suppressor of fused, Ptch; Patched, Smo; Smoothened, GliR: Gli repressor, Hh: hedgehog, GliA: Gli activator. Adapted from Hui and Angers (2011)

Due to whole genome duplication and further rearrangements, zebrafish have two *ihh* genes (*ihha* and *ihhb*), two *shh* genes (*shha* and *shhb*) (Gensure *et al.*, 2004). Moreover, four Glis have been identified in the zebrafish: Gli1 is a major activator with expression that only partially depends on Hh signaling (Karlstrom *et al.*, 2003; Ninkovic *et al.*, 2008), Gli2a and Gli2b have been described as minor repressors (Ke *et al.*, 2008) and Gli3 has both activator and repressor functions (Tyurina *et al.*, 2005).

1.2.2 Hedgehog signaling through primary cilia

In vertebrates, intracellular Hh signaling is highly dependent on the structural cellular component, the primary cilium (Huangfu and Anderson, 2005; Kim *et al.*, 2010; Roy, 2012). Primary cilia are organelles stabilized by microtubules that project from the cell surface of most vertebrate cells. Cilia are formed during the interphase of the cell cycle from the basal body, a modified centrosome, which associates to the plasma membrane (Wissam *et al.*, 2009). Since no protein synthesis occurs within the cilia during its assembly, a transport system is required to transfer proteins from the basal body to the distal tip of the cilia, and vice-versa. This process involves an ancient transport system that evolved along with the establishment of cilia in eukaryotic development, referred to as intraflagellar transport (IFT) (Rosenbaum and Witman, 2002). IFT is not only essential for the formation but also the maintenance of cilia. IFT is mainly controlled by two multimeric motor complexes, namely Kinesin2 and Dynein, and a number of IFT protein particles. Kinesin2 regulates

the anterograde movement of IFT particles from the base to the tip of the cilia, while Dynein regulates the retrograde movement from the tip to the base of the cilia (Rosenbaum and Witman, 2002; Scholey, 2008). The IFT proteins are composed of approximately 17 proteins arranged into two complexes, IFT-A and IFT-B, which are required, respectively, for the retrograde and anterograde movement within cilia. Therefore, the disruption of the IFT system can lead to a complete absence of cilia, or their stunted growth, with repercussions to their functionalities.

The core of the cilia, the axoneme, elongates from the basal body and extends towards the extracellular environment to form the cilium (Fig. 1.3). Based on structural composition and motility, there are three types of cilia: motile, nodal and primary cilia. Motile cilia consist of nine doublet microtubules and a central pair (9+2), which move relative to each other and cause the cilium to bend and therefore move. Motile cilia are commonly present in large number of cells and beat to cause fluid flow. Nodal cilia are present on cells of the embryonic node during development and can also beat but lack of the central pair of microtubules (9+0). Nodal cilia are responsible to cause preferential morphogen gradients helping to establish left-right body axis asymmetry during embryonic development. On the other hand, primary cilia, present in almost nearly all vertebrate cells, consist of nine doublet microtubules only (9+0) and are therefore non-motile (Wissam *et al.*, 2009).

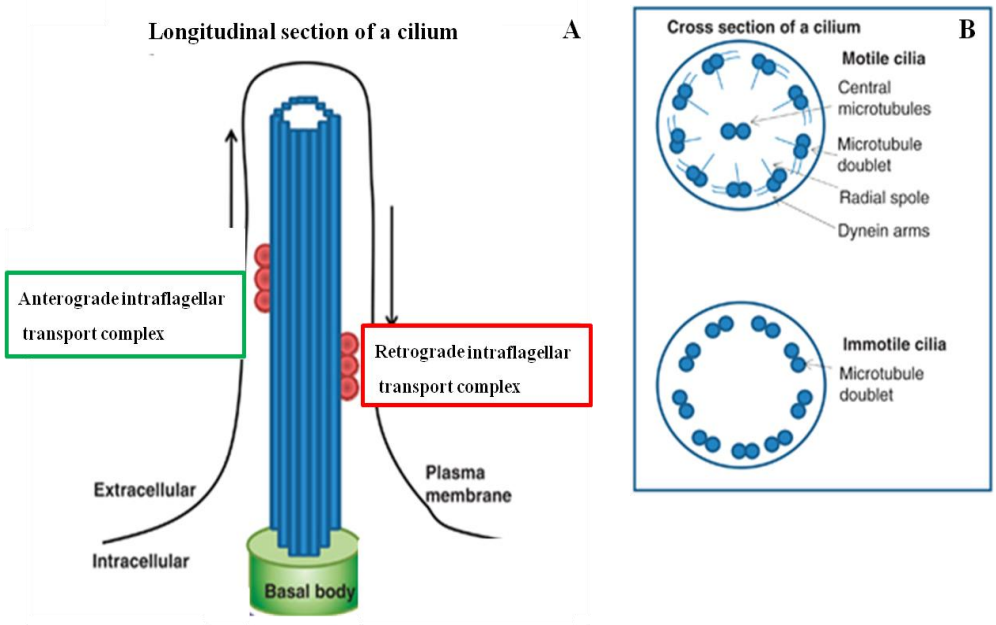

Figure 1.3 – Schematic representation of the cilia structure and microtubule arrangement. (**A**) A typical cilium consists of an axoneme of nine doublet microtubules. The axoneme arises from the basal body, a modified centrosome. Ciliary assembly and maintenance is accomplished via intraflagellar transport (IFT), which relies on the microtubule motor proteins kinesin2 and dynein to transport IFT protein complexes and their associated cargo up or down the microtubules (anterograde and retrograde transport, respectively). (B) Structural differences determine the motility of a cilium. Motile cilia consist of 9 doublet microtubules surrounding 2 inner singlet microtubules used to conduct force. Primary cilia are lacking both singlet microtubules and dynein arms. Adapted from Forsythe and Beales (2013).

Primary cilia, once considered vestigial structures, have been in recent years discovered as sensory organelles (Wissam *et al.*, 2009). Primary cilia main function is to act as chemo and mechano-sensors to sense fluids and mechanical stress, such as in the kidney cells (Wissam *et al.*, 2009). However, there are examples of primary cilia that detect osmolarity, temperature, and gravity (Wissam *et al.*, 2009). In addition, in some specialized cells, such as the photoreceptors and olfactory neurons, primary cilia have evolved to specialized functions in sensory perceptions (Wissam *et al.*, 2009). Primary cilia are therefore thought to be essential to concentrate signals and promote an efficient and rapid signal transduction within the cell, and allow specific protein interactions or modifications to occur (Wissam *et al.*, 2009). According to its capacity to sense extracellular signals, it is not surprising that this active organelle was recently described as essential to the coordination of several signaling pathways involved in both embryonic development and tissue homeostasis. The signaling pathways known to depend on primary cilia include: Platelet Derived Growth Factor Receptor α (PDGFRα) growth factor signaling, epidermal growth factor signaling, Wnt signaling, and Hh signaling (Eggenschwiler and Anderson, 2007; Goetz and Anderson, 2010).

Many components of the Hh pathway are enriched in the primary cilium and, in response to Hh stimulus, dynamically shuttle in or out this organelle. For example, in mouse, it has been shown that Smo is absent from primary cilia in limb bud resting cells, although in response to Shh stimulation Smo accumulates in the cilia tip (Corbit *et al.*, 2005). Smo translocation, which requires the IFT system, is essential to Hh signaling transduction (Corbit *et al.*, 2005). In addition, Ptc1 has been detected in primary cilia of mouse embryonic fibroblasts (MEFs) cultures and demonstrated to undergo internalization, moving out of the cilium, when bound to Hh (Rohatgi *et al.*, 2007). Also, Sufu, negative regulator of Hh signaling, has been shown to localize in primary cilia distal tip in the absence of Hh ligands (Haycraft *et al.*, 2005). Gli1, Gli2, Gli3A and Gli3R have as well been detected in primary cilia, indicating that Hh signaling transduction is reliant on primary cilium (May *et al.*, 2005; Eggenschwiler and Anderson, 2007). Furthermore, genetic screens in the mouse have revealed that mutations in components of the IFT machinery result in absence of cilia and consequently defective Hh signaling (Haycraft *et al.*, 2005). *Ift88* mutant mouse (also referred to as Tg737) demonstrated patterning defects in Hh-dependent tissues, including failure of ventral neurons specification and polydactyl limbs (Huangfu *et al.*, 2003; Huangfu and Anderson, 2005; Liu *et al.*, 2005), accompanied by reduction or absence of downstream Hh target genes expression. In the limb bud, it has been shown that Shh mainly functions to derepress Hh target genes expression through the decrease of Gli-R levels (Eggenschwiler and Anderson, 2007; Goetz and Anderson, 2010). Thus, a reduction of these levels would lead to a gain-of-function of Hh signaling phenotype. Accordingly, *ift88* mutant mice demonstrated an increase in the levels of full-length Gli3 protein, which would normally be processed to its repressor form in the absence of Hh pathway stimulation. Further, these mutants displayed a phenotype similar to those with *gli3* mutations. In addition, primary cultures

derived from *ift88* mutant mouse limb bud lacked of the responsiveness to Shh (Haycraft *et al.*, 2005; Eggenschwiler and Anderson, 2007). Therefore, cilia are not only essential to the processing of Gli transcription factors into their repressor form but to activation of Hh target genes transcription in response to Hh ligands (May *et al.*, 2005). Moreover, cilia are also essential to the activation of Gli full-length proteins. In *ift88* mutant mouse, a reduction of Gli2 activating form in the neural tube leads to a failure in the ventral neurons specification in a similar pattern to those with *shh* mutations (Huangfu *et al.*, 2003). Therefore, depending on the tissue and their dependence on GliA or GliR function, the loss of primary cilia can result in low or high Hh signaling activity as primary cilia are required to generate both activator and repressor forms of Gli transcription factors (Huangfu and Anderson, 2005; Liu *et al.*, 2005; May *et al.*, 2005).

The current view is that primary cilia are essential for normal Hh signaling transduction in all systems studied, including the mouse, *Xenopus*, chicken and zebrafish (Roy, 2012). In zebrafish, it has been shown that Smo, like in mouse, translocates to the primary cilium in response to Hh stimulation and that mutations that result in the disruption of Smo localization to this organelle cause a decrease in Hh pathway activity in zebrafish embryos (Aanstad *et al.*, 2009). Also, it has been demonstrated that Gli2 activating form translocates to the primary cilium of cells in the zebrafish embryo being this localization mediated through Hh pathway activity (Kim *et al.*, 2010). In *ptc1/ptc2* double mutant zebrafish, the levels of Gli2a are increased at the tip of the cilia, while in *smo* mutant zebrafish the levels are increased in the cilia basal bodies (Kim *et al.*, 2010). This implicates that in the absence of Hh signaling Gli2a is translocated from the tip of the cilia to the basal body and that Hh signaling regulation is therefore dependent on primary cilia.

Further studies have demonstrated that there is as well a ciliary defect associated with the deregulation of Hh signaling in the zebrafish (Huang and Schier, 2009). Huang and Schier (2009), using a maternal-zygotic zebrafish that lack *Ift88* gene (MZovl), revealed that zebrafish cilia mutants demonstrate defective neural patterning and somites formation, reflecting a disturbed Hh signaling activity (Huang and Schier, 2009). Nevertheless, while in mouse cilia mutants the expression of *ptc1*, a downstream Hh signaling target, is reduced or absent (Huangfu *et al.*, 2003), in zebrafish cilia mutants the expression of *ptc1* is reduced, and at the same time expanded, in different tissues depending on the tissue requirements for Hh signaling (Huang and Schier, 2009). As a result, cell types that require maximal Hh signaling activity are absent in both zebrafish and mouse cilia mutants, while cell types that depend on lower Hh signaling threshold levels are expanded in zebrafish but are absent in mouse cilia mutants. As an example, the motorneurons are specified in zebrafish cilia mutants (Huang and Schier, 2009) and absent in mouse *ift88* mutants (Huangfu *et al.*, 2003), suggesting that Hh signaling activity remains higher in zebrafish cilia mutants compared to mouse cilia mutants. Although, the nature of these differences between zebrafish and mouse Hh signaling activity remains not understood, one hypothesis argues that zebrafish *gli1* has greater basal level of Hh

signaling independent expression than mouse *gli1*, which results in greater levels of Hh signaling-independent pathway activation (Huang and Schier, 2009). As mentioned above, *gli1* transcription is not fully dependent on Hh signaling in the zebrafish (Karlstrom *et al.*, 2003; Ninkovic *et al.*, 2008), and therefore is expressed even in the absence of primary cilia, while *gli1* is inhibited in mouse cilia mutants (Eggenschwiler and Anderson, 2007). In particular, it has been demonstrated that zebrafish cilia mutants treated with *gli1* morpholinos show a reduced expression of *ptc1* compared to untreated zebrafish cilia mutants (Huang and Schier, 2009), suggesting that the expanded Hh pathway activity in zebrafish cilia mutants is indeed dependent on *gli1* basal expression. Hence, there is a conserved requirement of cilia for "normal" Hh signaling in the zebrafish; although it appears that only maximal Hh pathway activation is reduced in zebrafish cilia mutants.

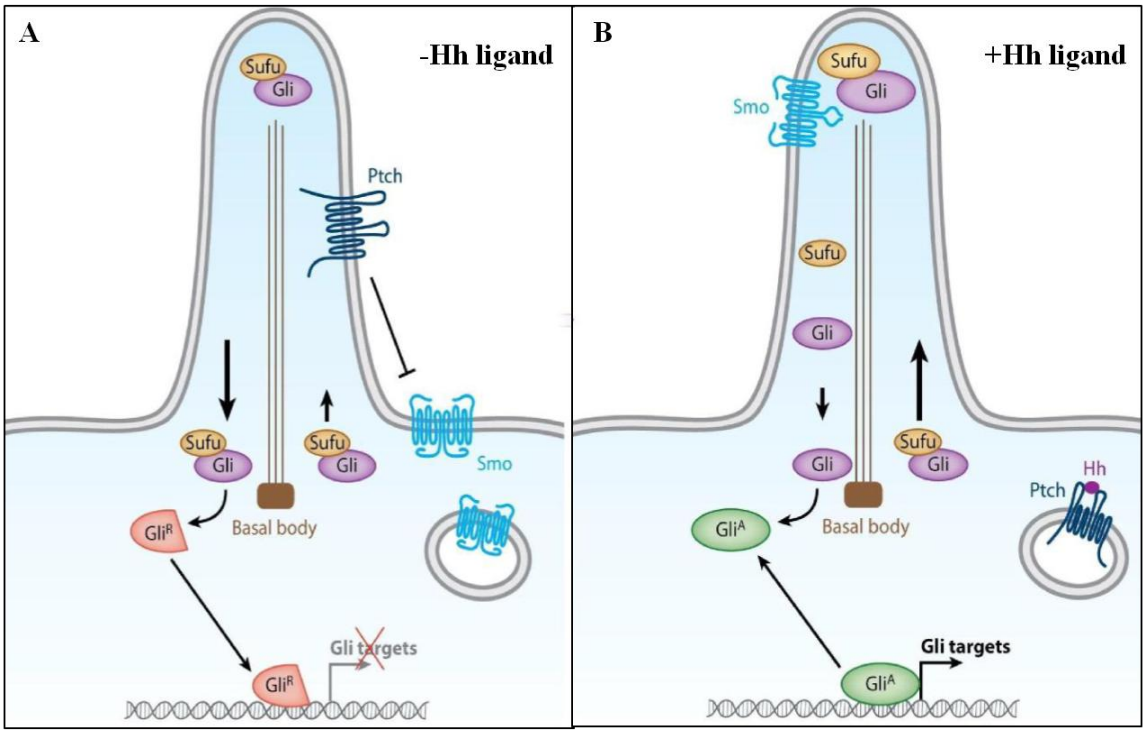

Figure 1.4 – The vertebrates Hedgehog signaling pathway. (**A**) In the absence of Hh ligand, Patched localizes in the cilia and represses Smoothened (Smo) activity by preventing its trafficking and localization to the primary cilium. Gli proteins are sequestered in the cytoplasm by several protein mediators including Sufu. Gli proteins undergo proteasomal cleavage and the resulting repressor form (GliR) translocate to the nucleus and inhibits transcription of Hh target genes. (**B**) On Hh ligand binding, Patched is displaced from the cilia, thereby allowing ciliary accumulation and activation of Smo. Activated Smo activates a signaling cascade that results in dissociation of Sufu-Gli complexes and translocation of an activated form of Gli (GliA) to the nucleus, where it induces expression of Hh target genes. SuFu: Suppressor of fused, Ptch; Patched, Smo; Smoothened, GliR: Gli repressor, Hh: hedgehog, GliA: Gli activator. Adapted from Hui and Angers (2011).

Therefore, in vertebrates, it has been postulated that in absence of Hh, Ptc localizes on the cell membrane of cilia and inhibits Smo from entering into to the primary cilium. Smo is trapped in intracellular vesicles. Gli proteins, at the tip of the cilium are then processed into repressor forms by Sufu. The Gli repressors associate with the IFT proteins and are transported in retrograde direction from the ciliary tip to the basal body. Upon stimulation of Hh, Ptc moves from the cilia. Smo is derepressed, associated with IFT proteins and transported in anterograde direction from the basal body towards the cilia tip. At the cilia tip interacts with Sufu and Gli proteins to regulate the Gli processing and promotes the formation of Gli activating forms. The Gli activating forms are then transported in retrograde direction and enter the nucleus to regulate the transcription of the downstream targets (Fig.1.4) (Eggenschwiler and Anderson, 2007; Goetz and Anderson, 2010).

1.3 The zebrafish *iguana* mutant

Iguana is a novel zinc finger protein, also referred to as the zebrafish homolog of Dzip1 (Daz interacting protein 1, mammalian protein) (Sekimizu *et al.*, 2004; Wolff *et al.*, 2004), that is required for normal Hh signaling transduction in the zebrafish embryo (Odenthal *et al.*, 2000; Sekimizu *et al.*, 2004; Wolff *et al.*, 2004).

The *iguana* (*igu*) gene was first associated to a role in Hh signaling regarding the several phenotypical traits that it shared with the zebrafish midline mutants (Brand *et al.*, 1996; Odenthal *et al.*, 2000), including ventral neural tube defects, U-shaped somites and malformations in the midline, and a characteristic ventral curved body phenotype (Brand *et al.*, 1996; Van Eeden *et al.*, 1996). Zebrafish midline mutants have been shown to encode components of the Hh signaling cascade, including *shha* (*syu*), *gli1* (*dtr*), *smo* (*smu*) and *gli2* (*yot*) (Brand *et al.*, 1996; Van Eeden *et al.*, 1996). *Iguana* mutant phenotype is unique among the midline mutants in that it demonstrates a reduction in downstream Hh signaling target genes expression in the ventral neural tube and expanded expression of Hh target genes in the somites (Sekimizu *et al.*, 2004), contrasting with the absence of Hh target genes expression in both neural tube and in the somites in zebrafish midline mutants. Reflecting this altered pattern of *ptc1* and *gli1* expression, the specification of Hh-dependent muscle cell types is also impaired in *iguana* mutant zebrafish (Wolff *et al.*, 2004). In zebrafish *iguana* mutant, cell types that require maximal levels of Hh signaling like the muscle pioneers are lost or reduced, while cells that require sub-maximal signaling activation like medial fast fibers (MFFs) are over produced (Wolff *et al.*, 2004). Therefore, *iguana* mutants exhibit both a gain and a loss-of-function of Hh signaling activity highly reminiscent to MZovl mutant zebrafish (Huang and Schier, 2009). The loss of Hh-dependent cell types could be explained by a reduction of Hh signaling activity in *iguana* embryos or an inability to respond to signals. On the other hand, the gain-of-function phenotype could be explained due to impairment of the negative regulation of the Hh signaling that is required for silencing the Hh target gene expression in the absence of Hh ligands. Using molecular approaches, it

has been shown that Iguana functions downstream of Smo and upstream of the transcription mediators Gli in the Hh signaling cascade (Sekimizu *et al.*, 2004; Wolff *et al.*, 2004). In the absence of Shh and Smo activity, the expansion in numbers of MFFs is unaffected in *iguana* mutant embryos, implicating that Iguana acts downstream of Smo. In addition, depletion of Sufu causes a further expansion in the number of MFFs, whereas over-expression of Sufu eliminates all Hh-dependent muscle cell types from *iguana* mutant embryos (Wolff *et al.*, 2004). Sufu acts to modulate the Gli protein function acting to inhibit their transcriptional activation (Merchant *et al.*, 2004). Therefore, it was proposed that GliA and GliR ratio was disrupted in the absence of Iguana activity and Iguana had a role in the modulation of the Gli protein function (Sekimizu *et al.*, 2004; Wolff *et al.*, 2004).

More recently, a role for the Iguana protein on primary ciliogenesis was discovered (Glazer *et al.*, 2010; Tay *et al.*, 2010). In particular, it has been demonstrated that the Iguana protein cell sub-localization is the cilia basal body and that Iguana is not required for the docking of the basal bodies but essential for the ciliary axoneme growth. Consequently, *iguana* mutant zebrafish lacks of the primary cilia (Glazer *et al.*, 2010; Tay *et al.*, 2010). Since the mechanism by which the Iguana protein regulates the Gli activator and repressor activities is unknown, it raised the possibility that it is a cilia-related role that explains its requirement for Hh signaling in the zebrafish and not a direct role as a component of the Hh signaling cascade (Glazer *et al.*, 2010; Tay *et al.*, 2010).

1.4 Aims

Previous work from our lab has shown that zebrafish larvae fin fold is ciliated and that upon amputation there is a ciliogenic event associated with the regenerative response (Lima, 2010). Based on the recent connection between primary cilia and Hh signal transduction (Huang and Schier, 2009), and since no one has previously characterized whether Hh signaling is induced upon amputation of the larval zebrafish fin fold and its functional requirement during its regeneration process, the main objective of this work is to determine the contribution of the Hh signaling pathway to the larval fin fold regenerative process. In a broad perspective, we want to assess whether the adult and larval zebrafish fin systems share the same requirement for Hh signaling during regeneration. In addition, we want to assess the importance of primary cilia as regulators of Hh signaling during the larval zebrafish fin fold regeneration process by analyzing the modulation of Hh signaling genes expression in the presence and absence of primary cilia, using the *iguana* mutant. Finally, our third objective is to determine the relevance of the cell proliferation that operates under the action of Hh signaling during regeneration by characterizing the zebrafish larvae fin fold regenerative process in the absence of cell proliferation.

Chapter 2: Materials and Methods

This chapter describes the methodologies adopted to achieve the aims and objectives stated in section 1.4 of Chapter 1.

2.1 Zebrafish lines and husbandry

Zebrafish (*Danio rerio*) embryos were obtained by natural spawning of AB *wild type* fish or by identified heterozygous carriers for *iguana* (*igu*^{tm79a}) (Brand *et al.*, 1996) or *smoothened* (*smu*^{b641}) (Varga *et al.*, 2001) mutation. *Igu*^{tm79a} is a point mutation coding for a premature stop codon, causing a non-functional truncated Iguana/Dzip1 protein (Brand *et al.*, 1996). As stated in section 1.2 of Chapter 1, Iguana/Dzip1 localizes to the basal body of the primary cilium and is required for its proper formation (Kim *et al.*, 2010). Therefore, homozygous *igu* mutant embryos lack of the primary cilia. On the other hand, *smu*^{b641} is point mutation that changes a glycine to an arginine residue in the second transmembrane domain of the Smoothened (Smo) protein, which severely affects the protein structure and function (Varga *et al.*, 2001). Smo is the downstream transducer of Hh signaling and consequently essential for activation of Hh signaling downstream targets transcription. Thus, homozygous *smu* mutant embryos have the Hh signaling pathway inhibited.

All zebrafish lines were maintained and raised according to standard protocols (Westerfield, 2000). Zebrafish embryos were staged by days post fertilization (dpf) at 28ºC and confirmed according to morphological criteria provided in Kimmel *et al.* (1995). Homozygous *igu* and *smu* mutant embryos were identified at 2dpf based on published descriptions (Brand *et al.*, 1996; Varga *et al.*, 2001) and Mendelian inheritance. In a heterozygous progeny pool of embryos, 25% should be wild type, 50% should be heterozygous for the specific mutation and 25% should be homozygous.

2.2 Fin fold amputation

Embryos were maintained in 1% embryo medium at 28ºC until amputation of the fin fold at 2dpf. Zebrafish embryos were then randomly selected as an uncut control group or to undergo amputation of the fin fold (experimental group). Prior to amputation, the chorions were removed and embryos were anesthetized in embryo media supplemented with 1% Tricaine (160 mg/ml) (MS222, Sigma Aldrich) for approximately 5 minutes. This allows immobilization of the embryo and reduces any possible discomfort caused by the procedure. Using a scalpel, the amputation of the fin fold was performed just posterior to the end of the notochord as previously described (Kawakami *et al.*, 2004) (Fig. 2.1). The amputation procedures were monitored using a Leica S8APO dissecting stereoscope. Subsequently, embryos were placed in 1% embryo medium or 1% embryo medium treated with drugs

or respective vehicles at 28ºC and allowed to regenerate their fin folds for several time points. Larvae were then collected and used for the following procedures.

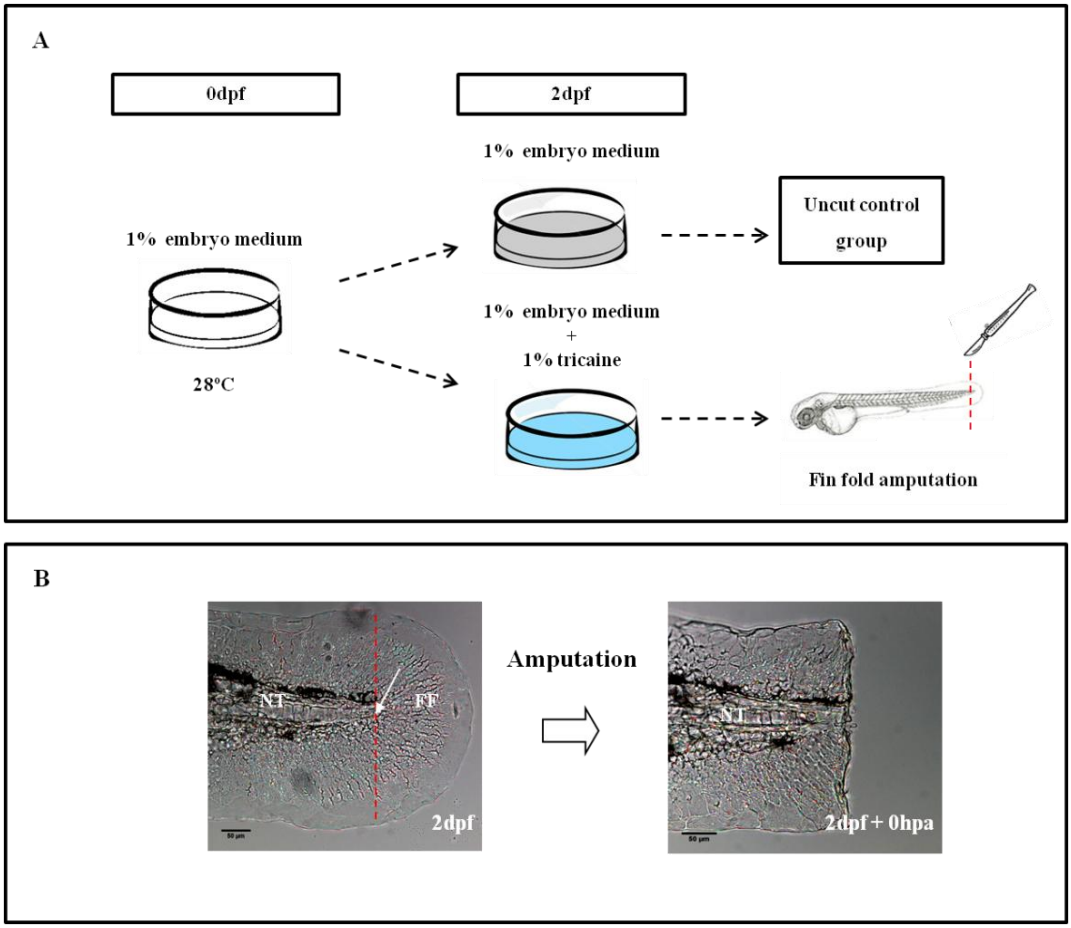

Figure 2.1 – **Methodology adopted to study the process of zebrafish larvae fin fold regeneration.** (A) Embryos were maintained in 1% embryo medium until the fin fold amputation stage (2dpf), when they were transferred to 1% embryo medium supplemented with 1% tricaine. (B) The fin fold was surgically ablated just posterior to the end of the notochord. The amputation plane is indicated by the red dotted line and the arrow indicates the most distal end of the notochord. NT – Notochord; FF – Fin Fold; Scale bar: 50 μm.

2.3 Drug treatments

2.3.1 Cyclopamine treatment

Cyclopamine, a small molecule that interferes with Smo receptor (Chen *et al.*, 2002), was used to inhibit Hh signaling during larval zebrafish fin fold regeneration. Cyclopamine (cat: C4116, Sigma-Aldrich) was resuspended in DMSO (dimethyl sulfoxide; cat: F515, Fynnzymes) to a 12mM stock solution based on published protocols (Hadzhiev *et al.*, 2007) and stored at -20ºC. Prior to treatment with cyclopamine, a solution of cyclopamine at the final concentration of 200μM was freshly prepared

from the stock solution through dilution in 1% embryo medium. The cyclopamine treatment was initiated following the amputation of wild type larvae fin fold at 2dpf and continued until 1-4 days post amputation (dpa). As vehicle control of the cyclopamine experiments, a group of larvae was treated with an equivalent amount of DMSO in embryo medium and analyzed at the same regenerative stages. Cyclopamine and DMSO treated larvae then processed for *in situ* hybridization (1-3dpa) and/or immunohistochemical detection of BrdU (2-4dpa).

2.3.2 Aphidicolin treatment

Aphidicolin, a pharmacological inhibitor of DNA polymerase α (Ikegami *et al.*, 1978), was used in this work to arrest cell proliferation during larval zebrafish fin fold regeneration. Aphidicolin (cat: A0781, Sigma Aldrich) was resuspended in DMSO (cat: F515, Fynnzymes) to a 3mM stock solution and stored at -20ºC. After the treatment with aphidicolin, a solution of aphidicolin at the final concentration of 30 or 50 50μM was prepared from the stock solution by dilution in 1% embryo medium. The aphidicolin treatment was initiated after amputation of wild type larvae fin fold at 2dpf and continued for 2, 3 and 4dpa. As positive control, a group of larvae was treated with an equivalent amount of DMSO in 1% embryo medium. Aphidicolin and DMSO treated larvae were processed for immunohistochemical detection of BrdU.

2.4 Detection of mitotic nuclei with Bromodeoxyuridine

2.4.1 Bromodeoxyuridine (BrdU) incorporation

Bromodeoxyuridine (BrdU) is an analogue of the DNA precursor thymidine. In proliferating cells the DNA has to be replicated before division can take place, which occurs during the S phase. If BrdU is administrated at this stage, cells will incorporate it into their DNA just like they would incorporate thymidine. Consequently, the number of cells incorporating BrdU is dependent on the frequency of cell proliferation of the tissue. The amount of BrdU incorporated can be detected using specific anti-BrdU antibodies immunohistochemically.

BrdU (cat: B5002, Sigma-Aldrich) was diluted in 1% embryo medium to a 2mM solution from a 10mg/ml stock solution dissolved in 1% PBS with 7mM NaOH. The patterns of cell proliferation, during the steps of the regeneration process, were analyzed following BrdU incubation for 6 hours preceding larvae fixation at specific regenerative endpoints per Kawakami *et al.* (2004).

2.4.2 Immunohistochemical detection of BrdU

For immunohistochemistry, larvae were fixed in fresh 4% PFA and incubated overnight at 4ºC. Subsequently, larvae were rinsed several times with PBS for 5 minutes each and dehydrated stepwise

through a series of methanol-PBS (from 25%, 50%, 75% and 100% methanol) gradient and storage at -20°C until processed. Immunohistochemistry experiments were performed according to the protocol presented in Appendix A.

2.5 *In situ* hybridization

In situ hybridization is a powerful technique for visualizing gene expression patterns in specific tissues. This technique allowed us to study the expression of several Hh signaling pathway genes at different stages of larvae fin fold regeneration (1dpa-3dpa) using specific digoxigenin-labeled (DIG) RNA probes.

2.5.1 RNA probes synthesis

The antisense mRNA probes used in this research were synthesized from the constructs listed at Table 2.1

Table 2.1 - Constructs used as DNA templates for mRNA probe synthesis.

Probe	References	Linearization site	RNA polymerase
gli1	(Karlstrom *et al.*, 2003)	*BamHI*	T7
gli2a	(Devine *et al.*, 2009)	*BamHI*	T7
gli3	(Devine *et al.*, 2009)	*NotI*	T7
ihha	(Eames *et al.*, 2011)	*SpeI*	T7
ihhb	(Eames *et al.*, 2011)	*HindIII*	T7
ptc1	(Lee *et al.*, 2008)	*SacII*	Sp6
ptc2	(Lewis *et al.*, 1999)	*ClaI*	T7
shh	(Poss *et al.*, 2000a)	*HindIII*	T7
smo	(Philipp *et al.*, 2008)	*NotI*	T7
sufu	(Hammond and Whitfield, 2009)	*EcoRI*	T7

Bacteria transformation

Each plasmid DNA was transformed into the *Escherichia coli* (*E. coli*) strain DH5α for further amplification and isolation: Frozen aliquots of competent cells were thawed on ice. Plasmid DNA

(1µl) was incubated with 100 µl of cells on ice for 30 minutes. A heat shock was applied at 42 ºC during 45 seconds (sec), followed by cooling on ice for 2 minutes. Next, 1ml of LB medium was added to the bacteria solution and the mixture was incubated for 1 hour with agitation at 37ºC. Bacteria were plated in solid medium (LB) containing ampicilin (100µg/µl) to select the transformed bacteria and grown overnight in an incubator at 37ºC, with the plates inverted.

Plasmid DNA Amplification

For plasmid DNA amplification, a single colony of transformed bacteria was collected using a micropipette tip and inoculated in a 15ml falcon containing 2ml of pre-warmed LB medium supplemented with ampicilin (100µg/µl). The mixture was grown in an incubator-shaker during 6 hours at 37ºC. Following the 6 hours incubation, 1,5ml of culture was transferred into a 1000ml Erlenmeyer containing 100ml of pre-warmed LB medium with 100µl of ampicilin and incubated overnight in a shaker- incubator at 37ºC.

Plasmid DNA Isolation

For small scale preparation of plasmid DNA, 2ml of a grown overnight bacterial culture of transformed competent cells was processed using the Wizard® Plus SV Minipreps DNA Purification System (Promega) according to the protocol suggested by the manufacturer (Appendix A). For large scale preparation of plasmid DNA, 100ml of a grown overnight bacterial culture of transformed competent cells was processed using the Roche Genopure Plasmid Midi Kit (Applied Science), according to the manufacturer's instructions (Appendix A). The extracted plasmid DNA was run on 1% Agarose (Sigma) gel to ensure the DNA integrity and quantified using the Nanodrop ®ND-1000 Spectrophotometer (Thermo Scientific).

Plasmid DNA Linearization

The plasmid DNA was linearized by digesting 5' of the probe sequence with an appropriate restriction enzyme (Promega/Roche) (Table 2.1) under optimal conditions specified by the manufacturers. In order to digest the plasmid DNA, the following reagents were mixed: 5µg of template DNA, 5µl of 10X NE Buffer, 5µl 10x BSA (100mg/ml), 1µl of restriction enzyme in a final volume of 50µl. The restriction mix was incubated for 3 hours at 37ºC. The restriction products were then validated by gel electrophoresis in 1% Agarose (Sigma) gel to confirm a single digestion by restriction enzyme. Afterwards, the Wizard SV Gel and PCR Clean-Up System Kit (Promega) was used to purify the restriction reaction products following the manufacturer's protocol. Plasmid DNA concentration was assessed against RNAse-free water using Nanodrop ®ND-1000 Spectrophotometer (Thermo Scientific).

Antisense mRNA Probe Transcription

The DIG labeled mRNA probes were synthesized with an appropriate RNA polymerase (T7 or SP6, Roche or Promega) (Table 2.1) following the manufacturer's guidelines. Briefly, approximately 1µg linearized plasmid DNA was added to 0.75M DTT, 10x DIG-NTP mix (Promega/Roche), RNAsin (Promega/Roche), 10x Transcription Buffer (Promega/Roche) in a final volume of 25µl with RNase free water. Finally, 20U of RNA polymerase was added and the reaction was incubated at 37ºC for 3 hours. RNA was purified by adding 20.5µl of RNAse free water, 2µl of 0.5M EDTA (pH 0.8), 2.5µl of 8M LiCl and 150µl of 100% cold ethanol and incubated at -20ºC for 2 hours. Subsequently, RNA was precipitated by centrifugation at 14680rpm for 30 minutes at 4ºC. The supernatant was removed and the pellet was washed with 1ml 70% cold ethanol. The precipitate was for a second time centrifuged at 14680 rpm during 15 minutes at 4ºC and the supernatant was removed. The pellet was then briefly air dried and resuspended in 30µl of 10 mM EDTA and stored at -20ºC. The probes were visualized by electrophoresis of 1µl on a 1% Agarose (Sigma) gel in order to validate its integrity and to ensure the amount of synthesized RNA probe. The RNA concentration was assessed against RNAse-free water using the Nanodrop®ND-1000 Spectrophotometer (Thermo Scientific).

2.5.2 Prevention of pigmentation development

In order to prevent pigment formation the embryos were treated with 1-phenyl-2-thiourea (PTU, 0.03mg/ml) (cat: P3755, Sigma-Aldrich) before the onset of pigmentation at 1dpf. 1% PTU was added to the embryo medium from a stock solution of 25% PTU. PTU inhibits melanogenesis by blocking all tyrosinase-dependent steps in the melanin pathway (Karlsson *et al.*, 2001) and the embryos remain transparent as long as the PTU treatment is continued. Therefore, the PTU solution was changed daily as needed.

2.5.3 Whole mount *in situ* hybridization

For whole mount *in situ* hybridization, larvae were fixed as previously described and stored at -20°C until processed. Whole mount *in situ* hybridizations were performed as described in Thisse and Thisse (2008) (Appendix A).

To validate our results, the *in situ* hybridization studies were performed at least three times with a minimum of ten larvae samples for each probe.

2.6 Histological analysis

Wild type larvae submitted to whole mount *in situ* hybridization were processed for sectioning and further microscopy analysis of the Hh signaling expressing cells. Larvae were rehydrated successively in 50% glycerol/PBS and 25% glycerol/PBS solutions for 5 minutes each and washed several times in PBS. The samples were then passed through a 15% sucrose/PBS solution allowing larvae to settle and incubated with a 30% sucrose/PBS solution at 4ºC overnight for cryoprotection. Afterwards, the samples were embedded in melted 15% gelatin (Sigma)/30% sucrose/PBS solution for an hour at 37ºC and further incubated at 4ºC on the appropriate support for solidification. Once the embedding blocks were solidified, they were trimmed into shape ready for sectioning. The trimmed blocks were frozen in isopenthane/liquid nitrogen and stored at -80ºC until sectioning. Longitudinal sections of 20µm stained fin folds of uncut controls and fin folds subjected to amputation were obtained using a Leica CM3050 S Cryostat and mounted on slides using Mowiol (Sigma).

2.7 Measurement of the regenerated area

To determine the regenerative outgrowth of *smu* mutants, cyclopamine and aphidicolin treated larvae in comparison to control conditions (*smu* sibling or DMSO, respectively), two measurements were collected: the fin fold regenerated area and the uncut fin fold area of larvae at the same developmental stages in each situation. To track the regenerative state until the end of the larvae fin fold regenerative process, the larvae were fixed in 4% paraformaldehyde (PFA) overnight and the fin folds were imaged on days 2, 3 and 4 post amputation using a Leica DM2500 bright field microscope with a 20x objective lens and a Leica DFC420 digital camera. The images were then processed in Image J (NIH) software.

Each image was set to scale with a calibration graticule under 20 x magnifications. The area measurements were obtained using the notochord as reference point to trace a line along the amputation plane and outlining the whole fin fold as depicted in Figure 2.2. The area of tissue posterior to this amputation plane presented the amount of regenerated tissue. Subsequently, the area measurements were acquired using the Measure tool of ImageJ (NIH) software. Images were adjusted for overall contrast and brightness in ImageJ software (NIH).

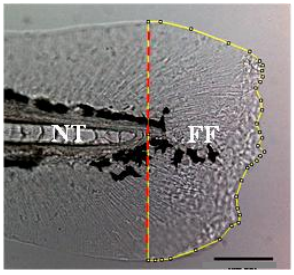

Figure 2.2 - Representation of the area measurement performed in ImageJ software. The image represents a zebrafish DMSO-treated larvae fin fold at 2dpa. The red line indicates the plane of amputation and the yellow line indicates the area of regenerated fin fold tissue. NT – notochord; FF – fin fold regenerated. Scale bar: 100 µm.

2.8 Data analysis and statistics

Since normal fin fold growth also occurs during regeneration of the larvae fin fold, the regenerated area was estimated at each stage as the ratio of the regenerated fin fold area related to the uncut area of larvae at the same developmental stage, in both control and experimental conditions. Subsequently, the mean and standard deviation were estimated for each ratio and plotted in graphs. Finally, the differences in control *versus* experimental conditions were analyzed using a two tailed Student t-test. Significance was established for p-values less than 0.05.

Calculation of descriptive statistics, plot design and t-tests were performed using Excell Microsoft Office 2007.

2.9 Image acquisition and processing

In situ hybridization stained larvae were cleared and mounted in 100% glycerol. Imaging was performed using a Leica Z6APO stereomicroscope equipped with a Leica DFC490 digital camera under a 36x objective lens. Detailed analysis of histological sections was obtained using a Leica DM2500 bright field microscope equipped with a Leica DFC429 digital camera under a 20x objective lens. High-resolution imaging of BrdU labeling was performed under a confocal scanning system (Zeiss LSM 510 Meta confocal microscope) equipped with an argon (Ar) laser with peak outputs of 488nm using a dry 20x objective lens. Confocal z-stacks were captured at 1.2 μm intervals and analysed with ImageJ (NIH) software. Figures were processed using Adobe Photoshop® CS6 Extended software (Adobe Systems) and/or ImageJ software (NIH).

2.10 Solutions and buffers

The following overview comprises general buffers and solutions. Other solutions are mentioned together with the methods.

1% Embryo Medium (10L)

50x 14,69g NaCl; 0.63g KCl; 2.43g CaCl2.2H2O; 4.07g MgSO4.7H2O; 1ml Methylene Blue

10x PBS (1L)

137mM NaCl; 2.7mM KCl; 4.3mM Na2HPO4.7H2O; 1.4mM KH2PO4

PBT

0.1% Tween20 in PBS

4% PFA

4gr of Paraformaldehyde in 100ml of PBS

Hybmix buffer solution

50% Formamide; 5x SSC; 0.1% Tween20; Citric Acid (pH=6.0); Heparin 50µg/ml; tRNA 500 µg/ml.

Blocking solution (ISH)

2% Goat serum, 2mg/ml BSA (in PBT solution)

NTMT buffer solution

0.1M Tris-HCl pH 9.5, 50mM MgCl2, 0.1M NaCl, 0.1% Tween 20

Staining Solution

50mg/ml NBT, 50mg/ml BCIP. (in NTMT solution)

20x SSC

3M NaCl (175.32g/l) and 0.3M Trinatrium citrate (88.23g/l)

Chapter 3: Results

The current chapter presents the results obtained while trying to answer the following questions: Is Hedgehog (Hh) signaling a key pathway in zebrafish larvae fin fold regeneration? Are primary cilia imperative for the Hh signal transduction in the regenerative context? Is cell proliferation absolutely necessary during zebrafish larvae fin fold regeneration? In order to answer these questions in a systematic way this chapter was divided into four major sections that comprise experiments carried out in wild type larvae (section 3.1), larvae with Hh signaling disrupted/inhibited (section 3.2) and mutant larvae that lack primary cilia (section 3.3). Finally there is an analysis on the regenerative capacity of the zebrafish larvae fin fold in the absence of cell proliferation.

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As stated in section 1.4 of Chapter 1, data from our lab has shown that zebrafish larvae fin fold is ciliated and that upon amputation an increase in number and length of cilia can be found in the fin fold reaching its maximum at 2 days post amputation (dpa). From this time point onwards, cilia number and length decrease to a baseline level until the architecture of the fin is restored at 3dpa (Lima, 2010). Based on preliminary results of electron microscopy of cilia cross-sections, the cilia in the zebrafish larvae fin fold are primary cilia (Pascoal *et al*., personal communication). Since primary cilia are involved in the signal transduction of key signaling pathways during embryo development (reviewed in Eggenschwiler and Anderson, 2007; Satir *et al*., 2010), the ciliogenesis event may be related to an increased susceptibility to respond to the lesion and allow the regeneration process to occur. Primary cilia have been described as central organelles essential for the Hh signal transduction in mammalian systems (reviewed in Eggenschwiler and Anderson, 2007) and more recently in zebrafish (Huang and Schier, 2009; Kim *et al*., 2010). Therefore, we aimed to study the role of the Hh signaling pathway during the process of zebrafish larvae fin fold regeneration.

3.1 Characterization of the expression pattern of Hedgehog pathway components during zebrafish larvae fin fold regeneration

The re-activation of the Hh signaling pathway has been described as a critical event for adult zebrafish caudal fin regeneration (Quint *et al*., 2002). However, the requirement of the Hh signaling pathway for larval fin fold regeneration is still unknown. To address this question a characterization of several Hh signaling pathway components was performed during the several stages of larvae fin fold regeneration. In order to perform this characterization, we analyzed by *in situ* hybridization (ISH) the expression of a selected group of genes intended to be representative of the whole Hh signaling

pathway. As will be described, this group of genes included *ihha*, *ihhb* and *shh* (Hh signaling pathway ligands), *patched1* and *patched2* (Hh signaling pathway receptors), *smoothened* (Hh signaling pathway transducer/activator), *suppressor of fused* (Hh signaling pathway repressor) and *gli1*, *gli2* and *gli3* (Hh signaling pathway transcription factors).

The fin fold amputation was always performed on zebrafish larvae at 2 days post fertilization (dpf), given that at this stage the larvae have undergone almost all morphological processes of development and the fin fold is already expanded (Kimmel *et al.*, 1995). According to what has been described in the larval fin fold system, regeneration occurs through three distinct phases: wound healing from 6 hours post amputation (hpa), blastema formation from 1-2 days post amputation (dpa) and regenerative outgrowth from 2-3dpa, with full regeneration of lost structures being observed at 3dpa (Kawakami *et al.*, 2004). Based on this knowledge, ISH studies were carried out on zebrafish larvae from 1dpa to 3dpa to scrutinize how Hh signaling expression is modulated throughout the fin fold regeneration process.

For each experiment, a separate group of uncut control larvae was also analyzed at the respective developmental stages of 3, 4 and 5dpf. This permitted the direct and simultaneous comparison of the expression of the Hh signaling genes in non-amputated and amputated larvae through the regeneration period. Expression in uncut controls was considered at a basal level. Therefore, it was assumed that a stronger expression level within the amputated fin folds, when compared to uncut controls, would indicate that gene transcriptional activation was induced in response to an amputation.

Expression pattern of the Hedgehog signaling ligands

The Hh signaling ligands are released into the extracellular environment to act as "instructors" and trigger cell fate decisions by inducing the Hh signaling cascade in the Hh ligands receiving cells (see section 1.2.1 of Chapter 1). During zebrafish larvae fin fold regeneration, *ihha* expression was found activated in the tip of the fin fold, consisting of the regenerating tissue, and seemed to expand to most proximal regions in the fin fold, as early as 1dpa (Fig.3.1 Panel A - A, A'). Also, at this stage *ihha* expression was weakly activated in the hypural cells region (see section 1.1.3 of Chapter 1), which have been described as essential to the normal development of the fin under the influence of Hh signaling (Hadzhiev *et al.*, 2007). In addition, strong activation of *ihha* expression was found throughout the fin fold at 2dpa (Fig.3.1 Panel A - B, B'), whereas it seemed to decrease in the same domain from 2 to 3dpa (Fig.3.1 Panel A - C, C'). This strongly suggests that *ihha* expression in the regenerating tissue is induced in response to an amputation and reaches a maximum of transcriptional activation at 2dpa, while from this time point onwards it decreases to a basal transcriptional level. In the same way, we found that *ihhb* expression was activated in the regenerating tissue and in the hypural cells region at 1dpa (Fig.3.1 Panel A - D, D') and strong transcriptional activation occurred

throughout the fin fold at 2dpa (Fig.3.1 Panel A - E, E'). Moreover, a decrease in *ihhb* expression was observed in the fin fold from 2 to 3dpa (Fig.3.1 Panel A - F, F'), suggesting that *ihhb* expression also reaches a maximum of transcriptional activation in the fin fold at 2dpa and decreases to a basal level of expression from the later stage of regeneration. In contrast, *shh* expression was initially activated in the tip of the notochord and in the hypural cells regions at 1dpa (Fig.3.1 Panel A- G, G') and was activated in the fin fold only at 2dpa (Fig.3.1 Panel A - H, H'). At this stage, *shh* expression was found strong throughout the larvae fin fold (Fig.3.1 Panel A - H, H'). Conversely, *shh* expression decreased to a basal transcriptional level similar to uncut controls at 3dpa (Fig.3.1 Panel A - I, I'). Hence, *shh* expression in the regenerating fin fold appears to be induced later than *ihh* genes expression and may be induced in response to their increased levels.

Expression pattern of the Hedgehog signaling receptors

The response of cells to Hh signaling is associated with expression of *patched (ptc)*, a transmembrane receptor in the Hh-receiving cell, which is directly up regulated in target tissues adjacent to those expressing *hh* ligands (Hooper and Scott, 2005). In vertebrates, there are two *ptc* receptors encoding genes: *ptc1* and *ptc2*. *Ptc1* is the major Hh signaling receptor and a downstream target of the Hh signaling cascade itself. *Ptc2* is co-expressed with *hh*, but its transcription is independent of Hh pathway activation. In the zebrafish, *ptc2* seems to be highly expressed in all Hh responsive tissues but is generally restricted to cells in close proximity to a source of Hh signal, whereas *ptc1* domain of expression extends from sources of Hh ligands (Lewis *et al.*, 1999).

As shown in Figure 3.1 Panel B, no differences were found in *ptc1* expression in regenerating and uncut control fin folds during the process of zebrafish larvae fin fold regeneration. At 1dpa, *ptc1* expression was restricted to the notochord and the neural tube in a similar pattern to the uncut control (Fig.3.1 A, A'). Subsequently, *ptc1* expression was activated in both regenerating and uncut control fin folds in the most distal end of the notochord at 2 and 3dpa, and 4dpf and 5dpf respectively (Fig.3.1 B-C'). *Ptc1* expression was never observed in the regenerating fin fold throughout the larvae fin fold regeneration process. This strongly suggests that Ptc1 may not play an important role during zebrafish larvae fin fold regeneration. Moreover, *ptc1* expression was absent from the hypural cells region in both regenerating and uncut control larvae, which indicates that Ptc1 may not as well be imperative to the normal development of the fin. On the other hand, *ptc2* expression was found weakly activated in the hypural cells region in regenerating larvae at 1dpa (Fig.3.1 D, D'), strongly activated in the tip of the fin fold and in the hypural cells region at 2dpa (Fig.3.1 E, E') and appeared to decrease in the fin fold from 2 to 3dpa (Fig.3.1 F, F'). This suggests that *ptc2* expression is induced in response to the larvae fin fold amputation and reaches a maximum of transcriptional activation in the fin fold at 2dpa in a similar pattern to the *hh* ligands genes expression.

Expression pattern of the Hedgehog signaling activator and repressor

When Hh ligands bind and inactivate Ptc, Smoothened (Smo), another transmembrane protein, initiates intracellular signal transduction events that lead to activation of the Gli family of transcription factors that control the output of Hh signaling. Hence, Smo accumulation on the cell surface in response to the increased Hh ligands levels is essential to Hh signal transduction (Corbit *et al.*, 2005; Milenkovic *et al.*, 2009). On the opposite, Suppressor of Fused (Sufu), a negative regulator of Hh signaling, prevents the activation of the Hh signaling cascade in the absence of Smo, sequestering and maintaining Gli proteins in the cytoplasm and promoting the processing of Gli repressor forms (Merchant *et al.*, 2004) (see section 1.2.1 of Chapter 1). Thus, it would be expected that during zebrafish larvae fin fold regeneration *smo* expression would be activated in the same domains as *hh* expressing cells and *sufu* expression would be activated in the same domains as *smo* as a negative feedback mechanism. In Figure 3.1 Panel C, we show that *smo* expression was indeed activated in the regenerating tissue and seemed to expand to most proximal regions of the fin fold at 1dpa (Fig.3.1 A, A') in a similar pattern to *ihh* genes expression. Thereafter, strong activation of *smo* was also observed in the fin fold and in the hypural cells region at 2dpa (Fig.3.2 B, B') and appeared to slightly decrease from 2 to 3dpa (Fig.3.1 B', C'). In the same way, *sufu* expression was weakly activated in the tip of the fin fold at 1dpa (Fig.3.1 D, D') and strongly activated in the fin fold and the hypural cells region at 2dpa (Fig.3.1 E, E') in a similar pattern to *smo* expression.

Expression pattern of the Hedgehog signaling transcription factors

The binding of the Gli transcription factors (Gli1, Gli2 and Gli3) to their cognate cis-acting elements, Smo or Sufu, modulates the expression of Hh signaling pathway target genes, which include *ptc1* and *gli1*. As previously mentioned (section 1.2.1 of Chapter 1), Gli1 functions exclusively as transcriptional activator. In the zebrafish, low level *gli1* expression is independent of Hh signaling, while *gli1* transcription becomes fully activated by Hh ligands to mediate its effects on Hh signaling target genes (Karlstrom *et al.*, 2003). Gli1, together with Gli2 is mostly responsible for providing prolonged cellular responses to Hh ligands. Conversely, Gli3 primarily acts as signaling repressor (Tyurina *et al.*, 2005).

During zebrafish larvae fin fold regeneration, *gli1* expression was weakly activated in few cells just beneath the amputation plane at 1dpa (Fig.3.1 Panel D- A, A') and this expression domain was maintained at 2dpa (Fig.3.1 Panel D - B, B'). At 3dpa, strong transcriptional activation of *gli1* was observed throughout the fin fold, namely in the new formed tissue (Fig.3.1 Panel D - C, C'). Since *gli1* is a direct readout of Hh signaling pathway activation, this strong *gli1* activation in the new tissue suggests that Hh signaling activation occurs during larvae fin fold regeneration.

On the other hand, *gli2* expression was activated in the regenerating tissue and in the hypural cells region at 1dpa (Fig.3.1 Panel D - D, D') in a similar pattern to the *ihh* ligands expression. In

addition, strong activation of *gli2* was observed in the regenerating tissue at 2dpa (Fig.3.1 Panel D - E, E'), which coincides with the peak of *hh* ligands expression at the same stage. Moreover, a decrease in *gli2* expression to a basal level was observed at 3dpa (Fig.3.1 Panel D - F, F'). Therefore, *gli2* expression appears to occur in coordination to *ihh* ligands expression.

Finally, *gli3* expression was found activated in the regenerating tissue early as 1dpa (Fig.3.1 Panel D - G, G'). *Gli3* expression was further enhanced in the same domain at 2dpa (Fig.3.1 Panel D - H, H') and reached a maximum of transcriptional activation in the fin fold, namely in the regenerating tissue, at 3dpa (Fig.3.1 Panel D - I, I'). This strong *gli3* activation at 3dpa coincides with the peak of *gli1* expression in the fin fold at the same stage (Fig.3.1 Panel D - C').

Together, our results showed that several Hh signaling components are up-regulated during zebrafish larvae fin fold regeneration soon as 1dpa and that this up-regulation may last until 3dpa. This strongly suggests that Hh signaling has a role to play in zebrafish larvae fin fold regeneration. Moreover, our results demonstrated that several Hh signaling components such as *ihha*, *ihhb*, *shh*, *smo*, *sufu*, *ptc2* and *gli2*, exhibit a strong transcriptional activation at 2dpa, which coincides with the peak of cilia during zebrafish larvae fin fold regeneration from previous work (Lima, 2010). A summary of the ISH results is depicted in Figure 3.2.

Following this characterization, a detailed analysis on the cell types expressing the Hh pathway genes through histological sections of both amputated and uncut control larvae would be important to clarify the stained tissues. However, due to the larvae fin fold tissue frailty upon ISH, it was not possible to obtain histological sections thin enough to scrutinize this type of information (Appendix B, Fig. B1). Hence, the histological protocol (section 2.6 of Chapter 2) needs further optimization.

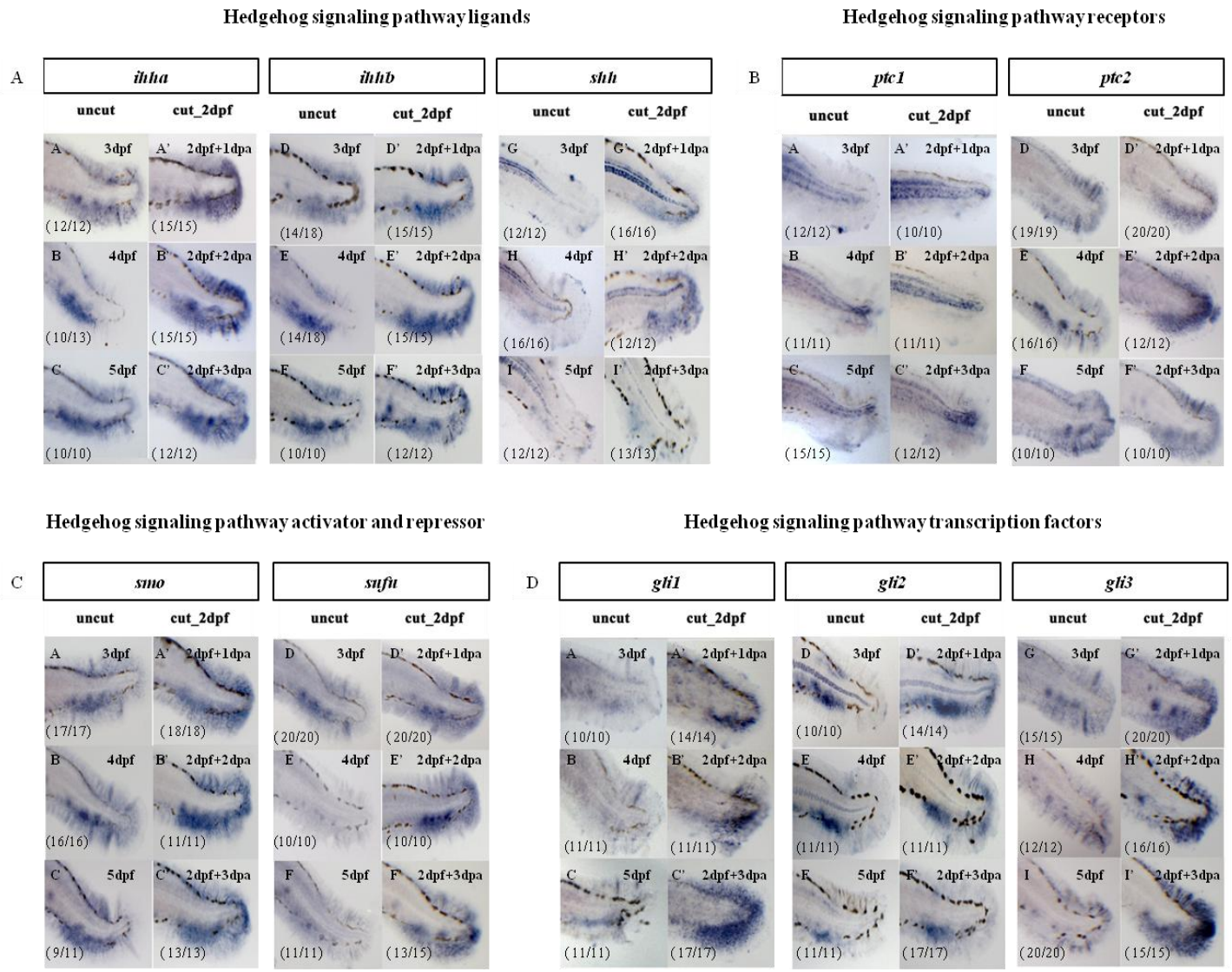


Figure 3.1 – Expression pattern of the Hedgehog signaling pathway genes during zebrafish larvae fin fold regeneration. (Panel A) Representative images of the Hh pathway ligands expression patterns during the three stages of regeneration (1-3dpa, A'-I') and respective uncut controls (3-5dpf, A-I). (Panel B) Representative images of the Hh pathway receptors expression patterns during the three stages of regeneration (1-3dpa, A'-F') and respective uncut controls (3-5dpf, A-F). (Panel C) Representative images of the Hh pathway activator and repressor expression patterns during the three stages of regeneration (1-3dpa, A'-F') and respective uncut controls (3-5dpf, A-F). (Panel D) Representative images of the Hh pathway transcription patterns expression patterns during the three stages of regeneration (1-3dpa, A'-I') and respective uncut controls (3-5dpf, A-I). All images are lateral views of the fin folds oriented anterior to the left, dorsal top, posterior to the right and ventral bottom (36x magnification).

A **Fin fold – *Wild type* (cut *versus* uncut)**

	ihha	*ihhb*	*shh*	*ptc1*	*ptc2*	*smo*	*sufu*	*gli1*	*gli2*	*gli3*
1dpa/ 3dpf										
2dpa/4dpf										
3dpa/5dpf										

Legend:

- Same level of expression
- Mild up-regulation
- Strong up-regulation

B **Hypural cells – *Wild type* (cut *versus* uncut)**

	ihha	*ihhb*	*shh*	*ptc1*	*ptc2*	*smo*	*sufu*	*gli1*	*gli2*	*gli3*
1dpa/3dpf										
2dpa/4dpf										
3dpa/5dpf										

C **Notochord– *Wild type* (cut *versus* uncut)**

	ihha	*ihhb*	*shh*	*ptc1*	*ptc2*	*smo*	*sufu*	*gli1*	*gli2*	*gli3*
1dpa/3dpf										
2dpa/4dpf										
3dpa/5dpf										

D

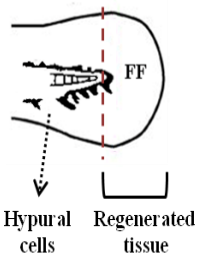


Figure 3.2 – Comparison on the Hedgehog pathway genes expression levels of amputated *versus* non-amputated zebrafish larvae fin folds (A) Representation of the Hh pathway genes expression in the larvae fin fold. (B) Representation of the Hh pathway genes expression in the hypural cells region. (C) Representation of the Hh pathway genes expression in the notochord (D) Drawings of the zebrafish larvae fin fold indicating the respective domains. FF – fin fold.

3.2 Impact of Hedgehog signaling inhibition on the larvae fin fold regeneration

Previous work from Quint *et al.* (2002) has shown that the zebrafish caudal fin regenerative process is dependent on Hh signaling activation and subsequent induced cell proliferation required to the caudal fin regenerative outgrowth (Quint *et al.*, 2002). Moreover, it demonstrated that Hh signaling inhibition results in the arrest of the zebrafish caudal fin regenerative outgrowth (Quint *et al.*, 2002). To determine whether the zebrafish larvae fin fold regenerative process is regulated by Hh signaling, two independent approaches were performed in this work. In a first approach, we studied the zebrafish larvae fin fold regenerative outgrowth in the absence of Hh signaling using a genetic zebrafish mutant line for smo (Barresi *et al.*, 2000a; Varga *et al.*, 2001), the Hh signaling transducer. In a second approach, the larvae fin fold regenerative process was analyzed through pharmacological treatment with an Hh signaling antagonist used to block Hh signaling in a temporally controlled manner.

3.2.1 The fin fold regenerative process in *smoothened* mutants

The slow muscle omitted mutant line (*smu*^{b641}) carries a mutation in the *smoothened* gene that results in a loss-of function variant of the receptor Smo (Barresi *et al.*, 2000a; Varga *et al.*, 2001). Consequently, *smu* mutants display strong phenotypes in all regions that are thought to depend on Hh signaling as the midline, the somites or the notochord, and are a helpful tool to study processes that are regulated through Hh signaling (Barresi *et al.*, 2000b; Varga *et al.*, 2001).

In order to obtain *smu* mutant embryos, heterozygous carriers' fish were crossed. *Smu* mutant embryos were sorted at 2dpf based on the characteristic phenotype which becomes apparent by the bulky U-shaped somites, the ventral body curvature, the smaller sized forebrain and the reduced interocular distance (Fig.3.3 B) (Varga *et al.*, 2001). *Smu* siblings that consist in 75% of the heterozygous carriers' progeny, exhibit a characteristic wild type phenotype (Fig.3.3 A).

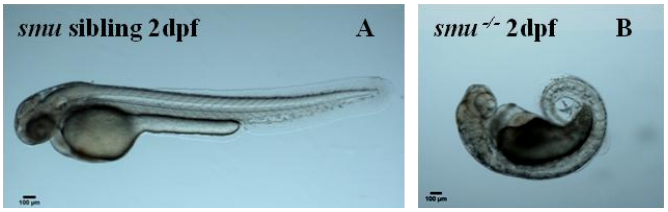


Figure 3.3 – *Smoothened* mutant and sibling zebrafish larvae at 2 days post fertilization. (A) *Smu* sibling larvae consist in 75% of the heterozygous carriers' progeny and resemble wild type larvae. (B) Homozygous *smu* mutants, which consist in 25% of the hetezygous carriers' progeny, are screened at 2dpf based on their characteristic phenotype which becomes apparent by the ventral body curvature. Images are lateral views. Magnification: 2 x. Scale bars: 50 um.

To evaluate whether Hh signaling is a requisite on the larval fin fold regenerative process, a characterization on the larvae fin fold regenerative outgrowth was performed on *smu* mutants in comparison to *smu* siblings which served as positive control. In order to perform this characterization, the larvae fin fold was amputated at 2dpf and allowed to recover for 2 and 3dpa (i.e. 4dpf and 5dpf, respectively), reflecting the larvae fin fold regenerative outgrowth time period (Kawakami *et al.*,

2004). Subsequently, to estimate the fin fold regenerative outgrowth, we measured the overall fin fold regenerated area at each stage, as previously described (see section 2.10 of Chapter2). To normalize the data according to the respective size of each larvae (*smu* mutant *versus smu* siblings) and control for possible influence of normal fin fold development during the regenerative period, we also measured the uncut fin fold area of both *smu*$^{-}$ mutant and *smu* sibling larvae at the same developmental stages. The regenerated area was, then, estimated at each stage based on the ratio of the regenerated area relative to the uncut fin fold mean area for *smu* mutant and *smu* sibling larvae.

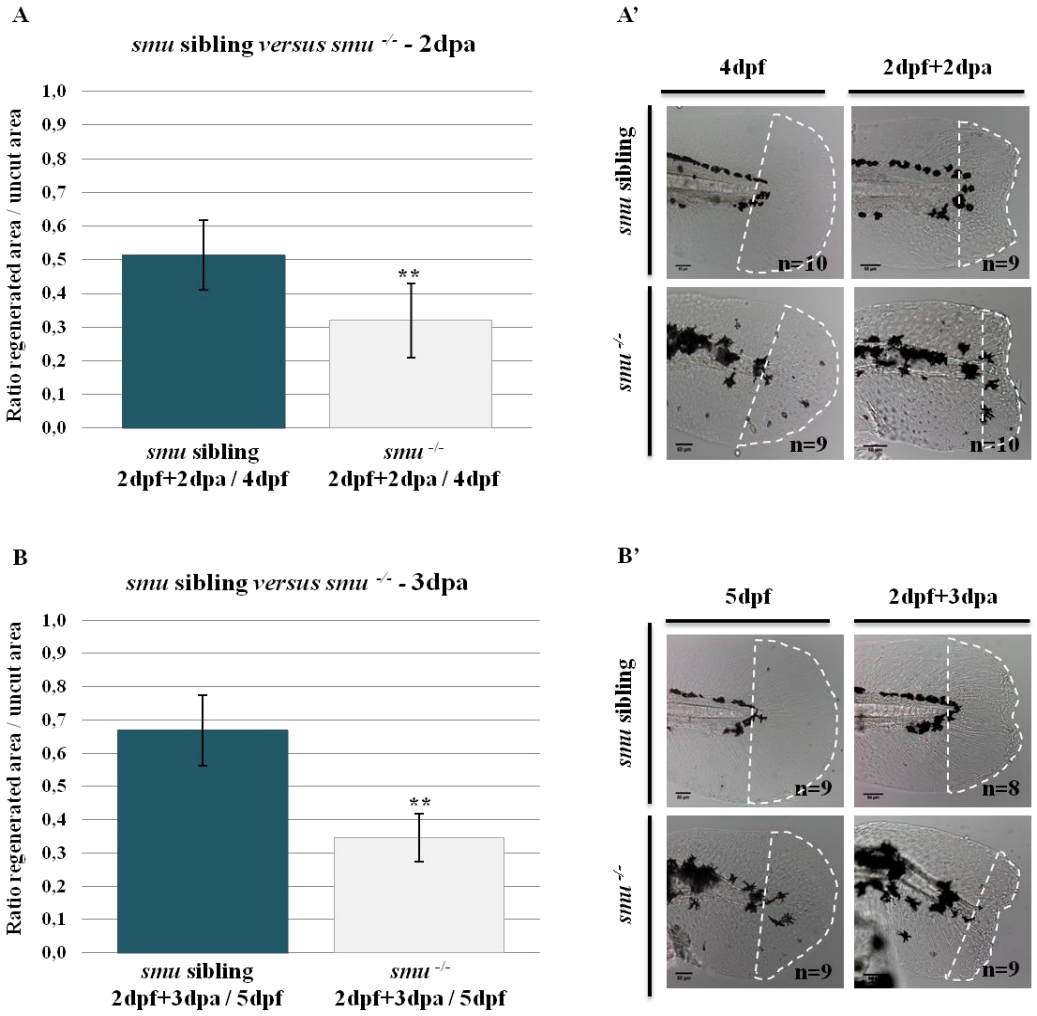


Figure 3.4 – Zebrafish *smu* mutant larvae are not able to recover their fin fold upon an amputation. Graphs showing the ratio of the regenerated fin fold areas in *smu* sibling versus *smu* mutant larvae at 2 (A) and 3 (B) days post amputation (dpa). S*mu* mutant larvae demonstrate a significant decrease in the regenerated fin fold area compared to *smu* siblings at 2dpa (A). The difference in the regenerated area in *smu* sibling and *smu* mutant larvae increases to 32% at 3dpa (B). At this point, regeneration of the fin fold is truncated in *smu* mutant larvae, while *smu* siblings display 67% of regenerated tissue. *Smu* sibling larvae do not complete the fin fold regenerative process within a 3 days period, as in a wild type situation. (A'-B') Bright field images represent lateral views of the larvae fin fold oriented posterior to the right, ventral to bottom. The white dotted lines represent the area measurements performed at each stage. Magnification: 20 x. Error bars: standard deviation. **: $p < 0.01$; Scale bars: 50 µm.

As shown in Figure 3.4, *smu* mutant larvae demonstrated a decrease in the regenerated fin fold area of approximately 20% compared to *smu* siblings at 2dpa (Fig.3.4 A). The regenerated area significantly decreased from 51±10% (mean ± standard deviation) in *smu* siblings to 32±11% in *smu* mutant larvae ($p=1.0 \times 10^{-3}$, t-test) (Fig.3.4.A, A'). Following 3dpa, the difference in the regenerated area between *smu* mutant and sibling larvae fin fold significantly increased to approximately 32% (Fig.3.4B). At this stage, s*mu* siblings continued to regenerate their fin fold and demonstrated an increase to 67±10% regenerated tissue, whereas regeneration was blocked in *smu* mutant larvae to just 35±7% fin fold regrowth ($p=8.8 \times 10^{-6}$, t-test) (Fig.3.4.B, B'). The experiment was reproduced one additional time and the same result was observed.

Given that *smu* mutant larvae display a severe phenotype and their lifespan is up to 5dpf, which corresponds to the stage of the truncation observed in the mutants fin fold regeneration, the hypothesis that the blockade in regeneration occurs due to a side effect of the mutant phenotype cannot be excluded. Furthermore, since the controls are not able to recover the amputated tissue within a 3 days period, as would be expected, these data cannot be trusted. Therefore, a second approach was followed to inhibit Hh signaling and confirm its role during zebrafish larvae fin fold regeneration.

3.2.2 The fin fold regenerative process in cyclopamine treated larvae

A standard methodology used to inhibit the Hh signaling pathway is the pharmacological treatment with cyclopamine (Incardona *et al.*, 1998; Taipale *et al.*, 2000). Cyclopamine is a steroidal compound that interferes with the Smo receptor, impairing the signal transduction of the Hh signaling cascade (Chen *et al.*, 2002) .

Data from our lab has already demonstrated that the initial events of the zebrafish larvae fin fold regeneration – wound healing and blastema formation – are correctly guided following cyclopamine treatment (Lima, 2010). To determine whether Hh signaling inhibition impacts larval fin fold regenerative outgrowth, a characterization on cyclopamine treated *versus* control larvae regeneration was performed at different stages of larvae fin fold regeneration. In order to perform this characterization, wild type larvae were exposed to 200μM cyclopamine subsequent to the fin fold amputation at 2dpf to ensure that only the regenerative process would be affected. Afterwards, larvae were allowed to recover for 2, 3 and 4 days in cyclopamine containing media to assess their regenerative capacity in the absence of Hh signaling through different stages. As a control, a separate group of wild type larvae treated with DMSO, a compound without known effects on the Hh signaling pathway, was also analyzed. Control larvae treated with DMSO were indistinguishable from wild type larvae.

In order to determine cyclopamine treated larvae fin fold regenerative outgrowth, the overall fin fold regenerated area was measured at each stage, in both control and cyclopamine treated larvae. Since normal fin fold development also occurs within the regenerative period, the uncut fin fold area

of larvae at the same developmental stages (i.e. 4, 5 and 6dpf) were measured to normalize the data at each stage, in both DMSO and cyclopamine conditions. The regenerated area in each stage was then estimated based on the ratio of the regenerated tissue area related to the uncut fin fold area for cyclopamine and DMSO treated larvae.

As depicted in Figure 3.5, larvae exposed to cyclopamine demonstrated a decrease in the regenerated fin fold area of approximately 22% compared to DMSO controls at 2dpa. The regenerated area significantly decreased from 75±10% (mean ± standard deviation) in DMSO controls to only 53±9% fin fold regrowth in cyclopamine treated larvae ($p=1.7 \times 10^{-6}$, t-test) (Fig.3.5 A, A'). At 3dpa, the difference in the regenerated area between cyclopamine and DMSO treated larvae was additionally increased to approximately 34%. DMSO controls demonstrated 94±8% of regenerated tissue, whereas cyclopamine treated larvae showed just 60±10% of regenerated tissue ($p = 5.8 \times 10^{-10}$, t-test) (Fig.3.5 B, B'). At 4dpa, regeneration in cyclopamine treated larvae was truncated to 56±11% fin fold regrowth, while DMSO controls recovered 99±8% of the amputated tissue ($p = 8.6 \times 10^{-10}$, t-test) (Fig.3.5 C, C'). Comparing the ratios of cyclopamine treated larvae regeneration at each stage through a Student t-test, we observed that tissue regeneration still occurs from 2 to 3dpa ($p = 3.5 \times 10^{-2}$, t-test), albeit is significantly decreased compared to DMSO treated larvae, while tissue regeneration in cyclopamine treated larvae was completely inhibited from 3 to 4dpa ($p = 2.8 \times 10^{-1}$, t-test). Therefore, the cyclopamine treatment led to a reduction and then to complete perturbation of the larvae fin fold regenerative outgrowth, similar to previous work on adult zebrafish (Quint *et al.*, 2002).

Subsequently, to determine whether cyclopamine impacts normal fin fold development, a Student t-test was used to compare the uncut fin fold areas of DMSO and cyclopamine treated larvae at each stage (4-6dpf). Accordingly, the normal fin fold development was also demonstrated to be impaired in cyclopamine treated larvae at all stages (4dpf: $p = 2.4 \times 10^{-7}$, 5dpf: $p = 1.3 \times 10^{-8}$, 6dpf: $p = 8.6 \times 10^{-9}$, t-test). Thus, cyclopamine treatment interferes not only with the growth of the regenerating tissue but with the normal fin fold development.

To ascertain whether the impairment in cyclopamine treated larvae fin fold regeneration was or not the result of the inhibition on cyclopamine treated larvae fin fold normal growth, we then assessed whether these larvae demonstrated significant fin fold growth throughout the different developmental stages (4, 5 and 6dpf) analyzed. This was performed by comparing the fin fold growth of cyclopamine treated larvae non-amputated using Student's t-test. Accordingly, no significant differences were demonstrated in cyclopamine treated larvae fin fold growth from 4 to 6dpf (4dpf *versus* 5dpf: $p = 9.6 \times 10^{-1}$, 5dpf *versus* 6dpf: $p = 9.7 \times 10^{-1}$, t-test). Thus, the impairment in cyclopamine treated larvae fin fold regeneration does not occur in consequence of the normal fin fold growth arrest.

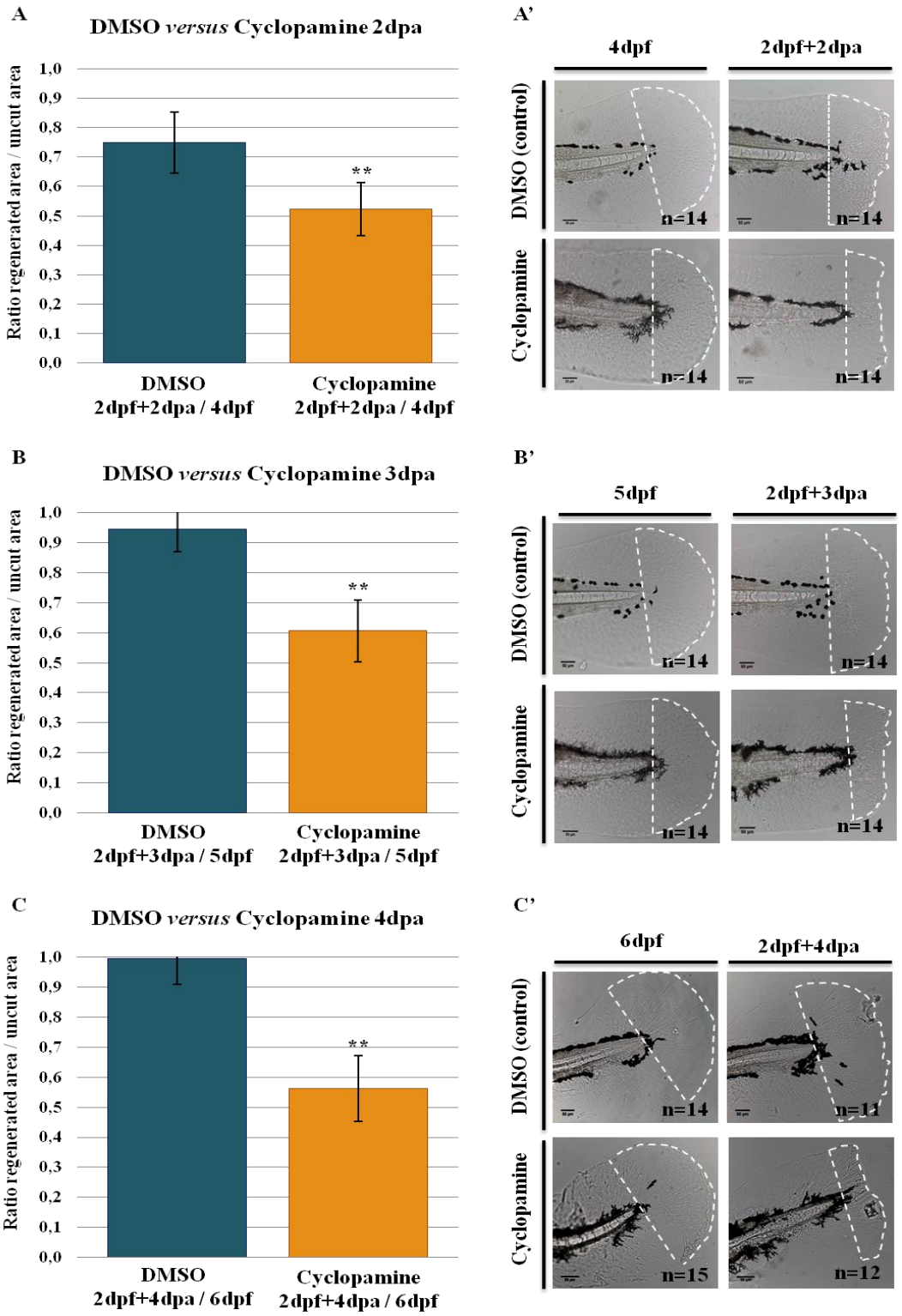


Figure 3.5 - Cyclopamine inhibits the regeneration of the zebrafish fin primordia. Graphs showing the ratio of the regenerated fin fold areas in DMSO versus cyclopamine treated larvae at 2 (A), 3 (B) and 4 (C) days post amputation (dpa). In cyclopamine treated larvae, the regenerated area significantly decreases compared to DMSO controls at 2dpa (A). Subsequently, the difference in the regenerated fin fold area in DMSO and cyclopamine treated larvae increases to 34% at 3dpa (B) and cyclopamine treated larvae are not able to recover the amputated tissue even at 4dpa (C). (A'-C') Bright field images represent lateral views of the larvae fin fold oriented posterior to the right, ventral to bottom. The white dotted lines represent the performed area measurements at each stage. Magnification: 20 x. Error bars: standard deviation. **: $p < 0.01$; Scale bars: 50 μm

In addition to these results, the cyclopamine treatment also led to an accumulation of melanophores (pigment cells) in the most distal end of the notochord in both uncut and amputated fin folds (Fig.3.5 A'-C'). This result is consistent to observations during regeneration of the adult zebrafish caudal fin upon exposure to cyclopamine, in which an accumulation of melanophores is observed in the most distal end of each regenerating bony ray (Quint *et al.*, 2002). Thus, together our results additionally demonstrate the power of the larval model system, whose ontogeny differs from the adult caudal tissue (Hadzhiev *et al.*, 2007), to unravel critical molecular signaling pathways essential for tissue regeneration.

3.2.2.1 Cyclopamine interferes with Hedgehog signaling expression in the novo tissue

To complement our cyclopamine data with a molecular analysis and ensure the specificities of the cyclopamine treatment, the mRNA expression of the previous selected Hh signaling components (see section 3.1) was analyzed in cyclopamine treated larvae through ISH. DMSO treated larvae served as positive control.

A common evaluation of the cyclopamine effects on the Hh signaling is the down-regulation of the Hh signaling downstream targets *gli1* and *ptc1*. Hence, we initially compared the changes in *gli1* and *ptc1* expression in DMSO treated *versus* cyclopamine treated larvae. The cyclopamine treatment led to a loss of *ptc1* expression in the notochord, in the somites borders and in the most distal end of the neural tube at 1dpa (Fig.3.6 Panel B, B') followed by a complete absence of *ptc1* expression throughout the whole fin fold at 2dpa (Fig.3.6 Panel B, D'). This observation is consistent to the loss of *ptc1* expression in the adult zebrafish caudal fin regeneration following the cyclopamine exposure (Quint *et al.*, 2002). At the same time, the cyclopamine treatment led to the loss of *gli1* expression in the fin fold domain consisting of the regenerating tissue at 1dpa (Fig.3.7 Panel B, B') and to a complete absence of *gli1* expression throughout the whole fin fold at 2dpa (Fig.3.7 Panel B, D'). The loss of *ptc1* and *gli1* expression at 2dpa coincides with the time period when cyclopamine interferes with the larvae fin fold regenerative process (see previous section), thus corroborating our results.

Since the expression of the Hh signaling target genes is absent from cyclopamine treated larvae fin fold from 2dpa, ISH studies for the remaining Hh signaling components were carried out at 1dpa and 2dpa. At 1dpa, no clear differences were observed in *ihha*, *ihhb* and *shh* expression in cyclopamine treated larvae compared to DMSO controls (Fig.3.6 Panel A, B', F', J'). Conversely, the cyclopamine treatment led to the loss of *ihha*, *ihhb* and *shh* expression in the fin fold domain consisting of the regenerating tissue at 2dpa (Fig.3.6 Panel A, D', H', L'), suggesting that cells at this domain are no longer competent to respond to the lesion after cyclopamine exposure. In agreement with the absence of *hh* ligands expression in the regenerating tissue, the expression of *ptc2* receptor and co-receptor *smo* was also lost from this domain early as 1dpa (Fig.3.6 Panel B, F'). Thus, cells of

the fin fold domain may no longer be competent to respond to Hh signaling following the cyclopamine treatment. Consequently, the expression of *sufu*, *gli2* and *gli3* was as well lost from the regenerating tissue in cyclopamine treated larvae from 1dpa (Fig.3.6 Panel A, F'; Panel B, F', I').

Therefore, together this dataset provides evidence on a continuing requirement for Hh signaling expression within the larvae regenerating fin fold.

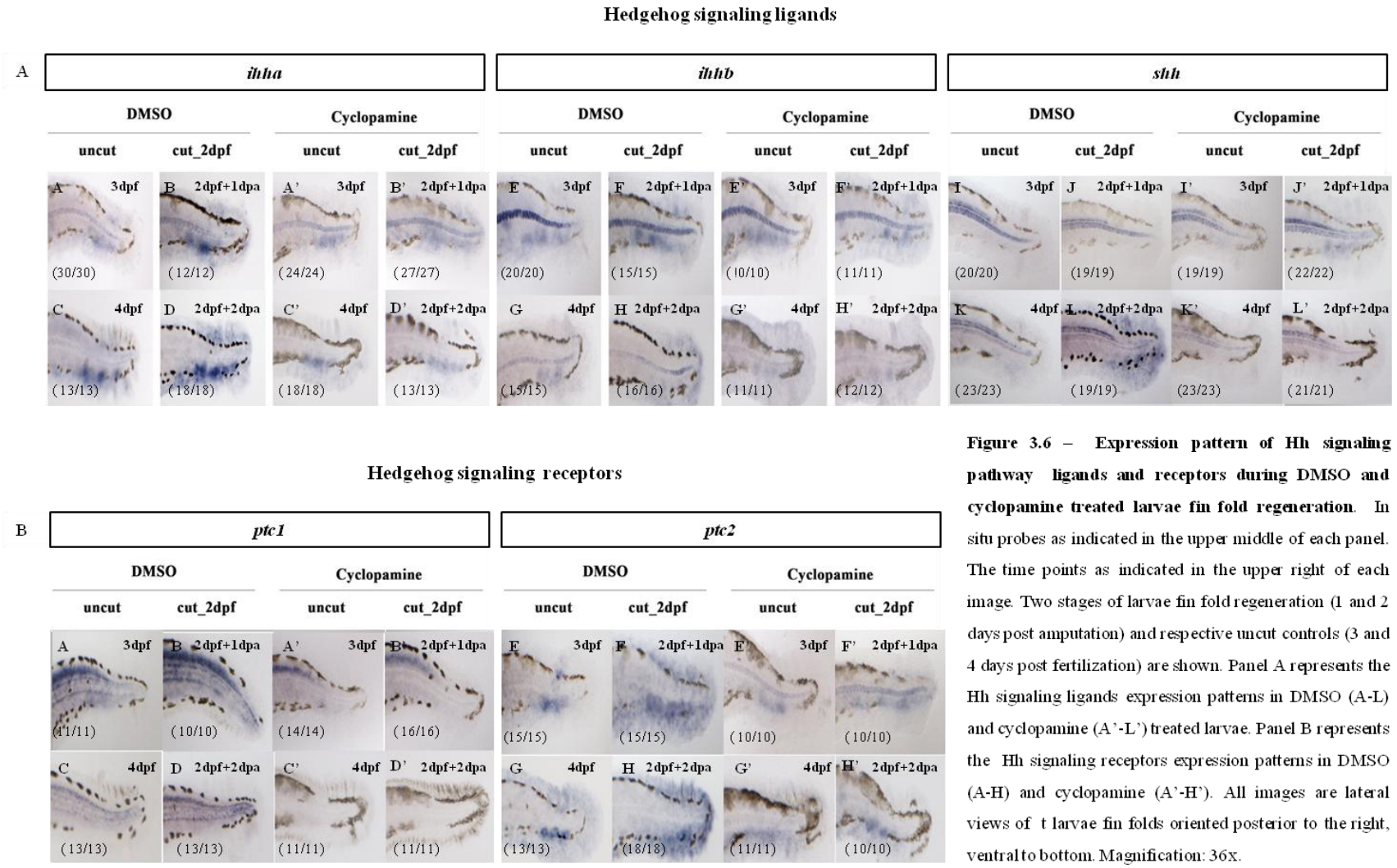


Figure 3.6 – Expression pattern of Hh signaling pathway ligands and receptors during DMSO and cyclopamine treated larvae fin fold regeneration. In situ probes as indicated in the upper middle of each panel. The time points as indicated in the upper right of each image. Two stages of larvae fin fold regeneration (1 and 2 days post amputation) and respective uncut controls (3 and 4 days post fertilization) are shown. Panel A represents the Hh signaling ligands expression patterns in DMSO (A-L) and cyclopamine (A'-L') treated larvae. Panel B represents the Hh signaling receptors expression patterns in DMSO (A-H) and cyclopamine (A'-H'). All images are lateral views of t larvae fin folds oriented posterior to the right, ventral to bottom. Magnification: 36x.

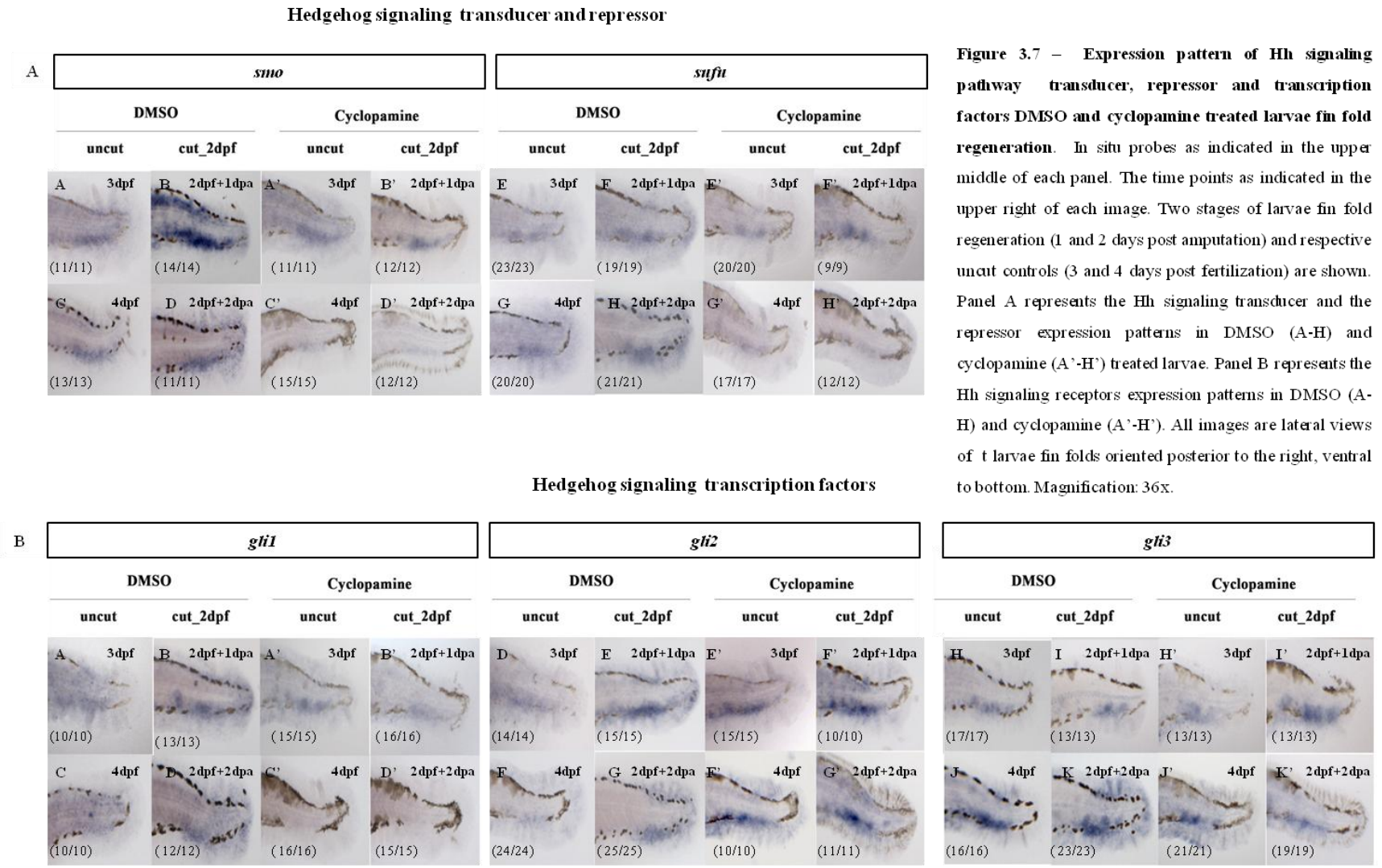


Figure 3.7 – Expression pattern of Hh signaling pathway transducer, repressor and transcription factors DMSO and cyclopamine treated larvae fin fold regeneration. In situ probes as indicated in the upper middle of each panel. The time points as indicated in the upper right of each image. Two stages of larvae fin fold regeneration (1 and 2 days post amputation) and respective uncut controls (3 and 4 days post fertilization) are shown. Panel A represents the Hh signaling transducer and the repressor expression patterns in DMSO (A-H) and cyclopamine (A'-H') treated larvae. Panel B represents the Hh signaling receptors expression patterns in DMSO (A-H) and cyclopamine (A'-H'). All images are lateral views of t larvae fin folds oriented posterior to the right, ventral to bottom. Magnification: 36x.

3.2.2.2 Cell proliferation is severely reduced following the cyclopamine treatment

Recent studies suggest that the process of zebrafish larvae fin fold regeneration is mediated by induced cell proliferation upon amputation (Kawakami *et al.*, 2004; Mateus *et al.*, 2012). According to Mateus *et al*. (2010), from 11hpa to 16hpa the proliferation rate significantly increases in regenerating fin folds when compared to non-amputated fin folds, and significantly decreases from 21hpa until the end of the fin fold regenerative process (Mateus *et al.*, 2012).

Proliferative cells are found throughout the fin fold in distal and proximal regions at 1dpa. From 2dpa until 54hpa, proliferative cells are located in most proximal regions of the fin fold near the notochord region (Kawakami *et al.*, 2004). In addition, a pool of highly proliferative cells localized in the posterior and ventral side of the notochord, termed hypural cells or ACFP (section 1.1.3 of Chapter 1), are found in both regenerating and non-amputated fin folds (Kawakami *et al.*, 2004). The hypural cells proliferative state is essential to the development of the adult zebrafish caudal fin, which is regulated by Hh signaling activity (Hadzhiev *et al.*, 2007).

It has been shown that inhibition of Hh signaling using cyclopamine arrests the zebrafish caudal fin regenerative outgrowth and severely reduces cell proliferation within the blastema (Quint *et al.*, 2002). To determine whether the same event is induced in cyclopamine treated zebrafish larvae, cell proliferation was analyzed from 2 to 3dpa and respective uncut stages of 4 and 5dpf. Cell proliferation was monitored through immunohistochemistry following BrdU pulse labeling for 6 hours prior to larvae fixation at the respective regenerative endpoints. Again, DMSO treated larvae served as positive control.

Consistent with previous reports, cell proliferation was detected in DMSO treated larvae in most proximal regions of the fin fold near the notochord at 2 and 3dpa (Kawakami *et al.*, 2004). Moreover, DMSO controls also showed a cluster of BrdU positive cells in the hypural cells domain in both uncut and regenerating fin folds at both stages (2-3dpa and 4-5dpf respectively) (Fig.3.8 A-B, A'-B'). In contrast, the cyclopamine treatment led to the absence of cell proliferation throughout the fin fold domain at 2 and 3dpa. In addition, the cyclopamine treatment also resulted in absence of cell proliferation in the hypural cells region (Fig.3.8 C-D, C'-D'), indicating that cyclopamine indeed interferes with the larvae fin fold development. In summary, these results suggest that the inhibition of Hh signaling through cyclopamine impairs cell proliferation in the larvae fin fold. Further, suggests that Hh signaling is the stimulus that promotes cell proliferation during zebrafish larvae fin fold regeneration.

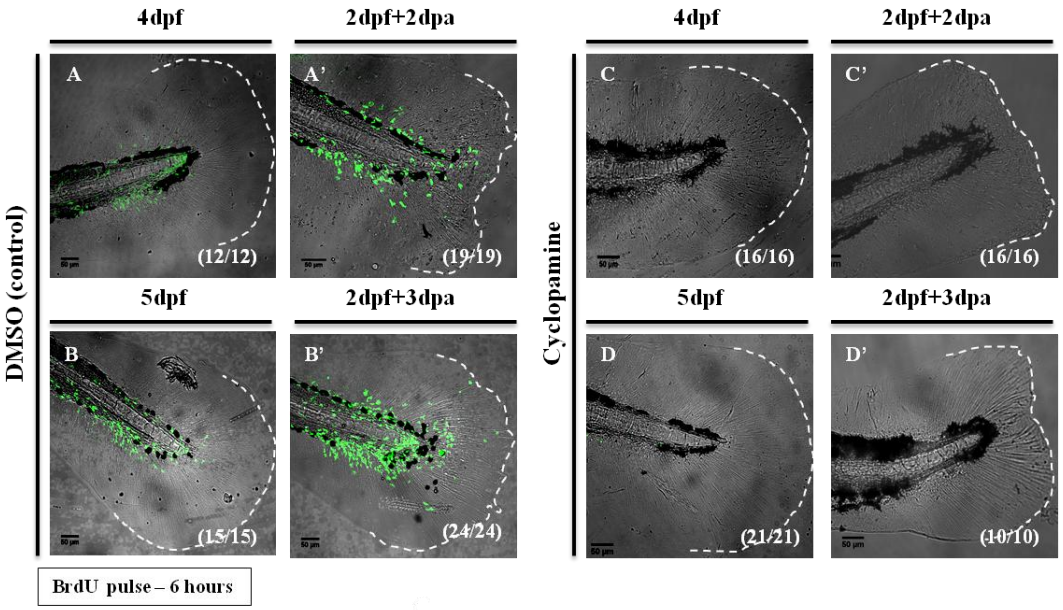


Figure 3.8 – Cell proliferation is inhibited in response to the cyclopamine treatment. DMSO treated larvae show a characteristic cell proliferation pattern. Cell proliferation is localized in most proximal regions of the fin fold near the notochord at 2 and 3dpa (A', B'). In addition, proliferative cells are also detected in the hypural cells domain in both regenerating (A', B') and non-amputated fin folds (A, B). Conversely, cyclopamine treated larvae are devoid of cell proliferation throughout the fin fold and in the hypural cells domain in both regenerating (C, D) and non-amputated fin folds (C', D'). Images are maximal intensity projections of confocal z-stacks. BrdU positive cells are shown in green. Lateral views of the larvae fin fold oriented posterior to the right, ventral to bottom. Magnification: 20x. Scale bars: 50 μm.

3.3 The *iguana* mutant regenerative process

The *iguana* gene was discovered in the zebrafish as required for Hh signaling, and encodes a zinc finger protein that is essential for differentiation of primary cilia (Sekimizu *et al.*, 2004; Wolff *et al.*, 2004; Kim *et al.*, 2010; Tay *et al.*, 2010). Previous work from our lab using an *iguana* mutant line (*igu*^{tm79a}), in which the formation of primary cilia is affected, has shown that regeneration of the larvae fin fold is not impeded in the absence of primary cilia (Lima, 2010). However, *iguana* mutant larvae fin fold regeneration occurs with a delay compared to wild type larvae at 2dpa. Consequently, *iguana* mutant larvae recover their fin folds structure and functionality just at 4dpa, whereas wild type larvae regenerate their fin folds within 3dpa (Lima, 2010). Taking this into consideration, a further characterization of the *iguana* mutant larvae fin fold regenerative process was performed in this work.

3.3.1 Hedgehog signaling is impaired during *iguana* mutant larvae fin fold regeneration

To assess whether the delay in the *iguana* mutant fin fold regeneration is the result of a perturbation in Hh signaling expression, we performed a characterization on the expression of the selected Hh signaling components (section 3.1) in *iguana* mutant larvae at the three regeneration stages (1-3dpa), through ISH. In order to perform this characterization, a group of *iguana* siblings, embryos of the same heterozygous carriers' posture, which demonstrate a wild type phenotype, were also analyzed. This permitted the direct and simultaneous comparison of the activation *versus* inhibition of the Hh signaling genes expression in the absence *versus* presence of primary cilia. It was assumed that stronger transcription activation of the analyzed genes in regenerating wild type siblings, when compared to *iguana* mutants, would indicate a down-regulation of these genes in *iguana* mutant larvae.

During zebrafish larvae fin fold regeneration, no differences were observed in *ihha* and *ihhb expression* in *iguana* mutant and wild type siblings at 1dpa. *Ihha* and *ihhb* expression was induced in the regenerating tissue and the hypural cells domain in both *iguana* mutant and sibling larvae (Fig.3.9 Panel A, D', J'). Conversely, a down-regulation of *ihha* and *ihhb* expression was observed in the regenerating tissue in *iguana* mutants compared to wild type siblings at 2dpa (Fig. 3.9 Panel A, E', K'). Since *ihha* and *ihhb* expression remained induced in the hypural cells domain, the *ihha* and *ihhb* down-regulation was specific to the new tissue. Thus, cells in the fin fold domain may not be competent to respond to lesion at 2dpa. On the other hand, strong *ihha* and *ihhb* expression was induced in the fin fold domain in *iguana* mutants at 3dpa (Fig.3.9 Panel A, F', L'), suggesting that cells at this domain regain the capacity to expand Hh signaling. In a similar pattern, down-regulation of *shh* expression was demonstrated in the regenerating tissue in *iguana* mutant compared to wild type siblings at 1dpa (Fig.3.9 Panel A, P'). At 2dpa, *shh* expression continued down-regulated in the fin fold in *iguana* mutant compared to wild type siblings (Fig.3.9 Panel A, Q'). In contrast, strong *shh* expression was activated in the fin fold in *iguana* mutant at 3dpa (Fig.3.9 Panel A, R'). Therefore, the

expression of Hh ligands appears to be impaired at 2dpa, which coincides with the peak of Hh ligands expression in wild type siblings fin fold regeneration

Regarding the expression of *ptc1*, downstream target of the Hh signaling, a clear down-regulation was demonstrated throughout the notochord and in the somites borders in *iguana* mutant in both regenerating and non-amputated fin folds compared to their wild type siblings. Conversely, strong *ptc1* expression was activated in the neural tube domain in *iguana* mutant compared to wild type siblings at the same stages (Fig.3.9 Panel B compare A-F and A'-F'). Since *ptc1* expression is a direct readout of the Hh signaling cascade, this suggests that Hh signaling activation is compromised in *iguana* mutant. In order to confirm the expanded *ptc1* expression domains it will be required to carry out histological sections of the stained structures. On the other hand, *ptc2* expression was absent from the regenerating tissue in *iguana* mutants at 1dpa (Fig.3.9 Panel B, J'). However, *ptc2* expression was activated in the fin fold in *iguana* mutants in a similar pattern to the wild type siblings at 2dpa (Fig.3.9 Panel B, K'). At this stage, *ptc2* expression was stronger in the tip of the fin fold domain consisting of the new tissue and appeared to expand to most proximal regions. Similar to wild type siblings, *ptc2* expression in the fin fold decreased from 2 to 3dpa in *iguana* mutant larvae (Fig.3.9 Panel B, K', L'). Thus, *ptc2* expression is only impaired at the initial stage of *iguana* mutant larvae fin fold regeneration. In the case of the co-receptor *smo*, no differences were demonstrated in *iguana* mutant and wild type siblings at 1dpa. At this stage, *smo* expression was activated in the regenerating tissue in both *iguana* mutant and wild type siblings (Fig.3.10 Panel A, D, D'). In contrast, *smo* expression demonstrated a down-regulation in the regenerating tissue and in the hypural cells region in *iguana* mutant compared to wild type siblings at 2dpa (Fig.3.10 Panel A, E, E'). This down-regulation of *smo* expression coincides with the down-regulation of *hh*-expressing cells in the new tissue. In addition, *smo* expression was strongly activated in the new tissue in *iguana* mutants in a similar pattern to the *hh* ligands expression at 3dpa (Fig.3.10 Panel A, F'). Therefore, *smo* expression is impaired in *iguana* mutant larvae and appears to be activated in response to Hh signaling. In contrast, no differences in *sufu* expression were observed in *iguana* mutants and wild type siblings through the process of fin fold regeneration. At 1dpa, *sufu* expression was activated in the regenerating tissue and in the hypural cells region in both *iguana* mutants and wild type siblings (Fig.3.10 Panel A, J, J'). At 2dpa, *sufu* expression was strongly activated in the same domains (Fig.3.10 Panel A, K, K'). At 3dpa, *sufu* expression was down-regulated in the new tissue in both *iguana* mutant and wild type siblings (Fig.3.10 Panel A, L, L'). Thus, *sufu* expression is unaffected in the absence of primary cilia.

Conversely, *gli1* expression was stronger in *iguana* mutants compared to wild type siblings throughout the larvae fin fold regenerative process. At 1dpa, *gli1* expression was activated throughout the fin fold in *iguana* mutants, while in wild type siblings was mainly activated in the regenerating tissue and in the hypural cells region (Fig.3.10 Panel B, D, D'). At 2dpa, *gli1* expression was lightly activated in the same domains in both *iguana* mutant and wild type siblings (Fig.3.10 Panel B, E, E'). At 3dpa, strong *gli1* expression was activated throughout the fin fold in *iguana* mutant, while in wild

type siblings this activation was stronger in the tip of the fin fold (Fig.3.10 Panel B, F, F'). Contrasting to *gli1*, *gli2* expression was absent from the regenerating tissue in *iguana* mutants at 1dpa (Fig.3.10 Panel B, J'). At 2dpa, *gli2* expression was weakly activated in the new tissue in *iguana* mutants, while a strong *gli2* expression was observed in wild type siblings (Fig.3.10 Panel B, K, K'). Conversely, strong *gli2* expression was activated in the fin fold and in the hypural cells region in *iguana* mutants at 3dpa (Fig.3.10 Panel B, L'). Therefore, *gli2* expression is impaired in *iguana* mutant larvae at the initial stages of fin fold regeneration, in a similar pattern to the one observed for *hh* ligands and *smo* expression.

Finally, a similar pattern of *gli3* expression was observed in *iguana* mutants and wild type siblings during the fin fold regenerative process. At 1dpa, *gli3* expression was activated in the regenerating tissue and in the hypural cells region in both *iguana* mutant and wild type sibling larvae (Fig.3.10 Panel B, P, P'). In addition, *gli3* expression was further activated in the same domains at 2dpa in *iguana* mutant and wild type siblings (Fig.3.10 Panel B, Q, Q'), reaching a maximum of transcriptional activation in the new tissue of both larvae at 3dpa (Fig.3.10 Panel B, R, R'). Notwithstanding, *gli3* expression levels were down-regulated in *iguana* mutant compared to wild type siblings at 2dpa and 3dpa. This indicates that *gli3* expression suffers a mild down-regulation during *iguana* mutant larvae fin fold regeneration.

In summary, this dataset suggests that Hh signaling expression is impaired during *iguana* mutant larvae fin fold regeneration. In particular, these data demonstrate that several Hh signaling components, including the *hh* (*ihha*, *ihhb*, *shh*) ligands, the downstream Hh signaling transducer *smo* and the *gli2* transcription factor are expressed with a delay compared to *iguana* siblings at 2dpa.

A qualitative comparison on the Hh pathway genes expression levels in *iguana* mutant *versus* *iguana* sibling larvae is provided in Figure 3.11.

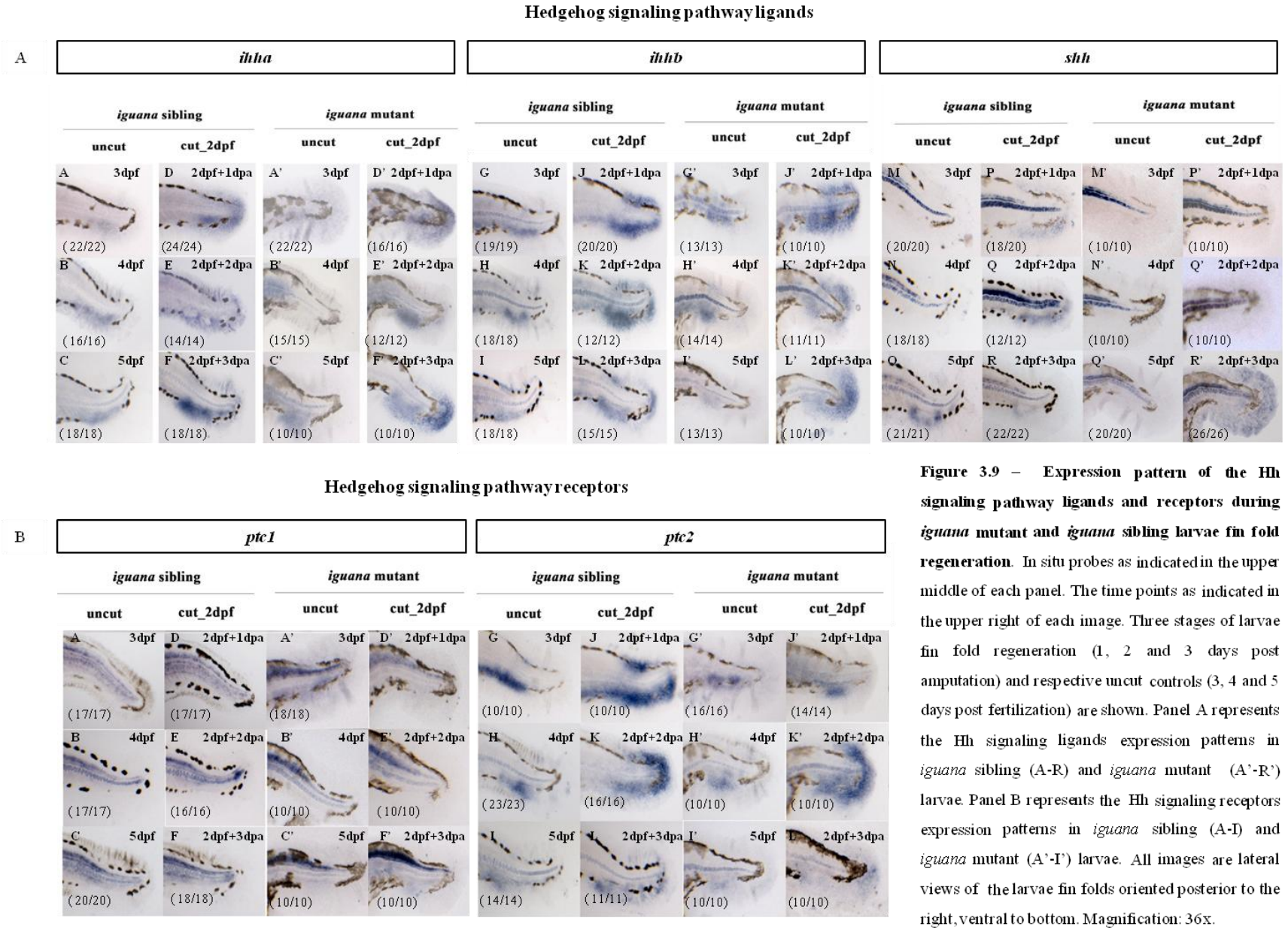


Figure 3.9 – Expression pattern of the Hh signaling pathway ligands and receptors during *iguana* mutant and *iguana* sibling larvae fin fold regeneration. In situ probes as indicated in the upper middle of each panel. The time points as indicated in the upper right of each image. Three stages of larvae fin fold regeneration (1, 2 and 3 days post amputation) and respective uncut controls (3, 4 and 5 days post fertilization) are shown. Panel A represents the Hh signaling ligands expression patterns in *iguana* sibling (A-R) and *iguana* mutant (A'-R') larvae. Panel B represents the Hh signaling receptors expression patterns in *iguana* sibling (A-I) and *iguana* mutant (A'-I') larvae. All images are lateral views of the larvae fin folds oriented posterior to the right, ventral to bottom. Magnification: 36x.

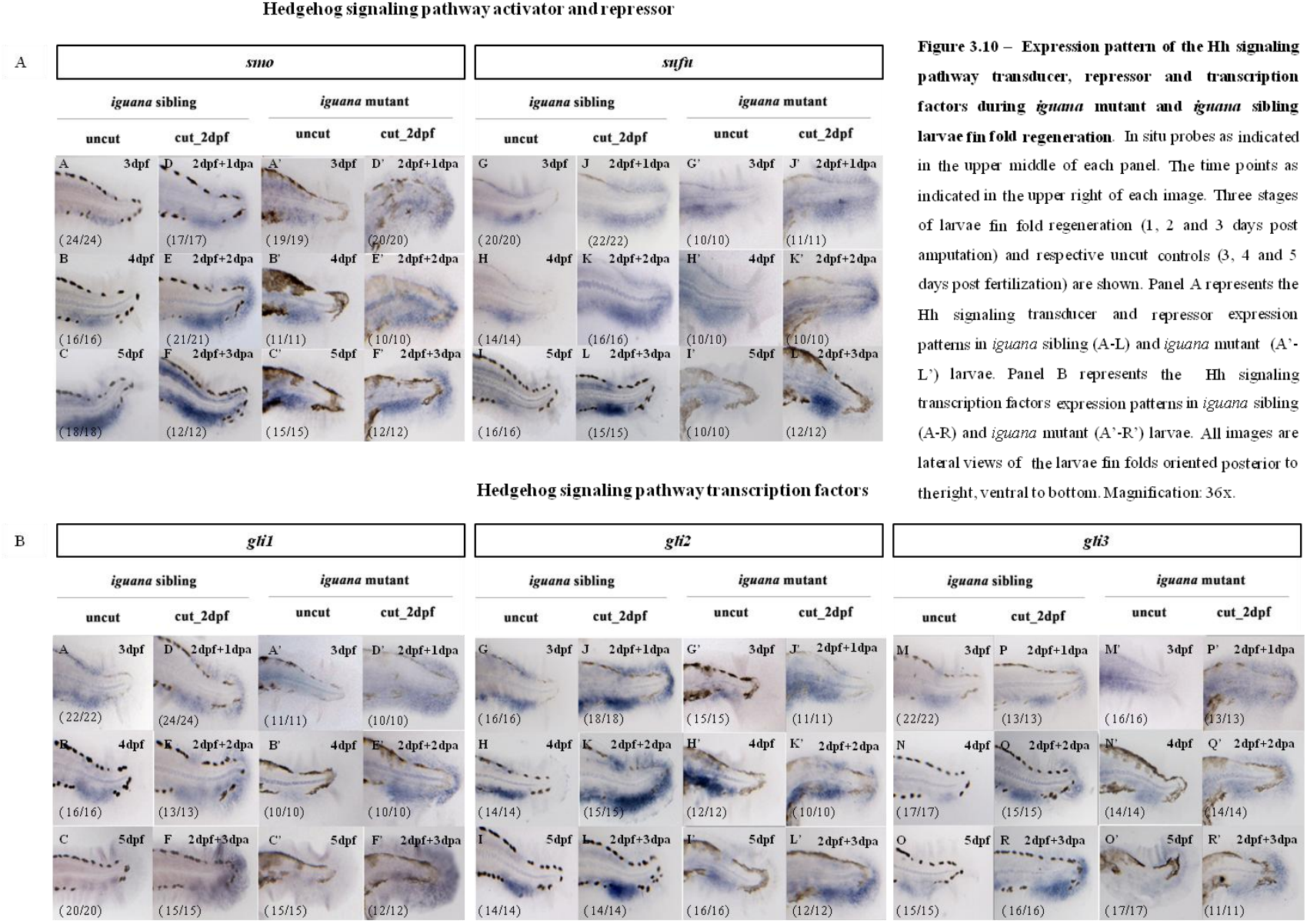


Figure 3.10 – Expression pattern of the Hh signaling pathway transducer, repressor and transcription factors during *iguana* mutant and *iguana* sibling larvae fin fold regeneration. In situ probes as indicated in the upper middle of each panel. The time points as indicated in the upper right of each image. Three stages of larvae fin fold regeneration (1, 2 and 3 days post amputation) and respective uncut controls (3, 4 and 5 days post fertilization) are shown. Panel A represents the Hh signaling transducer and repressor expression patterns in *iguana* sibling (A-L) and *iguana* mutant (A'-L') larvae. Panel B represents the Hh signaling transcription factors expression patterns in *iguana* sibling (A-R) and *iguana* mutant (A'-R') larvae. All images are lateral views of the larvae fin folds oriented posterior to theright, ventral to bottom. Magnification: 36x.

A Fin fold – *iguana* mutant (cut) *versus iguana* sibling (cut)

	ihha	*ihhb*	*shh*	*ptc1*	*ptc2*	*smo*	*sufu*	*gli1*	*gli2*	*gli3*
1dpa/3dpf										
2dpa/4dpf										
3dpa/5dpf										

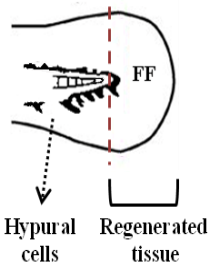
Legend:

- Same level of expression
- Mild up-regulation
- Strong up-regulation
- Mild down-regulation
- Strong down-regulation

B Hypural cells – *iguana* mutant (cut) *versus iguana* sibling (cut)

	ihha	*ihhb*	*shh*	*ptc1*	*ptc2*	*smo*	*sufu*	*gli1*	*gli2*	*gli3*
1dpa/3dpf										
2dpa/4dpf										
3dpa/5dpf										

D



C Notochord – *iguana* mutant (cut) *versus iguana* sibling (cut)

	ihha	*ihhb*	*shh*	*ptc1*	*ptc2*	*smo*	*sufu*	*gli1*	*gli2*	*gli3*
1dpa/3dpf										
2dpa/4dpf										
3dpa/5dpf										

Figure 3.11 – Comparison of the Hedgehog pathway genes expression levels on amputated *iguana* mutant *versus* amputated *iguana* sibling larvae. (A) Representation of the Hh pathway genes expression levels in *iguana* mutant *versus iguana* sibling larvae fin fold. (B) Representation of the Hh pathway genes expression levels in the hypural cells region of *iguana* mutant *versus iguana* sibling larvae. (C) Representation of the Hh pathway genes expression levels in the notochord of *iguana* mutant *versus iguana* sibling larvae. (D) Drawings of the zebrafish larvae fin fold indicating the respective domains. FF – fin fold.

3.3.2 *Iguana* mutant fin fold regenerative process requires no cell proliferation

To address whether cell proliferation is being correctly guided throughout the process of *iguana* mutant larvae fin fold regeneration, we next analyzed the *iguana* mutant cell proliferation pattern during the different stages of larvae fin fold regeneration (1-3dpa). Cell proliferation studies were monitored through immunohistochemical detection for BrdU as previously described (section 3.2.2.2). *Iguana* sibling proliferation pattern served as positive control. Since Hh signaling expression is impaired during *iguana* mutant larvae fin fold regeneration, it would be expected that cell proliferation was also being affected.

As shown in Figure 3.12, *iguana* siblings demonstrated a cell proliferation pattern similar to that described in Kawakami *et al.* (2004). Cell proliferation was observed in the hypural cells region in both regenerating and non-amputated fin folds (Fig.3.12 A-B'). Moreover, *iguana* siblings demonstrated cell proliferation in distal regions of the fin fold at 1dpa (Fig.3.12 A') and in most proximal regions near the notochord at 2dpa (Fig.3.12 B') and 3dpa (data not shown). Unexpectedly, *iguana* mutants showed an unusual pattern of cell proliferation. During *iguana* mutant fin fold development, 100% *iguana* mutants demonstrated no cell proliferation in the fin fold or in the hypural cells region (Fig.3.12 C-D). During *iguana* mutant fin fold regeneration, however, approximately 27% (3/11) *iguana* mutants demonstrated a small cluster of BrdU positive cells in the hypural cells regions at 1dpa (Fig.3.12C'), whereas 73% (8/11) showed no cell proliferation (Fig.3.12 C''). At 2dpa, the percentage of *iguana* mutants without cell proliferation in both the fin fold and the hypural cells region increased to 100% (16/16) (Fig.3.10D). Conversely, 50% *iguana* mutant larvae demonstrated again cell proliferation in the hypural cells region at 3dpa (data not shown). These data suggest that cell proliferation in *iguana* mutant larvae is simply induced at the level of the hypural cells domain and not in the regenerating fin fold tissue. Therefore, it is strongly suggested that *iguana* mutant larvae fin fold regeneration occurs in the absence of cell proliferation.

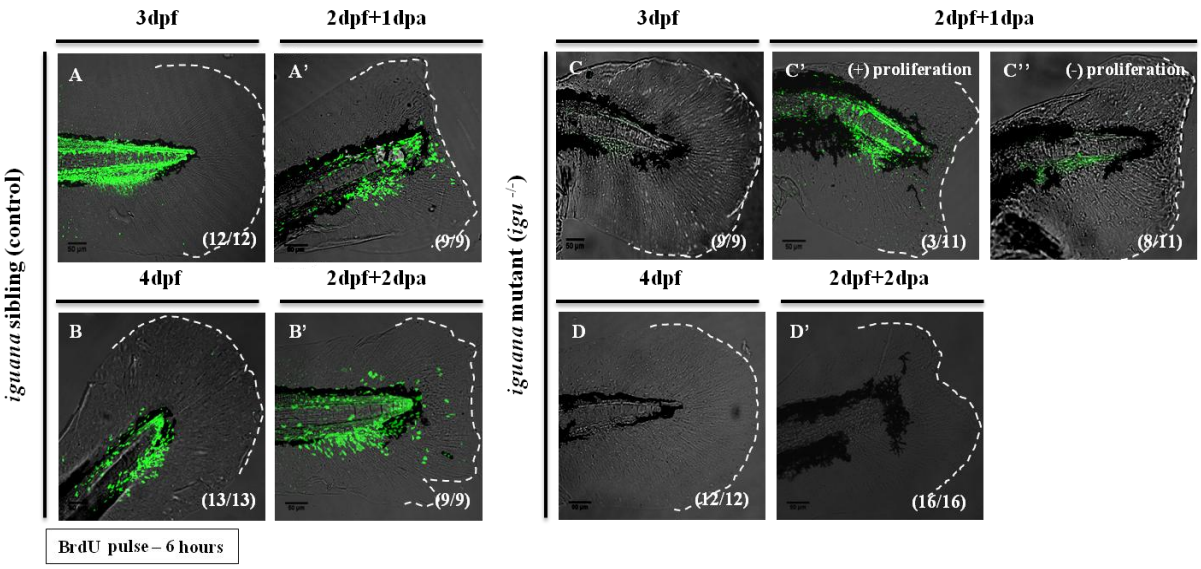


Figure 3.12 – Cell proliferation is absent from *iguana* mutant larvae fin fold. *Iguana* sibling larvae show a characteristic cell proliferation pattern. Cell proliferation is localized to most distal regions of the fin fold at 1dpa (A') and to most proximal regions near the notochord at 2dpa (B'). In addition, proliferative cells are also present in the hypural cells domain in both regenerating (A', B') and non-amputated fin folds (A, B). On the other hand, *iguana* mutant larvae are devoid of cell proliferation in the fin fold at 1 (C', C'') and 2dpa (D'). In contrast, 3/11 *iguana* mutant larvae demonstrate cell proliferation in the hypural cells domain at 1dpa (C', C''), while 16/16 *iguana* mutant larvae shown no cell proliferation. Images are maximal intensity projections of confocal z-stacks. BrdU positive cells are shown in green. Images represent lateral views of the larvae fin fold oriented posterior to the right, ventral to bottom. Magnification: 20x. Scale bars: 50 μm.

3.4 Impact of cell proliferation arrest on zebrafish larvae fin fold regeneration

In the light of the previous findings, we next asked whether the regenerative process of the larvae fin fold is possible without cell proliferation. To address this question a characterization of the fin fold regenerative capacity was performed on wild type larvae under a continuous inhibition of cell proliferation. In order to perform this characterization, larvae were exposed to aphidicolin, an inhibitor of the cell cycle (Ikegami *et al.*, 1978), following the amputation of the fin fold at 2dpf. Thereafter, we allowed their regeneration for 2, 3 and 4 days in aphidicolin containing media to evaluate their regenerative state during the different stages of regeneration. As a control, a group of wild type larvae treated with DMSO was also evaluated.

Initially, to be able to characterize and estimate the regenerative outgrowth without cell proliferation, we assessed the efficiency of the aphidicolin treatment to completely inhibit cell proliferation at a 30μM aphidicolin experimental concentration. Cell proliferation studies were conducted at each stage (2-4dpa) through BrdU incorporation and immuhistochemical detection of BrdU as previously described (section 3.2.3). As a result, 100% of the aphidicolin treated larvae demonstrated no cell proliferation throughout the fin fold and in the hypural cells region at 2dpa and respective uncut stage of 4dpf. On the other hand, the efficiency of the aphidicolin treatment to inhibit cell proliferation decreased at later stages of regeneration, indicating that aphidicolin was degraded or metabolized in the containing media throughout time. Proliferative cells were found in the hypural cells region and in most proximal regions of the fin fold in 31% aphidicolin treated larvae at 3dpa and 5dpf, and in 63% regenerating and 76% uncut aphidicolin treated larvae, respectively, at 4dpa and 6dpf (data not shown). Since our goal was to evaluate the regenerative outgrowth of the larvae fin fold without cell proliferation, another approach was tested.

In a second approach, the previous protocol was reproduced with a higher concentration of aphidicolin, namely 50μM aphidicolin, to determine whether a higher concentration would be required to effectively impair cell proliferation. Similar to the previous results, 100% aphidicolin treated larvae demonstrated no cell proliferation throughout the fin fold and the hypural cells region at 2dpa and 4dpf. However, the efficiency of the aphidicolin treatment to inhibit cell proliferation was again decreased at later stages of regeneration with only 58% aphidicolin treated larvae without cell proliferation at 3dpa and 5dpf. Besides, larvae mortality increased with the aphidicolin treatment to 100%, making it impossible to assess the inhibition of cell proliferation, at 4dpa and 6dpf. Therefore, another approach was tested.

Given that the first aphidicolin concentration tested was the most effective concentration in inhibiting cell proliferation without causing larvae mortality prior to 6dpf, that concentration was used in our third and last approach. In this third approach, larvae were exposed to 30μM aphidicolin shortly after the amputation of the fin fold and, in addition, the media containing aphidicolin was changed following 2 and 3days post amputation. This allowed us to ensure that the efficiency of the aphidicolin

treatment was maintained until the end of larvae fin fold regeneration. Consistent with the previous results, 100% aphidicolin treated larvae demonstrated no cell proliferation throughout the fin fold and the hypural cells region at 2dpa and 4dpf (Fig.3.13 D-D'). In addition, no proliferative cells were observed in 100% aphidicolin treated larvae at 3dpa and 4dpa, and respective uncut stages of 5 and 6dpf (Fig.3.13 E-F'). Therefore, the third approach was the most effective on inhibiting cell proliferation and for that reason the one used in our study. The efficiency of the aphidicolin treatment to inhibit cell proliferation in the three experimental approaches is summarized in Table 3.1.

Table 3.1 – Efficiency of the aphidicolin treatment to inhibit cell proliferation in the three experimental approaches

Larval stages	1st approach (30µM)	2nd approach (50µM)	3rd approach (30µM)
4dpf	100% (12/12)	100% (16/16)	100% (12/12)
2dpf+2dpa	100% (20/20)	100% (20/20)	100% (20/20)
5dpf	69% (9/13)	58% (11/19)	100% (18/18)
2dpf+3dpa	69% (12/17)	58% (12/20)	100% (23/23)
6dpf	24% (4/17)		100% (28/28)
2dpf+4dpa	37% (7/19)		100% (28/28)

dpf – days post fertilization; **dpa** – days post amputation. **%** denote the percentage of aphidicolin treated larvae without proliferative cells throughout the fin fold

As described above, wild type larvae were treated with aphidicolin or DMSO subsequent to the amputation of the fin fold and allowed to regenerate for 2, 3 and 4 days to ensure a complete regeneration and analyze their regenerative state at different stages. Next, to evaluate the regenerative capacity without cell proliferation we measured at each stage the full regenerated fin fold area, in both control and aphidicolin conditions. In addition, to normalize our data and control for possible influence of the normal growth of the fin, we also measured the uncut fin fold area of larvae at the same developmental stages, in both control and aphidicolin conditions. As previously described (section 3.2.1), the regenerated area was then estimated at each stage based on the ratio of the regenerated area related to the uncut fin fold area for both conditions. Hence, a direct comparison of DMSO treated *versus* aphidicolin treated larvae regeneration was possible and allowed us to assess the regenerative capacity in the presence *versus* absence of cell proliferation.

As shown in Figure 3.14, larvae exposed to aphidicolin demonstrated a decrease of approximately 20% regenerated area compared to controls upon 2dpa. The regenerated area significantly decreased from 71±9% (mean ± standard deviation) in DMSO treated larvae to 51±11% regrowth in aphidicolin treated larvae ($p=3.4 \times 10^{-5}$, t-test) (Fig.3.14 A, A'). On the opposite, aphidicolin treated larvae demonstrated an increase of approximately 31% regenerated area at 3dpa. The regenerated area increased to 85±11% in aphidicolin treated larvae whereas 92±8% of the original

fin fold was reconstituted by DMSO treated larvae (Fig.3.14 B, B'). At this stage, although a difference of approximately 7% regenerated area is shown between DMSO and aphidicolin treated larvae, the regenerated area increase in aphidicolin treated larvae was comparable to that treated by DMSO, according to Student's t-test ($p=7.5 \times 10^{-2}$, t-test). Finally, at 4dpa aphidicolin treated larvae successfully regenerated 92±8% of their fin fold and DMSO controls demonstrated a complete restoration of 99±8% amputated tissue ($p = 6.8 \times 10^{-2}$, t-test) (Fig. 3.14 C, C'). Therefore, the inhibition of cell proliferation does not restrain the larvae fin fold regenerative capacity, but promotes a delay in the larvae fin fold regeneration to 4 days upon amputation. This phenotype is highly reminiscent to the phenotype observed in *iguana* mutant larvae fin fold regeneration process (Lima, 2010).

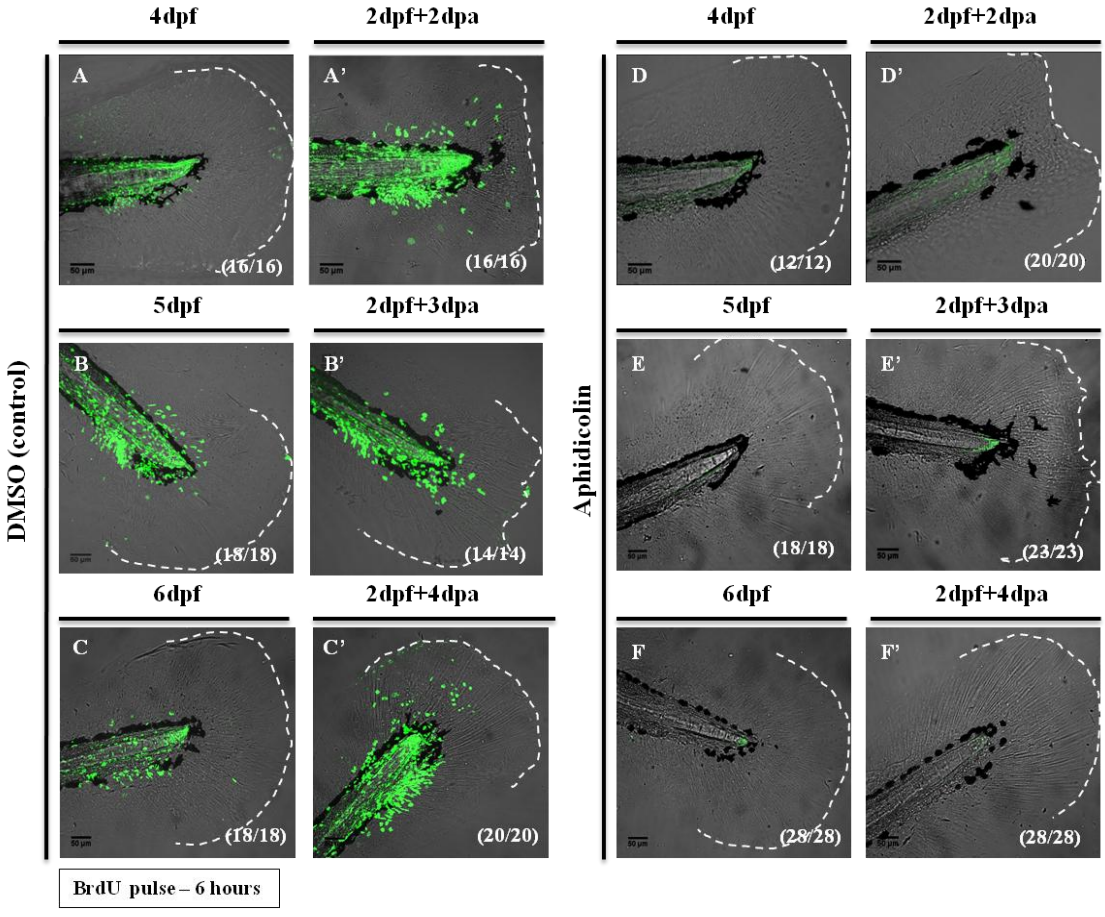


Figure 3.13 – The aphidicolin treatment completely inhibited cell proliferation throughout the larvae fin fold. Wild type larvae were exposed to DMSO or aphidicolin upon fin fold amputation and allowed to regenerate until 2, 3 and 4 days post amputation. Cell proliferation studies were performed on DMSO and aphidicolin treated larvae to confirm the inhibition of cell proliferation following the aphidicolin treatment. DMSO treated larvae show a normal cell proliferation pattern during larvae fin fold development (A-C) and regeneration (A'-C'). Conversely, cell proliferation was successfully inhibited in aphidicolin treated larvae at all analyzed stages (D-F, D'-F'). Images represent maximum intensity projections of confocal Z-stacks. Brdu positive cells are shown in green. Lateral views oriented posterior to the right, ventral to bottom. Magnification: 20x. Scale bars: 50 µm.

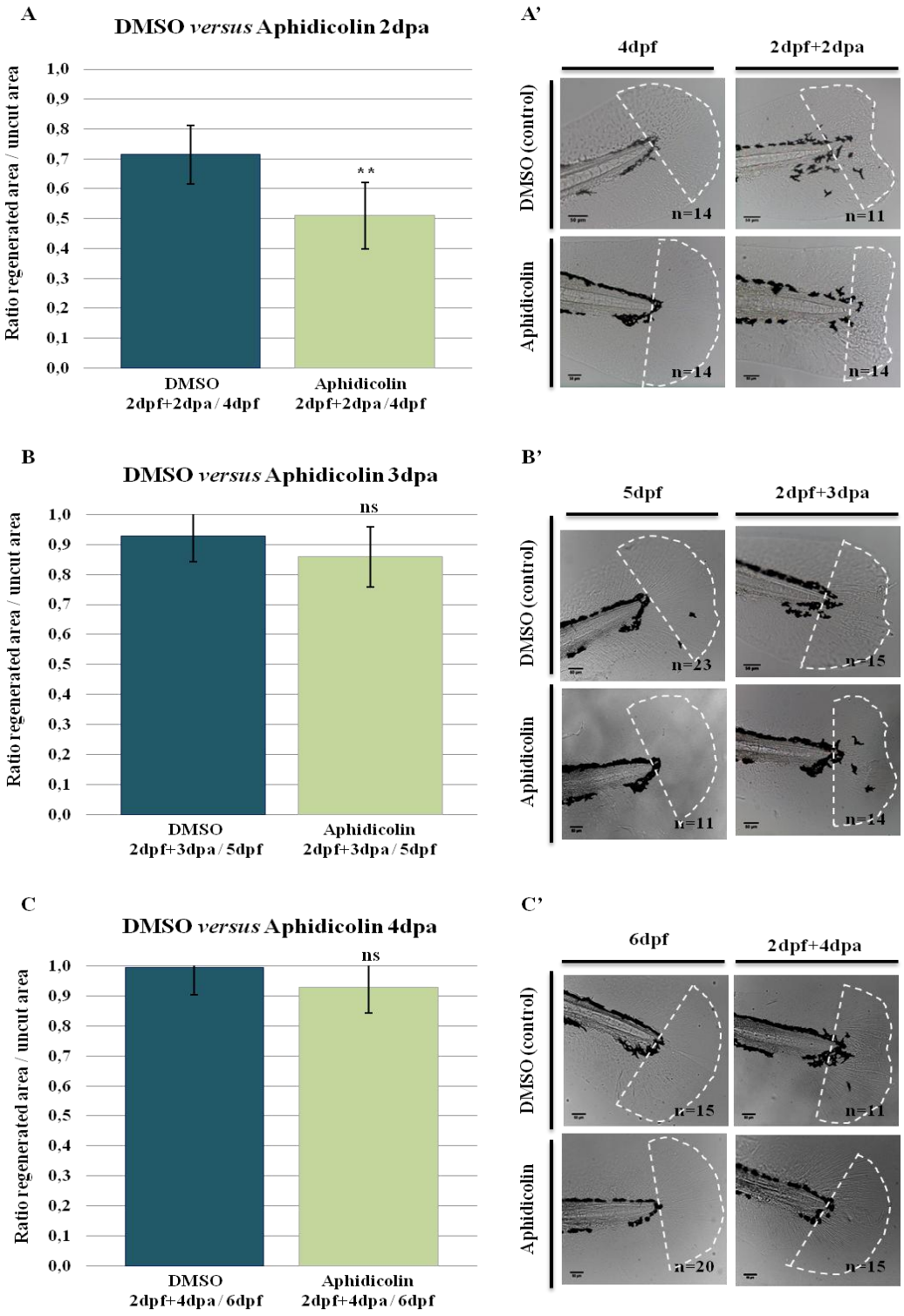


Figure 3.14- Cell proliferation is not required to the process of zebrafish larvae fin fold regeneration. Graphs showing the ratio of the regenerated areas of DMSO versus aphidicolin treated larvae at 2 (A), 3 (B) and 4 (C) days post amputation (dpa). The regenerated area significantly decreases in aphidicolin treated larvae compared to DMSO controls at 2dpa (A). On the other hand, the regenerated area in aphidicolin treated larvae increases to values statistical comparable to the DMSO controls at 3 (B), being the full restoration of the lost tissue observed at 4dpa (C). (A'-C') Representative bright field images of the larvae fin folds are shown (20x magnification). Lateral views are oriented posterior to the right, ventral to bottom. Error bars denote standard deviation. **: $p < 0.01$; ns: $p > 0.05$; Scale bars: 50 µm

To ensure the reliability of our results with aphidicolin, and control for a possible interaction with Hh signaling activity, we additionally analyzed the expression of *ptc1* in aphidicolin treated *versus* DMSO treated larvae through ISH at 2dpa. The 2dpa stage coincides with the perturbation observed in aphidicolin treated larvae fin fold regeneration. Accordingly, no differences in *ptc1* expression were observed in aphidicolin and DMSO treated larvae at 2dpa (appendix B, Fig. B2).

Chapter 4: Discussion

4.1 Hedgehog signaling and the regenerative process

While the essential role of the Hedgehog (Hh) signaling is well established in adult zebrafish caudal fin regeneration (Quint *et al.*, 2002), the involvement of Hh signaling in juvenile tissue regeneration is still unknown. Data from Kawakami *et al.* (2004) have suggested that adult and larval caudal fin tissue regeneration processes are similar in that they both form a specialized epithelial cover with a distinct molecular identity and that apparent activation of cell proliferation occurs in both in response to wounding. Moreover, it is thought that larval tissue regeneration, although being different from the adult and not containing an apparent cell differentiation process may have several core mechanisms, such as the initiation of regeneration and activation of morphogenetic program, in common with mature tissue regeneration (Yoshinari and Kawakami, 2011). A major goal of this work was, therefore, to investigate whether Hh signaling is involved in the regulation of the regenerative process in the zebrafish larvae fin fold.

4.1.1 Hedgehog signaling reactivation during zebrafish larvae fin fold regeneration process

Data from adult zebrafish have shown that amputation of the caudal fin stimulates the re-expression of Hh signaling pathway genes during regeneration (Laforest *et al.*, 1998; Quint *et al.*, 2002; Avaron *et al.*, 2006). In order to understand how Hh signaling is modulated in the larval zebrafish fin fold system, the expression of a selected group of Hh signaling pathway genes was analyzed during the different stages of zebrafish larvae fin fold regeneration (1-3dpa) through *in situ* hybridization (ISH). Accordingly, our results demonstrated that several Hh signaling components were reactivated in the zebrafish larvae fin fold during regeneration, suggesting that the Hh pathway might be important to regulate the regeneration process also in this system.

During zebrafish larvae fin fold regeneration, the Hh pathway ligands, *ihha* and *ihhb* were shown to be expressed in the larvae regenerating fin fold tissue as early as 1dpa and until 3dpa, while *shh* expression in this domain was just observed at 2dpa. This observation suggests that, like in the adult zebrafish caudal fin system (Laforest *et al.*, 1998; Quint *et al.*, 2002; Avaron *et al.*, 2006), the larval fin fold activate Hh ligands expression after amputation. However, since *ihha* and *ihhb* genes are expressed earlier and stronger than *shh* in the larval regenerating fin fold tissue, these results further suggests that *ihha* and *ihhb* may have a more prominent role during zebrafish larvae fin fold regeneration than *shh*. This observation differs from what has been described during regeneration in the adult zebrafish caudal fin system, in which *shh* has been demonstrated to be expressed around 30hpa in a subset of cells at the distal tip of each fin ray and to remain confined to the distal end of the

regenerate throughout the regeneration process (Laforest *et al.*, 1998; Quint *et al.*, 2002). Moreover, in the adult zebrafish caudal fin system, Shh is known to be a major influence in the blastema proliferation and in the caudal fin patterning after amputation (Laforest *et al.*, 1998; Quint *et al.*, 2002).

In addition to these results, our ISH studies further demonstrated that several downstream Hh signaling components, including *ptc2* receptor, co-receptor s*mo*, *sufu* and *gli2* transcription factor, were expressed in the larvae regenerating fin fold tissue as soon as 1dpa and to be up-regulated in the same domain at 2dpa. The expression of these genes occurs in coordination with *ihh* genes expression, suggesting that cells within the larvae regenerating fin fold tissue might be responding to *ihh* signals and therefore supporting a possible central role for *ihha* and *ihhb* genes during larval zebrafish fin fold regeneration. On the other hand, *gli3* transcription factor expression, although initially activated in the larvae regenerating fin fold tissue at 1dpa, was only shown to reach a maximum of transcriptional activation in this domain at 3dpa. This *gli3* expression activation at 3dpa coincides with the activation of *gli1* transcription factor expression in the zebrafish larvae fin fold during regeneration, suggesting both genes expression occurs in a coordinated manner during the zebrafish larvae fin fold regeneration process. Surprisingly, however, our ISH studies showed that *ptc1* expression, which has been described to be up-regulated in response to Hh signaling within the adult zebrafish regenerating fin rays closely adjacent to *shh*-expressing cells (Laforest *et al.*, 1998; Avaron *et al.*, 2006), was absent from the zebrafish larvae fin fold throughout the regenerative process. The absence of *ptc1* expression in the larvae fin fold regenerating tissue suggests that *ptc1* may not play an important role during the larval fin fold system regeneration, like it does in the adult zebrafish caudal fin system. (Laforest *et al.*, 1998; Quint *et al.*, 2002), thus, uncovering an additional divergence between adult and larval zebrafish fin regeneration processes.

Taking into consideration these results, a divergence between adult and larval zebrafish fin regeneration occurs regarding the downstream Hh signaling targets during both processes. Although, *ptc1* and *gli1* are known as the direct transcriptional targets of the Hh signaling pathway in many developmental and regenerative contexts (Briscoe and Therond, 2013), in this work we show that *ptc1* expression is never activated in the zebrafish larvae fin fold during regeneration and that *gli1* expression is just observed in this domain at 3dpa. These observations suggest that *ptc1* and *gli1* may not be the downstream targets of Hh signaling during the zebrafish larvae fin fold regeneration process, which contrast with the adult zebrafish caudal fin system. In this perspective, since *ptc2* and *gli2* are expressed in the regenerating fin fold tissue of the zebrafish larvae in a similar pattern with *ihha*, *ihhb* and the Hh signaling transducer *smo*, we argue that, at least in the larvae fin fold regenerative context, it is possible that *ptc2* and *gli2* are Hh signaling readouts.

Collectively, our ISH results demonstrate that several Hh signaling components are up-regulated in the zebrafish larvae fin fold during regeneration, suggesting that Hh signaling has an

important role to play during this process, as in the adult zebrafish caudal fin system. However, there are discrepancies between adult and larval zebrafish fin regeneration processes that strongly suggest that alternative strategies towards Hh signaling components regulation may exist between these processes, perhaps as a result of different organ ontogeny. This question, however, needs further characterization and confirmation.

It is important to note that for a preliminary analysis on the Hh signaling modulation during larvae fin fold regeneration, the ISH studies suited our purposes. However, the ISH is a non quantitative technique. In order to have a more precise idea of the expression levels of the different genes throughout the different stages of the zebrafish larvae fin fold regeneration, it would be necessary to perform a quantitative approach, for instance, a quantitative RT-PCR.

4.1.2 Hedgehog signaling regulates the regenerative outgrowth of the zebrafish larvae fin fold

Studies on the adult zebrafish caudal fin regeneration process have demonstrated that inhibition of Hh signaling by using cyclopamine, a compound that disrupts Hh signal transduction by acting on Smo (Chen *et al.*, 2002), ultimately leads to the inhibition of cell proliferation within the blastema and to the arrest of the zebrafish caudal fin regenerative outgrowth (Quint *et al.*, 2002). Here, in order to assess whether Hh signaling is a shared requirement to the larval fin fold system regenerative outgrowth, genetic and pharmacological approaches were performed to inhibit/disrupt the Hh signaling cascade.

Taking advantage of a zebrafish mutant line for Smo (*smu*^{b641}), the capacity of zebrafish larvae to regenerate its fin fold in the absence of Hh signaling activity was assessed from 2 to 3dpa, corresponding with the larvae fin fold regenerative outgrowth time period (Kawakami *et al.*, 2004). Accordingly, the results showed that *smu* mutant larvae, in which Hh signaling transduction is disrupted, have an accentuated decrease in the regenerated fin fold area of approximately 20% compared to wild type siblings at 2dpa and a consequent truncation in the regenerative process from this time point. This suggests that Hh signaling could be indeed important to the larvae fin fold regenerative process. Still, since *smu* mutant larvae exhibit a severe phenotype in consequence of the early loss of Hh signaling activity during development (Chen *et al.*, 2001), the hypothesis that the impairment in *smu* mutant larvae fin fold regeneration could be somehow a secondary effect of its phenotype could not be excluded. In addition, we observed that *smu* siblings were able to regenerate just 65±10% of their fin fold tissue following 3dpa, when the regenerative process should be completed. Since *smu* siblings consist of heterozygous and wild type larvae and phenotypically both types of larvae cannot be distinguished, we speculate that possibly only heterozygous larvae were analyzed in this experiment. Although up to now no phenotype has been associated to *smu* loss-of-function allele in heterozygosis, it could be possible that the wild type allele may not be able to completely compensate the loss of Smo in heterozygosis and lead to a mild phenotype during *smu*

sibling larvae fin fold regenerative process. This could somehow explain the odd phenotype observed in *smu* sibling larvae fin fold regenerative process at 3dpa. Nevertheless, since these results are doubtful it would be important to reproduce this experiment again and confirm the results concerning *smu* siblings. Further, it would be important to assess *smu* sibling fin fold regenerative capacity at 4dpa.

Since the results with zebrafish *smu* mutant larvae could not be trusted, the contribution of the Hh signaling pathway to the larvae fin fold regeneration process was, subsequently, pursued by using cyclopamine. The cyclopamine treatment permitted us to disrupt the Hh signaling activity upon wild type larvae fin fold amputation, ensuring that only the regenerative process would be affected and thus excluding secondary effects upon early loss of Smo activity that could be impacting *smu* mutant larvae fin fold regeneration. As a result, we found that the exposure to cyclopamine initially resulted in a reduction of the larvae fin fold growth at 2dpa and ultimately lead to a complete inhibition of the regenerative process from 3dpa. This non-regrowth phenotype is similar to the effect of the cyclopamine treatment in the adult zebrafish caudal fin regeneration (Quint *et al.*, 2002), suggesting that Hh signaling is a shared requirement to the zebrafish larvae fin fold regenerative outgrowth. In support of this result, we observed that the cyclopamine treatment lead to the complete inhibition of the Hh signaling target genes, *ptc1* and *gli1*, throughout cyclopamine treated larvae at 2dpa, coinciding with the perturbation in cyclopamine treated larvae fin fold regeneration. Moreover, the exposure to cyclopamine resulted the in the down-regulation of the several Hh signaling components throughout the larvae regenerating fin tissue from 1 and 2dpa, showing that the Hh signal transduction cascade was completely inhibited in cyclopamine treated larvae. Together, these results underscore our findings that cyclopamine impacts tissue regeneration by interfering with the normal functional role of Hh signaling during zebrafish larvae fin fold regeneration.

In addition, by analyzing BrdU incorporation within the cyclopamine treated larvae fin fold at 2 and 3dpa, we found that the inhibition of the regeneration process was accompanied with a complete inhibition of cell proliferation within the fin fold and in the hypural cells of these larvae. This suggests that Hh signaling is the trigger that instructs cells with proliferation cues during zebrafish larvae fin fold regeneration and that the larval fin fold system, therefore, request Hh signaling as a mitogenic stimulus during regeneration, as in the adult zebrafish caudal fin regeneration process (Quint *et al.*, 2002).

4.2 Hedgehog signaling in the zebrafish *iguana* mutant

Previous work from our lab has shown that the zebrafish larvae fin fold has primary cilia and that upon amputation these cilia prevalence and length changes during larvae fin fold repair (Lima, 2010). After amputation, the cilia in the larvae fin fold increase in number and length until 2dpa and decrease, from this time point onwards, to a basal level as the fin fold recovers its architecture and

functionality (Lima, 2010). Since primary cilia were shown to be involved in regulation of several signaling pathways important in vertebrates' development and tissue homeostasis (Eggenschwiler and Anderson, 2007), the ciliogenic event could be associated to an increased susceptibility to respond to the lesion and allow the regenerative process to occur. However, the *iguana* mutant, in which the formation of primary cilia is disrupted, is still able to regenerate its fin fold upon amputation, albeit with a delay compared to wild type siblings at 2dpa (Lima, 2010). This suggests that the presence of primary cilia is not essential to the zebrafish larvae fin fold regeneration process.

According to the results presented in this work, Hh signaling is essential to regulate the regeneration process of the zebrafish larvae fin fold. Given that primary cilia have been described as essential to regulate Hh signaling in vertebrate systems, a broad and systematic analysis on the expression of several Hh signaling pathway components, through ISH, was performed during different stages of *iguana* mutant zebrafish larvae fin fold regeneration (1-3dpa), to assess whether the delay in *iguana* mutant larvae fin fold regeneration could be the result of a perturbation in Hh signaling expression. As a result, several Hh signaling components, such as *ihha*, *ihhb*, *shh*, *smo*, *gli2*, were shown to be up-regulated in the regenerating fin fold tissue of *iguana* mutant larvae with a delay compared to wild type siblings at 2dpa. This delay coincided with the delay observed in *iguana* mutant larvae fin fold regeneration from previous work (Lima, 2010), suggesting that a possible impairment in Hh signaling occurs during *iguana* mutant larvae fin fold regeneration at this time point and possibly causes the delay. In addition, our results shown that *ptc1* expression is inhibited in the notochord, but up-regulated in the neural tube, in *iguana* mutant larvae compared to wild type siblings during both development and regeneration stages of the larvae fin fold. This observation indicates that both loss and gain of *ptc1* expression exists in *iguana* mutant larvae, respectively, in the notochord and the neural tube, suggesting once again that Hh signaling may be impaired in the *iguana* mutant. On the other hand, other Hh signaling components as *ptc2* and *gli3* showed just a mild down-regulation during *iguana* mutant larvae fin fold regeneration, whereas *sufu* and *gli1* components showed a similar expression pattern to wild type siblings at the same stages. Together, these results demonstrate that the expression of several Hh signaling components is impaired during the regeneration process of *iguana* mutant larvae fin fold, but not completely absent as observed in cyclopamine treated larvae where Hh signaling is blocked.

Although several studies have demonstrated that Hh signaling is impaired during the development of *iguana* mutant zebrafish embryos, and up or down-regulation of Hh signaling target genes is observed depending on Hh signaling requirements to specify the different tissues (Sekimizu *et al.*, 2004; Wolff *et al.*, 2004; Huang and Schier, 2009), this was the first time that Hh signaling expression was demonstrated to be impaired in *iguana* mutant zebrafish during the fin fold regeneration process context.

Taking into consideration that *iguana* mutant zebrafish larvae are still able to complete the fin fold regenerative process, even with a delay (Lima, 2010), and in cyclopamine treated larvae, where

Hh signaling is blocked, regeneration is completely disturbed, together our results led us to conclude that Hh signaling is possibly still active in *iguana* mutant cells, in the absence of primary cilia. This observation is highly reminiscent to what has been described during planarian regeneration (Rink *et al.*, 2009; Glazer *et al.*, 2010). In planarian, Hh signaling influences the anterior-posterior axis padronization that is required during the planarian regeneration process (Rink *et al.*, 2009). However, the absence of primary cilia in these organisms has no effect on this anterior-posterior axis padronization and consequently in its regenerative process, suggesting that Hh signaling regulation occurs independently of primary cilia (Rink *et al.*, 2009; Glazer *et al.*, 2010). Although planarians are invertebrate organisms, and the regulation of Hh signaling through primary cilia has only been described in vertebrate systems (Eggenschwiler and Anderson, 2007), this situation suggests a possibility of a similar regulation of Hh signaling independent of primary cilia during *iguana* mutant zebrafish larvae fin fold regeneration.

In *iguana* mutant cells, although primary cilia are not formed, the basal body is still present (Glazer *et al.*, 2010; Tay *et al.*, 2010). This can be sufficient to ensure the Hh signaling activity required to conduct the fin fold regenerative process in *iguana* mutant larvae in the absence of primary cilia. To address this question, it would be important determine whether *iguana* mutant cells are indeed responding to Hh signaling expression/activation, since it would support a mean of Hh signaling regulation independent of primary cilia. For instance, it would be important to activate or inactivate Smo, for instance using purmorphamine and cyclopamine respectively, during *iguana* mutant larvae fin fold regeneration and assess whether there is an impact in the regenerative process and in the Hh pathway genes expression.

4.3 The role of cell proliferation during zebrafish larvae fin fold regeneration

Regeneration of the zebrafish larvae fin fold has been reported to involve actively regulated cell proliferation and migration (Kawakami *et al.*, 2004; Mateus *et al.*, 2012). Accordingly, by labeling proliferative cells with BrdU for 6 hours at different stages of control zebrafish larvae fin fold regeneration, we also found extensive incorporation of this marker in the larvae fin fold and in the hypural cells region, consistent with previous works (Kawakami *et al.*, 2004; Mateus *et al.*, 2012). Therefore, we confirmed that cell proliferation is indeed triggered in response to the zebrafish larvae fin fold amputation.

As point out above, Hh signaling was shown to be the trigger that instructs cells with proliferation cues during regeneration of the zebrafish larvae fin fold, as its disruption leads to a complete absence of cell proliferation in cyclopamine treated zebrafish larvae. Taking this into consideration and that cyclopamine exerts a dose-dependent inhibition in cell proliferation within the blastema and consequently a dose-dependent inhibition in the adult zebrafish caudal fin outgrowth (Quint *et al.*, 2002), we at first reasoned that the absence of cell proliferation in cyclopamine treated

larvae could explain the arrest of larval fin fold outgrowth observed in this work. However, the cell proliferation pattern observed in *iguana* mutant larvae lead us to different assumptions regarding the role of cell proliferation during zebrafish larvae fin fold regeneration and the reason behind the perturbation in cyclopamine treated larvae fin fold regeneration.

In *iguana* mutant zebrafish larvae, no cell proliferation was observed at the fin fold level, either during regeneration or development stages. The few proliferative cells observed in *iguana* mutant larvae were restricted to the hypural cells region, in a transient manner, and only during regeneration. Cell proliferation in the hypural cells was detected in 27% *iguana* mutant larvae at 1dpa, it was absent from 100% *iguana* mutant larvae at 2dpa and again detected in the hypural cells of 50% *iguana* mutant larvae at 3dpa. One possible explanation for this transient activation of cell proliferation in the hypural cells region is that it could reflect the dynamics of Hh signaling expression during *iguana* mutant zebrafish larvae regeneration, which is manifested through a delay of *ihha, ihhb, shh*, *smo* and *gli2* genes expression at 2dpa. Still, it has been described that cell proliferation in the hypural cells region is not induced by amputation of the larval fin fold (Kawakami *et al.*, 2004) and that it is dependent upon Hh signaling from the notochord and/or floor plate during larval fin fold development (Hadzhiev *et al.*, 2007). Therefore, the hypural cells Hh dependency may be indirect. Moreover, the few proliferative cells detected in the hypural cells region are not sufficient to explain the *iguana* mutant capacity to completely recover its fin fold structure following amputation (Lima, 2010).

Regarding the absence of cell proliferation in *iguana* mutant larvae fin fold during regeneration, two explanations could be hypothesized. The fact that merely a percentage of *iguana* mutant zebrafish larvae demonstrate cell proliferation in the hypural cells region at 1dpa and 3dpa does not imply that cell proliferation at this region was absent in the remaining *iguana* mutant larvae. Instead, suggests that the BrdU pulse of 6 hours used for the cell proliferation assessment was not sufficient to detect cell proliferation in the hypural cells region in all *iguana* mutant zebrafish larvae. This suggests that cell proliferation in *iguana* mutant larvae may occur in a short period of time compared to wild type larvae. Therefore, it could be possible that, for some reason, cell proliferation in the fin fold of *iguana* mutant larvae exists in a different time window, than wild type larvae, that was not assessed in this work. To address this question, for example, cell proliferation could be assessed 12 hours prior to *iguana* mutant larvae fixation at the respective time points (1, 2 and 3dpa). On the other hand, another possible explanation for the absence of cell proliferation in the *iguana* mutant fin fold could be that cell proliferation is not necessarily required for regeneration of the zebrafish larvae fin fold. In order to test this second hypothesis, aphidicolin, a pharmacological compound that arrests the cell cycle (Ikegami *et al.*, 1978), was used to block cell proliferation during wild type larvae regeneration. Cell proliferation was completely inhibited in the fin fold, and even in the hypural cells region, as measured by the absence of BrdU incorporation. Recapitulating *iguana* mutant larvae fin fold regeneration from previous work (Lima, 2010), aphidicolin treated larvae presented a delay in regeneration compared to controls at 2dpa but were still able to recover their fin fold structure and functionality after 4dpa. This

suggests that cell proliferation is not essentially required to the zebrafish larvae fin fold regeneration process, and supports the hypothesis that *iguana* mutant larvae may be able to regenerate their fin folds possibly in the absence of cell proliferation.

Since cell proliferation seems not to be imperative to the zebrafish larvae fin fold regeneration, compensatory phenomena as cell migration or cell rearrangements may be happening during *iguana* mutant and aphidicolin treated larvae fin fold regeneration and would explain their capacity to conclude its regenerative process. The phenomenon of compensation has been described, for example, during zebrafish embryo gastrulation and axis elongation (Zhang *et al.*, 2008). A mutant zebrafish embryo (*tiy21*) for a negative regulator of the Anaphase Promoting Complex (APC) was shown to exhibit a mitotic block at the beginning of gastrulation (Zhang *et al.*, 2008). Despite this mitotic arrest, *tiy21* mutant embryos undergo gastrulation and axis elongation, indicating that cell proliferation is not absolutely required for the initial stages of the zebrafish embryo development. The *tiy21* mutants are on average 22% shorter than their wild type siblings, but exhibit larger somite cells and nuclei compared to their wild type siblings, which suggests that a compensatory growth mechanism takes place to allow axis elongation (Zhang *et al.*, 2008). Similar observations were observed when mitosis was blocked in zebrafish embryos at germ ring/early shield stage using a combination of hydroxyurea and aphidicolin (Zhang *et al.*, 2008). Thus, it is possible that in the absence of cell proliferation other growth mechanisms may occur, which in a normal condition would not be so important. A hint about compensatory phenomena during zebrafish larvae fin fold regeneration has also been provided by Mateus *et al.* (2012). Comparative analysis of epidermal growth and cell proliferation rates during zebrafish larvae fin fold regeneration have shown that epidermal growth increases by the time the cell proliferation rate significantly decreases, implying that epidermal growth occurs through a compensatory mechanism that does not requires cell proliferation (Mateus *et al.*, 2012). This supports the hypothesis that *iguana* mutant and aphidicolin treated larvae may also be able to regenerate their fin folds through a compensatory mechanism that functions in the absence of cell proliferation. This can only be confirmed analyzing cell rearrangement/movement during *iguana* mutant and aphidicolin treated larvae fin fold regeneration. One possible approach would be, for instance, perform histological sections on *iguana* mutant and aphidicolin treated larvae fin folds at the end of the regenerative process, stain the cells nuclei and compare the size of the nuclei relative to its uncut controls.

Taking into consideration that zebrafish larvae fin fold regeneration is possible without cell proliferation, it remained the possibility that the inhibition of cell proliferation in cyclopamine treated larvae would not be the critical event, resulting from Hh signaling inhibition, which leads to the arrest in the larvae fin fold regeneration. It is possible that other inherent processes regulated by Hh signaling are being inhibited with the cyclopamine treatment and are essential to the larvae fin fold regenerative process. Studies in other model systems have demonstrated that not all Hh-mediated epimorphic regeneration is simply mitogenic. Regeneration of the axolotl tail uses Hh signaling both

as mitogen and dorsoventral morphogen whereas axolotl limb utilizes Hh signaling predominantly as patterning agent (Schnapp *et al.*, 2005; Singh *et al.*, 2012). Also, in planarian Hh signaling regulates anterior-posterior polarity (Rink *et al.*, 2009). The difference between these animals and zebrafish may be that the zebrafish larvae fin fold has no dorsoventral polarity and so morphogenetic roles of Hh signaling do not exist. However, this suggests that Hh signaling may also be important to regulate other mechanisms during zebrafish larvae fin fold regeneration. Previous studies have shown that the cyclopamine treatment results in the formation of fewer or no actinotrichia along the regenerating caudal fin of the adult zebrafish (Quint *et al.*, 2002). Actinotrichia are structural components essential to the stabilization and structure of the fins (Zhang *et al.*, 2010). Actinotrichia are composed of collagen I and II and the correct expression of these collagens is essential for the fin formation by providing morphogenetic information (Duran *et al.*, 2011). It has been suggested that actinotrichia are important to assist the new tissue to migrate distally during adult zebrafish caudal fin regeneration (Quint *et al.*, 2002). Given the similarities between adult and larval zebrafish fin regenerative processes, it would be interesting to confirm whether actinotrichia are also affected in cyclopamine treated larvae.

Chapter 5: Conclusion

The results presented in this work provide evidence that the Hh signaling pathway has an essential role during zebrafish larvae fin fold regeneration. In particular, several Hh signaling genes were shown to be reactivated in the zebrafish larvae fin fold during regeneration. In addition, Hh signaling was demonstrated to be one of the key regulators of the zebrafish larvae fin fold regenerative outgrowth upon amputation, instructing the cells with proliferation cues, similar to the adult zebrafish caudal fin system (Quint *et al.*, 2002). Thus, it is demonstrated that, even though the zebrafish larvae fin fold structure has a different ontogeny from the adult zebrafish caudal fin (Yoshinari and Kawakami, 2011), there is a shared requirement for Hh signaling between adult and larval fin regeneration processes.

Furthermore, the results presented regarding *iguana* mutant and cyclopamine treated larvae suggest that Hh signaling may possibly be regulated independently of primary cilia during zebrafish larvae fin fold regenerative context. This question, however, will need further characterization.

Finally, in this work is for the first time demonstrated that cell proliferation may not be essentially required to the zebrafish larvae fin fold regenerative process.

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Appendix A – Supplementary Methods

Midi prep protocol

1. Centrifuge the bacterial cells from the culture grown in LB medium overnight (20 minutes – 14000 rpm) and discard the supernatant.
2. Carefully resuspend the pellet in 5ml Suspension Buffer and mix well.
3. Add 5ml Lysis Buffer to the suspension and mix gently by inverting the tube 6 to 8 times. Incubate 5 minutes.
4. Add 5ml chilled Neutralization Buffer to the suspension.
5. Immediately mix the suspension gently by inverting the tube 6 to 8 times until a homogenous suspension is formed. Incubate 5 minutes at 4ºC.
6. Clear the lysate by centrifugation (30 minutes – 14000 rpm) at 4ºC.
7. Put a folded filter into a funnel that has been inserted into a 50 ml plastic tube. Moisten the filter with 5ml of Equilibration Buffer. Load the lysate onto the wet, folded filter and collect the flow-through.
8. Mount a sealing ring into a column to fix the column in the collection tube. Insert one column into one collection tube. Equilibrate the column with 5 ml Equilibration Buffer. Allow the column to empty by gravity flow. Discard the flow-through.
9. Wash the column with 5ml Wash Buffer. Allow the column to empty by gravity flow. Discard the flow-through.
10. Repeat step 9.
11. Re-insert the column into a new collection tube. Elute the plasmid with 5 ml pre-warmed Elution Buffer. Allow the column to empty by gravity flow. The collected flow-through contains the plasmid.
12. Precipitate the eluted plasmid DNA with 5ml isopropanol. Centrifuge immediately (30 minutes – 14000 rpm) at 4ºC. Discard the supernatant.
13. Wash the plasmid DNA with 5ml chilled 70% ethanol. Centrifuge (20 minutes – 14000 rpm) and carefully remove ethanol from the tube with a pipette tip. Air-dry the plasmid DNA pellet for 15 minutes.
14. Carefully redissolve the plasmid DNA pellet in 30µl of TAE or Nuclease-free water.

(Roche Genopure Plasmid Midi Kit, Applied Science)

Mini prep protocol

Production of cleared lysate

1. Pellet 2ml of overnight culture for 5 minutes (14000 rpm).
2. Resuspend pellet with 250µl of Cell Resuspension Solution.
3. Add 250µl of Cell Lysis Solution to each sample; invert 4 times to mix.
4. Add 10µl of Alkaline Protease Solution; invert 4 times to mix. Incubate 5 minutes at room temperature.
5. Add 350µl of Neutralization Solution; invert 4 times to mix.
6. Centrifuge at 14000 rpm for 10 minutes at room temperature.

Binding of Plasmid DNA

7. Insert Spin Column into a Collection Tube. Decant cleared lysate into Spin Column.
8. Centrifuge at 14000 rpm for 1 minute at room temperature. Discard flowthrough, and reinsert Column into Collection Tube.

Washing

9. Add 750µl of Wash Solution. Centrifuge at 14000 rpm for 1 minute. Discard flowthrough and reinsert column into Collection Tube.
10. Repeat Step 9 with 250µl of Wash Solution.
11. Centrifuge at 14000 rpm for 5 minutes at room temperature.

Elution

12. Transfer Spin Column to a sterile 1.5ml microcentrifuge tube, being careful not to transfer any of the Column Wash Solution with the Spin Column.
13. Add 30µl of Nuclease-Free Water to the Spin Column. Centrifuge at 14000 rpm for 1 minute at room temperature.
14. Discard column, and store DNA at –20°C.

(Wizard® PlusSV Minipreps DNA Purification System, Promega)

Immunohistochemistry protocol used for BrdU detection

First day of protocol

1. Rehydrate through a methanol/PBST (PBS plus 0,1% Triton-X100) series (75%; 50%; 25%) for 5 min at room temperature
2. Incubate with 10µg/ml Proteinase K in PBST for 5 min at room temperature
3. Rinse several times with PBST
4. Refix in 4% PFA for 20 min
5. Wash several times in water
6. Wash 2x in 2N HCl, 0,1% Triton-X100 for 10 min
7. Incubate in 2N HCl, 0,1% Triton-X100 at 37ºC for 1 hour
8. Wash 2x in PBST for 10 min
9. Block with 5% goat serum in PBST for 1 hour at room temperature
10. Incubate with anti-BrdU antibody (monoclonal anti-mouse BrdU, cat: B2531, Sigma-Aldrich) (1:100 dilution in blocking solution) overnight at 4ºC.

Second day of protocol

11. Wash 2 times in PBST for 5 min
12. Wash in PBST for 2 hours
13. Incubate with secondary antibody conjugated with Alexa 488 antibody (Goat anti-mouse IgG, cat. A11001, Invitrogen) (1:500 dilution in blocking solution) for 2 hours at room temperature
14. Wash for 2 hours with 5-6 changes of PBST
15. Store in Mowiol (Sigma) at 4ºC.

Whole-mount *in situ* hybridization protocol (according to Thisse and Thisse, 2008)

Pre-treatments and Hybridization (1st day):

1. Rehydrate embryos through a Methanol/PBT series:
   - Wash 1x5min in Methanol /PBT (75% / 25%), allowing embryos to settle
   - Wash 1x5min in Methanol /PBT (50% / 50%), allowing embryos to settle
   - Wash 1x5min in Methanol /PBT (25% / 75%), allowing embryos to settle
2. Wash 4x5min with PBT.
3. Incubate with 10 µg/ml Proteinase K (PK) in PBT for 30min (for 2dpf), 35min (for 3dpf), 40min (for 4dpf) and 45min (for 5dpf)
4. Incubate in 4% PFA for 20 min, at room temperature.
5. Wash 5x5min with PBT.
6. Replace with pre- Hybridization (Hyb) solution and incubate at least 2 hours at 70ºC.
7. Denature probes at 70ºC during 10min.
8. Add pre-warmed RNA probes diluted in Hyb solution (1:200µl). Immediately place at 70ºC.
9. Incubate overnight at 70ºC (incubate the solutions for the 2nd day).

Post-Hybridization Washes (2nd day):

10. Wash embryos through stringent solutions at 70ºC:
    - Rinse in Hyb solution (100%)
    - Wash in Hyb /2x SSC (75% / 25%) for 15 min
    - Wash in Hyb /2x SSC (50% / 50%) for 15 min
    - Wash in Hyb /2x SSC (25% / 75%) for 15 min
    - Wash in 2x SSC (100%) for 15 min.
11. Wash embryos through the following solutions at room temperature:
    - Wash in 0,2x SSC for 2x30 min
    - Wash in 0,2x SSC /PBT (75% / 25%) for 10 min
    - Wash in 0,2x SSC /PBT (50% / 50%) for 10 min
    - Wash in 0,2x SSC /PBT (25% / 75%) for 10 min
    - Wash 2x10min with PBT solution.
12. Incubate for at least 1hour with Blocking Solution (section 2.10)
13. Incubate overnight with blocking solution + alkaline-phosphatase-conjugated anti-DIG Fab fragments (Roche) (1:5000) at 4ºC.

Post-antibody Washes and revelation (3rd day):

15. Rinse 1x with PBT solution.
16. Wash 6x15 minutes with PBT solution.

17. Wash 3x5 minutes with NTMT reaction buffer (section 2.5)
18. Incubate with detection solution (NTMT solution + NBT + BCIP) (Roche) for DIG labeled anti-sense RNA probes.
19. Once color had developed as desired, wash 3x5min with PBT to stop the reaction.
20. Post-fix with 4% PFA for 20 minutes.
21. Wash 3x5minutes with PBT.
22. Clear consecutively with 25% glycerol/PBS and 50% glycerol/PBS.
23. Store in 75% glycerol/PBS at 4ºC.

Appendix B – Supplementary Figures

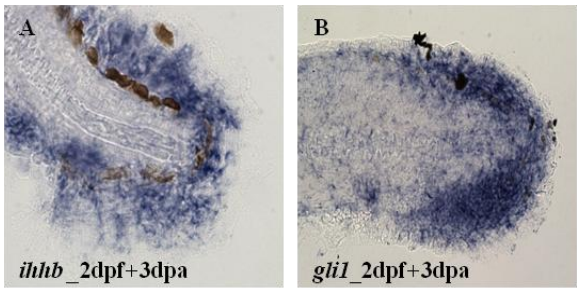

Figure B1 –Histological sections of wild type zebrafish larvae fin fold following whole mount *in situ* hybridization for *ihhb* and *patched1* genes. (A) Longitudinal section of the larvae fin fold stained for *ihhb* gene expression. (B) Longitudinal section of the larvae fin fold stained for *patched1* gene expression. Images are lateral views of the larvae fin folds oriented posterior to the right, ventral to bottom. Magnification: 20x.

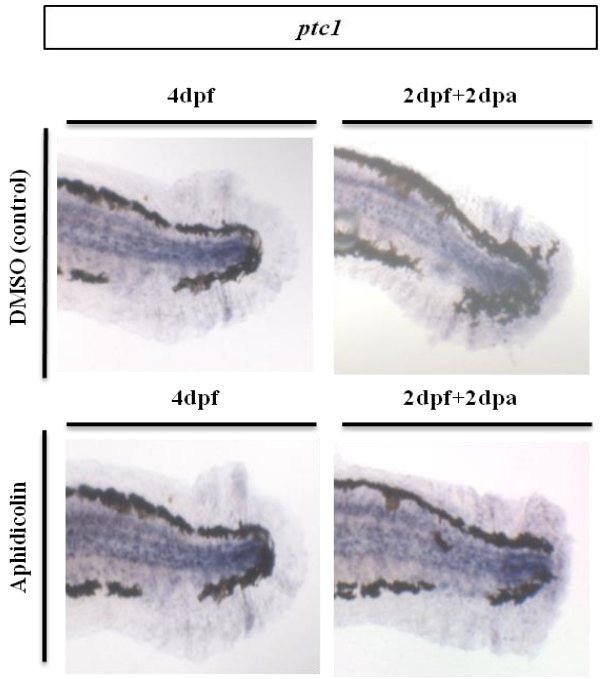

Figure B2 – *Patched1* gene expression in DMSO and aphidicolin treated larvae fin fold at 2 days post amputation (dpa). To control for a possible interaction between the aphidicolin treatment and Hh signaling during aphidicolin treated larvae fin fold regeneration, the expression of *ptc1* was assessed by *in situ* hybridization at 2dpa, which consists in the time period when regeneration is impaired in aphidicolin treated larvae compared to controls. As a result, no differences in *ptc1* expression are found between DMSO and aphidicolin treated larvae at this stage, thus confirming that aphidicolin has an impact just at the cell proliferation level. All images are lateral views of the larvae fin folds oriented posterior to the right, ventral to bottom. Magnification: 36x.