Introduction

What is regeneration?

In the simplest terms, regeneration is restoration of lost body part involving a broad range of processes (Bely and Nyberg, 2010). Regeneration is a postembryonic developmental process which is being operated under the familiar program of embryonic development (Gilbert, 2017). Regeneration can be defined differently in a context specific manner. It can cover the processes from tissue repair to asexual reproduction (Pirotte *et al*., 2016). Regeneration describes not only the replacement of skin cells or shark's teeth but also the generation of an entire organism from a small body part. These are the classical examples of either physiological (homeostatic) or reparative regeneration. Physiological regeneration describes the regular replacement of cells and tissues during homeostasis and aging, whereas reparative regeneration occurs in response to injury (Morgan, 1901; Seifert and Muneoka, 2018). Other than this, there are two other aspects based on which regeneration can be classified. These classifications have been discussed in the following section.

> "Development never ceases. Throughout life, we continuously generate new blood cells, epidermal cells, and digestive tract epithelium from stem cells. A more obvious recurrence of embryonic-like development is regeneration".
>
> (Gilbert, 2017)

Types of regeneration

Regeneration can occur at multiple levels of biological organization which have been summarized in Figure 1. The cell can be considered as the most basic unit of regeneration. The widely studied example of this is a severed axon (Seifert and Muneoka, 2018). Progressing a level higher, tissue regeneration has been reported in number of animals ranging from invertebrates to vertebrates. Regeneration of intestinal tissue in the sea cucumber *Holothuria glaberrima*, and well as repair of skin epithelium and gut lining of human are few examples of tissue regeneration (Ramírez-Gómez *et al*., 2008). Organ regeneration, especially of the internal organs has been studied extensively in variety of animals. Lens regeneration in newt and axolotl, liver regeneration in mammals and heart regeneration in zebrafish have been explored to great depths to understand the mechanisms of organ regeneration (Tsonis and Del Rio-Tsonis, 2004; Sugimoto *et al*., 2007; Suetsugu-Maki *et al*., 2012). Beyond organs, certain body structures such as tail, limb, fin, tentacles have

also been reported to regenerate upon loss of the appendages or injury (Bely and Nyberg, 2010). At last, the most complex of all organization levels, a whole animal from a small body part can undergo regenerative program. Planarians are well known for their elaborate regenerative capability for replacing large regions of missing structures (Agata *et al*., 2003; Reddien and Alvarado, 2004). However, the exact mechanism by which such large regions can regenerate, is still the enigma for regeneration biologists. Therefore, the next basis of classifying the regeneration is different mechanisms through which animals regain their lost body parts.

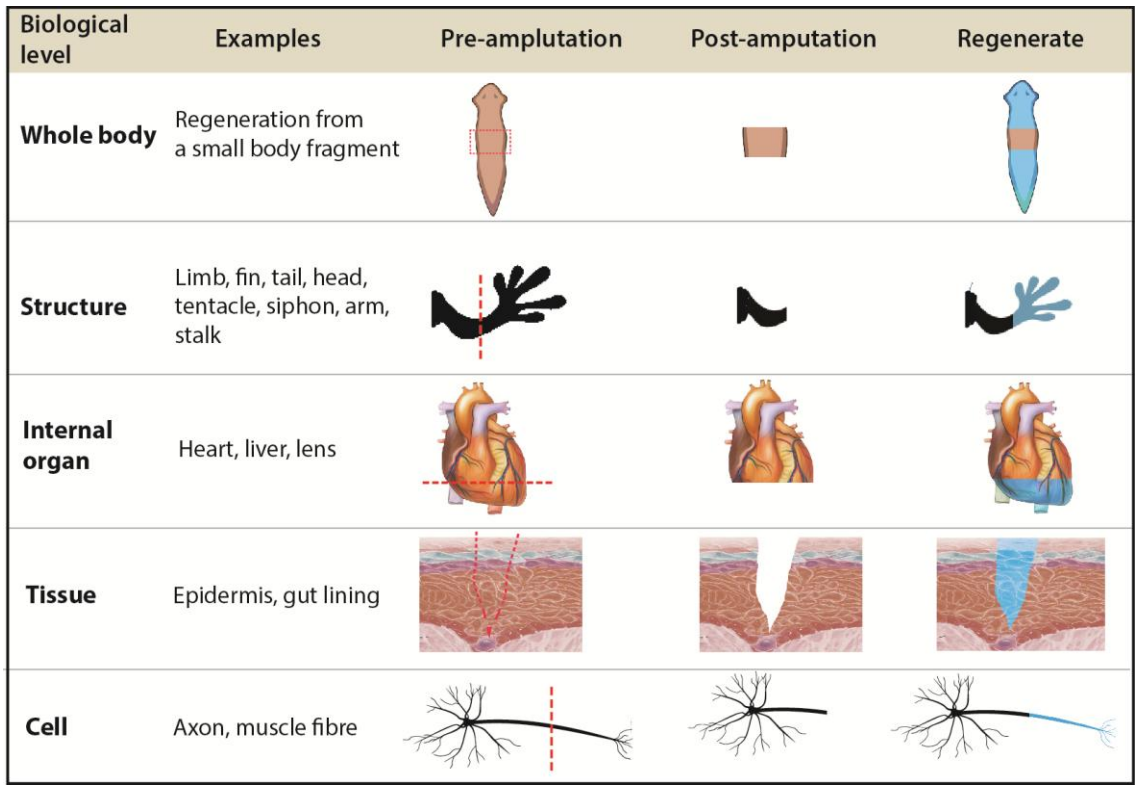

Figure 1: Types of regeneration at various biological organization levels. Red dotted line represents amputation plane. Blue colour indicates regenerated part (Adapted from: Bely and Nyberg, 2010)

Four different modes of regeneration have been listed by Gilbert (2017). First of those is *Morphallaxis* type of regeneration, wherein repatterning of the existing tissue occurs via transdifferentiation. There is little or no growth in this type of regeneration. On the contrary, *Compensatory regeneration,* for e.g., in case of mammalian liver, is achieved by cell divisions of existing differentiated cell and the new cells do not come from either stem cells or dedifferentiation of the adult cells. Third type is called the *Epimorphic regeneration*. In this process, the adult structure undergoes dedifferentiation in some species to form a mass

of undifferentiated cells called blastema, which then re-differentiates to form a new structure. Blastema formation is a peculiarity of epimorphic regeneration. Lastly, *Stem-cell mediated regeneration* allows an organism to regrow certain organs and tissues that have been lost through a resident population of stem cells. Regeneration of hair shafts from follicular stem cells in the hair bulge and the continual replacement of blood cells from the hematopoietic stem cells in the bone marrow are the well-studied examples of stem-cell mediated regeneration (Gilbert, 2017). All the four modes of regeneration have been represented in figure 2. Apart from these four different mechanisms of regeneration, there is a great difference in regenerative capability across the animal phylogeny.

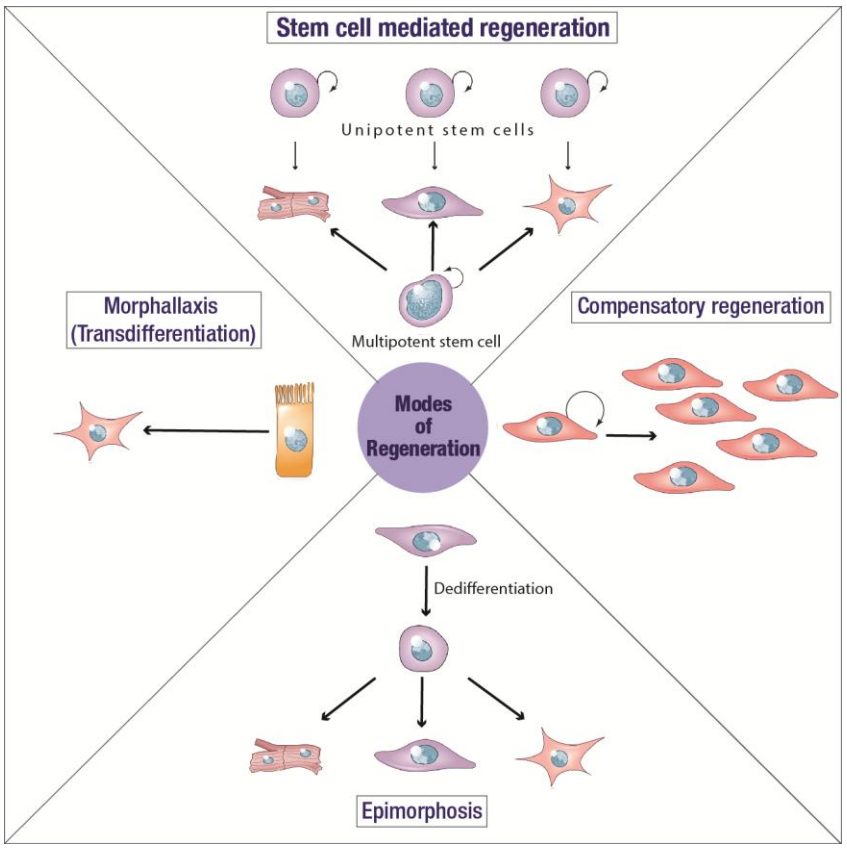

Figure 2: Modes of regeneration (Adapted from Gilbert, 2017)

Regeneration across animal kingdom

The phylogenetic distribution of regeneration shows that regeneration first arose in the early animals coinciding with evolution of multi-cellularity (Bely and Nyberg, 2010). Regeneration has been documented to be present from porifera to mammals although with varying capacity. Though remarkable progress has been made towards understanding the evolution of regeneration, many questions remain unanswered. For example, can

regeneration arise anew in the taxa it is not currently present? Why is regeneration present in those groups where it does not offer any obvious advantages for an animal's survival? (Bely and Nyberg, 2010). In order to answer such queries, regeneration biologists have explored the phenomenon in diverse animals exhibiting regeneration process. Therefore, intuitively, few animals showing a particular mode of regeneration have been used as representative model organisms. Such model organisms include Hydra from phylum Cnidaria, Planaria from phylum Platyhelminthes, Zebrafish from class Pisces, axolotl and *Xenopus* from class amphibian as well as Lizards from class Reptilia. The following text presents the existing knowledge on the characteristic aspects of regeneration in each of these organisms.

Hydra regeneration

Swiss researcher Abraham Trembley in 1744 had noted that a tube-like animal with a delicate array of tentacles could completely regenerate its head after amputation (Lenhoff *et al*., 1986). Since then, Hydra has been one of the prominent models to study regeneration for almost over 270 years (Li *et al*., 2015). *Hydra* can regenerate entire polyp from small body fragments. Hydra reflects the most basic type of regenerative mechanism of morphallaxis. Chera *et al*. (2009) have reported that a burst of apoptosis is triggered at the end of regenerating head fragment after mid-gastric transection in Hydra. These interstitial apoptotic cells help in establishing head organizer via secretion of Wnt3 ligand. Wnt3 induced β-catenin triggers apoptosis-induced compensatory proliferation of interstitial cells and subsequently causes remodelling in the epithelial cells. Such Wnt/β-catenin signalling induced proliferation indicates the involvement of epimorphosis during hydra regeneration. Moreover, the mechanism of apoptosis-induced compensatory proliferation has also been observed in other regenerating animals proving the conservation of this attribute even in higher vertebrates (Li *et al*., 2015).

Planarian regeneration

The history of planarian regeneration is almost nearly 240 years old. Peter Simon Pallas, for the first time, had reported the magnificent regenerative capacity of the tiny marine animals now called as planarians (Pallas, 1766). Amongst variety of species of planaria, *Dugesia japonica* and *Schmidtea mediterranea,* can regrow all missing body parts after transection, regardless of how many times or where they are cut along the axis of the head to the tail and therefore these two species are intensively studied (Liu *et al*., 2013). As far as the mode of

regeneration in planarians is concerned, Morgan (1898) had illustrated that planarians regenerate in entirely within two months after amputation by morphallaxis. Later on, the presence of blastema was reported during planarian regeneration by Tanaka and Reddien (2011). In the recent past, it has been observed that blastema does not arise from dedifferentiation, rather it is formed from pluripotent stem cells called clonogenic neoblasts (cNeoblast). These cNeoblast cells are a set of pluripotent cells in flatworms that serve as stem cells to replace the aging cells of the adult body (Adler and Alvarado, 2015). Umesono *et al*. (2013) have suggested that extracellular signal-related kinase (ERK) signalling pathway, creates a spatial gradient that decreases along the head to tail axis. It was suggested that the Wnt/β-catenin pathway exerts its effects by negatively modulating ERK signalling. Hence, it was reasoned that the balance between anterior ERK signalling and posterior β-catenin signalling might vary across planarian species, resulting in radical differences in their capacity to regenerate heads (Li *et al*., 2015). Therefore, planaria serves as an excellent model for understanding intricacies of multiple modes of regeneration.

Regeneration in Zebrafish

The teleost fish represent a unique vertebrate with a remarkable capability to regenerate appendages, cardiovascular system and central nervous system structures (Li *et al*., 2015; Pfefferli and Jaźwińska, 2015). Zebrafish has been an amenable teleost fish model for standard molecular and genetic manipulations. Additionally, there are few more advantages of using zebrafish as a model organism, such as: they have a short generation time, can be raised with ease and maintained in a large numbers; the availability of complete genome sequence; well-developed transgenesis and knockdown techniques etc. (Poss *et al*., 2003). Amongst all the organs of zebrafish, heart and fin regeneration have been studied extensively by regeneration biologists. Zebrafish heart regeneration occurs by cardiomyocyte dedifferentiation and proliferation upon amputation of 20% of the ventricle (Jopling *et al*., 2010). By using three different models including genetic ablation (Wang *et al*., 2011), cryocauterization (González-Rosa *et al*., 2011) and hypoxia/reoxygenation (Parente *et al*., 2013), zebrafish have been shown to regenerate the heart completely although at different rates. Another well-studied structure for regeneration in zebrafish is caudal fin. Following amputation of caudal fin, a series of events take place to regrow the fin by 2-4 weeks depending upon water temperature (Pfefferli and Jaźwińska, 2015). Another example of a teleost fish that has also been used in our laboratory for regenerative

research is *Poecilia latipinna.* The details of *P. latipinna* caudall fin regeneration are given elsewhere in this chapter.

Axolotl limb regeneration

Newts and salamanders are impressive regenerators with an extensive capability of regenerating limbs, tails, lower jaw, brain, spinal cord and the ventricle of the heart (Li *et al*., 2015). Spallanzani (1769) had first published a thesis addressing the limb regeneration in amphibian. Since then, the regeneration of complex organs became popular among biologists. Limb regeneration in amphibians has been reported to occur via epimorphic regeneration (Whited and Tabin, 2009). Recent advancement in the study of limb regeneration reveals the involvement of Wnt/β-catenin signalling during limb regeneration in both axolotl and *Xenopus* (Kawakami *et al*., 2006; Yokoyama *et al*., 2007). Other than limb, some amphibians have retained the ability of lens regeneration as well. Lens regeneration in adult newts is a well-understood process of natural transdifferentiation (Eguchi, 1988; Barbosa-Sabanero *et al*., 2012).

Lizard tail regeneration

Amongst reptiles, regeneration of lost tail is common to many but not all species of lizards. Most species including scincids (skinks), gekkotans (geckos), lacertids (wall lizards), and anoles are capable of tail regeneration at all stages of post-natal life (Gilbert *et al*., 2015). Lizards, being the only amniotes capable of scar-free wound healing in adult life make an important model to study epimorphic regeneration. Besides, they are closer to mammals in the hierarchy of evolution (Alibardi and Lovicu, 2010; Hutchins *et al*., 2014). Therefore, regulatory mechanisms underlying the tail regeneration may be extrapolated to the possibilities of scar-free wound healing in mammals. Although, achieving that is a distant goal and requires a lot of further research. Regenerative mechanism involved in tail regeneration is an ever growing and active area of investigation. Tail regeneration in lizard is a classical example of epimorphosis. The key structure of epimorphic regeneration called blastema has been characterized very well during tail regeneration in leopard gecko (*Eublepharis macularius*) (McLean and Vickaryous, 2011; Delorme *et al*., 2012). Moreover, role of Wnt/β-catenin signalling during tail regeneration in *Hemidactylus flaviviridis* has also been reported similar to other regenerating animals (Buch *et al*., 2017).

Caudal fin regeneration in *Poecilia latipinna*

P. latipinna is an apposite animal model to study epimorphic regeneration of caudal fin for a number of reasons and has been a model of choice in our lab. Caudal fin has several ideal properties due to which it has been used to study regeneration. Firstly, caudal fin being largest external appendage located at the posterior end of the body makes it accessible for surgery and imaging. Secondly, injury to caudal fin does not compromise the animal's survival. Moreover, the architecture of caudal fin is relatively symmetrical and simple (Poss *et al*., 2003; Pfefferli and Jaźwińska, 2015). *P. latipinna* caudal fin possesses 16-18 fin rays (lepidotrichia), connected to each other by interray tissues. Each lepidotrichium consists of a pair of concave hemirays surrounded by a monolayer of scleroblasts, similar to zebrafish (Tal *et al*., 2010). The interray tissue is comprised of mesenchymal cells, blood vessels, melanocytes, fibroblast cells as well as nerves. The entire multi-ray fin is covered by an epithelial layer (Tal *et al*., 2010). An illustration of caudal fin has been depicted in Figure 3.

Sequence of events in epimorphic regeneration

Following amputation of caudal fin, a series of regenerative stages are initiated resulting into complete restoration of all the tissue types. A detailed account of all the events following fin amputation has been detailed in Chapter 1. A brief timeline of major events is presented here. Within few hours post-amputation (hpa), the epidermis covers the wound surface. By 24hpa, the first landmark structure of epimorphic regeneration namely wound epithelium (WE) is formed. The WE turns into apical epithelial cap (AEC) by 48hpa during caudal fin regeneration in *P. latipinna* (Murawala *et al*., 2017). Beneath the AEC, blastema is formed at the distal end of each ray. Each blastema (BL) serves as an individual unit for regeneration of each fin ray. Cellular and molecular interactions between AEC and BL further orchestrate the epimorphic regeneration of caudal fin. The blastemal cells proliferate and redifferentiate into various cell types to regain the caudal fin. The signs of early differentiation can be seen at 4dpa during caudal fin regeneration in *P. latipinna*. Thereafter, the regenerate keeps growing until it achieves the proper length. The early stages of epimorphic regeneration following amputation of caudal fin have been illustrated in Figure 3. The entire process of epimorphic regeneration in caudal fin of *P. latipinna* has been recently published by us (Patel *et al*., 2019b).

Early epimorphosis: Structural, cellular and molecular aspects

Epimorphic regeneration is characterized by formation of two key structures: wound epithelium (WE) and blastema (Gilbert *et al*., 2015). Removal of WE have been shown to delay the process of regeneration (Thornton, 1957) and preventing formation of the WE inhibits regeneration (Mescher, 1976). Similarly, formation and maintenance of blastema has also been reported to be crucial for appendage regeneration (Brockes and Kumar, 2005; Stoick-Cooper *et al*., 2007).

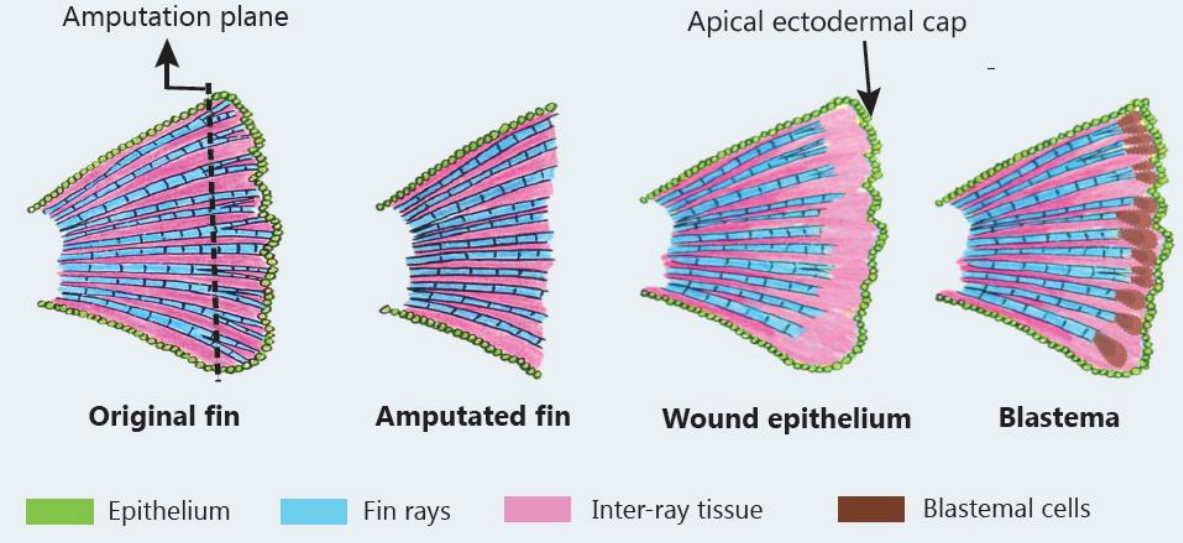

Figure 3: Stages of early epimorphosis during caudal fin regeneration

Therefore, in the present study these two stages of epimorphic regeneration were studied in depth. Further, formation of structures, such as WE and BL, is governed by various cellular processes including cell proliferation, migration, apoptosis, inflammation, patterning as well as extracellular matrix (ECM) remodelling of existing tissues (Simkin *et al*., 2015; Lehoczky, 2017). A transient inflammatory response is initiated at injury site following amputation during zebrafish caudal fin regeneration, which plays an essential role in tissue regeneration (Hasegawa *et al*., 2017). Simultaneously, the tissues at the amputation plane are retracted back by traction forces over which epithelial cells migrates and covers the wound within few hours of amputation (Nechiporuk and Keating, 2002). In order to provide space for further growth of regenerate, dynamic remodelling of ECM has been reported during caudal fin regeneration in zebrafish, found analogous to during newt skeletal and heart muscle regeneration (Govindan and Iovine, 2015). Following this, intensive cell proliferation has also been reported to be involved for maintenance of blastema during both

zebrafish fin as well as lizard tail regeneration (Lee *et al*., 2009; Gilbert *et al*., 2015). In order to balance the cell cycle turn over, certain degree of apoptosis has also been shown during WE and BL formation during zebrafish fin regeneration (Gauron *et al*., 2013).

All the cellular processes involved in regeneration of an organ are tightly regulated by signalling pathways, most often multiple and interconnected. Many, if not of most of these, signalling molecules are conserved among the species of regenerating animals. Moreover, regeneration being a post-embryonic developmental process, is presumed to reactivate those signalling pathways which are employed during embryonic development. Such pathways include Wnt, Transforming growth factor-β (TGF-β)/Bone morphogenetic protein (BMP), Sonic hedgehog (Shh), Fibroblast growth factor (FGF) and Notch signalling. Many studies have already been initiated in this direction to investigate the role of each signalling pathway during epimorphosis, using different model organisms. FGF Signalling has been reported to be involved in all the three stages of epimorphic regeneration during zebrafish fin regeneration as well as in *Xenopus* tadpole and lizard tail regeneration (Lin and Slack, 2008; Alibardi and Lovicu, 2010; Tal *et al*., 2010). Similarly, Wnt has also been essential for regeneration in Hydra, Planaria, Zebrafish, *Xenopus*, Newt and Lizard (Bely and Nyberg, 2010). Shh and BMP pathway have been studied too, for their involvement during regeneration in various animals (Tal *et al*., 2010; Gilbert *et al*., 2015). The key signalling pathways involved in regeneration have been detailed in following sections.

FGF pathway

Fibroblast growth factors belong to a large family of growth factors having 23 ligand members. They carry out variety of functions by binding and activating the FGFR receptor in an HS (heparan sulfate)-dependent manner. There are four FGFR genes (FGFR1–FGFR4) that encode receptors consisting of three extracellular immunoglobulin domains (D1–D3), a single-pass transmembrane domain and a cytoplasmic tyrosine kinase domain (Beenken and Mohammadi, 2009; Hui *et al*., 2018). Binding of FGF ligand to FGFR along with the HS leads to dimerization of FGFRs. FGFRs being protein tyrosine kinases are transphosphorylated at the tyrosine residues of their intracellular domains. The signalling downstream to this phosphorylation event will depend on the functional FGF:FGFR:HSGAG complex (Beenken and Mohammadi, 2009). This is one of the

mechanisms by which FGFs exert diverse biological functions. The general signalling pathway for FGF-FGFR interaction has been illustrated in Figure 4.

FGFs have been reported to participate in pivotal processes such as embryonic development, angiogenesis, wound healing and regulation of endocrine secretion (Kuo *et al*., 2014; Aimi *et al*., 2015; Herriges *et al*., 2015; Owen *et al*., 2015). Fgf6 knockout mice have been shown to exhibit defective muscle regeneration, with significantly increased fibrosis following a freeze–crush injury (Floss *et al*., 1997). Furthermore, inhibition of FGF signalling during fin regeneration in zebrafish has been demonstrated to prevent further outgrowth of the regenerate (Poss *et al*., 2000).

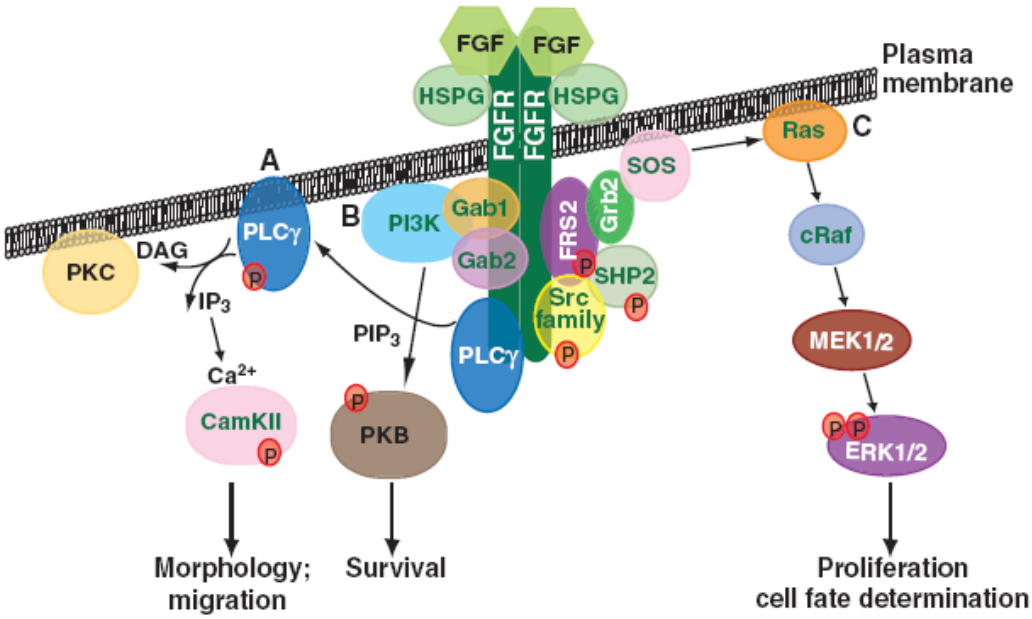

Figure 4: Overview of FGF signalling pathway (Source: Dorey and Amaya, 2010)

In fact, cooperative inputs of Bmp and Fgf (Fgf2, Fgf8 and Bmp7) signalling have been shown to induce tail regeneration in urodele amphibians (Makanae *et al*., 2016). These same signals have been shown to participate during gill regeneration in *Ambystoma mexicanum* by Saito *et al*. (2019). Nacu *et al*. (2016) have also shown that FGF8 and Shh provide complementary signals during salamander limb regeneration. Knockdown of Fgf8 and Bmp7 from dorsal root ganglia by dsRNA interference could impair blastema induction in limb regeneration in *A. Mexicanum* (Satoh *et al*., 2016). Requirement of Fgf20 has been reported to be essential for morphogenesis of the regeneration of epidermis and for

mesenchymal proliferation during blastema formation (Whitehead *et al*., 2005). Recently Shibata *et al*. (2016) have performed a detailed study on involvement of FGF signalling during zebrafish fin regeneration wherein they have reported the presence of Fgf20 in early epidermis as well as Fgf3/10a in the regenerating blastema.

Sonic hedgehog pathway

The hedgehog (HH) pathway comprises of three ligands: Sonic hedgehog (SHH), Desert hedgehog (DHH), and Indian hedgehog (IHH) in mammals. The family includes a 12-pass transmembrane receptor Patched1 (PTCH1); a G-protein-coupled receptor- like seven-pass transmembrane protein Smoothened (SMO); and three transcription factors namely GLI1, GLI2, and GLI3 (Ingham and Placzek, 2006). Shh is one of the three vertebrate hedgehog genes (Indian, Desert, and Sonic) homologous to the Drosophila hedgehog (Choudhry *et al*., 2014). There are two major ways by which shh pathway can be activated. The canonical pathway is activated by ligand-dependent interaction or through receptor-induced signalling and non-canonical is operated by activation downstream of smoothened (Blotta *et al*., 2012). In absence of Shh ligand, the activity of the Smo is inhibited by Ptch1. Inhibition of Smo activity leads to sequestration of GLI2 and GLI3 into cytoplasm, where they are phosphorylated and processed by PKA, CK1, and GSK3β. Phosphorylated GLI2 and GLI3 are processed by the proteasome into repressor forms ($GLI2^R$ and $GLI3^R$). These repressed forms of GLI2 and GLI3 are translocated to nucleus, where they repress the expression of Shh target genes (Wu *et al*., 2017; Carballo *et al*., 2018). In contrast, binding of Shh ligand inactivates Ptch1 receptor relieving the repression of Smo. Activated Smo relieves the suppression of GLI2 and GLI3, which will now maintain their full length and bypass phosphorylation. The activated forms of GLI2 and GLI3 ($GLI2^A$ and $GLI3^A$) translocate to the nucleus and induce the expression of Shh target genes (Wu *et al*., 2017). GLI1, by contrast, lacks the N-terminal repressor domain and functions exclusively as an activator. Therefore, Gli1 gene is one of the targets of Shh signalling and hence amplifies the response to the signal (Ingham and Placzek, 2006; Lee *et al*., 2016). A pictorial representation of Shh signalling has been appended at the end of this section (Figure 5).

The Shh signalling pathway has been involved intensively during development for intracellular communication (Carballo *et al*., 2018). It has been studied extensively for its role in regulation of cell proliferation as well as patterning of the appendages (Ingham and Placzek, 2006). In addition, Shh signalling plays crucial role in tissue homeostasis as well

as in regenerative response to injury in a number of organs (Lee *et al*., 2016). Shh has been shown to regulate angiogenesis and myogenesis during post-natal skeletal muscle regeneration (Straface *et al*., 2009). Inhibition of shh signalling using cyclopamine during limb regeneration in axolotls caused loss of digits (Roy and Gardiner, 2002). Their results have also demonstrated that Shh signalling and function are conserved during regeneration as well as development of limb in vertebrates. There are numerous reports on involvement of Shh pathway during fin regeneration in zebrafish. Quint *et al*. (2002) has shown that ectopic expression of shh or bmp2 in the blastema of regenerating fin exhibited excess bone deposition and abnormal patterning in the regenerate. Similar observations were made by Laforest *et al*. (1998), wherein dynamic expression of *shh* plays a pivotal role in bifurcation of regenerating rays in zebrafish. Further, *shh* mRNA has been reported to express in the blastema of caudal fin between 1.5 to 2dpa (Simoes *et al*., 2014).

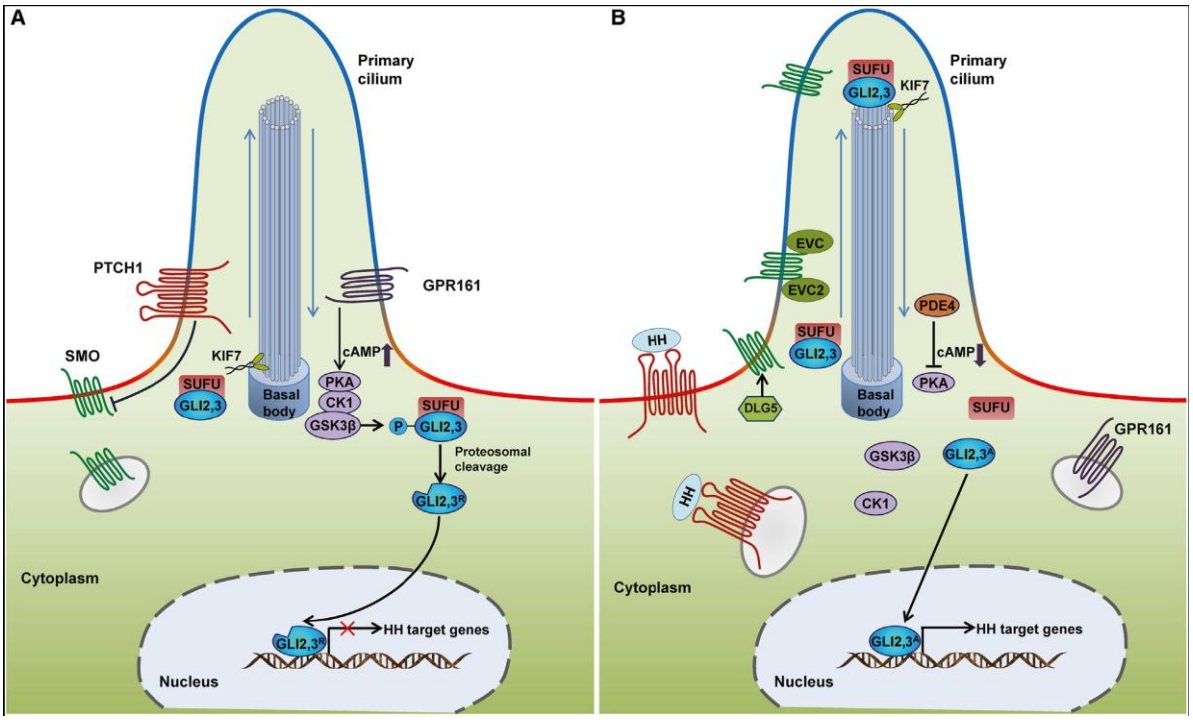

Figure 5: The basic Shh signalling in mammals in absence and presence of ligand (Source: Wu *et al*., 2017)

Wnt/β-catenin pathway

Wnt signalling pathway harbour 19 Wnt genes, falling into 12 conserved Wnt subfamilies in most mammalian genome including humans. Out of these, at least 11 subfamilies occur in the genome of Cnidaria exhibiting the crucial role of Wnts throughout the animal kingdom (Kusserow *et al*., 2005; Clevers and Nusse, 2012). The extra-cellular Wnt signal stimulates

several intra-cellular signal transduction cascades, including the canonical or Wnt/β-catenin dependent pathway and the non-canonical or β-catenin-independent pathway which can be

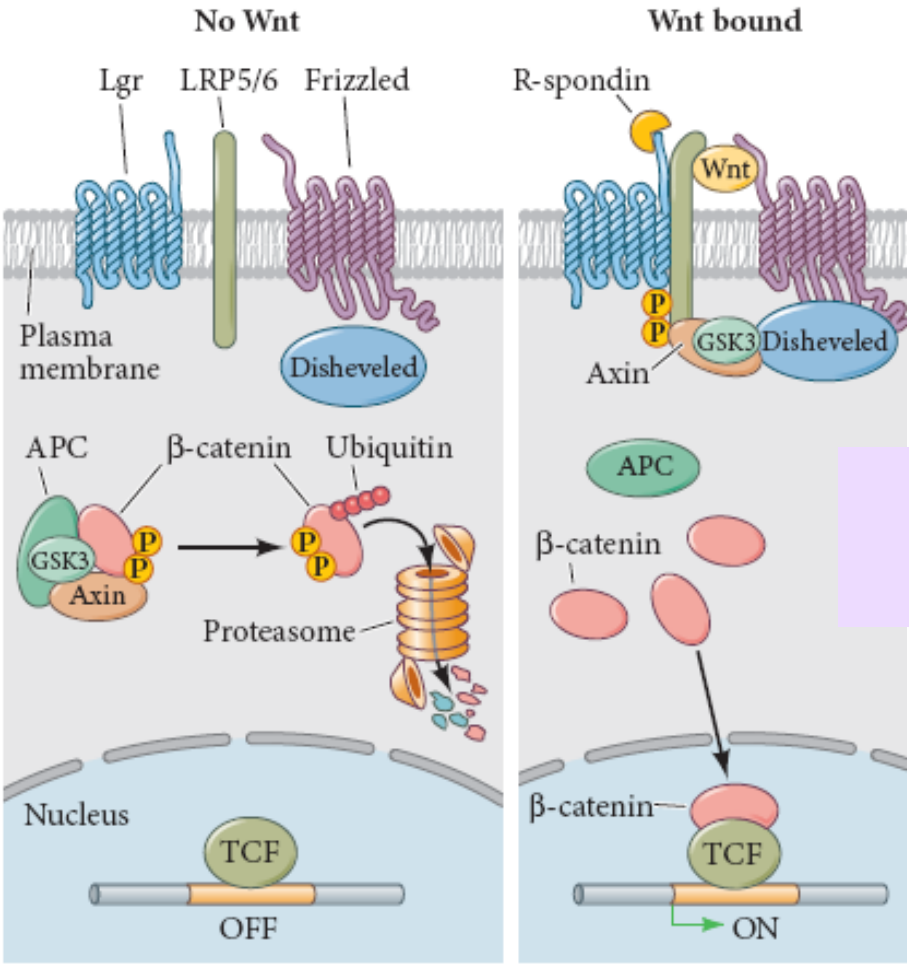

Figure 6: Canonical Wnt signalling pathway (Source: Gilbert, 2017)

divided into the Planar Cell Polarity pathway and the Wnt/Ca^{2+} pathway (Habas and Dawid, 2005). In the absence of Wnt, GSK3β bound to axin, phosphorylates β-catenin bound to both axin and by Axin-casein kinase1a-adenomatous polyposis coli (APC). Phosphorylated β-catenin undergoes ubiquitination and subsequent degradation through the F-box protein β-TRCP, which is part of an SCF ubiquitin ligase complex. In the presence of the Wnt signal, the activated Dishelleved (Dsh) protein binds to axin and inhibits β-catenin phosphorylation, which in turn inhibits its ubiquitination and consequent degradation. The stabilization of β-catenin in the presence of a Wnt signal leads to transcription of Wnt responsive genes (Lee *et al*., 2003). The Wnt1 class ligands (Wnt2, Wnt3, Wnt3a, and Wnt8a) function via the canonical Wnt/β-catenin signalling pathway whereas non-canonical Wnt signalling is initiated by the Wnt5a type (Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7a, and

Wnt11). Both canonical and non-canonical Wnt pathways regulate crucial cell functions across species such as survival, apoptosis, proliferation, cell fate decision, cell motility and cytoskeletal rearrangements (Van den Heuvel *et al*., 1993; Wehrli *et al*., 2000; Logan and Nusse, 2004). The on and off state of Wnt/β-catenin signalling pathway has been illustrated in figure 6.

Wnt/β-catenin signalling is observed to be activated in the regenerating zebrafish tail fin for formation and subsequent proliferation of the progenitor cells of the blastema (Stoick-Cooper *et al*., 2007). The involvement of Wnt/β-catenin signalling has also been reported in the proliferation of cells during regeneration of mammalian muscle, liver and bone (Sodhi *et al*., 2005; Zhong *et al*., 2006). β-catenin induced by Wnt3 has been shown to causes epithelial cells to undergo remodelling to replace the missing head after decapitation in hydra (Chera *et al*. 2009). RNA interference (RNAi)-mediated knockdown of Dlac-β-catenin-1, the Wnt signal transducer have been demonstrated to rescue the regeneration of fully functional heads on tail pieces, indicating the contribution of the Wnt/β-catenin signalling pathway in the inhibition of head regeneration in *D. Lacteum* (Liu *et al*., 2013).

TGF-β/BMP pathway

The TGF-β superfamily consists of a large family of polypeptide growth factors with 33 members (Zhang, 2018). These can be phylogenetically divided into two main groups: the TGF-β/Activin and BMP/growth and differentiation factor (GDF) as reported by De Caestecker (2004). They are further subdivided into seven related sub-groups (Table 1) based on their structural similarity as well as evolutionary relationship with other animals (Newfeld *et al*., 1999). Ligands are synthesized as large precursors which undergo proteolytic cleavage, releasing the pro-domain from the active domain. The carboxy-terminal region of ligands binds to receptor.

The characteristic feature of TGF-β superfamily receptors is the three-finger toxin fold in the ligand-binding extra-cellular domain, a single trans-membrane and an intracellular serine threonine kinase domain. These are divided into two groups, the types I and II receptors. The common nomenclature of type I receptors is Activin receptor-like kinases (Alks) and of type II receptors is based on their dominant ligand interactions (Ten Dijke *et al*., 1994). The detailed classification of type I and type II receptors along with their ligand-binding properties have been listed in Table 2.

Subgroup	Ligand	Alternative name
TGF-β	TGF-β1 TGF-β2 TGF-β3	
Activins	Activin A Activin B Activin AB	Activin β_A– β_A β_B –β_B β_A –β_B
Inhibins	Inhibin A Inhibin B	Inhibin α-Activin β_A α–β_B
Nodal	Nodal	Ndr
Lefty	Lefty1 Lefty2	Lefty A/EBAF Lefty B
BMP2/4	BMP2 BMP4	BMP2A BMP2B
BMP5/6/7	BMP5 BMP6 BMP7 BMP8A BMP8B	 Vgr1/DVR6 OP1 OP2 OP3/PC8
GDF1	GDF1 GDF3	 Vgr2
GDF5/6/7	GDF5 GDF6 GDF7	CDMP1 CDMP2/BMP13 BMP12
BMP9/10	BMP9 BMP10	GDF2
BMP3	BMP3 BMP3b	Osteogenin GDF10/Sumitomo-BIP
GDF9	GDF9 GDF9b GDF15	 BMP15 MIC1/PLAB/PTGFB/PDF
GDF8	GDF8 GDF11	Myostatin BMP11
MIS	MIS	MIS/AMH

Table 1: Classification of ligands of TGF-β superfamily into subgroups with their alternative names

Receptor	Alternative names	Ligands
Type I receptors		
Alk1 group		
Alk1	TSR1/SKR3/ACVR1	TGF-β, Activin A
Alk2	TSK7L/ActRIA/SKR1	TGF-β, Activin A, MIS, BMP6/7
Alk3 group		
Alk3	BMPRI/BMPRIA/BRK1/ Tfr11/ACVRLK3	BMP2/4, BMP6/7
Alk6	BMPRIB/BRK2	BMP2/4, GDF5/6, GDF9b, MIS, BMP6/7
Alk5 group		
Alk4	ActRIB/ACVR1B/SKR2	Activin A, GDF1, Nodal, GDF11,
Alk5	TGF-BRI/SKR4	TGF-β
Alk7		Nodal
Type II receptors		
TGF-βRII		TGF-β
BMPRII	BRK3/T-Alk	Inhibin A (with TGFβRII), BMP2/4, BMP6/7, GDF5/6, GDF9B
ActRII	ACVR2	Activin A, Inhibin A/B, GDF1, Nodal, BMP2, BMP6/7, GDF5, GDF9b, GDF8/11
ActRIIB	ACVR2B	Activin A, Inhibin A/B, Nodal, BMP2, BMP6/7, GDF5, GDF8/11
MISRII	AMHR2	MIS

Table 2: Nomenclature of TGF-β superfamily receptors with their ligand-binding properties (Source: Caestecker, 2004)

Receptor-ligand interactions and downstream signalling

Binding of ligand to the constitutively active type II receptor which then recruits the type I receptor and trans-phosphorylate the type I receptor. This phosphorylated type I receptor subsequently phosphorylates the receptor-activated smad (R-Smad) proteins. Activated R-smads associate with common mediator smads (Co-Smad) and enter the nucleus. The complexes of R-Smads and Co-Smads accumulates in the nucleus where they either positively or negatively regulate the transcription of a battery of target genes (Xiao *et al*.,

2007; Heldin and Moustakas, 2016; Miller *et al*., 2019). Traditionally, the TGF-β family has been split in two pathways, with TGF-βs, Nodal and activin leading to the phosphorylation of Smad2 and Smad3 (Smad2/3), whereas the BMPs and some of the GDF proteins induce phosphorylation of Smad1, Smad5 and Smad8 (Smad1/5/8) (Schmierer and Hill, 2007). The third class of Smad proteins, the inhibitory Smads (I-Smad), comprises of Smad6 and Smad7. I-Smads have been reported to regulate TGF-β in negative fashion in a feedback loop. I-Samds recruit ubiquitin ligases Smurf1/2 or protein phosphatase I to inactivate receptor complex (Kavsak *et al*., 2000; Shi *et al*., 2004). Additionally, the I-Smads may also interfere with formation of the R-Smad/Co-Smad complex, inhibit co-activator recruitment by the R-Smads or interfere with Smad–DNA complex formation (Zhang *et al*., 2007; Ross and Hill, 2008). The overall signalling pathway for TGF-β/ BMP has been summarized in figure 7.

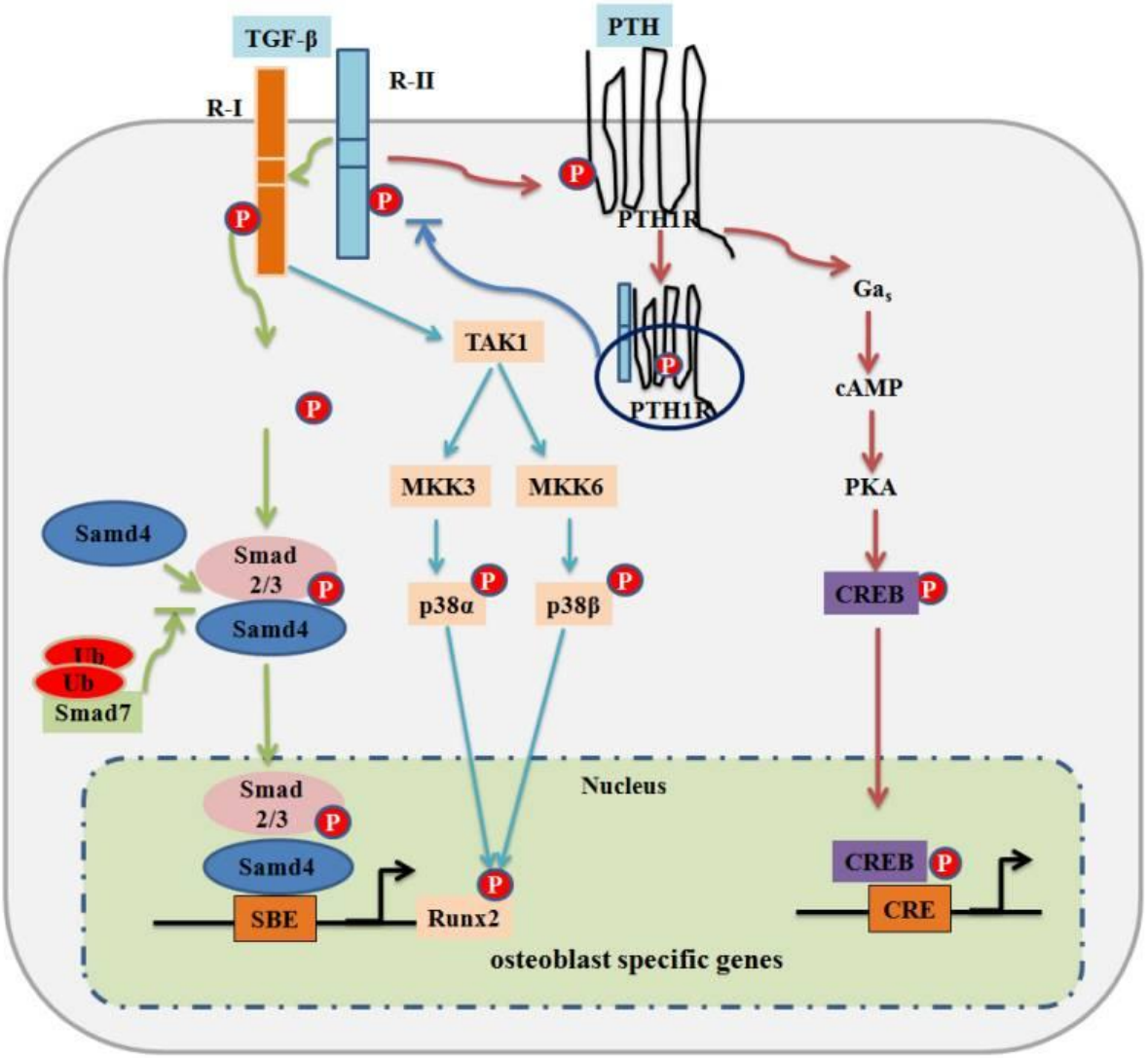

Figure 7: Canonical Smad-dependent TGF-β/BMP pathway (Source: Chen *et al*., 2012)

Other than canonical signalling, TGF-β family members have shown the evidences of cell-type dependent regulation of alternative non-canonical signalling pathways. Different members of TGF-β family have been reported to activate MAPK signalling pathways, ERK, JNK, p38 MAPK, PI3 kinase and PKC. These signals may exert diverse downstream responses in different cell types or may augment the Smad-dependent responses (De Caestecker, 2004; Zhang, 2017, Zhang 2018). In fact, TGF-β/BMP pathway has been studied for the extensive cross-talk with other signalling pathways such as Wnt, Shh, Notch, IL, TNF-α and IFN-γ (Guo and Wang, 2009).

As mentioned earlier, all these pathways have been implicated during various cellular and molecular processes governing regeneration. TGF-β plays pivotal role during embryonic development and adult tissue homeostasis (Miller *et al*., 2019). Inhibition of BMP signalling during zebrafish fin regeneration has been shown to disrupt fin growth as well as scleroblast differentiation and function (Smith *et al*., 2006). Beck *et al*. (2006) have shown that noggin induced inhibition of BMP pathway significantly reduces cell proliferation in the notochord and spinal cord of the tail, and of the blastema in the limb bud during tail and limb regeneration in *Xenopus* tadpole. BMP and FGF signalling in synergistic manner have been reported to induce tail regeneration in urodele amphibian by Makane *et al*. (2016). Previous studies from our lab also suggests role of BMP during caudal fin regeneration in *P. latipinna* (Rajaram *et al*., 2016, 2017). However, there are only few selective ligands and receptors of TGF-β pathway that have been studied for their involvement during epimorphosis. Therefore, an initiative was made in current study to obtain a temporal profile of TGF-β family members as well as of other pathways too, during caudal fin regeneration in *P. latipinna*. The further part of the study was focused on TGF-β/BMP pathway for understanding its role during caudal fin regeneration. Inhibition of signalling pathways using specific inhibitors have been a widely accepted approach in research. Hence, in the current study TGF-β/BMP pathway was inhibited using a specific inhibitor LDN193189 in chapter 2 and chapter3.

LDN193189 as an inhibitor of TGF-β/BMP pathway

The kinase activity of type I receptors (ALKs) of TGF-β superfamily has always been an attractive target to inhibit this signalling pathway (Yingling *et al*., 2004; Hong and Paul, 2009). There are many small molecule inhibitors of ALKs have been developed since past

few years (Vogt *et al*., 2011; Horbelt *et al*., 2015). Compound C was identified as an inhibitor of AMP-activated protein kinase to hamper BMP induced Smad and non-Smad signalling by targeting the BMP type I receptors ALK1, ALK2, ALK3 and ALK6. Later on, compound C was renamed as dorsomorphin (DM) for its potential to induce dorsalization in zebrafish embryos (Paul *et al*., 2008; Boergermann *et al*., 2010). Such dorsalized axial pattern, upon exposure to DM reflected the same phenotype of BMP-pathway mutants (Mullins *et al*., 1996). Unfortunately, DM shows off-target effect on receptor tyrosine kinases for VEGF and PDGF (Paul *et al*., 2008; Hao *et al*., 2010). On the other hand, LDN193189, derivative of DM shares the central pyrazolo [1,5-a] pyrimidine moiety with DM and target BMP type I receptors with increased potency and specificity (Cuny *et al*., 2008; Paul *et al*., 2008). The structure of LDN193189 is shown in Figure 8. The *in vitro* kinome assay of LDN193189 has been shown to inhibit ALK-1 to -6 as well as ActRIIA in the range of 0.5-210nM (Sanvitale *et al*., 2013). Boergermann *et al*. (2010) have reported that LDN193189 treatment to C2C12 cells decreased the protein levels of p-Smad 1/5/8 in dose dependent manner. LDN193189 has also been tested for its potential inhibitory effect on GDF8 induced phosphorylation of Smad 2/3 (Horbelt *et al*., 2015). Due to hampered TGF-β/BMP signalling, it can affect various cellular and molecular processes which are vital to development as well as homeostasis. Owing to such properties of LDN193189 as a specific inhibitor of TGF-β/BMP pathway, it was used in the current study to ablate the signalling. The dose of 2.5mg/kg of body weight was considered based on the previous studies conducted by Rajaram *et al*. (2016).

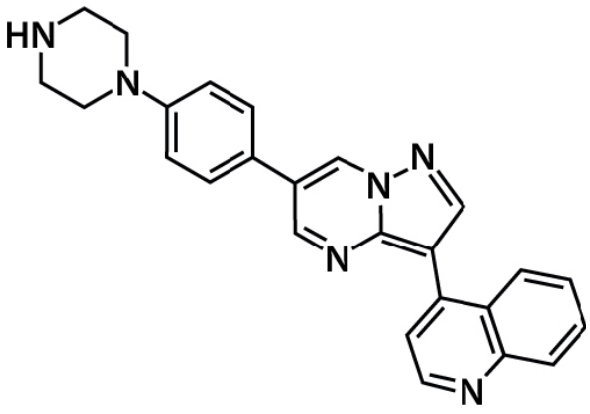

Figure 8: Structure of LDN193189

Thus, owing to the fundamental and critical role of signalling pathways in regeneration, with a hypothesis that a resolute understanding of the molecular components, their actions and governing principles of epimorphic regeneration can lead to extrapolations useful in the field of regenerative medicine in mammals. In order to achieve this, the study was divided into three specific objectives as mentioned below:

1) Study the progressive morphogenesis and temporal expression pattern of signalling molecules during the early stages of epimorphosis
2) Effect of inhibition of TGF-β/BMP pathway on proliferation and inflammatory response along with associated signalling molecules during wound epithelium stage
3) Influence of ablation of TGF-β/BMP pathway on developmentally significant signalling molecules as well as cell cycle turn over and ECM remodelling during blastema stage