

# Insights into the role of the Wnt signaling pathway in the regeneration of animal model systems

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**ABSTRACT** Regeneration enables the regrowth and restoration of missing body parts. It is a common phenomenon among animals. However, only some species exhibit remarkable regeneration capabilities and can regenerate organs such as limbs, lenses or hearts. Regeneration has been widely studied, thereby giving rise to new fields, such as regenerative medicine. Furthermore, regeneration has the potential to be applied to the human body. However, the molecular mechanisms governing this process should be elucidated first. Recent advancements in research methods have led to the identification of numerous signaling pathways involved in regeneration. One of them, the Wnt transduction pathway, is an ancient and evolutionarily conserved pathway that plays an important role in both embryonic development and regeneration. The Wnt pathway plays an important role during the regeneration process, as it is implicated in cell fate determination, cell migration, cell polarity and adult cell homeostasis. To date, two major Wnt pathways have been identified: the canonical ( $\beta$ -catenin dependent) pathway and the non-canonical pathway. The latter pathway can be further divided into planar cell polarity, the Wnt/Ca2+ pathway and the JNK pathway. In this review, we summarize the current state of knowledge regarding the Wnt signaling pathway and its role in regeneration, with a particular emphasis on key model species.

**KEYWORDS:** regeneration, Wnt/ $\beta$ -catenin, signalling pathway, model organisms

## Introduction

Regeneration is a common phenomenon in the animal kingdom that enables the restoration of missing body parts after injury or amputation; as a result, damaged structures are perfectly or near-perfectly replaced (Gilbert, 2000). Although some form of regeneration exists in almost all species, remarkable regenerative capabilities are observed only in a few taxa, such as cnidarians, crustaceans and salamanders. Humans have very poor regenerative abilities, and only a few types of tissue can regenerate. Nevertheless, people have been fascinated with this process since the 18th century. In recent years, there have been considerable advancements in the field of regenerative medicine, which aims to restore tissues, organs or even entire body parts. To achieve this, the first step should be full elucidating the molecular components underlying the regeneration

process. In this review, we attempt to compile existing knowledge regarding a small part of the regeneration process.

The process of regeneration varies widely in terms of complexity, underlying mechanisms and magnitude (Tsonis, 2000). Four major modes can be distinguished: morphallaxis, compensatory regeneration, stem cell-mediated regeneration and epimorphosis. Morphallaxis, which is observed in *Hydra*, is repatterning of existing tissues. Compensatory regeneration occurs when differentiated cells divide but maintain their functions (neither dedifferentiation nor stem cells are involved). For example, in the mammalian liver, stem cell-mediated regeneration allows for regrowth of certain tissues, such as blood cells from hematopoietic stem cells in the bone marrow. Epimorphosis enables the formation of a new structure from an undifferentiated mass of cells that later redifferentiates; this process is typical in amphibian limb regeneration (Iismaa *et al.*, 2018).

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In brief, epimorphic regeneration is accomplished by cell dedifferentiation and the formation of a regeneration blastema that proliferates and redifferentiates into new body parts (Fig. 1B). Generally, the regeneration process can be divided into three phases: wound healing, dedifferentiation and redevelopment. Within a few hours following amputation or injury, the wound is sealed by the rapid migration of epithelial cells that form the wound epidermis (no scar) (Brookes, 1997). In the next few days, all the cells undergo dedifferentiation and lose their characteristics. This cell mass is known as the regeneration blastema. In addition, the wound epidermis thickens and forms the apical epidermal cap (AEC). Growth of the blastema depends on the presence of AECs, nerves and ion currents (Altizer et al., 2002). It elongates for the next several days through mitotic activity. During the second phase, which is unique to limb regeneration, spatial and temporal patterns of gene expression differ from those observed in development; then, in the third phase, the expression of genes shows similarities with the developing limbs (Gardiner et al., 2002). Although gene functions are conserved during development and regeneration, there are still many differences in expression patterns.

The development of sophisticated research tools previously led to the identification of the main components taking part in the regeneration of species with high regeneration potential, such as planarians, newts, and zebrafish. These studies revealed evolutionarily conserved signaling pathways involved in the process, including the Wnt (Clevers et al., 2014), Notch (Raya et al., 2003), Hedgehog (Hh) (Torok et al., 1998), BMP (Molina et al., 2007),

and JAK/STAT pathways (Herrera and Bach 2019). Although there are many other pathways that play a considerable role in regeneration, this review will focus on the Wnt pathway, which has been identified to be essential for the regeneration of *Hydra*, Planaria, newts, zebrafish, *Drosophila* and even mouse or human liver. The highly conserved Wnt pathway has a critical function during development and regeneration. Although Wnt genes are extant in the earliest metazoans, such as sponges, placozoans and ctenophores, they have not been described in any unicellular eukaryotes (Holstein et al., 2011). Recent research advancements have helped to better understand its components, signals and consequences resulting from its perturbations.

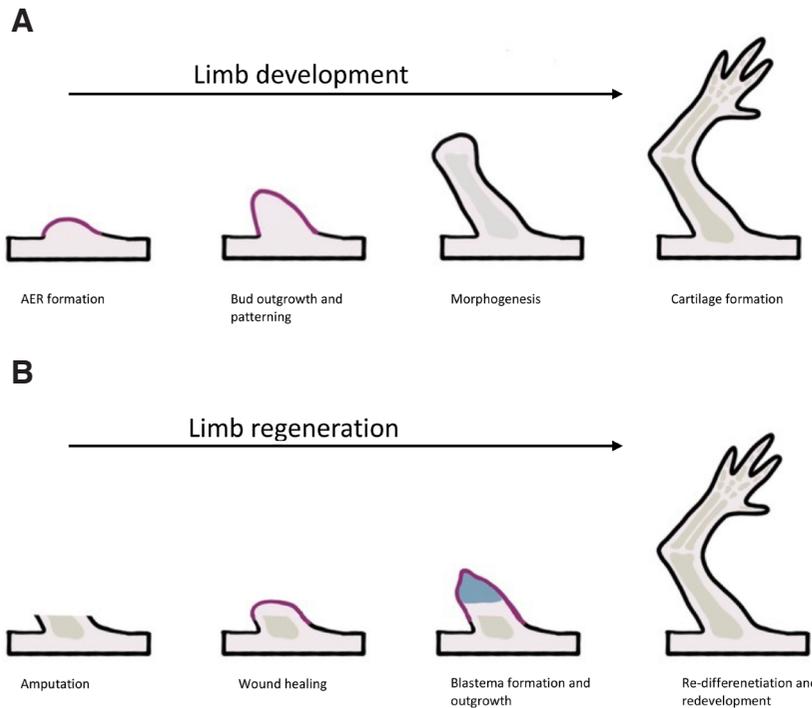
In this review, we characterize the main components of the Wnt signaling pathway and summarize the current state of knowledge regarding the role that the Wnt transduction pathway plays in the regeneration of vertebrates. Finally, we identify gaps in knowledge and indicate possible directions for future research.

## Wnt signaling

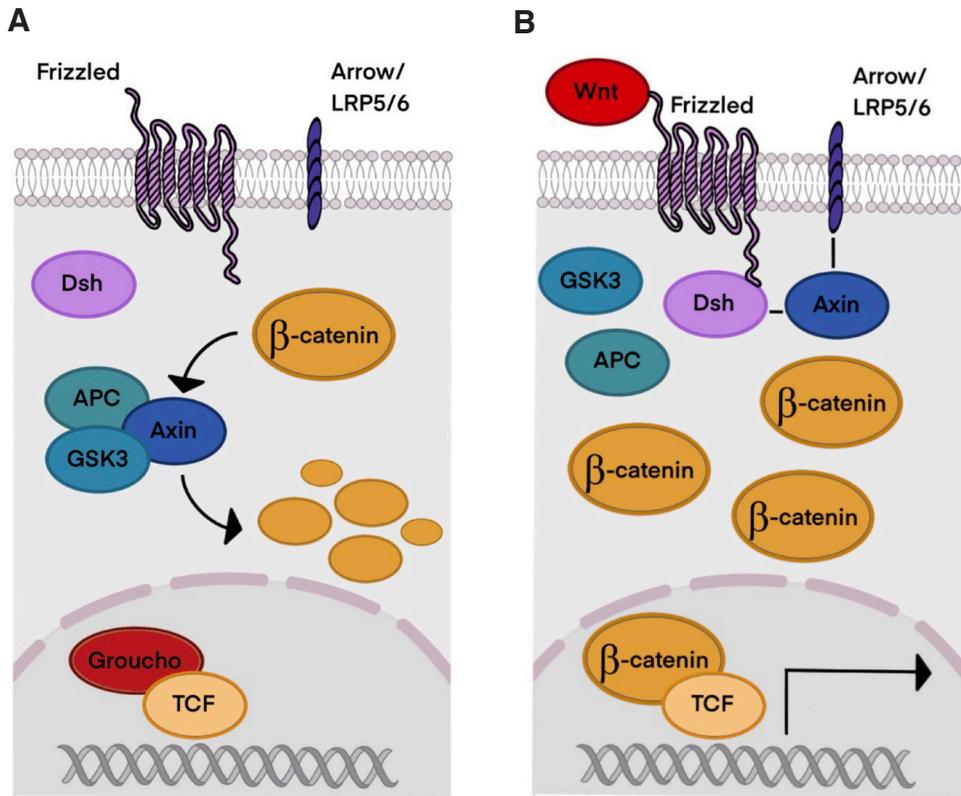
Wnt genes are characterized by their structure, and they encode palmitoylated proteins with a characteristic cysteine pattern. It has been shown that cysteine is critical for their function, while palmitate is essential for signaling. In the Wnt/ $\beta$ -catenin pathway, Wnts bind to the cysteine-rich domain of the seven-transmembrane-span protein Frizzled (Fz). Additionally, Wnt signaling also requires a molecule from the LRP family (*arrow* in *Drosophila*, LRP5 or LRP6 in vertebrates). Dishevelled (Dsh), a cytoplasmic protein, transduces Wnt signals through direct binding between Dsh and Fz. Wnt signaling leads to phosphorylation of Dsh. LRP may also interact with a cytoplasmic component of the Wnt pathway: Wnts induce phosphorylation of LRP, allowing it to dock Axin. Wnt binding to Fz and LRP enhances the direct interaction between Axin and Dsh, modifying the protein complex that regulates  $\beta$ -catenin concentration in the cell (Huelsken and Behrens, 2002).

In the absence of Wnt signaling (Fig. 2A),  $\beta$ -catenin is phosphorylated by the kinases and glycogen synthase kinase 3 (GSK3). This interaction is facilitated by the scaffolding proteins Axin and anaphase-promoting complex (APC), which together form a  $\beta$ -catenin degradation complex. Phosphorylated  $\beta$ -catenin is then ubiquitinated and degraded by the proteasome. Without Wnt signaling in the nucleus, TCF acts as a repressor of Wnt target genes by forming a complex with Groucho. Additionally, the Wnt pathway may be suppressed by multiple inhibitors, of which Dickkopf (Dkk) is one of the most potent, as it binds to LRP and other transmembrane molecules, which makes it unavailable for Wnt reception.

Activation of the Wnt pathway prevents phosphorylation of  $\beta$ -catenin and hence its degradation, leading to the disruption of the degradation complex and accumulation of  $\beta$ -catenin. When Wnt ligand binds to its receptor, it induces a conformational modification of the receptor, which leads to phosphorylation by protein kinases and inhibition of GSK3



**Fig. 1. Comparison of limb development (A) and limb regeneration (B).** Limb development starts with bud formation, followed by apical ectodermal ridge (AER) formation. The next phase is bud outgrowth and patterning. Later, morphogenesis starts, followed by chondrocyte condensation, and finishing with cartilage formation. Limb regeneration starts with wound healing, then the blastema forms and cells proliferate. At the end, re-differentiation and redevelopment is observed.



**Fig. 2. The Wnt/β-catenin signaling pathway. (A)** Without Wnt signaling β-catenin concentration remains low because of its degradation and ubiquitination. **(B)** When Wnt signaling is active, β-catenin accumulates in the cell and nucleus, initiating transcription of Wnt target genes. Abbreviations: LRP5/6, Low density lipoprotein receptor-related protein 5/6; Dsh, Dishevelled; APC, adenomatous polyposis coli protein; GSK3, glycogen synthase kinase 3; TCF, T cell factor.

(Clevers *et al.*, 2014). Nuclear accumulation of β-catenin causes conversion of the TCF repressor complex into a transcriptional activator complex. Groucho is replaced with CBP, which may bind β-catenin/TCF as a coactivator (Fig. 2B). In summary, β-catenin forms a complex with the LEF/TCF transcription factor that results in transcriptional activation of Wnt target genes. Wnt target genes control a wide range of biological processes, and they are involved in embryonic and larval patterning and homeostasis in adults. Even loss of a single Wnt gene can create dramatic phenotypes (Logan and Nusse 2004). Disruption of this pathway can lead to degenerative diseases and cancer in humans (Katoh 2005; Yin *et al.*, 2016). Although in some cases Wnt signals may be transduced independently of β-catenin and although Frizzled and Dishevelled play crucial roles, this review is concentrated on the β-catenin-mediated pathway.

## Wnt signaling pathway in regeneration

### Hydra

*Hydra* is a freshwater cnidarian with high regeneration potential, which is why it is ubiquitously used as a model species in many laboratories. *Hydra* can regenerate the foot, basal and apical head via morphallactic (without cell proliferation) or epimorphic modes. In comparison with vertebrates, due to its simple anatomy, it is relatively easy to study; consequently, there is much available data, including data on the Wnt signaling pathway (Mehta and Singh 2019).

In Cnidaria, 13 Wnt families have been identified, eleven of which were found in *Hydra* and include genes involved in both canonical and non-canonical pathways (Lengfeld *et al.*, 2009a). During *Hydra* embryogenesis, most Wnt genes are expressed, while during asexual reproduction, all of them are expressed in the initial stages of bud formation. Their expression ratio varies between ectoderm and endoderm for each gene. Most of the genes are strongly expressed in the ectoderm, and then their expression shifts to the endoderm.

Numerous studies have shown that Wnt signaling is pivotal for *Hydra* head regeneration (Philipp *et al.*, 2009). Cells close to the amputation plane undergo apoptosis. During the short time window, apoptotic cells are filled with Wnt3a, and it is likely that it is already present in dying cells. Then, Wnt3 released by apoptotic cells activates the β-catenin pathway. Moreover, the course of the regeneration process in *Hydra* varies depending on the amputation level. Following decapitation, direct redevelopment is induced as a result of Wnt3 signaling from epithelial cells (Fig. 3A). In the case of mid-gastric amputation, Wnt3 signaling is initiated in interstitial cells, and activation in

epithelial cells occurs later (3 h post-amputation in comparison to 1.5 h post-amputation after decapitation) (Galliot and Chera 2010). Silencing Wnt3 or β-catenin inhibits head regeneration (Chera *et al.*, 2009). β-Catenin is required for both head and foot regeneration. On the other hand, overexpression of β-catenin results in regeneration of extra heads and feet (Güfler *et al.*, 2018).

The remaining Wnt genes are activated sequentially. Following decapitation, Wnt1 and Wnt16 are upregulated at 3 hours post-amputation (hpa), and then Wnt9, Wnt10a and Wnt7 start at 6 hpa. In the case of mid-gastric bisection, Wnt7, Wnt9, Wnt10a and Wnt16 are expressed at 12 hpa and Wnt1 at 24 hpa (Lengfeld *et al.*, 2009a). Furthermore, head regeneration from the mid-gastric level depends upon two waves of Wnt3 signaling. Although the course of the Wnt signaling pathway in *Hydra* regeneration is quite well documented, there are still some unanswered questions. For example, how apoptosis directs Wnt3 release and how interstitial Wnt3 signaling leads to Wnt3 expression in the endodermal epithelial cells.

Based on functional analysis, research indicated that eight Wnt genes are involved in the formation and maintenance of the head organizer (Hobmayer *et al.*, 2000), such as Wnt1, -3, -7, -11 and probably Wnt2, which plays a role in the early stages of axis formation. As mentioned before, Wnt3 acts as a primary ligand; it not only induces head formation but is also crucial for axis formation. In contrast, Wnt5 acts as a putative inhibitor of the Wnt/β-catenin pathway, repressing head growth. It remains

unclear whether Wnt5 acts on the canonical, non-canonical or both pathways, even though different studies have indicated that Wnt5 is involved in the non-canonical pathway (PhilippPhilipp et al., 2009). Another inhibitor, Sp5, represses Wnt3 expression a few hours after bisection, preventing ectopic head formation but providing enough time to instruct head formation. Interestingly, Sp5 most likely autorepresses when reaching high intracellular levels; however, more evidence should be acquired (Vogg et al., 2019).

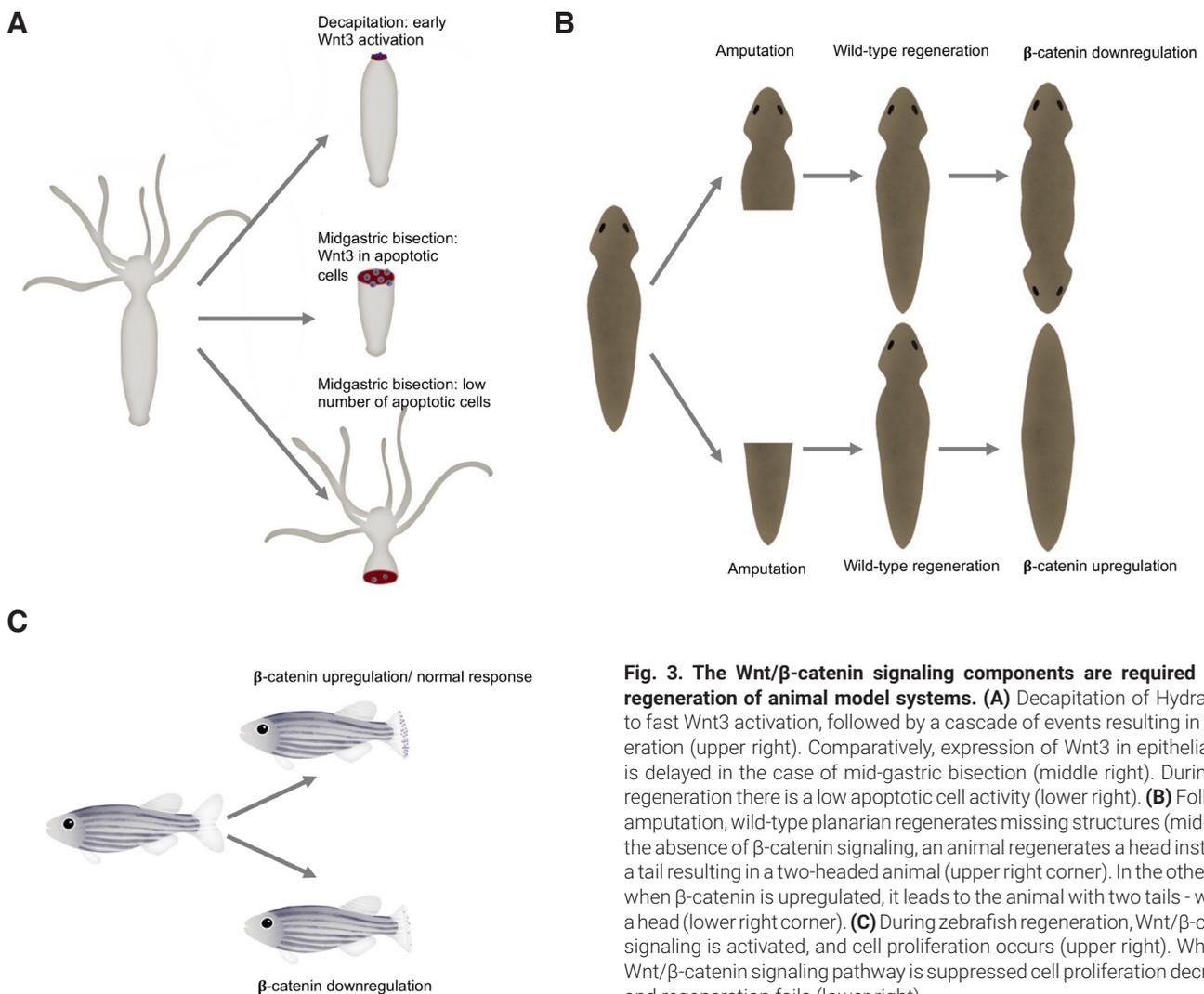
As stated above, there are various non-canonical pathways. Their role in the regeneration process has not been studied as extensively as the canonical pathway. Nevertheless, Philipp et al., (2008) have taken a first step toward that direction. They showed that Wnt/ $\beta$ -catenin signaling is required for the activation of the non-canonical pathways. The JNK pathway is involved in lateral cell intercalation. Inhibition of the JNK pathway stops tentacle formation, which could be due to morphogenetic tissue movements. Interestingly, it does not influence positional information.

In other species, hierarchical dependency of the genes involved in various signaling pathways has been observed. In the case of Hydra, Notch signaling is required for the activation of Wnt3 and  $\beta$ -catenin, and its inhibition represses Wnt3 and  $\beta$ -catenin expression; therefore, the head organizer does not form. This raises a

question: how is the Wnt pathway related to other pathways during regeneration? Research on Hydra provides useful information about animal regeneration and the role of the Wnt pathway in that process. Additionally, previous studies indicate the significance of the evolutionarily conserved Wnt pathway, suggesting that molecular mechanisms in Hydra regeneration might have applications in other species.

### Planaria

Planaria (Platyhelminthes) are freshwater free-living flatworms with bilateral symmetry and a nervous system. They have astonishing regenerative potential, as they can regrow any part of their body. They regenerate via cell proliferation (with blastema formation) and morphallaxis (Reddien and Sánchez Alvarado, 2004). The Wnt signaling pathway has been identified in Planaria as one of the most important pathways during the regeneration process. To date, nine Wnt genes have been found. It seems that the Wnt/ $\beta$ -catenin pathway determines head-tail polarity during regeneration. Intriguingly, the  $\beta$ -catenin anterior-posterior specification system is present in adult tissue. Knockdown of  $\beta$ -catenin results in regeneration of the animal with two heads (with no tail) (Fig. 3); moreover, in that case, the animal may grow numerous sideways



**Fig. 3. The Wnt/ $\beta$ -catenin signaling components are required in the regeneration of animal model systems. (A)** Decapitation of Hydra leads to fast Wnt3 activation, followed by a cascade of events resulting in regeneration (upper right). Comparatively, expression of Wnt3 in epithelial cells is delayed in the case of mid-gastric bisection (middle right). During foot regeneration there is a low apoptotic cell activity (lower right). **(B)** Following amputation, wild-type planarian regenerates missing structures (middle). In the absence of  $\beta$ -catenin signaling, an animal regenerates a head instead of a tail resulting in a two-headed animal (upper right corner). In the other case, when  $\beta$ -catenin is upregulated, it leads to the animal with two tails - without a head (lower right corner). **(C)** During zebrafish regeneration, Wnt/ $\beta$ -catenin signaling is activated, and cell proliferation occurs (upper right). When the Wnt/ $\beta$ -catenin signaling pathway is suppressed cell proliferation decreases and regeneration fails (lower right).

TABLE 1

## REGENERATING ORGANS AND BODY PARTS IN DESCRIBED ORGANISMS

Body parts/organs/ tissues	Hydra	Planaria	Zebrafish	Xenopus	Axolotl
Head	Lengfeld <i>et al.</i> , 2009	Petersen and Reddien, 2009a			
Tail		Petersen and Reddien, 2009a	Gemberling <i>et al.</i> , 2013	Lin and Slack 2008	Brookes and Kumar 2008
Limbs/fins/ feet	Lengfeld <i>et al.</i> , 2009		Gemberling <i>et al.</i> , 2013	Lin and Slack 2008	Brookes and Kumar 2008
Heart			Zhao <i>et al.</i> , 2019	Liao <i>et al.</i> , 2017	Cano Martínez <i>et al.</i> , 2010
Eyes (retina)			Gemberling <i>et al.</i> , 2013	Beck <i>et al.</i> , 2009	Suetsugu-Maki <i>et al.</i> , 2012
Muscles			Gemberling <i>et al.</i> , 2013	Lin <i>et al.</i> , 2012	McCusker and Gardiner 2011
Bones			Gemberling <i>et al.</i> , 2013	Lin and Slack 2008	McCusker and Gardiner 2011
Spine			Gemberling <i>et al.</i> , 2013	Edwards-Faret <i>et al.</i> , 2017	Sabin <i>et al.</i> , 2019
Liver			Gemberling <i>et al.</i> , 2013		Ohashi <i>et al.</i> , 2021
Kidney			Gemberling <i>et al.</i> , 2013	Krneta-Stankic <i>et al.</i> , 2017	
Pancreas			Gemberling <i>et al.</i> , 2013		
Brain			Gemberling <i>et al.</i> , 2013	Lee-Liu <i>et al.</i> , 2017	Roy and Gaten, 2008

facing heads (Petersen and Reddien, 2009b). In contrast, when  $\beta$ -catenin is upregulated, it may result in regeneration of posterior structures. Moreover,  $\beta$ -catenin silencing in uncut adult animals triggered the transformation of a tail into a head. Hence,  $\beta$ -catenin is required for the regeneration of posterior structures; it acts as a switch to specify and maintain anteroposterior identity (Gurley *et al.*, 2008). It is possible that the secret behind planarian abilities to regenerate lies in the application of embryonic signaling profiles during adult tissue homeostasis (Tanaka and Weidinger, 2008). A similar situation takes place in the absence of Evi (Evenness, also known as Wntless): its depletion caused the growth of posterior heads. However, Evi is also necessary for the proper regeneration of the brain and ventral brain cords in Planaria (Adell *et al.*, 2009). Interestingly, Wnt signaling is pivotal for antero-posterior brain patterning in various planarian species (Kobayashi *et al.*, 2007).

WntP-1 is one of the genes required for tail formation; typically, it is expressed posteriorly. Surprisingly, at the beginning of regeneration, it is expressed anteriorly and posteriorly in all kinds of wounds, indicating its role in the process. Another gene, WntP-2, is activated afterward in posterior wounds, as it requires the expression of WntP-1 and  $\beta$ -catenin (de Robertis, 2010). Both WntP-1 and WntP-2 have a role in polarity, which seems independent and instructive for tail formation. It is possible that neoblasts are active after Wnt-mediated polarity to implement further tail formation (Petersen and Reddien, 2009a). WntP-3 and WntP-11 are also expressed posteriorly; however, expression of WntP-2-1 is restricted anteriorly in heads. These data suggest that the AP axis in Planaria is controlled by anterior Wnt inhibition and posterior Wnt activation (Petersen and Reddien, 2009b). Petersen and Reddien (2009a) found that none of the Wnt genes result in posterior head regeneration, likely due to redundancy among these genes. Nonetheless, double knockdown of WntP-1 and WntP-2 boosted the rate of posterior head development (de Robertis, 2010).

As mentioned in the Hydra paragraph, there is a certain hierarchy of transduction pathways. For example, in the case of Planaria, an early regenerative response is activated by Hedgehog (Hh) signaling acting upstream of Wnt (de Robertis, 2010). Experiments activating the Hh pathway led to the overexpression of WntP-1 and hence regeneration of tails instead of heads.

Research on Planaria contributed substantially to our understanding of the Wnt signaling pathway's role in the wounding response, regeneration and stem cell proliferation. Among others, tissue identity determination is a considerable function of Wnt proteins.

### Zebrafish

Zebra fish (*Danio rerio*) is a teleost freshwater fish native to South Asia. As a lower vertebrate, it constitutes a perfect model species for regeneration studies since it has the ability to regrow numerous tissues, such as fins, retina, pancreas, liver, kidney, spinal cord, brain and even heart (Table 1) (Gemberling *et al.*, 2013). Moreover, considering that many human genes have zebrafish orthologs (Howe *et al.*, 2013), new knowledge regarding the mechanisms of zebrafish regeneration gives hope for applying zebrafish to the human body in the future (Poss *et al.*, 2003). Wnt signaling is required for the regeneration of various tissues in zebrafish. For example, during retinal regeneration, a canonical pathway is necessary for the proliferation of Müller glia (Meyers *et al.*, 2012). During bone regeneration, epithelial to mesenchymal transformation of osteoblasts depends on Wnt/ $\beta$ -catenin signaling, which is necessary to generate proliferative pre-osteoblasts (Stewart *et al.*, 2014).

As human hearts have no ability to regenerate by themselves, knowledge about regeneration among other species may be very valuable for regenerative medicine, such as zebrafish, which can regrow up to 20% of their cardiac muscle (Plackett 2021). Moreover, unlike other regenerating organisms, teleost fish can regenerate hearts throughout their life. They constitute a good model for regeneration studies, as some aspects of their heart physiology are similar to those of mammals; however, these fish hearts only have two chambers, which makes them easier to work with. Following heart injury, transcriptional responses occur within a few days, and many genes expressed during development are upregulated. Nevertheless, the role of the Wnt pathway has yet to be thoroughly investigated. Just a few years ago, researchers only suspected that the Wnt pathway takes part in heart regeneration; however, tools available at the time did not allow verification (Ozhan and Weidinger 2015). Fortunately, since then, significant technological advances have been made, and the role of the Wnt pathway in cardiac regeneration has been extensively reviewed by Li *et al.*, (2022). They state that Wnt and Wnt antagonists are involved in various processes during cardiac injury repair; furthermore, non-canonical or Wnt-independent processes contribute to cardiac repair. Heart injury in zebrafish results in the expression of Wnt inhibitors such as Dkk1 and Dkk3 and secreted Frizzled-related protein 1 (sFrp1) and sFrp2 (Peng *et al.*, 2021). Wnt inhibition is beneficial for cardiac recovery, as it supports cardiomyocyte dedifferentiation and proliferation and prevents scarring (Zhao *et al.*, 2019). Developing tools allowing for Wnt antagonist employment without affecting other pathways will be crucial for treating human diseases.

Another impressive ability that regenerative medicine would like to apply to the human body involves zebrafish's potential to regenerate the spinal cord. In contrast to cardiac injury, during spinal cord repair, the Wnt/ $\beta$ -catenin pathway is pivotal for fish recovery, specifically for axon regrowth and functional recovery. It was found that overexpression of Wnt antagonists such as Dkk1 inhibits processes including locomotory recovery, axon regeneration, and glial bridge formation. Furthermore, spinal cord transection leads to the upregulation of Wnt/ $\beta$ -catenin in non-neural cells. It is essential as it controls the composition of the lesion site extracellular matrix, and it induces expression of col12a1a/b and Collagen XII: a promoter of axonal regeneration. Interestingly, inhibition of Wnt signaling impacts col12a1a/b in a delayed manner, thereby sustaining its expression and resulting in successful regeneration (Wehner et al., 2015, 2017). Additionally, Strand et al., (2016) demonstrated that the role of this pathway is conserved in larval and adult zebrafish, indicating that using developing fish for spinal cord injury research might have advantages over adult fish.

#### Zebrafish fin regeneration

The first implication of Wnt signaling in zebrafish regeneration was made by Poss et al., (2000), who observed the expression of *lef1*, a Wnt target gene. Initial research on zebrafish was not focused on the members of the Wnt family but on the target genes. Except for *lef1*, *msxb* (marker of blastema formation) and other genes were also downregulated by Dkk overexpression, (Kawakami et al., 2006) and their silencing led to regeneration failure. As in other animals described above, in epimorphic regeneration of zebrafish, Wnt/ $\beta$ -catenin signaling plays a crucial role, and its signals can be detected even 3 h following the injury. Ligand activating the canonical pathway, *wnt10* is expressed very early during regeneration. It has been demonstrated that the same gene activates the Wnt pathway during limb development. Activation of Wnt/ $\beta$ -catenin signaling may be a conserved trait, as a similar pattern was observed during zebrafish heart regeneration.

Ligand expression does not have to result in activation of transcription; however, it leads to the expression of Wnt target genes (Stoick-Cooper et al., 2007). This leads to the expression of genes from the fibroblast-like growth factor (FGF) family that, together with the canonical pathway, regulate blastema proliferation. After blastema formation, the non-canonical pathway is activated by the expression of Wnt5 genes and is maintained throughout the process (Poss et al., 2000).

Expression of Wnt inhibitors in transgenic fish after wound healing but before blastema formation results in complete regeneration failure. Subsequent experiments expressing Dkk during the outgrowth phase proved that Wnt/ $\beta$ -catenin signaling is required not only for blastema formation but also for its maintenance. On the other hand, overexpression of Wnt8 led to a significant increase in blastema mesenchyme proliferation; however, it did not influence fin length. Intriguingly, fish with the mutated copy of Axin (a potent inhibitor of the Wnt pathway) regenerated significantly longer fins than wild-type fish. This aspect of Wnt/ $\beta$ -catenin signaling in regeneration was reported for the first time, and it sheds new light on its function. As mentioned above, after the canonical and non-canonical pathways are activated, they have opposing roles in the process. Wnt5b activates the non-canonical pathway; when this gene is overexpressed, proliferation is reduced, resulting in regeneration failure. Moreover, it produces similar phenotypes as

in the case of Dkk overexpression. The lack of this gene's expression did not cause a patterning effect or inappropriate growth, suggesting that it acts in a negative feedback loop to modulate Wnt/ $\beta$ -catenin signaling.

In previous chapters, we mentioned the hierarchy of various pathways. Work on zebrafish indicated that Wnt/ $\beta$ -catenin acts upstream of FGF and is necessary to maintain FGF signaling (Stoick-Cooper et al., 2007). Later studies implied that Wnt/ $\beta$ -catenin signaling affects regeneration in an indirect way, as it regulates other key pathways, including the Hh, BMP, retinoic acid (RA), Igf, Notch and Activin pathways (Wehner et al., 2014). In summary, zebrafish injury activates the expression of Wnt ligands (such as Wnt10a), which initiate Wnt/ $\beta$ -catenin signaling. This leads to the expression of FGF genes, which together with Wnts prompt blastema formation and thus regeneration. In the meantime,  $\beta$ -catenin-independent signaling is triggered, which regulates  $\beta$ -catenin signaling. It is likely that these two pathways form a loop that guarantees accurate level, duration, and location of  $\beta$ -catenin signaling. Wnt and FGF signaling are assumed to be the main pathways that govern epimorphic fin regeneration in zebrafish (Tal et al., 2010).

#### Xenopus

*Xenopus laevis* is another ubiquitously used model organism in numerous studies worldwide. It has a remarkable regeneration capacity as a tadpole (Table 1), but its ability to regenerate significantly decreases after metamorphosis (Muneoka et al., 1986). After limb injury, young post-metamorphosis adults can regenerate only spike-like structures. Even at later stages when regeneration capacity is partially attenuated,  $\beta$ -catenin can promote regeneration.

As in many other species, in a dose-dependent manner, Wnt signaling is necessary for AP patterning during *Xenopus* gastrulation, and it also constitutes a transforming morphogen to form the central nervous system (Kiecker and Niehrs, 2001). Although both  $\beta$ -catenin and non-canonical pathways are involved in cardiogenesis, little is known about their involvement in heart regeneration (Hoppler and Conlon 2020).

In recent years, the role of Wnt/ $\beta$ -catenin signaling in the regeneration process has been widely researched. Wnt/ $\beta$ -catenin signaling is essential for tadpole early-stage limb regeneration, especially during apical epithelial thickening and blastema formation (Yokoyama et al., 2007). Without it, initiation of tadpole limb regeneration will fail, while  $\beta$ -catenin can promote tail outgrowth (Lin and Slack, 2008). On the other hand, at the later stages of regeneration, Wnt signaling is still involved but not needed, as tadpoles with the heat-shocked expressed Dkk still managed to regenerate (at least partially!). According to Yokoyama et al., (2007), even though many ligands activate  $\beta$ -catenin signaling, only Wnt3a activates the pathway in both developing and regenerating limbs. Its expression pattern suggests that it contributes to the formation of AECs. Similar expression patterns were observed in both tadpoles and froglets (young-metamorphosed adults). Thus, it could be assumed that the absence of Wnt signaling during regeneration of these two stages will have similar effects. As previously explained, the lack of Wnt signaling in tadpoles will result in regeneration failure; however, the situation is slightly different in the case of froglets.

Previous research established that limb regeneration requires the presence of nerves (Suzuki et al., 2005). Surprisingly, even when Wnt signaling is blocked, froglets can still regenerate spike structures. Yokoyama et al., (2011) reported that nerve signals can

functionally substitute for Wnt/ $\beta$ -catenin signaling during froglet regeneration. Nerve signals may involve members of other families, such as FGF, compensating for impaired Wnt signaling. Consequently, they can stimulate blastema formation. On the other hand, Wnt signaling can rescue defects caused by partial innervation. It is conspicuous that while inhibition of Wnt/ $\beta$ -catenin signaling is not sufficient to block regeneration, total denervation will entirely hinder this process. These facts support the hypothesis that nerve signals are more pivotal for the initiation of froglet limb regeneration because nerve signals can activate more target genes involved in regeneration than Wnt/ $\beta$ -catenin signaling. This paper confirms that nerve signals and Wnt/ $\beta$ -catenin have overlapping functions in the initiation of the regeneration process. To our knowledge, no prior studies have examined the mutual implications of nerve and Wnt/ $\beta$ -catenin signaling. It is clear that nerves play a role in regeneration; however, more studies are needed to understand the complete correlation between nerve signaling, Wnt and other pathways. To apply these data to the practical aspects of regenerative medicine, it is particularly recommended to focus future investigations on other model species.

Another fascinating aspect of regeneration in *Xenopus* is the role of the non-canonical pathway. Tail regeneration requires Wnt5a, which is mediated by the Wnt/JNK pathway. Studies have shown that Wnt/JNK plays a role in the distal specification of the regenerating tail. Sugiura *et al.*, (2009) deduced that Wnt/ $\beta$ -catenin and Wnt/JNK are required to form the distal epidermis. Nevertheless, as described in the above chapter, there is strong evidence that canonical and non-canonical pathways antagonize each other (Stoick-Cooper *et al.*, 2007). For that reason, future research should examine whether an antagonistic relationship between these pathways occurs in the case of *Xenopus* tail regeneration.

Resembling zebrafish, during *Xenopus* regeneration, Wnt/ $\beta$ -catenin signaling acts upstream of FGF, which has a noteworthy implication (Slack *et al.*, 2008)). Suppression of the Wnt pathway stops the expression of some Fgf genes in the blastema (Ng *et al.*, 2002), although others imply that they are not directly affected (Yokoyama *et al.*, 2007). In comparison with Wnt signaling, FGF signaling occurs quite late and is supposedly necessary for the late outgrowth stage. In contrast, the BMP pathway is upstream of both Wnt and FGF and is required for their maintenance (Lin and Slack, 2008). Another factor influencing the Wnt pathway and hence *Xenopus* tail regeneration is reactive oxygen species (ROS). ROS inhibition is followed by a decrease in Wnt/ $\beta$ -catenin signaling, which leads to a reduction in cell proliferation and compromises tail regeneration (Love *et al.*, 2013). Even so, there are still gaps in knowledge regarding elements that impact the Wnt pathway.

In recent years, most discussions regarding *Xenopus* regeneration have focused solely on the tail and limb, yet *Xenopus* can regenerate other tissues and organs. *Xenopus* is a well-established and vital model for eye development and regeneration studies, as its eye structure is similar to that of humans. Additionally, *Xenopus* can regenerate both the retina and lens, even after metamorphosis (Tseng, 2017). As mentioned, tail and limb regeneration in *Xenopus* rely on epimorphosis. However, in eye regeneration, another process can be distinguished: transdifferentiation of the cornea to the lens. Wnt has been shown to be one of the pathways involved in lens regeneration, and several of its components were significantly upregulated in that process (Day and Beck, 2011). Malloch *et al.*, (2009) suggested that Wnt, among other pathways such as BMP,

Hedgehog, FGF, and TGF-beta signaling, plays an important role in regeneration. Although the Wnt pathway in retinal development has been extensively studied, its detailed role in *Xenopus* has yet to be elucidated.

Lin *et al.*, (2012) documented that Wnt and FGF signals are compulsory for spinal cord and muscle regeneration. Generally, different types of tissues undergo regeneration independently; therefore, they require specific signaling. Nevertheless, there seems to be a relationship in the regeneration of these tissues. Although regeneration might proceed even in the absence of the spinal cord, inhibition of that process reduces the volume of the regenerated muscles and affects a notochord. Whereas attenuation of the Wnt and Fgf signals in muscles may reduce their size, it will not block spinal cord regeneration. As in many other organisms or tissue types, overexpression of Dkk1 caused reduction or complete inhibition of spinal cord regeneration, which resulted in tail regeneration failure (Lin *et al.*, 2012). In addition to these exciting insights, little is known about the role of Wnt signaling in the regeneration of other tissue types.

### Urodele amphibians

Urodeles: Newts and salamanders are the only tetrapod vertebrates that can fully regenerate injured limbs throughout their life cycle. They also have the ability to regenerate tails, lenses, retina, spinal cord and heart tissue (Table 1) (Brockes and Kumar 2008). Hence, they offer a functional model for studying molecular and cellular regeneration mechanisms. Analysis of amphibian regeneration is the focus of regenerative biology with the anticipation that once we understand them, we will be able to replicate it in mammalian limbs (Stocum and Cameron, 2011). One of the most popular urodele model species used in regenerative studies is *Ambystoma mexicanum*, commonly known as an axolotl. As axolotl limbs are anatomically similar to those of humans, knowledge of their regeneration can provide valuable information for regenerative medicine (Haas and Whited, 2017).

Axolotl hearts have two atria and an avascular ventricle. After heart injury, they can replace lost tissues, starting with clot-preventing blood loss, followed by fibrin and collagen deposits. Axolotls can functionally and structurally regenerate within 60-90 days, and the response is related to the formation of fibrotic scars, replaced by new cardiomyocytes (Vivien *et al.*, 2016). They can also regenerate their spinal cord via ependymoradial glial cell proliferation and differentiation (Khyeam *et al.*, 2021). Although heart and spinal cord regeneration in urodele amphibians is very impressive, there is little knowledge regarding the role of the Wnt pathway in these processes.

Nevertheless, they are absorbing material for analysis of the Wnt pathway: not only does its genome have orthologs representing all Wnt classes, but it also has a complete set of vertebrate Wnt genes (Nowoshilow *et al.*, 2018). It has been well established that the Wnt signaling pathway can not only promote axolotl limb regeneration but is also necessary in both adults and embryos. Reduction of Wnt signaling can alter AEC formation, which causes defects in the regeneration process (Kawakami *et al.*, 2006). Although axolotls are highly regenerative and possess all Wnt genes, the expression patterns and function during regeneration have been characterized for only a few of them.

There have been many reports suggesting that Wnt functions in specifying the AP axis, while data regarding the DV axis remain

limited (Mehta and Singh, 2019). It has been demonstrated that in amniotes, Wnt7a expression is restricted to the dorsal limb bud, and its inhibition leads to ventralization of the dorsal limb mesoderm, indicating that Wnt7a plays a role in specifying the DV axis (Cyan et al., 1997). However, in axolotl regeneration, Wnt7a is expressed in the epithelium and mesenchyme of the blastema in a diffuse manner, inferring that it has a different function than in amniotes (Shimokawa et al., 2013). It seems that although AP patterning is similar in amphibians and amniotes, there are major differences in DV patterning (Christen and Slack, 1998). Therefore, this information highlights the need for more detailed functional analysis of Wnt7 and the mechanisms governing the formation of the DV axis.

Wnt5a and Wnt5b were detected in regenerating limb blastemas. Wnt5a was expressed during late dedifferentiation (prior blastema formation) and the early digit stage. Wnt5b expression was observed in the mesenchyme of a medium bud when blastema had already formed. Overexpression of Wnt5a inhibited dedifferentiation by diminishing the expression of genes related to that process. As in other described species, these genes are expressed distally, where they inhibit Wnt/ $\beta$ -catenin signaling, allowing for the differentiation of the distal limb structures (Ghosh et al., 2008). A similar pattern can be observed when the Wnt pathway is blocked by the expression of Axin or Dkk (Kawakami et al., 2006).

As can be presumed based on the previous data, Wnt/ $\beta$ -catenin signaling does not work in a uniform way across the entire regeneration process. It has been reported that a lack of Wnt/ $\beta$ -catenin signaling may inhibit regeneration; however, little is known about the opposite situation. What happens when Wnt/ $\beta$ -catenin is chemically activated? It has been shown that in some cases, overexpression of Wnt genes enhances regeneration (Stoick-Cooper et al., 2007); however, it may have various effects depending on the regeneration phase. Wischin et al., (2017) investigated how chemical activation of this pathway influences the key regeneration stages. Treatment at the early stage of wound healing completely blocked regeneration, and at the later stage, it resulted in denervated phenotypes, thereby inhibiting regeneration. Treatment applied after blastema formation caused skeletal malfunctions, but it did not influence cell proliferation. This study illustrates that Wnt/ $\beta$ -catenin signaling controls skeletal morphogenesis, which is consistent with the processes occurring during limb development (Tamamura et al., 2005).

It has been noted that in other species, such as zebrafish, Wnt signaling is necessary in the late wound healing stage but before blastema formation. Studies on axolotl embryos demonstrated that during tail regeneration, Wnt signaling is relatively delayed, and it is not required for early wound healing (Ponomareva et al., 2015). It seems that there is a lack of data regarding details of the regeneration early stages and a more detailed screen would be useful in order to evaluate the exact role of the Wnt signaling. It has been established that the early phase of regeneration is significant for the further course of this process, but more research is needed to determine the involvement of particular Wnt genes.

Most models of axolotl regeneration have studied the limb and tail; however, as stated before, urodele amphibians exhibit the ability to regenerate a wider range of tissues. At present, there are no data regarding the role of the Wnt pathway in these processes. Even so, axolotls are not the only animals that were analyzed with an emphasis on Wnt signaling. Newts are another well-investigated group that includes numerous species with the ability to regenerate (Caubit et al., 1997). Except for tail and limb research, there

is evidence that the Wnt pathway is involved in lens regeneration. According to earlier research, initiation of lens regeneration occurs only in the dorsal iris. Wnt/ $\beta$ -catenin pathway initiates the lens developmental program in pigmented epithelium. There are many Wnt genes taking part in newt development, but only two of them were detected during regeneration: Wnt2b (canonical pathway) in the dorsal half of the iris, and Wnt5a (non-canonical pathway) in both the dorsal and ventral parts of the iris (Hayashi et al., 2006). These data support not only that lens development from the dorsal iris is dependent on Wnt signals but also that there is a variation in Wnt signaling in the regeneration of different tissues or organs. Remarkably, the addition of exogenous Wnt3a accelerates lens regeneration and promotes the development of larger lenses. A similar event takes place after the addition of Wnt5a, although the effect was weaker than in the case of Wnt3a. Based on the patterns of Wnt gene expression, it can be inferred that Wnt signaling is required for acquiring positional information.

### Mammals and birds

Mammals have very limited regenerative capacity, but they have the ability to regenerate some tissues, such as bones, skeletal muscles or skin. Adult mice and humans can regenerate the distal tip of the terminal phalanges. Unfortunately, their limbs do not regenerate after amputation at a more proximal level. Currently, modern medicine offers solutions such as replants or bionic appendages. Nevertheless, scientists continuously explore mammalian regeneration, and there is considerable evidence supporting the significance of the Wnt/ $\beta$ -catenin transduction pathway. The role of the Wnt pathway in development has been well documented, and it has been established that it is involved in numerous processes (Vainio et al., 1999; Stump et al., 2003; Zhang et al., 2011; el Wakil and Lalli, 2011; Buikema et al., 2013). In mice, Wnt/ $\beta$ -catenin participates in liver regeneration, as  $\beta$ -catenin promotes hepatocyte proliferation (Li et al., 2019) and supports cochlear regeneration (Samarajeewa et al., 2018).

It has been shown that during mouse heart development, Hippo and Wnt pathway interactions restrict cardiomyocyte proliferation and regulate heart size (Heallen et al., 2011). Following myocardial infarction, reduced levels of  $\beta$ -catenin or overexpression of Wnt11 improved the recovery of cardiomyocyte function (Hoppler and Conlon 2020).

In different systems, Müller glial cells have the potential to become progenitor cells: various pathways contribute to the formation of Müller glia-derived progenitor cells (MGPCs) and neuronal regeneration in the retina. In the zebrafish retina, the Wnt pathway activates the formation of MGPCs and neuronal regeneration. During retina development in chicks, Wnt signaling maintains an undifferentiated state of neural stem cells in peripheral regions of the retina;  $\beta$ -catenin must be downregulated to allow retinal regeneration from ciliary marginal tissues at the peripheral edge of the developing neural retina. In healthy retinas, the Wnt pathway is not active, and nuclear  $\beta$ -catenin is absent in Müller glia. In the case of retinal damage, as in the zebrafish model, the Wnt pathway is activated in Müller glia and MGPCs, while Wnt inhibition results in a reduced number of proliferating MGPCs (Osakada et al., 2007). Gallina et al., (2016) noted that there might be significant differences between how Wnt signaling regulates MGPC formation in mammals, birds and fish. The authors' research showed complex relations among different

pathways and how they can vary. One example involves *Ascl1*, which promotes neurogenesis in the developing retina. In the chick retina, inhibition of Wnt signaling following NMDA treatment results in an *ascl1a* increase and does not affect cell specification. In zebrafish, *ascl1a* is expressed within four hours after injury and works downstream of Wnt signaling during reprogramming of Müller glia into MGPCs. On the other hand, *ascl1a* is not upregulated in rodent retinas, indicating the presence of factors or signaling pathways that override changes in *ASCL1a*, preventing neuronal differentiation (potentially Jak/Stat, BMP or Notch). In summary, Wnt signaling stimulates proliferation during MGPCs formation in the chick retina without influencing the differentiation of the progeny produced by MGPCs (Gallina *et al.*, 2016).

Deer antlers constitute one of the most impressive regeneration examples in mammals.  $\beta$ -catenin regulates antler progenitor cell survival and fate (Mount *et al.*, 2006). Another function of Wnt signaling in mammals is to sustain tissue renewal; this pathway is responsible for continuous tissue replenishment and maintenance over a lifetime (Clevers *et al.*, 2014). Generally, Wnt signaling is

activated during injuries of various mammalian organs, such as lungs, kidneys or skin, and this process has been reviewed in detail by Bastakoty and Young (2016). They reported that although the Wnt/ $\beta$ -catenin pathway obviously contributes to regeneration, its complexity and numerous factors (timing, cell type, target) make it challenging to apply in therapeutics.

## Discussion

Phylogenetic data suggest that the Wnt signaling pathway present in bilaterians first appeared earlier in metazoan evolution (Lengfeld *et al.*, 2009). The number of Wnt genes ranges from 11 to 19 in deuterostomes and from 4 to 9 among protostomes (Fig. 4) due to the loss of family members during their evolution (Guder *et al.*, 2006). Wnt is an evolutionarily conserved pathway, and its function is also conserved during regeneration. As shown above, Wnt signaling plays a critical role in almost every studied regeneration system, including cnidarians, fish, frogs, and salamanders. As shown in Table 2, the role of many Wnt genes in regeneration has

TABLE 2

### SUMMARY OF WNT GENES AND THEIR FUNCTION IN VARIOUS MODEL SPECIES

	Hydra	Planaria	Zebrafish	Xenopus	Axolotl
Wnt1	bud formation (Lengfeld <i>et al.</i> , 2009)	head formation (de Robertis, 2010)	✓	✓	✓
Wnt2	axis formation (Lengfeld <i>et al.</i> , 2009)	AP positional information (Petersen and Reddien, 2009a)	✓	✓	✓
Wnt2B		✓	✓	✓	✓
Wnt3	primary ligand (Lengfeld <i>et al.</i> , 2009)	AP positional information (Petersen and Reddien, 2009b)	✓	✓	✓
Wnt3A	primary ligand (Lengfeld <i>et al.</i> , 2009)		outgrowth in basal layer of wound epidermis (Poss <i>et al.</i> , 2000)	blastema formation (Yokoyama <i>et al.</i> , 2007)	✓
Wnt4		✓	✓	(Sugiura <i>et al.</i> , 2009)	✓
Wnt4B			ligand required for $\beta$ -catenin reporter activity (Strand <i>et al.</i> , 2016)		
Wnt5A	inhibition of the canonical pathway (Philipp <i>et al.</i> , 2009)	patterning mediolateral axis (Gurley <i>et al.</i> , 2008)	modulation of the regeneration rate (Stoick-Cooper <i>et al.</i> , 2007)	regenerative outgrowth; activation of JNK pathway (Sugiura <i>et al.</i> , 2009)	inhibition of the canonical pathway (Ghosh <i>et al.</i> , 2008)
Wnt5B			modulation of the regeneration rate (Stoick-Cooper <i>et al.</i> , 2007)	✓	✓
Wnt6		✓		✓	✓
Wnt7A	bud formation (Lengfeld <i>et al.</i> , 2009)	✓	✓	✓	specification of the DV axis (Shimokawa <i>et al.</i> , 2013)
Wnt7B				✓	✓
Wnt7C				✓	
Wnt8A	Lengfeld <i>et al.</i> , 2009		cell proliferation (Stoick-Cooper <i>et al.</i> , 2007)	(Sugiura <i>et al.</i> , 2009)	✓
Wnt8B			✓	✓ ligand	✓
Wnt9A	bud formation (Lengfeld <i>et al.</i> , 2009)				✓
Wnt9B				✓	✓
Wnt10A	bud formation (Lengfeld <i>et al.</i> , 2009)		activation of the canonical pathway (Stoick-Cooper <i>et al.</i> , 2007)	✓ ligand	✓
Wnt10B			✓	✓	✓
Wnt11	bud formation (Lengfeld <i>et al.</i> , 2009)	AP positional information, patterning the posterior midline (Petersen and Reddien, 2009b)	✓	(Sugiura <i>et al.</i> , 2009)	✓
Wnt16	bud formation (Lengfeld <i>et al.</i> , 2009)			✓	✓

The ✓ symbol indicates that a gene was detected in a particular species; however, whether it is involved in regeneration or the function it plays remains unknown. Second column (P) indicates whether a particular gene acts via canonical (C) or non-canonical (NC) pathway, although in some cases there are discrepancies between different authors (Croce and McClay, 2008; Ackers and Malgor, 2018).

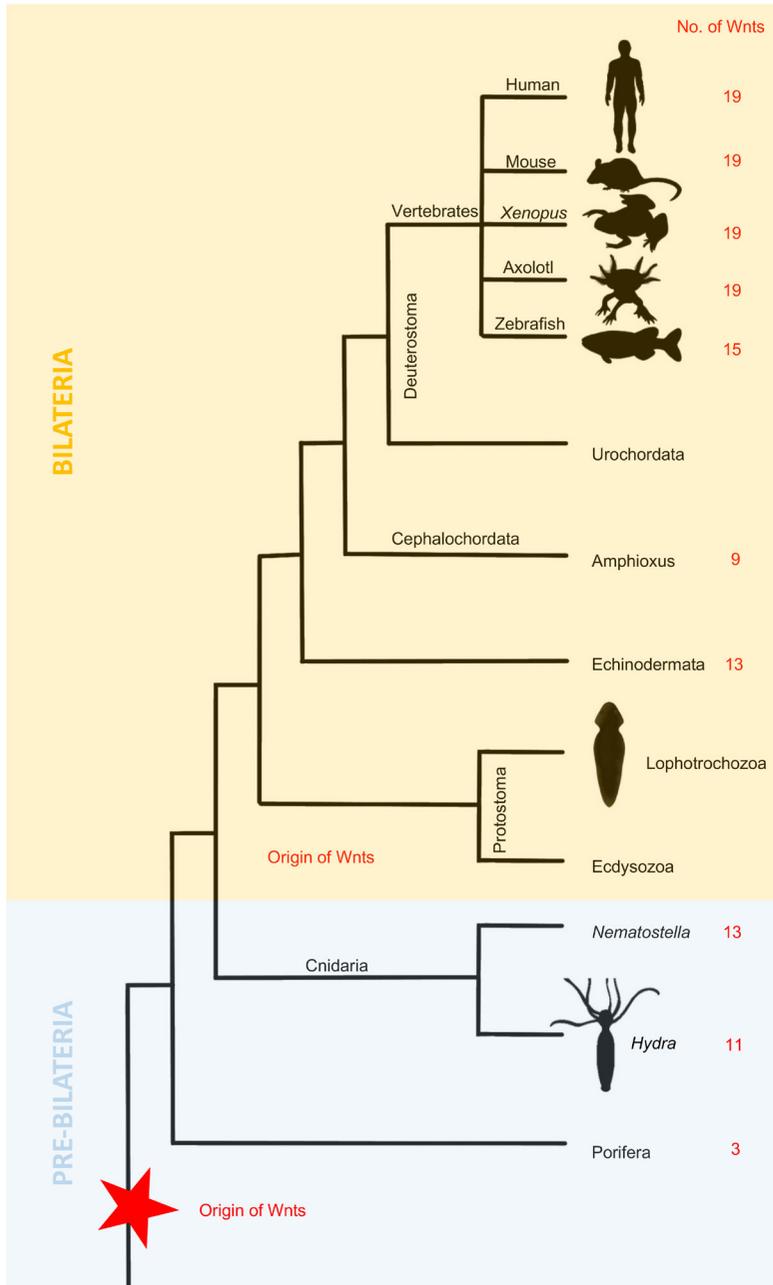
yet to be described. There are also differences among various species. It is clear that the function of more genes has been elucidated in animals with relatively simple body structures, such as Hydra. Interestingly, these species have fewer Wnt genes, most likely lost during evolution. Although some stages of limb regeneration seem equivalent to limb development, there are still significant discrepancies. Wnt gradients occurring during regeneration are not the same as those in early embryos. Therefore, we cannot say that hierarchical development is entirely consistent with regeneration.

Bilaterians can be divided into deuterostomes and protostomes (Ecdysozoa and Lophotrochozoa). Although there are major differ-

ences between these phyla, it seems that Wnt/ $\beta$ -catenin signaling specifies the anteroposterior axis in all of them, suggesting that it might be an ancient trait present in the ancestor of all bilaterians (Fig. 4). A theory has been derived promoting that whole-body regeneration is probably an ancestral trait lost in most animal lineages, while structural regeneration might be *de novo* adaptation (Slack, 2017). The ancestral role of Wnt signaling is the specification and regulation of posterior aspects of the AP axis. There is a high resemblance in the expression of Wnt genes and their antagonists among many deuterostome species concerning AP polarity (Petersen and Reddien, 2009a). One example is an evolutionarily ancient  $\beta$ -catenin protein that in many metazoan species acts as a molecular switch responsible for polarity specification (Gurley et al., 2008). Its critical function is deeply conserved, as it controls AP polarity in species such as *C. elegans* (Ackley, 2014), *Drosophila* (Siegfried and Perrimon, 1994), *Gryllus bimaculatus* (Nakamura et al., 2007) and many others.

The Wnt/ $\beta$ -catenin transduction pathway is evolutionarily conserved, and it plays a similar role in the regeneration of various animals; moreover, similar molecular mechanisms underline the regeneration of anatomically distinct species. Notwithstanding, the involvement of dedifferentiation and stem cell activation in the generation of progenitor cells during regeneration differs between organisms (Stoick-Cooper et al., 2007). Part of Wnt/ $\beta$ -catenin signaling in dedifferentiation and cell activation in blastema remains vague. Numerous data presented in this paper have shown that in various systems, Wnt signaling may be activated at different stages (initial or later part of the process), and little is known regarding what controls the activation of Wnt signaling. For example, Kawakami et al., (2006) indicated that the primary role of Wnt signaling resides at the early stages of regeneration, such as AEC formation (by controlling numerous transcription factors). Yokoyama et al., (2007) stated that the Wnt/ $\beta$ -catenin pathway is essential during early stages, but it is not required after blastema formation. Some genes are expressed later during the outgrowth phase (Poss et al., 2000). There might also be functional differences between Wnt genes in various species (Table 1); therefore, more studies are needed to compare their function in different animals as well as within the same species but for different tissues or organs. What is clear, however, is that Wnt/ $\beta$ -catenin signaling plays a main role in many regenerative processes. It is well established that in the regeneration of vertebrates, the Wnt pathway is crucial in the organization of cell polarity. A further, potentially open question regards the role of the  $\beta$ -catenin-independent pathway and its role in cell fate determination.

Some of the data released in recent years are contradictory to older information, especially regarding gene function. For instance, some of the genes taking part in zebrafish tail regeneration (Stoick-Cooper et al., 2007) were not detected in similar experiments before, and the function of some genes was not assigned correctly. At first, it was thought that Wnt5 stimulates blastema formation (Poss et al., 2000, 2003), whereas currently, we know that Wnt5 operates in the opposite way to  $\beta$ -catenin signaling



**Fig. 4. Evolution of Wnt signaling during Metazoan expansion.** The number of Wnt genes increased from the first Metazoans to the Cnidarians and were preserved in basal Deuterostomes, while Protostomes are characterized by the loss of Wnt genes.

by reducing proliferation (Stoick-Cooper *et al.*, 2007). We believe that such a discrepancy results from the emergence of new, more precise research techniques, and we can assume that more detailed knowledge will also appear in the future. Furthermore, with the advancement of complex research tools (genome sequencing, transgenics, CRISPR, etc.) there are completely new perspectives awaiting in the field of regeneration.

We believe that experiments on organisms that constitute model systems in evolutionary studies could significantly explain some of the issues raised in this article. One such animal is amphioxus, cephalochordate, which represents the earliest evolutionary lineage of chordates (Liang *et al.*, 2019; Bertrand *et al.*, 2021). Although some of the amphioxus Wnt genes were characterized (not described here as it exceeds the scope of this paper), it is evident that there are still many aspects that can be revealed thanks to the position of amphioxus in the evolutionary tree (Somorjai *et al.*, 2012, 2018; Somorjai, 2017). Therefore, we would like to encourage scientists to explore possibilities for unconventional research materials, for instance, among non-model species, as they can also be a useful source of information.

Data emerge daily; however, we still need a full view of how the Wnt pathway interacts with others. For example, while some findings suggest that Wnt acts upstream of numerous other pathways (Wehner *et al.*, 2014), others state something completely opposite (Blum and Begemann, 2012). Accordingly, there is an obvious need for more detailed work regarding this topic.

Finally, there is no doubt that Wnt/ $\beta$ -catenin signaling regulates key signaling pathways involved in regeneration, and this pathway is required regardless of age and developmental stage. Scientists currently extensively study its application in cancer research (Barker and Clevers, 2006; Kahn, 2014) and regenerative medicine (de Santis *et al.*, 2018).

It is currently being developed in the direction of stem cell therapies and *ex vivo* organs. It is an exciting approach, as traditional transplants have many limitations, such as insufficient suitable donors and post-transplant complications such as graft failures. Therefore, a novel technology such as *ex vivo* organs based on stem cell therapy might offer an alternative solution (Luijmes *et al.*, 2022). There have been attempts to perform transplants of bioengineered organ scaffolds in animal systems (Wagner *et al.*, 2013). Wnt/ $\beta$ -catenin signaling has been proven to have numerous applications in development and regeneration; hence, scientists working in this field naturally pay much attention to it (Majidinia *et al.*, 2018). In an experiment where *ex vivo* liver was cultured in the presence of Wnt-3A, embryonic cultures were proliferating with minimal apoptosis. The study showed that Wnt enhances stem cell proliferation, which supports hepatocyte transdifferentiation and promotes biliary survival (Hussain *et al.*, 2004). Gentile and Garcovich (2019) reported that stem cells had been used in therapies supporting hair regrowth and contributing to hair follicular regeneration. They discovered that the Wnt pathway is one of the key factors stimulating hair growth through cellular proliferation and suppression of apoptotic cues. It seems that activation of the Wnt pathway is a promising instrument to support stem cell renewal, tissue homeostasis and repair, as well as preventing aging. Nevertheless, safety issues should also be considered, as stem cells rely on various interactions and crosstalk of different pathways; therefore, Wnt activation may lead to dysregulation of mechanisms that keep stem cells inactive, especially since Wnt

overactivation can lead to cancer. Ultimately, Wnt activators are still in the early stages of research, and more data are needed. Additionally, Wnt antagonists can be applied to promote stem cell expansion *in vivo* as well as *ex vivo* (Bonnet *et al.*, 2021).

In summary, recent findings provide an optimistic perspective for the future of regenerative medicine, as one day, knowledge about the Wnt transduction pathway might contribute to the application of human tissue regeneration. The rapid development of sophisticated tools and increasing understanding of the Wnt pathway provide promising opportunities. Despite the challenges, we believe it is just a matter of time before therapies using the Wnt/ $\beta$ -catenin pathway will be applied. Further investigation of the Wnt pathway's role in regeneration is necessary.

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#### Conflicts of interest

The authors declare no competing interests.

#### Ethical approval

This review was performed using previously published results, without additional experimentation involving human participants or animals.

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