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Molecular Characterization of Foot Regeneration in *Hydra oligactis*

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## Abstract

The freshwater cnidarian *Hydra* possess remarkable regeneration abilities, being capable of whole-body regeneration. The simple anatomy of a *Hydra* polyp is comprised by one axis with a head at one extreme and a foot at the other, connected by a body column. While *Hydra vulgaris* can regenerate its head and foot when bisected, the closely-related species *Hydra oligactis* demonstrates a notable foot regeneration defect. This difference provides an opportunity for comparative research to uncover the molecular processes involved in foot regeneration. The focus of this thesis is to compare the injury response mechanisms between these two *Hydra* species to shed light on the mechanisms of foot regeneration. I used RNA *in situ* hybridization to identify activated bZIP TFs during early wound response, which showed differences in the expression of *cre3/1*. Moreover, I analyzed the expression of Wnt signaling genes through RNA *in situ* hybridization and RNA-seq, in regenerating head and foot tissue uncovering differences in the timing of transcriptional activation. In addition, I performed interspecies lateral grafting between *H. oligactis* and *H. vulgaris* and confirmed that the organizer signal is conserved in both species. Importantly, I tested the impact of Alsterpaullone, a Wnt signaling agonist on foot regeneration in *H. oligactis*, and found that ALP treatment promotes foot regeneration. Furthermore, RNA-seq analysis of ALP treated *H. oligactis* points at the relevance of specific Wnt genes, underscoring the crucial role of the Wnt signaling pathway in foot regeneration in *Hydra*.

## Zusammenfassung

Das Süßwasser-Nesseltier *Hydra* verfügt über bemerkenswerte Regenerationsfähigkeiten und ist in der Lage, den gesamten Körper zu regenerieren. Die einfache Anatomie eines *Hydra*-Polypen besteht aus einer Achse mit einem Kopf an einem Ende und einem Fuß am anderen Ende, die durch eine Körpersäule verbunden sind. Während *Hydra vulgaris* ihren Kopf und Fuß regenerieren kann, wenn sie halbiert wird, weist die eng verwandte Art *Hydra oligactis* einen bemerkenswerten Fußregenerationsdefekt auf. Dieser Unterschied bietet die Möglichkeit für vergleichende Forschung, um die molekularen Prozesse aufzudecken, die an der Fußregeneration beteiligt sind. Der Schwerpunkt dieser Arbeit liegt auf dem Vergleich der Verletzungsreaktionsmechanismen zwischen diesen beiden *Hydra*-Arten, um Aufschluss über die Mechanismen der Fußregeneration zu geben. Ich habe RNA *in situ* Hybridisierung verwendet, um aktivierte bZIP-TFs während der frühen Wundreaktion zu identifizieren, die Unterschiede in der Expression von *cre3/1* zeigten. Darüber hinaus analysierte ich die Expression von Wnt-Signalgenen durch RNA-*in situ* Hybridisierung und RNA-Seq in regenerierendem Kopf- und Fußgewebe und deckte Unterschiede im Zeitpunkt der Transkriptionsaktivierung auf. Darüber hinaus habe ich eine laterale Interspezies transplantation zwischen *H. oligactis* und *H. vulgaris* durchgeführt und bestätigt, dass das Organisatorsignal in beiden Arten konserviert ist. Wichtig ist, dass ich den Einfluss von Alsterpaullon, einem Wnt-Signalagonisten, auf die Fußregeneration bei *H. oligactis* getestet habe und festgestellt habe, dass die ALP-Behandlung die Fußregeneration fördert. Darüber hinaus weist die RNA-Seq-Analyse von ALP-behandeltem *H. oligactis* auf die Relevanz spezifischer Wnt-Gene hin und unterstreicht die entscheidende Rolle des Wnt-Signalwegs bei der Fußregeneration in *Hydra*.

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# 1. INTRODUCTION

*Hydra* is a genus of small freshwater Cnidarians that possess a simple anatomy that consists of a head and a foot linked by a central body column. The species *Hydra vulgaris* is recognized for its impressive regenerative abilities. The regenerative potential of this species ranges from the ability to grow back their entire body from tissue fragments containing just 300 epithelial cells (Shimizu et al., 1993) to actual de novo patterning of a whole *Hydra* even when the polyps are dissociated into a solution of individual cells (Technau et al., 2000; Technau & Holstein, 1992). When *H. vulgaris* is bisected by cutting it at the mid-section of the body column, both halves can regenerate a whole animal, with the top half growing a new foot and the bottom half growing a new head (H. R. Bode, 2003). Conversely, the closely-related species *H. oligactis*, when bisected, the lower half regenerates a head, but the upper half exhibits a very low rate of foot regeneration compared to *H. vulgaris* (S. A. H. Hoffmeister, 1991). Head regeneration in *H. vulgaris* has been the object of much study in the past, however, little is known about the pathways that regulate the formation of the foot in response to injury. The differences between these two species of *Hydra* presents an opportunity for conducting comparative studies to uncover the molecular mechanisms of foot regeneration. In this thesis, I specifically aimed to identify the molecular differences in response to injury between *H. oligactis* and *H. vulgaris*, to elucidate the mechanisms of foot regeneration.

This chapter will provide an introduction to the study by first discussing the background and context, followed by the research problem, the research aims and questions, and finally the significance and limitations.

## 1.1 General overview of *Hydra* regeneration

The process of regeneration in animals has been typically classified as either epimorphic or morphallactic. Epimorphic regeneration occurs when animals produce a specialized tissue that contains a large number of undifferentiated cells, called the blastema, this tissue is responsible for the regeneration of the missing organ or appendage. In contrast to epimorphic regeneration, in morphallaxis, the regrowth takes place without any cell proliferation by rearranging cells at the site of injury (Agata et al., 2007a; Sunderland, 2010). *Hydra* has been regarded as a prototype for

morphallactic regeneration due to findings indicating that cell proliferation is not essential for *Hydra's* regenerative process (M. P. Bode & Bode, 1984; Campbell, 1976; CLARKSON & WOLPERT, 1967; Cummings & Bode, 1984; Gierer, 1977; Graf & Gierer, 1980; Hicklin & Wolpert, 1973; Marcum & Campbell, 1978; PARK et al., 1970; M. P. Bode & Bode, 1984; Gierer, 1977; Graf & Gierer, 1980). A key aspect of *Hydra's* regeneration is that the body column has the capacity to differentiate into more specialized structures like the head and foot (Martínez & Bridge, 2012; Reddy, Gungi, & Unni, 2019).

There are three main stages to *Hydra's* regeneration process, these are: 1) injury response, 2) pre-patterning, and 3) differentiation (J. Cazet et al., 2021a; H. O. Petersen et al., 2015). When *Hydra* is mechanically damaged, the first stage involves wound closing. Also, some of the genes expressed during this stage may trigger the regeneration process. Following this initial phase there is pole-specific gene expression, thus this stage is considered pre-patterning, and it is characterized by early formation of the regenerating tissue. Finally, during the last stage, the development of the missing structures is observed (H. O. Petersen et al., 2015; Tursch et al., 2022a). After an injury, when the mesoglea pulls away from the wound location (Shimizu et al., 2002), and the injury site is sealed by endo- and ectodermal cells, the initial response involves extensive phosphorylation and dephosphorylation processes (H. O. Petersen et al., 2015). During this phase, various signaling pathways including components of the MAPK pathway and Wnt signaling among others are activated, initiating cellular processes such as apoptosis, proliferation, and differentiation (Gufler et al., 2018a; Hobmayer et al., 2000; H. O. Petersen et al., 2015). In short, amputation triggers a generic injury response that includes regulatory mechanisms required to ultimately trigger the activation of structure-specific patterning pathways. However, how the general injury response resolves into structure-specific responses are not well understood.

## **1.2 Regenerative capacity in *Hydra oligactis***

Oral regeneration in *Hydra* has been well-investigated, however aboral regeneration is less well understood. *Hydra oligactis* species exhibits a significantly reduced ability to regenerate its foot, as documented almost a century ago in Tripp (1928) and Weimer (1928) studies. In 1991,

Hoffmeister found that a likely cause for diminished foot regeneration capabilities in *H. oligactis* lies in a difference in the gradient of head associated molecules through the body column, which may leave little space for a foot morphogenetic zone (S. A. H. Hoffmeister, 1991). However, the molecular identity of the foot organizer and critical players in foot regeneration remains unclear.

### **1.3 Head organizer in *Hydra***

*Hydra*'s patterning is controlled by a Wnt signaling center, which functions as the organizer during gastrulation (Holstein, 2022). The adult hypostome expresses Wnt ligands such as *wnt1*, -3, -7, -9/10a, -9/10c, -11, and -16. These ligands are also present during the formation of a new head organizer in head regeneration and bud formation (Lengfeld et al., 2009a). Research on the head organizer identity has put forward the role of certain factors such as *wnt3* that meet the criteria to act as a secreted organizer that specifically contributes to the induction and formation of the primary body axis, which in turn may affect the early formation of the foot organizer (Hobmayer et al., 2000; Lengfeld et al., 2009b; Technau et al., 2000). High expression of *wnt3*, TCF (the downstream transcriptional activator of Wnt signaling), and beta-catenin is co-localized in the hypostome of *Hydra*, and through transplantation experiments, it has been shown that they have the ability to induce a secondary body axis (Broun et al., 2005a; Broun & Bode, 2002; Browne, 1909; Holstein, 2022; Lengfeld et al., 2009b). Furthermore, a study on *wnt3* promoter uncovered a reciprocal relationship between  $\beta$ -Catenin and *wnt3*, facilitated by the existence of TCF-sites in the *wnt3* promoter. Conversely, *wnt3* contributes to the stabilization of  $\beta$ -Catenin (Nakamura et al., 2011). The TCF-binding sites found in the *wnt3* promoter enable an autocatalytic self-amplification mechanism for *wnt3*/ $\beta$ -Catenin, which is essential for preserving the head organizer (Holstein et al., 2003; Nakamura et al., 2011). These molecular feedback mechanisms closely resemble the self-enhancement of an "activator" proposed by Gierer and Meinhardt in their non-linear reaction-diffusion model, which involves autocatalytic short-range interactions (Gierer & Meinhardt, 1972; Meinhardt, 1982; Meinhardt & Gierer, 2000). The model revolves around a short-range autocatalytic substance called the activator, which controls the generation of its long-range antagonist, the inhibitor. The activation of Wnt/ $\beta$ -Catenin signaling is mostly in line

with the model's expectations for the activator, but the specific nature of the long-range inhibitor is still unknown.

#### **1.4 Structure and regeneration of the aboral side in *Hydra oligactis***

The foot in *Hydra* is a specialized structure (Dübel et al., 1987) consisting of mucous-secreting basal disc cells, which are characterized by the presence of peroxidase activity (Hoffmeister-Ullerich et al., 2002). The foot regeneration process in *Hydra* involves the differentiation of body column cells in the peduncle region into basal disc cells, which rapidly occurs upon removal of the basal tissue. This differentiation process is influenced by the location of the tissue within the peduncle, with cells in the lower peduncle showing a higher commitment to becoming basal disc cells (Kobayakawa & Kodama, 2002). The success rate of regeneration has been measured in relation to the location of the cut site. The foot regeneration ability of *H. oligactis* was assessed by amputating them at varying distances from the head, specifically at 3/4, 1/2, and 1/4 of the body length. Foot regrowth success rates were 100% within 96 hours post-amputation, 30% within 96 hours post-amputation, and 20% within 96 hours post-amputation, respectively. These findings indicate that in comparison to *H. vulgaris*, *H. oligactis* exhibits a significantly steeper gradient in foot regrowth potential (S. A. H. Hoffmeister, 1991).

The research on the regeneration ability of *Hydra* mainly focused on head regeneration, to the point that in 2009 Chera et al., reported that after injury, apoptotic cells kickstart the development of the head and they connected Wnt signaling specifically to the apoptotic cells located on the apical side (Chera et al., 2009). Furthermore, the researchers found a profound asymmetry in the activation of apoptosis and Wnt expression between both oral and aboral wound sites (Galliot, 2013). However, recent data indicates that apoptosis and the initial activation of Wnt/ $\beta$ -Catenin are actually similar in both head and foot regenerates in *Hydra vulgaris*. In *Hydra*, during regeneration the MAPK and Wnt signaling pathways are universally up-regulated, irrespective of position, and there is initially detectable injury-induced Wnt expression in both head and foot regenerating tissues (J. Cazet et al., 2021a; Gufler et al., 2018a; Tursch et al., 2022a). During the initial stage of foot regeneration, a temporary activation of Wnt signaling is necessary to enhance aboral patterning. However, as foot regeneration progresses, a specific

set of genes associated with the head region must be suppressed (J. Cazet et al., 2021a). In the early stages of aboral regeneration, there is an increase in a BMP signaling ligand and a potential BMP inhibitor, indicating the involvement of BMP signaling in the foot organizer. Maintaining and regenerating *Hydra* anatomy may require a strong interaction between Wnt/ $\beta$ -catenin at the oral end and BMP signaling at the aboral end as Wenger and colleagues performed RNA-seq of regenerating *Hydra* and found that there is an interaction between the Wnt/ $\beta$ -catenin and BMP pathways in the regeneration of the head and foot in *H. vulgaris* (Wenger et al., 2019.).

### **1.5 Modulation of head and foot regeneration in *Hydra***

How can the regeneration of the aboral side in *Hydra* be suppressed through treatment or manipulation? Could the principles for the lack of foot regeneration in *H. oligactis* be similar to those when foot regeneration is extrinsically blocked in *H. vulgaris*? A recent study showed that prolonged exposure to H<sub>2</sub>O<sub>2</sub> inhibits both head and foot regeneration, due to extensive DNA damage and downregulation of genes crucial for regeneration. More specifically, when bisected *Hydra* were exposed to H<sub>2</sub>O<sub>2</sub> for a long time, they showed a significant downregulation of genes responsible for activating the Wnt pathway, which are essential for head regeneration. This downregulation seemed to occur due to  $\beta$ -catenin failed to be both stabilized and transiently localized in the nucleus. Likewise, genes involved in foot regeneration also underwent downregulation when exposed to H<sub>2</sub>O<sub>2</sub> for an extended period (Haval et al., 2020). Multiple studies have demonstrated that suppressing genes involved in Wnt signaling activation, crucial for regeneration, inhibits both head and foot regeneration. In the upcoming section, I will briefly outline some key discoveries from these research studies including treatment with b-catenin inhibitor, iCRT14, UV-induced inactivation of the Wnt pathway, *sp5* silencing, astacin knockdown, TSP and SETD8 inhibition impact on regeneration in *Hydra*.

In 2018 Gufler observed that inhibition of Wnt activity through a b-catenin inhibitor, iCRT14, resulted in blocked head and foot regeneration. In this work they found that treating early regenerates with iCRT14 disrupted the expression of genes with a role in position-specific patterning programs, specifically genes associated with the head identity of *Hydra* (Gufler et al., 2018a).

In another study, it was demonstrated that UV-induced inactivation of the Wnt pathway, due to excess hydrogen peroxide exposure, inhibited both head and foot regeneration (Krishnapati et al., 2016).

In addition to multiple external influences that all commonly influence Wnt activity, such as iCRT treatment, excessive H<sub>2</sub>O<sub>2</sub>, and UV exposure, which alter head and foot regeneration, there are internal modulators involved with Wnt genes that influence head and foot regeneration. In 2018, Mattias Vogg demonstrated that the expression of *sp5* transcription factor is positively modulated by Wnt/b-catenin signaling in *Hydra* at both the head and foot, however its expression becomes head-specific in later stages of regeneration. They found that the knockdown of *sp5* results in a multiheaded phenotype in *Hydra* suggesting that *sp5* functions as a transcriptional repressor of *wnt3*, but the authors did not report or assess the effect of *sp5* knockdown on foot regeneration (Vogg et al., 2019a).

In addition, Berenica Ziegler<sup>2021</sup> presented another instance of Wnt signaling impact on patterning through the activity of astacin-7. Recently, it has been revealed that the activity of Wnt is managed at the protein level by Astacin proteolysis activity (Ziegler et al., 2021). *Hydra* astacin-7 (HAS-7) is a proteinase from the astacin family, with a role in maintaining a single head organizer by breaking down *wnt3*. Knocking down *has-7* by siRNA halts *wnt3* breakdown in the head tissue and leads to a double axis phenotype, which can be reversed by simultaneous *wnt3* knockdown. They reported that a mathematical model and experimental promoter analysis indicated that there is an indirect regulation of HAS-7 by beta-Catenin.

In addition to HAS-7, another factor secreted by *Hydra* that has recently been found to have Wnt modulating functions is Thrombospondin (TSP). Thrombospondins (TSPs) are glycoproteins with multiple domains that play a role in maintaining tissue homeostasis and restructuring. In *Hydra*, TSP is a significant component of the mesoglea, and its expression corresponds to the dynamics of  $\beta$ -Catenin during head regeneration. TSP functions as a negative regulator of organizer formation, but the mechanism by which TSP inhibits Wnt signaling has yet to be examined (Lommel et al., 2018).

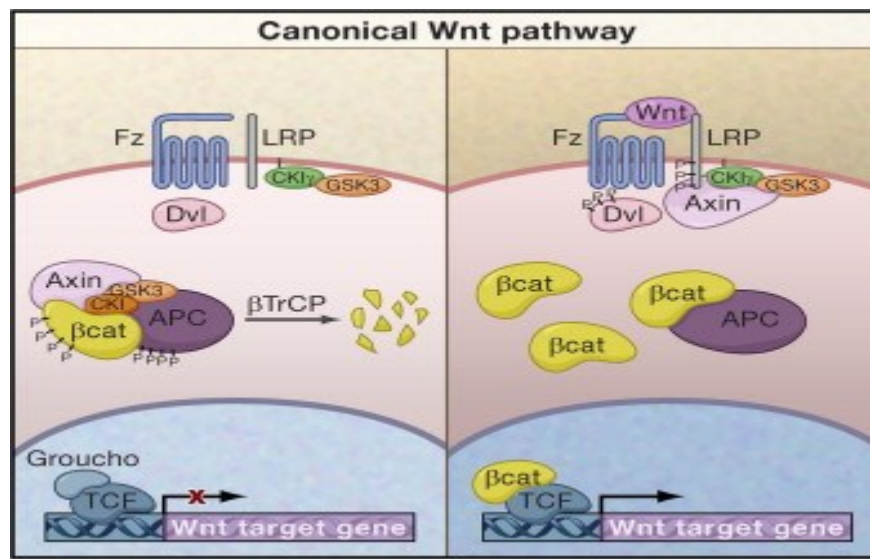
Recently, an enzyme named SETD8 has been discovered that plays a crucial role in Wnt-induced regeneration, with the Wnt pathway governing the activity of both this enzyme and the modification of its target (Gungi et al., 2023). SETD8 serves as a monomethyltransferase enzyme that regulates cellular functions through its activity in posttranslational modifications (PTMs) (Deribe et al., 2010; Gungi et al., 2023). In their study, Gungi and colleagues discovered that the preserved connection between SETD8 and  $\beta$ -catenin is essential for *Hydra*'s regeneration process, as it adjusts the related gene regulatory system. In *Hydra*, it has been demonstrated that the suppression of SETD8 function prevents the regeneration of head and foot. The expression of SETD8 in *Hydra* increases when the Wnt signaling pathway is activated, and it directly associates with  $\beta$ -catenin in the cell nucleus. SETD8's function is crucial for the activation of genes downstream of the Wnt/ $\beta$ -catenin signaling pathway. The inhibition of SETD8 does not morphologically affect the earliest step of regeneration, wound healing. However, at a molecular level, dysregulation occurs in various Wnt signaling components, apoptosis-related genes, and Jun kinase upon the inhibition of SETD8 (Gungi et al., 2023). The SETD8 might help expand our knowledge of regulatory interactions associated with Wnt signaling in *H. oligactis*.

In summary all these findings emphasize the complex connection between the head organizer, Wnt signaling, and the regeneration of body parts in *Hydra*. Various studies suggest that downregulation of the Wnt pathway and foot-specific genes are strong candidates to hinder foot regeneration in *H. oligactis* (Gufler et al., 2018b; Gungi et al., 2023; Haval et al., 2020; Krishnapati et al., 2016; Lommel et al., 2018; Vogg et al., 2019b; Ziegler et al., 2021). Delving further into the topic of regeneration will come after a brief overview of the Wnt signaling pathway. This is particularly important because Wnt's activity is thought to act as a morphogen, indicating that it is a long-range signal influenced by its concentration (Logan & Nusse, 2004; Zecca et al., 1996).

## **1.6 Canonical Wnt pathway**

The Wnt group of secretive glycolipoproteins participates in regulating gene expression and has a vital function in directing cell growth, cell polarity, cell activity, cell adhesion, and cell fate determination during embryonic growth and tissue stability (Logan & Nusse, 2004). Upon activation of the Wnt receptor, three distinct pathways are believed to be activated: the canonical

Wnt/ $\beta$ -catenin cascade, the noncanonical planar cell polarity (PCP) pathway, and the Wnt/ $\text{Ca}^{2+}$  pathway (Clevers, 2006). One of the most researched Wnt pathways is the canonical Wnt signaling pathway. This pathway works by controlling the level of the transcriptional co-activator  $\beta$ -catenin, which in turn regulates key developmental gene expression programs (MacDonald et al., 2009) (Figure 1).



**Figure 1** (Clevers, 2006): (Left panel) When no ligand binds to Wnt receptor complexes, CK1 and GSK3 $\alpha/\beta$  phosphorylate  $\beta$ -catenin. E3 ubiquitin ligase complex recognizes phosphorylated  $\beta$ -catenin. After ubiquitination,  $\beta$ -catenin is quickly targeted for destruction by the proteasome. In the nucleus, Groucho binding to TCF (T cell factor) suppresses the transcription of Wnt target genes. (Right panel) Upon Wnt binding to Wnt receptor complexes, the Frizzled (Fz)/LRP coreceptor complex triggers the activation of the canonical signaling pathway. Fz binds with Dsh, a cytoplasmic protein that operates upstream of  $\beta$ -catenin and the kinase GSK3 $\beta$ . Wnt signaling has an impact on the phosphorylation of Dishevelled (Dsh). It is believed that Wnts initiate the phosphorylation of LRP by GSK3 $\beta$  and casein kinase I- $\gamma$  (CK1 $\gamma$ ), which governs the recruitment of Axin. The displacement of Axin from the destruction complex due to its recruitment leads to the stabilization of  $\beta$ -catenin. Within the nucleus,  $\beta$ -catenin displaces Groucho from Tcf/Lef in order to support the transcription of Wnt target genes (Clevers, 2006).

The activation of Wnt signaling occurs when Wnt proteins bind to their dimeric cell surface receptors, which are made up of frizzled proteins with seven transmembrane segments and LRP5/6 (Bhanot et al., 1996; Wehrli et al., 2000). After binding to their receptors, the cytoplasmic protein dishevelled (Dvl) gets recruited, phosphorylated, and activated (Wallingford & Habas, 2005). Activated Dvl inactivates the destruction complex. The destruction complex is made up of

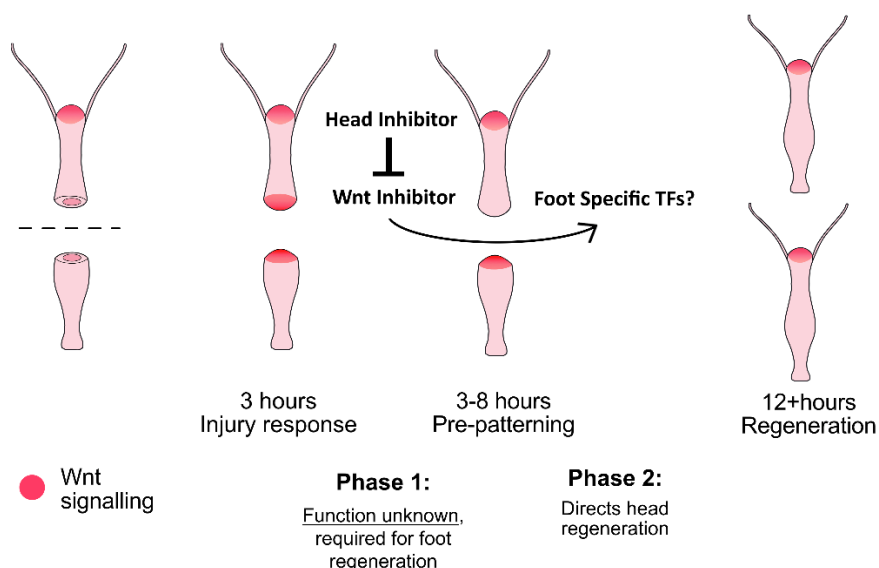
the tumor suppressor protein Axin, which serves as the foundation of this complex by directly engaging with all the other components— $\beta$ -catenin, the tumor suppressor protein APC, and the two kinase families (CK1 $\alpha$ , - $\delta$ , - $\epsilon$  and GSK3 $\alpha$  and - $\beta$  [discussed in (Price, 2006)]). Dvl activation causes GSK-3 $\beta$  to dissociate from Axin, resulting in the inhibition of GSK-3 $\beta$  (Wallingford & Habas, 2005). Then, the inactivation of the "destruction complex" hinders the phosphorylation and degradation of  $\beta$ -catenin. Stabilized nonphosphorylated  $\beta$ -catenin accumulates and moves to the nucleus, binding to the LEF/TCF (lymphoid enhancer factor/T cell factor) transcription factors, which leads to activation of various target genes (Behrens et al., 1996; Molenaar et al., 1996; van de Wetering et al., 1997).

## **1.7 Injury effect**

In 1982, MacWilliams and colleagues demonstrated that two key factors seem crucial in the head regeneration process. One factor involves the levels of head activation and head inhibition in the tissue (Hicklin & Wolpert, 1973) and another is the injury effect (MacWilliams, 1982). The determination of head regeneration occurs when the head inhibition level (S value) drops below a particular threshold value compared to the head activation level (P value) (Hicklin & Wolpert, 1973). MacWilliams in 1983 proposed the "injury effect" model based on transplantation observations. According to his model, injury can trigger an increase in activator in normal *Hydra*. However, the model did not contain any details about the molecular properties of the activating or inhibiting factors (MacWilliams, 1983). The Juliano lab put forward a new model in the context of *Hydra* regeneration by adding the molecular mechanism (J. Cazet et al., 2021a), which significantly enhanced the previous model that only explained the theoretical components and their role in the injury-induced activation of head patterning (Gierer & Meinhardt, 1972; Kobatake & Sugiyama, 1989; MacWilliams, 1983; Newman, 1974). Juliano lab suggested that the injury effect documented in previous research was the result of injury-induced Wnt signaling pathway (Figure 2).

## 1.8 Injury induced Wnt signaling activity in *Hydra*

Following a mechanical injury, signals for the injury are produced and sent to create patterning cues with accurate positional memory. Previous evidence from the Juliano lab suggests that immediately after injury, Wnt signaling is transcriptionally activated by bZIP transcription factors (TFs) such as *jun*, *fos*, and *CREB*. Injury-induced MAPK activation triggers body axis formation in *Hydra* through default Wnt signaling, with sustained activation of Wnt signaling in the head regenerate and transient activation in the presumptive foot tissue (J. Cazet et al., 2021a; Tursch et al., 2022a). The main point in Juliano lab's model is that regardless of tissue context, injury triggers a Wnt signaling cascade by directly increasing the expression of the Wnt ligands *wnt9/10c* and *wnt3* through injury-responsive basic leucine zipper transcription factors. Wnt signaling then inhibits injury-responsive transcription factors. Although Wnt signaling can self-amplify, this only happens during head regeneration. The self-amplification is prevented during foot regeneration by an unknown inhibitor originating from pre-existing oral tissue.



**Figure2:** The proposed model by Cazet et al illustrates the activity of canonical Wnt signaling after mid-gastric bisection in *Hydra*. The activation of canonical Wnt signaling occurs in response to injuries, regardless of the type of injury, and this activation has the potential to create a new head organizer through a self-enhancing feedback process. In the context of foot regeneration, inhibitory signals from existing head tissue block the Wnt feedback loop and prevent ectopic head formation. The activation of transcription factors specific to the foot may be primarily triggered by the suppression of Wnt signaling, or at least connected to the Wnt signaling inhibition.

In 2022 Tursch et al., presented a model to explain how ROS and Calcium release and MAPKs activity are related to Wnt signaling. It was proposed that injury result in the release of ROS and calcium, which subsequently impact the phosphorylation status of MAPKs. Erk and stress-responsive MAPKs display an antagonistic interaction, maintaining a balance between apoptosis and the expression of *wnt9/10c*, *wnt3* and *wnt7* (Tursch et al., 2022a). Given the importance of injury-induced Wnt gene activation, among the different signaling pathways involved in injury response in *Hydra*, the upcoming sections will focus on dissecting the role of Wnt gene activity pattern during regeneration.

### **1.9 The early activated canonical Wnt signaling components**

In 2009, Lengfeld identified the presence of ten Wnt genes in *H. vulgaris*, in addition to the previously described *wnt3*, but the specific functions of these different Wnt genes remain a matter of speculation. It was proposed that certain Wnt genes, like *wnt1*, -3, -7, -11, and possibly *wnt2*, were connected to the head organizer. The experiment involving the transplantation of *wnt3*-soaked tissue, which could induce secondary axes in host polyps, indicated there is a crucial role to *wnt3* in the formation of organizers (Lengfeld et al., 2009b). Lately, subsequent activation of Wnt genes during head regeneration suggested distinct functions for each Wnt gene. Transcriptomic analysis and qRT-PCR conducted in separate studies revealed that *wnt9/10c* occupies a prominent position in the Wnt activation cascade, with *wnt3* being subsequently expressed following *wnt9/10c* (J. Cazet et al., 2021a; Tursch et al., 2022a). In a study from 2021, Cazet and colleagues demonstrated that various key components of the canonical Wnt signaling pathway, such as *wnt9/10c*, *wnt3*, *wnt7*, *wntless*, *dishevelled*, *b-catenin*, and *sp5*, are all upregulated during the early symmetric phase of regeneration.

### **1.10 Morphogenesis following transcriptional symmetry break**

The transcriptional response at the start of head and foot regeneration is initially symmetrical (Gufler et al., 2018b; Wenger et al., 2019.). This response involves the activation of canonical Wnt signaling components like *wnt9/10c*, *wnt3*, *wntless*, *dishevelled*, *b-catenin*, and *sp5*, which play a crucial role in relaying injury signals and initiating the activation of other Wnt genes needed for

patterning. However, the expression of genes that encourage the shift to the morphogenesis stage begins only when Wnt expression diminishes in the foot tissue and continues to rise in the head to facilitate head organizer formation and head patterning (J. Cazet et al., 2021b). The process of morphogenesis and cell differentiation involves noticeable changes in restructuring of the mesoglea, and the transformation of epithelial cells into hypostomal cells and tentacle cells at the oral end, and into basal disk cells of the foot at the aboral end (S. Hoffmeister & Schaller, 1985; Philipp et al., 2009a; Technau & Holstein, 1995). Foot specification can be identified by the activation of genes like *anklet* and *CnNK-2*, while Wnt expression is no longer observable during this period (Amimoto et al., 2006; Forman & Javois, 1999; Grens et al., 1996b). In contrast, the development of the head relies heavily on continuous Wnt expression to support the successful shaping of oral structures (J. Cazet et al., 2021b; Hobmayer et al., 2000; Technau & Holstein, 1995; Tursch et al., 2022b).

All in all, this data suggests that the Wnt signaling pathway serves a dual purpose in the regeneration of *Hydra*, participating in both the response to injury and the establishment of oral and aboral patterns (Tursch et al., 2022b). Injury specific Wnt expression might be necessary to grant the tissue competency for regeneration, but the mechanism for that has yet to be discovered. During the subsequent phase, regeneration becomes specific to the positional context, and at this stage, Wnt/ $\beta$ -Catenin is still necessary to guarantee successful head patterning, but it has been observed to be suppressed in the presumptive foot. In this thesis, I will examine the important canonical Wnt signaling components both for symmetrical transcription during early injury response and symmetry break during pre-patterning phase in order to determine whether the lack of responsiveness of the injured aboral tissue observed in *H. oligactis* is dependent upon Wnt activity.

So, to summarize the critical points about Wnt activity during regeneration, certain bZIP TFs play a crucial role in initiating regeneration in the process of early wound response in *Hydra* tissue regeneration. They relay injury signals and trigger the transcriptional activation of Wnt ligands required for arranging the regenerating tissue. Initially, Wnt is activated in a non-specific manner, and later, it is activated specifically according to the position in the pattern formation phase. Activation of the early Wnt genes is driven by MAPK signaling instead of older notion of apoptosis

being in charge. The early Wnt gene activity is carefully coordinated with a stable gradient based on  $\beta$ -Catenin along the primary body axis, which helps to maintain axial polarity and triggers the activation of additional Wnts in the regenerating head (J. Cazet et al., 2021b; Tursch et al., 2022b).

### **1.11 Significance & limitation of the project**

Understanding the regeneration of *Hydra* may seem easy due to its simple body plan, but there are significant gaps in knowledge regarding the dynamics of canonical Wnt signaling genes. In this sense, the specific roles of each Wnt ligand are not known at this time. With numerous Wnt ligands, it remains uncertain whether they have context-specific functions or they may all have additive effects on regeneration. Alternatively, they might have different phase-dependent roles, for instance only some may participate in the symmetry-breaking event that results in the formation of two distinct structures, the head and foot, following the *Hydra*'s bisection. This raises the question of how activation of Wnt signaling results in two distinct regeneration outcomes.

The conserved genetic programs responsible for *Hydra* regeneration offer not only valuable insight into *Hydra*'s exceptional abilities but also offer clues about potential mechanisms found in other regenerative animals (Karin & Clevers, 2016). Studying the reasons behind the inability of the aboral side of *Hydra* to regenerate may provide important insights into the barriers to regenerative capability in organisms with more complex structures and limited regenerative capacity (Birnbaum & Alvarado, 2008). Although there is no consensus on whether regeneration mechanisms are widely conserved throughout evolution, the core network of molecular interactions in *Hydra* may explain some of the mechanisms that drive regeneration in vertebrates (Holstein et al., 2003). For example, Wnt expression following injury was detected in all bilaterian phyla, including ancient acoel, planarian species, and flatworms (Agata et al., 2007b; C. P. Petersen & Reddien, 2009; Srivastava, 2021; Wurtzel et al., 2015) as well as mammals (Liu et al., 2022). Despite the importance of Wnt signaling in regeneration, our knowledge of how Wnt signaling becomes activated after an injury and the precise roles of its various components remains incomplete.

Studying *Hydra* is also insightful in relation to the fact that the Cnidaria clade is the sister taxa to bilaterians, which provides a valuable framework for examining the development of the metazoan

wound response (Laumer et al., 2018). This is supported by the several genomic traits, which align with those of the most recent common ancestor (Holstein, 2022; Philippe et al., 2009).

Their genome contains important components of major signaling pathways, including all significant elements of the Wnt pathways including 11 Wnt genes out 19. This complexity in molecular level which is similar to that found in bilaterians (Chapman et al., 2010; Holstein, 2022) makes findings from regeneration studies in *Hydra* applicable to bilaterians. In brief, given the highly conserved injury-induced activation loop in *Hydra* and the presence of 11 Wnt genes in *Hydra* (Lengfeld et al., 2009a), studying the naturally impaired aboral regeneration in *Hydra* will reveal insights that go beyond the regeneration of its own species.

This study has five main limitations, which I will briefly outline. One of them is the limited time point for RNA-sequencing results. We observed gene expressions based on RNA sequencing at 0-, 3-, 12-, and 48 hours post-amputation. We acknowledge the potential significant peak of expressions between 3 to 12 and 12 to 48 hours post-amputation. Other limitations include being blind to the primary injury signals, calcium and ROS role in transducing injury signals into intracellular cues and being blind to cross-talk of the Wnt signaling pathway with other signaling pathways such as BMP. Also, our research neglected to consider the influence of epigenetic factors that could be significant in determining whether to begin regeneration or repair tissue following an injury. Last but not least, the effect of heterogeneity in our bulk RNA-seq can iron out the peaks of up and down regulations at any of our time points, which we refer to as our reference to interpret the activity of each gene.

## **1.12 Hypothesis and aims of the project**

To identify the early injury bZIP TFs activity at the wound site of bisected *H. oligactis*, I performed an RNA in situ hybridization test. The aim was to compare the pattern and intensity of bZIP TFs activity in *H. oligactis* and *H. vulgaris* and investigate their potential contribution in foot regeneration deficiency in *H. oligactis*. Next, I compared the early activity of Wnt components in the regenerating tips of the head and foot. Based on findings from *H. vulgaris*, I expected the activity of early Wnt-associated genes to be similar at the head and foot regenerating tips. At the

cellular level, I examined the apoptosis activity in *H. oligactis* as one of the early events following injury and compared the timing and pattern with *H. vulgaris*.

The divergence we observed in *H. oligactis* results compared to *H. vulgaris* encouraged us to perform bulk RNA-seq analysis in an extended time course, 0, 3, 12, and 48hpa, to evaluate the expression pattern of selective genes involved in the early injury response and pre-patterning phase. Low and delayed expression patterns of important Wnt-associated genes triggered the question of whether treatment with Alsterpaullone could improve foot regeneration in *H. oligactis*. Previous studies show that treating *Hydras* with the glycogen synthase kinase-3 $\beta$  inhibitor ALP (Broun et al., 2005b) can cause ectopic activation of the Wnt pathway. This results in an overall increase in nuclear  $\beta$ -Catenin, enabling tissue in the gastric region to form heads and develop multiple ectopic tentacles. Although, this might conflict with our goal of enhancing foot regeneration, the fact that throughout this process, there is a scattered expression of canonical Wnt ligands, Frizzled receptors (Philipp et al., 2009b), and Wnt downstream targets (Watanabe et al., 2014) hinted that limited ALP treatment may make up for missing messages caused by low wnt expression. Indeed, our ALP treatment results for enhancing foot regeneration in *H. oligactis* were promising enough to perform additional RNA-seq analysis to look for the treatment effect on genes with an essential role in injury response. Also, we performed inter-species lateral grafting to ensure the similarity between the head organizer molecular identity between the two species. We plan to identify key players in foot regeneration deficiency in *H. oligactis* by comparing the activity of specific genes in the two *Hydra* at various stages of regeneration. Our initial inquiry focuses on whether the bZIP TFs are correctly activated in the immediate response to injury post-amputation. Subsequently, we aim to investigate if the injury signal is transmitted to Wnt signaling and foot-specific TFs. Finally, we seek to assess the efficacy of ALP treatment in enhancing foot regeneration.

## 2. Materials and Methods

### 2.1 Gene isolation and Cloning

For probe preparation first I performed RNA isolation from *Hydra* head and foot tissue to detect the expression pattern of the genes of interest. I checked the quality of the isolated RNA by gel electrophoresis and A260/A280 absorbance ratio. RNA with A260/A280 absorbance ratio more than 2 as acceptable quality for pure RNA was used to synthesize complementary DNA (cDNA) by reverse transcription. I used this cDNA as a template to amplify a region of the genes of interest. The primers are provided in Supplementary Files-2. The PCR cycle consisted of 98 °C for 30s, 33 cycles of 98 °C for 10 s, 60 °C for 30 s, 72 °C for 1 min, and finally 72 °C for 5 min and the PCR product was checked by 2% agarose gel. The PCR product from the last step ligated into the pGem vector using the pGEM-T easy vector kit (PROMEGA ®). To calculate the mass of insert required for a ligation reaction with an insert: vector ratio of 3:1, we considered the vector length of 3015bp and the vector mass of 50ng. The insert DNA lengths were as follows: Jun 700bp, Fos 609bp, Cre3l1 926bp, Wnt3 599bp, and Wnt9/10c 763bp. The ligated plasmids transformed into TOP10 competent cells. A purified plasmid extraction sent for sequencing to confirm the identity of the cloned sequence. Confirmed plasmids used to amplify the region of interest by PCR. This product was the template to obtain a DIG-labeled RNA probe using the DIG RNA labeling kit (SP6/T7) (ROCHE ®).

### 2.2 *Hydra* culture

All experiments were conducted using *Hydra vulgaris*, AEP strain and *Hydra oligactis*, Innsbruck female 12 strain (provided by Prof. Bert Hobmeyer), they were grown in *Hydra* medium at 18°C with 12 hours light cycle. The animals were fed five times per week with freshly hatched larvae of *Artemia* nauplii for 4-8 hours and then were transferred into fresh *Hydra* medium. Animals were starved for at least 48 hours prior to all experiments detailed in this thesis.

## 2.3 Whole Mount *in situ* Hybridization

Whole Mount *in situ* Hybridization was conducted on *Hydra oligactis* Innsbruck female 12 strain, based on a previously described protocol (Siebert et al., 2019). Animals fixed with 4% PFA of different post-injury stages (0, 3, 12, and 48hours after amputation) are rehydrated by sequential steps into an aqueous buffer. Afterwards, animals were digested with Proteinase-K (0.08 mg/ml in PBT). This reaction was stopped with Glycine (4mg/ml in PBT). Animals were washed in PBT followed by Triethanolamine + Acetic acid and re-fixed by 4% formaldehyde to prevent dissociation of the animal. The animals were washed with PBT, followed by 2XSSC and by incubation in pre-hybridization solution (Hyb solution + sheared salmon sperm). Then Animals were hybridized with the DIG-labeled probes in Hyb solution at 65 °C for 48 hours. Probes were washed away and incubated with Anti-DIG FAB fragments. Next antibodies were washed away to proceed with colorimetric detection of antibody binding with NTB/BCIP. The reaction stopped when a purple staining was visible in the animals.

## 2.4 Imaging

After performing colorimetric RNA in situ hybridization to identify the location of the expressed gene of interest, we utilized a Leica DM5000B microscope (camera Leica DFC310FX) or Leica M165C digital stereomicroscope (camera MC170HD).

## 2.5 Apoptosis

In order to determine the timing for the onset of apoptosis at the injury site in *H. oligactis*, I bisected 15 animals for each time point and stained them with 1.6µM acridine orange at different time points: 0.5, 1, 3, 4, and 4.5 hours post amputation (hpa).

To visualize the apoptotic cells in different regeneration time points, we stained bisected animals using acridine orange based on a modified protocol for acridine orange staining (Cikala et al., 1999). First, we collected 15 *H. oligactis* and 15 *H. vulgaris* in separate batches for each regeneration time point, 0.5, 1, 3, 4, and 4.5hpa. We bisected them and incubated head and foot regenerating mid body bisected animals in 1 ml 1.6 mM acridine orange diluted in *Hydra* medium

for 2 min in the dark. Straight away, they washed twice in *Hydra* medium and analyzed them microscopically.

## **2.6 Alsterpaullone treatment**

To investigate the effect of ALP treatment on foot regeneration, we bisected batches of (*n* animals) *H. oligactis* with either 2.5, 5 or 10  $\mu$ M ALP for 3 or 6 hours after amputation. All concentrations of ALP were dissolved in DMSO, the final concentration of DMSO in *Hydra* media was adjusted to 0.05% DMSO. Control animals were kept in DMSO 0.05% during the indicated times after injury. Foot regeneration was monitored daily for 6 days.

## **2.7 Lateral Grafting of *Hydra***

The head organizer, located near the hypostome, plays a central role in axial patterning and regeneration in *Hydra* and I used lateral grafting of tentacles in *Hydra* to compare the head organizer's properties in *H. vulgaris* and *H. oligactis*. To perform lateral grafting of the *Hydra* head organizer tissue I used polyps of *H. oligactis* Innsbruck strain and watermelon (WM; GFP ectoderm and RFP endoderm). Their size and shape made them distinguishable. I first amputated the donor polyp's head just below the tentacle ring. I then bisected the head to create a slice containing a tentacle. Next, I made an incision in the side of the body column of the host *Hydra*. I inserted the donor tissue piece into the incision wound in the host polyp and left the grafted polyp undisturbed for about 30 minutes to allow the tissue to partially fuse together. Afterwards, I transferred them to an 18°C incubator for overnight recovery.

## **2.8 RNA extraction to prepare RNA-seq library**

We extracted RNA from regenerating tissue of treated and non-treated *H. oligactis* at 0, 3, 12 and 48 hours after amputation (hpa). The *H. oligactis* (strain Innsbruck female12) used in these experiments were fed five times a week. Before midbody bisection, 40 whole, bud-free *H. oligactis* were starved for two days. We bisected them in batches of 10 to lower inconsistency in amputation and collection times. A second foot regeneration time course for *H. oligactis* was obtained after treatment with 5  $\mu$ M ALP for 3 hpa treatment performed according to the protocol

as was described above. The bisected animals continued their regeneration for 0, 3, 12, and 48 hpa. Approximately 1/3 of the total length of regenerating tips were cut from the head and foot regenerating tips and frozen in Trizol at -80°C. RNA extractions were done on four biological replicates of 40 animals each. RNA was extracted according to the Trizol/chloroform protocol and DNA contamination was eliminated by DNase I and finally a cleanup was performed using an RNA Clean and Concentrator kit. Purified RNA samples were sent to Novogene in Sacramento, CA to be sequenced.

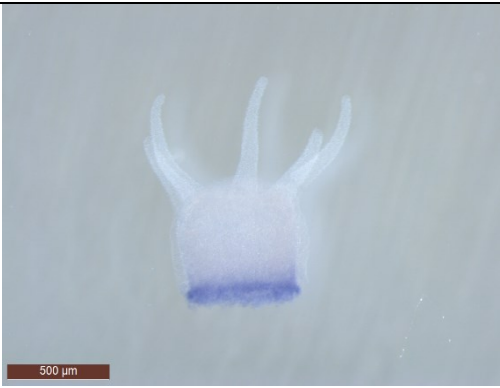
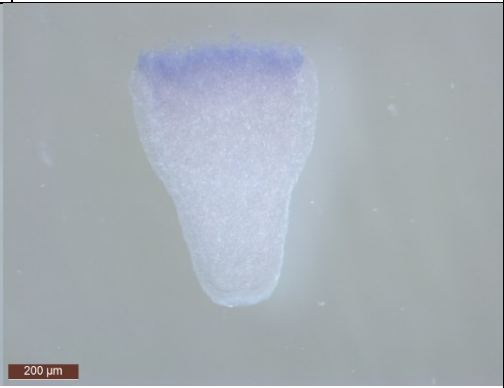
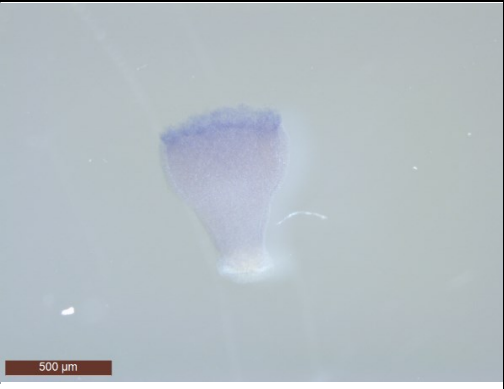
## **2.9 RNA-seq data processing and differential gene expression analysis**

We utilized Trimmomatic to eliminate sequencing adapters and low-quality base calls from the raw sequencing data. RSEM was used to map reads to a reference transcriptome, and to calculate counts per million. A reference transcriptome for *Hydra oligactis* was downloaded from HydraAtlas.unige.ch (Wenger et al., 2019.). BUSCO was used to evaluate redundancy of the reference transcriptome, this analysis indicated there is 26.3% duplicated genes, hence EviGene pipeline for reference gene reduction was used. After running EviGene the level of duplication was 2.4%, this reduced transcriptome was used as a reference for mapping. Counts per million directly from RSEM were normalized and analyzed for differentially expressing genes using edgeR. We used quasi-likelihood tests of count data fitted to a negative binomial generalized log-linear model with an FDR cutoff of 1e-2 to find differentially expressed genes. In our RNA-seq analysis, we utilized annotations from a recent study that provided comprehensive resources, including genome assemblies for *Hydra oligactis* and the AEP strain of *Hydra vulgaris*, among others (J. F. Cazet et al., 2023). All scripts used are provided in Supplementary Files-1.

### 3. Results

#### 3.1 basic leucine zipper (bZIP) transcription factors expression in bisected in *H. oligactis*

A published work from the Juliano Lab suggest that following a mid-gastric bisection in *Hydra*, injury-responsive basic leucine zipper (bZIP) transcription factors transcriptionally activate Wnt pathway components as part of a general injury response. In *H. vulgaris*, several transcription factors (TFs) containing bZIP domains, such as CREB, jun, and fos are upregulated transiently by injury at 3 hours post amputation (hpa). Subsequently, several genes from the Wnt signaling pathway are transcriptionally activated at both sides of a bisected wound during early injury response, this expression up-regulation becomes specific to head regeneration starting as early as 8 hpa. In brief, the current model for the transcriptional activation of the Wnt signaling pathway involves transcriptional activation by general injury-response bZIP transcription factors (TFs) jun, fos, and CREB (J. Cazet et al., 2021b). To understand why foot regeneration fails in *H. oligactis*, I sought to characterize the timing and localization of expression of some of the key genes involved in the general injury response in *H. vulgaris*. For this purpose, I bisected 20 *H. oligactis* polyps and evaluated early injury response at 3 hours post amputation (hpa) by using RNA *in situ* hybridization of TFs fos, jun and cre3l1. The expression patterns of jun and fos, clearly show that these TFs are transcriptionally activated by 3hpa at both sides of the wound (Figure 3 A-B). However, RNA *in situ* hybridization of cre3l1 shows a uniformly-distributed punctate pattern across the body column, characteristic of interstitial stem cells, it also showed no obvious increase in the expression in the injury adjacent tissue (Figure3 C).

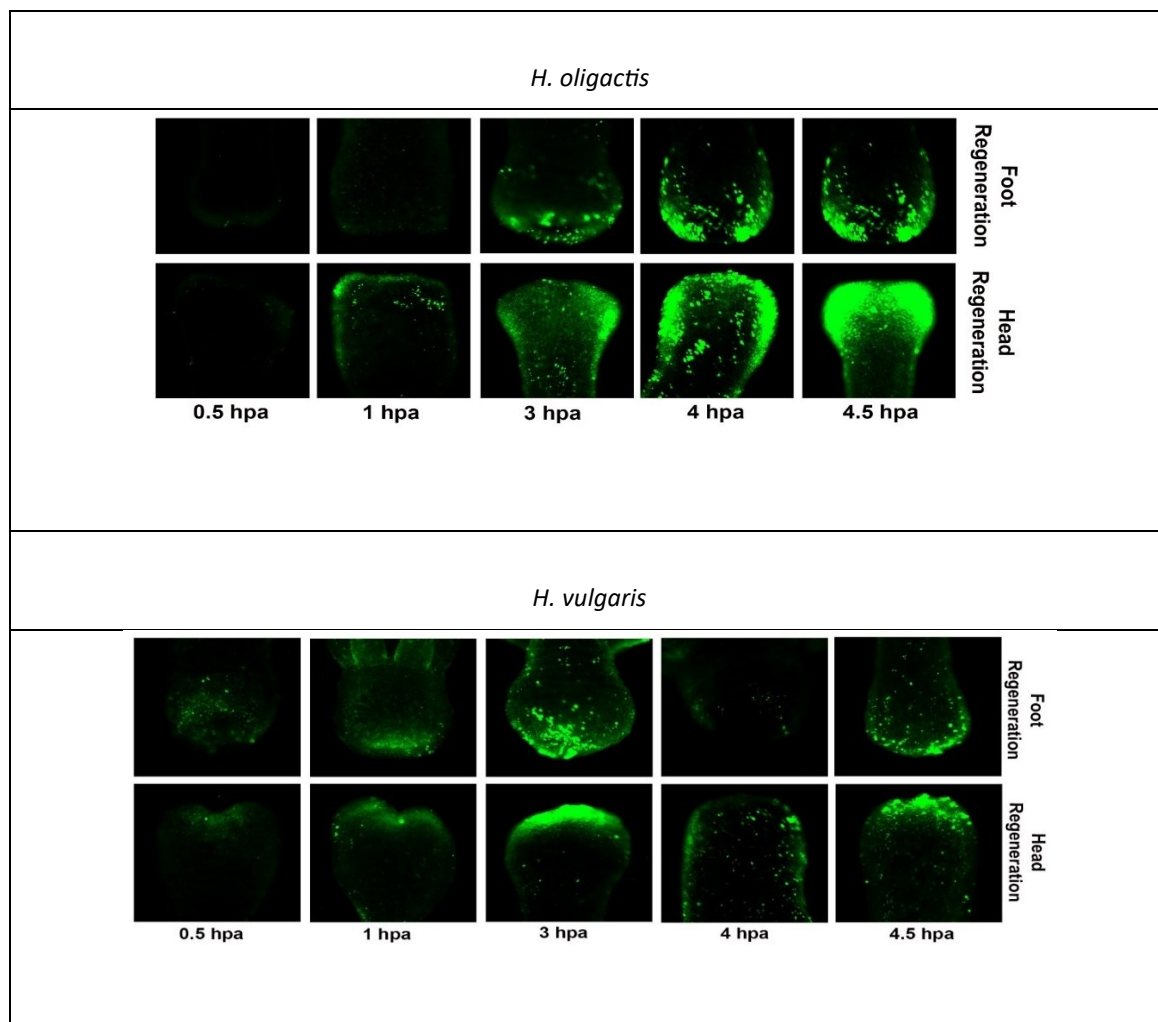
|                  | RNA <i>in situ</i> Hybridization of bZIP TFs in bisected <i>H. oligactis</i> (3hpa) |                                                                                      |
|------------------|-------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|
|                  | Foot regenerating tip                                                               | Head regenerating tip                                                                |
| A. <i>Fos</i>    |    |    |
| B. <i>Jun</i>    |   |   |
| C. <i>cre3l1</i> |  |  |

**Figure3:** RNA *in situ* hybridization of *jun*, *fos* and *cre3l1* in *H. oligactis*. (The images are modified.) A: Expression pattern of *fos* in head and foot regenerating tips at 3hpa. B: Expression pattern of *jun* in head and foot regenerating tips at 3hpa. C: Expression pattern of *cre3l1* in head and foot regenerating tips at 3hpa.

### 3.2 Activation of apoptosis as a part of a generic wound response

In *H. vulgaris*, it has been shown that apoptosis is elicited readily in the tissue adjacent to an injury on both sides of the wound. To identify differences in the physiology of the general injury response between both *Hydra* species, I characterized the distribution of apoptotic cells at head and foot regenerating tips by acridine orange staining. In order to determine the timing for the

onset of apoptosis at the injury site in *H. oligactis*, I bisected 15 animals for each time point and stained them with 1.6 $\mu$ M acridine orange at different time points: 0.5, 1, 3, 4, and 4.5 hours post amputation (hpa). I observed a limited amount of apoptosis at 0.5, and 1. At 3h I observed large variation in the amount of apoptosis between samples, some of samples showed very high level of apoptosis while some of them were not very different than bisected samples at 0.5, and 1h. However, by 4 and 4.5h apoptosis was consistently activated at both head and foot regenerating tips (Figure4).



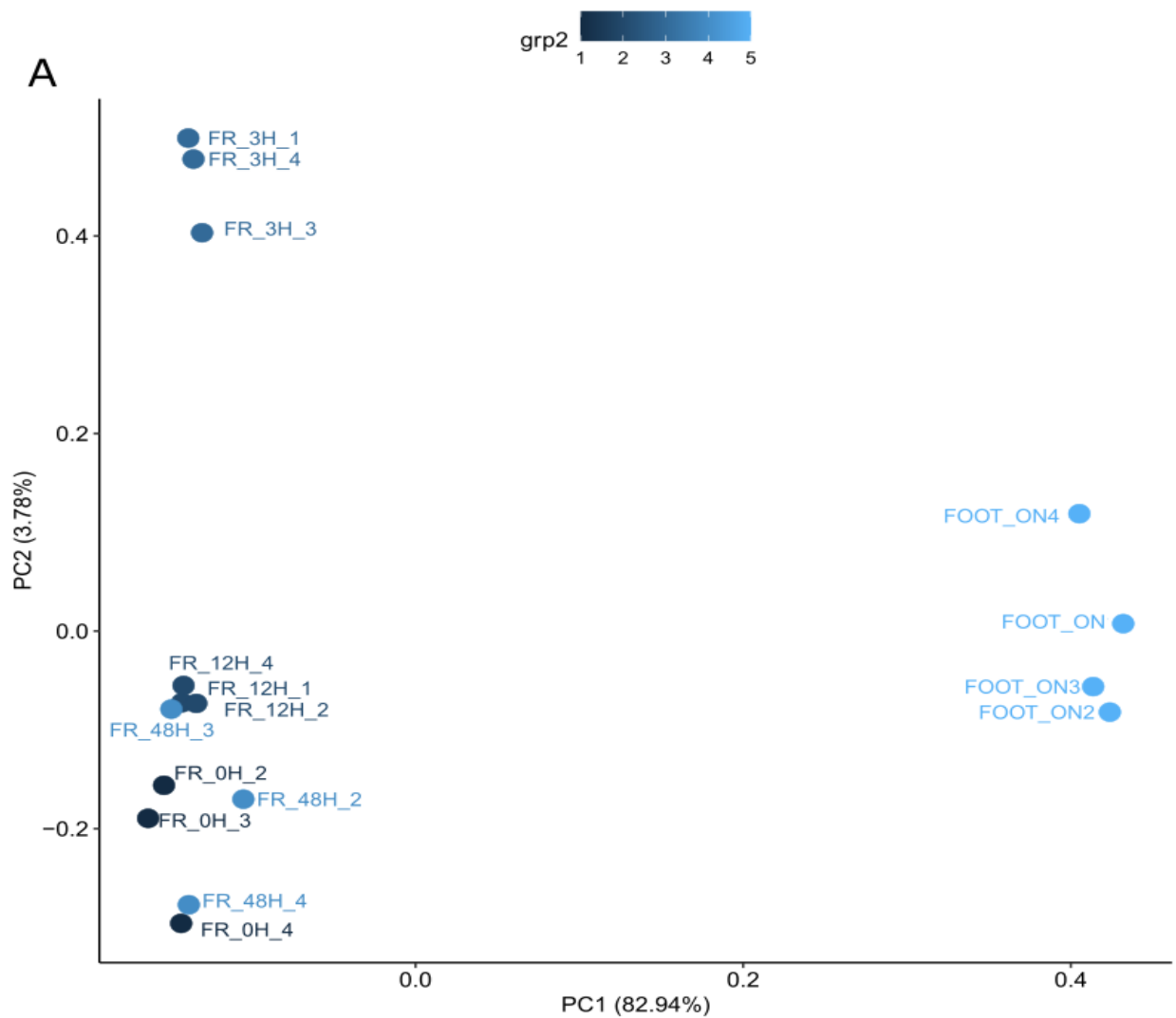
**Figure4:** Apoptotic cells in bisected *H. oligactis* and *H. vulgaris* during head and foot regeneration time course stained with acridine orange.

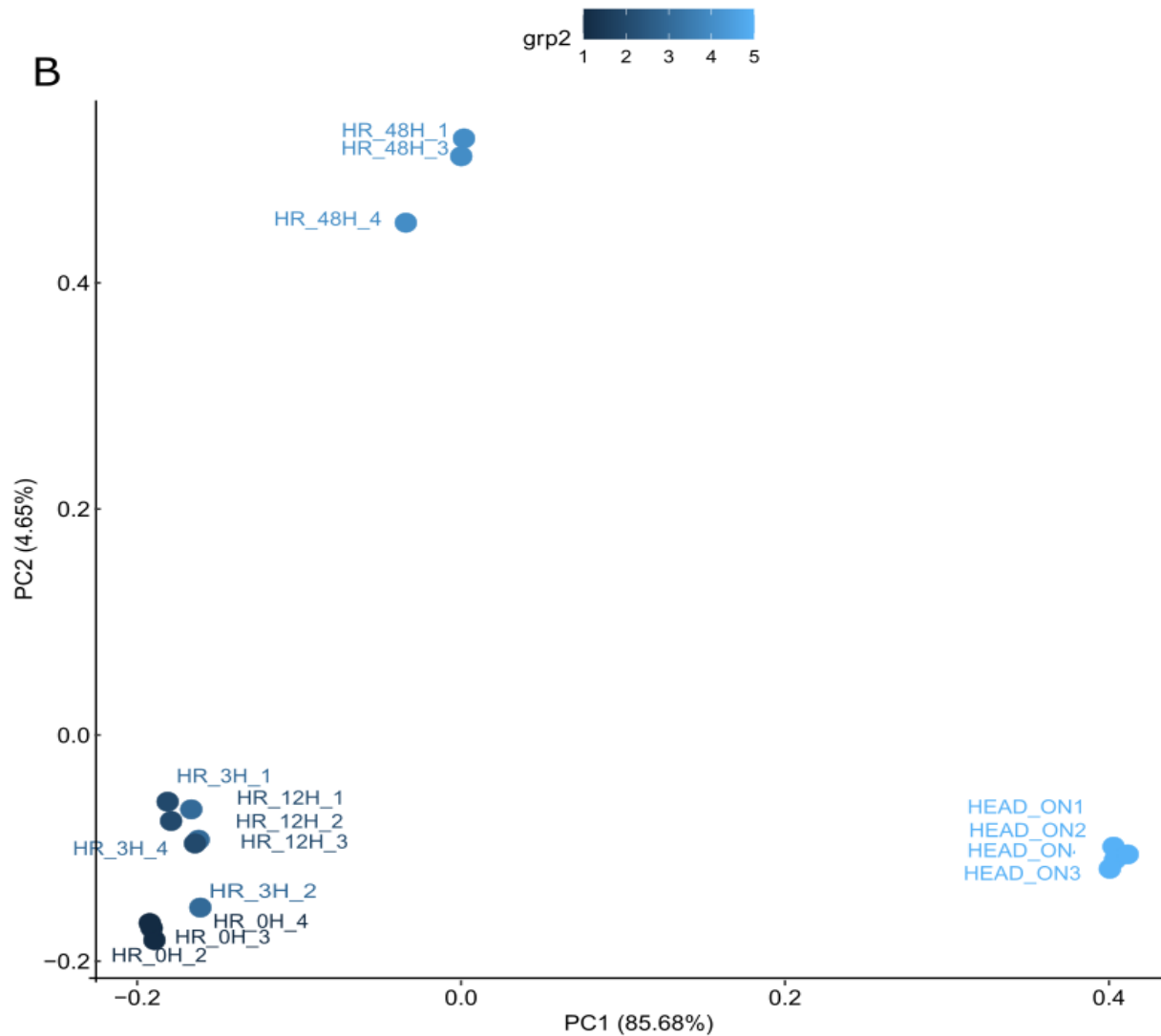
### 3.3 Transcriptional response following mid-body bisection in *H. oligactis*

#### 3.3.1 Global PCA analysis of transcriptional activity in head and foot regenerating tips

A previous study by (J. Cazet et al., 2021b) showed that in *H. vulgaris* the transcriptional response following mid-body bisection in *Hydra* demonstrates similar patterns in head and foot regenerating tips at 3 hpa, but becomes tissue-specific by 8 hpa. To understand the molecular mechanisms that differ between *H. vulgaris* and *H. oligactis*, I sought to characterize a regeneration time course in *H. oligactis* using RNA-seq. We made RNA-seq libraries at 0, 3, 12, and 48 hours following mid-gastric bisection on both sides of the wound. I performed RNA-seq data analysis to identify differences in the transcriptional events after injury in *H. oligactis* and *H. vulgaris*. By applying Principal Component Analysis (PCA), I observed that the first principal component (PC1) accounts for the maximum variance, and in our foot regenerating PCA (Figure 5A), PC1 covers 82.94% of the dataset. It represents the differences in gene expression between the body column and the foot tissue, as it is evident in foot tissue differentiating from the other samples in far advanced areas along PC1. The foot tissue samples are scattered in the middle of PC2, so the genes pulling the PC2 dimension do not affect it much; they are just at the basal level. All the genes pulling the foot sample toward the end of PC1 are probably foot-specific. The PC2 represents 3.78% of the variance in the dataset and seems to set the 3 hpa tissue apart from the other samples because only the 3 hpa samples are mainly elongated along PC2. The 3hpa samples are going through early general injury response activities, so they are in a very different state of gene expression compared to 0, 12, and 48hpa, which each represents a different phase of regeneration. Foot regenerating tissue at 3h separates from the rest of the time points in PC2; the genes responsible for the variation in this component represent mainly general injury response gene expression rather than foot-specific gene expression. Foot regenerating tissue at 0, 12, and 48 hpa group together with minimum gene expression changes in either of the two principal components. This analysis showed that at the 12h and 48h time points, foot regenerating tissue does not follow foot trajectories along principal component 1. We were expecting at 12hpa to still have the influence of the injury response on gene expression while approaching the pre-patterning phase, while what we observe is that unexpectedly, it goes back

to almost the level of 0h. In conclusion, interestingly, at 12hpa, like 48hpa, the foot regenerating tissue gene expression profile again becomes similar to 0hpa. This result suggests that at 12h, gene expression profiles resemble that in the body column. Considering that *H. oligactis* is deficient in foot regeneration, it is expected that the 48hpa expression profile resembles that of the body column (Figure5A).





**Figure5:** A) The Principal Component Analysis (PCA) of foot and head regeneration tissue during regeneration time course of 0, 3, 12, and 48hpa. B) The Principal Component Analysis (PCA) of head and head regeneration tissue during regeneration time course of 0, 3, 12, and 48hpa.

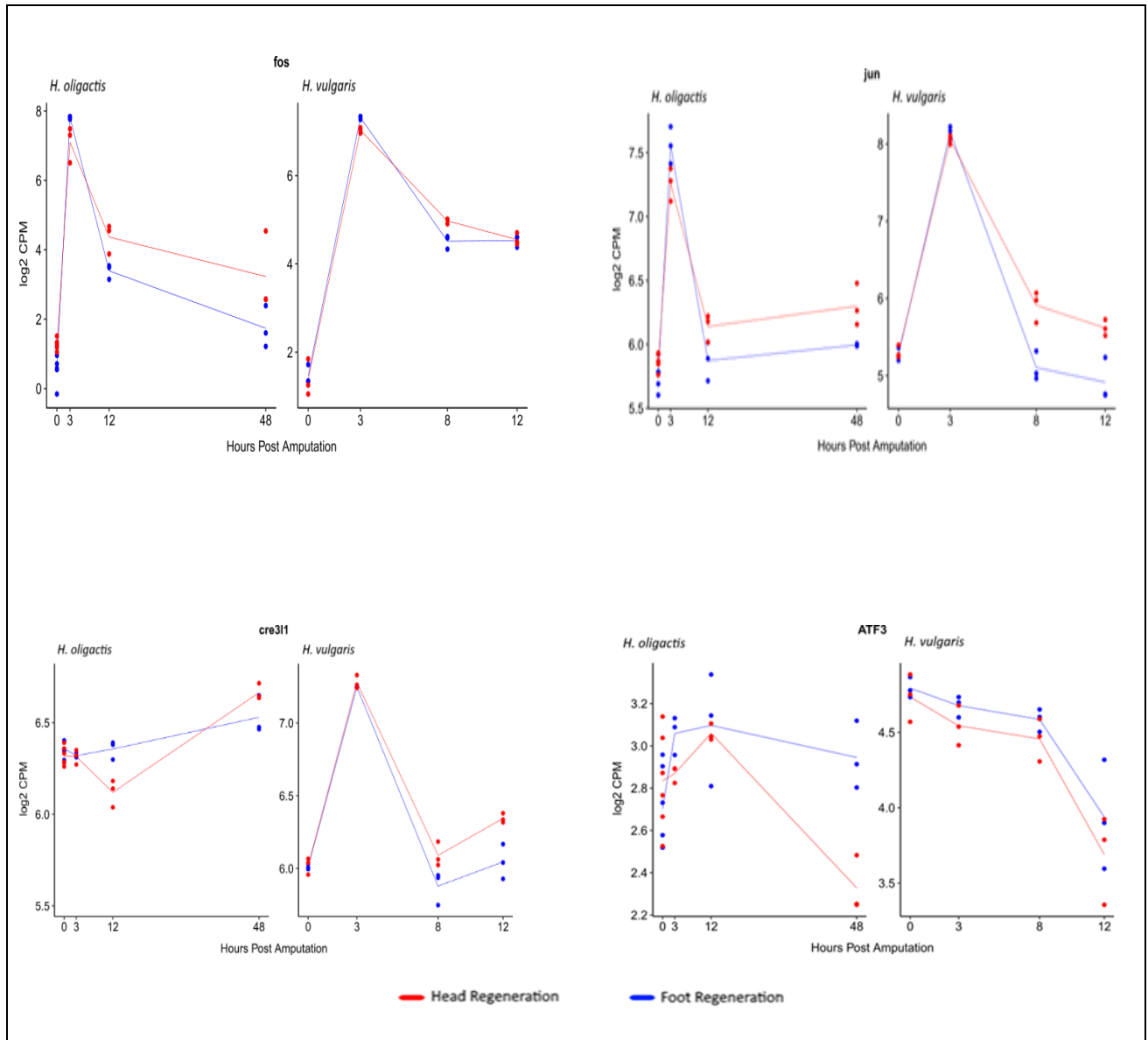
The PCA analysis of head regenerating tissue (Figure5B) shows that 0, 3, and 12h are grouped together with zero to minimum head-specific gene activity. In PCA analysis of head regenerating tissue, unlike the foot regenerating PCA plot, the PC2 does not represent injury response genes, as 48hpa tissue is advancing in both PC1 and PC2 directions. At 48h, the head regenerating tissue is becoming head so it is advancing along PC1 but also changing in another direction, alongside PC2. It is important to note that the head regeneration timeline differs between *H. oligactis* and *H. vulgaris*. It is apparent that in 48hpa, the regenerating tissues are becoming heads. However,

still they are not very close to the head profile because they start showing tentacles by 72hpa, whereas in *H. vulgaris*, that happens earlier. For example, some *H. vulgaris* at 24hpa have already established a tentacle. By 48hpa, they all have developed their heads.

### **3.3.2 RNA expression of bZIP TFs during head and foot regeneration in *H. oligactis***

I looked into the expression of *jun*, *fos*, *cre3l1* and another member of bZIP TFs, ATF3 (Vinson et al., 1993) as general injury-response bZIP transcription factors (TFs) (J. Cazet et al., 2021b; Tursch et al., 2022a) and compared their expression pattern with the in-situ results. RNA *in situ* results correlated well with our RNA-seq data analysis and previous RNA-seq data for *H. vulgaris*, which clearly showed an upregulation of *fos* and *jun* at 3hpa for both types of regenerating tissues and by 12hpa the gene activity was subsided to almost the level of expression at 0hpa (Figure6).

CREB-like TF is also a bZIP TF, which putatively bind the cAMP response element sequence (CRE). RNA-seq data analysis for *cre3l1* in *H. vulgaris* shows a peak of expression at 3hpa and then at 8hpa is back to the expression level of before the injury. While in *H. oligactis*, there is no strong up-regulation throughout the time course of regeneration at neither the head regenerating tip nor the foot regenerating tip (Figure6). In *H. oligactis*, ATF3, a member of the bZIP transcription factor family, displays a consistent increase in expression from 0 to 12hpa, unlike *H. vulgaris* (Figure6). Contrary to other ATF family members, ATF3 primarily functions as a transcriptional repressor. It is suggested that the distinct ATF binding to Wnt promoters may regulate their expression during the early stages of regeneration in *Hydra*. This regulation prevents premature up-regulation of patterning wnts like *wnt3* and *wnt7* during initial regeneration (Tursch et al., 2022a). As a result, the contrasting expression patterns of this transcription factor in *H. oligactis* and *H. vulgaris* could potentially impact patterning events during foot regeneration in *H. oligactis*.



**Figure6:** RNA expression plots depicting average normalized RNA-seq read counts for bZIP transcription factors (TFs) *fos*, *jun*, *cre3l1* and *ATF3* in log<sub>2</sub> counts per million (log<sub>2</sub>CPM) during head and foot regeneration.

### 3.4 RNA expression of apoptosis marker genes in *H. oligactis* during regeneration

To confirm the results obtained with the acridine orange assay I looked for apoptosis marker genes in the RNA-seq dataset for *H. oligactis* regeneration time-course and compared their expression pattern with those seen in *H. vulgaris*. In *H. vulgaris* apoptotic markers including *caspase-3*, *bcl-like-6*, and *p53* are symmetrically upregulated at 3hpa during head and foot regeneration (J. Cazet et al., 2021b). Among them, *bcl-like6* is upregulated in head and foot regenerating tips at 3hpa in *H. oligactis*, however, it is not symmetrical and the upregulation at

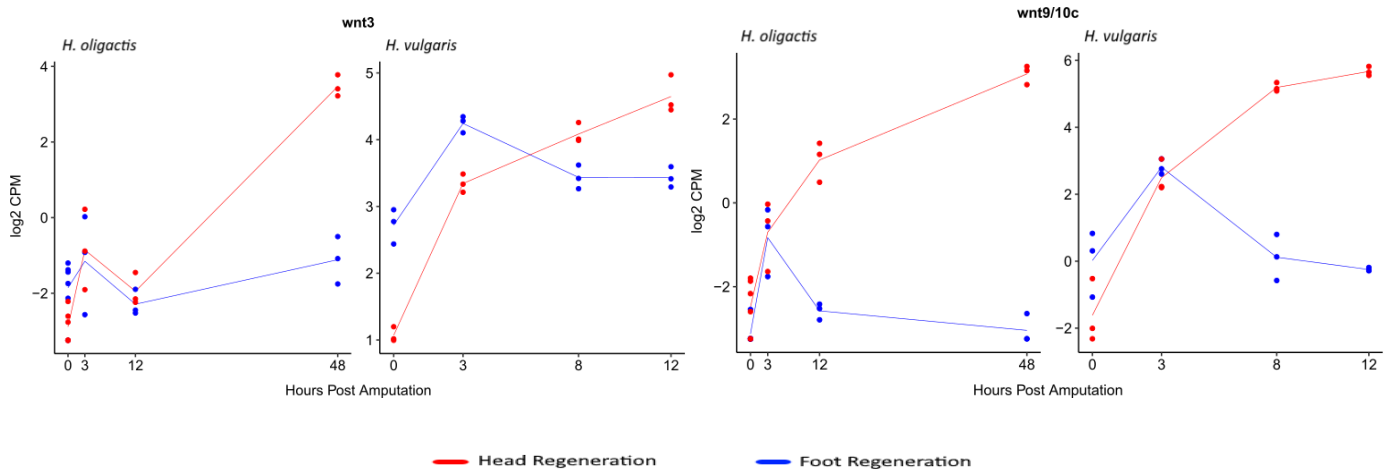
3h in foot is higher than the upregulation in head regenerating tip. P53 is symmetrically upregulated in head and foot regenerating tips at 3hpa in *H. vulgaris*. However, in *H. oligactis* upregulation of *p53* at the foot regenerating tip is not observed until 12hpa. *Caspase-3* which is upregulated at head and foot regenerating tips in *H. vulgaris*, did not show a significant upregulation in *H. oligactis*. In addition, other apoptosis-associated genes such as caspase-8, prolyl endopeptidase FAP(g14219), and Endoribonuclease ZC3H12A(g5828) were symmetrically upregulated in head and foot regenerating tips in *H. oligactis* while there was not any upregulation in head and foot regenerating tips in *H. vulgaris*. In summary, it seems like although apoptosis occurs in *H. oligactis* at both head and foot regenerating tips, it is not clear how the apoptosis process is regulated through genes expression alone in both *Hydra* (Supplementary Figure 1).

### **3.5 RNA expression of canonical Wnt signaling components during *H. oligactis* regeneration**

Multiple studies revealed that beside *wnt3* and *wnt9/10c*, several canonical Wnt signaling genes, including *wnt7*, *wntless*, *dishevelled*, *b-catenin*, and *sp5* are upregulated during the early injury-response phase of regeneration at both the oral and aboral wounds in *H. vulgaris* (J. Cazet et al., 2021b; H. O. Petersen et al., 2015; Tursch et al., 2022a). These genes were shown to be highly-expressed in head tissue in *H. vulgaris* according to a structural enrichment analysis using a single cell expression atlas of this species(J. F. Cazet et al., 2023). To identify any differences in the transcriptional activation of the Wnt signaling pathway between *H. oligactis* and *H. vulgaris*, I compared the pattern of expression of the previously identified Wnt genes in both *Hydra* species from their RNA-seq datasets.

Based on our RNA-seq data for *H. oligactis*, we observed that the expression patterns of *wnt3* during the regeneration process differed between the foot regenerating tip and the head regenerating tissue (Figure7). In the foot regenerating tip, there was an initial increase in expression but at a low scale, while in the head regenerating tissue, there was upregulation at 3 hpa, which is similar to *H. vulgaris*. However, the expression level at 12hpa was lower than at 3hpa, resulting in a different expression pattern in head regenerating tissue compared to *H.*

*vulgaris*. This lack of upregulation at 12hpa for *wnt3* in *H. oligactis* may be due to a delay. Overall, it appears that *wnt3* expression in *H. oligactis* is low and delayed. The expression pattern of *wnt9/10c* is similar to that of *H. vulgaris*, with the only difference being a lower expression level (Figure7).



**Figure7:** RNA expression plots depicting average normalized RNA-seq read counts for *wnt3* and *wnt9/10c* in log<sub>2</sub> counts per million (log<sub>2</sub>CPM) during head and foot regeneration.

I conducted RNA *in situ* Hybridization for *wnt3* and *wnt9/10c* (Figure8). My results for *H. oligactis* show that the signal for *wnt9/10c* was very faint and there was no visible *wnt3* in the time-course of regeneration, while they are both visibly expressed at the hypostome of intact *H. oligactis*. These results also were agreeable with the low expression seen in the RNA-seq data. The expression patterns for *wnt3* and *wnt9/10c* in *H. oligactis* are similar to those observed in *H. vulgaris*. However, the expression level for both wnt ligands is very low, in the case of *wnt3* this may be the reason it was not detectable through RNA *in situ* hybridization.

| RNA <i>in situ</i> Hybridization of <i>wnt3</i> and <i>wnt9/10c</i> in <i>H. oligactis</i> |                                                                                    |                                                                                     |
|--------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|
|                                                                                            | Foot regenerating tip                                                              | Head regenerating tip                                                               |
| <i>wnt9/10c</i>                                                                            |   |   |
| <i>wnt3</i>                                                                                |  |  |

**Figure8:** In situ hybridization of *wnt3* and *wnt9/10c* in *H. oligactis* at 3hpa and in intact animals.

Furthermore, I analyzed the expression of *wnt7*, *wntless*, *dishevelled*,  $\beta$ -*catenin*, and *sp5* during the regeneration time course at 0, 3, 12, and 48hpa. In *H. vulgaris*, *wnt7* rises at 3 hpa in foot regenerating tissue and decreased at 12 hpa compared to 3 hpa. In *H. oligactis* foot regenerating tips show a raise at 3 hpa was insignificant and at 12mhpa the expression level was similar to 48 hpa. In head regenerating tissue the expression of this gene is sightly upregulated at 3 hpa, however it drops by 12hpa, only to increase again by 48hpa. However, the comparison between *H. vulgaris* and *H. oligactis* RNA-seq datasets showed that the expression pattern of head specific genes in general is similar in *H. oligactis* but for *wntless* and  $\beta$ -*catenin* the up-regulation observed from 0 hpa to 3 hpa is small in magnitude, in *dishevelled* is low and delayed and in *sp5* is identical to that of *H. vulgaris* (Supplementary Figure 2).

Previous studies in *H. vulgaris* have suggest that TCF is necessary for the shift from the general wound response to head and foot-specific transcription(J. Cazet et al., 2021b). In *H. vulgaris* TCF is involved in upregulation of several wnt components like *sFRP*, *frizzled4/9/10*, *axin*, and TCF

itself in head regenerating tissue, in addition, it is involved in the downregulation of the head-specific genes *brachyury1* and  $\beta$ -*catenin* in foot regenerating tissue (J. Cazet et al., 2021b; Gufler et al., 2018a). To understand how the downstream activation of the Wnt signaling pathway impacts regeneration in *H. oligactis*, I compared the expression pattern of TCF between 0 to 12 hpa in *H. vulgaris* and *H. oligactis*. The foot regenerating tip of *H. oligactis* did not show an increase in expression from 3 hpa to 12 hpa, contrarily to what was observed in *H. vulgaris*. The higher expression at 48 hpa suggests that the upregulation occurred later.

The expression of genes downstream of *TCF* such as *axin* is similar in the foot regenerating tip in both *Hydra* but *sFRP*, *fzd4/9/10* have different expression pattern in *H. oligactis* (Supplementary Figure 2). For *sFRP3* from 0 to 3 hpa, there was no increase in foot regeneration. In foot regeneration from 3 hpa to 12 hpa, we observed a decrease in head regeneration and an increase in foot regeneration. Given the general delay in Wnt activation in *H. oligactis*, a plausible explanation for this difference is that the increase in foot regeneration is actually delayed and it matches the one observed from 0 to 3 hpa in *H. vulgaris*. In *H. vulgaris*, the expression of *fzd4/9/10* at both the head and foot regenerating tips decreases from 0 hpa to 3 hpa, but in *H. oligactis* there was an increase. Additionally, in *H. vulgaris* there was an increase from 3 hpa to 12 hpa, but there was a decrease instead in *H. oligactis*. In addition, in *H. oligactis* head regenerating tip the expression of these genes is lower compared to *H. vulgaris*. In general, while the expression pattern of certain crucial head-specific genes in *H. oligactis* resembles that of *H. vulgaris*, it is possible that the late or diminished upregulation of key genes such as *wnt3*, *wnt7*, and TCF, which affect the expression of downstream genes, has altered the expression of genes responsible for the patterning phase in *H. oligactis*.

One of the crucial genes that plays a significant role in cell fate decisions in the head regenerating tissue is *brachyury1*. In *H. vulgaris*, *brachyury1* is an early head-specific gene. Its importance in foot regeneration is that it is inhibited by TCF in the foot-regenerating tip. In *H. oligactis*, RNA-seq data shows that the *brachyury1* expression pattern from 0 to 12 hpa is not significantly different from *H. vulgaris*, and only the activation is delayed (Supplementary Figure 2).

In general, two key points to note are that the Wnt signaling activated by injury is significantly reduced in *H. oligactis*, and there is a delay in the activation of Wnt genes during regeneration compared to *H. vulgaris*. The divergent expression of certain Wnt signaling genes indicates that it might contribute the lack of foot regeneration in *H. oligactis*.

### 3.6 RNA expression of Wnt regulatory genes during head and foot regeneration

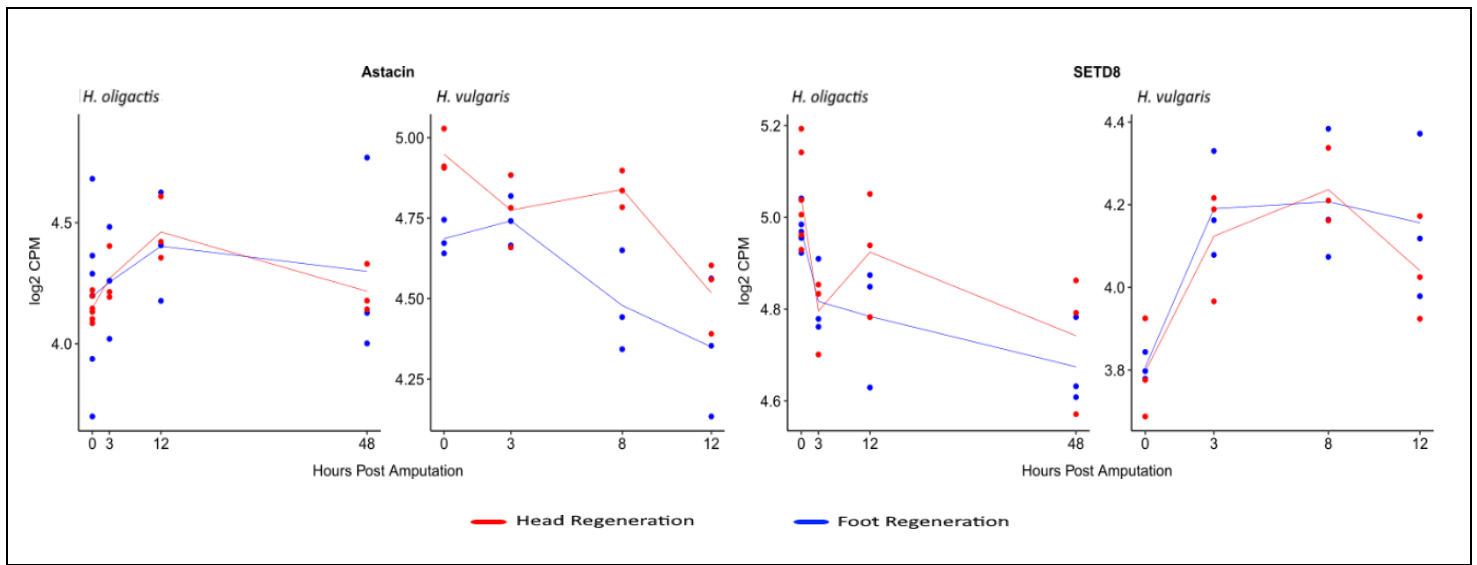
Numerous researches have presented evidence that reducing the activity of genes involved in the Wnt pathway, which is essential for regeneration, hinders both head and foot regrowth. For instance, studies have demonstrated this by inhibiting beta catenin (Gufler et al., 2018a) suppressing *sp5* (Vogg et al., 2019a), knockdown of Astacin with a negative regulatory role in Wnt (Ziegler et al., 2021), and inhibiting SETD8 and knockdown of TSP (Lommel et al., 2018). Alongside *sp5* and *brachyury1*, both of which have Wnt regulatory functions I analyzed the expression profiles of additional members of the Wnt regulatory system, such as *Hydra* astacin protease-7 (HAS-7) discussed in Ziegler et al. (2021), Thrombospondin (TSP) documented by Lommel et al. (2018), and SETD8 researched by Akhila Gungi (2023).

The expression of Astacin(g24277) varies between *H. vulgaris* and *H. oligactis* during the regeneration of their head and foot. In *H. vulgaris*, the expression decreases from 3 hpa to 12 hpa, whereas in *H. oligactis*, it increases in both the head and foot regenerating tips (Figure9). The upregulation of Astacin during the early regeneration time course because of Astacin's ability to inhibit the protein stability of recombinant Wnt3 from *Hydra* (Ziegler et al., 2021) is essential. It may be involved in the impaired foot regeneration in *H. oligactis*.

In the results of our RNA-seq analysis, we identified five genes labeled as Thrombospondin. Of the five genes, only one exhibited elevated expression in foot regenerating tips at 12hpa, resembling *H. vulgaris*. Two genes did not display increased expression at 12hpa, one demonstrated delayed upregulation, and one showed minimal upregulation (Supplementary Files-3). TSP likely serves as a feedback inhibitor of Wnt signaling in *H. vulgaris* (Lommel et al., 2018), so the lack of its upregulation might result from low Wnt expression being unable to activate TSP. So, low expression of TSP may indicate the low Wnt activity impact in *H. oligactis* that influences downstream regulatory genes activity.

In *H. vulgaris*, the expression of SETD8 from 0 to 3hpa in head and foot regenerating tips increases, and in foot regenerating tip stays at the same level of expression at 8hpa and 12hpa. In opposite *H. oligactis*, the expression of *SETD8* decreases from 0 to 3hpa in head and foot regenerating tips, and in the foot, regenerating tips continue to decline from 3 to 12hpa (Figure9). As inhibiting SETD8 activity has been found to abolish head and foot regeneration in *Hydra* (Gungi et al., 2023) downregulation of SETD8 in *H. oligactis* may contribute to foot regeneration deficiency.

These findings underline the complex relationship between the head organizer, Wnt signaling, and the regeneration of different body parts in *Hydra*.



**Figure9:** RNA expression plots depicting average normalized RNA-seq read counts for Astacin and SETD8 in log<sub>2</sub> counts per million (log<sub>2</sub>CPM) during head and foot regeneration in *H. oligactis* and *H. vulgaris*.

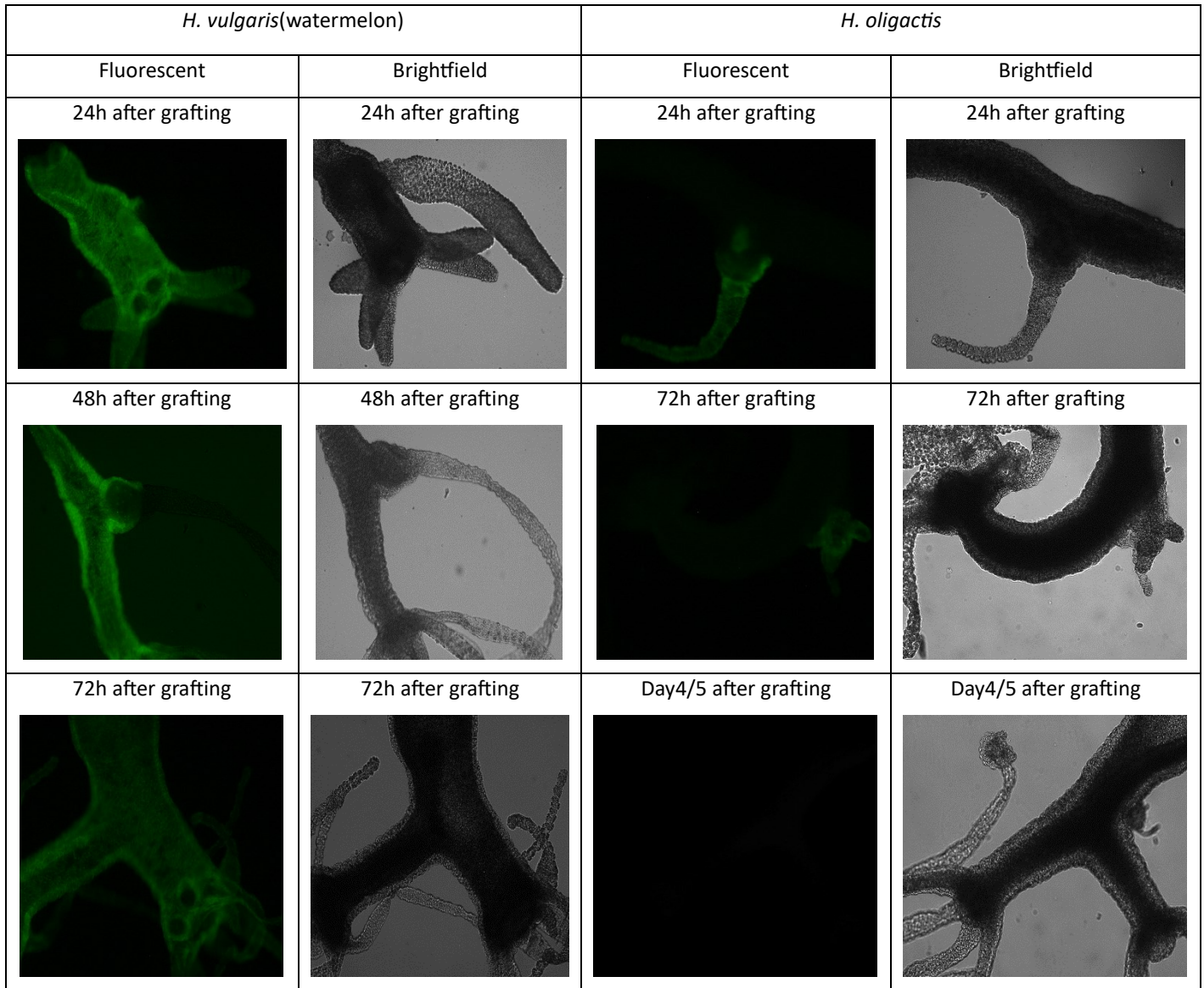
### 3.7 Interspecies lateral grafting of head organizer

Considering the differences in Wnt signaling activation between *H. vulgaris* and *H. oligactis*, an important question arises, do the head organizers in *H. oligactis* and *H. vulgaris* conserve their molecular identity in both species? To answer this, I used a classic *Hydra* experiment where the head organizer of a *Hydra* polyp is transferred to the body column of another otherwise intact *Hydra*. When the grafted tissue contains an active head organizer, the host *Hydra* will develop a lateral head or secondary axis. To test the conservancy of the head organizer signal between *H.*

*oligactis* and *H. vulgaris*, I grafted a tentacle of *H. vulgaris* laterally to mid body section of *H. oligactis* and vice versa (Figure10). A secondary axis was induced in 9.8% of grafted *H. vulgaris* and 20% of *H. oligactis* from the grafted tissue (Table1). These results suggest that there is a high conservation between the Wnt signaling pathway components between the two species. Moreover, unpublished data of intraspecies grafting of head-regenerating tissue performed by the Juliano Lab shows that the formation of the head organizer in *H. oligactis* is delayed compared to *H. vulgaris*. This finding is in agreement with the delayed expression patterns observed for Wnt signaling in *H. oligactis*.

| Result of interspecies grafting between <i>H. oligactis</i> and <i>H. vulgaris</i> |                                      |                            |                             |                          |                          |
|------------------------------------------------------------------------------------|--------------------------------------|----------------------------|-----------------------------|--------------------------|--------------------------|
|                                                                                    | Total number of grafted <i>Hydra</i> | remained grafted in 3-hour | Remained grafted in 24-hour | Rate of abnormality<br>┐ | Secondary axes formation |
| <b><i>H. oligactis</i></b><br>(grafted a tentacle from <i>H. vulgaris</i> )        | 120                                  | 78(65%)                    | 67(55.8%)                   | 5(4%)                    | 24(20%)                  |
| <b><i>H. vulgaris</i></b><br>(grafted a tentacle from <i>H. oligactis</i> )        | 132                                  | 63(47.7%)                  | 57(43.1%)                   | 6(4.5%)                  | 13(9.8%)                 |

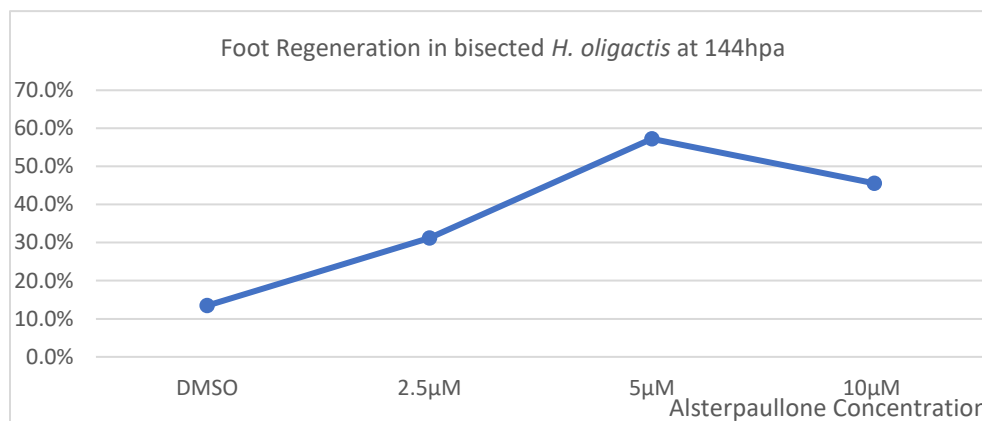
**Table1:** The table represents the quantitative results of interspecies lateral grafting, providing the success rate in grafting progress during 3h, 24h, and 72h for *H. vulgaris* and 3h, 24h, and 4/5 days for *H. oligactis*. Out of 120 *H. oligactis* and 132 *H. vulgaris* grafted, some *Hydra* rejected the grafted tentacle in 3h and 24h after grafting; the numbers provided in the table show the rate of successful grafting. Among the total grafted *Hydra*, 24 *H. oligactis* and 13 *H. vulgaris* formed a second axis from the donated tentacle. Additionally, 5 *H. oligactis* and 6 *H. vulgaris* from the total grafted *Hydra* developed an abnormality in the position of grafting rather than a second axis.



**Figure10:** Interspecies lateral grafting to examine the compatibility of head organizer between the two species of *H. oligactis* and *H. vulgaris*. The images were captured at 24 hours, 48 hours, and 72 hours for *H. vulgaris*, while for *H. oligactis*, the imaging times were 24 hours, 72 hours, and day 4 or 5 due to slower progress. The imaging results for *H. vulgaris* at 24 hours, 48 hours, and 72 hours are displayed in both Fluorescent and brightfield modes on the left panel. On the right panel, the imaging results for *H. oligactis* at 24 hours, 48 hours, and day 4 or 5 are shown in both fluorescent and brightfield modes. Ectopic Wnt activation by Alsterpaullone treatment during regeneration

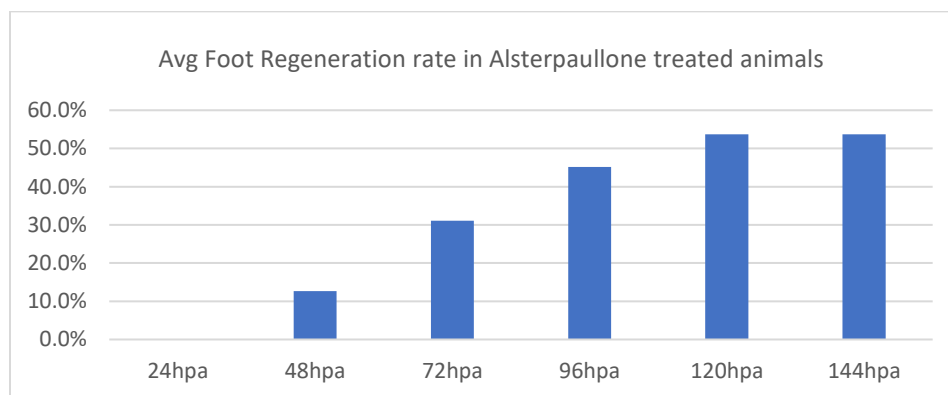
Given that *H. oligactis* displays a considerable delay in the transcriptional activation of several genes in the Wnt signaling pathway compared to *H. vulgaris*, we hypothesized that the lack of injury-induced Wnt signaling is a key factor in the *H. oligactis* foot regeneration defect. To test this hypothesis, I used the Wnt agonist Alsterpaullone to chemically activate Wnt signaling in bisected *H. oligactis* in the aboral injury site and monitored the foot regeneration rate for 144

hours. To determine the optimal concentration and exposure time for foot regeneration with Alsterpaullone treatment, I tested 2.5, 5, and 10  $\mu\text{M}$  concentrations of Alsterpaullone in DMSO for 3 hours of exposure after amputation (Figure11).



**Figure11.** Comparison of foot regeneration rate in Alsterpaullone gradients and DMSO treatment.

The foot regeneration rate in animals treated with DMSO for 3 hours as control animals was 13.5%, similar to untreated animals. The lowest concentration of Alsterpaullone that we checked for its effect on foot regeneration was 2.5 $\mu\text{M}$ , with a 31.2%-foot regeneration outcome. This rate for 5  $\mu\text{M}$ , the following concentration I checked, was 57.2%, four times higher than the control animals. The following concentration I checked was 10 $\mu\text{M}$ , and the foot regeneration percentage dropped to 45.6% in that concentration. In summary, animals treated with 5  $\mu\text{M}$  Alsterpaullone for 3 hours after injury showed the highest rate of foot regeneration at 57%, while only 9.8% of untreated animals regenerated their feet (Figure12).



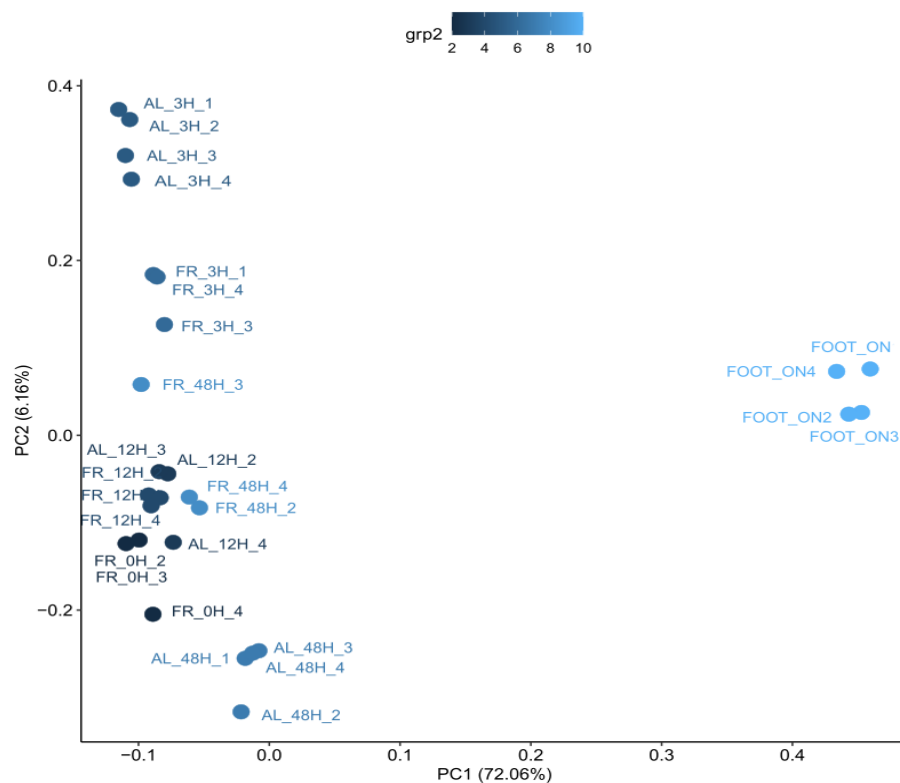
**Figure 12.** Daily monitoring foot regeneration in 5 $\mu\text{M}$  Alsterpaullone for 3hours.

### 3.8 Transcriptional response following Alsterpaullone treatment in bisected *H. oligactis*

Alsterpaullone, an inhibitor of glycogen synthase kinase-3 (GSK-3), activates the canonical Wnt pathway by stabilizing beta-catenin (Kunick et al., 2004). However, the impact of activated canonical Wnt signaling during *Hydra's* foot regeneration is unclear. To identify the transcriptional changes that result in foot regeneration rescue after Alsterpaullone treatment, I extracted RNA from *H. oligactis* foot regenerating tissue from treated with 5  $\mu$ M Alsterpaullone for 3 hours at 0, 3, 12 and 48 hpa to perform RNA-seq.

#### 3.8.1 Global PCA analysis following Alsterpaullone treatment in bisected *H. oligactis*

I applied principal component analysis (PCA) to globally compare gene expression of 0, 3, 12 and 48h regeneration time course, I included foot tissue to identify differences between differentiated foot tissue and the different stages during regeneration (Figure 13).



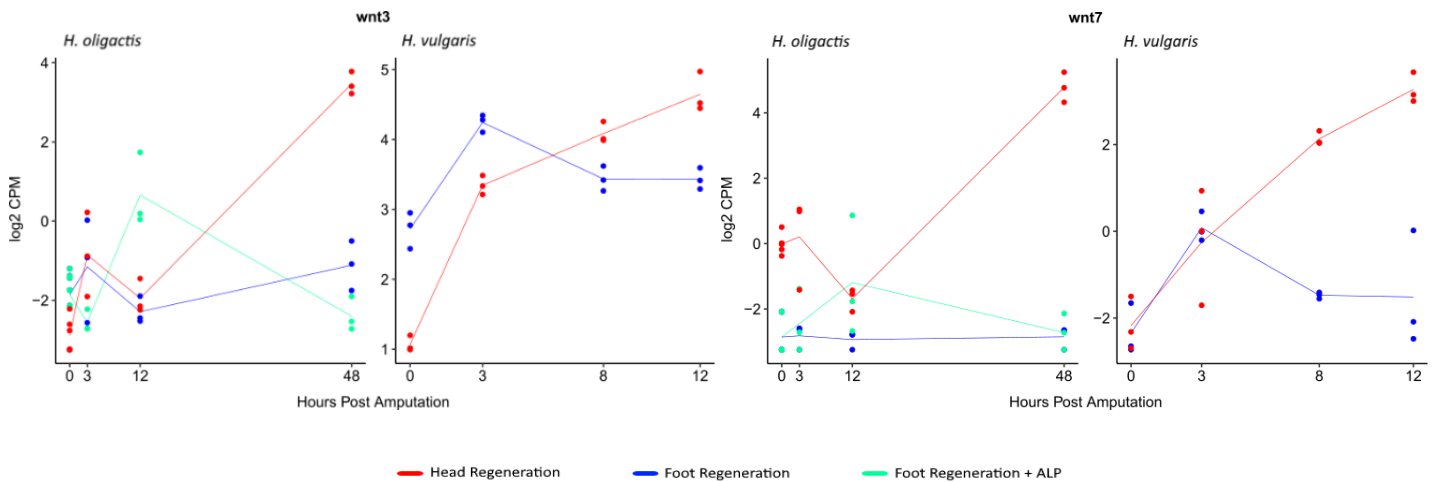
**Figure13:** PCA analysis of gene expression in untreated vs ALP treated foot regenerating tips in *H. oligactis* at 0, 3, 12 and 48h time course.

In the principal component analysis (PCA) of foot regenerating tissue, both untreated and ALP-treated samples show a large cluster of replicates at 0 hpa, as well as at 12 hpa for both control and ALP-treated tissues. This suggests that the gene expression at 12 hpa in the ALP-treated tissues reverts back to a pattern more akin to the profile of 0 hpa, similar to that of the untreated *Hydra*. Therefore, the ALP treatment does not induce gene expression conducive to foot formation at 12 hpa. The 3 hpa tissues display movement along the PC2 direction, with the ALP-treated tissues exhibiting greater displacement compared to the untreated ones, implying a more significant change in gene expression at 3hpa between the control and treated tissue. At 48hpa, the most noticeable contrast is seen with non-treated tissue in terms of differentiation. This is because the non-treated tissue still closely resembles gene expression at 0hpa and continues to exhibit characteristics of a body column, whereas the ALP-treated tissue at 48hpa shows a shift in behavior, leaning more towards the transcriptional profile of a foot, thus beginning to develop feet. It's important to note that the 48hpa time point represents an intermediate stage, as some tissue will eventually undergo foot regeneration at 96hpa or even 144hpa. Therefore, it's crucial that by 48hpa, the tissues are already progressing towards foot development.

### **3.8.2 RNA expression of Wnt components following Alsterpaullone treatment in bisected *H. oligactis***

Given that ALP stabilizes *b-catenin*, resulting in a strong activation of the wnt pathway, I sought to determine if the essential members of the Wnt pathway were significantly more activated with ALP treatment in the foot regenerating side. The important observation among canonical Wnt signaling pathway components in untreated *Hydra* was that the upregulation in foot regenerating tip at 3hpa was missing for *wnt7* and *dvl2*, and in *wnt3* upregulation was very low; otherwise, *beta-catenin* and head-specific genes such as *sp5* and *wntless* were similar to the expression of these genes in *H. vulgaris*. To evaluate the effect of ALP treatment in *H. oligactis*, I compared the expression patterns of the genes that I had previously examined in untreated *Hydra* with those in treated *Hydra*, thereby assessing the impact of ALP treatment on the activation of the Wnt pathway (Supplementary Figure 3).

*wnt9/10c* gene expression pattern is similar in treated and untreated *H. oligactis*. It is upregulated at 3hpa and then downregulated only at the foot regenerating tip by 12hpa. The only difference between *H. vulgaris* and *H. oligactis*, is that *H. oligactis* shows a lower expression level which was not affected by ALP treatment (Supplementary Figure 3). Similarly, in *H. vulgaris*, *wnt3* is upregulated at 3 hpa in head and foot regenerating tips and by 12hpa it becomes slightly downregulated in foot regenerating tips. In untreated *H. oligactis*, the pattern of expression is already similar to *H. vulgaris* and only the expression is very low. In ALP treated *H. oligactis* *wnt3* in foot regenerating tip shows an upregulation at 12 hpa but this upregulation is delayed and the level of expression is low compared to what was previously observed in *H. vulgaris* for this gene (Figure14). Likewise, in *H. vulgaris*, *wnt7* is upregulated at 3hpa and then downregulated by 12hpa in foot regenerating tips. In untreated *H. oligactis*, the expression of *wnt7* during the regeneration time course is steady and in ALP-treated *Hydra* the expression is modestly up-regulated at 3 and 12 hpa (Figure14). In general, treatment of *H. oligactis* with ALP does not make gene expression patterns of earliest Wnt genes more similar to *H. vulgaris* compared to untreated ones.



**Figure14:** RNA expression plots depicting average normalized RNA-seq read counts for *wnt3* and *wnt7* in log<sub>2</sub> counts per million (log<sub>2</sub>CPM) during head and foot regeneration in treated and untreated *H. oligactis* and *H. vulgaris*.

The expression of *wntless*, *dishevelled*, *sp5* and  $\beta$ -catenin in *H. vulgaris* and untreated *H. oligactis* is similar and ALP treatment enhanced and prolonged the expression in *wntless* and  $\beta$ -catenin at 12hpa and also increased the expression of *dishevelled* and *sp5* at 3hpa (Supplementary Figure

3). Furthermore, I checked some of the Wnt signaling TCF-dependent genes including *sFRP(fz8)*, *frizzled4/9/10*, *axin*, and TCF itself (Supplementary Figure 3). During *Hydra* foot regeneration, *sFRP(fz8)* like other head specific genes show context-dependent expression variations. The expression of this gene in *H. vulgaris*, ALP-treated and non-treated foot regenerating tip of *H. oligactis* shows a similar pattern, and treatment has no significant effect on its expression pattern. However, the expression of *sFRP3* in foot regenerating tip of treated *H. oligactis* at 12hpa is significantly increased, while this increase does not result in the expression pattern resembling that observed in *H. vulgaris*. The overall expression pattern of *fzd4/9/10* gene in both *Hydra* species is similar and ALP treatment did not have a significant effect. The expression pattern of *axin* in *H. vulgaris* and untreated *H. oligactis* is similar, while ALP treatment increases the upregulation seen at 3hpa, however this results in a different expression pattern in treated *H. oligactis* compared to *H. vulgaris*. Regarding TCF expression in *H. oligactis*, the upregulation at 12hpa in foot regenerating tips that was observed in *H. vulgaris* is missing, and it seems to happen in a delayed manner. But ALP treatment increases the upregulation at 3hpa without making expression pattern more similar to *H. vulgaris*. The expression of *brachyury1* in foot regenerating tips of *H. oligactis* is comparable to *H. vulgaris*, but at a lower level, and treatment with ALP does not increase its similarity to *H. vulgaris*. In conclusion, this comparative analysis of gene expression in time course of foot regeneration in *H. oligactis* in the two conditions, revealed an increase in the transcriptional activation of Wnt signaling activity in the Alp-treated *H. oligactis* at the foot regenerating tip, reaching its highest point at 12 hpa. The timing of this response differs from that of *H. vulgaris*, however, the burst of transcription of Wnt signaling genes mimics what has been observed for *H. vulgaris*, this transcriptional event is likely crucial for enabling foot regeneration to occur.

### **3.9 RNA expression of foot specific TFs in bisected *H. oligactis***

After injury in *Hydra*, the establishment of the foot organizer involves complex molecular and cellular processes, including the expression of specific genes such as *distal-less(dlx2)*, *nk-2*, *gata-3-like*, and *foxd2-like*. Several studies have shown that bisection triggers expression of these foot-specific TFs during the foot patterning phase (J. Cazet et al., 2021; Grens et al., 1996; Hemmrich

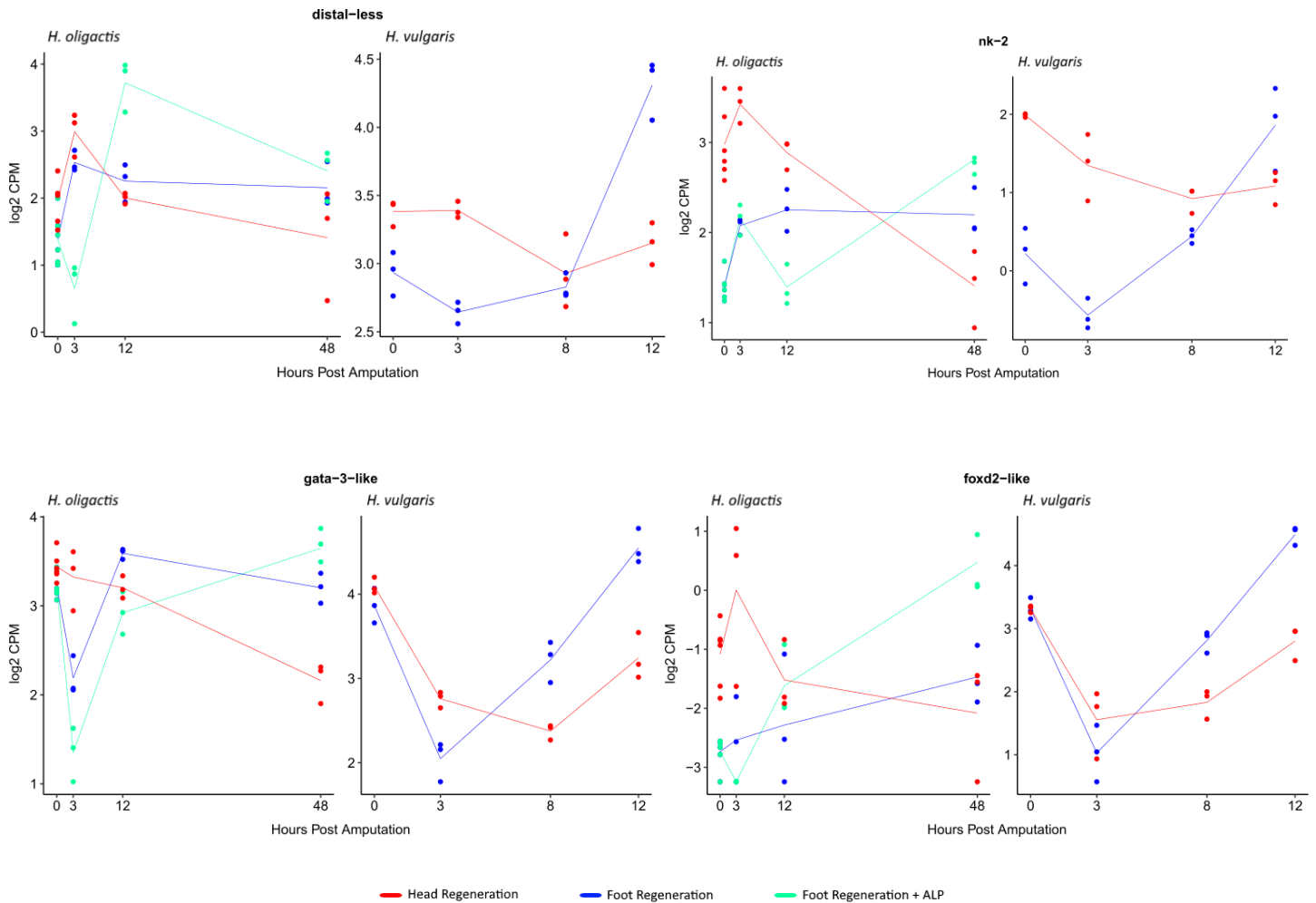
et al., 2012). A knockdown experiment showed that early foot markers *distal-less* and *gata-3* play crucial roles in foot patterning (Ferenc et al., 2021). To determine the role of these genes in the foot regeneration defect in *H. oligactis*, I compared the gene expression of *gata-3-like*, *nk-2*, *foxd2-like*, and *distal-less* in *H. vulgaris* and *H. oligactis* during the time course of regeneration. The general expression pattern of these genes in *H. vulgaris* shows a drop in the expression levels at 3hpa followed by upregulation at the foot regenerating tip (Figure15). Among these four foot-specific genes, the most similar expression pattern between the two species was observed in *gata3-like* with a drop at 3hpa and increased expression at 12hpa. In *H. oligactis* at 12pha compared to 0hpa, the expression of *gata-3-like* in the foot regenerating tip is higher than 0hpa, and in the head regenerating tip is lower than 0hpa and that is the pattern that also was observed in *H. vulgaris*. However, their up-regulation in the foot regenerating tip at 12 hpa is not significantly different from the expression level obtained for head regenerating tissue at the same time point. In *H. oligactis* not only the expression of *nk-2*, *foxd2-like* and *distal-less* does not drop at 3 hpa but there is a marked upregulation of these genes at this time point. At the head regenerating tip, the expression of these genes goes down by 12 hpa in both species. However, at 12 hpa *nk-2* and *foxd2-like* expression level in the head regenerating tip is still higher than at the foot regenerating tip, and eventually, at 48 hpa, the expression becomes foot specific. Despite the increase in expression at 3 hpa, unlike what has been reported for *H. vulgaris*, the fact that the levels of *nk-2* and *foxd2-like* are higher at 12 hpa than at 3hpa suggests that the overall pattern of these genes resembles that of *H. vulgaris*. However, in the case *distal-less*, there is an upregulation at 3 hpa, unlike what we observed in *H. vulgaris*, the expression level at 12hpa in foot regenerating tips decreases in *H. oligactis*. To sum up, out of the four foot-specific TFs, namely *distal-less*, *nk-2*, *gata-3-like*, and *foxd2-like*, only *gata3-like* showed an increase in expression during the patterning phase. The increase in expression of *foxd2-like* and *nk-2* was minimal, and *distal-less* displayed a distinct expression pattern in comparison to *H. vulgaris* (Figure15).

### 3.10 RNA expression of foot specific TFs in bisected *H. oligactis* following Alsterpaullone treatment

To investigate the effect of Alsterpaullone in the establishment of the foot organizer in *H. oligactis*, I compared the patterns of expression of foot-specific genes in DMSO and ALP-treated injured animals with untreated animals (Figure15).

*nk-2* and *distal-less* genes are foot-associated TFs that are involved in foot regeneration in *Hydra* by regulating patterning and morphogenesis, both of these genes have been shown to be upregulated at 12 hpa in bisected *H. vulgaris*. In *H. oligactis* the expression of *nk-2* at 3h in treated and non-treated *Hydra* is similar and I observed a decrease in the expression at 12 hpa in ALP-treated animals which makes the treated animal even more distinct from the expression pattern of *H. vulgaris*. In ALP-treated animals, *nk-2* is significantly upregulated by 48 hpa compared to untreated animals. This delayed upregulation is what we expected from *nk-2* as a foot-specific TF to occur in the hypothetical patterning phase at 12 hpa. Although the upregulation at 12 hpa in intact animals and treated animals is missing there is delayed upregulation at 48 hpa in treated animals. *Hydra* foot-specific genes, *dlx2* and *foxd2*, expression pattern was affected by ALP treatment in *H. oligactis*, making it similar to the expression pattern observed in *H. vulgaris*. In *H. vulgaris* and in ALP-treated *H. oligactis*, the expression of these genes drops at 3hpa and it markedly increases at 12hpa while in untreated *H. oligactis*, *dlx2* is upregulated at 3hpa and by 12hpa which is putatively the patterning phase the expression of this gene goes down to lower level than expression level at 3hpa. The level of expression of *dlx2* in treated *H. oligactis* increases considerably from 3 to 12 hpa and it is significantly higher compared to untreated *Hydra*. So, the expression of *dlx2* in ALP-treated *H. oligactis* is similar to that seen in *H. vulgaris*. Transcription factors *gata-3-like* and *foxd2-like* have recently found to be involved in foot regeneration in *H. vulgaris*. In *H. vulgaris*, these genes become upregulated at 12 hpa. In untreated *H. oligactis*, *gata-3-like* shows a similar pattern to the one observed in *H. vulgaris*, and in treated animal the drop at 3hpa and the delayed rise at 48hpa display a larger fold change value. Overall, out of the four foot-specific TFs mentioned earlier, the upregulation in the patterning phase is enhanced in *foxd2-like*, and *distal-less* expression became similar to that of *H. vulgaris* due to ALP treatment. In

contrast, the treatment downregulated *nk-2* expression at the patterning phase, making it less similar to *H. vulgaris*.

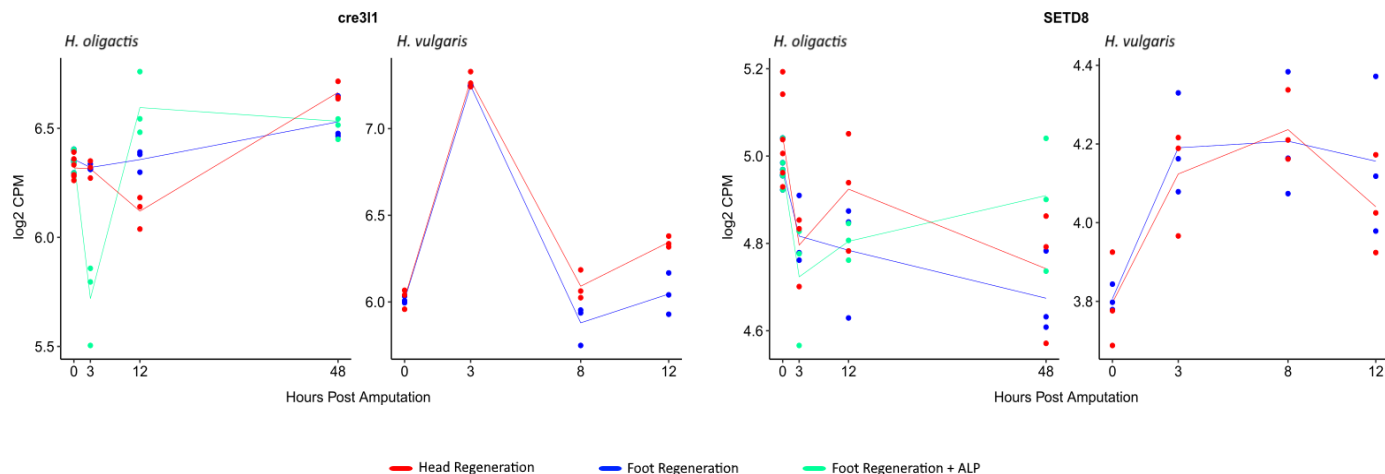


**Figure15:** Comparing the foot specific TFs expression pattern during regeneration time course in *H. oligactis* and *H. vulgaris*.

### 3.11 Alsterpaullone treatment impact on cre3l1 TF and two genes with Wnt regulatory properties TSP and SETD8

To get a broader overview of the gene expression changes in ALP-treated *H. oligactis*, I compared upregulated and downregulated genes at contrasted time points and uncovered a noticeable

overlap in the gene expression of one bZIP TF, *cre3l1*, and two genes with Wnt regulatory properties SETD8 (Figure16) and TSP (Supplementary Files-3), in ALP-treated *H. oligactis* and *H. vulgaris*.



**Figure16:** RNA expression plots depicting average normalized RNA-seq read counts for *cre3l1* and *SETD8* in  $\log_2$  counts per million ( $\log_2$ CPM) during head and foot regeneration in treated and untreated *H. oligactis* and *H. vulgaris*.

The upregulation of *cre3l1* bZIP TF was influenced by ALP treatment, leading to an increase at 12 hpa. In *H. vulgaris*, the expression of *cre3l1* is heightened at 3 hpa, whereas in *H. oligactis*, no such elevation is detected. As a result, the ALP treatment brings the expression of *cre3l1* closer to that of *H. vulgaris*. About TSP, in our RNA-seq findings, we identified five genes labeled as Thrombospondin. Upon observing ALP treated *Hydra*, we noted that all five genes annotated as Thrombospondin showed upregulation in the treated animals. While in untreated *Hydra* out of these, only one exhibited an increase in foot regenerating tips at 12hpa, which was akin to *H. vulgaris*. Two of these genes did not display upregulation at 12hpa, one demonstrated delayed upregulation, and one had very minimal upregulation.

SETD8 expression in *H. oligactis* decreases from 0 to 12 hpa. Our findings indicate that the expression of SETD8 is intrinsically suppressed in *H. oligactis*. Interestingly, treatment with ALP leads to an increase in the expression of these genes, as noted in *H. vulgaris*.

It appears that ALP treatment has an overall effect in modulating a diverse set of genes, some which may be involved in head and foot specification and some may play a role in early regulation

of canonical Wnt signaling pathway. Among all the genes affected by the treatment, the ones involved in the regulation of the canonical Wnt signaling pathway might have a more potent effect on gene modulation. Given that any difference in the expression level or expression pattern of regulatory genes may possibly display an accumulative effect and significant role in the overall balancing the gene networks involved in regeneration.

## 4. Discussion

### 4.1 Early injury response following a mid-gastric bisection in *H. oligactis*

#### 4.1.1 Activation of injury-responsive bZIP TFs

Previously published data from Juliano Lab indicates that after a mid-gastric bisection in *Hydra*, injury-responsive bZIP TFs trigger the transcriptional activation of the Wnt pathway as an integral part of the overall injury response (Cazet et al., 2021). In *H. oligactis* the expression pattern of bZIP TFs *jun* and *fos*, was indistinguishable from that in *H. vulgaris* in both RNA *in situ* hybridization results and RNA-seq data. However, the expression of *cre3/1*, another bZIP TF, seems to be restricted to interstitial stem cells in *H. oligactis* as shown by RNA *in situ* hybridization, with no noticeable increase in the expression at the tissue adjacent to an injury site at 3 hours post amputation (hpa). The expression pattern of *cre3/1* matches the distribution of interstitial stem cells in *Hydra*, although we did not conduct any experiments to inform the type of cell expressing this transcript, this could be done by searching for interstitial stem cells gene markers and performing a double *in situ* hybridization for such markers and *cre3/1*.

Our RNA seq data showed that in *H. oligactis*, it appears that the injury does not affect the expression of *cre3/1* while in *H. vulgaris*, the expression of *cre3/1* is increased at 3 hpa similarly to that of *jun* or *fos*. Studies conducted earlier have suggested that MAPK signaling plays a crucial role in responding to injury. These studies have also highlighted the phosphorylation of multiple mitogen-activated kinases (MAPKs) following injury, which aligns with the observations we have made regarding *jun* and *fos* activity (Cazet et al., 2021; Petersen et al., 2015; Tursch et al., 2022). In *H. oligactis*, the increased expression of *jun* and *fos* suggests that the injury signal activates some bZIP TFs, but not *cre3/1*. Differences in *cre3/1* activation between the two species of *Hydra*

may account for their contrasting regenerative abilities. When the Cre3l1 transcription factor (TF) and other bZIP TFs are active, these may induce the transcriptional activation of Wnt signaling components. Therefore, the unspecificity of Cre3l1 expression at the injury site may result in hindered Wnt signaling activity. Based on our findings, ALP treatment enhances foot regeneration in *H. oligactis*, while it also results in a moderate increase in *cre3l1* expression, opening up the possibility that the null up-regulation of *cre3l1* upon injury is directly involved in the foot regeneration defect in *H. oligactis*. that connected, then the absence of *cre3l1* upregulation could contribute to the foot deficiency in *H. oligactis*. The activation of *cre3l1* and foot regeneration should be further studied through experiments such as *cre3l1* knockdown in *H. vulgaris*. Additional research is needed to establish a connection between *cre3l1* activation and foot regeneration.

Moreover, I observed that other Wnt ligands are still up-regulated during general injury response unlike *wnt3*, such is the case of *wnt9/10c* which according to most recent data this gene might be at the top of the Wnt activation cascade (Lengfeld et al., 2009; Tursch et al., 2022). In this regard, the *wnt9/10c* expression may be regulated by different bZIP TFs than *wnt3*. This might result from differences in the regulatory regions of the various Wnt ligands, something that could be confirmed by both in-silico analysis and functional experiments analyses of the promoter region of these genes. An in-silico study in 2022 provides some evidence suggesting another bZIP TF, ATF3, with a differential affinity to Wnt promoters, presumptively regulates the serial activation of injury-responsive Wnt genes (Tursch et al., 2022). Their hypothesis proposed that ATF3 primarily functions as a transcriptional repressor, and by binding to Wnt promoters with a different affinity, ATF3 could control the expression of these promoters in *Hydra*, thus preventing the early increase of patterning Wnts such as *wnt3* and *wnt7* during the initial stages of regeneration. They suggested that the ATF3 binds to the *wnt7* and *wnt3* promoters with a higher probability than *wnt9/10c*. Interestingly, in *H. oligactis*, ATF3 expression rises from 0 to 12hpa, contrarily to what happens in *H. vulgaris*, this might be suppressing the transcription of *wnt3* and *wnt7* in *H. oligactis*. Independent RNA-seq experiments (Cazet et al., 2021; Petersen et al., 2015) have shown that a network of bZIP TFs, including ATFs, Fos, Jun, and CREB, is up-regulated during *Hydra* regeneration, and our result show that at least the expression pattern of two of these TFs,

namely *cre3l1* and ATF3, is different in *H. oligactis*. The relationship between genes and TFs is complex, therefore further functional research is required to understand the specific or indirect impact of each element on the phenotype.

A possible explanation for the observed expression pattern of *wnt3* and *wnt7* in *H. oligactis*, is that *cre3l1* is the direct regulator of *wnt3* in response to injury, and the failure to activate *cre3l1* in addition to repressive effect of highly activated ATF3 result in Wnt signaling defects in *H. oligactis*. Given the crucial role of *wnt3* in head-organizing activity (as mentioned by (Hobmayer et al., 2000; Lengfeld et al., 2009; Technau et al., 2000; Tursch et al., 2022; Vogt et al., 2019)) the activation of *wnt3* on a minuscule level has the potential to significantly influence the patterning phase of regeneration. The siRNA mediated knockdowns demonstrated the significance of *wnt9/10c*, *wnt3* and *wnt7* by inhibiting both head and foot regeneration (Tursch et al., 2022). The expression of *wnt3* and *wnt7(wnt2B)* in *H. oligactis* is similar to that in *H. vulgaris* but at a very low scale, suggesting that they may have a targeted function in the foot regeneration deficiency in *H. oligactis*.

Overall, differences in *cre3l1* and ATF3 activation between the two *Hydra* species may contribute to their varying regenerative capabilities. However, there is a need for stronger evidence from functional experiments like *cre3l1* and ATF3 knockdown in *H. vulgaris* to establish a link between early injury response, *cre3l1* activation and the expression of *wnt3* and *wnt7*.

#### **4.1.2 Regeneration-specific activation of Wnt signaling**

Subsequently, we assessed the injury response progression from bZIP TFs activation to early wnt genes expression. In *H. vulgaris* position-independent Wnt genes, *wnt9/10c*, *wnt3*, *wnt7(wnt2B)*, *wntless*, *dishevelled*, *b-catenin*, and *sp5* are among the first to be activated during early response regeneration (Cazet et al., 2021; Tursch et al., 2022). The pattern of expression for *wnt9/10c* and *sp5* expression in *H. oligactis* resembles that of *H. vulgaris*. While the pattern of *wnt3* expression was similar in both *Hydra* species, the level of *wnt3* expression was very low in *H. oligactis* and not even detectable in our *in-situ* hybridization experiments. Another case of contrasting expression pattern between these two *Hydra* species is *wnt7(wnt2B)*, while in *H. oligactis* it does not change during regeneration time course, it shows an increase in expression shortly after injury

in *H. vulgaris*. Subsequently, during the patterning phase, its expression becomes head-specific and decreases at the foot regenerating tip. In *H. oligactis*, *wnt7(wnt2B)* eventually becomes head-specific, with an important delay. Given the established importance of early Wnt expression for head and foot regeneration, these differences in the expression of Wnt genes might account for the low foot regeneration rate observed in *H. oligactis*. The fact that *wnt3* and *wnt7(wnt2B)* are not induced by injury in *H. oligactis* and that these two genes are highly expressed at the regenerated head suggests that *H. oligactis* are capable of expressing these genes, but for reasons that are not clear, they are not activated by injury.

## **4.2 The general injury response transitions to head and foot specific gene transcription**

In a previous study, it was suggested that TCF plays a crucial part in the shift from the general injury response to head and foot-specific gene transcription (J. Cazet et al., 2021b). In my results, the increase in the expression of TCF in *H. oligactis* foot regenerates, occurred at a later time compared to *H. vulgaris*. If the delay in the expression of TCF is important in *H. oligactis* foot regeneration deficiency, then we should observe some deviation in TCF functionality. In *H. vulgaris*, previous research has shown that treatment with the TCF inhibitor iCRT14, prevents the downregulation of the head-specific genes like *brachyury1* at the foot regenerating tip (J. Cazet et al., 2021b; Gufler et al., 2018b). Contrastingly, in *H. oligactis* the expression of *brachyury1* in foot regenerating tips at 12hpa is not downregulated. The down regulation of *brachyury1* being delayed aligns with a delay in the activation of TCF. Furthermore, iCRT14 experiments have shown that TCF inhibition blocked the expression of foot regeneration-specific TFs (Cazet et al., 2021). Among the previously described four foot-specific TFs, *gata3-like*, *distal-less*, *foxd2-like*, and *nk-2*, in *H. oligactis* only *gata3-like* exhibited upregulation during the patterning phase. The upregulation of *foxd2-like* and *nk-2* was minimal, and there was a lack of upregulation in *distal-less*. The delayed TCF is once again in line with the pattern. However, TCF-associated function regarding bZIP activity in both *Hydra* is similar. In the same iCRT14 experiment, the decrease in bZIP TFs following the early upregulation during initial injury response was also indicated to be linked to TCF activity, which is clearly evident in *H. oligactis*. In summary, the transcriptional

differences observed between *H. oligactis* and *H. vulgaris* at foot regenerating tips may be the result of delayed expression of TCF. However, considering the complex network of interaction between genes and their regulators, further investigations must be carried out to clarify this connection.

### 4.3 Wnt regulatory network activation upon injury

In my research so far, I have been analyzing whether bZIP TFs show increased activity during the phase of injury response. Additionally, I have been studying the expression of Wnt members and the potential influence of TCF on Wnt signaling. Next, I examined the function of specific Wnt regulatory genes in *H. oligactis*, which were previously documented in *H. vulgaris*. This is important because the expression of regulatory genes may be an essential contribution to the divergent Wnt gene expression in *H. oligactis*. After an injury, there has been demonstrated an increase in the activity of many components within the Wnt regulatory system, including *sp5*, and *brachyury1* among others (Guder et al., 2006; Gufler et al., 2018; Lommel et al., 2018; Vogg et al., 2019). Two additional secreted factors from *Hydra* have recently been found to have functions in modulating Wnt: *Hydra* astacin protease-7 (HAS-7) (Ziegler et al., 2021) and Thrombospondin (TSP) (Lommel et al., 2018) and an enzyme known as SETD8 exhibiting activity related to posttranslational modifications. In the following, I will summarize the results obtained from our RNA-seq data regarding these specific genes and the possible interpretation of their gene expression outcome.

The behavior of *sp5* in *H. oligactis* is similar to that of *H. vulgaris* in the regeneration time course where we examined the expression pattern. Therefore, it is unlikely that *sp5* plays a negative role in *H. oligactis* regeneration. The downregulation of *brachyury1* is delayed in foot regenerating tips in *H. oligactis*. This delay may contribute to axial regeneration in *H. oligactis*, but interpreting the effect of delayed *brachyury1* downregulation on foot regeneration deficiency is challenging without further experiments specifically targeting *brachyury1*.

Among the astacin associated genes, the one that was annotated as Astacin (g24277), the expression levels differed between *H. vulgaris* and *H. oligactis* during head and foot regeneration. In *H. vulgaris*, the expression decreased from 3 hpa to 12 hpa, while in *H. oligactis*, it increased in

both head and foot regenerating tips. The early upregulation of Astacin, known to inhibit the protein stability of recombinant *wnt3* from *Hydra*, as discussed in (Ziegler et al., 2021), may contribute in the deficiency of foot regeneration in *H. oligactis*. Astacin's increasing expression pattern in *H. oligactis*, as opposed to *H. vulgaris*, suggest that the elevated activity of Astacin could lead to overregulation of the *wnt3* ligand at the protein level. However, the extent of the prevalence of this mechanism and its precise effect on the Wnt pathway remains uncertain at this point, as well as how it specifically modulates them.

TSP was discovered in a mesoglea proteome screen conducted in *Hydra*. As one of the major components of mesoglea, it is co-expressed with *wnt3* at the tip of the head organizer (Lommel et al., 2018). In our RNA-seq results, we found five genes annotated as Thrombospondin. Among them, only one showed an increase in foot regenerating tips at 12hpa, similar to *H. vulgaris*. Two of these genes did not show upregulation at 12hpa, one had delayed upregulation, and one had very low upregulation. TSP is a target of  $\beta$ -Catenin in *Hydra* (Lommel et al., 2018) and we observed that all five Thrombospondin annotated genes were upregulated in ALP treated animals. These results are in line with the findings from a previous study that demonstrated that ALP-treated animals exhibited an up-regulation of TSP in focal areas at ectopic tentacles (Lommel et al., 2018), mirroring the observed pattern for *wnt3* (Nakamura et al., 2011). Through fluorescent dual color ISH for TSP and *wnt3*, they inferred that TSP expression seems to be influenced by the signaling activity status of the Wnt pathway. Considering the enhanced foot regeneration rate in ALP-treated animals, it is possible that the low and delayed expression of important Wnt genes such as *wnt3* may not be adequate to induce the expression of TSP. This is significant because the expression of TSP likely serves as a feedback inhibitor of Wnt signaling in *H. vulgaris* (Lommel et al., 2018). So, when subjected to ALP treatment, TSP exhibits enhanced activity, causing its downstream genes to function similarly to those of *H. vulgaris* in axial patterning. This leads to improved foot regeneration in animals treated with ALP compared to those that are untreated. So, could low and delayed TSP expression potentially impart the foot regeneration deficiency in *H. oligactis*? To verify TSP's involvement in aboral regeneration, one approach could involve designing an experiment to silence TSP gene in *H. vulgaris* and then evaluating the rate of foot

regeneration. The TSP silencing experiment's outcome in *H. vulgaris* could be used to assess the low and delayed TSP results in *H. oligactis*.

Furthermore, I compared the expression of a specific Wnt regulatory gene, SETD8, which demonstrated varying levels of expression in both species of *Hydra*. In 2023, Akhila Gungi showed that in *Hydra*, the expression of SETD8 is increased at the mRNA and protein levels when the Wnt signaling pathway is activated. They observed that even though the initial phase of regeneration, known as wound healing, does not show any visible changes when SETD8 is inhibited, several molecular changes occur. These changes involve dysregulation of various Wnt signaling components, apoptosis-related genes, Jun kinase among others (Gungi et al., 2023). It is noteworthy that SETD8 collaborates with  $\beta$ -catenin to modulate the gene regulatory network associated with regeneration and inhibiting SETD8 activity has been found to abolish head and foot regeneration in *Hydra* (Gungi et al., 2023). Interestingly, in *H. oligactis*, the expression of SETD8 decreases from 0 to 12 hpa. Our results show that the expression of SETD8 is inherently inhibited in *H. oligactis* and this downregulation of SETD8 may contribute to foot regeneration deficiency. Interestingly, ALP treatment up-regulates the expression of this genes, as observed in *H. vulgaris*, making it a potential candidate to participate in foot regeneration rescue. To verify the potential impact of downregulated SETD8 levels on the foot regeneration deficiency in *H. oligactis*, an experiment involving the treatment of SETD8 protein could be conducted to assess its possible effect on foot regeneration rescue. However, the additive effect of various components with Wnt regulatory function on regeneration capacity of *Hydra* is not well understood.

Given the multitude of regulatory factors involved in fine tuning the regeneration process, drawing conclusions based on a limited set of genes could potentially be misleading. Nonetheless, to comprehend the roles of the different players involved in foot regeneration deficiency in *H. oligactis*, it is undoubtedly essential to analyze the relationships between various gene expression levels and the potential input of each of them in the regenerative capacity of aboral side in *H. oligactis*.

## 4.4 Apoptosis in *H. oligactis* regeneration

Apoptosis has been reported to be activated during early regeneration in *Hydra*. We observed apoptosis to be symmetrically induced at both head and foot regenerating tips in *H. oligactis*, as it has been previously reported in *H. vulgaris* (Cazet et al., 2021). In this regard the result we obtained is consistent with the results demonstrated in previous studies, indicating that apoptosis activation in *Hydra* is similar in both head and foot regenerates (Cazet et al., 2021; Gufler et al., 2018; Tursch et al., 2022). This questions the model of apoptosis-mediated Wnt release (Chera et al., 2009), which emphasized that apoptosis exclusively occurs at the head regenerating tip.

However, apoptosis in *H. oligactis* peaked at a later time compared to *H. vulgaris*, potentially contributing to the extended timeline for injury response in *H. oligactis*. Additionally, I observed variations in the expression of apoptosis marker genes between the two *Hydra* species, suggesting some differences in the regulation of the apoptosis process through gene expression in *Hydra*. However, the lack of significant deficiency in apoptosis in *H. oligactis* aboral injuries does not support apoptosis as a primary cause of foot regeneration deficiency in *H. oligactis*.

## 4.5 Alsterpaullone effect on foot regeneration in *H. oligactis*

### 4.5.1 The impact of Alsterpaullone treatment on Wnt signaling components

In *Hydra*, Alsterpaullone triggers the activation of the canonical Wnt signaling pathway, resulting in the increased expression of  $\beta$ -catenin and *wnt3* (Aide Macias-Muñoz, 2022; Broun et al., 2005; Guder et al., 2006; Vogg et al., 2019). So, we hypothesized that if the low expression of *wnt3* plays an essential role in foot regeneration deficiency, by interrupting the serial activation of Wnt genes, then ALP treatment would be effective in foot rescue. In untreated animals, foot regeneration was found to be 9.8%, whereas it increased to 57% in *Hydra* treated with ALP according to our findings. The expression of *wnt3* and *wnt7*(*Wnt2B*) in treated *H. oligactis* was comparable to *H. vulgaris*, although not identical. In treated *H. oligactis* foot regenerating tips, *wnt3* showed an upregulation at 12 hpa instead of at 3hpa. In addition to this upregulation being delayed, the level of expression was low. About *wnt7*(*wnt2B*) in untreated *H. oligactis*, the expression of

*wnt7(wnt2B)* did not change significantly during the regeneration time course while in ALP-treated *Hydra* the expression was modestly up-regulated at 12 hpa. In general, treatment of *H. oligactis* with ALP is effective on *wnt3* and *wnt7(Wnt2B)* expression but it did not result in upregulation of Wnt genes during early injury response as in *H. vulgaris*. However, ALP treatment either increased or prolonged the expression of *wntless*, *dishevelled*, *sp5*, and *beta-catenin* during the course of regeneration. We did not observe the upregulation of TCF at 12hpa in the foot regenerating tips of *H. oligactis* following ALP treatment, unlike what we observed in *H. vulgaris*.

Instead, there was an early peak in upregulation at 3 hpa due to the treatment. The close coordination of *Hydra* Wnt genes,  $\beta$ -Catenin, and TCF in the head organizer suggests that their roles are closely interconnected (Cazet et al., 2021; Nakamura et al., 2011; Tursch et al., 2022). Maybe the increased activity of *wnt3* and *wnt7 (wnt2B)*, even though it is low and occurs later, and the early activation of TCF, despite not matching the expression of these genes in *H. vulgaris*, still affects foot patterning. However, additional experiments are needed to confirm this.

It has been reported that treatment with ALP represses the activity of the foot organizer by generating ectopic apical organizers along the body column due to the proximity of the head organizer (Wenger et al., 2019.). In 2019 Wenger and colleagues discovered that when Wnt/ $\beta$ -catenin signaling is ectopically activated in *Hydra*, basal differentiation is not sustained and basal regeneration is inhibited, which contradicts the findings of our ALP treatment. My findings are particularly intriguing, as ALP treatment resulted in a higher rate of foot regeneration in *H. oligactis*. However, it is noteworthy that the protocol I used here to treat *H. oligactis* with ALP treatment differs from what has been reported in the literature. In brief, I exposed *Hydra* to 5  $\mu$ M ALP for 3 hours right after amputation, whereas published works used the same concentration to expose animals for one or two days. This discrepancy in exposure time may have a substantial impact on the outcomes. So, my initial hypothesis regarding Wnt activation role in foot regeneration was supported by my results with ALP treatment, given that my results indicate that extended activation of the Wnt pathway enhances foot regeneration in *H. oligactis*.

#### 4.5.2 The impact of Alsterpaullone treatment on some of the Wnt regulatory genes

In ALP-treated *Hydra*, our RNA-seq analysis of the aboral regenerating tissue in *H. oligactis* confirmed an increase in the expression of some critical Wnt members such as *wnt7*(*wnt2B*), *wnt3*, *dvl2*, *sFRP3*, *sp5*, and more importantly genes with regulatory function like TSP, SETD8. Another significant effect of ALP treatment in *H. oligactis* is the increased expression of *cre3l1*, making its pattern similar to that of *H. vulgaris*. One explanation about the positive effect of ALP on foot regeneration in *H. oligactis* is that the low expression of Wnt genes in untreated *Hydra* does not induce the upregulation of Wnt regulatory genes. Treatment with ALP increases the expression of Wnt-associated genes, leading to the activation of Wnt-regulatory genes, mimicking the axial regenerative behavior of *H. vulgaris*.

#### 4.5.3 The impact of Alsterpaullone treatment on some of the foot specific TFs

After being injured, *Hydra* undergoes complex molecular and cellular processes to establish the foot organizer, involving the expression of specific genes such as *distal-less*, *nk-2*, *gata-3-like*, and *foxd2-like*. These genes may play crucial roles in foot patterning in *Hydra*. Previous studies indicate that the bisection of *Hydra* triggers the expression of these foot-specific TFs during the foot patterning phase. Among these genes, *distal-less* exhibits a distinct expression pattern compared to *H. vulgaris*. It is noteworthy that ALP treatment resulted in *distal-less* expression becoming similar to that of *H. vulgaris*. The results we obtained align with a 2019 study conducted by Puli Chandramouli Reddy, which utilized ALP treatment. In their research, they identified transcription factors (TFs) such as DLX and TLX (Gorget), which are closely related members of the ANTP class, as well as other Wnt/ $\beta$ -catenin signaling TF targets. Additionally, they proposed a potential role for these TFs in organizer functions or associated pattern formation in complex organisms (Reddy et al., 2019). It is uncertain whether ALP treatment impacts foot regeneration by modulating foot-specific gene expression or if modulation of an earlier pathway is necessary.

#### 4.6 Conclusion and outlook:

The aim of this study was to investigate the mechanisms behind the foot regeneration deficiency in *H. oligactis*. To address this question I compared the injury response between *H. oligactis* and

*H. vulgaris* as a model for successful regeneration. The comparison between the two species showed that they generally responded similarly to injury and during pre-patterning phase, with a few noticeable differences such as reduced or delayed upregulation and, in some cases, missing expression. For instance, there were differences in the way *cre3l1* and *ATF3* TFs were expressed during the early injury response, as well as variations in the expression of *wnt3*, *wnt7*, *wnt9/10c*, Wnt regulatory genes and foot-specific TFs during pre-patterning. The occurrence of apoptosis, as discovered in the immediate events following injury (Petersen et al., 2015), was triggered symmetrically at both the head and foot regenerating tips, but the peak of induction was delayed compared to *H. vulgaris* which is consistent with the delayed expression pattern seen in other aspects of *H. oligactis* regeneration. ALP treatment has a foot regeneration rescue property, but it has a wide range of impacts on gene expression, making it difficult to single out the exact expression changes involved in foot regeneration deficiency. However, it is very likely that low *wnt3* expression plays a role in foot regeneration, especially considering its role in axial patterning in many species. Interspecies lateral grafting between *H. oligactis* and *H. vulgaris* revealed the head organizing signal conserved between species. The high levels of *wnt3* expression at the head regenerating tips show that *H. oligactis* potentially could express *wnt3* at the foot regenerating tip, but for any reason that might be connected to the activity of bZIP TFs, the expression of *wnt3* is not triggered. The importance of early high expression of *wnt3* for the sequential activation of other Wnt genes involved in patterning indicates that low *wnt3* expression may disrupt the patterning by interrupting the domino effect of the Wnt regulatory network. So, insufficient *wnt3* expression may act as a primary factor in the deficiency of foot regeneration. In that case, the reason for the weak trigger of *wnt3* expression in foot regenerating tips may be the activity of bZIP TFs.

To validate this hypothesis, the next step should be silencing *cre3l1* and *atf3* through RNAi in *H. vulgaris* and monitoring *wnt3* expression level and foot regeneration potential of the knockdown animals. If such an intervention results in low *wnt3* expression and a lower foot regeneration rate, a subsequent experiment should test the foot regeneration potential in a *wnt3* knockdown of *H. vulgaris*. However, if silencing of these TFs in *H. vulgaris* does not influence *wnt3* expression level, that would suggest that the expression of *wnt3* relies on the activity of different TFs.

Nevertheless, this other TF may appear as up-regulated in my RNA-seq analysis of ALP-treated tissue in *H. oligactis*. The core of our hypothesis is that transcriptional activation of the Wnt signaling pathway immediately following an injury is essential for foot regeneration. This response is seemingly missing in *H. oligactis*, as we noticed lower and delayed expression of certain crucial Wnt ligands. Even though injury response genes, like *jun* and *fos*, become active at 3 hpa, it is possible that the level and timing of Wnt signaling transcriptional activation are insufficient to support the subsequent regeneration events. In *H. oligactis*, *wnt3* and *wnt7* are examples of genes with low or delayed expression. These genes, which are typically expressed at the hypostome, could also be crucial for the onset of foot regeneration. For foot regeneration, activation of Wnt signaling genes surpassing a certain threshold may be required to actually jumpstart regeneration. Interestingly, head regeneration at the oral wound site overcomes the low or delayed Wnt expression because, possible due to the lack of a Wnt-organizing center that may suppress Wnt activity elsewhere. Essentially, this would allow Wnt transcriptional activation to accumulate in some cells, rendering them potential hypostomes. However, the initial wnt activation timeframe is missed in the context of foot regeneration, and the existing Wnt organizing center in the hypostome prevents foot regeneration.

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## 6. Supplementary Files and Figures

**Supplementary Files-1:** All the scripts we used to process the original RNA-seq reads.

**Supplementary Files-2:** Primer sequences containing the sequences of the primers used in this study.

**Supplementary Files-3:** The table containing contrasted time points (F3vF0, F12vF0, F48vF0, ALP3vALP0, ALP12vALP0, ALP48vALP0, H3vH0, H12vH0, H48vH0) for the expression of TSP in *H. oligactis* and *H. vulgaris*.

**Supplementary Figure 1:** RNA expression plots depicting average normalized RNA-seq read counts for *caspase-3c*, *bcl-like-6*, and *p53*, prolyl endopeptidase FAP, and Endoribonuclease ZC3H12A(g5828) in log<sub>2</sub> counts per million (log<sub>2</sub>CPM) during head and foot regeneration time course of 0, 3, 12, and 48hpa in *H. oligactis* and *H. vulgaris*.

**Supplementary Figure 2:** RNA expression plots depicting average normalized RNA-seq read counts for *wnt7*, *wntless*, *dishevelled*, *beta-catenin*, *sp5*, TCF, *sFRP(fz8)*, *frizzled4/9/10*, *axin*, *sFRP3*, and *Brachyury1* in log<sub>2</sub> counts per million (log<sub>2</sub>CPM) during head and foot regeneration time course of 0, 3, 12, and 48hpa in *H. oligactis* and *H. vulgaris*.

**Supplementary Figure 3:** RNA expression plots depicting average normalized RNA-seq read counts for *wnt9/10c*, *wnt7*, *wntless*, *dishevelled*, *beta-catenin*, *sp5*, TCF, *sFRP(fz8)*, *frizzled4/9/10*, *sFRp3*, and *axin* in log<sub>2</sub> counts per million (log<sub>2</sub>CPM) during head and foot regeneration time course of 0, 3, 12, and 48hpa in ALP treated *H. oligactis* and *H. vulgaris*.

## Supplementary files-1:

This file includes all the scripts I used to process the original RNA-seq reads, which consisted of ~20 millions fasta reads per sample, to generate an expression matrix. This pipeline consists of Trimmomatic, rsem and EdgeR. I first used Trimmomatic to filter out the adapters surrounding the inserts, polyA tails, and low-quality base calls. To map the reads I used RSEM (RNA-Seq by Expectation Maximization). The typical RSEM process involves the execution of two commands (rsem-prepare-reference and rsem-calculate-expression), which streamlines the application of Bowtie for aligning reads. The rsem-calculate-expression script manages both the alignment of reads to reference transcript sequences and the determination of the relative abundances. The Bowtie alignment algorithm is used by RSEM as the default option to align reads(Li & Dewey, 2011). Once the expression matrix is created, R was utilized to normalize the counts matrix, add annotations and perform differentially expressed genes analysis. Below, I have included the scripts to conduct principal component analysis (PCA) and to generate single gene expression pattern plots.

1. This first section which includes Trimming and mapping was run in a linux based cluster environment.

# This code requires:

# Trimmomatic (V. 0.36)

# Read files should be formatted as such:

# prefix\_R#\_001.fastq.gz

# The only argument needed for this script is the unique sample ID

prefix="\$1"

# create an initial fastqc report of paired end reads

# This will be used to determine the effectiveness of the trimming

echo "initial fastqc report"

fastqc -o . -t 2 rawdata.Body2/"\$prefix"\_L001\_R\*.fastq.gz

mv "\$prefix"\_L001\_R1\_001\_fastqc.html "\$prefix"\_R1\_unfiltered\_fastqc.html

mv "\$prefix"\_L001\_R2\_001\_fastqc.html "\$prefix"\_R2\_unfiltered\_fastqc.html

mv "\$prefix"\_L001\_R1\_001\_fastqc.zip "\$prefix"\_R1\_unfiltered\_fastqc.zip

mv "\$prefix"\_L001\_R2\_001\_fastqc.zip "\$prefix"\_R2\_unfiltered\_fastqc.zip

#Remove adapter sequences and stretches of low-quality base calls

#It also sets the minimum read size to 32

echo "filtering reads"

java -jar \$TRIMMOMATIC\_HOME/trimmomatic-0.36.jar PE -threads 16 -phred33 \

rawdata.Body2/"\$prefix"\_L001\_R1\_001.fastq.gz rawdata.Body2/"\$prefix"\_L001\_R2\_001.fastq.gz \

```

data.trimmed.Body/"$prefix"_R1_P.fastq.gz data.trimmed.Body/"$prefix"_R1_UP.fastq.gz \
data.trimmed.Body/"$prefix"_R2_P.fastq.gz data.trimmed.Body/"$prefix"_R2_UP.fastq.gz \
ILLUMINACLIP:rawdata/TruSeq3-PE.fa:2:30:10 LEADING:3 TRAILING:3 \
SLIDINGWINDOW:4:15 MINLEN:32
rm data.trimmed.Body/"$prefix"_R2_UP.fastq.gz
# concatenate all data for R1 including reads not found in R2
cat data.trimmed.Body/"$prefix"_R1_P.fastq.gz data.trimmed.Body/"$prefix"_R1_UP.fastq.gz > \
    data.trimmed.Body/"$prefix"_R1_A.fastq.gz
# create a fastqc report of filtered reads
# you should see: high phred scores across the entire
# read length, a read length distribution that reflects
# expected length for RNA-seq data and no overrepresented sequences
# looking at the GC distribution can also give you a
# sense of bacterial contamination (curvibacter GC% is around 60)
echo "post-filtering fastqc report"
fastqc -o . -t 2 data.trimmed.Body/"$prefix"_R1_A.fastq.gz
# Maps reads to the reference H. oligactis transcriptome
# and count the number of aligned reads per transcript
echo "mapping reads"
# Rsem will output a genes.results file that will be used downstream to
# generate the read count matrix
rsem-calculate-expression --num-threads 16 --no-bam-output \
    --bowtie2 \
    data.trimmed.Body/"$prefix"_R1_A.fastq.gz \
    reducedRef/HoCR "$prefix"_RNA_A
#rm "$prefix"*isoforms.results
#rm -r "$prefix"*.stat
echo "done"
#once we have the gene.results output we can turn them into a matrix
rsem-generate-data-matrix *.genes.results > expressionMatrix.txt

2. The following code is intended for use in R programming. The initial section of the script requires the
count matrix generated above as input. It then creates a DGE object, normalizes the counts, and adds
annotations. Subsequently, there are scripts for generating principal component analysis (PCA) and
producing single gene expression pattern plots.
#Last edited on 27_July2024
library(edgeR)

```

```

library(limma)
library(rstudioapi)
library(ggfortify)
library(readxl)
library(ggfortify)
library(ggplot2)
library(ggpubr)
library(cluster)

setwd("D:/SergioZoom/ZoomMeetings/Annotations")
#setwd("C:/Users/Sahar/Desktop/R")

pval <- 5e-2
foldChange <- 0

counts = read.table("innsbruckMatrix.txt", header=TRUE, row.names = 1, sep="\t", comment.char="")
colnames(counts) = c("FR_OH_1", "FR_OH_2", "FR_OH_3", "FR_OH_4", "HR_OH_1",
  "HR_OH_2", "HR_OH_3", "HR_OH_4",
  "FR_3H_1", "FR_3H_2", "FR_3H_3", "FR_3H_4", "HR_3H_1", "HR_3H_2",
  "HR_3H_3", "HR_3H_4",
  "AL_3H_1", "AL_3H_2", "AL_3H_3", "AL_3H_4", "FR_12H_1", "FR_12H_2",
  "FR_12H_3", "FR_12H_4",
  "HR_12H_1", "HR_12H_2", "HR_12H_3", "HR_12H_4", "AL_12H_1", "AL_12H_2", "AL_12H_3",
  "AL_12H_4",
  "FR_48H_1", "FR_48H_2", "FR_48H_3", "FR_48H_4", "HR_48H_1", "HR_48H_2",
  "HR_48H_3", "HR_48H_4",
  "AL_48H_1", "AL_48H_2", "AL_48H_3", "AL_48H_4", "FOOT_ON1", "FOOT_ON2",
  "FOOT_ON3", "FOOT_ON4",
  "HEAD_ON1", "HEAD_ON2", "HEAD_ON3", "HEAD_ON4")

counts[c("FR_OH_1", "HR_OH_1", "FR_3H_2", "HR_3H_3", "FR_12H_3", "HR_12H_4", "AL_12H_1", "HR_48H_2")] <-
NULL

counts = counts[!grepl("i", colnames(counts))]
#Add IDs as column
counts$ID <- rownames(counts)
#define treatment groups
factor1 = substr(colnames(counts[1:length(counts)-1]), 1, 1)
factor2 = substr(colnames(counts[1:length(counts)-1]), 4, 4)

```

```

grp = interaction(factor1,factor2)
#Call groups and build a DGE object
#Extract group names leaving the replicate number out
grp = sub(".$", "", colnames(counts[1:length(counts)-1]))
#define treatment groups
# grp <- factor(c(rep("F0", 4), rep("H0", 4),
#       rep("F3", 4), rep("H3", 4), rep("AL3", 4),
#       rep("F12", 4), rep("H12", 4), rep("AL12", 4),
#       rep("F48", 4), rep("H48", 4), rep("AL48", 4),
#       rep("foot",4), rep("head",4)))
#Create the DGE object
dge <- DGEList(counts = counts[,1:(ncol(counts)-1)], group = grp, genes = counts$ID)
#exclude lowly expressed genes from the analysis
#(at least six samples have at least two counts per million)
keep <- rowSums(cpm(dge)>=2) >= 3
dge <- dge[keep, , keep.lib.sizes=F]
#calculate normalization factors for each sample
normdge <- calcNormFactors(dge)
normdge = estimateCommonDisp(normdge)
normdge$common.dispersion
#Define groups for DGE analysis
design <- model.matrix(~0+grp, data = normdge$samples)
colnames(design) <- gsub("grp","",colnames(design))
#calculate differential gene expression
normdge <- estimateGLMRobustDisp(normdge,design)
fit <- glmFit(normdge, design, robust = T)
#define pairwise comparisons to be used
my.contrasts <- makeContrasts(
  F3vF0 = `FR_3H` - `FR_0H`,
  F12vF0 = `FR_12H` - `FR_0H`,
  F48vF0 = `FR_48H` - `FR_0H`,
  F48vF12 = `FR_48H` - `FR_12H`,
  F48Vfoot= `FR_48H` - `FOOT_O`,
  H3vH0 = `HR_3H` - `HR_0H`,
  H12vH0 = `HR_12H` - `HR_0H`,

```

```

H48vH0 = `HR_48H` - `HR_OH`,
H48vH12 = `HR_48H` - `HR_12H`,
H48vhead = `HR_48H` - `HEAD_O`,
ALP3vALP0 = `AL_3H` - `FR_OH`,
ALP12vALP0 = `AL_12H` - `FR_OH`,
ALP48vALP0 = `AL_48H` - `FR_OH`,
ALP48vALP12 = `AL_48H` - `AL_12H`,
ALP48vfoot = `AL_48H` - `FOOT_O`,
HR0vFR0 = `HR_OH` - `FR_OH`,
HR3vFR3 = (`HR_3H` - `HR_OH`) - (`FR_3H` - `FR_OH`),
HR12vFR12c0 = (`HR_12H` - `HR_OH`) - (`FR_12H` - `FR_OH`),
HR48vFR48c0 = (`HR_48H` - `HR_OH`) - (`FR_48H` - `FR_OH`),
HR3vAR3 = (`HR_3H` - `HR_OH`) - (`AL_3H` - `FR_OH`),
HR12vAR12c0 = (`HR_12H` - `HR_OH`) - (`AL_12H` - `FR_OH`),
HR48vAR48c0 = (`HR_48H` - `HR_OH`) - (`AL_48H` - `FR_OH`),
FR3vAR3 = (`FR_3H` - `FR_OH`) - (`AL_3H` - `FR_OH`),
FR12vAR12c0 = (`FR_12H` - `FR_OH`) - (`AL_12H` - `FR_OH`),
FR48vAR48c0 = (`FR_48H` - `FR_OH`) - (`AL_48H` - `FR_OH`),
FootvHead = `FOOT_O` - `HEAD_O`,
FootvF0 = `FOOT_O` - `FR_OH`,
FootvH0 = `FOOT_O` - `HR_OH`,
HeadvF0 = `HEAD_O` - `FR_OH`,
HeadvH0 = `HEAD_O` - `HR_OH`,
levels=design)

```

```
# glmTreat instead of glmQLFTest
```

```
# This method does a binomial regression over fold change values, and calculates an FDR directly onto #the
differences in FC that genes might have at any expression levels. This tends to give more #importance to the real
#differences in expression, while still taking into account variability.
```

```
#We will use this function to make our pairwise comparisons
```

```
#function to generate the results objects for a named pairwise comparison
```

```
fc = 0
```

```
fdr = 5e-2
```

```
genResTables <- function(x,fdr,fc) {
```

```
  #pull full results
```

```
  y <- glmTreat(fit, contrast = my.contrasts[,x], lfc = log2(1.2), null = "interval")
```

```

y <- topTags(y, n=Inf)
#extract results table
z <- y$table
z$ID <- rownames(z)
z <- merge(z,annotations, by= "ID", all.x = T)
#z <- na.omit(z)
#z$FDR <- p.adjust(z$PValue, method = "BH")
#significance filters
z.DG <- z[z$FDR <= fdr,]
z.DG.up <- z.DG[z.DG$logFC > fc,]
z.DG.down <- z.DG[z.DG$logFC < -fc,]
#return the four results objects
return(list(z,z.DG,z.DG.up,z.DG.down))
#return(list(z))
}

full.annotation <- read.csv("fullannotation.csv", stringsAsFactors = F, row.names = 1)
annotationsA <- read.csv("annotationsA.csv", stringsAsFactors = F, row.names = NULL)
annotateHuman <- read.csv("oligToHSedit.csv", stringsAsFactors = F, row.names = NULL)
oligToDove <- read.csv("oligToDove.csv", stringsAsFactors = F, row.names = NULL)
oligToDove$ID <- oligToDove$H_oligactisCR
oligToDove["H_oligactisCR"] <- NULL
annotations <- merge(annotationsA, annotateHuman, by = "ID", all.x = T)
annotations <- merge(annotations, oligToDove, by = "ID", all.x = T)
annotations <- merge(annotations, full.annotation, by = "H_vulgaris105", all.x = T)
#PARIWISE COMPARISONS
#generate all results objects
#systematize object naming based on contrast names
for (i in 1:length(colnames(my.contrasts))) {
  resList <- genResTables(colnames(my.contrasts)[i],fdr,fc)
  assign(colnames(my.contrasts)[i], resList[[1]])
  assign(paste0(colnames(my.contrasts)[i],".DG"), resList[[2]])
  assign(paste0(colnames(my.contrasts)[i],".DG.up"), resList[[3]])
  assign(paste0(colnames(my.contrasts)[i],".DG.down"), resList[[4]])
}
#Save Results so Far!!

```

```

#We also want to save the normalized counts (for plotting purposes)
normalizedCounts <- as.data.frame(cpm(normdge, normalized.lib.sizes=T, log = T))
normalizedCounts$ID <- rownames(normalizedCounts)
normCounts <- merge(normalizedCounts, annotations, by="ID", all.x = T)
write.csv(normCounts, file = "Results.innsbruck.treat/normCountsinnbrucktreat_27July2024.csv")
#save results dataframes, the DGE object, Annotations, and the normalized counts
#save results from all contrasts
saveThese <- ls(pattern = paste(colnames(my.contrasts),collapse = " | "))
# save gene lists in a csv (more git friendly format)
lapply(saveThese, function(x) write.csv(eval(parse(text=x)), file = paste0("Results.innsbruck.treat/", "", x, ".csv")))
#save contrasts table to help make sense of file names
write.csv(my.contrasts, file = "Results.innsbruck.treat/contrasts_27July2024.csv")
saveThese <- c(saveThese,"normdge","normalizedCounts")

save(file = "Results.innsbruck.treat/RNA_DGE_27July2024.RData", list = saveThese)
#####

3. This section includes calculation and plotting o a Principal Component Analysis
# Start PCA script here
#pca libraries are found on top.
#install.packages(ggfortify)
normalizedCounts <- as.data.frame(cpm(normdge, normalized.lib.sizes=T, log = T))
normalizedCounts[,c("HR_0H_2","HR_0H_3","HR_0H_4","HR_3H_1","HR_3H_2","HR_3H_4","HR_12H_1","HR_12
H_2","HR_12H_3","HR_48H_1","HR_48H_3","HR_48H_4","HEAD_ON1","HEAD_ON2","HEAD_ON3","HEAD_ON4",
"AL_3H_1","AL_3H_2","AL_3H_3","AL_3H_4","AL_12H_2",
"AL_12H_3","AL_12H_4","AL_48H_1","AL_48H_2","AL_48H_3","AL_48H_4")] <- NULL
reg.pca <- t(normalizedCounts)
tmp <- normalizedCounts
reg.pca <- prcomp(reg.pca, center = T, scale = F)
autoplot(reg.pca, label=T, x = 1, y = 2)
aa<-t(normalizedCounts[,])
#Create new grp with the indicated number of samples
tmp1 = substr(colnames(normalizedCounts[1:length(normalizedCounts)]), 1, 1)
tmp2 = substr(colnames(normalizedCounts[1:length(normalizedCounts)]), 4, 4)
grp2 = interaction(tmp1,tmp2)
aa<-cbind(aa,grp2)
pdf("PCA_innsbruckFR_27July2024.pdf", width = 8.3, height = 8.3)

```

```

p <- autoplot(reg.pca, label=T, x = 1, y = 2, data=aa, colour="grp2", size=4)
g.load <- reg.pca$rotation
s.load <- reg.pca$x
pc1 <- scale(s.load[,1], center = T, scale = T)
p <- p + theme_pubr(base_size = 2, legend=c("bottom"))
print(p)
dev.off()

#end PCA script here

#####

#Recover sets of genes affecting PC1 and 2 FR
#PC3.genes <- data.frame(sort((reg.pca$rotation[, "PC1"]), decreasing=TRUE)[1:100])
#PC4.genes <- data.frame(sort((reg.pca$rotation[, "PC2"]), decreasing=TRUE)[1:100])
#PC3.genes$ID <- rownames(PC1.genes)
#PC4.genes$ID <- rownames(PC2.genes)
#PC3.genes <- merge(PC1.genes, annotations, by = "ID", all.x=T)
#PC4.genes <- merge(PC2.genes, annotations, by = "ID", all.x=T)
#write.csv(PC3.genes, file="PC3_FR_25July2024.csv")
#write.csv(PC4.genes, file="PC4_FR_25July2024.csv")

```

#### 4. Single gene expression pattern plotting

```

library(dplyr)
library(stringr)
library(ggpubr)
library(ggplot2)
gene.OLI <- c("R031122c0g1_i02")
Input = "mean.Counts.OLI.csv"
mean.Counts.OLI <- read.csv(Input, sep = ",", header = TRUE, stringsAsFactors = FALSE, row.names = NULL)
Input = "all.Counts.OLI.csv"
all.Counts.OLI <- read.csv(Input, sep = ",", header = TRUE, stringsAsFactors = FALSE, row.names = NULL)
Input = "mean.Counts.AEP.csv"
mean.Counts.AEP <- read.csv(Input, sep = ",", header = TRUE, stringsAsFactors = FALSE, row.names = NULL)
Input = "all.Counts.AEP.csv"
all.Counts.AEP <- read.csv(Input, sep = ",", header = TRUE, stringsAsFactors = FALSE, row.names = NULL)
#Filter for gene of interest in H. oligactis foot regeneration counts
mean.gene.Counts.OLI <- mean.Counts.OLI %>%
  filter(str_detect(ID, paste(gene.OLI, collapse = "|")))
all.gene.Counts.OLI <- all.Counts.OLI %>%
  filter(str_detect(ID, paste(gene.OLI, collapse = "|")))
#Filter for gene of interest in H. vulgaris foot regeneration counts
mean.gene.Counts.AEP <- mean.Counts.AEP %>%
  filter(str_detect(ID, paste(mean.gene.Counts.OLI$H_vulgaris105, collapse = "|")))
all.gene.Counts.AEP <- all.Counts.AEP %>%
  filter(str_detect(ID, paste(mean.gene.Counts.OLI$H_vulgaris105, collapse = "|")))
#Create matrix with H. oligactis gene expression data by column for plotting

```

```

tmp.OLI <- as.data.frame(t(mean.gene.Counts.OLI[,2:16])); colnames(tmp.OLI) <- c("counts");
tmp.OLI$time <- rep(c(0,3,12,24,48),3);
tmp.OLI$group<-c(rep("FR",5),rep("ALP",5),rep("HR",5))
#Create matrix with AEP gene expression data by column for plotting
tmp.AEP <- as.data.frame(t(mean.gene.Counts.AEP[,2:9])); colnames(tmp.AEP) <- c("counts");
tmp.AEP$time <- rep(c(0,3,8,12),2);
tmp.AEP$group<-c(rep("FR",4),rep("HR",4))
#Include all replicates for dot plots H. oligactis
tmpcol.OLI <- as.data.frame(t(all.gene.Counts.OLI[,2:55])); colnames(tmpcol.OLI) <- c("counts");
tmpcol.OLI$time <- rep(c(0,0,0,0,0,0,3,3,3,12,12,12,24,24,24,48,48,48),3)
tmpcol.OLI$group<-c(rep("FR",18),rep("ALP",18),rep("HR",18))
#Including all replicates for dot plots H. vulgaris
tmpcol.AEP <- as.data.frame(t(all.gene.Counts.AEP[,2:25])); colnames(tmpcol.AEP) <- c("counts");
tmpcol.AEP$time <- rep(c(0,0,0,3,3,3,8,8,8,12,12,12),2)
tmpcol.AEP$group<-c(rep("FR",12),rep("HR",12))
#time (hours) labels
p1 <- ggplot(data=tmp.OLI, aes(x=time,y=counts, color=group)) + geom_line(show.legend = FALSE, size = 0.8) +
  scale_color_manual(values = c("#00FF99","blue","red")) +
  theme_pubr(legend = "none") +
  ggtitle(c(mean.gene.Counts.OLI$G_Names)) +
  # ylim(-3.5,6) +
  xlab(NULL) + ylab(NULL) + scale_x_continuous(breaks = c(0,3,12,24,48))
p2 <- ggplot(data=tmp.AEP, aes(x=time,y=counts, color=group)) + geom_line(show.legend = FALSE, size = 0.8) +
  scale_color_manual(values = c("blue","red")) +
  theme_pubr(legend = "none") +
  ggtitle(c("H. vulgaris",mean.gene.Counts.AEP$ID)) +
  xlab(NULL) + ylab(NULL) + scale_x_continuous(breaks = c(0,3,8,12))
#Now we add on top of the plots geom_points to represent variation in biological replicates
p1 <- p1 + geom_point(data=tmpcol.OLI, aes(color=group, shape = ".", size = .5))
p2 <- p2 + geom_point(data=tmpcol.AEP, aes(color=group, shape = ".", size = .5))
p3 <- ggplot(data=tmp.AEP, aes(x=time,y=counts, color=group, legend = "right")) + geom_line() +
  scale_color_manual(values = c("#0000FF", "#00FF99", "red")) +
  theme(legend.text = element_text(size = 12), legend.title = element_text(size = 12))
legnd<-get_legend(p3)
#pdf("classgenes.swiss.pdf", width = 7, height = 4.5)
p4 <- ggarrange(p1,p2, ncol = 2, nrow=1)
p4
#dev.off()

```

## Supplementary files-2:

| Primer Name  | Sequence                               | length | Tm   |
|--------------|----------------------------------------|--------|------|
| jun.Fw2      | AAACGGAGGTCGATCAGCAG                   | 20     | 57.6 |
| jun.Rv2      | TGTCCTGTGCATCCAAATCG                   | 21     | 57   |
| jun.Rv2.T7   | TAATACGACTCACTATAGTGTCCTGTGCATCCAAATCG | 39     | 64.5 |
| fos.Fw1      | TGCACCAAGAAGTACCTAACCA                 | 22     | 56.9 |
| fos.Rv1      | CGATACTGCGGTTTCCTGC                    | 20     | 57   |
| fos.Rv1.Sp6  | ATTTAGGTGACACTATAGCGATACTTGCGGTTTCCTGC | 38     | 64.3 |
| cr3l.Fw2     | GTCTCAACTTCGTCTCCAGCT                  | 21     | 56.9 |
| cr3l.Rv2     | CCCTCTTCTCCAAGGCTTCC                   | 20     | 56.9 |
| cr3l.Rv2.T7  | TAATACGACTCACTATAGCCCTCTTCTCCAAGGCTTCC | 38     | 63.4 |
| w9/10cFW_3   | AACACGGAATGGATTCTTCG                   | 20     | 53.3 |
| w9/10cRv_3   | GCTGACAGGTCTTGCAATCA                   | 20     | 55.6 |
| T7w9/10cRv_3 | TAATACGACTCACTATAGGCTGACAGGTCTTGCAATCA | 38     | 64.3 |
| wnt3FW_1     | AACTGTGGATGGCTCTTGGG                   | 20     | 57.5 |
| wnt3RV_1     | GTTGCAGTTTCCCGACGTTT                   | 20     | 56.9 |
| SP6wnt3RV_1  | ATTTAGGTGACACTATAGGTTGCAGTTTCCCGACGTTT | 38     | 64.1 |
| sp6 promoter | ATTTAGGTGACACTATAG                     | -      | -    |
| T7 promoter  | TAATACGACTCACTATAG                     | -      | -    |

### Supplementary files-3:

|                     | ID        | F3v0  | F8v0  | F12v0 | F48v0 | ALP3v0 | ALP12v0 | ALP48v0 | H3v0 | H8v0  | H12v0 | H48v0 |
|---------------------|-----------|-------|-------|-------|-------|--------|---------|---------|------|-------|-------|-------|
| <i>H. oligactis</i> | g26102    | 0.12  | NA    | 0.14  | 0.21  | 0.67   | 1.38    | 1.38    | 0.07 | NA    | 0.45  | 0.27  |
| <i>H. oligactis</i> | g26102    | 0.17  | NA    | 0.06  | 0.12  | -0.06  | 2.46    | 2.46    | 0.06 | NA    | 0.05  | 0.22  |
| <i>H. oligactis</i> | g11553    | 0.79  | NA    | 0.99  | 0.59  | 0.93   | 2.80    | 2.80    | 0.35 | NA    | 1.07  | 0.79  |
| <i>H. oligactis</i> | g19382    | -0.05 | NA    | -0.58 | -0.16 | -0.58  | 2.63    | 2.63    | 0.14 | NA    | -0.89 | 0.61  |
| <i>H. oligactis</i> | g11553(2) | 0.18  | NA    | 0.63  | 0.60  | 0.61   | 5.49    | 5.49    | 0.13 | NA    | 0.84  | 0.38  |
| <i>H. vulgaris</i>  | g11553    | 0.05  | -0.05 | 0.13  | NA    | NA     | NA      | NA      | 0.12 | -0.04 | -0.31 | NA    |

**F3v0:** The fold change value of contrasted 3hpa versus 0hpa foot regenerating tip.

**F8v0:** The fold change value of contrasted 8hpa versus 0hpa foot regenerating tip.

**F12v0:** The fold change value of contrasted 12hpa versus 0hpa foot regenerating tip.

**F48v0:** The fold change value of contrasted 48hpa versus 0hpa foot regenerating tip.

**ALP3v0:** The fold change value of contrasted 3hpa versus 0hpa ALP treated foot regenerating tip.

**ALP12v0:** The fold change value of contrasted 12hpa versus 0hpa ALP treated foot regenerating tip.

**ALP48v0:** The fold change value of contrasted 48hpa versus 0hpa ALP treated foot regenerating tip.

**H3v0:** The fold change value of contrasted 3hpa versus 0hpa head regenerating tip.

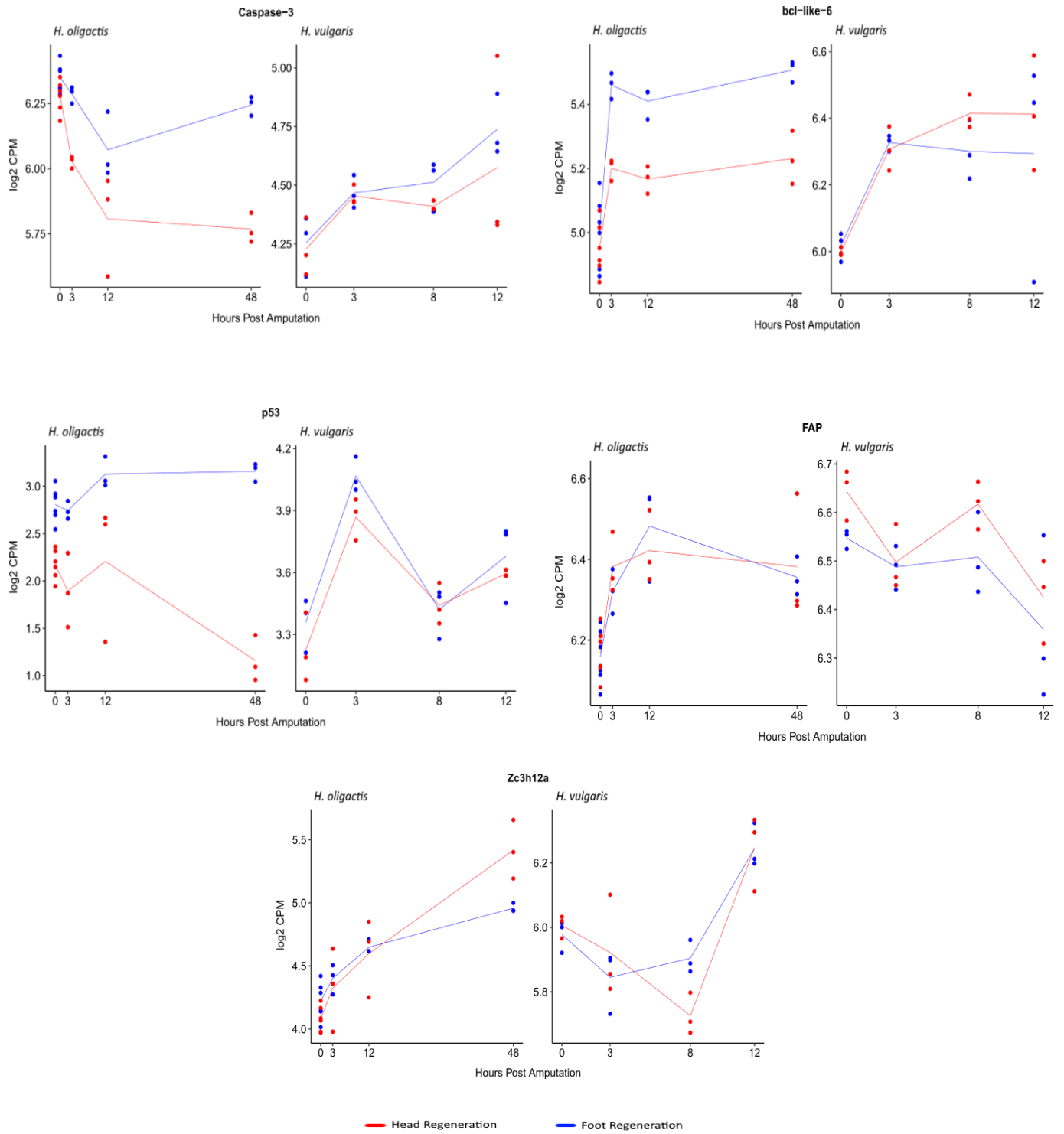
**H8v0:** The fold change value of contrasted 8hpa versus 0hpa head regenerating tip.

**H12v0:** The fold change value of contrasted 12hpa versus 0hpa head regenerating tip.

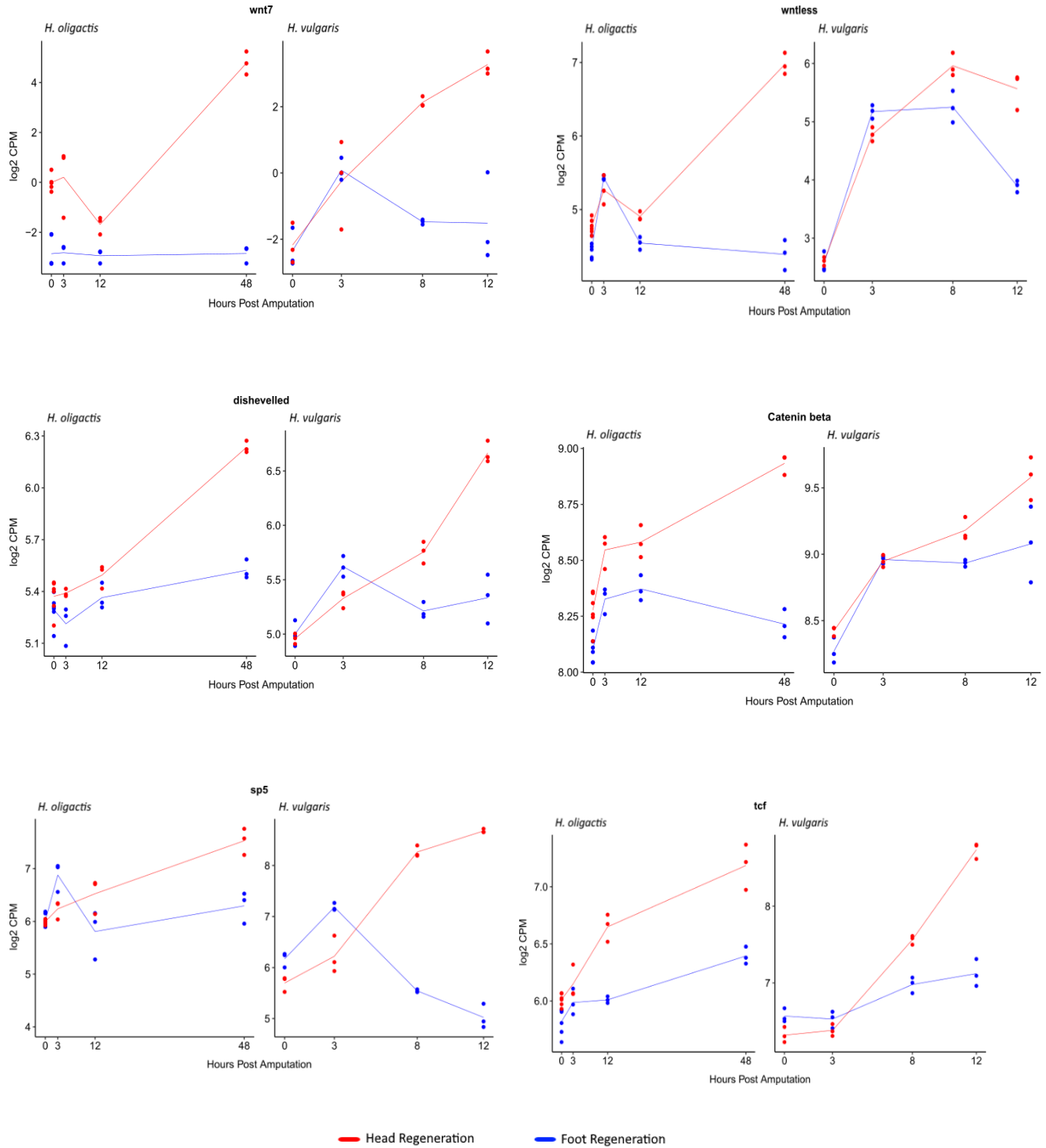
**H48v0:** The fold change value of contrasted 48hpa versus 0hpa head regenerating tip.

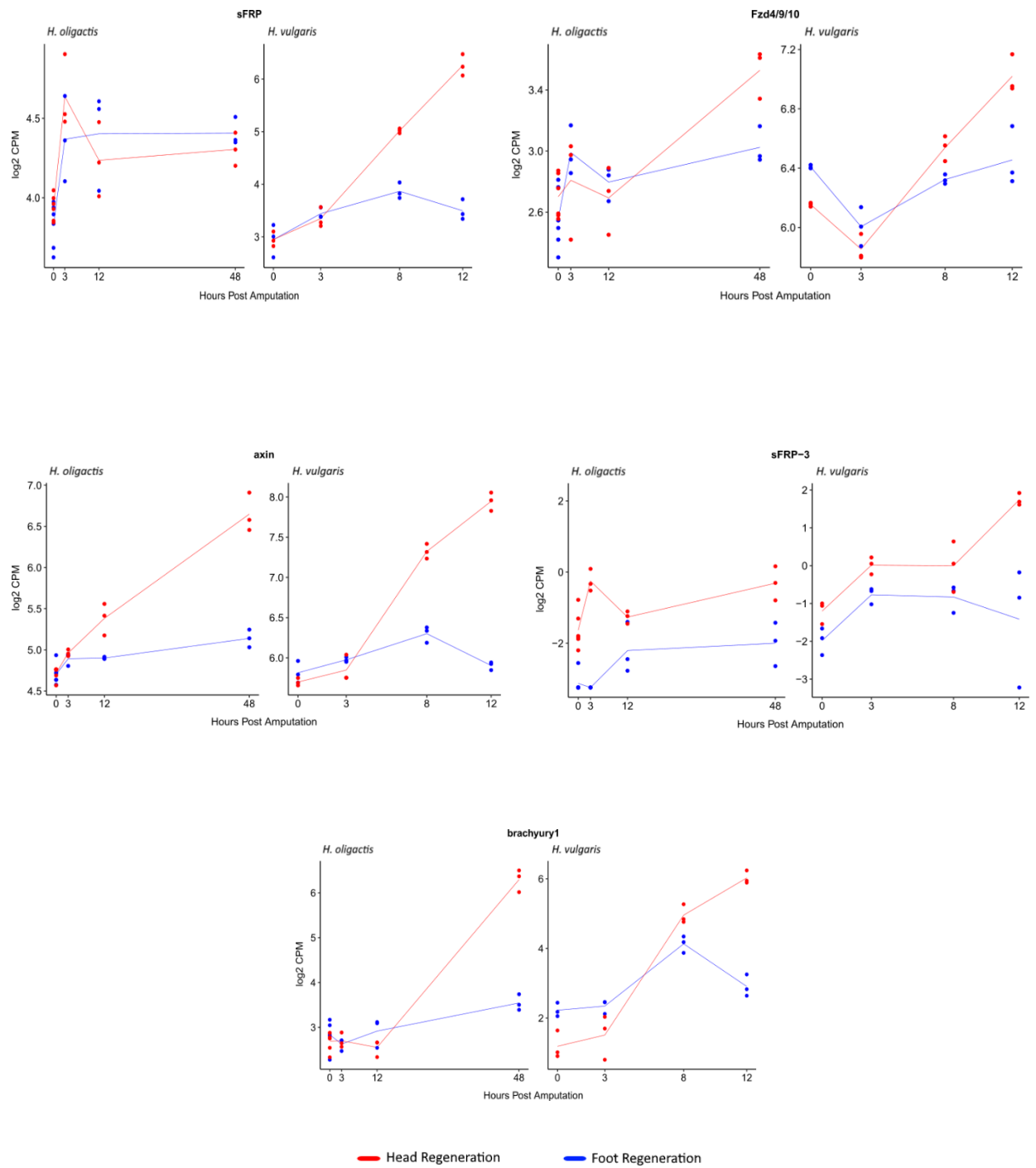
**NA:** Not Available

Supplementary Figure 1-3

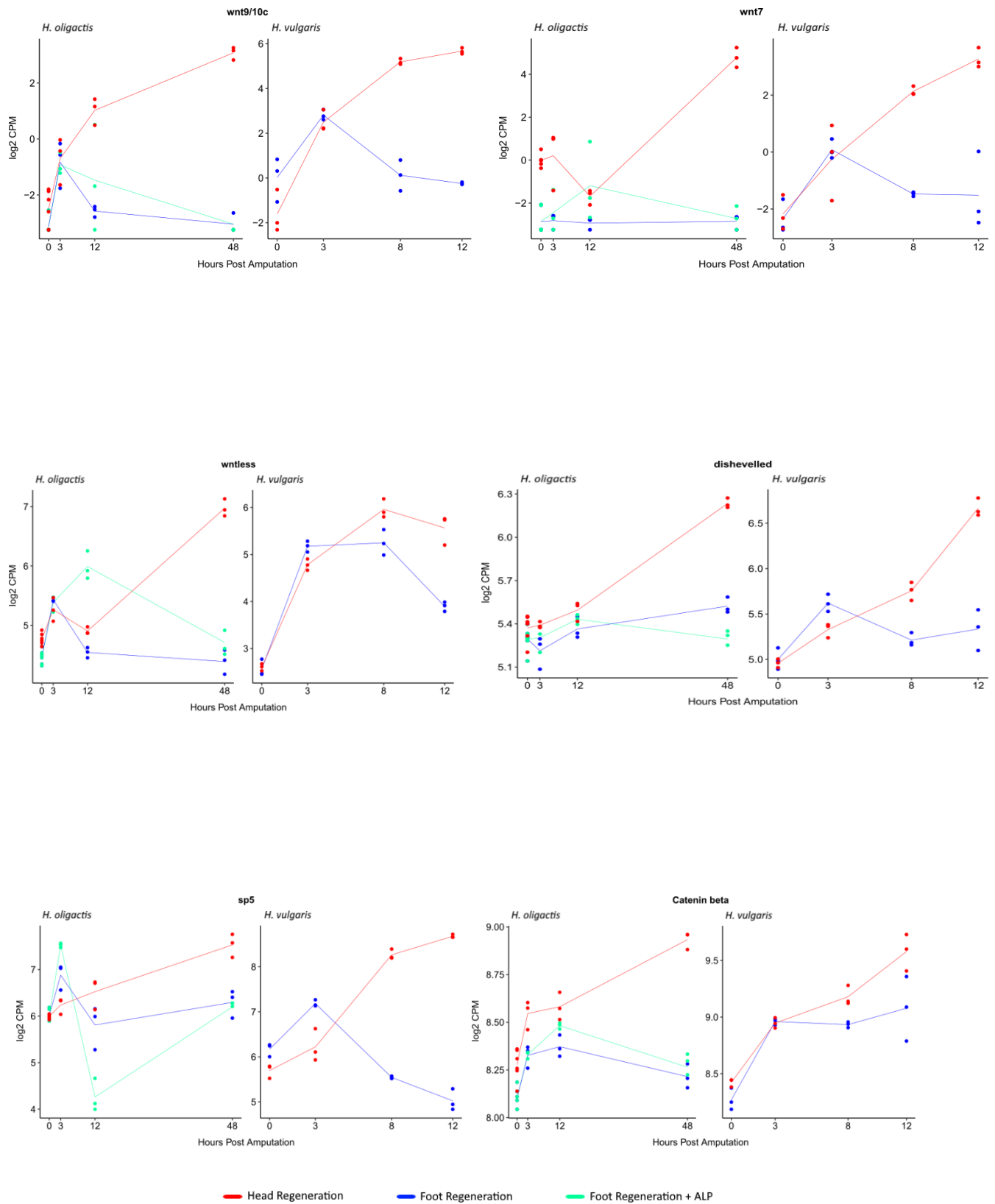


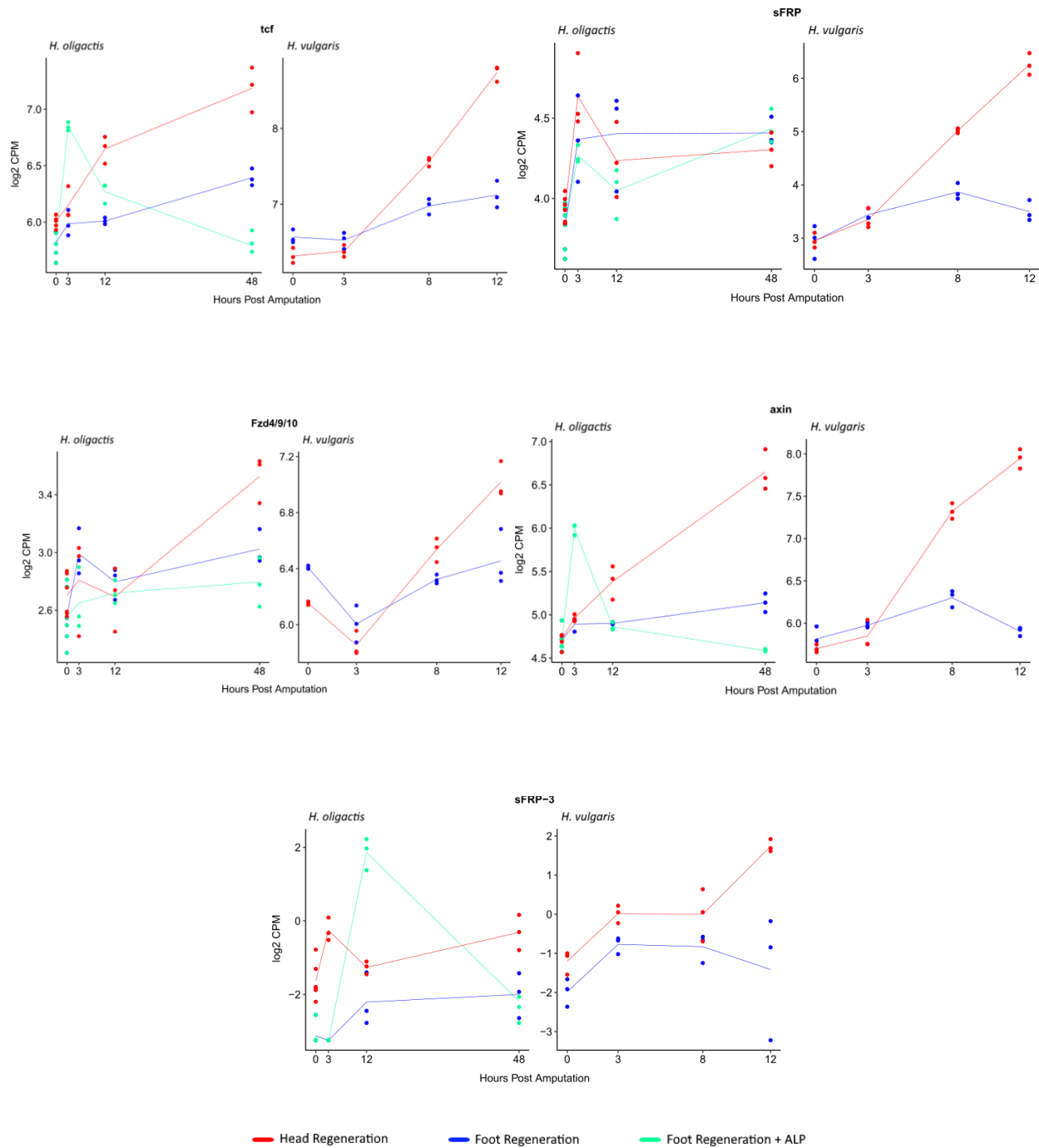
**Supplementary Figure 1:** RNA expression plots depicting average normalized RNA-seq counts per million for *caspase-3*, *bcl-like-6*, and *p53*, prolyl endopeptidase FAP, and E3 ubiquitin-protein ligase (*ZC3H12A*) in log<sub>2</sub> counts per million (log<sub>2</sub>CPM) during head and foot regeneration time course (0, 3, 12, and 48 hpa) in *H. oligactis* and *H. vulgaris*.





**Supplementary Figure 2:** RNA expression plots depicting average normalized RNA-seq counts per million for *wnt7*, *wntless*, *dishevelled*, *beta-catenin*, *sp5*, *TCF*, *sFRP(fz8)*, *frizzled4/9/10*, *axin*, *sFRP3*, and *Brachyury1*, during head and foot regeneration time courses (0, 3, 12, and 48 hpa) in *H. oligactis* and *H. vulgaris*.





**Supplementary Figure 3:** RNA expression plots depicting average normalized RNA-seq counts per million for *wnt9/10c*, *wnt7*, *wntless*, *dishevelled*,  $\beta$ -catenin, *sp5*, *TCF*, *sFRP(fz8)*, *frizzled4/9/10*, *sFRp3*, and *axin* during head and foot regeneration time courses (0, 3, 12, and 48 hpa) in DMSO and ALP treated *H. oligactis* and *H. vulgaris*.