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The Hydra model of Wnt-based regeneration

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The *Hydra* model: disclosing an apoptosis-driven generator of Wnt-based regeneration

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The *Hydra* model system is well suited to decipher the mechanisms underlying adult regeneration, opening the possibility to characterize those that have been robust enough to be maintained across evolutionary time. The detailed analysis of the activation of the Wnt-βcatenin pathway in bisected *Hydra* actually shows that the route taken to regenerate a structure as complex as the head dramatically varies according to the amputation level. When decapitation induces a direct re-development thanks to Wnt3 signaling from the epithelial cells, head regeneration after mid-gastric section relies first on Wnt3 signaling from the interstitial cells that undergo apoptosis-induced compensatory proliferation and secondarily activate Wnt3 signaling in the epithelial cells. The relative distribution between stem cells and head progenitor cells is strikingly different in these two contexts indicating that the pre-amputation homeostatic conditions define and constrain the route that bridges wound healing to the re-development program of the missing structure.

A need for new model systems to tackle the principles of regeneration

The variability of regeneration along the individual life cycle and in animal phyla

Regenerative potentials in the animal kingdom are not at all uniformly distributed among the different phyla and are even highly variable between closely related species [1, 2]. Moreover, in the same individual, the regenerative potential dramatically varies according to its developmental stage, sexual maturity and age. If we follow the typical sequence of life stages, the earliest regenerative processes after fertilization correspond to 'embryonic regulation', often leading to full body regeneration, as characterized initially in sea urchin embryos and later in *Xenopus*, chick and mammalian embryos. Later, at larval stages, many species such as insects (cricket, *Drosophila*) or amphibian tadpoles can regenerate organs and appendages. After hatching or birth, juvenile organisms (that, by definition, have not yet reached sexual maturity) often exhibit some regenerative potential – an example being human young children that can regenerate distal phalanges [3]. Finally in adulthood – that is, once sexual maturity is reached – the number of species that can replace complex structures is pretty limited, although widely spread among all phyla (see **Box 1**).

Adult regeneration can actually be subdivided into two classes, paedomorphic when sexual and somatic developments are uncoupled in time (see **Glossary**), or truly adult when sexual and somatic developments are kept tightly linked. As a general rule, the regenerative potential is usually higher when somatic development is not fully achieved. However, even when unable to regenerate complex structures requiring restoration of a functional tridimensional shape (usually relying on blastema formation and

Glossary

Apical head regeneration: regeneration of the *Hydra* head that is initiated in the upper body column where decapitation takes place.

Apoptosis-induced compensatory proliferation: The capacity for cells undergoing apoptosis to promote the proliferation of the neighboring cells by releasing growth factors.

Basal head regeneration: regeneration of the *Hydra* head that is initiated in the mid-gastric body column where bisection is performed.

Blastema: Transient structure formed during regeneration characterized by a localized and time-limited massive proliferation of progenitor cells, with self-organizing activity. Blastemas do not form in mammals after appendage or organ amputation.

Canonical Wnt pathway: this pathway is activated by the binding of Wnt signals to the Frizzled receptor, leading to the activation of Dishevelled, the inactivation of the axin/GSK3/APC complex, the stabilization of β-catenin that can then reach the nucleus to interact with the TCF/LEF transcription factor.

Compensatory proliferation: cell proliferation induced upon injury or tissue damage to repair the altered structure.

Epimorphic regeneration: reestablishment upon amputation of a spatial structure with identical shape and function thanks to the formation of a blastema, a self-organizing system.

Hypostome: most apical region of the *Hydra* polyp, shaped as a dome that surrounds the mouth opening.

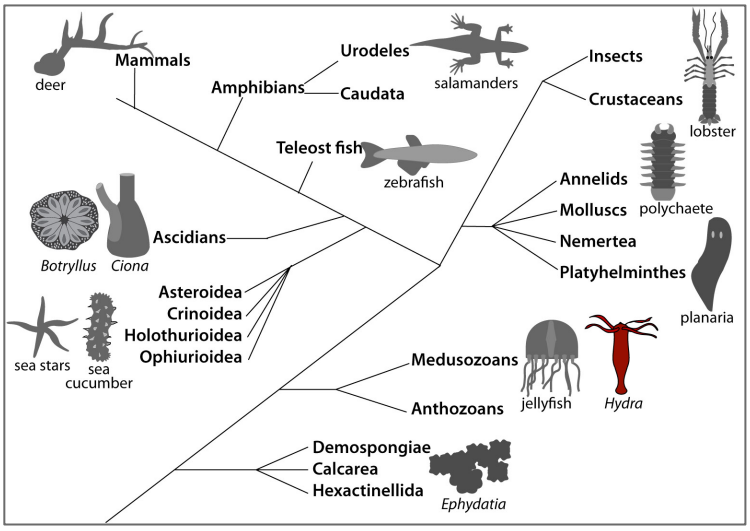
Morphallactic regeneration: reestablishment upon amputation of a spatial structure with identical shape and function in the absence of cell proliferation. Apical head regeneration provides a typical example of morphallaxis.

Non-canonical Wnt pathway: this pathway that activates Planar Cell Polarity or Ca2+ signaling, also involves Frizzled and Dishevelled but acts independently of β-catenin.

Paedomorphic: sexually mature organisms that exhibit juvenile traits as a consequence of either premature sexual development (progenesis) or delayed somatic development (neotenic). The regeneration potential is often higher in paedomorphic species, e.g. *Hydra* that never develops as a medusa, and axolotl that does not undergo metamorphosis provide examples of paedomorphic regeneration.

growth), most animal species exhibit cell renewal and tissue repair. All these processes rely on the combination of cell proliferation, cell death and cell differentiation but whereas tissue repair and regeneration are induced upon external forces (injury, tissue damage, amputation), cell renewal rather responds to an endogenous regulation [4].

The questions are then, what is common between all these regenerative processes? Would it be possible to identify some principles for adult regeneration – that is, when developmental processes are turned off? What model systems are best suited to uncover these principles? On the one hand, vertebrates do not provide an easy context to identify the core processes underlying regenerative processes because mammals do not form blastema upon injury and because vertebrate genomes are highly complex (having undergone several rounds of duplication), a situation that often leads to redundancy between paralogous genes. On the other hand, the two most popular invertebrate model systems, nematode and *Drosophila*, are not the most suitable for the study of adult regeneration as these organisms exhibit in adulthood no cell renewal or limited to the gut. Therefore with the aim of inducing organ regeneration in humans, there is a clear need for invertebrate model systems that highlight the principles of regeneration, and more specifically for those regulating adult regeneration.



Box 1: Phylogenetic tree showing the animal phyla that contain species with high regenerative potential. All species schematized here provide useful model systems to study the biology of adult stem cells and the mechanisms supporting adult regeneration [1, 2, 31, 57, 70]. By contrast Caudata (*Xenopus*) and Insects (*Drosophila*, cricket) only regenerate their appendages at the larval stage but provide valuable model systems to test the impact of extinguishing developmental processes on regenerative programs [73] and to dissect the genetic pathways involved in apoptosis-induced compensatory proliferation [58-60]

Strengths of the *Hydra* model system

Since the mid-eighteenth century, *Hydra* has been recognized as a popular animal model system to study body regeneration [5]. *Hydra* is a freshwater hydrozoan, which belongs to the Cnidaria, a phylum that, together with Ctenophora, is a sister group to Bilateria [6]. In contrast to most hydrozoans, *Hydra* lost the medusa stage and should therefore be considered as a paedomorphic organism. *Hydra* exhibits a simple anatomy, basically a tube shape with, at one extremity, a head region, formed of a dome (named hypostome) surrounding the unique mouth/anus opening and tentacles inserted at the base of this dome. At the other extremity, a basal disc (also named foot) secretes a mucous that helps the animal to attach to the substrate **(Figure 1A)**. Despite this simple anatomy, *Hydra* is already equipped to elaborate complex behaviors based on neuromuscular transmission [7]. Its two tissue layers contain a dozen cell types that correspond to the basic cell types shared by eumetazoans: typical epithelial cells that also differentiate myofibrils, gland cells that secrete digestive enzymes (also named 'pancreatic cells'), mucous cells, sensory-motor neurons and interneurons named ganglion cells [8]. In addition, cnidarians differentiate phylum-specific mechano-sensory cells named cnidocytes (or nematocytes) that resemble the bilaterian mechano-sensory cells thanks to their cnidocil, but also differentiate, as a phylum-specific trait, a venom capsule (the nematocyst or cnidocyst). Beside somatic differentiation, gametogenesis and sexual development share some common rules with bilaterian species [9, 10].

Adult *Hydra* possess the potential for multiple developmental fates **(Figure 1B)** and shares numerous features with the planaria *Schmidtea mediterranea*, another classical model system for the study of regeneration [11]. Indeed, in adulthood, both models show: *1) a continuous tissue replacement* due to a stock of mitotically active stem cells (unique in planarians – the neoblasts –, triple in *Hydra* – the ectodermal epithelial, endodermal epithelial and interstitial stem cells*; 2) a stock of adult pluripotent stem cells* that produce throughout the life of the animals both germ cells and somatic cells [12-15] (a rather special case in eumetazoans that usually segregate their germ cells during early embryonic development); *3) an efficient asexual reproduction* (budding in *Hydra,* fission in planaria); *4) the amazing property to regenerate* almost any missing part of the body after injury; *5) an apparent lack of aging*, at least as long as the animals are kept asexual [16-18].

Molecular and cellular tools to dissect the mechanisms underlying regeneration have been developed recently in both model systems. Among these, RNA interference (RNAi) obtained by feeding the animals with bacteria producing double-stranded RNAs, provides an easily amenable, incremental and harmless method (initially established in the nematode [19, 20]) to transiently silence gene expression in adult organisms [21-23]. Also in *Hydra* the recent possibility to establish transgenic strains allows investigators to follow live the cellular remodeling that takes place

after amputation [24-26]. Finally in both models, the cloning of targeted cDNAs together with the sequencing of ESTs and genomes definitively proved that the signaling pathways at work during programmed cell death [27-30] and development [31, 32] are highly conserved and active in these two species too. Therefore the analysis of the mechanisms that support regeneration in *Hydra* and planarians should help identify some principles of regeneration that are shared between cnidarians and bilaterians.

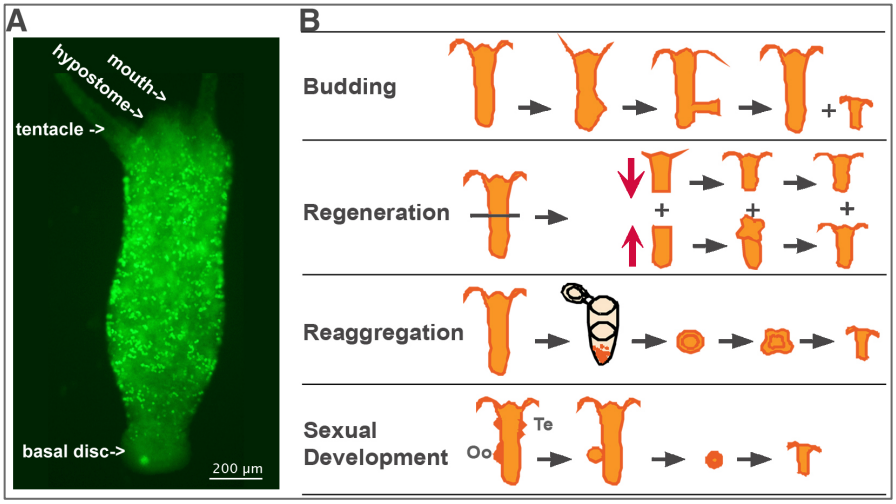


Figure 1: A) Hydra polyps show a high density of fast-cycling interstitial cells in their body column whereas no cycling activity is observed at the extremities. BrdU-positive cells were detected here after a 2 hours labeling. B) The various developmental potentials available in the adult polyp: Upon regular feeding, the animals undergo asexual reproduction through budding; as initially reported by A. Trembley (1744) [5], they regenerate any missing part after bisection of the body column performed at any level, and, as discovered more recently, these animals can also regenerate after tissue dissociation and reaggregation of the cells [40]. Finally, sexual development takes over when natural conditions (feeding, temperature) become too severe: the parental polyps before dying produce embryos that are protected by a thick cuticle. These survive up to the time when more favorable conditions (usually in the following Spring) allow them to conclude their development and hatch [9].

Work over the past 10 years has shown that *Hydra* relies on the activation of the Wnt-βcatenin pathway to regenerate its head [33-39]. However this activation dramatically varies according to the amputation level [39]: Indeed after decapitation *Wnt3* is rapidly and strongly up-regulated in the epithelial cells [33, 37], whereas after mid-gastric bisection the Wnt3 protein is first detected in the interstitial cells that undergo *apoptosis-induced compensatory proliferation* (see GLOSSARY) and subsequently *Wnt3* is up-regulated in the epithelial cells [37, 39]. This single or dual mode of Wnt-βcatenin activation in fact reflects two different processes for regenerating a head, *morphallactic* after decapitation, *epimorphic-like* after mid-gastric bisection (see GLOSSARY). Together with data obtained in *Drosophila* imaginal discs and in mouse adult tissues, these results suggest that 1) apoptosis-induced compensatory proliferation provides an evolutionarily-conserved mechanism to launch a regenerative response, 2) distinct regenerative routes may exist to reactivate the same structure-specific developmental process, 3) the cellular status at the time of injury provides significant constraints on the selection of the regenerative route that triggers this reactivation.

How to translate injury into a regenerative code ?

Plasticity of regeneration in *Hydra*

That the *Hydra* model system is highly amenable to address this question is supported by the vast cellular knowledge based on transplantation, cell-lineage and quantitative cellular analyses that has been accumulated over the past 40 years [8, 12, 40-45]. These studies identified the differentiation pathways of most cell types in homeostatic and regenerative conditions.

In addition, the molecular work performed over the past 20 years has started to dissect the genetic pathways at work during budding, regeneration and reaggregation as the canonical Wnt pathway for maintaining and re-establishing apical organizer activity [33-39], the non-canonical Wnt pathway for cellular evagination processes [38], the MAPK-CREB pathway for triggering head regeneration [46], the BMP/chordin pathway for axis patterning [47, 48], the FGF pathway for bud detachment [49], the Notch pathway for differentiating some interstitial cell lineages [50].

Following bisection at mid-gastric level, the two *Hydra* halves immediately initiate at the wound an asymmetric process whereby the upper half undergoes foot regeneration, a process that takes less than two days, whereas the lower half initiates a more complex process that leads to head regeneration in three days. Biochemical, cellular and molecular analyses indeed confirmed that these two regions that were adjacent and identical prior to mid-gastric bisection, immediately undergo dramatically different reorganizations to become foot- and head-regenerating tips, as evidenced by the analysis of phosphorylation patterns, kinase activities [46, 51] and cellular remodeling [39]. As a main feature, head regeneration from the mid-gastric level relies on apoptosis-induced compensatory proliferation, whereas no apoptosis and no cellular proliferation is observed in foot-regenerating tips.

But beside these two types of regeneration that can be compared in tissues originating from the same animal, morphological and immunological analyses also showed that head regeneration after decapitation (80% body length, named apical head regeneration)

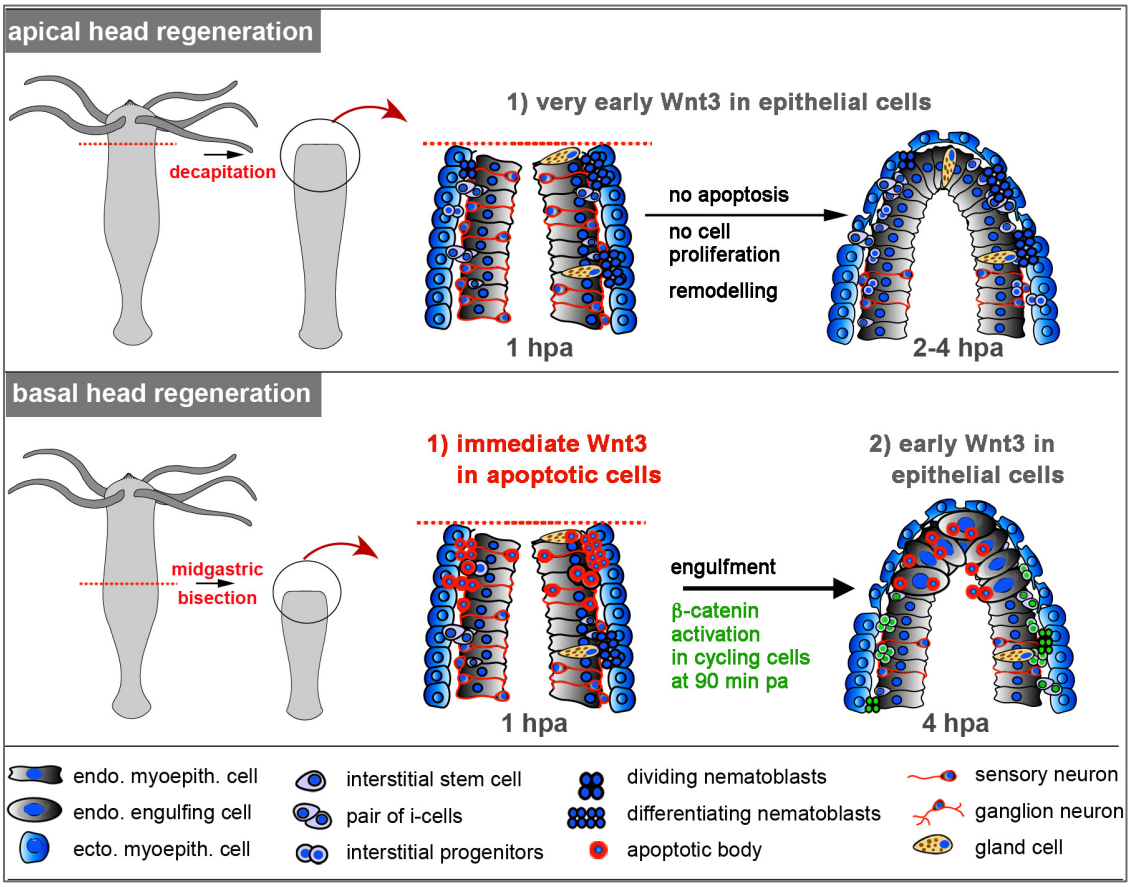


Figure 2: Scheme showing the specific features of apical (upper panel) and basal (lower panel) head regeneration in *Hydra*. For each context, the regenerating tip is depicted, showing the two cell layers, ectoderm (ecto.) and endoderm (endo.), that comprise myoepithelial cells and interstitial cells (i-cells). The red broken line represents amputation plane. During apical head regeneration, the epithelial cells rapidly up-regulate Wnt3 expression, and the remodeling takes place in the absence of cell death or enhanced cell proliferation (morphallaxis). During basal head regeneration, cells from the interstitial lineage (neurons, nematoblasts) immediately undergo cell death while the endodermal epithelial cells transiently lose their epithelial organization and engulf the apoptotic bodies (it should be noted that a similar loss of epithelial organization was previously described during early reaggregation [83]). The apoptotic cells (red cells) provide an immediate but transient source of Wnt3 that activates the Wnt-βcatenin pathway in the surrounding cycling cells (green cells), which rapidly divide (epimorphic-like response). hpa: hours post-amputation.

and head regeneration after mid-gastric section (50% body length, named basal head regeneration) are different. After decapitation, the tentacles differentiate first, whereas, after mid-gastric bisection, the differentiation of the hypostome precedes that of the tentacles [52]. In fact, after decapitation, head regeneration is initiated from a region that, prior to amputation, is populated with apical progenitors actively dividing and differentiating, until they migrate or get displaced to the tentacles. By contrast, after mid-gastric bisection, head regeneration takes place in a less determined region where proliferating stem cells are not yet committed to an apical or basal fate [8]. Therefore *Hydra* provides a model system where at least three distinct types of regeneration can be studied, i) *foot regeneration* that does not require a complex morphogenesis and hence could be considered as a form of tissue repair, ii) *apical head regeneration* and iii) *basal head regeneration* that follow two distinct routes that differ in their dependence upon cell division – morphallactic after decapitation but epimorphic-like after mid-gastric bisection. As previously mentioned the Wnt-βcatenin pathway also shows distinct modes of activation after decapitation or mid-gastric bisection [37, 39], suggesting that its regulation is under the influence of the cellular niche where regeneration is initiated.

Activation of the canonical Wnt pathway leads to head organizer activity in *Hydra*

Thanks to the sequencing of the *Hydra* and *Nematostella* (sea anemone) genomes [32, 53], the repertoire of the cnidarian genes involved in upstream Wnt ligand signaling is well known. Phylogenetic analyses unexpectedly proved that most of the 13 *Wnt* families present in deuterostomes already diversified in the common ancestor of cnidarians and bilaterians, with 12 and 8 of these 13 families identified in *Nematostella* [54, 55] and *Hydra* [37], respectively. These families represent both canonical (Wnt1, Wnt3, Wnt11) and the non-canonical (Wnt4, Wnt5, Wnt8) pathways.

In *Hydra*, seven out of eight of these families are expressed in the hypostome of the adult head and are upregulated in the head-regenerating tips, at the time the organizer activity is rising, as evidenced by transplantation experiments [35, 37, 42]. Four types of functional studies indeed support the role of the Wnt signals in head formation: first ectopic activation of β-catenin signaling in the body column through alsterpaullone treatment induces ectopic head

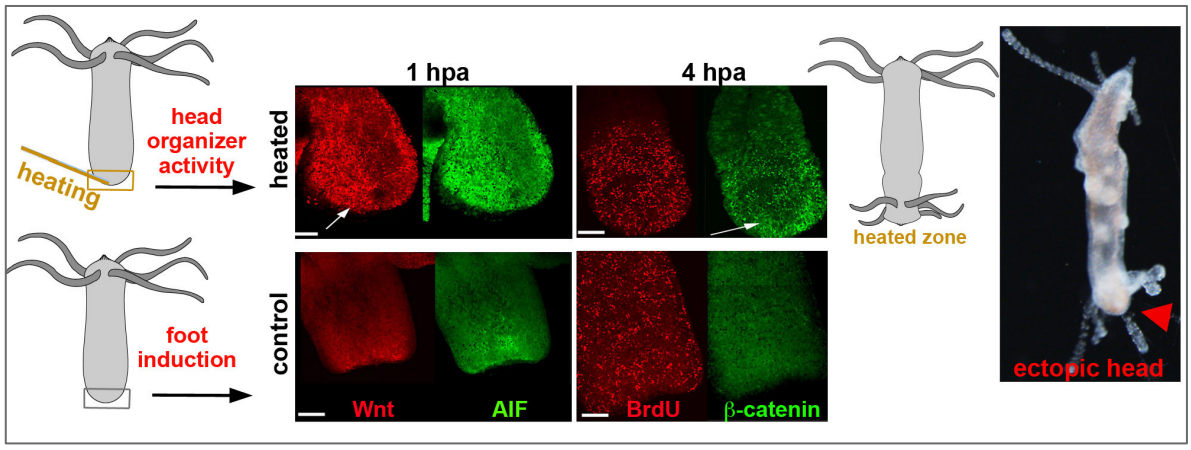


Figure 3: Ectopic apoptosis suffices to force a foot-regenerating tip to regenerate a head. Local heating of a foot-regenerating tip with a flame-heated glass capillary (brown line) immediately after bisection induces apoptosis, as detected with antibody staining against apoptosis inducing factor (AIF). The apoptotic cells produce a transient source of Wnt3 signaling detected here at 1 hpa (red, arrow), which leads to the nuclear translocation of β-catenin in the BrdU-positive cycling cells, detected here at 4 hpa (green, arrow), and 2 days later to ectopic head formation ('biheaded *Hydra*', red arrowhead). Scale bars = 50 µm.

organizer activity [35]. And at least *Wnt3*, *Wnt5* and *Wnt8* are upregulated in such tissues [35, 38]. Second a slice of gastric tissue exposed to Wnt3 protein from mouse or *Hydra* origin for 3 hours and grafted back at the same position in a non-treated host, forms more frequently a secondary axis [37]. Third it is possible to significantly enhance head regeneration in the head-regeneration-deficient strain *reg-16* by treating these polyps with Wnt3 for 20 hours after decapitation [37]. Finally silencing *wnt3* or *β-catenin* by RNAi abolishes head regeneration [39].

Interestingly the activation of seven different *Wnt* genes is sequential in head-regenerating tips, with noticeable differences between apical and basal head regeneration: after decapitation *Wnt3* appears first, already very strongly at 1.5 hpa, together with *Wnt11* highly restricted and *Wnt9/10c* very weak, whereas after mid-gastric bisection, *Wnt3* also appears first but later (3 hpa), much more diffuse and at rather low level together with *Wnt9/10c*, here quite strong and *Wnt11* very weak. Then two successive waves of upregulation are rapidly detected after decapitation (*Wnt1* and *Wnt16* from 3 hpa, *Wnt9/Wnt10a* and *Wnt7* at 6 hpa) whereas *Wnt7*, *Wnt9/Wnt10a* and *Wnt16* show up only at 12 hpa and *Wnt1* at 24 hpa after mid-gastric bisection [37]. The fact that *wnt3* is actually the first among the *Wnt* genes to be up-regulated after decapitation suggested to the authors that Wnt3 is instructive in the formation of the head organizer. However the temporal, spatial and quantitative differences in *Wnt(s)* gene expression after decapitation and mid-gastric bisection suggest that if the Wnt signals control head regeneration through a coordinated crosstalk, it is likely not the same crosstalk in these two contexts.

The regulation of the activation of the Wnt3-bcatenin pathway is only partially known in *Hydra*, however it appears quite different after decapitation and after mid-gastric bisection. In fact head regeneration from the mid-gastric level requires two successive and dependent waves of Wnt3 signaling, an immediate one taking place in the interstitial cell lineage, followed by a second one that requires transcription and is restricted to the epithelial cells **(Figure 2)**. For clarity, we will refer to these as the '*immediate interstitial Wnt3 signaling'* and the '*early epithelial Wnt3 signaling'* respectively. By contrast, after decapitation, a single wave of Wnt3 signaling appears necessary, delivered very early after amputation by the epithelial cells where the *Wnt3* gene is upregulated **(Figure 2)**. We assume that the *'very early epithelial Wnt3 signaling'* after decapitation or the '*early epithelial Wnt3 signaling'* after mid-gastric bisection play similar functions as, in both contexts, this epithelial Wnt3 expression remains sustained all along the regenerative process and later in the adult head, suggesting that it is required to maintain the activity of the head organizer.

Apoptotic cells provide a source of Wnt3 signaling in *Hydra*

The first regulation of the Wnt-βcatenin pathway during *Hydra* head-regeneration after mid-gastric bisection can be detected as early as 15 minutes, when cells from the interstitial lineage (neurons, nematocytes, progenitors, but not the epithelial cells), located close to the amputation plane undergo a massive but transient wave of apoptosis [39]. This apoptotic process is specific to head regeneration as on the other side of the cut, which will regenerate a foot, the number of apoptotic cells remains very low at all time points. During a very short time-window, these apoptotic cells appear to be filled with Wnt3a-positive speckles. However, this immediate and transient overexpression of Wnt3 protein is likely not under transcriptional control as the *wnt3* upregulation becomes detectable only a few hours later and in the endodermal epithelial cells [33, 37, 39]. A putative scenario is that the Wnt3 protein is already present in the dying cells, potentially sequestered within a protein complex. Upon injury and induction of apoptosis, this complex would dissociate, either through an active mechanism that would cause the release of the Wnt3 protein, or through the elimination of an inhibitory signal that keeps Wnt3 masked in homeostatic

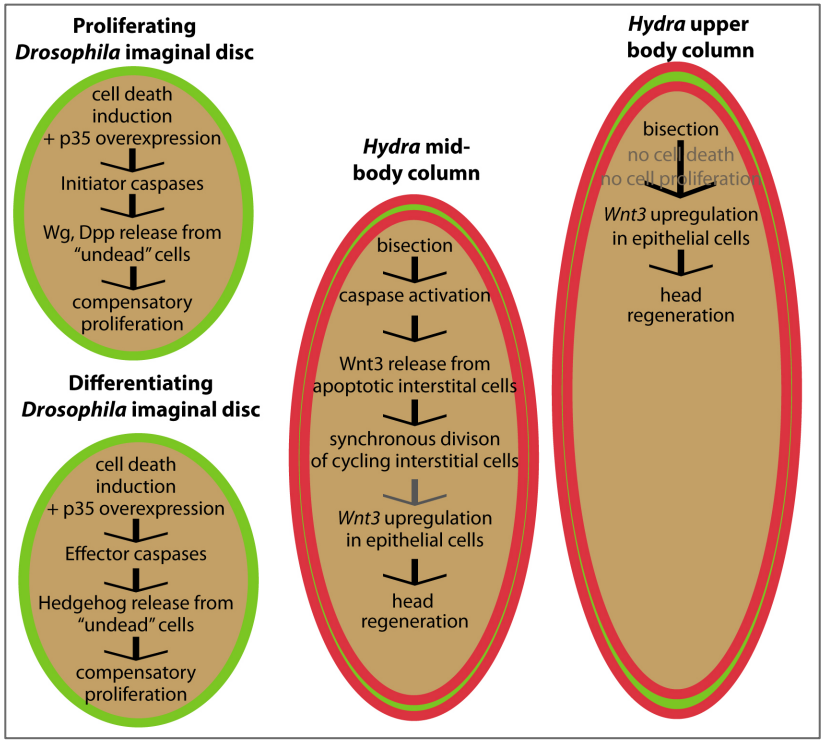


Figure 4: Scheme showing the tissue-dependent plasticity of apoptosis-induced compensatory proliferation in *Drosophila* [58, 69] and in *Hydra* [37-39]. In *Drosophila* larvae, cell death is locally induced genetically and the concomitant overexpression of the p35 apoptosis inhibitor prevents apoptosis termination, maintaining the apoptotic cells in an "undead" status [59]. In *Hydra*, bisection performed at mid-gastric level leads to a massive apoptosis of interstitial cells on the head-regenerating side, which is not the case after decapitation. The release of Wnt3 by the apoptotic cells is necessary to observe the secondary Wnt3 up-regulation in the epithelial cells but the role of the cycling interstitial cells is not established yet (grey arrow).

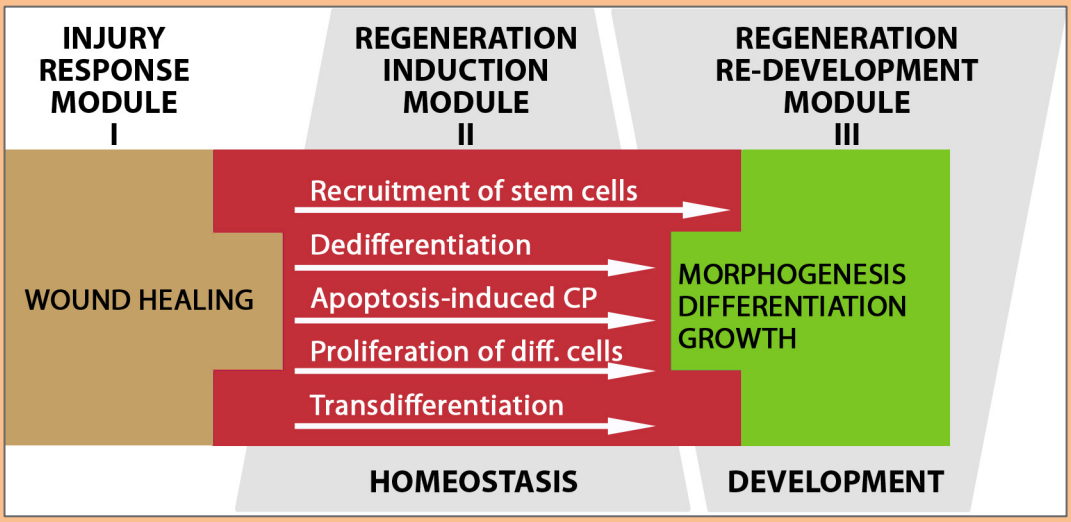
conditions. Further studies should tell us whether the glycosylation and lipidation processes that control Wnt3/Wg biogenesis in bilaterians are also at work during *Hydra* regeneration [56].

Subsequently, the Wnt3 protein released by the interstitial apoptotic cells activates the β–catenin pathway in the interstitial progenitor cells located underneath the amputation plane. The stabilized β–catenin translocates into the nucleus between 1 and 1.5 hours post-amputation (hpa), inducing a wave of mitotic division in the responsive cells of the stump [39]. As a result, the immediate cellular event coordinated by the Wnt-βcatenin pathway is the synchronization of the proliferating interstitial cells, suggesting an essential role for Wnt3 signaling in the switch that allows the wound healing process to trigger a complex regeneration program.

Interestingly, the level of apoptosis seems to be crucial for launching this Wnt3-dependent head regeneration program as evidenced by three distinct types of assays [39]. First, the pharmacological inhibition of apoptosis inhibits the first (interstitial) and the second (epithelial) waves of Wnt3 signaling together with the synchronous division of interstitial progenitors. Also there is no head regeneration at all when apoptosis is inhibited whereas foot regeneration is not affected. Second head regeneration can be fully rescued in polyps pharmacologically inhibited for apoptosis by simply adding Wnt3 of mouse origin in the medium for a few hours. This treatment could replace the lack of immediate interstitial Wnt3 release, leading to β–catenin nuclear translocation in interstitial cycling progenitors. Finally, when the level of apoptosis was experimentally raised in the foot-regenerating tip upon local heating, Wnt3 was similarly overproduced by the apoptotic cells, the Wnt-βcatenin pathway was activated in cycling progenitors, leading to ectopic head regeneration by forcing foot-regenerating tips to adopt a head fate **(Figure 3)**. Again, this assay was designed to demonstrate the essential role of the immediate interstitial Wnt3 signaling to launch head regeneration after mid-gastric bisection. Surprisingly this immediate interstitial Wnt3 signaling was never detected after decapitation or after bisection in the lower body column (SC and Ghila, unpublished). Further studies should help identify i) how mid-gastric bisection leads to an asymmetric activation of apoptosis, ii) how entry into apoptosis directs Wnt3 release, iii) how this immediate interstitial Wnt3 signaling leads to Wnt3 upregulation in the endodermal epithelial cells, iv) what are the other signals that participate in this process.

The *Hydra* model confirms the link between apoptosis-induced compensatory proliferation and regeneration

Although the capacity to undergo regeneration is widespread among members of the animal kingdom, whether there is a core sequence of cellular processes that similarly drives regeneration in evolutionarily distant regenerative contexts remains an open question [1, 2, 31, 57]. Recently, the apoptosis-induced compensatory proliferation was proposed to provide a conserved mechanism to trigger regeneration in contexts as different as the *Drosophila* larva regenerating its imaginal discs after cell death induction [58-60], the *Xenopus* tadpole regenerating its tail after amputation [61], the *Hydra* polyp regenerating its head after mid-gastric bisection [39], or the mouse repairing its skin after damage and liver after partial hepatectomy [62]. In each of these contexts it is suspected that the apoptotic cells support the compensatory proliferation of their neighbors via non-apoptotic functions of their caspases [63]. In the planaria regenerating its body after transverse sectioning, two successive waves of apoptosis were recorded, an



Box Fig.1: Scheme depicting the tri-modular structure of regenerative processes.
A complete and efficient regenerative response to tissue injury, degeneration or amputation requires the activity of three successive modules, possibly partially overlapping: 1) *the injury response with* the activation of repair genes, 2) *the regeneration induction module,* which is the focus of this review, and 3) *the regeneration re-development module*. Cellular processes supporting modules I and III are not listed. Among these three modules, the regeneration induction module is likely the most variable one, as either recruitment of stem cells, and/or dedifferentiation, and/or apoptosis-induced compensatory proliferation, and/or direct proliferation of differentiated cells, and/or transdifferentiation can be activated. This is evidenced by species regenerating homologous structures following different routes (for instance salamanders and *Xenopus* tadpoles regenerating their appendages), by the absence of a unique route for a given species regenerating distinct structures, or even by organisms that follow several routes to regenerate the same structure as *Hydra* regenerating its head differently from apical or basal positions. This variability actually reflects the constraints applied by both the developmental status of the organism and the pre-injury homeostatic conditions of the injured tissue on this module. Indeed the length of this module is directly influenced by the intensity of the developmental and aging processes on-going in the regenerating organism [84, 85]: basically no need for a bridge between wound healing and re-development in the embryo, but a bridge that gets longer with the closing of the developmental processes and the progressive appearance of aging in the adult. Also the homeostatic status at the time and place of injury defines which cellular tools can be activated upon injury. Once recruited these tools can combine to activate the third regeneration re-development module (green). This module might be less plastic as re-development of a given structure / organ often relies on the signaling used during organogenesis [86-89].

early one, localized to the vicinity of the wound, possibly related to blastema formation, and a later one, systemic, related to body remodelling [28, 30]. Hence it remains to establish whether the apoptotic cells also promote cell proliferation in planarians.

The confirmation that apoptotic cells actually release themselves the signals that trigger proliferation of the surviving neighbouring cells came in 2004 with the possibility genetically to produce 'undead' cells in *Drosophila* wing discs – that is, cells where the caspase inhibitor p35 is overexpressed to allow the cells to start, but not to terminate, the apoptotic process [64-66]. In this context, the immortalized apoptotic cells indeed release signaling molecules after apoptosis induction, signals that trigger a proliferative response, although part of the effect might be linked to the undead status of these cells [67]. Surprisingly enough, the same signals are released by the apoptotic cells in *Drosophila* and *Hydra*, wingless (wg) and Wnt3, respectively, inducing compensatory proliferation in the neighbouring cells through the activation of the β–catenin pathway [39]. In mice, cell death-induced regeneration requires the activation of the "Phoenix" pathway, which involves the production of arachidonic acid and prostaglandins [62]. Prostaglandins actually closely interacts with the Wnt pathway to induce stem cell proliferation in mammals [68] and further work will tell us whether similar crosstalk also takes place in non-mammalian regenerative contexts.

How many routes to launch a regenerative process?
Interestingly it has been found that the mitogens released by the apoptotic cells can vary depending on the status of the dying cell, as specified by its pre-injury homeostatic niche. Illustrative of this concept is the case of the *Drosophila* eye imaginal disc, where the undead apoptotic cells upregulate *wnt* and *dpp* when proliferative as in the anterior part of the eye disc or in the wing discs, but upregulate Hedgehog (Hh) and not Wnt or Dpp when differentiated as in the posterior part of the eye imaginal disc [69]. These two types of niche-dependent signaling actually reflect two different forms of apoptosis-induced compensatory proliferation: in proliferating tissues, the release of Wnt or Dpp requires the activation of the initiator caspase Dronc (caspase 9), whereas, in differentiating tissues, Hh release relies on the activation of the effector caspases DrIce and DcpI [69].

Again one can see an interesting parallel with *Hydra*, where the processes leading to head regeneration are immediately different according to the cellular status of the tissue where bisection is performed **(Figure 4).** After mid-gastric bisection, but not after decapitation, *Hydra* regenerates its head by using the apoptosis-induced compensatory proliferation process. The mid-

gastric region and the upper body column from which basal and apical regeneration are initiated, respectively, are actually dramatically different at the cellular level: the mid-gastric region contains predominantly stem cells, whereas the upper body column is an active site of proliferation for the progenitors that undergo differentiation [8]. These data strongly suggest that, as in *Drosophila*, the cellular environment where injury takes place influences the way in which the regenerative response is launched.

More generally, this strongly suggests that the regenerative route taken after injury is actually dramatically influenced by the homeostatic context [70]. On the basis of the above argument, we propose here a tri-modular organization of regenerative processes that would be applicable to most if not all contexts **(Box Fig.1)**: we see regeneration as a highly dynamic bridging process between two boundary markers – the wound healing process and the re-development of the missing structure – which definitively need to be connected [71]. This bridging process that we name "regeneration induction module" is well defined in classical model systems of regeneration: In urodeles regenerating their appendages, dedifferentiation of specific cell types plays a major role to provide pools of progenitors that form the blastema [72] whereas in *Xenopus* tadpoles regenerating their tail or their limbs, dedifferentiation is not observed and recruitment of stem cells seems predominant [73]. So closely-related species regenerating homologous structures, make use of distinct routes within the induction module, indicating that this phase of the regenerative process is actually quite plastic. In *Xenopus*, a limited amount of apoptosis is necessary for the growth zone to proliferate and regenerate [61] but it is currently unknown whether apoptosis-induced growth also contributes to blastema formation in urodeles. Finally another way to regenerate a tissue or an organ is to use transdifferentiation of differentiated cells that traverse or not the cell cycle [74]. A recent report showed that adult mice efficiently regenerate their pancreatic β-islets after massive lineage-specific apoptosis by reprogramming their α-islets, proving the plasticity of these cells [75]. Here again the intensity of the apoptotic process seems to play a role but the signaling function of the apoptotic cells remains to be investigated. In *Hydra* transdifferentiation takes place after decapitation but it is currently unclear whether it works as a driving force or as a consequence of the head regenerative process [26].

Concluding remarks

The technical difficulties linked to the study of regeneration relied for long on the absence of classical genetics in vertebrate and invertebrate species with strong regenerative potential. Also re-development during regeneration makes use of the signaling cascades that drive embryogenesis or organogenesis, therefore any non-conditional approach is unsuitable. Finally genetic approaches need to be combined to highly reliable analyses of cell lineages and cell behaviors, which is rather difficult when these behaviors are as transient as apoptosis [76]. Significant advances were recently made in that direction in the salamanders [72], the *Xenopus* [73] and the zebrafish [77-80]. In non-classical invertebrates (planarians, *Hydra* but also crustaceans [81], cricket [82]) timely gene silencing by RNAi offers the possibility to finely dissect the molecular mechanisms underlying the cellular remodeling linked to regeneration. These strategies should soon provide extensive comparative data that will tell us for instance how and when caspases are recruited for non-apoptotic tasks to promote regeneration. They should also help identify the various signals released by these apoptotic cells and elucidate the different role(s) these signals play in early regeneration, driving cell proliferation but also possibly amplifying rare cell behaviors such as transdifferentiation.

The data discussed here suggest that the process bridging injury to the structure-specific re-developmental program is highly plastic, between species and even within a given species. We anticipate that this plasticity actually reflects the pre-injury homeostatic conditions that favor one or the other route. If future investigations confirm this model, one might be able to precisely predict from the homeostatic conditions of a given tissue / organ / structure the endogenous molecular and cellular tools available to launch a regenerative response. This would be of utmost interest to design regenerative strategies.

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