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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 for the eludication of the mechanisms underlying regeneration in the adult, and an understanding of the core mechanisms is likely to cast light on pathways conserved in other species. Recent detailed analyses of the activation of the Wnt-β-catenin pathway in bisected Hydra shows that the route taken to regenerate a structure as complex as the head varies dramatically according to the level of the amputation. When decapitation induces direct redevelopment due to Wnt3 signaling from epithelial cells, head regeneration after mid-gastric section relies first on Wnt3 signaling from interstitial cells, that undergo apoptosis-induced compensatory proliferation, and subsequently on activation of 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

# Variability of regeneration over the life cycle and in different animal phyla

Regenerative potential in the animal kingdom is not uniformly distributed among the different phyla and can be highly divergent even between closely related species [1,2]. Moreover, in the same individual, regenerative potential varies dramatically 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

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number of species that can replace complex structures is very limited, although widely spread across all phyla (Box 1).

Adult regeneration can be subdivided into two classes: paedomorphic, when sexual and somatic developments are uncoupled in time (Glossary), and truly adult in animals that have reached both sexual and somatic maturity. As a general rule, regenerative potential is usually higher when somatic development is not yet complete. However, even when unable to regenerate complex structures requiring restoration of a functional tridimensional shape (usually relying on blastema formation and 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 by external forces

#### Glossary

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

**Blastema:** a transient structure formed during regeneration that is characterized by 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  $\beta$ -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: re-establishment upon amputation of a spatial

structure with identical shape and function due to the formation of a blastema, a self-organizing system.

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Apoptosis-induced compensatory proliferation: the capacity of cells undergoing apoptosis to promote the proliferation of 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.

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

**Morphallactic regeneration:** re-establishment 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 activates Planar Cell Polarity or  $Ca^{2+}$ signaling, and also involves Frizzled and Dishevelled but acts independently of  $\beta$ -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. *Hydra*, that never develops as a medusa, and axolotl, that does not undergo metamorphosis, provide examples of paedomorphic regeneration.

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(injury, tissue damage, amputation), cell renewal is subject to 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 in which 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 because these organisms exhibit no cell renewal in adulthood, or this is restricted to the gut. Therefore, with the aim of facilitating steps towards organ regeneration in humans, there is a clear need for invertebrate model

#### Box 1. Species with high regenerative potential

All species schematized here (Figure I) provide useful model systems for studying 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 for testing the impact of extinguishing developmental processes on regenerative programs [73] and for dissecting the genetic pathways involved in apoptosis-induced compensatory proliferation [58-60].

In Figure II complete and efficient regenerative response to tissue injury, degeneration or amputation, requires the activity of three successive modules, possibly partially overlapping: (i) the injury response with the activation of repair genes, (ii) the regenerationinduction module, the focus of this review, and (iii) the regeneration re-development module. Cellular processes supporting modules I and III are not listed. Of these three modules, the regeneration-induction module is likely to be the most variable because 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, and even by organisms that follow several routes to regenerate the same structure, such as the different pathways of Hydra head regeneration from apical or basal positions. This variability 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 ongoing in the regenerating organism [84,85]: basically there is no need for a bridge between wound healing and re-development in the embryo, but in the adult a bridge is required, and one that becomes progressively longer with the closing of the developmental processes and the progressive appearance of aging. Homeostatic status at the time and place of injury also defines which cellular tools are activated upon injury. Once recruited these tools can combine to activate the third regeneration re-development module (green). This module might be less plastic because redevelopment of a given structure/organ often relies on the signaling used during organogenesis [86-89].



Figure I. Phylogenetic tree showing the animal phyla that contain species with high regenerative potential.



systems that highlight the principles of regeneration, and more specifically for those regulating adult regeneration.

#### 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 belonging 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 phylumspecific mechano-sensory cells named cnidocytes (or nematocytes) that resemble the bilaterian mechano-sensory cells in virtue of their cnidocil. Upon stimulation this cnidocil leads to the external discharge of an intracellular venom capsule (the nematocyst or cnidocyst). In addition to somatic differentiation, gametogenesis and sexual development share some common rules with bilaterian species [9,10].



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 2 hours of labeling. (b) The different 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].

Adult Hydra possess the potential for multiple developmental fates (Figure 1b) and share numerous features with the planarian flatworms, another classical model system for the study of regeneration [11]. Indeed, in adulthood both models show: (i) continuous tissue replacement due to a stock of mitotically-active stem cells (a single cell type in planarians - the neoblasts - but comprising three different types in Hydra - the ectodermal epithelial, endodermal epithelial and interstitial stem cells), (ii) a stock of adult pluripotent stem cells that produce both germ cells and somatic cells throughout the life of the animals [12-15] (a rather special case in eumetazoans that usually segregate their germ cells during early embryonic development), (iii) efficient asexual reproduction (budding in Hydra, fission in planaria), (iv) an amazing ability to regenerate almost any missing part of the body after injury, and (v) 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), achieved 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]) for transiently silencing gene expression in adult organisms [21–23]. Also in Hydra, the recent possibility of establishing transgenic strains now allows investigators to follow in real time 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 has definitively demonstrated that the signaling pathways at work during programmed cell death [27-30] and development [31,32] are also highly conserved in both two species. Therefore the analysis of the mechanisms that support regeneration in Hydra and planarians should help to identify some principles of regeneration that are shared between cnidarians and bilaterians.

Work over the past 10 years has shown that Hydra relies on the activation of the Wnt- $\beta$ -catenin pathway to regenerate its head [33-39]. However the patterns of activation vary markedly according to the level of amputation [39] – after decapitation Wnt3 is rapidly and strongly upregulated 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 (Glossary) and subsequently Wnt3 is upregulated in epithelial cells [37,39]. This single or dual mode of Wnt- $\beta$ -catenin activation reflects two different processes for regenerating a head, morphallactic after decapitation, and epimorphiclike after mid-gastric bisection (Glossary). Together with data obtained in Drosophila imaginal discs and in mouse adult tissues, these results suggest (i) apoptosis-induced compensatory proliferation provides an evolutionarilyconserved mechanism to launch a regenerative response, (ii) distinct regenerative routes can reactivate the same structure-specific developmental process, and (iii) 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 body of knowledge based on transplantation, cell-lineage and quantitative cellular analyses that has 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, molecular studies performed over the past 20 years on the genetic pathways at work during budding, regeneration and reaggregation have highlighted six central pathways: (i) the canonical Wnt pathway for maintaining and re-establishing apical organizer activity [33-39], (ii) the non-canonical Wnt pathway for cellular evagination processes [38], (iii) the MAPK-CREB pathway for triggering head regeneration [46], (iv) the BMP/chordin pathway for axis patterning [47,48], (v) the FGF pathway for bud detachment [49], and (vi) the Notch pathway for differentiating some interstitial cell lineages [50].

Following bisection at mid-gastric level, the two Hydra halves immediately initiate an asymmetric process at the wound 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 have confirmed that these two regions, that were adjacent and identical prior to midgastric bisection, immediately undergo markedly different reorganization to become foot- and head-regenerating tips, respectively, 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 or enhanced cell proliferation is observed in foot-regenerating tips.

In addition to these two types of regeneration, that can be compared in tissues originating from the same animal, morphological and immunological analyses have revealed that the mechanisms underlying head regeneration after decapitation (80% body length, named apical head regeneration) are very different from those involved in head regeneration after mid-gastric section (50% body length, named basal head regeneration). 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 progenitor cells that actively divide and differentiate until they migrate to (or are displaced to) the tentacles. By contrast, head regeneration after mid-gastric bisection 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 complex morphogenesis and hence could be considered as a form of tissue repair, and (ii) *apical head regeneration* and (iii) *basal head regen* 

eration 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– $\beta$ -catenin 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 headorganizer activity in Hydra

The sequencing of the *Hydra* and *Nematostella* (sea anemone) genomes [32,53] has allowed the repertoire of the cnidarian genes involved in upstream Wnt ligand signaling to be firmly established. Phylogenetic analyses unexpectedly proved that most of the 13 *Wnt* families present in deuterostomes had already diversified in the common ancestor of cnidarians and bilaterians, with 12 and 8 of these 13 families being present in *Nematostella* [54,55] and *Hydra* [37], respectively. These families represent both the 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 their expression is upregulated in the head-regenerating tips, at the time the organizer activity is rising, as demonstrated 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  $\beta$ catenin signaling in the body column through alsterpaullone treatment induces ectopic head organizer activity associated with upregulation of Wnt3, Wnt5 and Wnt8 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 nontreated host, more frequently forms a secondary axis [37]. Third, it is possible to enhance head regeneration significantly in the head-regeneration-deficient strain reg-16 by treating these polyps with Wnt3 for 20 hours after decapitation [37]. Finally, silencing *wnt3* or  $\beta$ -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 hours post-amputation (hpa), together with highly restricted expression of Wnt11 and very weak expression of Wnt9/10c, whereas after mid-gastric bisection, Wnt3 also appears first but later (3 hpa), and is much more diffuse and at a rather low level, together with Wnt9/ 10c, here quite strong and Wnt11 very weak. Two successive waves of upregulation are then rapidly detected after decapitation (Wnt1 and Wnt16 from 3 hpa, Wnt9/Wnt10a and Wnt7 at 6 hpa) whereas Wnt7, Wnt9/Wnt10a and Wnt16 appear only at 12 hpa and Wnt1 at 24 hpa after mid-gastric bisection [37]. The fact that wnt3 is the first of the Wnt genes to be upregulated after decapitation suggested 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 Wnt

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signals control head regeneration through a coordinated crosstalk, it is probably not the same crosstalk in these two contexts.

The regulation of the activation of the Wnt3–β-catenin pathway is only partially known in Hydra, however it appears quite different after decapitation and after midgastric bisection. In fact, head regeneration from the midgastric level requires two successive and dependent waves of Wnt3 signaling, an immediate one taking place in the interstitial cell lineage, followed by a second that requires transcription and is restricted to the epithelial cells (Figure 2). For clarity we will refer to these as *'immediate* interstitial Wnt3 signaling' and '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 midgastric bisection play similar functions because, in both contexts, this epithelial Wnt3 expression remains sustained throughout 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

First changes in Wnt signaling during Hydra head-regeneration can be detected as early as 15 minutes after midgastric bisection, 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 because, 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 probably not under transcriptional control because wnt3 upregulation only becomes detectable 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 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  $\beta$ -catenin pathway in the interstitial progenitor cells located underneath the amputation plane. The stabilized  $\beta$ -catenin translocates into the nucleus between 1 and 1.5 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- $\beta$ -catenin pathway is the synchronization of the pro-



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 upregulate Wnt3 expression, and 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 or enhanced cell proliferation (morphallaxis). During basal head regeneration 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–β-catenin pathway in the surrounding cycling cells (green cells), which rapidly divide (epimorphic-like response). hpa, hours post-amputation.

liferating 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, pharmacological blockade of apoptosis inhibits the first (interstitial) and the second (epithelial) waves of Wnt3 signaling as well as the synchronous division of interstitial progenitors. In addition, head regeneration is abolished when apoptosis is inhibited, whereas foot regeneration is not affected. Second, head regeneration can be fully rescued in polyps in which apoptosis is pharmacologically inhibited by simply adding mouse Wnt3 to 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, and the Wnt-β-catenin 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 immediate interstitial Wnt3 signaling in launching head regeneration after mid-gastric bisection. Surprisingly, this immediate interstitial Wnt3 signaling was not detected after decapitation or after bisection in the lower body column (S.C. and L. Ghila, unpublished). Further studies should help to establish (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, and (iv) the identity of the other signals participating in this process.

#### The Hydra model confirms the link between apoptosisinduced compensatory proliferation and regeneration

Although the capacity to undergo regeneration is widespread among members of the animal kingdom, it remains an open question whether there is a core sequence of cellular processes that similarly drives regeneration in evolutionarily distant regenerative contexts [1,2,31,57]. Recently, the apoptosis-induced compensatory proliferation was proposed to provide a conserved mechanism to



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, arrowed), and this 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.

trigger regeneration in contexts as different as the regeneration of imaginal discs by Drosophila larvae after cell death induction [58-60], tail regeneration after amputation in Xenopus tadpoles [61], Hydra polyp head regeneration after mid-gastric bisection [39], and skin repair after damage and liver regeneration after partial hepatectomy in mouse [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 planaria body-regeneration after transverse sectioning, two successive waves of apoptosis were recorded: an early wave, localized to the vicinity of the wound, possibly related to blastema formation, and a later systemic wave related to body remodeling [28,30]. Hence it remains to be established whether the apoptotic cells also promote cell proliferation in planarians.

The confirmation that the apoptotic cells themselves release the signals that trigger proliferation of surviving neighbouring cells came in 2004 when it was found possible to produce genetically 'undead' cells in Drosophila wing discs – that is, cells in which the caspase inhibitor p35 is overexpressed, so allowing 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 neighbouring cells through the activation of the  $\beta$ -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 interact closely 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 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 very 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 strikingly different at the cellular level: the mid-gastric region contains predominantly stem cells, whereas the upper body column is an active site for proliferation of the progenitors that undergo differentiation [8]. These data strongly suggest that, as in Droso*phila*, the cellular environment where injury takes place influences the way in which the regenerative response is launched.

More generally, this argues that the regenerative route taken after injury is markedly 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 (Figure II in Box I), 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 the 'regeneration-induction module', is well defined in classical model systems of regeneration – in urodeles regenerating their



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 massive apoptosis of interstitial cells on the head-regenerating side, and this is not the case after decapitation. The release of Wnt3 by the apoptotic cells is necessary to observe the secondary Wnt3 upregulation in the epithelial cells, but the role of the cycling interstitial cells has not yet been established (4th grey arrow).

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 to predominate [73]. Closely related species regenerating homologous structures thus 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  $\beta$ -islets after massive lineage-specific apoptosis by reprogramming their  $\alpha$ -islets, demonstrating 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. Transdifferentiation in Hydra takes place after decapitation, but it is currently unclear whether it operates as

a driving force or as a consequence of the head-regeneration process [26].

#### **Concluding remarks**

The study of regeneration has for many years been constrained by the absence of classical genetics in vertebrate and invertebrate species with strong regenerative potential. In addition, re-development during regeneration makes use of the signaling cascades that drive embryogenesis and/or organogenesis, and therefore any non-conditional approach is unsuitable. Genetic approaches also need to be combined with highly reliable analyses of cell lineages and behaviors, and this is rather difficult when these behaviors are as transient as apoptosis [76]. Significant advances were recently made in that direction in salamanders [72], Xenopus [73] and zebrafish [77-80]. In non-classical invertebrates (planarians and Hydra, and also crustaceans [81] and cricket [82]) time-restricted gene silencing by RNAi will make it possible to dissect in detail the molecular mechanisms underlying 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 cellular 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 predict precisely, from the homeostatic conditions of a given tissue/organ/structure, the endogenous molecular and cellular tools that launch a regenerative response. This would be of utmost interest for the design of regenerative strategies.

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