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REVIEW

Injury-induced asymmetric cell death as a driving force for head regeneration in *Hydra*

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Abstract The freshwater *Hydra* polyp provides a unique model system to decipher the mechanisms underlying adult regeneration. Indeed, a single cut initiates two distinct regenerative processes, foot regeneration on one side and head regeneration on the other side, the latter relying on the rapid formation of a local head organizer. Two aspects are discussed here: the asymmetric cellular remodeling induced by mid-gastric bisection and the signaling events that trigger head organizer formation. In head-regenerating tips (but not in foot ones), a wave of cell death takes place immediately, leading the apoptotic cells to transiently release Wnt3 and activate the β-catenin pathway in the neighboring cycling cells to push them through mitosis. This process, which mimics the apoptosis-induced compensatory proliferation process deciphered in *Drosophila* larvae regenerating their discs, likely corresponds to an evolutionarily conserved mechanism, also at work in *Xenopus* tadpoles regenerating their tail or mice regenerating their skin or liver. How is this process generated in *Hydra*? Several studies pointed to the necessary activation of the extracellular signal-regulated kinase (ERK) 1–2 and mitogen-activated protein kinase (MAPK) pathways during early head regeneration. Indeed inhibition of ERK 1–2 or knockdown of *RSK*, cAMP response element-binding protein (*CREB*), and CREB-binding protein (*CBP*) prevent injury-induced apoptosis and head regeneration. The current scenario involves an asymmetric activation of the MAPK/CREB pathway to trigger injury-induced apoptosis in the interstitial cells and in the epithelial cells a CREB/CBP-dependent transcriptional activation of early genes essential for head-organizing activity as *wnt3*, *HyBra1*, and *prdl-a*. The question now is how bisection in the rather uniform central region of the polyp can generate this immediately asymmetric signaling.

Keywords Hydra regeneration · Injury-induced apoptosis · MAPK/CREB/CBP pathway · Asymmetric signaling · Apoptosis-induced compensatory proliferation

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Introduction

Hydra, a model for regenerative studies since 270 years

Regeneration is a widespread phenomenon in metazoan phyla although submitted to multiple variations across evolution, including extensive losses in mammals (Sanchez Alvarado and Tsonis 2006; Brockes and Kumar 2008; Bely and Nyberg 2010; Galliot and Chera 2010). If one assumes that the high regenerative potential of basal metazoans (porifers and cnidarians) was at least partially maintained in bilaterian species, then a systematic comparative analysis of the cellular and molecular mechanisms that drive regeneration in a variety of species should inform us about these plesiomorphic elements. Among the different models used in regenerative studies (Fig. 1), *Hydra* deserves special consideration as this freshwater cnidarian polyp provided the first convincing evidences that an animal can regenerate any missing part of its body after bisection (Trembley 1744; Ratcliff 2012). A brief introduction to *Hydra* anatomy is necessary to approach the paradigmatic value of this little animal (for reviews, see Steele 2002; Galliot et al. 2006; Bosch 2009): *Hydra* polyps display a tube shape, with a unique oral–aboral axis, terminated at the apex by the head region, formed of a ring of tentacles and a dome named hypostome centered on the mouth opening, and at the basis a basal disc. *Hydra* tissues are formed of two parallel myoepithelial cell layers named ectoderm and endoderm,

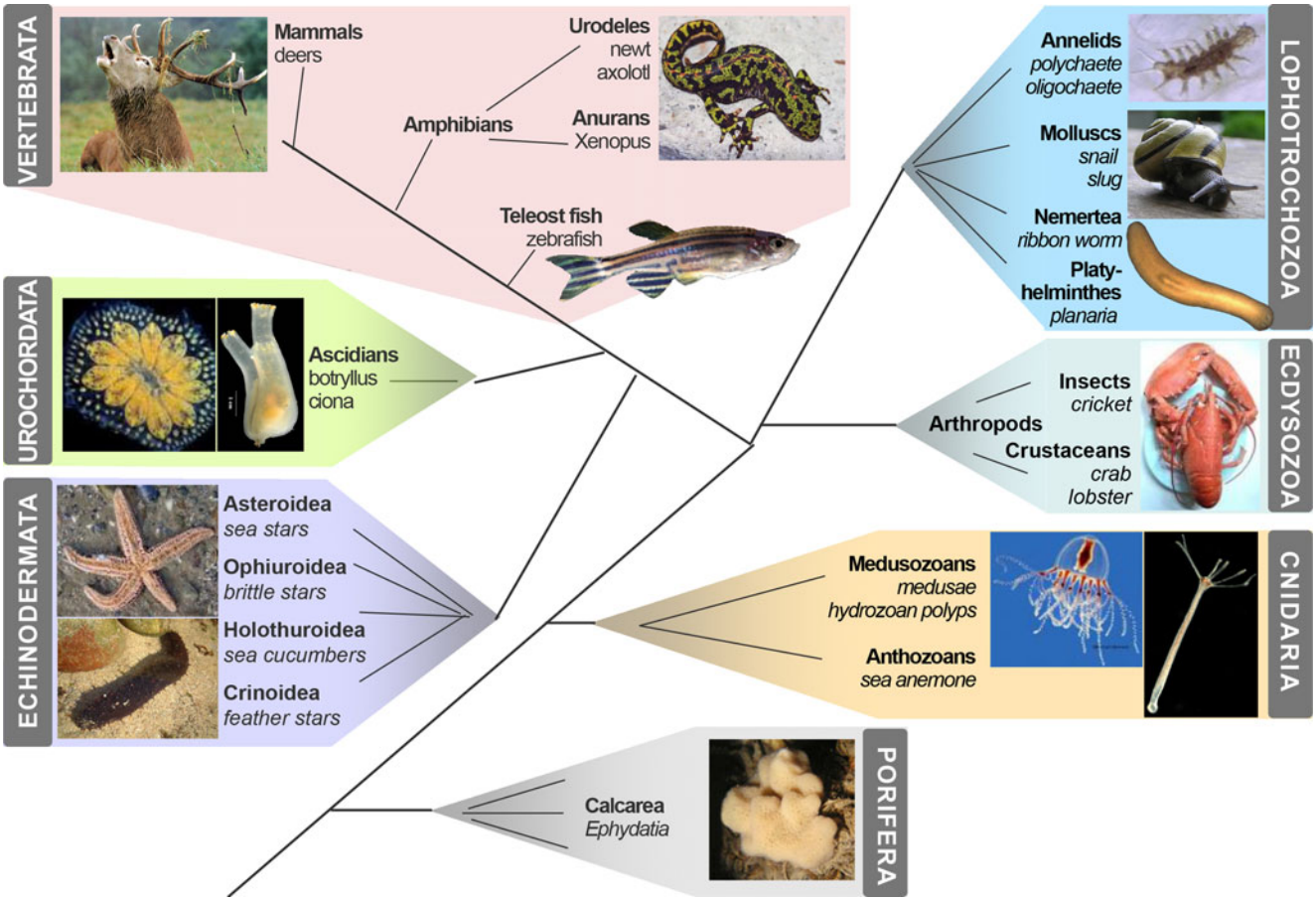

Fig. 1 Phylogenetic tree showing the animal phyla that contain species with high regenerative potential, either as larvae or as adult (adapted from Galliot and Ghila 2010)

separated by an extracellular matrix named mesoglea (Sarras 2012). A dozen of distinct cell types differentiate from three distinct populations of stem cells, epithelial ectodermal, and epithelial endodermal that provide epithelial cells for the ectoderm and the endoderm, respectively, and interstitials that are multipotent.

These interstitial stem cells are located between the ectodermal epithelial cells; they cycle fast, every day on average, providing a variety of distinct somatic cell types, as the gland cells that migrate to the endoderm, the sensory neurons, the ganglion neurons, the stinging cells (nematocytes), but also the germ cells when the animal follows the sexual cycle (Bode 1996; David 2012; Nishimiya-Fujisawa 2012). The distribution of these interstitial stem cells is not homogenous along the body column. They are predominantly located in the central part of the animal, whereas the progenitors actively migrate towards the extremities where they terminally differentiate (David and Plotnick 1980) (Fig. 2). By contrast, the two epithelial cell populations cycle more slowly than the interstitial cells, every 3 or 4 days, and also stop dividing at the extremities. They are considered as unipotent as they provide epithelial cells with specific features at the extremities. In the tentacles, ectodermal epithelial cells differentiate in battery cells, and in the basal disc, they provide the mucus basal cells (Hobmayer et al. 2012). In the endoderm, the myoepithelial cells, also named digestive cells, line the body column and perform the digestion of the nutrients together with the gland cells. Interestingly, the body column is a highly plastic tissue as once bisected it rapidly transforms into an organizer.

Bisection transforms gastric tissue into a head organizer

In 1909, Ethel Browne, a student in Thomas Morgan's laboratory performed careful and well-controlled transplantation experiments between pigmented and unpigmented polyps from the *Hydra viridissima* species. That way, she discovered that a piece of an adult head grafted onto the body column of a second *Hydra* is able to induce the formation of a secondary axis by recruiting cells from the host (Browne 1909). Similarly, she found that head-regenerating tips and the presumptive head region of a growing bud induce a secondary axis when grafted onto a host. This process, named induction (although Ethel Browne

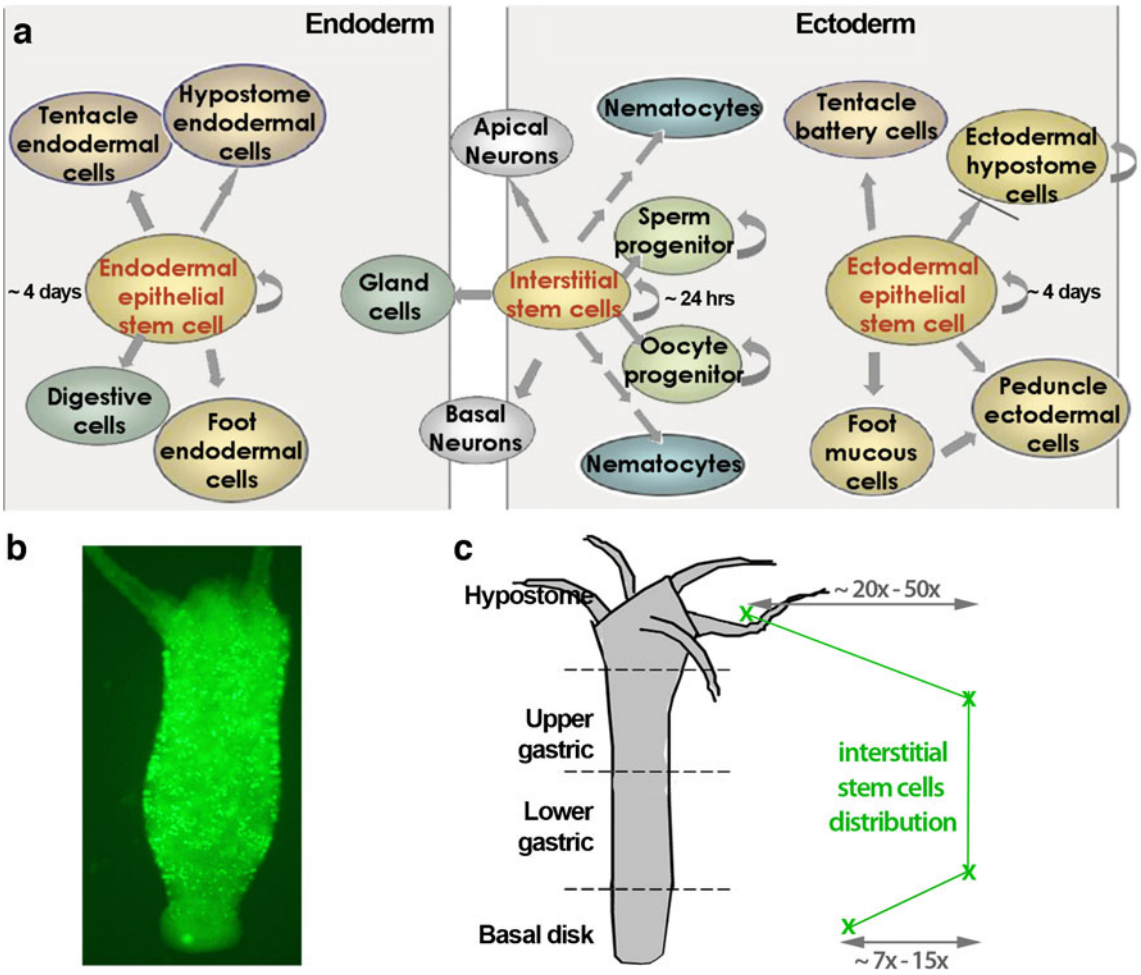

Fig. 2 Stem cells and cell types in *Hydra.* **a** Distribution of the different cell types and stem cells (*red*) in the ectodermal (*left*) and endodermal (*right*) layers. **b** Cycling cells detected in intact *Hydra* after 2 h of BrdU incubation. Note the absence of labeled cells at the extremities. **c** Distribution of interstitial stem cells in the body column as measured by David and Plotnick in a clone-forming unit assay performed on nitrogen mustard aggregates (David and Plotnick 1980). Note the uniform distribution of stem cells in the central part of the body column and the drastic drop at the extremities

did not use this word), was rediscovered 15 years later by Hans Spemann and Hilde Mangold in developing vertebrates (Spemann and Mangold 1924). They applied the same strategy for testing vertebrate embryonic tissues, using this time two closely related new species (*Triturus*), one pigmented and the other unpigmented. Thanks to this procedure they convincingly showed that the dorsal lip of the blastopore is able to recruit cells of the host and induce the formation of a secondary axis when grafted on another gastrula. They thus proved that this region of the blastopore behaves as an embryonic organizer. In the publication of this landmark discovery, the authors unfortunately did not mention the primary discovery of this phenomenon in *Hydra*, although Hans Spemann had read and annotated the publication of Ethel Browne and Hilde Mangold had performed some experiments on *Hydra* (Lenhoff 1991).

Along the twentieth century, transplantation approaches continued to be applied to *Hydra*, becoming precise enough to quantify the strength of the organizing activity in multiple contexts (for review see Shimizu 2012). To assess the temporal and spatial regulation of the organizing activity in head-regenerating tips, Harry MacWilliams performed systematic transplantation assays at various time points after bisection to test the organizing activity in regions located at a variable distance of the bisection plane (MacWilliams 1983). As a short summary, MacWilliams showed that the head-organizing activity starts rising in the head-regenerating tip about 3 h after mid-gastric bisection, reaching a plateau after 10 h, to remain stable for about 2 days (Fig. 3). Interestingly, this organizing activity is first restricted to the head-regenerating tip, and from the second day on, it distributes as a gradient along the apico-basal axis.

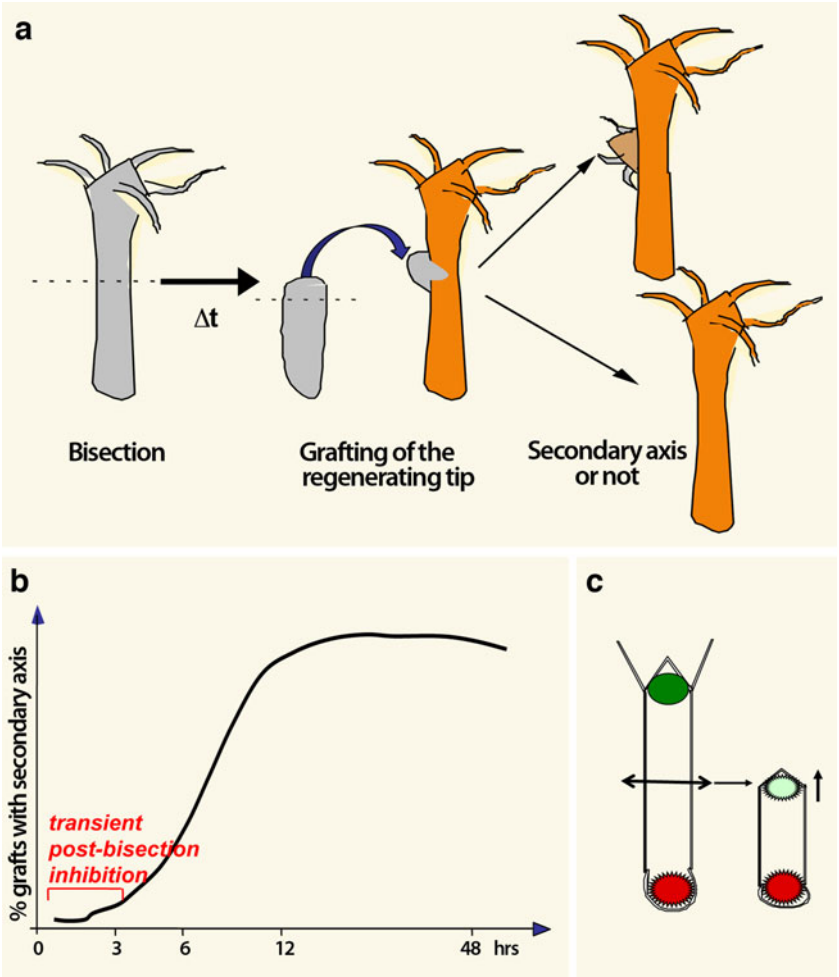

Fig. 3 Transplantation analysis of head organizer formation in head-regenerating *Hydra* (after MacWilliams 1983). **a** Head-regenerating tips are grafted laterally on the body column of the host at various time points after bisection, and formation of an ectopic axis is detected after 2 days. **b** The analysis of the proportion of grafts inducing a secondary axis shows a transient post-bisection inhibition, followed by an increase in head-organizing activity to reach a plateau value about 10 h after bisection, which remains stable for 2 days. **c** Organizers in intact (*left*) and head-regenerating halves (*right*). Homeostatic head organizer: *dark green*; regenerating head organizer: *light green*; foot organizer: *red*

The question was then to identify the early molecular and cellular changes that take place in the regenerating tip, leading first to the formation of a head organizer, and subsequently to the de novo formation of the missing apical structures. Recent data highlighted two distinct aspects of this question: first the cellular remodeling that transforms a piece of adult gastric tissue into an organizer and second the asymmetric signaling that supports these changes in response to bisection. Indeed, the *Hydra* body column is a rather homogenous tissue in the mid-gastric region, from which asymmetric responses are immediately generated upon bisection, leading to the adoption of two distinct fates, head regeneration on the lower half and foot regeneration on the upper one.

Cellular remodeling in head-regenerating stumps

Asymmetric injury-induced apoptosis after mid-gastric bisection

To identify the cellular remodeling that takes place at the time the organizer is forming in head-regenerating tips, head- and foot-regenerating tips were macerated at regular time points after bisection and their cellular composition was compared to regions of the body column that do not exhibit organizer activity. This approach detected a massive cell death in head-regenerating tips, maximal between 30 and 60 min after bisection, affecting 50 % of the cells. By contrast in the foot-regenerating tips, cell death remained very limited and slightly delayed (<7 %, maximal at 2 h) (Chera et al. 2009b). When tissues originating from the body column of intact animals or located far from the wound were examined, then less than 1 % of the cells were found apoptotic, proving that this immediate wave of apoptosis is restricted to the tip. Interestingly only the interstitial progenitors and their derivatives including neurons and nematocytes undergo apoptosis, while the epithelial cells remain intact, proving that distinct cell types sense and respond quite differently to the pro-apoptotic signals (Reiter et al. 2012).

Evidences for this asymmetric response of head- versus foot-regenerating tips came from the quantitative analysis of the surviving cell types over the first 16 h following bisection. The rapid disruption and loss of the nerve net in head-regenerating tips but not in foot-regenerating tips was also confirmed by immunodetection of the nerve net with the

anti β-tubulin antibody: the head-regenerating tips look "empty" with no nerve cells over the first 24 h post-amputation (hpa), whereas the foot-regenerating tips display almost no alteration of their nerve net. Subsequently, progenitors that migrate towards the wound progressively refill the head-regenerating tips, providing de novo-differentiated neurons that can be detected at 32 hpa (Miljkovic-Licina et al. 2007).

The endodermal epithelial cells perform the engulfment function

Although the epithelial cells located in the vicinity of the wound do not undergo apoptosis, they however, participate in this process, especially the endodermal digestive cells that engulf the surrounding apoptotic bodies. During that process, they exhibit strong modifications of their shape, similar to those observed during reaggregation (Murate et al. 1997), rapidly losing their intercellular contacts and their apico-basal polarity, to transiently take an ovoid shape. In the subsequent 4 to 8 h, these endodermal epithelial cells that now contain each of them several apoptotic bodies (easily identified thanks to their bright DNA content) regain their typical epithelial organization (see Supplemental Figs. 1 and 2 in Chera et al. 2009b and Fig. 4i in Chera et al. 2009a). This dynamic process was well visualized in the transgenic *Icy1* strain, whose interstitial cells constitutively express green fluorescent protein (GFP) (Khalturin et al. 2007): after amputation, the digestive cells located in head-regenerating tips were found loaded with GFP+ apoptotic bodies (Chera et al. 2009b). Interestingly numerous studies have shown that the endodermal epithelial cells located at the tip express signaling molecules and transcription factors involved in the formation of the head organizer (Gauchat et al. 1998; Technau and Bode 1999; Galliot and Miller 2000; Hobmayer et al. 2000; Kaloulis et al. 2004; Chera et al. 2007; Lengfeld et al. 2009). However, the role of engulfment in the head regeneration process remains to be investigated: these digestive cells certainly act as scavenger, clearing the animal from the apoptotic bodies, but their exposure to the content of the interstitial cells might also modulate their intracellular signaling and help them reprogram from digestive to organizer cells.

Injury-induced apoptosis is necessary for head regeneration

The extent and timing of this immediate wave of apoptosis in head-regenerating tips strongly suggest that it plays a role in the formation of the head organizer. To test the putative function of cell death, complementary strategies were designed, either to inhibit the apoptotic process or to induce it ectopically. All these approaches affected the head regeneration process (Table 1). The first and most direct way to inhibit cell death was to prevent caspase activity in regenerating *Hydra:* for this purpose, animals were treated for a short period of time (90 min before amputation and up to 90 min after) with the Z-VAD-fmk pan-caspase inhibitor (Graczyk 2002). Previous studies performed by the group of Charlie David and Angelika Boettger in Munich had proven that the genetic circuitry supporting apoptosis is well-conserved across eumetazoans and that, indeed, caspase inhibitors significantly reduce caspase activity in *Hydra* (Cikala et al. 1999; Lasi et al. 2010). This short exposure to Z-VAD-fmk was sufficient to efficiently inhibit apoptosis: 75 % of the bisected animals exposed to Z-VAD-fmk no longer regenerate their head and actually die within the next days. This result indicated that caspase activity is necessary for the regenerative process.

Apoptotic cells provide a transient source of signaling in *Drosophila*

Since the 1970s, it was known that dying cells can trigger the proliferation of their neighbors, a process firstly identified by developmental biologists who had noticed in *Drosophila* larvae that irradiation of their imaginal discs can induce regeneration through compensatory proliferation (Haynie and Bryant 1977; Bergmann and Steller 2010) and secondly by radiologists who had understood that irradiated tumoral tissues also lead to compensatory proliferation, proposing the name of "altruistic cell death" to describe this phenomenon (Kondo 1988; Li et al. 2010). If apoptosis is necessary to trigger compensatory proliferation or regeneration, then it means that either the apoptotic cells themselves deliver some signals (active model) or that the tissue senses the absence of the cells that died and react by replacing them (passive model) (Ryoo et al. 2004; Simon et al. 2009). To test which model would be valid, it was necessary to set up specific tools to monitor the signaling activity of dying cells given the fast kinetics of the apoptosis process.

A decade ago, *Drosophila* geneticists established a novel and elegant strategy whereby they created "undead cells": such cells are obtained by concomittantly inducing apoptosis in growing imaginal discs (usually by inhibiting inhibitors of the initiator caspase drONC) and expressing the p35 baculoviral protein, an inhibitor of the effector caspases drICE. That way, cells that enter apoptosis cannot make use of their effector caspases and become arrested in the apoptotic process. Thanks to this strategy, it was possible to characterize the signaling molecules produced in the undead cells upon initiator caspase activation. In 2004, three different groups published convergent results, showing that apoptotic cells of the wing imaginal discs release signaling molecules, namely wg and dpp, which promote cell proliferation in their vicinity (Huh et al. 2004; Perez-Garijo et al.

Table 1 Different effects obtained on head-regenerating halves after exposure to caspase inhibitors (Z-VAD), MEK inhibitors (U0126), or after RNAi knockdown of the *Wnt3*, *β-catenin*, *RSK*, *CREB*, and *CBP* genes

	Injury-induced apoptosis	β-catenin nuclear translocation	Synchronous cell division in the tip	Cell proliferation along the body column	Migration of precursor cells towards wound	Wound healing	Head regeneration
Head-regenerating tips after mid-gastric bisection	(+) 30–60 min	(+) 60–90 min	(+) ~4 hpa	(+)	(+)	(+)	100 %, 50–60 h
Z-VAD-fmk 20 μM	(−)	(−)	(−)	Low	(+)	(−)	25 %
Z-VAD-fmk + Wnt3	(−)	(+)	(+)	(+)	(+)	(+)	(+)
Wnt3 (RNAi) 3×	(?)	(−)	(−)	Very low		(−)	(−)
β-catenin (RNAi) 3×	(?)	(−)	(−)	None		(−)	(−)
U0126 20 μM	(−)	nd	(−)	Very low		(−)	(−)
RSK (RNAi) 1×	(−)				(+)	(−)	+41 h
2×		nd	nd	nd			+138 h
3×	(−)						Lethal
CREB (RNAi) 1×	(−)	(−)	(−)	(+)	(+)	(−)	+50 h
2×		(−)	(−)	None			+149 h
3×	(−)						Lethal
CBP (RNAi) 1×	(−)				(+)	(−)	+64 h
2×		nd	nd	nd			+154 h
3×	(−)						Lethal
Heat-induced ectopic apoptosis		(+)	nd	(+)	nd	(+)	(+)

For RNAi knockdown experiments, animals were fed with dsRNAs repeatedly and the number of feedings is indicated (1×, 2×, or 3× exposures to dsRNAs). The sign (?) in Wnt3(RNAi) 3× and β-catenin(RNAi) 3× animals indicates that the level of injury-induced apoptosis could not be evaluated as these animals showed already high levels of homeostatic apoptosis. Note that animals exposed 3× to RSK dsRNAs lack RSK and also CREB and CBP proteins, whereas animals exposed 3× to CREB dsRNAs lack CREB and CBP proteins. The last row indicates the effects recorded on foot-regenerating halves briefly heated to ectopically induce a high level of apoptosis at the wound. (For detailed results see Chera et al. 2009b, 2011)

2004; Ryoo et al. 2004). These results definitely provided a link between apoptosis and compensatory proliferation and pointed to an active model involving the non-apoptotic functions of caspases (Kuranaga and Miura 2007; Fan and Bergmann 2008; Martin et al. 2009).

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

In 2004, we observed for the first time in *Hydra* regenerating their heads a massive cell death restricted to head-regenerating tips, but we also noted adjacent to this apoptotic area a denser zone of proliferating cells, identified thanks to BrdU labeling performed immediately after amputation. We immediately suspected some link between these two processes as inhibition of apoptosis would affect the formation of the proliferative zone. However, we were unable to characterize this link until Miguel Torres (Madrid) pointed to us the recent results obtained in *Drosophila*. We obviously thought that a similar mechanism might operate in *Hydra* and we started to investigate whether the apoptotic cells would provide a source of transient signaling in head-regenerating tips. The laboratory of Thomas Holstein's had shown the very early activation of the canonical Wnt-signaling pathway during head regeneration (Hobmayer et al. 2000). Therefore, we first searched for modulations of Wnt3 expression and β-catenin activation in head-regenerating tips—thanks to antibodies raised against the Wnt3 and β-catenin mammalian proteins that proved to cross-react with the *Hydra* cognate proteins. Indeed, we detected a transient overexpression of Wnt3 in dying cells, at early stages of apoptosis, suggesting that these cells release wnt3. In fact, as soon as the cells reach an advanced apoptotic stage, the Wnt3 signal can no longer be detected (Chera et al. 2009b).

This transient release of Wnt3 by the apoptotic cells is difficult to explain, as the epithelial cells are the main providers of Wnt3 at least when detected at the RNA level (Lengfeld et al. 2009). However in *Hydractinia*, a hydrozoan colonial polyp related to *Hydra*, the Wnt3 β-catenin pathway is activated in interstitial cells where it plays a key role in the maintenance of stem cells (Teo et al. 2006). In *Hydra* head-regenerating tips after mid-gastric bisection, we suspect that activation of caspases in the interstitial cells

induces a modification of a preexisting complex that contains Wnt3. This is assumed from the following observations: first *Hydra* treated with the pan-caspase inhibitor Z-VAD-fmk does not show any Wnt3 release, and second, we did not detect any upregulation of the *Wnt3* gene within the first 2 h following mid-gastric bisection (Chera et al. 2009b). Although we cannot exclude a very fast upregulation of the *Wnt3* gene in cells undergoing cell death, we rather suspect that some Wnt3 protein is stored in the interstitial cells, masked in a complex where it is not immuno-detectable. Upon injury, caspases activation would allow Wnt3 release. This hypothesis remains to be proven, and beside Wnt3, additional signals might also be released by the apoptotic cells.

Activation of β-catenin signaling in response to cell death in *Hydra*

To assess the role of this apoptosis-induced Wnt3 signal, we measured the activation of the β-catenin pathway in head-regenerating tips and indeed identified a clear nuclear translocation of β-catenin in the neighboring cells. The apoptosis-induced Wnt3 signal peaks between 30 and 60 min after bisection, while the nuclear translocation of β-catenin is detected between 60 and 90 min after bisection, showing thus a temporal and spatial correlation between the two events. In addition, the cells that exhibit an activation of β-catenin are actually cycling cells as evidenced by the colocalization of nuclear β-catenin with BrdU labeling. A detailed analysis of these BrdU-positive cells indicates that β-catenin activation pushes the cycling cells through mitosis (Chera et al. 2009b). Four hours after amputation, one can see two distinct regions in the stump: the most superficial empty zone as previously reported (Holstein et al. 1991) that corresponds to the apoptotic zone, about 100 μm thick, and the proliferating zone where cycling progenitors accumulate (Fig. 4a, b). These progenitors accumulate in fact as a result of several mechanisms: first migration of progenitors towards the wound (Khalturin et al. 2007) and second their rapid division upon activation of the β-catenin pathway by the apoptotic cells. All together, these experiments suggested that very similar mechanisms operate in the *Drosophila* wing disc and in *Hydra* regenerating its head after mid-gastric bisection.

Apoptosis-induced compensatory proliferation via β-catenin signaling leads to head regeneration

So far, the scenario was rather correlative and functional manipulations were needed to test its validity. The first approach aimed at rescuing head regeneration in Z-VAD-fmk-treated animals by simply adding recombinant Wnt3 in the *Hydra* medium. Indeed exposing animals to exogenous Wnt3 efficiently rescues β-catenin activation in Z-VAD-fmk-treated *Hydra* with a dose-dependent effect. The efficiency of this Wnt3 treatment was observed at the molecular level (β-catenin nuclear translocation) and also at the process level as the animals first survived the Z-VAD-fmk treatment and second regenerated new heads, even faster than control animals and often with ectopic tentacles (Chera et al. 2009b). This is in agreement with the expected role of Wnt3 as a main head-inducing signal (Lengfeld et al. 2009).

As a second approach, we induced ectopic apoptosis in foot-regenerating tips where the level of injury-induced apoptosis is low, to see whether higher levels of apoptosis would suffice to induce ectopic activation of β-catenin signaling and ectopic head regeneration. To induce a higher level of apoptosis, we briefly heated the wound of foot-regenerating tips immediately after bisection without destroying the tissues (Fig. 4c-f). We indeed detected in a large proportion of the animals an apoptotic zone that was sensitive to Z-VAD-fmk (proving that caspases are activated) and that led to ectopic cell proliferation as evidenced by BrdU labeling and ultimately to ectopic head regeneration (Chera et al. 2009b). The heat-induced apoptosis appeared to trigger a signaling identical to that observed in head-regenerating tips, as ectopic apoptosis led to Wnt3 activation at the tip and subsequently to β-catenin nuclear translocation.

To prove that the same signaling is at work in ectopic and physiological apoptosis, we developed an assay named the splitting assay, where the wound of upper halves was first heated (to induce ectopic high level of apoptosis), and then at various time points, each regenerating animal was separated in two longitudinal halves, bisected along the apico-basal axis. Out of these two halves, one was kept alive to monitor the outcome of the regenerative process (the formation of an ectopic head or that of a basal disc) while the second half was fixed at early time points and analyzed at the molecular level. When fixed 30 min after bisection, the heat-induced upregulation of Wnt3 was tested, when fixed 2 h after bisection, the nuclear translocation of β-catenin in BrdU+ cells was detected (Chera et al. 2009b). This assay allowed us to match for each animal the heat-induced activation of the Wnt pathway and the ectopic head regeneration. Indeed, we found for each animal a perfect correlation between these two events in locally heated foot-regenerating animals, indicating that the same mechanism takes place physiologically and experimentally, in head-regenerating tips showing endogenous apoptosis and in foot-regenerating tips submitted to heat-induced apoptosis. However, the question of the signaling triggering a high level of apoptosis on one

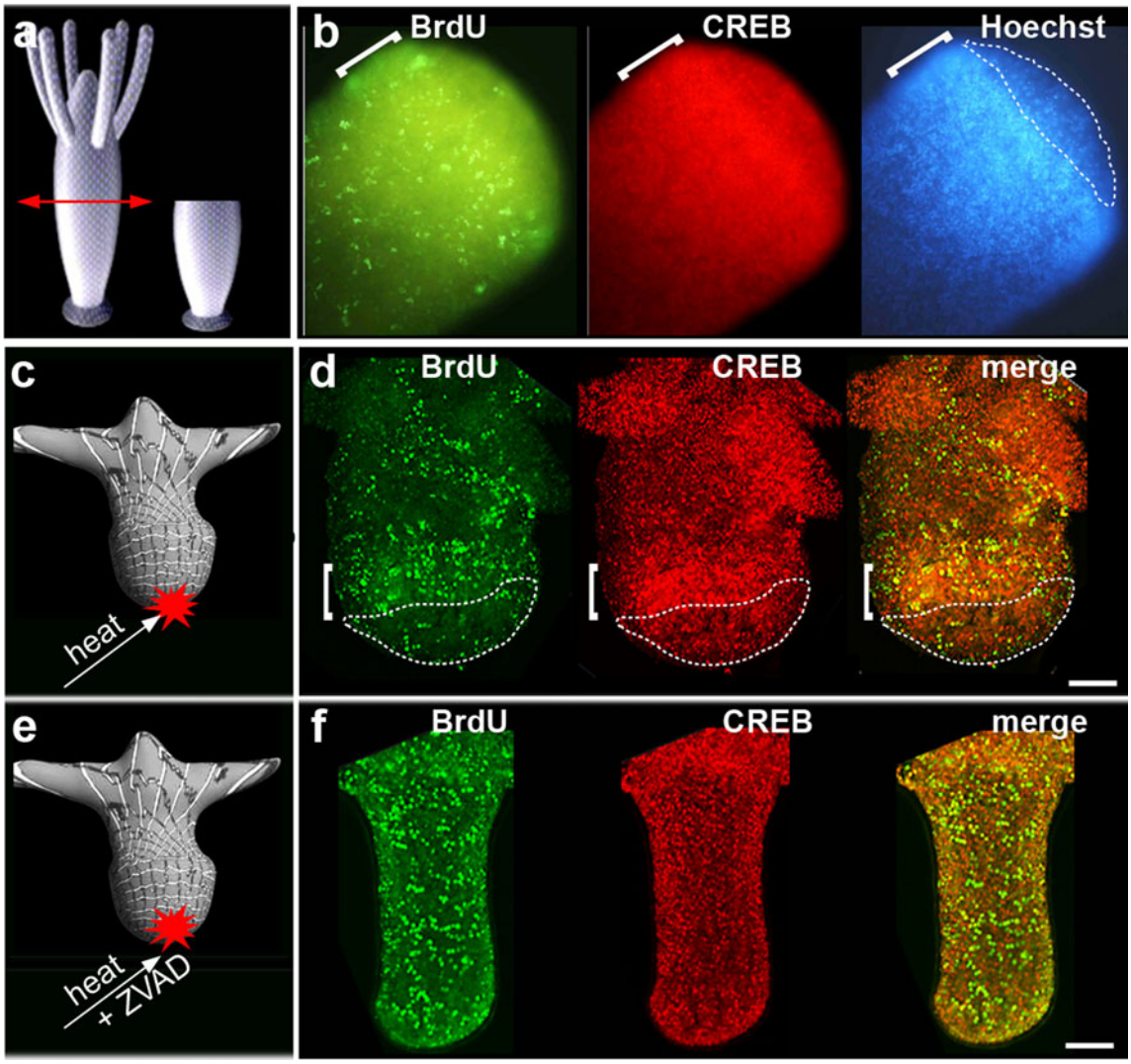

Fig. 4 Apoptotic and proliferative zones in head-regenerating halves (**a**, **b**) and heated foot-regenerating halves (**c**–**f**). *Hydra* was bisected at mid-gastric level (**a**), BrdU-labeled for 2 h after bisection, fixed at 4 hpa, immunodetected with anti-BrdU (*green*) and anti-hyCREB (*red*) and stained with Hoechst (shown only in **b**). In **b** and **d**, the *dashed lines* circle the apoptotic zone and the *brackets* indicated the proliferative zone. In **c**–**f**, the regenerating tips of upper halves were immediately heated after bisection to induce ectopic apoptosis. In **e**, **f** heating was followed by a 90-min ZVAD exposure. Note the absence of apoptosis and proliferative zones in **f**. In **f**, tentacles were removed prior to picturing. *Scale bar*, 100 μm. For details, see in Chera et al. (2009b)

side of the wound and a low level of apoptosis on the other side remained open.

Bisection immediately induces the asymmetric activation of signaling pathways

Activation of STK, Pi3K, ERK, and MAPK pathways during head but not foot regeneration

To identify the signaling pathways supporting the establishment and the activity of the organizer that arises in head-regenerating tips, pharmacological and RNAi knock-down approaches were performed. Both showed that activation of STK (Cardenas et al. 2000; Cardenas and Salgado 2003; Manuel et al. 2006; Arvizu et al. 2006), GSK3 (Broun et al. 2005), PKC, phosphoinositide 3-kinase (Pi3K), extracellular signal-regulated kinase (ERK) 1–2 (Manuel et al. 2006; Arvizu et al. 2006), mitogen-activated protein kinase (MAPK), and ribosomal S6 kinase (RSK) (Kaloulis et al. 2004; Chera et al. 2011) are indeed necessary over the first hours following bisection (see Table 2 and Fig. 5). Interestingly, the activation of STK, ERK 1–2, PI3K, MEK, and RSK is asymmetric, unnecessary for foot regeneration but required for head regeneration, as deduced from biochemical analyses that the RSK as an early substrate of asymmetric phosphorylation event (Kaloulis et al. 2004).

Table 2 Inhibition of the STK, PKC, Pi3K, ERK 1–2, and MEK kinases affect head regeneration but not foot regeneration

Target kinases	Inhibitors	IC	Head regeneration	Foot regeneration	Reference
Src-TK	PP1/ AGL1872	1 μM 3 days	100 % inhibited after decapitation	No inhibition	(Cardenas et al. 2000)
	PP2/ AG1879*	1 μM 3 days	100 % inhibited after decapitation	No inhibition	(Cardenas and Salgado 2003)
PKC	Sphingosine	2 μM 3 days	100 % inhibited after decapitation	No inhibition	(Cardenas et al. 2000)
	Staurosporine	100 nM 3 days	100 % inhibited after decapitation	No inhibition	(Cardenas et al. 2000)
	H7	25 μM 3 days	100 % inhibited after decapitation	No inhibition	(Cardenas et al. 2000)
PI3K	LY294002*	6.6 μM 3 days	100 % inhibited after decapitation	No inhibition	(Manuel et al. 2006; Arvizu et al. 2006)
ERK 1–2	Apigenin*	4.2 μM 3 days	100 % inhibited after decapitation	No inhibition	(Manuel et al. 2006; Arvizu et al. 2006)
MEK1 MEK2	U0126*	20 μM 3 h	95 % inhibited after mid-gastric section	No inhibition	(Kaloulis et al. 2004; Chera et al. 2011)
	PD98059	20 μM 3 h	15 % inhibited after mid-gastric section	20 % inhibition	(Kaloulis et al. 2004)
	PD98059*	50 μM 3 days	80 % inhibited after decapitation	No inhibition	(Manuel et al. 2006; Arvizu et al. 2006)
	Olomoucine	40 μM 3 days	80 % inhibited after decapitation	No inhibition	(Manuel et al. 2006)

Note that most head regeneration experiments were performed after decapitation (~70 % body length). Asterisks indicate kinase inhibitors that exhibit their complete effect only when given immediately after bisection and show lower effects or no effect at all when given 6 or 10 h after bisection. For treatments performed for 3 h, animals were exposed to the drugs from 90 min before bisection up to 90 min after bisection

The study of these kinases allowed the group of Luis Salgado to investigate two interesting aspects of head-organizing activities in *Hydra* : The first question was to discriminate between the formation and the maintenance phases of the head organizer during regeneration: the STK, Pi3K, ERK 1–2, and MAPK pathways are clearly involved in the formation of the head organizer and not for the maintenance of its activity as when these inhibitors are given several hours after bisection, they lose their inhibitory effect, proving that when organizer activity is set up, the activity of these kinases is no longer necessary (Arvizu et al. 2006). Indeed after decapitation, apigenin, LY294002, and PD98059 that inhibit ERK 1–2, PI3K, and MEK, respectively, need to be added within the first 6 h to show an effect on head regeneration (Manuel et al. 2006; Arvizu et al. 2006). Similarly, a short pulse of U0126 given for 90 min before and after mid-gastric bisection suffices to inhibit injury-induced apoptosis, phosphorylation of the CREB transcription factor, and head regeneration (Kaloulis et al. 2004; Chera et al. 2011).

The second question concerns the comparative analysis of the signaling activities that support head-organizing activities in the homeostatic (i.e., in the head of the polyp) and the regenerative contexts. To address this question, Arvizu et al. performed lateral transplantation experiments that measured the organizer activity of animals exposed to kinase inhibitors for 48 h. When heads from intact animals treated for 48 h were grafted, a fully efficient organizer activity was recorded, similar to that obtained with heads from untreated polyps, indicating that exposure to a single kinase inhibitor does not affect the organizer activity in homeostatic conditions. By contrast, when the apical tips of decapitated polyps exposed to one or the other kinase inhibitors for 48 h were grafted, the organizer activity was totally missing, demonstrating that the STK, Pi3K, ERK 1–2, and MEK kinases are necessary for the formation of the head organizer after decapitation (Arvizu et al. 2006).

However, in most of these studies, the role of these signaling pathways was investigated exclusively after decapitation and not after mid-gastric bisection (Table 2). In fact, several lines of evidences suggest that launching a head regeneration program upon decapitation or upon mid-gastric bisection is not identical (Technau and Holstein 1995; Kaloulis 2000; Galliot and Chera 2010). The fact that injury responses vary according to the bisection level is not so surprising as the tissue composition differs dramatically between the upper body column, made of progenitors ready to terminally differentiate and the mid-gastric region, densely packed in interstitial stem cells (David and Plotnick 1980) (Fig. 2c). As a consequence, the analysis of the immediate

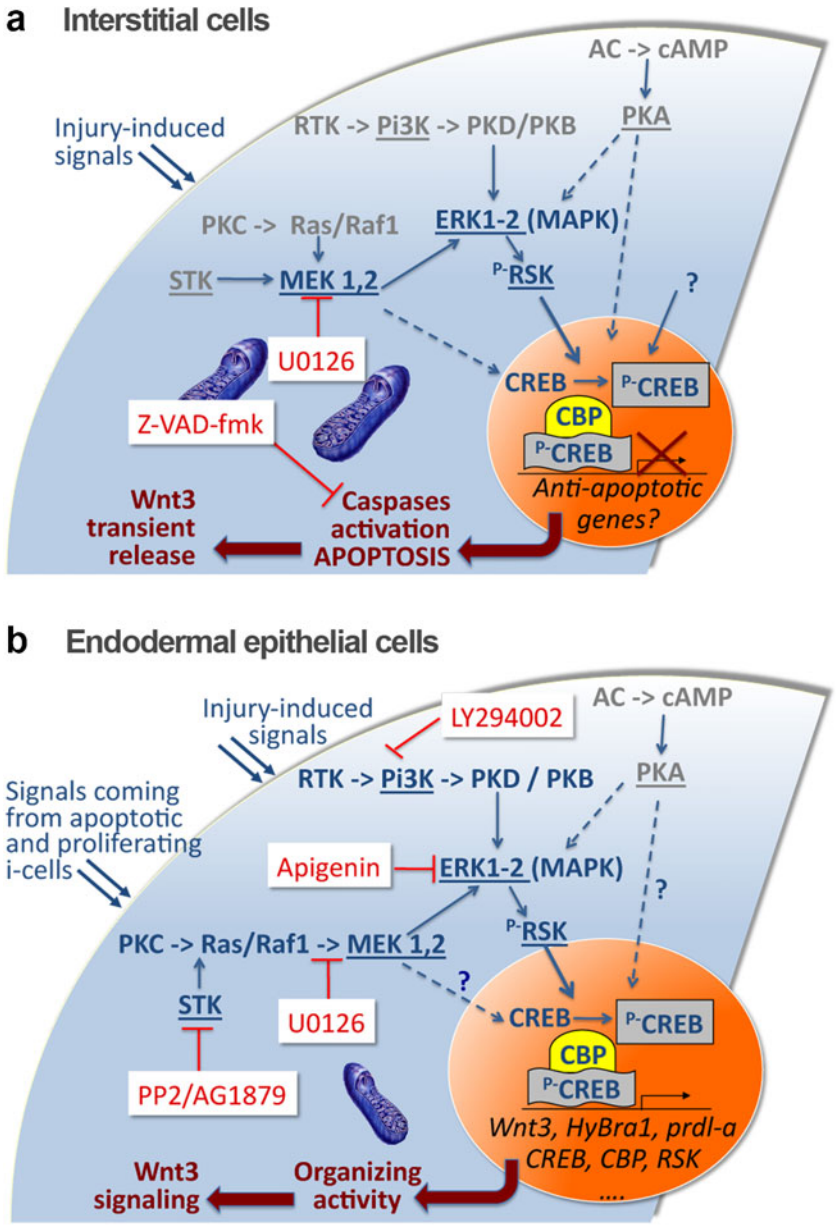

Fig. 5 Summary scheme showing the signaling pathways activated upon bisection in head-regenerating tips, in the interstitial cells that undergo injury-induced apoptosis immediately after mid-gastric bisection (**a**, top), and in the endodermal epithelial cells that develop a de novo head-organizing activity in few hours (**b**, bottom). After mid-gastric bisection (50 % body length), interstitial cells and their derivatives undergo injury-induced apoptosis as a result of the asymmetric MEK-dependent phosphorylation of RSK and CREB (Kaloulis et al. 2004; Chera et al. 2009b, 2011). The mechanism leading to cell death possibly involves the asymmetric silencing of anti-apoptotic genes maintained active in homeostatic conditions along the body column (W. Buzgariu, S. Reiter unpublished). As a result of caspase activation, signaling molecules as Wnt3 are transiently released leading to the activation of β-catenin signaling in the surrounding cycling progenitors followed by their mitotic division (not depicted here; see in Chera et al. 2009b; Galliot and Chera 2010). A couple of hours after bisection, a series of early genes are upregulated in the endodermal epithelial cells, including the Wnt3 gene that contains CREs in its regulatory sequences (Gauchat et al. 1998; Technau and Bode 1999; Hobmayer et al. 2000; Kaloulis et al. 2004; Chera et al. 2007; Chera et al. 2009b; Lengfeld et al. 2009; Chera et al. 2011; Nakamura et al. 2011). By contrast, after decapitation (80 % body length), head injury-induced apoptosis and apoptosis-induced compensatory proliferation are not observed (Galliot and Chera 2010) and Wnt3 seems to be directly upregulated in the epithelial cells (Lengfeld et al. 2009). Among the pharmacological inhibitors that affect the early phase of head regeneration but not foot regeneration (see Table 2), only U0126 was tested after mid-gastric bisection (Cardenas and Salgado 2003; Kaloulis et al. 2004; Arvizu et al. 2006; Manuel et al. 2006; Chera et al. 2011)

signaling triggered by mid-gastric bisection offers the possibility to decipher the mechanisms that generate an asymmetric signaling in few minutes from a homogenous tissue, but comparative analysis of this response to that

obtained after decapitation should help understand how the homeostatic background can influence the activation of a regenerative program.

Immediate and asymmetric activation of the MAPK/CREB pathway

To decipher the signaling cascades underlying head regeneration after mid-gastric bisection, we focused on the posttranslational regulation of CREB, a transcription factor initially characterized thanks to the modulations of its DNA-binding pattern during the first hours of regeneration (Galliot et al. 1995). As posttranslational regulation through phosphorylation is critical for CREB activity (Mayr and Montminy 2001), the level of CREB phosphorylation as well as the activity of the different kinases that bind to CREB were analyzed (Kaloulis et al. 2004). Immunodetection assays performed against CREB and phosphorylated CREB (P-CREB) detected a significantly higher level of P-CREB in the endodermal cells of head-regenerating tips than in the foot ones in the first hours following bisection. The role of P-CREB in the immediate phase of head regeneration was confirmed first by pharmacological approaches, as exposure to the MEK inhibitor U0126 that inhibits CREB phosphorylation but also injury-induced apoptosis and finally head regeneration without affecting foot regeneration (Kaloulis et al. 2004). Among the kinases that display a temporal and spatial regulation after bisection, p80 showed an enhanced activity and a hyperphosrylated status in head-regenerating halves, but not in foot-regenerating ones already 20 min after bisection. Further biochemical evidences identified this p80 CREB kinase as the RSK, itself regulated by the MAPK pathway. Indeed animals RNAi knocked-down for *RSK*, *CREB*, or *CBP* lack injury-induced apoptosis, exhibit wound healing defects as well as a significant delay in head regeneration, until expression of these genes resumes (Chera et al. 2011) (see details in Table 1). All together, these data indicate that a functional MAPK/CREB pathway is required for injury-induced apoptosis and for head organizer formation (Fig. 5). Two aspects require further investigations: the signals that lead to an immediate phosphorylation of MEK, ERK 1–2, and RSK in head-regenerating tips but not in foot-regenerating ones and the process followed by the MAPK/CREB pathway to trigger apoptosis. One possibility would be the injury-induced CREB-dependent downregulation of anti-apoptotic genes as previously reported for the CREB-related gene ATF3 in mammals (Hua et al. 2006).

Early genes in the head organizer and putative regulation by the ERK/MAPK/CREB pathway in the epithelial cells

The formation of the head organizer during the first hours after bisection occurs concomittantly with the local upregulation of genes encoding transcription factors as the paired-like homeobox gene *prdl-a* (Gauchat et al. 1998; Galliot and Schmid 2002), the T-box gene *HyBra1* (Technau and Bode 1999), the high-mobility group gene *Tcf* that interacts with β-catenin in response to Wnt activation (Hobmayer et al. 2000), the zinc finger gene *cnnos2* (Mochizuki et al. 2000), the bZIP gene *CREB* (Chera et al. 2007), and the multifunctional chromatin regulator *CBP* (Chera et al. 2011). Within the same time window, a number of genes encoding signaling molecules are also upregulated, as *Wnt3* and its antagonist *hydkk1/2/4* (Hobmayer et al. 2000; Guder et al. 2006; Lengfeld et al. 2009), *BMP5-8b* (Reinhardt et al. 2004), as well as genes encoding kinases as *PKB* (Herold et al. 2002), *PKC2* (Hassel et al. 1998), and *RSK* (Chera et al. 2011). STK, Pi3K, ERK, and MAPK activation is linked to the formation of the head organizer after decapitation although their respective role in the regulation of gene expression remains unknown. After mid-gastric bisection, *HyBra1* and *prdl-a* are no longer upregulated when CREB phosphorylation is inhibited (Kaloulis et al. 2004). Similarly, *RSK*, *CREB*, and *CBP* require a functional pathway to maintain their level of expression and their head regeneration-specific upregulation (Chera et al. 2011). The recent functional dissection of the regulatory sequences of *Wnt3* identified an autoregulatory element as well as a repressor element that restricts *Wnt3* expression to the organizer region, but also binding sites for CREB (Nakamura et al. 2011). Therefore, beside their participation in injury-induced apoptosis in the interstitial cells, RSK, CREB, and CBP also likely play in the epithelial cells a key role to modulate the expression of the early head regeneration genes that are essential for the establishment of the head organizer (Fig. 5).

Perspectives

The data discussed in this review definitely show that foot regeneration and head regeneration, despite sharing a similar wound healing response, are immediately different. Our prediction is that foot regeneration corresponds to a process that is close to tissue repair, whereas head regeneration requires the activation of a complex morphogenetic process. Transplantation studies identified three stages in the setting up of the de novo head organizer after bisection: a first one, immediate and negative, named post-cutting inhibition, followed by a second one, when the organizer activity is rising in the stump, and finally a third one characterized by its plateau value (MacWilliams 1983). One can see from the recent studies discussed here that the first period immediately after bisection is actually extremely dynamic, both at the cellular and molecular levels, with posttranslational modifications that on one hand affect cell behaviors, but on the other hand likely modulate the level of expression of the genes required for the formation of the head organizer. Therefore, this immediate/

early stage does not seem to correspond to a "post-cutting inhibition" but should rather be viewed as an unsteady phase in the formation of the head organizer, at a time when its activity cannot be detected by transplantation yet. Subsequently, once the early genes produce the required amount of proteins, the activity of the head organizer starts to be detected upon transplantation until it becomes steady.

Injury-induced apoptosis, which is immediately induced after bisection certainly belongs to the first unsteady stage. However, its role might be more complex. Here, we viewed apoptosis as an additional signaling tool to launch head regeneration, the dying cells providing signals to modulate the behavior of their neighbors. This is an "inductive or active" view of injury-induced apoptosis, similar to that described in *Drosophila* or in mice (Ryoo et al. 2004; Bergmann and Steller 2010; Li et al. 2010). However, injury-induced apoptosis can also be viewed as "suppressive or passive," as a way to transiently interrupt the crosstalk between two or several cell types, here the interstitial cells that die upon injury and the epithelial cells that survive. This suppressive hypothesis is valid when cell death leads to the destruction of cells that in homeostatic conditions send signals and regulate the behavior of their neighbors (Simon et al. 2009). In *Hydra*, this scenario might also be valid as the interstitial cells seemingly repress the morphogenetic activity of the epithelial cells: Twenty years ago, Sugiyama and Wanek could rescue head regeneration in the head regeneration-deficient strain reg-16 by eliminating the interstitial cells by colchicine treatment (Sugiyama and Wanek 1993). They measured the organizer activity of this mutant strain by transplantation and interpreted this phenotype as the result of an excessive repression of the morphogenetic potential of the epithelial cells by the interstitial cells. Thus, a transient and local modulation of the crosstalk between cell populations with distinct morphogenetic potentials might be critical for launching regeneration. These two modes of activity of injury-induced apoptosis, inductive and suppressive, are not mutually exclusive, and they might in fact be sequential, with a very transient inductive mode, corresponding to the apoptotic process per se, and a longer suppressive mode, persisting as long as the dying cells are not replaced.

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