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CELL DEATH: A PROGRAM TO REGENERATE

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Abstract

Recent studies in *Drosophila*, *Hydra*, planarians, zebrafish, mice, indicate that cell death can open paths to regeneration in adult animals. Indeed injury can induce cell death, itself triggering regeneration following an immediate instructive mechanism, whereby the dying cells release signals that induce cellular responses over short and/or long-range distances. Cell death can also provoke a sustained de-repressing response through the elimination of cells that suppress regeneration in homeostatic conditions. Whether common properties support what we name “regenerative cell death”, is currently unclear. As key parameters, we review here the injury pro-apoptotic signals, the signals released by the dying cells, the cellular responses and their respective timing. ROS appears as a common signal triggering cell death through MAPK and/or JNK pathway activation. But the modes of ROS production vary, from a brief pulse upon wounding to repeated waves as observed in the zebrafish fin where ROS supports two peaks of cell death. Indeed regenerative cell death can be restricted to the injury phase, as in *Hydra, Drosophila,* or biphasic, immediate and delayed, as in planarians and zebrafish. The dying cells release in a caspase-dependent manner a variety of signaling molecules, cytokines, growth factors, but also prostaglandins or ATP as recorded in *Drosophila*, *Hydra*, mice and zebrafish respectively. Interestingly the ROS producing cells often resist to cell death, implying a complex paracrine mode of signaling to launch regeneration, involving ROS-producing cells, ROS-sensing cells that release signaling molecules upon caspase activation, and effector cells that respond to these signals by proliferating, migrating, and/or differentiating.

1. Introduction to regenerative cell death

Regeneration of body parts, appendages or internal organs needs the orchestration in time and space of several genetic programs, from wound healing to complete restoration of the missing part, with reestablishment of the initial function. This reactivation of developmental programs is easily triggered by injury in a wide variety of species, while in others, like most adult mammals, lesion/amputation leads to wound healing with scar formation (Sanchez Alvarado and Tsonis, 2006; Brockes and Kumar, 2008; Tanaka and Reddien, 2011; Wood and Eming, 2012). This observation suggests that injury activates distinct signaling cascades in these two contexts, either a signaling restricted to wound

healing, or a signaling giving rise to wound healing and full restoration of tissues, organs, appendages (Murawala et al., 2012). If true, a detailed inventory of the injury-induced signals throughout the animal kingdom should provide valuable pieces of information to understand how developmental programs can be reactivated upon wounding, and thus contribute to the development of regenerative medicine.

Although regeneration is a widespread phenomenon among metazoans, a limited number of model systems actually provide the conditions for a parallel analysis of wound healing in the absence or presence of regenerative processes. Among them, we find the *Hydra* polyps and the planarian non-parasitic flatworms that can both regenerate any missing part of their body (Galliot and Ghila, 2010; King and Newmark, 2012), the adult holothuries (sea cucumbers) that regenerate their gut as well as their nervous system (San Miguel-Ruiz et al., 2009; Mashanov and Garcia-Arraras, 2011), *Drosophila* that provides a genetic model for epithelial wound healing at embryonic, larval and adult stages (Galko and Krasnow, 2004; Razzell et al., 2011), but also regenerates its imaginal discs as larva (Bryant, 1971; Bergantinos et al., 2010b), and repair its midgut as adult (Amcheslavsky et al., 2009; Buchon et al., 2009b).

Among vertebrates, the teleost fish (e.g. zebrafish) that regenerate their fins, heart and brain (Nakatani et al., 2007; Kizil and Brand, 2011; Choi and Poss, 2012), the *Xenopus* tadpoles that regenerate their appendages (Slack et al., 2008), and the adult urodeles (axolotl and newts) that perfectly heal their wounds while regenerating their appendages and a variety of organs (Brockes and Kumar, 2002; Murawala et al., 2012), also provide valuable models for this comparison. By contrast, adult mammals maintain their tissues through active self-renewal and can repair some tissues (skin, liver, bone, lung) but most of them are unable to regenerate the 3D organization of their organs or appendages, and repair their wounds with scars. Moreover these tissue repair capacities decline upon aging (see in (Sousounis et al., 2013).

At adulthood, size, morphology complexity, tissue complexity and cellular behaviors largely vary between regenerative models. The body structure of *Hydra* polyps and planarians, although not similar, is relatively simple, comprising 12 to 15 differentiated cell types and large stocks of stem cells (Steele, 2002; Reddien and Sanchez Alvarado, 2004). Regeneration assays are usually performed on adult animals about 10 mm long, however in both species, the adult body size dramatically varies according to the feeding rhythm, even though regeneration remains highly robust during starvation (Romero and Baguna, 1991; Galliot and Ghila, 2010; Gonzalez-Estevez et al., 2012). By contrast adult *Drosophila,* which exhibit a complex anatomy, do not renew much of their somatic tissues, mainly their gut that is used as a model of tissue repair (Buchon et al., 2013).

At the other end of the spectrum, limbs of axolotls are several centimetres long, exhibit a complex 3D structure with tissues composed of a variety of differentiated cell types, which regenerate the missing structures from lineage-restricted progenitors (Tanaka and Reddien, 2011). Time provides a useful scale to measure these differences in complexity of regenerative processes, e.g. head regeneration takes about three days in *Hydra,* a couple of weeks in planarians, whereas limb regeneration in urodeles takes about two months. However despite this heterogeneity, regeneration relies on a succession of common steps from injury to regenerative outgrowth, i.e wound healing, blastema formation, differentiation and growth, and the duration of the initial phases (wound healing, induction of the regenerative program) appear similar **(Fig. 1**).

In most regenerative contexts, progenitor cells proliferate to form a blastema, and two non-mutually exclusive mechanisms operate to produce this pool of progenitors, either by recruiting stem cells located in proximity or at distance of the lesion site, or by inducing the dedifferentiation of the cells located in the vicinity of the lesion (Brockes and Kumar, 2008; Kragl et al., 2009; Poss, 2010). In both situations, the tissues that provide progenitor cells are subjected to an important histolysis, a process affecting tissues subjacent to the wound over a 1-2 mm distance in vertebrates (Weiss and Rosenbaum, 1967). Although initially described in the notochord and cartilage of metamorphic toads by Carl Vogt in 1842, and subsequently repeatedly reported in developmental as well as non-developmental contexts (Clarke and Clarke, 1996), the role of Programmed Cell Death or apoptosis in developmental processes was then neglected and re-discovered 120 years later (Kerr et al., 1972).

In regenerative studies cell death was considered as a side effect of injury and has not been taken into consideration until recently. In 1977, Haynie and Bryant reported that despite a 60% cell loss induced by irradiation, *Drosophila* wing imaginal discs were able to fully recover their size and shape through intensive cell proliferation (Haynie and Bryant, 1977). In parallel radiologists irradiating tumoral tissues (skin) of patients noted that dying cells induce proliferation of their

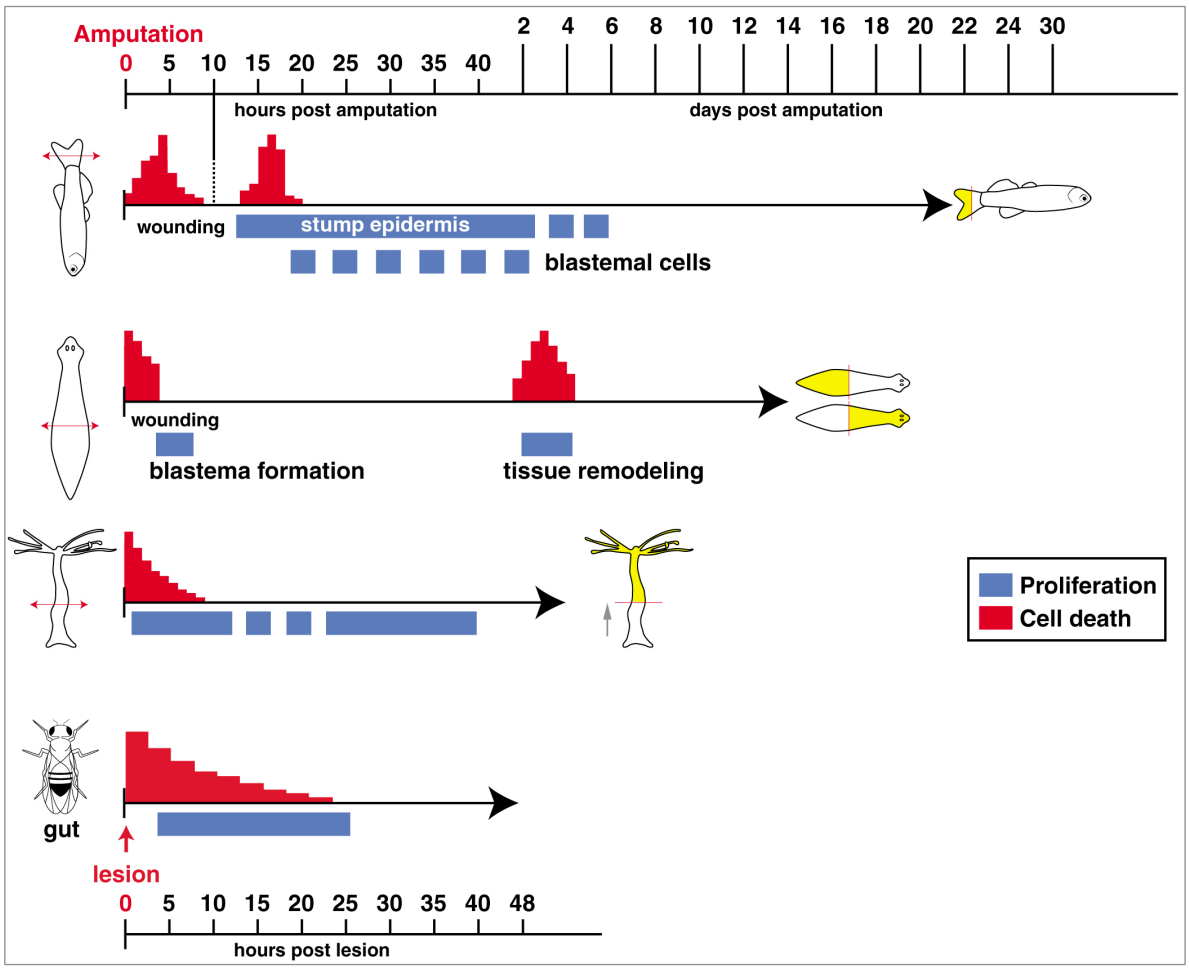

Figure 1: Injury-induced cell death (red) and cell proliferation (blue) events in *Danio rerio* regenerating its caudal fin, *Schmidtea mediterranean* regenerating its body *Hydra vulgaris* regenerating its head after mid-gastric bisection, *Drosophila melanogaster* repairing its midgut. Fin regeneration in *Danio*, completed in three weeks, shows two peaks of apoptosis, the second one being required for the proliferation of the stump epidermis and the recruitment of blastema cells (Gauron et al., 2013). Body regeneration in *Schmidtea* (planarian), completed in 10-14 days, also exhibits two peaks of apoptosis, the second one associated with a round of proliferation, is involved in tissue remodelling (Pellettieri et al., 2010). Head regeneration in *Hydra*, completed in three days, displays one burst of apoptosis, directly promoting cell division (Chera et al., 2009). Infection with pathogenic bacteria leads to cell death of enterocytes in adult *Drosophila*, which stimulates the proliferation of intestine stem cells and the repair of the gut (Amcheslavsky et al., 2009; Buchon et al., 2009b). Time scales are indicated at the top and bottom; regenerated structures are colored yellow.

neighboring cells and proposed the concept of "altruistic cell death" (Kondo, 1988). Thanks to a genetic strategy designed in *Drosophila* larval imaginal discs to produce "undead" cells, i.e. cells where PCD is induced by activating the initiator caspases but simultaneously inhibited by expressing the baculovirus protein p35 that inhibits the effector caspase (Ryoo et al., 2004; Huh et al., 2004; Perez-Garijo et al., 2004), cells remain indefinitely apoptotic and the concept of apoptosis-induced proliferation, via JNK activation and release of growth factors by the dying cells, was evidenced (see in (Bergmann and Steller, 2010; Morata et al., 2011; Worley et al., 2012). However it should be stressed that in both contexts, *Drosophila* imaginal discs and mammalian irradiated tissues, cell death is artificially induced.

The repair and/or regenerative contexts where injury induces cell death are numerous, in vertebrates as well as in invertebrates. One can cite adult newts regenerating their retina (Kaneko et al., 1999) or their forelimb (Vlaskalin et al., 2004), flatworms regenerating their body (Hwang et al., 2004; Pellettieri et al., 2010), *Xenopus* tadpoles regenerating their tail (Tseng et al., 2007), *Hydra* regenerating their head (Chera et al., 2009), adult *Drosophila* repairing their midgut (Amcheslavsky et al., 2009; Buchon et al., 2009b), mice regenerating their skin or their liver (Li et al., 2010b), sea cucumbers regenerating their gut (Mashanov et al., 2010) or their radial nerve cord (Mashanov et al., 2013), adult zebrafish regenerating their fins (Gauron et al., 2013).

When cell death appears instrumental for regeneration, we name the process “regenerative cell death”. In several of these contexts (*Hydra, Drosophila* larvae, zebrafish, mice), it was shown that caspase activation is actually necessary for triggering the signaling that drives cellular remodeling and regeneration (Bergmann and Steller, 2010), and then one can talk of *regenerative apoptosis.* However, in contexts where caspase activation takes place, one observes a large heterogeneity in terms of cell death timing, signals inducing cell death, signals released by the dying cells, cellular responses driven by these signals. To tentatively evidence some common rules between the various contexts where regenerative apoptosis operates, we propose here a comparative analysis of this process in vertebrate as well as invertebrate contexts, focusing on i) the timing of injury-induced cell death, ii) the injury signals that drive cell death, iii) the caspase-dependent signaling turned on in the dying cells, and finally iv) the cellular responses induced by the signals released by the dying cells.

2. What timing for spontaneous cell death during regeneration?

A SINGLE IMMEDIATE WAVE OF CELL DEATH IN *HYDRA* REGENERATING ITS HEAD

Since 1744 *Hydra* provides a simple model for regeneration studies, historically the first one (Galliot, 2012). *Hydra* is a tube shape freshwater cnidarian, about 10 mm long, with an apical pole, named head, centered on the mouth/anus and surrounded by a ring of tentacles, and a basal disc at the other extremity. *Hydra* consists of two cell layers named ectoderm and endoderm, separated by an acellular layer named mesoglea. The myoepithelial cells that form an epidermis in the ectoderm and a gastrodermis in the endoderm, arise from two distinct stem cell populations. Moreover the multipotent stem cells, located in the ectoderm, provide sensorymotor and ganglia neurons, stinging cells, and germ cells in the ectoderm, but also nerve cells and gland cells in the endoderm.

After midgastric bisection, regeneration of the apical half (also named head) is observed in about three days, of the basal half (also named foot) in less than three days (**Fig. 1**). The analysis of the immediate cellular remodeling induced upon bisection showed a massive wave of cell death in head-regenerating tips, peaking after one hour, while remaining limited in foot-regenerating tips (Chera et al., 2009). This asymmetric wave of apoptosis plays a key role in the initiation of head regeneration, as dying cells transiently release Wnt3, which activates the b-catenin pathway in the surrounding progenitors and promotes Wnt3 expression in the epithelial cells.

Interestingly the cells that undergo cell death belong to the interstitial lineage exclusively, either as progenitors or as differentiated cells (neurons, nematocytes, gland cells). In fact, only a mild increase in the number of epithelial dying cells was noted in the vicinity of the wound, indicating that the epithelial cells in *Hydra* are extremely resistant to cell death, whatever the way used to induce cell death (Reiter et al., 2012). Inhibition of apoptosis leads to abortion of the head regeneration process in a large number of animals, whereas induction of apoptosis in the foot regenerating part leads to ectopic head formation (Chera et al., 2009). Heat-shock induced cell death in non-regenerating *Hydra* does not lead to ectopic heads, or ectopic regenerating structures. Therefore high level of cell death in regenerating tips suffices to convert them to head regeneration, but cell death in the absence of wounding is not sufficient to trigger a regenerative program (Chera et al., 2009).

Hydra regeneration is a rapid process and it is possible that two peaks observed in planaria and zebrafish are superimposed or even merged in *Hydra*. There is actually a second phase of cell proliferation during head regeneration, starting about 24 hours after amputation, involving all stem cell populations, epithelial and interstitial (Holstein et al. 1991; Miljkovic-Licina et al. 2007). This wave of cell proliferation is restricted to the presumptive head region, supporting the differentiation of the apical nervous system and the patterning of the regenerating head; it requires the expression of transcription factors as the paired-like homeobox gene *prdl-a* and the parahox gene *cnox-2* (Gauchat et al. 1998; Miljkovic-Licina et al. 2007). A wave of cell death preceding this second phase of cell proliferation was never recorded, but also was never thoroughly monitored.

TWO DISTINCT PEAKS OF CELL DEATH IN PLANARIAN REGENERATING ITS BODY

The freshwater planarian *Schmidtea mediterranea* is able to fully regenerate its body axis in about two weeks after gastric bisection. Regeneration does not involve a dedifferentiation process but the mobilisation of well-identified stem cells, the neoblasts. In response to amputation the neoblasts, which are spread all over the body, increase their mitotic rate and migrate to the wound site where they stop proliferating and form a blastema. Then blastemal cells differentiate to replace the missing structures (Reddien and Sanchez

Alvarado, 2004). Two distinct phases of cell death are observed during planaria regeneration, the first wave is strictly localised, taking place during the first four hours in a 100 μm thick area on both sides of the bisection plane, whereas the second one, recorded three days later, is diffuse (Hwang et al., 2004; Pellettieri et al., 2010). The first peak is not specific to regeneration, rather linked to wound healing as also observed after a small incision. In contrast the second peak of cell death is specific to regeneration.

The widely spread second peak of cell death exhibits a magnitude that depends on the amputation level along the animal axis and on the regeneration type: The number of dying cells is very high in planarians regenerating their head or trunk, but close to the baseline level in animals regenerating their tail (Pellettieri et al., 2010). This second episode of cell death affects exclusively the differentiated cells, as neoblasts do not undergo cell death during regeneration. Hence this second apoptotic event suggests some systemic response to wounding, which allows the remodelling of preexisting structures (Pellettieri et al., 2010). Interestingly these two peaks of cell death match the modulations of mitotic activity observed at 4-8 hours post-amputation (hpa) and 2-3 days post-amputation (dpa), with a relative minimum at 24 hpa (Salo and Baguna, 1984; Wenemoser and Reddien, 2010). Hence in planarians, injury-induced cell death is tightly regulated in time and space, a regulation that correlates with the proliferative responses. However the signaling function of the apoptotic cells remains unclear, particularly regarding the regulation of neoblast proliferation.

TWO PEAKS OF CELL DEATH IN THE ADULT ZEBRAFISH REGENERATING ITS CAUDAL FIN

All teleost fish regenerate organs and appendages at adulthood (Nakatani et al., 2007) and do so at a moderate speed, faster than amphibians, but slower than *Hydra*. The caudal fin regeneration is a popular model in which the amputated structure is replaced thanks to the formation of a blastema. This process involves the recruitment of progenitor cells through de-differentiation of the stump mesenchymal cells (Nakatani et al., 2007). Blastema formation includes four successive steps, the two first ones restricted to the wound epidermis, the two last ones involving the formation and the function of the blastema (Santos-Ruiz et al., 2002; Gauron et al., 2013).

During the first step (from 0 to 10 hpa), the lateral epidermis migrates to cover the wound in the absence of any cell proliferation and connective tissues form a clot. During this period, massive cell death is detected at the amputation plane, both apoptotic and non apoptotic, but not specific to regeneration as also observed during skin wound healing (Gauron et al., 2013). Then, at the time of wound closure (10 hpa) the level of cell death gets transiently back to that observed in non-amputated fins. Soon after, a second burst of cell death, mainly apoptotic this time, arises in the stump epidermis, up to five ray segments away from the amputation plane (about one mm). This second burst of cell death is both specific and necessary to regeneration, as not observed during mere wound healing, and preventing blastema formation when inhibited (Gauron et al., 2013). Indeed cells from the same compartment, the stump epidermis, start to proliferate as a consequence of apoptosis (Gauron et al., 2013), an essential step as any treatment reducing epidermal proliferation impairs blastema formation (Dufourcq and Vriz, 2006; Jazwinska et al., 2007).

The third phase starts around 15 hpa with the recruitment of the prospective blastemal cells from the stump mesenchyme. These cells re-enter the cell cycle and, while dividing, migrate towards the wound. This second proliferative event, which directly concerns the blastemal cells, is the criterion used to identify progenitor cells that have dedifferentiated and contribute to blastema formation. Even if in fish (Christen et al., 2010) and amphibians (Maki et al., 2009; Jhamb et al., 2011), these progenitor cells express stem cell markers as *klf4, pou5f1, sox2* etc…, an *in vivo* visualization of the recruitment process is currently missing. Finally at 36 hpa, the functional blastema is formed and a morphogenetic field is established (Poleo et al., 2001; Santos-Ruiz et al., 2002), with complex signaling networks that further regulate proliferation and patterning (Stoick-Cooper et al., 2007a).

In summary, during zebrafish adult caudal fin regeneration, cell death occurs twice, first linked to the initial wound-healing process, and second as a burst strictly regulated in time and space in the wound epidermis, essential to blastema formation. Notably apoptosis does not occur in the mesenchyme and is no longer detected once the blastema is formed (Fig. 1). This implies that cells from the epidermis are the only cells to be sensitive to apoptosis-inducing signals, whereas the mesenchymal/blastemal cells are resistant to them, either actively expressing anti-apoptotic factors and/or unable to undergo apoptosis.

The role of injury-induced cell death is at least double, first directly triggering the proliferation of the stump epidermis, and later involved in the recruitment of blastemal cells to promote their entry into S-phase. The signaling pathways

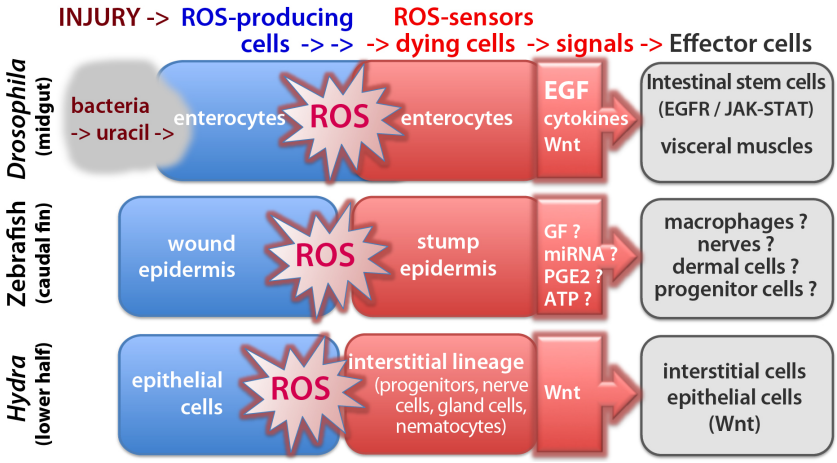

Figure 2: Paracrine signaling linked to regenerative cell death
Adult *Drosophila* repairing its midgut, adult zebrafish regenerating its caudal fin or *Hydra* polyps regenerating their apical half, produce Reactive Oxygen Species (ROS) as an injury-induced signal that induces cell death and activate regeneration. Note that in *Drosophila,* the same cell type, enterocytes, but not necessarily the same cells, produce high levels of ROS and undergo cell death. In zebrafish, the delayed ROS producion by the wound epidermis triggers cell death in the stump epidermis. In *Hydra* the epithelial cells at the wound edge that produce ROS resist to cell death. The signals that are transiently emitted by the apoptotic cells (red background) instruct the effector cells (grey background) involved in the regenerative processes (see text for details).

engaged by the apoptotic cells for this latter role are still elusive. As in *Drosophila* larvae regenerating their imaginal disc or adult *Drosophila* repairing their gut, apoptosis in regenerating zebrafish likely engage short- and long-distance responses.

Cell death in enterocytes supports gut repair in adult *Drosophila*

Adult *Drosophila* is able to maintain its midgut through self-renewal in homeostatic conditions, but also to repair it in response to injury (Jiang and Edgar, 2011; Buchon et al., 2013; Lee and Brey, 2013). Upon enteric bacterial infection, or after ingestion of cytotoxic agents, or upon expression of pro-apoptotic genes, the enterocytes leave the epithelium through delamination, then undergo cell death. The stressed enterocytes release signals, namely EGF that promotes the rapid asymmetric division of the intestine stem cells, which self-renew and provide progenitors ready to differentiate (Amcheslavsky et al., 2009; Buchon et al., 2009b; Jiang et al., 2011). In case of oral infection with pathogenic bacteria, this massive wave of cell death can affect up to 50% of the enterocytes, induced as early as 30 minutes after exposure to bacteria and persisting for hours, as long as bacterial infection is present (Buchon et al., 2010).

Sustained cell death in holothuria regenerating their gut or their nervous system

Echinoderms can regenerate external body parts (arms, spines) and internal organs (gut, visceral mass, nervous system) following self-induced (autonomy) or predator related mutilations. In some species a piece of arm detached from the body can regenerate the whole body, which in the zoo of regenerative models put echinoderms on the podium of champions.

After evisceration, gut regeneration relies on the remodelling of the remaining tissues. Cell death and cell proliferation have been documented in the see cucumber *Holothuria glaberrima* regenerating its gut (Mashanov and Garcia-Arraras, 2011; Mashanov and Garcia Arraras, 2013). Upon elimination of the intestine, cell death is observed in about 5% of the cells in the outer layer (mesothelium) during the first week, while surviving cells undergo dedifferentiation, cell cycle re-entry, proliferation and differentiation of a new luminal epithelium (Mashanov et al., 2010). The signaling function of these dying cells has not been reported yet, however most mesothelial cells strongly express anti-apoptotic genes as survivin and mortalin, suggesting that injury-induced cell death is tightly controlled.

A recent RNAseq transcriptional profiling during intestinal regeneration in the sea cucumber *Apostichopus japonicas* did not identify changes related to cell death regulation except a down-regulation of the effector of apoptosis gelsolin (Sun et al., 2013). A similar cell death pattern was recorded in *Holothuria glaberrina* regenerating its radial nerve cord (central nervous system), with about 4% of the cells located in the lesion area becoming TUNEL positive, representing a 20x increase in cell death during the first post-injury week, most of these cells being glial and nerve cells (Mashanov et al., 2013).

In conclusion, three main features emerge from this comparative analysis of the timing of regenerative cell death: (1) Cell death is observed in the vicinity of the wound, but it can

also be recorded far from the injury site, implying a long distance relay for the pro-apoptotic signals, (2) cell death is immediately detected after injury, but in some contexts, a second delayed wave of cell death is observed, with a pattern distinct from the first wave, (3) a restricted subset of cells are sensitive to the cell death inducing signals linked to injury, as only few cell types undergo cell death in an injured tissue.

3. Injury signals triggering regenerative cell death

Several types of molecules have been characterized as immediate transcription-independent damage signals, including the Ca^{2+} ion and the nucleoside triphosphate ATP, which separately or together can activate NADPH enzymes to produce the oxidizer hydrogen peroxide (H_2O_2) (Cordeiro and Jacinto, 2013). At high intra-cellular levels H_2O_2 acts as a potent cell death inducer through MAPK phosphatase inactivation and sustained JNK activation (Kamata et al., 2005; Circu and Aw, 2010). However if the role of these damage signals in wound healing processes is well established (for review see (Cordeiro and Jacinto, 2013), their role in regenerative processes is largely unknown. We review here recent data indicating that ROS signaling is involved in regenerative cell death.

A direct link between ROS production and induction of cell death in regenerative contexts was first shown in adult *Drosophila* repairing their midgut (for review see (Buchon et al., 2013; Lee and Brey, 2013). Briefly, the pathogenic bacteria release in the intestinal lumen large amounts of uracil, a signal that directly activates the NADPH oxidase DUOX in enterocytes (ECs) leading to ROS production by these cells (Ha et al., 2009; Lee et al., 2013). ROS have a microbicidal function in homeostatic conditions, but high levels of ROS also elicit the delamination and death of damaged ECs as a response to pathogenic infection (Buchon et al., 2009a). These damaged ECs release cytokine-like signals that induce the proliferation of the neighboring intestine stem cells (**Fig. 2**). In the presence of non-lethal pathogenic bacteria, lower levels of uracil produce lower levels of ROS that lead to the replacement of the stressed enterocytes by quick differentiation of enteroblasts (Lee et al., 2013). Thus ROS levels appear critical for directing distinct types of cellular response in the *Drosophila* midgut.

In zebrafish larvae, amputation of the caudal fin induces an immediate and transient wave of ROS production by the DUOX enzyme, and these ROS molecules act at several levels, attracting the leucocytes towards the wound and promoting the regrowth of sensory axons (Niethammer et al., 2009; Rieger and Sagasti, 2011). In adult zebrafish regenerating their caudal fin, ROS are also rapidly detected at the wound but this time ROS production is sustained, reaching a peak value between 12 and 16 hpa. In this period, ROS signaling triggers two parallel events, JNK activation and a delayed burst of apoptosis observed between 15 and 18 hpa (Gauron et al., 2013). Both apoptosis and JNK activation are essential for blastema formation, however it should be noted that here apoptosis does not seem to rely on JNK activation, as still present when JNK is pharmacologically inhibited.

In mammalian cells high levels of ROS inactivate MAPK phosphatases, and this inactivation leads to the sustained activity of the JNK and p38 MAP kinases, which activate caspases (Kamata et al., 2005; Ravindran et al., 2011). But oxidative stress also leads to cell death via activation of the upstream MAPK activator, the Apoptosis signal-regulated kinase (ASK). In the absence of stress, thioredoxin, a small oxido-reductase enzyme, maintains ASK inactive and/or promotes its degradation, two processes that are no longer efficient in the presence of ROS (Saitoh et al., 1998; Liu and Min, 2002; Finkel, 2011; Ray et al., 2012). In *Hydra*, injury-induced cell death in head-regenerating tips relies on the activation of the MAPK/CREB pathway (Kaloulis et al., 2004; Chera et al., 2011), a process that appears to be ROS-dependent as firstly ROS are produced within minutes upon bisection at the wound edges (**Fig. 3**) and secondly such high levels of ROS are required for injury-induced

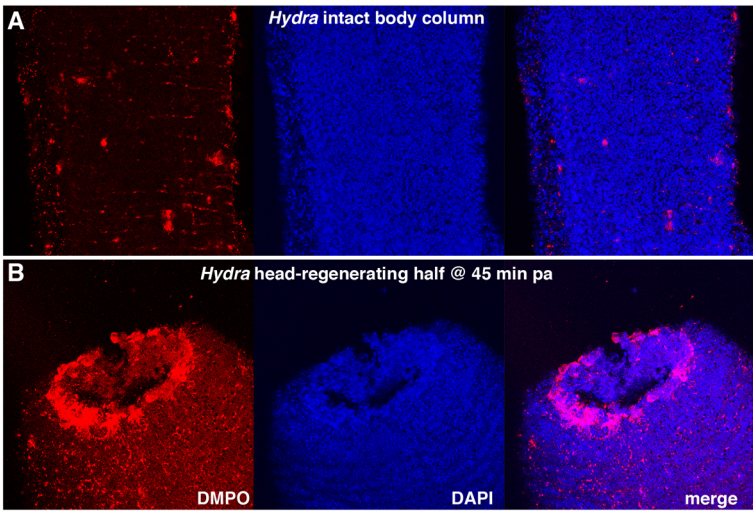

Figure 3: ROS signals in intact and bisected *Hydra*
ROS were detected by DMPO spin trap immunostaining in intact (A) or bisected (B) *Hydra* polyps (*sf-1* strain). In homeostatic conditions (A), few cells constitutively produce ROS along the body column. Upon bisection (B) ROS are produced locally at the wound edges, detected

MAPK activation and injury-induced apoptosis (Reiter et al. unpublished).

In *Xenopus tropicalis* tadpole regenerating its tail and in the axolotl regenerating its limbs, proteomic and microarray analyses indicate that the NADPH metabolic pathway is induced shortly after amputation, suggesting that oxidative signaling participates in blastema formation (Rao et al., 2009; Love et al., 2011; Knapp et al., 2013). Indeed a recent study showed that amputation-induced high levels of ROS are necessary, not for wound healing, but for tail regeneration in *Xenopus laevis* tadpoles (Love et al., 2013). Interestingly this sustained production of ROS leads to the activation of the Wnt pathway, which in turn promotes cell proliferation and axial growth. Regenerative cell death was not investigated in this study, although apoptosis was previously reported to be present and necessary within the first day following tail amputation in *Xenopus* tadpoles (Tseng et al., 2007). One possible scenario would speculate that the death of either ROS-producing or ROS-sensing cells provide signals that instruct the tail regenerative program. Alternatively ROS signaling can directly activate Wnt signaling in the absence of any cell death via nucleoredoxin (Funato et al., 2006).

All together these data indicate that ROS levels efficiently control induction of regenerative cell death. One critical question that arises from this scenario is the regulation of ROS sensing / ROS resistance, as ROS sensitivity appears quite variable among the distinct cell types present in a given wounded tissue. In the *Drosophila* midgut, the enterocytes that produce high levels of ROS might be distinct from the damaged enterocytes that sense ROS and delaminate; in *Hydra* regenerating its head and in the adult zebrafish regenerating its fin, the ROS sensing cells that enter regenerative cell death, are distinct from the ROS producing cells (**Fig. 2**). Differential ROS sensing is well known in the immune system where ROS produced by monocytes induce apoptosis of Natural Killer cells but have no effect on lymphocytes (Hansson et al., 1996). Possible regulators of ROS sensitivity are the levels of O_2 consumption and metabolic rate.

During amphibian metamorphosis, the different cell types exhibit different sensitivities to thyroid hormones, namely to triiodothyronine (T3) that controls cell proliferation and apoptosis (Puzianowska-Kuznicka et al., 2006; Ishizuya-Oka, 2011). T3 levels are controled by deiodinases that metabolize thyroid hormones, via deiodinase 2 (D2) for activation, and deiodinase 3 (D3) for inactivation (Gereben et al., 2008). This regulation is possibly at work in zebrafish regenerating fins as D3 activity was found enhanced during the early phase of regeneration and required for blastema formation (Kester et al., 2009; Bouzaffour et al., 2010). Further studies will tell whether the D2 and D3 expression levels correlate with cell sensitivity or cell resistance to ROS-induced apoptosis, and whether local modulations of deiodinase levels control ROS-induced apoptosis and subsequent proliferation.

4. Caspase activation, a versatile tool to regulate regeneration

To promote regenerative processes, cell death can elicit two distinct mechanisms, one active, instructive, through the caspase-dependent release of signals by the dying cells, and another passive, de-repressing, through the injury-induced elimination of cells that in homeostatic conditions deliver inhibitory signals on “pro-regenerative” neighboring cells (Ryoo et al., 2004; Simon et al., 2009). Experimental evidences accumulated over the past 10 years have shown that both mechanisms potentially co-exist in the same organism, even in the same regenerative context where they can add their successive effects (see below). Indeed these two mechanisms cover different time windows, rather quick and transient for the instructive mechanism, vanishing when apoptotic bodies are metabolized, while the de-repressing mechanism can persist over hours or days, as long as the cells that repress the regenerative potential of their neighbors are not replaced by new ones. Finally, we also briefly mention in this section some contexts where injury-induced cell death is toxic for regeneration.

ACTIVE, INSTRUCTIVE MECHANISM OF REGENERATIVE CELL DEATH

Developmental biologists using imaginal discs in *Drosophila* larvae and radiologists measuring the effects of irradiation in mammalian adult tissues, have proven the validity of this instructive mechanism by characterizing in dying cells the production of caspase-dependent signals that modulate the behavior of neighboring cells or of even more distant cells (reviewed in (Bergantinos et al., 2010b; Bergmann and Steller, 2010; Worley et al., 2012; Zimmerman et al., 2013). The different signals released by the apoptotic cells after injury are depicted in **Fig. 4.**

Growth factors: Wnts, Dpp, hedgehog, EGF

To decipher the role of induced cell death in *Drosophila* larval imaginal discs, several groups designed an ingenious genetic strategy whereby the initiator caspase Dronc and the p35 baculovirus protein that inhibits the effector caspases Drice / Dcp-1 were co-activated in well-

defined regions of the wing disc, pushing thus the cells to enter apoptosis but simultaneously blocking the completion of the apoptotic program (Huh et al., 2004; Ryoo et al., 2004; Perez-Garijo et al., 2004; Wells et al., 2006). This strategy showed that "undead" cells, which produce mitogenic paracrine signals, namely the classical morphogens Decapentaplegic (Dpp) and Wingless (Wg), trigger proliferation of their neighbors thanks to the activation of Dronc, JNK and p53 (for review see (Bergmann and Steller, 2010; Morata et al., 2011).

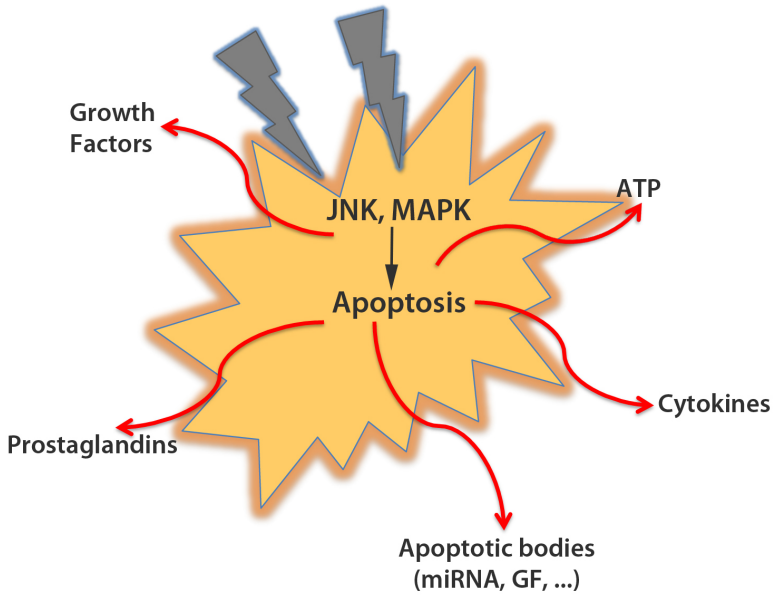

Figure 4: Active instructive mode of regenerative cell death
Scheme depicting a cell submitted to injury-induced signals (dark grey) activating the JNK and/or the MAPK pathways, which trigger cell death. This apoptotic cell potentially releases a variety of signals involved in tissue repair and/or regeneration: growth factors as Wnt, BMPs, hedgehog, EGF, but also prostaglandins, apoptotic bodies that potentially contain miRNA and signaling molecules, cytokines, purines (ATP). Cellular outcomes of this paracrine signaling are diverse, including cell proliferation, cell migration, immune response and neuronal activity.

However these studies characterized apoptosis-induced proliferation in wing discs where epithelial cells are all proliferating, differentiating later during pupation. When apoptosis-induced proliferation was tested in eye imaginal discs, where proliferating and differentiating cells co-exist but are separated by the morphogenetic furrow, a distinct mechanism was identified in the differentiating cells. This mechanism relies on the activation of the effector caspases DrICE / Dcp-1 and the release of Hedgehog (and not Dpp or Wg) to trigger cell cycle re-entry of their undifferentiated cell-cycle arrested neighbors (Fan and Bergmann, 2008b). Hence different mechanisms can drive apoptosis-induced proliferation, and the choice between these mechanisms is influenced by the status of the tissue where apoptosis takes place, proliferating or differentiating (Fan and Bergmann, 2008a).

To make life a little bit more complex, it also appeared that the "undead cell" strategy likely introduces some bias due to the persistant ectopic activation of JNK and the continuous delivery of growth factors. In fact in this experimental paradigm, apoptosis-induced proliferation can occur in imaginal discs mutated for Wg and Dpp, suggesting that these signals are actually dispensable (Morata et al., 2011). The Gal80 genetic system provides an alternative strategy, which allows the transient expression of pro-apoptotic genes by simply raising the temperature, triggering cell death for selected periods of time. Upon a 40 hours cell death period, Wg was detected in the dying cells of the wing disc as expected, but was also found largely produced by the regenerating tissue where Wg stabilizes dMyc, leading to cell proliferation and regeneration (Smith-Bolton et al., 2009).

A similar approach where cell death was induced for shorter periods of time, detected activated JNK in the healing tissue, and two periods of cell proliferation, first immediate and transient in the injury area, and then delayed and sustained, affecting the growth of the whole disc (Bergantinos et al., 2010a). In a third approach a massive and prolonged (40-48 hours) induction of cell death led to an immediate systemic response of the whole disc, which rapidly regenerates the damaged regions thanks to JNK signaling, allowing the apoptotic cells to induce proliferation over long distance, again in the absence of Wg or Dpp contribution (Herrera et al., 2013).

All together these data point to JNK activation as necessary for the regeneration of the proliferating fly imaginal disc, but they also highlight some variability in the responses to injury-induced cell death depending on the extent and duration of cell death, with limited defects inducing a short-range signaling relying on Dpp and Wg signals on one side, and extended defects inducing a Dpp- and Wg-independent long-range signaling,

on the other side. As recently stated "*compensatory proliferation"* is distinct from "*apoptosis-induced proliferation*": In the first case, cell proliferation (i) does not rely on the signals produced by the dying cells, (ii) engages the entire region/organ that senses tissue loss, with the proliferative cells migrating towards the damaged territory (Herrera et al., 2013).

In the second case, cell proliferation is restricted to the damaged area and relies on the mitogenic signals released by the apoptotic cells (Mollereau et al., 2013). Compensatory proliferation, so far poorly understood, could actually respond to a passive, de-repressing mechanism of regenerative cell death (see below). Although obtained in larval tissues artificially manipulated to trigger cell death, these results brought major conceptual advances on the various modes of regeneration and on the importance of the non-apoptotic functions of caspases.

In *Drosophila* larvae, normal apoptotic cells in imaginal discs also produce Wg whereas in adult *Drosophila,* stressed enterocytes produce EGF as main signal to trigger proliferation of intestine stem cells (see above), indicating that this damage-induced growth factor production is not an experimental artifact. In *Hydra*, the apoptotic cells that are spontaneously produced in the head-regenerating tips upon mid-gastric bisection, also release growth factors. These dying cells release Wnt3, a signal that activates β-catenin signaling in the proliferating neighboring interstitial cells, and promote thus their rapid division (Chera et al., 2009). This early signaling is required for head regeneration, as head regeneration is inhibited by the presence of the caspase inhibitor z-vad-fmk in the medium, but rescued by adding the recombinant Wnt3 protein.

Cell death also promotes the up-regulation of *Wnt3* expression in the surrounding epithelial cells, which is required for developing a sustained head organizing activity, responsible for apical patterning (Lengfeld et al., 2009). In bilaterians, Wnt signaling is required for blastema formation in the adult zebrafish regenerating its caudal fin (Stoick-Cooper et al., 2007b), in the *Xenopus* tadpole regenerating its tail (Lin and Slack, 2008) or its limb (Yokoyama et al., 2007), in the planarian regenerating its body (Gurley et al., 2010). However a link between injury-induced apoptosis and Wnt activation has not been established in these latter contexts.

Prostaglandins (PG9)E2

During apoptosis, caspases activate the Ca2+-independent phospholipase A2 (iPLA2) and trigger the production of prostaglandin (PG) E2, which in turn induces proliferation (Li et al., 2010b). This PGE2-dependent induction of cell proliferation nearby the dying cells is essential for skin wound healing and for liver regeneration in mice (Li et al., 2010b). It is worth noting that in the zebrafish hematopoietic stem cells, PGE2 directly modulate the activity of the Wnt pathway. This direct PGE2/Wnt interaction, which is essential for liver and caudal fin regeneration in the adult zebrafish, is thus not restricted to the hematopoietic system and might be a shared property of regenerative tissues (Goessling et al., 2009). This induction of cell proliferation via apoptosis/PGE2 also exerts some unexpected effect on tumor cells. In anti-cancer chemotherapy and radiotherapy in particular, the major goal is to induce apoptosis of tumor cells. However recent study demonstrated that dying cells stimulate tumor cell repopulation through a compensatory mechanism dependent on caspase and PGE2 signaling (Huang et al., 2011; Mao et al., 2013). Hence apoptosis-induced PGE2 signaling is involved in both regeneration and cancer progression.

Purinergic signaling via ATP

Apoptotic cells during healing release ATP through the caspase-dependent activation of the pannexin-1 channel (Chekeni et al., 2010). The purinergic pathway is a widespread mode of signaling, present in both the plant and the animal kingdoms. Extracellular ATP can activate the P2X and P2Y receptors on adjacent cells, or act via adenosine receptors or translocators once hydrolysed by the ecto-nucleotidases CD39 and CD73 (also called nt5e). These two pathways are not mutually exclusive and are subject to considerable crosstalk (Stagg and Smyth, 2010). During regeneration, both extracellular ATP and adenosine are able to stimulate proliferation *in vivo* (Lin et al., 2007), with extracellular ATP mediating proliferation through MAPK activation (Tu et al., 2000). Extracellular purines (ATP or adenosine) can signal either directly to induce cell proliferation, or indirectly by regulating the immune response to damage or nerve transmission.

ATP release also participates in the “find me signal”, which attracts phagocytes to the lesion site (Elliott et al., 2009). Infiltration of macrophages is required for blastema formation in newts regenerating their limb regeneration (Godwin et al., 2013) as well as in adult zebrafish regenerating their caudal fin and their heart (Li et al., 2012; Huang et al., 2013). Within the first 24 hpa these macrophages release proteases, growth factors and cytokines of importance for tissue remodelling and stimulation of proliferation. Finally, ATP is an essential molecule in the communication between neuron and glia (Abbracchio et al., 2009), an essential function in

systems where regeneration is nerve dependent (Singer, 1974; Geraudie and Singer, 1985; Kumar and Brockes, 2012).

Apoptotic bodies as signaling vehicles for miRNA

During apoptosis, blebbing of the cell membrane induces the formation of apoptotic bodies (also named microparticles) loaded with molecules. Upon calcium activation, these microparticles spread into the tissue (Hoang et al., 2011), which allow them to carry biological information from apoptotic to non-apoptotic cells. Indeed apoptotic bodies that derive from endothelial cells, contribute to the recruitment of progenitor cells thanks to the microRNA-126 (miR-126) they contain, which triggers the production of the chemokine CXCL12 in the recipient cells (Zernecke et al., 2009). A recent review focusing on microRNA transportation points to the efficacy of this mode of intercellular communication in various tissues (Vickers and Remaley, 2012). Interestingly the CXCL12 (also named SDF1) chemokine not only recruits progenitors to protect from atherosclerosis, but also participates in epimorphic fin regeneration by interacting with the FGF pathway (Dufourcq and Vriz, 2006; Bouzaffour et al., 2009). These data indicate that the apoptotic bodies likely play an important role in the paracrine signaling supporting tissue repair and regeneration. This mode of signaling can persist over long periods of time, longer than the signals directly released by the apoptotic cells.

PASSIVE, DE-REPRESSING MECHANISM OF REGENERATIVE CELL DEATH

Neurotransmitters as inhibitors of tissue repair in urodeles

In the adult newt brain, the drug-induced elimination of dopaminergic neurons leads to the transient proliferation of ependymal progenitors and their subsequent differentiation into new dopaminergic neurons (Parish et al., 2007). Further studies evidenced a direct negative paracrine control of neurogenesis by dopamine, as the ependymal progenitors, which express the dopamine receptor transiently resume cell proliferation in the absence of dopamine, but become quiescent upon dopaminergic signaling, when novel dopaminergic neurons differentiate (Berg et al., 2011). This discovery of a suppressive role of dopamine on the cycling activity of progenitors might be generalized to other neurotransmitters, each of them inhibiting a specific subset of stem cells (Berg et al., 2013). This mechanism does not seem to operate in mammals as evidenced by the development of the Parkinson disease upon degeneration of dopaminergic neurons. But understanding where lie the differences between mammals and urodeles, would offer new ways to use cell death and manipulate neuronal regeneration.

The Hydra interstitial cells produce signals repressing regeneration

In *Hydra*, the epithelial cells from the ectodermal and endodermal layers suffice to drive head regeneration (Marcum and Campbell, 1978; Sugiyama and Fujisawa, 1978). However in homeostatic conditions the interstitial cells and their derivatives (nerve cells, stinging cells – nematocytes, nematoblasts - and gland cells) appear to repress the morphogenetic potential of these epithelial cells. This negative control was evidenced in the head-deficient *reg-16* strain, where the elimination of the interstitial cells could restore head regeneration (Sugiyama and Wanek, 1993). This “over-repression” of head regeneration by the interstitial cells in this strain was confirmed by transplantation experiments. Therefore we speculate that in wild-type conditions, beside an instructive mode of regenerative cell death (i.e. the transient release of growth factors as Wnt3 by the dying cells), the elimination of the nerve cells, gland cells, nematoblasts and interstitial progenitors in head-regenerating tips, which persists for about 30 hours, contribute to head regeneration by de-repressing locally the morphogenetic potential of the epithelial cells (Galliot, 2013). Candidate suppressors of regeneration in *Hydra* are neuropeptides that might replace cytokine signaling in cnidarians (Bosch and Fujisawa, 2001), but also inhibitors of Wnt signaling that are expressed in gland cells along the body column (Guder et al., 2006).

CELL DEATH AS A TOXIC FORCE PREVENTING REGENERATION

In the chick embryo, neurogenesis of the central nervous system takes place until day 15 and regeneration of the neural tube is fully efficient until day 13, a process that does not rely on cell death but rather on the plasticity of neuronal progenitors (Whalley et al. 2009). However from day 13, i.e. when angiogenesis takes place, injury induces massive apoptosis of the nervous system and the presence of dying cells prevents regeneration (Ferretti and Whalley, 2008). These data indicate that apoptosis can act as a brake rather than as a promoter of regeneration.

Here again it would be interesting to evaluate whether the negative role of cell death is strictly linked to a quantitative parameter, i.e. the level of regeneration inhibition correlates with the level of cell death, and/or wheter a different type of cell death is actually responsible for this negative role. In *Holothuria glaberrina* regenerating its gut or its radial nerve cord (central nervous system),

the cells involved in the regenerative process overexpress genes that appear to protect them from cell death, either mortalin and survivin in the mesothelium of the gut, or LTR retrotransposons in the glial cells (Mashanov et al., 2010; Mashanov et al., 2012). If confirmed, these results indicate that injury induces transcriptional regulations that restrict the level or the extent of cell death and thus ensure the conditions for an efficient regeneration.

5. A complex paracrine control of regeneration through cell death

Immediate injury-induced cell death is a direct response to tissue injury, i.e. observed at the wound leading edge independently of any regenerative process, and thus considered as part of the wound healing process itself (Cordeiro and Jacinto, 2013). By contrast, when a later round of cell death is observed, it appears specific to regeneration as observed in planarians and zebrafish (Pellettieri et al., 2010; Gauron et al., 2013). Interestingly in *Hydra* the immediate massive wave of cell death recorded in head-regenerating tips (Chera et al., 2009) does not belong to the wound healing process as first it is not induced upon wounding, and second it is asymmetric, high on one side of the cut (head regenerating tips), but low on the other side (foot-regenerating tips). In *Drosophila* where immunological, metabolic and developmental studies led to a minutious dissection of the injury-induced signaling involved in midgut repair, the level of injury signals (uracil in case of pathogenic bacterial infection) directly modulates the level of ROS production, which when high leads to cell death and stem cell activation (Lee et al., 2013; Buchon et al., 2013).

Several questions arise from the studies discussed above. A first one concerns the injury-induced damages: are they qualitatively different when injury triggers wound healing, or when injury triggers a regenerative response? Or alternatively, are these two distinct types of injury responses the result of the same signaling operating at different levels, e.g. low levels triggering wound healing, and high levels a more complex signaling, necessary and sufficient to launch a regenerative response? The mechanisms at work in each context probably integrate qualitative and quantitative parameters as suggested by the recent model proposed by Lee et al. (2013) where a combination of intricate factors regulate gut homeostasis and gut repair in the adult *Drosophila*: the nature of the injury signal that varies with the pathogenicity of the bacterial strains, the duration of DUOX activation and the resulting ROS levels.

A second important question concerns the specific features of cell death that drive regenerative processes. Although examples of compensatory proliferation induced by radiotherapy or chemotherapy are well established, it is generally assumed that dying cells do not systematically produce mitogenic factors that trigger proliferation of their neighbors. Therefore what are the properties of cell death that allow the delivery of signaling molecules by the dying cells, as such as the neighboring tissues change their behavior or even undergo reprogramming, thus promoting regeneration?

A series of studies in *Drosophila* larvae have demonstrated the importance of the non-apoptotic functions of caspases in developing tissues (see above) and in cell fate specification (Kanuka et al., 2005; Kuranaga and Miura, 2007; Kuranaga, 2012; Hyman and Yuan, 2012). Therefore activation of caspases and not cell death itself might be necessary to trigger a regenerative response. A recent study performed on human fibroblasts has shown that activation of caspases 3 and 8 in the absence of cell death is required for iPS reprogramming (Li et al., 2010a). As in *Drosophila*, the non-apoptotic functions of caspases might regulate the cellular plasticity required for tissue repair and regeneration.

A third type of questions relates to the various modes of injury-induced signaling in regenerating organisms. Injury-induced caspase activation can be local and immediate, but it can also be delayed or propagated to sites distant from injury (Gauron et al., 2013; Lee and Miura, 2013). In adult *Drosophila* lesions of the cuticle are sensed far from the initial lesion as evidenced by the distant redox-dependent responses recorded in neuronal or intestinal tissues, both necessary for the survival of the whole animal (Nam et al., 2012; Takeishi et al., 2013).

Similar systemic wound response might be at work in the delayed and distant cell death responses as recorded in zebrafish, with ROS signaling at long distance to promote caspase activity in specific cell types (enterocytes in *Drosophila*, epidermal cells in zebrafish and), while other cells are refractory to caspase induction. What are the mechanisms that propagate injury signals to distant sites? What are the molecular properties that maintain some cells resistant to cell death and others highly sensitive? Answers to these questions will open opportunities to manipulate injury-induced ROS signaling, caspase activation and cell death in damaged tissues, organs, or appendages to possibly unlock and activate regenerative processes in mammalian contexts.

Abbreviations

dpa: days post-amputation
Dpp: Decapentaplegic (*Drosophila* growth factor that belongs to the BMP / TGF-β family)
DUOX: Dual Oxidase
EC: enterocytes
EGF: Epithelial Growth Factor
hpa: hours post-amputation
JNK: c-Jun N-terminal kinase
MAPK: Mitogen-Activated Protein Kinase
NADPH oxidase: nicotinamide adenine dinucleotide phosphate-oxidase
PCD: Programmed Cell Death
PG: prostaglandin
ROS: Reactive Oxygen Species
TUNEL: Terminal deoxynucleotidyl transferase dUTP Nick End Labeling
Wg: wingless (*Drosophila* growth factor that belongs to the Wnt family)

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