Biochimica et Biophysica Acta 1793 (2009) 1432–1443

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Review

Autophagy in *Hydra*: A response to starvation and stress in early animal evolution

Simona Chera [1], Wanda Buzgariu [1], Luiza Ghila, Brigitte Galliot *

Department of Zoology and Animal Biology, Faculty of Sciences, University of Geneva, 30 Quai Ernest Ansermet, CH-1211 Geneva 4, Switzerland

ARTICLE INFO

Article history:
Received 26 December 2008
Received in revised form 12 March 2009
Accepted 22 March 2009
Available online 9 April 2009

Keywords:
Hydra starvation
Hydra regeneration
Kazal-type serine protease inhibitor
RNA interference
Cnidarian autophagy genes
Evolution

ABSTRACT

The *Hydra* polyp provides a powerful model system to investigate the regulation of cell survival and cell death in homeostasis and regeneration as *Hydra* survive weeks without feeding and regenerates any missing part after bisection. Induction of autophagy during starvation is the main surviving strategy in *Hydra* as autophagic vacuoles form in most myoepithelial cells after several days. When the autophagic process is inhibited, animal survival is actually rapidly jeopardized. An appropriate regulation of autophagy is also essential during regeneration as *Hydra* RNAi knocked-down for the serine protease inhibitor *Kazal-type* (SPINK) gene *Kazal1*, exhibit a massive autophagy after amputation that rapidly compromises cell and animal survival. This excessive autophagy phenotype actually mimics that observed in the mammalian pancreas when SPINK genes are mutated, highlighting the paradigmatic value of the *Hydra* model system for deciphering pathological processes. Interestingly autophagy during starvation predominantly affects ectodermal epithelial cells and lead to cell survival whereas *Kazal1*(RNAi)-induced autophagy is restricted to endodermal digestive cells that rapidly undergo cell death. This indicates that distinct regulations that remain to be identified, are at work in these two contexts. Cnidarian express orthologs for most components of the autophagy and TOR pathways suggesting evolutionarily-conserved roles during starvation.

1. *Hydra*, a suitable model system to investigate autophagy

The freshwater *Hydra* belongs to Cnidaria, a phylum that emerged as a sister group of bilaterians (Fig. 1A). *Hydra* polyps display a simple tube shape with at the oral extremity an apical dome named hypostome centered on the mouth opening and surrounded by a ring of tentacles, and at the aboral extremity a basal disc that secretes some mucous allowing the animal to attach to the substrate (Fig. 1B). *Hydra* has the amazing potential to regenerate any missing structures after bisection and to reproduce asexually through budding upon regular feeding [1–3]. Moreover, after dissociation into cell suspension, the polyps are able to re-aggregate, forming new animals that progressively restore their initial shape [4]. A section through the body plane of the *Hydra* polyps (Fig. 1C) reveals two cell layers, the ectoderm on the outside and the endoderm or gastrodermis, separated by an extracellular matrix layer named mesoglea. The cells forming these two layers derive from three distinct stem cell populations, the ectodermal myoepithelial cells, the endodermal myoepithelial cells and the interstitial cells whose derivatives are scattered among the epithelial cells in both layers. These three stem cell lineages continuously divide in the adult polyp, giving rise to a limited number of differentiated cell types (Fig. 1D).

In the body column the endodermal myoepithelial cells carry out the digestive function and are thus named digestive cells. At the extremities, the myoepithelial cell lineages provide in the ectodermal and endodermal layers foot- and head-specific cells. The interstitial cell lineage is quite unique as it produces all along the life of the animal somatic cells, i.e. glands cells, nerve cells, mechanoreceptor cells (nematocytes or cnidocytes) as well as gametes [5]. Representative cell types of the body column are depicted in Fig. 1E. Interestingly, these cells do not distribute randomly along the body axis but according to patterns that are specific to each cell type. The progenitor cells either migrate or get displaced along the body axis towards the extremities as such as terminally differentiated cells expressing specific features are found in the tentacles and the basal disk where they ultimately get sloughed off.

Hydra is carnivorous, feeding usually on crustacean nauplii, from *Daphnia* in ponds or desalted brine shrimps in the laboratory. *Hydra* actively catches its food thanks to its sophisticated nervous system. This nervous system is a nerve net with high density at the oral pole that includes sensory and ganglia nerve cells, as well as numerous nematocytes on the tentacles. Although *Hydra* polyps can survive starvation for weeks, the experimental evidence concerning autophagy in *Hydra* and more generally in non-bilaterian animals (Porifera, Ctenophora, Cnidaria) are currently limited.

Autophagy activation was actually discovered in *Hydra* by pure serendipidity when we decided to use *Kazal1,* a Kazal-type serine protease inhibitor (SPINK) gene that is expressed in the gland cells, to adapt the RNAi feeding method to *Hydra* [6]: after repeated exposures

* Corresponding author.
E-mail address: brigitte.galliot@zoo.unige.ch (B. Galliot).
[1] Both authors equally contributed.

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doi:10.1016/j.bbamcr.2009.03.010

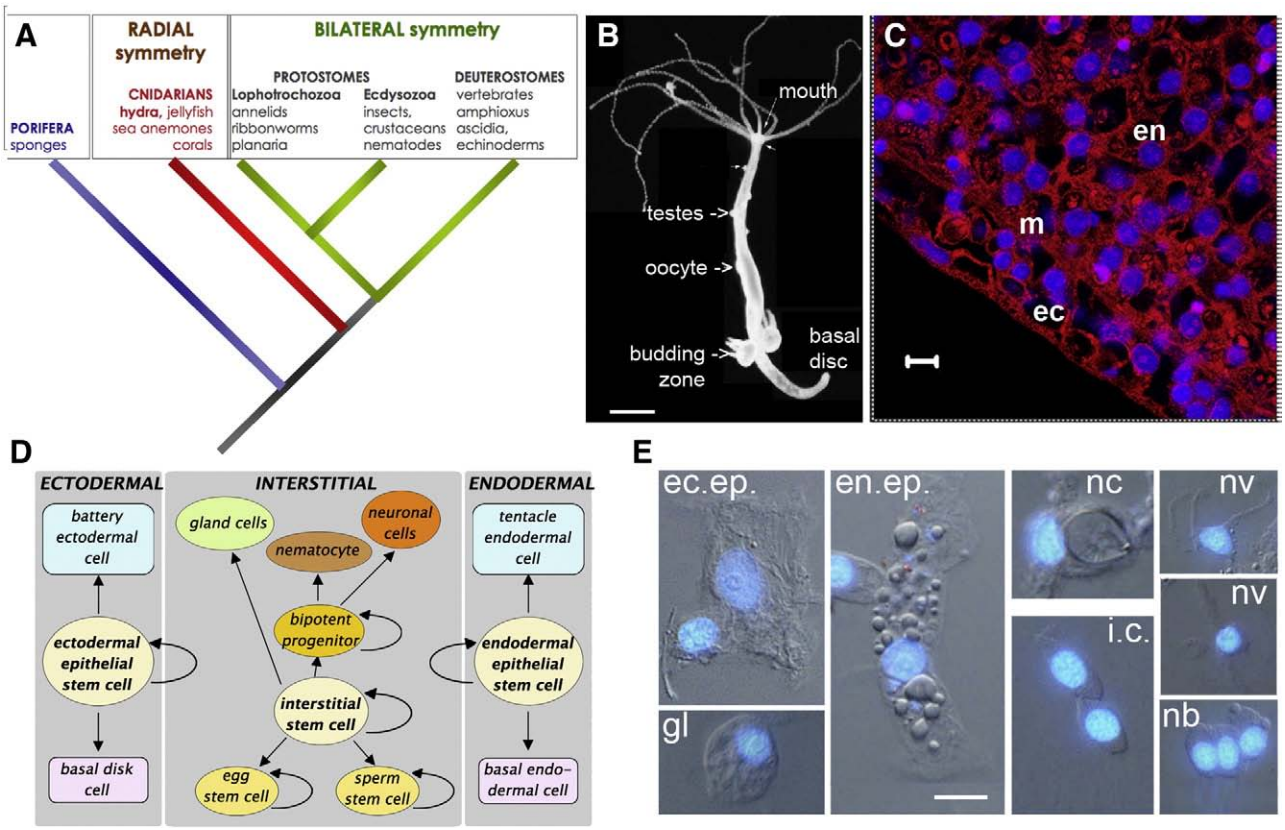

Fig. 1. Phylogenetic position, anatomical structure and cellular organization of the hydra polyp. (A) Position of *Hydra* (Cnidaria, hydrozoan) in the metazoan tree. (B) Tubular anatomy of the *Hydra* polyp with a ring of tentacles surrounding the mouth at the oral pole, an extended body column and a basal disc at the aboral pole allowing the animal to attach. Upon regular feeding *Hydra* reproduces asexually through budding. In the budding zone two buds are visible here, together with male and female gonads. Scale bar: 1 mm. (C) Confocal view of the ectodermal (ec) and endodermal (en) cell layers stained with MitoFluor 589 and Hoechst. The extracellular matrix named mesoglea (m) is visible as a thick line that separates the two cell layers. Scale bar: 10 μm. (D) Scheme adapted from [5] depicting the derivatives of the three distinct stem cell populations in *Hydra*. Foot and head specialized epithelial cells differentiate from ectodermal and endodermal epithelial stem cells, whereas interstitial stem cells give rise to somatic cell types that include gland cells, nematocytes, neurons, as well as germ cells. (E) DIC views of *Hydra* cell types after maceration and Hoechst staining (blue): the ectodermal epithelial cell (ec.ep.) and endodermal epithelial cells (en.ep., also named digestive cells) are large cells that contain myofibers at the basal pole, the interstitial cells (i.c.) are multipotent stem cells and progenitors for the nematocytes (nc) with their typical vacuole filled with toxins and ready for discharge, for the nerve cells, either sensory or ganglia (nv), for the gland cells (gl) that are restricted to the endoderm and for the germ cells (not shown). The cell type identification is based on morphological and molecular criteria described in Refs. [7,38,41,56]. Scale bar: 10 μm.

to dsRNAs the digestive cells exhibited large vacuoles engulfing cytoplasmic organelles and their confluency rapidly led to cell death and subsequently animal death [7]. In *Kazal1* knocked-down *Hydra* this cellular phenotype, which was interpreted as an excessive autophagy, was obtained after several days when animals were kept intact but in less than 1 h when the animals were bisected. Interestingly a similar autophagy phenotype was observed in newborn mice when the related gene *SPINK3* was mutated [8]. It was also suspected that the similarity between mammals and *Hydra* concerning the SPINK function might extend to the regeneration context as injury of the adult pancreas immediately upregulates *SPINK3* expression [9], whereas *Hydra* bisection immediately activates *Kazal1* in regenerating tips [7]. All together these results indicated that the control of the level of autophagy is essential for cytoprotection and cell survival in homeostatic and regenerative contexts, likely involving a genetic circuitry that was conserved since early metazoans [10].

But this first study about autophagy in *Hydra* did not investigate whether autophagy is induced upon starvation and required for animal survival. Upon starvation animals rapidly stop budding and progressively decrease in size [11] proving that they efficiently adapt their metabolism to the nutrient-depleted environment. Indeed autophagy occurs in food-deprived *Hydra* as 60% of the digestive cells contain autophagic vacuoles after 15 days of starvation; those vacuoles are not observed in regularly fed or shortly starved animals [11]. This study also showed that pharmacological agents known to inhibit or induce autophagy accordingly modulate the level of the LC3/ATG8 lipidated protein in either fed or starved *Hydra*, suggesting a conservation of the genetic circuitry regulating autophagy across evolution.

2. Conservation of the genetic machinery regulating autophagy in cnidarians

The cellular basis of autophagy and the components of the genetic circuitry driving autophagy are highly conserved from yeast to mammals [12–14]. More than 20 proteins are involved in the autophagic flux in mammals and yeast, encoded by the *ATG* genes that act at specific stages of the process. Concerning the regulation of the starvation-dependent autophagy, the nutrient sensor TOR (Target of Rapamycin) kinase is also evolutionarily-conserved. We report here on the *Hydra* and/or *Nematostella* orthologous genes characterized as individual genes or identified in the ESTs projects (Fig. 2).

2.1. Nutrient sensing activation, TOR regulation and vesicle induction

Nutrient abundance tightly controls animal growth and budding rate in *Hydra* (see below) whereas evidence concerning the role of growth factor are currently lacking. In yeast and bilaterians, the major player in this regulation is the conserved serine/threonine kinase TOR. In mammals, mTOR acts downstream to the PI3K/Akt signaling pathway to activate protein synthesis and cell growth [15] and together with the modulators raptor and LST8 forms the TOR complex

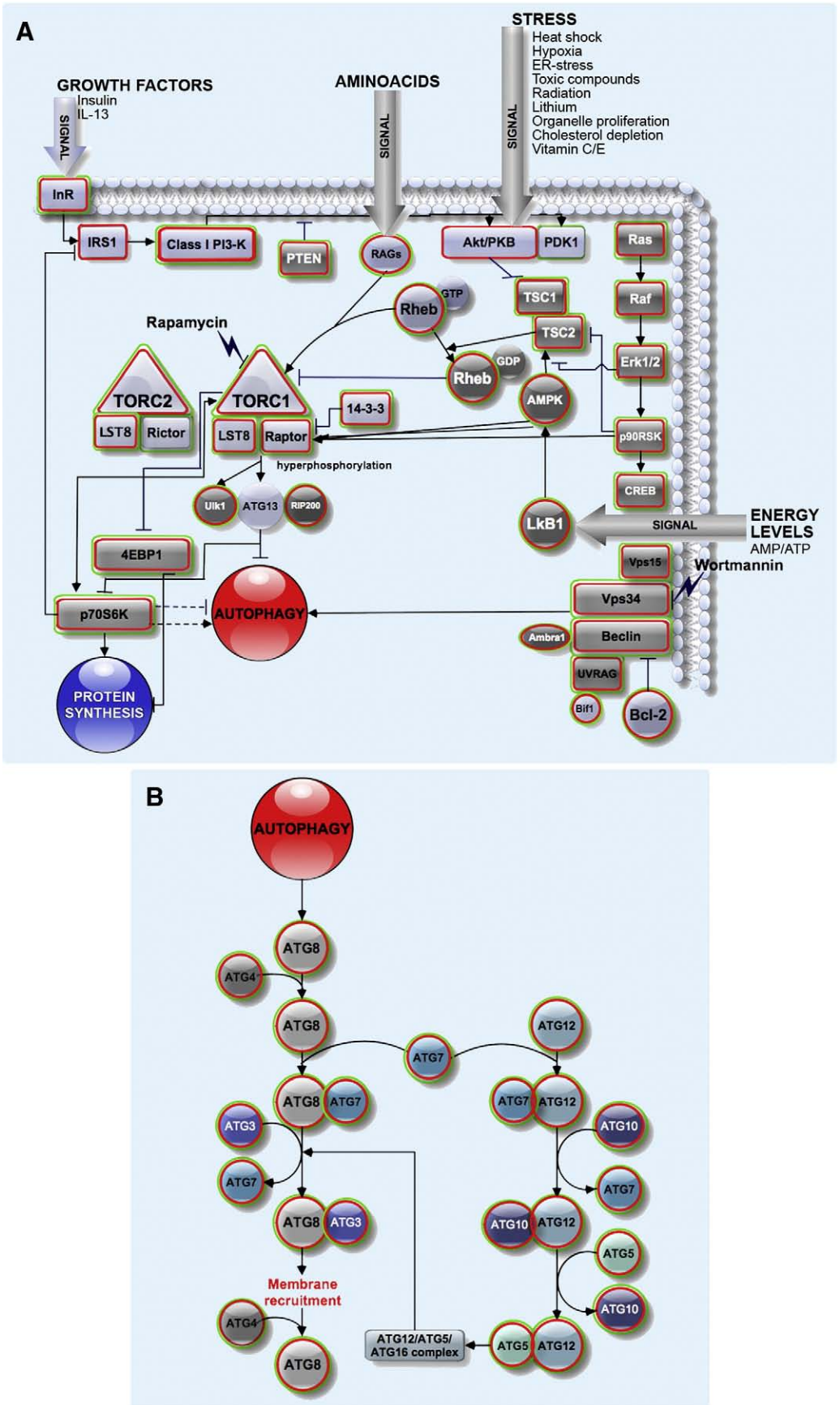

Fig. 2. Conservation of the TOR and autophagy pathways in cnidarians. (A) Control and regulation of the TOR pathway as currently described in vertebrates. (B) The autophagy pathway as described in yeast. Red-circled genes were identified in *Hydra* (Cnidaria, hydrozoan) and green-circled ones in *Nematostella* (Cnidaria, anthozoan).

1 (TORC1). The control of TOR activity implies the AMP-activated protein kinase (AMPK) and the tumor-suppressor proteins TSC1 and TSC2, which negatively regulate the TOR activator RHEB, a small Ras-like GTPase. Moreover the 14-3-3-proteins, involved in numerous signaling pathways, also control mTORC1 activity [16]. In addition to the PI3K/Akt signaling pathway, the Ras-MAPK pathway can also regulate mTOR, as the 90kD ribosomal S6 kinase RSK can phosphorylate TSC2 and raptor [17], thereby activating TOR activity. In nutrient-rich conditions and in the presence of growth factors, mTOR is activated by RHEB and phosphorylates its targets S6 kinase (S6K1/2) and 4EBP1, whereas under nutrient-poor condition, the heterodimer formed by TSC1–TSC2 maintains RHEB in a GDP-bound state, blocking mTOR activation and inducing autophagy. Moreover AMPK can also phosphorylate TSC2 and raptor when activated upon metabolic stress by a low ATP/AMP ratio, thereby inhibiting mTOR. In yeast, when TOR is inhibited, Atg13 is dephosphorylated and its association with Atg1 and Atg17 induces autophagy. This activation mechanism is different in mammals, as the formation of the Atg13–Atg1(ULK1)–FIP200 complex is not influenced by nutrient conditions [18,19]. However the incorporation of mTORC1 in this complex that leads to autophagy suppression, is nutrient-dependent. Hence mTOR, besides its role on protein synthesis and cell growth [20] exerts a key control on autophagy regulation in both yeast and mammals.

The PI3K/Akt pathway is well conserved in *Hydra* where the AKT gene was characterized [21] and a partial PI3K cDNA identified (WashU *Hydra* EST Project). However the regulatory role of this pathway on autophagy during nutrient deprivation remains to be tested. As anticipated, genes encoding for most autophagy regulators were also identified in *Hydra*: the metabolic sensor *TOR*, but also the components of TORC1, *Raptor* and *LST8*, the TOR regulators *RHEB* and *TSC2*, and the TOR target *Atg1* (Fig. 2, WB, unpublished), supporting the view that autophagy activation and induction of the double membrane formation is well conserved in metazoans. Concerning the putative regulation by growth factors, three genes encoding insulin-like peptides have been found in *Hydra* [22], possibly acting through the insulin receptor gene *HTK7* [23]. The presence of insulin-like peptides and candidate receptors suggest that the HTK7/PI3K/Akt pathway regulates *Hydra* survival during starvation as in other animal systems.

2.2. Vesicle nucleation

Autophagy begins with the sequestration of a portion of the cytoplasm into a membranous organelle called a phagophore [24]. Phagophore formation requires the activation of the class III PI3K Vps34, which depends on the formation of a multiprotein complex, composed in mammals of beclin1 (ATG6 in yeast), UVRAG (UV irradiation resistance-associated gene), Ambra1, Bcl-2, Bif 1 and Vps15. Orthologs to all these proteins exist in fly and nematode, and we have now identified *Hydra* orthologs for Vps34, Beclin1 and UVRAG, one of the positive regulator of autophagy at this level [25]. The other positive regulator of autophagy, the WD-40 domain-containing protein named Ambra1 [26] was not found but genes encoding WD-40 domains are present in *Hydra* [27]. Beclin1 is a key regulator of phagophore formation due to its interaction with Bcl-2 at the BH3 domain: in nutrient-rich condition Bcl-2 binds Beclin1 and prevents its activity, thereby inhibiting autophagy. Upon starvation the cellular Bcl-2–Beclin1 complex dissociates through the phosphorylation of Bcl-2 by the stress-activated kinase JNK1 [28]. JNK and members of the Bcl-2 gene family are expressed in *Hydra* [29–31] and represent thus obvious candidates for this regulation (Fig. 2).

2.3. Vesicle elongation and autophagosome formation

Once phagophore formation is initiated, the elongation of the double-layer membranes and maturation of the vesicles take place under the combined action of two highly conserved ubiquitin-like conjugation pathways, the Atg12 conjugation system (Atg10, Atg12, Atg5 and Atg16) and the Atg8 lipidation system (Atg8, Atg3 and Atg7). The Atg8 or LC3 (mammalian Atg8 ortholog) lipidation pathway involves the conversion of the proLC3 protein to LC3-I through the Atg4-dependent proteolysis and the sequential action of the E1-like enzyme Atg7 and the E2-like enzyme Atg3. In this way, the soluble form LC3-I is converted by lipidation to the phosphatidyl-ethanolamine conjugated form LC3-II, which is anchored to the phagophore and autophagosome membranes. These conjugation systems are widely conserved in eukaryotes including cnidarians: the *Atg10, LC3/Atg8*, *Atg3* and *Atg7* ortholog genes have been identified in *Hydra*, their deduced protein sequences showing a high level of conservation (WB, unpublished) offering hence the possibility to use cross-reacting heterologous antibodies [11].

2.4. Docking and fusion of autophagosomes with the lysosomes

After maturation, the autophagosomes reach the lysosomes by using a dynein-reliant transport; the outer membrane of the autophagosome fuses then with the lysosomal membrane to form vacuoles named autolysosomes. The inner membrane is broken and the acidic lysosomal hydrolases degrades the engulfed cytosolic material, generating nucleotides, aminoacids and free fatty acids that are used for macromolecular and ATP synthesis. This fusion and degradation steps rely on mechanisms and components that are not specific for the autophagic flux but linked to the endosomal network. Again these components are highly conserved across evolution.

3. Induction of autophagy upon starvation in *Hydra* leads to cell survival

3.1. The cellular adaptation to starvation in Hydra

Hydra polyps are amazingly resistant to starvation as they are able to survive long periods of food depletion, even longer than four weeks. In daily-fed animals, the interstitial cells complete the cell cycle within 24 h, whereas the epithelial cells need three to five days [32]; the continuously dividing and differentiating epithelial and interstitial pools participate in the budding process, giving rise to a new polyp. After few days of starvation, the budding process that requires a regular feeding diet, stops and the animals decrease in size, becoming thinner and shorter than the regularly fed polyps. After feeding resumption, the animals start to re-grow and recover in several days its size and the ability to bud (Fig. 3A). Previous work showed that starvation does not dramatically modify the cell cycle length implying that cell proliferation in *Hydra* is only weakly modulated by the feeding diet [33]. In fact in starved *Hydra*, the total number of cells remains more or less constant whereas the epithelial and interstitial cells continue to divide almost at the same pace as in fed *Hydra*. To explain this paradox, Bosch and David proposed a unique mechanism that would regulate both the cell number and the animal survival. They made the observation that the number of digestive cells containing DNA-positive phagocytic vacuoles significantly increased in *Hydra* receiving low-feeding diet and assumed that the cells produced in excess through mitosis became pycnotic and engulfed by the digestive cells [33]. Later on, once the morphological features of apoptosis were clearly characterized, they proposed that the response to the feeding diet was predominantly regulated through apoptosis rather than changes in the cell proliferation rate [34]. However the recent evidence for starvation-induced autophagy in *Hydra* [11] impose a re-evaluation of the respective contribution of apoptosis and autophagy for survival during starvation.

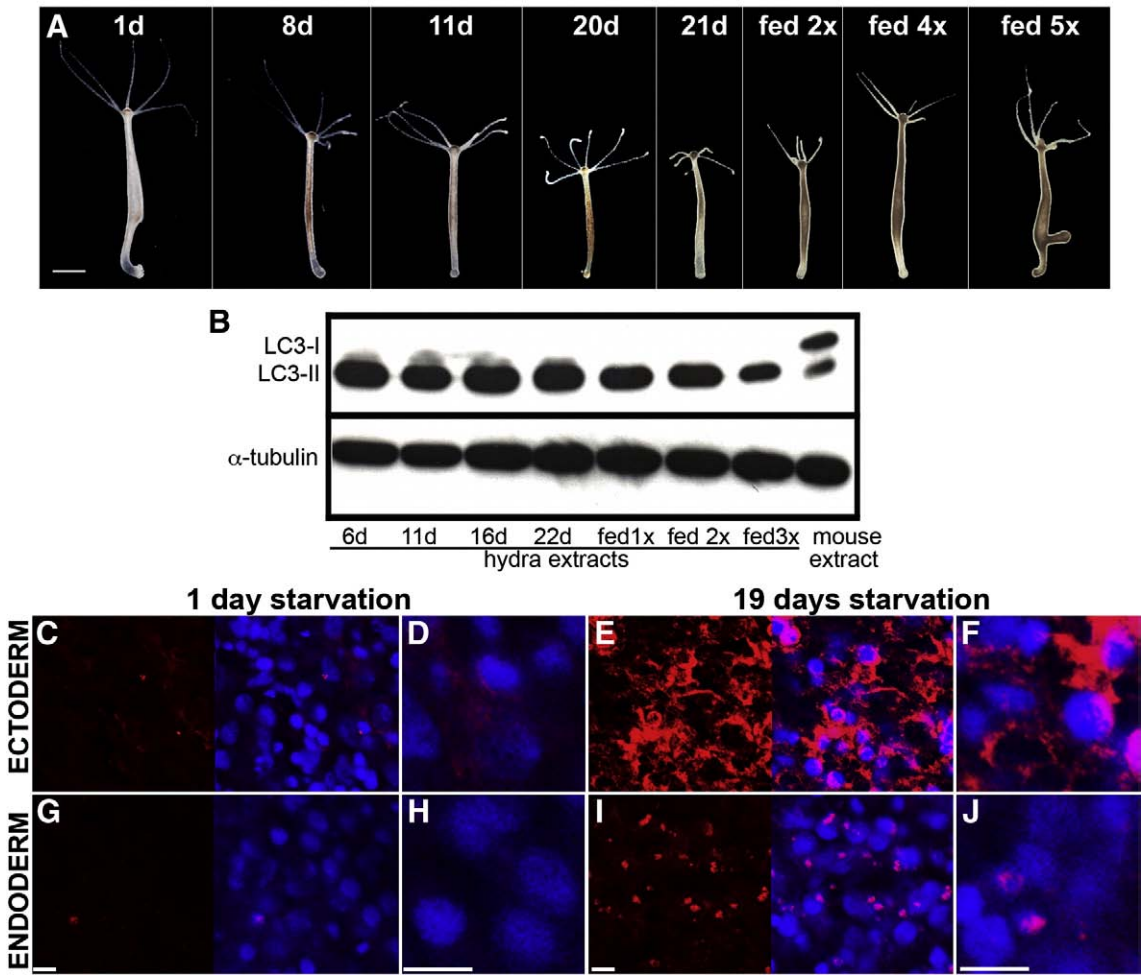

Fig. 3. The feeding diet modulates hydra morphology and autophagy levels. (A) Decrease in animal size during 21 days of starvation and rapid re-growth after the resumption of regular feeding. In food-deprived environment the animal stops budding, a diet-dependent asexual form of reproduction that is reestablished after only few feedings. At the indicated time points, the same animal was kept relaxed and pictured with a stereomicroscope. Scale bar: 1 mm. (B) Western blot analysis showing high levels of LC3-II protein during starvation and a progressive reduction after re-feeding. The anti-LC3 antibody (Cell Signaling) recognizes only the LC3-II form in *Hydra* but the two forms, LC3-I and LC3-II in the mouse embryo extract. (C–J) Detection of autophagy in the ectodermal (C–F) and endodermal (G–J) layers during starvation. After 1 day of starvation weak LC3+ vacuoles are already detected in the ectodermal layer (D) but none in the endodermal one (H). After 19 days of starvation (E, F, I, J) numerous LC3+ vacuoles (red) are observed in both layers with LC3+ conglomerates that are few and large in the endodermal cells (I,J) but abundant and smaller in the ectoderm (E, F). LC3 is also detected in membranes of epithelial ectodermal cells (E, F). Nuclei are Hoechst-stained (blue). D, F, H, J: enlarged views of C, E, G, and I respectively. Scale bar: 10 μm.

3.2. LC3/ATG8 as a reliable marker of starvation-induced autophagy in Hydra

The LC3/ATG8 protein, which is cleaved and lipidated upon binding to the membrane of the autophagic vacuole providing a reliable marker of autophagy from yeast to mammals [35], is indeed well suited to monitor autophagy in *Hydra*. In fact the abundance of the lipidated form LC3-II in cellular and biochemical analyses reflects well the number of autophagosomes [36] although in a non-dynamic fashion [35]. Two cross-reacting anti-LC3/ATG8 antibodies provide convergent results when tested on *Hydra* extracts: the LC3-II form was detected at higher levels in starved than in fed *Hydra* whereas its amount rapidly decreased when the animals were re-fed (Fig. 3B and [11]), the recovery from starvation being actually a fast process as a single feeding was sufficient to significantly reduce the LC3-II level.

Anti-LC3 immunochemistry performed on whole mount animals is also highly informative as it shows distinct modulations of the LC3 pattern in the two cell layers during starvation. In the ectoderm, the LC3+ vesicles are small and rather weak after 1 day (Fig. 3C, D) but numerous and large after 19 days, providing a diffuse intense staining (Fig. 3E, F). Epithelial ectodermal cells often contain at that stage irregularly shaped LC3+ conglomerates interpreted as large autolysosomes, formed after an extended period of starvation. In the endoderm, LC3 staining is not detectable at all after short starvation period, i.e. 1 day (Fig. 3G, H), but becomes intense with large LC3+ vacuoles after 19 days (Fig. 3I, J). Hence induction of autophagy takes place much earlier in the ectoderm than in the endoderm, whereas it is present in both layers after 19 days although with slightly different LC3+ patterns. Moreover a LC3 membrane staining was noted in the ectoderm but not in the endoderm. The fact that this membrane staining is only visible in the ectodermal myoepithelial cells and not in the endodermal ones is surprising. One possible hypothesis would be that the Atg8 lipidation system is already at work during phagophore formation in the cells were the autophagy rate is maximal. If confirmed, this would point to the different properties of the autophagy process in the two cell layers.

3.3. Dramatic increase in the number of autophagic vacuoles upon starvation

Autophagy can be monitored at the cellular level in *Hydra*, as tissues can be macerated, a classical technique in the field that

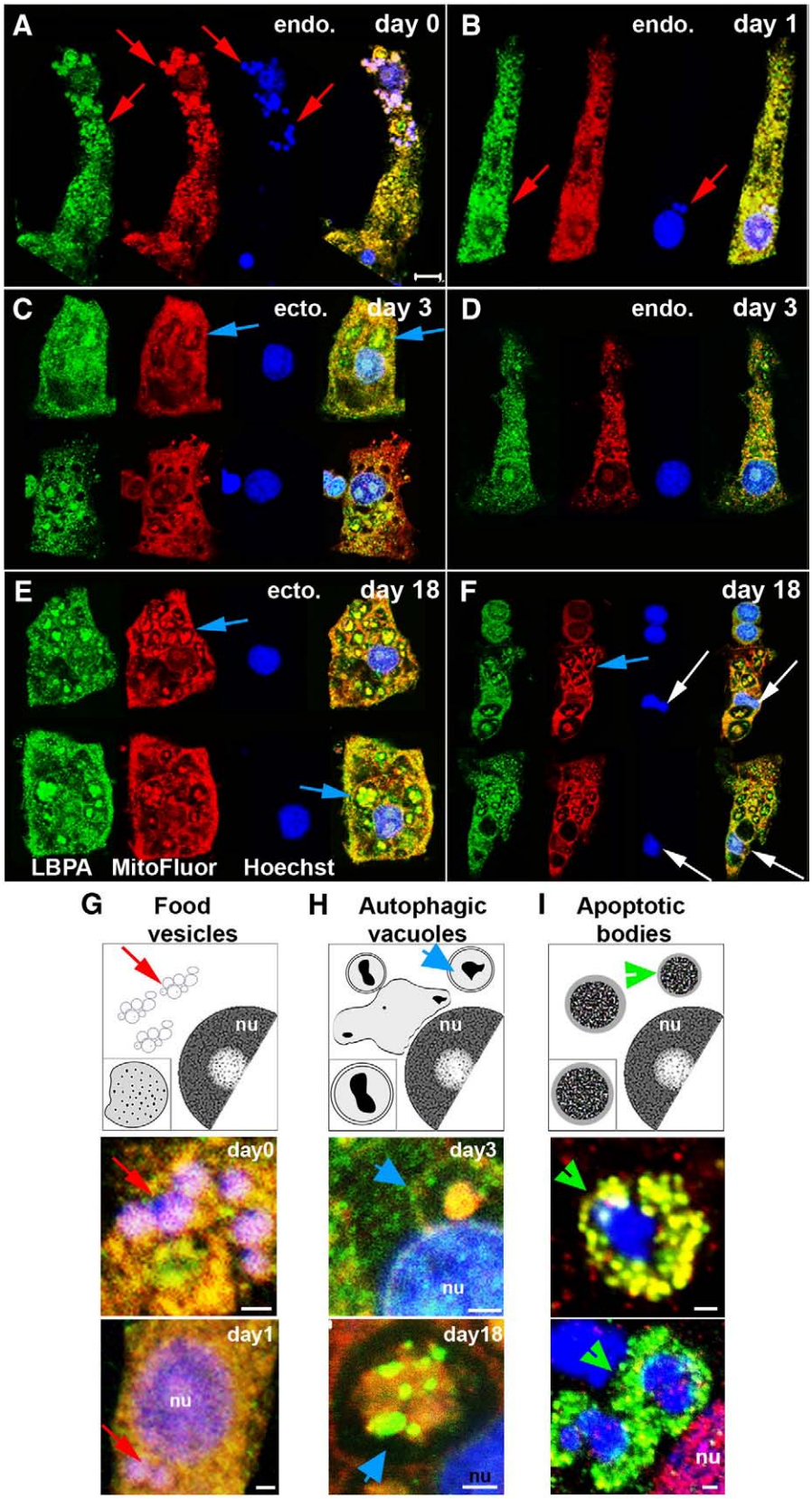

Fig. 4. Formation of autophagic vacuoles in myoepithelial cells during starvation. (A–H) After several weeks of daily feeding *Hydra vulgaris* were macerated either on the last feeding day (day 0 in A, G) or after one (B, G), three (C, D) or 18 (E, F, H) days of starvation and stained with anti-LBPA (green), MitoFluor Red 589 (red) and Hoechst (blue) that detect late endosomes, mitochondria and DNA respectively. In daily-fed animals, the food vesicles are strongly Hoechst-labeled and observed in the apical part of the digestive cells (A, G, red arrows). Their number drastically decreases over the first 24 h of starvation (B, G, red arrows). After 3 days of starvation autophagosomes (blue arrows) are observed in the ectodermal (ecto. C) and endodermal (endo. D, H) myoepithelial cells. E, F, H) After 18 days, the large autophagic vacuoles contain conglomerates of sequestered cytoplasmic material and occupy almost entirely the cytoplasmic space. In the digestive cells, they compress the nuclei (F, white arrows). Note the absence of autophagic vacuoles in the interstitial cells (F, pair at the top). I) For comparison, engulfed apoptotic bodies (green arrowheads) contain condensed DNA (Hoechst-stained, blue) and are surrounded by a cytoplasmic rim (immunostained with anti-RSK, green). Scale bars: 10 μm (A–F), 2 μm (G–I).

preserves cell morphology and thus allows to characterize modulations in each cell type and quantify the different cell populations [37]. Given the multiple functions of the digestive cells, i.e. in food digestion, phagocytosis of apoptotic bodies and autophagy, well-defined criteria have to be used to distinguish between these different types of vacuoles. These criteria, initially established on ultrastructural analyses [38,39], rely on histochemical and immunological analyses that specifically detect lysosomes (Lysotracker red-LTR), mitochondria (MitoFluor 589), late endosome (LBPA) found in the autophagic vacuoles, DNA (Hoechst, DAPI) [40] and anti-RSK immunostaining. The RSK hydra protein, orthologous to the vertebrate p90RSK protein (SC, unpublished) is expressed in most cell types [41] and is present in the membranes and content of the autophagic vacuoles [7,11].

In *Hydra*, the ingested food is primarily processed in the gastric cavity, where the enzymes released by the gland cells contribute to its partial degradation, resulting in smaller food fragments that are engulfed by the digestive cells through phagocytosis. Therefore, soon after feeding the apical pole of these cells is filled up with food vesicles (Fig. 4A, G) that are relatively small (<3 μm) with a fuzzy content, strongly stained with DNA dyes [40]. Their number progressively decreases as the content of these food vesicles is gradually degraded over the next hours, and fewer food vesicles with weaker DNA staining can be detected 24 h after feeding, often located in the vicinity of the nucleus (Fig. 4B, G). After an extended period of starvation, some of these vesicles persist as residual bodies, enclosing fine aggregates or fibers in a partially degraded stage, very weakly Hoechst-stained (not shown).

Autophagosomes are membrane-circled vacuoles usually larger than food vesicles (>4 μm). They appear after few days of starvation in epithelial cells where the punctuated distribution of mitochondria and lysosomes across the cytoplasmic space that is normally observed in regularly fed *Hydra*, is markedly altered (Fig. 4A, B, D). As typical features, the content of these autophagosomes is RSK+, often as conglomerates, and includes mitochondria and endosomes [7]. Moreover the Lysotracker staining of their membranes identifies these vacuoles as autolysosomes [11]. In starving animals, autophagic vacuoles are detected as early as 3 days after the last feeding predominantly in the ectodermal epithelial cells (Fig. 4C, D, H). After 18 days of starvation, most epithelial cells contain numerous, large autolysosomes with sequestred cytoplasmic material in different degradation phases including organelles like mitochondria and endosomes that colocalize (Fig. 4E, F, H). Moreover as a consequence of the adaptation to the nutrient availability, progressive morphological changes occur in these digestive cells, namely a drastic reduction in cell size (compare cells in Fig. 4A, B and F) and a compression of the nucleus by the vacuoles. Those occupy most of the cytoplasmic space, forcing the nucleus to lose its ovoid shape (Fig. 4F, white arrows). Surprisingly, the ectodermal epithelial cells exhibit neither cell size reduction nor nuclear compression over the same period of time (compare Fig. 4C and E).

In contrast to the food vesicles and autophagic vacuoles, the apoptotic phagosomes identified in the cytoplasm of the digestive cells have a characteristic, spherical shape, usually larger than 6 μm in diameter. They are formed of highly condensed chromatin that homogeneously and strongly stains Hoechst and are surrounded by a cytoplasmic rim that strongly expresses RSK (Fig. 4I). RSK never stains the membranes of the autophagic vacuoles or the outlines of the food vesicles. The proportion of epithelial cells engulfing apoptotic bodies remains low and stable along the starvation period. In contrast, the number of cells with autophagic vacuoles progressively increases along the starvation period, with rather distinct "survival" responses of the two epithelial cell layers upon prolonged starvation, as despite an intense autophagy process in both, only the endodermal epithelial cells show a dramatically-altered morphology.

4. Induction of autophagy upon *Kazal1* silencing leads to cell death

RNA interference (RNAi) recently implemented in *Hydra* offers the possibility to study the autophagy process when potential regulators of autophagy are knocked-down. Two questions are open to investigation: how similar are the autophagy processes in starvation- versus gene-silencing induced autophagy? How similar are the autophagy processes in homeostatic versus regenerating contexts? The incremental feeding RNAi method actually allows the follow-up of a progressive gene silencing in parallel with starvation during several weeks and, as such, permits a comparative analysis of autophagy in these different contexts. However the starvation condition during the RNAi procedure is not strictly identical to that observed in food-depleted animals as *Hydra*, which indeed do not receive their usual food, can gather some nutrients from the bacteria used for the production of dsRNAs. Therefore this RNAi starvation condition can be considered as "pseudo-starvation" given that the level of autophagy is lower than that observed in animals strictly starved for the same period.

4.1. The starvation-induced autophagy slowly becomes lethal in Kazal1 (RNAi) hydra

The analysis of the autophagy process in *Kazal1(RNAi) Hydra* turned out to be highly informative [7,10]. *Kazal1* is specifically expressed in the acinar-like gland cells along the body column and is immediately up-regulated in the gland cells located in regenerating tips after bisection. In intact hydra, the proportions of digestive cells containing autophagic vacuoles remain the same in control (*L4440 (RNAi)*) and *Kazal1(RNAi)* polyps up to 7× exposures to dsRNAs, implying that the progressive reduction in *Kazal1* expression does not modulate the level of starvation-induced autophagy in intact polyps (Fig. 5E). However the dynamic and cellular features of autophagy progressively become quite different between control and *Kazal1 (RNAi) Hydra.* Following the 3rd feeding the *Kazal1(RNAi)* digestive cells contain a larger number of autophagosomes (Fig. 5A, B), which subsequently fuse and disrupt the cell morphology causing cell death. As a main consequence, the *Kazal1(RNAi) Hydra* start dying following the 5th feeding. Interestingly, this excessive autophagy phenotype observed in *Kazal1(RNAi)* animals never affects the ectodermal epithelial cells, which are highly autophagic during starvation.

The *Kazal1(RNAi)* animals also present a disrupted morphology of their gland cells with fusion of the secretory vacuoles. But these cells, which do not display typical autophagosomes, very rapidly died and a clear involvement of the autophagy process could not be established. In the *SPINK3(−/−)* newborn mice, the autophagic cell death affected both cell types, pancreatic acinar and digestive cells, possibly as a result of the lack of inhibition of the digestive enzymes that are produced by the exocrine cells and released in the lumen [8]. A similar mechanism likely explains the fact that autophagic cell death is restricted to the digestive cells in *Hydra*, the ectodermal ones being protected by the mesoglea. These observations indicate that autophagy can act as a presumptive cell survival mechanism during starvation, with neither cell death nor animal death at least over a 24 days period, but can also trigger massive cell death and animal death when the disruption of the fine tuned balance of the autophagy process is altered as upon *Kazal1* silencing.

4.2. The amputation stress leads to lethal excessive autophagy in Kazal1(RNAi) hydra

The amputation stress actually appears as another parameter of autophagy regulation as bisection dramatically enhances the excessive autophagy syndrome in *Kazal1(RNAi) Hydra:* after 3× exposures to dsRNAs, the level of autophagy is increased up to 25× in

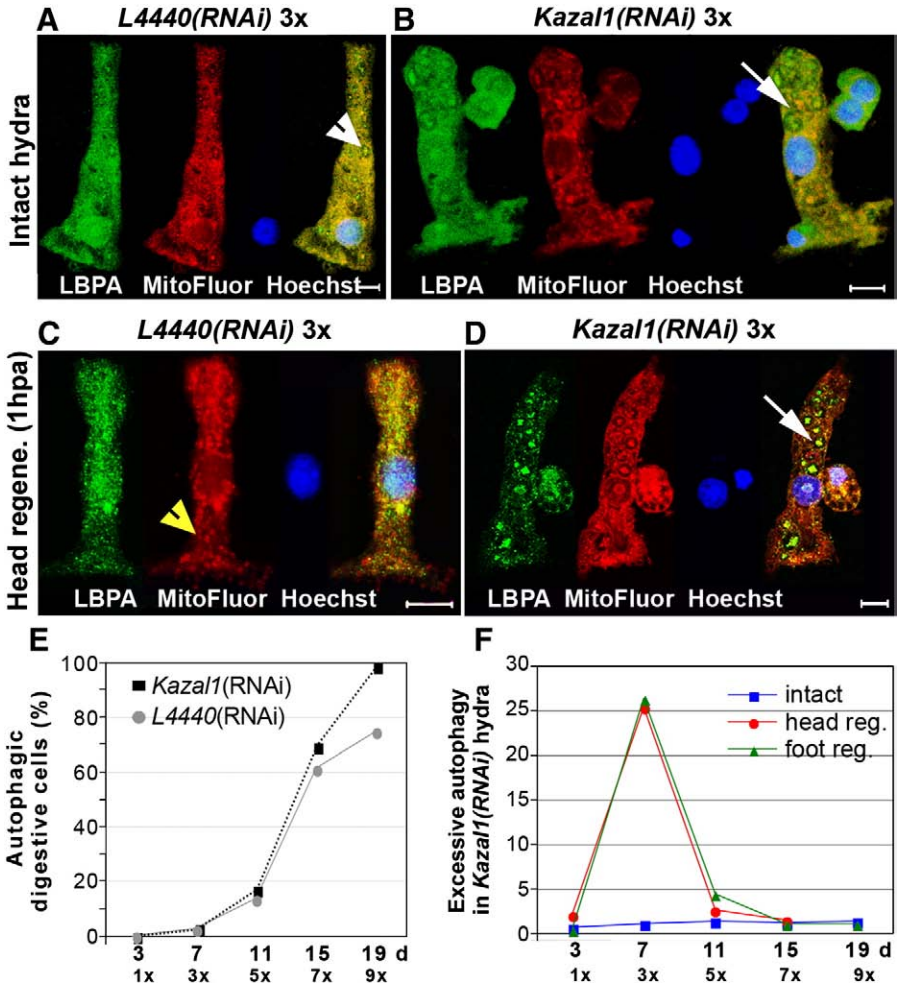

Fig. 5. Amputation-induced autophagy in the digestive cells of *Kazal1(RNAi) Hydra*. (A, B) Confocal views of digestive cells from intact *Hydra* macerated after 3× exposures to control (L4440 vector, A) or L4440-*Kazal1* (B) dsRNAs and stained with MitoFluor Red 589 (red), anti-LBPA antibody (green) and Hoechst (blue). During the RNAi procedure *Hydra* are starved but partially feed on bacteria that produce dsRNAs (pseudo-starvation). The small autophagic vacuoles observed in L4440 *Hydra* (A, arrowhead) likely result from this pseudo-starvation condition. In contrast larger vacuoles are observed in cells from *Kazal1(RNAi) Hydra* (B, arrow). Note the absence of vacuoles in interstitial cells. (C, D) Confocal views of digestive cells isolated at one hpa from head-regenerating halves bisected after 3× exposures to dsRNAs and stained as above. The cytoplasm of *Kazal1(RNAi)* digestive cells contains numerous, large autophagic vacuoles (arrow) which start to fuse in the perinuclear region (D). Note in control cells (C) the absence of vacuoles and the punctuated pattern of mitochondria and late endosomes (yellow arrowhead). Scale bar: 10 μm. (E) Graph showing the gradual increase in autophagy in *Kazal1(RNAi)* and *L4440(RNAi)* intact *Hydra* after repeated dsRNA exposures (1×, 3×, …) represented on the abscissa (the corresponding pseudo-starvation period is expressed in days). The percentage of autophagic cells among 600 digestive cells is reported for each condition. (F) Graph showing the ratio between the number of autophagic digestive cells in *Kazal1(RNAi) Hydra* over that detected in *L4440(RNAi) Hydra* after repeated dsRNA exposures (abscissa as in E). These ratios were counted in intact *Hydra* (blue squares, ratio of the values depicted in E), in head- (red circles) and foot- (green triangles) regenerating halves. A dramatic increase (25 fold) in the number of autophagic cells is observed in regenerating *Hydra* exposed 3× to *Kazal1* dsRNAs. This dramatic difference in autophagy between *Kazal1(RNAi)* and *L4440(RNAi) Hydra* is no longer detected after 5× exposures probably because of the starvation-induced autophagy. Nevertheless the qualitative differences described in A–D, namely the number, size and confluency of autophagic vacuoles per cell, were amplified after 3× exposures, leading to a drastic cell size decrease followed by cell death in *Kazal1(RNAi)* but not *L4440(RNAi) Hydra*. After 5× dsRNA exposures, *Kazal1(RNAi)* rapidly die after amputation, whereas the *L4440(RNAi) Hydra* survive the amputation stress [7].

regenerating *Kazal1(RNAi) Hydra* when compared to that observed in control animals (Fig. 5C, D, F). But autophagy is only transient and the animals can survive. However after 5× exposures, this amputation-induced excessive autophagy becomes irreversible and the polyps die in few hours, proving the essential role played by *Kazal1* in surmounting the amputation stress and keeping the level of autophagy compatible with cell survival during the initial hours of regeneration.

This first report about autophagic cell death in intact and regenerating *Hydra* underlines the fact that the cytoprotective functions played by Kazal1 face much more stringent constraints in regenerative than in homeostatic contexts. We believe that *Kazal1* silencing does not affect the regeneration process *per se* but rather the conditions required for regeneration, the survival of digestive cells from the regenerating tips being critical for the formation of the transient organizer in regenerating tips and completion of the regeneration program [7,10]. Similar cytoprotective role for the SPINK proteins was also observed in mammals where the mouse *Spink-3* gene is rapidly activated in the adult injured pancreas [9]. All these results suggest a conserved self-preserving role for Spink-related protease inhibitors from *Hydra* to humans and strengthen the value of the *Hydra* polyp as a model to study physiological and pathological aspects of autophagy.

5. Pharmacological modulations of autophagy in regenerating *Hydra*

Tissue repair and regeneration of organs, appendages and body parts are present in all animal phyla, involving processes like tissue remodeling and cell proliferation that rely on evolutionary conserved signaling pathways (see in [42]). Two model systems deploy body regeneration: cnidarian polyps and planarians. In contrast to *Hydra*

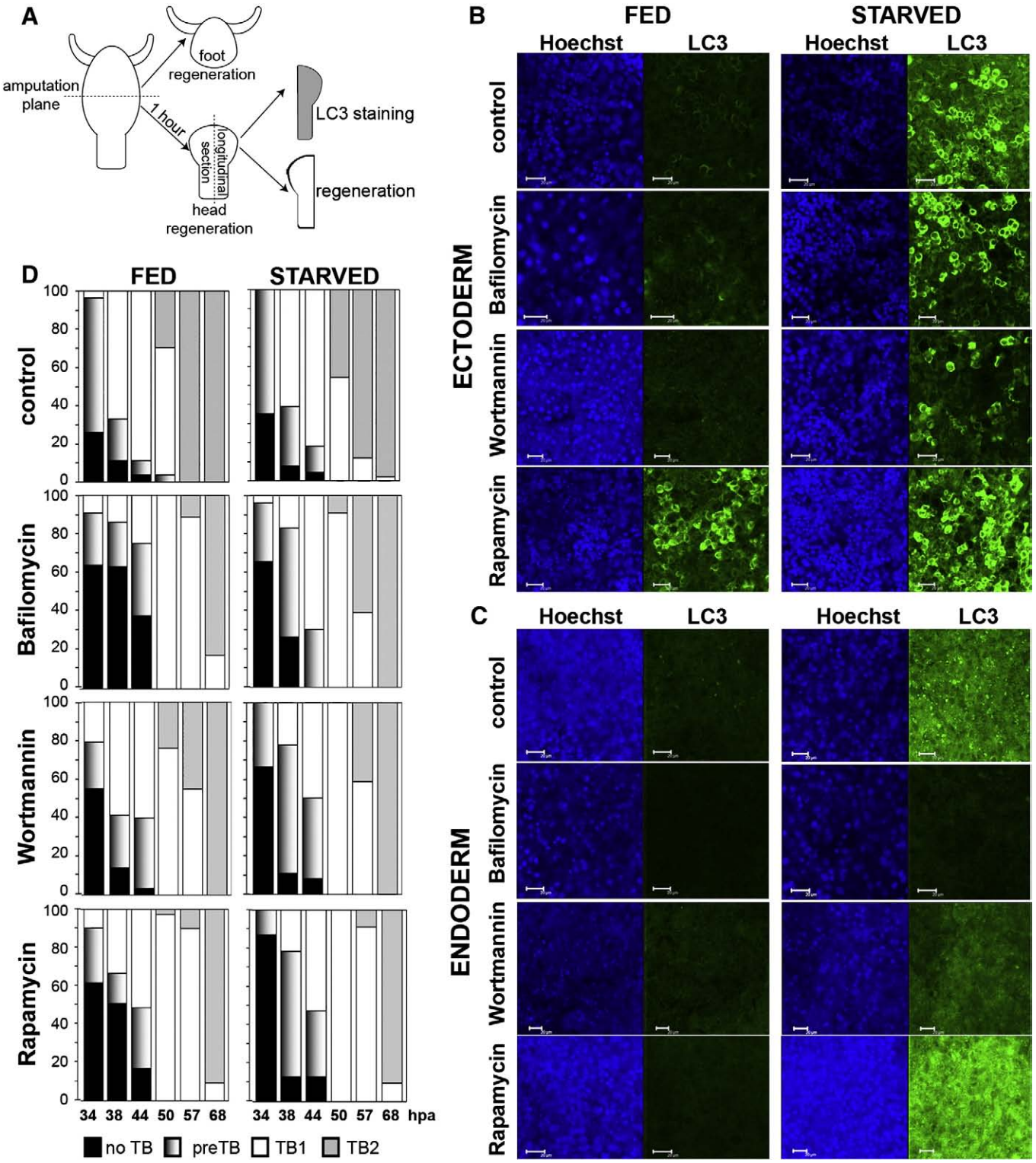

Fig. 6. Pharmacological modulations of autophagy slightly affect the head regeneration process in both fed and starved hydra. (A) Scheme depicting the splitting-assay procedure to detect in the same *Hydra* the early cellular alterations and the efficiency of the head regeneration process after drug treatment. One hour after bisection the head-regenerating half is bisected longitudinally, one longitudinal half is fixed and LC3 immunostained while the other that heals immediately is left alive to regenerate. (B, C) Detection of LC3 expression in either daily-fed or 17 days starved regenerating *Hydra* exposed to Bafilomycin (0.1 μM), Wortmannin (1 μM) or Rapamycin (10 μM). Drug treatments are initiated 2 h prior to amputation and stopped with fixation at 1 hpa or 2 hpa. In daily-fed *Hydra*, LC3+ cells are only detected in the ectoderm of Rapamycin-treated *Hydra* (B, left), undetectable in the endoderm (C, left). In contrast in starved *Hydra*, LC3+ cells are detected in the ectoderm in all conditions although reduced in *Hydra* exposed to Wortmannin (B, right). In the endoderm, LC3 expression is low in untreated *Hydra* but massive (saturating the field) in Rapamycin-treated *Hydra* (C, right), suggesting that autophagy is strongly induced in these conditions. Scale bar: 20 μm. (D) Regeneration kinetics of the *Hydra* depicted in B, C. The emergence of tentacle buds (TB) was scored at indicated time points to follow the head regeneration process. Starvation does not significantly modify the head regeneration process, slightly faster in fed *Hydra*. Bafilomycin delays head regeneration, specially the early phase in fed *Hydra*. Wortmannin also slightly delays head regeneration, similarly in fed and starved *Hydra*. Rapamycin is the most potent drug, affecting the early (no TB), early-late (preTB) and late (TB1, just emerged) phases in fed and starved *Hydra* (TB2 corresponds to the advanced stage when at least two tentacle rudiments are longer than the head diameter).

where excessive autophagy prevents body regeneration, recent evidences suggest that autophagy is actually required for planaria remodeling during regeneration [43]. However bisected *Hydra* taken 1 h post-amputation (hpa) and intact *Hydra* actually display similar LC3 pattern: in daily-fed condition, LC3 remains low in both cell layers whereas after 17 days of starvation, LC3 staining is high in the ectoderm, slightly weaker in the endoderm that is already thinner at that stage (compare Fig. 6B, C left to Fig. 3C). This lack of modulation of the LC3 pattern between intact and regenerating hydra suggests that, whatever the starvation status, autophagy is either not or only very transiently regulated immediately after bisection. Pharmacological agents that in yeast and mammals modulate autophagy either as inducer (Rapamycin) or as inhibitors (Wortmannin, Bafilomycin), provide efficient tools to investigate the putative physiological role of autophagy in *Hydra* head regeneration as modulations of the LC3 pattern and kinetics of the regenerative process can be monitored in the same animal briefly exposed to one or the other drug (Fig. 6A).

5.1. Rapamycin induce autophagy in the ectodermal layer of fed and starved Hydra

Rapamycin that works as a derepressor of autophagy, efficiently induces autophagy in intact *Hydra* when applied at relative high concentrations [11], likely by inhibiting TOR activity as exemplified in *Drosophila* [44]. At 10 μM, the ectoderm of either fed or starved regenerating *Hydra* is strongly LC3+ implying a dramatic activation of autophagy (Fig. 6B). In the endodermal layer such activation seems possible in starved but not in fed *Hydra* where the LC3 levels remain undetectable (Fig. 6C). These results suggest that the ectodermal layer is more prone to autophagy induction than the endodermal one in fed-regenerating *Hydra*, which mimics the starvation induction of autophagy. Nevertheless this treatment similarly delays the early phases of head regeneration in both fed and starved *Hydra* (Fig. 6D), possibly as a result of a transiently excessive autophagy.

5.2. The autophagy inhibitors Wortmannin and Bafilomycin slightly delay head regeneration

Wortmannin is a specific covalent inhibitor of all three classes of PI3 kinases, potentially inhibiting the class I that activates PKB/Akt, but also the class III, Vps34, that acts on the formation of the sequestration membrane. Wortmannin also induces apoptosis in *Hydra* possibly as a result of PKB/Akt inhibition and BAD dephosphorylation [34]. Using similar conditions, we noted that autophagy is drastically inhibited in starved regenerating *Hydra* treated with Wortmannin as demonstrated by the reduced LC3 staining in both cell layers (Fig. 6B, C). This treatment actually delays head regeneration, more pronounced in starved than in fed animals (Fig. 6D) suggesting that some level of autophagy is required to achieve an efficient regeneration in starved conditions. However given the pleiotropic effect of Wortmannin, further evidence are required to confirm this hypothesis.

Bafilomycin is an inhibitor of the final step of the degradation process, impairing the formation of autolysosomes [45]. In intact *Hydra*, the continuous exposure to Bafilomycin (100 nM) differently affects the daily-fed and starved *Hydra*: the former ones dye within 24 h whereas the latter ones survive, smaller but with intact morphology [11]. During regeneration, Bafilomycin treatment enhances the ectodermal LC3 staining only in starved polyps (Fig. 6B) probably as a consequence of the blockade of the autophagy process elicited upon starvation [35]. Bafilomycin delays the early phases of head regeneration in both conditions, especially in fed *Hydra* that, as in homeostatic conditions, are more sensitive: at 57 hpa, the fed-regenerating polyps are still delayed compared to the starved ones (Fig. 6D). As autophagy was never detected in daily-fed *Hydra*, these results suggest that Bafilomycin exerts some autophagy-independent negative effect on the metabolism of fed *Hydra*.

In amputated starved *Hydra* where the level of homeostatic autophagy is high, the three drugs apparently affect the autophagy process, as deduced from the modulated levels of LC3 expression. In amputated daily-fed *Hydra* where autophagy is undetectable, Bafilomycin and to a lesser extent Wortmannin, actually delay the early and early-late phases of head regeneration. One explanation would be that these inhibitory drugs affect some other cellular processes, which in the case of Wortmannin might be linked to excessive apoptosis [34]. But they might also prevent some limited amputation-induced autophagy, transiently activated in regenerating tips to protect the cells from the amputation stress. This physiological wave of autophagy, not identified so far, could be searched on macerated cells from regenerating halves. In contrast in daily-fed *Hydra*, Rapamycin induces autophagy, like reported in yeast, fly and mammals [46,47] and also delays the head regeneration process, suggesting that a tight control of the level of autophagy is required to keep optimal the conditions for cell survival after amputation. However there is currently no clear evidence for a regeneration-specific role for autophagy in *Hydra*. In fact these drug-induced modulations of the head regeneration process remain reversible and mild compared to the dramatic post-amputation autophagic cell death resulting from *Kazal1* silencing [7,10]. These experiments also show that, as during starvation, the drug-induced modulations of autophagy occur first and predominantly in the ectoderm.

6. Crosstalk between the MAPK, TOR and autophagy pathways during head regeneration

During regeneration, numerous regulatory genes that encode for transcription factors, signaling molecules exhibit tightly-controlled regulation in head-regenerating stumps [48]. Early after bisection, many of these genes are expressed in the endodermal cells that contribute to the formation of the organizer and need to be protected from an excessive autophagy. The autophagy-specific genes actually show some regulation during head regeneration as *TOR* expression that remains constant in the first 4 hpa, progressively decreases over the next 32 hpa and is finally dramatically up-regulated between 32 and 48 hpa (Fig. 7A). In parallel, the *LC3* and *ATG3* genes are transiently up-regulated within the first 4 h of regeneration, and return to their original level at 12 hpa. Surprisingly, *ATG10* expression decreases markedly after 4 hpa and remains low during the whole regeneration period.

These modulations are compatible with an activation of the autophagic degradation pathway in early regeneration, when *LC3* and the lipidating enzyme *ATG3* are up-regulated. A transient up-regulation of the *LC3*/*ATG8* gene was also observed in the fat bodies of *Drosophila* following autophagy induction at the end of larval development [35]. In the late phase of regeneration, the clear up-regulation of *TOR* expression suggests that TOR is required for the growth phase that follows the patterning process. In contrast the gradual down-regulation of *TOR* gene expression between 4 and 36 hpa might indicate a derepression of autophagy during that period, but also, given the multiple roles played by TOR, its participation in the early phase of the regeneration program, independently of autophagy. In summary autophagy might take place at the early and early-late phases of regeneration when TOR is low, but inhibited at the late phase of regeneration.

In *Hydra* the immediate activation of the RSK kinase is required for launching the head regeneration program [49]. Similarly to bilaterians where the MAPK pathway likely impinge on the TOR and autophagy pathways [17], we observed that knocking-down *RSK* leads to a dramatic decrease in *TOR* mRNA levels, correlating with an increased expression of *LC3*, *ATG3* and *ATG10* (Fig. 7B). If we assume that *TOR* down-regulation likely affects the phosphorylation of its target proteins, we expect then an increase in the autophagic process in these conditions. Indeed the analysis of the digestive cells at 1 and 4

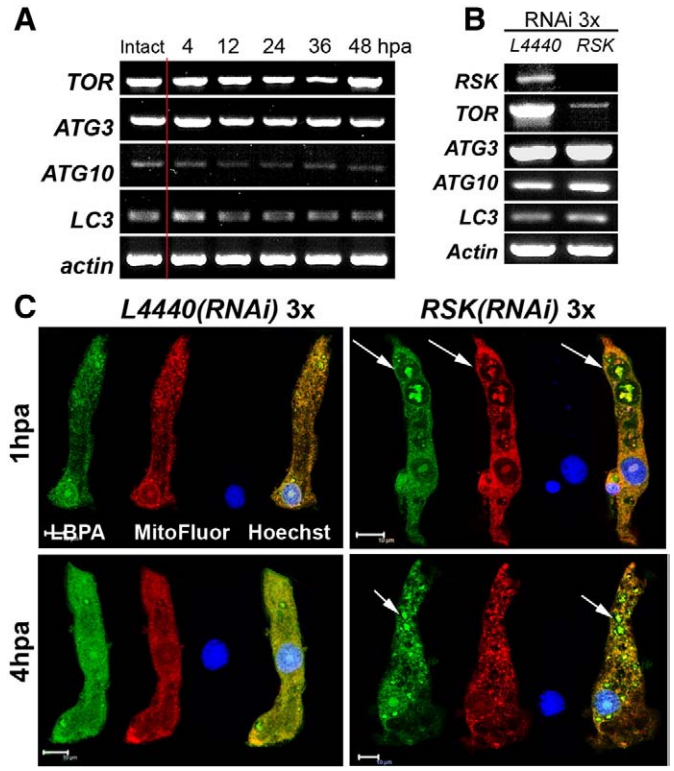

Fig. 7. *RSK* silencing affects *TOR* expression and induces amputation-driven autophagy. (A) Modulations of gene expression at indicated time points after mid-gastric section performed on 3 days starved *Hydra*. Gene expression was detected by semi-quantitative RT-PCR. (B) *TOR* and the autophagy genes *ATG10* and *LC3* show opposite modulations of their expression in *RSK*(RNAi) *Hydra* at 4 hpa. Semi-quantitative RT-PCR was performed on head-regenerating halves bisected after 3× exposures to dsRNAs, corresponding to 7 days of pseudo-starvation. The dramatic down-regulation of *TOR* and concomitant up-regulation of *ATG10* and *LC3* suggest an increase in autophagy upon *RSK* silencing. (C) Confocal views of digestive cells isolated from regenerating halves of *Hydra* exposed 3× to *RSK* and *L4440* dsRNAs. After maceration at 1 and 4 hpa, the cells were stained with Mitofluor Red 589 (red), anti-LBPA (green) and Hoechst 33342 (blue). Note the fast formation of autophagosomes in *RSK(RNAi) Hydra* after amputation. Scale bar: 10 μm.

hpa confirmed the presence of numerous autophagic vacuoles in *RSK (RNAi) Hydra* cells (Fig. 7C). In most cells the autophagy phenotype appears transient, as evidenced by the drastic reduction in the size of the autophagosomes at 4 hpa (Fig. 7C). Nevertheless, in few cells, the autophagy phenotype is actually enhanced, the autophagic vacuoles occupying most of the cytoplasm and starting to fuse (not shown). This is reminiscent of the *Kazal1* autophagy phenotype, which is first transient and reversible, and subsequently, when silencing is complete, leads to autophagic cell death [7]. Hence further functional analyses of the MAPK pathway should highlight the crosstalk that takes place between *TOR* signaling and autophagy at the time cells need cytoprotection after amputation.

7. Concomitant but distinct roles for autophagy and apoptosis in starving *Hydra*?

The initial work performed in yeast established that autophagy plays a fundamental role to supply the nutrient source under unfavorable conditions [50]. Subsequently, work performed in *C. elegans*, fly and mammals confirmed that nutrient withdrawal or food depletion also stimulates autophagy in metazoans, contributing to cell and animal survival during starvation as the nutrients obtained from the autophagic degradation pathway provide the substrates for both bioenergetics and biosynthetic demand [51–53]. Some recent evidence reviewed here show that *Hydra* also rapidly adapt to extended periods of starvation by triggering an autophagic response. In starved animals, most epithelial cells contain autophagic vacuoles, which are almost absent in the regularly fed animals. In contrast, during the starvation process, epithelial cells that contain phagosomes with apoptotic bodies appear during the first days following the last feeding but their number is low, as in our experiments the percentage of digestive cells with engulfed apoptotic bodies does not exceed 2%. These results are in agreement with those previously reported where up to 1.4% epithelial cells exhibit an increase in their phagocytotic activity upon starvation, compared to 0.2% in heavily-fed *Hydra* [33].

Moreover the evolution of these two cellular contingents along starvation is dramatically different: both processes are likely launched at the same time, i.e. after 2 days of starvation, but one process appears quite stable with time, as evidenced by a rather constant proportion of cells with apoptotic bodies, whereas the other process, autophagy, is highly dynamic, affecting more and more cells with an increased number of autophagic vacuoles per cell upon long-lasting food depletion. Hence a highly significant difference between the respective sizes of the autophagic cell contingent (>70% after 19 days) and the engulfing cell contingent (<2%) progressively emerges, suggesting that survival during starvation rather relies on the autophagy-derived nutrients than on the phagocytosed apoptotic bodies. In fact as previously discussed by David et al. [33,54] apoptosis upon starvation might be the main mechanism to control cell number, by eliminating the excess cells that are produced in the absence of animal growth and animal budding. But given the fact that most epithelial cells are progressively recruited for autophagy, we doubt that apoptosis provides the source of nutrients during starvation. In conclusion, we anticipate that apoptosis and autophagy likely support distinct tasks during starvation, apoptosis keeping constant the cell number and autophagy maintaining the conditions for cell and animal survival. To elucidate the links that exist between apoptosis and autophagy in starving *Hydra*, further studies based on the functional dissection of the autophagic and apoptotic genetic circuitries are required.

8. Conclusions and perspectives

The discovery of similar cellular and molecular basis of autophagy in phylogenetically different species proved that the response to starvation was a surviving strategy well conserved across evolution from yeast to mammals [13]. The results discussed in this review indicate that the basic cellular, biochemical and genetic aspects of autophagy are also at work in the cnidarian *Hydra*. Autophagy is strongly activated in starving *Hydra*, appearing as the main survival strategy after food depletion. In that context the myoepithelial cells of the two cell layers undergo autophagy, but the ectodermal ones obviously differ from the endodermal ones as they respond faster to the starvation signals and never undergo autophagic cell death. The need for a tight regulation of autophagy in *Hydra* was proved not only in starvation, but also during regeneration, during the critical hours that follow bisection. In physiological conditions, there might be a mild autophagy in the early phase of the regeneration process as autophagy genes are up-regulated, and both inhibitors and activators of autophagy delay regeneration, suggesting that a tightly tuned level of autophagy is needed. However this physiological amputation-induced autophagy remains to be characterized.

In contrast a deficient regulation of autophagy at the time of the amputational stress results in massive autophagic death of the digestive cells, highlighting the essential cytoprotective role played by proteins like Kazal1, a serine protease inhibitor that prevents excessive autophagy in *Hydra* in a fashion similar to that reported in mammals. In that context the autophagy that affects exclusively the digestive cells, appears to involve a regulation that is different from that recorded during starvation. Hence the control of autophagy in *Hydra* appears rather complex. Two tools need be developed to dissect these regulatory networks in a close future: a transgenic *Hydra* [55] that would constitutively express the chimeric GFP-LC3 protein to monitor live the modulations in autophagy, and a RNAi screen that

would identify the molecular mechanisms supporting autophagy in the different contexts. Hence *Hydra* provides a new powerful experimental paradigm to study the physiological and pathological responses to starvation, stress and regrowth.

Acknowledgements

We are very grateful to Robbie Loewith for critical reading and excellent advices, and to two anonymous reviewers for constructive criticism. The work in our laboratory is funded by the Swiss National Foundation, the Geneva State, the Claraz Donation and the Academic Society of Geneva.

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