The ULK1 kinase, a necessary component of the pro-regenerative and anti-aging machinery in Hydra

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A R T I C L E  I N F O

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A B S T R A C T

Hydra vulgaris (Hv) has a high regenerative potential and negligible senescence, as its stem cell populations divide continuously. In contrast, the cold-sensitive H. oligactis (Ho, CS) rapidly develop an aging phenotype under stress, with epithelial stem cells deficient for autophagy, unable to maintain their self-renewal. Here we tested in aging, non-aging and regenerating Hydras the activity and regulation of the ULK1 kinase involved in autophagosome formation. In vitro kinase assays show that human ULK1 activity is activated by Hv extracts but repressed by Ho,CS extracts, reflecting the ability or inability of their respective epithelial cells to initiate autophagosome formation. The factors that keep ULK1 inactive in Ho,CS remain uncharacterized. Hv_Basel1 animals exposed to the ULK1 inhibitor SBI-0206965 no longer regenerate their head, indicating that the sustained autophagy flux recorded in regenerating Hv_AEP2 transgenic animals expressing the DsRed-GFP-LC3A autophagy tandem sensor is necessary. The SBI-0206965 treatment also alters the contractility of intact Hv_Basel1 animals, and leads to a progressive reduction of animal size in Hv_AEP2, similarly to what is observed in ULK1(RNAi) animals. We conclude that the evolutionarily-conserved role of ULK1 in autophagy initiation is crucial to maintain a dynamic homeostasis in Hydra, which supports regeneration efficiency and prevents aging.

1. Introduction

Since the discovery that specific genes directly regulate the lifespan of animals, two invertebrate models, the roundworm C. elegans and the fruit fly D. melanogaster, as well as the yeast S. cerevisiae, have played a key role in our understanding of the genetic mechanisms of aging (Friedman and Johnson, 1988; Cuervo, 2008). More recently, some organisms used as models in the study of the mechanisms of regeneration have been shown to be valid for the study of aging mechanisms, particularly those equipped with large stocks of adult stem cells (Austad, 2009; Murthy and Ram, 2015). Among these, the simple freshwater polyp called Hydra, which belongs to Cnidaria, a sister group of Bilateria (Fig. 1A), is known for its remarkable regenerative capabilities (Galliot, 2013; Schenkelaars et al., 2018). Among these, the simple freshwater polyp called Hydra, which belongs to Cnidaria, a sister group of Bilateria (Fig. 1A), is known for its remarkable regenerative capabilities (Galliot, 2013; Schenkelaars et al., 2018). Among these, the simple freshwater polyp called Hydra, which belongs to Cnidaria, a sister group of Bilateria (Fig. 1A), is known for its remarkable regenerative capabilities (Galliot, 2013; Schenkelaars et al., 2018).

Hydra is a tube-shaped animal that differentiates a head at the apical end, a dome-shaped structure called hypostome centered on the mouth opening and surrounded by a ring of tentacles. At the basal end, the animal is equipped with a disc, also called a foot, which allows the animal to attach itself to substrates (Fig. 1B). Hydra consists of two tissue layers, the epidermis and the gastrodermis, which contain three distinct populations of adult stem cells that continuously self-renew under homeostatic conditions. The two epithelial stem cell (ESCs) populations are either epidermal when located in the outer layer (eESC), or gastrodermal when located in the inner layer (gESC) (Fig. 1B). ESCs have a slow cell cycle, renewing every three to four days, while interstitial stem cells (ISCs), which are exclusively located in the outer layer, have a much faster cell cycle, renewing every 24 to 30 hours (Holstein and David, 1990; Holstein et al., 1991; Buzgariu et al., 2014). One remarkable characteristic of Hydra is their ability to withstand weeks of starvation as the cyclic activity of their stem cells is continuously maintained (Otto and Campbell, 1977; Bosch and David, 1984). One key process to

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maintain homeostasis in response to starvation is an inducible flux, named macroautophagy (named here autophagy) that generates nutrients. In *Hydra*, autophagy is indeed rapidly induced in ESCs upon starvation (Buzgariu et al., 2008; Chera et al., 2009; Galliot et al., 2018).

Autophagy consists in a dynamic flux where autophagic vacuoles form in response to a number of environmental conditions (starvation, stress, drugs …), these autophagosomes subsequently fuse to lysosomes to allow the self-digestion of their content, providing nutrients and clearing potentially toxic aggregates (Mizushima et al., 2010; Yu et al., 2018) (Fig. 1C). The molecular actors of the autophagy process were initially identified in yeast mutants that could not survive long-term starvation (Ohsumi, 2001; Matecic et al., 2010). The link between autophagy and aging has subsequently been established in a variety of animal models. It appears that a tightly-tuned balance of the autophagy flux contributes to prevent aging by allowing the production of nutrients and the clearance of protein, lipidic and nucleic acid aggregates in response to different types of stress (Cuervo, 2008; Levine and Kroemer, 2008).

In *Hydra*, the components of the autophagy machinery are highly conserved, predominantly expressed in the epithelial stem cells (ESCs) where autophagy can easily be induced (Buzgariu et al., 2008; Chera et al., 2009; Tomczyk et al., 2020). In a recent study, we found that ESCs are deficient for autophagy in the *H. oligactis* Cold Sensitive (*Ho_CS*) strain, unable to maintain their cycling activity when animals face stressful situations such as gametogenesis or loss of interstitial cells (Tomczyk et al., 2020). As a consequence, these animals rapidly undergo aging, i.e. they lose their ability to regenerate, stop reacting to their environment, stop eating, show tissue atrophy and die within three to four months (Yoshida et al., 2006; Tomczyk et al., 2019, 2020).

**Fig. 1.** *Hydra*, a model system for aging and regeneration studies. (A) Schematic representation of the phylogenetic position of the *Hydra* genus within metazoans. *Hydra vulgaris* and *Hydra oligactis* are two distinct species, whereas the North American (NA) *H. vulgaris* and the Eurasian *H. vulgaris Pallas* are classically considered as forming a unique species. (B) Anatomy of the adult *Hydra* polyp. The brown-colored part of the animal corresponds to the region where all epithelial cells are stem cells that continuously proliferate. The enlargement on the right shows the typical bilayered structure of the animal, with epidermal epithelial stem cells (eESCs) on the outside and gastrodermal ones (gESCs) on the inside, separated by an extracellular matrix called mesoglea. The third population of stem cells, called interstitial cells, are located in the epidermal layer. (C) Scheme depicting the successive stages of the autophagy flux with the formation of pre-autophagosomal vacuoles (PAS), the elongation of phagophores until they close to form autophagosomes that sequester cytoplasmic content, then fuse with lysosomal vacuoles (red) to form autolysosomes whose content is degraded. The components of the ULK1 and Beclin-1 (BECN1) complexes involved in autophagosome formation are evolutionarily-conserved (Mizushima, 2010) and ubiquitously expressed in *Hydra* (Figures S1-S6). PE: phospho-ethanolamine; PI: phospho-inositol; PI-EP: PI-ethanolamine.
Hydra, we exposed Hv animals to the ULK1 inhibitor SBI-0206965 (Egan et al., 2015) and we knocked-down ULK1 expression. Both approaches alter the contractility of intact animals and affect animal size, two typical aging signs, while SBI-0206965 also blocks apical regeneration. This study highlights the importance of ULK1 activity in autophagosome formation and maintenance of fitness and regeneration in Hydra.

2. Methods

2.1. Hydra culture, survival and regeneration experiments

The *H. vulgaris* AEP (*Hv*AEP, strain AEP2), *H. vulgaris* Basel (*Hv*Basel, strain BaselI), *H. vulgaris* sf-1 (*Hv*sf1), *H. oligactis* Cold Resistant (*Ho*CR) and *H. oligactis* Cold Sensitive (*Ho*CS) strains were mass cultured at 18 °C ± 0.5 °C in Hydra Medium (HM: 1 mM NaCl, 1 mM CaCl₂, 0.1 mM KCi, 0.1 mM MgSO₄, 1 mM Tris – HCl pH 7.6) and fed 3x a week with freshly hatched *Artemia* nauplii (Watson Aquaculture). The *Hv*AEP2 and *Hv*BaselI strains were characterized by their microsatellite pattern (Schenkelars et al., 2020). Animal survival was followed on cohorts of 6 × 10 animals per condition placed in six-well dishes (Greiner Bio-One). To induce gametogenesis *Ho*CS and *Ho*CR animals were transferred to 10 °C ± 0.3 °C and then fed twice a week. For regeneration experiments, animals were fed twice a week prior to bisecation, then starved for 2 days, bisected at mid-gastric position and staged as in (Bossert and Galliot, 2012).

2.2. Phylogenetic and RNA-seq analyses

For phylogenetic reconstruction of the ULK1 and Beclin-1 gene families, related sequences representative of a wide diversity of phyla were collected via Basic Local Alignment Search Tool (blastP software) performed on the Uniprot (www.uniprot.org) and NCBI (blast.ncbi.nlm.nih.gov/) databases. *Hydra vulgaris* and *Hydra oligactis* sequences were also retrieved from HydrATLAS (hydratlas.unige.ch). 33 ULK1-related protein sequences were aligned using the software MAFFT (mafft.cbrc.jp/alignment/server/) (Katoh and Yamada, 2019) and 38 Beclin-1 related protein sequences were aligned with the software Muscle Align (www.ebi.ac.uk/Tools/msa/muscle/). The phylogenetic trees were built with PhyML 3.0 (Guindon et al., 2010) using the LG substitution model with 4 transversion 1 transition ratio, 8 substitution rate categories and tested with 100 bootstraps (www.hiv.lanl.gov/content/sequence/PHYML/interface.html).

The large-scale production of RNA-seq profiles related to *H. vulgaris* (spatial distribution along the body axis, temporal regulation during regeneration, enrichment in the different stem cell types) is described and analyzed in (Wenger et al., 2019), while details about the production of RNA-seq data related to *H. oligactis* (*Ho*CS and *Ho*CR animals maintained at 18 °C or at 10 °C) are reported in (Tomczyk et al., 2020). All *Hydra* sequences and RNA-seq profiles can be retrieved from HydrATLAS (https://hydratlas.unige.ch).

2.3. Drug treatments: MG1312, SBI-0206965, bafilomycin-A1, rapamycin

To induce autophagy immediately prior to live imaging, polyps were treated with the proteasome inhibitor MG1312 (SelleckChem, S2619) diluted to 10 μM in HM from a 10 mM stock solution in DMSO. Control animals were exposed to DMSO 0.05%. For longer treatments, animals were exposed to MG1312 1 or 5 μM as indicated. To prevent initiation of autophagy, animals were treated with the ULK inhibitor SBI-0206965 (Medkoo, 407159) (Egan et al., 2015) diluted in DMSO from 1 to 7 μM, given either continuously or as pulses as described. To block autolysosome formation (Yamamoto et al., 1998), animals were treated with bafilomycin-A1 (Baf-A1) (Enzo Life Sciences, BML-CM110—0100), up to 200 nM diluted in HM. To induce autophagy, animals were exposed to rapamycin (LC Laboratories) 0.8 μM diluted in HM, given continuously from day-2 after transfer to 10 °C and the drug was changed 3x a week. See recapitulative Table 1.

2.4. Transient expression of the mCherry-eGFP-LC3A autophagy sensor

The construction of the hyActin:mCherry-eGFP-hyLC3A tandem sensor (named mCherry-eGFP-LC3A) is described in (Tomczyk et al., 2020). To ensure the optimal expression level in electroporated animals, the plasmidic DNA was prepared with the Endotoxin free Maxi prep kit (Qiagen), resuspended at 1 μg/μl and stored at −20 °C (Miljkovic et al., 2002). The day of electroporation (EP) 20 polys per condition were washed 8 h after feeding 3x in distilled water and incubated for 45 min in distilled water. The animals were then transferred into the EP cuvettes (Biorad) and the water was replaced by 200 μL DNA solution diluted 0.1 μg/μl in 10 mM Hepes (Sigma, H3375). After 5 min incubation, animals were electroporated with the GenePulsXcell (Bio-Rad) as in (Watanabe et al., 2014) with Voltage 150 V, 2 pulses separated by 1 s, each pulse 50 ms long. Immediately after EP, the animals were transferred to a 10 cm diameter plastic dish prefilled with 50 mL HM. To prevent the toxic effect of the dying cells released by the animals, animals were kept in large volumes during the recovery time. The next day, animals were washed in HM, and on day two post-EP, the animals that show an intense fluorescence of 10–15 eSCs in the central body column were selected under a stereo-microscope to continue with the imaging process.

2.5. Production of the DsRed-GFP-LC3A transgenic line

Male and female animals of the *H. vulgaris* AEP strain were selected from the mass culture and placed together to produce fertilized embryos. The hyActin:DsRed-GFP-hyLC3A plasmid was injected into one-cell stage embryos as described in (Wittlieb et al., 2006). Of the 194 injected embryos, 23 hatched and 2 exhibited a DsRed-GFP fluorescence in ESCs. Two chimeric lines were produced, one expressing the reporter in gastrodermal EGCs (ga-LC3A) and the other in epidermal EGCs (ep-LC3A).

2.6. Live imaging of cells expressing the autophagy sensor in whole Hydra

Time-lapse imaging was performed on a 3i Marianas spinning disk confocal microscope. 3–5 selected animals were pre-incubated for 10–15 min in 2% urethane (Sigma, U2500) or 0.5 μM linalool (Sigma, L2602—100 G), then transferred to a 35 mm dish suitable for inverted microscope (Cellvis, D35—20.1.5-N) prefilled with HM containing MG132 10 μM and the anesthetic, and oriented as such as the cells of interest are placed in direct contact with the cover slip. The laser power was kept as low as possible to avoid bleaching, typically 40 with exposure time 100 ms for cells from *H. vulgaris* and camera intensification set up to 1000. The optimal resolution of autophagosome and autolysosomes in *Hydra* was obtained with 1 μm Z-steps. Z-stacks were acquired over a 150–200 μm depth as *Hydra* epithelial cells are rather large, with the first and last slices “blurry”, i.e. with no visible autophagic vacuoles. The images in tiff format were then treated with the Imaris software to number the quantity of puncta in each cell. For animals expressing the tandem sensor and treated with drugs or DMSO prior to imaging, the drug was washed-away and the animals were anesthetized and treated as above. For the imaging of regenerating transgenic animals, the amputated halves were embedded for the duration of the recording in 1% low melting Agarose layer poured in the imaging dish and imaged at indicated time points as indicated above. For the staining of lysosomes, we used the Lysotracker Deep Red fluorescent dye (ThermoFisher, L12492) diluted to the final concentration of 50 nM in HM. Three *Hy BaseI* animals previously electroporated with mCherry-eGFP-LC3A plasmid were incubated in 50 nM Lysotracker Deep Red for 30 min in the dark. Next, the animals were washed three times with HM and imaged with the spinning disk confocal microscope as described above.
**Table 1**

Summary table showing the physiological, pharmacological and genetic modulations that impact the signs of aging and autophagy in various Hydra strains.

| Condition | H. vulgaris (Hv_Basel1) | H. vulgaris (Hv_AEP2) | H. oligactis (Ho_CS) | H. oligactis (Ho_CR) |
|-----------|-------------------------|-----------------------|---------------------|---------------------|
| Physiological aging | nd | (−) | (−) | (−) |
| Starvation (1–9 days) | (−) | (−) | (−) | (−) |

**Detection of the autophagy flux**

| Condition | H. vulgaris (Hv_Basel1) | H. vulgaris (Hv_AEP2) | H. oligactis (Ho_CS) | H. oligactis (Ho_CR) |
|-----------|-------------------------|-----------------------|---------------------|---------------------|
| Autophagy inhibition & Hydra fitness | MGI-32 treatment increases the autophagy flux (−) | 0.2 mM (−) | 0.2 mM (−) | nd |
| Autophagy inhibition & Hydra fitness | Bafilomycin A1 inhibits the autophagy flux (−) | 5 μM (−) | 5 μM (−) | 1 μM (−) |
| Autophagy inhibition & Hydra fitness | Bafilomycin A1 treatment decreases muscle contractility (−) | 6 μM (−) | nd | 1 μM (−) |
| Autophagy inhibition & Hydra fitness | SBI-0206965 treatment decreases muscle contractility (−) | 6 μM (−) | nd | 1 μM (−) |

**ULK1(RNAi) & Hydra fitness**

| Condition | H. vulgaris (Hv_Basel1) | H. vulgaris (Hv_AEP2) | H. oligactis (Ho_CS) | H. oligactis (Ho_CR) |
|-----------|-------------------------|-----------------------|---------------------|---------------------|
| Loss of contraction | 3x EPs (−) | 5x EPs (−) | nd | nd |
| Inhibition of apical regeneration | 3x EPs (−) | 5x EPs (−) | nd |

Decrease in contractility, decrease in animal size and loss of regeneration are considered as signs of aging (Tomczyk et al., 2020). BafA1-induced blockade of the autophagy flux was evidenced either with the autophagy sensor, or with the increased p62 levels (Tomczyk et al., 2020; this work). Hv_AEP2 animals did not survive SBI-0206965 treatments (5 μM or 7 μM) given for three periods of three days separated by a one-day release (not shown). (−): no effect; (−): effect; nd: experiment not done.

2.7. Quantification of red and green fluorences in gastroleral epithelial cells from transgenic animals expressing the DsRed-eGFP-hyLC3A sensor (Quant-IM assay)

Two animals from a cohort of intact animals, treated or not with MG132, or from cohorts of regenerating animals were selected for each condition. The tissue of interest was dissected with scalpel, then disso...
3. Results

3.1. Analysis of ULK1 and Beclin-1 expression during regeneration and induction of aging

Two main nutrient sensing pathways, TORC and AMPK, regulate the entry into autophagy through the Serine/threonine-protein kinase ULK1, which once activated, phosphorylates components of the ULK1 complex to initiate the formation of autophagophores, the precursors of autophagosomes (Fig. 2A) (Russell et al., 2013; Hosokawa et al., 2009; Papinski and Kraft, 2016). To verify that Hydra polyplys do express the elements of this machinery, we analyzed the transcriptomic resources available for Hydra and found three ULK1-related sequences and a single Beclin-1 sequence. The phylogenetic analysis shows that Hydra ULK1 (seq40808): is orthologous to the ULK1 and ULK2 vertebrate sequences, to Unc51 from C. elegans, to the ATG1 sequences from plant and yeast (Fig. S1). The two other Hydra ULK sequences (seq46768 /T2M57 and seq32486 /T2M8D5) are orthologous to the bilaterian ULK3 and ULK4 sequences respectively. The human ULK1 protein includes four domains: a kinase domain, a LC3-interaction region (LIR), an AMPK interacting region and an ATG13/FIP200 interacting regions. The kinase domain and the LIR are highly conserved in Hydra ULK1, the EAT / MIF domains more distantly related (Fig. S2).

The Beclin-1 (BECN1) protein, a key regulator of entry into autophagy but also maturation of autophagosomes (Russell et al., 2013; Sun et al., 2015; Papinski and Kraft, 2016; Menon and Dhamija, 2018), is evolutionarily-conserved (Fig. S3), showing a typical tripartite structure (BH3 motif, coiled-coiled domain and evolutionary-conserved domain) with most regulatory phosphorylation sites present in the Arabidopsis, Hydra, C. elegans, Drosophila and human sequences, including the Ser15 and Ser30 residues that are ULK1 target sites (Fig. 2A, Fig. S4). The components of the ULK1 complex (ULK1, RB1CC1, ATG13 and ATG101) and Beclin-1 complex (BECN1, ATG14, PIK3C3 and PIK3R4 named Vps34 and Vps15 in yeast respectively) are also highly conserved, suggesting that these complexes are functional in Hydra. As anticipated, we found the level of Beclin-1 phosphorylation increased after exposing Hv Basell and Hv AEP2 animals to an overnight MG132 treatment that induces autophagy by inhibiting proteasomal degradation (Fig. 2B).

The analysis of the RNAseq expression profiles available on Hydratlas (https://hydratlas.unige.ch) shows that ULK1 is expressed at high levels along the body axis, about twice higher in the apical region (Fig. S5A) and enriched in the three stem cell populations, although at higher level in ESCs (Fig. S5C). By contrast, BECN1 is expressed at higher levels along the body column and enriched in all stem cell populations, but higher in the interstitial stem cells. During regeneration, ULK1 is upregulated between one and 8 hpa, at highest level during apical regeneration after mid-gastric bisection (Fig. S5B). This upregulation is consistent with a role for ULK1 in the activation of the autophagy flux during the early and early-late phases of regeneration. All the other components of the ULK1 and PI3K-III complexes are also expressed along the body axis, in the three stem cell populations and rather steadily during apical and basal regeneration (Fig. S5).

We also analyzed the regulation of ULK1 during the aging process, and detected a transient up-regulation at 14 and 26 days after transfer to 10 °C in the Ho CR strain that does not undergo aging (Fig. S6). In the Ho CS strain that does undergo aging, this up-regulation is delayed, between 26 and 32 days after transfer to 10 °C and at a higher level than in Ho CR. This finding is consistent with the general but transient up-regulation of the autophagy genes, two weeks after transfer to 10 °C for Ho CR, and about 12 days later for the Ho CS strain where autophagy remains limited. Therefore, the signals that activate the entry into autophagy seem to be sensed by the two strains, however with delay and inefficiency in Ho CS animals.

3.2. Differential regulation of ULK1 activity in extracts from aging and non-aging Hydra

To investigate the regulation of ULK1 activity in Hydra, we first performed an in vitro kinase assay where the phosphorylation level of human Beclin-1 at Ser15 was measured in the presence of Hydra extracts. Unfortunately, Hydra ULK1 activity was undetectable in kinase assays (not shown), therefore we decided to rather monitor the activity of putative ULK1 modulators in Hydra extracts. To do this, we designed an in vitro assay where Hydra extracts are co-incubated with the recombinant human ULK1 and Beclin-1 proteins (Fig. 2C). The ULK1 activity is deduced from the level of phospho-Beclin-1 detected by immunoblotting with anti-Beclin-1 and anti-phospho-Beclin-1 antibodies (red arrowheads in Fig. 2D-F).

We first compared the activity of Hydra extracts produced from daily fed or 9-day starved animals maintained at 18 °C in the presence of Hv extracts, the human ULK1 activity is maintained very high, slightly higher than that observed in the absence of extracts, suggesting that Hv extracts rather play a positive role on ULK1 activity, similar after one or nine days of starvation (Fig. 2D, compare lanes 3–6 to lanes 7–10). However, at the highest concentration (4 μg), ULK1 activity is no longer enhanced (lanes 6 and 10). By contrast, extracts from fed Ho CR animals inhibit human ULK1 activity, an inhibition that is dose-dependent (lanes 2–6) and no longer detected when Ho CR extracts are prepared from 9-day starved animals as deduced from higher phospho-Beclin-1 levels (Fig. 2D, compare lanes 3–6 to lanes 7–10).

Similarly extracts from fed Ho CS animals repress human ULK1 activity in a dose-dependent manner, a repressive effect that is less intense when extracts are prepared from 9-day starved Ho CS animals, although the level of phospho-Beclin-1 is lower than that observed in the presence of Ho CR extracts. These results point to a similar constitutive repression of ULK1 activity in both Ho strains when compared to Hv, a repressive activity possibly released with starvation in Ho CR animals but to a much weaker extent in Ho CS animals. In Hv and Ho strains, a 16 -h MG132 treatment, which is supposed to activate autophagy, only slightly increase ULK1 activity (Fig. 2E, compare lanes 13–16 to lanes 17–20).

In contrast, chronic treatment with rapamycin given for 13 days in Ho CS and Ho CR animals maintained at 10 °C appears to de-repress ULK1 activity, particularly when extracts of Ho CS are added (Fig. 2F, compare lanes 23–26 to lanes 27–30), suggesting that Ho CS animals transduce some autophagy-inducing signals via ULK1. The summary of the results (Fig. 2G), indicate in the upper part that the constitutive modulators that regulate ULK1 kinase activity are very different, rather activating in Hv, while inhibitory in Ho CR and strongly inhibitory in Ho CS. Also, the lower part shows that the response to starvation varies between the different strains and species, with already a maximal ULK1 activity after one day of starvation in Hv, an activation of ULK1 activity during starvation in Ho CR, compared to a limited one in Ho CS. Taken together, these results suggest that Ho CS produce inhibitory substances that repress ULK1 activity, probably blocking the induction of autophagy. In addition, we cannot rule out an alternative mechanism whereby the lack of ULK1 activity results from the absence of an activator, including activating modifications such as AMPK-phosphorylation of ULK1 (Kim et al., 2011) as the Ser317 and Ser775 are conserved in Hydra ULK1.
Fig. 2. In vitro analysis of ULK1 activity in the presence of extracts prepared from *H. vulgaris* (AEP2) or *H. oligactis*. 

A. Schematic view of the evolutionarily-conserved regulation of autophagy initiation via ULK1-dependent phosphorylation of Beclin-1. The two ULK1 phospho-sites are conserved in *Hydra* Beclin-1. 

B. Western analysis detecting phospho-Beclin-1 levels in WCE prepared from *H. vulgaris* exposed or not overnight to MG132 (5 μM). *Hydra* Beclin-1 molecular weight is 52 kDa (arrowhead). 

C. Design of the in vitro kinase assay where human Beclin-1 is phosphorylated at Ser15 by human ULK1 enzyme in the presence of *Hydra* extracts prepared from animals either starved, or exposed to drugs (MG132 or rapamycin), or induced for aging. 

D–F. Analysis of the ULK1-dependent phosphorylation levels of human Beclin-1 (red arrowheads) tested in the presence of *Hydra* extracts prepared from animals either starved for one or nine days (D), or treated with MG132 (5 μM) for 16 h (E), or maintained at 10 °C and treated or not with rapamycin (0.8 μM) for 13 days (F). In panels D–F, the ULK1 inhibitor SBI-0206965 (20 μM) was added during the in vitro reaction to detect phospho-Beclin-1 in the absence of ULK1 (lanes 2, 12, 22). 

G. Summary of the obtained results: extracts from *H. vulgaris* (Hv_AEP2) rather enhance ULK1 activity whereas extracts from *H. oligactis* inhibit in a dose-dependent manner Beclin-1 phosphorylation (panel D, lanes 3–6). Note the stronger inhibition by extracts from *Ho_CS* when compared to *Ho_CR*. A 9-day starvation at 18 °C promotes ULK1 activity in *Ho_CR*, at a lower level in *Ho CS* (panel D, compare lanes 3–6 to lanes 7–10). ULK1-dependent phosphorylation levels of Beclin-1 are only mildly increased when extracts from *Hydra* treated with MG132 are added, with no significant differences between *H. vulgaris*, *Ho_CR* and *Ho_CS* (panel E, compare lanes 13–16 to lanes 17–20). Rapamycin treatment of *Ho_CS* leads to a mild increase in phospho-Beclin-1 levels (panel F, compare lanes 23–26 to lanes 27–30).
Mechanisms of Ageing and Development 194 (2021) 111414

3.3. Transient expression of the mCherry-eGFP-hyLC3A autophagy sensor to monitor the autophagy flux in live Hydra

To characterize the autophagy flux in animals exposed to autophagy modulators (Fig. 1C), we adapted to Hydra a mCherry-GFP-LC3A tandem sensor used in mammalian cells (Pankiv et al., 2007; Tomczyk et al., 2020). The fluorescence-labelled LC3A protein that is anchored in the membrane of autophagic vacuoles, fluoresces green in the autophagophores and forming autophagosomes where the mCherry is not fluorescent yet, orange/yellow in mature autophagosomes, red only in autophagolysosomes where the acidic environment quenches the GFP fluorescence while the red fluorescence remains stable (Fig. 3A, B). To evidence the sensitivity of this tandem reporter, we treated the electroporated animals with the proteasome inhibitor MG132 known as an autophagy-enhancing drug (Ding et al., 2007). We recorded by live imaging with a spinning disk confocal microscope every 15 min one to three cells per animal over 75 min (Fig. 3A). When magnified, these positive cells show fluorescent puncta that exhibit an MG132-induced variation of their fluorescence, yellow-green in autophagosomes to orange and red in autolysosomes, reflecting the progression through the autophagy flux.

To quantify the kinetics of the progression of the autophagy flux, we counted at each time-point the number of red and green vacuoles and found that autolysosomes correspond to 10–15% of fluorescent puncta at the onset of the MG132 treatment, reaching almost 90% of puncta after 75 min, without any obvious change in the total number of puncta (Fig. 3B). This rapid change in the ratio autophagosomes over autolysosomes reflects a fast progression through the autophagy flux, i.e. a fast turnover of autophagosomes to autolysosomes upon proteasome inhibition induced by the MG132 treatment. Electroporation of this sensor as a plasmid is efficient but the number of cells expressing the sensor are limited: two days after the electric pulse is applied, about 60% Hv_Basel1 animals express this autophagy sensor, usually in epidermal ESCs, with 5–20 fluorescent cells per animal that can be co-immunodetected with the anti-mCherry and anti-GFP antibodies (Fig. 3C, D). We also validated that red puncta correspond to autolysosomes by co-staining the mCherry-GFP-LC3A positive cells with a lysotracker dye (Fig. 3E, arrows).

Next, we tested the cellular phenotype obtained when the transformation of autophagosomes to autolysosomes is blocked upon exposure to bafilomycin-A1 (Baf-A1), known to prevent autophagosome-lysosome fusion in mammalian cells (Yamamoto et al., 1998). We applied Baf-A1 for 18 h on animals previously electroporated with the autophagy sensor, and then imaged the animals in the presence of MG132. As anticipated, we noted in cells from animals exposed to the combination Baf-A1/MG132 the accumulation of green/yellow autophagic vacuoles and very few red ones when compared to cells from animals exposed only to MG132 (Fig. 3F). The quantification of this assay shows that in animals exposed to Baf-A1, the proportion of autolysosomes (red autophagic vacuoles) is dramatically reduced, about three-fold (from 50 % to 17 %) while the total number of vacuoles does not vary significantly (Fig. 3G). These results demonstrate that the autophagy sensor transiently expressed in Hydra epithelial cells can provide distinct cellular phenotypes that accurately reflect the status of the autophagy flux, with imaging data that can be readily quantified.

3.4. Monitoring of the autophagy flux in transgenic Hv_AEP2 animals that constitutively express the DsRed-eGFP-LC3A sensor

To follow the autophagic flux in regenerating animals, we produced two transgenic lines that constitutively expresses the DsRed-GFP-hyLC3A tandem autophagy sensor in the epithelial layers, either epidermal (ep-LC3A) or gastrodermal (ga-LC3A) (Fig. 4A). A Western blot analysis shows the higher level of GFP expression in ga-LC3A when compared to ep-LC3A, suggesting a stronger level of expression of the transgene in the gastrodermal layer (Fig. 4B). This result is consistent with the higher red fluorescence observed in ga-LC3A animals when compared to ep-LC3A ones (Fig. 4A). A closer view of both ep-LC3A and ga-LC3A animals shows the chimeric structure of this transgenic line, the LC3A positive cells being intermingled with non-fluorescent cells (Figs. 4C, S7A). Among the LC3A cells, three distinct fluorophores can be identified: green corresponding to novel autophagosomes where DsRed is not fluorescent yet, yellow/orange corresponding to mature autophagosomes where both DsRed and GFP are fluorescent, and red corresponding to autolysosomes where GFP fluorescent is quenched. This result suggests that autophagic vacuoles are rather synchronized in a given cell. As expected, the addition of Baf-A1 for 60 h leads to the accumulation of ga-LC3A cells containing mature autophagosomes (yellow cells) (Fig. 4C). By contrast, the MG132 treatment of ep-LC3A animals does not seem to modify the autophagy flux (Fig. S7A), indicating that Hv_AEP2 animals are poorly sensitive to this drug when compared to Hv_Basel1 animals (Table 1).

To quantify the proportion of autolysosomes versus autophagosomes in LC3A cells, we established a test we named Quant-IM: after dissection from the live polyp, the tissue was briefly dissociated in diluted trypsin solution and cells spread on slides for picturing and quantification with Imaris. The obtained data are then tidied and visualized (Fig. 4D). The microscopic analysis of the Baf-A1 treated ga-LC3A cells immediately after trypsin dissociation shows the almost complete absence of autolysosomes, confirming the blockade of the autophagy flux as observed in whole transgenic Hydra (Fig. 4E). As anticipated, the Quant-IM quantification of the green fluorescence shows a significant increase after Baf-A1 treatment (Fig. 4F). The Quant-IM analysis of the ga-LC3A cells in the apical, central and basal regions detects similar levels of green fluorescence, suggesting a similar autophagy flux along the body axis (Fig. 4G).

3.5. Changes of the autophagy flux in regenerating Hydra

To monitor the autophagy flux during regeneration, we either left ep-LC3A animals untreated or treated them for 16 h with MG132 to sensitize the tissue, or with a combination of MG132 and Baf-A1 to evidence a potential blockade of the autophagy flux. Then we bisection the animals at mid-gastric position and imaged the halves every 12 h, focusing on the region adjacent to the bisection zone (Fig. 5A, B). During the regeneration time-course we noted in untreated animals at 24 h post-amputation (hpa) numerous cells fluorescing red in the zone adjacent to the bisection plane, reflecting a potential increase in the autophagy flux (Fig. 5B, red arrowheads). This increase, similar in apical and basal regenerating halves, was not noted at earlier or later time-points. The same effect was observed in MG132-treated animals although temporarily and spatially enhanced, maximal at 24 hpa but also detected at 13, 37 and 48 hpa. The MG132 drug delivered continuously on amputated animals appears more efficient than on intact ones, indicating that the cuticle that covers the epidermis in intact Hv_AEP2 animals is an obstacle to the diffusion of the drug across the epidermal layers. In ep-LC3A animals exposed to MG132 and Baf-A1 the red fluorescence was spatially and temporarily reduced when compared to that observed in MG132-treated animals, similar to that observed in untreated animals (Fig. 5B). Incidentally, untreated animals showed the emergence of tentacle rudiments at 37 hpa (Fig. 5B, white arrowheads), signing a successful apical regeneration process, whereas tentacle rudiments were not observed in MG132 and MG132/Baf-A1-treated animals. This analysis suggests that the autophagy flux is up-regulated during regeneration, seemingly reaching a maximal level 24 h after bisection.

We also analyzed the autophagy flux in ga-LC3A transgenic animals bisected at mid-gastric position. The Quant-IM quantification of green fluorescence in ga-LC3A cells from the basal-regenerating tips (region R1), apical-regenerating tips (region R2) as well as in the sub-jacent body column region (region R3) dissected one, 24 and 48 h after bissection.
Fig. 3. Analysis of autophagosome formation in live Hydra expressing the tandem sensor mCherry-GFP-LC3A.

(A) Structure of the mCherry-GFP-LC3A autophagy tandem sensor designed to discriminate between early autophagosomes (green), mature autophagosomes (yellow) and autolysosomes (red). HyActin: promoter of the Hydra actin gene (1345 bp); LC3A: Hydra LC3A coding sequence (Tomczyk et al., 2020). Two days after electroporation (EP), the mCherry-GFP-LC3A sensor is expressed cells in several epidermal epithelial cells of intact Hv_Basel1 polyps pictured at the spinning disk confocal microscope. At low magnification, cells expressing the reporter construct appear mainly yellow; at higher magnification, green or yellow vacuoles (autophagosomes) as well as red ones (autolysosomes, right panel) are visible.

(B) Quantification of the green/yellow and red puncta detected in the cell shown in A. After one-hour exposure to the proteasome inhibitor MG132 (10 μm), most autophagosomes have transformed to autolysosomes, reflecting the progression through the autophagic flux.

(C) Immunodetection of mCherry (red) and eGFP (green) proteins in animals electroporated two days earlier with the mCherry-GFP-LC3A sensor. Animals were pictured with a wide-field Leica DM5500 microscope. Note that all positive cells equally express the two proteins (arrows).

(D) Number of cells expressing the mCherry-GFP-LC3A sensor two days after electroporation in 12 Hv_Basel1 and 11 Hm-105 animals. (E) Live imaging with a spinning disk confocal microscope of an epithelial cell expressing the mCherry-GFP-LC3A sensor stained with lysotracker (far red, shown in white, purple in the merge view). Scale bar: 50 μm.

(F) Hv_Basel1 animals expressing the mCherry-eGFP-LC3A sensor for 2 days were treated with Baf-A1 (100 nM) together with MG132 (1 μm) for 18 h prior to live imaging. Five distinct cells were pictured in live animals with the spinning disk confocal microscope, showing the formation of autolysosomes (red arrowheads) after MG132 treatment and the persistence of the yellow/green vacuoles (yellow arrowheads) after the combined MG132 and Baf-A1 treatment. Scale bar: 25 μm.

(G) Quantification with Imaris software of the autophagic vacuoles detected in the cells shown in F. In panels D and G, mean values are represented with error bars that correspond to SEM values. The p values were determined with the unpaired t-test.
Fig. 4. Bafilomycin-induced arrest of autophagy in transgenic Hv_AEP2 animals expressing the DsRed-GFP-LC3A protein.

(A) Transgenic (Tg) Hv_AEP2 animals constitutively expressing the DsRED-GFP-LC3A chimeric protein either in the epidermal epithelial layer (ep-LC3A) or in the gastrodermal epithelial layer (ga-LC3A) pictured live with a wide-field Leica DM5500 microscope. DsRed: Discosoma red fluorescent protein. Scale bar: 500 μm.

(B) Western analysis detecting the DsRED-GFP-LC3A protein in transgenic animals (MW = 69 kDa).

(C) Enlarged views of the body column of ep-LC3A transgenic animals treated or not with Bafilomycin-A1 (100 nM) for 60 h before dissection of the body column and immediate imaging done with a spinning disk confocal microscope. Note that the DsRED-GFP-LC3A positive cells fluoresce green in novel autophagosomes where DsRed is not fluorescent yet, yellow/orange when mature autophagosomes are fluorescent for both GFP and DsRed, or red in autolysosomes where GFP fluorescence is quenched.

(D) Schematic view of the Quant-IM procedure to quantify the green/yellow or red fluoroses in 300–500 cells after trypsin tissue dissociation. Imaging + data analysis:
1. Quantification (Imaris)
2. Data tidying (dplyr/R)
3. Data visualization (ggplot2/R)

(E) Green and red fluoroses detected in single ga-LC3A cells obtained from animals exposed or not to Baf-A1 as in panel C, trypsin dissociated and immediately pictured with a spinning disk confocal microscope (63x). Scale bar: 20 μm.

(F) Quant-IM measurement of green fluorescence in ga-LC3A cells from animals exposed to DMSO or Bafilomycin-A1 as in panel C. Median and individual values are represented with box plots and the p values were determined with the unpaired t-test with the Welch correction. **** p < 0.0001; ns: not significant.

(G) Quantification of green fluorescence in apical, central and basal tissues of ga-LC3A transgenic animals represented as in F.
bisection detects similar levels in these three regions with no obvious temporal regulation (Figs. 5C, S7B). This lack of modulations does not fit the results observed microscopically; one possible explanation might be the thickness of the tissue slices (about 250 μm) used for the quantification. We anticipate that the cells located at the wound edge, which show a higher number of autolysosomes, are diluted with cells where the autophagy flux is not modulated.

Fig. 5. Pharmacological inhibition of the autophagy flux prevents apical regeneration.

(A) Schematic representation of the experimental procedure. Regularly fed transgenic ep-LC3A animals were exposed for 16 h prior to mid-gastric bisection and continuously after bisection to DMSO (0.05 %), MG132 (1 μM), or MG132 (1 μM) and bafilomycin A1 (Baf-A1, 100 nM). (B) Animal halves undergoing basal and apical regeneration (BR, AR) were imaged every 12 h after bisection with a spinning disk confocal microscope. The amputation plane is indicated with a dashed line, the wound with a yellow dashed circle. Note the predominance of red-fluorescent cells (red arrowheads) in the area adjacent to the bisection plane. White arrowheads indicate the emergence of the tentacle rudiments. Scale bar: 100 μm. (C) Quantification of the green fluorescence of ga-LC3A cells obtained after trypsinisation of three regions, each about 250 μm thick, dissected from regenerating animals as depicted on the left. Each region was submitted to the Quant-IM procedure at indicated timepoints after mid-gastric bisection. The median values obtained in three independant experiments are shown and the individual values are depicted in box plots in supplemental Figure S9B. (D, E) Impact on apical regeneration of the pharmacological blockade of the autophagy flux in Hv_Basel1. In D, one-day starved animals were exposed to Baf-A1 at indicated concentrations for one day, then bisected and left in the drug up to 24 hours post-amputation (hpa). In E, one-day starved animals were exposed to SBI-0206965 at indicated concentrations for three days, then bisected and left in the drug up to 24 hpa. In D and E, animals were imaged at 72 hpa with an Olympus stereo microscope. In panel E, mean values from three independent experiments are represented with error bars corresponding to SEM values. Scale bars: 500 μm.
3.6. Pharmacological inhibition of ULK1 and lysosome fusion prevents apical regeneration

We tested and compared in Hv_Basel1 animals, which are generally more sensitive to drugs, the effect on apical regeneration of SBI-0206965 and Baf-A1, two drugs that affect autophagosome and autolysosome formation respectively (Fig. 5D, 5E). Baf-A1 was provided at three distinct concentrations to animals exposed for one day before amputation and during the early phase of regeneration up to 24 hours post-amputation (hpa) (Fig. 5D). We detected a clear inhibition of apical regeneration when Baf-A1 was delivered at 200 nM, with 85% animals lacking apical structures after six days. When given at 100 nM, we noted a milder effect with a 20% reduction in apical regeneration six days after amputation. Next, we tested the impact of ULK1 inhibition induced by SBI-0206965 on apical regeneration (Fig. 5E). When given 7 μM, 95% of the animals had not regenerated their head after four days, and we noted a 50% inhibition when the drug was given 5 μM. Both drugs show a dose-dependent inhibition of apical regeneration, with an almost complete blockade of regeneration at the highest concentration, indicating that a functional autophagy flux is necessary for an efficient apical regeneration.

3.7. Impact of inhibiting ULK1 activity in homeostatic H.vulgaris

Next, we wanted to test the effects of a prolonged inhibition of ULK1 activity on intact animals. We first compared the survival rate of cohorts of Ho_CS and Hv_sf-1 animals continuously exposed to low concentrations of the ULK1 inhibitor SBI-0206965 (0.5 or 1 μM) (Fig. 5E). When kept starved at 18 °C, we noted the expected 40-day difference in survival period between Ho_CS and Hv_sf1 animals but measured no effect of the ULK1 inhibitor on the survival of the Ho_CS and Hv_sf-1 cohorts (Fig. S8A). We also tested the survival rate of Ho_CS animals maintained at 10 °C and exposed or not to SBI-0206965 alone, rapamycin alone or both drugs combined (Fig. SBB). As expected, treatment with rapamycin prolonged survival, but the addition of SBI-0206965 did not modify the observed survival. We also treated Ho_CS and Ho_CR animals maintained at 18 °C with SBI-0206965, bisected at mid-gastric position after four days of starvation. The treatment was given for three days before bisection and then continuously. As expected, we observed a slower and less efficient regeneration process in Ho_CS versus Ho_CR animals but no delay or blockade of apical regeneration in animals treated with SBI-0206965 (Fig. SBC). These negative results suggested that the SBI-0206965 treatment at low concentration does not efficiently inhibit ULK1 kinase activity in Ho_CS, Ho_CR or Hv_sf1 animals. However, SBI-0206965 given at higher concentrations was toxic for Ho_CS animals.

Next, we designed an approach where SBI-0206965 was given at higher concentrations (3 and 6 μM) for repeated 40 h pulse treatments separated by a 55 h drug-free period (Fig. 6A-C). Our previous analysis of aging in Hydrad had shown that in parallel to the loss of regeneration, a decrease in contractility occurs rapidly after induction of aging in Ho_CS as well as a decrease in body size (Tomczyk et al., 2019; Tomczyk et al., 2020). To follow the decrease in body length, we selected Hv_Basel1 animals and treated them as described above over a three-week period, i. e. a series of six 40 h treatments of SBI-0206965, each treatment followed by a drug-free period during which the animals are pictured to measure their size and their spontaneous contractions recorded on video for 20 min. Note that the animals were left unfed during this 23-day period. As expected, we observed a progressive decrease in animal length, significantly enhanced in SBI-0206965 treated animals already after three treatments when given at 6 μM, after five treatments when given at 3 μM (Fig. 6B). By contrast, the number of spontaneous contractions was similar between animals exposed six times to SBI-0206965 or not, with three contractions per hour as median values at day-23. As animal size is maintained in Hydra by the replacement of the various cell types through the continuous self-renewal of epithelial and interstitial stem cells, these results suggest that a prolonged inhibition of ULK1 activity negatively impacts the self-renewal of stem cells, as observed in aging Ho_CS animals.

3.8. Impact of knocking-down ULK1 expression in homeostatic H.vulgaris

We also tested the impact of knocking-down ULK1 expression by RNA interference (Fig. 6D-6I). Hv_AEP2 animals were electroporated (EP) five times and analyzed two days later. When the mCherry-GFP-LC3A sensor was added to the fifth EP (Fig. 6D), the autophagosome formation proved to be efficient in mCherry-GFP-LC3A+ cells from scramble(RNAi) animals but not in the mCherry-GFP-LC3A- cells from ULK1(RNAi) animals (Fig. 6D). Also, we noted a decrease in the size of animals exposed to ULK1 siRNAs by about 30% (Fig. 6E). We tested the efficiency of each siRNA sequence in Hv_AEP2 animals and found siRNA-A quite inefficient while siRNA-B and siRNA-C tested independently lead each to a 55% decrease in ULK1 expression after three EPs (Fig. S9). Similarly, ULK1(RNAi) experiments performed in Hv_Basel1 animals electroporated three times with a mixture of siRNA-B and siRNA-C led to a 60% reduction of expression (Fig. 6F).

We tested the impact of ULK1(RNAi) on animal size in Hv_Basel1 animals and did not record a significant decrease (Fig. 6G). This result suggests that five electroporations are necessary to produce a sustained ULK1 knock-down that leads to animal size reduction. By contrast, we recorded a clear decrease in the number of spontaneous contractions, from 12 per hour in control animals to three per hour as median values in ULK1(RNAi) Hv_Basel1 animals (Fig. 6H). We noted that the contractility is reduced in animals electroporated three times when compared to intact animals that were not submitted to any electric shock (compare with values shown in Fig. 6C, top panel). Finally, we also tested the impact of ULK1(RNAi) knock-down on apical regeneration, in Hv_AEP2 as well as Hv_Basel1 animals but never obtained a significant effect (not shown). This lack of impact is likely due to the too transient ULK1 knock-down obtained after three EPs. After five EPs, animals become too small to undergo apical regeneration in healthy conditions. These results show that a transient and partial but significant silencing of ULK1 is sufficient to impact both the size and the spontaneous contractile activity of Hv animals.

4. Discussion

The aging phenotype in Ho_CS animals reported by Tomczyk et al. (2020) share features with mammalian aging, like loss of somatic stem cells, deterioration of the muscular network, alterations of the nervous system, loss of active behaviors such as feeding, morphological changes, global loss of health. Cellular investigations have shown that this phenotype resulted from a deficient autophagy, a phenotype that could be mimicked in H. vulgaris animals by silencing WIP2, a gene involved in autophagosome formation (Dooley et al., 2014). However, the molecular mechanism(s) responsible for the observed deficient autophagy in Ho_CS animals remained unidentified. The aim of the present study was to highlight the potential molecular actor involved in autophagy deficiency. The obtained results point to the regulation of ULK1 activity, which is repressed in Ho_CS animals and does induce signs of aging in H. vulgaris when inhibited pharmacologically or genetically. It is important to note that the strains used in this study exhibit some variations in terms of size, drug sensitivity and sexual versus asexual differentiation (Table 1): for example, animals from the Hv_AEP2 strain are less sensitive to drug treatments than Hv_Basel1 ones, whereas RNAi efficiency obtained via electroporation of siRNAs targeted against ULK1 is similar. Also, so far only the Hv_AEP2 strain that readily produces gametes, can be used to produce transgenic lines.

4.1. The regulation of ULK1 kinase in the context of autophagy deficiency in aging Hydra

By using in vitro kinase assays to study the regulation of the ULK1
4.2. The autophagy tandem sensor, expressed either transiently or constitutively in live Hydra, provides us with a valuable tool to monitor the autophagy flux

In this study, we used the mCherry-eGFP-LC3 tandem sensor to measure the autophagy flux in live animals. The strength of this tool is its ability to discriminate autophagic vesicles based on the GFP protein, whose fluorescence is extinguished in an acidic environment such as this occurs in the autolysosome. However, the eGFP (Enhanced Green Fluorescent Protein, used here) has been shown to retain some weak fluorescence at pH 4–5, a pH close to that of the autolysosome. This may lead to misinterpretation of the data, as some autolysosomes might be confused with autophagosomes. Some new synthetic monomeric green fluorescent proteins have a higher sensitivity to acidic pH and improved versions of tandem sensors for autophagy have been produced, such as mTagRFP-mWasabi-LC3 (Zhou et al., 2012) or pHluorin-mKate2-LC3 (Tanida et al., 2014). These new versions of the autophagy tandem sensor might improve the discrimination between autophagosomes and autolysosomes in live Hydra exposed to environmental changes or undergoing developmental processes.

We also used transgenic animals that constitutively express the autophagy tandem sensor DsRED-eGFP-LC3A to follow modulations of the autophagy flux during Hydra regeneration. The main advantage of using transgenic animals, even chimeric, is the large number of cells expressing the tandem sensor and the possibility to qualitatively and quantitatively monitor the autophagy flux during developmental processes or in response to environmental changes over long periods of time. This type of monitoring is temporally limited in electroporated animals for two reasons, firstly because the migration of the electroporated cells towards the extremities takes place within few days, and secondly because the expression of the sensor fades out as the plasmidic DNA gets diluted with each mitotic event. As a consequence, the expression of the tandem sensor can only be observed transiently, over two weeks at most.

Transgenic animals can be submitted to a quantitative assay (QuantIM) designed to quantify in a large number of live cells obtained after a...
fast dissociation of transgenic tissues, the status of the autophagy flux as deduced from the respective percentages of autophagosomes and autolysosomes. An increase in the percentage of autolysosomes is interpreted as a shorter autophagosomal period and thus an acceleration of the autophagy flux. In the conditions tested here, a significant modulation could be observed after Baf-A1 treatment.

4.3. A double-edge role for autophagy in regenerative processes

Compared to aging studies, the impact of autophagy on regenerative processes remains much less characterized (García-Prat et al., 2016; Tettamanti et al., 2019). Here we show that the autophagy flux is activated in the epidermal layer of regenerating tips, either apical or basal, reaching a maximum level one day after bisection. The fact that H. vulgaris animals knocked-down for WIPI2, lose their ability to regenerate, indicates that autophagy plays a positive necessary role on Hydra regeneration (Tomczyk et al., 2020). We confirm this finding here by inhibiting apical regeneration in HcBasel animals with the ULK1 inhibitor SBI-0206965 that prevents autophagosome formation, as well as with Baf-A1 that prevents the fusion of autophagosomes with lysosomes to form autolysosomes.

However, previous findings indicate that the levels of autophagy need to be tightly controlled: indeed when autophagy is chronically activated in Hydra, homeostasis gets progressively altered and a massive cell death is observed immediately after amputation, leading to the rapid death of the animals (Chera et al., 2006). This cytotoxic effect of massive autophagy was revealed when Kazal1, a gene coding for a serine protease inhibitor, was silenced leading to an excessive self-digestion phenotype noted in glandular and digestive epithelial cells. A similar protease inhibitor, was silenced leading to an excessive self-digestion mass, massive autophagy was revealed when autophagy flux. In the conditions tested here, a significant modulation of autophagy through LC3-GFP autophagy sensor, autophagy is induced two days after caudal zonation and a double-edge role for autophagy in regenerative processes could be observed after Baf-A1 treatment. 

5. Contributors

NS performed the regeneration and in vivo imaging experiments, DC contributed to the biochemical and molecular analyses, CP produced the mCherry-eGFP-LC3A transgenic line, NS prepared the figures, NS and BG wrote and revised the manuscript, BG supervised the study.

Declaration of Competing Interest

All authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.mad.2020.111414.

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