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METHODS TO INVESTIGATE AUTOPHAGY DURING STARVATION AND REGENERATION IN HYDRA

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ABSTRACT

In hydra, the regulation of the balance between cell death and cell survival is essential to maintain homeostasis across the animal and promote animal survival during starvation. Moreover, this balance also appears to play a key role during regeneration of the apical "head" region. The recent finding that autophagy is a crucial component of this balance, strengthens the value of the *Hydra* model system to analyze the implications of autophagy in starvation, stress response and regeneration. We describe here how we adapted to *Hydra* some established tools to monitor steady-state autophagy. The ATG8/LC3 marker used in biochemical and immunohistochemical analyses showed a significant increase in autophagosome formation in digestive cells after 11 days of starvation. Moreover, the maceration procedure that keeps intact the morphology of the various cell types, allows the quantification of the autophagosomes and autolysosomes in any cell type, thanks to the detection of the MitoFluor or LysoTracker dyes combined with the anti-LC3, anti-LBPA and/or anti-RSK (ribosomal S6 kinase) immunostaining. The classical activator (rapamycin) and inhibitors (wortmannin, bafilomycin A_1) of autophagy also appear to be valuable tools to modulate autophagy in hydra, as daily-fed and starved hydra display slightly different responses. Finally, we show that the genetic circuitry underlying autophagy can be qualitatively and quantitatively tested through RNA interference in hydra repeatedly exposed to double stranded RNAs.

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THE VALUE OF THE HYDRA MODEL SYSTEM FOR INVESTIGATING AUTOPHAGY

Hydra is a freshwater polyp, which belongs to Cnidaria (Fig. 1A), a phylum positioned as a sister group to the bilaterians, having separated early during animal evolution. Despite their apparent simplicity, cnidarian animals possess a sophisticated neuro-muscular system and display an amazing potential for regeneration (Galliot and Schmid, 2002; Holstein et al., 2003; Bosch, 2007). Since the genomic era, cnidarian species actually currently emerge as new model systems to investigate fundamental biological questions in cell biology, developmental biology and evolution (Technau et al., 2005; Kamm et al., 2006; Seipel and Schmid, 2006; Momose and Houliston, 2007). Hydra polyps are characterized by a radial symmetry with an oral-aboral polarity along their unique body axis; they display a tube shape with a mouth opening at the top, surrounded by a ring of tentacles, whereas the basal disk allows the animal to attach to substrates (Fig. 1B). The body wall comprises two layers, ectodermal and endodermal, which all together contain about a dozen cell types (Lentz, 1966), which derive from three distinct stem cell populations, ectodermal myoepithelial, endodermal myoepithelial and interstitial cells (Dubel et al., 1987; Bode, 1996; Steele, 2002; Galliot et al., 2006). The myoepithelial cells form continuous epithelia and differentiate into head- and foot-specific myoepithelial cells, whereas the interstitial cells provide different nerve cells, mechanoreceptor cells (stinging cells, named nematocytes or cnidocytes), gland cells and gametes when the animals follow the sexual cycle. The spatial distribution of these highly differentiated cells occurs as a consequence of the continuous division of stem cells in the body column and the active migration or displacement of the committed/precursor cells towards the extremities.

Hydra has the amazing ability to regenerate any missing part (foot and head) in several days after amputation, a property identified in the 18th century by Abraham Trembley (Trembley, 1744). In fact whatever the level and the number of amputation along the body axis, each piece of tissue, except tentacles, has the potential to reform a new hydra. This potential that is dissected at the cellular and molecular levels since decades, was shown to rely on signaling pathways that are evolutionarily conserved (Galliot and Schmid, 2002; Steele, 2002; Holstein et al., 2003; Galliot et al., 2006; Miljkovic-Licina et al., 2007) although less conserved peptides probably also play a role (Fujisawa, 2003; Bosch, 2007). Moreover hydra can reproduce asexually through budding (Otto and Campbell, 1977) and, after dissociation, is able to re-aggregate and rebuild the initial shape (Gierer et al., 1972). This unique developmental plasticity among multi-cellular organisms has made hydra one of the classical model organisms to investigate the molecular and cellular basis of regeneration. In homeostatic conditions the hydra polyp can survive an extended period without any feeding, up to several weeks. During this time, the starving animals shrink from the adult size to a half size (Fig. 1B), while the cell cycle length of epithelial cells lengthen (Bosch and David, 1984). However an imbalance between the tissue growth rate and cell cycle length was observed: The overproduced cells actually die and are phagocytosed by the neighboring cells, providing hence a nutrient source for the surviving animals (Bosch and David, 1984). This is in contrast with the heavy feeding condition when hydra cells from all three stem cell compartments continuously divide and the growing rate of the tissues tightly follows the cell cycling rate. Although a systematic study concerning macroautophagy in hydra and autophagy-mediated cell death has not been made yet, this evidence suggests that survival of starving animals relies on the latter. In any case the respective roles of autophagic cell death and apoptosis remain to be established in the future.

In fact, a first analysis of autophagy in hydra has been recently reported in animals where *Kazal1*, an evolutionarily-conserved gene encoding a serine protein-kinase inhibitor Kazal-type (SPINK) was knocked down upon RNA interference (Chera et al., 2006). This study shows the formation of autophagosomes in both gland cells and digestive cells (endodermal myoepithelial cells) in intact polyps where *Kazal1* transcripts are no longer detected. This autophagy process immediately affects the homeostatic condition as evidenced by the decreased budding rate. In *Kazal1*(RNAi) hydra, the autophagic cells progressively shrink, their number drastically decrease and the animals die. During regeneration, the expression of *Kazal1* is dramatically enhanced in regenerating tips. *Kazal1* upregulation immediately

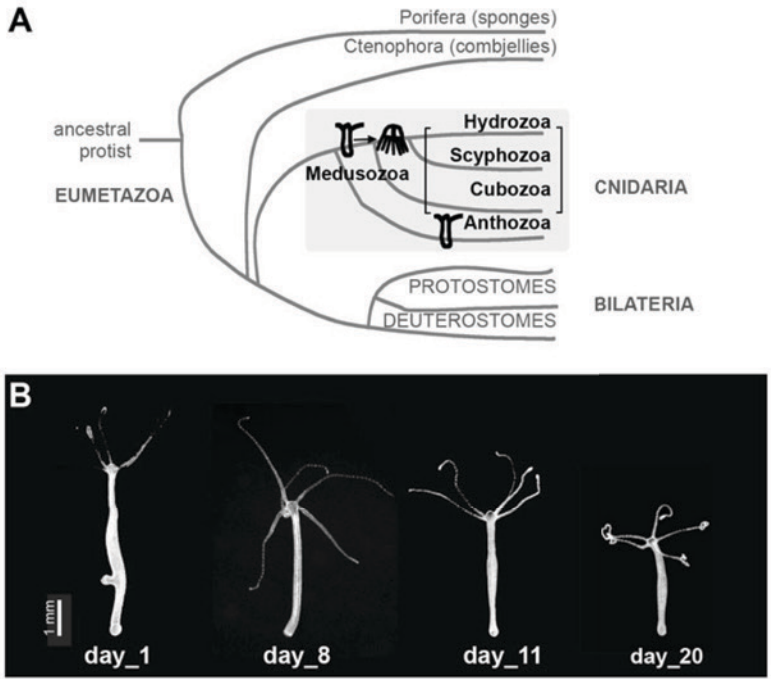

Figure 1: A) Position of hydra (Cnidaria, hydrozoan) in the metazoan tree. B) Dramatic reduction in hydra size upon 20 days starvation. The animals were kept relaxed at the indicated time points and pictured using a stereomicroscope. Bar: 1 mm.

after amputation appears to be essential to prevent excessive autophagy, as hydra knocked-down for *Kazal1* display massive autophagy in the regenerating tips as early as one hour after amputation and, following repeated exposures to *Kazal1* double stranded RNAs (dsRNAs), survive only a few hours the amputation.

This study indicates that the autophagy process in hydra is strictly controlled in both homeostatic and regenerative contexts, as a long-standing increase in autophagy prevents asexual reproduction (budding) and slowly leads to the death of intact animals, whereas the absence of regulation of the amputation-induced autophagy in the regenerative context results in rapid animal death. Moreover, we propose that the cytoprotective role played by *Kazal1* immediately after amputation actually allows the activation of the regenerative process (Galliot, 2006). Therefore, systematic cellular and molecular studies that would dissect the regulative pathways and the function of autophagy in these two contexts are required. One question would be to learn how similar are the autophagy processes in the starvation and regenerative contexts. We anticipate that such studies will no doubt identify some evolutionarily conserved mechanisms that regulate autophagy in most metazoan species when they starve. In addition, such studies could also provide the starting point for comparing the regulation of stress-induced autophagy in regenerating and non-regenerating species. Given the growing importance of autophagy in pathological and homeostatic conditions, the need to define valuable comparative tools to measure and study autophagy in different model systems is necessary. Here we present and discuss the respective values of some morphological, immunochemical and biochemical methods, which allow the monitoring of autophagy in hydra. We show that specific autophagy markers as well as pharmacological agents that are widely used in other model systems indeed provide reliable tools in hydra. Moreover the RNA interference (RNAi) method developed in our laboratory is well suited for dissecting in quantitative terms the genetic circuitry underlying the regulation and function of autophagy in hydra.

EXPERIMENTAL PARADIGMS TO FOLLOW AUTOPHAGY IN HYDRA

1.- Biochemical detection of autophagy in hydra

LC3-II as a marker to monitor starvation-induced autophagy in hydra

Although the number of methods to monitor autophagy is limited, one should consider the fact that some methods monitor the steady-state level of autophagy whereas others measure the autophagic flux (Klionsky et al., 2008). In this report we will consider exclusively the former ones. One of the most widely used biochemical method to characterize autophagy in steady-state conditions is the detection of the LC3/Atg8 protein. LC3 is synthesized as a precursor, proLC3, which is converted into LC3-I by proteolysis and then modified by lipidation to the phosphatidylethanolamine-conjugated form, LC3-II. Once lipidated, LC3-II is anchored to the phagophore and autophagosomes membranes. It is well accepted that the amount of LC3-II detected in western analysis,

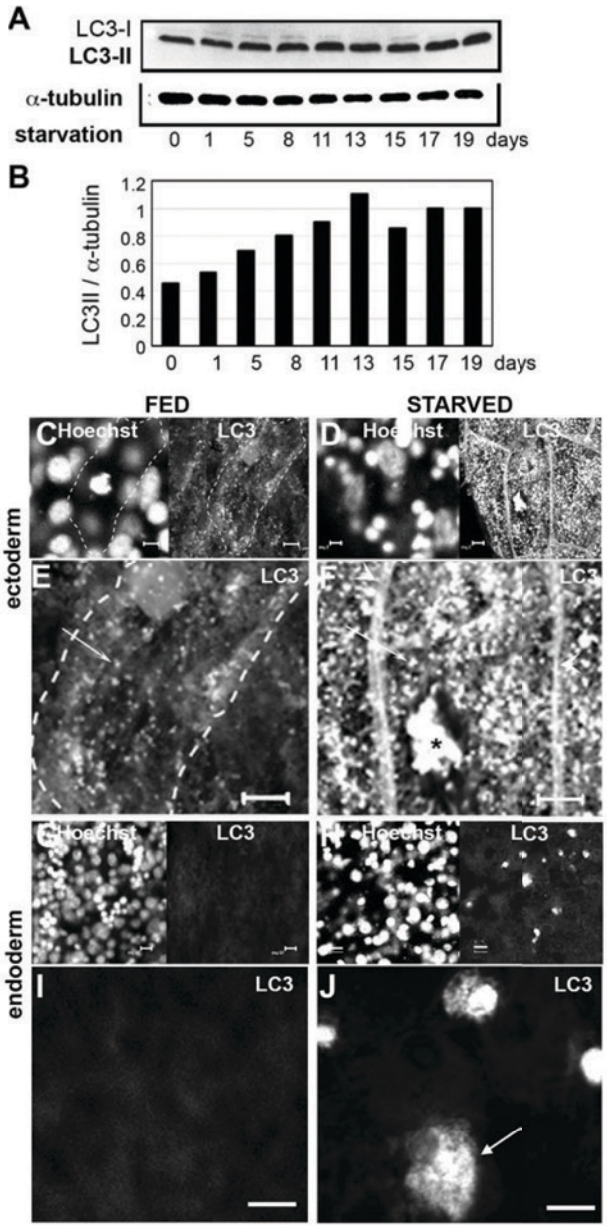

Figure 2: Increase in LC3-II expression levels during starvation. A, B) Western blot analysis showing the progressive increase in LC3-II level during the first two weeks of starvation (the LC3-I band is faintly seen above the LC3-II band). During the third week, a plateau level was reached assuming that the maximal rate of LC3-II turnover was attained. A) The same blot was detected first with anti-LC3 antibody and then with anti-α-tubulin as described in Protocol 1. B) For each time point, the LC3-II and α-tubulin bands are quantified with the ImageJ software and the LC3-II amount normalized against α-tubulin is represented (arbitrary units). C-J) LC3 immunostaining of the ectodermal (C-F) and endodermal (G-J) layers of daily-fed (C, E, G, I) and starved (D, F, H, J) polyps as described in Protocol 2. C-F) Note the weak LC3 staining in the ectodermal epithelial cells of daily-fed polyps (C, E, dashed line) contrasting with the strong LC3 signal detected in the same cells of starved animals (D, F, arrow), particularly in cell membranes (arrowhead) and abundant small cytoplasmic vacuoles (arrow). The asterisk marks an artifact. G-J) LC3 staining is completely absent in the endodermal layer of daily-fed hydra (G, I) but in starved polyps (H, J) reveals the presence of large LC3-specific vacuoles (J, arrow). Scale C, D, G, H: 10 µm; E, F, I, J: 5 µm.

correlates with the number of autophagosomes (Kabeya et al., 2000).

In starving and daily-fed hydra, endogenous LC3 is detected as two bands in western blot analysis: LC3-I at a molecular mass around 17 kD and LC3-II at approximately 14 kD (Fig. 2A). The level of LC3-I is very weak compared with the LC3-II isoform, which is not surprising as the selected antibody more easily detects LC3-II than LC3-I (Settembre et al., 2008). Moreover LC3-I is more labile, as it is more sensitive to the extraction condition than LC3-II; finally hydra tissues might contain a limited amount of LC3-I protein. Therefore, the amount of LC3-II is normalized against α-tubulin and the samples are compared based on the relative index of LC3-II amount. In starved animals, a progressive increase in LC3-II level can be observed. After 13 days of starvation, the LC3-II value exceeds a two-fold increase when compared to the LC3-II value measured in daily-fed hydra (Fig. 2B). At later time-points, a plateau value is recorded. As LC3-II relative index is considered as an indicator of autophagosome formation, this indicates that the autophagic process reaches its maximal rate of turnover within two weeks of starvation.

Hydra culture and starvation conditions:

1. Culture about 500 hydra (*H. vulgaris,* Basel strain) in 2 liters square dishes (20 cm long, Roth) covered by 1 liter of hydra medium (HM: 1 mM NaCl, 1mM $CaCl_2$, 0.1 mM KCl, 0.1 mM $MgSO_4$, 1 mM Tris pH 7.6) at 18°C.
2. Feed animals every day between 9 and 10 a.m. with freshly hatched nauplii (larvae) of *Artemia salina*.
3. 6 to 8 hours after feeding, transfer the animals in a 2 liter becher and wash them in three successive 800 ml glass bechers containing each 200 ml HM. Use large glass pipettes for transferring the animals from one becher to the other, before plating them in a clean HM pre-filled culture dish. Under these conditions the cultures have a doubling time of 3.5 days (David and Campbell, 1972).
4. For starvation experiments, select 100 large budless hydra per condition the day following the last feeding to provide a homogeneous animal population and plate 50 hydra per 10 cm diameter plastic dish filled with 50 ml HM (1 ml HM /hydra).
5. Let them starve for the indicated periods of time (2, 5, 8, 11, 13, 15, 17 and 19 days). During this period, wash the animals every other day and carefully remove any debris or dead animals. Discard the culture in case of yeast or bacterial infection.
6. Stop animal culture on the same day for all conditions to prepare the extracts.

Protocol 1: Whole cell protein extract and LC3-II detection in Western blot

1. Wash 100 hydra per condition in three successive 800 ml glass bechers containing each 200 ml HM as above. Finally transfer hydra to 2 ml eppendorf tubes and briefly wash them in 2 ml pre-cooled lysis buffer (LB) containing 50 mM HEPES, 150 mM NaCl, 2.5 mM $MgCl_2$, 0.5 mM DTT, 10% glycerol, 1% Triton x100, 25 mM NaF, 20 mM β-glycerophosphat, 1mM Na_3VO_4, 0.1 mg/ml PMSF and the complete protease inhibitor cocktail (Roche Applied Science).
2. Remove the last wash and resuspend the animals in 200 µl LB, homogenize on ice through a 2.5 gauge needle 20-30 times and centrifuge at 14,000 g for 10 minutes. Collect the supernatant fractions, aliquot them (25 µl), freeze in liquid nitrogen and store at -80°C.
3. Measure the protein content on duplicates (1 µl each) with the Bradford method by reading the optical densities of the samples at 595 nm. Extrapolate the protein concentrations from a Bovine Serum Albumin (BSA) calibration curve read at the same time.
4. Mix 17 µg of each extract with 2x loading buffer (62.5 mM Tris, pH 6.8, 4% SDS, 20% glycerol, 0.1% bromophenol blue, 200 mM β-mercaptoethanol), boil for 3 minutes, load onto a 15% denaturing SDS-PAGE gel and migrate 1 hour at 200 V.
5. Transfer the proteins onto a Hybond PVDF membrane (GE-Amersham) in a wet transfer system unit (Biorad) for 30 minutes at 25 V.
6. After transfer, block the membrane for 3 hours at room temperature (RT) in PBS, 0.2% Tween 20 (TBS) containing 5% non-fat milk.
7. Incubate the membrane overnight (ON) at 4°C with a rabbit polyclonal anti-LC3 antibody 1:1,000 (Novus Biological, NB910-40752).
8. Wash the membrane 4 times in TBS, 5% nonfat-milk at RT.
9. Incubate for 1 hour at RT with horseradish peroxidase (HRP)-conjugated anti-rabbit 1:10,000 (Promega W401B).
10. Wash 4 times in TBS, 5% nonfat-milk, and 2 times in TBS at RT.
11. Detect the peroxidase activity using the enhanced chemiluminescent (ECL) blotting reagent (GE-Amersham) on hyperfilm ECL (Amersham Bioscience).
12. For α-tubulin detection as a loading control, strip the membrane in 62 mM Tris, 0.2% Tween 20, 200 mM β-mercaptoethanol for 30 minutes in 3 steps at RT and reprobe with the α-tubulin 1:5,000 (Sigma, T5168) antibody as above. Detect with an HRP-coupled anti-mouse antibody 1:3,000 (Biorad, 170-6516).

Scan the films and perform a densitometric analysis with ImageJ software (http://rsb.info.nih.gov/ij/). Quantify the area of LC3 and α-tubulin bands and normalize against α-tubulin. The results can be expressed either as the relative index of LC3-II amount normalized against α-tubulin (Fig. 2) or as percentage from the fed control value (Fig. 4).

2. Analysis of autophagy in hydra tissues

Autophagosome identification on whole mount hydra:

The LC3 immunostaining performed on whole mount polyps shows clear differences between regularly fed (3 days starvation) and starved polyps (24 days starvation) observed in both ectodermal and endodermal layers (Fig. 2C-2J). In the ectodermal layer of regularly fed polyps the LC3 staining is almost

absent, with weakly LC3-positive small granules present in the cytoplasm of the epithelial cells (Fig. 2C, zoom in 2E, arrow). In contrast, in starved animals, the ectodermal LC3 immunostaining is very intense (Fig. 2D, zoom in 2F), strongly labeling membranes (Fig. 2F arrowhead) and cytoplasmic vacuoles (Fig. 2F, arrow) of the myoepithelial cells. Some of these cells also display conglomerates of LC3 granules observed in the vicinity of the nucleus (Fig. 2F, asterisk). However, the most striking differences in LC3 staining are observed in the endodermal layer of starved and daily-fed polyps. In the endodermal cells of regularly fed polyps we do not record any LC3 staining (Fig. 2G right, 2I), whereas in starved polyps we note large conglomerates that correspond~~ed~~ to vacuoles (Fig. 2H right, 2J arrow). Furthermore, the endoderm of starving polyps appears thinner, rippled and disrupted.

Protocol 2: LC3 immunostaining on whole mount hydra

1. Relax the 1 to 30 polyps for 90 seconds in 1 ml 2% urethane/water and fix them ON at 4°C in 500 µl 4% formadehyde /50% ethanol.
2. Wash out the fixative with 4x 10 minutes washes in PBS and subsequently block the endogenous peroxidase activity by incubating the animals in 500 µl 3% H_2O_2 for 30 minutes at RT.
3. Next, block the samples with 500 µl 2% BSA/PBS and incubate with the rabbit polyclonal anti-LC3 antibody (Novus Biological, NB910-40752, 1:200) ON at 4°C.
4. Wash out the first antibody with 4x10 minutes washes in PBS and incubate the hydra for 4 hours at RT with the anti-rabbit HRP 1:100 from the Tyramide Amplification Kit (Molecular Probes, T-30954).

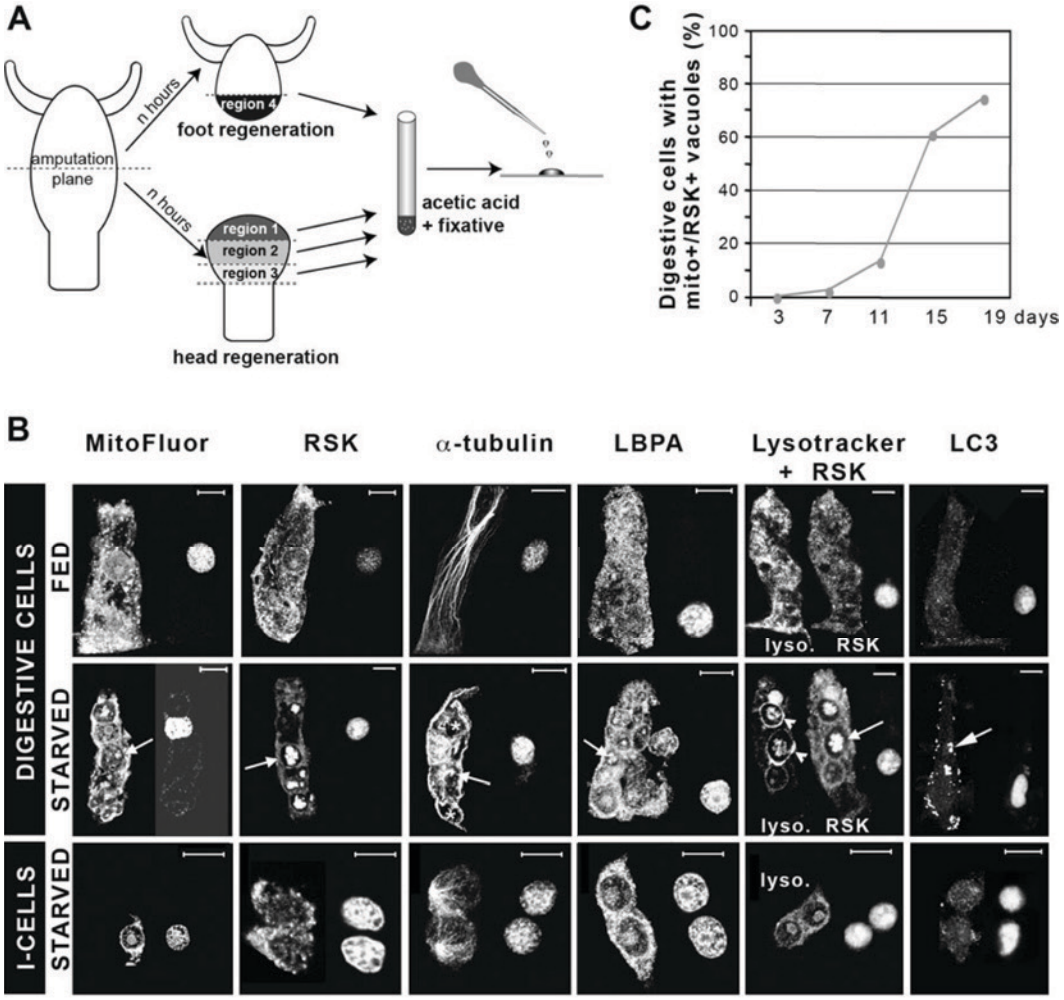

Figure 3. Maceration procedure as a tool to monitor autophagy in hydra cells. A) Scheme depicting the maceration procedure in hydra. Intact or regenerating polyps are incubated with acetic acid and glycerol followed by fixation with PFA as described in Protocol 4. For monitoring autophagy during regeneration, hydra are bisected and left to regenerate for the desired amount of time (n hours); the entire regenerative half or the regions of interest are then collected and macerated. B) Detection of autophagic vacuoles in cells from daily-fed or 14 days starved animals with MitoFluor, anti-RSK, anti α-tubulin, anti-LBPA, LysoTracker (lyso.) combined to anti-RSK (RSK), and anti-LC3 as described in Protocol 5. For each cell, the Hoechst staining is shown on the right. Autophagic vacuoles are missing in the digestive cells of fed animals (upper row) as well as in the interstitial cells (i-cells) of starved animals (lower row) but are numerous in the digestive cells of starved hydra (arrows). Note the disturbed α-tubulin network of these cells (third column asterisk) and the strong LysoTracker staining at the margins of the RSK-positive vacuoles (arrowheads). Furthermore note the large LC3 precipitates in the cytoplasm of starved cells (6th column, arrow). Scale bars: 10 µm. C) Graph showing the dramatic increase during starvation in the proportion of digestive cells containing large vacuoles whose content was labeled with both MitoFluor and anti-RSK antibody (mito+/RSK+). Approximately 600 digestive cells were counted for each condition. Scale bars: 200 µm (A); 8 µm (B).

5. Wash the samples 4x10 minutes in PBS and incubate them with tyramide as described in the Tyramide Amplification Kit protocol (Molecular Probes, T-30954) for 16-18 minutes at RT.
6. Immediately wash the hydra 2x10 minutes in PBS, counterstain with 500 µl Hoechst 33342 1 µg/ml in H_2O (Molecular Probes, H3570) and block for 1 hour in H_2O_2 3% at RT.
7. Wash out the blocking reagent twice in PBS, once in water, and mount the samples on untreated glass slides in 50 µl Mowiol 4-88 (Sigma 81381).
8. Picture and scan at the confocal microscope (SP2 AOBS Leica) at the proper wavelength depending on the type of fluorochrome linked to tyramide.

Protocol 3: LysoTracker Red (LTR) staining in fixed whole mounts

LysoTracker Red is a vital fluorescent acidotropic dye that accumulates in acidic vacuoles including functional lysosomes and autolysosomes. However LTR is not specific to the autophagic process as it also labels endosomes and phagosomes by virtue of their low internal pH. Therefore it necessary to combine this approach with a specific autophagic marker to trace with high fidelity the autophagic process (Bampton et al., 2005). As a first approach to detect autophagic vacuoles in hydra cells, we tested LTR staining in three distinct conditions: on fixed whole mount animals (Protocol 3), on macerated tissues (Protocol 5) combined with immunostaining (Fig. 3) and on live hydra (Protocol 7) in the presence or absence of rapamycin (Fig. 5). Concerning the first approach, the large LTR+ vacuoles remain stained after fixation with aldehydes and LTR can thus be used on animals stained live and immediately fixed. However, we do not find the results highly informative (not shown) when compared either to anti-LC3 immunostaining (Fig. 2) or to LTR live staining with live recording (Fig. 5).

1. Incubate in the dark 10 polyps for 15 minutes in 2.5 µM LysoTracker Red DND-99 (Molecular Probes, L 7528) diluted in 500 µl HM at RT.
2. Wash out the dye several times with HM; relax the animals in 2% urethane for 90 seconds and fix them in 4% PFA diluted in HM for 15 minutes at 4°C.
3. Wash out the fixative in 3x 5 minutes PBS washes at RT.
4. Stain the nuclei for 10 minutes in 0.1 µg/ml Hoechst 33342 diluted in PBS; wash several times in PBS, once in water and mount the animals with Mowiol 4-88 on untreated slides.
5. Record the LTR fluorescence with a Leica SP2 AOBS confocal microscope under a 40x oil objective lens. Excitation at 543 nm is provided by an argon/krypton laser.

3. Analysis of autophagy in hydra cells

The maceration procedure

Another approach used to monitor autophagy in steady state conditions in hydra is based on the immunocytochemical detection of markers for autophagosome formation on macerated tissues. In fact, the maceration technique developed previously (David, 1973) allows the identification and quantification of each different cell type as upon maceration with acid acetic and glycerol, and subsequently fixation with paraformaldehyde (PFA), the hydra tissues are completely dissociated into individual cells or small clusters, which retain their *in vivo* morphology. Given the fact that only small amounts of tissues are required, specific regions along the axis of the animals can be isolated and macerated as for example the different regions of the regenerating halves (Fig. 3A) (Chera et al., unpublished). Moreover this maceration procedure can be combined with dye staining or immunostaining. That way the specific hallmarks of autophagy can be identified and followed in the different cell types undergoing autophagy.

Gelatin coated slides protocol

Prepare the gelatin solution (in water):

1. Prepare a 0.5% gelatin and 0.1% chrom alum solution in water and mix on the magnetic agitator using a magnetic bar.
2. Microwave for 30 sec and mix gently the mixture to avoid bubbles, repeat the procedure until the solution is clear (3 to 5 min).
3. Filter the solution through filter paper. This gelatin solution can be reused up to one month.

Preparing the slides:

1. Insert the slides in the Universal slide racks and place the racks inside clean black boxes (Roth, T214.1).
2. Wash the slides one time in ethanol 100% for 1 minute.
3. Dry the slides and boxes for 30 min using ventilation.
4. Poor the 40°C gelatin solution in the boxes.
5. Insert the slide racks and incubate them for 2 minutes with the gelatin solution.
6. Extract the racks from boxes and dry them using ventilation.
7. Store the slides in closed boxes at RT for several weeks.

Protocol 4: Maceration technique (after David, 1973)

1. Wash hydra 3x in HM as described in Protocol 1, transfer 10 animals into tubes and incubate them at RT in 100 µl 7% glycerol, 7% acetic acid, H_2O. From time to time, vortex the samples mildly until the polyps dissociate and no visible tissue fragments are observed in the tube (30 – 40 min).
2. Fix the cell suspension by adding an equal volume (100 µl) of 8% PFA. Mix and let them stand in the fixative for 30 minutes at RT.
3. Add 20 µl of 10% Tween 80 to the cell suspension and spread 50 µl over an area of about 2 cm^2 (2 x 1) on freshly prepared gelatin-coated slides. Let the cells air-dry on the bench at least 40 hours at RT.
4. Once dried, either proceed immediately for immunostaining or store the slides at -20°C for long periods.

Dye staining and immunostaining of macerated tissues

Vacuoles that contain degraded organelles are characterized as autophagosomes or autolysosomes thanks to the detection of autophagosomal and

lysosomal markers, whose presence indicates the induction or maintenance of an autophagic process (Klionsky et al., 2008). To identify reliable markers of autophagy in hydra, we detect in macerates prepared from both starved and fed hydra two organelle dyes, LysoTracker Red DN99, which accumulates in the acidic compartment of the living cells, and MitoFluor Red589 (Molecular Probes), which labels mitochondria and membranes in both live and fixed cells. Moreover we test several antibodies targeted against the Atg8/LC3 protein (Novus Biological NB100-2220) (Settembre et al., 2008), 6C4-lysobisphosphatidic acid (anti-LBPA, a generous gift from Jean Grünberg) (Kobayashi et al., 1998), ribosomal S6 kinase (anti-RSK, BD Transduction Laboratory, R23820) (Chera et al., 2007) and anti-α-tubulin (Sigma T5168).

As criteria to quantify the induction of the autophagic process during starvation, we quantify the number of digestive cells that display large vacuoles containing MitoFluor-positive organelles and RSK-positive conglomerates as depicted in Fig. 3B (1^{st} and 2^{nd} columns, arrows) at 3, 7, 11, 15 and 19 days of starvation (Fig. 3C). Whereas the number of cells containing such type of vacuoles is very low in regularly fed animals (Fig. 3B, upper row), starvation induces a dramatic increase in their number, markedly between 11 and 15 days of starvation, when 60% of the cells appear affected (Fig. 3C). This result confirms that autophagy massively occurs after 11 days of starvation as noted in the western analysis of LC3-II (Fig. 2A, 2B). Beside digestive cells, autophagy is also detected in myoepithelial cells from the ectodermal layer (not shown) but not in derivatives from the interstitial cell lineage (Fig. 3B, lower row and not shown).

In addition to these two markers, the anti-α-tubulin staining reveals the strong disorganization of the microtubule filaments following prolonged starvation (Fig. 3B third column, asterisk). Moreover, the late-endosomal marker LBPA shows a pattern similar to that obtained with MitoFluor in starved cells (Fig. 3B fourth column, arrows) labeling endosomal structures located within large vacuoles. The LysoTracker staining provides a sharp staining of the periphery of these large vacuoles (Fig. 3B, fifth column, arrowheads), implying that those vacuoles correspond to autolysosomes. When the cells are co-stained with LysoTracker and the RSK antibody, we note that the content of these LysoTracker-positive vacuoles is also intensely RSK-positive (Fig. 3B, fifth column, arrow) indicating that RSK is indeed a reliable marker for detecting the advanced stages of autophagy. Furthermore, the anti-LC3 staining detects numerous sizeable LC3 precipitates in the cytoplasm of the epithelial cells of starved polyps (Fig 3B, last column, arrow). These are absent or reduced in size in the cells of regularly fed polyps, confirming that anti-LC3 is a rather specific marker of autophagy in hydra cells, to be used either alone or in combination with other markers (not shown).

Protocol 5: Dye labeling and immunostaining on macerated samples:

Note that LysoTracker Red DND-99, used on live animals, and MitoFluor 589, used on fixed cells, emit with similar wavelengths and cannot be combined.

1. Transfer 10 polyps per condition in 24 wells dish and incubate them in 2.5 µM LysoTracker Red (Molecular Probes, L 7528) diluted in 500 µl HM for 15 minutes at RT.
2. Wash the animals 4 times in 1 ml HM, and macerate as indicated in Protocol 4.
3. Once cells are dried on slides, wash them 2x 10 minutes in 1 ml PBS, block in 1 ml PBS, 2% BSA for 1 hour at RT.
4. Incubate ON at 4°C with the primary antibodies: anti-RSK 1:1,000 (Transduction Laboratories, cat 610226), anti-LC3 1:200 (Novus Biological, NB910-40752), anti α-tubulin 1:2,000 (Sigma, T5168) and anti 6C4-phospholipd LBPA (1:50) (Kobayashi et al., 1998).
5. Wash the slides several times in PBS, incubate them for 2 hours at RT in the secondary antibody coupled with AlexaFluor dyes (AlexaFluor 555 or AlexaFluor 488) 1:600.
6. Wash the slides in PBS, stain them in Hoechst 33342 diluted in water 1 µg/ml (Molecular Probes) for 5 minutes, wash 2x 10 minutes in PBS, 1x 2 minutes in water and mount with 30 µl Mowiol.
7. When required, stain with the dye MitoFluor 589, 200 nM diluted either in water or in PBS (Molecular Probes) for 20 minutes before Hoechst staining.

4. Pharmacological modulations of autophagy in hydra

Autophagic degradation is a multi-step process, which is regulated by a number of pathways. As a consequence, the autophagy process can be modulated by pharmacological agents that act at various stages. The inhibitors wortmannin, 3-methyladenine and LY294002 act at the sequestration step (Petiot et al., 2000) whereas most other inhibitors impair the formation and function of autolysosomes by inhibiting lysosomal enzymes or vesicle fusion, or by elevating lysosomal pH (Klionsky et al., 2008). The main limitation in the use of these pharmacological agents is their low specificity; in most cases they are not exclusively targeted to autophagy, implying that the non-autophagic effects should be carefully considered. As a first approach we test several of these modulators on intact hydra that are either daily-fed or maintained starved. For each context, we follow the drug-induced morphological modifications (Fig. 4A) and detect the level of LC3-II by western analysis (Fig. 4B). In the case of rapamycin, we also detect the formation of acidic vesicles in live hydra stained with LTR (Fig. 5).

Rapamycin, a candidate activator of autophagy in daily-fed hydra

The TOR (Target of Rapamycin) protein negatively controls autophagy in yeast and mammals. It is a serine/threonine kinase, which belongs to the PI3K-related family and exerts a central role in nutrient-sensing signal transduction, regulation of translation and cell-cycle progression (Blommaart et al., 1995; Noda and Ohsumi, 1998; Kamada et al., 2000; Klionsky et al., 2005). The lipophilic macrolide antibiotic rapamycin induces autophagy by forming a complex with the immunophilin FK506-binding protein 12 (FKBP12), which then binds to and inactivates mTOR, leading to a derepression of autophagy. To verify whether rapamycin affects the level of autophagy in hydra, starved and daily-fed animals are exposed for 12 hours to different concentrations of rapamycin (0.1 µM, 1 µM and 10 µM). Surprisingly, we observe in both

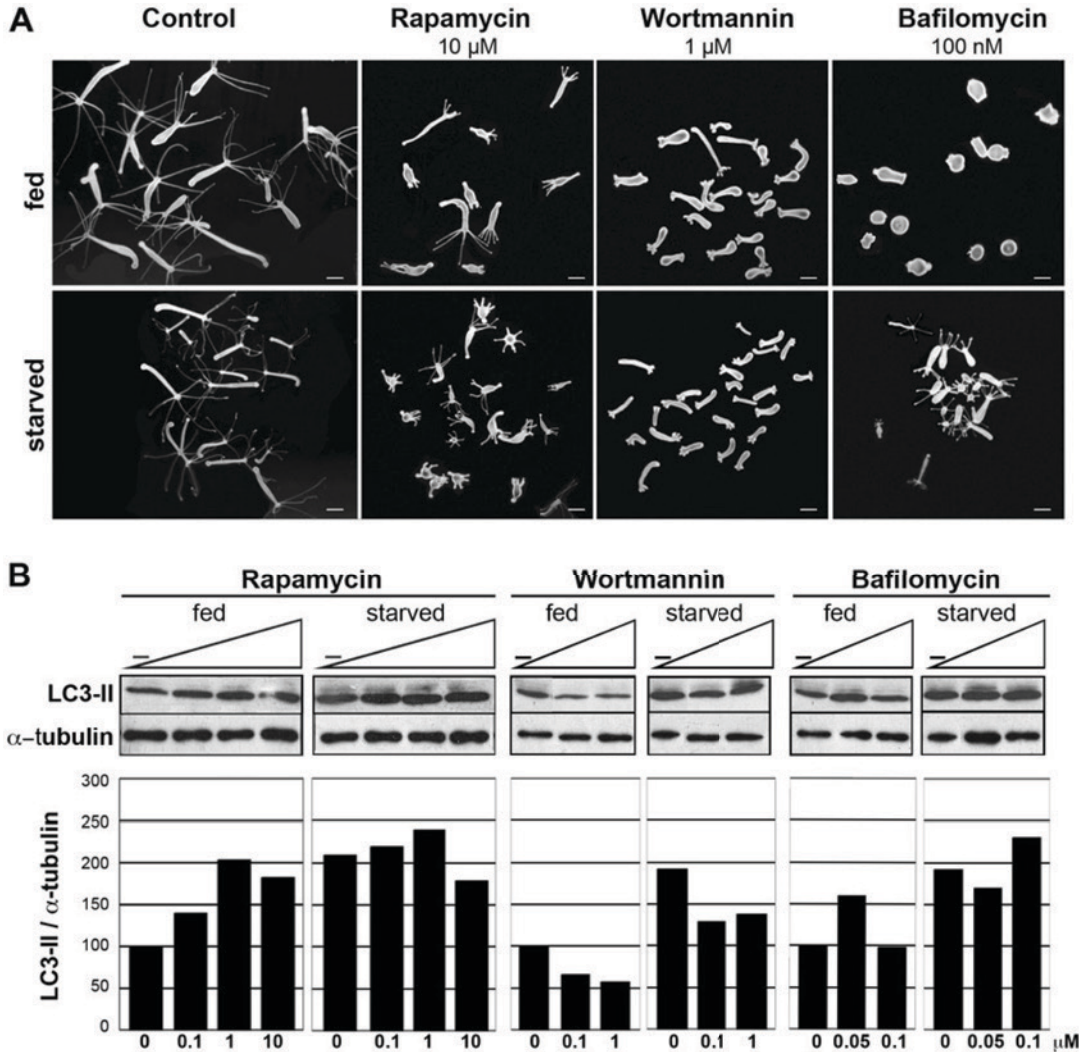

Figure 4: Drug-induced modulations of morphology and autophagy in daily-fed and starved hydra. A) Morphological changes induced in hydra either daily-fed (upper row) or starved for 19 days (lower row) after 12 hours exposure to rapamycin (10 μM), wortmannin (1 μM) or bafilomycin A_1 (100 nM) as described in Protocol 6. Note the constriction of the lower body column in rapamycin-treated hydra, the toxic effect of wortmannin and the resistance of starved hydra to the toxic effect of bafilomycin A_1; these are reduced in size but display a normal shape with full tentacles. Scale bar: 1 mm. B) Western blot analysis of the LC3-II content in daily-fed and 19 days starved hydra exposed to rapamycin (0.1, 1 and 10 μM), wortmannin (0.1 and 1 μM) and bafilomycin (50 and 100 nM) as in A (the LC3-I band is faintly seen above the LC3-II band). The relative index of LC3-II amount was normalized against α-tubulin by densitometric analysis with ImageJ software and is expressed as percentage from the fed control value.

starved and daily-fed hydra, a rather unusual morphological change, as the polyps become strongly constricted in the lower part of the body column (Fig. 4A, 2nd column). This change is actually even more obvious in starved hydra. In both contexts, hydra are reduced in size but are in good shape, with intact tentacles, reacting to mechanical stimulus by contracting as do untreated hydra. Concerning the LC3-II amount (Fig. 4B), fed and starved hydra show a gradual increase up to 1 μM, indicating an increase in the number of autophagosomes, although with a lower increment in starved than in fed polyps. When levels of rapamycin increase to 10 μM, the LC3-II amount decreases in both fed and starved animals. This last result was not expected and requires further investigation to determine whether the level of autophagy is indeed reduced in this condition. In fact, the LC3-II content is not a definitive indicator of the total autophagic flux and further experiments where exposure to rapamycin is performed in the presence of lysosomal inhibitors such as pepstatin A, E64d and leupeptin, should provide more information about the LC3 turnover rate. In addition side effects of high rapamycin concentration on mTOR signaling cannot be excluded, as rapamycin is able to repress protein translation, arrest the cell cycle and alter cell size. Moreover, upon chronic exposure to rapamycin, the rapamycin–insensitive TORC2 complex can be destabilized, affecting downstream signaling through Akt1 (Sarbassov et al., 2006).

Wortmannin, a candidate inhibitor of autophagy in daily-fed and starved hydra

The autophagic flux can be inhibited by targeting the sequestration event, which requires phosphatidylinositol 3-kinase (PI3K) activity. Wortmannin, a furanosteroid metabolite of the fungi *Penicillium funiculosum* is a specific PI3K inhibitor that inhibits autophagy at the sequestration step (Arcaro and Wymann, 1993; Blommaart et al., 1997). When hydra, either daily-fed or starved, are treated with wortmannin at 1 μM for 12 hours, they rapidly showdramatic morphological changes with a reduced body size and loss of their tentacles (Fig. 4A, 3rd

column). At lower concentration (0.1 µM), the animals are not affected to the same degree (not shown). In fact, immunoblot analysis shows that the level of the membrane-bound LC3-II is significantly lowered when starved and daily-fed animals are exposed to 0.1 and 1 µM wortmannin (Fig. 4B). Interestingly, the starved and daily-fed animals are inhibited in the same manner by wortmannin, at the morphological and biochemical levels, implying a similar sensitivity to this pharmacological agent.

Bafilomycin A_1, a candidate inhibitor of autophagy in daily-fed and starved hydra:

Bafilomycin A_1, a macrolide antibiotic isolated from Streptomyces sp. is an extremely potent inhibitor of the vacuolar type H^+-ATPases (Bowman et al., 1988). Bafilomycin A_1 plays an important role in maintaining the acidic environment of the endosomes, lysosomes, and other secretory vesicles. At low concentrations (in the nanomolar range), bafilomycin A_1 increases the pH of acidic vesicles by disrupting the proton gradient and may prevent the fusion of autophagosomes with lysosomes. As a consequence, an accumulation of the autophagosomes can be observed (Yamamoto et al., 1998). However other data indicate that Bafilomycin blocks the activity of lysosomal hydrolases but does not affect fusion (Fass et al., 2006).

In contrast to wortmannin, daily-fed and starved hydra display different sensitivities to bafilomycin A_1. In both cases, exposure to 200 nm bafilomycin is rapidly lethal whereas exposure to 50 nM does not alter the animal size and shape (not shown). However exposure to 100 nM bafilomycin is clearly more toxic for the daily-fed hydra than for the starved ones, the former becoming dramatically scrubby, with short and buttoned tentacles (Fig. 4A, compare the upper and lower panels in the 4th column). At the biochemical level, the level of LC3-II remaind unchanged or decreased (other experiment) in daily-fed hydra exposed to 100 nM bafilomycin that were actually dying, but increased in daily-fed hydra exposed to 50 nM bafilomycin (Fig. 4B). In starved animals, a significant increase in LC3-II level is noticed only at 100 nM. These results suggest that bafilomycin can inhibit autophagy in hydra although at different concentrations in daily fed and starved hydra, the former ones being more sensitive thn the latter.

Protocol 6: Hydra treatment with Rapamycin, Wortmannin and Bafilomycin

1. Dissolve rapamycin, wortmannin and bafilomycin (LC Laboratories) in DMSO to obtain 10 mM (rapamycin, wortmannin) and 1 mM (bafilomycin) stock solutions. Aliquot and store at -20°C. Freshly dilute the stock solutions in HM prior any hydra treatment.
2. Dilute in 10 ml HM each drug, rapamycin (0.1, 1, 10 µM), wortmannin (0.1, 1, 10 µM), bafilomycin (50, 100, 200 nM) and 0.1% DMSO for the control condition. Fill 9 ml of each solution in the wells of 6 well dish plate
3. Wash 30 hydra per condition in HM and transfer them into 1.5 ml tubes; aspirate HM, add 1 ml of the diluted drug solution.
4. Dispatch the hydra of each condition in the corresponding wells of the 6 well dish plate. Keep the dish in the dark for 12 hours.
5. After treatment, wash hydra several times in HM, picture them live using a stereomicroscope (Leica MZ16FA) and prepare total protein extracts as in Protocol 1.

Lysotracker Red staining in live animals

To assess autophagy-induced modulations of the acidic compartment (including all the above mentioned vacuoles), starved and daily-fed hydra are loaded with LysoTracker Red and examined with a stereomicroscope after 12 hours exposure to 10 µM rapamycin (Fig. 5). In daily-fed animals, only a few number of highly fluorescent LTR spots are observed along the body axis, which corresponds to cells with an increased lysosomal-derived activity (Fig. 5A). The red intense fluorescence detected in the tentacles is attributed to nematocytes (not shown). In starved animals, the number of LTR-positive vacuoles increases along the body column (Fig. 5B). When hydra are exposed to rapamycin, numerous LTR bright spots are observed spread all over the body, in the ectoderm as well as in the endoderm of starved and daily-fed hydra (Fig. 5C, 5D). Therefore, rapamycin exposure significantly increases the number of acidic vacuoles when compared to non-treated animals, but this increase is more pronounced in the daily-fed than in the starved animals. This result is consistant with the level of LC3-II detected in rapamycin-treated hydra after western analysis.

Protocol 7: LysoTracker Red staining in live animals

1. Treat 10 hydra, either daily-fed or 21 days starved, with rapamycin (10 µM) or 0.1% DMSO alone for

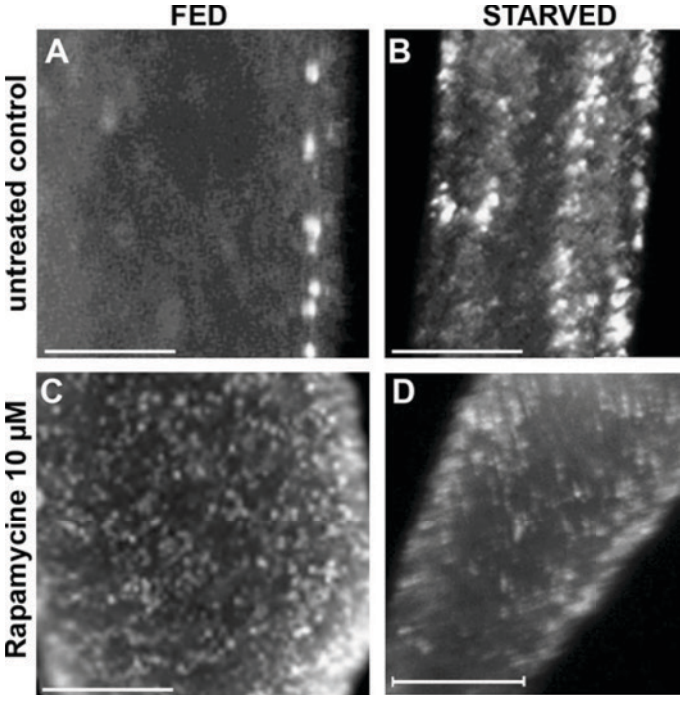

Figure 5: LysoTracker detection in live hydra. Live intact hydra either daily-fed (A, C) or starved for 24 days (B, D) were stained with LTR as described in Protocol 7. After 12 hours rapamycin treatment, both daily-fed (C) and starved (D) hydra showed numerous bright LTR spots spread all over the body. This increase in LTR signal was more evident in daily-fed hydra than in the starved condition. Bars: 200 µm (A, B); 400 µm (C, D).

12 hours as described in Protocol 6.

2. Incubate them for 15 minutes in 1 µM Lysotracker Red diluted in 500 µl HM.
3. Wash out the dye several times in HM.
4. To picture live hydra, keep them relaxed in 0.5% urethane diluted in HM under a fluorescence stereomicroscope (Leica MZ16FA equipped with a DFC300 FX Leica camera). The red fluorescence is measured with a Texas Red filter.

5. Functional identification of autophagy regulators

RNA interference obtained through hydra dsRNAs feeding is a potent functional tool

The dsRNA-mediated genetic interference approach has been successfully applied to the hydra model system, opening the possibility to study the signaling pathways and the genetic circuitry regulating homeostasis, stress response and head regeneration through loss-of-function assays (Galliot et al., 2006; Galliot et al., 2007). Among the different RNAi procedures (injection, soaking, electroporation or feeding), the most suitable for our system is based on the regular feeding of animals with bacteria that produce dsRNAs and are embedded in agarose; these could be administrated to animals over long periods without any toxic effects (Chera et al., 2006). Moreover, we show that gene silencing through RNAi is well adapted for deciphering the epistatic relationships between candidate genes expressed in a given cellular process, thus making it possible to decipher the genetic circuitry underlying its regulation (Miljkovic-Licina et al., 2007).

Knocking-down *Kazal1* expression leads to excessive autophagy in hydra

To decipher the regulation and function of the autophagy process in hydra and especially its potential role during hydra regeneration, we silenced the *Kazal1* gene that encodes a serine protease inhibitor related to the vertebrate SPINK genes. *Kazal1* is expressed along the body column in the gland cells that are located in the endodermal cell layer and secrete the digestive enzymes. *Kazal1* is not expressed in the head and foot regions (Fig. 6A left panel). Recurring feedings with *Kazal1* dsRNA completely abolish *Kazal1* expression in the gland cells of the *Kazal1(RNAi)* animals (Fig. 6A right panel). Surprisingly, at the cellular level these animals exhibit large vacuoles in their gland cells but also in the neighboring digestive cells, the endodermal epithelial cells. These vacuoles contain cytoplasmic organelles (Chera et al., 2006) and RSK+ conglomerates as shown in Fig. 6B, and are thus identified as autophagosomes. The monitoring of digestive cells over a long period of time (19 days) where dsRNAs exposures are repeated (up to 9 feedings) shows a dramatic increase in the number of vacuolated cells in *Kazal1(RNAi)* hydra compared to control polyps maintained under the same starvation conditions (Fig. 6C upper panel). In *Kazal1(RNAi)* hydra, we also note additional morphological alterations of the cells that underwent massive autophagy, such as their detachment from the basal membrane, loss of epithelial polarity, disruption of the microtubule cytoplasmic organization, fusion of the autophagic vacuoles forming giant vacuoles and cell size decrease. All together these changes are interpreted as a progressive process leading to an excessive autophagic degradation and finally to cell death (Chera et al., 2006).

Interestingly, the chronic excessive autophagic event identified in intact *Kazal1(RNAi)* animals is dramatically enhanced one hour after amputation at the regenerating tip of head- but also foot-regenerating animals exposed at least 3 times to dsRNAs (Fig. 6C middle and lower panels). Moreover, after 7x feedings the *Kazal1*(RNAi) hydra become unable to regenerate, suggesting that the protection of the endodermal cells against excessive autophagy is an absolute requirement for regeneration (Chera et al., 2006; Galliot, 2006). However, the physiological role of autophagy during hydra regeneration was not tested in this study and remains unknown. Preliminary studies suggest that, surprisingly, in regularly fed animals autophagy is not required for either head or foot regeneration (Buzgariu, unpublished). Nevertheless it is possible that the time-point chosen for monitoring the amputation-induced autophagy (1 hour post-amputation) was not appropriate, as it might be a very rapid and transient event.

Protocol 8: Production of Kazal1 knocked-down hydra upon RNA interference

The RNAi procedure detailed here was initially described in (Chera et al., 2006):

1. The *Kazal-1* cDNA was inserted in the pPD129.36 (L4440) double T7 (www.addgene.org/pgvec1?f=c&plasmidid=1654&cmd=viewseq) and transformed into HT115(DE3) bacteria. This strain is tetracycline resistant, it lacks RNAse III and can express IPTG-inducible T7 polymerase http://wormbase.org/db/gene/strain?name=HT115(DE3);class=Strain. Such constructs should be verified regularly through sequencing as this vector is unstable and frequently recombines.
2. As negative control for the RNAi feeding procedure, use either pPD129.36 (L4440) (no insert, empty L4440 vector) or pLT61.1 vector (L4440 *unc-22*) www.addgene.org/pgvec1?f=c&identifier=1690&atqx=pLT61.1&cmd=findpl. The pLT61.1 construct produces dsRNAs targeted against the *unc-22* nematode gene. It is considered as a standard control by labs that adapted the RNAi feeding strategy from *C. elegans* (Timmons et al., 2001) to planarians or hydra (Newmark et al., 2003; Chera et al., 2006).
3. Transform into HT115(DE3) competent cells two days before starting the RNAi experiment and plate the transformed bacteria on ampicillin (50 µg/ml) + tetracycline (12.5 µg/ml) agar plates. Do not use glycerol stocks, always retransform.
4. Inoculate one colony in 4 ml starter culture in Terrific Broth (Sambrook and Russell, 2001) containing ampicillin 50 µg/ml, tetracycline 12.5 µg/ml (TBAT) and grow ON (>16 hours) at 37°C in long glass tubes vigorously shaken (250 rpm).
5. For each construct, start duplicated pre-cultures by adding 200 µl of the starter culture to 2 ml TBAT in 14 ml tubes (Falcon, 2059). Grow the pre-culture for 90 min at 37°C to reach OD_{595} = 0.4.
6. Induce with IPTG (400 µM final) and grow for 4 hours at 37°C vigorously shaken. Do not overgrow the culture: dead bacteria do not produce dsRNAs. Do not increase the IPTG concentration as the

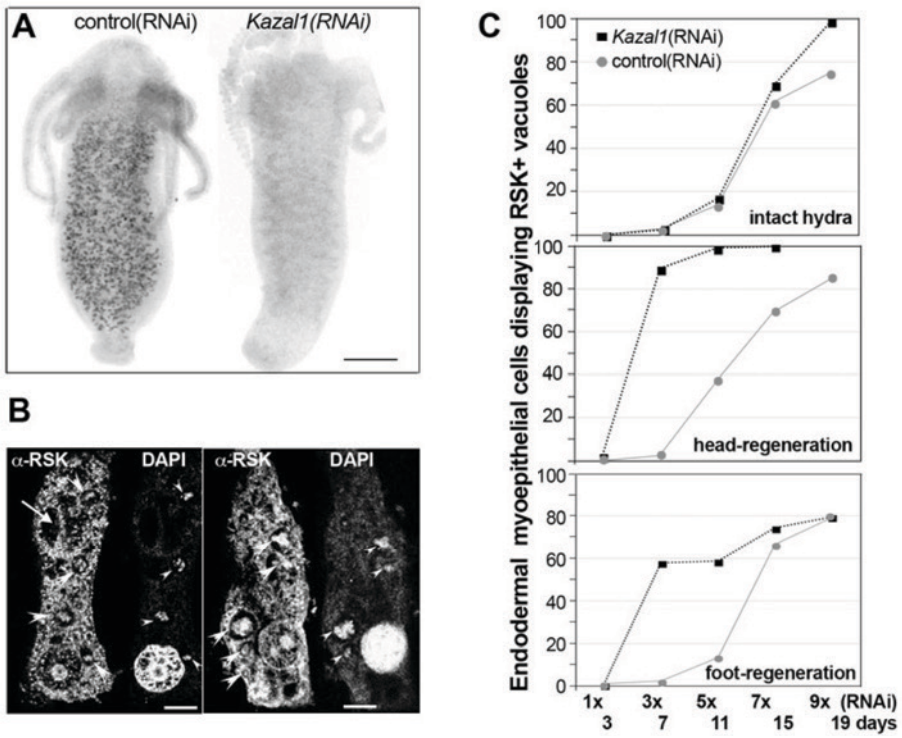

Figure 6: Silencing the hydra Kazal-1 serine-protease inhibitor induced autophagy.
A) *Kazal-1* expression detected in gland cells is knocked down in hydra polyps exposed five times to *Kazal1* dsRNAs (*Kazal1(RNAi)* 5x, right) but normal in hydra exposed five times to control dsRNAs (control(RNAi) 5x, left) as described in Protocol 8. B) Detection of the strongly vacuolated phenotype in the digestive cells from head- (left) and foot- (right) regenerating tips of *Kazal1(RNAi)* 5x hydra. Anti-RSK immunostaining and DAPI staining were performed on tips macerated one hour after amputation. Note the large intracellular spaces (arrows) that characterize the digestive cells and the presence of numerous RSK+ vacuoles identified as autophagosomes (large arrowheads). Small arrowheads indicate artifactual DAPI staining. C) Graphs depicting the gradual increase in the proportion of the digestive cells displaying RSK+ vacuoles in intact (upper panel), head-regenerating (middle panel) and foot-regenerating (lower panel) hydra. For each context, two hydra populations were compared, the control(RNAi) one, repeatedly exposed to vector dsRNAs (grey curve) that shows the progressive formation of autophagosomes upon starvation, and the *Kazal1*(RNAi) one, repeatedly exposed to *Kazal1* dsRNAs (black curve). None of these populations were fed during the experiment but both received every other day the bacterial-agarose mixture and were amputated on alternate days. Note the dramatic increase in the proportion of cells containing autophagosomes after three exposures to *Kazal1* dsRNAs in regenerating hydra. For each condition 600 digestive

RNAi phenotypes will become hypomorphic, http://genomebiology.com/2000/2/1/RESEARCH/0002/table/T2

7. Melt 500 µl/ sample 1% low melting agarose (LMP Qbiogene) in HM and keep it pre-heated at 37°C. Alternatively, the agarose solution can be stored in 50 ml tubes at 37°C for weeks.
8. Transfer the bacterial culture to 2 ml Eppendorf tubes and spin them at 5,000 rpm for 10 min at 4°C in a pre-cooled centrifuge.
9. For each construct, resuspend the pellet of one tube in 100 µl HM and transfer into a second tube to resuspend that pellet (that way, the bacteria of both tubes are collected in 100 µl final volume).
10. Add 100 µl pre-heated 1% low melting agarose and keep the bacteria-agarose mixture on ice until it gets solidified (almost immediately).
11. Collect 50 to 70 healthy hydra starved for 2 days and transfer them into 1.5 ml tubes. Adjust the volume to 250 µl HM and add 250 µl HM, 10 mM Tris, pH 7.5.
12. Add glutathione to 100 µM final concentration (2 µl of a 25 mM stock), and incubate for 5 min. Glutathione efficacy can possibly be checked with a stereomicroscope before grinding the agar; if efficient, the hydra polyps have a widely open mouth.
13. Transfer the bacteria-agarose solidified mix onto the outside of an artemia filter (Hobby Dohse) and grind it with the help of a glass slide. Collect the mixture on the inside of the filter with the edge of a glass slide, and serve it to the animals for 1.5 hours.
14. Gently shake the tubes every 10 – 15 minutes as hydra tend to clump on top of each other, decreasing their capability to eat the dsRNA-producing bacteria.
15. After 90 minutes, extensively wash the hydra with HM until the agarose chunks are removed, usually 10x, first in tubes, then in dishes. Repeat the washing the next day.
16. The day following each feeding, record animal survival, animal shape, animal size and budding rate (budding rate over a week is usually quite informative).
17. Repeat RNAi feeding every other day.
18. Gene knock-down is best proven by RT-PCR or in situ hybridization performed after several dsRNAs exposures as described previously (Miljkovic-Licina et al., 2007).

19. To monitor autophagy in *Kazal1*(RNAi) regenerating hydra, perform mid-gastric section the day following each dsRNA exposure, and one hour after amputation, isolate with a scalpel the different regions to be macerated as depicted in Fig. 3A.
20. Detect the autophagy phenotype by macerating either one intact hydra or ten regenerating tips and stain the cells with dye and anti-RSK.

STAINING	Macerates	Whole-mount	Western	References
Mitochondrial dye **MitoFluor Red 589 (Molecular Probes)**	1. Label fixed cells for 20 minutes; sequestration in autophagosomes	2. Label fixed tissues for 10 minutes; sequestration in autophagosomes	-	1. Chera et al. 2006 2. unpublished
Lysosome dye **LysoTracker Red (LTR, Molecular Probes)**	Label live hydra for 15 min prior to maceration; strongly stains outlines of autolysosomes	Label live hydra for 15 minutes; Starvation and rapamycin patterns	-	This work
Anti-LC3 antibody **(Novus laboratory)**	Autophagosomes in myoepithelial cells	Starvation-induced pattern	LC3-I (+/-) LC3-II (++)	This work
6C4 anti-LBPA antibody **(Gruenberg's lab)**	Late endosomes; sequestered in autophagosomes	nd	nd	Kobayashi et al 1998; Chera et al. 2006
Anti-RSK antibody **(Transduction Laboratoires)**	1) autophagosomes 2) autolysosomes	Starvation-specific pattern?	not tested during starvation	Chera et al. 2006
Anti-α-tubulin antibody **(Sigma)**	1) Disruption of the tubulin network 2) α-tubulin inside the autophagosomes	Not of interest for autophagy	Not of interest for autophagy	Chera et al. 2006 This work
Anti-RSK antibody + LTR	autolysosomes	nd	-	This work

Table 1: Tools to monitor autophagy in hydra

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

In this work we detail several methods to investigate the autophagy process in daily-fed and starved hydra at three distinct levels: biochemical, tissue and cellular as summarized in Table 1. Among the different dyes and antibodies tested here, we show that the conventional anti-LC3 antibody used in other model systems is indeed fully appropriate to detect and measure autophagy in hydra, in immunostainings performed either on western blots (Fig. 2, Fig. 4) or on whole mounts (Fig. 2) or on macerated tissues (Fig. 3). The analysis of autophagic vacuoles on macerated tissues actually appears very well suited for quantitative analyses, more precise and more reliable than dye staining or immunostainings performed on whole mount animals, which are nevertheless advantageous to provide information about regional differences in the regulation of autophagy (Fig. 2B). The cellular analysis of macerated tissues also appears far more sensitive than western analysis. As an example, a maximal two fold increase in LC3-II is detected over the first 13 days of starvation on western blot, reaching then a plateau value (Fig. 2A), whereas the increase in the proportion of digestive cells containing MitoFluor+/RSK+ vacuoles is actually dramatic after 11 days of starvation, starting from 0% at day 3, reaching 10% at day 11, 60% at day 15 and almost 80% at day 19 (Fig. 3B). Moreover, the combination of the anti-RSK antibody with the LysoTracker Red dye also provides a very useful tool to identify autolysosomes and distinguish them from autophagosomes. When macerates are stained with LTR and RSK, 88% of the vacuoles display LTR+ outlines and 78% also contain RSK+ conglomerates as shown in Fig. 3C. We assume that those LTR+ and LTR+/RSK+ vacuoles correspond to autolysosomes, whereas the large vacuoles that are RSK+ only (about 8%) correspond to autophagosomes.

One potential problem in the monitoring of autophagy in the digestive cells (endodermal epithelial cells) of regularly fed animals is the presence of digestive vacuoles. These are large vacuoles (1 µm) in intimate contact with the cell membrane, which usually contain fine granules or filaments (Lentz, 1966). As dyes or antibodies easily penetrate into the digestive vacuoles, it is essential that the pattern generated by this nonspecific staining is clearly discriminated. In fluorescence, the digestive vacuoles have no clear outlines and their content is homogenously fuzzy as no specific binding of the antibody or dye takes place. For animals exposed to dsRNAs, the identification of digestive vacuoles is easy as they often contain small chunks of agarose that exhibit a mild auto-fluorescence, making them clearly visible.

A second problem is the presence of apoptotic phagosomes in the cytoplasm of endodermal epithelial cells, as these cells carry out the efferocytosis (phagocytosis of apoptotic cells) of the apoptotic bodies when the surrounding cells die by apoptosis (Chera et al., submitted). The internalized apoptotic bodies have a characteristic spherical shape,

containing a highly condensed chromatin surrounded by a thin layer of cytoplasm that is strongly RSK-positive. The RSK staining is intimately "coating" the condensed chromatin of the apoptotic bodies. In contrast the autophagosomes are characterized by a cleft between the outlines of the vacuole and their content. Moreover, RSK appears strongly positive on conglomerates located inside the autophagosome but never stains the autophagosome membrane. Therefore, with these criteria, the distinction between digestive vacuoles, autophagosomes and apoptotic phagosomes should be quite obvious for the trained microscopist.

The analysis of LC3-II levels after exposure to pharmacological agents shows that rapamycin at moderate concentrations (0.1 µM, 1 µM) induces autophagy more efficiently in daily-fed than in starved hydra, where the level of autophagy is already increased. The decrease in LC3-II levels observed at higher concentration (10 µM) in Western analysis might correspond to a massive autophagy with an extensive degradation of LC3-II bound to autolysosomes as suggested by the significant increase of the acidic compartment in live hydra exposed to same concentration of rapamycin (Fig. 5C, 5D). The concomitant increase in the autophagic flux should be tested in the presence of lysosomal protease inhibitors, which partially inhibit LC3-II degradation. In contrast wortmannin inhibits autophagy similarly in daily-fed and starved hydra when used at 0.1 µM and 1 µM concentrations. Concerning bafilomycin A_1, it is interesting to note that daily-fed hydra appear far less resistant to bafilomycin A_1 toxicity than starved hydra, as if high levels of autophagy are protective in that context. In all cases, autophagy is primarily regulated in the endodermal myoepithelial cells that carry out the digestive function in homeostatic conditions. Nevertheless, we also report here that starvation (Fig. 2C-2F) and rapamycin treatment (Fig. 5) can likely induce autophagy in ectodermal myoepithelial cells.

Given the potential of the hydra model system to highlight the function(s) of autophagy in homeostatic and developmental contexts, there is a clear need for establishing methods that would measure the flux of autophagy. Indeed, as reporter constructs can be expressed in adult polyps through electroporation (Miljkovic et al., 2002), the next step would be therefore to more precisely monitor the regulation of autophagy in hydra by using reporter constructs that prove to be highly efficient in other model systems, such as the chimeric GFP-LC3 construct to monitor cleavage of GFP from LC3, or the use of lysosomal protease inhibitors along with LC3-II turnover (Klionsky et al., 2008).

Beside the regulation of autophagy in the context of homeostasis, there is also the need to investigate more precisely the physiological role of autophagy during regeneration. We previously showed that an excessive autophagy is deleterious for cell survival after amputation (Chera et al., 2006; Galliot, 2006). More recently we showed that a massive wave of apoptosis is taking place in head-regenerating tips soon after amputation and that this apoptotic event is required to trigger the head regeneration program (Chera et al., submitted). Therefore, numerous questions are currently pending: Is autophagy induced at any time of the regeneration process in the wild-type context? Is a low level of autophagy helpful or detrimental for the regeneration process? The expression and functional analysis of the genes that regulate the autophagy process should address these questions and provide useful information for understanding the core mechanisms underlying regenerative processes in the animal kingdom.

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ABBREVIATIONS:

BSA:	bovine serum albumin
dsRNAs:	double stranded RNAs
HM:	hydra medium
LTR:	LysoTracker Red
ON:	overnight
PBS:	phosphate buffered saline
PFA:	paraformaldehyde
PI3K:	phosphatidylinositol 3-kinase
RNAi:	RNA interference
RT:	room temperature
SPINK:	Serine Protease Inhibitor Kazal-type
TBS:	Tween phosphate buffered saline

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