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Molecular Characterization
of Autophagic Responses
Part B

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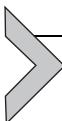
> CHAPTER TWENTY-TWO

Monitoring Autophagic Responses in *Caenorhabditis elegans*

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Contents

Abstract

Autophagy, from the Greek *auto* (self) and *phagy* (eating), is a self-degradative process critical for eukaryotic cell homeostasis. Its rapidly responsive, highly dynamic nature renders this process essential for adapting to and offsetting acute/harsh conditions such as starvation, organelle dysfunction, and deoxyribonucleic acid (DNA) damage. Autophagy involves an intricate network of interacting factors with multiple levels of control. Importantly, dysregulation of autophagy has been linked to numerous debilitating pathologies, including cancer and neurodegenerative conditions in humans. Methods to monitor and quantify autophagic activity reliably are essential both for studying the basic mechanisms of autophagy and for dissecting its involvement in disease. The nematode *Caenorhabditis elegans* is a particularly suitable model organism to effectively

visualize and study autophagy, in vivo, in a physiological and pathological context due to its optical transparency, experimental malleability, and precise developmental and anatomical characterization. Here, we outline the main tools and approaches to monitor and measure autophagic responses in *C. elegans*.
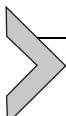
1. INTRODUCTION

Caenorhabditis elegans is a simple invertebrate animal model that offers a particularly versatile platform to dissect fundamental cellular and molecular processes. Technological advances in genetic engineering and optical microscopy, coupled with the unique features of the nematode, including its transparency, unsurpassed developmental and anatomical characterization, and genetic malleability, have facilitated studies of physiological responses in vivo, at both the organismal and cellular level. Macroautophagy, hereafter referred to as *autophagy*, is part of such a physiological response to nutrient deprivation and other stressors. Autophagy entails the formation of a double-membrane-bound vesicle, the autophagosome, which ultimately fuses with the lysosome, a lytic enzyme-containing organelle, where autophagosomal contents are degraded. These include aggregated proteins (general autophagy) or damaged organelles (selective autophagy), which are destroyed via the autophagic pathway, in a highly controlled manner. Perturbed or aberrant autophagy can lead to severe pathology, such as neurodegeneration, cancer, and accelerated aging. The relevant basic mechanisms underlying both physiological and pathophysiological regulation of autophagy in various contexts can be readily studied in *C. elegans*.

While autophagy was initially considered to be a bulk degradation process, it is now clear that several highly selective types of autophagy are important for cell function and survival. Specific proteins and protein modifications mediate cargo recognition by the core autophagic machinery. For example, appropriate receptors/adaptors direct selective autophagy toward regulatory proteins or organelles such as mitochondria that are targeted for degradation. In vivo imaging and monitoring the levels and subcellular localization of autophagy components under both normal and stress conditions are essential for elucidating the involvement of autophagy in the relevant responses. In this chapter, we survey tools and resources that can be used to examine different types of autophagy (both general and selective) in *C. elegans*. In addition, we detail methodologies that have been developed to visualize autophagic processes in live animals under various conditions of interest.

2. REPORTERS FOR AUTOPHAGY

2.1 Core Autophagic Components

The nematode proteins LGG-1 and LGG-2 are homologous to Atg8/mammalian LC3B (cytosolic microtubule-associated protein 1 light chain 3-MAP1LC3) and are involved in autophagosome formation. Conversion of LC3B-I to its lipidated form, LC3B-II, occurs during its incorporation in the preautophagosomal and autophagosomal membrane. LC3B is used to visualize autophagosomes as cytoplasmic puncta under autophagy-inducing conditions (Wu et al., 2015). This ubiquitinlike modifier interacts with autophagic substrates containing an LC3-interacting-region (LIR) motif, the simplified version of which is F/W/Y-x-x-L/I/V. For efficient identification of proteins containing LIR motifs, two recent online tools, iLIR and SLiM, have been developed, which can be used to predict new LC3-interacting proteins that are potential substrates of autophagy (Kalvari et al., 2014; Popelka & Klionsky, 2015). The specific, differential roles of the closely related *C. elegans* LGG-1 and LGG-2 proteins remain largely elusive. Both proteins can be imaged in vivo by generating full-length green fluorescent protein (GFP) or DsRed translational reporter fusions. Upon autophagy induction, these proteins become lipidated and decorate the autophagosome. This conversion causes the diffuse cellular distribution pattern to become distinctively punctate. Autophagy induction can then be assessed by the number of LGG-1 and or LGG-2 GFP/DsRed positive puncta per cell (Keith et al., 2016). In principle, reporter fusions can be directed for expression in different cell types of interest where monitoring of autophagy is desired. Extrachromosomal transgenes can be utilized, although genomic integration of reporter constructs is recommended to minimize expression variability among animals and phenomena of mosaicism. Even when integrated, reporter constructs may show idiosyncratic expression. For example, the widely used *adIs2122* [p$_{lgg-1}$GFP::LGG-1; *rol-6(su1006)*] transgenic reporter strain shows expression mainly in seam cells, hypodermis, and intestinal cells (Klionsky et al., 2016).

Proteins of the WIPI family also colocalize and form distinct puncta, together with LC3 and ULK1 (Itakura & Mizushima, 2010). WIPI proteins are required for omegasome to autophagosome formation under autophagy-inducing, nutrient-deprived conditions. The absence of the *C. elegans* WIPI-4 homolog EPG-6 causes accumulation of LGG-1 and abortive early autophagic structures (Lu et al., 2011). The GFP::EPG-6 reporter has been used to monitor autophagy at embryonic stages and is widely expressed in

pharyngeal, body wall muscle, and neuronal cells. This protein is not itself a substrate of autophagy; thus, it can be effectively used as an early step marker to visualize the autophagic response without its levels being affected/diminished.

2.2 Receptors/Adaptors for Selective Autophagy

Autophagic receptors mediate the initial recognition of substrates such as organelles for selective autophagic degradation. Contrary to the abovementioned example, the *C. elegans* homolog of mammalian SQSTM-1/p62, SQST-1 (SeQueSTosome-related protein) has a dual role as an autophagic receptor/adaptor, but also as an autophagic substrate being degraded via autophagy. Visualization of SQST-1 is quite challenging under control conditions at larval and adult stages, most probably due to the rate of basal autophagic flux. However, this is a suitable autophagy reporter under conditions where autophagy is suppressed and SQST-1 levels are elevated in tissues such as the epidermis, neurons, and intestine (Zhang et al., 2015). Other autophagic substrates used as translational reporters to observe autophagic responses include the nematode-specific W07G4.5::GFP fusion, which is expressed both in the cytosol and the nucleus of intestinal cells. Its expression is accentuated in animals with compromised autophagy (Lin et al., 2013).

Mitochondria are energy-producing organelles found in eukaryotic cells; fluctuations in their numbers have been reported in diverse pathologies (Palikaras, Lionaki, & Tavernarakis, 2015). The regulation of mitochondrial mass is highly dynamic due to the balance between mitochondrial biogenesis and mitochondria selective autophagy (mitophagy) that takes place either by direct interaction of LC3 with mitochondrial membrane proteins or via autophagic adaptors such as p62 or optineurin (OPTN). Mitophagy is implemented as a response to dysfunctional or unneeded mitochondria. Two main tools have been developed to monitor mitophagy in *C. elegans*. The first manipulates the core autophagic component LGG-1, tagged with either GFP or DsRed, and a mitochondria-tagged DsRed or GFP, respectively, which can be targeted to different tissues for instance body wall muscle cells, depending on the promoter that drives expression of the transgene (Palikaras et al., 2015). Using this double reporter, the extent of colocalization of LGG-1 puncta and mitochondria, referred to as *mitoautophagosomes*, can be measured under different autophagy–inducing/hindering conditions analyzed in this section and Section 3, and shown in Fig. 1. This can be applied to other types of selective autophagy as well, such as endoplasmic reticulum (ER)-phagy by using an ER-specific reporter or a
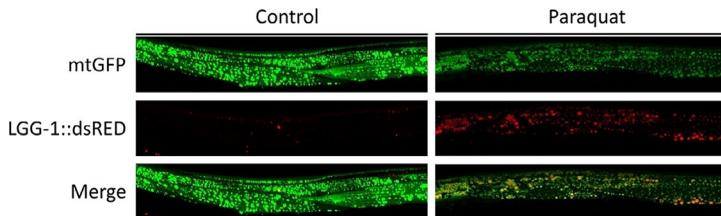
Fig. 1 LGG-1::DsReD colocalization with mtGFP in control and paraquat-treated worms. *Adapted from Palikaras, K., Lionaki, E., & Tavernarakis, N. (2015). Coordination of mitophagy and mitochondrial biogenesis during ageing in C. elegans.* Nature, *521(7553), 525–528.* http://dx.doi.org/10.1038/nature14300.
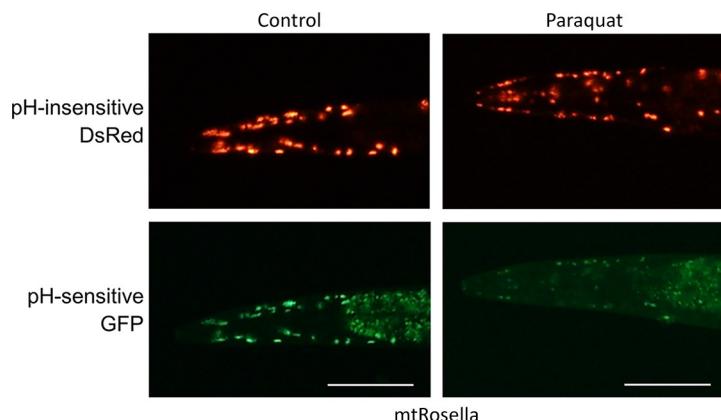

Fig. 2 mtRosella expression in control and paraquat-treated worms. *Adapted from Palikaras, K., Lionaki, E., & Tavernarakis, N. (2015). Coordination of mitophagy and mitochondrial biogenesis during ageing in C. elegans.* Nature, *521(7553), 525–528.* http://dx.doi.org/10.1038/nature14300.

reporter for the mammalian ER–phagy receptor ortholog in *C. elegans* (Khaminets et al., 2015).

The second tool takes advantage of the Rosella biosensor, a reporter initially used in the unicellular organism *Saccharomyces cerevisiae*. This construct, which was adapted for *C. elegans*, contains a fused, fast-maturing, and pH–insensitive variant of DsRed and a pH–sensitive variant of GFP (Rosado, Mijaljica, Hatzinisiriou, Prescott, & Devenish, 2008). Targeting to any organelle/cellular compartment (in this case, the mitochondrion, mtRosella) indicates the level of acidity of the environment/vacuole in which it is contained. Thus, under the autophagy–inducing conditions described here, the level of GFP/DsRed ratio is reduced when mitochondria are included in the autophagolysosome (Fig. 2). Furthermore, an even more refined approach to more accurately quantifying selective autophagy is

Table 1 Fluorescent Reporter Fusion Proteins, and *C. elegans* Strains Expressing the Respective Transgenes for Monitoring General and Selective Autophagy

Reporter	Nonselective Autophagy	Selective Autophagy	References
DsRed:: LGG–1 or GFP:: LGG–1	Ex011[p$_{lgg-1}$ DsRed::LGG–1; *rol-6(su1006)*] *adIs2122*[p$_{lgg-1}$ GFP::LGG–1; *rol-6(su1006)*]	R1284: N2;*Is* [p$_{myo-3}$mtGFP];Ex011 [p$_{lgg-1}$DsRed::LGG–1]	Kang, You, and Avery (2007); Palikaras et al. (2015)
Rosella biosensor	—	IR1631: N2;*Ex003* [p$_{myo-3}$TOMM-20:: Rosella]	Palikaras et al. (2015)
p$_{hlh-30}$HLH– 30::GFP	JIN1679 *jinEx10* [p$_{hlh-30}$*hlh-30::gfp*; *rol-6 (su1006)*]	—	Lapierre et al. (2013)
W07G4.5:: GFP	bpIs239[*w07g4.5:: gfp, unc-76*]	—	Lin et al. (2013)

the construction of translational reporters for selective autophagy receptors or adaptors. Moreover, double-transgenic animals expressing either a core autophagic reporter, GFP::LGG–1, or the Rosella biosensor (as previously described), together with selective autophagy receptors, can also be generated (Table 1). In the case of mitophagy, the protein ortholog of NIX/BNIP3L and BNIP3 (the Nip3–like protein X/Bcl-2 and the adenovirus E1B-interacting protein) in *C. elegans* has been identified as DCT–1 (DAF–16/FOXO Controlled, germline Tumor affecting-1) (Zhang et al., 2012). This outer mitochondrial membrane protein is a critical regulator of the interplay between mitochondrial biogenesis and degradation through mitophagy. For instance, generation of a strain carrying a double reporter (LGG–1 fused with GFP as indicated previously, and DCT–1 fused with DsRed) will allow direct detection of colocalization between core autophagy components and a selective factor. Other selective factors include PDR–1/PARK2 and PINK–1/PINK1. Lipophagy, the degradation of lipid droplets by macroautophagy, has been a technically challenging task. More traditional techniques include monitoring by measuring lipid storage abundance by BODIPY and Nile Red staining, which have low specificity as they can also stain lysosomes. Coherent anti-Stokes Raman scattering microscopy is a more recent, noninvasive, label-free imaging method for monitoring lipid metabolism (Folick, Min, & Wang, 2011).

2.3 Transcriptional Activation

Upon autophagy-inducing conditions, besides the increased protein degradation, there are significant transcriptional alterations. The autophagic transcriptional program is mainly regulated by HLH-30, an ortholog of the mammalian TFEB, which is a helix–loop–helix transcription factor. Under autophagy-inducing stimuli, which cause the inhibition of LET-363/TOR, HLH-30 translocates to the nucleus to induce transcription of autophagy-related genes (Lapierre et al., 2013). Thus, evaluation of HLH-30 nuclear translocation can be done by confocal imaging of nematodes expressing the integrated translational fusion p$_{hlh-30}$HLH-30::GFP (Table 1). In this case, the percentage of animals with GFP localized in the nucleus is assessed. Moreover, another core transcription factor linking autophagy with aging is PHA-4/FOXA, which positively regulates *unc-51*/Ulk1 transcription. However, contrary to HLH-30::GFP translocating to the nucleus upon autophagy induction, PHA-4::GFP nuclear levels remain constant (Panowski, Wolff, Aguilaniu, Durieux, & Dillin, 2007).

3. AUTOPHAGY INDUCTION AND SUPPRESSION

3.1 Environmental Stress

Upon nutrient limitation or starvation, *C. elegans* upregulates autophagy to cope with this unfavorable condition, as do all other eukaryotes. Receptors send the signal to intracellular nutrient sensitive kinases such as LET-363/ TOR, which is inhibited, causing the disinhibition of UNC-51/ULK1. Then vesicle nucleation begins and is mediated by the PI3K class III lipid kinase complex, including LET-512/VPS34 and BEC-1/BECLIN-1 (Fujioka et al., 2014). Autophagosome elongation and maturation involve two ubiquitin-like conjugation systems encompassing, the first, ATG5, LGG-3/ATG12, and ATG16, and the second, LGG-1(2)/MAP1LC3, ATG3, and ATG7. These steps are followed by autophagosome fusion with the lysosome. Nutrient deprivation is the most standard and evolutionarily conserved approach to upregulate the autophagic response.

Other assays that induce both general and selective macroautophagy are crowding, hypoxia, heat, and oxidative and DNA damage-induced stress. Thermotolerance has been used as an in vivo approach to activating autophagy in diverse organisms ranging from plants to nematodes, as it has been shown to induce transcription of autophagic genes, autophagosome accumulation, and increased mitochondrial degradation (Palikaras et al., 2015;

Zhou, Wang, Yu, & Chen, 2014). Similarly, DNA damage induced by ultraviolet irradiation (UV–B or UV–C) has also been shown to activate autophagy in *C. elegans* (Meyer & Bess, 2012). Moreover, oxidative stress induced by paraquat and mitochondrial stress by carbonyl cyanide *m*–chlorophenylhydrazone (CCCP), which is a chemical inhibitor of oxidative phosphorylation in mitochondria, both lead to mitochondrial defects, ultimately stimulating mitophagy.

3.2 Genetic Induction and Inhibition

Genetic manipulations, either knockouts or knockdowns, that influence longevity have been repeatedly associated with autophagy, and in some cases, autophagy is required for their effects on life span. For example, the *eat-2(ad1116)* mutant is a dietary restriction genetic model, and like other feeding-defective mutants, it has been shown to have naturally higher levels of autophagy in both larvae and adults, ultimately extending life span. Along similar lines, mutations in the gene encoding the insulin–IGF-1–like receptor abnormal Dauer Formation 2 (DAF–2) extend the *C. elegans* life span and activate autophagy. This life span extension requires both the activity of DAF–16/FOXO and autophagy. Notably, DAF–16 itself is dispensable for autophagy activation in *daf-2* mutants, and autophagy is not sufficient to promote longevity. Likewise, autophagy is required for life span extension by dietary restriction in *C. elegans*. Moreover, suppression of autophagy prevents life span extension by dietary restriction and TOR inhibition, which is known to mediate, at least in part, the beneficial effects of dietary restriction on longevity (Hansen et al., 2008).

Genetic inhibition of autophagy can also be performed with both mutants and knockdowns. Mutants of the autophagic machinery are usually nonviable, very sick, or have severe growth delay, causing technical difficulties in developmental synchronization. A list of mutants is provided in Table 2 (Cheng et al., 2013). Animals carrying mutations in *bec-1* (involved in vesicle nucleation) are not viable as homozygotes, while mutations in the *unc-51 gene* are viable but cause movement difficulties and defects in autophagy such as mislocalization of GFP::LGG-1. Alternatively, a mosaic analysis using the *bec-1(−)*; BEC-1::GFP transgenic strain could be applied (Takacs-Vellai et al., 2005). Further, *let-363* mutation inhibits the TOR pathway but cannot be effectively used, as it causes L3 developmental arrest (Vellai et al., 2003). On the other hand, the *lgg-2 (tm5755)* mutant is viable and reaches adulthood, although mutant phenotypes can be masked by

Table 2 *C. elegans* Genes That Are Routinely Targeted to Influence Autophagy, and the Corresponding Alleles or Genetic Manipulations Typically Used

Mammalian Homolog	*C. elegans* Gene	Genetic Manipulation	References
Beclin 1	*bec-1*	RNAi	Takacs–Vellai et al. (2005)
VPS–34	*let-512*	RNAi	Cheng et al. (2013)
WIPI3/4	*epg-6*	bp242	Lu et al. (2011)
ULK–1 (ATG1)	*unc-51*	RNAi	Hansen et al. (2008)
ATG2	*atg-2*	bp576	Cheng et al. (2013)
ATG4	*atg-4*	bp501	Cheng et al. (2013)
ATG5	*atg-5*	RNAi	Cheng et al. (2013)
ATG7	*atg-7*	bp422	Cheng et al. (2013)
LC3B (ATG8)	*lgg-1 & lgg-2*	RNAi	Cheng et al. (2013)
Sqstm1/p62	*sqst-1*	ok2892	Cheng et al. (2013)
HLH–30	*hlh-30*	hlh-30(tm1978)	Cheng et al. (2013)
IGF–1R	*daf-2*	daf-2(e1370)	Cheng et al. (2013)
TOR	*let-363*	RNAi	Vellai et al. (2003)
SH2D1B	*eat-2*	eat-2(ad1116)	Hansen et al. (2008)

functional redundancy or compensation by the LGG–1 protein. It is thus generally recommended that genetic inhibition of autophagy be performed by RNA interference (RNAi) knockdown of these genes after completion of development (larval stage 4) in order to examine the molecular and functional effects of autophagic ablation in itself. This is readily carried out in nematodes by feeding them with bacteria that have been transformed with double-stranded RNA (dsRNA)-expressing plasmid for specific knockdown of autophagy genes such as *bec-1*, *atg-7*, *vps-34*, *unc-51*, *lgg-1*, and *atg-18*.

3.3 Chemical Inhibition

Chemical suppression of autophagy has been widely used in higher eukaryotes. Cell culture experiments are usually conducted to investigate the effects of exogenous agents on autophagic responses, as there are no paracrine effects or boundaries that can affect the readout of the experiment. In vivo

GFP::LGG-1
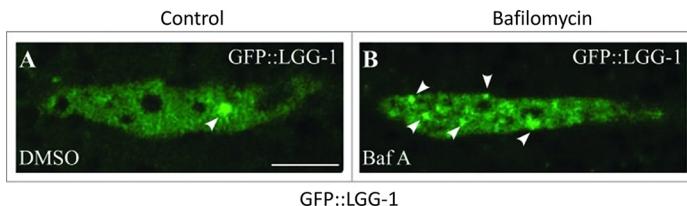
Fig. 3 LGG-1::GFP puncta (*arrowhead*) in seam cells of (A) control (DMSO) and (B) bafilomycin-treated worms. *Aadapted from Zhang, H., Chang, J. T., Guo, B., Hansen, M., Jia, K., Kovacs, A. L., … Wu, F. (2015). Guidelines for monitoring autophagy in Caenorhabditis elegans.* Autophagy, 11*(1), 9–27.* http://dx.doi.org/10.1080/15548627.2014.1003478.

experiments need much higher concentrations for longer periods of time. Bafilomycin A1 is a V–ATPase inhibitor that blocks the autophagic flux acutely by inhibiting autolysosome acidification and autophagosome–lysosome fusion. Practically, this means that autophagic substrates cannot be degraded in the lysosome, and thus the total autophagic pool can be imaged or quantified. Two ways have been proposed to administer bafilomycin A1 to the animals, feeding and injection (Saha et al., 2015; Wilkinson et al., 2015; also see Fig. 3). Feeding requires the bleaching of adult worms carrying eggs, followed by egg hatching in liquid media containing a high concentration of bafilomycin A1; 24 h later, worms are transferred to nematode growth medium (NGM) plates containing bafilomycin until used for imaging. Alternatively, bafilomycin A1 can be injected to adult worms and after 2 h, there is a distinct increase in GFP::LGG–1 puncta (Klionsky et al., 2016). This is an interesting autophagic inhibitor, as it blocks late steps of autophagy after autophagosome formation, permitting visualization of autophagic substrates, which are otherwise rapidly degraded, preventing imaging.

4. EXPERIMENTAL CONSIDERATIONS

There are certain parameters during experimental design that should be considered. Measuring autophagic responses at different developmental stages can be critical as to the readout of the experiment. Synchronization of animals is an essential prerequisite, as aggregates degraded by autophagy vary with age. Note that when using certain mutants, the rate of growth is slower. Autophagy levels change with age, although whether it declines or increases during aging is still a matter of scientific debate (Chapin, Okada, Merz, & Miller, 2015). Moreover, most of the studies have been performed

and perfected in embryos and early larval stages, where cells are more easily visualized. Although autophagy is high during early development, one should be cautious about the scientific question raised. For instance, if monitoring mitophagy, different parameters should be taken into consideration, as the mitochondrial number is low and mitochondria are still morphologically and consequently functionally immature during development. Mitophagy measurements are not ideal at that age. Also, after a certain age, it is important to discriminate functional effective autophagy. Changes in the number of autophagosomes and the abundance of autophagic substrates are merely indications. Monitoring different steps of the autophagic pathway, such as autophagy initiation, autophagosome formation/maturation, and degradation, is critical. Autophagosomes visualized as GFP:: LGG-1 puncta may increase in number as a result of either increased autophagosome formation or decreased degradation, i.e., autophagic flux. This pitfall can be resolved by using bafilomycin A1, a specific V-ATPase inhibitor blocking lysosomal acidification, among others (hence autophagic turnover). In addition, autofluorescence increases with age, especially in the worm intestine, making it difficult to discriminate between an actual signal and background noise. Confocal microscopy reduces background noise. GFP::LGG-1 can also increase as a result of technical issues, such as unwanted starvation of the animals, contamination of the agar plate where they are maintained, and the anesthetic used after 5–10 min of treatment (Zhang et al., 2015).

5. EXPERIMENTAL PROCEDURES

5.1 Reagents/Equipment

- Worm pick
- Eppendorf tubes
- Tube rotator
- NGM agar plates: Weigh 3 g of NaCl (Merck, Nottingham, UK; cat. no. 1.06404.1000), 2.5 g of bactopeptone (BD Biosciences, San Jose, California, USA; cat. no. 211677), 0.2 g of streptomycin kept at 4°C (Sigma; cat. no. S-6501), 17 g of agar (Merck; cat. no. 1.01614) and add 0.8 L of distilled water. Autoclave with 500 mL of distilled water. While mixing, allow cooling for 1 h before adding 1 mL of 5 mg/mL cholesterol (SERVA Electrophoresis GmbH, Heidelberg, Germany; cat. no. 1701) in EtOH, 1 mL 1 M MgSO$_4$, 1 mL 1 M CaCl$_2$ (Sigma; cat. no. C-5080), (Sigma; cat. no. M-7506), 1 mL 10 mg/mL nystatin

(Sigma; cat. no. N–3503), 25 mL 1 M phosphate buffer with pH 6.0, and add up to 1 L of distilled water.

- M9 buffer for starvation: Weigh 3 g of KH_2PO_4 (Merck; cat. no. 1.04873.1000), 6 g of Na_2HPO_4 (Merck; cat. no. 1.06586.0500), 5 g of NaCl (Merck; cat. no. 1.06404.1000), and dissolve to 1 L of distilled water. Autoclave and add 1 mL of sterile 1 M $MgSO_4$ (sterile; Sigma; cat. no. M–7506). Store M9 buffer at 4°C.
- Levamisole: Weigh 1.2 g of levamisole (Sigma–Aldrich, St. Louis, Missouri, USA; cat. no. 196142) and add in 10 mL of distilled sterile water. Store levamisole solution at 4°C.
- Dimethyl sulfoxide cell culture grade BC (DMSO; Applichem, Maryland Heights, MO, USA; cat. no. A3672.0250).
- 50 μM of bafilomycin A1 in 0.2% DMSO (Sigma–Aldrich; cat. no. B1793).
- *C. elegans* strains: R1284: N2;*Is*[p$_{myo\text{-}3}$mtGFP];Ex011[p$_{lgg\text{-}1}$DsRed:: LGG–1] and IR1631: N2;Ex003[p$_{myo\text{-}3}$TOMM–20::Rosella].
- 20°C incubator.
- UV cross–linker (BIO–LINK–BLX–E365, Vilber Lourmat).
- Zeiss AxioObserver Z1 confocal microscope.
- Zeiss ZEN 2012 software.
- Volocity High–Performance 3D imaging software (PerkinElmer, Waltham, Massachusetts, USA).
- Microscope slides $75 \times 25 \times 1$ mm (Marienfeld, Lauda-Koenigshofen, Germany; cat. no. 10 006 12).
- Microscope cover glass 18×18 mm (Marienfeld; cat. no. 01 010 30).
- Prism software package (GraphPad software).

5.2 Methodology

The following protocol outlines the basic steps to image selective autophagy of mitochondria in vivo in adult worms. First, synchronization of the strains N2;*Is*[p$_{myo\text{-}3}$mtGFP];Ex011[p$_{lgg\text{-}1}$DsRed::LGG–1] and IR1631: N2;Ex003 [p$_{myo\text{-}3}$TOMM–20::Rosella] is necessary to avoid variation due to developmental age differences. The first step is bleaching of gravid adult hermaphrodites, followed by egg hatching onto NGM plates seeded with *Escherichia coli* OP50 bacteria. In order to detect basal autophagy of mitochondria at different ages, injection of 50 μM of bafilomycin A1 at d1, d2, d4, d6, d10, and d14 of adulthood should be done, as a control injection of DMSO should be performed. Feeding with bafilomycin A1 requires a concentration

of 100 μg/mL from egg hatching until adulthood, which is both costly and increases variability, due to dependence on feeding conditions. Then, 2 h later, worms are collected with a worm pick, transferred to an empty NGM plate for 5 min to remove excess bacteria, and then transferred onto a levamisole droplet to immobilize worms on a microscope slide (approximately 13 μL). A coverslip is then gently placed on top and sealed around. Quick handling is essential at this point to avoid DsRed::LGG-1 puncta formed as a result of levamisole incubation.

A second alternative is to examine the autophagic flux under nutrient deprivation. In this case, all of these steps should be followed, but with two main alterations. First, egg laying is done on plates seeded with OP50 bacteria that have been UV-C irradiated for 15 min (0.5 J) using a UV cross-linker (BIO-LINK—BLX-E365, VilberLourmat). In this scenario, worms will have food to grow, but the bacteria will be dead; thus, when transferring to an empty NGM plate for acute starvation, no bacteria will be able to grow. In fact, even a small amount of OP50 can hinder the process, completely changing the results of the stress assay. Starvation can vary from 6 to 12 h, and bafilomycin can be administered 2 h before imaging. A more extreme approach to caloric restriction can be performed ideally for 24 h in an eppendorf tube containing M9 buffer on a rotator; however, this can cause bagging in gravid adults, and worms might not survive.

Image capturing using a confocal microscope (Zeiss AxioObserver Z1), for snapshots of single worms, as well as *Z*-stacks of single-body-wall muscle cells (1 μm step) is recommended. The mtRosella reporter fusion, expressed in body-wall muscle cells, is best imaged and quantified at the single-worm level, while colocalization between DsRed::LGG-1 and mitochondria-targeted GFP requires imaging at the cellular level. Lens, magnification, exposure time, resolution, laser intensity, and gain should be kept constant. Analysis of images can be performed directly using the Volocity software. Conversion is automatically done by transferring the Zeiss format images to a new folder of Volocity. Next, the pixel intensity of each fluorescent reporter/animal can be measured by encircling the worm, followed by choosing the Measurements panel, which contains the mean pixel intensity for each channel. Normalization can then be performed by dividing pixel intensity to the fluorescent area, and thereafter, the GFP to DsRed ratio can be calculated. For DsRed::GFP and mitoGFP colocalization assessment, in the Measurements panel, there is a "find spots" choice where, for each fluorescent spot on the image the pixel intensity is given. The list provided

shows spots/puncta that contain a number representing pixel intensity over a certain manually selected threshold where it is apparent there is colocalization. After standardizing the thresholds, repetition for each body wall muscle cell should be done. All calculations can be performed using Microsoft Excel 2011 software package (Microsoft Corporation, Redmond, Washington, USA). Statistical analysis and graph design can be performed using the Prism software package (GraphPad Software). For comparison, use Student's t-test with a cutoff $p < 0.05$. For each experiment/biological replicate, examine 70 animals minimum (mtRosella) or 50 body-wall muscle cells (DsRed::LGG-1;mtGFP).
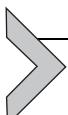
6. CONCLUDING REMARKS

The growing interest in selective forms of autophagy that are differentially regulated in various tissues has triggered the development of an arsenal of versatile tools that allow monitoring of both general and cargo-specific autophagy in *C. elegans*. The availability of reliable and quantitative methods for monitoring autophagy is a prerequisite for shedding light on the molecular mechanisms underlying autophagic responses in living cells and organisms. Delineating the role of autophagy at the organismal level is pivotal for the development of therapeutic interventions to treat several human pathologies, including neurodegenerative disorders. *C. elegans*-specific approaches and resources, combined with analogous methodologies developed in other organisms, including mice, will be instrumental toward facilitating progress in this direction.

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