Monitoring autophagy in muscle stem cells

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ABSTRACT

Autophagy is critical not only for the cell's adaptive response to starvation but also for cellular homeostasis, by acting as quality-control machinery for cytoplasmic components. This basal autophagic activity is particularly needed in postmitotic cells for survival maintenance. Recently, basal autophagic activity was reported in skeletal muscle stem cells (satellite cells) in their dormant quiescent state. Satellite cells are responsible for growth as well as for regeneration of muscle in response to stresses such as injury or disease. In the absense of stress, quiescence is the stem cell state of these cells throughout life, although which mechanisms maintain long-life quiescence remain largely unknown. Our recent findings showed that autophagy is necessary for quiescence maintenance in satellite cells and for retention of their regenerative functions. Importantly, damaged organelles and proteins accumulated in these cells with aging and this was connected to age-associated defective autophagy. Refueling of autophagy through genetic and pharmacological strategies restored aged satellite cell functions, and these finding have biomedical implications. In this chapter, we describe different experimental strategies to evaluate autophagic activity in satellite cells in resting muscle of mice. They should facilitate our competence to investigate stem cell functions both during tissue homeostasis as in pathological conditions.

Keywords:

stem cell, satellite cell, skeletal muscle, autophagy, quiescence, aging

1- INTRODUCTION

All organisms undergo continuous tissue and cell renovation. This, in part, occurs to replace old components with fresh, better-quality ones. Such “cellular renovation” requires synthesis of new constituents but also degradation of pre-existing, often damaged materials, which can serve as building blocks for “the *novo* synthesis”[1]. Autophagy is a process whereby intracellular cytosolic components including both macromolecules, such as proteins, glycogens, lipids and nucleotides, and organelles, such as mitochondria, peroxisomes, ribosomes and endoplasmic reticulum among others, are degraded within the lysosome [2-8]. Lysosomes are cellular organelles that contain acid hydrolases (proteases, lipases, nucleases, etc.) that break down macromolecules internalized inside this organelle. Once the macromolecules are degraded, their essential components can be transferred back to the cytosol for the building of new cellular material.

In mammals there are three types of autophagy: macroautophagy, microautophagy and chaperone-mediated autophagy. Macroautophagy, which will be simply referred to here as autophagy, is mediated by the autophagosome. A small portion of the cytoplasm is engulfed by an isolated membrane or phagophore, leading to formation of a double-membrane vesicle called autophagosome. Then, the autophagosome fuses with the lysosome, giving rise to an autolysosome, and materials inside the autophagosome are degraded by lysosomal hydrolases. Thus, autophagy is considered a dynamic process that comprises the following sequential steps: initiation, nucleation of the autophagosome membrane, recognition and trapping of the substrate, transport of the loaded autophagosomes towards the lysosomes, fusion of both vesicles and ultimately degradation of the cargo [9] (Figure1).

Although autophagy can be a non-selective degradation pathway where intracellular components are randomly engulfed, under some conditions and in response to certain signals, autophagy is a highly selective process by recognizing and eliminating components like mitochondria (mitophagy), peroxisomes (pexophagy)[10], ER portions (reticulophagy)[11], ribosomes (ribophagy)[12], lipid droplets (lipophagy)[13], pathogens (xenophagy)[14] or ubiquitinated-aggregates (aggrephagy)[15]. Most of these pathways require the action of autophagy adaptors, like p62, that act as linkage between the autophagosome (interacting with LC3-II proteins) and the substrate (binding to its ubiquitin chain)[16-18].

The most fundamental and evolutionary conserved role of autophagy is the supply of amino acids in the "adaptation response" to starvation conditions for new protein synthesis and energy production[19,20]. However, under conditions of stress, such as mitochondrial damage, ER stress, hypoxia, redox stress, accumulation of aggregated proteins or pathogen-associated molecular patterns (PAMPs), autophagy might also be induced as part of the cellular stress-response mechanism [8].

Growing evidence points to the importance of basal autophagy, operating constitutively at low rate, even under a nutrient-rich environment, and to its key role in global turnover of cellular components including organelles. Consistent with this, constitutive autophagy acts as the quality-control machinery for cytoplasmic components, being crucial for cellular homeostasis. The importance of constitutive autophagy is specially critical in postmitotic cells like neurons, that require an effective and basal autophagy activity to maintain cell homeostasis and survival[21,22]. More recently, quiescent muscle stem cells (also called *satellite cells*) have also been shown to require constitutive autophagy to maintain their stemness status (García-Prat, *in press*). In this context, autophagy has been demonstrated to operate in two different scenarios. In the first one, a recent study reported that suppression of autophagy inhibits activation of young satellite cells from quiescence upon injury[23]. The authors proposed that

autophagy is induced by Sirt1 during satellite cell activation to provide the nutrients necessary to meet bioenergetic demands during the transition from quiescence to activation[23]. Of note, this study proposed a relative lack of nutrient availability to induce autophagy during the satellite cell activation phase, mimicking starvation-induced autophagy, a process necessary for cellular adaptation to nutritional stress, similar to what was described in hematopoietic stem cells[24]. In a second scenario, we have demonstrated that young quiescent satellite cells (in resting muscle) display a basal autophagy flux, and that this basal activity preserves cell integrity and fitness over time. Through Atg7 (one of the essential genes for the formation of the autophagosomes) genetic deletion in young satellite cells, we have shown that constitutive autophagy, which declines during aging, functions as a cytoprotective and cellular quality control mechanism to balance protein and organelle homeostasis, which is essential to maintain both the population of satellite cells and their functional properties. These findings reflect the relevance of autophagy in stem cells homeostasis regulation.

Because of the low number of quiescent satellite cells, the peculiarities of their isolation in a resting state and their particular morphology (very low proportion of cytoplasm), the study of the autophagic process in these stem cells and the consequences of its impairment, is therefore challenging. In this chapter, we review a variety of methods developed to study autophagy in quiescent muscle stem cells.

2- MATERIALS

2.1 Isolation of quiescent satellite cells by fluorescence-activated cell sorting (FACS)

1. DMEM (Dulbecco's Modified Eagle Medium) high glucose, supplemented with 1% penicillin/streptomycin (P/S) and 10% Fetal Bovine Serum (FBS).
2. Lysis Buffer (BD Pharm lyse, 555899)
3. FACS Buffer: Phosphate Buffered Saline (PBS) 1X, 5% Goat Serum (Gibco, 16210-064)
4. Antibodies: PE/Cy7 anti-mouse/human Ly-6A/E (Sca-1) (Biolegend, 108114), PE/Cy7 anti-mouse/human CD11b (Biolegend, 101216), α-7 integrin R-Phycoerythrin (AbLab, 53-0010-05), Alexa Fluor 647 Rat anti-mouse CD34 (BD Pharmingen, 560230).
5. Digestion mix: 0.8% collagenase D, 0.125% Trypsin in DMEM 1% Penicillin/Streptomycin (four limb muscles of 1 mouse require 100 mL of digestion mix)
6. DAPI, stock solution 1 mg/mL, final concentration 1μg/mL

2.2 Autophagic flux determination: Turnover of LC3-II by Western blotting of isolated quiescent satellite cells

1. FACS Buffer: PBS 1X, 5% Goat Serum (Gibco, 16210-064)
2. Antibodies: PE/Cy7 anti-mouse/human Ly-6A/E (Sca-1) (Biolegend, 108114), PE/Cy7 anti-mouse/human CD11b (Biolegend, 101216), α-7 integrin R-Phycoerythrin (AbLab, 53-0010-05), Alexa Fluor 647 Rat anti-mous CD34 (BD Pharmingen, 560230),
3. Bafilomycin A1: 10 nM final concentration
4. Immunoprecipitation (IP) Buffer: 50 mM TrisHCl, 150 mM NaCl, 1% NP-40, 5 mM EGTA, 5 mM EDTA, 20 mM NaF.
5. IP Working Soultion (for 10 mL): 1 tablet of protease inhibidor (ComplteMini, Roche, 11836153001), 50 μl phosphatase inhibitor Cockatil 1 (Sigma, P2850), 50 μl phosphatase inhibitor Cocktail 2 (Sigma, P5726), NaVanadate, 0,1 mM final concentration, PMSF, 1 mM final concentration, Glicerophosphate, 10 mM final concentration.

6. Resolving gel 12% (2 gels): 8.6 mL ddH20, 6 mL 40% acrylamide, 3 mL 1,5 M Tris HCl, pH 8.8, 200 μl 10% SDS, 200 μl 10% APS, 8 μl TEMED

7. Stacking gel 5% (2 gels): 5.9 mL ddH_20, 1 mL 40% acrylamide, 1 mL 1.0 M Tris HCl, pH 6.8, 80 μl 10% SDS, 80 μl 10% APS, 8 μl TEMED.

8. Running Buffer (pH 8.3): 0.025 M Tris-HCl, 0.192 M Glycine, 0.1% SDS.

9. Transfer Buffer: 0.025 M TrisHCl , 0.192 M Glycine, 20% Methanol

10. Blocking Solution: 5% milk, TBS-T

11. Antibody solution: 5% BSA, TBS-T

12. PVDF membrane

13. TBS-T: TBS, 1X (150 mM NaCl, 10 mM Tris.HCl, pH 7.4), 0.05% Tween

14. 5X Laemmli Buffer: 100 mM Tris pH 6.8, 32% Glycerol, 2% SDS, 0.05% β-mercaptoethanol, 0.1% Bromopehnol Blue

15. Antibodies: anti-LC3 (Novus Biologicals NB100-2331), Anti-Tubulin (Sigma, T-6199), Polyclonal Goat anti-rabbit Immunoglobulins/HRP (Dako, P0448), Alexa fluor 680 Goat anti-mouse IgG (Invitrogen, A21058), anti-p62/SQSTM1 antibody produced in rabbit (Sigma, P0067)

2.3 Autophagic flux determination: GFP-LC3 fluorescence microscopy of isolated quiescent satellite cells

1. FACS Buffer: PBS 1X, 5% Goat Serum (Gibco, 16210-064)

2. Antibodies: PE/Cy7 anti-mouse/human Ly-6A/E (Sca-1) (Biolegend, 108114), PE/Cy7 anti-mouse/human CD11b (Biolegend, 101216), α-7 integrin R-Phycoerythrin (AbLab, 53-0010-05), Alexa Fluor 647 Rat anti-mouse CD34 (BD Pharmingen, 560230)

3. Bafilomycin A1, 10 nM final concentration (Sigma, B1793)

4. 8-well glass slides (Thermo Scientific, 177402)

5. Poly L-lysin

6. PBS 1X, 0.02% azide
7. Fixation solution: 4% paraformaldehyde (PFA) in phosphate buffer 0.1 M
8. Mowiol mounting medium

2.4 Autophagic flux determination: Immunostaining analysis of freshly-isolated quiescent satellite cells

1. Blocking Solution: 10% Goat serum (Gibco, 16210-064), 10% FBS (Capricorn Sientific, FBS-12A) in PBS 1X
2. Washing buffer: PBS 1X, 0.01% Tween (Sigma, P1379)
3. Permeabilization buffer: PBS 1X, 0,5% Triton (Sigma, T8787)
4. DAPI, stock solution 1 mg/mL, final concentration 1μg/mL
5. Antibodies: anti-p62/SQSTM1 antibody produced in rabbit (Sigma, P0067), mouse monoclonal antibody to LC3 (NanoTools, 5F10), LAMP-1 (Santa cruz Biotechnology, sc-19992)

2.5 Autophagic flux determination: Immunostaining analysis of GFP-LC3 quiescent satellite cells in tissue sections

1. Cassettes 15 x 15 x 5 mm (Tissue-Tek Cryomold, Sakura)
2. OCT (Tissue-Tek, Sakura)
3. Blocking Solution 1: 1/50 MOM mouse IgG blocking reagent (Vector, MKB-2213), 0,01% Triton, PBS 1X
4. Blocking Solution 2: 10% Goat Serum, 10% FBS, PBS 1X
5. Washing buffer: PBS 1X, 0,01% Triton
6. Permeabilization buffer: PBS 1X, 0.5% Triton
7. DAPI, stock solution 1 mg/mL, final concentration 1μg/mL
8. Fixation solution: 4% paraformaldehyde (PFA) in phosphate buffer 0.1M.
9. Mowiol mounting medium

2.6 Autophagic flux determination: Flow cytometry analysis of GFP-LC3 quiescent satellite cells

1. FACS Buffer: PBS 1X, 5% Goat Serum (Gibco, 16210-064)
2. Antibodies: PE/Cy7 anti-mouse/human Ly-6A/E (Sca-1) (Biolegend, 108114), PE/Cy7 anti-mouse/human CD11b (Biolegend, 101216), α-7 integrin R-Phycoerythrin (AbLab, 53-0010-05), Alexa Fluor 647 Rat anti-mous CD34 (BD Pharmingen, 560230)
3. Bafilomycin A1, 10 nM final concentration (Sigma, B1793)
4. DAPI, stock solution 1 mg/mL, final concentration 1 μg/mL

2.7 Autophagic flux determination: Fluorescence microscopy analysis of cell expressing the mRFP-GFP-LC3 plasmid

1. Growth medium: HAM'S F-12 (Biowest, l0140-500), 20% FBS (Capricorn Sientific, FBS-12A), 1% L-Glutamine (Biowest, X0550-100), 1% P/S (Biowest, L0022-100)
2. Bafilomycin A1, 10 nM final concentration
3. DAPI, stock solution 1 mg/mL, final concentration 1 μg/mL (Invitrogen, D1306)
4. Coating Solution: 0.1 mg/mL of Collagen (Becton Dickinson, 354236), 0,02N acetic acid (Sigma, 320099)
5. Fixation buffer : 4% PFA in phosphate buffer 0.1M.
6. Mowiol mounting mediuml
7. Lipofectamine 3000 (Life Techonologies, L3000001)
8. mRFP-GFP-LC3 plasmid [25]

2.8 Transmission electron microscopy (TEM)

1. Fixation buffer: 2% paraformaldehyde/2.5% glutaraldehyde in phosphate buffer (0.1M; pH 7.4)

2.9 Transcriptional regulation: q-RT-PCR

1. RNeasy Micro kit (Qiagen, 74004)
2. SuperScript III Reverse Transcriptase (Invitrogen 1674043)
3. LightCycler 480 System using Light Cycler 480 SYBR Green I Master reaction mix (Roche Diagnostic Corporation)
4. Primers (Sigma) (see table 1)

2.10 Transcriptional regulation: Global gene expression analysis of quiescent satellite cells

1. RNeasy Micro kit (Qiagen, 74004)
2. Agilent SurePrint G3 Mouse GE 8x60K (Agilent Technologies, G4852A)

2.11 Autophagy modulation: (in vivo and in vitro treatments)

1. Activators: Rapamycin (LC Laboratories, R-5000), Spermidine (Sigma, S2626), LV-Atg7[26]. Lentivirus infection reagents: Retronectin (Takara, T100A), Hank's Balanced Salt Solution (HBSS), Bovine Serum Albumins (BSA) and Hepes.
2. Inhibitiors: Bafilomycin A1 (Sigma ,B1793), chloroquine (Sigma, C-6628), E64d (Sigma, E3132), pepstatin A (Sigma, P-5318), leupeptin (Sigma, L2884).

2.12 Mouse models useful for monitoring autophgay in quiescent satellite cells

1. Tamoxifen (Sigma, T5648)
2. Corn Oil

3- METHODS

3.1 Isolation of quiescent satellite cells by FACS

Muscle stem cells (satellite cells) are located surrounding the basal lamina and outside the myofiber plasma membrane in a quiescent state. These small stem cells were first described in adult muscle in 1961 by Alexander Mauro[27], using electron microscopy. This technique also revealed other morphological attributes: large nuclear-to-cytoplasmic ratio, few organelles and condensed interphase chromatin. This morphology is in harmony with the notion that most satellite cells, in healthy unstressed muscles, are mitotically quiescent (arrested in the G0 phase of the cell cycle) and transcriptionally inactive. The steps for isolation of these cells by FACS (see Figure 2a) are described below:

1. Muscles from fore and hind limbs are collected in cold DMEM 1% P/S into 50 mL falcon tubes.
2. Decant all the muscles collected in a petri dish and remove DMEM 1% P/S completely.
3. Mince muscles with scissors.
4. Mince muscles further with razor blades.
5. Collect minced muscles into a 50 mL falcon tube and add cold DMEM 1% P/S. Leave muscle sediment and remove DMEM 1% P/S, discarding floating fat pieces. Repeat this step to further clean the sample from non-muscle pieces.
6. In the last wash, remove DMEM 1% P/S as much as possible and split the minced muscle into two 50 mL falcon tubes.
7. Add 10 mL of the prepared digestion mix (collagenase/trypsin) to each tube.
8. Incubate 25 min at 37ºC in a shaking water bath.
9. At the end of the digestion, leave the tube for 5 min on ice to let the sample sediment.
10. Transfer the digestion supernatant to a new 50 mL falcon tube on ice.
11. Add 10 mL of the digestion mix again and incubate 25 min at 37ºC.

12. Collect the new digestion supernatant and pool it with the supernatant from the first digestion round.
13. Steps 6 to 12 are repeated 2 more times.
14. If some pieces of muscle still remain, decant the sample on a petri dish for a new mincing process until no more are seen, and repeat one round of digestion. At the end of the 4rth round of digestion, all muscle tissue should be digested.
15. Add 5 mL of fetal bovine serum (FBS) to each 50 mL falcon tube to block the digestion.
16. Filter the supernatant with 100 and 70 μM filters.
17. Centrifuge the supernatant 10 min at 50 g and at 4ºC.
18. Collect the supernatant and discard the pellet (optional: the pellet can be washed and the supernatant collected and pooled with the previous supernatants).
19. Centrifuge at 1700 rpm for 15 min at 4ºC, repeat 3 times. The supernatant is discarded at each round and the pellet is resuspended gently in cold DMEM 1% P/S.
20. After 3 centrifugations at 1700 rpm, pellets from both tubes are pooled together in cold DMEM 1% P/S, and passed through a 40 μM filter.
21. Centrifuge at 1700 rpm for 15 min at 4ºC.
22. Resuspend the pellet in 3 mL of 1X Lysis Buffer and incubate at 4ºC for 10 min (protect from light). Before centrifugation add cold DMEM 1% P/S up to 50 mL.
23. Centrifuge at 1700 rpm for 15 min at 4ºC.
24. Discard the supernatant and resuspend the pellet in 1 mL of cold DMEM 1% P/S.
25. Count the number of cells for each sample.
26. Centrifuge at 1700 rpm for 15 min at 4ºC and resuspend the pellet at $1 \cdot 10^4$ cells/μl ($1 \cdot 10^6$ cells in 100μl) in FACS Buffer.
27. Staining with antibodies:

a. Negative selection: Sca1-PECy7 and CD11b-PECy7 (0.5 μl/100μl FACS Buffer)
b. Positive selection: α-7 integrin-PE (1 μl/100μl FACS Buffer) and CD34-APC (1.5 μl/100μl FACS Buffer)
c. Controls: Single staining and FMO controls are required to set up the gates

28. Incubate the cells with antibodies for 20 min at 4ºC, protected from light.
29. Centrifuge at 1700 rpm for 15 min at 4ºC.
30. Discard the supernatant and resuspend the cell bulk in 1 mL of FACS Buffer for sample sorting.
31. Add DAPI (final concentration 1 μg/mL) 5 min prior FACS to detect and exclude dead cells.
32. Collect $Sca1^-/CD11b^-/CD34^+/\alpha7\text{-integrin}^+$ satellite cells in Eppendorf tubes with 500 μl of FACS Buffer at 4ºC.

3.2 Autophagic flux determination: Introduction

The term “autophagic flux” refers to the dynamic process of autophagy, including all the steps described before (see Figure 1). The simple determination of numbers of autophagosomes is insufficient for an overall estimation of autophagic activity. Given that the autophagosome is an intermediate structure in a dynamic pathway, the number of autophagosomes observed at any specific time point depends on the balance between the rate of their generation and the rate of their conversion into autolysosomes[28]. Thus, accumulation of autophagosomes may represent either autophagy induction or, alternatively, suppression (dysfunction) of steps in the autophagy pathway downstream of autophagosome formation (as observed in satellite cells of advanced age) (Laura García-Prat, *in press*). The main biological autophagy marker used is the microtubule-associated protein LC3-II. LC3-I is normally located in the cytoplasm but is cleaved and lipidated by phosphatidylethanolamine when

incorporated into the autophagosome inner leaflet of the membrane as LC3-II[29]. Thus, for autophagic flux determination, it is important to use inhibitors of autophagosome clearance to discern between increased autophagy or impaired autophagosome clearance. Currently, several compounds are used. In particular, we use Bafilomycin A1 (a vacuolar H^+-ATPase inhibitor) (see Figure 3a) -but other lysosomal inhibitors can be used, like chloroquine and NH_4Cl (both impairing lyosomal acidification) or protease inhibitors (like pepstatin A, E64d or leupeptin).

Another widely used autophagy marker is p62, also called sequestrome 1 (SQSTM1), which binds directly LC3 and GABARAP (Atg8 orthologues) proteins via a short LC3 interaction region (LIR). This may serve as a mechanism to deliver selective autophagic cargo for degradation in lysosomes. The p62 protein is itself degraded by autophagy, acting as a marker of cargo degradation and is, consequently, useful to study autophagic flux as a complementary technique to LC3-II turnover [19,30,31,17]. Thus, when autophagy is activated, p62 levels decrease, while autophagy inhibition leads to p62 accumulation [29].

3.3 Autophagic flux determination: LC3-II turnover analysis by Western blotting:

1. After step 29 of the protocol for isolation of quiescent satellite cells, transfer and divide the bulk of cells (already stained with the antibodies) into two Eppendorf tubes (500 μl/each).
2. Add FACS Buffer up to 1mL.
3. Treat the cells with Bafilomycin A1 (10 nM) or DMSO in FACS Buffer prior to FACS analysis for 4 hours at 37ºC in the incubator.
4. Add DAPI (final concentration 1 μg/mL) 5 min prior FACS to detect and exclude dead cells.
5. Collect 200.000 satellite cells ($Sca1^-/CD11b^-/CD34^+/\alpha7\text{-integrin}^+$) from each sample in Eppendorf tubes with 500 μl of FACS Buffer at 4ºC.

6. Centrifuge at 14.000 g for 5 min at 4ºC.
7. Remove the supernatant as much as possible. At this step, samples can be stored at -80ºC.
8. Add 100 μl of IP Working Solution in each Eppendorf tube for cell lysis
9. Ensure complete cell lysis by incubating suspensions for 45 min at 4ºC with shaking.
10. Remove remaining cell debris by centrifuging at 14.000 g for 15 min at 4ºC. The supernatant contains the proteins and can be quantified using the standard Bradford Assay. However, since quiescent satellite cells contain small amounts of proteins, and since the same number of satellite cells has been used for each sample, the protein quantification step can be omitted.
11. Prepare samples for gel loading: 40 μl of each sample with 5X Laemmli Buffer (10 μl).
12. Incubate samples for 5 min at 95ºC.
13. Prepare the stacking gel at 5% and the resolving gel at 12%, use 1.5 mm gel and 10-well comb to be able to load 50 μl of sample.
14. Load the maximum sample volume (around 50 μl)
15. Run the samples at 100 V with Running Buffer.
16. Transfer at 100 V for 1 h, using PVDF membrane and Transfer Buffer.
17. Block membrane with Blocking Solution for 1 h at RT. The membrane can be divided in different parts by cutting it horizontally at different levels, to allow Western blotting of proteins with different molecular weights, by using different antibodies.
18. Incubate O/N with the primary antibody (anti-LC3) in Antibody Solution. Dilution 1/200.
19. Wash 3X with TBS-T.
20. Incubate with the secondary HRP antibody for 2 h at RT, protected from light.

21. Wash 3X with TBS-T.
22. Detect HRP using ECL reagent and X-ray film. Ensure that the exposures are in the linear range.
23. Wash 3X with TBS-T.
24. Incubate O/N with the primary antibody used as loading control (Tubulin) in Antibody Solution.
25. Wash 3X with TBS-T.
26. Incubate with infrared secondary antibody for 2 h at RT, protected from light. Dilution 1/4000 in Odyssey Blocking Buffer (PBS).
27. Wash 3X with TBS.
28. Odyssey infrared imager (LI-COR) is used for antibody detection.
29. Quantify the bands using imageJ. LC3-I is usually detected on a gel at a molecular mass around 16kD, and LC3-II at approximately 14 kD (see Figure 2b).

See Note 1.

3.4 Autophagic flux determination: GFP-LC3 fluorescence microscopy of isolated quiescent satellite cells

1. After step 29 of the protocol for quiescent satellite cell isolation from muscles of GFP-LC3 mice, transfer and divide the bulk of cells (already stained with the antibodies) into two Eppendorf tubes (500 μl on each).
2. Add FACS Buffer up to 1 mL.
3. Treat the cells with Bafilomycin A1 (10 nM) or DMSO in FACS Buffer prior to FACS analysis for 4 h at 37ºC in the incubator.
4. Add DAPI (final concentration 1 μg/mL) 5 min prior FACS to detect and exclude dead cells.
5. Collect $Sca1^-/CD11b^-/CD34^+/\alpha7\text{-integrin}^+$ satellite cells in Eppendorf tubes with 500 μl of FACS Buffer at 4ºC.

6. Coat 8-wells glass slide with Poly L-lysin 30 min at room temperature.
7. Remove Poly L-lysin (it can be reused).
8. Add cell suspension (10.000 to 15.000 cells/well). Ensure that cell suspension is evenly distributed in the well; if necessary, add PBS1X.
9. Spin the slides 5 min at 50 g.
10. Remove supernatant from each well.
11. Add 4% PFA (200 μl) in each well. Incubate 10 min at RT.
12. Remove 4% PFA and perform 2 washes with PBS 1X. At this point, slides can be stored at 4ºC with PBS 1X 0.02% azide.
13. Mount slides with mowiol.
14. Analyze fluorescence in images obtained with Leica SPE confocal laser scanning microscope and Superresolution microscopy using stimulated emission depletion (STED), Leica TCS SP5 STED (Objective HCX PL APO 100x/1.4 Oil STED; zoom 5).
15. The number and percentage of cellular area occupied by GFP-LC3 puncta can be determined on digital images with Fiji and the cell image analysis software CellProfiler.

See Note 2.

3.5 Autophagic flux determination: Immunostaining analysis of quiescent satellite cells

We can combine GFP-LC3 fluorescence analysis of quiescent satellite cells with other autophagy markers to assess for their colocalization (Figure 3). For instance, impairment of autophagy in several cell types is accompanied by accumulation of p62 and Ubiquitin aggregates that stain positive for LC3 (Figure 3b). In addition, LAMP-1 (lysosome marker) can be used to study the different phases of the autophagy process (Figure 3c). Co-localization between p62 or ubiqitin and GFP-LC3 indicate a selective cargo of autophagy recognized by an autophagosome. Co-localization of GFP-LC3

with LAMP-1 (always in the presence of lysosomal inhibitors to avoid LC3-II degradation) is a marker of autolysosomes.

1. After step 12 of the previous protocol, perform the following steps.
2. Permeabilize cells with PBS 1X 0.5% Triton for 15 min at RT.
3. Wash 3X with PBS 1X.
4. Add Blocking Solution for 30 min at RT.
5. Incubate with primary antibody in Blocking Solution O/N at 4ºC.
6. Wash 3X with PBS 1X, 0.01% Tween
7. Incubate with secondary antibody in PBS 1X, 0.01% Tween 1 h 30 min at RT (seat in dark).
8. Wash 3X 1X, 0.01% Tween.
9. Add DAPI (final concentration 1 μg/mL) for 5 min at RT.
10. Wash 3X PBS 1X, 0.01% Tween.
11. Mount with mowiol.
12. Analyze fluorescence, by doing confocal images taken using Leica SPE confocal laser scanning microscope system and Superresolution microscopy using stimulated emission depletion (STED), Leica TCS SP5 STED (Objective HCX PL APO 100x/1.4 Oil STED; zoom 5).

See Note 3.

3.6 Autophagic flux determination: Immunostaining analysis of GFP-LC3 quiescent satellite cells in tissue sections

1. Collect muscles from GFP-LC3 mice and put them in cassettes with OCT.
2. Freeze the muscles in isopentane cooled with liquid nitrogen and stored at -80ºC until analysis.
3. Cut the muscle samples in 10 μm sections with a cryostat.

4. Fix muscle sections with 4% PFA 12 min on ice.
5. Wash 3X with PBS 1X for 5 min.
6. Permeabilize with PBS 1X, 0.5% Triton for 20 min at RT.
7. Wash 3X with PBS 1X for 5 min.
8. Add Blocking Solution 1 for 1 h at RT.
9. Add Blocking Solution 2 for 20 min at RT.
10. Incubate primary antibody in Blocking Solution 2, O/N at 4ºC.
11. Wash 3X with PBS 1X, 0.01% Triton for 5 min.
12. Incubate secondary antibody in Blocking Solution 2, for 2 h at RT (protected from light).
13. Wash 3X with PBS 1X, 0.01% Triton for 5 min.
14. Add DAPI (final concentration 1 μg/mL) for 5 min at RT.
15. Wash 3X with PBS 1X, 0.01% Triton for 5 min.
16. Mount with mowiol.
17. Analyze fluorescence in images obtained with Leica SPE confocal laser scanning microscope system and Superresolution microscopy using stimulated emission depletion (STED) Leica TCS SP5 STED (Objective HCX PL APO 100x/1.4 Oil STED; zoom 5). Acquisition is performed using Leica Application or LAS AF software (Leica).

See Note 4.

3.7 Autophagic flux determination: Flow cytometry analysis of GFP-LC3 quiescent satellite cells

1. After step 29 of the protocol for quiescent satellite cell isolation from muscles of GFP-LC3 mice, transfer and divide the bulk of cells (already stained with the antibodies) into two Eppendorf tubes (500 μl on each).
2. Add FACS Buffer up to 1 mL.

3. Treat the cells with Bafilomycin A1 (10 nM) or DMSO in FACS Buffer prior to FACS analysis for 4 h at 37ºC in the incubator.
4. Add DAPI (final concentration 1 μg/mL) for 5 min prior FACS to detect and exclude dead cells.
5. Analyze GFP-LC3 fluorescence in FACS LSR Fortesa (Becton Dickinson) of 10.000 satellite cells ($Sca1^-/CD11b^-/CD34^+/\alpha7\text{-integrin}^+$) for each sample.
6. GFP-LC3 fluorescence signal is achieved by determining the mean fluorescence intensity (MFI) of the whole histogram signal for satellite cells and compared to the corresponding negative control sample (no GFP-LC3) in an overlaid histogram[32].
7. For autophagy flux determination, relative changes in linear scaled MFI are compared between samples with or without Bafilomycin A1 (see Figure 4b).

See Note 5.

3.8 Autophagic flux determination: Fluorescent microscopy analysis of mRFP-GFP-LC3 plasmid

Measuring autophagy flux through this method is based on the concept of lysosomal quenching of GFP. GFP is a stably folded protein and relatively resistant to lysosomal proteases. However, the low pH inside the lysosome quenches the fluorescent signal of GFP, which makes it difficult to trace the delivery of GFP-LC3 to lysosomes. In contrast, RFP exhibits more stable fluorescence in acidic compartments, and mRFP-LC3 can readily be detected in autolysosomes. By exploiting the difference in the nature of these two fluorescent proteins (i.e., lysosomal quenching of GFP fluorescence versus lysosomal stability of RFP fluorescence), autophagic flux can be morphologically traced with an mRFP-GFP-LC3 tandem construct[25] (see Figure 4c).

1. After step 31 of the protocol for quiescent satellite cell isolation, satellite cells ($Sca1^-/CD11b^-/CD34^+/\alpha7\text{-integrin}^+$) are collected in Eppendorf tubes with 500 μl of Growth Medium (GM).
2. Coat 8-well glass slides with Coating Solution (0.1 mg/mL collagen in sterile water and 0.02N acetic acid).
3. Plate sorted satellite cells in coated glass slides and culture them in GM.
4. After 4 days in culture, transfect satellite cells with mRFP-GFP-LC3 plasmid[33] using Lipofectamine 3000.
5. After 48 h of plasmid transfection, add Bafilomycin A1 (10 nM) or DMSO in the culture medium and incubate cells for 4 h at 37ºC.
6. Fix cells with 4% PFA for 10 min at RT.
7. Wash 3X with PBS 1X.
8. Stain cell nuclei with DAPI (final concentration 1 μg/mL) in PBS 1X for 5 min at RT.
9. Wash 3X with PBS 1X.
10. Mount with mowiol.
11. Analyze fluorescence in images obtained with Leica SPE confocal laser scanning microscope system and Superresolution microscopy using stimulated emission depletion (STED) Leica TCS SP5 STED (Objective HCX PL APO 100x/1.4 Oil STED; zoom 5)
12. Co-localization of mRFP-LC3 and GFP-LC3 puncta is determined on the maximum projection of three Z-sections using Fiji automated macro pipeline calculating single and double-positive autophagosomes (Figure 4c).

See Note 6.

3.9 Transmission electron microscopy (TEM)

TEM is a valid and important method both for qualitative and quantitative analysis of changes in various autophagic structures. Autophagosomes have a double membrane

and contain cytosol and/or organelles that look morphologically intact, while autolysosomes usually have only one limiting membrane, and contain cytoplasmic material and/or organelles at various stages of degradation. In quiescent satellite cells, as explained above, images with very high resolution will be required for proper analysis of the presence of autophagosomes/autolysosomes due to the reduced size and cytoplasmic cell content.

1. Intact muscles are collected and fixed with 2% paraformaldehyde/2,5% glutaraldehyde in phosphate buffer (0.1 M, pH7.4), as fast as possible.
2. Process sample for TEM analysis following standard procedures.
3. Images were acquired using a Jeol 1010 microscope, working at 80 kv and equipped with a CCD Megaview III camera.
4. Identification of satellite cells in skeletal muscle by electron miscoscopy was based on cell size, content of heterochromatin and position with respect to basal lamina.

3.10 Transcriptional regulation of autophagy: Introduction

Analysis of the transcriptional regulation of the autophagy gene network can also provide valuable information about possible alterations in the autophagic system. We chose a list of autophagic-related genes involved in different steps of the autophagic machinery, from autophagic initiation to lysosomal clearance.

3.11 Transcriptional regulation of autophagy: RT–qPCR

1. After step 31 of the protocol for quiescent satellite cell isolation, satellite cells ($Sca1^-/CD11b^-/CD34^+/\alpha7\text{-integrin}^+$) are collected in Eppendorf tubes with 500 μl of FACS Buffer.
2. Centrifuge Eppendorf tubes at 14.000 g for 5 min.
3. Remove supernatant.
4. Perform total RNA extraction using RNeasy Micro kit following manufacturer's protocol.

5. Complementary DNA (cDNA) is synthesized from total RNA using SuperScript III Reverse Transcriptase according to manufacturer's protocol.
6. Real-time PCR reactions are performed on a LightCycler 480 System using Light Cycler 480 SYBR Green I Master reaction mix and specific primers.
7. Thermocycling conditions: initial step of 10 min at 95 °C, then 50 cycles of 15 s denaturation at 94 °C, 10 s annealing at 60 °C and 15 s extension at 72 °C.
8. Reactions must be run in triplicate, and automatically detected threshold cycle (Ct) values are compared between samples.
9. Transcripts of the ribosomal protein L7 or GAPDH housekeeping genes can be used as endogenous control, with each unknown sample normalized to L7 or GAPDH content.
10. Primers of autophagy-related genes (mouse) (see Table1)

3.12 Transcriptional regulation of autophagy: Global gene expression analysis of quiescent satellite cells

1. After step 31 of the protocol for quiescent satellite cell isolation, satellite cells ($Sca1^-/CD11b^-/CD34^+/\alpha7\text{-integrin}^+$) are collected Eppendorf tubes with Lysis Buffer.
2. Extract total RNA using RNeasy Micro kit according to manufacturer's protocol.
3. Analyze transcriptome with Agilent SurePrint G3 Mouse GE 8x60K high-density microarray slides.
4. Raw data must be taken from the Feature Extraction output files and corrected for background noise using the normexp method.
5. Normalize data using cyclic loess, and identify differentially expressed genes using AFM 4.0[34] for all pairwise comparisons.
6. Use quantile normalization to assure comparability across samples.
7. Analyze differential expression analysis on non-control probes with an empirical Bayes approach on linear models (limma).

8. Results must be corrected for multiple testing according to the False Discovery Rate (FDR) method.
9. Statistical analysis can be done with the Bioconductor project (http://www.bioconductor.org/) in the R statistical environment.
10. Gene ontology analysis functional annotation using DAVID[35]
11. Venn diagrams can be generated using BioVenn[36].

3.13 Autophagy modulation by in vivo and in vitro treatments: Introduction

Autophagy is induced as a response to both extracellular stress conditions (nutrient deprivation, hypoxia, and oxidative stress) and intracellular stress conditions (endoplasmic reticulum stress, accumulation of damaged organelles, and aggregation of proteins). The large number of stimuli able to trigger autophagy implies the involvement of multiple signaling pathways in autophagosome formation.

3.14 Autophagy modulation by in vivo and in vitro treatments: Activation

Autophagy upregulation may have therapeutic benefits in a range of diseases. New research related to autophagy activators has become a hot topic owing to their potential clinical value[37]. We chose pharmacological and genetic methods for activation of autophagy:

- Pharmacological :

1- Rapamycin is a natural product with potent antifungal and immunosuppressive activities. It forms a complex with the immunophilin FK506-binding protein 12 (FKBP12), which then stabilizes the raptor-mTOR association and inhibits the kinase activity of mTOR. As an inhibitor of mTOR, rapamycin has been widely reported in the literature to induce autophagy both in vivo and in vitro[38].

For in vivo induction of autophagy in quiescent satellite cells:

Inject mice intraperitonally with Rapamycin (4 mg/kg) or vehicle every day for 2 weeks.

For in vitro induction of autophagy in satellite cells:

Add Rapamycin (100 ng/ml final concentration) or vehicle (DMSO) in cell media for 48 h.

2- Spermidine is a polyamine compound found in citrus fruit and soybean, which has recently been shown to increase lifespan of yeast, nematodes and flies in an autophagy-dependent manner[39]. Spermidine acts as an acetylase inhibitor that stimulates autophagy independently of SIRT1 and mTOR in human and yeast as well as in nematodes[38,40].

For in vivo induction of autophagy in quiescent satellite cells:

Treat mice with Spermidine (3 mM final concentration) in drinking water for 2 weeks. Water should be changed every two days.

For in vitro induction of autophagy in satellite cells:

Add Spermidine (5 μM) or vehicle (DMSO) in cell media for 48 h.

See Note 7.

- Genetic: LV-Atg7

The autophagy-related genes and their products are named as ATG and Atg, respectively. Once the pagophore has been formed, the membrane structure expands to sequester materials to form autophagosome; this process is mediated by two ubiquitin-like conjugation systems, the Atg12-Atg5 and Atg8 conjugation systems. Of these Atgs, Atg7 is an E1-like activating enzyme that activates Atg12 for its conjugation with Atg5 as well as the Atg8 family proteins for their conjugation with phosphatidylethanolamine. Thus, Atg7 is essential for autophagosome formation[41].

Ex vivo infection of freshly-isolated quiescent satellite cells with LV-ATG7[26]

1. After step 31 of the protocol for quiescent satellite cell isolation, satellite cells ($Sca1^-/CD11b^-/CD34^+/\alpha7\text{-integrin}^+$) are collected in Eppendorf tubes with 500 μl of Growth Medium (GM).

2. Coat petri dishes for cell culture with Coating Solution (0.1mg/mL of Collagen in sterile water and 0.02 N acetic acid).
3. Plate sorted satellite cells in coated petri dishes and culture them with GM.
4. Centrifuge petri dishes at 50 g for 5 min
5. After 22 h, coat new petri dishes with Retronectin (10 μg/mL) for 2 h at 37ºC
6. Recover Retronectin and wash with 2% BSA for 30 min at RT.
7. Wash with HBSS, 2,5% Hepes 1M.
8. Transfer satellite cells from Collagen to Retronectin-coated dishes.
9. Add LV-Atg7[26] or LV-control (10^5 pfu, final concentration) in cell media
10. Centrifuge petri dishes at 50 g for 5 min.
11. Culture cells with Lentivirus O/N.
12. Change cell media.
13. At that point, infected satellite cells can be transplanted in vivo or cultured in vitro for new experiments.

See Note 8.

3.15 Autophagy modulation by in vivo and in vitro treatments: Inhibition

Autohagy could potentially be suppressed at any stage of autophagic flux. To do so, many chemical inhibitors have been identified and used in various cells and animal models. However, most chemical inhibitors of autophagy are not entirely specific, and caution should be taken when interpreting the findings obtained with the use of these compounds, especially regarding their dose and incubation time[37]. We chose pharmacological methods for inhibition of autophagy:

1- Bafilomycin A1. Vacuolar-type H (+)-ATPases (V-ATPases) are found within the membranes of many organelles including lysosomes, endosomes, and secretory vesicles, where they play a variety of roles crucial for organelle function. Bafilomycin A1 is a specific inhibitor of V-ATPases in cells, which inhibits the acidification of

lysosomes and endosomes and consequently the activity of their enzymes that are active at acidic pH. Bafilomycin A1 was reported to prevent maturation of autophagic vacuoles by inhibiting the fusion between autophagosomes and lysosomes; however, contradictory results have also appeared; nevertheless, this alternative effect does not affect the interpretation of the autophagic flux[37].

Bafilomycin A1 is used at 10 nM for 4 h at 37ºC.

2- Chloroquine. Chloroquine is a lysosomal lumen alkalizer and is used to block the autophagic progress by imparing lysosomes[37].

Cholorquine is used at 50 mg/mL for 4 h at 37ºC.

3- Protease inhibitors. The lysosome is the ultimate degradative autophagic compartment in the cell. Protease inhibitors block autophagy at the step of degradation of cytoplasm enclosed in lysosomes, and causes accumulation of autolysosomes and/or many cytoplasmic inclusions in the central vacuoles. Leupeptin is a naturally occurring protease inhibitor that inhibits cysteine, serine and threonine peptidase.

Lysosomal cathepsins, which are enclosed in lysosomes, help maintain the homeostasis of the cell's metabolism by participating in the degradation of autophagic bodies. E64d and pepstatin A are two autophagy inhibitors that function by suppressing lysosomal proteases. E64d is a membrane-permeable inhibitor of cathepsin B, H, and L, whereas pepstatin A is an inhibitor of cathepsin D and E[37].

Cocktail of protease inhibitors used: Pepstatin A 10 μM, Leupeptin 100 μM, E64d 10 μM, for 4 h at 37ºC.

3.16 Mouse models useful for monitoring autophagy in quiescent satellite cells: GFP-LC3 transgenic mice

The GFP-LC3 transgenic mouse was first described by Mizushima et al., (2004)[42]. Detection of GFP-LC3 is not only simple but also generally highly specific. Specificity for GFP-LC3 puncta was previously determined by several studies. This transgenic

mouse contains an enhanced GFP (EGFP)-LC3 cassette inserted between the CAG promoter (cytomegalovirus immediate-early (CMV) enhancer and chicken β-actin promoter) and the SV40 late polyadenylation signal[43]. GFP-LC3 fragment is randomly integrated into the mouse genome, but does not affect the functions of other genes[44].

For initial experiments, it is recommended to maintain GFP-LC3 mouse colony in heterozygosis, because in order to distinguish true GFP-LC3 signals from background autofluorescent signals, it is important to compare transgenic mice with wild-type (non-fluorescent) siblings. After finishing the initial experiments (or if littermate control is not necessary), GFP-LC3 mice can be maintained in homozygosis[43].

See Note 9.

3.17 Mouse models useful for monitoring autophagy in quiescent satellite cells: Specific deletion of Atg7 gene in muscle stem cells

1- Constitutive deletion. Atg7-floxed mouse ($Atg7^{f/f}$) was previously described in Komatsu et al., 2005[41]. To examine the consequences of deleting Atg7 in satellite cells from the embryonic stage, we bred $Atg7^{f/f}$ mice with transgenic mice expressing the Cre recombinase under the control of the Pax7 promoter[45].

2- Inducible deletion. For inducible deletion of the Atg7 gene in Pax7-expressing cells (satellite cells), $Atg7^{f/f}$ mice were bred with transgenic mice expressing the Cre recombinase under the control of the Pax7 promoter, and in the presence of tamoxifen[46].

Tamoxifen administration: one injection per day for 4 days of 5 mg/25 g body weight. (10 mg/ml in corn oli).

4- NOTES:

1. Which is the best indicator of autophagy? The amount of LC3-II, the LC3-II/LC3-I ratio or LC3-II/(LC3-I+LC3-II) ratio is now used. Levels of LC3-II should be compared preferably to tubulin or any other housekeeping protein instead to LC3-I.

2. Quiescent satellite cells have low cytoplasm content, and autophagosomes are hard to discern in such small cytoplasm. For this reason, high objectives with good resolution are necessary. In addition, doing several z-sections can improve their visualization. One needs to be able to see the increased number of autophagosomes in Bafilomycin-treated samples (see Figure 3a). Video resconstructions of autophagosomes can be generated in Imaris software using full confocal z-stacks of each satellite cell. Z-stacks must be previously imported to Fiji software for background adjustments and then deconvolved using the blind-deconvolution wizard of Huygens software. Eventually, endogenous LC3 might also be detected by immunofluorescence with antibodies against LC3, but the sensitivity is much lower and detection in most of the cases is not feasible. Whenever possible it is recommended to use GFP-LC3 mice.

3. Co-localizations can be determined on digital images Fiji, according to[47], with respect to the total cellular area. The Pearson's coefficient (r) is used to analyze the correlation of the intensity values of green and red pixels in dual-channels images. This coefficient measures the strength of the linear relationship between the intensities in two images calculated by linear regression and ranges from 1 to -1, with 1 standing for complete positive correlation.

4. For analysis of quiescent satellite cells in muscle section, we need to use a satellite cell marker, such as Pax7, for its identification (Figure 4a). Analysis of autophagosomes in quiescent satellite cells on muscle sections is very difficult because of the small cytoplasm and the localization adjacent to the myofiber (Figure 4a). This analysis would be an additional/complementary approach, but not a definitive one. Once more, to improve our analysis, we can use other antibodies as autophagy markers, such as LAMP-1, p62 and ubiquitin.

5. MFI refers to the fluorescence intensity of each event (in average) of the selected cell population, in the chosen fluorescence channel.

6. With this tandem construct, autophagosomes and autolysosomes are labeled with yellow (i.e., mRFP and GFP) and red (i.e., mRFP only) signals, respectively (Figure 4c). Results can be expressed as the percentage of yellow or red puncta from total (yellow+red) puncta. In Bafilomycin-treated samples, yellow puncta should be increased.

7. To assess autophagy induction, Bafilomycin A1 treatment (4 h at 37ºC) is necessary to observe the increase in autophagosome accumulation.

8. To assess rapidly autophagy induction by LV-ATG7 infection, GFP-LC3 satellite cells can be infected, and the increase in GFP-LC3 fluorescence can be monitored by flow cytometry between samples with or without Bafilomycin A1 treatment. Alternatively, in other cell types, LV-Beclin 1 has also been used as a genetic activator of autophagy[48,49].

9. Some cautions should be taken regarding the use GFP-LC3 transgenic mice. GFP-LC3 can be incorporated into protein aggregates independently of autophagy[50]. LC3 localization should be carefully interpreted in cells having protein aggregates or inclusion bodies, such as cells defective in autophagy machinery[21,41].

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TABLES:

Table1:

Gene	Forward primer	Reverse primer
Atg7	TCTGGGAAGCCATAAAGTCAGG	GCGAAGGTCAGGAGCAGAA
Map1lc3	TTATAGAGCGATACAAGGGGGAG	CGCCGTCTGATTAT

Beclin1	ATGGAGGGGTCTAAGGCGTC	TCCTCTCCTGAGTTAGCCTC
Bnip3l	TTGGGGCATTTTACTAACCTTG	TGCAGGTGACTGGTGGTACTAA
Atg12	TTCGCTCCACAGCCCATTTC	TCCCCGGAACGAGGAACTC
Atg4b	ATTGCTGTGGGGTTTTTCTG	AACCCCAGGATTTTCAGAGG
Cathepsin	GTGGACTGTTCTCACGCTCAAG	TCCGTCCTTCGCTTCATAGG
p62	CCCAGTGTCTTGGCATTCTT	AGGGAAAGCAGAGGAAGCTC
Bnip3	TTCCACTAGCACCTTCTGATGA	GAACACCGCATTTACAGAACAA
Vps34	TGTCAGATGAGGAGGCTGTG	CCAGGCACGACGTAACTTCT
Gabarapl1	GGACCACCCCTTCGAGTATC	CCTCTTATCCAGATCAGGGACC

FIGURE LEGENDS:

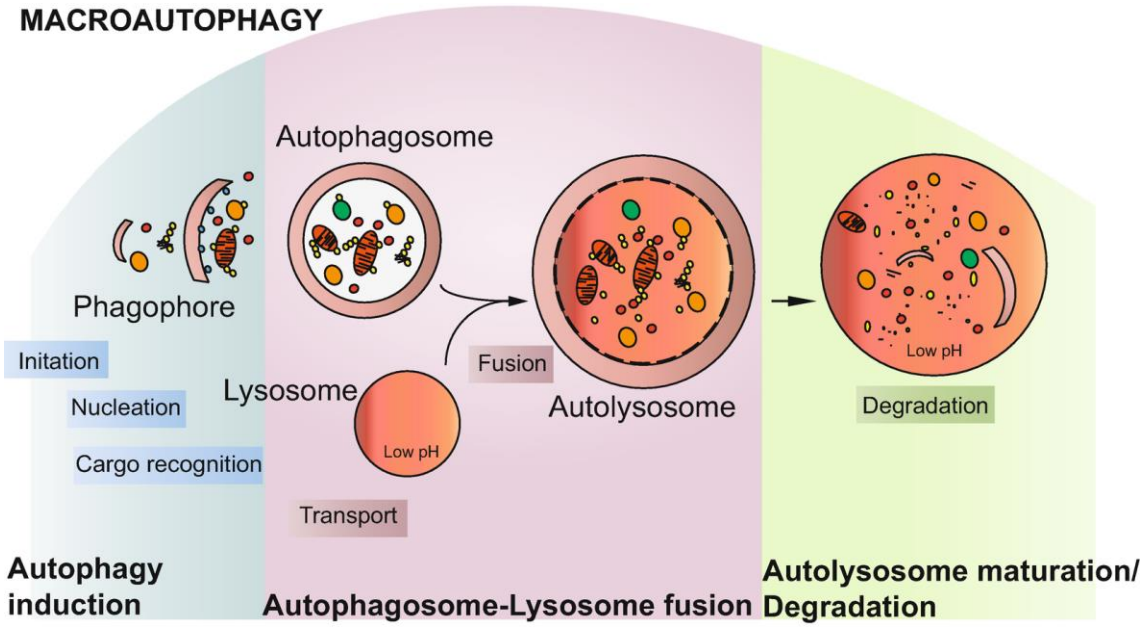

Figure 1. The process of autophagic flux.

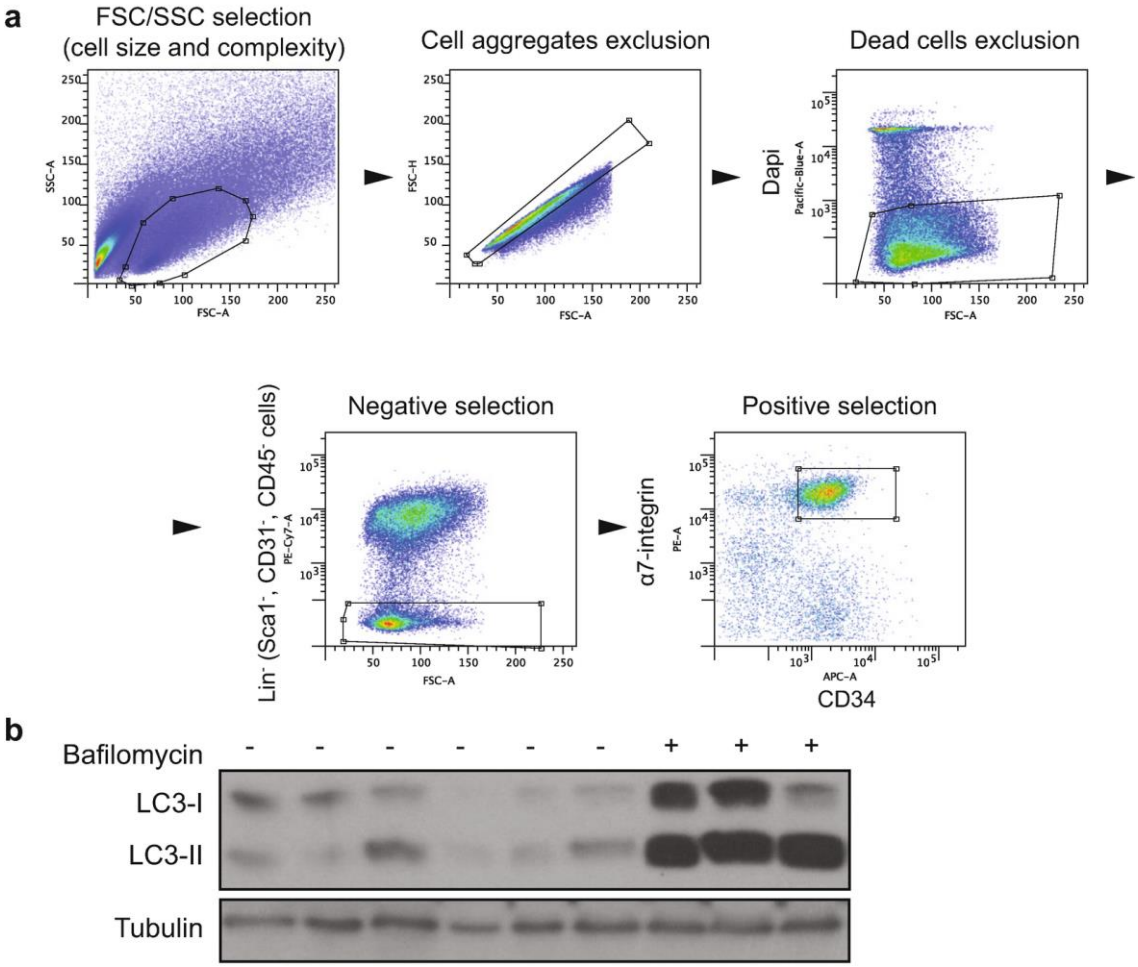

Figure 2. **a**, Representative example of the FACS strategy and gating scheme to isolate quiescent satellite cells from resting muscles of wild type (WT) mice. **b,** LC3 and Tubulin protein quantity by western blot analysis of young WT satellite cells treated with Bafilomycin A1 or vehicle for 4 h prior to analysis.

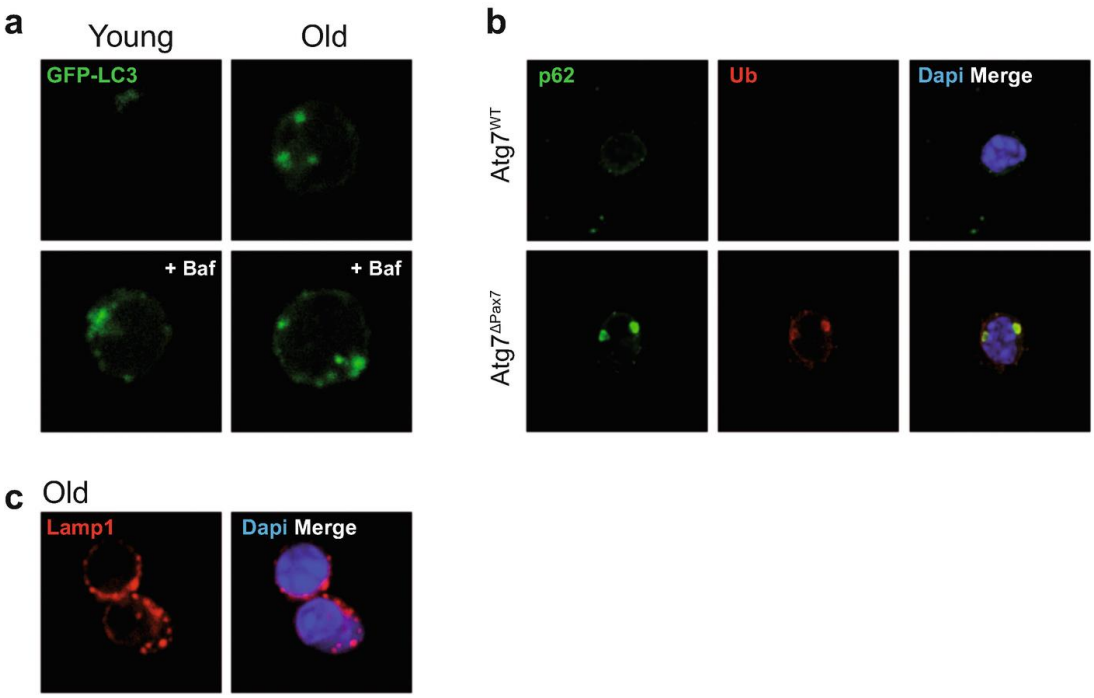

Figure 3. **a**, GFP-LC3 autophagosomes in quiescent satellite cells. Z projections. **b**, p62 and Ub immunostaining in quiescent satellite cells freshly isolated from $Atg7^{WT}$ and $Atg7^{\Delta Pax7}$ mice. **c**, LAMP-1 immunostaining in quiescent satellite cells freshly isolated from old mice.

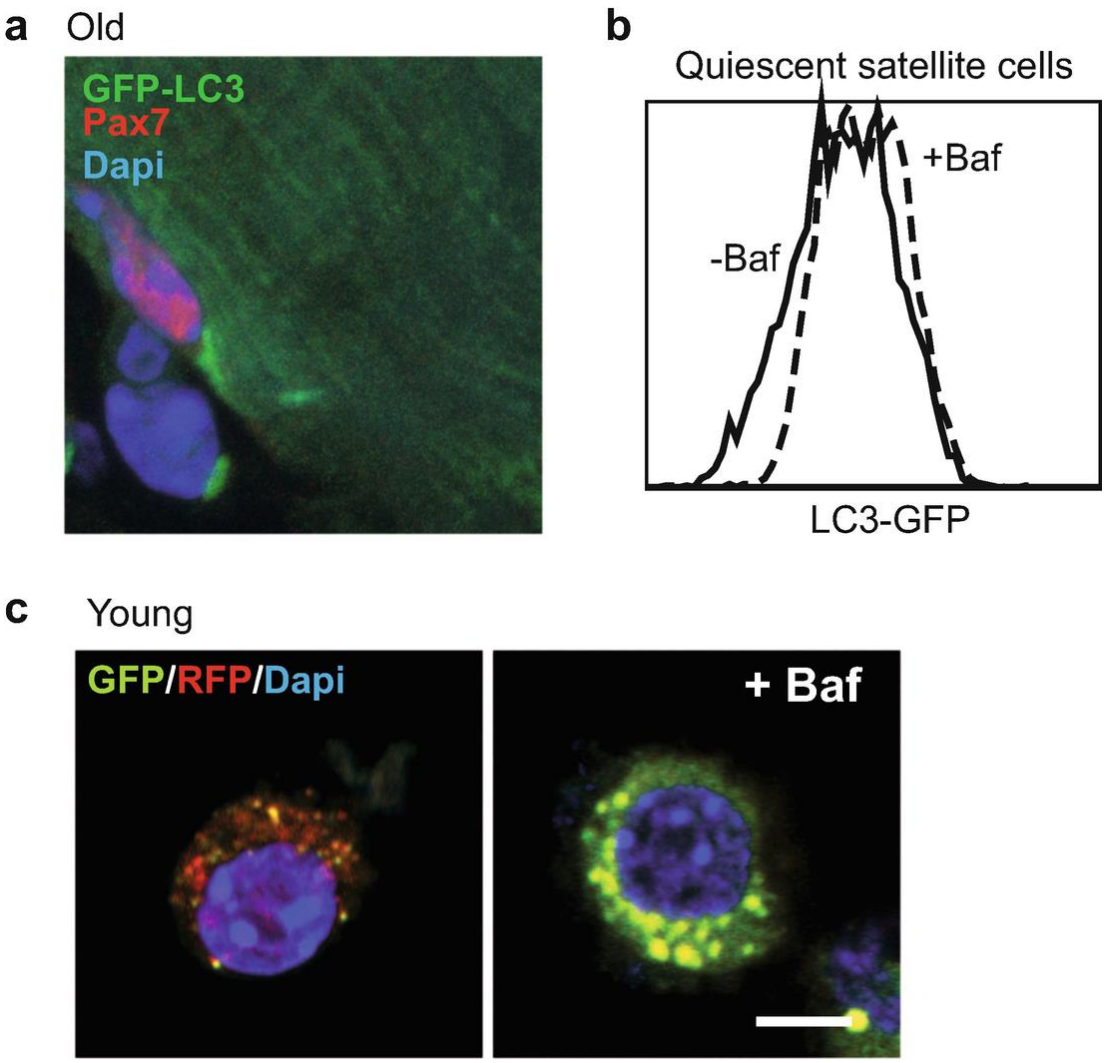

Figure 4. a, Pax7 and GFP immunostaining on tissue sections from resting TA muscles of old GFP-LC3 mice. **b**, Autophagy flux analyzed by flow cytometry in quiescent satellite cells isolated by FACS from young mice. Satellite cells were treated with Bafilomycin A1 (+Baf) or vehicle for 4 h prior to analysis. **c**, mRFP-GFP-LC3 plasmid was transfected into young satellite cells, for detecting autophagosomes (yellow signal) and their maturation into autolysosomes (red signal), and treated with Bafilomycin A1 or vehicle for 4 h prior to cell fixation. 3 z projections are shown.

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