

In vivo analysis of autophagy using GFP-LC3 transgenic mouse

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METHOD

Maintenance of GFP-LC3 transgenic mice

GFP-LC3 transgenic mice are recommended to be maintained as heterozygous for most experiments because it is important to compare the transgenic mice with wild-type littermates in order to distinguish the GFP-LC3 signals from autofluorescent signals.

Genotyping of GFP-LC3 transgenic mice

1. Cut 0.5 cm of the tail and put it in a 1.5 ml tube.
2. Add 100 μ l of tail digestion solution containing 0.2 mg/ml proteinase K.
3. Incubate the tails at 55°C overnight or 8 h.
4. Vortex and boil the tails for 5 min.
5. Centrifuge the tails at 15,000 $\times g$ for 10 min.
6. Mix the following components for PCR reaction: 1 μ l of the supernatant, 0.2 μ l of 100 μ M primer 1, 0.2 μ l of 100 μ M primer 2, 0.2 μ l of 100 μ M primer 3, 1.6 μ l of 2.5 mM dNTP mix, 2 μ l of 10x PCR buffer, 0.2 μ l of rTaq, and 14.6 μ l of distilled water.

Primer 1: 5'-ATAACTTGCTGGCCTTTCCACT-3'

Primer 2: 5'-CGGGCCATTTACCGTAAGTTAT-3'

Primer 3: 5'-GCAGCTCATTGCTGTTCTCAA-3'

7. Run the following PCR program:
 - a. Step 1: 94°C for 4 min
 - b. Step 2: 94°C for 0.5 min
 - c. Step 3: 60°C for 0.5 min
 - d. Step 4: 72°C for 1 min
 - e. Step 5: Repeat steps 2-4, 30 cycles
 - f. Step 6: 72°C for 1 min
 - g. Step 7: 4°C
8. Run 5 μ l of each reactant on a 2% agarose gel and decide genotypes based on band size (Figure 1). Primer 1 and 2 provides 250 bp band for a Tg

allele. Primer 1 and 3 provides 350 bp band for a non-Tg allele.

NOTE Genotyping can also be carried out by examining GFP signals by GFP macroscopy.

Nutrient starvation (optional)

To detect starvation-induced autophagosome formation, mice are deprived of food for 24 or 48 h (10 a.m. to 10 a.m.). They have free access to drinking water.

Tissue preparation and fixation

1. Anesthetize mice. We usually use 2% avertin (2,2,2-tribromoethanol) by intraperitoneal injection of about 0.125 ml for each 10 g of mouse body weight (250 mg/kg, i.p.).

2. Perfuse mice through the left ventricle with approximately 3 times the volume (body weight) of 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS). We usually use a peristaltic pump equipped with a 27G perfusion needle for whole body perfusion.

NOTE Fresh tissues can be directly dipped into PFA without whole body perfusion. In such case, however, quick fixation is required to avoid autophagy artificially induced during sample preparation.

3. Remove the tissues and post-fix them in the same PFA for an additional 4 h or overnight at 4°C.

4. Immerse the tissues with 15% sucrose in PBS for 4 h, followed by 30% sucrose in PBS for at least 4 h or overnight.

NOTE This step is important to prevent ice-crystal artifact during freezing.

5. Embed the tissues using OCT compound, freeze them on the surface of liquid nitrogen, and store them in -80°C.

Preparation of cryosections

1. Section the tissues at 5-7 μm thickness using cryostat at around -20°C.

NOTE The optimal cutting temperature must be determined for each tissue.

2. Collect a section on a glass slide and air dry it for 30 min at room temperature. They can be stored at -80°C or -20°C.

3. Wash the cryosections on a slide glass in PBS and remove excess PBS

from a slide glass.

NOTE The nuclei can be stained by incubating the cryosections with Hoechst in PBS (0.1 µg/ml) for 5 min. Wash them twice with PBS after staining.

NOTE Immunohistochemistry using antibodies can be performed before mounting if necessary.

4. Add 5-10 µl of *SlowFade* Gold antifade solution onto slides and mount them with cover glasses. Seal the edges of the cover glass with nail polish. Store them at 4°C.

Immunofluorescence microscopy of GFP-LC3

1. Select 60x (or 100x) oil-immersion objective lens. We usually use a standard wide-field fluorescence microscopy (Olympus IX81) equipped with a cooled charge-coupled device camera.

2. Place a slide glass on the stage and focus by transmitted light imaging (differential interference contrast). Select a dichroic filter set (GFP or fluorescein isothiocyanate [FITC]).

3. Take images. Fluorescent exposure should be as short as possible to prevent photo bleaching. Isolation membranes and autophagosomes can be detected as cup-shaped and ring-shaped structures positive for GFP-LC3.

Precautions about the interpretation of GFP-positive puncta

1. Cells frequently possess autofluorescent puncta including lipofuscin granules that can be detected by a green filter set. There are at least two methods to distinguish GFP-LC3 puncta from these autofluorescence signals (Mizushima et al. (2004)). One method is to take control images using an unrelated filter set (RFP or Cy5) in addition to images through a green (GFP or FITC) filter set. True GFP-LC3 signals should be detected specifically by a green filter set but not by an unrelated filter set (Figure 3). Another method is to compare samples from GFP-LC3 mice with those from wild-type littermates. Specific GFP-LC3 signals must be detected only in samples from GFP-LC3 mice.

2. GFP-LC3 and endogenous LC3 are sometimes incorporated into intracellular protein aggregates, such as inclusion bodies formed in

autophagy-deficient neurons, hepatocytes, or senescent fibroblasts, or those induced by polyQ expression independently of autophagy (Kuma et al. (2007)). In neonatal *Atg5^{-/-};GFP-LC3⁺* mice, GFP-LC3 puncta were not detected in most tissues including the heart (Figure 4F). However, larger and intense GFP-LC3 puncta appeared in some tissues such as the liver, dorsal root ganglion, and pituitary gland (Figure 4B, D, and H). Moreover, overexpression of GFP-LC3 itself can generate aggregates. Because these protein aggregates and inclusion bodies are frequently ubiquitinated, it is useful to stain ubiquitin to distinguish them from autophagosomes (Kuma et al. (2007)). Additional methods using electron microscopy should be considered to identify autophagosomes.

3. The number of GFP-LC3 puncta at steady state can be reflected not only by autophagosome formation (induction), but also by autophagosome degradation. For example, suppression of lysosomal degradation can increase the number of GFP-LC3-positive autolysosomes. On the other hand, rapid fusion of autophagosomes with lysosomes can result in a decrease of the number of GFP-LC3 puncta. Thus, autophagy flux should be measured by administering a lysosomal inhibitor such as chloroquine or colchicine, the latter of which is a microtubule depolarizing agent that suppresses autophagosome maturation to autolysosomes (please see "Overview" by Mizushima). Immunostaining of lysosomal proteins such as Lamp1 is useful to distinguish autophagosomes from autolysosomes.

MATERIALS

GFP-LC3 transgenic mice

GFP-LC3#53 mice (C57BL/6 background, RBRC No. RBRC00806; MSM background, RBRC01227; BALBc background, RBRC01413; C3H background, RBRC01414) published in Mizushima et al. (2004) are distributed from Riken BioResource Center (http://www2.brc.riken.jp/cache_all/RBRC00806). Background strain of GFP-LC3 mice is C57BL/6J Jcl.

REAGENTS

- Potassium chloride (Wako, 163-03545)
- Tris (hydroxymethyl) aminomethane (Nacalai, 35434-21)

- Magnesium chloride hexahydrate (Wako, 135-00165)
- Tween-20 (MP biomedical, 103168)
- NP-40 (Nacalai, 23640-94)
- Proteinase K (Sigma, P6556)
- TaKaRa rTaq (TakaRa, R001A)
- Avertin (2,2,2-Tribromoethanol, Sigma, T48402)
- 2-methyl-2-butanol (Nacalai, 02730-35)
- *SlowFade*® Gold antifade (Life technologies, S36936)
- Hoechst 33342 (Wako, 346-07951)
- Sodium chloride (Wako, 197-01667)
- Disodium hydrogenphosphate 12-water (Wako, 196-02835)
- Potassium dihydrogen phosphate (Wako, 169-04245)
- 10x DPBS (Life technologies, 14200-075)
- Paraformaldehyde (Wako, 162-16065)
- Sodium hydroxide (Wako, 197-02125)

EQUIPMENT

- Peristaltic pump (ATTO, AC-2110)
- 27G perfusion needle (TERUMO, SV-27DL)
- Cryostat (CM3050 S, Leica, Deerfield, IL)
- Tissue-Tek OCT compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan)
- Thermal cycler (Biometra, T3000)
- Micro cover glass (Matsunami, 24 x 24 mm, 0.12 - 0.17 mm)
- Micro slide glass (Matsunami, S2441)
- Fluorescence microscope (Olympus, IX81) equipped with a 60X PlanApo N oil immersion lens (Olympus, 1.42NA), a charge-coupled device camera (Hamatsu Photonics, ORCA ER), U-MGFPHQ mirror unit for GFP observation, and U-MWIG2 mirror unit for checking autofluorescence.

REAGENT PREPARATION

Tail digestion solution

50 mM KCl, 10 mM Tris-HCl (pH8.4), 2.5 mM MgCl₂, 0.45% Tween-20, and 0.45% NP-40. Autoclave and store it at room temperature. Add 1/200 volume of

20 mg/ml proteinase K (final concentration, 0.2 mg/ml) before use.

2% (w/vol) avertin solution

For 80x (1.6 g/ml) stock solution, dissolve 1.6 g of avertin with 1 ml of 2-methyl-2-butanol overnight. Dilute 80x stock solution 1:80 with PBS and store it in dark bottle at 4°C.

PBS

For 25x PBS stock solutions, dissolve 2000 g of sodium chloride, 725 g of disodium hydrogenphosphate 12-water, 50 g of potassium chloride, and 50 g of potassium dihydrogen phosphate in 10 L of distilled water. Dilute 25x DPBS 1:25 with distilled water.

4% (w/vol) paraformaldehyde/PBS

For 5x stock solutions, dissolve 200 g of paraformaldehyde in 600 ml of distilled water and add 4 ml of 10N NaOH. The solution need to be carefully heated (use a stirring hot-plate in a fume hood) to dissolve. Adjust the volume to 1000 ml with distilled water and stored at -30°C. Aliquot to avoid repeated freeze and thaw. For 1x working solutions, thaw 5x stock solutions at 65°C and dilute 1:5 with PBS. For 10N NaOH preparation, dissolve 8 g of sodium hydroxide in 20 ml of distilled water.

TROUBLESHOOTING TIPS

1. No GFP-LC3 puncta are detected.

Take a positive control using the liver, heart, or skeletal muscles from GFP-LC3 mice starved for at least 24 h. A number of intense GFP-LC3 puncta can be clearly observed in these tissues. In some tissues such as the lens, however, expression level of GFP-LC3 is relatively low. In such cases, increase of the exposure time can clearly visualize GFP-LC3 puncta. Note that GFP-LC3 expression is extremely low in some cells such as endocrine pancreatic cells (islet cells), some blood cells (erythrocytes etc), and type I alveolar cells (Mizushima et al. (2004)).

2. No change of the number of GFP-LC3 puncta by starvation.

Autophagy in mice with starvation or fed *ad libitum* should be analyzed in the morning at the same time (ex. 10 a.m.) because mice do not eat food in the daytime, which causes mild starvation even in control mice.

3. GFP-LC3 signals disappear with ethanol fixation or paraffin processing. It is known that GFP loses fluorescence by ethanol treatment (Ward W. (2005)). GFP cannot tolerate ethanol dehydration, which is required for paraffin processing. In these cases, immunohistochemistry of LC3 or GFP should be considered.

REFERENCES

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Mizushima N., Yamamoto A., Matsui M., Yoshimori T., and Ohsumi Y. (2004). In vivo analysis of autophagy in response to nutrient starvation using transgenic mice expressing a fluorescent autophagosome marker. *Mol. Biol. Cell* 15, 1101–1111.

Ward W. (2005). Biochemical and physical properties of green fluorescent protein. In: *Green fluorescent protein*. 2nd edition ed. by M. Chalfie and S. R. Kain. Wiley Interscience 39–65.

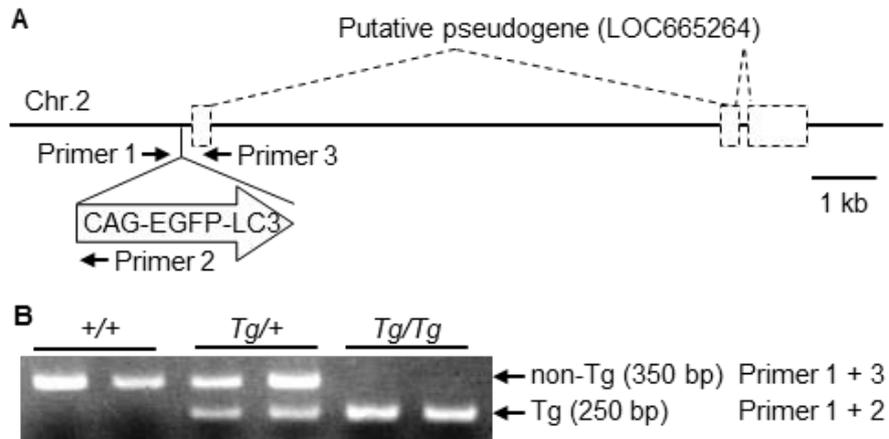


Figure 1. Genotyping of GFP-LC3 mice by PCR. (A) CAG-EGFP-LC3 is inserted into chromosome 2. Positions of Primers 1 to 3 are indicated as arrows. (B) Representative band patterns of wild-type (+/+), heterogenous (*Tg*/+), and homogenous (*Tg*/*Tg*) GFP-LC3 mice are shown. These images were modified from data published in Kuma et al. (2008).

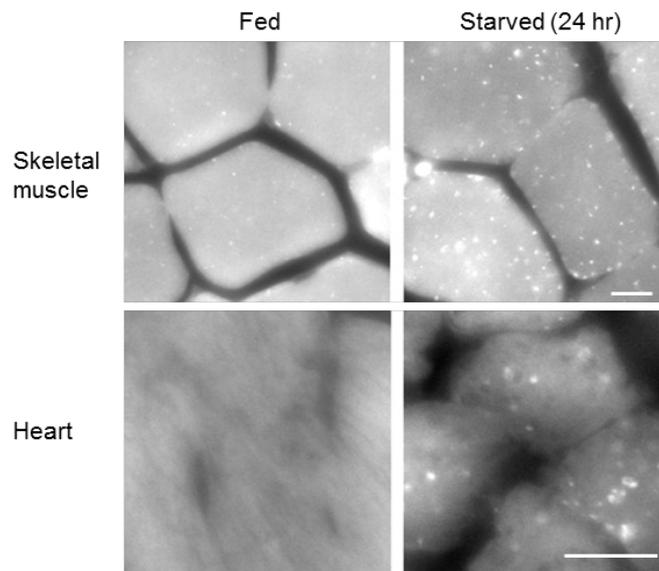


Figure 2. Autophagy in muscle tissues of GFP-LC3 mice. GFP signals in transverse sections of skeletal muscle (extensor digitorum longus muscles) and heart muscles at 0- and 24-h starvation are shown. Bar, 10 μ m. These images were modified from data published in Mizushima et al. (2004).

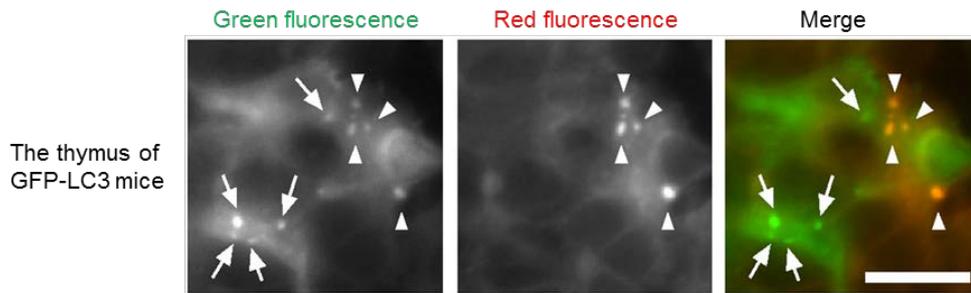


Figure 3. Difference of GFP-LC3 and autofluorescence signals. The medulla of the thymus of GFP-LC3 mice was analyzed for green and red fluorescence using a GFP and RFP filter set, respectively. GFP-LC3-specific signals (arrows) can be distinguished from autofluorescence signals (arrowheads). Bar, 10 μm . These images were modified from data published in Mizushima et al. (2004).

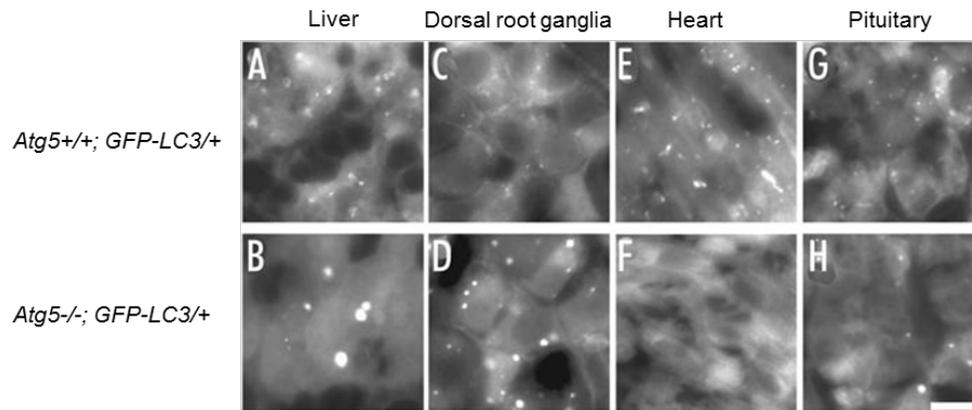


Figure 4. GFP-LC3 signals in wild-type and *Atg5*^{-/-} neonatal mice. Several organs were prepared from 6-h *Atg5*^{+/+}; *GFP-LC3*^{+/+} or *Atg5*^{-/-}; *GFP-LC3*^{+/+} neonates. Large and intense GFP-LC3 signals were detected even in autophagy-deficient liver (B), dorsal root ganglia neurons (D), and anterior lobe of the pituitary gland (H). Bar, 10 μ m. These images were modified from data published in Kuma et al. (2007).