

Autophagy-monitoring and autophagy-deficient mice

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ABSTRACT

Discovery of yeast autophagy-related (*ATG*) genes and subsequent identification of their homologs in other organisms have enabled researchers to investigate physiological functions of macroautophagy/autophagy using genetic techniques. Specific identification of autophagy-related structures is important to evaluate autophagic activity, and specific ablation of autophagy-related genes is a critical means to determine the requirements of autophagy. Here, we review currently available mouse models, particularly focusing on autophagy (and mitophagy) indicator models and systemic autophagy-related gene-knockout mouse models.

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Introduction

In the past decade, knowledge of the physiological roles of autophagy in mammals has increased. Autophagy plays important roles in many physiological and disease processes, including starvation adaptation, quality control of intracellular proteins and organelles, development, tumor suppression, prevention of neurodegeneration, and immunity.¹ For analysis of autophagy in vivo, 2 types of gene-modified mouse models have played critical roles: “autophagy-monitoring mice” and “autophagy-deficient mice.” Monitoring the autophagic process and measuring autophagic activity are critical to investigate the function of autophagy. In the past, electron microscopy was mainly used to monitor autophagy because specific autophagosome markers were lacking. Soon after the identification of MAP1LC3/LC3 (microtubule-associated protein 1 light chain 3), a mammalian homolog of yeast Atg8, as an autophagosome marker,² transgenic mice systemically expressing EGFP-LC3 (GFP-LC3 mice) were generated.³ By using GFP-LC3 mice, observation and quantification of autophagy in vivo became drastically simplified, and this transgenic mouse line has been widely used for monitoring autophagy.

Another tool that has greatly contributed to understanding the role of autophagy in vivo is mice deficient in specific *Atg* genes. It is notable that analysis of autophagy-deficient mice has led to conceptual findings in this field. For example, the concept of “intracellular quality control” has been established based on the observation of mice with neuron-specific and liver-specific deletion of *Atg5* or *Atg7*. The importance of autophagy in quality control of proteins and organelles is difficult to observe in yeast, probably due to dilution of damaged cellular components because of their rapid cell division. In mouse models, autophagy deficiency in the brain and liver causes accumulation of ubiquitinated proteins, damaged organelles,

and autophagy-specific substrates such as SQSTM1/p62. These findings suggest that autophagy is critical for preventing the toxic accumulation of damaged proteins and organelles in mammals. In addition, autophagic dysfunction has been implicated in many pathological processes including human diseases. Here, we discuss the advanced utilization of autophagy-monitoring and autophagy-deficient mice for analysis of the physiological roles of autophagy in vivo.

Monitoring and measuring autophagy in vivo

The ‘core’ *ATG* genes

From yeast genetic studies, 41 *ATG* genes have been identified. Among them, *ATG1* through *ATG10*, *ATG12* through *ATG14*, *ATG16* through *ATG18*, *ATG29*, and *ATG31* are required for efficient autophagosome formation. These 18 ‘core’ *ATG* genes consist of several functional units; (i) Atg1 kinase and its regulators (Atg1, Atg13, Atg17, Atg29, Atg31), (ii) the phosphatidylinositol 3-kinase (PtdIns3K) complex (Vps30/Atg6, Atg14), (iii) the Atg12 conjugation system (Atg5, Atg7, Atg10, Atg12, Atg16), (iv) the Atg8 conjugation system (Atg3, Atg4, Atg7, Atg8), (v) Atg9, and (vi) the Atg2-Atg18 complex. Many of these proteins are conserved in mammals, and identification of the corresponding genes provides the means to assess autophagic activity and study the role of autophagy. There are several steps for the complete pathway of autophagy: initiation, phagophore expansion, autophagosome maturation, fusion with the vacuole/lysosome, cargo degradation in the lysosome and efflux. Therefore, it is important to assess autophagic activity at each step or to monitor flux (from initiation to degradation). Among the *ATG* proteins, LC3, one of the Atg8 homologs in mammals, is established as an autophagosome marker and

many of the 'core' *Atg* genes have been knocked out for understanding the physiological role of autophagy in mammals.

GFP-LC3 transgenic mice

Mammals have several counterparts of yeast *ATG8*, one of the core *ATG* genes; MAP1LC3A, MAP1LC3B, MAP1LC3B2, MAP1LC3C and GABARAP, GABARAPL1, GABARAPL2. The LC3 and GABARAP family proteins are particularly useful for monitoring autophagy. Among the proteins in this family, LC3B is the most widely used marker. Precursor forms of the LC3 and GABARAP family proteins are specifically cleaved at the carboxyl terminus by ATG4 family proteins to form LC3-I (or GABARAP-I, etc.; we will refer exclusively to LC3 hereafter), which has an exposed carboxyl terminal glycine that is conjugated to phosphatidylethanolamine to form LC3-II. LC3-II is tightly bound to both the inner and outer surface of the

phagophore and autophagosomal membrane. When the autophagosome fuses with the lysosome, LC3-II on the outer membrane is again cleaved by ATG4 proteins and released from the membranes. By contrast, LC3-II on the inner membrane is degraded by lysosomal enzymes. Therefore, the autophagosome is monitored by tracking LC3.

Immunostaining or fluorescent tagging is used for detection of LC3. EGFP-tagged LC3 was the first probe used to monitor autophagy (Fig. 1).² Because LC3 is cleaved at the carboxyl terminus, EGFP should be fused at the N terminus of LC3. With this probe, autophagosomes are easily detected as fluorescent puncta or sometimes as ring-shaped structures. Although monitoring GFP-LC3 puncta is the most popular assay, it has a limitation. The fluorescent signal of GFP is quenched quickly under the low pH conditions in lysosomes. Therefore, it is difficult to determine how much GFP-LC3 is delivered to lysosomes unless quenching of GFP is inhibited by lysosomotropic reagents. Due

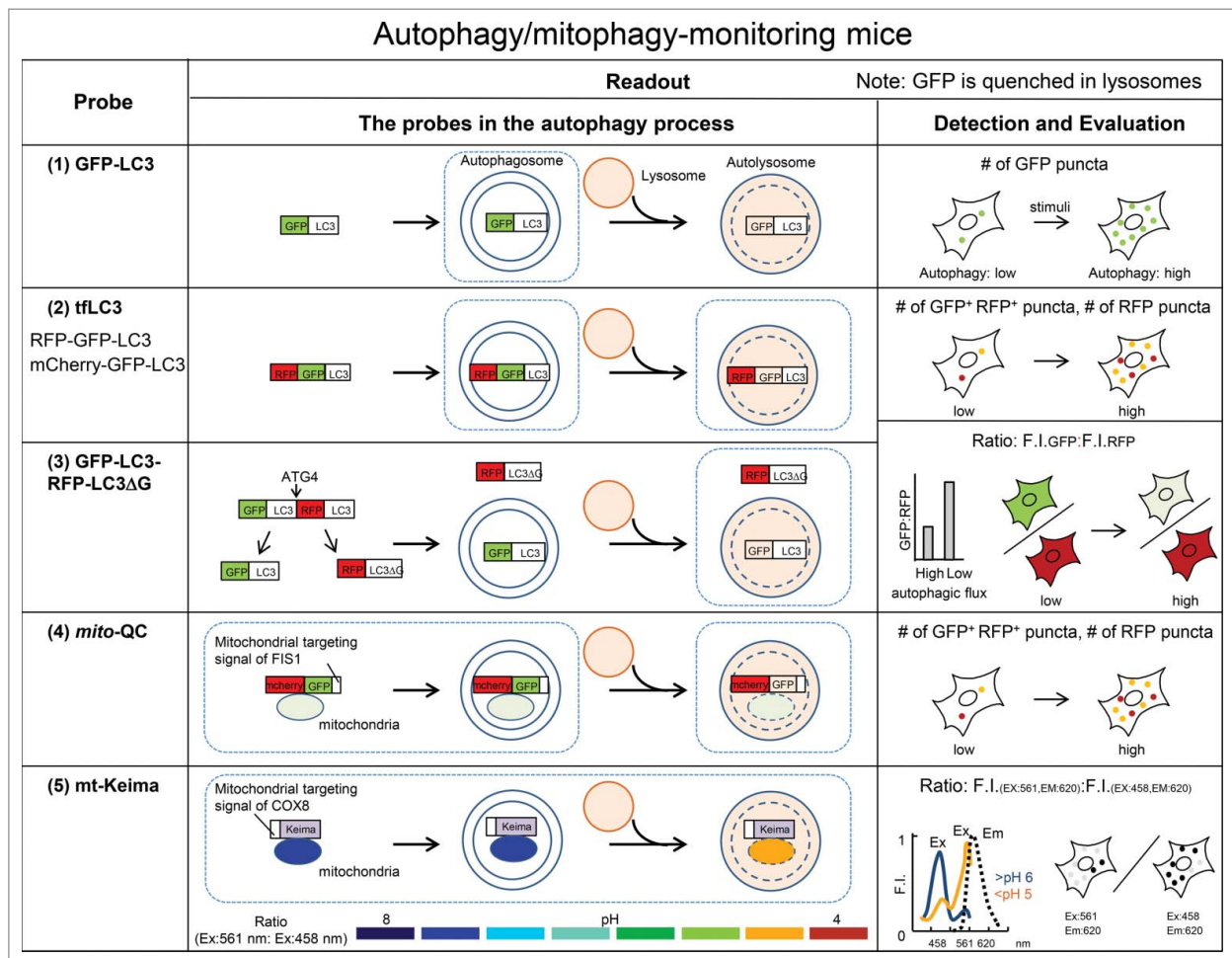


Figure 1. Transgenic mice expressing autophagy probes for monitoring autophagy. (1) GFP-LC3 mice express EGFP-LC3 for labeling phagophores and autophagosomes. The number of GFP puncta is counted before and after stimulation. (2) tflc3 mice express RFP-EGFP-LC3 or mCherry-EGFP-LC3 for measuring autophagy flux. The numbers of GFP⁺ RFP⁺ puncta (yellow) and RFP puncta (red) are counted. Yellow puncta represent phagophores and autophagosomes, and red puncta represent autolysosomes. (3) GFP-LC3-RFP-LC3ΔG mice express GFP-LC3-RFP-LC3ΔG for measuring autophagy flux. The reduction of the fluorescence intensity of GFP indicates autophagy flux because GFP-LC3 is degraded through autophagy (and quenched) as a substrate. The reduction of the fluorescence intensity of GFP-LC3 in the total cells is measured and corrected by the fluorescence intensity of RFP-LC3ΔG in the total cells as an internal control. (4) *Mito-QC* mice express mCherry-EGFP fused with the FIS1 mitochondrial targeting sequence for monitoring mitophagy. The numbers of GFP⁺ RFP⁺ (yellow) puncta and RFP puncta (red) are counted. Yellow puncta represent mitochondria, and red puncta represent mitochondria in the lysosomes. (5) *Mt-Keima* mice express Keima (pH-dependent fluorescent protein) fused with the COX8 mitochondrial-targeting sequence for monitoring mitophagy. *Mt-Keima* is excited predominantly by 458-nm light in a neutral environment (mitochondria), and by 561-nm light in an acidic environment (lysosome). The ratio of the 561-nm:458-nm excited Keima fluorescence intensity indicates the delivery of mitochondria to the lysosome. Squares with a blue dotted-line show the stages detected with the probes. F.I., fluorescence intensity.

to this limitation, a simple increase in the number of GFP-LC3 puncta is not sufficient to conclude that autophagy is activated. The increased number of autophagosomes could represent either induction of autophagy or impairment of autophagosome-lysosome fusion or lysosomal degradation. Therefore, it is critical to distinguish between these options by treatment with lysosomal inhibitors.

The use of EGFP-LC3 has also been applied to mice. Transgenic mice expressing GFP-LC3 in all tissues under the control of the constitutive *Cag* promoter have been widely used.³ The presence of autophagosomes in mouse tissues can be directly monitored by fluorescence microscopy analysis of cryosections. GFP-LC3 mice were validated by intercrossing with *Atg*-deficient mice.⁴ However, in contrast to cells in culture, it is more challenging to measure autophagy flux with transgenic mice. For example, chloroquine has been used for monitoring autophagic flux in mice, but chloroquine treatment is not as robust as has been demonstrated in culture cells, and the efficiency is altered among organs,⁵ probably due to detoxification of intraperitoneally administered inhibitors, barriers for their access into the tissues such as the blood-brain barrier, and other factors. Therefore, reporter mice expressing probes that could measure autophagic flux without using pharmacological inhibitors of lysosome function have been anticipated (see below).

TfLC3 transgenic mice

Because red fluorescent protein (RFP) is more resistant to quenching in the lysosome than EGFP, tandem fluorescent-tagged LC3 (tfLC3) reporters such as mRFP-EGFP-LC3 and mCherry-EGFP-LC3 have been developed as single-molecule probes to detect autophagosomes and autolysosomes specifically.⁶ In green and red merged images with this probe, autophagosomes are labeled with yellow (mRFP and GFP) and autolysosomes are labeled with red (mRFP only) signals. Therefore, the entire process of autophagy can be traced morphologically with this probe. When autophagy is activated, the numbers of both yellow (autophagosomes) and red (autolysosomes) puncta increase; by contrast, when autophagosome maturation into autolysosomes is blocked, only the number of yellow puncta (autophagosomes) increases.⁶

These probes have also been applied to *in vivo* assays by virus-mediated intracranial administration of tfLC3 and have been used to generate transgenic tfLC3 mice.⁷⁻⁹ tfLC3 mice were validated by examining the response to starvation and drugs such as rapamycin, which are established autophagy-inducing conditions. In the heart, autophagy is increased by ischemia-reperfusion injury through an oxidative stress-dependent mechanism.⁹ Also in renal proximal tubules, autophagy is activated after ischemia-reperfusion injury, which might be for clearance of damaged organelles and remodeling of tubular cells during renal repair.⁸

GFP-LC3-RFP-LC3ΔG transgenic mice

Another reporter model for assessing autophagic flux is GFP-LC3-RFP-LC3ΔG mice.¹⁰ The GFP-LC3-RFP-LC3ΔG probe was developed for accurate quantification of autophagic flux by detecting a reduction of GFP-LC3 signals in the lysosomes.

When expressed in cells, GFP-LC3-RFP-LC3ΔG is cleaved by endogenous ATG4, producing equimolar amounts of GFP-LC3 and RFP-LC3ΔG. GFP-LC3 on the autophagosomal inner membrane is delivered into the lysosome and quenched. By contrast, RFP-LC3ΔG is not lipidated due to lack of the carboxyl-terminal glycine, but remains in the cytosol and serves as an internal control. Thus, the GFP:RFP ratio is reduced if autophagic activity is enhanced. In the case of GFP-LC3 and tfLC3 probes, LC3 is used as a marker for labeling autophagosomal structures, and the readout is the number of GFP-LC3 and of RFP-LC3 puncta. In contrast, with the GFP-LC3-RFP-LC3ΔG probe, GFP-LC3 is used as a substrate for autophagic degradation (and quenching of the GFP signal), and the readout is the fluorescence intensity of GFP and RFP in the total cell population. The GFP:RFP ratio can be measured using a fluorescence microscope, flow cytometer, or microtiter plate reader.

The GFP-LC3-RFP-LC3ΔG probe can also be used to monitor autophagic flux *in vivo*. Zebrafish and mice expressing GFP-LC3-RFP-LC3ΔG were generated (the mouse model only expresses an adequate amount of the probe in muscles). Zebrafish expressing GFP-LC3-RFP-LC3ΔG were validated by intercrossing with *Atg*-deficient animals. Using this probe, it has been successfully observed that autophagy flux is induced by fertilization in both zebrafish and mouse eggs. One notable advantage of this probe is that it is capable of measuring basal autophagy activity. Because basal autophagic activity is usually very low and fluctuates at different time points dependent on nutrient availability and other factors, it is difficult to evaluate by a “snapshot” assay. This probe can overcome these inherent difficulties because it can measure basal autophagic flux as an overall cumulative index. The reporter mouse revealed that the level of autophagy is relatively high in fast-twitch type II fibers compared with slow-twitch type I fibers in the muscle of fed mice. As basal autophagy plays an important role in quality control *in vivo*, the ability to measure basal autophagy is a strong advantage of this probe.

Although both GFP-LC3-RFP-LC3ΔG and tfLC3 probes are available to measure autophagic flux without requiring the use of lysosomal inhibitors, which is a great advantage especially in mouse experiments, they also have their own limitations. A limitation of GFP-LC3-RFP-LC3ΔG is that the time base for analysis is in hours, because this probe quantifies autophagic flux as the reduction of the GFP signal in a whole cell. Conversely, the tfLC3 probe measures autophagic flux by detecting the number of tfLC3 puncta, which is clearly observed within 30 min after starvation treatment in cultured cells. Therefore, tfLC3 could be more suitable for measuring flux in short-term experiments.

It has been reported that tfLC3 is also available for measuring autophagic flux by detecting the GFP:RFP ratio, similar to GFP-LC3-RFP-LC3ΔG.^{11,12} However, the lysosome-targeted RFP of tfLC3 was not originally designed to be used as an internal control; RFP is less stable in the lysosome compared with that in the cytosol, especially in long-term experiments. Therefore, RFP-LC3ΔG should be more suitable for an internal control than tfLC3 for measuring the GFP:RFP ratio. It is important to choose an appropriate method and, ideally, a combination of at least 2 methods would be preferred.

Mitophagy-monitoring mice

Selective degradation of mitochondria by autophagy, termed mitophagy, can be a selective pathway to remove damaged mitochondria, and could be a major means of mitochondrial quality control. However, the importance of mitophagy *in vivo* remains unclear. So far, 2 mouse models have been developed for monitoring mitophagy: mt-Keima transgenic mice and *mito-QC* transgenic mice.^{13,14} A mitochondria-targeted form of Keima (mt-Keima), a ratiometric fluorescence probe, is engineered by fusing a tandem repeat of the COX8 pre-sequence with Keima.¹⁵ Keima is a lysosome-resistant fluorescent protein that is activated in a unique manner. mt-Keima is excited predominantly by 458-nm light in a neutral environment (mitochondria), and by 561-nm light in an acidic environment (lysosome). The ratio of the 561-nm:458-nm excited Keima fluorescence intensity indicates the delivery of mitochondria to lysosomes, i.e., the mitophagic activity.

Transgenic mice expressing mt-Keima have been generated.¹³ mt-Keima mice were validated by intercrossing *Atg7*-deficient mice and checking colocalization with organelles such as mitochondria and lysosomes in mouse embryonic fibroblasts and tissues. As lysosomal activity should be maintained to produce correct acidic signals, it is important to maintain tissue viability during microscopy observation. Using mice expressing mt-Keima, it has been shown that mitophagy can be induced by several stimuli/stresses such as hypoxia, mitochondrial DNA mutations, and tumors, and it can be reduced by high-fat diet, aging, and HTT (huntingtin) expression. In heart, mitophagy is transiently activated and then suppressed in response to pressure overload.¹⁶

Another probe for monitoring mitophagy is *mito-QC*.¹⁷ *Mito-QC* is engineered by fusing the mitochondrial targeting sequence of the outer mitochondrial membrane proteins FIS1 to a tandem mCherry-GFP tag (mCherry-GFP-FIS1; *mito-QC*). As with autophagosomes labeled with tFLC3, the cytoplasmic mitochondria display both red and green signals. Upon mitophagy, mitochondria are delivered to lysosomes and *mito-QC* produces only red signals. Transgenic mice harboring *mito-QC* in the *Rosa26* locus are generated.¹⁴ *mito-QC* mice were also validated by checking colocalization with organelles such as mitochondria, lysosomes, and autophagosomes in tissues. Validity was further confirmed by electron microscopy analysis in tissues. Using mice expressing *mito-QC*, it has been shown that basal mitophagic activity differs among tissues and cell types. The kidneys are a major site of mitophagy, and cardiomitophagy is activated during development in mice.¹⁴

Although both mt-Keima and *mito-QC* mouse models are valuable resources and their utility is based on a similar concept that mitophagy is detected by a change in pH, they have different advantages and limitations. The limitation of the mt-Keima mouse is that the Keima protein is incompatible with fixation, and freshly excised organs are required for observation. Therefore, this probe is incompatible with performing immunohistochemical experiments. Another limitation of mt-Keima is that the excitation spectra between acidic and neutral environments are not completely separated, but this problem will be improved by genetic alterations in the structure of Keima in the future.

There is a growing interest in the physiological and pathological role of mitophagy. Assessing mitophagic activity in diseased states in which mitophagy is thought to be involved (e.g.,

neurodegenerative diseases and heart diseases) by crossing the mitophagy monitoring-mice with pathological mouse models may provide valuable insights into the role of mitophagy *in vivo*.

Analysis of the role of autophagy using autophagy-deficient mice

Analysis of *Atg* gene knockout mice has greatly contributed to understanding the physiological roles of autophagy *in vivo*. There are approximately 20 core autophagy-related genes involved in autophagosome formation in mammals. So far, 14 of them have been knocked out in mice (Table 1, Fig. 2). These *Atg* knockout mice die at different stages: (1) some die *in utero*, (2) some are born alive but die within 1 d of birth, and (3) some show no obvious abnormalities. *atg4b*^{-/-}, *atg4c*^{-/-}, *lc3b*^{-/-}, *gabarap*^{-/-}, *ulk1*^{-/-}, and *ulk2*^{-/-} mice show no obvious (or weak) phenotypic effects. This is probably because these *Atg* genes have homologs that function redundantly. Mice deficient in nonredundant genes involved in the ATG conjugation systems and in *ulk1*^{-/-} *ulk2*^{-/-} (double-knockout mice) were neonatal lethal, and mice deficient in nonredundant genes not involved in the ATG conjugation systems were embryonic lethal. The reasons for the phenotypic differences between *Atg* mutant mice are not well understood. Some of the autophagy receptors involved in selective autophagy have also been knocked out in mice.

Deletion of *Atg* genes in the conjugation systems

Systemic knockout mice of *Atg* genes in the conjugation system

The *Atg12* and *Atg8* conjugation systems consist of 8 *Atg* proteins in yeast (*Atg3*, *Atg4*, *Atg5*, *Atg7*, *Atg8*, *Atg10*, *Atg12*,

Table 1. Mouse defects associated with *Atg* gene knockouts (systemic knockout mouse).

<i>Atg</i> gene	Survival time	Phenotypes	Ref. of the phenotypes
<i>Becn1</i>	E8.5	Defects in proamniotic canal closure, (spontaneous tumors in heterozygotes)	(28,29)
<i>Pik3c3/Vps34</i>	E8.5	Fail to form mesoderm, reduced cell proliferation	(30)
<i>Atg9a</i>	Embryonic lethal	Growth retardation	(31)
<i>Rb1cc1</i>	E14.5-E16.5	Defective heart and liver development	(27)
<i>Atg13</i>	E17.5	Growth retardation Defective heart development	(26)
<i>Ulk1/2</i>	Neonatal lethal	Impaired lung function Die within one d after birth, morphologically normal, reduced amino acids levels, suckling defect	(25)
<i>Atg3</i>	Neonatal lethal		(17)
<i>Atg5</i>	Neonatal lethal		(18)
<i>Atg7</i>	Neonatal lethal		(19)
<i>Atg12</i>	Neonatal lethal		(20)
<i>Atg16l1</i>	Neonatal lethal		(21)
<i>Atg4b</i>	Viable	Balance dysfunction	(54)
<i>Atg4c</i>	Viable	Increased susceptibility to carcinogen-induced fibrosarcomas	(16)
<i>Map1lc3b</i>	Viable	No obvious abnormality	(14)
<i>Gabarap</i>	Viable	No obvious abnormality	(15)
<i>Ulk1</i>	Viable	Increased reticulocyte number, delayed mitochondrial clearance	(55)
<i>Ulk2</i>	Viable	No obvious abnormality	(56)

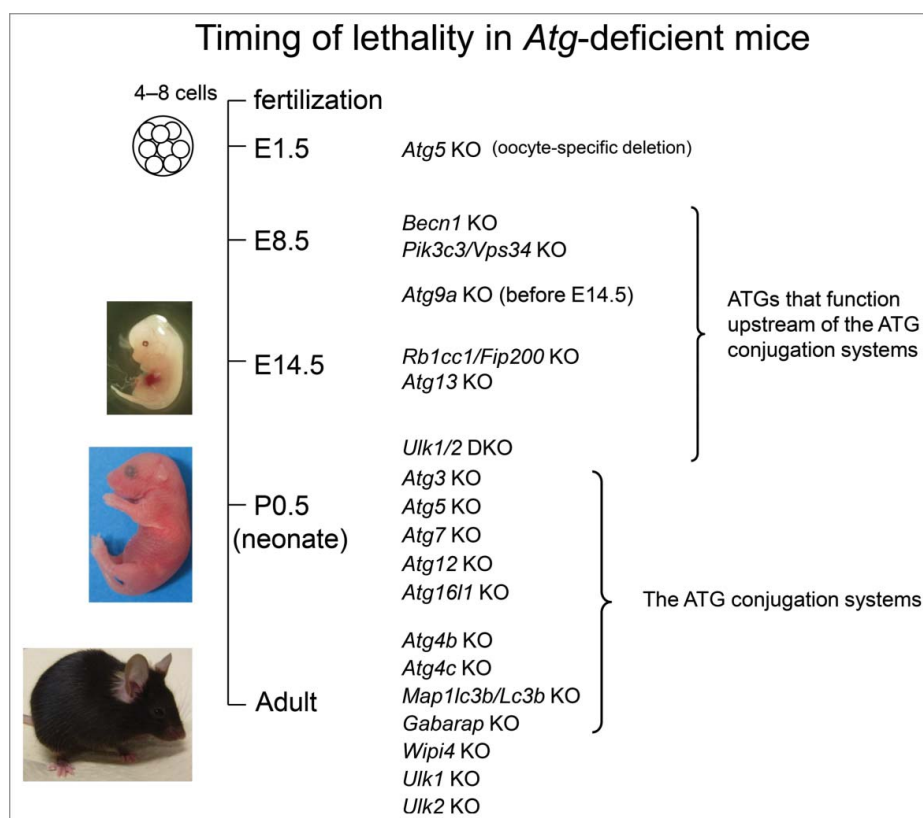


Figure 2. Timing of lethality in autophagy-deficient mice. Mice deficient in genes involved in the ATG conjugation system (*atg3*^{-/-}, *atg5*^{-/-}, *atg7*^{-/-}, *atg12*^{-/-}, *atg1611*^{-/-}) and *ulk1/2* DKO mice are born with normal morphology but die within 1 d after birth. Mice deficient in genes functioning upstream of the ATG conjugation system (*rb1cc1*^{-/-}, *atg13*^{-/-}, *becn1*^{-/-}, *uvrag*^{-/-}, *pik3c3/vps34*^{-/-}, *atg9a*^{-/-}) die in utero. Others (*atg4b*^{-/-}, *atg4c*^{-/-}, *lc3b*^{-/-}, *gabarap*^{-/-}, *ulk1*^{-/-}, *ulk2*^{-/-}) show no obvious (or weak) phenotypic effects.

Atg16). Mice deficient in each of these proteins (except *Atg10*) have been generated. Among them, *lc3b*^{-/-}, *gabarap*^{-/-}, *atg4b*^{-/-}, and *atg4c*^{-/-} mice show no obvious (or weak) phenotypic effects.^{18–20} Mice deficient in nonredundant genes involved in the 2 conjugation systems (*atg3*^{-/-}, *atg5*^{-/-}, *atg7*^{-/-}, *atg12*^{-/-}, *atg1611*^{-/-}) are born developmentally normal but die within 1 d of birth.^{4,21–24} Autophagy is massively induced immediately after birth as an adaptation response to starvation due to the sudden interruption of the transplacental nutrient supply.⁴ During this neonatal starvation period, amino acid supply through autophagic degradation of ‘self’ proteins is important for the maintenance of amino acid levels.^{4,21,22} However, the exact cause of death had remained unknown. Recently, it was reported that neuronal dysfunction caused by autophagy deficiency, resulting in suckling failure, is the primary cause of the neonatal lethality of *Atg5*-null mice; neuron-specific expression of ATG5 can rescue these mice.²⁵ Neonates defective in autophagy suffer from and die as a result of a combination of serious malnutrition due to a lack of self-derived nutrients and suckling failure. Therefore, autophagy plays 2 critical roles in neonates: prevention of neuronal dysfunction and adaptation to starvation.

It has been considered that neonatal lethality is the phenotype of autophagy-deficient mice because it is commonly observed in mice deficient in *Atg* genes encoding proteins involved in the conjugation systems; however, another possibility has emerged. Recently, it was reported that autophagosomes could be formed even in the absence of the ATG conjugation

systems, although at a reduced rate.²⁶ The autophagosomes in ATG conjugation system-deficient cells could fuse with lysosomes; however, degradation of the inner membrane of autophagosomes was significantly delayed. Thus, even though autophagic activity in ATG conjugation system-deficient cells is strongly suppressed, a very low level of autophagic activity might remain, leading to a milder phenotype observed in ATG conjugation system-deficient mice compared with those with deletion of upstream *Atg* genes, such as *Rb1cc1* or *Atg13* (Fig. 2).²⁶

Systemic *Atg7* and *Atg5* knockout in adult mice

For physiological analysis, conditional tissue-specific *Atg* gene knockout mice have been mainly used, because embryonic or neonatal lethality is observed in conventional *Atg* gene knockout mice. However, it is also possible to investigate autophagy in the whole body in adult mice by using conditional *Atg*-deficient mice in combination with *Ubc-CreERT2* or *atg5*^{-/-}; *Eno2-Atg5* mice.^{25,27} *Ubc-CreERT2* mice are used for tamoxifen (TAM)-inducible Cre-mediated deletion of floxed genes throughout the body. By administration of TAM to *Atg7*^{+/F}; *CreERT2* mice at 8 wk of age, a near-complete and sustained loss of ATG7 protein is accomplished in 2 wk. By 6–12 wk after deletion at 8 wk of age, many histological abnormalities are observed in *atg7*^{Δ/Δ} mice, and more than 90% of these mice die within 12 wk after deletion. The majority of *atg7*^{Δ/Δ} mice die because of neurodegeneration or bacterial infection. *atg7*^{Δ/Δ} mice have reduced body size and body weight with less fat and

lean body mass. Liver abnormalities, splenic enlargement, testicular degeneration, low white adipose tissue, degenerative changes in muscle, small myofibers, and pancreatic damage are observed in these animals. These abnormalities are also observed in *atg5*^{-/-}; *Eno2/NSE-Atg5* mice (*Eno2* is a neuron-specific promoter) and other tissue-specific *Atg* knockout mice. In the brain, the numbers of pyramidal neurons and Purkinje cells are decreased, and progressive motor and behavioral deficits are observed. In addition, *atg7*^{Δ/Δ} mice cannot survive 24-h fasting due to severe hypoglycemia. Therefore, both adult and neonatal mice require autophagy for adaptation to starvation.

Another mouse model deleting *Atg* in the whole body in adult mice corresponds to the *atg5*^{-/-}; *Eno2-Atg5* mice referred to above. *atg5*^{-/-}; *Eno2-Atg5* mice were generated to rescue *Atg5*-null mice from neuronal damage and neonatal lethality.²⁵ Expression of exogenous ATG5 only in the brain under control of the *Eno2* promoter is sufficient to rescue *Atg5*-null mice. The majority of these rescued mice survive for between 8 wk and 8 mo. Although the cause of lethality in *atg5*^{-/-}; *Eno2-Atg5* mice has not been established, the wide window of death indicates that these mice might die due to several reasons besides abnormalities in the brain, which is the main cause of death of *atg7*^{Δ/Δ} mice. In histological analyses, *atg5*^{-/-}; *Eno2-Atg5* mice show similar abnormalities to *atg7*^{Δ/Δ} mice. Additional previously uncharacterized abnormalities observed in *atg5*^{-/-}; *Eno2-Atg5* mice are morphological changes related to inflammation in many tissues (liver, small intestine, kidney, lung, adipose tissues, spleen, lymph nodes), atrophy in organs regulated by sex hormones (testis, seminal vesicle, ovary, uterus, pituitary gland, salivary gland), and iron-deficiency anemia. SQSTM1/p62 (sequestosome 1), a selective substrate of autophagy, accumulated in all organs examined at 8 wk of age. Accumulation of phosphorylated SQSTM1 causes hyperactivation of the transcription factor NFE2L2/NRF2 (nuclear factor, erythroid derived 2, like 2); however, significant increases in phosphorylated SQSTM1 and NFE2L2 are observed only in the liver and skeletal muscle. Ubiquitinated proteins clearly accumulate only in the liver, heart, skeletal muscle, and pancreas, suggesting that autophagy-dependent protein quality control is important in these organs. This finding also indicates that there may be causes, other than accumulation of phospho-SQSTM1 and ubiquitinated proteins, for the abnormalities observed in other organs. Utilization of these mouse models will provide new insights into the physiological roles of autophagy.

Deletion of *Atg* genes not involved in the conjugation systems

ULK1/2 DKO mice die during the neonatal period similar to the ATG conjugation system-deficient mice.²⁸ Lung function is impaired in *ULK1/2* DKO neonates. All mice deficient in *Atgs* that are not part of the ATG conjugation systems (*atg13*^{-/-}, *rb1cc1*^{-/-}, *atg9a*^{-/-}, *becn1*^{-/-}, *vps34*^{-/-}), except *ULK1/2* DKO animals as noted above, die at different stages of embryonic development. *atg13*^{-/-} mice die at embryonic d 17.5 (E17.5) of development with myocardial growth defects, which are similar to those observed in *rb1cc1*^{-/-} mice.²⁹ *rb1cc1*^{-/-} mice die at E14.5 with abnormalities of the heart and liver.³⁰ In cultured fibroblasts, sensitivity to TNF (tumor necrosis factor)-induced

apoptosis is enhanced by deletion of *Atg13* or *Rb1cc1*. *becn1*^{-/-} mice die at E8.5 and *becn1*^{+/-} mice show a high incidence of spontaneous tumors such as lymphomas, hepatocellular carcinomas, and adenocarcinoma of the lung.^{31,32} *pik3c3/vps34*^{-/-} mice die at E8.5 with severely reduced cell proliferation.³³ *atg9a*^{-/-} mice also die in utero.³⁴ These findings show that timing of lethality and phenotypes are non-uniform among these mutants and clearly different from the phenotypes observed in mice lacking the ATG conjugation systems.

One explanation for these phenotypic differences could be that these proteins are multifunctional. ULK1/*Atg1* has important roles in neuronal function.³⁵ In *Caenorhabditis elegans*, the *unc-51 (atg1)*-mutant displays defects in axonal elongation and transport, and uncoordinated movement.³⁶⁻³⁹ These defects are considered to be independent of autophagy as they are not observed in other *atg* mutants or following silencing of *Atgs*. The importance in neuronal function of ULK has also been reported in *Drosophila melanogaster* and mammals.⁴⁰⁻⁴² RB1CC1 is also a multifunctional protein and regulates cell size, proliferation, migration, and apoptosis by interacting with PTK2, PTK2B, TSC1, TP53, MAP3K5, and TRAF2.⁴³⁻⁴⁵ BECN1 is involved not only in autophagy as part of a complex with ATG14, PIK3C3/VPS34, and PIK3R4/p150, but also in regulation of the endocytic pathway as part of a complex with UVRAG, PIK3C3/VPS34, and PIK3R4.⁴⁶ PIK3C3/VPS34 plays roles in membrane trafficking, phagocytosis, cytokinesis, and nutrient sensing.⁴⁷ These non-autophagic functions would be essential for embryogenesis. Another explanation could be that embryonic lethality is the true phenotype of complete suppression of autophagy by deletion of these *Atg* genes, which function upstream of the ATG conjugation systems as discussed above.

Deletion of receptors involved in selective autophagy

Although autophagy was originally characterized as a nonselective bulk degradation system, under certain conditions autophagosomes engulf cytosolic materials selectively. This type of autophagy, termed selective autophagy, is classified according to its cargo: aggrephagy, mitophagy, xenophagy, reticulophagy, ferritinophagy, and glyco-phagy for aggregate proteins, surplus and damaged mitochondria, invasive microbes and viruses, endoplasmic reticulum, ferritin, and glycogen respectively.⁴⁸ Selective autophagy modulates the quality and quantity of organelles and removes cytotoxic structures, and also regulates the amount of specific proteins such as SRC kinase, a key component of focal adhesions, and KEAP1 (kelch-like ECH-associated protein 1), an adaptor component of a CUL3 (cullin 3)-based ubiquitin E3 ligase, because both are substrates of autophagy. Specific receptors ensure autophagic selectivity. When autophagic cargo such as damaged mitochondria appear in the cytoplasm, they may be tagged with ubiquitin, leading to assembly of receptor proteins that bind to both marker molecules and ATG8 family proteins around the cargo. Some receptors such as SQSTM1 and OPTN are phosphorylated during selective autophagy, which enhances their binding to labeled cargo.⁴⁹⁻⁵² Gene expression of several receptors with a transmembrane region such as BNIP3L/NIX is induced under certain conditions, and the gene products are directly targeted to

Table 2. Mouse defects in receptors involved in selective autophagy.

Receptors	Types of selective autophagy	Phenotypes of systemic knockout mice	Reference
SQSTM1/p62	Aggrephagy, mitophagy, xenophagy, pexophagy, specific proteins (e.g., KEAP1)	Mature-onset obesity	(57,58)
NBR1	Aggrephagy, pexophagy	Age-dependent increase in bone mass and bone mineral density	(59)
TOLLIP	Aggrephagy	Less production of the proinflammatory cytokines after low dose of IL1B/IL-1 β and LPS treatment	(60)
OPTN	Mitophagy, xenophagy	Susceptible to infection with <i>Salmonella</i> , <i>Citrobacter</i> -induced colitis, and <i>E. coli</i> -induced peritonitis	(61,62)
CALCOCO2/ NDP52	Mitophagy	—	—
SMURF1	Mitophagy, xenophagy	Age-dependent increase in bone mass accompanied by enhanced osteoblast activity	(63)
BCL2L13	Mitophagy	—	—
BNIP3L/NIX	Mitophagy	Anemia with reduced mature erythrocytes and compensatory expansion of erythroid precursors	(64,65)
BNIP3	Mitophagy	Decreased ischemia-induced myocardial apoptosis	(66)
FUNDC1	Mitophagy	—	—
FAM134B	Reticulophagy	Neurodegeneration in peripheral sensory nerves	(67)
NCOA4	Feritinophagy	Mild microcytic hypochromic anemia associated with iron overload	(68,69)
TAX1BP1	Xenophagy	Age-dependent inflammatory cardiac valvulitis and skin dermatitis, premature death, and hypersensitivity to low doses of TNF and IL1B	(70)
TECPR1	Aggrephagy, mitophagy, xenophagy	No gross phenotypic abnormalities	(71)
STBD1	Glycophagy	—	—
CBL	Specific protein (active SRC)	Changes in hemopoietic profiles, splenomegaly, extensive extramedullary hemopoiesis, and mammary duct hyperplasia	(72)
TRIMs	Specific proteins	Refer to ref. 73	(73)

specific cargo. After the translocation of these receptors to their cargo, some core ATG proteins such as RB1CC1 also recognize the targets—though detailed molecular mechanisms largely remain unclear—beginning the process of autophagosome formation around the targets.^{49,53} Thus, cargo labeling as well as the transfer of receptors to cargo are essential for selective autophagy. To date, a large number of receptors have been identified in higher eukaryotes, and systemic knockout mice for more than 10 genes encoding receptors have been generated and analyzed. In contrast to *Atg* gene-null mice, receptor-knockout mice that have been produced to date are viable and exhibit relatively mild phenotypes. The phenotypes of these knockout mice for representative receptors are summarized in Table 2.

Future studies

Although autophagy-monitoring and autophagy-deficient mice have led to a huge increase in knowledge about the functions of autophagy in mammals, many aspects of the physiological roles of autophagy in vivo remain unclear. For example, the role of autophagy in nutrient metabolism is still controversial. The concept that autophagy is important for nutrient supply during starvation was proposed long ago; however, the exact role of autophagy has yet to be established. Selective organelle degradation by autophagy such as mitophagy has been considered to be related to disease, but the physiological significance is still largely unknown. Taking advantage of mouse models will help us to understand these issues.

Autophagy-deficient mice show various abnormalities in various organs. For example, apoptosis and neurodegeneration

are observed in the brain, whereas proliferation/tumor growth was observed in the liver. Autophagy is important for quality control and elimination of proteins and organelle, but loss of this function might affect different organs in different ways. For example, although ablation of autophagy causes accumulation of SQSTM1 in both liver and brain, the phenotypes observed in the liver, but not in the brain, are largely depends on the accumulation of SQSTM1. Likewise, sensitivity to accumulation of damaged mitochondria and misfolded proteins due to impaired autophagy might be different among organs. These tissue-specific functions of autophagy should be investigated in more detail in the future.

In the meantime, it has become apparent that the autophagy machinery is not as simple as previously thought. The core *Atg* gene knockout mice show different phenotypes, but the underlying mechanisms are not elucidated. Some, or probably all, ATG proteins may have autophagy-independent functions, whereas some ATG proteins may not be essential even for canonical autophagy. These findings have led to an important notion that we may not be able to determine the specific function of autophagy simply by deletion of one *Atg* gene. Confirmation of results using another model system would be helpful. Nevertheless, these valuable mouse models are essential for analysis of the role of autophagy.

Abbreviations

ATG	autophagy-related
CreERT2	tamoxifen-inducible Cre recombinase
DKO	double knockout
EGFP	enhanced green fluorescent protein

Em	emission
Ex	excitation
PtdIns3K	phosphatidylinositol 3-kinase
RFP	red fluorescent protein
TAM	tamoxifen
tfLC3	tandem fluorescent-tagged LC3
TNF	tumor necrosis factor

Disclosure of potential conflicts of interest

The authors declare that they have no conflicts of interest related to the contents of this article.

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