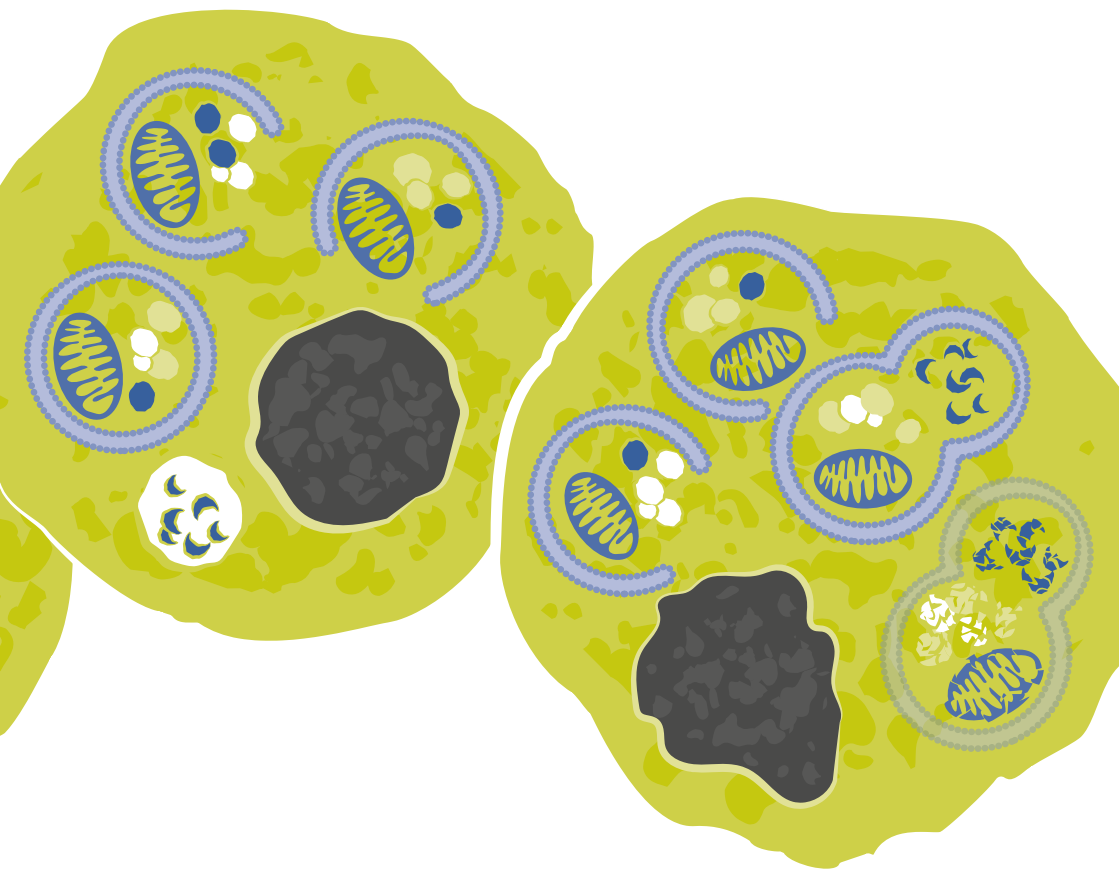


Tools for cell death series 2

Autophagy



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Introduction to cell death

Cell death happens when a biological cell fails to maintain essential life functions. This may be the result of the natural process of development or protection against cellular defects, of normal cells replacement, or factors such as disease or localized injury.

Cell death can be classified according to its morphological appearance (such as apoptotic or necrotic), enzymological criteria (with or without involvement of distinct proteases), functional aspects (programmed or accidental) or immunological characteristics (immunogenic or non-immunogenic).

In this guide, we want to examine the role of autophagy, a self-degradation process that has been historically linked to non-apoptotic cell death.

Autophagy

Autophagy (from the Greek “*auto*”-self and “*phagein*”-to eat) is a general term for processes by which dysfunctional cellular components are degraded inside the cell through the action of lysosomes. Components of the cytoplasm, including organelles, are sequestered and moved into the lumen of lysosomes/vacuoles where they are broken down into basic components and returned to the cytosol for reuse.

Under physiological conditions, autophagy has a number of vital roles such as maintenance of the amino acid pool during starvation, preimplantation development, clearance of intracellular microbes, and regulation of innate and adaptive immunity (Mizushima *et al.*, 2010).

Autophagy has been widely implicated in many pathophysiological processes such as cancer, metabolic and neurodegenerative disorders as well as cardiovascular and pulmonary diseases. It also has an important role in aging and exercise (Choi *et al.*, 2013).

One of the characteristic features of autophagy is its dynamic regulation; cellular autophagic activity is usually low under basal conditions, but there are a number of stimuli that can lead to its upregulation. Nutrient starvation is the most well-known inducer of autophagy, but other biological stress stimuli such as hypoxia or energy depletion, hormonal stimulation or pharmaceutical agents can increase autophagy in the cells as well.

Autophagy is a common morphological feature in dying cells, and therefore it was presumed for a long time to be a cell death pathway. Recent investigations show that one of its major functions is to work on keeping cells alive under stressful conditions that could otherwise lead to cell death.

Types of autophagy

There are currently three types of autophagy in mammalian cells: macroautophagy, microautophagy and chaperone-mediated autophagy, macroautophagy being the most extensively studied type ([Glick *et al.*, 2010](#)).

Macroautophagy

Macroautophagy is the main autophagic pathway, generally referred to as autophagy. It is characterized by the delivery of cytoplasmic cargo to the lysosome through an intermediary double membrane-bound vesicle, known as autophagosome, which fuses with the lysosome to form an autolysosome.

Throughout this guide we will review the cellular and molecular mechanism involved in macroautophagy (referred to as autophagy from now on), as well as methods and assays which can be used to monitor autophagy.

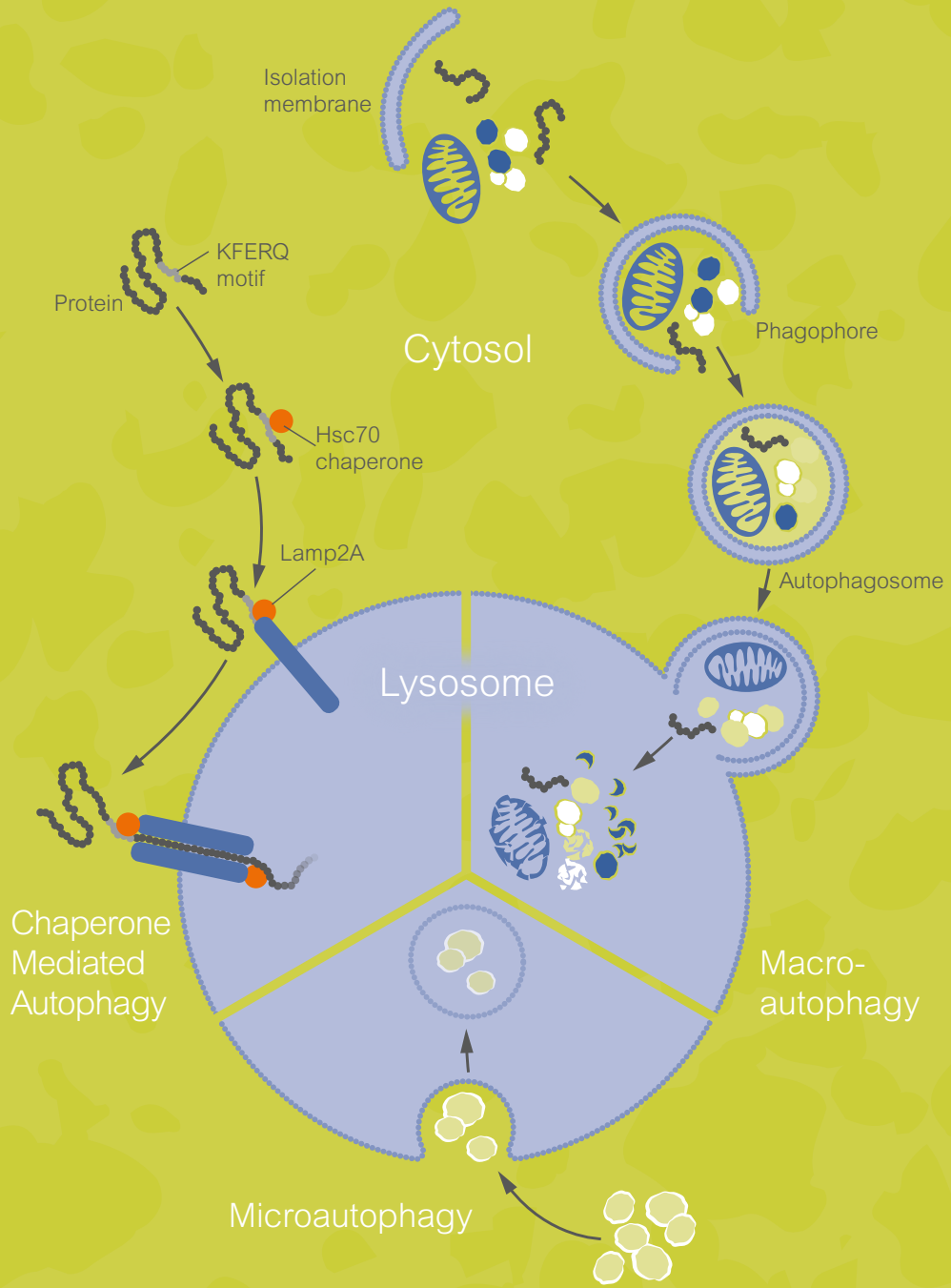
Microautophagy

Microautophagy, on the other hand, involves the direct engulfment of cytoplasmic cargo into the lysosome through invagination of the lysosomal membrane. Microautophagy is important in the maintenance of organellar size, membrane homeostasis and cell survival under nitrogen restriction ([Li *et al.*, 2012](#)).

Chaperone-mediated autophagy

Chaperone-mediated autophagy (CMA) involves the direct translocation of cytoplasmic proteins across the lysosomal membrane in a complex with chaperone proteins that are recognized by the lysosomal membrane receptor LAMP-2A (lysosomal-associated membrane protein 2A), resulting in their unfolding and degradation.

Figure 1. Types of Autophagy

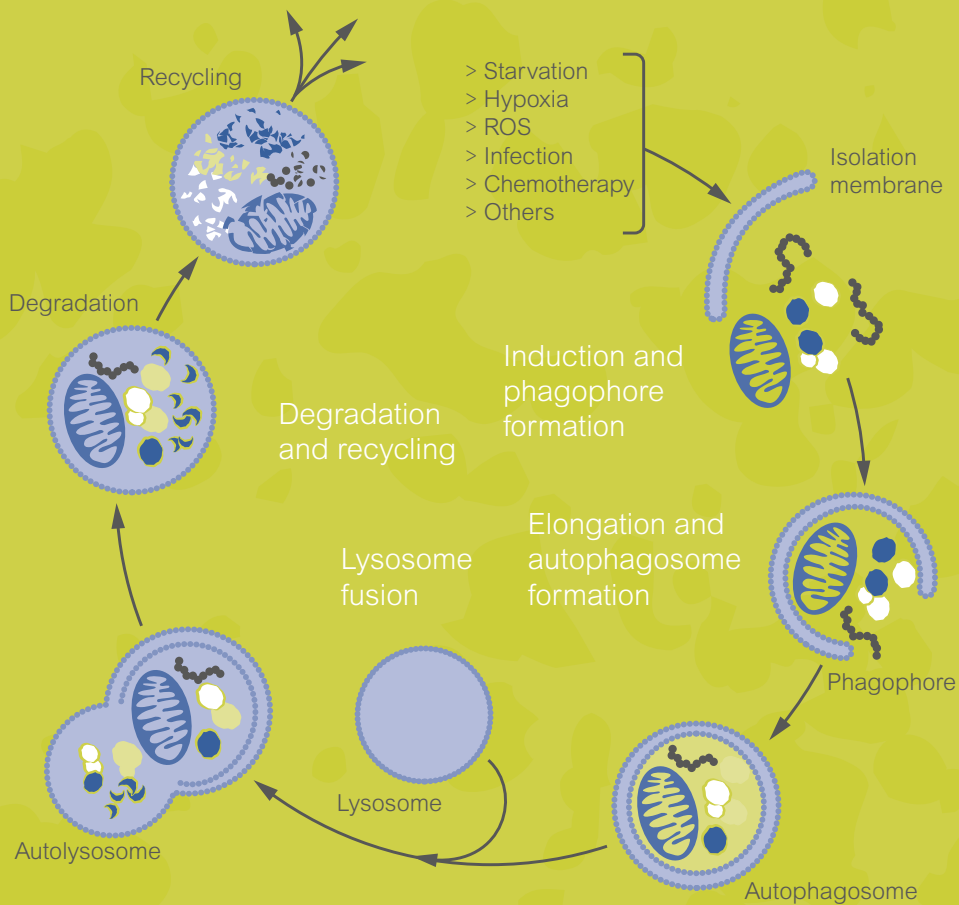


Autophagy: Cellular and molecular mechanisms

Autophagy is a tightly regulated pathway which at basal level has an important housekeeping role allowing cells to eliminate damaged or harmful components through catabolism and recycling to maintain nutrient and energy homeostasis. Autophagy is also a major protective mechanism which allows cells to survive in response to multiple stress conditions such as: nutrient or growth factor deprivation, hypoxia, reactive oxygen species, DNA damage or intracellular pathogens ([Levine & Kroemer, 2008](#)).

The process of autophagy begins with an expanding membrane structure termed the phagophore which enwraps portions of the cytoplasm. This leads to the formation of a double-membrane sequestering vesicle, termed the autophagosome. Autophagosomes subsequently fuse with lysosomes, releasing their inner compartment to the lysosomal lumen. The inner membrane part of the autophagosome, together with the enclosed cargo, is degraded, and the resulting macromolecules are released into the cytosol through lysosomal membrane permeases for recycling ([Mizushima, 2007](#)).

Figure 2. Autophagy process - overview



Induction and phagophore formation

The molecular mechanism of autophagy involves several conserved Atg (autophagy –related) proteins. In response to various stimuli (nutrient starvation, hypoxia, ROS, infection, DNA damage and others) a unique flat membrane (phagophore) is formed. Two protein complexes are involved in this initiation step: one complex contains the class III PI3 K/Vps34, Atg6/Beclin1, Atg14, and Vps15/p150, and the other complex includes a serine/threonine kinase Atg1/ULK1 which is an essential positive regulator of autophagosome formation. When nutrients are abundant, binding of the ULK1 complex by the mammalian target of rapamycin (mTOR) complex 1 (mTORC1) inhibits autophagy. The mTORC1 is an important regulator of cell growth and metabolism. It is composed of five subunits that include Raptor, which binds ULK1, and mTOR, a serine-threonine kinase. By phosphorylating ULK1, mTOR inhibits autophagy initiation. In starvation, mTORC1 dissociates from the ULK1 complex, freeing it to trigger autophagosome nucleation and elongation. The kinase activity of Atg1 requires the function of two other autophagy proteins, Atg13 or Atg8 and Atg17 ([Badadani, 2012](#)).

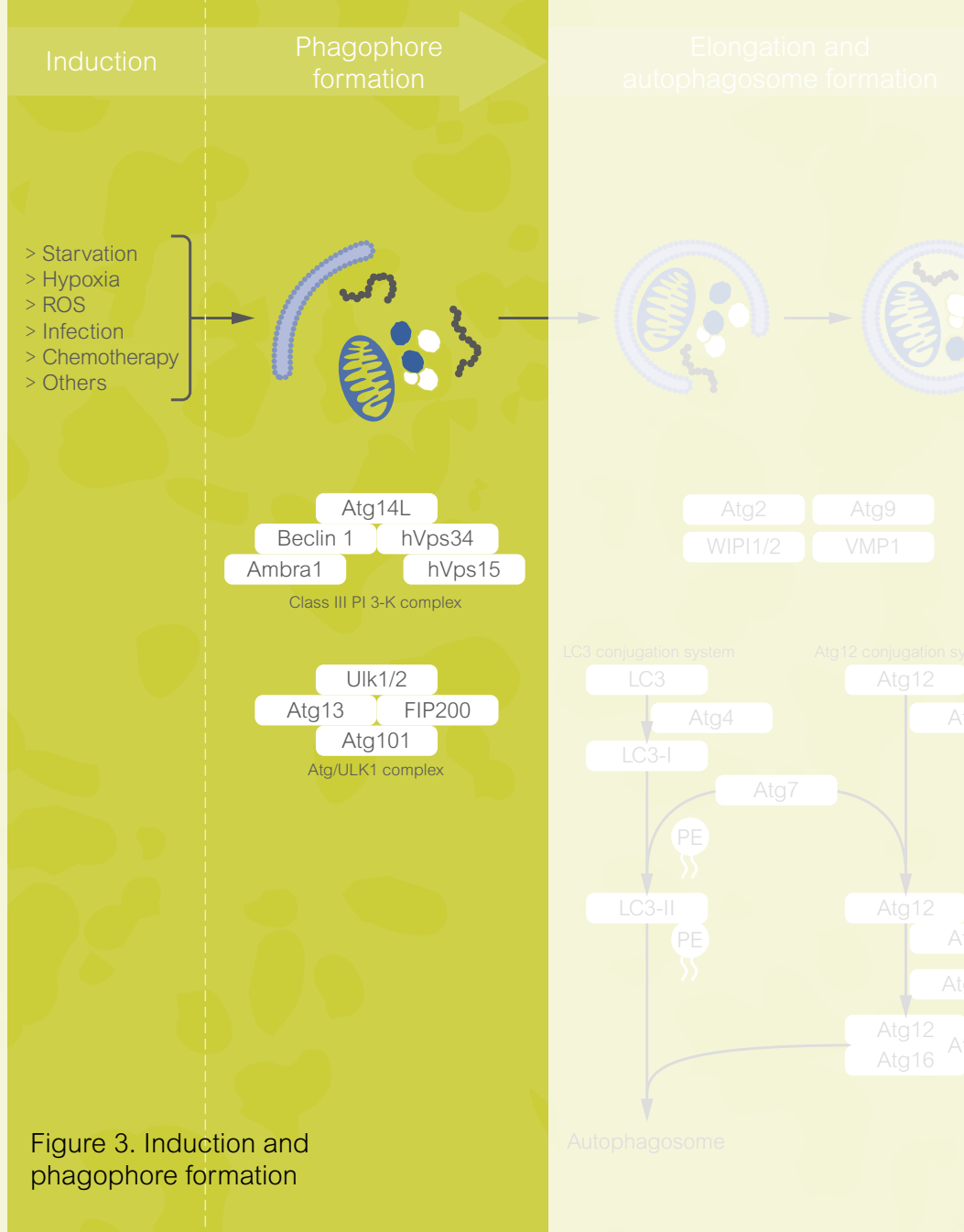


Figure 3. Induction and phagophore formation

Phagophore elongation and autophagosome formation

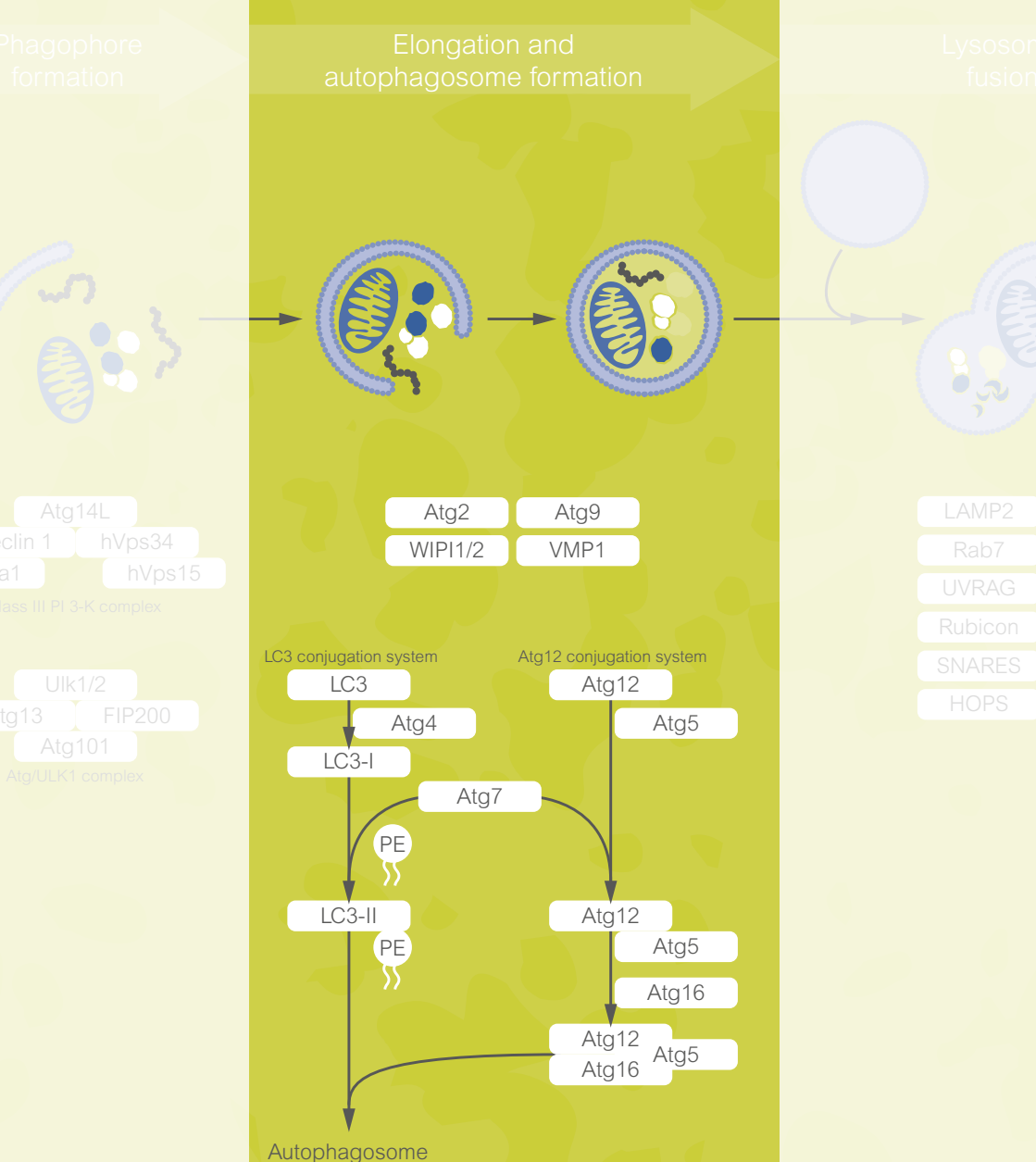
The elongation of the phagophore results in the formation of autophagosome, which is typically a double-membraned organelle. This step is a simple sequestration, and no degradation occurs. Phagophore elongation and autophagosome completion requires two ubiquitin-like conjugation pathways, both catalyzed by Atg7.

The first leads to the conjugation of Atg5-Atg12 which then form a multimeric complex with Atg16L. The Atg5-Atg12-Atg16L complex associates with the outer membrane of the extending phagophore (Glick *et al.*, 2010, Kaur & Debnath, 2015). The association of Atg5-Atg12-Atg16L complexes is thought to induce curvature into the growing phagophore through asymmetric recruitment of processed LC3B-II. Atg5-Atg12 conjugation is not dependent on activation of autophagy and once the autophagosome is formed, Atg5-Atg12-Atg16L dissociates from the membrane, making conjugated Atg5-Atg12 a relatively insufficient marker of autophagy (Barth *et al.*, 2010).

The second ubiquitin-like system involved in autophagosome formation, results in the processing of microtubule-associated protein light chain 3 (LC3B), which is encoded by the principal mammalian homologue of the yeast Atg8. Upon autophagy induction LC3B, expressed in most cell types as a full-length cytosolic protein, is proteolytically cleaved by Atg4 to generate LC3B-I. LC3B-I is activated by Atg7 and then transferred to Atg3 followed by conjugation to phosphatidylethanolamine (PE) to generate processed LC3B-II.

Processed LC3B-II is recruited onto the growing phagophore and its integration is dependent on Atg5-Atg12. Unlike Atg5-Atg12-Atg16L, LC3B-II is found on both the inner and the outer surfaces of the autophagosome, where it is required for the expansion and completion of the autophagic membrane and plays a role in selecting cargo for degradation as well as in the fusion of the autophagosome with the lysosome. LC3B-II has been proposed to act as a receptor for selective substrate, p62/SQSTM1 (Bjørkøy *et al.*, 2005). During autophagy the synthesis and processing of LC3 is increased. Recent research indicates that the deacetylation and cytosolic translocation of a nuclear pool of LC3 is required for its lipidation with PE during starvation-induced autophagy (Huang *et al.*, 2015). After the closure of the autophagosome Atg16-Atg5-Atg12 complex dissociates from the autophagosome whereas a proportion of LC3B-II remains covalently bound to the membrane and therefore LC3B-II could be used as a marker to monitor levels of autophagy in cells. Now the completed autophagosome is ready for fusion with the lysosome (Badadani, 2012). Various sources, including mitochondria, the Golgi complex, and the ER, are proposed to be the origins of the autophagosomal membrane (He & Klionsky, 2010). However, it is not completely clear by what mechanism the additional membranes are delivered to and fused with the growing phagophore. Recent studies demonstrate that self-multimerization of Atg9 may facilitate membrane tethering and/or fusion (He *et al.*, 2008). Atg9 is the only identified integral membrane protein required for autophagosome formation.

Figure 4. Autophagosome formation



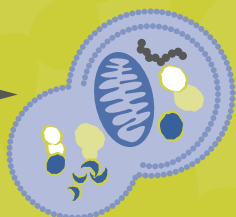
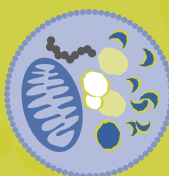
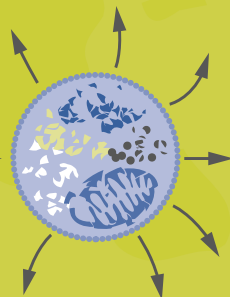
Fusion, degradation and recycling

When autophagosome formation is completed, LC3B-II attached to the outer membrane is cleaved from PE by Atg4 and released back to the cytosol. However, the retrieval and uncoating mechanisms of other Atg proteins remain to be studied. Autophagosome-lysosome fusion is mediated by the same machinery that is involved in homotypic vacuole membrane fusion. In mammalian cells, the fusion event is thought to require the lysosomal membrane protein LAMP-2 and the small GTPase Rab7. In yeast, the machinery consists of the Rab family GTPase Ypt7 (the homolog of Rab7), the NSF homolog Sec18, the SNARE (N-ethylmaleimide-sensitive factor attachment protein receptors) proteins Vam3, Vam7, Vti1, and Ykt6, the class C Vps/HOPS complex proteins, and two other proteins, Ccz1 and Mon1.

After fusion, degradation of the inner vesicle is dependent on a series of lysosomal/vacuolar acid hydrolases, including proteinases A and B (encoded by PEP4 and PRB1, respectively) and the lipase Atg15 in yeast and cathepsin B, D (a homolog of proteinase A), and Lin in mammalian cells. The resulting small molecules from the degradation, particularly amino acids, are transported back to the cytosol for protein synthesis and maintenance of cellular functions under starvation conditions. The identification of Atg22, together with other vacuolar permeases (such as Avt3 and Avt4) as vacuolar amino acid effluxers during yeast autophagy, has helped in the understanding of the mechanisms of nutrient recycling; these permeases represent the last step in the degradation and recycling process (He & Klionsky, 2010).

Lysosome
fusion

Degradation
and recycling



LAMP2

Rab7

UVRAG

Rubicon

SNARES

HOPS

Atg22

Figure 5. Fusion, degradation and recycling

Autophagy regulation

Amino acid and insulin/growth factor signals are thought to converge on mTOR, which is a master regulator of nutrient signaling. Indeed, treatment with inhibitors of TOR such as rapamycin and CCI-779 induces autophagy in yeast and even in animals. However, not all autophagy signals are transduced through mTOR; some amino acid signaling can suppress autophagy in an mTOR-independent manner. More recently, small-molecule enhancers of the cytostatic effects of rapamycin (called SMERs) were identified to induce autophagy, which acts independently of mTOR (Mizushima, 2007).

In addition to insulin and amino acid signaling, the involvement of many other factors in autophagy regulation has been reported. These include Bcl2, reactive oxygen species (ROS), calcium (Høyer-Hansen *et al.*, 2007), AMP-activated protein kinase (AMPK), BNIP3, p19 ARF, DRAM, calpain, TRAIL, FADD, and myo-inositol-1,4,5-triphosphate (IP3) (Mizushima, 2007). Other regulators of autophagy include UVRAG (UV irradiation resistance-associated gene) which interacts via the coiled-coil region of Beclin 1. UVRAG was shown to be a member of the class III PI3-kinase complex and a positive regulator of autophagy. Also, Ambra1, which is primarily expressed in neural tissues and is indispensable for normal neural tube development, has been found to be a Beclin 1-interacting protein and was shown to positively regulate Beclin 1-dependent autophagy.

Autophagy and disease

The autophagic response has been described in various pathophysiological situations, including neurobiology, cancer, cardiovascular disease, and infectious diseases. However, its role in diseases is not fully understood as it can act in different conditions to work towards cell survival or induce cell death.

Cancer

The role of autophagy in cancer is complex and may differ depending on the tumor type and conditions. Autophagy was first linked to cancer through the role of Beclin 1 (Liang *et al.*, 1999), which is essential for autophagy pathway and has been mapped to tumor susceptibility. Since then, a number of tumor-suppressor proteins that are involved in control of autophagy have been identified (e.g. p53, Bcl2, PTEN etc.). Autophagy is also required for normal embryonic, neonatal and adult development (Qu *et al.*, 2007; Kuma *et al.*, 2004). Therefore, it would seem reasonable to assume that a decrease in autophagy would lead to tumor progression (Meijer & Codogno, 2004; Gozuacik & Kimchi, 2004; Shintani & Klionsky, 2004). There are many studies that suggest up-regulation of autophagy can either be a cell-protective mechanism or in fact an alternative cell-death mechanism given that in cancer cells the apoptosis pathway is commonly altered (Meijer & Codogno, 2004).

The current consensus is that autophagy has a dual role in cancer. In normal cells, autophagy protects cells from transforming into tumor cells by clearing reactive oxygen species (ROS), organelle abnormalities and reducing DNA damage. However, once established, the tumor cells exploit the autophagic mechanism to provide a way for them to overcome nutrient-limiting conditions and facilitate tumor growth. Studies show that autophagy can modulate the tumor microenvironment by promoting angiogenesis, supply nutrients, and modulate inflammatory response (Yang *et al.*, 2015)

Many types of cancer cells including pancreatic and lung cancers are highly reliant on autophagy activation to meet growth demands. Tumor cells can meet the high metabolic demand of growth by

autophagic activation using metabolite turnover and absorption, inhibition of apoptosis caused by mitochondrial malfunction and ROS production (Yang *et al.*, 2011).

Tumor resistance to radiotherapy and chemotherapy is also associated with autophagy. Various tumor associated animal models have confirmed that autophagy inhibition has the effects of enhancing chemosensitivity and promoting tumor regression. These findings make autophagy inhibition a viable strategy for therapeutic intervention.

Chloroquine and its derivatives are the most widely tested in clinical trials either alone or in combination with chemotherapy or targeted agents. These trials have been showing some promising results providing for some much needed strategies for treatment (Yang *et al.*, 2011).

Neurodegenerative diseases

Neurodegenerative diseases are characterized by accumulation of mutant or toxic proteins (Ravikumar *et al.*, 2002; Ravikumar *et al.*, 2004). It has been shown that autophagic pathway helps in cell survival by removing unwanted cellular organelle and protein aggregates.

In neurons, although autophagy-related proteins (ATGs) are highly expressed, markers for autophagic activity are low compared with other cell types. There is growing evidence to suggest its importance in neural homeostasis (Shehata *et al.*, 2012). It has been demonstrated that disruption of autophagy specific genes in neural cells lead to neurodegeneration (Komatsu *et al.*, 2006; Hara *et al.*, 2006). PARP1 has been shown to act as a sensor in neurons under acute stress, which can either promote cell survival through autophagy or induce necrotic cell death (Nikoletopoulou *et al.*, 2014).

Autophagy also plays a very important role in the regulation of adult neurogenesis and synaptic plasticity in the brain. Autophagy interacts with many pathways that are known to underlie synaptic plasticity and memory and can respond to environmental clues to help regulate synaptic growth and plasticity (Hernandez *et al.*, 2012). There is recent evidence to suggest that change in constitutive autophagy in neurons can impact the number and quality of synaptic plasticity and regulation of presynaptic signaling. Many neurodevelopmental disorders are associated with dysfunction in autophagy related pathways (e.g. mTOR). Autophagy is also required for maintenance and function of adult neural stem cells. Ablation of genes essential for autophagy lead to progressive loss of neural stem cells and impairment of neural differentiation (Wang *et al.*, 2013).

Cardiovascular diseases

Cardiovascular disease is the leading cause of mortality through the developed world. Autophagy has been reported in almost all differentiated cells including the cardiovascular system. Autophagic pathway is essential for normal maintenance, repair, and adaptation of the heart tissue. Therefore, autophagic deficiencies have been associated with a variety of cardiac pathologies.

Changes in autophagic flux are seen in essentially all forms of heart disease and in all cell types. It can either be beneficial or aid in disease progression. Ischemic heart disease is characterized by lack of nutrients and oxygen to the myocardium. Studies looking at models of ischemia have shown that autophagy functions as a pro-survival mechanism by adapting to changing metabolic needs and eliminate damaged mitochondria, which can release ROS and initiate apoptosis to prevent any tissue damage. Inhibition of autophagy can exacerbate cardiac dysfunction and remodeling (Kanamori *et al.*,

2011). Once oxygen and nutrients are restored, autophagy can either be adaptive or detrimental to the tissue. Some studies have shown that reperfusion following an ischemic attack leads to an increase in autophagy but an impairment of autophagosome clearance leading to cell death (Ma *et al.*, 2012).

Deficiency of the autophagy gene LAMP-2 was shown to cause Danon disease. Patients have an increase of autophagic vacuoles leading to cardiomyopathy (Nishino *et al.*, 2000), a phenotype seen in mice with inactivation of the LAMP-2 protein (Tanaka *et al.*, 2000).

Autophagic vacuoles have been described in the heart tissue of patients with idiopathic dilated cardiomyopathy (Kostin *et al.*, 2003). Cardiac hypertrophy is characterized by an increase in cardiomyocyte size, disarrangement of sarcomeric structure, enhanced protein synthesis and re-expression of fetal genes (Harvey & Leinwand, 2011). Cardiac hypertrophy is accomplished by dysfunction of numerous signaling pathways that are involved in autophagy. PI3K/AKT, GSK3, MAPKs and mTOR pathways have all been associated in regulation of cardiomyocyte autophagy (Li *et al.*, 2015).

Autophagy acts as a defense mechanism against aging of cardiomyocytes. The heart develops cardiac hypertrophy and diastolic dysfunction with aging when autophagy is inhibited (Linton *et al.*, 2015).

Recent studies have showed that miRNAs play an important role in regulating cardiac autophagy (Li *et al.*, 2014) and there are growing number of studies targeting autophagy regulating miRNAs as a potential strategy in the treatment of cardiac disorders.

Further study of the autophagic pathway will lead to increased understanding of the regulation of this critical homeostatic pathway and identify new therapeutic potentials for cardiac disease.

Infectious disease

Autophagy plays a key role in immune defense against invading bacteria and pathogens. Upon infection, autophagy regulates inflammation, antigen presentation and micro-organism capture and degradation (Levine *et al.*, 2011).

Autophagy is induced by pattern recognition receptors and, through autophagic adaptors, it provides a mechanism for the elimination of intracellular microorganisms. Autophagy controls inflammation through regulatory interactions with innate immune signaling pathways, by removing endogenous inflammasome agonists and through effects on the secretion of immune mediators.

Moreover, autophagy contributes to antigen presentation and to T cell homeostasis, and it affects T cell repertoires and polarization (Deretic *et al.*, 2013).

Autophagy detection

The cells respond to changes in the extracellular environment by regulating autophagic flux to meet intracellular metabolic demands. The impact of autophagic flux on cell death and human pathologies therefore demands accurate tools. It is important to measure the autophagic flux through the entire autophagy pathway to correctly interpret the results of autophagic activity. The autophagic substrates need to be monitored dynamically over time to verify that they have reached the correct endpoint of degradation.

In 2008 and 2012, DJ Klinosky and collaborators ([Klinosky *et al.*, 2008](#); [Klinosky *et al.*, 2012](#)) published a comprehensive guide to help standardize how autophagy should be monitored. These guidelines were created to help establish criteria that are essential for reliable detection of autophagy in various experimental systems. The methods below are created using their guidelines for each of these techniques.

An important step towards understanding the role autophagy plays in these diseases is to establish assays and screening systems to measure autophagic flux rapidly and precisely. This becomes more important as we explore new methods to manipulate autophagy for therapeutic purposes.

Markers of autophagy

There are no absolute criteria for determining autophagic status that are applicable in every biological or experimental context. The key issue is to differentiate between the often transient changes in the amount of autophagosomes due to increased induction and from accumulation due to inefficient completion of autophagy.

Autophagy is always present at a baseline level in a given system and the key to accurately analyze autophagic flux is to measure change from baseline activity under various conditions.

Table 1. Key markers of autophagy

Key Markers	Function	Role in Autophagy
Atg1/ULKs	Protein kinase, phosphorylated by mTORC1	Involved in autophagy induction and possibly in phagophore formation
Atg2	Interacts with Atg18/WIP14	Possibly involved in phagophore formation
Atg3	E2-like enzyme involved in Atg8/LC3 lipidation	Involved in phagophore expansion
Atg4	Cysteine protease involved in processing and delipidation of Atg8/LC3	Involved in phagophore expansion
Atg5	Ubiquitin-like modifier that covalently binds to Atg12, generating the Atg12–Atg5 conjugate to lipidate Atg8	Involved in phagophore expansion
Atg6/beclin 1	Inhibited by binding to Bcl2. Component of various PI(3)K complexes. Interacts with PIK3C3/VPS34 to signal the onset of autophagy	Involved in autophagy induction and phagophore formation

Key Markers	Function	Role in Autophagy
Atg7	E1-like enzyme involved in LC3 ubiquitin chain	Involved in phagophore expansion
Atg8/LC3s	Ubiquitin-like protein	Involved in phagophore expansion and autophagosome formation
GABARAP	Ubiquitin-like modifier that conjugates to LC3 to PE	Involved in a later stage of autophagosome maturation
GABARAPL1/Atg8L/GEC1	Ubiquitin-like modifier	Involved in a later stage of autophagosome maturation
GABARAPL2/GATE-16/GEF2	Ubiquitin-like modifier involved in intra-Golgi traffic	Involved in a later stage of autophagosome maturation
Atg9	Transmembrane protein involved in directing membrane to the phagophore	Involved in the induction of autophagy and phagophore formation
Atg10	E2-like enzyme for the ubiquitin-like conjugation system that mediates the formation of Atg12–Atg5 conjugate	Involved in phagophore expansion
Atg12	Ubiquitin-like protein that conjugates to Atg5 to lipidate Atg8	Involved in phagophore expansion
Atg13	Binding partner and regulator of Atg1/ULKs, phosphorylated by mTORC1	Involved in autophagy induction and possibly phagophore formation
Atg14	Component of the PI(3)K complex I. Required for both basal and inducible autophagy. Essential for the autophagy-dependent phosphorylation of Beclin1/Atg6	Involved in autophagy induction and possibly phagophore formation. Associated with phagophore and mature autophagosome
Atg16/Atg16L1	Interacts with Atg12-Atg5 conjugate to form a large complex, which mediates LC3 lipidation on autophagosomal membranes	Involved in phagophore expansion
Atg17/FIP200	Binding partner and regulator of Atg1/ULKs, scaffold for ULK1 and Atg13	Involved in autophagy induction and possibly phagophore formation
Atg18/WIPiS	PIP3P-binding protein that functions downstream the Class III PI3-kinase complex and upstream the Atg12 and LC3 ubiquitin-like complexes	Involved in phagophore and autophagosome formation
Ambra1	Regulator of the PI(3)K and Atg1/ULK complexes. Interacts with Beclin1	Involved in autophagy induction
ZFYVE1/DFCP1	PtdIns3P-binding protein localized in Golgi and ER, the later population being involved in omegasome formation	Involved in autophagy induction and possibly phagophore formation

Key Markers	Function	Role in Autophagy
VMP1	Transmembrane protein	Involved in regulation of autophagy induction
DRAM1	Lysosomal modulator induced by activated p53/TP53	Involved in autolysosome formation
Vps34/PtdIns3PKC3	Component of various PI(3)K complexes	Involved in autophagy induction and phagophore formation
SQSTM1/p62	Ubiquitin-binding scaffold protein which colocalizes with ubiquitinated protein aggregates. P62/SQSTM1 binds directly to LC3 and GABARAP family proteins	Involved in autophagy regulation and possibly autophagosome formation
mTORC1	Central cell-growth regulator which upon activation, phosphorylates Atg13, preventing it from entering the ULK1 kinase complex	Negative regulator of autophagy
AMPK	Key energy sensor which regulates cellular metabolism to maintain energy homeostasis	Positive regulator of autophagy

Visual identification

Visually analysis using techniques such as Transmission Electron Microscopy (TEM) is one of the most widely used and important method to monitor autophagy. It is also one of the most problematic due to misinterpretations mostly due to challenges in classification of compartments into discrete morphological subset.

It is very important to take care in the selection of sample to be analyzed and its fixation method. Perfusion fixation of the tissue is recommended when studying autophagy.

The accurate identification of the autophagosome is essential for a useful analysis. It is also recommended to consider monitoring autophagy using at least two time points. The advantage of this approach is that it can provide accurate information on flux even when the autophagosomes are abnormal.

- The phagophore and autophagosomes contain few, or no detectable intramembrane particles (IMPs) when compared with lysosomes.
- Autophagosomes that become fused with an endosome and a lysosome contain an increased density of IMPs in the membrane.
- Autolysosomes generally have only a single membrane because the inner membrane is degraded by the lytic enzymes in addition to the engulfed material.

The best approach is to estimate the volume occupied by autophagic structures (as percentage of cytoplasmic or cellular volume, using volumetric morphometry/stereology) in at least 20 cell profiles per sample (the number needed should be dictated by some form of power analysis, which indicates that the data are significant).

Product highlight:

CytoPainter LysoOrange Indicator Reagent
(ab176827)

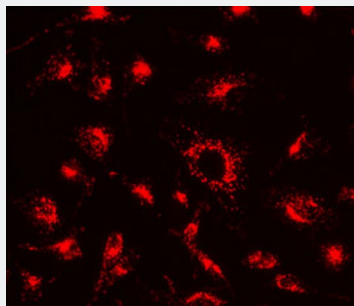
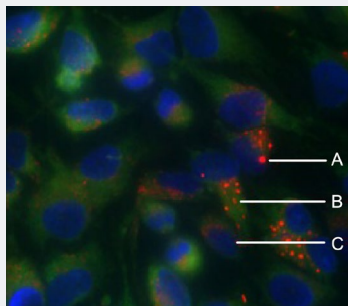


Image of HeLa cells stained with Abcam's CytoPainter LysoOrange Indicator Reagent (ab176827). Also available in other colours.

CytoPainter Lysosome/ER/NuclearStaining Reagent (ab139488)



Live cells treated with Chloroquine and stained with Organelle Reagent II showing composite of (A) lysosomes in red, (B) endoplasmic reticula in green, and (C) nuclei in blue.

ATG8/LC3 detection

Western Blotting

Atg8/LC3 is an excellent marker for autophagy. It belongs to ubiquitin-like protein family and exist in non-lipidated and lipidated forms called LC3-I and LC3-II respectively. LC3 (Atg8) is converted from proLC3 into a cleaved form, LC3-I. This processed LC3-I is finally modified into the PE-conjugated form, LC3-II. LC3-II/Atg8-PE is the only protein marker that is reliably associated with completed autophagosomes. In western blot experiments, mammalian marker LC3-I migrates at approximately 18 kDa and LC3-II at approximately 16 kDa when both are detectable.

Because of the dynamic nature of the autophagic process there are several important factors to consider when performing western blotting.

Atg8/LC3-II detection

1. Samples Types

- LC3-II levels are dependent on tissue and cell type.
- Variation in the amounts of LC3-I vs. LC3-II expression in the different tissues can present technical problems. LC3-I is very abundant in brain tissue.
- Asynchronous and heterogeneous cell populations can present with variable results when analyzing LC3 by western blotting.
- Cells can exhibit low levels of LC3-II due to an intense autophagic flux and tumor-derived cells can have high basal levels of LC3-II (e.g. MDA-MB-231).
- Autophagy can be induced by transfection or nucleofection. The amount of autophagy induced by transfection of a control empty vector can be too high to make any meaningful reading.

- f. siRNA transfection does not show any induction of autophagy; but transfection of plasmid shRNA or plasmid expression constructs causes increase in autophagy. It is recommended to use endotoxin-free DNA to help address this problem.
- g. If transfection efficiency is too low, it may be necessary to use methods, such as fluorescence microscopy, that allow autophagy to be monitored in single cells.
- h. Certain media components have shown effects on basal autophagy levels.

2. Visual detection of LC3-II

- a. Autophagosome accumulation and amount of LC3-II are not well correlated using high resolution microscopy.
- b. Formation of LC3-II is not sufficient evidence for autophagy. Deletion of genes involved in formation of LC3-II (e.g. Beclin) does not alter levels of LC3-II even though autophagy is substantially reduced.
- c. LC3-II is also associates with the membranes of certain non-autophagic structures.
- d. LC3 can be recruited directly to bacteria or virus containing phagosome membranes under certain immune activating conditions.

3. Antibodies

- a. LC3-I may be less sensitive to detection by certain anti-LC3 antibodies.
- b. LC3 is expressed as four isoforms in mammalian cells, LC3A, LC3B, LC3B2 and LC3C, which exhibit different tissue distributions.
- c. Certain commercial anti-LC3B antibodies also recognize LC3A, but do not recognize LC3C, which share less sequence homology. It is important to note that LC3C possesses in its primary amino acid sequence the DYKD motif that is recognized with a high affinity by [anti-DDDDK tag antibodies](#), such as [anti-DDDDK tag antibody \(ab1162\)](#).

4. SDS-PAGE

- a. Not all of the autophagy-related proteins are required for Atg8/LC3 processing, including lipidation.
- b. PVDF membranes are better suited for stronger LC3-II retention than nitrocellulose membranes.
- c. Triton X-100 does not efficiently solubilize LC3-II in some systems. Heating in the presence of 1% SDS, or analysis of membrane fractions is recommended.
- d. LC3-II levels should be compared with actin or other appropriate “housekeeping” proteins and not to LC3-I.
- e. No special treatments are needed for the separation of mammalian LC3-I from LC3-II.

Product highlight:

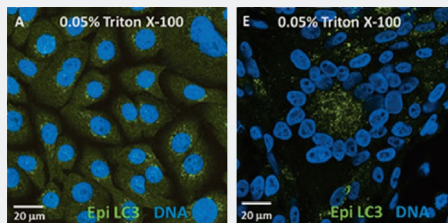
Anti-LC3B antibody (ab48394)



Western Blot image of Mouse hepatocytes treated with 20 µg/ml of poly(I:C) for 12hr using anti-LC3B antibody (ab48394).

Image courtesy of an anonymous Abreview.

Anti- MAP1LC3A RabMAb antibody (ab52768)



Immunocytochemistry/Immunofluorescence analysis of human keratinocytes (panel A) and VZV-infected melanoma cells (panel E) labeling MAP1LC3A with ab52768.

Image from Buckingham EM et al., Biotechniques. 2014;57(5):241-4. Fig 1.; doi: 10.2144/000114226.

GABARAP-PE detection

1. GABARAPL1 should be analyzed by western blot using high concentration (15%) gels.
2. GABARAP migrates faster than GABARAPL1.
3. It is very difficult to differentiate between GABARAPL1 and GABARAPL3 proteins, which have 94% identity and their expression patterns are apparently identical. GABARAPL3 lacks an ortholog in mice.

Atg8 (yeast) detection

1. Atg8 and Atg8–PE can be resolved by including 6M urea in the SDS-PAGE separating gel, or by using a 15% resolving gel without urea.
2. Atg8 in plants can be resolved by using urea combined with prior treatment of the samples with (or without) Phospholipase D. Phospholipase D removes the PE moiety from Atg8.
3. Atg8–PE migrates faster than unconjugated Atg8 during SDS-PAGE, even though the former has a larger molecular mass.
4. It is also possible to label cells with radioactive ethanolamine, followed by autoradiography to identify Atg8–PE, and a C-terminal peptide can be analyzed by mass spectrometry to identify the lipid modification at the terminal glycine residue.

Turnover of LC3-II/ATG8–PE

There are very important differences between monitoring the steady-state level of Atg8/LC3 and autophagic flux. The autophagic flux is monitored by following Atg8/LC3 in the absence and presence of autophagy inhibitors, and/or by examining the autophagy-dependent degradation of appropriate substrates.

Lysosomal degradation can be prevented through the use of the following –

- Protease inhibitors - Pepstatin A and E-64d.
- Compounds such as bafilomycin A1, chloroquine or NH_4Cl that neutralize the lysosomal pH.
- Agents that can block autophagosomes and lysosome fusion (note that bafilomycin A1 will ultimately cause a fusion block as well as neutralize the pH).
- Knock down or knock out of LAMP2 to block the fusion of autophagosomes and lysosomes.

Table 2. Autophagy inhibitors

AbID	Inhibitors	Description	Target point
ab120497	Bafilomycin A1	Highly potent, selective (V)-ATPase inhibitor	Autophagosome formation
ab120366	(±)-BAYK8644	Potent L-type Ca^{2+} channel activator	mTOR-independent signaling pathway
ab142116	Chloroquine diphosphate	Antimalarial; apoptosis and autophagy inhibitor	Autophagolysosome
ab144227	Concanamycin A (Folimycin)	Potent, selective vacuolar-type H^+ -ATPase inhibitor. Macrolide antibiotic agent.	Autophagosome formation
ab141254	DBeQ	Selective, potent, reversible and ATP competitive p97 ATPase inhibitor	Autophagosome formation
ab144048	E-64d (Aloxistatin)	Irreversible cell-permeable cathepsin B and L inhibitor	Autophagolysosome
ab120243	LY 294002	PI3K inhibitor	Autophagosome formation
ab120841	3-Methyladenine	Selective PI3K inhibitor	Autophagosome formation
ab120630	Nocodazole	Microtubule inhibitor	Autophagosome formation
ab141416	Pepstatin A	Potent selective aspartic protease inhibitor	Autophagolysosome
ab142205	Spautin-1	Cell-permeable USP10 and USP13 inhibitor	Autophagosome formation
ab120143	Paclitaxel	Anticancer agent	Autophagosome formation
ab141475	Vinblastine sulfate	Potent microtubule dynamics inhibitor	Autophagosome formation
ab120148	Wortmannin	PI3K inhibitor	Autophagosome formation
ab144590	Xanthohumol	Valosin-containing protein inhibitor. Potent chemopreventive agent.	Autophagosome formation

AbID	Inhibitors	Description	Target point
ab143032	Acetylcysteine (N-Acetyl-L-cysteine)	ROS inhibitor.	Autophagosome formation
ab141404	Leupeptin hemisulfate	Reversible competitive serine/ cysteine protease inhibitor	Autophagolysosome
ab120093	Cycloheximide	Protein synthesis inhibitor	Autophagosome formation
ab120827	Hydroxychloroquine sulfate	Anti-inflammatory agent	Autophagolysosome

For the full list of autophagy inhibitors please visit our website: www.abcam.com/autophagy-inhibitors

Factors to consider while using inhibitors of autophagy –

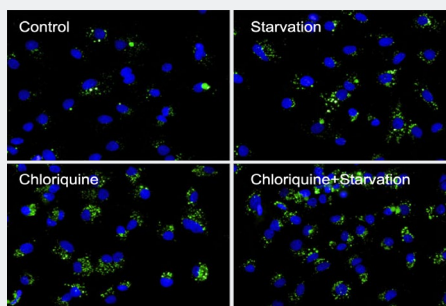
1. Increase in the levels of LC3-II in the presence of bafilomycin A1 indicates presence of autophagic flux. However, a lack of increased LC3-II levels in the presence of bafilomycin A1 suggests a block in autophagy at the terminal stages.
2. It is essential to avoid assay saturation when using inhibitors of autophagy.
3. The duration of the bafilomycin A1 treatment needs to be relatively short (1–2h) to allow comparisons of the amount of LC3 that is lysosomally degraded over a given time frame.
4. Control experiments are important to demonstrate the efficacy of this approach in each experimental context.

Autophagic flux can also be measured by the amount of cytosolic LC3-IIs. The ratio between LC3-I and LC3-IIs can provide a more accurate measure of autophagic flux than ratios based on the total level of LC3-II.

One key advantage of this approach is that it does not require the presence of autophagic or lysosomal inhibitors to block the degradation of LC3-II.

Product highlight:

Autophagy Detection Kit (ab139484)



Fluorescent microscopy analysis showing nucleus (blue nuclear stain; DAPI filter) and autophagic vesicles (green, FITC filter) in control HepG2 cells or cells starved in serum free medium for 5 hours, treated with 0.1 mM chloroquine for 24 hours or starved and chloroquine treated for 5 hours.

Luminescence based assays

Autophagic flux can be monitored based on the turnover of LC3-II, by utilizing a luminescence-based assay. A reporter assay based on the degradation of Luciferase-LC3 fusion proteins. LC3- turnover can thus be estimated by monitoring the change in the ratio of luciferase between the two cell populations. Luc-LC3-assay can be used to estimate autophagic flux at a single time point by defining the luciferase activities in cell extracts. The live cell luciferase substrate makes it possible to monitor changes in autophagy activity in living cells in real time.

- Soluble form of LC3-II (i.e., LC3-IIs) is not observed in many standard cell types including HeLa, HEK 293 and PC12.
- LC3-IIs/ LC3-I ratio must be analyzed using the cytosolic fractions rather than the total homogenates.
- LC3-II turnover can be a result of partial autophagy block and care should be taken in interpreting the results as disruption in autophagy will still result in an increase in LC3-II.
- It is necessary to follow LC3-II turnover if other substrates are being monitored simultaneously.
- Inhibitor concentration and incubation times are very important when using lysosomal protease inhibitor. In contrast to the protease inhibitors, chloroquine (10mM) or bafilomycin A1 (1–100nM) can be added to cells immediately prior to autophagy induction.

GFP-ATG8/LC3 detection

The GFP-Atg8/LC3 processing assay is another convenient way to monitor autophagy. When GFP-LC3 is delivered to a lysosome/vacuole, Atg8/LC3 is sensitive to degradation but GFP protein is relatively resistant. Therefore, the appearance of free GFP on western blots can be used to monitor autophagic flux. But there are considerations and limitations of using this method and it is not as easy to quantify as enzyme-based assays. Some of the factors are listed below -

Lysosomal pH is a critical factor in the detection of free GFP to monitor flux.

Free GFP fragments are only detectable in the presence of non-saturating levels of lysosomotropic compounds or when autophagic/degradative machinery allows the accumulation of the proteolytic fragment.

There are some limitations of using free GFP to detect autophagy -

- The assay depends on cell type and culture conditions.
- GFP is more sensitive to mammalian lysosomal hydrolases than of the yeast vacuole.
- When the lysosomal pH becomes particularly low, free GFP is undetectable.
- Immunoblotting should be included as a control to address the stability of non-lysosomal GFP such as GFP-LC3-I.
- It should also be noted that free GFP can be detected when cells are treated with non-saturating doses of lysosomal inhibitors such as chloroquine, E-64d and bafilomycin A1.
- The saturating concentrations of these lysosomal inhibitors vary in different cell lines, and it would be better to use a saturating concentration of lysosomal inhibitors when performing an autophagic flux assay. Therefore, caution must be exercised in interpreting the data using this assay; it would be helpful to combine an analysis of GFP-LC3 processing with other assays such as the monitoring of endogenous LC3-II by western blot.
- Lysosomal inhibition needs to be carefully controlled. Prolonged inhibition of lysosomal hydrolases (6h) is likely to induce a secondary autophagic response triggered by the accumulated undigested autophagy cargo.

Fluorescence microscopy

The use of cell imaging allows rapid and quantitative measures of the number of LC3 puncta and their relative number in individual or mixed cell types. Immunofluorescence microscopy method utilizes a fluorescent protein tag at its N terminus. Autophagy is measured by observing increase in punctate LC3 or GFP-LC3.

The tagged fusion proteins are useful for *in vivo* studies and live cell imaging experiments using transgenic models. Different tags have limitations and are recommended for different applications.

Table 3. Key tags that can be used in fluorescence microscopy

Tag	Requirements	Recommendation
GFP/EGFP	Requires Neutral pH	Not recommended in live cell imaging. Can be used in paraformaldehyde fixed cells
PA-GFP	Photon activation	Can induce DNA damage and produce additional autophagy
mRFP/mCherry	Photostable at acidic pH. Overexpression can be toxic	mCherry signal can persist in acidic environment
Dendra2	Photoswitchable – Low phototoxic activation, efficient chromophore maturation	Recommended for live cell imaging
Venus (YFP variant)	Brighter than mRFP, less sensitive to pH	Recommended for live cell imaging

Viral vectors can also be used to transfect fusion protein GFP-LC3. Care must be taken as viral vectors can also activate stress pathways in some cells and possibly induce autophagy.

There are some limitations when using GFP as a tag for monitoring autophagy -

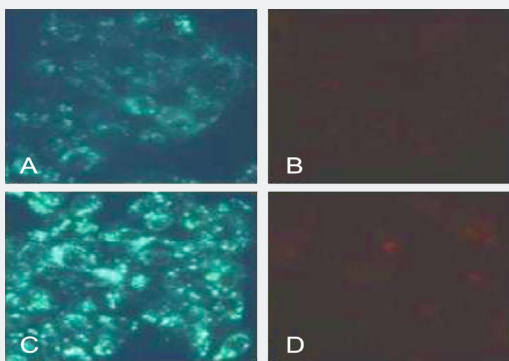
- GFP tag is large relative to the size of LC3 and the fusion protein LC3-GFP can behave differently from the native protein.
- GFP is not native in cells and can be targeted for degradation, which has obvious implications when studying autophagy.
- Some forms of GFP tend to oligomerize, which may interfere with protein function and/or localization.
- EGFP inhibits polyubiquitination and may cause defects in other cellular processes.

Leupeptin is an inhibitor of lysosomal cysteine and serine proteases that can help stabilize the GFP-LC3 signal during fluorescence microscopy, especially under conditions of induced autophagic flux.



Product highlight:

Autophagy/Cytotoxicity Dual Staining Kit (ab133075)



Ab133075 uses monodansylcadaverine (MDC), a fluorescent compound used as a probe for detection of autophagic structures in cultured cells.

A: MDC staining of untreated HepG2 cells that show basal level of autophagy

B: PI staining of untreated HepG2 cells

C: MDC staining of HepG2 cells treated with 10 μ M Tamoxifen that show increase in autophagy

D: PI staining of HepG2 cells treated with 10 μ M Tamoxifen.

Immunocytochemistry or immunohistochemistry procedures can utilize anti-LC3/Atg8 antibodies for the detection of endogenous proteins without the need for transfection and/or the generation of a transgenic organism. This method helps avoid potential overexpression artefacts. If the endogenous amount is below the level of detection, exogenous construct is recommended provided the system is amenable to introduction of exogenous genes.

It is important to consider the use of stable transfectants vs. transient transfections.

Table 4. Key advantages of stable and transient transfection

Stable Transfectants	Transient transfections
Reduced background	Higher gene expression
Eliminates artefacts	Immediate effects of the transfection
All cells express the construct	Double transfections
Screen for clones with best signal/noise ratio	Optimize DNA concentration

The following must be considered when using this method -

- It is recommended to include additional assays to measure autophagy and not rely solely on monitoring GFP-LC3.
- It is better to determine the number of puncta corresponding to GFP-LC3 on a per cell basis rather than simply the total number of cells displaying puncta.
- Software programs can help count the puncta including ImageJ, Imaris or CellProfiler.
- Although it is possible to detect changes in the size of GFP-Atg8/LC3 puncta by fluorescence microscopy, it is not possible to correlate size with autophagy activity without additional assay methods. Therefore, size determinations are not recommended as a method for monitoring autophagy.
- Quantification of the fluorescence intensity of GFP-Atg8/LC3 at specific puncta provides a valid measure of protein recruitment. But the number of puncta visible will be influenced by both the level of expression of GFP-LC3 in a given cell and by the exposure time of the microscope.
- In many cell types it is essential to establish a cut-off value for the number of puncta per cell in conditions of “low” and “high” autophagy.
- When using automated analysis methods, care must be taken to manually evaluate parameters used to establish background cut off values for different treatment conditions and cell types and it is critical that the researchers also specify the baseline number of puncta that are used to define “normal” or “low” autophagy.
- GFP-LC3 can associate with aggregates, especially when expressed at high levels. Inhibition of autophagy *in vitro* and *in vivo* leads to the accumulation of these aggregates, suggesting a role for autophagy in mediating their clearance.
- One way to control for background levels of puncta is to determine fluorescence from untagged GFP.
- Controls are needed to verify that the transfection protocol does not artificially induce GFP-LC3 puncta or cause LC3 aggregation.

Tandem mRFP/mcherry-GFP fluorescence microscopy

As explained in the previous method, the GFP signal is sensitive to the acidic and/or proteolytic conditions of the lysosome lumen, whereas the mRFP is more stable. This method enables simultaneous estimation of both the induction of autophagy and flux through autophagic compartments using a tandem monomeric RFP-GFP-tagged LC3. Based on the fluorescence the status of the LC3 protein is determined –

- Yellow color – presence of GFP and mRFP fluorescence indicates a compartment that has not fused with a lysosome, such as the phagophore or an autophagosome.
- Red color - mRFP signal without GFP corresponds to an autolysosome.

This method can also be used to monitor autophagy in high-throughput drug screening studies and can be automated.

An alternative dual fluorescence assay involves the Rosella biosensor. This assay monitors the uptake of material to the lysosome/vacuole and complements the use of the tandem mRFP/ mCherry-GFP reporter. The assay is based upon the genetically encoded dual color-emission biosensor Rosella, a fusion between a relatively pH-stable fast-maturing RFP variant, and a pH sensitive GFP variant.

When targeted to specific cellular compartments or fused to an individual protein, the Rosella biosensor provides information about the identity of the cellular component being delivered to the vacuole/lysosome for degradation. Importantly, the pH-sensitive dual color fluorescence emission provides information about the environment of the biosensor during autophagy of various cellular components.

The Rosella assay has not been tested in a wide range of mammalian cell types and sensitivity and the specificity of the assay must be verified independently.

Care must be taken when using the tandem fusion protein method to detect autophagy –

- The motion of LC3/Atg8 puncta are difficult to measure in live imaging experiments.
- Conventional confocal microscopy may not allow visualization of colocalized mRFP/mCherry-GFP puncta.
- The use of mTagRFP-mWasabi-LC3 chimera is recommended, as mTagRFP is brighter than mRFP1 and mCherry, and mWasabi is brighter than EGFP. Overexpression of mTagRFP-mWasabi-LC3 does not appear to be toxic to HeLa cells.
- If used fixed cells, the tissue or cell samples must be properly processed to avoid losing the acidic environment of the autolysosomes.
- Monomeric green fluorescent protein is more acid sensitive than EGFP and is well suited for assaying autophagic flux using this method.
- Another caution in the interpretation of the tandem fluorescent marker is that colocalization of GFP and mRFP/mCherry might also be seen in the case of impaired proteolytic degradation within autolysosomes, or altered lysosomal pH.
- Expression of tandem mRFP-GFP-LC3 is toxic to some cancer cell lines relative to GFP-LC3 or RFP-LC3.
- The cytotoxicity of DsRed and its variants such as mRFP1 is associated with downregulation of BCL2L1/Bcl-X(L).

Flow and multispectral imaging cytometry

Flow cytometry and multispectral cytometry is a method to analyze autophagy at medium- to high-throughput. The advantage of this approach is that larger numbers of cells can be analyzed with regards to GFP-LC3 puncta, cell morphology and/or autophagic flux, and concomitant detection of surface markers can be included, potentially providing more robust data than is achieved with other methods.

Since EGFP-LC3 is a substrate for autophagic degradation, total fluorescence intensity of EGFP-LC3 can be used to indicate levels of autophagy in living mammalian cells. When autophagy is induced, the decrease in total cellular fluorescence can be precisely quantified in large numbers of cells to obtain robust data.

Multispectral imaging flow cytometry allows characterization of single cells within a population by assessing a combination of morphology and immunofluorescence patterns, thereby providing

statistically meaningful data. These approaches make it possible to capture high-content images of cells in flow (up to 1,000 cells/sec), and are particularly useful for cells that grow in suspension.

The preparation of single cell suspensions causes significant levels of plasma membrane disruption and injury that can induce further autophagy.

Therefore, staining for plasma membrane permeabilization (e.g., cell death) before vs. after isolation is an important control.

Optimization of image analysis permits the study of cells with heterogeneous LC3 puncta, thus making it possible to quantify autophagic flux accurately in situations that might disturb normal processes (e.g., microbial infection).

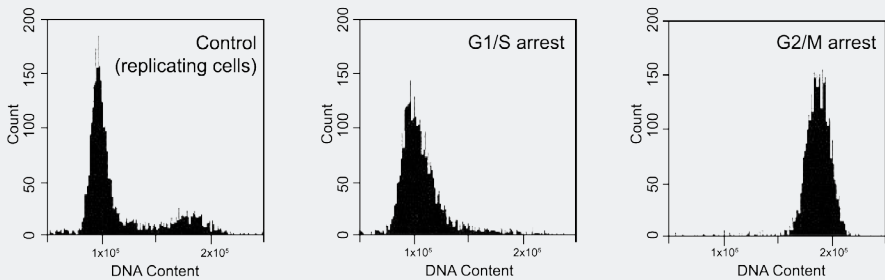
Soluble EGFP-LC3-I can be depleted from the cell by a brief saponin extraction so that the total fluorescence of EGFP-LC3 then represents that of EGFP-LC3-II alone. Since EGFP-LC3 transfection typically results in high relative levels of EGFP-LC3-I, this treatment significantly reduces the background fluorescence due to non-autophagosome associated reporter protein. By comparing treatments in the presence or absence of lysosomal degradation inhibitors, subtle changes in the flux rate of the GFP-LC3 reporter construct can be detected.

Saponin extraction method should include carefully titrated saponin concentrations and times of treatment in order to ensure specific extraction of LC3-I in their systems.

Cell membrane permeabilization with digitonin and extraction of the non-membrane bound form of LC3 allows combined staining of membrane-associated LC3-II protein and any markers for detection of autophagy in relation to other cellular events/ processes.

Product highlight:

Propidium Iodide Flow Cytometry Kit (ab139418)



ab139418 on HeLa cells treated with Thymidine and Nocodazole.

- A Untreated HeLa cells with the expected distribution of cells with 2N, 2N-4N and 4N DNA content.
- B HeLa cells treated with Thymidine have predominantly 2N DNA content. Thymidine inhibits DNA synthesis.
- C HeLa cells treated with Nocodazole are predominantly have predominantly 4N DNA content.

A number of methods have been developed that allow the determination of autophagic flux using the fluorescence-activated cell sorter.

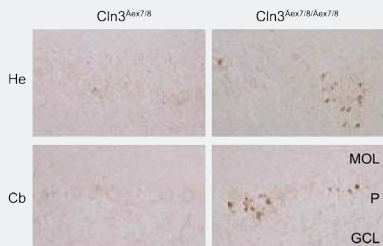
Immunohistochemistry

Immunodetection of autophagic proteins (particularly LC3 and BECN1) is also used as prognostic factor in various human carcinomas, including lymphoma, breast carcinoma, endometrial adenocarcinoma, head and neck squamous cell carcinoma, hepatocellular carcinoma, gliomas, non-small cell lung carcinomas, pancreatic and colon adenocarcinomas, as well as in cutaneous and uveal melanomas. One of the main challenges of using immunohistochemistry for LC3 detection is that in some tissues this protein can be localized in structures other than autophagosomes including lipid droplets. Thus, immunodetection of LC3 in cytoplasmic granules is not sufficient to monitor autophagy *in vivo*.

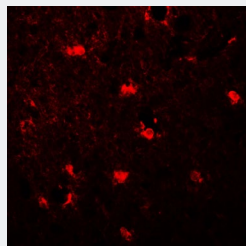
Importantly, this kind of assay should be performed as recommended by the Reporting Recommendations for Tumor Marker Prognostic Studies (Altman *et al.*, 2012).

Product highlight:

Anti-LC3B N-terminal antibody (ab168831)



Anti-LC3C C-terminal antibody (ab191574)



Immunohistochemical analysis of wild-type (Cln3^{+/+}) or homozygous Cln3^{Aex7/8} (Cln3^{Aex7/8/Aex7/8}) paraffin-embedded murine brain sections labeling LC3B using ab168831 at a 1/50 dilution.

Immunohistochemical analysis of paraffin-embedded human brain tissue labeling LC3C with ab191574 at 20 µg/mL.

SQSTM1/p62 and related LC3 binding protein detection

SQSTM1/p62 serves as a link between LC3 and ubiquitinated substrates and is efficiently degraded by autophagy. Thus, the level of SQSTM1/p62 proteins can be used to monitor autophagic flux.

SQSTM1/p62 amount is generally considered to inversely correlate with autophagic activity. Accumulation of SQSTM1/p62-positive inclusions during immunocytochemistry or elevated p62 levels on western blots are frequently used as signs of autophagy impairment.

Although the measurement of the cellular SQSTM1/p62 level appears to correlate well with other markers of autophagic flux, it is important to note the following -

- SQSTM1/p62 is degraded by both the autophagy and ubiquitin-proteasome system, and its level may be increased when the proteasome is inhibited.
- Besides LC3, SQSTM1/p62 contains domains that interact with several signaling molecules, indicating that SQSTM1/p62 may have other functions with regards to its role in autophagy.
- It is necessary to examine endogenous SQSTM1/p62 because overexpression of this protein tends to self-aggregate and may no longer indicate autophagy activity.
- SQSTM1/p62 can be transcriptionally upregulated under certain conditions.

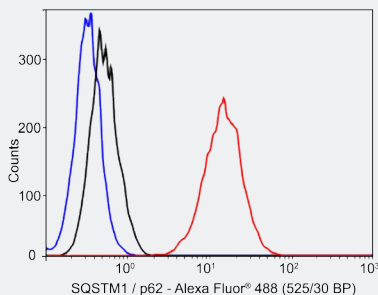
Measurement of the SQSTM1/p62 levels must be performed in combination with other methods such as LC3-II turnover.

SQSTM1/p62 aggregates are insoluble in NP40 or Triton X-100 lysis conditions. Therefore, in order to conclusively establish SQSTM1/p62 degradation by autophagy, SQSTM1/p62 levels in both Triton X-100 soluble and insoluble fractions need to be determined upon treatment with autophagy inducers or autophagy inhibitors.

Product highlight:

Anti-SQSTM1/p62 [EPR4844] RabMAb antibody (ab109012)

SQSTM1/p62 full length Protein (ab132366)



Overlay histogram showing HeLa cells stained with ab109012 (red line). Isotype control antibody (black line) was rabbit IgG (monoclonal) used under the same conditions. Unlabeled sample (blue line) was also used as a control.



12.5% SDS-PAGE analysis of ab132366 stained with Coomassie Blue.

TOR, AMPK and ATG1/ULK1 detection

Both mTORC1 and AMPK are able to oppositely regulate ULK1 (and ULK2) kinase activity by direct phosphorylation. Under glucose starvation AMPK promotes autophagy by directly activating ULK1 through phosphorylation. Under nutrient sufficiency, high TORC1 activity inhibits ULK1 by phosphorylating alternate ULK1 residues and preventing the interaction between ULK1 and AMPK.

TORC1 is an autophagy suppressive regulator which integrates growth factors, nutrient and energy signals. TORC1 activity can be monitored by analyzing the phosphorylation of its substrates such as EIF4EBP1 (at Thr37 and Thr46) and RPS6KB1 (at Thr389) using phospho-specific antibodies. Generally a decrease in TORC1 activity is a good measure for autophagy induction, however:

- TORC1 selectivity and sensitivity to its substrates depend on its integrity and configuration. Various stimuli may affect TORC1 integrity and therefore a decrease or increase in the phosphorylation status of one TORC1 substrate does not necessarily reflect changes in others, including ULK1. Therefore reliable antibodies should be used to directly assess the phosphorylation state of ULK1.
- Furthermore, TORC1 activity does not necessarily prevent autophagy induction, because there are TORC1- independent mechanisms that induce autophagy.
- Also, under conditions of ongoing starvation, persistent autophagy induction can cause negative feedback which reactivates TORC1.

TORC1 must be used in combination with other assays which directly measure autophagy.

AMPK is a multimeric serine/threonine protein kinase. The enzymatic activity of AMPK depends on phosphorylation at Thr172 of the alpha subunit and its activity can be monitored using a

phospho-specific antibody in western blotting. However, the role of AMPK in autophagy is complex and depending on cell type and metabolic conditions AMPK can suppress autophagy (in liver cells) or stimulate autophagy (e.g., fibroblasts, colon carcinoma cells and skeletal muscles).

ULK1 has been identified as a direct target for TORC1 and AMPK; however, there is not a consensus on the specific residues of ULK1 which are targeted by AMPK and TORC1.

Activation of the ULK1 complex is one of the initial steps of autophagy induction. The phosphorylation status of ULK1 at the activating sites (Ser317, Ser467, Ser555, Ser637, Ser777 or Thr574) or dephosphorylation at the inhibiting sites (Ser638, Ser757) can be used to monitor autophagy induction by using phospho - specific antibodies in western blotting and immunoprecipitation. When using ULK1 complex assembly to monitor autophagy induction it is important to note:

- Endogenous levels of ULK1 are low in many systems.
- ULK1 is phosphorylated by multiple kinases and the amount of phosphorylation can vary during autophagy induction.

Therefore, it can be difficult to detect phospho-ULK1 in western blotting without prior assay optimization.

Using ULK1 kinase activity as a tool to monitor autophagy is yet limited because further studies identifying physiological substrates of ULK1 are needed to understand how ULK1 dependant phosphorylation affects the initiation of the autophagy and therefore, is not a direct assay for autophagy.

Additional autophagy related markers

ATG9

Atg9 is the only known transmembrane protein required for autophagy and is proposed to deliver membrane to the phagophore structures and autophagosomes. Atg9 shuttles to and from the phagophore assembly site. Atg9 localizes distinctively to multiple discrete vesicles throughout the cytoplasm, compared to the other Atg proteins which are mainly detected in a single punctum or diffusely within the cytosol. Atg9 vesicles are 30–60 nm in diameter and contain approximately 30 molecules of Atg9 (Yamamoto *et al.*, 2012). These vesicles fuse to form the preautophagosomal structure, and subsequently Atg9 becomes integrated into the outer autophagosomal membrane. After fusion of autophagosomes with the lysosome or vacuole, Atg9 is recycled.

Atg12-Atg5/Atg16L1 complex associates with the phagophore and can be detected by immunofluorescence. During starvation there is an increased proportion of cells with punctate Atg5, Atg12 and Atg16L1, whereas under physiological conditions, the endogenous proteins are predominantly diffusely distributed throughout the cytoplasm. Autophagosome formation inhibitors, used in mammalian cells assays, result in blocking of the starvation induced puncta. Monitoring conjugation of Atg12-Atg5 may not be useful way to measure autophagy induction because it appears that in some mammalian cells all Atg12 and Atg5 proteins exist in the conjugated form and at least in short term starvation the expression levels don't seem to change. Atg16L1 is located on the phagophore, but not on the completed autophagosome, therefore can be used to monitor early steps of the process by immunostaining, Immune-TEM or by the use of tagged Atg16L1.

ATG14

Atg14 localizes primarily on the phagophore and can be detected by immunostaining and used as a

phagophore marker. However, since Atg14 is not exclusively localized on the phagophore, but can be detected on the autophagosome and on the ER, its detection should be performed together with other phagophore and autophagosome markers.

ATG18

Atg18/WIP1 family proteins bind to phosphatidylinositol 3-phosphate (PtdIns3P) that is present at both the phagophore and the autophagosome. When autophagy is initiated WIP1 and WIP2 bind PtdIns3P and accumulate at membranes such as ER where they participate in the autophagosome formation. Upon starvation or rapamycin administration, autophagy activity is increased and the number of cells which display WIP1 puncta at the autophagosomal membrane is also increased. Respectively, under autophagy inhibited conditions, reduced number cells display WIP1 puncta when studied by fluorescence microscopy or immunostaining. It may be possible to use the formation and disappearance of WIP1 puncta to monitor autophagy but it is important to note that overexpression of WIP1 and WIP2 can cause formation of aggregates which are stable in the presence of PtdIns3K inhibitors.

BECN1

BECN1 and PIK3C3 are involved in the complex which signals the onset of autophagy and could be used to monitor autophagy. BECN1 is inhibited by its binding to anti-apoptotic protein Bcl2. Autophagy is induced when BECN1 is released from Bcl2, however, it is important to note that autophagy can also be induced in a BECN1-independent manner. BECN1 and PIK3C3 can be part of multiple complexes and monitoring their localization should be used cautiously. Upon autophagy stimulation BECN1 and PIK3C3 form aggregates at the region of the Golgi complex and therefore BECN1-GFP puncta can be used as marker for autophagy induction by immunofluorescence. However:

- GFP moiety can interfere with the correct localization of BECN1 so appropriate controls and inhibitors should be used in the experiments.
- In BECN1-independent autophagy such aggregates are not formed despite the cells ability to express BECN1 (assessed by western blotting).

DRAM1

DRAM1 (DNA-Damage Regulated Autophagy Modulator 1) is a small hydrophobic protein with six transmembrane domains. Elimination of DRAM1 by siRNA blocks autophagy which indicates its importance in this process. Detection of DRAM1 RNA during autophagy is easy by qRT-PCR, however DRAM1 protein measurement and detection is very difficult because of its hydrophobicity and small size.

General considerations for autophagy detection

Detection of autophagy is a complex process and there is no one perfect method to measure autophagosome numbers or autophagic flux. We recommend the use of multiple assays whenever possible rather than relying on the results from a single method.

The table below (Table 5) highlights some of the recommended methods for monitoring autophagy in a wide range of systems.

Table 5. Recommended methods for monitoring autophagy

Method	Description
Electron Microscopy	Quantitative electron microscopy, immune-TEM; monitor autophagosome number and/or volume
Atg8/LC3 western blotting	Western blot. The analysis is performed in the absence and presence of lysosomal protease or fusion inhibitors to monitor flux; an increase in the LC3-II amount in the presence of the inhibitor is usually indicative of flux
GFP-Atg8/LC-3 lysosomal delivery and proteolysis	Western blot ± lysosomal function or degradation inhibitors; the generation of free GFP indicates lysosomal/vacuolar delivery
GFP-Atg8/LC3 fluorescence microscopy	Fluorescence microscopy, flow cytometry to monitor vacuolar/lysosomal localization. Also, increase of in punctuate GFP-Atg8/LC3 or Atg18/WIPI
Tandem mRFP/mCherry-GFP fluorescence microscopy	Flux can be monitored as a decrease in green/red (yellow) fluorescence (phagophores, autophagosomes) and an increase in red fluorescence (autolysosomes)
SQSTM1/p62 and related LC3 binding protein turnover	The amount of SQSTM1 increases when autophagy is inhibited and decreases when autophagy is induced
mTOR, AMPK and Atg1/ULK1 kinase activity	Western blot, immunoprecipitation or kinase assays
Autophagic sequestration assays	Lysosomal accumulation by biochemical or multilabel fluorescence techniques and TEM with a maturation inhibitor
Turnover of autophagic compartments	Electron microscopy with morphometry/stereology
Autophagosome-lysosome colocalization	Fluorescence microscopy
Sequestration and processing assays in plants	Chimeric RFP fluorescence and processing, and light and electron microscopy
Tissue fractionation	Centrifugation, western blot and electron microscopy
Degradation of endogenous lipofuscin	Fluorescence microscopy

When deciding which methods to use for their experiment, researchers need to keep in mind that the appropriate assay will depend on the question being asked and the system being used.

We hope that this summary, based on the recommendations by DJ Klinosky and others (Klinosky *et al.*, 2012) is useful in helping you decide the best assay(s) for your experiment.

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