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Methods in Mammalian Autophagy Research

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Abstract

Autophagy has been implicated in many physiological and pathological processes. Accordingly, there is a growing scientific need to accurately identify, quantify, and manipulate the process of autophagy in cells. However, as autophagy involves dynamic and complicated processes, it is often analyzed incorrectly. In this Primer, we discuss methods to monitor autophagy and to modulate autophagic activity, with a primary focus on mammalian macroautophagy.

Introduction

The past decade has witnessed an explosion of research on a fundamental cell biology pathway called autophagy (Greek for “self-eating”). The discovery of evolutionarily conserved genes (initially identified in yeast) that are required for autophagy has enabled scientists to uncover a vast array of homeostatic, developmental, and other physiological functions of autophagy. Moreover, increasing evidence suggests that the deregulation of autophagy may contribute to a broad spectrum of mammalian diseases (Levine and Kroemer, 2008; Mizushima et al., 2008). Consequently, there is a rapidly growing need among scientists to be able to accurately detect autophagy and to study its function in diverse biological processes, especially in mammalian systems.

Research in mammalian autophagy has been historically plagued by two major considerations. First, there has been the challenge of capturing a “dynamic process” with “static measurements,” and the inherent limitations associated with making biological inferences based on such measurements. Second, there has been the challenge of separating “form” from “function,” and avoiding the common pitfall of assigning physiological functions to autophagy based on its detection (or lack thereof) in a given physiological setting. These two challenges likely underlie many of the misconceptions in our historical understanding of the functions of mammalian autophagy. For example, certain neurodegenerative and myodegenerative diseases were initially believed to result, at least in part, from increased autophagy (based on microscopic visualization of increased numbers of early intermediates in the pathway) when, in reality, the accumulation of early intermediates in such diseases likely represents a block in later stages of the autophagy pathway (Levine and Kroemer, 2008; Mizushima et al., 2008;

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Rubinsztein, 2006). Autophagy, a common morphological feature in dying cells, was also often erroneously presumed to be a cell death pathway, whereas it now seems clear that one of its major functions is to fight to keep cells alive under stressful "life-threatening" conditions (Kroemer and Levine, 2008).

These historical challenges in mammalian autophagy research have been partially overcome by applying advances in the elucidation of autophagy's molecular mechanisms to the development of new methods in autophagy research. Accordingly, within the past decade, numerous new techniques have been developed both (1) to monitor autophagy as a dynamic process and (2) to modulate autophagy in order to probe its functions in a given cellular process. The aim of this Primer is to provide a critical overview of currently available techniques in mammalian autophagy research and the limitations in their interpretation. More detailed information on each technique can also be found in other reviews (Klionsky et al., 2008a; Mizushima, 2004; Mizushima and Yoshimori, 2007; Rubinsztein et al., 2009).

The Primer's Primer on Autophagy

Autophagy is a general term for processes by which cytoplasmic materials including organelles reach lysosomes for degradation (Levine and Kroemer, 2008; Mizushima et al., 2008; Rubinsztein, 2006). Among the three types of autophagy (macroautophagy, microautophagy, and chaperone-mediated autophagy), the most extensively studied is macroautophagy. Chaperone-mediated autophagy involves the direct translocation of cytosolic proteins across the lysosomal membrane, which requires protein unfolding by chaperone proteins. Microautophagy involves inward invagination of lysosomal membrane, which delivers a small portion of cytoplasm into the lysosomal lumen.

Macroautophagy (simply referred to as autophagy hereafter) is the pathway that will be the focus of this Primer. This pathway is conserved from yeast to mammals, and is mediated by a special organelle termed the autophagosome. Upon induction, a small vesicular sac called the isolation membrane or phagophore elongates and subsequently encloses a portion of cytoplasm, which results in the formation of a double-membraned structure, the autophagosome (Figure 1 and Figure 2). Then, the outer membrane of the autophagosome fuses with a lysosome (to form an autolysosome), leading to the degradation of the enclosed materials together with the inner autophagosomal membrane. The endosome can also fuse with the autophagosome (to form an amphisome) before fusion with the lysosome. Amino acids and other small molecules that are generated by autophagic degradation are delivered back to the cytoplasm for recycling or energy production. The methods described below detect different stages of the autophagy pathway (e.g., early autophagosome, autolysosome, autophagic degradation products) and should be used coordinately with each other to determine whether an increase in intermediates in the pathway represents a true increase in autophagic degradation or rather, a block in the completion of the autophagic pathway (Figure 3 and Figure 4).

Under physiological conditions, autophagy has a number of vital roles such as maintenance of the amino acid pool during starvation, preimplantation development, prevention of neurodegeneration, antiaging, tumor suppression, clearance of intracellular microbes, and regulation of innate and adaptive immunity (Cecconi and Levine, 2008; Deretic and Levine, 2009; Levine and Kroemer, 2008; Mizushima et al., 2008; Rubinsztein, 2006). One of the characteristic features of autophagy is its dynamic regulation; cellular autophagic activity is usually low under basal conditions, but can be markedly upregulated by numerous stimuli. The most well-known inducer of autophagy is nutrient starvation, both in cultured cells and in intact organisms, ranging from yeast to mammals. Besides starvation, autophagy can also be activated by other physiological stress stimuli (e.g., hypoxia, energy depletion, endoplasmic reticulum stress, high temperature, and high-density conditions), hormonal stimulation, pharmacological

agents (e.g., rapamycin and other compounds discussed below), innate immune signals, and in diseases such as bacterial, viral, and parasitic infections, acute pancreatitis, heart disease, and protein aggregopathies. Conversely, autophagy suppression is also often associated with certain diseases, including a subset of cancers, neurodegenerative disorders, infectious diseases, and inflammatory bowel disorders, and a decline in autophagy function is a common feature of aging. Given this strong association between autophagy and different physiological and pathophysiological processes, there is an increasing need for scientific methods in autophagy research that reliably determine (1) whether autophagy is present, upregulated, or suppressed in a given biological context and (2) whether (and how) basal autophagy and/or modified autophagy contribute mechanistically to the physiological or pathophysiological process under investigation.

The execution of autophagy involves a set of evolutionarily conserved gene products, known as the Atg proteins, that are required for the formation of the isolation membrane and the autophagosome (herein referred to as the “autophagic machinery”) (Table 1). The process of autophagosome formation involves two major steps: nucleation and elongation of the isolation membrane. The ULK/Atg1 kinase complex, the autophagy-specific PI3-kinase complex, and PI(3)P effectors and their related proteins are important for the nucleation step, whereas the Atg12- and LC3/Atg8-conjugation systems are important for the elongation step. In addition, other proteins required for autophagosome-lysosomal fusion, lysosomal acidification, and lysosomal digestion, and regulatory signals that integrate environmental cues with the autophagic machinery are involved in autophagy. Details of the molecular regulation and machinery of autophagy have been reviewed elsewhere (He and Klionsky, 2009;Longatti and Tooze, 2009). In mammalian cells, most of the Atg proteins are observed on isolation membranes (e.g., ULK1/2, Atg13, FIP200, Atg101, Beclin 1, Atg14, LC3, Atg12, Atg16L1) but not on complete autophagosomes (Longatti and Tooze, 2009) (Figure 1,Table 1). To date, only microtubule-associated protein light chain 3 (LC3), a mammalian homolog of yeast Atg8, is known to exist on autophagosomes, and therefore, this protein serves as a widely used marker for autophagosomes (Figure 1 and Figure 4B) (Kabeya et al., 2000;Mizushima et al., 2004). The identification of the autophagic machinery has greatly facilitated the detection of autophagy (through LC3-based biochemical and microscopic assays), as well as the ability to experimentally manipulate the autophagy pathway (through knockout or knockdown of autophagy genes or the expression of dominant negative autophagy proteins). The autophagy pathway can also be manipulated with agents that regulate autophagosome formation or subsequent degradation steps (Figure 1).

Monitoring Cellular Autophagic Activity

A common misconception is the notion that increased numbers of autophagosomes in cells invariably correspond to increased cellular autophagic activity. Given that the autophagosome is an intermediate structure in a dynamic pathway, the number of autophagosomes observed at any specific time point is a function of the balance between the rate of their generation and the rate of their conversion into autolysosomes. Thus, autophagosome accumulation may represent either autophagy induction or, alternatively, suppression of steps in the autophagy pathway downstream of autophagosome formation. For example, compared to basal levels of autophagy (Figure 3A), autophagy activation is expected to result in an increase in the numbers of all autophagic structures (i.e., isolation membranes, autophagosomes, and autolysosomes) (Figure 3B). If any step upstream of autophagosome formation is blocked, the numbers of all autophagic structures are decreased (Figure 3C). In contrast, the blockade of any step downstream of autophagosome formation increases the number of autophagosomes while decreasing the number of autolysosomes (Figure 3D). Notably, in two physiologically opposite scenarios, autophagy activation (and increased autophagic degradation) (Figure 3B) and blockade of a downstream step in autophagy (and decreased autophagic degradation) (Figure

3D), there is an increase in the number of autophagosomes. Therefore, the simple determination of numbers of autophagosomes is insufficient for an overall estimation of autophagic activity. Rather, different methods often need to be used in concert to distinguish between basal levels of autophagy, induction of autophagy, suppression of upstream steps of autophagy, and suppression of downstream steps of autophagy. The term “autophagic flux” is used to denote the dynamic process of autophagosome synthesis, delivery of autophagic substrates to the lysosome, and degradation of autophagic substrates inside the lysosome and is a more reliable indicator of autophagic activity than measurements of autophagosome numbers. In subsequent sections, we will discuss different methods for monitoring the number of autophagosomes and for monitoring autophagic flux.

Monitoring the Number of Autophagosomes

Three principal methods are presently used to monitor the number of autophagosomes, including electron microscopy, light microscopy detection of the subcellular localization of LC3, and biochemical detection of the membrane-associated form of LC3.

Electron Microscopy

The most traditional method is electron microscopy, and in fact, mammalian autophagy was originally discovered in the late 1950s by electron microscopists studying the lysosome. At the ultrastructural level, an autophagosome is defined as a double-membraned structure containing undigested cytoplasmic contents, which has not fused with a lysosome (Figure 2 and Figure 4A). Autophagosomes often enclose intracellular organelles such as mitochondria and fragments of the endoplasmic reticulum (ER). As this definition is straightforward, it is usually easy to identify autophagosomes, or at least those organelles that envelop cellular contents. It is less clear how to interpret double-membraned structures that specifically enwrap intracellular pathogens: the life cycle of some pathogens may involve transit through autophagic-like intermediate structures (without eventual delivery to the lysosome), whereas in other cases, intracellular pathogens are truly captured by the autophagy pathway for lysosomal degradation. Another consideration is that the classic definition of an autophagosome includes the visualization of different cellular contents inside the double-membraned structure. Although this may be the case in “bulk autophagy,” there is increasing evidence for organelle-specific autophagy, including pexophagy, mitophagy, ribophagy, and reticulophagy (van der Vaart et al., 2008). Thus, the morphological criteria used to identify an autophagosome may need to be revised to incorporate recent evidence for pathogen-specific and organelle-specific autophagy in mammalian cells.

In contrast to an autophagosome containing cellular cargo (which is usually easy to identify), the distinction of autolysosomes from other cellular membranous compartments is often more difficult. The autolysosome is a hybrid organelle generated by the fusion of an autophagosome and a lysosome (an endosome can also be involved), which has a single limiting membrane and contains cytoplasmic materials at various stages of degradation. At early stages, the inside materials can be recognized as having originated from cytoplasm. However, if degradation proceeds too far, it is not easy to determine whether the inside materials are of intracellular origin. Furthermore, it is often difficult to distinguish autolysosomes from endocytic compartments (heterophagic vacuoles) or from certain other vacuoles/structures of unknown origin. In particular, vacuoles with no or scarce inside materials should not be judged as “autophagic” structures. Other typical examples of misinterpretation are discussed in more detail in another review (Eskelinen, 2008). Thus, although electron microscopy is a powerful tool, it is not a perfect method and it is limited in its potential for application to functional studies as described below.

Fluorescence Microscopy

The assessment of autophagosome number by electron microscopy requires considerable specialized expertise, and is becoming increasingly replaced by light microscopic and biochemical methods that are more widely accessible to researchers in different fields. As noted above, the mammalian autophagy protein, LC3, is a marker of autophagosomes (Figure 1 and Figure 4B). Among the four LC3 isoforms, LC3B is most widely used. Soon after synthesis, nascent LC3 is processed at its C terminus by Atg4 and becomes LC3-I, which has a glycine residue at the C-terminal end. LC3-I is subsequently conjugated with phosphatidylethanolamine (PE) to become LC3-II (LC3-PE) by a ubiquitination-like enzymatic reaction. In contrast to the cytoplasmic localization of LC3-I, LC3-II associates with both the outer and inner membranes of the autophagosome (Figure 4B). After fusion with the lysosome, LC3 on the outer membrane is cleaved off by Atg4 and LC3 on the inner membrane is degraded by lysosomal enzymes, resulting in very low LC3 content in the autolysosome. Thus, endogenous LC3 or GFP-LC3 is visualized by fluorescence microscopy either as a diffuse cytoplasmic pool or as punctate structures that primarily represent autophagosomes (Figure 5A).

Although the number of punctate LC3 or GFP-LC3 structures per cell is usually an accurate measure of autophagosome number, this assay has some potential experimental pitfalls. First, there is potential for subjectivity, and a uniform approach needs to be established and applied by the investigator, both with respect to the method of quantitation and the criteria for defining a “puncta.” The number of punctate structures can be counted visually (by an observer blinded to experimental condition) or automatically determined using computerized software image analysis programs (e.g., Top Hat algorithm of MetaMorph version 7.0 by Molecular Devices, and G-Count by G-Angstrom). Although puncta number is markedly increased after autophagy induction, small numbers of punctae are also observed even under normal conditions (Figure 5A). Therefore, “percentage of cells with GFP-LC3 punctae” is not an appropriate indicator (theoretically, it should be 100% in most cell types), unless a clear threshold can be established that effectively distinguishes “autophagy active” and “autophagy inactive” states; if so, results should then be expressed as the “percentage of cells with more than a certain number of punctae.” In general, it is preferable to quantify the “average number of GFP-LC3 punctae per cell” in all cells in the population under evaluation. The “total area of GFP-LC3 punctae per cell” may also be analyzed with image analysis software, but in such a case, it is particularly important to rule out experimental artifacts due to the formation of large GFP-LC3 aggregates (see below).

A second potential pitfall with the detection of LC3 or GFP-LC3 punctate structures as a method to monitor autophagosome numbers is the observation that GFP-LC3, and probably even endogenous LC3, can be easily aggregated if overexpressed or coexpressed with other aggregate-prone proteins (Kuma et al., 2007). GFP-LC3 aggregates are often indistinguishable from true autophagosomes by fluorescence microscopy. However, certain precautions can be exercised to reduce the possibility of GFP-LC3 aggregation. The use of stable GFP-LC3 transformants is highly recommended, so that one can select clones that express appropriate levels of GFP-LC3 without artificial aggregation. When the GFP-LC3 construct is used in transient transfection experiments, caution should be exercised to avoid high levels of expression that result in artificial aggregation. Furthermore, it is possible to experimentally distinguish between nonspecific GFP-LC3 incorporation into aggregates versus GFP-LC3 incorporation into autophagosomes with the use of a C-terminal glycine mutant GFP-LC3 that is defective in ubiquitin-like conjugation with phosphatidylethanolamine (GFP-LC3G120A) as a negative control (Tanida et al., 2008). In settings where autophagosome numbers are truly increased (versus non-specific GFP-LC3 aggregation), one should see increased wild-type

GFP-LC3 punctae but not increased mutant GFP-LC3G120A punctae (assuming that both wild-type and mutant GFP-LC3 forms are expressed at comparable levels).

The GFP-LC3 labeling method has been successfully applied to in vivo mammalian autophagy research by generating transgenic GFP-LC3 transgenic mice (Mizushima et al., 2004). Similar approaches have also been used in model organisms, including *Drosophila* (Rusten et al., 2004; Scott et al., 2004), nematodes (Meléndez et al., 2003), plants (Yoshimoto et al., 2004), and zebrafish (He et al., 2009). In GFP-LC3 transgenic mice, GFP-LC3 is ubiquitously expressed under the control of the CAG promoter, and the accumulation of GFP punctae (which represent autophagosomes) is observed in almost all tissues after a 24 hr fasting period (Figure 5B). One exception seems to be the brain, where the accumulation of GFP-LC3 punctae is not observed even after 48 hr of starvation. This may reflect the lack of autophagy regulation by nutritional conditions or alternatively, the very rapid turnover of autophagosomes in the brain. In addition to the systemic GFP-LC3 transgenic mice, tissue-specific transgenic mice expressing GFP-LC3 and mCherry-LC3 have also been generated (Iwai-Kanai et al., 2008; Zhu et al., 2007). These systemic and tissue-specific models have been successfully used to show reductions in autophagosome numbers in mice deficient in autophagy genes. They have also been used to show increases in autophagosome numbers under disease and stress conditions, for example, in hepatocytes expressing an α1-antitrypsin Z variant (Kamimoto et al., 2006), in degenerating Purkinje cell axons with a mutation that enhances excitotoxicity (Wang et al., 2006), and in heart muscle subjected to pressure overload or overexpressing a mutant αβ-crystallin protein (Tannous et al., 2008; Zhu et al., 2007). Thus, the monitoring of GFP-LC3 punctae in GFP-LC3 transgenic mice is a powerful method to assess whether different physiological and pathophysiological stimuli regulate autophagosome numbers in vivo.

Biochemical Assays

In addition to its utility in fluorescence microscopy assays, LC3 is also useful in biochemical assays to assess autophagosome numbers. The conversion from endogenous LC3-I to LC3-II and from GFP-LC3-I to GFP-LC3-II can be detected by immunoblotting with antibodies against LC3 and GFP, respectively. Although the actual molecular weight (MW) of LC3-II (a PE-conjugated form) is larger than that of LC3-I, LC3-II (apparent MW is 14 kD) migrates faster than LC3-I (apparent MW is 16 kD) in SDS-PAGE because of extreme hydrophobicity of LC3-II (this is often misunderstood as “processing” because of the apparent size reduction) (Figure 4C and Figure 6A). The amount of LC3-II usually correlates well with the number of autophagosomes (or more precisely, in theory, the amount of autophagic membrane labeled with LC3-II) (Kabeya et al., 2000). However, not all LC--II is present on autophagic membranes, and, importantly, some population of LC3-II seems to be ectopically generated in an autophagy-independent manner. For example, a significant amount of LC3-II is detectable in FIP200- and Atg14-deficient mouse embryonic fibroblasts (Hara et al., 2008; Matsunaga et al., 2009), in Beclin 1-deficient embryonic stem cells (Matsui et al., 2007) and in cells with RNA interference (RNAi)-mediated suppression of Beclin 1, Atg13, Atg14, and Vps34 (Hosokawa et al., 2009; Itakura et al., 2008; Matsui et al., 2007; Zeng et al., 2006), even though autophagosome formation and autophagic flux (see below) are completely or profoundly inhibited. Similarly, in yeast, Atg8 lipidation occurs in *atg1, 2, 6, 9, 13, 14, 16*, and *17* mutants (Suzuki et al., 2001). Therefore, in settings where certain components of the autophagic machinery are inactivated (genetically or pharmacologically), it is still possible that autophagy is suppressed even if LC3-II is detected. In such cases, other approaches including GFP-LC3 labeling methods and autophagic flux assays (described below) are required to assess autophagic activity.

Cautions

It should be noted that certain approaches sometimes used in the literature are not considered appropriate measures of autophagosome numbers (or autophagic activity) by most experts in the field. For example, the number and activity of lysosomes is not a reliable general indicator of autophagy, although it may work well in *Drosophila*. Therefore, at least for mammalian cells, we do not recommend the use of LysoTracker, Acridine orange, or monodansylcadaverine (MDC). (MDC was originally proposed to be a specific autophagosome indicator but was later demonstrated to have higher affinity for lysosomes [Bampton et al., 2005; Mizushima, 2004].) Second, although mammalian LC3, yeast Atg8, and certain other autophagy genes may be transcriptionally upregulated in response to stress conditions that induce autophagy (He and Klionsky, 2009), there is no clear evidence that autophagic activity per se is transcriptionally upregulated. Moreover, Atg proteins are constitutively expressed in sufficient amounts, and their posttranslational modifications and/or associations with other members of the autophagic machinery, rather than regulation of their expression levels, seems to be critical for their activity in the autophagy pathway. Therefore, Atg mRNA or protein expression levels are not considered appropriate indicators for monitoring autophagy.

Monitoring Autophagic Flux

The methods described in the above section are useful to evaluate the number of cellular autophagosomes, which generally–but not always–is an indicator of the level of cellular autophagic activity. As discussed above (and illustrated in Figure 3), the accumulation of autophagosomes is not always indicative of autophagy induction and may represent either the increased generation of autophagosomes and/or a block in autophagosomal maturation and the completion of the autophagy pathway. A similar concern is also true for the measurement of LC3-II. For example, if cells are cultured with chloroquine, an agent that impairs lysosomal acidification, LC3-II accumulates even under normal (nonstarved) conditions because turnover of LC3-II by basal autophagy is blocked (Figure 6A). Thus, one cannot distinguish between bona fide induction of autophagy (e.g., by starvation) and impairment of autophagolysosomal maturation simply by measuring autophagosome numbers (e.g., by electron microscopy or by light microscopy detection of LC3 or GFP-LC3 puncta) or by measuring levels of LC3-II (by immunoblot analysis). In most experimental settings, it is necessary to distinguish whether autophagosome accumulation is due to autophagy induction or rather a block in downstream steps, by performing “autophagic flux” assays (Figures 4D–4H) that distinguish between these two possibilities. It should be noted that the quantitation of relative numbers of autophagosomes and autolysosomes observed by electron microscopy can help discriminate between autophagy activation (in which both autophagosomes and autolysosomes are increased in numbers) and a block in autophagosome maturation (which is associated with an increase in autophagosome numbers without a change in autolysosome numbers) (Figure 4A). However, electron microscopy does not provide direct information about lysosomal degradation of autophagic substrates and, therefore, we do not formally classify it as an “autophagic flux” assay.

LC3 Turnover Assay

One of the principal methods in current use to measure autophagic flux is the monitoring of LC3 turnover, which is based on the observation that LC3-II is degraded in autolysosomes. As described above, if cells are treated with lysosomotropic reagents such as ammonium chloride, chloroquine, or bafilomycin A_1, which inhibit acidification inside the lysosome or inhibit autophagosome-lysosome fusion, or with inhibitors of lysosomal proteases such as E64d and pepstatin A, the degradation of LC3-II is blocked, resulting in the accumulation of LC3-II (Tanida et al., 2005). Accordingly, the differences in the amount of LC3-II between samples in the presence and absence of lysosomal inhibitors represent the amount of LC3 that is delivered to lysosomes for degradation (i.e., autophagic flux) (Figure 4D) (Klionsky et al.,

2008a; Mizushima and Yoshimori, 2007; Rubinsztein et al., 2009). For example, levels of LC3-II are increased by treatment with chloroquine even under nonstarvation conditions (compare lanes 1 and 2, Figure 6B). However, the difference in LC3-II levels in the presence and absence of chloroquine is larger under starvation conditions (compare lanes 3 and 4, Figure 6B), indicating that autophagic flux is increased during starvation.

Although the LC3 turnover assay is theoretically straightforward, in some experimental settings, it can be challenging to obtain meaningful results and reliably detect autophagic flux. This may in part reflect the highly sensitive nature of the assay; in particular, a high rate of flux can be detected even during basal conditions (for example, in HeLa cells grown in normal media), which makes it difficult in such settings to detect additional changes in LC3 turnover upon autophagy upregulation. Therefore, we propose that the measurement of LC3 turnover should not be the single “sine qua non” for evaluating autophagic flux. Rather, it should be viewed as a reliable indicator of autophagic flux in some settings, but in other settings, autophagic flux may need to be measured by a combination of other techniques (described below) that will yield more useful information.

Degradation of LC3 and Selective Substrates

As LC3 is degraded by autophagy, the disappearance of total LC3 is paradoxically a good indicator of autophagic flux (Figure 4E). Even the amount of LC3-II, which increases transiently upon induction of autophagy, is decreased after longer periods of autophagy activation (e.g., more than 2 hr of starvation) (Mizushima and Yoshimori, 2007). Similarly, cells under starvation exhibit a large number of GFP-LC3 puncta, but the cytoplasmic GFP-LC3 signal and the GFP-LC3 nuclear signal (the significance of which is unknown) are both reduced after autophagy induction (Figure 5A). This reduction of total GFP-LC3 expression can be quantitatively and sensitively monitored by flow cytometry (Shvets et al., 2008). Thus, the amount of total cellular LC3 that can be quantitated by immunoblot analysis or flow cytometry (or observed qualitatively by fluorescence microscopy) inversely correlates with autophagic flux. One important implication of this relates to the interpretation of LC3 staining in histological studies. Increased LC3 staining is sometimes interpreted as evidence for autophagy activation; however, such an observation may instead indicate that autophagy is suppressed, resulting in decreased autolysosomal degradation of LC3.

Besides LC3, levels of other autophagy substrates can be used to monitor autophagic flux. Classically, autophagy was considered to be a random degradation system, but recent studies have revealed that several specific substrates are preferentially degraded by autophagy, of which the best studied example is p62 (also known as SQSTM1/sequestome 1). p62 is selectively incorporated into autophagosomes through direct binding to LC3 and is efficiently degraded by autophagy (Figure 4E) (Bjørkøy et al., 2005); thus, the total cellular expression levels of p62 inversely correlate with autophagic activity. For example, a starvation-induced reduction in p62 levels is not observed in autophagy-deficient cells, and instead, p62 accumulates (Mizushima and Yoshimori, 2007). In numerous studies, the measurement of cellular p62 appears to correlate well with other parameters of autophagic flux, and overall, this assay seems quite promising. However, it should be noted that it is not yet clear whether p62 is degraded solely through autophagy or partially through the ubiquitin-proteasome pathway. Moreover, p62, as well as LC3, can be transcriptionally regulated during autophagy (He and Klionsky, 2009; Nakaso et al., 2004), which may confound the interpretation of p62 and LC3 levels as indicators of autophagic flux. Given these potential limitations, we recommend that the measurement of autophagic substrate levels be used in combination with other independent experiments to evaluate autophagic flux.

Delivery of mRFP-GFP-LC3 to the Lysosome

Another useful assay to measure autophagic flux is based on the concept of lysosomal quenching of GFP in GFP-labeled autophagic substrates such as LC3 (Figure 4F). GFP is a stably folded protein and relatively resistant to lysosomal proteases. However, the low pH inside the lysosome quenches the fluorescent signal of GFP, which makes it difficult to trace the delivery of GFP-LC3 to lysosomes; indeed, most GFP-LC3 punctate signals do not colocalize with lysosomes (Bampton et al., 2005; Kabeya et al., 2000). In contrast, RFP (and other red fluorescent proteins, such as mCherry) exhibits more stable fluorescence in acidic compartments (Katayama et al., 2008), and mRFP-LC3 can readily be detected in autolysosomes (Table 2). By exploiting the difference in the nature of these two fluorescent proteins (i.e., lysosomal quenching of GFP fluorescence versus lysosomal stability of RFP fluorescence), autophagic flux can be morphologically traced with an mRFP-GFP-LC3 tandem construct (Figure 4F and Figure 5C) (Kimura et al., 2007). With this novel construct, autophagosomes and autolysosomes are labeled with yellow (i.e., mRFP and GFP) and red (i.e., mRFP only) signals, respectively. If autophagic flux is increased, both yellow and red punctae are increased; however, if autophagosome maturation into autolysosomes is blocked, only yellow punctae are increased without a concomitant increase in red punctae. Although this assay can be used as an indicator of autophagic flux, it does not provide precisely the same information as other flux assays that directly measure endpoints of lysosomal degradation. This method depends on the acidification and degradation capacity of the lysosome. It is, therefore, sometimes possible that autolysosomes are observed as yellow, depending on the activity of lysosomal enzymes and the speed at which the acidic lysosomal pH quenches the GFP signal.

GFP-LC3 Cleavage Assay

Although GFP fluorescence is quenched by the acidic pH environment inside the lysosome, GFP is still detectable by immunoblotting and is more stable than the GFP-LC3 fusion protein (which is partially degraded upon reaching the lysosome, resulting in the appearance of a free GFP fragment) (Gao et al., 2008; Hosokawa et al., 2006). Therefore, another assay to measure autophagic flux is the detection of the free GFP fragment that is generated by degradation of GFP-LC3 in the autolysosome by immunoblotting with an anti-GFP antibody (Figure 4G). Although this assay has been fairly widely used in yeast cells expressing GFP-Atg8, experience is more limited in mammalian cells. In some cases, depending on the activity and acidity of lysosomes (which may differ between cell types), this assay may not be successful as a result of GFP degradation in the autolysosome.

Long-Lived Protein Degradation

One of the most traditional methods to evaluate autophagic flux, developed in the 1970s, is the measurement of bulk degradation of long-lived proteins (Figure 4H). In this assay, cells are cultured with isotope-labeled amino acids (usually [^{14}C]- or [^{3}H]-valine or leucine) for a long duration (several hours to several days) to label long-lived proteins, followed by a short incubation period without isotope-labeled amino acids to wash out radiolabeled short-lived proteins, which are primarily degraded by the proteasome. After treatment with an autophagy-inducing stimulus, the cellular release of degraded proteins (measured as tricholoracetic acid-soluble radioactivity in the culture supernatant) is quantified. This may be the most quantitative assay, because it provides a precise numerical readout that reflects the fate of all long-lived cellular proteins and avoids the pitfalls associated with measuring a single autophagic substrate. To ensure that one is truly measuring the contribution of “autophagic degradation” (versus other potential pathways that may contribute to long-lived protein degradation), it is standard practice to compare degradation rates between samples cultured in the presence or absence of an autophagy inhibitor (e.g., 3-methyladenine [3-MA]). One weakness of this assay is that its

interpretation depends entirely on the specificity and efficiency of the autophagy inhibitors used (discussed below).

Cautions

As reviewed in this section, several different assays are presently available to monitor autophagic flux, including the LC3 turnover assay, measurements of total levels of autophagic substrates (e.g., LC3, GFP-LC3, or p62), analysis of the mRFP-GFP-LC3 color change, measurement of free GFP generated from GFP-LC3, and measurement of lysosome-dependent long-lived protein degradation. The utility and limitations of each of these assays may vary somewhat in different cell types and in different experimental contexts; therefore, we propose that the choice of assays used to measure autophagic flux be "custom-tailored" accordingly. As the limitations of the individual assays are largely nonoverlapping, we recommend using a combination of different assays to measure autophagic flux. In this manner, it should be possible to reliably monitor autophagic flux (and thereby autophagic activity) in most mammalian tissue culture settings. Unfortunately, only a limited number of methods have been reported for monitoring autophagic flux in vivo (Iwai-Kanai et al., 2008). Although p62 levels have been reported to increase in the tissues of autophagy-deficient mice (Komatsu et al., 2007), it is not yet known how p62 levels correlate with autophagy induction in vivo. Similar to the GFP-LC3 transgenic mice that have been a useful tool for monitoring autophagosome numbers in vivo, it should be possible to generate GFP-RFP-LC3 transgenic mice that can be used to monitor autophagic flux in vivo. A more difficult, but extremely important, challenge will be to develop assays to measure autophagic flux in patients (and in blood and tissue samples from patients).

Inhibition of Autophagic Activity

To fully understand a given biological process, it is usually critical to perform experiments to modulate the activity of the process. One of the most serious problems in current autophagy research is that we still lack highly specific autophagy inhibitors and activators. Nonetheless, several modulators are now available, and genetic manipulation techniques have also provided powerful tools (Figure 1) (Rubinsztein et al., 2007). In this section and the subsequent section, we discuss different pharmacological and genetic approaches to inhibit and activate autophagy, respectively.

As autophagosome formation requires class III PI3-kinase activity, one of the most commonly used pharmacological approaches to inhibit autophagy in vitro involves the use of PI3-kinase inhibitors such as wortmannin, LY294002, or 3-MA (Blommaart et al., 1997; Itakura et al., 2008; Matsunaga et al., 2009). However, it should be noted that all of these reagents can inhibit both class I PI3-kinase activity (which inhibits autophagy) as well as class III PI3 kinase activity (which is required for autophagy) (Knight et al., 2006), and some of the PI3-kinase inhibitors, such as LY294002 and wortmannin, also inhibit mTOR (an autophagy-inhibitory molecule) by targeting its ATP binding site (Brunn et al., 1996). Moreover, as PI3-kinases (both class I and class III) regulate diverse cell signaling and membrane trafficking processes, these PI3-kinase inhibitors are not autophagy specific. Another concern is that 3-MA, which is used at very high concentrations to inhibit autophagy (usually 10 mM), can target other kinases and affect other cellular processes such as glycogen metabolism, lysosomal acidification (Caro et al., 1988), endocytosis (Punnonen et al., 1994), and the mitochondrial permeability transition (Xue et al., 2002). Indeed, 3-MA can suppress proteolysis even in Atg5-deficient cells, suggesting that its effects on protein degradation extend beyond its role in autophagy inhibition (Mizushima et al., 2001).

Although PI3-kinase inhibitors block the formation of autophagosomes, the other major pharmacological inhibitors in experimental use block later stages of autophagy (Figure 1).

Microtubule-disrupting agents (e.g., vinblastine and nocoda-zole) inhibit autophagosome-lysosome fusion, a step in the pathway that requires microtubules (Jahreiss et al., 2008;Kimura et al., 2008). Final degradation of autophagic cargo inside autolysosomes can also be inhibited by ammonium chloride, bafilomycin A_1, and lysosomal protease inhibitors such as E64d and pepstatin A (Figure 1). Although bafilomycin A_1 was originally reported to inhibit autophagosome-lysosome fusion (Yamamoto et al., 1998), a recent study suggests that it primarily affects intralysosomal degradation by inhibiting acidification, at least under certain conditions (Klionsky et al., 2008b).

One major limitation is that disruption of microtubules and inhibition of lysosomal degradation affect other cellular processes besides autophagy such as mitosis and endocytosis. Accordingly, as with the PI3-kinase inhibitors, caution needs to be exercised in interpreting phenotypes observed with such treatments. Given the lack of specificity of these currently available autophagy inhibitors, we recommend that investigators avoid drawing conclusions about the functions of autophagy based upon studies that rely uniquely upon the pharmacological inhibition of autophagy. Rather, pharmacological studies should be combined with genetic approaches to more specifically inhibit the autophagy pathway.

More specific inhibition of the autophagy pathway can be achieved by knockout or knockdown of different *ATG* genes. To date, autophagy deficiency/reduction has been confirmed in cells lacking Atg3 (Sou et al., 2008), Atg5 (Mizushima et al., 2001), Beclin 1 (Qu et al., 2003; Yue et al., 2003), Atg7 (Komatsu et al., 2005), Atg9a (Saitoh et al., 2009), Atg16L1 (Cadwell et al., 2008; Saitoh et al., 2008), FIP200 (Hara et al., 2008) and Ambra1 (Fimia et al., 2007). The knockout of Atg4C (Mariño et al., 2007), LC3B (Cann et al., 2007), and ULK1 (Kundu et al., 2008) results in mild phenotypes in vivo, probably because related isoforms compensate for the gene deficiency. Therefore, these genes should not be used as first choice RNAi targets for autophagy knockdown experiments (although small interfering RNA [siRNA] against ULK1 is effective at least in certain cell types (Chan et al., 2007). Another consideration with RNAi-mediated approaches to autophagy inhibition is that certain Atg proteins (e.g., Atg5; Hosokawa et al., 2006) still function normally in autophagy when present at very low levels; in such cases, RNAi-mediated silencing will require nearly complete suppression of protein expression to observe effective autophagy inhibition. Thus, when performing autophagy gene knockdown experiments, we recommend that investigators not only confirm effective knockdown of autophagy protein expression levels with each siRNA, but also confirm effective inhibition of the autophagy pathway using a known autophagy-inducing stimulus such as starvation.

Another successful approach for genetic inhibition of autophagy involves the utilization of dominant-negative mutant autophagy proteins. These include kinase-dead mutants of ULK1 (ULK1 ^{K46N}, ULK1 ^{K46R}, and ULK1 ^{K46I}) (Chan et al., 2009; Hara et al., 2008), Atg4B ^{C74A} (Fujita et al., 2008a), and the coiled-coil region of Atg16L1 (Fujita et al., 2008b). In addition, the overexpression of wild-type Atg12 and Atg16L1 (Fujita et al., 2008b) and a conjugation-defective Atg5 mutant (Atg5 ^{K130R}) (Pyo et al., 2005) result in dominant-negative effects. As the effect of these dominant-negative mutants is sometimes context and/or cell-type specific, it is important to confirm the inhibitory effect in each experiment.

Of note, most *ATG* gene deletions or dominant-negative mutant autophagy proteins block initiation of autophagosome formation (Figure 1, Table 1). However, abnormally elongated membranes are observed in *Atg3* knockout cells (Sou et al., 2008), *Atg5* knockout cells (Mizushima et al., 2001;Nishiyama et al., 2007), and Atg4B^{C74A}-expressing cells (Fujita et al., 2008a), suggesting that these factors, which belong to the Atg12 and Atg8/LC3 conjugation systems, may also be important for the complete closure of autophagosomes (Figure 1).

In theory, autophagy gene knockdown/knockout represents a more specific approach than pharmacological agents to inhibit autophagy. However, one important cautionary note is that the Atg proteins may not be entirely specific for autophagy; they may have autophagy-independent functions, including a role in cell death, endocytosis, and immunity-related GTPase trafficking (Kroemer and Levine, 2008; Virgin and Levine, 2009). Moreover, divergent functions for Atg proteins are suggested by the nonoverlapping phenotypes of different *ATG* gene knockout mice. The phenotypes of targeted mutant mice deficient in Atg3 (Sou et al., 2008), Atg5 (Kuma et al., 2004), Atg7 (Komatsu et al., 2005), Atg9a (Saitoh et al., 2009), and Atg16L1 (Saitoh et al., 2008) are essentially the same (neonatal lethality), whereas embryonic lethality is observed in mice deficient in Beclin 1 (Qu et al., 2003; Yue et al., 2003), FIP200 (Gan et al., 2006), and Ambra1 (Fimia et al., 2007). Thus, given the possibility that any individual Atg protein may also have autophagy-independent functions, a combination of different genetic approaches (i.e., targeting different genes that act at different steps in the autophagy pathway) is advised to increase the likelihood that observed phenotypes in the setting of autophagy gene knockdown/ knockout are truly due to inhibition of autophagic activity.

Activation of Autophagic Activity

There is growing interest in activators of autophagy, not only for research purposes but also for potential therapeutic purposes. Similar to the case with autophagy inhibitors, there are several different methods to activate autophagy, but they lack complete specificity for the autophagy pathway. As stated above, the most potent known physiological inducer of autophagy is starvation, which exerts effects both in vitro and in vivo (withdrawal of amino acids is more potent than withdrawal of serum or growth factors in vitro) (Figure 1). In most cell lines, autophagy induction can be observed within 1 hr of amino acid withdrawal; a notable exception is that certain tumor cell lines may be resistant to starvation-induced autophagy. Another approach to activate autophagy is through the modulation of nutrient-sensing signaling pathways. The best target is mTOR, which is a potent suppressor of autophagy. Rapamycin, an inhibitor of mTOR, and its analogs, such as CCI-779, activate autophagy both in vitro and in vivo (Ravikumar et al., 2004). One limitation of rapamycin in mammalian cells is that it seems to only exert partial effects at least with respect to autophagy induction and 4E-BP1 dephosphorylation (Thoreen et al., 2009). Recently developed ATP-competitive inhibitors of mTOR such as Torin1 (Thoreen et al., 2009) and PP242 (Feldman et al., 2009) show stronger inhibitory activity and may have more promise as autophagy-inducing agents. Of note, starvation and mTOR inhibition are not specific inducers of autophagy; these treatments affect a wide range of cellular responses, particularly protein synthesis and cellular metabolism, in addition to autophagy activation.

Several mTOR-independent autophagy activators have also been reported (Figure 1). Lithium induces autophagy through inhibition of inositol monophosphatase (which is mTOR-independent), but it also attenuates autophagy through glycogen synthase kinase-3β inhibition (which is mTOR-dependent) (Sarkar et al., 2008); therefore, if used to activate autophagy, lithium should always be combined with mTOR inhibitors. BH3 mimetics such as ABT737 (and similar compounds in pre-clinical development) induce autophagy by competitively disrupting the interaction between Beclin 1 and Bcl-2 or Bcl-X_L (Maiuri et al., 2007). Trehalose and small-molecule enhancers of rapamycin (SMERs) also induce autophagy by mechanisms that are not well understood (Sarkar et al., 2007a;Sarkar et al., 2007b). Two screens of FDA-approved compounds identified additional agents that induce autophagy in an mTOR-independent manner, including fluspirilene, trifluoperazine, pimozide, nicardipine, niguildipine, loperamide, and amiodarone (Zhang et al., 2007), and verapamil, minoxidil, and clonidine (Williams et al., 2008). To achieve maximal autophagy induction either in vitro (or in vivo in patients), it may be desirable to combine reagents that act in an mTOR-independent

manner with rapamycin or other mTOR inhibitors to achieve additive effects in autophagy activation.

Concluding Remarks

We have discussed currently available techniques and methods to monitor and modulate autophagy in mammalian cells. There is no perfect method to measure autophagosome numbers or to measure autophagic flux, and no perfect method to specifically activate or inhibit the autophagy pathway. Accordingly, it should be emphasized that there is no single "gold standard" for methods to monitor or modulate autophagic activity. Rather, one should consider the use of several different concurrent methods (with nonoverlapping limitations) to accurately assess the status and functions of autophagic activity in any given biological setting. With further elucidation of the molecular mechanisms of autophagy, it is anticipated that better assays will be developed to monitor autophagy and more specific agents will be developed to modulate autophagy. Such advances will be critical for an improved understanding of the biological functions of autophagy and for the successful development of therapies that modulate autophagy for use in clinical medicine.

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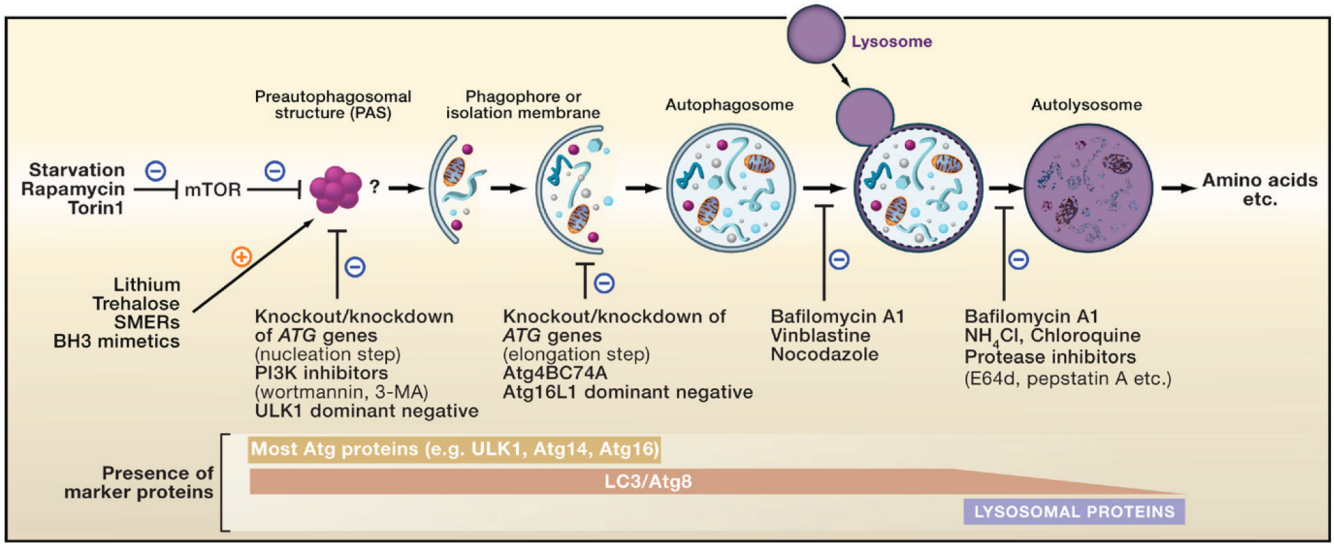

Figure 1. The Process of Macroautophagy

A portion of cytoplasm, including organelles, is enclosed by a phagophore or isolation membrane to form an autophagosome. The outer membrane of the autophagosome subsequently fuses with the lysosome, and the internal material is degraded in the autolysosome. In yeast, autophagosomes are generated from the preautophagosomal structure (PAS), which has not yet been identified in mammalian cells. A partial list of treatments and reagents that modulate autophagy are indicated. Notably, lithium may also inhibit autophagy through mTOR activation. Atg proteins that have thus far been identified on isolation membranes include ULK1/2, Atg5, Beclin 1, LC3, Atg12, Atg13, Atg14, Atg16L1, FIP200, and Atg101.

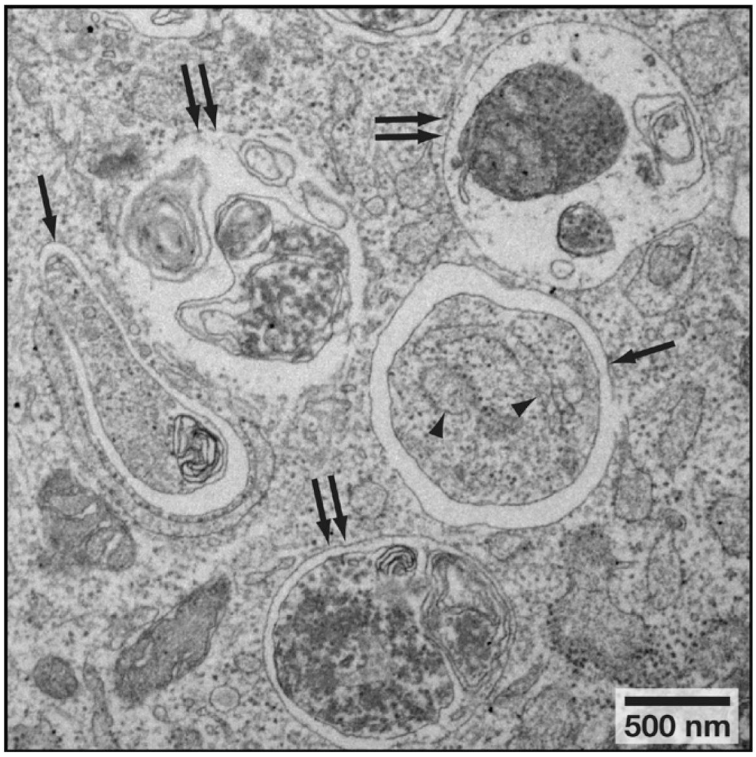

Figure 2. Autophagosome and Autolysosome Morphology

Electron microscopic analysis of nutrient-starved mouse embryonic fibroblasts. Arrows indicate autophagosomes and double arrows indicate autolysosomes/amphisomes. Arrowheads indicate fragments of endoplasmic reticulum inside the autophagosome. (Image courtesy of Chieko Kishi.)

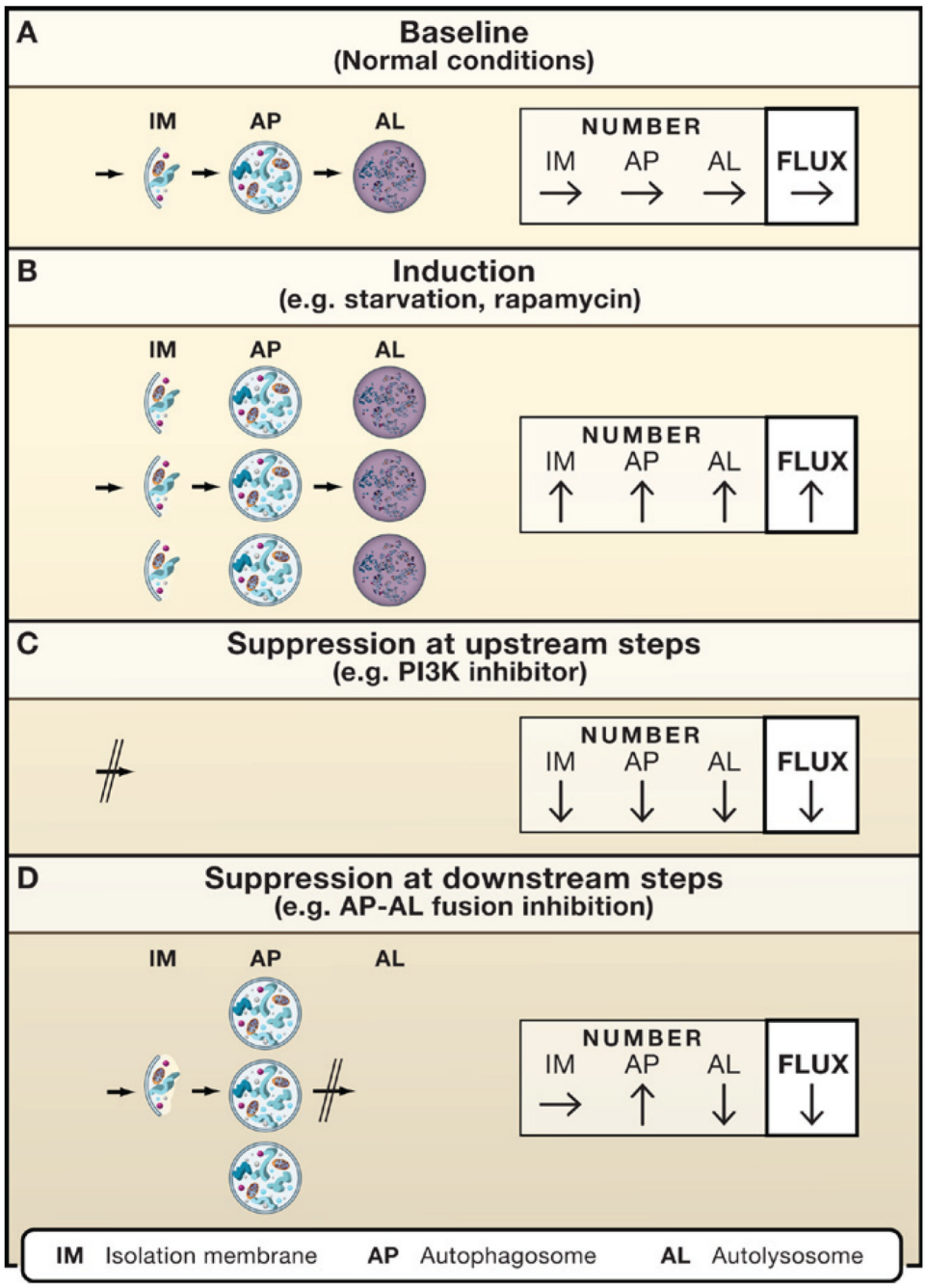

Figure 3. Dynamic Regulation of Autophagy and Cellular Accumulation of Different Autophagic Structures

Depicted are the relative amounts of isolation membrane (IM), autophagosomes (AP), and autolysosomes (AL).

(A) Under normal conditions, basal autophagy occurs.

(B) When autophagy is induced, for example by starvation, there is an increase in all types of autophagic structures.

(C) When autophagy is suppressed at any step upstream, none of the autophagic structures are generated.

(D) When autophagy is suppressed at any step after complete closure of the autophagosome, only autophagosomes accumulate.
Autophagic flux increases in (B), but decreases in (C) and (D).

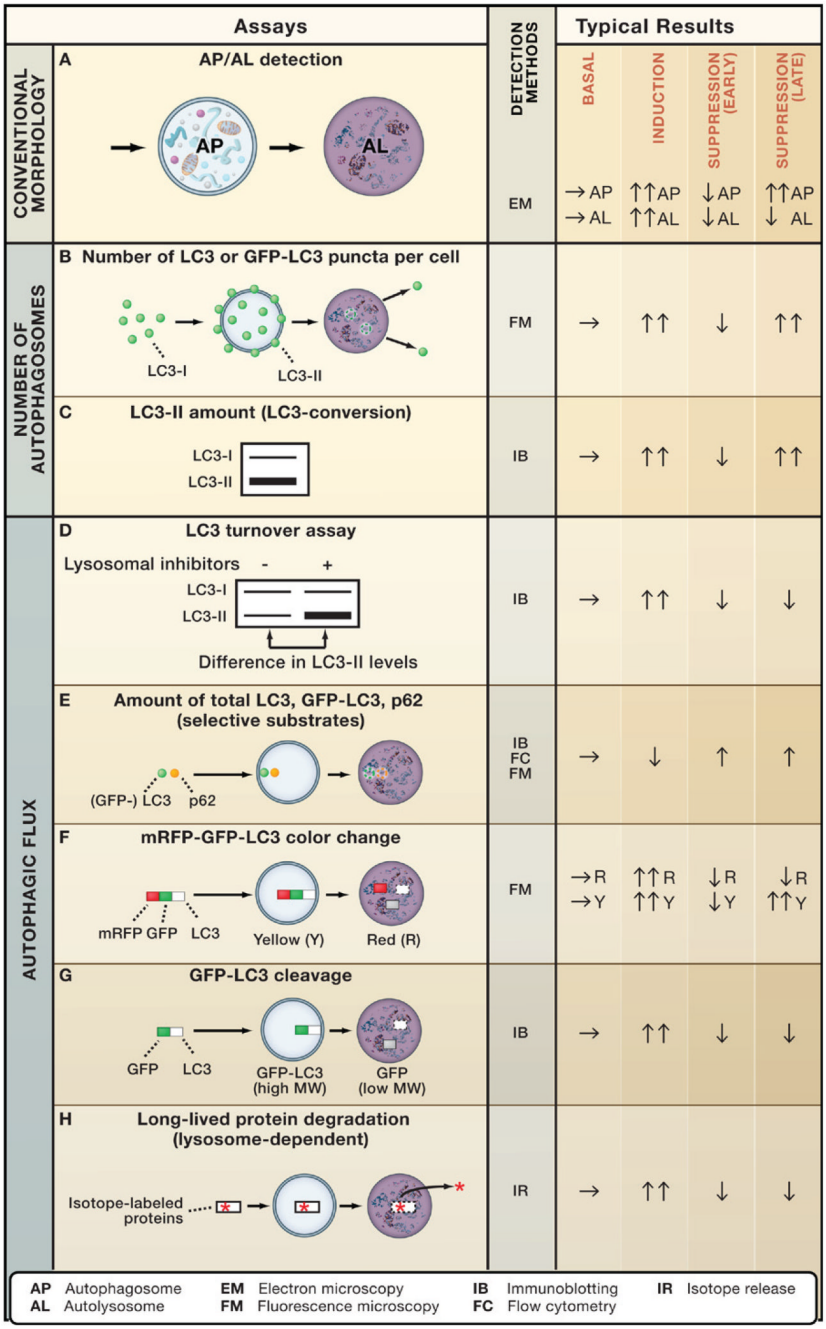

Figure 4. Methods for Monitoring Autophagosome Number and Autophagic Flux

(A) Detection of autophagosomes and autolysosomes by conventional electron microscopy.

(B) The GFP-LC3 (or endogenous LC3) puncta formation assay counts the average number of punctate structures per cell by fuorescence microscopy.

(C) Detection of the conversion of LC3-I (cytosolic form) to LC3-II (membrane-bound lipidated form) by immunoblotting.

(D) In the LC3 turnover assay, degradation of LC3-II inside the autolysosome is estimated by the comparison of two samples with and without lysosomal inhibitor treatment.

(E) Degradation of autophagy-selective substrates such as LC3 and p62 is detected by immunoblotting (LC3 is part of the autophagy machinery rather than a true substrate but is

selectively degraded by autophagy). The degradation of GFP-LC3 can also be quantifed by flow cytometry.

(F) Detection of autophagosomes labeled with a yellow signal (mRFP-GFP-LC3) and their maturation into autolysosomes labeled with a red signal (after quenching of GFP fuorescence in the lysosome).

(G) Detection of the GFP fragment generated by the degradation of GFP-LC3 inside autolysosomes by immunoblotting with an anti-GFP antibody.

(H) Measurement of long-lived protein degradation that is suppressed by autophagy inhibitors.

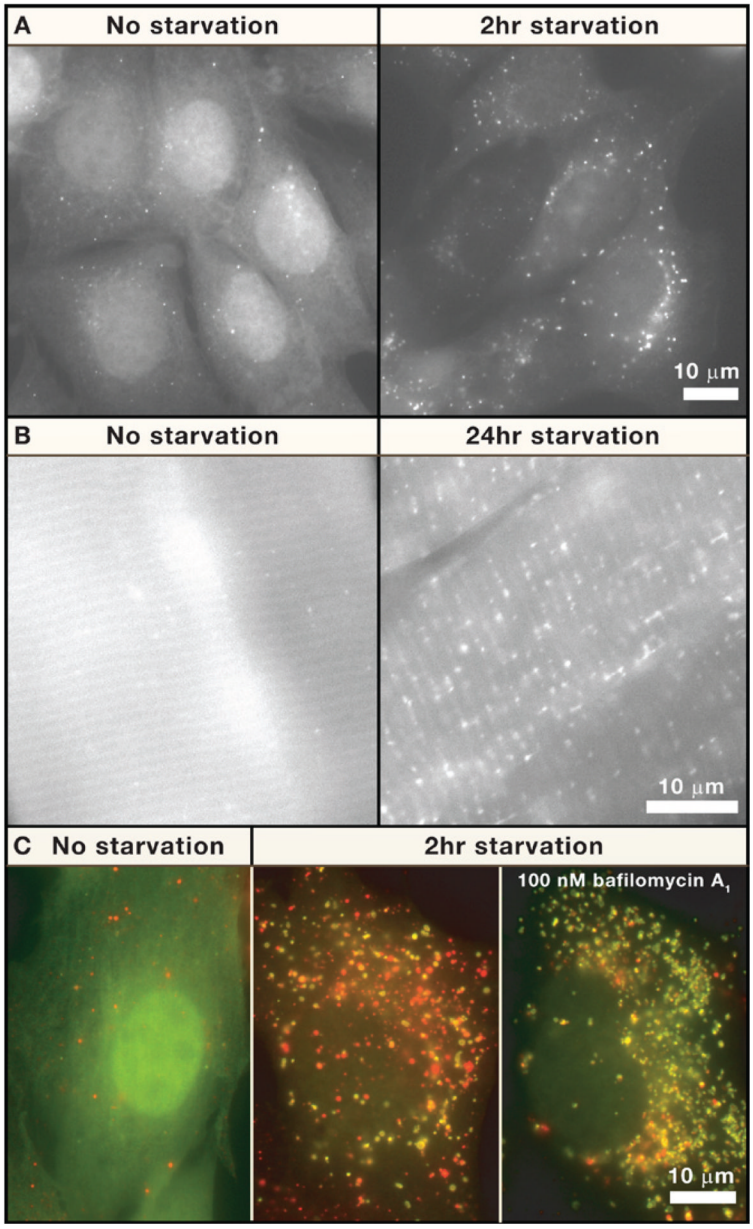

Figure 5. GFP-LC3 and mRFP-GFP-LC3 Puncta Formation Assays

(A) NIH 3T3 cells in culture stably expressing GFP-LC3 with (right) or without (left) 2 hr of starvation (depletion of both amino acids and serum). There is not only an increase in GFP-LC3 puncta number, but also a decrease in total GFP-LC3 fluorescent signals during the 2 hr incubation period.

(B) Example of an analysis of GFP-LC3 transgenic mice. Skeletal muscle (extensor digitorum longus) and heart muscle samples were prepared from GFP-LC3 transgenic mice before (left) or after (right) 24 hr of starvation.

(C) Mouse embryonic fibroblasts expressing mRFP-GFP-LC3 (left) were subjected to starvation treatment (2 hr) with (right) or without (middle) 100 nM bafilomycin A_1 to inhibit autophagosome/lysosome fusion. Note that both yellow (autophagosome) and red (autolysosome) puncta increase in the middle panel, whereas most puncta in the right panel

are yellow (autophagosome). Scale bars represent 10 μm. (Images in A and C courtesy of Eisuke Itakura.)

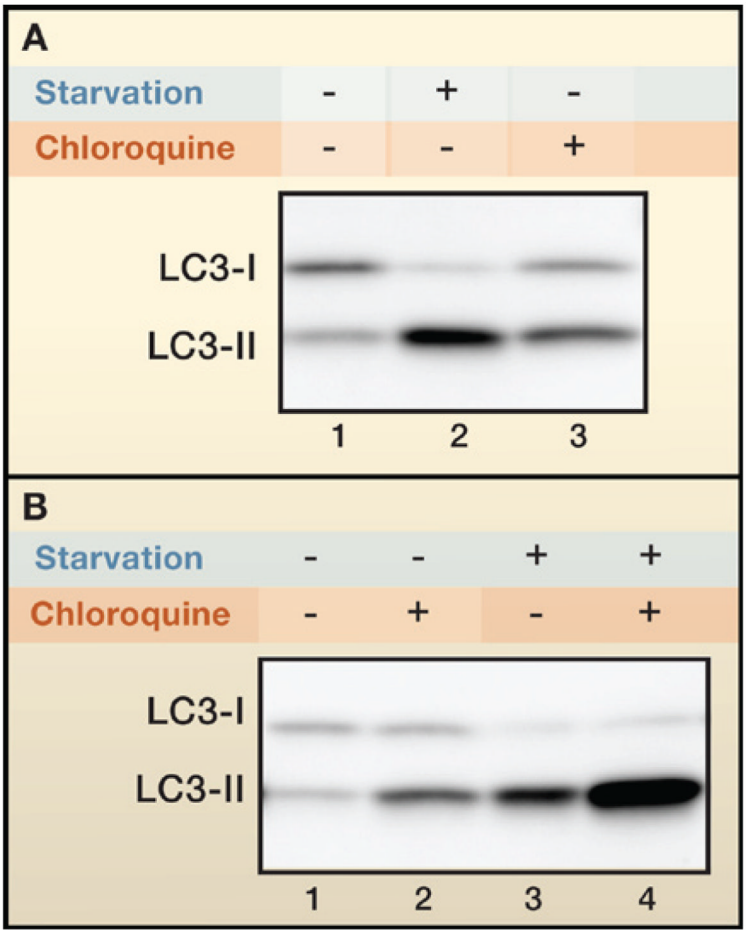

Figure 6. LC3 Conversion and Turnover Assays

(A) Steady-state levels of LC3 expression. Mouse embryonic fibroblasts were cultured in regular Dulbecco's modified Eagle's medium (DMEM) culture medium (lane 1), DMEM without amino acids and serum (lane 2), and regular DMEM containing 20 μM chloroquine (lane 3) for 1 hr. Cell lysates were subjected to immunoblot analysis with an anti-LC3 antibody. The positions of LC3-I and LC3-II are indicated.

(B) LC3 turnover assay. Cells were cultured as in (A), and the difference in LC3-II levels between samples with and without chloroquine was compared under nonstarvation and starvation conditions. (Data courtesy of Akiko Kuma.)

Table 1

Key Proteins in Mammalian Autophagosome Formation

	Mammalian Protein	Yeast Ortholog	Feature	Function
Nucleation Step				
ULK/Atg1 complex	ULK1, ULK2	Atg1	Protein kinase, phosphorylated by mTORC1	This complex is negatively regulated by mTORC1 in a nutrient-dependent manner. Upon autophagy induction, this complex translocates to early autophagic structures. Although FIP200 and Atg13 are known to be phosphory-lated by ULK1, physiologically relevant substrates remain unknown. FIP200 and Atg101 may have functions similar to yeast Atg17, 29, and 31, although they show no se-quence similarity with these proteins.
	Atg13	Atg13	Phosphorylated by (m)TORC1	
	FIP200	-	Scaffold for ULK1/2 and Atg13	
	Atg101	-	Interacts with Atg13	
	-	Atg17, 29, 31	Interacts with Atg13	
Class III PI3-kinase complex	Vps34	Vps34	PI3-kinase	Beclin 1 is negatively regulated by Bcl-2 through direct binding. This complex produces PI(3)P, most likely on the ER membrane. Vps34, p150, and Beclin 1 are shared with the UVRAG complex, which seems to function in the late endocytic pathway. Rubicon negatively regulates autophagosome-lysosome fusion through interaction with the UVRAG complex.
	p150	Vps15	Myristoylated	
	Beclin 1	Vps30/Atg6	BH3-only protein, interacts with Bcl-2	
	Atg14	Atg14	Autophagy-specific subunit	
	Ambra1	-	Interacts with Beclin 1	
Others	Atg2	Atg2	Interacts with Atg18 in yeast	DFCP1 forms an “omegasome” on the ER, where other Atg proteins are assembled. Atg9, WIPIs, and VMP1 are present on autophagic membrane. Atg9 also exists in other compartments such as endosomes and the Golgi apparatus.
	Atg9	Atg9	Transmembrane protein	
	WIPI1-4	Atg18	PI(3)P-binding proteins	
	DFCP1	-	PI(3)P-binding ER protein	
	VMP1	-	Transmembrane protein	
Elongation Step				
Atg12-conjugation system	Atg12	Atg12	Ubiquitin-like, conjugates to Atg5	The Atg12-Atg5-Atg16(L) dimer is important for LC3/Atg8-phosphatidylethanolamine (PE) conjugation. This complex is

	Mammalian Protein	Yeast Ortholog	Feature	Function
				present on the outer side of the isolation membrane and is essential for proper elongation of the isolation membrane.
	Atg7	Atg7	E1-like enzyme	
	Atg10	Atg10	E2-like enzyme	
	Atg5	Atg5	Conjugated by Atg12	
	Atg16L1	Atg16	Homodimer, interacts with Atg5	
LC3/Atg8-conjugation system	LC3 (GATE-16, GABARAP)	Atg8	Ubiquitin-like, conjugates to PE	The formation of LC3/Atg8-PE conjugates and their deconjugation by Atg4 is important for isolation membrane elongation and/or complete closure. LC3/Atg8 is present on both inner and outer membranes of the autophagosome, and also serves as an adaptor for selective substrates such as p62, NBR1, and the yeast mitophagy protein, Atg32.
	Atg4A-D	Atg4	LC3/Atg8 C-terminal hydrolase, deconjugating enzyme	
	(Atg7)	(Atg7)	(E1-like enzyme)	
	Atg3	Atg3	E2-like enzyme	

Table 2

Materials Discussed In This Primer

Addgene	
GFP-LC3 plasmid	http://www.addgene.org/pgvec1?f=c&cmd=showcol&colid=623
mRFP-GFP-LC3 (ptfLC3) plasmid	
Atg4BC74A plasmid (pmStrawberry-Atg4BC74A)	
Riken BioResource Center, Cell Bank	
Atg5−/− mouse embryonic fibroblast	http://www2.brc.riken.jp/lab/cell/detail.cgi?cell_no=RCB2711&type=1
Atg5+/+ mouse embryonic fibroblast	http://www2.brc.riken.jp/lab/cell/detail.cgi?cell_no=RCB2710&type=1
Riken BioResource Center, Experimental Animal Division	
GFP-LC3 mice	http://www2.brc.riken.jp/lab/animal/detail.cgi?reg_no=00806
Atg5+/− mice	http://www2.brc.riken.jp/lab/animal/detail.cgi?reg_no=02231
Atg5flox mice	http://www2.brc.riken.jp/lab/animal/detail.php?brc_no=RBRC02975
Atg7+/− mice	http://www2.brc.riken.jp/lab/animal/detail.php?brc_no=RBRC02760
Atg7flox mice	http://www2.brc.riken.jp/lab/animal/detail.php?brc_no=RBRC02759
Atg3+/− mice	http://www2.brc.riken.jp/lab/animal/detail.php?brc_no=RBRC02761