[Autophagy 3:3, 181-206; May/June 2007]; ©2007 Landes Bioscience

Review

Methods for Monitoring Autophagy from Yeast to Human

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Original manuscript submitted: 01/30/06
Manuscript accepted: 08/04/06

Previously published online as an *Autophagy* E-publication:
http://www.landesbioscience.com/journals/autophagy/abstract.php?id=3678

KEY WORDS

assay, lysosome, protein degradation, protein targeting, trafficking, vacuole

ABBREVIATIONS

GFP	green fluorescent protein
MDC	monodansylcadaverine
TAKA	transport of Atg9 after knocking-out Atg1
Ams1	α-mannosidase
Ape1	aminopeptidase I
Atg	autophagy-related
LC3	microtubule associated protein 1 light chain 3
PE	phosphatidylethanolamine
3MA	3-methyladenine

ACKNOWLEDGEMENTS

The authors thank Yang Cao and Dr. Julie Legakis for helpful comments. This work was supported by Public Health Service grant GM53396 to D.J. Klionsky, by grants from the Norwegian Cancer Society to P.O. Seglen, and by Public Health Service grant AG21904 to A.M. Cuervo.

ABSTRACT

The increasing interest in autophagy in a wide range of organisms, accompanied by an ever-growing influx of researchers into this field, necessitates a good understanding of the methodologies available to monitor this process. In this review we discuss current approaches that can be used to follow the overall process of autophagy, as well as individual steps, from yeast to human. The majority of the review considers methods that apply to macroautophagy; however, we also consider alternative types of degradation including chaperone-mediated autophagy and microautophagy. This information is meant to provide a resource for newcomers as well as a stimulus for experienced researchers who may be prompted to develop additional assays to examine autophagy-related pathways.

INTRODUCTION

Macroautophagy is one of the major degradative pathways in eukaryotic cells, and it is the only one with the capacity to degrade entire organelles.[1-3] This process is ubiquitous among eukaryotes and has been characterized from yeast to man.[4] Various developmental pathways utilize macroautophagy,[5] and it is also associated with certain pathophysiological conditions.[6,7] These roles for macroautophagy have been the focus of recent reviews and will not be discussed in this article.

A number of protein components required for macroautophagy have been identified within the past decade.[8,9] The functions of these proteins are still being determined and have also been the subject of several reviews.[10-13] This information, combined with our morphological understanding of macroautophagy, allows the process to be divided into eight steps (Fig. 1). These steps begin with induction,[14,15] a signal transduction event that triggers the nucleation of a sequestering membrane or phagophore (Fig. 2), which leads to the formation of a cytosolic double-membrane vesicle that sequesters cytoplasm, the hallmark of macroautophagy.[16] Microautophagic processes also involve sequestration of a portion of cytoplasm; however, this occurs at the surface of the lysosome/vacuole.[17] The expansion of the phagophore and completion (Fig. 3) of the sequestering vesicle, the autophagosome, probably involve some type of membrane delivery event.[18] During this time, most of the protein components may cycle on and off the forming autophagosome because only two of the autophagy-related (Atg) proteins, Atg8 and Atg19, appear to remain associated with the completed vesicle. The role of Atg8 is not known, but it is considered to be a structural protein that is critical in autophagosome formation, whereas Atg19 is a receptor that functions in cargo recognition and packaging (Fig. 4).[19,20] The recycling of Atg9 may be highly regulated and is certainly complex because it is a transmembrane protein (Fig. 5; Legakis JE, Yen W-L, He C, Yorimitsu T, Monastyrska I, Klionsky DJ, unpublished results).[21] Fusion of the autophagosome with the lysosome/vacuole utilizes components that are common with all other fusion events that involve these organelles including SNARE and Rab proteins as well as components of the class C Vps/HOPS machinery (step 6, Fig. 1).[9,22] The breakdown of the autophagic cargo within the lysosome/vacuole lumen and the subsequent efflux through permeases allows the resultant macromolecules to be reused in the cytosol for metabolic reactions (steps 7 and 8, Fig. 1).

Because of the increasing connections made between autophagy and human physiology, this field has garnered increased attention, which in turn has led to renewed interest in methods for monitoring this process. Three general types of methods have been predominant: (1) microscopy, (2) biochemical methods, and (3) detection of protein modifications through SDS-PAGE and western blotting (Table 1). Microscopy,

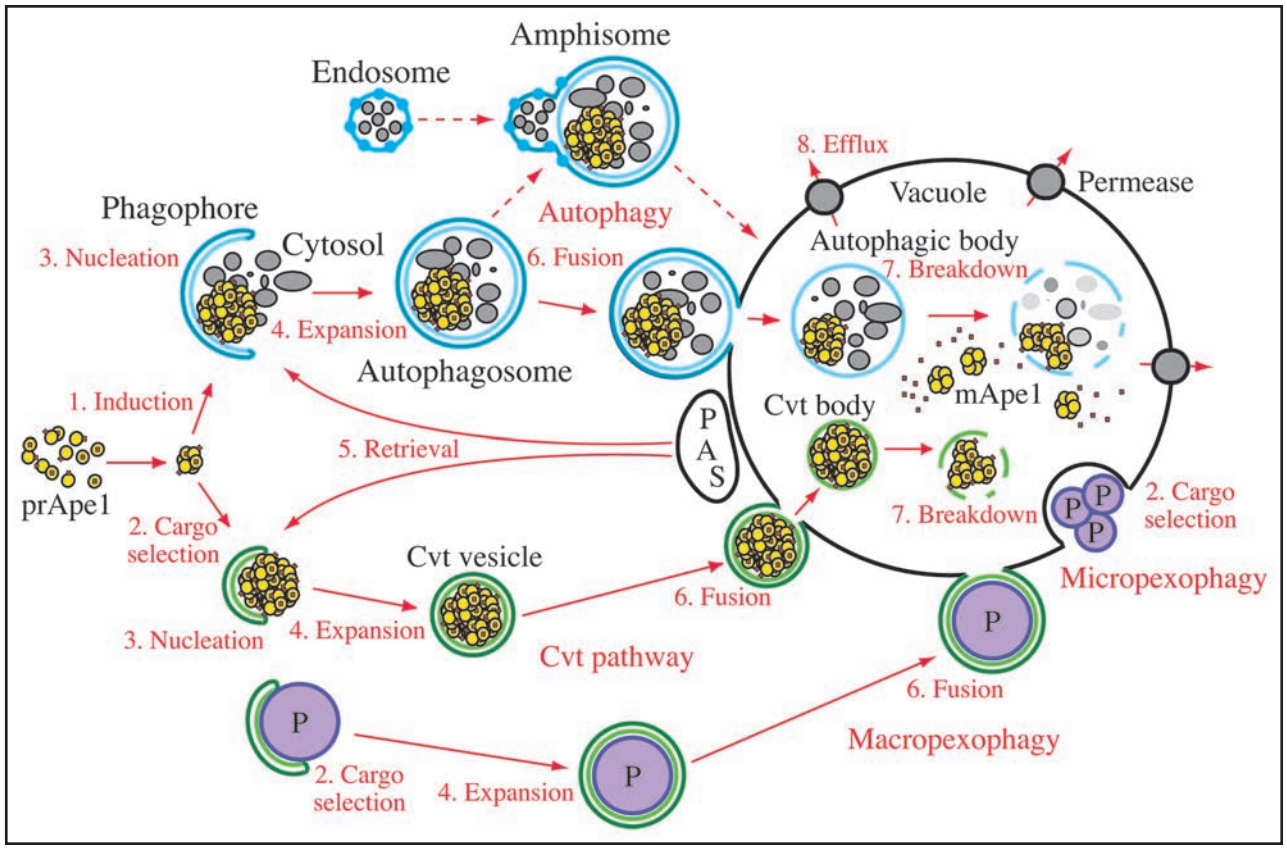

Figure 1. Schematic overview of autophagy-related processes. Non-specific autophagy can be separated into seven steps: (1) Induction; (3) Vesicle nucleation; (4) Vesicle expansion and completion; (5) Retrieval; (6) Fusion; (7) Breakdown; (8) Efflux. Specific types of autophagy include an additional step (2) of cargo recognition and packaging. The cytoplasm to vacuole targeting (Cvt) pathway and pexophagy are two examples of specific autophagy. See text for additional details. PAS, phagophore assembly site/preautophagosomal structure; prApe1 and mApe1, precursor and mature form of aminopeptidase 1, respectively. This schematic primarily depicts a yeast cell, but includes information from mammalian cells (e.g., the amphisome). This figure was modified from a figure previously published in reference 10 and is reproduced by permission of The Company of Biologists, copyright 2005.

in particular electron microscopy, has been utilized for the longest period of time, reflecting the fact that biochemical and molecular methods are more recent developments; however, microscopy is still indispensable for the characterization of autophagic organelles. Quantitative studies of autophagy were greatly facilitated by the realization that a major fraction of the degradation of long-lived endogenous protein, easily followed by a pulse-chase radiolabeling approach,[23] was due to macroautophagy, which could be specifically suppressed by amino acids or 3-methyladenine.[24,25] Even more specific quantitative measurements of autophagy, and of individual steps in the autophagic-lysosomal pathway, became possible with the introduction of biochemical assays that measured the transfer of electroinjected or endogenous (enzymatic) probes from the cytosol to sedimentable autophagic organelles such as autophagosomes and lysosomes.[26-32]

With the identification of the molecular components of the macroautophagic machinery, a range of new assays became available that relied on specific properties of the Atg proteins. In addition, some assays were developed or adapted specifically to monitor autophagy based on the particular process being investigated. For example, the filter trap assay is utilized to detect defects in autophagic clearance of protein aggregates;[33] this use stems from the relatively recent studies that implicate autophagy in neurodegeneration and the removal of inclusion bodies.[34,35]

Table 1 **Methods for monitoring autophagy**

Assay Type	Available Assay
1. Microscopy	Acridine orange (F)[a], Autophagic body (E,F,L) and autophagosome (E,F) formation and accumulation, GFP-Atg8 localization (E,F), GFP-LC3 (F), LysoTracker Red (F), MDC (F), Precursor Ape1 localization (F), Sequestration of cytosolic markers (F), TAKA assay (F)
2. Biochemical	Ams1 activity, Ape1 activity, Atg1 kinase activity, Peroxisomal enzyme inactivation, Pho8Δ60 activity, Protein degradation, Sequestration of cytosolic markers
3. Protein modification	Active site CtoS[b], Ape1 processing, Ape1 synthesis, Atg8-GFP processing, Atg8–PE formation and membrane association, Atg8 synthesis, Atg12–Atg5 conjugation, Atg13 phosphorylation, Fox3 degradation, GFP-Atg8 and GFP-LC3 processing, LC3-I conversion, LC3 C-terminal processing, LC3-II turnover, Pex14 processing, Protease protection

[a]E, Electron microscopy; F, fluorescence microscopy; L, light microscopy. GFP, green fluorescent protein; MDC, monodansylcadaverine; TAKA, Transport of Atg9 after knocking-out Atg1; Ams1, α-mannosidase; Ape1, aminopeptidase I. [b]Mutation of cysteine to serine.

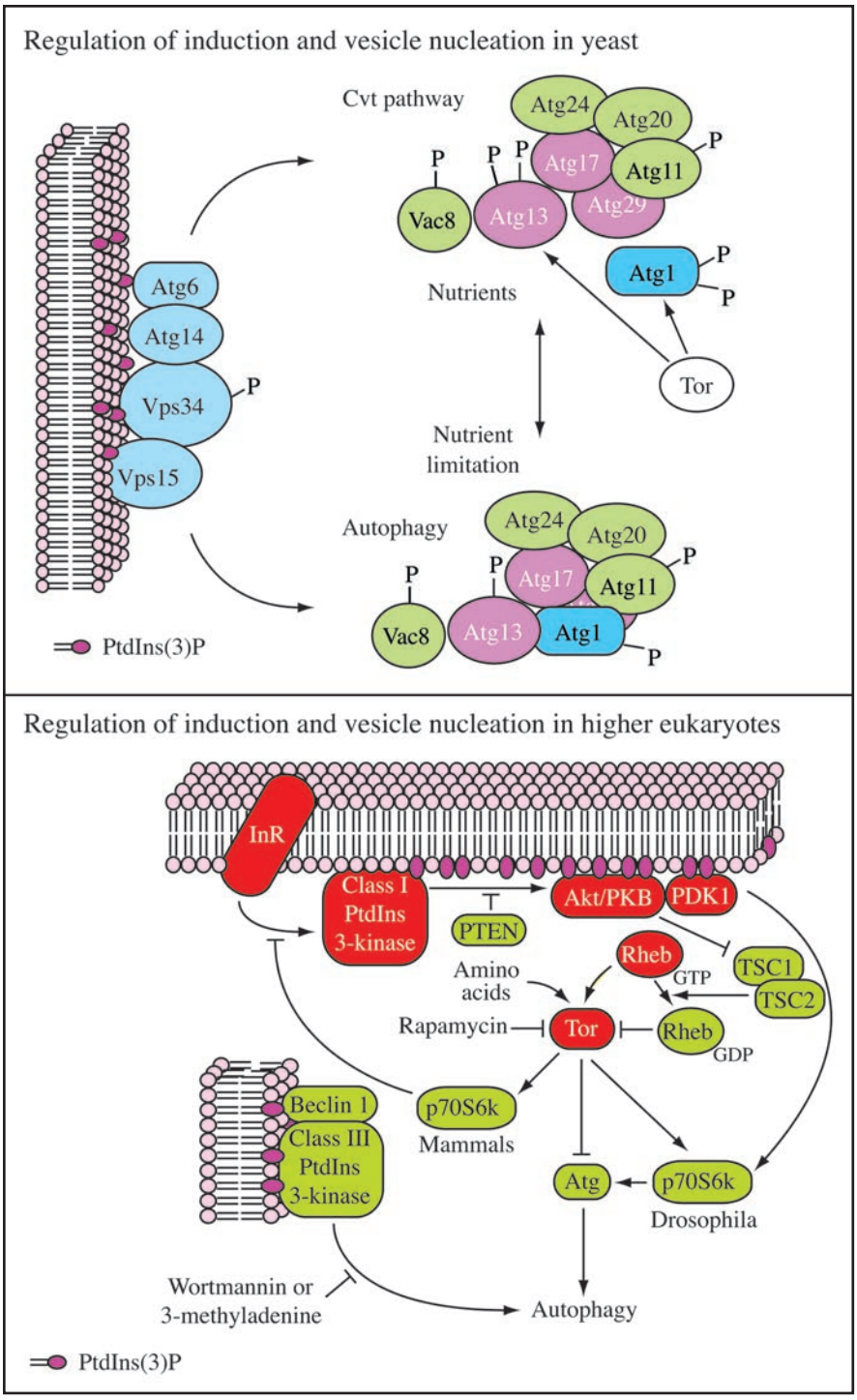

Figure 2. Machinery involved in signal transduction events that induce autophagy (step 1 of Fig. 1) and nucleate formation of the sequestering vesicle (step 3). In yeast (top), the Atg1 kinase is part of a complex that is composed of proteins that are relatively specific for autophagy (Atg17, Atg29 and possibly Atg13) or the Cvt pathway (Atg11, Atg20, Atg24 and Vac8). The phosphorylation of at least Atg13 is nutrient-dependent and the highly phosphorylated form appears to have a lower affinity for Atg1. The Atg1 and Atg13 proteins appear to be phosphorylated in a Tor- and protein kinase A-dependent manner. In eukaryotes (bottom), Tor is regulated by a class I PtdIns 3-kinase that acts through a regulatory cascade of kinases and phosphatases to inhibit autophagy. In this case, in addition to Atg proteins, other targets of Tor such as p70S6 kinase also contribute to the modulation of autophagy. This figure was modified from a figure previously published in reference 10 and is reproduced by permission of The Company of Biologists, copyright 2005.

With the increase in assays it is now possible to monitor many of the individual steps of macroautophagy (Table 2). Clearly the assignment of some assays to one particular category is arbitrary. For example, assays such as those measuring degradation monitor not just breakdown but also reflect the functionality of previous steps. Furthermore, most of the Atg molecular marker assays can only be utilized in fungal systems, particularly yeasts, although Atg8/LC3 has become an important tool in the study of mammalian macroautophagy as well (Table 3).

Previous reviews have covered some of the assays mentioned in this article;[36,37] however, those reviews have focused on higher eukaryotes. As a consequence, no reviews have included the wide array of assays available in yeast, or assays that are specific to plants. In addition, because many of these techniques are currently restricted to yeasts, previous reviews have generally excluded most of the assays that measure specific functions of individual Atg proteins. In contrast, the present review is fairly inclusive, dealing with the yeast assays as well as assays used in higher eukaryotes. Specific protocols are not provided and the descriptions and discussions are only meant to give a brief indication of the relative complexity of the procedure. The primary literature should be consulted for complete details. The methods are presented below according to the autophagic pathways, steps and organelles to which they apply.

THE MACROAUTOPHAGIC PATHWAY

Autophagic-lysosomal protein processing/degradation. The original definition of autophagy[38] corresponds to the process now often referred to as macroautophagy, a term that is becoming increasingly useful to distinguish the bulk sequestration of normal cytoplasm[39,40] from the many specialized autophagic functions that have been characterized in recent years, such as chaperone-mediated autophagy,[41] pexophagy,[3] xenophagy,[42] mitophagy,[43] etc. In the present article, the term autophagy will be used synonymously with macroautophagy unless otherwise specified.

(Macro)autophagy is a largely nonselective process; any endogenous or introduced cytoplasmic marker can, therefore, in principle be used to monitor the overall activity of the autophagic-lysosomal pathway, where autophagic sequestration is usually the rate-limiting step. Because protein is the major biochemical constituent of cytoplasm, protein degradation is both a logical and convenient way of measuring autophagy, although it should be noted that RNA degradation can be used for this purpose as well.[44]

The obvious drawback of protein degradation measurements is that they do not just reflect autophagic activity, but a number of other proteolytic processes as well, such as proteasomal degradation (the major alternative pathway), other forms of autophagy (chaper-

Table 2 **Steps of autophagy that can be monitored**

Step of Autophagy	Assay	Represents/Requires[a]
1. Induction	Atg8 synthesis	Signaling
	Atg13 dephosphorylation	Tor inactivation
	GFP-LC3	Signaling
	LC3-I conversion to LC3-II	Signaling
	LysoTracker Red	Acidified compartments
	Monodansylcadaverine	Acidified compartments
2. Cargo recognition/ packaging	Ams1 activity	Atg19, Atg11, Cytoskeleton
	Ape1 processing/activity	Atg19, Atg11, Cytoskeleton
	Precursor Ape1 localization	Atg19, Atg11, Cytoskeleton
3. Vesicle formation	Acridine orange	Vesicle formation machinery
	Active site CtoS	Atg7 activation, Atg3 and Atg10 conjugation
	Atg1 kinase activity	Atg1, Atg11, Atg13, Atg17 function
	Atg8-GFP processing	Atg4 cleavage
	Atg12–Atg5 conjugation	Atg7, Atg10 conjugation
	Atg8–PE formation	Atg3 conjugation, Atg4, Atg7
	Atg8–PE membrane association	Atg3 conjugation, Atg4, Atg7
	AP formation/accumulation	Vesicle formation machinery
	Electron microscopy	Vesicle formation machinery
	GFP-Atg8 localization	Atg3 conjugation, Atg4, Atg7, PAS formation
	GFP-LC3	Vesicle formation machinery
	LC3 C-terminal processing	Atg4 cleavage
	LysoTracker Red	Vesicle formation machinery
	Monodansylcadaverine	Vesicle formation machinery
	Protease-protection	AP/Cvt vesicle completion
	Sequestration of cytosolic markers	AP formation
4. Retrieval	TAKA assay	Atg1, Atg2, Atg13, Atg18, Atg23, Atg27, Cytoskeleton
5. Fusion/ Breakdown	AB/AP formation and accumulation	Atg15, Pep4, Prb1, Fusion machinery, Vacuolar pH
	Electron microscopy	Atg15, Pep4, Prb1, Fusion machinery, Vacuolar pH
	Fox3 degradation	Pexophagy
	GFP-Atg8 localization	Atg15, Pep4, Prb1, Fusion machinery, Vacuolar pH
	GFP-Atg8/LC3 processing	Membrane delivery, AB lysis
	LC3-II turnover	Complete autophagy
	Peroxisomal enzyme inactivation	Pexophagy
	Pex14-GFP processing	Pexophagy
	Pho8Δ60 activity	Bulk delivery
	Protein degradation	Bulk delivery
6. Efflux	Ape1 (or other protein) synthesis	Permease function

[a]These assays do not necessarily represent one step of autophagy, and this information is only included as a general guide. AB, autophagic body; AP, autophagosome; Prb1, proteinase B. CtoS, mutation of cysteine to serine.

one-mediated autophagy), nonautophagic lysosomal degradation (related, for example, to endocytosis) and the activity of various cytoplasmic proteinases (calpains, mitochondrial proteinases, etc.). One way to increase the specificity is to preferentially measure the degradation of long-lived proteins, which tend to be degraded relatively more by autophagy than by the other pathways (Fig. 6). The general strategy is to use a pulse-chase technique, where the cellular protein is labeled with a radioactive amino acid and given a sufficiently long chase period (for example, 4 h) to ensure that a major fraction of the short-lived proteins has been degraded, as indicated by the attainment of a constant (linear) rate of radioactivity release.[23,45] Because of the long half-life of the proteins being analyzed in this assay, the labeling (pulse) should be long enough (generally greater than 10 h) to guarantee that a considerable fraction of the long-lived pool of proteins is labeled. To avoid metabolism of the released amino acid, the protein radiolabeling should be performed with an amino acid that is poorly metabolized and easily exchanged across the plasma membrane (such as the branched-chain amino acids), and to avoid reincorporation the chase should be done in the presence of an excess of unlabeled amino acid. [^{14}C]Valine is the perfect choice for this purpose, and has the additional advantage of (unlike leucine) not interfering with autophagic sequestration.[45,46] In cell cultures, 1–2 mM unlabeled valine during the chase and degradation measurement will usually suffice;[47] in perfused livers, higher concentrations (15 mM) have been found necessary.[23] In suspensions of freshly isolated hepatocytes, valine equilibrates so rapidly across the plasma membrane that the addition of unlabeled valine is not really required. The method can be further simplified by injecting rats with [^{14}C]valine intravenously 24 h before cell isolation, thus obviating the need for a chase period.[45]

It should be noted that the distinction between long-lived and short-lived proteins is far from absolute: Nearly all cellular proteins are sequestered and degraded by the autophagic-lysosomal pathway to approximately the same extent.[29] On the other hand, their degradation by other proteolytic mechanisms is highly individual, accounting for the large differences in the turnover (half-life) of specific proteins. The overall nonlysosomal protein degradation can be estimated through the use of inhibitors of lysosomal proteolysis, such as ammonium chloride or other weak bases, that neutralize the acidic intralysosomal pH required for optimal activity of all lysosomal proteases.[49] Surprisingly, the overall nonautophagic protein degradation is quite invariant at 0.5–1.5%/h, depending somewhat on the cell type. A substantial fraction (20–100%, depending on the relative autophagic activity) of the degradation of even long-lived proteins will, therefore, reflect non-autophagic proteolysis.[25]

To achieve a more specific measurement of autophagic protein degradation, the use of inhibitors is helpful. Amino acid mixtures,[24,48] and in some cases serum,[49] can suppress autophagic degradation completely and quite selectively, although rapid metabolism of amino acids may make the suppression transient.[25] The drug 3-methyladenine (3MA) is an effective and selective autophagy inhibitor that will inhibit autophagic protein degradation completely and stably at high concentrations (5–10 mM), with no detectable effects on other proteolytic pathways.[25,49] It was suggested that 3MA inhibited non-autophagic protein degradation in Atg5-deficient mouse cells (Atg5 is required for autophagosome expansion and completion;

Table 3 **Autophagic assays used in different model systems**

System[a]	Assay
Arabidopsis	AB formation and accumulation (light microscopy), Atg8 synthesis, Electron microscopy, GFP-Atg8, Plant-specific assays
C. elegans	AB formation and accumulation (light microscopy), Electron microscopy, Viability
Dictyostelium	Development, Electron microsocpy, GFP-Atg8, Protein degradation, Viability
Drosophila	Electron microscopy, LC3-I conversion to LC3-II, LC3-II turnover, LysoTracker Red, Viability
Mammals	Acridine orange, Active site CtoS, Electron microscopy, Filter trap, GFP-LC3 puncta formation, GFP-LC3 processing, LC3-I conversion to LC3-II, LC3 C-terminal processing, LC3-II turnover, LysoTracker Red, Monodansylcadaverine, Peroxisomal enzyme inactivation, Protein degradation, Sequestration of cytosolic markers, Viability
Protozoa	Electron microscopy, Monodansylcadaverine, Sequestration of cytosolic markers
Yeast	Active site CtoS, Ams1 activity, Ape1 processing and activity, Ape1 synthesis, Atg1 kinase activity, Atg8-GFP processing, Atg8–PE formation and membrane association, Atg8 synthesis, Atg12–Atg5 conjugation, Atg13 phosphorylation/dephosphorylation, AB/AP formation and accumulation, Development (sporulation), Electron microscopy, Fox3 degradation, GFP-Atg8 localization, GFP-Atg8 processing, GFP-SKL, Peroxisomal enzyme inactivation, Pex14-GFP processing, Pho8Δ60 assay, Precursor Ape1 localization, Protein degradation, Protease-protection, TAKA assay, Viability

[a]The inclusion of a system means that the assay has been used in the system in the published literature. Some assays could presumably be used in organisms other than as indicated above. CtoS, mutation of cysteine to serine; AB, autophagic body.

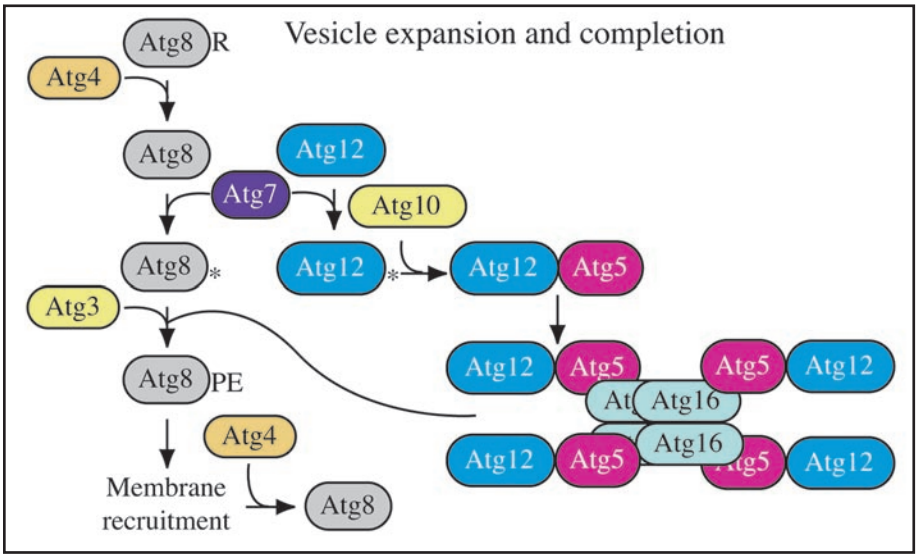

Figure 3. Two systems involving ubiquitin-like proteins participate in formation of the sequestering vesicles of macroautophagy (step 4 of Fig. 1). Atg8 (and its mammalian homologue LC3) is processed at the C terminus by the protease Atg4 to expose a glycine residue. The processed Atg8 and Atg12 are both activated independently by Atg7, a homologue of the ubiquitin activating enzyme. Atg8 and Atg12 are then conjugated by another protein, Atg3 or Atg10, respectively, which functions analogously to an ubiquitin conjugating enzyme, linking them to phosphatidylethanolamine or Atg5. Atg8–PE becomes attached to membrane, whereas Atg12–Atg5 bind Atg16, which multimerizes to form a large complex. This figure was modified from a figure previously published in reference 10 and is reproduced by permission of The Company of Biologists, copyright 2005.

see Fig. 3), but because some autophagosome formation was still indicated in those cells,[50] an effect on residual, Atg5-independent autophagy would seem more likely.

Although 3MA is a nontoxic compound that does not impair cellular energy metabolism, protein synthesis or viability,[25,51] its mechanism of action as an inhibitor of class III phosphatidylinositol (PtdIns) 3-kinases[52] and its chemical properties as an adenine analogue enable it to interfere with a variety of cellular processes,[53,54] including DNA synthesis (P.O. Seglen, unpublished results) and fluid-phase endocytosis.[55,56] An inhibition of cellular chloroquine uptake by 3MA was interpreted as an effect on lysosomal pH,[53] but no direct effect was seen on isolated lysosomes, the effect has not been confirmed[56] and 3MA did not impair the ability of lysosomes to degrade endocytosed proteins in intact cells.[25] Although care should be exercised in interpreting secondary effects of 3MA, the drug can be safely used to distinguish between autophagic and nonautophagic protein degradation (Fig. 7).

Some long-lived proteins appear to be degraded exclusively by autophagy,[29,31] and could probably be used in specific assays of autophagic protein degradation. Measuring the decay of these slowly turning over proteins by activity assays[29] or immunoprecipitation[31] does not provide adequate accuracy due to the high background, but the use of a recombinant protein tagged with a suitable adduct, released and measurable upon intralysosomal hydrolysis, might be an approach worth pursuing, although particular attention should be paid to the expression level of the recombinant tagged protein, to avoid overloading protein folding and targeting mechanisms.

Autophagic lactolysis. There is one assay, autophagic lactolysis, that specifically measures overall autophagic-lysosomal degradation. [^{14}C]Lactose, electroinjected into isolated hepatocytes, is not degraded in the cytoplasm because there is no β-galactosidase in this location; this disaccharide can only be degraded by autophagic transfer to lysosomes, where it is hydrolyzed by the lysosomal β-galactosidase.[57] The formation of radiolabeled monosaccharides (measured, for example, by column chromatography) will thus specifically reflect the activity of the autophagic-lysosomal pathway, as confirmed by its complete inhibition by 3MA (Fig. 7C).[30] The rate of autophagic lactolysis can, alternatively, be calculated (by difference) by comparison with a nonhydrolyzable sugar such as [^{3}H]raffinose.[58] Electroinjected [^{14}C]lactose can also be used to measure individual steps in the autophagic-lysosomal pathway, as

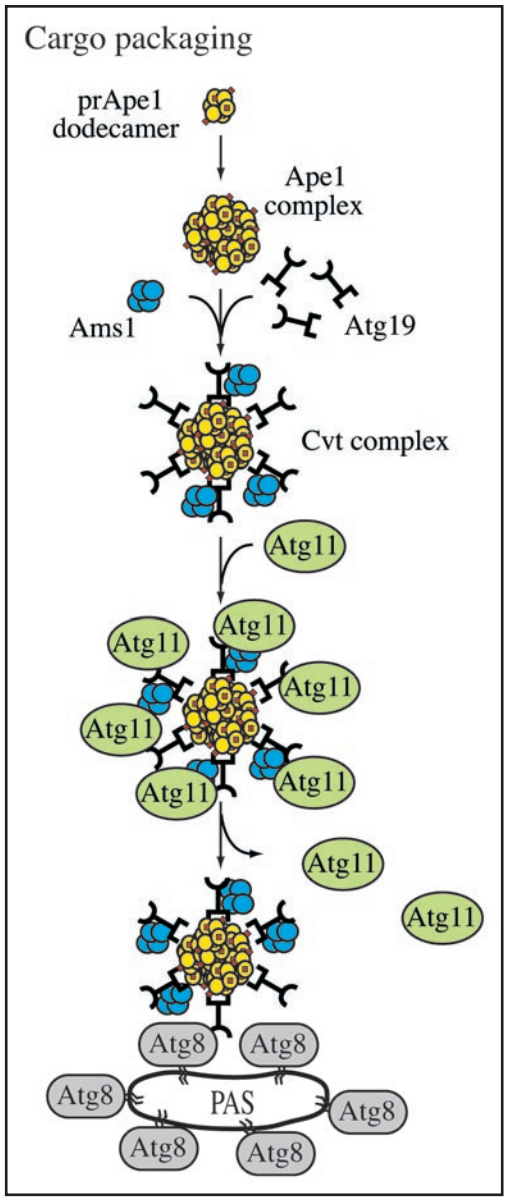

Figure 4. Cargo recognition and packaging (step 2 of Fig. 1). Macroautophagy is generally considered to be nonspecific, sequestering bulk cytoplasm; however, there are examples of specific autophagy such as the Cvt pathway and pexophagy. In the Cvt pathway, prApe1 forms a large complex that interacts with the receptor Atg19 via the prApe1 propeptide. Atg19 also binds Atg11 and these two proteins are needed to move the complex to the site of vesicle formation, the phagophore assembly site/preautophagosomal structure (PAS). Atg19 subsequently binds Atg8–PE, which may trigger completion of the vesicle. This figure was modified from a figure previously published in reference 10 and is reproduced by permission of The Company of Biologists, copyright 2005.

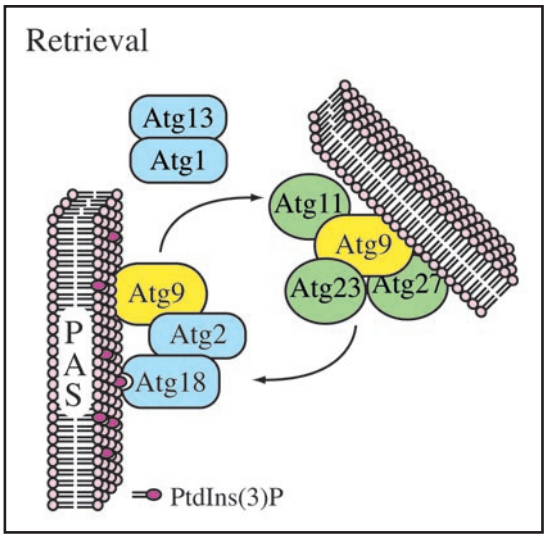

Figure 5. Retrieval of Atg9 from the forming autophagosome (step 5 of Fig. 1). The majority of the proteins involved in formation of the sequestering vesicles appear to be retrieved because they are not found associated with the completed autophagosome or Cvt vesicle. Most of these proteins are soluble and may simply dissociate from the membrane; however, retrieval of the transmembrane protein Atg9 involves several other Atg proteins. Atg9 and Atg27 are integral membrane proteins; however, this figure does not portray their topology. This figure was modified from a figure previously published in reference 10 and is reproduced by permission of The Company of Biologists, copyright 2005.

discussed below. Although the autophagic lactolysis assay cannot be used in yeast cells, which have a cytoplasmic β-galactosidase, it should be universally applicable to mammalian cells.

Autophagic sequestration: General principles. The most direct way of monitoring autophagic activity is by autophagic sequestration assays, which measure the accumulation of autophagocytosed cytoplasmic markers in autophagic organelles. By using markers resistant to lysosomal degradation, or by blocking lysosomal degradation with suitable inhibitors, there is no need to distinguish between the various autophagic organelles (autophagosomes, amphisomes and lysosomes), or to worry about autophagic-lysosomal turnover: The initial sequestration step will be rate-limiting, and the rate of accumulation will reflect the rate of autophagic sequestration.

Any endogenous or introduced cytoplasmic component can, in principle, be used in an autophagy assay, provided it is sufficiently membrane-impermeant to stay inside the autophagic organelles once it has entered. Proteins (and other high-molecular mass cytoplasmic constituents) will qualify, and can usually be quantitatively measured by immunoblotting or an enzymatic assay. Exogenous probes offer more freedom in terms of detectability (for example, by radioactivity or fluorescence), but one will need to overcome the obstacle of getting a membrane-impermeant substance into the cell across the plasma membrane. Various methods are available, such as microinjection,[59] lipofection,[60] electropermeabilization,[61] transient surface injury (blebbing),[62,63] or endocytosis followed by endosome rupture.[64]

A second basic requirement of a sequestration assay is the separation of autophagic vacuoles from cytosol. This can be done by homogenization of the cells in isotonic, buffered sucrose, followed by centrifugation and pelleting of the sedimentable components (including the autophagic organelles), preferably through a dense cushion to achieve a clean separation. Working with isolated hepatocytes, we have found electrodisruption in ion-free sucrose followed directly by low-speed centrifugation of the "cell corpses" through a dense cushion to be a very rapid and convenient alternative to homogenization, suitable for the processing of many small samples.[61] For attached cells in culture, plasma membrane permeabilization with a pore-forming agent such as streptolysin O[65] or plasma membrane lysis by gentle treatment with a detergent such as saponin[66] may be used for selective extraction of cytosol.

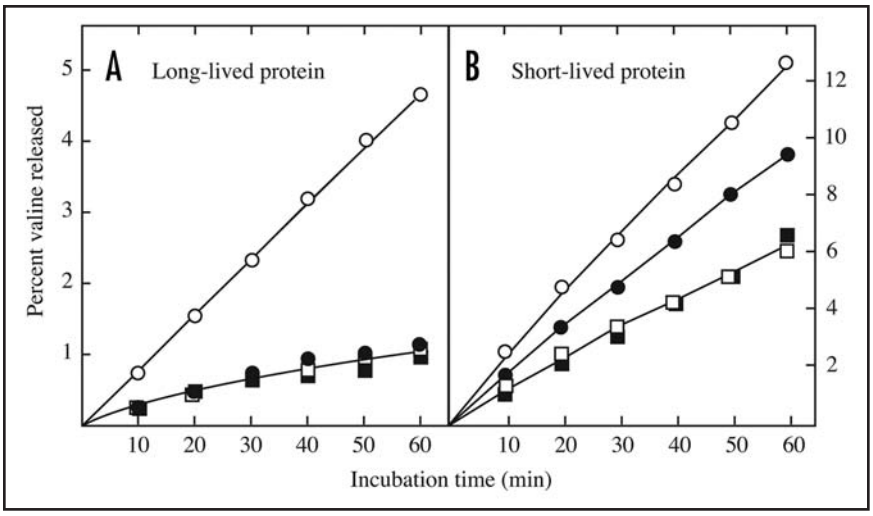

Figure 6. Degradation of long-lived and short-lived protein in isolated rat liver cells. Rats were given [^{14}C]valine intravenously 24 h before the isolation of hepatocytes to preferentially label long-lived protein (A), or the isolated hepatocytes were incubated with [^{14}C]valine for 1 h in suspension to preferentially label short-lived protein (B). After a 30-min preincubation (to remove soluble [^{14}C]valine), the release of [^{14}C]valine from labeled protein was measured in an amino acid-free medium for maximal autophagic activity (open circles); with a complete amino acid mixture to suppress autophagy (closed circles); with the acidotropic drug propylamine (10 mM) to inhibit lysosomal activity (open squares); or with both amino acids and propylamine (closed squares). Note the large fraction of long-lived protein degradation that is suppressed by amino acids; this fraction represents autophagy, and accounts for nearly all of the propylamine-sensitive (lysosomal) protein degradation. Modified, with permission, from reference 247.

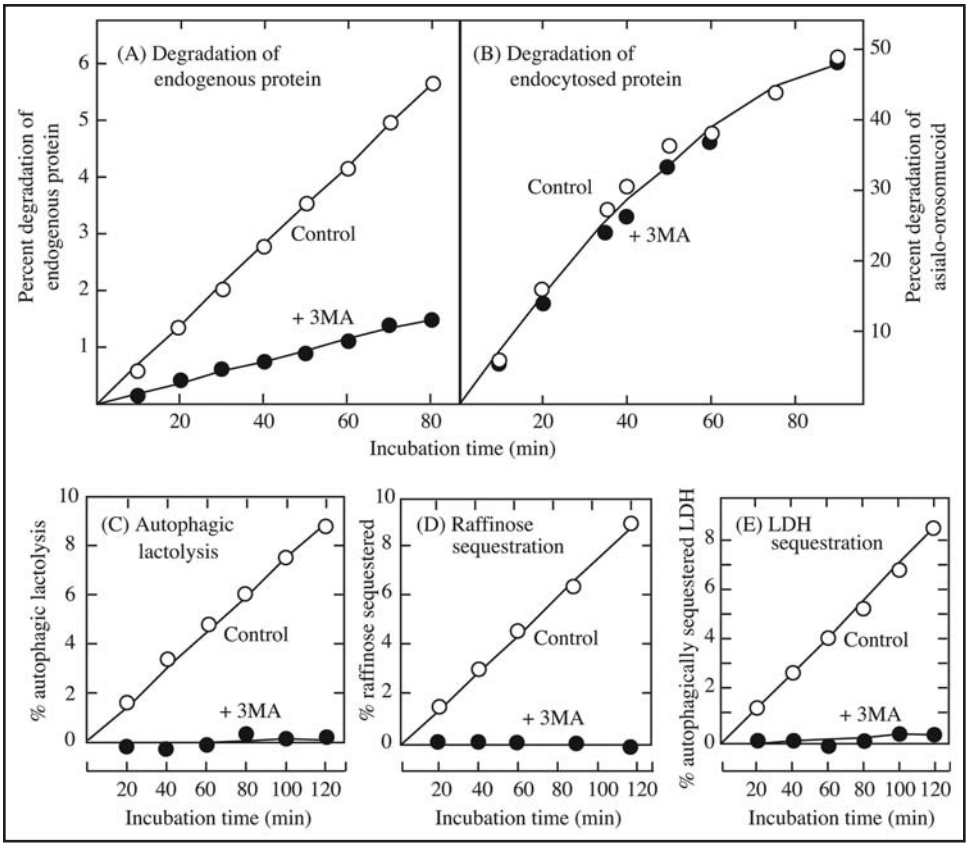

Figure 7. Endogenous protein degradation, autophagic lactolysis and autophagic sequestration of raffinose or LDH as assays for autophagy. The autophagy inhibitor, 3-methyladenine (3MA, 10 mM) was added to isolated hepatocytes to examine its effect on (A), degradation of long-lived (24 h labeled) endogenous protein; (B) degradation of endocytosed asialo-orosomucoid; (C) hydrolysis of electroinjected [^{14}C]lactose; (D) sequestration of electroinjected [^{3}H]raffinose; and (E) sequestration (in the presence of leupeptin, an inhibitor of lysosomal protein degradation) of endogenous lactate dehydrogenase (LDH). Open symbols, control (incubation in an amino acid-free saline medium without additions); closed symbols, 3-methyladenine (10 mM) included. Data assembled, with permission, from references 248 and 30 and various unpublished experiments.

Some of the more commonly used autophagic sequestration assays will be briefly discussed below.

Sequestration of radioactive sugars. The first biochemical autophagy assay was based on autophagic sequestration following electroinjection of the membrane-impermeant, nonmetabolizable (in mammalian cells) disaccharide [^{14}C]sucrose,[61] eventually in combination with selective digitonin extraction to avoid the inclusion of mitochondrial sugar uptake.[26] A simpler assay was provided by the trisaccharide [^{3}H]raffinose, which was excluded from mitochondria, thus obviating the need for digitonin.[27] Electroinjected [^{3}H]raffinose could be shown to accumulate in sedimentable cell fractions by a sequestration process that was virtually completely suppressible by 3MA (Fig. 7D).[27,29] Electroinjected sugars (in particular, raffinose) have been used in several laboratories to study autophagy in mammalian cells,[67-71] but cannot be used in yeast cells due to their rapid metabolism.

Sequestration of cytosolic enzymes or other macromolecules. A long-lived enzyme such as lactate dehydrogenase (LDH), which turns over exclusively by autophagy, is a suitable endogenous sequestration probe. LDH begins to accumulate in lysosomes (as part of the sedimentable cell fraction) as soon as its degradation there is blocked, for example, by an endoproteinase inhibitor such as leupeptin (Fig. 7E).[29] The inhibition of lysosomal degradation does not affect the autophagic sequestration step, and the large cytosolic pool from which sequestered LDH is drawn does not change with time (autophagy reduces the cytosolic volume, not the concentrations of cytosolic components), allowing LDH to accumulate for hours at a constant rate.[29] Autophagy studies based on LDH sequestration usually measure LDH by an activity assay,[72,73] but immunoblotting can also be used, as in a yeast autophagy assay employing another long-lived enzyme, fatty acid synthase,[31] and in the detection of an autophagically sequestered myc-tagged antigenic neomycin phosphotransferase fragment.[74]

Visualization of autophagic sequestration in the light/fluorescence microscope is achievable with dye-conjugated macromolecules (or, in principle, oligosaccharides), provided these can be introduced into cells. Schaible et al.[63] showed that Texas Red-conjugated dextrans could be loaded by mechanical plasma membrane injury (glass bead rolling or syringe shearing) into *Leishmania*-infected mouse macrophages, where they were subsequently autophagocytosed and observable in autophagosomes and parasitophorous vacuoles (lysosomes). Stacey and Allfrey similarly visualized the autophagic sequestration of microinjected, rhodamine-conjugated proteins in HeLa cells.[59] With presently available recombination technologies, the autophagic sequestration of, for example, green fluorescent protein (GFP) or a GFP-conjugated cytosolic protein,[74] would seem like an attractive alternative. With any of these methods, the visibility of autophagic organelles can be greatly improved by gently permeabilizing the cells after the completion of autophagy, using, for example, electrodisruption[61] or saponin treatment[66] to reduce cytosolic background fluorescence.

The yeast alkaline phosphatase assay. The yeast gene *PHO8* encodes vacuolar alkaline phosphatase, a resident hydrolase.[75] Pho8 contains an N-terminal transmembrane domain that acts as an internal uncleaved signal sequence allowing translocation into the endoplasmic reticulum (ER); like most resident vacuolar hydrolases, Pho8 is delivered to the vacuole through a portion of the secretory pathway.[76] The altered Pho8Δ60 protein lacks the N-terminal transmembrane domain, so it is no longer able to enter the ER, and instead remains in the cytosol. The only way for Pho8Δ60 to reach the vacuole is through autophagy.[32] If it is delivered to the vacuole, the C-terminal propeptide of Pho8Δ60 can be removed in a proteinase B (Prb1)-dependent manner, generating the mature active enzyme. This conversion can be conveniently measured either enzymatically (Fig. 8A) or through a molecular mass shift monitored by SDS-PAGE (Fig. 8B). Pho8Δ60 activity is typically very low in vegetative conditions, presumably reflecting the basal level of autophagy, and is substantially increased following induction of autophagy. Optimally, the enzyme assay should be performed in a strain that is deleted for *PHO13*, the gene encoding a cytosolic alkaline phosphatase, and where the *PHO8* locus has been replaced with *Pho8Δ60*. One advantage of the SDS-PAGE assay is that processing can be followed in a strain deleted only for *PHO8* (this deletion is not essential but is helpful because mature Pho8 migrates at the same position as unprocessed Pho8Δ60), where Pho8Δ60 is expressed on a plasmid; however, anti-Pho8 antiserum is required. Because the sequestration step is rate limiting in the sequence of events that ultimately results in Pho8Δ60 activation, the Pho8Δ60 assay may serve as a measure of autophagic sequestration in yeast cells; however, because the assay depends on delivery of Pho8Δ60 to the vacuole, it will not detect autophagic sequestration in the absence of autophagosome-vacuole fusion.

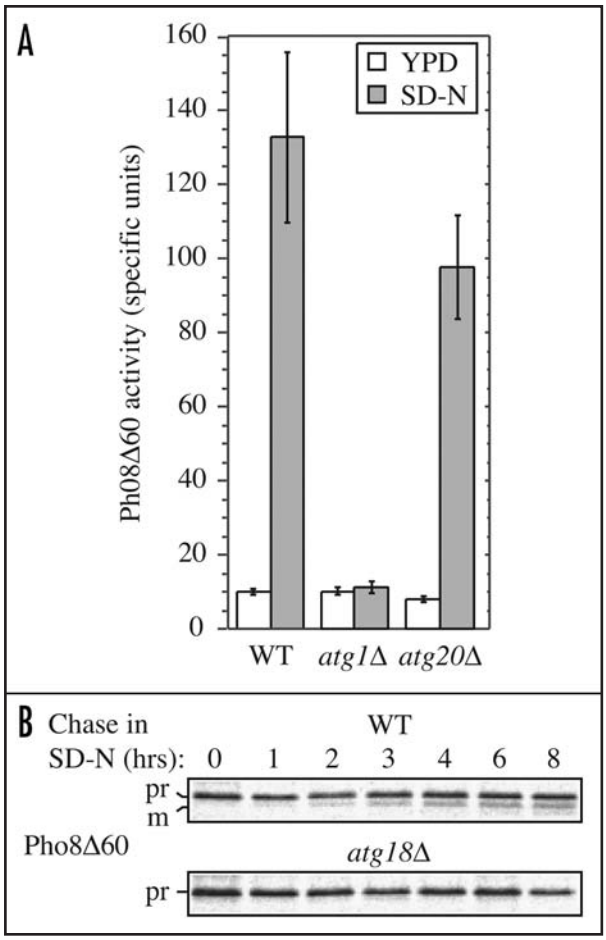

Figure 8. The Pho8Δ60 assay measures nonspecific autophagy in yeast. (A) The wild type, *atg1*Δ and *atg20*Δ strains were grown in YPD (rich medium) and shifted to SD-N (starvation medium lacking nitrogen) for 4 h. Samples were collected and protein extracts assayed for alkaline phosphatase activity. The *atg20*Δ mutant is defective for the Cvt pathway but not autophagy, whereas *atg1*Δ is defective for both. This figure was modified from data previously published in reference 184, and is reproduced by permission of the American Society for Cell Biology, copyright 2005. (B) Pulse/chase labeling and immunoprecipitation can be used to follow Pho8Δ60 processing. Wild type and *atg18*Δ cells expressing Pho8Δ60 from a plasmid were pulse-labeled in SMD (synthetic medium, equivalent to rich medium in this context) for 10 min, and subjected to a nonradioactive chase in SD-N for the indicated times. The kinetics of Pho8Δ60 processing were analyzed by immunoprecipitation followed by SDS-PAGE and autoradiography. The precursor and mature forms of Pho8Δ60 are indicated. This figure was modified from data previously published in reference 208, and is reproduced by permission of the American Society for Cell Biology, copyright 2001.

Atg8/LC3-II accumulation. Atg8 and its mammalian homologue, LC3, are unique markers of the autophagic-lysosomal pathway, in that their lipidated (phosphatidylethanolamine [PE]-conjugated) forms (LC3-II in mammalian cells) appear to be predominantly associated with autophagic organelles.[77,78] In mammalian cells, forced expression of GFP-conjugated LC3-II has been widely used to detect autophagic organelles in the microscope as fluorescent dots appearing, for example, upon amino acid starvation.[50] The advantage of GFP-LC3-II as an autophagy marker, compared to sequestered cytosolic enzymes, is that the cytoplasmic background is low, thus obviating the need to permeabilize or fractionate the cells, although the removal of cytosol will improve detectability.[66] It is important to note, however, that caution must be exercised when interpreting data from analysis of GFP-LC3 due to the tendency of LC3-GFP to aggregate.[79] In particular overexpression resulting from transient transfection, or expression in the presence of intracellular protein aggregates (such as polyQ-associated inclusion bodies) can lead to LC3 punctate structures that do not represent autophagosomes.[79]

By blocking its lysosomal degradation with proteinase inhibitors, Atg8–PE/LC3-II will, like sequestered cytosolic enzymes, accumulate within the vacuole/lysosome, thus offering a potential means of quantifying autophagic activity.[80] Quantification can be performed

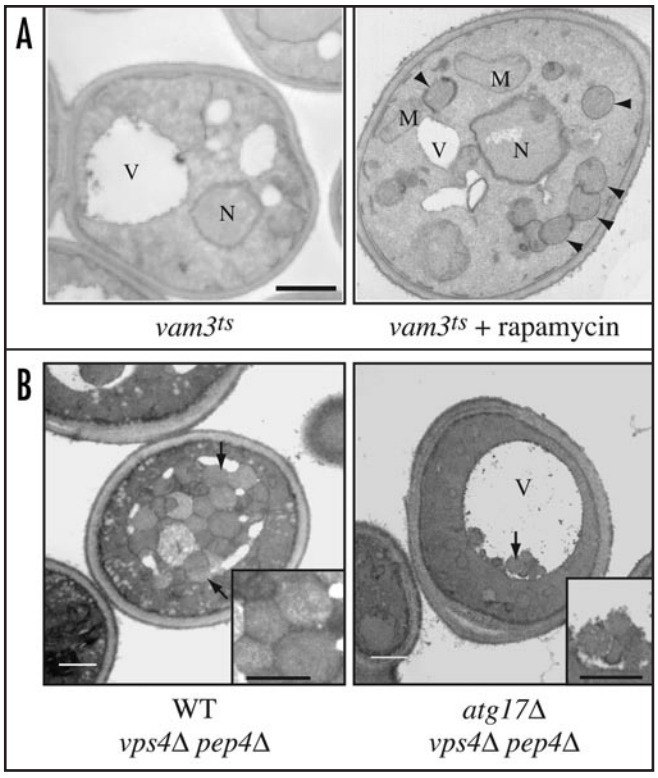

Figure 9. Autophagosome and autophagic body formation and accumulation. (A) Autophagosomes can be detected in mutants defective in fusion with the vacuole. A *vam3ts* strain was grown to mid log stage at 26˚C, then shifted to 38˚C. The cells were then treated with rapamycin (0.2 μg/ml final concentration) or mock drug vehicle and incubated for 1.5 hours at 38˚C prior to permanganate fixation for electron microscopy. Arrowheads denote autophagosomes. The bar represents 1 μM. This figure was modified from data previously published in reference 177, and is reproduced by permission of the American Society for Cell Biology, copyright 2000. (B) The *atg17*Δ mutant generates fewer and smaller autophagosomes/autophagic bodies. Wild type (*vps4*Δ *pep4*Δ) and *atg17*Δ (*vps4*Δ *pep4*Δ) strains were grown to mid log stage in YPD and transferred to SD-N medium for 4 h. Cells were fixed with permanganate and examined by electron microscopy. Arrows mark the locations of autophagic bodies. The bars in the main images and insets (2X magnification) represent 0.5 μm. N, nucleus; V, vacuole; M, mitochondria. This figure was modified from data previously published in reference 184, and is reproduced by permission of the American Society for Cell Biology, copyright 2005.

by densitometric scanning of western blots,[80] or by counting the number of fluorescent dots per cell (counting the fraction of Atg8–PE/LC3-II-positive cells is also used, but is much less accurate).[66] However, there are several problems connected with the interpretation of Atg8–PE/LC3-II accumulation: Atg8–PE/LC3-II is associated with all types of autophagic organelles, including the preautophagosomal phagophores,[78] and its formation/lipidation/delipidation kinetics are not well understood, a problem compounded by the often poor detectability of the unlipidated form, LC3-I, by currently available antibodies.[81] The sequestration step (autophagosome formation) is, therefore, not necessarily rate-limiting for Atg8–PE/LC3-II accumulation, hence the accumulation does not necessarily reflect the rate of autophagic sequestration. Furthermore, it is not clear whether all autophagosome-associated LC3-II enters the lysosome, or whether LC3-II on the outer autophagosomal surface[78] is disposed of (delipidated and degraded) by nonlysosomal mechanisms, as is the case with Atg8–PE. Unlike the autophagic sequestration of cytosolic markers, or overall autophagic-lysosomal protein degradation, the rate of Atg8–PE/LC3-II accumulation tends to decline rapidly,[66,80] probably reflecting the fact that it is influenced by several processes other than autophagic sequestration.

Accumulation of autophagic organelles. One of the most widely used ways to monitor autophagy is by the counting of autophagic organelles. The counting of GFP-LC3-positive dots in the fluorescence microscope has already been discussed; more traditional methods include the quantification of lysosomes (which are turned into larger and more active autolysosomes during autophagy) in the fluorescence microscope after staining[82,83] or of autophagosomes and other autophagic organelles in the electron microscope.[24,48] Apart from the problems associated with unequivocal organelle identification, discussed below, these morphological methods are at best semi-quantitative, in that they usually measure the steady-state levels of organelles within a continuous autophagic-lysosomal flux rather than a sequestration-dependent end-stage accumulation. Under such conditions, an accumulation of, for example, autophagosomes, would not necessarily reflect an increase in autophagic activity, but could equally well be due to a reduced fusion of autophagosomes with endosomes or lysosomes.[84] The fact that the autophagic-lysosomal pathway is characterized by a series of fusions means that morphometric recordings of the volume fractions of autophagic compartments are more meaningful than the counting of organelle numbers, but maximum information is obtained by doing both.[85] It should be emphasized, however, that even the volume fractions are strongly flux-dependent, and, moreover, subject to influence by endocytic influx independent of autophagic activity.

In yeast cells, several adequate morphological end-point assays are actually available: In strains defective in fusion with the vacuole, such as *ypt7*Δ or *vam3*Δ, the accumulation of autophagosomes can be observed by electron microscopy (Fig. 9A), or more easily by monitoring GFP-Atg8 fluorescence. The accumulation of autophagosomes in a wild type strain would indicate a defect in fusion.

A second morphological yeast autophagy assay is based on autophagic bodies, the single-membrane intravacuolar vesicles that result from the fusion of autophagosomes with the vacuolar limiting membrane (Fig. 1). Autophagic bodies are normally degraded within the vacuole lumen in a process that is dependent on Prb1 activity;[86] presumably, Prb1 is needed to activate other hydrolases that act directly upon the autophagic body lipid membrane. The vacuoles of wild type cells appear essentially devoid of autophagic bodies, whereas these structures accumulate under starvation conditions (or following treatment with rapamycin, a macrolide antibiotic that inhibits Tor kinase resulting in autophagic induction) in strains lacking Pep4 or Prb1 activity, or in the presence of the serine protease inhibitor PMSF. The appearance of autophagic bodies can be detected by light microscopy in yeast due to the relatively large size of the vacuole; this analysis is not practical for mammalian cells due to the smaller size of the lysosome. Analysis of autophagic body accumulation is a simple, but very qualitative (because it is usually done as a static analysis, and it is difficult to determine the actual number of autophagic bodies in the entire vacuole), assay for autophagy. In addition, the absence of autophagic bodies (comparing cells in the absence and presence of inhibitors) can be used as a measure of vesicle lysis, or, more precisely, breakdown. Quantification of autophagic body accumulation and size is possible when the analysis is carried out with electron microscopy (Fig. 9B). It may be helpful to delete the *VPS4* gene to prevent the appearance of intravacuolar vesicles derived from the fusion of multivesicular bodies with the vacuole.

INHIBITION OF AUTOPHAGY

The autophagic-lysosomal pathway can be blocked at several points;[28,30,87] each block providing important information about an individual step in the pathway. To assess the secondary consequences of autophagy, a complete shutdown of the whole pathway may be desirable, which is most cleanly achieved by blocking autophagy at or before the sequestration step. Several agents, genetic manipulations and conditions are available for this purpose, each with its characteristic spectrum of side effects, thus allowing a choice of minimal complications relative to the secondary process one wants to investigate.

Amino acids. Increased protein degradation in response to amino acid deficiency is a major function of autophagy, making amino acids both the most important physiological inhibitors of autophagy and a useful experimental tool.[88] Probably the most common way of turning autophagy off and on in experimental studies with mammalian cells is by switching cell cultures between a rich medium containing serum and high levels of amino acids (which cooperate at several levels in suppressing autophagy), and a nutrient-poor medium (buffered saline). This is not a very clean experimental approach, however, because several autophagy-regulatory signaling pathways may be altered at the same time: The removal of serum growth factors switches off the autophagy-suppressive pathway dependent on PtdIns 3-kinase class I,[73] and if an energy substrate (glucose or pyruvate) is not included, a decline in the ATP/AMP ratio may switch on an autophagy-regulatory metabolic stress pathway dependent on the AMP-activated protein kinase (AMPK).[89] To obtain a pure nitrogen starvation (amino acid) effect, serum proteins and glucose should be included in the starvation medium, or a comparison should be made between cells reincubated in the presence or absence of amino acids.

Although a complete, balanced amino acid mixture is usually employed for autophagy suppression,[48,90] not all amino acids are autophagy-suppressive, meaning that simpler mixtures of regulatory amino acids can be used.[90-92] Leucine clearly plays a major role,[46,91] and tryptophan, tyrosine, phenylalanine and histidine also contribute significantly to the inhibition of autophagic sequestration in hepatocytes.[93] In addition, asparagine partially inhibits amphisome-lysosome fusion;[30,94] glutamine inhibits intralysosomal protein degradation by acting as an ammonia donor,[95] and alanine modulates the effect of the other amino acids by an as yet undefined mechanism.[96]

The suppression of autophagic sequestration by amino acids is poorly understood: although the rapamycin-sensitive lipid/protein kinase, mTOR, clearly plays a major role,[88] the effects of amino acids on autophagy are not always rapamycin-sensitive,[97] and the additive or synergistic effects of amino acids in different combinations[93] may indicate that several mechanisms are operative. Given the specific metabolic profiles of different cell types, their sensitivities towards autophagy-suppressive amino acids are also likely to differ, hence no universally effective set of regulatory amino acids has yet been devised.[98] For the time being, a complete, balanced amino acid mixture is probably the best choice. It should be noted, however, that because amino acids are rapidly metabolized, their autophagy-suppressive effect is less sustained than the effect of, for example, 3MA, discussed below.[99]

3-methyladenine and other PtdIns 3-kinase inhibitors. The autophagy-suppressive effects of 3MA and some other methylaminopurines and mercaptopurines[61,100] were originally discovered on the basis of a side effect of the protein synthesis inhibitor, puromycin, on protein degradation.[101,102] As discussed above, high concentrations of 3MA (5–10 mM) will block autophagic sequestration completely in all mammalian cell types studied (Fig. 7), with litte or no effect on other endogenous degradation mechanisms or on basic cellular functions such as energy metabolism, protein synthesis or cellular viability.[25,51] However, 3MA may interfere with other cellular processes independent of autophagy,[53-56] hence its secondary effects must be interpreted with caution. 3MA has been shown to be a PtdIns 3-kinase inhibitor,[52] and probably blocks autophagic sequestration by inhibiting the class III PtdIns 3-kinase, mVps34.[73] Other PtdIns 3-kinase inhibitors, such as wortmannin and LY294002, are more potent autophagy suppressants than 3MA,[52] but they are also strong inhibitors of class I PtdIns 3-kinases and thus likely to be less specific. Vps34 is required for phagophore formation in yeast[103,104] and appears to be involved in the regulation of mammalian autophagy as well,[105,106] although its precise role remains to be clarified.

Okadaic acid and related toxins. Okadaic acid and several other protein phosphatase-inhibitory algal toxins have been found to suppress autophagic sequestration completely in isolated rat hepatocytes.[67,107] The toxin effects appear to be mediated by overphosphorylation of the AMP-activated-protein kinase (AMPK), are shared by the AMPK activator, AICAR,[89] and are accompanied by extensive fragmentation of the cellular keratin and plectin cytoskeleton networks.[108,109] How these cytoskeletal alterations relate to the suppression of autophagy is not clear, but the possibility should be considered that the autophagy suppression may be secondary, in particular because AMPK has been found to have the opposite (stimulatory) effect on autophagy in yeast.[110] Although the toxins are remarkably effective autophagy suppressants, their protein phosphatase-inhibitory properties induce a generalized overphosphorylation of cellular proteins[111] and eventually apoptotic cell death,[112] making them probably the least specific of the autophagy suppressants available.

Thapsigargin and calcium chelators. Autophagic sequestration can be virtually completely suppressed by thapsigargin and other inhibitors of the calcium pump in the ER.[113] These drugs cause a rapid depletion of calcium from the ER cisternae, an effect that to some extent can be mimicked by calcium chelators. Nothing is known about why intracisternal calcium is required for autophagy, but an attractive possibility is that it needs to be present in the intracisternal space of the sequestering phagophore. This inhibitory effect may also conform with the view that at least one source of the phagophore and autophagosome membrane is the ER/early secretory pathway.

Cyclic AMP. High concentrations of cAMP have been shown to suppress autophagy both in yeast[114,115] and in mammalian cells.[116,117] The effect of the cyclic nucleotide is apparently mediated by protein kinase A, which can phosphorylate several of the proteins (Atg1, Atg13 and Atg18) involved in phagophore formation.[118] In hepatocytes, cell-permeable cAMP analogues (dibutyryl-cAMP), activators of adenylyl cyclase (forskolin) and inhibitors of cAMP phosphodiesterases (theophylline; 3-isobutyl-1-methylxanthine) can suppress autophagic sequestration virtually completely without affecting, for example, general energy metabolism,[116] which may make them useful as inhbitors of the overall autophagic-lysosomal pathway.

Acidotropic amines. Acidotropic drugs have long been known to disturb lysosomal function by acting as intralysosomal proton-trapping agents that raise the lysosomal pH and hence suppress the activity of lysosomal acid hydrolases.[87,119] The discovery that ammonia could inhibit a major fraction of endogenous proteolysis

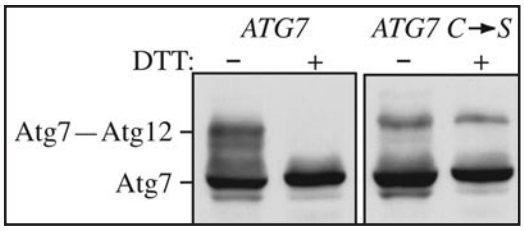

Figure 10. The Atg7 cysteine to serine (C to S) mutation can be used to monitor function of the Atg7 protein. *Pichia pastoris* cells transformed with HA-tagged wild type or mutant Atg7 were grown in methanol-containing medium and then adapted to glucose for 3 h. Aliquots were solubilized in 2% SDS and boiled for 3 min (-DTT) or solubilized in SDS with 1.5% DTT and boiled for 5 min (+DTT). Following SDS-PAGE, the samples were probed with anti-HA antibodies to detect HA-tagged Atg7. The C to S mutation allows the detection of the Atg7–Atg12 ester intermediate even in the presence of DTT. This figure was modified from data previously published in reference 138 and is reproduced by permission of the American Society for Cell Biology, copyright 1999.

by functioning as a lysosomotropic inhibitor[95,120,121] was in fact a crucial argument for the involvement of lysosomes in intracellular protein degradation and for the delineation of the autophagic-lysosomal pathway.[45,122,123] The addition of 10–20 mM NH_4Cl is still a widely used, and very effective, method for inhibition of autophagic-lysosomal protein degradation, not only via macroautophagy but through all types of autophagic pathways. Other weak bases, like methylamine and propylamine, are as effective as ammonia,[124] and have the advantage of being metabolically inert, thus not disturbing cellular nitrogen metabolism. Chloroquine, on the other hand, also widely used as a lysosomotropic agent, is a reactive drug with enzyme-inhibitory effects in addition to its acidotropic properties.[119] All lysosomotropic amines will, however, induce swelling of lysosomes and other acidic compartments, thus interfering with cellular processes such as endocytosis,[125] secretion[126] and even locomotion.[127]

Genetic knockouts and knockdowns. Because the *ATG* genes are essential for autophagy in yeast, mutational knockouts of these genes will not only affect the individual step regulated by the respective protein, but will also usually block the whole autophagic-lysosomal (vacuolar) pathway.[128,129] Certain *atg* knockouts have been found to suppress autophagy in mammalian (mouse) cells as well as in yeast; this is the case with the mammalian *ATG6* homologue, *beclin 1*[130] and the mammalian *ATG5* and *ATG7* homologues (the latter two being generated with a Cre/flox system).[50,131,132] However, the formation of some autophagosome-like structures was still detectable in cells from homozygous Atg5 knockout mice,[50] suggesting, perhaps, that the Atg requirements may not be as stringent in mammalian cells as in yeast cells.

The recent introduction of the use of small interfering RNAi for blocking protein expression in mammalian cells has allowed efficient supression of autophagy in cultured mammalian cells by targeting Atg8/LC3, Atg6/Beclin 1 and the mammalian homologues of Atg5, Atg10 and Atg12.[133,134] Some aspects to consider in the application of this approach for blocking autophagy include those related to the use of RNAi in general (i.e., off target effects, stability of the blockage, etc.).

Mutational knockouts of genes encoding important autophagy-inducing protein kinases may also have the potential to block the entire autophagic-lysosomal pathway. Thus, knockout of the yeast eIF2α kinase, Gcn2, completely blocks nitrogen starvation-induced autophagy in yeast cells, and knockout of the mammalian eIF2α kinase, PKR, blocks the induction of autophagy in mouse fibroblasts by HSV-1 virus.[135] Similarly, dominant-negative mutations in death-associated protein kinases (DAPKs) prevent autophagy induction by nitrogen starvation and other stimuli in various mammalian cells.[136]

Active site cysteine to serine mutation. In most organisms autophagy employs two conjugation systems involving the ubiquitin-like proteins Atg8 and Atg12 (Fig. 3).[12] Conjugation of Atg8 requires, in order of function, Atg4 (cysteine protease), Atg7 (activating enzyme) and Atg3 (conjugating enzyme) to covalently attach PE. Conjugation of Atg12 requires Atg7 and Atg10 (conjugating enzyme) to covalently attach Atg5. Various assays can be used to ascertain the functionality of each step of the process (Table 2). Atg7 forms a thioester intermediate with Atg8 and Atg12 independently. The Atg7–Atg12 intermediate can be detected by removing the reducing agent during sample preparation and SDS-PAGE, or more easily by using a mutant where the active site cysteine residue has been replaced by a serine, resulting in a stable ester bond (Fig. 10),[137,138] verifying the functionality of Atg7 and to some extent that of Atg12. Mutation of the active site cysteine to serine in Atg3 was used to detect a stable interaction between Atg3 and Atg8, although in this case it was also necessary to overexpress Atg7 to generate a sufficient pool of activated Atg8.[139] A similar mutation in Atg10 allowed the detection of the normally transient Atg10–Atg12 intermediate in yeast[140] and mice,[141] and the same approach was used to form a stable intermediate between Atg7 and the three mammalian Atg8 homologues, LC3, GATE-16 and GABARAP.[142] Finally, it should be noted that interactions between substrates and particular components of the conjugation machinery can also be detected through yeast two-hybrid assays.[143,144]

AUTOPHAGIC-LYSOSOMAL ORGANELLES

Atg8/LC3, a general marker of autophagic organelles. The most widely used autophagic organelle marker at present is Atg8 and its mammalian homologue, LC3; often expressed as the respective fluorescent GFP conjugates. Prior to lipidation, GFP-Atg8 is dispersed within the cytosol. Thus, dispersed GFP-Atg8 can be seen in mutants that are defective in the Atg8 conjugation reaction (Fig. 11A), and also in mutants defective in Atg12 conjugation where Atg8–PE is unstable. Although it is not known where conjugation occurs, the lipidated protein GFP-Atg8–PE, is commonly used to mark the phagophore assembly site/preautophagosomal structure (PAS, Fig. 11A; see below). Atg8–PE is one of two Atg proteins that have been shown to remain associated with the completed autophagosome or the equivalent vesicles used in the cytoplasm to vacuole targeting (Cvt) pathway (Fig. 1), and thus also serves as a marker for these structures (the other is Atg19, which is only present in yeast). For example, in mutants defective in fusion with the vacuole, including *ypt7*Δ or *vam3*Δ, GFP-Atg8–PE accumulates within autophagosomes. In wild type cells, GFP-Atg8–PE can be seen dispersed within the vacuole lumen, serving as a general marker for delivery via the Cvt or autophagy pathways. It can also be detected inside Cvt or autophagic bodies in strains that are defective in intravacuolar vesicle breakdown such as *pep4*Δ (Fig. 11B). Thus, the GFP-Atg8 chimera can be used to follow the localization or accumulation of autophagosomes, the PAS or autophagic bodies, depending on the conditions or strains being monitored.

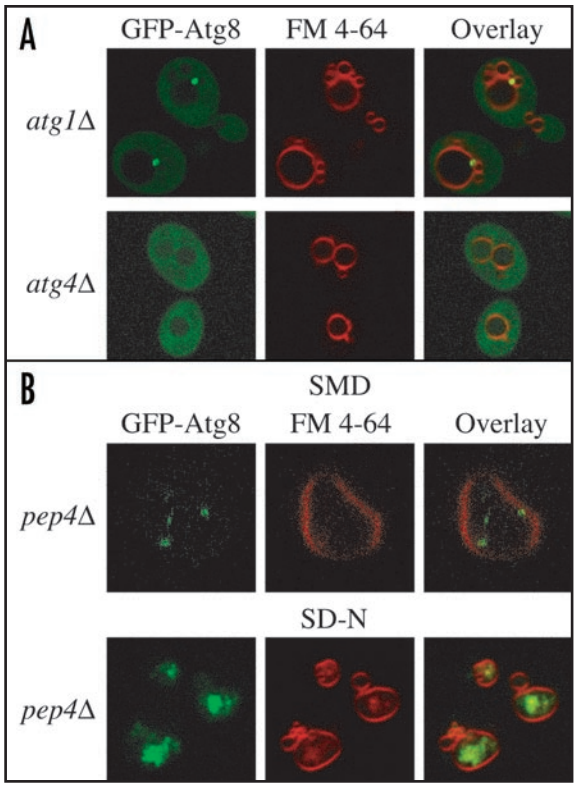

Figure 11. GFP-Atg8 localization. (A) Atg4-dependent conjugation to phosphatidylethanolamine is required for the association of GFP-Atg8 to punctate, perivacuolar structures (the PAS). The *atg1*Δ and *atg4*Δ yeast strains were transformed with a plasmid encoding GFP-Atg8. The cells were grown to mid log stage in SMD medium and then labeled with FM 4-64 (to stain the vacuole), shifted to SD-N medium for 3 h, and examined by confocal microscopy. In *atg1*Δ cells, GFP-Atg8 is recruited to punctate structures, whereas in the Atg8 processing-defective *atg4*Δ cells, GFP-Atg8 appears uniformly distributed in the cytosol. (B) A GFP fusion to the N-terminus of Atg8 (GFP-Atg8) serves as a functional marker for the Cvt and autophagy pathways. GFP-Atg8 labels Cvt and autophagic bodies inside the vacuole. The *pep4*Δ strain was transformed with a plasmid encoding GFP-Atg8 and grown to mid log phase in SMD medium and then labeled with FM 4-64. One sample was shifted to SD-N for 3 h and the cells were examined by confocal microscopy. In nutrient-rich conditions, GFP-Atg8 accumulates inside Cvt bodies in *pep4*Δ cells and inside autophagic bodies when cells are cultured in SD-N. This figure was modified from data previously published in reference 190, and is reproduced by permission of the American Society for Cell Biology, copyright 2001.

As with Atg8, GFP-LC3 can be used to monitor the localization of the protein.[37] An increase in GFP-LC3 punctate dots may reflect the induction of autophagy and also the function of the vesicle-forming machinery (Fig. 12). Two different counting methods have been used to quantify the appearance of GFP-LC3 puncta: The number of GFP-LC3-positive dots per cell and the number of cells with positive dots. The former is probably more representative of the extent of autophagy. GFP-LC3 punctate dots mostly mark autophagosomes and phagophores[37] but are also associated with (auto)lysosomes as confirmed by counterstaining GFP-LC3-expressing cells with anti-CD63 (endosomal/lysosomal marker) antibody followed by indirect immunofluorescence.[145] Furthermore, a portion of the lysosome-associated LC3, which is localized on the cytosolic side of the lysosomal membrane, seems quite stable in this location, and may not be a direct reflection of the extent of autophagy, as it can be detected at this site even when macroautophagy is blocked for 4 hours (Kaushik S, Wang Y, Martinez-Vicente M, Cuervo AM, unpublished results). Overexpression of GFP-LC3 does not appear to interfere with autophagy, so the chimeric marker can be used in transient transfections, although transgenic mice expressing GFP-LC3 have recently been generated.[146] It is possible to use other fluorescent protein-tagged Atg proteins in mammalian cells, and presumably in other systems. Fluorescent chimeras of Atg5, Atg6/Beclin 1 and Atg16 have been observed in mammalian cells.[50,143,147,148] In general, care should be exercised to determine whether the altered expression of an Atg protein(s) causes a change in the level of autophagy.

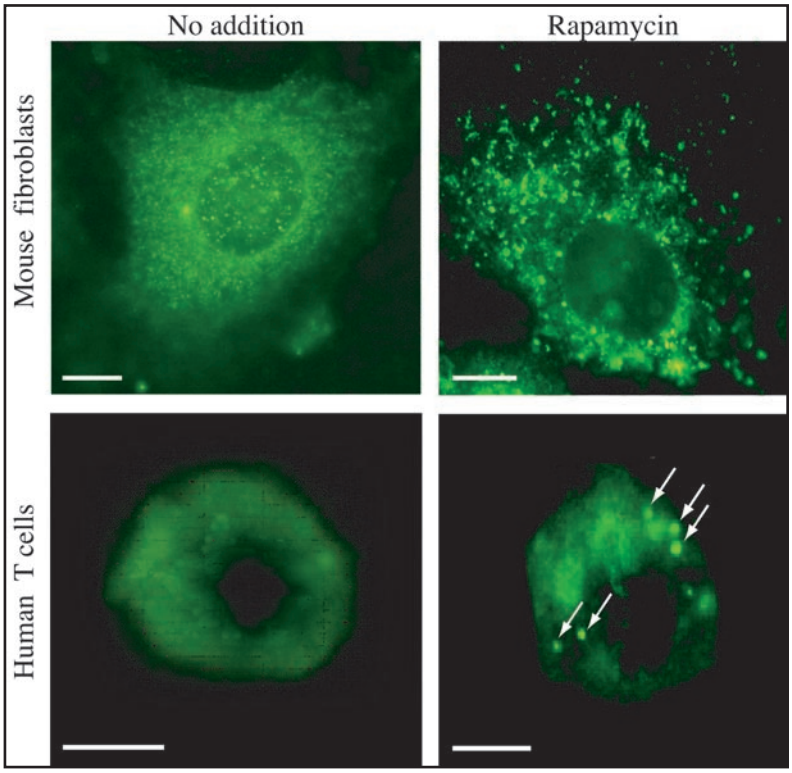

Figure 12. LC3 staining in different cell types. Mouse fibroblasts and human T cells untreated or treated with 100 mM rapamycin for 4 h were subjected to immunofluorescence with a selective antibody against LC3. LC3-stained autophagic vacuoles in T cells are indicated with arrows. Quantification of 20 cells similar to the ones shown here indicated the presence after rapamycin treatment of 165 ± 8 vesicles per fibroblasts and 6 ± 2 vesicles per T cell. Bar, 5 mm.

Atg8 and LC3 are often upregulated during autophagy (Fig. 13) and they remain associated with the completed autophagosome; however, in pathological conditions in which autophagosome clearance is impaired and autophagosomes persist longer in the cytosol, it is possible to find autophagosomes denuded of surface LC3, suggesting that the protein may eventually be detached from the membrane of this vesicle, in agreement with the Atg4-dependent removal of Atg8 from the outer surface of the autophagosome in yeast.[149] The change in LC3 levels is typically modest compared to Atg8, with many cell lines and tissues displaying a two-fold or lower increase. Following delivery to the lysosome/vacuole the populations of these proteins

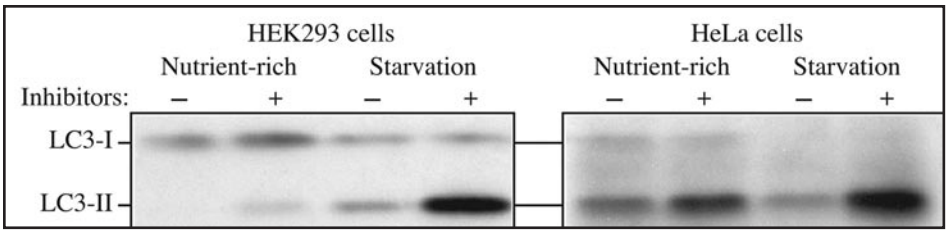

Figure 13. LC3-I conversion and LC3-II turnover. HEK293 and HeLa cells were cultured in nutrient-rich medium (DMEM containing 10% FCS) or incubated for 4 h in starvation conditions (Krebs-Ringer medium) in the absence (-) or presence (+) of E64d and pepstatin at 10 μg/ml each (Inhibitors). Cells were then lysed and the proteins resolved by SDS-PAGE. Endogenous LC3 was detected by immunoblotting. This figure was modified from data previously published in reference 80, and is reproduced by permission of Landes Bioscience, copyright 2005.

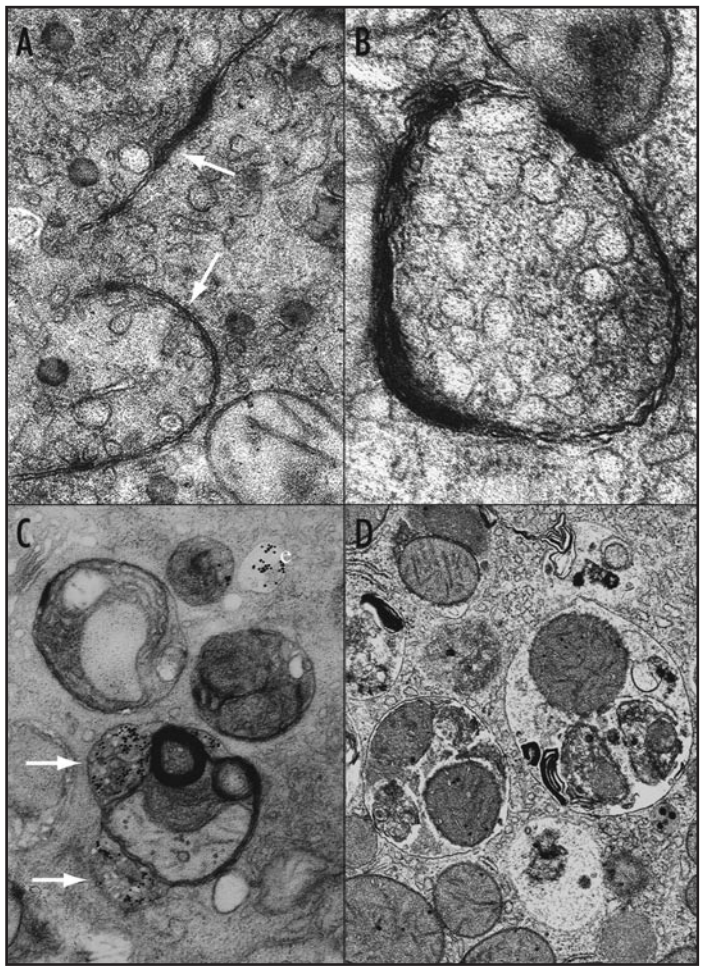

Figure 14. Autophagic organelles in rat hepatocytes. (A) Phagophores (arrows). (B) Autophagosome; notice the thick left wall suggesting sequestration by several stacked phagophores. (C) Amphisomes in leupeptin-treated cells which have been allowed to endocytose gold particles into their endosomes (e); one amphisome can be seen to be the fusion product of several endosomes (arrows) and autophagosomes. (D) Lysosomes, mostly autolysosomes, containing recognizable remnants of autophagocytosed mitochondria and other cytoplasmic elements. Data assembled, with permission, from references 85 and 167 and unpublished experiments.

that were present within the lumen are degraded. The competing synthesis and degradation processes complicate any analysis of Atg8/LC3. For example, high levels of these proteins may indicate autophagic induction, but could also reflect a block in any subsequent stage of autophagy including vesicle formation, fusion with the lysosome/vacuole or degradation of the autophagic body, which would cause the protein to accumulate. Accordingly, it is necessary to monitor turnover of these proteins in the absence and presence of lysosomal/vacuolar protease inhibitors, as opposed to simply following the steady state level.[80] For example, in HeLa cells in the absence of protease inhibitors the level of LC3-II appears to decrease 4 h after shifting to starvation conditions; however, when the experiment is carried out in the presence of inhibitors it is clear that the level actually increases and that the apparent decrease was due to lysosomal proteolysis (Fig. 13). Finally, Rab24 has also been proposed recently as a possible autophagic marker; in response to starvation this protein relocates from its usual ER-Golgi location to cup- and ring-shaped vesicular structures that are positive for LC3.[150]

The phagophore assembly site (preautophagosomal structure; PAS). In yeast cells, a number of Atg proteins have been found, by immunostaining or GFP conjugation, to colocalize to a dot-like organelle known as the phagophore assembly site or preautophagosomal structure (PAS).[151,152] The PAS apparently functions as an assembly site, from which nascent phagophores (isolation membranes) emanate in the form of membrane vesicles or cisternae equipped with autophagy-specific proteins and protein conjugates.[13] The PAS is not membrane-enclosed,[153] and is difficult to recognize in the electron microscope, but is easily detected in the fluorescence microscope as the major site of concentrated Atg localization (Fig. 11A). Almost any Atg protein (Atg1, Atg2, Atg7, Atg9, etc.) can serve as a PAS marker, preferably those not associated with other autophagic or nonautophagic organelles (Atg5, Atg8, Atg12, Atg16, Atg19); although in practice, most Atg proteins are detected primarily at the PAS. The primary exceptions that should be avoided as PAS markers are Atg9, Atg23 and Atg27, which localize to the PAS, but also to additional sites within the cell (Yen W-L, Legakis JE, Klionsky DJ, unpublished results).[21,154,155]

No clear equivalent of the PAS has yet been observed in mammalian cells, perhaps indicating that phagophore assembly takes place in a more distributed fashion than in yeast cells.

Phagophores. In mammalian cells, phagophores are easily recognized ultrastructurally as compressed (electron-dense), curving cisternae in the process of enclosing part of the cytoplasm. The same structural appearance is observed in the walls (delimiting membranes) of completed autophagosomes, which derive from the phagophores. Once this characteristic membrane ultrastructure is recognized, phagophores can also frequently be observed as flat (straight), condensed or stacked sheets (Fig. 14A).[16,85] Sometimes the cisternal space opens to produce a "double-membrane" structure; in most cases this is probably an artifact of the sample preparation.[156] In freeze-fracture electron microscopy images, phagophore membranes have the same characteristically smooth appearance as in the autophagosome walls, being essentially devoid of intramembrane particles (transmembrane proteins).[156,157]

Phagophores can also be detected by the presence of Atg5, anchored to the phagophore membrane in an Atg12–Atg5-Atg16 complex. Because the complex detaches upon closure of the autophagosome, all three components should, in principle, be ideal

as specific phagophore markers.[50] Although Atg5 is diffusely distributed throughout the cytoplasm of mammalian cells, a GFP-Atg5 conjugate (expressed in Atg5-deficient cells) is sufficiently concentrated on phagophores to make these organelles detectable as dots or crescent-shaped structures in the fluorescence microscope. In the electron microscope, early phagophores can be recognized as heavily Atg5-immunogold-labeled, crescent-shaped vesicles that eventually expand to enclose a portion of the cytoplasm.[50] The expansion phase probably involves the coalescence of smaller vesicles into large cisternal sheets.[156] In some cells, large phagophores can be observed as stacks or compacted balls, possibly representing storage forms that are unpacked upon the initiation of sequestration.[85,99]

Atg8/LC3 is also associated with phagophores, anchored as a lipid conjugate (LC3-II in mammalian cells), and may serve as a phagophore marker in the electron microscope. Gold-immunolabelling of Atg8 has thus been used to identify yeast phagophores,[158] which have otherwise been less well characterized than in mammalian cells. In the light microscope, the association of Atg8/LC3 with Cvt vesicles (in yeast cells) as well as with completed autophagic organelles (autophagosomes, amphisomes and lysosomes/vacuoles) precludes its use as a specific visual phagophore marker. In biochemical studies (subcellular fractionation and western blotting), on the other hand, Atg8/LC3 may be a useful marker of any autophagic organelle, including the phagophore.

Autophagosomes. Autophagosomes are easily recognized in the electron microscope by their contents of completely normal cytoplasm, morphologically indistinguishable from the surrounding cellular environment. Any contents alteration indicates that fusion with an endosome or a phagosome has occurred, causing cytoplasmic denaturation and turning the organelle into an amphisome or (auto)lysosome.[16] The autophagosome is delimited by a compressed, electron-dense cisterna (derived from the phagophore) that is often (artificially) swollen to reveal the appearance of a double membrane (sometimes multiple, if several stacked phagophores have performed the sequestration; Fig. 14B).[85,157] The double membrane is often used as a defining criterion for the autophagosome, which may be misleading, because membranes that are part of the contents of amphisomes or lysosomes may often be apposed to their delimiting membrane, giving a false "double membrane" impression. In freeze-fracture ultrastructural studies, autophagosomes can be recognized by their smooth (particle-free) delimiting membranes.[157,159]

There is no specific biochemical marker that will distinguish autophagosomes from other autophagic organelles. In the absence of good ultrastructure (light/fluorescence microscopy, cryoelectron microscopy), it is, therefore, necessary to use a combination of positive and negative markers to make the desired distinctions. For example, in cryoelectron microscopy immunogold labeling studies, a relatively degradation-resistant cytosolic enzyme such as superoxide dismutase (SOD) can be used to mark all autophagic vacuolar organelles (autophagosomes, amphisomes and autolysosomes); an endocytosed protein such as bovine serum albumin (BSA) to mark endosomes, amphisomes and lysosomes; and a lysosomal membrane protein, for example, lysosome associated membrane protein type 1 or 2 (LAMP-1 or -2), to mark autophagolysosomes and lysosomes. The combination of positive and negative markers will then identify endosomes (SOD-/BSA+/LAMP-), autophagosomes (SOD+/BSA-/LAMP-), amphisomes (SOD+/BSA+/LAMP-), autolysosomes (SOD+/BSA±/LAMP+) and heterolysosomes (SOD-/BSA+/LAMP+).[85] A similar approach can be applied to fluorescence microscopy studies, using, for example, LC3-II, as a marker for all types of autophagic vacuolar organelles, an acidotropic dye (for example, acridine orange, LysoTracker Red or monodansylcadaverine) to mark acidic organelles (endosomes, amphisomes and lysosomes), and a lysosomal membrane protein marker to identify the lysosomes. Acid hydrolases (cathepsins, acid phosphatase) are also frequently used as lysosome markers, but as with the LAMPs, they may be present at low levels in endosomes and amphisomes as well.[160] In summary, there is not yet a single marker for each of the autophagic and endocytic compartments that could be used to highlight a particular organelle, but combinations of multiple markers, such as the ones described here, taking into account their enrichment in particular compartments, are helping to track the autophagic process in mammalian cells.

Purification of autophagosomes. Density gradient methods have a long-standing tradition as a means of separating autophagic organelles from cytosol and other cytoplasmic fractions.[161-163] Whereas sucrose gradients are hypertonic in the relevant density range, tending to cause most organelles to cluster in the same region of the gradient, isotonic gradients of Percoll or iodinated compounds like metrizamide, nycodenz or iodixanol provide a good separation of autophagic organelles from mitochondria.[27,164,165] Autophagosomes, amphisomes and lysosomes have similar densities, but the lysosomes can be eliminated by selective, substrate-mediated lysis.[166] By combining such lysis with the stepwise application of various gradient media, and by using the fusion inhibitor vinblastine to specifically induce the accumulation of autophagosomes, a procedure for the preparation of very pure autophagosomes from rat liver cells has been developed.[165]

Amphisomes. Amphisomes are autophagic/endocytic organelles formed by the fusion between autophagosomes and early or late endosomes.[94,167] Because this organelle both contains acid hydrolases and a proton pump contributed by the endosomal fusion partner,[58] its cytoplasmic contents are rapidly denatured, making the amphisome ultrastructurally different from its surrounding cytoplasm, unlike the autophagosome. The presence of a single, thin delimiting membrane, and of endosomal markers, may further aid to distinguish amphisomes from autophagosomes (Fig. 14C).[85] However, to distinguish amphisomes from early (auto)lysosomes with moderately degraded cytoplasmic contents, the absence of lysosomal markers is the only criterion available. As discussed above, enrichment in specific lysosomal membrane proteins is more useful for this purpose than enrichment in acid hydrolases.

Amphisomes can be partially purified by a modification of the autophagosome preparation procedure,[165] using partial inhibitors of amphisome-lysosome fusion (leupeptin or asparagine) rather than vinblastine to induce autophagic organelle accumulation. The resulting preparation from rat liver cells has been shown to be a 50:50 mixture of amphisomes and autophagosomes.[167]

Lysosomes. As the end destination of the autophagic-lysosomal pathway, lysosomes are frequently used to monitor autophagy. Needless to say, not all lysosomal changes are due to autophagy, but in those experimental situations where marked alterations in autophagic activity are known to take place, they will usually be reflected in corresponding alterations in the lysosomal population, which may consequently serve as a proxy measure of autophagy. When autophagy is switched on, both the number and average volume of lysosomes will usually rise, but not necessarily proportionally, making the overall volume fraction of the lysosomal compartment (or, better still, the volume fraction of the total autophagic-lysosomal compartment) the most appropriate indicator.[48,84]

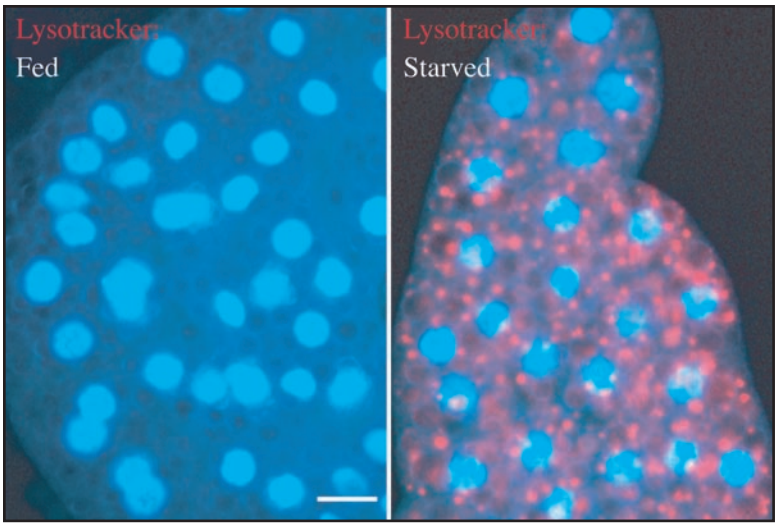

Figure 15. LysoTracker Red stains lysosomes and can be used to monitor autophagy in *Drosophila*. Live fat body tissues from *Drosophila* were stained with LysoTracker Red (red) and Höechst 33342 (blue) to stain the nucleus. Tissues were isolated from fed (left) or 3 h starved (right) animals. Bar; 25 μm. This figure was modified from data presented in reference 175, Developmental Cell, 7, Scott RC, Schuldiner O, Neufeld TP, Role and regulation of starvation-induced autophagy in the *Drosophila* fat body, pp. 167-78, copyright 2004, with permission from Elsevier.

Lysosomes are extremely heterogeneous with regard to ultrastructure, and can assume almost any size or shape, posing, for example, as lamellar bodies, storage granules or tubular "nematolysosomes" (Fig. 14D).[168] Inactive lysosomes can usually be recognized in the electron microscope as small, electron-dense organelles, and autolysosomes by their contents of cytoplasmic material at various stages of degradation. Active autolysosomes have a lower density than the small, inactive lysosomes, allowing the progression of autophagy to be monitored as a lysosomal density shift in isotonic gradients.[169] The lysosomal degradation stage (the time since the autophagic input) can be measured quite precisely as the intralysosomal ratio between a rapidly degraded cytosolic enzyme such as carbonic anhydrase III and a poorly degradable enzyme such as CuZn-superoxide dismutase.[85,170] "Early" lysosomes cannot, however, be distinguished from amphisomes unless a lysosomal membrane protein is included as a marker. In the absence of autophagy, active lysosomes ("endolysosomes" or "phagolysosomes") may be morphologically indistinguishable from endosomes, becasue even completely electron-lucent vacuoles may be lysosomal in nature.

The burden of an increased autophagic influx will tend to stimulate lysosomal proton pumping, thus establishing a correlation between autophagic activity and overall lysosomal acidity under some conditions. A number of fluorescent acidotropic dyes are available for staining of lysosomes on the basis of acidity, such as acridine orange (AO),[171] LysoSensor Blue DND-167,[172] monodansylcadaverine (MDC)[173] and LysoTracker Red.[174] These dyes are able to cross the lysosomal membrane as uncharged molecules, but become protonated and trapped in the acidic interior of the lysosome.[87] The intensity of lysosomal staining, detected by fluorescence microscopy, will be roughly proportional to lysosomal acidity. Because the general increase in lysosomal acidity accompanying increased autophagy will increase the detectability of the lysosomes, quantification can be performed by counting the number of detectable lysosomes per cell (Fig. 15)[175] as well as by measuring the overall fluorescence intensity.

It must be emphasized that acidotropic dyes not only stain lysosomes, but also other acidic organelles, such as endosomes and amphisomes, albeit (usually) with lower intensity, reflecting the higher average pH of the latter. Presumably, an increased endocytic influx to the lysosomes may stimulate proton pumping and promote lysosomal acidity much like autophagy. It is, therefore, important to carry out additional, and more specific tests for autophagy to verify that any detected structures are truly reflecting an autophagic response. Although MDC was originally reported to be a specific marker for autophagic organelles in general,[176] this acidotropic dye does not stain autophagosomes, which are neutral, and subsequent studies have made it clear that it is not specific for autolysosomes either, but will stain lysosomes in general.[37,145]

A number of methods for lysosome purification have been described. We have found it convenient to apply the autophagosome preparation procedure[165] (omitting, obviously, the lysosome lysis step) to cells pretreated with 3-methyladenine to suppress the formation of autophagosomes and amphisomes. The result is a very pure preparation of lysosomes.[167]

INDIVIDUAL STEPS IN MACROAUTOPHAGY

Induction: Tor and the Atg1-Atg13 protein kinase complex. The switching on of autophagy upon nitrogen (amino acid) starvation or growth factor deprivation appears to be mediated by inhibition of the Tor protein kinase in yeast as well as in mammalian cells.[88,114] In yeast, both a stimulation of de novo phagophore formation (through Atg gene expression) and an activation of preexisting phagophores (cycloheximide-resistant autophagy) appears to be involved;[10,177] in mammalian cells an activation mechanism would seem to predominate, because autophagy can proceed at a high rate in the absence of new protein synthesis (i.e., in the presence of cycloheximide) for a considerable length of time.[102] Tor is ivolved in too many cellular activities to serve as a meaningful autophagy indicator on its own, but its downstream effects on Atg13 and (secondarily) Atg1 are quite autophagy-specific.

Atg13 is part of a putative multi-protein complex that includes the protein kinase Atg1,[178,179] and Atg13 appears to modulate Atg1 kinase activity.[180] In addition, Atg13 is a phosphoprotein that undergoes rapid changes in phosphorylation in response to nutrient conditions;[179,180] Atg13 is hyperphosphorylated in rich media and partially dephosphorylated when autophagy is induced (Fig. 16). The phosphorylation is mediated by Tor kinase, but it is not known whether this is direct or indirect. At present, an Atg13 homolog has not been positively identified in higher eukaryotes except for plants. In yeast, however, monitoring Atg13 dephosphorylation would appear to be an adequate way of following induction.

Atg1 is a serine/threonine protein kinase;[181] kinase activity has been measured in vitro[180] and its autophosphorylation activity both in vitro[181] and in vivo.[182] The in vitro kinase assay has shown that maximal activity requires Atg13, Atg17 and even Atg11,[180] all of which are thought to interact with Atg1. The main drawback of either assay at present is that the in vivo targets of Atg1 are not completely known. Accordingly, the in vitro assay uses myelin basic protein as an artificial substrate. The putative mammalian Atg1 homologs, Ulk1 and Ulk2, have so far not been shown to play clear roles in autophagy, although movement of mammalian Atg9 is influenced by Ulk1.[182a] The major function of the Atg1-Atg13 complex

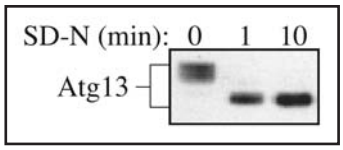

Figure 16. The Atg13 dephosphorylation assay measures autophagy induction. Cells containing overexpressed Atg13 were grown in SMD and then shifted to SD-N at time 0. Samples were collected at the indicated time points, and Atg13 detected by immunoblot. Atg13 is hyperphosphorylated in rich media and rapidly, partially dephosphorylated after shifting to starvation conditions. This figure was modified from data previously published in reference 179 (Scott SV, Nice DC III, Nau JJ, Weisman LS, Kamada Y, Keizer-Gunnink I, Funakoshi T, Veenhuis M, Ohsumi Y, Klionsky DJ. Apg13p and Vac8p are part of a complex of phosphoproteins that are required for cytoplasm to vacuole targeting. J Biol Chem 2000; 275:25840-9, and is reproduced by permission of the American Society for Biochemistry and Molecular Biology and Elsevier.

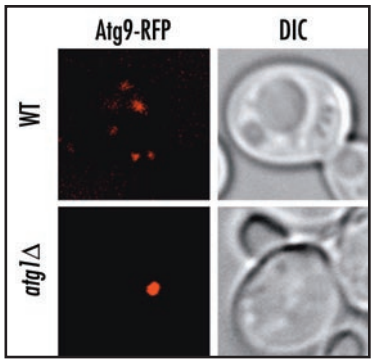

Figure 17. The transport of Atg9 after knocking out Atg1 (TAKA) assay. Wild type and *atg1*Δ strains carrying a genomic Atg9-RFP fusion were grown to mid log phase in SMD medium and imaged by fluorescence microscopy. Atg9-RFP is present in multiple punctate dots in wild type cells, but accumulates at the PAS in the *atg1*Δ mutant. DIC, differential interference contrast. This figure was modified from data previously published in reference 226, and is reproduced by permission of the American Society for Cell Biology, copyright 2005.

may be in the acquisition and processing of membrane material for phagophore formation, see below.

Acquisition of phagophore membrane: Role of Atg9. The source of membrane material for phagophore formation has always been a controversial question: In mammalian cells, rough and smooth ER, Golgi cisternae and the plasma membrane have all been suggested as membrane sources, but the issue is still unresolved. In yeast, the transmembrane protein, Atg9 and the peripheral membrane protein, Atg23 shuttle between the PAS and other cellular locations such as the mitochondria and the ER, and are probably involved in the transport of membrane material from these sources to the PAS.[18,21] Whereas most of the Atg proteins reside at least transiently at the PAS, and can only be detected by fluorescence microscopy at this site,[151,152] Atg9 and Atg23 display localization in multiple peripheral punctate structures in addition to the PAS (Fig. 17).[154,155] Both proteins appear to undergo a cycling process whereby they transit between these two locations.[183] The retrieval of Atg9 and Atg23 from the PAS requires Atg1; in an *atg1*Δ strain, both proteins accumulate at the PAS (Fig. 17). This finding makes it possible to perform an epistasis assay to determine whether a protein functions in Atg9 localization prior to Atg1.[184,185] That is, if a second mutation in the *atg1*Δ background causes Atg9 to display its localization in multiple dots, the respective protein presumably acts before Atg1 and is required for Atg9 delivery to the PAS (anterograde transit). Conversely, if Atg9 accumulates at the PAS in the double mutant strain, the other protein would act at the same stage or after Atg1 (retrograde transit). The assay consists of monitoring the transport of Atg9 after knocking out *ATG1* (TAKA assay), using a fluorescent Atg9 chimera, and is typically carried out in a double mutant strain, where one of the mutations is *atg1*Δ.

Atg8 synthesis. Atg8/LC3 plays a key role both in the autophagic process and as a marker protein for autophagy. Although formation of small autophagosomes and some low, basal autophagic activity is detectable in its absence,[50,158,177,186] Atg8/LC3 is required for phagophore expansion, the formation of large autophagosomes and a high rate of autophagy. In yeast, this requires new protein synthesis,[86,177] and among the several Atg proteins upregulated during autophagy, the greatest change in expression is seen with Atg8.[158,187] The levels of this protein may increase by 10-fold or more under starvation conditions.[188] Monitoring the change in total Atg8 levels is thus one way to follow the induction of autophagy in yeast (in mammalian cells, the pool of preexisting LC3 is apparently sufficient to keep autophagy going for quite a while, as discussed above). There is, however, one important caveat to keep in mind with regard to this approach. Because Atg8 is degraded in the vacuole while new Atg8 protein is being synthesized, it is problematic to use this method to measure autophagy through western blotting, unless degradation is inhibited (as discussed above). That is, a high level of Atg8 would indicate that autophagy has been induced, but it could also reflect a block in the later stages of the process. By examining Atg8 levels in the presence and absence of a vacuolar protease inhibitor, it is possible to look at both the change in Atg8 levels and the turnover of the protein. In other words, a high level of Atg8–PE in the presence of protease inhibitor but a lower level in its absence would indicate both autophagic induction and normal turnover of Atg8 within the vacuole, further reflecting normal fusion of autophagosomes and breakdown of the autophagic body in addition to degradation of Atg8.

Atg12–Atg5 conjugation. The binding of Atg8/LC3 to the phagophore membrane is dependent on the presence of an Atg12–Atg5-Atg16 complex. Atg12 (21 kDa) is an ubiquitin-like protein that is covalently attached to Atg5 (34 kDa), forming a higher molecular mass conjugate (70 kDa).[189] This process is dependent upon the Atg7 (activating enzyme) and Atg10 (conjugating enzyme) proteins. Atg5 and Atg12 do not appear to be highly antigenic (personal observations); however, either protein can be epitope-tagged for detection by western blotting. Tagging at the C terminus of Atg12 should be avoided because the C-terminal glycine is involved in the conjugation reaction.

Atg8/LC3 processing and delipidation: Role of Atg4. In order to attach to the phagophore membrane, Atg8 needs to be proteolytically processed and conjugated to PE. The processing is performed by the cysteine proteinase Atg4, which removes a C-terminal arginine to expose the penultimate glycine, making Atg8 susceptible to activation by Atg7. Atg4 also removes PE from the Atg8–PE that is located on the outer surface of the completed autophagosome. Fusion of GFP to the C terminus of Atg8 allows the detection of Atg4 function in the cytosol; the appearance of free GFP, or the disappearance of full-length Atg8-GFP indicates Atg4 activity (Fig. 18).[190]

The mammalian homolog of Atg8, LC3, undergoes a similar C-terminal processing event to that seen with Atg8.[78] A C-terminal processing assay can be used to analyze mammalian Atg4 homologues by carrying out in vitro reactions with purified Atg4 and

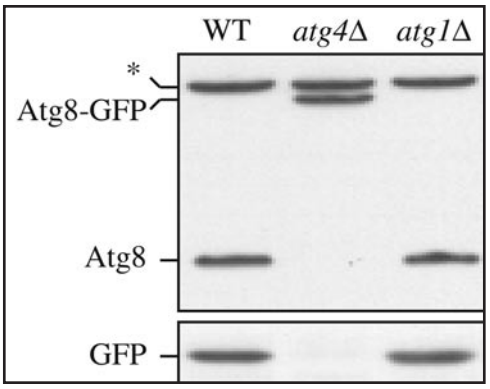

Figure 18. The Atg8-GFP processing assay measures Atg4 function. Wild type, *atg4*Δ and *atg1*Δ strains transformed with a centromeric plasmid encoding Atg8-GFP were grown to midlog phase in SMD. Protein extracts were prepared and analyzed by immunoblot using antiserum to Atg8 (top panel) or GFP (bottom panel). Atg4 proteolytically processes the Atg8 C terminus. This processing occurs in the cytosol, not in the vacuole, as can be seen with the *atg1*Δ control strain. The asterisk (*) denotes a background band. This figure was modified from data previously published in reference 190, and is reproduced by permission of the American Society for Cell Biology, copyright 2001.

Atg8 homologues that have been tagged at the C terminus with the myc epitope.[191] As an alternative, the ability of Atg4 to delipidate LC3-II can be monitored via the resulting change in mobility during SDS-PAGE.[191]

Atg8/LC3 lipidation. A two-step process catalyzed by the E1-like enzyme, Atg7, and the E2-like enzyme, Atg3 is involved in the PE conjugation required for attachment of Atg8/LC3 to the phagophore membrane. Western blotting without and with subcellular fractionation can be used to follow Atg8/LC3 conjugation. The attachment of PE causes a molecular mass shift that can be observed through SDS-PAGE (Fig. 19). Unexpectedly, the lipidated form of Atg8/LC3 migrates more rapidly than the nonlipidated species, and resolution of the two forms requires a high percentage gel (12.5–15%) with 6 M urea.[144,192] Yeast mutants defective in autophagosome formation or in vacuolar degradation, such as *pep4*Δ, accumulate higher levels of Atg8–PE. In contrast, some mutants display low levels of the lipidated protein even in a *pep4*Δ background because this species is unstable.[144] Atg8 is a hydrophilic protein and localizes to the supernatant fraction prior to conjugation. Attachment of PE causes Atg8 to become membrane-associated, which is easily followed by cell lysis and separation of membrane and soluble fractions.[192]

In mammalian cells, conversion of the unlipidated and soluble form, LC3-I, to a lipidated species, LC3-II, is a widely used autophagy assay.[78] One problem with this analysis, however, is that the level of detectable LC3-I, as well as the overall increase in LC3, is highly cell line- and tissue-dependent.[146] For example, in HEK293 cells maintained in nutrient-rich conditions, the primary form of the protein is LC3-I, whereas in HeLa cells it is LC3-II. Thus, it would be possible to monitor the conversion to LC3-II in the former but not the latter (Fig. 13). In addition, simply monitoring LC3-II levels does not provide an unequivocal assessment of autophagy as discussed above. Furthermore, LC3-II may be more easily detected than LC3-I in western blots.[81] One final complication is that lipidated Atg8/LC3 is unstable in the absence of the Atg12–Atg5 conjugate,[81] so that reduced levels of the lipidated species may reflect a defect in the other conjugation system.

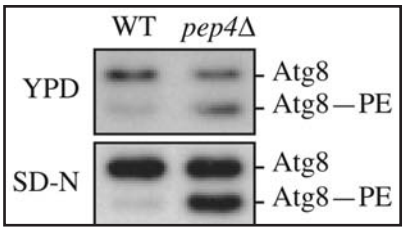

Figure 19. Atg8–PE formation. Wild type cells or a mutant defective in vacuolar degradation (*pep4*Δ) were grown in YPD to early mid log phase and then starved for 3 h in SD-N. Atg8–PE was separated from Atg8 in 12% SDS PAGE gels containing 6% Urea. This figure was modified from data previously published in reference 144, and is reproduced by permission of the American Society for Cell Biology, copyright 2004.

Autophagic organelle fusion. Subsequent to formation (closure) of the autophagosome, which can be monitored by the various sequestration assays discussed above, the autophagosome can fuse with an endosome to form an amphisome,[94] which subsequently fuses with a lysosome to have its contents degraded; or, the autophagosome may fuse directly with a lysosome (Fig. 1).[193] Very little work has been done to study these various fusion steps, in particular because it has not yet been possible to reconstitute them under cell-free conditions. It is, however, possible to address them indirectly by the use of suitable inhibitors. For example, because amphisome-lysosome fusion is partially blocked by asparagine,[94] preincubation of hepatocytes with asparagine will cause an accumulation of electroinjected [^{14}C]lactose in amphisomes. Upon subsequent incubation in the absence of this reversible inhibitor, the sugar will be transferred to lysosomes, allowing the amphisome-lysosome fusion step to be assayed.[30] By preloading autophagosomes with radioactive sugar, for example, in the presence of vinblastine, it may be possible to monitor autophagosome-endosome fusion[94] or autophagosome-lysosome-fusion.[57] Similarly, sugar preloading of the endosomes can be used to study, for example, endosome-lysosome fusion.[194]

The intralysosomal degradation step. The organelle preloading technique discussed above can also be used to study the intralysosomal degradation step directly, independently of preceding autophagic steps. Lysosomes can be loaded, for example, with [^{14}C]lactose, in the presence of a reversible acidotropic agent such as propylamine that will prevent acid hydrolysis; upon subsequent drug withdrawal (preferably in the presence of inhibitors to block further autophagic influx), the intralysosomal lactose will be degraded by the lysosomal β-galactosidase.[30] This strategy has been used to study the energy requirement of intralysosomal hydrolysis.[28] In principle, any lysosome substrate can be used for this purpose.

GFP-Atg8 cleavage. The use of a C-terminal GFP-Atg8 conjugate to monitor Atg8 processing (Atg4 function) was discussed above. By fusing, instead, GFP to the N terminus of Atg8, the generation of free GFP can be used as a measure of intralysosomal hydrolysis. Upon delivery of such a GFP-Atg8–PE complex to the vacuole, the Atg8–PE moiety is rapidly degraded, whereas the released GFP remains relatively stable.[187] Accordingly, the appearance of free GFP can be used to monitor the Cvt pathway or autophagy (Fig. 20).[184,185] Because only the inner membrane of autophagosomes or Cvt vesicles enters the vacuole, this assay can be used to specifically monitor the degradation of that membrane, or the efficiency of lysis of autophagic bodies. This method can also be used with GFP-LC3 in at least some mammalian cell lines.[195]

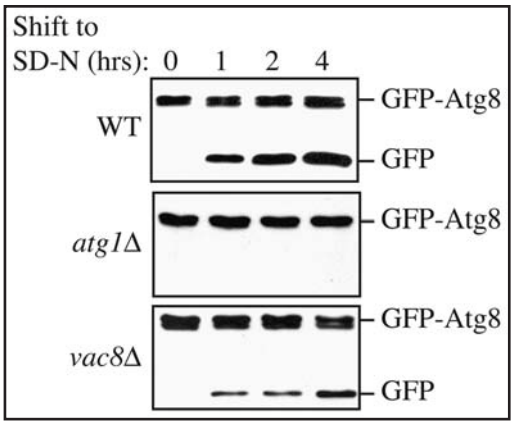

Figure 20. GFP-Atg8 processing can be used to monitor delivery of autophagosomal membrane by autophagy, as well as autopahgic body lysis. Wild type, *atg1*Δ and *vac8*Δ strains expressing GFP-Atg8 were grown in SMD lacking auxotrophic amino acids and shifted to SD-N medium. At the indicated times aliquots were removed, the proteins precipitated with TCA and resolved by SDS-PAGE. Full-length GFP-Atg8 and free GFP were detected by immunoblot using anti-GFP antibodies. The band running just below full-length GFP-Atg8 is a cross-contaminant. The *vac8*Δ mutant displays a partial defect in autophagy. This figure was modified from data previously published in reference 184, and is reproduced by permission of the American Society for Cell Biology, copyright 2005.

MICROAUTOPHAGY

Microautophagy in mammalian cells refers to the formation of lysosomal membrane invaginations, observed in the electron microscope, which are believed to be pinched off and degraded intralysosomally along with their contents of sequestered cytoplasm.[39] The process is thought to contribute to the overall autophagic degradation of cytoplasm in intact cells,[196,197] and has been claimed to be reconstituted under cell-free conditions,[198,199] but its physiological significance or molecular properties have never been well characterized.

The invagination and degradation of vacuolar membrane containing cytosol has also been demonstrated in *S. cerevisiae*, as a process distinct from micropexophagy (see below).[200,201] Microautophagy does not appear to directly require Atg proteins, and its exact physiological role is not known; it may play a role in removing the outer autophagosome membrane from the vacuole limiting membrane following fusion. The process can be measured in vitro by the uptake of FITC-dextran, or through the appearance of autophagic tubes (invaginations) detected by fluorescence microscopy of FM 4-64-stained vacuoles. Measuring the activity of protease-resistant luciferase following in vitro sequestration, and monitoring autophagic tube formation by electron microscopy are alternative assays.[200,201]

Piecemeal microautophagy of the nucleus (PMN). PMN is a specific type of autophagy in which portions of the nucleus are invaginated within the yeast vacuole and degraded.[202] This process is distinct from macroautophagy and does not appear to rely on Atg proteins, although it does require Vac8. The process can be observed through fluorescence microscopy of Vac8 and Nvj1, as colocalized regions of the nucleus blebbing into FM 4-64-stained vacuoles (or through electron microscopy), and also by monitoring degradation of Nvj1.

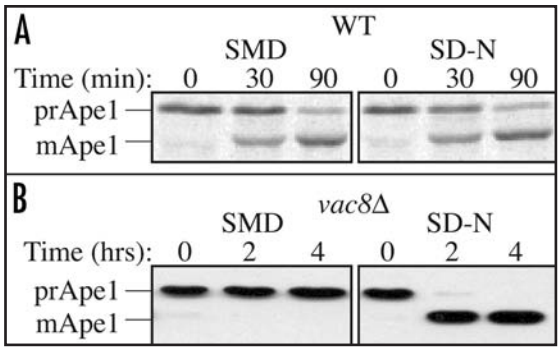

Figure 21. The Ape1 processing assay can be used to monitor kinetics of transport by the Cvt and autophagy pathways. (A) Cells from a wild type strain were grown in SMD to mid log phase and then subjected to a radioactive pulse for 10 min with ^{35}S-methionine. After labeling, the cells were reisolated and resuspended in either SMD (Cvt pathway) or SD-N (autophagy) and chased for the indicated times (min). Following TCA precipitation, protein extracts were prepared and subjected to immunoprecipitation with anti-Ape1 serum. The positions of precursor (pr) and mature (m) Ape1 are indicated. This figure was modified from data previously published in reference 179, (Scott SV, Nice III DC, Nau JJ, Weisman LS, Kamada Y, Keizer-Gunnink I, Funakoshi T, Veenhuis M, Ohsumi Y, Klionsky DJ. Apg13p and Vac8p are part of a complex of phosphoproteins that are required for cytoplasm to vacuole targeting. J Biol Chem 2000; 275:25840-9.) Apg13p and Vac8p are part of a complex of phosphoproteins that are required for cytoplasm to vacuole targeting, pp. 25840-9, 2000, and is reproduced by permission of the American Society for Biochemistry and Molecular Biology and Elsevier, copyright 2000. (B) The *vac8*Δ mutant is defective in the Cvt pathway and can be used to monitor autophagy through a western blot kinetic analysis. *vac8*Δ cells were grown in SMD and shifted to SD-N for the indicated times to induce autophagy. At each time point, protein extracts were prepared, resolved by SDS-PAGE and analyzed by western blot. This figure was modified from data previously published in reference 184, and is reproduced by permission of the American Society for Cell Biology, copyright 2005.

It should be noted that whole nuclei can also be subject to autophagic sequestration, but in this case by the macroautophagic pathway.[156]

THE CVT PATHWAY

The cytoplasm to vacuole targeting (Cvt) pathway shares many molecular characteristics and Atg proteins with macroautophagy (Fig. 1), but has a number of distinct properties that can be used for specific assay purposes.

Ape1 processing and activity. Aminopeptidase I (Ape1) is a resident hydrolase located in the vacuole lumen. It is initially synthesized as a cytosolic precursor (prApe1) containing an N-terminal propeptide segment that keeps the enzyme inactive and also contains a domain that binds the Atg19 receptor.[203] Precursor Ape1 is delivered to the vacuole through either macroautophagy or the Cvt pathway, depending upon the nutrient conditions. Upon delivery to the vacuole, the propeptide is enzymatically removed generating the mature, active enzyme. The maturation event can be measured by following a molecular mass shift through SDS-PAGE or enzymatically. The former can be seen through a radioactive pulse/chase analysis, allowing a determination of kinetics (Fig. 21A). Delivery of prApe1 to the vacuole requires the receptor protein Atg19 as well as Atg11.[19,204] These proteins allow import of prApe1 to be rapid and efficient—the kinetics of import are essentially identical under vegetative or starvation conditions, having a half-time of 45–60 min for conversion to the mature form.[203,205] This means

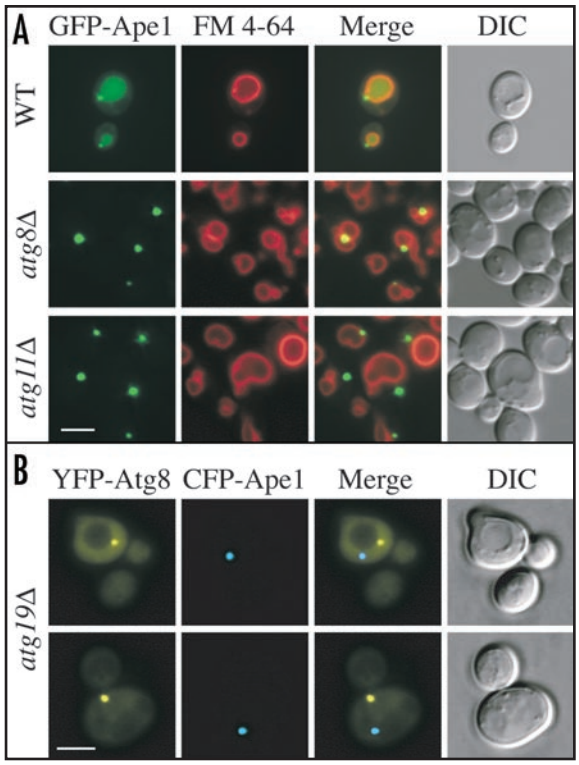

Figure 22. Precursor Ape1 localization. (A) Localization of Ape1 by fluorescence microscopy of wild type, *atg8*Δ and *atg11*Δ strains expressing GFP-Ape1. Cells were grown to mid log phase, labeled with FM 4-64 and examined with fluorescence and DIC microscopy. In the wild type strain, GFP-Ape1 can be seen at the PAS and within the vacuole lumen. The *atg8*Δ mutant accumulates GFP-Ape1 at the PAS, whereas the *atg11*Δ mutant accumulates GFP-Ape1 away from the PAS. Bar; 5 μm. (B) CFP-Ape1 does not colocalize with YFP-Atg8 in *atg19*Δ cells. Cells coexpressing CFP-Ape1 and YFP-Atg8 were grown in SMD medium to mid log phase and examined with fluorescence and DIC microscopy. Bar; 5 μm. This figure was modified from data presented in reference 20, Shintani T, Huang W-P, Stromhaug PE, Klionsky DJ. Mechanism of cargo selection in the cytoplasm to vacuole targeting pathway. Developmental Cell 2002; 3:825-37, copyright 2002, with permission from Elsevier.

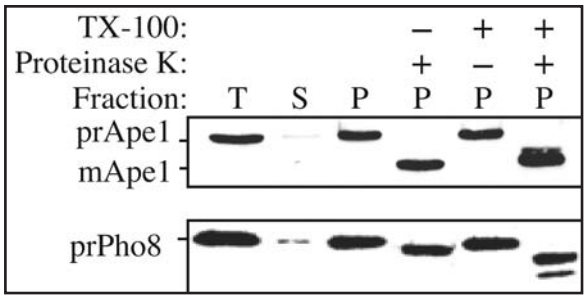

Figure 23. Protease-protection assay. The *atg18*Δ *pep4*Δ strain was grown to mid log phase in SMD. Cells were converted to spheroplasts and osmotically lysed. The 5,000 x g pellet fraction was resuspended in lysis buffer, then mixed with equal volumes of lysis buffer, 40 μg/ml proteinase K, 0.4% TX-100, or proteinase K plus 0.4% TX-100 and incubated on ice for 30 min. The reactions were stopped by mixing with 20% TCA. Samples were resolved by SDS-PAGE and examined by immunoblot with serum to Ape1 and Pho8 as indicated. Even in the absence of detergent, the prApe1 in *atg18*Δ cells was digested to its mature form by proteinase K. Sensitivity of the Pho8 cytosolic domain in the absence of detergent reflects efficient spheroplast lysis, whereas protection of the Pho8 propeptide indicates integrity of the vacuole. This figure was modified from data previously published in reference 208, and is reproduced by permission of the American Society for Cell Biology, copyright 2001.

that prApe1 processing can be used to monitor specific autophagy. Certain mutants, such as *vac8*Δ, block prApe1 processing only under vegetative conditions.[179] When autophagy is induced in the *vac8*Δ mutant, prApe1 is delivered to the vacuole with normal kinetics. Thus, the kinetics of processing can be monitored through a western blot analysis under these conditions (Fig. 21B). As indicated above, it is also possible to monitor vacuolar delivery of prApe1 through an enzyme assay.[206]

Precursor Ape1 localization. In general, it is possible to follow the localization of any of the Atg proteins; however, in most cases these data are not particularly informative because most of these proteins can only be detected diffusely in the cytosol and at the PAS. Precursor Ape1 forms a complex in the cytosol that appears to be independent of any of the Atg proteins.[20,185] The localization of this complex at the PAS, however, requires the Atg19 and Atg11 proteins (Fig. 22). Thus, by monitoring the localization of prApe1 by itself or comparing it to another protein such as Atg8, it is possible to obtain information about the steps of cargo recognition and packaging.

Protease-protection. A protease-protection or protease-sensitivity assay can be used to assess the completeness of the autophagosome or Cvt vesicle, and is also used in the measurement of chaperone-mediated autophagy (see below). One such assay that is convenient in yeast monitors the marker protein prApe1. Precursor Ape1 is resistant to exogenous protease when it is contained within a completed vesicle. In contrast, the propeptide is sensitive to degradation if prApe1 is accessible. The assay consists of osmotic lysis of spheroplasts under conditions that retain the integrity of subcellular compartments, followed by the addition of protease in the absence and presence of detergent.[207] The most important aspect of this assay is the use of an appropriate control. One possibility is to use a mutant such as *ypt7*Δ that accumulates prApe1 within completed vesicles as a protease-resistant control;[207] most *atg* mutants can serve as controls for protease-sensitivity. The disadvantage of this approach is that the control is external to the experimental sample. An alternate and preferred control is to use Pho8.[208] Precursor Pho8 contains a short cytosolic domain as well as a C-terminal propeptide. The propeptide is removed in the vacuole lumen, but will be retained in the *pep4*Δ background. The vacuole is relatively fragile, so conditions that maintain the integrity of the vacuole are likely to also preserve the integrity of an autophagosome or Cvt vesicle. The two terminal domains of prPho8 allow an assessment of spheroplast lysis and vacuole integrity; the cytosolic domain should be completely susceptible to degradation by exogenous protease following efficient spheroplast lysis, whereas the lumenally-oriented propeptide should only be degraded if the vacuole limiting membrane has been compromised such as with the addition of detergent (Fig. 23).

Ams1 activity. α-Mannosidase (Ams1) is a resident vacuolar hydrolase that is delivered to this organelle through the Cvt and autophagy pathways.[209] Ams1 does not appear to require proteolytic processing to become activated; the protein does not contain a propeptide. As a result, vacuolar delivery can only be followed by enzyme assay[210] following vacuole purification,[211] or qualitatively by fluorescence microscopy of a chimeric protein.[209] In addition, Ams1 is synthesized at substantially lower levels than Ape1, which is the standard marker protein for the Cvt pathway (see Ape1 processing and activity). For these two reasons, it is not a convenient marker for autophagy and the Cvt pathway. Nonetheless, Ams1 import requires the same specificity components as prApe1, including Atg19, and thus serves as another marker of specific autophagy.

CHAPERONE-MEDIATED AUTOPHAGY

At present, chaperone-mediated autophagy (CMA) has only been detected in mammalian cells; the closest equivalent may be the vacuolar import and degradation (Vid) pathway in yeast.[212,213] CMA activity can be measured in confluent cells in culture using pulse/chase experiments as described above, as the increase in the degradation rates of long-lived proteins after removing the serum from the culture medium.[214,215] In most mammalian cells, macroautophagy is activated during the first hours of serum removal, but formation of autophagic vacuoles does not occur after 4–6 h of starvation. After this, CMA activity increases progressively, reaching maximal activation at about 10 h after the removal of serum, and remains at this level for the rest of the starvation period.[216,217] CMA-dependent degradation is defined under those conditions as that activated by serum removal, which is sensitive to ammonium chloride (the same as any other form of autophagy), but insensitive to PtdIns 3-kinase inhibitors (which block macroautophagy).[215] Note that metabolic labeling coupled with the use of various inhibitors is essential for this kind of study, because the redundancy of the proteolytic systems in mammalian cells means that the total levels of CMA-substrate proteins measured by immunoblot do not necessarily reflect changes in their degradation through this pathway. For example, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is one of the established substrates of CMA, and its binding or uptake/degradation have been measured to follow different steps of this process;[218,219] however, GAPDH can be degraded by multiple pathways.

The most unequivocal method to measure CMA is by directly tracking the translocation of substrate proteins into isolated lysosomes using protease-protection assays similar to those described above.[41,213,218] CMA can be reconstituted in vitro using isolated lysosomes, and both substrate binding and translocation can be determined.[219,220] To measure binding, lysosomes are incubated with the substrate protein in an isotonic medium supplemented with hsc70, the cytosolic chaperone that targets CMA-susbstrates to lysosomes, and nucleotides. Binding of substrate to the lysosomal membrane can be determined as the amount of substrate pulled down with the lysosomes, because any protein internalized by the lysosomes will be rapidly degraded (the half-life of substrate proteins in the lysosomal lumen is about 5 min). Translocation of substrate proteins can be tracked using lysosomes that have been treated with protease inhibitors. The amount of substrate translocated into the lysosomal lumen can be determined by comparing the level of total substrate (bound plus translocated) to the substrate that is resistant to cleavage by an exogenously added protease (Fig. 24).

An indirect method to evaluate CMA activity in those tissues or cells in which lysosomal isolation is not feasible, is to analyze changes in the population of lysosomes responsible for CMA, which is defined as those containing lys-hsc70 (the lysosomal chaperone required for substrate translocation) in their lumen. This population can be detected by immunoelectron microscopy with an antibody directed against hsc70[218,219] or by immunofluorescence as the number of vesicles containing hsc70 and LAMP-2A (the lysosomal receptor for CMA; note that only the 2A variant of the LAMP-2 proteins is related to CMA).[221] It is important to point out that changes in total cellular levels of CMA-related components, namely the lysosomal receptor LAMP-2A and hsc70, do not necessarily correlate with changes in CMA, because both proteins are also present in other cellular compartments and play other roles in the cell. It is thus necessary to determine that the increase or decrease in these components takes place in the lysosome-associated fraction.[222]

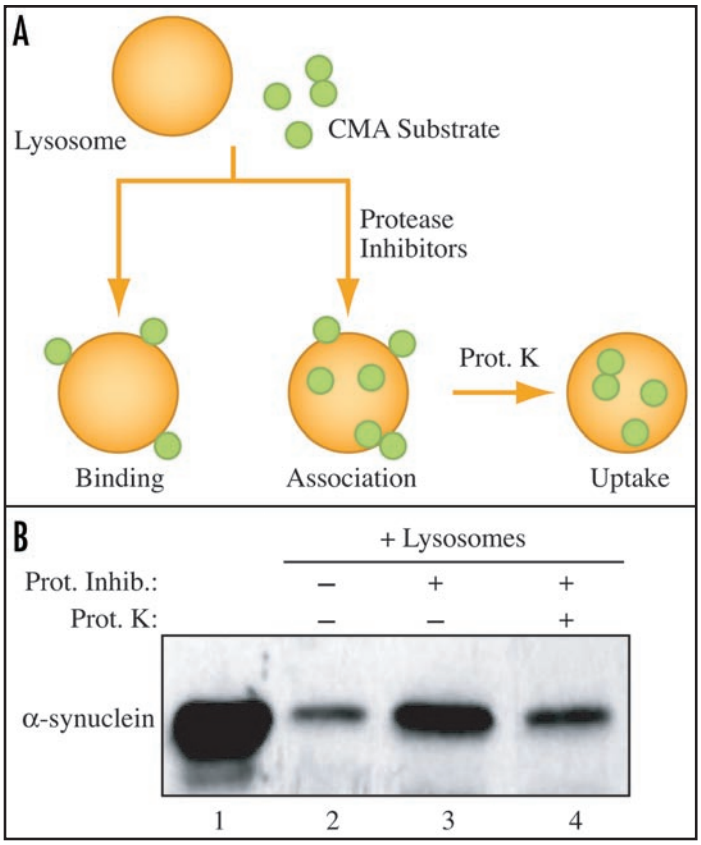

Figure 24. Protease protection assay for monitoring chaperone-mediated autophagy (CMA). (A) Binding and translocation of CMA-substrate proteins into lysosomes can be reproduced in an *in vitro* system using isolated lysosomes supplemented with the corresponding chaperones and an energy-regenerating system. In the absence of protease inhibitors the substrate translocated into the lysosomal lumen is rapidly degraded and only those proteins bound to the lysosomal membrane can be detected when lysosomes are recovered by centrifugation (binding). When the lysosomal proteolytic activity is inhibited, the lysosomes contain both the substrate bound to the lysosomal membrane and the substrate that has translocated inside the lysosomal lumen (association). The amount of translocated substrate can be identified as those proteins that are resistant to treatment with exogenous proteases (Prot. K), which will degrade only the substrate that is not protected by the lysosomal membrane (uptake). (B) Immunoblot showing binding, association and uptake of α-synuclein into isolated rat liver lysosomes. Lane 1 shows the input.

PEXOPHAGY

Fox3 degradation. 3-oxoacyl-coenzyme A thiolase, or Fox3, is a marker enzyme of the peroxisome matrix. When yeast such as *S. cerevisiae* are grown on glucose, peroxisome number and Fox3 levels are quite low. If the cells are shifted to oleic acid as the sole carbon source (or if methylotrophic yeasts such as *Pichia pastoris* or *Hansenula polymorpha* are shifted to methanol), peroxisomes proliferate and Fox3 levels increase. When peroxisome-induced cultures are subsequently shifted back to glucose or to starvation conditions, the peroxisomes are rapidly and specifically degraded by pexophagy, a type of specific autophagy.[223] Following the loss of Fox3 by western blotting can be used to monitor the breakdown process (Fig. 25). Transcription of the *FOX3* gene is controlled by an oleate response element resulting in a greater than 10-fold decrease in expression in the presence of glucose.[224] Accordingly, the loss of Fox3 can be followed kinetically by western blot as there is very little new synthesis occurring under

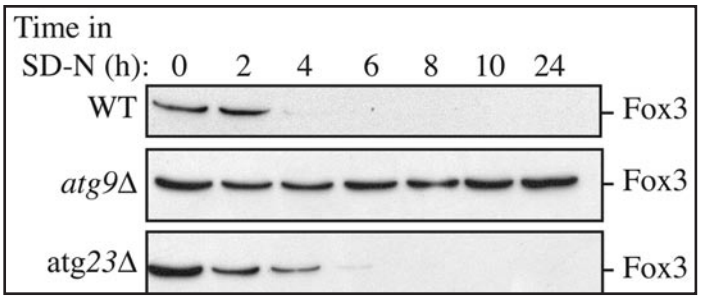

Figure 25. Fox 3 degradation assay for monitoring pexophagy in *S. cerevisiae*. Cells from wild type, *atg9*Δ and *atg23*Δ strains in the BY4742 background were grown under peroxisome-inducing conditions (oleic acid) and shifted to SD-N for 24 h. At the indicated times, protein extracts were prepared and subjected to immunoblot analysis using anti-Fox3 antiserum. The *atg23*Δ mutant is not defective in pexophagy. This figure was modified from data previously published in reference 155, Tucker KA, Reggiori F, Dunn WA Jr, Klionsky DJ. Atg23 is essential for the cytoplasm to vacuole targeting pathway and efficient autophagy but not pexophagy. J Biol Chem 2003; 278:48445-52, copyright 2003 and is reproduced by permission of the American Society for Biochemistry and Molecular Biology and Elsevier.

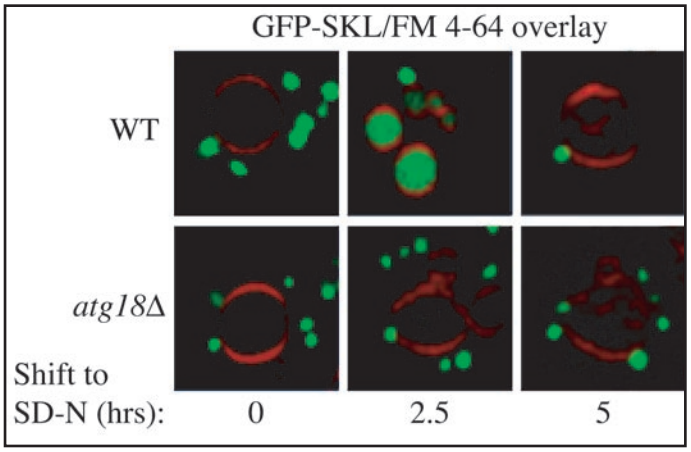

Figure 26. The GFP-SKL pexophagy assay. Cells from wild type and *atg18*Δ strains were grown to mid log phase in SMD and transferred to oleic acid-containing medium to induce peroxisome proliferation, labeled with FM 4-64 to stain the vacuole and subsequently transferred to SD-N to induce degradation of the excess peroxisomes. Cells were examined by scanning confocal microscopy after incubation in SD-N at the indicated times. A diffuse fluorescence can be seen within the vacuole lumen of the wild type strain at 2.5 h, reflecting lysis of the peroxisomes. This signal dissipates by 5 h, indicating degradation of the GFP-SKL. Peroxisomes accumulate outside of the vacuole during pexophagy in the *atg18*Δ mutant. This figure was modified from data previously published in reference 208, and is reproduced by permission of the American Society for Cell Biology, copyright 2001.

these conditions.[225] It should be noted that the degradation of peroxisomes when cells are shifted to a nitrogen starvation medium (SD-N) is rapid and specific, and cannot be accounted for by bulk autophagy; thus, it presumably reflects a specific process.[225] Furthermore, there are practical advantages to using SD-N as opposed to glucose to induce pexophagy. The disadvantage of using glucose is that the cells will grow and dilute peroxisomes due to cell division. This must be taken into account when following protein loss by western blot.

GFP-SKL. One of the most convenient microscopy assays to monitor pexophagy is achieved by labeling peroxisomes with a fluorescent protein modified to express the type 1 peroxisomal targeting signal of serine-lysine-leucine at the C terminus.[208] As noted for the Fox3 assay above, when yeasts are grown in media with oleic acid or methanol as the sole carbon source peroxisomes proliferate. Subsequently shifting to glucose or starvation conditions results in peroxisome degradation (Fig. 26). This assay can be modified by placing the GFP gene under the control of a regulable promoter such as the oleate response element, to prevent further synthesis after shifting to pexophagy conditions. Finally, as noted for Fox3, growth in glucose to induce pexophagy has the disadvantage of causing cell growth and subsequent dilution of peroxisomes; however, this is less of a problem with microscopy assays than it is with western blot.

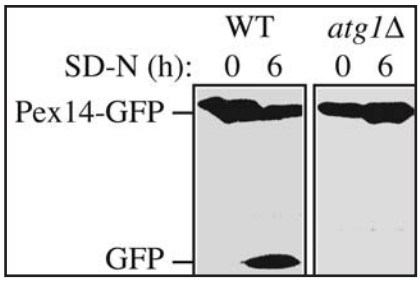

Figure 27. The Pex14-GFP processing assay to monitor pexophagy. The Pex14-GFP and Pex14-GFP *atg1*Δ strains were grown in medium containing oleic acid, then shifted to SD-N for 6 h. Protein extracts were prepared from cells before and after nitrogen starvation, resolved by SDS-PAGE and, after western blot, membranes were probed with anti-GFP monoclonal antibody (Covance, Princeton, NJ). The positions of the full-length Pex14-GFP fusion protein and free GFP are indicated. The *atg1*Δ mutant is defective in pexophagy and does not generate free GFP. This figure was modified from data previously published in reference 226, and is reproduced by permission of the American Society for Cell Biology, copyright 2005.

Pex14-GFP processing. Pex14 is one component of the import machinery that is located in the peroxisomal membrane; Pex14-GFP serves as an alternative marker to Fox3 or GFP-SKL for this organelle.[226] During pexophagy, the GFP domain is cleaved from the chimera in the vacuole. Thus, the appearance of free GFP is a measure of pexophagy (Fig. 27), similar to the way in which it is a measure of autophagy in the GFP-Atg8 assay; however, in this case the GFP is a measure of the cargo rather than the autophagosome membrane. The advantage of Pex14-GFP relative to Fox3 or GFP-SKL is that it is easier to measure the appearance of free GFP than it is to accurately follow the loss of Fox3 or GFP-SKL, especially when the level of pexophagy is low.

Peroxisomal enzyme inactivation. In addition to the other methods for monitoring pexophagy, there are various assays that rely on the loss of enzyme activity that occurs when peroxisomes are degraded. The most commonly used assay is loss of alcohol oxidase (AOX) activity.[227,228] The degradation of this enzyme can also be followed through western blot. Alcohol oxidase is not present in *S. cerevisiae*, but can be detected in methylotrophic yeasts such as *Candida boidinii*,[229] *Pichia methanolica*,[230] *Pichia pastoris*[231,232] and *Hansenula polymorpha*.[233] Degradation of formate dehydrogenase and/or catalase has also been examined in *H. polymorpha*, *P. pastoris* and *P. methanolica*.[230,232,234] The loss of isocitrate lyase in *Yarrowia lipoplytica*[235] and *Aspergillus nidulans*,[236] dihydroxyacetone synthase in *H. polymorpha*[237] and amine oxidase in *Y. lipolytica*[235] is due to pexophagy. Degradation of peroxisomal fatty acyl-CoA oxidase and catalase has been followed in rat hepatocytes.[238]

AUTOPHAGY IN DEVELOPMENT

Autophagy is required for certain developmental programs, and these can be monitored to follow the overall autophagic process.[5] For example, yeast *atg* mutants are defective in sporulation.[239] *Dictyostelium discoideum* autophagy mutants display a range of phenotypes including the inability to form multicellular aggregates, or defects in fruiting body formation.[240,241] In *Drosophila*, autophagy is needed for pupae formation, and in *C. elegans* for entry into the dauer stage of the life cycle.

AUTOPHAGY IN PLANTS

Autophagy can be monitored in plants or plant cells using some of the different assays described in this review;[242] however, there are also several assays that are specific to plants,[243,244] which are grouped in this section.

Bolting. The transition from a rosette form to one with an elongated stem is accelerated in autophagy-defective plants.

Chlorosis and senescence. The rosette leaves of *atg* mutant plants undergo earlier senescence and display accelerated chlorosis, observed as a more rapid conversion from green to yellow, reflecting the degradation of chloroplasts and the subsequent loss of chlorophyll. One caveat to this assay is that there is no evidence that degradation of the chloroplast occurs through autophagy, which fits with the acceleration in chlorosis in the absence of autophagic activity; the mechanism of chlorosis is not known.

Root elongation. Overall slower rates of growth in *atg* mutant plants can be followed by measuring root elongation.

Seed/Silique production. *Arabidopsis atg* mutants develop fewer inflorescence branches and display reduced production of seeds or seed pods.

AUTOPHAGIC CELL DEATH

Defects in autophagy cause reduced life-span and lead to a loss in viability. Thus, measuring cell or organismal survival is one method for monitoring the overall process of autophagy. One problem with this assay is that a loss of viability may be due to factors other than an autophagy defect. In addition, loss of viability may be considered a phenotype rather than an assay. Nonetheless, measuring the autophagy-dependent loss of viability has been used successfully in *C. elegans*,[245] *D. discoideum*,[241] mammals[246] and yeast.[128,129]

Two general methods have been used to monitor viability in yeast. In the first approach, yeast strains are grown on rich medium (YPD) and then replica plated onto plates lacking nitrogen, but containing the vital dye phloxine B.[129] After incubation for approximately 3–4 days, the colonies are examined for a red appearance that indicates uptake of the dye, loss of viability, and a potential defect in autophagy. One caveat with this method is that the plates should be observed daily because wild type strains will take up the dye to some extent, eventually becoming difficult to distinguish from mutants.

The second method relies on growth and incubation in liquid media. Strains are grown in rich medium (YPD) in flasks, then shifted to nitrogen starvation medium (SD-N).[207] Aliquots of the culture are spread onto YPD plates in triplicate starting immediately after the shift and continuing for several days. Viable colonies are counted after 2–3 days growth on the YPD plates. Strains such as *atg1*Δ show essentially complete loss of viability after 2–3 days incubation in SD-N, whereas other mutants display decreasing viability over the course of seven or more days. A wild type strain should always be examined in parallel because the viability of the control strain may be quite variable depending in part on the density of the culture prior to the shift to SD-N.

CONCLUSION

This review should make it clear that a large number of assays are currently available to monitor various steps and types of autophagy. At the same time, it should be obvious that no single method is perfect or sufficient, and it is better to combine multiple approaches for a rigorous evaluation of autophagic mechanisms. The current assays include microscopy, biochemical approaches and analysis of specific Atg proteins. The majority of these assays can be used in yeast, but many are applicable to higher eukaryotes. The development of additional methods for the latter will enhance our ability to monitor autophagy for potential therapeutic uses. We hope that the information we have provided will be of use to newcomers to the field, and will stimulate additional analyses of the autophagic process.

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