



## Guidelines for the use and interpretation of assays for monitoring autophagy

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# Guidelines for the use and interpretation of assays for monitoring autophagy

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**Keywords:** autolysosome, autophagosome, flux, LC3, lysosome, phagophore, stress, vacuole

**Abbreviations:** 3-MA, 3-methyladenine; AMPK, AMP-activated protein kinase; Ape1, aminopeptidase I; Atg, autophagy-related; AV, autophagic vacuole; CLEAR, coordinated lysosomal enhancement and regulation; CLEM, correlative light and electron microscopy; CMA, chaperone-mediated autophagy; Cvt, cytoplasm-to-vacuole targeting; DQ-BSA, dequenched bovine serum albumin; e-MI, endosomal microautophagy; EBSS, Earle's balanced salt solution; FACS, fluorescence-activated cell sorter; GFP, green fluorescent protein; IMP, intramembrane particle; LAMP2, lysosome-associated membrane protein 2; LAP, LC3-associated phagocytosis; LC3, microtubule-associated protein 1 light chain 3 (MAP1LC3); MDC, monodansylcadaverine; mRFP, monomeric red fluorescent protein; MTOR, mechanistic target of rapamycin; NVJ, nucleus-vacuole junction; RPS6KB, ribosomal protein S6 kinase, 70kDa, polypeptide 2; PAS, phagophore assembly site; PE, phosphatidylethanolamine; PMN, piecemeal microautophagy of the nucleus; PtdIns3K, phosphatidylinositol 3-kinase; PtdIns3K class III; Rluc, *Renilla reniformis* luciferase; SD, standard deviation; SOD, superoxide dismutase; TEM, transmission electron microscopy; tFLC3, tandem fluorescent LC3; TORC1, TOR complex I; TR-FRET, time-resolved fluorescence resonance energy transfer; UPR, unfolded protein response

In 2008 we published the first set of guidelines for standardizing research in autophagy. Since then, research on this topic has continued to accelerate, and many new scientists have entered the field. Our knowledge base and relevant new technologies have also been expanding. Accordingly, it is important to update these guidelines for monitoring autophagy in different organisms. Various reviews have described the range of assays that have been used for this purpose. Nevertheless, there continues to be confusion regarding acceptable methods to measure autophagy, especially in multicellular eukaryotes. A key point that needs to be emphasized is that there is a difference between measurements that monitor the numbers or volume of autophagic elements (e.g., autophagosomes or autolysosomes) at any stage of the autophagic process vs. those that measure flux through the autophagy pathway (i.e., the complete process); thus, a block in macroautophagy that results in autophagosome accumulation needs to be differentiated from stimuli that result in increased autophagic activity, defined as increased autophagy induction coupled with increased delivery to, and degradation within, lysosomes (in most higher eukaryotes and some protists such as *Dictyostelium*) or the vacuole (in plants and fungi). In other words, it is especially important that investigators new to the field understand that the appearance of more autophagosomes does not necessarily equate with more autophagy. In fact, in many cases, autophagosomes accumulate because of a block in trafficking to lysosomes without a concomitant change in autophagosome biogenesis, whereas an increase in autolysosomes may reflect a reduction in degradative activity. Here, we present a set of guidelines for the selection and interpretation of methods for use by investigators who aim to examine macroautophagy and related processes, as well as for reviewers who need to provide realistic and reasonable critiques of papers that are focused on these processes. These guidelines are not meant to be a formulaic set of rules, because the appropriate assays depend in part on the question being asked and the system being used. In addition, we emphasize that no individual assay is guaranteed to be the most appropriate one in every situation, and we strongly recommend the use of multiple assays to monitor autophagy. In these guidelines, we consider these various methods of assessing autophagy and what information can, or cannot, be obtained from them. Finally, by discussing the merits and limits of particular autophagy assays, we hope to encourage technical innovation in the field.

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## Introduction

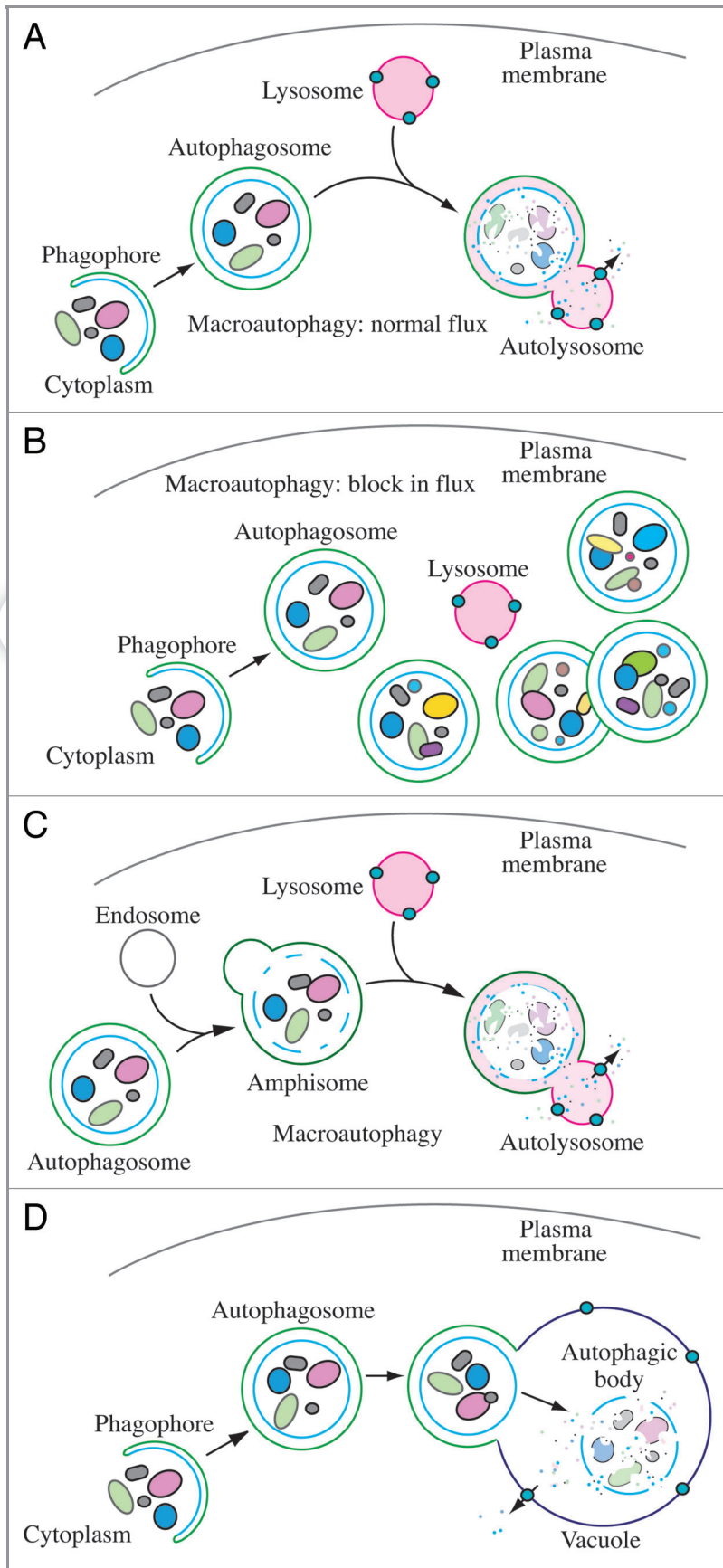
Many researchers, especially those new to the field, need to determine which criteria are essential for demonstrating autophagy, either for the purposes of their own research, or in the capacity of a manuscript or grant review.<sup>1</sup> This is an important issue, particularly considering that each of us may have his/her own opinion regarding the answer. Unfortunately, the answer is in part a “moving target” as the field evolves,<sup>2</sup> and this can be extremely frustrating for researchers who may think they have met those criteria, only to find out that the reviewers of their paper have different ideas. Conversely, as a reviewer, it is tiresome to raise the same objections repeatedly, wondering why researchers have not fulfilled some of the basic requirements for establishing the occurrence of an autophagic process. In addition, drugs that potentially modulate autophagy are increasingly being used in clinical trials, and screens are being performed for new drugs that can modulate autophagy for therapeutic purposes. Clearly it is important to determine whether these drugs are truly affecting autophagy based on a set of accepted criteria. Accordingly, we describe here a basic set of contemporary guidelines that can be used by researchers to plan and interpret their experiments, by clinicians to evaluate the literature with regard to autophagy-modulating therapies, and by both authors and reviewers to justify or criticize an experimental approach.

Several fundamental points must be kept in mind as we establish guidelines for the selection of appropriate methods to monitor autophagy.<sup>1</sup> Importantly, there are no absolute criteria for determining autophagic status that are applicable in every biological or experimental context. This is because some assays are inappropriate, problematic or may not work at all in particular cells, tissues or organisms.<sup>3-6</sup> In addition, these guidelines are likely to evolve as new methodologies are developed and current assays are superseded. Nonetheless, it is useful to establish guidelines for acceptable assays that can reliably monitor autophagy in many experimental systems. It is important to

note that in this set of guidelines the term “autophagy” generally refers to macroautophagy; other autophagy-related processes are specifically designated when appropriate.

For the purposes of this review, the autophagic compartments (Fig. 1) are referred to as the sequestering (pre-autophagosomal) phagophore (previously called the isolation or sequestration membrane<sup>7,8</sup>),<sup>9</sup> the autophagosome,<sup>10</sup> the amphisome (generated by the fusion of autophagosomes with endosomes, also referred to as an acidic late autophagosome<sup>11</sup>),<sup>12</sup> the autolysosome (generated by fusion of autophagosomes or amphisomes with a lysosome), and the autophagic body (generated by fusion and release of the internal autophagosomal compartment into the vacuole in fungi and plants; autophagic bodies are not formed within lysosomes/autolysosomes because these lytic organelles are typically smaller than autophagosomes<sup>13</sup>).<sup>8,10</sup> One critical point is that autophagy is a highly dynamic, multi-step process. Like other cellular pathways, it can be modulated at several steps, both positively and negatively. An accumulation of autophagosomes [measured by transmission electron microscopy (TEM) image analysis, as fluorescent GFP-MAP1LC3 (GFP-LC3) dots, or as LC3 lipidation on a western blot], could, for example, reflect induction of autophagy, reduction in autophagosome turnover,<sup>14-16</sup> or the inability of turnover to keep pace with increased autophagosome formation (Fig. 1).<sup>17</sup> For example, inefficient fusion with endosomes and/or lysosomes, respectively, or perturbation of the transport machinery,<sup>18</sup> would inhibit autophagosome maturation to amphisomes or autolysosomes, whereas decreased flux could also be due to inefficient degradation of the cargo once fusion has occurred.<sup>19</sup>

Accordingly, the use of autophagy markers such as LC3-II needs to be complemented by assays to estimate overall autophagic flux, or flow, to permit a correct interpretation of the results. That is, autophagic activity includes not just the increased synthesis or lipidation of Atg8/LC3 (LC3 is a mammalian homolog of yeast Atg8), or an increase in the formation of autophagosomes, but, most importantly, flux through the entire



**Figure 1.** Schematic model demonstrating the induction of autophagosome formation when turnover is blocked vs. normal autophagic flux, and illustrating the morphological intermediates of macroautophagy. (A) The initiation of autophagy includes the formation of the phagophore, the initial sequestering compartment, which expands into an autophagosome. Completion of the autophagosome is followed by fusion with lysosomes and degradation of the contents, allowing complete flux, or flow, through the entire pathway. This is a different outcome than the situation shown in (B) where induction results in the initiation of autophagy, but a defect in autophagosome turnover due, for example, to a block in fusion with lysosomes or disruption of lysosomal functions will result in an increased number of autophagosomes. In this scenario, autophagy has been induced, but there is no or limited autophagic flux. (C) An autophagosome can fuse with an endosome to generate an amphisome, prior to fusion with the lysosome. (D) Schematic drawing showing the formation of an autophagic body in plants and fungi. The large size of the plant and fungal vacuole relative to autophagosomes allows the release of the single-membrane autophagic body within the vacuole lumen. In cells that lack vacuolar hydrolase activity, or in the presence of inhibitors that block hydrolase activity, intact autophagic bodies accumulate within the vacuole lumen and can be detected by light microscopy. The lysosome of most higher eukaryotes is too small to allow the release of an autophagic body.

system, including lysosomes or the vacuole, and the subsequent release of the breakdown products. Therefore, autophagic substrates need to be monitored dynamically over time to verify that they have reached the lysosome/vacuole, and, when appropriate, are degraded. By responding to perturbations in the extracellular environment, cells tune autophagic flux to meet intracellular metabolic demands. The impact of autophagic flux on cell death and human pathologies therefore demands accurate tools to measure not only the current flux of the system, but also its capacity,<sup>20</sup> and its response time, when exposed to a defined insult.<sup>21</sup>

One approach is to measure the rate of general protein breakdown by autophagy.<sup>8,22</sup> Alternatively, it is possible to arrest the autophagic flux at a given point, and then record the time-dependent accumulation of an organelle, an organelle marker, a cargo marker or the entire cargo at the point of blockage; however, the latter assumes there is no feedback of the accumulating structure on its own rate of formation.<sup>23</sup> Along the same lines, one can follow the time-dependent decrease of an autophagy-degradable marker (with the caveat that the potential contribution of other proteolytic systems needs to be experimentally addressed). In theory, this can be achieved by blocking autophagic sequestration at specific steps of the pathway (e.g., blocking further

induction or nucleation of new phagophores) and by measuring the decrease of markers distal to the block point.<sup>14,16,24</sup> The key issue is to differentiate between the often transient accumulation of autophagosomes due to increased induction, from accumulation due to inefficient completion of autophagy, by measuring both the levels of autophagosomes at static time points and by addressing changes in the rates of autophagic degradation of cellular components.<sup>19</sup> Both processes have been used to estimate “autophagy,” but unless the experiments can relate changes in autophagosome numbers to a direct or indirect measurement for autophagic flux, they may be difficult to interpret.<sup>25</sup> A general caution regarding the use of the term “steady state” is warranted at this point. It should not be assumed that an autophagic system is at steady-state in the strict biochemical meaning of this term, as this implies that the level of autophagosomes does not change with time, and the flux through the system is constant. In these guidelines, we use steady-state to refer to the baseline range of autophagic flux in a system that is not subjected to specific perturbations that increase or decrease that flux.

Autophagic flux refers to the entire process of autophagy including the delivery of cargo to lysosomes (via fusion of the latter with autophagosomes or amphisomes) and its subsequent breakdown and release of the resulting macromolecules back into the cytosol (this may be referred to as productive or complete autophagy). Thus, increases in the level of phosphatidylethanolamine (PE)-modified Atg8/LC3 (Atg8-PE/LC3-II), or even the appearance of autophagosomes are not measures of autophagic flux per se, but can reflect the induction of autophagic sequestration and/or inhibition of autophagosome or amphisome clearance. Also, it is important to realize that while formation of Atg8-PE/LC3-II appears to correlate with the induction of autophagy, we do not know, at present, the actual mechanistic relationship between Atg8-PE/LC3-II formation and the rest of the autophagic process; indeed, it may be possible to execute “self-eating” in the absence of LC3-II.<sup>26</sup> As a final note, we also recommend that researchers refrain from the use of the expression “percent autophagy” when describing experimental results, as in “The cells displayed a 25% increase in autophagy.” In contrast, it is appropriate to indicate that the average number of GFP-Atg8/LC3 puncta per cell is increased or a certain percentage of cells display punctate GFP-Atg8/LC3 that exceeds a particular threshold (and this threshold should be clearly defined in the methods), or that there is a particular increase or decrease in the rate of degradation of long-lived proteins, as these are the actual measurements being quantified.

In the previous version of these guidelines,<sup>1</sup> the methods were separated into two main sections—steady-state and flux. In some instances, a lack of clear distinction between the actual methodologies and their potential uses made such a separation somewhat artificial. For example, fluorescence microscopy was initially listed as a steady-state method, although this approach can clearly be used to monitor flux as described in this article, especially when considering the increasing availability of new technologies such as microfluidics. Furthermore, the use of multiple time points and/or lysosomal fusion/degradation inhibitors can turn even a typically static method such as TEM into one that monitors flux.

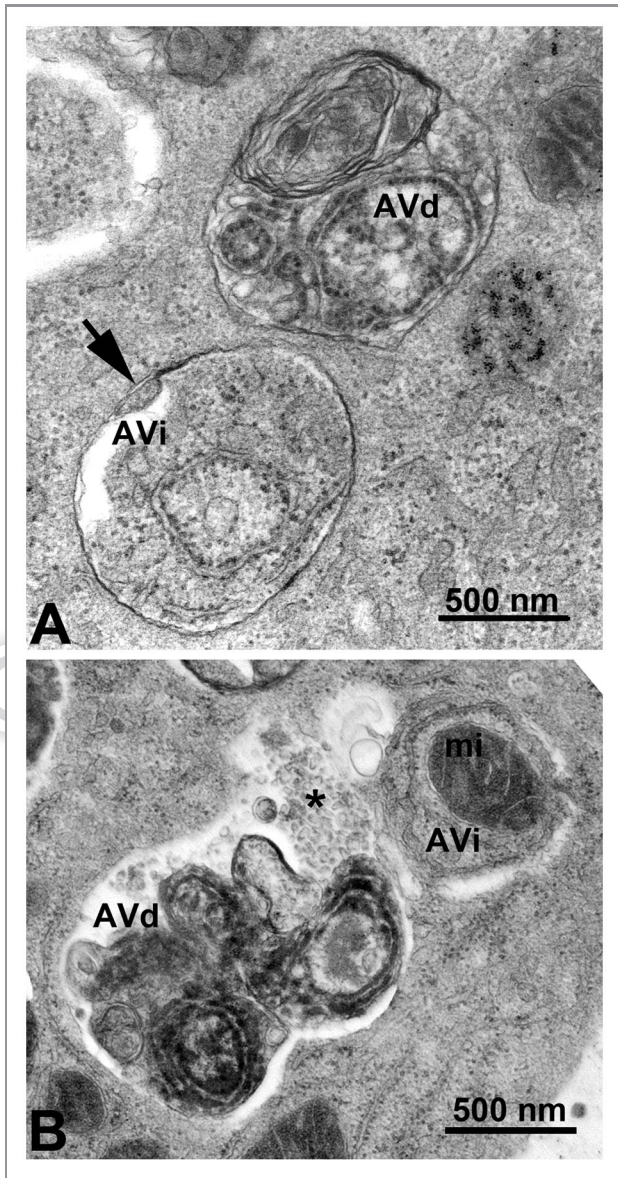
Therefore, although we maintain the importance of monitoring autophagic flux and not just induction, this revised set of guidelines does not separate the methods based on this criterion. Readers should be aware that this article is not meant to present protocols, but rather guidelines, including information that is typically not presented in protocol papers. For detailed information on experimental procedures we refer readers to various protocols that have been published elsewhere.<sup>27-42</sup>

Collectively, we propose the following guidelines for measuring various aspects of selective and nonselective autophagy in eukaryotes.

## A. Methods for Monitoring Autophagy

**1. Transmission electron microscopy.** Autophagy was first detected by TEM in the 1950s (reviewed in ref. 8). The focal degradation of cytoplasmic areas sequestered by the phagophore, which matures into the autophagosome, is the morphological hallmark of autophagy. TEM can be used to monitor both selective and nonselective autophagy. In the case of selective autophagy, the cargo should correspond to the specific substrate being targeted for sequestration—bulk cytoplasm is essentially excluded. In contrast, during nonselective autophagy, the content of the autophagosome is morphologically identical to the cytoplasm, containing similar densities of ribosomes, and intact sequestered organelles are clearly identifiable. Therefore, the use of TEM is a valid and important method both for the qualitative and quantitative analysis of changes in various autophagic structures that sequentially form the phagophore, autophagosome, amphisome, autolysosome and autophagic body (Fig. 1).<sup>43</sup> The maturation from the phagophore through the autolysosome is a dynamic and continuous process,<sup>44</sup> and, thus, the classification of compartments into discrete morphological subsets can be problematic; however, some basic guidelines can be offered.

Autophagosomes (also referred to as initial autophagic vacuoles, AVi) have a double membrane that is usually at least partly visible as two parallel membrane bilayers separated by an electron-lucent cleft (Fig. 2A).<sup>45,46</sup> Autophagosomes contain cytosol and/or organelles that look morphologically intact, i.e., similar to the cytosol and organelles elsewhere in the cell.<sup>43,47</sup> Amphisomes<sup>48</sup> can sometimes be identified by the presence of small internal vesicles inside the autophagosome/autophagic vacuole (AV).<sup>49</sup> These internal vesicles are delivered into the lumen by fusion with multivesicular endosomes. Late/degradative autophagic vacuoles and autolysosomes (AVd) usually have only one limiting membrane, and contain cytoplasmic material and/or organelles at various stages of degradation (Fig. 2A and B).<sup>43,47</sup> It should be emphasized that not all vesicles containing electron-dense amorphous material are autolysosomes. The cytoplasmic origin of the contents must still be morphologically identifiable, if morphology is the only criterion that is being used for the identification of autolysosomes. For many biological and pathological situations, examination of both early and late autophagic structures yields valuable data regarding the overall autophagy/lysosomal status in the cells.<sup>17</sup> Along these lines, it is possible to use immunocytochemistry to follow particular cytosolic proteins



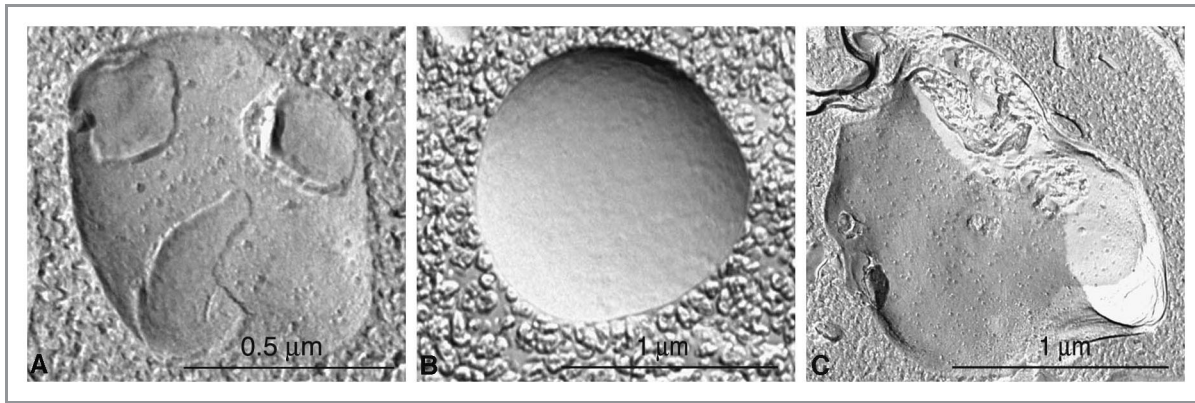
**Figure 2.** TEM images of autophagic vacuoles in isolated mouse hepatocytes. (A) One autophagosome or early autophagic vacuole (AVi) and one degradative autophagic vacuole (AVd) are shown. The AVi can be identified by its contents (morphologically intact cytoplasm, including ribosomes, and rough endoplasmic reticulum), and the limiting membrane that is partially visible as two bilayers separated by a narrow electron-lucent cleft, i. e., as a double membrane (arrow). The AVd can be identified by its contents, partially degraded, electron-dense rough endoplasmic reticulum. The vesicle next to the AVd is an endosomal/lysosomal structure containing 5-nm gold particles that were added to the culture medium to trace the endocytic pathway. (B) One AVi, containing rough endoplasmic reticulum and a mitochondrion, and one AVd, containing partially degraded rough endoplasmic reticulum, are shown. Note that the limiting membrane of the AVi is not clearly visible, possibly because it is tangentially sectioned. However, the electron-lucent cleft between the two limiting membranes is visible and helps in the identification of the AVi. The AVd contains a region filled by small internal vesicles (asterisk), indicating that the AVd has fused with a multivesicular endosome. mi, mitochondrion. Image provided by E.-L. Eskelinen.

such as CuZn superoxide dismutase (SOD) and carbonic anhydrase to determine the stage of autophagy; the former is much more resistant to lysosomal degradation.<sup>50</sup> In some autophagy-inducing conditions it is possible to observe multi-lamellar membrane structures in addition to the conventional double-membrane autophagosomes. The nature of these structures is not fully understood. They may indeed be multiple double layers of phagophores<sup>51</sup> and positive for LC3,<sup>52</sup> or mere artifacts of fixation.

TEM observations of platinum-carbon replicas obtained by the freeze fracture technique can also supply useful ultrastructural information on the autophagic process. In quickly frozen and fractured cells the fracture runs preferentially along the hydrophobic plane of the membranes, allowing characterization of the limiting membranes of the different types of autophagic vacuoles and visualization of their limited protein intramembrane particles (IMPs, or integral membrane proteins). Several studies have been performed using this technique on yeast,<sup>53</sup> as well as on mammalian cells or tissue, first on mouse exocrine pancreas,<sup>54</sup> then on mouse and rat liver,<sup>55,56</sup> mouse seminal vesicle epithelium<sup>24,51</sup> or cancer cell lines (e.g., breast cancer MDA-MB-231)<sup>57</sup> to investigate the various phases of autophagosome maturation, and to reveal useful details about the origin and evolution of their limiting membranes.<sup>2,8,58-60</sup>

The phagophore and the limiting membranes of autophagosomes contain few, or no detectable, IMPs (Fig. 3A and B), when compared with other cellular membranes and to the membranes of lysosomes. In subsequent stages of the autophagic process the fusion of the autophagosome with an endosome and a lysosome results in increased density of IMPs in the membrane of the formed autophagic compartments (amphisomes, autolysosomes; Fig. 3C).<sup>8,24,53-56,61,62</sup> Autolysosomes are generally delimited by a single membrane because, in addition to the engulfed material, the inner membrane is degraded by the lytic enzymes. Similarly, the limiting membrane of autophagic bodies in yeast and plants is also quickly broken down under normal conditions. Autophagic bodies can be stabilized, however, by the addition of phenylmethylsulfonylfluoride (PMSF) or genetically by the deletion of the yeast *PEP4* gene. Thus, another method to consider for monitoring autophagy in plants and yeast is to count autophagic bodies by TEM using at least two time points. The advantage of this approach is that it can provide accurate information on flux even when the autophagosomes are abnormally small.<sup>63,64</sup> Thus, although a high frequency of “abnormal” structures presents a challenge, TEM is still very helpful in analyzing autophagy.

**Cautionary notes:** Although TEM is one of the most widely used methodologies to monitor autophagy, it is also one of the most problematic due to misinterpretations mostly deriving from methodological artifacts.<sup>45,46,65,66</sup> Care in the choice of sample to be analyzed is critical to the success of TEM studies for autophagy. Whereas fixation of *in vitro* samples is relatively straightforward, fixation of excised tissues requires care to avoid sampling a nonrepresentative or uninformative section of tissue. For instance, if 95% of a tumor is necrotic, TEM analysis of the necrotic core may not be informative, and if the sampling is



**Figure 3.** Different autophagic vesicles observed after freeze fracturing in cultured osteosarcoma cells after treatment with the autophagy inducer voacamine.<sup>59</sup> (A) Early autophagosome delimited by a double membrane. (B) Inner monolayer of an autophagosome membrane lacking protein particles. (C) Autolysosome delimited by a single membrane rich in protein particles. In the cross-fractured portion (on the right) the profile of the single membrane and the inner digested material are easily visible. Images provided by S. Meschini, M. Condello and A. Giuseppe.

from the viable rim, this needs to be specified when reported. Ex vivo tissue should be fixed immediately and systematically across samples to avoid changes in autophagy that may occur simply due to elapsed time ex vivo. It is recommended that for tissue samples, perfusion fixation should be used when possible. For yeast, rapid freezing techniques such as high pressure freezing followed by freeze substitution (i.e., dehydration at low temperature) may be particularly useful.

Due to the high potential for sampling artifacts, careful selection of appropriate nonbiased methods of quantification and morphometric/stereological analyses is essential.<sup>67-69</sup> Data obtained simply by scoring for the presence or absence of autophagic vacuoles (autophagosomes, autolysosomes) in the section of a cell leads to unreliable results due to variability in cell areas, and autophagic vacuole profiles in the sections. It is more reliable to quantify autophagosome (and/or autolysosome) profiles per total cytoplasmic or cellular area in sections, which still includes an unaccounted variability in the profile size of the autophagic element. The best approach is to estimate the volume occupied by autophagic structures (as percent of cytoplasmic or cellular volume, using volumetric morphometry/stereology) in at least 20 cell profiles per sample (the number needed should be dictated by some form of power analysis, which indicates that the data are significant).<sup>20,65,68,70,71</sup> During quantification it is important to make sure that each imaged cell profile is captured and scored at the same magnification, and that every cell profile in the thin section has an equal probability to be included in the counting.

The accurate identification of the autophagosome is a prerequisite for a valid analysis. An additional complication, however, is that maturation of metazoan autophagosomes involves a transition from a double-membrane compartment to single-membrane structures (i.e., amphisomes and autolysosomes).<sup>72</sup> In addition, not all double-membrane structures are autophagosomes. Thus, double membranes cannot be relied upon as the sole means for the ultrastructural identification of autophagy-related structures, and it is important to employ expert analysis to avoid misinterpretation of micrographs.<sup>46,65,66</sup> In some cases,

it may be prudent to employ tomographic reconstructions of the TEM images to confirm that the autophagic compartments are spherical and are not being confused with endomembrane cisternae or damaged mitochondria with similar appearance in thin-sections (e.g., see ref. 73), but this is obviously a time-consuming approach requiring sophisticated equipment. In addition, interpretation of tomographic images can be problematic. For example, starvation-induced autophagosomes should contain cytoplasm (i.e., cytosol and possibly organelles), but autophagosome-related structures involved in specific types of autophagy should show the selective cytoplasmic target, but may be relatively devoid of cytoplasm. Such processes include selective peroxisome or mitochondria degradation (pexophagy or mitophagy, respectively),<sup>74,75</sup> targeted degradation of pathogenic microbes (xenophagy),<sup>76-81</sup> as well as the yeast biosynthetic cytoplasm-to-vacuole targeting (Cvt) pathway.<sup>82</sup> Furthermore, some pathogenic microbes express membrane-disrupting factors during infection (e.g., phospholipases) that disrupt the normal double-membrane architecture of autophagosomes.<sup>83</sup> It is not even clear if the sequestering compartments used for specific organelle degradation or xenophagy should be termed autophagosomes or if alternate terms such as pexophagosome,<sup>84</sup> mitophagosome and xenophagosome should be used, even though the membrane and mechanisms involved in their formation may be identical to those for starvation-induced autophagosomes; for example, the double-membrane vesicle of the Cvt pathway is referred to as a Cvt vesicle.

It can also be difficult to determine whether material present within a phagosomal structure reflects self-eating, or is from a heterophagic (consumption of components from outside the cell) process. A prominent example is related to apoptosis. Apoptotic bodies from neighboring cells are readily phagocytosed by surviving cells of the same tissue.<sup>85,86</sup> Immediately after phagocytic uptake of apoptotic bodies, phagosomes have double limiting membranes. The inner one is from the plasma membrane of the apoptotic body and the outer one is that of the phagocytizing cell. The early heterophagic vacuole formed in this way may

appear similar to an autophagosome or, in a later stage, an early autolysosome in that it contains recognizable cytoplasmic material. A major difference, however, is that the surrounding membranes are the thicker (plasma membrane type), rather than the thinner sequestration membrane type (9–10 nm, vs. 7–8 nm, respectively).<sup>66</sup> A good feature to distinguish between autophagosomes and double plasma membrane-bound structures is the lack of the distended empty space (characteristic for the sequestration membranes of autophagosomes) between the two membranes of the phagocytic vacuoles. In addition, engulfed apoptotic bodies usually have a larger average size than autophagosomes.<sup>87</sup> The problem of heterophagic elements interfering with the identification of autophagic ones is most prominent in cell types with particularly intense heterophagic activity (such as macrophages, and amoeboid or ciliate protists). Special attention has to be paid to this problem in cell cultures or *in vivo* treatments (e.g., with toxic or chemotherapeutic agents) causing extensive apoptosis.

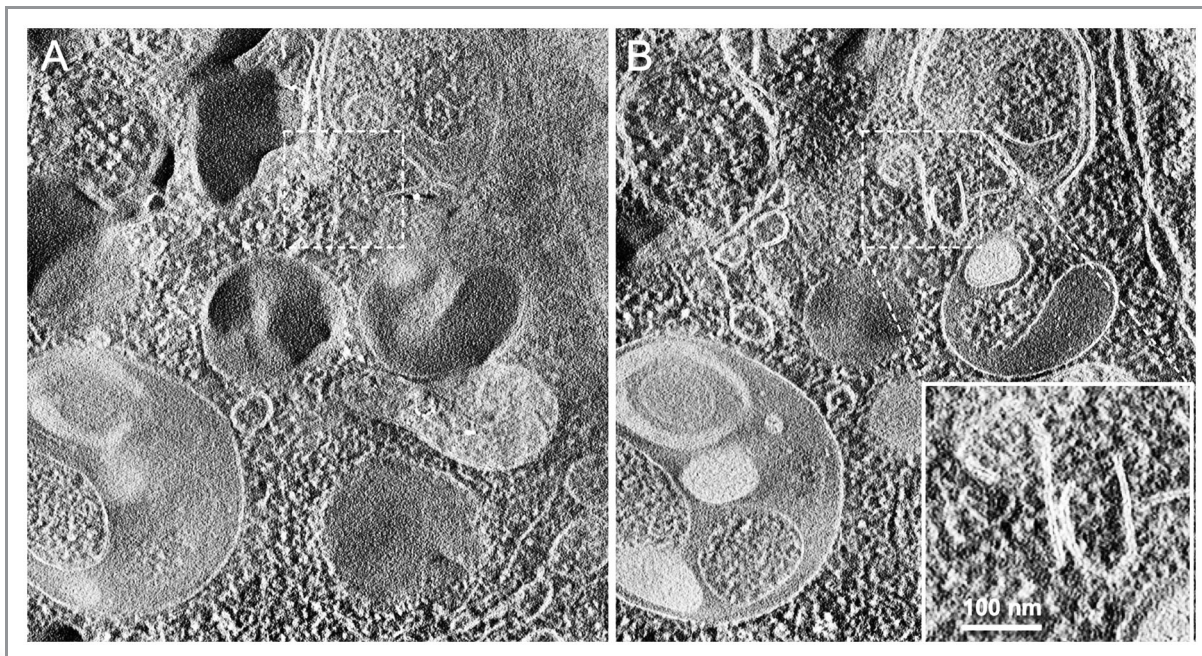
To decide about the lytic nature of a vacuolar compartment, demonstration of the presence of lysosomal enzymes by traditional (enzyme) cytochemistry or immunocytochemistry is also feasible for identifying post-fusion autophagic compartments. However, when heterophagy and autophagy are going on in parallel, the fusion of secondary lysosomes from both sources happens without distinction. The result will be a mixture of degradative products that may be derived both from heterophagy and autophagy, making it impossible to determine the proportion derived from a single process.

There are numerous structures in cells that resemble, or can be confused with, autophagic vesicles. The most common organelles are mitochondria, and ER, and also (depending on their structure) plastids in plants. Due to the cisternal structure of the ER, double membrane-like structures surrounding mitochondria or other organelles are often observed after sectioning,<sup>88</sup> but these can also correspond to cisternae of the ER coming into and out of the section plane.<sup>45</sup> If there are ribosomes associated with these membranes they can help distinguish them from the ribosome-free double-membrane of the phagophore and autophagosome. Observation of a mixture of early and late autophagosomes that is modulated by time point of collection and/or brief pulses of bafilomycin A<sub>1</sub> (a V-ATPase inhibitor) to trap the cargo in a recognizable early state<sup>41</sup> increases the confidence that an autophagic process is being observed. Criteria for distinguishing specialized structures such as melanosomes containing electron dense pigment from autophagosomes should be specified prior to scoring and included in a methods section when reporting the results. Considering that swollen mitochondria can encompass most of the internal structure of small autophagosomes, the possibility exists for scoring errors in every study. To minimize the impact of such errors, exact specification of autophagic elements must be applied. Efforts should be made to clarify the nature of questionable structures by extensive preliminary comparison in many test areas. Elements that still remain questionable should be categorized into special groups and measured separately. Should their later identification become possible, they can be added to the proper category or, if not, kept separate.

Uncertainties of identification and special features of the autophagic process may be clarified by immuno-TEM with gold-labeling,<sup>89,90</sup> using antibodies, for example, to cargo proteins of cytoplasmic origin and to LC3 to verify the autophagic nature of the compartment. Although labeling of LC3 can be difficult, good antibodies exist to visualize the GFP moiety of GFP-LC3 reporter constructs.<sup>91</sup> Antibodies against an abundant cytosolic protein will result in high background labeling; however, organelle markers work well. Because there are very few characterized proteins that remain associated with the completed structure, the choices for confirmation of its autophagic nature are limited. Furthermore, autophagosome-associated proteins may be cell type-specific. At any rate, the success of this methodology depends on the quality of the antibodies and also on the TEM preparation and fixation procedures utilized. With immuno-TEM, authors should provide controls showing that labeling is specific. This may require a quantification of staining over different cellular compartments.

In addition, statistical information should be provided due to the necessity of showing only a selective number of sections. Again, we note that for quantitative data it is preferable to use proper volumetric analysis rather than just counting numbers of sectioned objects. On the one hand, it must be kept in mind that even volumetric morphometry/stereology only shows either steady-state levels, or a snapshot in a changing dynamic process. Such data by themselves are not informative regarding autophagic flux, unless performed over multiple time points. Alternatively, investigation in the presence and absence of flux inhibitors can reveal the dynamic changes in various stages of the autophagic process.<sup>14,20,92,93</sup> For example, if the turnover of autolysosomes is very rapid, a low number/volume will not necessarily be an accurate reflection of low autophagic activity. On the other hand, quantitative analyses indicate that autophagosome volume in many cases does correlate with the rates of protein degradation.<sup>94-96</sup> One potential compromise is to perform whole cell quantification of autophagosomes using fluorescence methods, with qualitative verification by TEM,<sup>97</sup> to show that the changes in fluorescent puncta reflect increases in autophagic structures.

One additional caveat with TEM, and to some extent with confocal fluorescence microscopy, is that the analysis of a single plane within a cell can be misleading and may make the identification of autophagic structures difficult. Confocal microscopy and fluorescence microscopy with deconvolution software (or with much more work, 3-dimensional TEM) can be used to generate multiple/serial sections of the same cell to reduce this concern; however, in some cases where there is sufficient structural resolution, analysis of a single plane with multiple cells can suffice given practical limitations. Newer EM technologies, including focused ion beam dual-beam EM, should make it much easier to apply three-dimensional analyses. An additional methodology to assess autophagosome accumulation is correlative light and electron microscopy, CLEM, which is helpful in confirming that fluorescent structures are autophagosomes.<sup>98,99</sup> Along these lines, the new mini Singlet Oxygen Generator (miniSOG) fluorescent flavoprotein, which is less than half the



**Figure 4.** Cryoelectron microscopy can be used as a three-dimensional approach to monitor the autophagic process. Two computed sections of an electron tomogram of the autophagic vesicle-rich cytoplasm in a hemophagocyte of a semi-thin section after high-pressure freezing preparation. The dashed area is membrane-free (A) but tomography reveals newly formed phagophore-like membranes (B). Image published previously<sup>104</sup> and provided by M. Schneider and P. Walter.

size of GFP, provides an additional means to genetically tag proteins for CLEM analysis under conditions that are particularly suited to subsequent TEM analysis.<sup>100</sup> Combinatorial assays using tandem mRFP-GFP-LC3 (see *Tandem mRFP/mCherry-GFP fluorescence microscopy*) along with static TEM images should help in the analysis of flux and the visualization of cargo structures.<sup>101</sup> Another technique that has proven quite useful for analyzing the complex membrane structures that participate in autophagy is three-dimensional electron tomography,<sup>102,103</sup> and cryoelectron microscopy (Fig. 4). Finally, although an indirect measurement, a comparison of the ratio of autophagosomes to autolysosomes by TEM can support alterations in autophagy identified by other procedures.<sup>105</sup> In this case it is important to always compare samples to the control of the same cell type and in the same growth phase, as the ratio of autophagosome/autolysosome varies in a cell context-dependent fashion, depending on their clearance activity. It may also be necessary to distinguish autolysosomes from telolysosomes/late secondary lysosomes (the former are actively engaged in degradation, whereas the latter have reached an end point in the breakdown of luminal contents and are also referred to as residual bodies; see *Tissue fractionation*) because lysosome numbers generally increase when autophagy is induced.

With regard to immunohistochemistry using SOD1 as a marker to follow autophagy, it should be noted that a portion of the CuZn superoxide dismutase is also associated with various organelles, including the ER, mitochondria and peroxisomes. In addition, the wild-type SOD1 protein can be oxidized, and this form (SODox) is associated with sporadic cases of amyotrophic

lateral sclerosis.<sup>106</sup> Thus, the oxidized form of SOD1 might interfere with autophagy, and, if so, it may not be a good choice for a marker to monitor steady-state autophagic flux.

**Conclusion:** EM can be an extremely informative method for monitoring autophagy; however, it must be performed with extreme caution and rigor to avoid bias, and to ensure correct identification and quantification of autophagic compartments. With TEM, immunogold labeling is strongly recommended as it generally provides the most unequivocal results. Whenever possible, EM should not be the sole method used to monitor autophagy, but rather should be complemented by additional assays as described in this article.

**2. Atg8/LC3 detection and quantification.** Atg8/LC3 is the most widely monitored autophagy-related protein. In this section we describe multiple assays that utilize this protein, separating the descriptions into several subsections for ease of discussion.

*a. Western blotting and ubiquitin-like protein conjugation systems.* The Atg8/LC3 protein is a ubiquitin-like protein that can be conjugated to PE (and possibly to phosphatidylserine<sup>107</sup>). In yeast and several other organisms, the conjugated form is referred to as Atg8-PE. The mammalian homologs of Atg8 constitute a family of proteins subdivided in two subfamilies: LC3 (microtubule-associated protein 1 light chain 3) and GABARAP (GABA<sub>A</sub> receptor-associated protein). The former is comprised of LC3A, B, B2 and C, whereas the latter family includes GABARAP, GABARAPL1/Atg8L/GEC1 (GABA<sub>A</sub> receptor-associated protein like 1/Glandular Epithelial Cell 1), GABARAPL2/GATE-16/GEF2 (GABA<sub>A</sub> receptor-associated protein like 2/Golgi-associated ATPase enhancer of 16 kDa/ganglioside



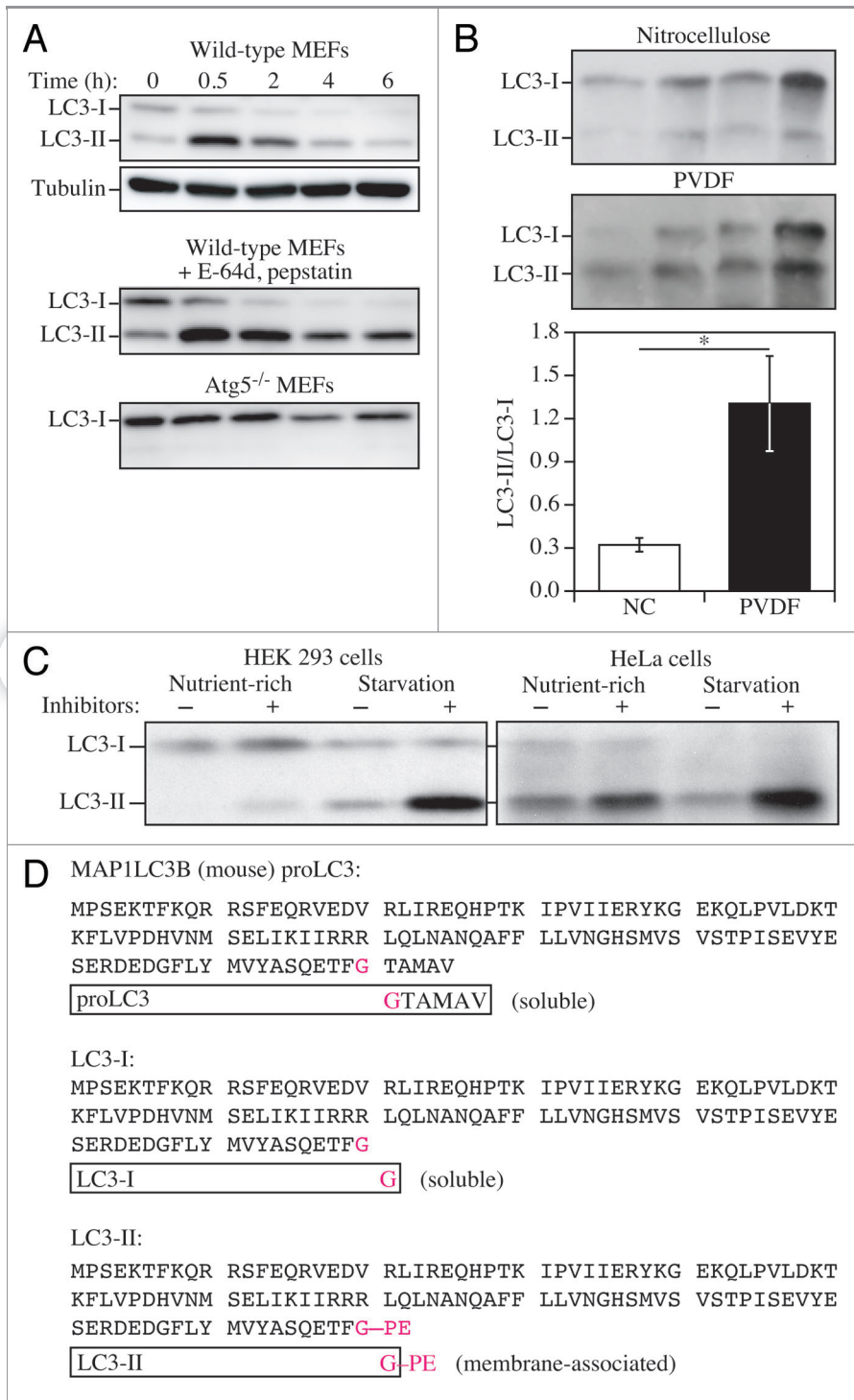
expression factor 2) and GABARAPL3 (GABA<sub>A</sub> receptor-associated protein like 3).<sup>108</sup> The nonlipidated and lipidated forms are usually referred to as LC3-I and LC3-II, or GABARAP and GABARAP-PE, etc. The positions of both Atg8/LC3-I (approximately 18 kDa) and Atg8-PE/LC3-II (approximately 16 kDa) should be indicated on western blots whenever both are detectable.

The mammalian Atg8 homologs share from 29% to 94% sequence identity and have all, apart from GABARAPL3, been demonstrated to be involved in autophagosome biogenesis.<sup>109</sup> The LC3 proteins are involved in phagophore formation, with participation of GABARAP subfamily members in later stages of autophagosome formation, in particular phagophore elongation and closure.<sup>110</sup> Nevertheless, in most published studies, LC3 has been the primary Atg8 homolog examined in mammalian cells and the one that is typically characterized as an autophagosome marker per se, making this factor the most relevant for this discussion (note that although this protein is referred to as “Atg8” in many other systems, for simplicity we primarily refer to it here as LC3 to distinguish it from the yeast protein). LC3, like the other Atg8 homologs, is initially synthesized in an unprocessed form, proLC3, which is converted into a proteolytically processed form lacking amino acids from the C terminus, LC3-I, and is finally modified into the PE-conjugated form, LC3-II (Fig. 5). Atg8-PE/LC3-II is the only protein marker that is reliably associated with completed autophagosomes, but is also localized to phagophores. In yeast, Atg8 amounts increase at least 10-fold when autophagy is induced.<sup>113</sup> In mammalian cells, however, the total levels of LC3 do not necessarily change in a predictable manner, as there may be increases in the conversion of LC3-I to LC3-II, or a decrease in LC3-II relative to LC3-I if degradation of LC3-II via lysosomal turnover is particularly rapid. Both of these events can be seen sequentially in several cell types as a response to total nutrient and serum starvation. In cells of neuronal origin a high ratio of LC3-I to LC3-II is a common finding.<sup>114</sup> For instance, SH-SY5Y neuroblastoma cell lines display only a slight increase of LC3-II after nutrient deprivation, whereas LC3-I is clearly reduced. This is likely related to a high basal autophagic flux, as suggested by the higher increase in LC3-II when cells are treated with NH<sub>4</sub>Cl,<sup>115,116</sup> although cell-specific differences in transcriptional regulation of LC3 may also play a role. The pattern of LC3-I to LC3-II conversion seems not only to be cell specific, but also related to the kind of stress to which cells are subjected. For example, the same SH-SY5Y cells display a strong increase of LC3-II when treated with the mitochondrial uncoupler CCCP, a well-known inducer of mitophagy. Thus, neither assessment of LC3-I consumption nor the evaluation of LC3-II levels would necessarily reveal a slight induction of autophagy (e.g., by rapamycin). Also, there is not always a clear precursor/product relationship between LC3-I and LC3-II, because the conversion of the former to the latter is cell type-specific and dependent on the treatment used to induce autophagy. Accumulation of LC3-II can be obtained by interrupting the autophagosome-lysosome fusion step (e.g., by depolymerizing acetylated microtubules with vinblastine, or by raising the lysosomal pH with the lysosomal proton pump inhibitor

bafilomycin A<sub>1</sub>) or by inhibiting lysosome-mediated proteolysis (e.g., with the cysteine protease inhibitor E-64d, the aspartic protease inhibitor pepstatin A, or chloroquine<sup>117</sup>). Western blotting can be used to monitor changes in LC3 amounts (Fig. 5);<sup>25,118</sup> however, even if the total amount of LC3 does increase, the magnitude of the response is generally less than that documented in yeast. It is worth noting that since the conjugated forms of the GABARAP subfamily members are usually undetectable without induction of autophagy in mammalian cells,<sup>119</sup> these proteins might be more suitable than LC3 to study and quantify subtle changes in the autophagic flux.

In most organisms, Atg8/LC3 is initially synthesized with a C-terminal extension that is removed by the Atg4 protease. Accordingly, it is possible to use this processing event to monitor Atg4 activity. For example, when GFP is fused at the C terminus of Atg8 (Atg8-GFP), the GFP moiety is removed in the cytosol to generate free Atg8 and GFP. This processing can be easily monitored by western blot.<sup>120</sup> It is also possible to use assays with an artificial fluorogenic substrate, or a fusion of LC3B to phospholipase A(2) that allows the release of the active phospholipase for a subsequent fluorogenic assay,<sup>121</sup> and there is a FRET-based assay utilizing CFP and YFP tagged versions of LC3B and GABARAPL2 that can be used for high-throughput screening.<sup>122</sup> Another method to monitor ATG4 activity *in vivo* uses the release of *Gussia* luciferase from the C terminus of LC3 that is tethered to actin.<sup>123</sup> Note that there are four Atg4 homologs in mammals, and they have different activities with regard to the Atg8 subfamilies of proteins.<sup>124</sup> ATG4A is able to cleave the GABARAP subfamily, but has very limited activity toward the LC3 subfamily, whereas ATG4B is apparently active against most or all of these proteins. The ATG4C and ATG4D isoforms have minimal activity for any of the Atg8 homologs. In particular because a C-terminal fusion will be cleaved immediately by Atg4, researchers should be careful to correctly specify whether they are using GFP-Atg8/LC3 (an N-terminal fusion, which can be used to monitor various steps of autophagy) or Atg8/LC3-GFP (a C-terminal fusion, which can only be used to monitor Atg4 activity).<sup>125</sup>

**Cautionary notes:** There are several important caveats to using Atg8/LC3-II or GABARAP-PE to visualize fluctuations in autophagy. First, changes in LC3-II amounts are tissue- and cell context-dependent.<sup>112,126</sup> Indeed, in some cases, autophagosome accumulation detected by TEM does not correlate well with the amount of LC3-II (Tallóczy Z, de Vries RLA, Sulzer D, unpublished results; Eskelinen E-L, unpublished results). This is particularly evident in those cells that show low levels of LC3-II (based on western blotting) because of an intense autophagy flux that consumes this protein,<sup>127</sup> or in cell lines having high levels of LC3-II that are tumor-derived, such as MDA-MB-231.<sup>112</sup> Conversely, the detectable formation of LC3-II is not sufficient evidence for autophagy, without careful quantification. For example, homozygous deletion of *Becn1* does not prevent the formation of LC3-II in embryonic stem cells even though autophagy is substantially reduced, whereas deletion of *Atg5* results in the complete absence of LC3-II (see Fig. 5A and Supplemental Data in ref. 128). The same is true for the generation of Atg8-PE in yeast in the absence of *ATG6*



**Figure 5.** LC3-I conversion and LC3-II turnover. (A) Expression levels of LC3-I and LC3-II during starvation. *Atg5*<sup>+/+</sup> (wild-type) and *Atg5*<sup>-/-</sup> MEFs were cultured in DMEM without amino acids and serum for the indicated times, and then subjected to immunoblot analysis using anti-LC3 antibody and anti-tubulin antibody. E-64d (10  $\mu$ g/ml) and pepstatin A (10  $\mu$ g/ml) were added to the medium where indicated. Positions of LC3-I and LC3-II are indicated. The inclusion of lysosomal protease inhibitors reveals that the apparent decrease in LC3-II is due to lysosomal degradation as easily seen by comparing samples with and without inhibitors at the same time points (the overall decrease seen in the presence of inhibitors may reflect decreasing effectiveness of the inhibitors over time). Monitoring autophagy by following steady-state amounts of LC3-II without including inhibitors in the analysis can result in an incorrect interpretation that autophagy is not taking place (due to the apparent absence of LC3-II). Conversely, if there are high levels of LC3-II but there is no change in the presence of inhibitors this may indicate that induction has occurred but that the final steps of autophagy are blocked, resulting in stabilization of this protein. This figure was modified from data previously published in reference 25 and is reproduced by permission of Landes Bioscience, copyright 2007. (B) Lysates of 4 human adipose tissue biopsies were resolved on two 12% polyacrylamide gels, as described previously.<sup>111</sup> Proteins were transferred in parallel to either a PVDF or a nitrocellulose membrane, and blotted with anti-LC3 antibody, and then identified by reacting the membranes with an HRP-conjugated anti-rabbit IgG antibody, followed by ECL. The LC3-II/LC3-I ratio was calculated based on densitometry analysis of both bands. \**p* < 0.05. (C) HEK 293 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 E-64d and pepstatin at 10  $\mu$ g/ml each (Inhibitors). Cells were then lysed and the proteins resolved by SDS-PAGE. Endogenous LC3 was detected by immunoblotting. Positions of LC3-I and LC3-II are indicated. In the absence of lysosomal protease inhibitors, starvation results in a modest increase (HEK 293 cells) or even a decrease (HeLa cells) in the amount of LC3-II. The use of inhibitors reveals that this apparent decrease is due to lysosome-dependent degradation. This figure was modified from data previously published in reference 112 and is reproduced by permission of Landes Bioscience, copyright 2005. (D) Sequence and schematic representation of the different forms of LC3B. The sequence for the nascent (proLC3) from mouse is shown. The glycine at position 120 indicates the cleavage site for ATG4. After this cleavage, the truncated LC3 is referred to as LC3-I, which is still a soluble form of the protein. Conjugation to PE generates the membrane-associated LC3-II (equivalent to Atg8-PE).

(see Fig. 7 in ref. 129). Thus, it is important to remember that not all of the autophagy-related proteins are required for Atg8/LC3 processing, including lipidation.<sup>129</sup> Vagaries in the detection and amounts of LC3-I vs. LC3-II present technical problems. For example, LC3-I is very abundant in brain tissue, and the intensity of the LC3-I band may obscure detection of LC3-II, unless the polyacrylamide crosslinking density is optimized. Conversely, certain cell lines have much less visible LC3-I compared with LC3-II. In addition, tissues may have asynchronous and heterogeneous cell populations, and this may present challenges when analyzing LC3 by western blotting.

Second, LC3-II also associates with the membranes of non-autophagic structures. For example, some members of the  $\gamma$ -protocadherin family undergo clustering to form intracellular tubules that emanate from lysosomes.<sup>130</sup> LC3-II is recruited to these tubules, and appears to promote or stabilize membrane expansion. Furthermore, LC3 can be recruited directly to bacteria-containing phagosome membranes under certain immune activating conditions, for example, TLR-mediated stimulation, in a process designated LC3-associated phagocytosis (LAP)<sup>131,132</sup> and also to apoptotic cell-containing phagosome membranes,<sup>133,134</sup> macropinosomes,<sup>133</sup> and to single-membrane entotic vacuoles.<sup>133</sup> TEM analysis of murine macrophage-like RAW 264.7 cells infected with *Burkholderia pseudomallei* reveals that intracellular bacteria colocalize with GFP-LC3 puncta. However, the TEM analysis further shows that bacteria are either free in the cytosol, or sequestered in single-membrane phagosomes rather than within canonical double-membrane autophagosomes.<sup>135</sup> Therefore, in studies of infection of mammalian cells by bacterial pathogens the identity of the LC3-II-labeled compartment as an autophagosome should be confirmed by a second method, such as TEM. It is also worth noting that autophagy induced in response to bacterial infection is not directed solely against the bacteria but can also be a response to remnants of the phagocytic membrane.<sup>136</sup> Similar cautions apply with regard to viral infection, as coronaviruses induce the formation of double-membrane vesicles that are coated with LC3-I, and this nonlipidated form of LC3 plays an autophagy-independent role in viral replication.<sup>137</sup> Along these lines, with herpes simplex virus infection, an LC3<sup>+</sup> autophagosome-like organelle derived from nuclear membranes is observed that contains viral proteins.<sup>138</sup>

Third, caution must be exercised in general when evaluating LC3 by western blotting, and appropriate standardization controls are necessary. For example, LC3-I may be less sensitive to detection by certain anti-LC3 antibodies. Moreover, LC3-I is more labile than LC3-II, being more sensitive to freezing-thawing and to degradation in SDS sample buffer, so fresh samples should be heated and assessed as soon as possible and should not be subjected to repeated freeze-thaw cycles. A general point to consider when examining transfected cells concerns the efficiency of transfection. A western blot will detect LC3 in the entire cell population, including those that are not transfected. Thus, if transfection efficiency is too low, it may be necessary to use methods, such as fluorescence microscopy, that allow autophagy to be monitored in single cells. The critical point is that the analysis of the gel shift of transfected LC3 or GFP-LC3 can be employed to follow LC3 lipidation only in readily transfected cells.<sup>139</sup>

When dealing with animal tissues, western blotting of LC3 should be performed on frozen biopsy samples homogenized in the presence of general protease inhibitors (Isidoro C, personal communication; see also *Human*).<sup>140</sup> Caveats regarding detection of LC3 by western blotting have been covered in a review.<sup>25</sup> For example, PVDF membranes may result in a stronger LC3-II retention than nitrocellulose membranes, possibly due to a higher affinity for hydrophobic proteins (Fig. 5B; Kovsan J, Rudich A,

personal communication), and Triton X-100 may not efficiently solubilize LC3-II in some systems.<sup>141</sup> Heating in the presence of 1% SDS, or analysis of membrane fractions,<sup>142</sup> may assist in the detection of this protein.

Another important issue concerns the quantification of changes in LC3-II. The previous version of these guidelines specifically stated that the levels of LC3-II should be compared with actin (and here we would modify this to include other appropriate “housekeeping” proteins) and not to that of LC3-I. As a general rule, this still holds true, but there are some exceptions. For example, in some cases actin levels decrease when autophagy is induced, so that it may be more appropriate to determine the ratio of LC3-II to LC3-I. Either method has its potential advantages and disadvantages. For example, if the amount of LC3-I is high relative to LC3-II (as in brain tissues, where the LC3-I signal can be overwhelming), it can be difficult to quantify the change in LC3-II relative to LC3-I. Conversely, by ignoring the level of LC3-I in favor of LC3-II the researcher may miss part of the overall picture of the cellular autophagic response.

Fourth, LC3 is expressed as four isoforms in mammalian cells, LC3A, LC3B, LC3B2 and LC3C,<sup>143,144</sup> which exhibit different tissue distributions, and it may be necessary to use different antisera or antibodies that distinguish among these isoforms. A point of caution along these lines is that the increase in LC3A-II vs. LC3B-II levels may not display equivalent changes in all organisms under autophagy-inducing conditions, and it should not be assumed that LC3B is the optimal protein to monitor.<sup>145</sup> This supports the important notion that the LC3 isoforms display different functions, but these are yet to be fully elucidated. The commercialized anti-LC3B antibodies also recognize LC3A, but do not recognize LC3C, which shares less sequence homology. It is important to note that LC3C possesses in its primary amino acid sequence the DYKD motif that is recognized with a high affinity by anti-FLAG antibodies. Thus, the standard anti-FLAG M2 antibody can detect and immunoprecipitate over-expressed LC3C, and caution has to be taken in experiments using FLAG-tagged proteins (Biard-Piechaczyk M, Espert L, personal communication).

In addition, it is important to keep in mind the presence of the other subfamily of Atg8 proteins, the GABARAP subfamily (see above).<sup>109,146</sup> Certain types of mitophagy induced by BNIP3L/NIX are highly dependent on GABARAP and less dependent on LC3 proteins.<sup>147</sup> Furthermore, commercial antibodies for GABARAPL1 also recognize GABARAP,<sup>108</sup> which might lead to misinterpretation of experiments, in particular those using immunohistochemical techniques. The problem with cross-reactivity of the anti-GABARAPL1 antibody can be overcome when analyzing these proteins by western blot because they can be resolved during SDS-PAGE using high concentration (15%) gels, with GABARAP migrating faster than GABARAPL1 (Boyer-Guittaut M, personal communication). We therefore advise caution in choosing antibodies for western blotting and immunofluorescence experiments and in interpreting results based on stated affinities of antibodies unless these have been clearly determined. As with any western blot, proper methods of quantification must be used, which are, unfortunately, often not

well disseminated, and the readers are referred to an excellent paper on this subject (see ref. 148). Unlike the other members of the GABARAP family, almost no information is available on GABARAPL3, perhaps because it is not yet possible to differentiate between GABARAPL1 and GABARAPL3 proteins, which have 94% identity. As stated by the laboratory that described the cloning of the human *GABARAPL1* and *GABARAPL3* genes,<sup>146</sup> their expression patterns are apparently identical. It is worth noting that *GABARAPL3* is the only gene of the *GABARAP* subfamily that seems to lack an ortholog in mice.<sup>146</sup> *GABARAPL3* might therefore be considered as a pseudogene without an intron that is derived from *GABARAPL1*. Hence, until new data are published, *GABARAPL3* should not be considered as the fourth member of the *GABARAP* family.

Fifth, in non-mammalian species, the discrimination of Atg8-PE from the nonlipidated form can be complicated by their nearly identical SDS-PAGE mobilities and the presence of multiple isoforms (e.g., there are 9 in Arabidopsis). In yeast, it is possible to resolve Atg8 (the nonlipidated form) from Atg8-PE by including 6 M urea in the SDS-PAGE separating gel,<sup>149</sup> or by using a 15% resolving gel without urea (Reggiori F, personal communication). Similarly, urea combined with prior treatment of the samples with (or without) phospholipase D (that will remove the PE moiety) can often resolve the ATG8 species in plants.<sup>150,151</sup> It is also possible to label cells with radioactive ethanolamine, followed by autoradiography to identify Atg8-PE, and a C-terminal peptide can be analyzed by mass spectrometry to identify the lipid modification at the terminal glycine residue. Furthermore, Atg8-PE aberrantly migrates faster than unconjugated Atg8 during SDS-PAGE, even though the former has a larger molecular mass. Special treatments are not needed for the separation of mammalian LC3-I from LC3-II.

Finally, we would like to point out that one general issue with regard to any assay is that it could introduce some type of stress—for example, mechanical stress due to lysis, temperature stress due to heating or cooling a sample, or oxidative stress on a microscope slide, which could lead to potential artifacts including the induction of autophagy.<sup>152</sup> This point is not intended to limit the use of any specific methodology, but rather to note that there are no perfect assays; special care should be taken with cells in suspension, however, as the stress resulting from centrifugation can induce autophagy. Therefore, it is important to verify that the positive (e.g., treatment with rapamycin, torin1 or other inducers) and negative (e.g., inhibitor treatment) controls behave as expected in any assays being utilized. Similarly, plasmid transfection or nucleofection can result in the potent induction of autophagy (based on increases in LC3-II or SQSTM1 degradation). In some cell types, the amount of autophagy induced by transfection of a control empty vector may be so high that it is virtually impossible to examine the effect of enforced gene expression on autophagy (Levine B, personal communication). This effect is generally not observed with siRNA transfection; however, it is an issue for plasmid shRNA transfection and for plasmid expression constructs. The use of endotoxin-free DNA reduces, but does not eliminate, this problem. Finally, the precise composition of media components

can have profound effects on basal autophagy levels and may need to be modified empirically depending on the cell lines being used.

**Conclusion:** Atg8/LC3 is often an excellent marker for autophagy; however, it must be kept in mind that there are multiple LC3 isoforms, there is a second family of mammalian Atg8-like proteins (GABARAPs), and antibody affinity (for LC3-I vs. LC3-II) and specificity (for example, for LC3A vs. LC3B) must be considered and/or determined.

*b. Turnover of LC3-II/Atg8-PE.* Autophagic flux can be measured by inferring LC3-II/Atg8-PE turnover by western blot (Fig. 5C)<sup>112</sup> in the presence and absence of lysosomal, or vacuolar, degradation. The relevant parameter in this assay is the difference in the amount of LC3-II in the presence and absence of saturating levels of inhibitors, which can be used to examine the transit of LC3-II through the autophagic pathway; if flux is occurring, the amount of LC3-II will be higher in the presence of the inhibitor.<sup>112</sup> Lysosomal degradation can be prevented through the use of protease inhibitors (e.g., pepstatin A and E-64d), compounds such as bafilomycin A<sub>1</sub>, chloroquine or NH<sub>4</sub>Cl that neutralize the lysosomal pH,<sup>18,114,153,154</sup> or by treatment with agents that block fusion of autophagosomes with lysosomes (note that bafilomycin A<sub>1</sub> will ultimately cause a fusion block as well as neutralize the pH<sup>155</sup>).<sup>156</sup> Alternatively, knocking down or knocking out lysosomal-associated membrane protein 2 (LAMP2) represents a genetic approach to block the fusion of autophagosomes and lysosomes [for example, inhibiting LAMP2 in myeloid leukemic cells results in a marked increase of GFP-LC3 dot formation and endogenous LC3-II protein compared with control cells upon autophagy induction during myeloid differentiation (Tschan MP, unpublished data)].<sup>157</sup> This approach, however, may be complicated by the compensatory upregulation of macroautophagy that occurs when chaperone-mediated autophagy is blocked,<sup>158</sup> unless the LAMP2B isoform is specifically knocked down.<sup>159</sup>

Generally, an increase in the levels of LC3-II observed with a particular treatment condition in the presence of bafilomycin A<sub>1</sub>, compared with the treatment alone, is indicative of some degree of flux through the system (i.e., compound/drug treatment plus bafilomycin A<sub>1</sub> should result in a higher amount of LC3-II than compound/drug treatment alone); however, a treatment condition increasing LC3-II on its own that has no difference in LC3-II in the presence of bafilomycin A<sub>1</sub> compared with treatment alone may suggest a block in autophagy at the terminal stages.<sup>6</sup> This procedure has been validated with several autophagy modulators.<sup>160</sup> With each of these techniques, it is essential to avoid assay saturation. The duration of the bafilomycin A<sub>1</sub> treatment needs to be relatively short (1–2 h) to allow comparisons of the amount of LC3 that is lysosomally degraded over a given time frame under one treatment condition to another treatment condition. Positive control experiments using treatment with known autophagy inducers, along with bafilomycin A<sub>1</sub> vs. vehicle, are important to demonstrate the utility of this approach in each experimental context. The same type of assay monitoring the turnover of Atg8-PE can be used to monitor flux in yeast, by comparing the amount of Atg8

present in a wild-type vs. a *pep4Δ* strain following autophagy induction.<sup>161</sup>

An additional methodology for monitoring autophagy relies on the observation that a subpopulation of LC3-II exists in a cytosolic form (LC3-IIs) in some cell types.<sup>162</sup> The amount of cytosolic LC3-IIs and the ratio between LC3-I and LC3-IIs appears to correlate with changes in autophagy and may provide a more accurate measure of autophagic flux than ratios based on the total level of LC3-II.<sup>162</sup> The validity of this method has been demonstrated by comparing autophagic proteolytic flux in rat hepatocytes and hepatoma cells. One advantage of this approach is that it does not require the presence of autophagic or lysosomal inhibitors to block the degradation of LC3-II.

Finally, autophagic flux can be monitored based on the turnover of LC3-II, by utilizing a luminescence-based assay. For example, a reporter assay based on the degradation of *Renilla reniformis* luciferase (Rluc)-LC3 fusion proteins is well suited for screening compounds affecting autophagic flux.<sup>163</sup> In this assay, Rluc is fused N-terminally to either wild-type LC3 (LC3wt) or a lipidation-deficient mutant of LC3 (G120A). Since Rluc-LC3wt, in contrast to Rluc-LC3-G120A, specifically associates with the autophagosomal membranes, Rluc-LC3wt is more sensitive to autophagic degradation. A change in autophagy-dependent LC3-turnover can thus be estimated by monitoring the change in the ratio of luciferase activities between the two cell populations expressing either Rluc-LC3wt or Rluc-LC3-G120A. In its simplest form, the Rluc-LC3-assay can be used to estimate autophagic flux at a single time point by defining the luciferase activities in cell extracts. Moreover, the use of a live cell luciferase substrate makes it possible to monitor changes in autophagic activity in living cells in real time. This method has been successfully used to identify positive and negative regulators of autophagy from cells treated with microRNA, siRNA and small molecule libraries.<sup>163-166</sup>

**Cautionary notes:** The main caveat regarding the measurement of LC3-IIs/LC3-I is that this method has only been tested in isolated rat hepatocytes and H4-II-E cells. Thus, it is not yet known whether it is generally applicable to other cell types, and a soluble form of LC3-II (i.e., LC3-IIs) is not observed in many standard cell types including HeLa, HEK 293 and PC12. In addition, the same concerns apply regarding detection of LC3-I by western blotting. It should be noted that the LC3-IIs/LC3-I ratio must be analyzed using the cytosolic fractions rather than the total homogenates. Furthermore, the same caveats mentioned above regarding the use of LC3 for qualitatively monitoring autophagy also apply to the use of this marker for evaluating flux.

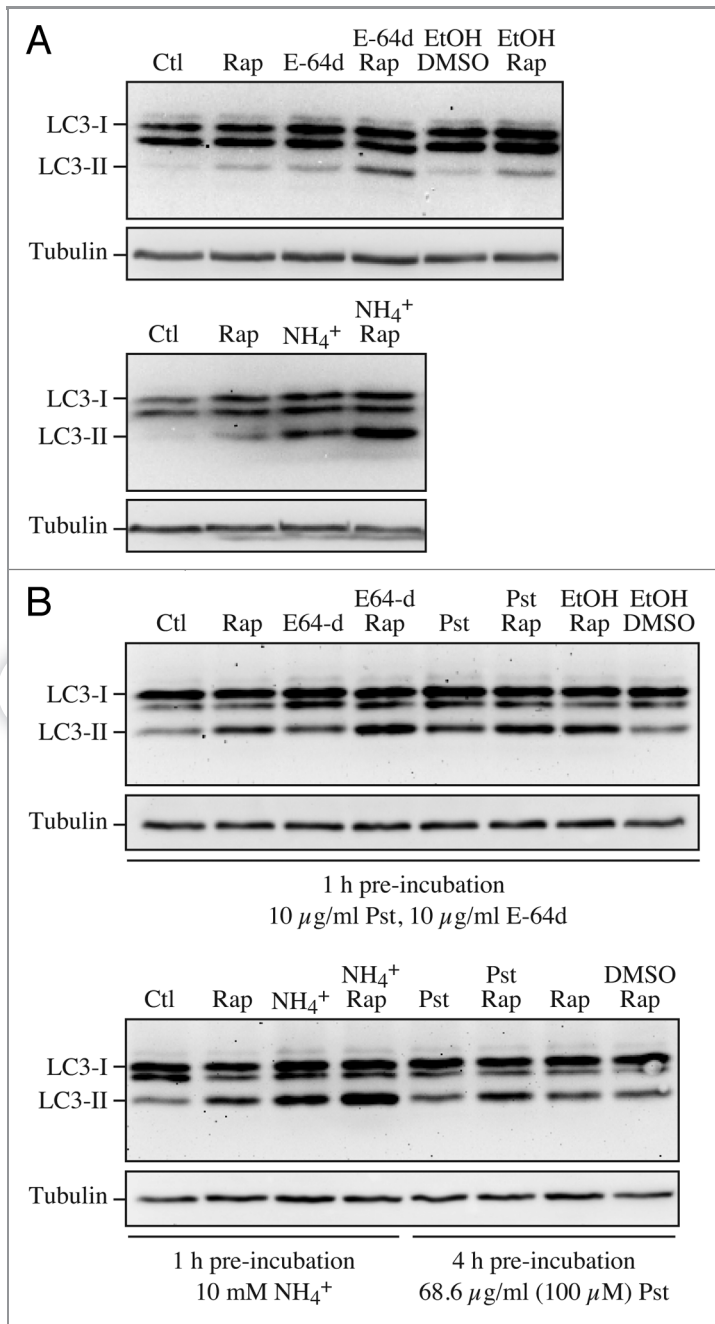
The use of a radioactive pulse-chase analysis provides an alternative to lysosomal protease inhibitors,<sup>113</sup> although such inhibitors should still be used to verify that degradation is lysosome-dependent. In addition, drugs must be used at concentrations and for time spans that are effective in inhibiting fusion or degradation, but that do not provoke cell death. Thus, these techniques may not be practical in all cell types or in tissues from whole organisms where the use of protease inhibitors is problematic, and where pulse labeling requires artificial short-term

culture conditions that may induce autophagy. Another concern when monitoring flux via LC3-II turnover may be seen in the case of a partial autophagy block; in this situation, agents that disrupt autophagy (e.g., bafilomycin A<sub>1</sub>) will still result in an increase in LC3-II, which may be interpreted as the complete absence of a block resulting from the mutant or compound being tested. Thus, care is needed in interpretation. Furthermore, for characterizing new autophagy modulators, it is ideal to test autophagic flux at early (e.g., 4 h) and late (e.g., 24 h) time-points, since in certain instances, such as with calcium phosphate precipitates, a compound may increase or decrease flux at these two time-points, respectively.<sup>167</sup> Finally, many of the chemicals used to inhibit autophagy, such as bafilomycin A<sub>1</sub>, NH<sub>4</sub>Cl (see *Autophagy inhibitors and inducers* below) or chloroquine, also directly inhibit the endocytosis/uncoating of viruses (Smith DR, personal communication), and other endocytic events requiring low pH, as well as exit from the Golgi (Tooze S, personal communication) and as such should be used only with extreme caution in studies investigating autophagy-virus interactions.

One additional consideration is that it may not be absolutely necessary to follow LC3-II turnover if other substrates are being monitored simultaneously. For example, an increase in LC3-II levels in combination with the lysosomal (or ideally autophagy-specific) removal of an autophagic substrate (such as an organelle<sup>168,169</sup>) that is not a good proteasomal substrate provides an independent assessment of autophagic flux. However, due to the fact that LC3 might be coupled to endosomal membranes and not just autophagosomes, and the levels of well-characterized autophagosome substrates such as SQSTM1 can also be affected by proteasome inhibitors,<sup>170</sup> it is probably prudent to monitor both turnover of LC3-II and an autophagosome substrate in parallel.

Another issue relates to the use of protease inhibitors (see *Autophagy inhibitors and inducers* below). When using lysosomal protease inhibitors, it is of fundamental importance to assess proper conditions of inhibitor concentration and time of pre-incubation to ensure full inhibition of lysosomal cathepsins. In this respect, 1 h of pre-incubation with 10 μg/ml E-64d is sufficient in most cases, since this inhibitor is membrane permeable and rapidly accumulates within lysosomes. On the other hand, pepstatin A is membrane impermeable (ethanol or preferably DMSO must be employed as a vehicle) and requires a prolonged incubation (> 8 h) and a relatively high concentration (> 50 μg/ml) to fully inhibit lysosomal cathepsin D (Fig. 6). An incubation of this duration, however, can be problematic due to indirect effects (see *GFP-Atg8/LC3 lysosomal delivery and proteolysis*). Also, note that the relative amount of lysosomal cathepsins B and D is cell-specific and changes with culture conditions. In contrast to the protease inhibitors, chloroquine (10 μM) or bafilomycin A<sub>1</sub> (1–100 nM) can be added to cells immediately prior to autophagy induction.

**Conclusion:** It is important to be aware of the difference between monitoring the steady-state level of Atg8/LC3 and autophagic flux; the latter can be determined by following Atg8/LC3 in the absence and presence of autophagy inhibitors, and/or by examining the autophagy-dependent degradation of appropriate



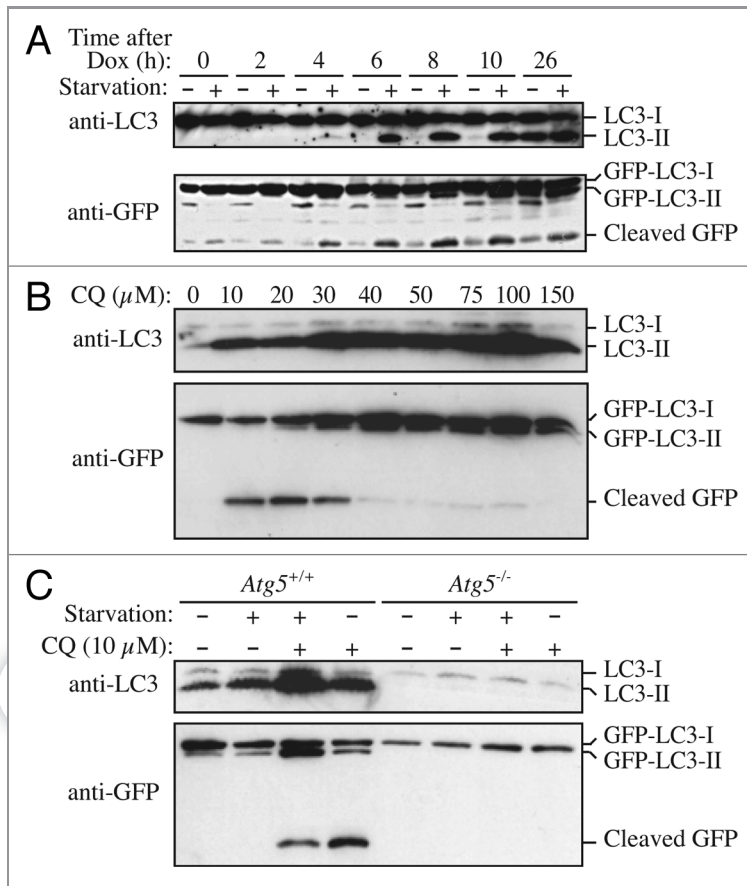
**Figure 6.** Effect of different inhibitors on LC3-II accumulation. SH-SY5Y human neuroblastoma cells were plated and allowed to adhere for a minimum of 24 h, then treated in fresh medium. Treatments were as follows: rapamycin (Rap), (A) 1 μM, 4 h or (B) 10 μM, 4 h; E-64d, final concentration 10 μg/ml from a 1 mg/ml stock in ethanol (EtOH); NH<sub>4</sub>Cl (NH<sub>4</sub><sup>+</sup>), final concentration 10 mM from a 1 M stock in water; pepstatin A (Pst), final concentration 10 μg/ml from a 1 mg/ml stock in ethanol, or 68.6 μg/ml from a 6.86 mg/ml stock in DMSO; ethanol or DMSO, final concentration 1%. Pre-incubations in (B) were for 1 or 4 h as indicated. 10 mM NH<sub>4</sub>Cl (or 30 μM chloroquine, not shown) were the most effective compounds for demonstrating the accumulation of LC3-II. E-64d was also effective in preventing the degradation of LC3-II, with or without a preincubation, but ammonium chloride (or chloroquine) may be more effective. Pepstatin A at 10 μg/ml with a 1 h pre-incubation was not effective at blocking degradation, whereas a 100 μM concentration with 4 h pre-incubation had a partial effect. Thus, alkalinizing compounds are more effective in blocking LC3-II degradation, and pepstatin A must be used at saturating conditions to have any noticeable effect. Images provided by C. Isidoro. Note that the band running just below LC3-I at approximately 17.5 kDa may be a processing intermediate of LC3-I; it is detectable in freshly prepared homogenates, but is less visible after the sample is subjected to a freeze-thaw cycle.

substrates. In particular, if there is any evidence of an increase in LC3-II (or autophagosomes), it is essential to determine whether this represents increased flux, or a block in fusion or degradation through the use of inhibitors such as chloroquine or bafilomycin A<sub>1</sub>.

*c. GFP-Atg8/LC3 lysosomal delivery and proteolysis.* GFP-LC3B (hereafter referred to as GFP-LC3) has also been used to follow flux. First, when GFP-Atg8 or GFP-LC3 is delivered to a lysosome/vacuole the Atg8/LC3 part of the chimera is sensitive to degradation, whereas the GFP protein is relatively resistant to hydrolysis (note, however, that GFP fluorescence is quenched by low pH; see *GFP-Atg8/LC3 fluorescence microscopy* and *Tandem mRFP/mCherry-GFP fluorescence microscopy*). Therefore, the

appearance of free GFP on western blots can be used to monitor lysis of the inner autophagosome membrane and breakdown of the cargo in metazoans (Fig. 7A),<sup>161,171,173</sup> or the delivery of autophagosomes to, and the breakdown of autophagic bodies within, the yeast and plant vacuole.<sup>151,161,174</sup> Reports on Dictyostelium and mammalian cells highlight the importance of lysosomal pH as a critical factor in the detection of free GFP that results from the degradation of fused proteins. In these cell types, free GFP fragments are only detectable in the presence of nonsaturating levels of lysosomotropic compounds (NH<sub>4</sub>Cl or chloroquine) or under conditions that attenuate lysosomal acidity; otherwise, the autophagic/degradative machinery appears to be too efficient to allow the accumulation of the proteolytic fragment (Fig. 7B and C).<sup>36,172</sup> Hence, a reduction in the intensity of the free GFP band may indicate reduced flux, but it may also be due to efficient turnover. Using a range of concentrations and treatment times of compounds that inhibit autophagy can be useful in distinguishing between these possibilities.<sup>175</sup> Since the pH in the yeast vacuole is higher than that in mammalian or Dictyostelium lysosomes, the levels of free GFP fragments are detectable in yeast without the necessity of using lysosomotropic compounds.<sup>29</sup> Additionally, in yeast the diffuse fluorescent haze from the released GFP moiety within the vacuole lumen can be observed by fluorescence microscopy.

The movement of GFP-LC3 to lysosomes also can be monitored by fluorescence microscopy, although, as mentioned above, the GFP fluorescent signal is more sensitive to acidic pH than other fluorophores (see *GFP-Atg8/LC3 fluorescence microscopy*). A time-course evaluation of the cell population showing GFP-LC3 puncta can serve to monitor the autophagy flux, since a constant increase in the number of cells accumulating GFP-LC3 puncta is suggestive of defective fusion of autophagosomes



**Figure 7.** GFP-LC3 processing can be used to monitor delivery of autophagosomal membranes. (A) *Atg5*<sup>-/-</sup> MEFs engineered to express *Atg5* under the control of the Tet-off promoter were grown in the presence of doxycycline (10 ng/ml) for one week to suppress autophagy. Cells were then cultured in the absence of drug for the indicated times, with or without a final 2 h starvation. Protein lysates were analyzed by western blot using anti-LC3 and anti-GFP antibodies. The positions of untagged and GFP-tagged LC3-I and LC3-II, and free GFP are indicated. This figure was modified from data previously published in reference 171, FEBS Letters, 580, Hosokawa N, Hara Y, Mizushima N, Generation of cell lines with tetracycline-regulated autophagy and a role for autophagy in controlling cell size, pp. 2623–2629, copyright 2006, with permission from Elsevier. (B) Differential role of unsaturating and saturating concentrations of lysosomal inhibitors on GFP-LC3 cleavage. HeLa cells stably transfected with GFP-LC3 were treated with various concentrations of chloroquine (CQ) for 6 h. Total lysates were prepared and subjected to immunoblot analysis. (C) CQ-induced free GFP fragments require classical autophagy machinery. Wild-type and *Atg5*<sup>-/-</sup> MEFs were first infected with adenovirus GFP-LC3 (100 viral particles per cell) for 24 h. The cells were then either cultured in regular culture medium with or without CQ (10  $\mu$ M), or subjected to starvation in EBSS buffer in the absence or presence of CQ for 6 h. Total lysates were prepared and subjected to immunoblot analysis. (B and C) are modified from the data previously published in reference 172.

with lysosomes, and conversely a decline implies that GFP-LC3 is consumed within newly formed autolysosomes. In either case, it can be problematic to use GFP fluorescence to follow flux, as new GFP-LC3 is continuously being synthesized. A potential solution to this problem for following fluorescence is to use a photoactivatable version of the fluorescent protein,<sup>176</sup> which allows this assay to be performed essentially as a pulse/chase analysis. Another alternative is to follow flux using GFP-LC3

fluorescence by adding lysosomal protease or fusion inhibitors to cells expressing GFP-LC3 and monitoring changes in the number of puncta. In this case, the presence of lysosomal inhibitors should increase the number of GFP-LC3-positive structures, and the absence of an effect on the total number of GFP-LC3 puncta or on the percentage of cells displaying numerous puncta is indicative of a defect(s) in autophagic flux.<sup>177</sup> The combination of protease inhibitors (to prevent the degradation of GFP) or compounds that modify lysosomal pH such as NH<sub>4</sub>Cl or chloroquine, or compounds such as bafilomycin A<sub>1</sub> or others that block fusion of autophagosomes with lysosomes (e.g., vinblastine) may be most effective in preventing lysosome-dependent decreases in GFP-LC3 puncta. However, because the stability of GFP is affected by lysosomal pH, we advise the use of protease inhibitors whether or not lysosomotropic compounds or fusion inhibitors are included (although lysosomotropic compounds should help stabilize GFP by neutralizing the pH in the lysosome, they do not have an immediate effect on lysosomal hydrolase activity).

**Cautionary notes:** The GFP-Atg8 processing assay is used routinely to monitor autophagy in yeast. One caveat, however, is that this assay is not always performed in a quantitative manner (for example, western blot exposures need to be in the linear range). Accordingly, an enzymatic assay such as the Pho8 $\Delta$ 60 assay may be preferred (see *Autophagic protein degradation*),<sup>178,179</sup> especially when the differences in autophagic activity need to be precisely determined; however, appropriate caution must be used as with any enzyme assay regarding, for example, substrate concentrations and linearity (note that an equivalent assay has not been developed for higher eukaryotic cells).

The main limitation of the GFP-LC3 processing assay in mammalian cells is that it seems to depend on cell type and culture conditions (Hosokawa N, Mizushima N, unpublished data). Apparently, GFP is more sensitive to mammalian lysosomal hydrolases than to the degradative milieu of the yeast vacuole. Alternatively, the lower pH of lysosomes relative to that of the vacuole may contribute to differences in detecting free GFP. Under certain conditions [such as Earle's balanced salt solution (EBSS)-induced starvation] in some cell lines, when the lysosomal pH becomes particularly low, free GFP is undetectable because both the LC3-II and free GFP fragments are quickly degraded.<sup>172</sup> Therefore, if this method is used it should be accompanied by immunoblotting including controls to address the stability of nonlysosomal GFP such as GFP-LC3-I. It should also be noted that free GFP can be detected when cells are treated with nonsaturating doses of lysosomal inhibitors such as chloroquine, E-64d and bafilomycin A<sub>1</sub>. The saturating concentrations of these lysosomal inhibitors vary in different cell lines, and it would be better to use a saturating concentration of lysosomal inhibitors when performing an autophagic flux assay.<sup>172</sup> Therefore, caution must be exercised in interpreting the data using this assay; it would be helpful to

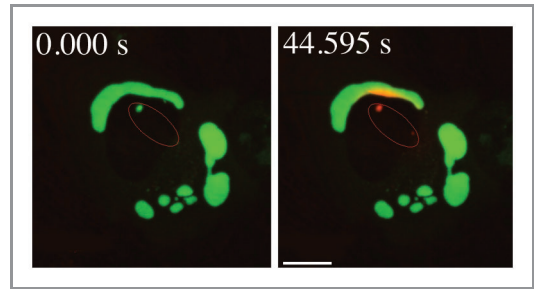
combine an analysis of GFP-LC3 processing with other assays such as the monitoring of endogenous LC3-II by western blot.

Along these lines, a caution concerning the use of the EGFP fluorescent protein for microscopy is that this fluorophore has a relatively neutral pH optimum for fluorescence,<sup>180</sup> so that its signal may diminish quickly during live cell imaging due to the acidic environment of the lysosome. It is possible to circumvent this latter problem by imaging paraformaldehyde-fixed cultures that are maintained in a neutral pH buffer, which retains EGFP fluorescence (Kleinman M, Reiners JJ, personal communication). Alternatively, it may be preferable to use a different fluorophore such as monomeric red fluorescent protein (mRFP) or mCherry, which retain fluorescence even at acidic pH.<sup>181</sup> On the one hand, a putative advantage of mCherry over mRFP is its enhanced photostability and intensity, which are an order of magnitude higher (and comparable to GFP), enabling acquisition of images at similar exposure settings as are used for GFP, thus minimizing potential bias in interpretation.<sup>182</sup> On the other hand, caution is required when evaluating the localization of mCherry fusion proteins during autophagy due to the persistence of the mCherry signal in acidic environments; all tagged proteins are prone to show enrichment in lysosomes during nonspecific autophagy of the cytoplasm, especially at higher expression levels. In addition, red fluorescent proteins (even the monomeric forms) can be toxic due to aggregation.<sup>183</sup> Dendra2 is an improved version of the green-to-red photoswitchable fluorescent protein Dendra, which is derived from the octocoral *Dendronephthya sp.*<sup>184</sup> Dendra2 is capable of irreversible photoconversion from a green to a red fluorescent form, but can be used also as a normal GFP or RFP vector. This modified version of the fluorophore has certain properties including a monomeric state, low phototoxic activation and efficient chromophore maturation, which make it suitable for real-time tracking of LC3 and SQSTM1 (Fig. 8; Kaarniranta K, personal communication). Another alternative to mRFP or mCherry is to use the Venus variant of YFP, which is brighter than mRFP and less sensitive to pH than GFP.<sup>186</sup>

The pH optimum of EGFP is important to consider when using GFP-LC3 constructs, as the original GFP-LC3 marker<sup>187</sup> uses the EGFP variant, which may result in a reduced signal upon the formation of amphisomes or autolysosomes. An additional caveat when using the photoactivatable construct PA-GFP<sup>180</sup> is that the process of activation by photons may induce DNA damage, which could, in turn, elicit induction of autophagy. Also, GFP is relatively resistant to denaturation, and boiling for 5 min may be needed to prevent the folded protein from being trapped in the stacking gel during SDS-PAGE.

As noted above (see *Western blotting and ubiquitin-like protein conjugation systems*), Atg4 cleaves the residue(s) that follow the C-terminal glycine of Atg8/LC3 that will be conjugated to PE. Accordingly, it is critical that any chimeras be constructed with the fluorescent tag at the N terminus of Atg8/LC3.

Finally, lysosomal inhibition needs to be carefully controlled. Prolonged inhibition of lysosomal hydrolases (> 6 h) is likely to induce a secondary autophagic response triggered by the accumulated undigested autophagy cargo. This secondary autophagic response can complicate the analysis of the autophagy flux,



**Figure 8.** Movement of activated pDendra2-hp62 (orange) from the nucleus (middle) to the aggregate in ARPE-19 cells, revealed by confocal microscopy. Cells were exposed to 5  $\mu$ M MG132 for 24 h to induce the formation of perinuclear aggregates.<sup>185</sup> The cells were then exposed to a UV pulse (the UV-induced area is shown by red lines that are inside of the nucleus) that converts Dendra2 from green to red, and the time shown after the pulse is indicated. SQSTM1/p62 is present in a small nuclear aggregate, and is shuttled from the nucleus to a perinuclear large protein aggregate (detected as red). Scale bar, 5  $\mu$ m. Image provided by K. Kaarniranta.

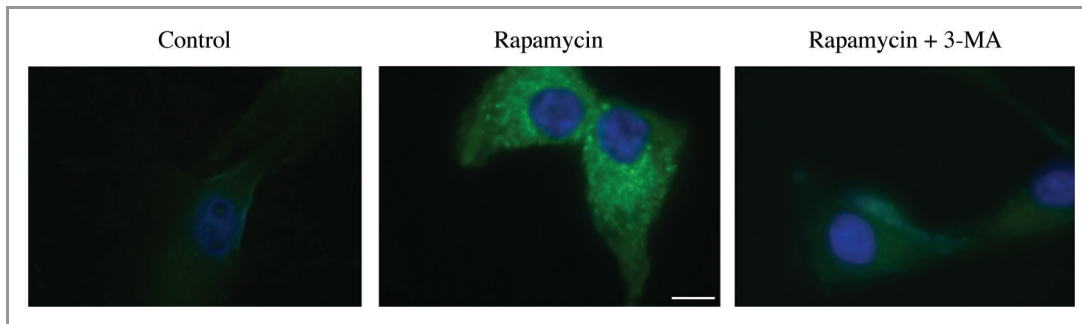
making it appear more vigorous than it would in the absence of the lysosomal inhibitors.

**Conclusion:** The GFP-Atg8/LC3 processing assay (the generation of free GFP within the vacuole/lysosome) is a convenient way to monitor autophagy, but it does not work in all cell types, and is not as easy to quantify as enzyme-based assays.

*d. GFP-Atg8/LC3 fluorescence microscopy.* LC3B, or the protein tagged at its N terminus with a fluorescent protein such as GFP (GFP-LC3), has been used to monitor autophagy through indirect immunofluorescence or direct fluorescence microscopy (Fig. 9), measured as an increase in punctate LC3 or GFP-LC3.<sup>187,188</sup> The detection of GFP-LC3/Atg8 is also useful for in vivo studies using transgenic organisms such as *Caenorhabditis elegans*,<sup>189</sup> *Dictyostelium discoideum*,<sup>190</sup> filamentous ascomycetes,<sup>191-195</sup> *Ciona intestinalis*,<sup>196</sup> *Drosophila melanogaster*,<sup>197-199</sup> *Arabidopsis thaliana*,<sup>200</sup> *Leishmania major*<sup>202,203</sup> and mice.<sup>126</sup> It is also possible to use anti-LC3/Atg8 antibodies for immunocytochemistry or immunohistochemistry,<sup>140,204-209</sup> procedures that have the advantages of detecting the endogenous protein, obviating the need for transfection and/or the generation of a transgenic organism, as well as avoiding potential artifacts resulting from overexpression (for example, high levels of overexpressed GFP-LC3 can result in its nuclear localization, although the protein can still relocate to the cytosol upon starvation), but we note that it is not always possible to detect endogenous Atg8/LC3. The use of imaging cytometry allows rapid and quantitative measures of the number of LC3 puncta and their relative number in individual or mixed cell types, using computerized assessment, enumeration, and data display (e.g., see refs. 142 and 210). In this respect, the alternative use of an automated counting system may be helpful for obtaining an objective number of puncta per cell. For this purpose, the WatershedCounting3D plug-in for ImageJ may be useful.<sup>211,212</sup>

Monitoring the endogenous protein, however, obviously depends on the ability to detect it in the system of interest. If the endogenous amount is below the level of detection, the use of





**Figure 9.** Changes in the detection and localization of GFP-LC3 upon the induction of autophagy. U87 cells stably expressing GFP-LC3 were treated with PBS, rapamycin (200 nM), or rapamycin in combination with 3-MA (2 mM) for 24 h. Representative fluorescence images of cells counterstained with DAPI (nuclei) are shown. Scale bar, 10  $\mu$ m. This figure was modified from Figure 6 published in Badr et al. Lanatoside C sensitizes glioblastoma cells to tumor necrosis factor-related apoptosis-inducing ligand and induces an alternative cell death pathway. *Neuro-Oncology* 2011, 13:1213–24, by permission of Oxford University Press.

an exogenous construct is warranted. In this case, it is important to consider the use of stable transformants vs. transient transfections. On the one hand, stable transformants may have reduced background resulting from the lower gene expression, and there is also the advantage of eliminating artifacts resulting from recent exposure to transfection reagents (see below). Furthermore, with stable transformants more cells can be easily analyzed because nearly 100% of the population will express tagged LC3. On the other hand, one disadvantage of stable transfectants is that the integration sites cannot always be predicted, and expression levels may not be optimal. Therefore, it is worth considering the use of stable episomal plasmids that avoid the problem of unsuitable integration.<sup>181</sup> An important advantage of transient transfection is that this approach is better for examining the immediate effects of the transfected protein on autophagy, although it restricts the length of time that the analysis can be performed, and consideration must be given to the induction of autophagy resulting from exposure to the transfection reagents (see below). In addition, a double transfection can be used (e.g., with GFP-LC3 and the protein of interest) to visually tag the cells that express the protein being examined. In conclusion, there is no simple rule for the use of stable vs. transient transfections. When stable transfections are utilized, it is worthwhile screening for clones that give the best signal-to-noise ratio, and when transient transfections are used, it is worthwhile optimizing the GFP-LC3 DNA concentration to give the best signal-to-noise ratio. In clones, the uniformity of expression of GFP-LC3 also makes “thresholding” when scoring puncta-positive cells (see below) much easier. However, there is also a need to beware of the frequent unrepresentative behavior of mammalian cell lines when selecting a single cell clone from a parental pool; therefore, it may be better to use a pool of multiple selected clones to avoid possible artifacts that can arise from the selection and propagation of individual clones from a single transfected cell (although the use of a pool is also problematic as its composition will change over time). Optimization, together with including the appropriate controls, will help overcome the effects of the inherent variability in these analyses.

An additional use of GFP-LC3 is to monitor colocalization with a target during autophagy-related processes such as organelle

degradation or the sequestration of pathogenic microbes.<sup>213–215</sup> Preincubation of cells stably expressing GFP-LC3 with leupeptin can help stabilize the GFP-LC3 signal during fluorescence microscopy, especially under conditions of induced autophagic flux. Leupeptin is an inhibitor of lysosomal cysteine and serine proteases and will therefore inhibit degradation of membrane-conjugated GFP-LC3 that is present within autolysosomes.

Cautionary notes: Quantification of autophagy by measuring GFP-LC3 puncta (or LC3 by immunofluorescence) can be more tedious, depending on the method used (e.g., high-throughput image analysis can obviously be rapid and efficient), than monitoring LC3-II by western blot; however, the former may be more sensitive and quantitative. Ideally, it is preferable to include both assays and to compare the two sets of results. In addition, if GFP-LC3 is being quantified, it is better to determine the number of puncta corresponding to GFP-LC3 on a per cell basis (or per cell area basis if the cell population is in culture or the cell composition of the tissue is not homogenous, as well as in the case of plant cells, which tend to grow) rather than simply the total number of cells displaying puncta. This latter point is critical because even in nutrient-rich conditions, cells display some basal level of GFP-LC3 puncta, unless they are lacking autophagy-related genes (and even in the latter case it is possible to get puncta of GFP-LC3 depending on the specific conditions). There are, however, practical issues with counting puncta manually and reliably, especially if there are large numbers per cell [although this may be more accurate than relying on a software program, in which case it is important to ensure that only appropriate dots are being counted; applicable programs include ImageJ, Imaris, which may be more accurate (Ktistakis NT, personal communication), and the open-source software CellProfiler<sup>216</sup>]. Moreover, when autophagosome-lysosome fusion is blocked, larger autophagosomes are detected, possibly due to autophagosome-autophagosome fusion. Although it is possible to detect changes in the size of GFP-Atg8/LC3 puncta by fluorescence microscopy, it is not possible to correlate size with autophagy activity without additional assay methods. Size determinations can be problematic by fluorescence microscopy unless careful standardization is performed,<sup>217</sup> and size estimation is not

recommended as a method for monitoring autophagy; however, it is possible to quantify the fluorescence intensity of GFP-Atg8/LC3 at specific puncta, which does provide a valid measure of protein recruitment.<sup>218</sup>

In addition to autophagosome size, the number of puncta visible to the eye will also be influenced by both the level of expression of GFP-LC3 in a given cell and by the exposure time of the microscope, if using widefield microscopy. In many cell types it may be possible to establish a cut-off value for the number of puncta per cell in conditions of “low” and “high” autophagy.<sup>219</sup> This can be tested empirically by exposing cells to autophagy-inducing and -blocking agents. Thus, cell populations showing significantly greater proportions of cells with autophagosome numbers higher than the cut-off in perturbation conditions compared with the control cells could provide quantitative evidence of altered autophagy. It is then possible to score the population as the percentage of cells displaying numerous autophagosomes. This approach will only be feasible if the background number of puncta is relatively low. For this method, it is particularly important to count a large number of cells and multiple representative sections of the sample (probably on the order of 50 or more, preferably in at least three different trials, depending on the particular system and experiment, but the critical point is that this determination should be based on statistical power analysis). Accordingly, high-content imaging analysis methods are extremely applicable to provide reliable values. Such methods enable quantification of GFP-LC3 puncta (or overall fluorescence intensity) in thousands of cells per sample (e.g., see refs. 164, 175 and 220). When using automated analysis methods, care must be taken to manually evaluate parameters used to establish background cutoff values for different treatment conditions and cell types. Another note of caution is that treatments affecting cell morphology, leading to the “rounding-up” of cells for example, can result in apparent changes in the number of GFP-LC3 puncta per cell. To avoid misinterpretation of results due to such potential artifacts, manual review of cell images is highly recommended.

To allow comparisons by other researchers attempting to repeat these experiments, it is critical that the authors also specify the baseline number of puncta that are used to define “normal” or “low” autophagy. Furthermore, the cells should also be counted using unbiased procedures (e.g., using a random start point followed by inclusion of all cells at regular intervals), and statistical information should be provided for both baseline and altered conditions, as these assays can be highly variable. One possible method to obtain unbiased counting of GFP-LC3 puncta in a large number of cells is to perform multispectral imaging flow cytometry (see *Autophagic flux determination using flow and multispectral imaging cytometry*).<sup>221</sup> This method can also be used for endogenous LC3, and, therefore, is useful for non-transfected primary cells.<sup>222</sup> Multispectral imaging flow cytometry allows characterization of single cells within a population by assessing a combination of morphology and immunofluorescence patterns, thereby providing statistically meaningful data.<sup>223</sup> For adherent cell cultures, one caution for flow cytometry is that the techniques necessary to produce single cell suspensions can cause significant

injury to the cells, leading to secondary changes in autophagy. Therefore, staining for plasma membrane permeabilization (e.g., cell death) before vs. after isolation is an important control.

An important caveat in the use of GFP-LC3 is that this chimera can associate with aggregates, especially when expressed at high levels in the presence of aggregate-prone proteins, which can lead to a misinterpretation of the results.<sup>224</sup> Of note, GFP-LC3 can associate with ubiquitinated protein aggregates;<sup>225</sup> however, this does not occur if the GFP-LC3 is expressed at low levels (Rubinsztein DC, unpublished observations). These aggregates have been described in many systems, and are also referred to as Aggresome-Like Induced Structures or ALIS,<sup>225-227</sup> dendritic cell ALIS,<sup>228</sup> p62 bodies/sequestosomes<sup>229</sup> and inclusions. Inhibition of autophagy in vitro and in vivo leads to the accumulation of these aggregates, suggesting a role for autophagy in mediating their clearance.<sup>225,226,230-232</sup> One way to control for background levels of puncta is to determine fluorescence from untagged GFP.

The adaptor protein SQSTM1/p62 is required for the formation of ubiquitinated protein aggregates in vitro (see *SQSTM1/p62 and related LC3 binding protein turnover assays*).<sup>229</sup> In this case, the interaction of SQSTM1 with both ubiquitinated proteins and LC3 is thought to mediate delivery of these aggregates to the autophagy system.<sup>233,234</sup> Many cellular stresses can induce the formation of aggregates, including transfection reagents,<sup>225</sup> or the introduction of foreign DNA (especially if the DNA is not extracted endotoxin free). SQSTM1-positive aggregates are also formed by proteasome inhibition or puromycin treatment. Calcium phosphate transfection of COS7 cells or lipofectamine transfection of MEFs (Pinkas-Kramarski R, personal communication), primary neurons (La Spada AR, personal communication) or neuronal cells (Chu CT, personal communication) transiently increases basal levels of GFP-LC3 puncta and/or the amount of LC3-II. One solution is to examine GFP-LC3 puncta in cells stably expressing GFP-LC3; however, as transfection-induced increases in GFP-LC3 puncta and LC3-II are often transient, another approach is to use cells transfected with GFP, and cells subjected to a mock time-matched transfection as background (negative) controls. A lipidation-defective LC3 mutant where glycine 120 is mutated to alanine is targeted to these aggregates independently of autophagy (likely via its interaction with SQSTM1, see above) and as a result this mutant can serve as another valuable control.<sup>225</sup> When carrying out transfections it may be necessary to alter the protocol depending on the level of background fluorescence. For example, changing the medium and waiting 24 to 48 h after the transfection can help to reduce the background level of GFP-LC3 puncta that is due to the transfection reagent (Colombo MI, personal communication). Similarly, when using an mCherry-GFP-SQSTM1 double tag (see *Tandem mRFP/mCherry-GFP fluorescence microscopy*) in transient transfections it is best to wait 48 h after transfection to reduce the level of aggregate formation and potential inhibition of autophagy (Johansen T, personal communication). Another consideration is that in addition to transfection, viral infection can activate stress pathways in some cells and possibly induce autophagy, again emphasizing the importance of appropriate controls, such as control viruses expressing GFP.<sup>235</sup>

Ubiquitinated protein aggregate formation and clearance appear to represent a cellular recycling process. Aggregate formation can occur when autophagy is either inhibited or when its capacity for degradation is exceeded by the formation of proteins delivered to the aggregates. In principle, formation of GFP-LC3-positive aggregates represents a component of the autophagy process. However, the formation of GFP-LC3-positive ubiquitinated protein aggregates does not directly reflect either the induction of autophagy (or autophagosome formation), or flux through the system. Indeed, formation of ubiquitinated protein aggregates that are GFP-LC3 positive can occur in autophagy-deficient cells.<sup>225</sup> Therefore, it should be remembered that GFP-LC3 puncta likely represent a mix of ubiquitinated protein aggregates in the cytosol, ubiquitinated protein aggregates within autophagosomes and more “conventional” phagophores and autophagosomes bearing other cytoplasmic cargo (this is one example where CLEM could help in resolving this question). In *Dictyostelium*, inhibition of autophagy leads to huge ubiquitinated protein aggregates containing SQSTM1 and GFP-Atg8, when the latter is co-expressed; the size of the aggregates and the typical presence of only one structure per cell make them easily distinguishable from autophagosomes. Saponin treatment has been used to reduce background fluorescence under conditions where no aggregation of GFP-LC3 is detected in hepatocytes, GFP-LC3 stably-transfected HEK 293<sup>235</sup> and human osteosarcoma cells, and in nontransfected cells;<sup>236</sup> however, treatment with saponin and other detergents can provoke artifactual GFP-LC3 puncta formation,<sup>237</sup> and controls need to be included in such experiments in light of these findings. In general, it is preferable to include additional assays that measure autophagy rather than relying solely on monitoring GFP-LC3. In addition, we recommend that researchers validate their assays by demonstrating the absence or reversal of GFP-LC3 puncta formation in cells treated with pharmacological or RNA interference-based autophagy inhibitors (Table 1). For example, 3-MA is commonly used to inhibit starvation- or rapamycin-induced autophagy,<sup>256</sup> but some data indicate that this compound can also have stimulatory effects (see *Autophagy inhibitors and inducers*).<sup>257</sup>

Another general limitation of the GFP-LC3 assay is that it requires a system amenable to the introduction of an exogenous gene. Accordingly, the use of GFP-LC3 in primary non-transgenic cells is more challenging. Here again, controls need to be included to verify that the transfection protocol itself does not artifactually induce GFP-LC3 puncta or cause LC3 aggregation. Furthermore, transfection should be performed with low levels of constructs, and the transfected cells should be followed to determine (1) when sufficient expression for detection is achieved, and (2) that during the time frame of the assay, basal GFP-LC3 puncta remain appropriately low. In addition, the demonstration of a reduction in the number of induced GFP-LC3 puncta under conditions of autophagy inhibition is helpful. For some primary cells, delivering GFP-LC3 to precursor cells by infection with recombinant lentivirus, retrovirus or adenovirus,<sup>258</sup> and subsequent differentiation into the cell type of interest, is a powerful alternative to transfection of the already differentiated cell type.<sup>91</sup>

To implement the scoring of autophagy via fluorescence microscopy, one option is to measure pixel intensity. Since the expression of GFP-LC3 may not be the same in all cells—as discussed above—it is possible to use specific imaging software to calculate the standard deviation (SD) of pixel intensity within the fluorescence image and divide this by the mean intensity of the pixels within the area of analysis. This will provide a ratio useful for establishing differences in the degree of autophagy between cells. Cells with increased levels of autophagic activity, and hence a greater number of autophagosomes in their cytosol, are associated with a greater variability in pixel intensity (i.e., a high SD). Conversely, in cells where autophagy is not occurring, GFP-LC3 is uniformly distributed throughout the cytosol and a variation in pixel intensity is not observed (i.e., a low SD) (Campanella M, personal communication).

Although LC3-II is primarily membrane-associated, it is not necessarily associated with autophagosomes as is often assumed; the protein is also found on phagophores, the precursors to autophagosomes, as well as on amphisomes and phagosomes (see *Western blotting and ubiquitin-like protein conjugation systems*).<sup>132,259,260</sup> Along these lines, yeast Atg8 can associate with the vacuole membrane independent of lipidation, so that a punctate pattern does not necessarily correspond to autophagic compartments.<sup>261</sup> Thus, the use of additional markers is necessary to specify the identity of an LC3-positive structure; for example, ATG12-ATG5-ATG16L1 would be present on a phagophore, but not an autophagosome. In addition, the site(s) of LC3 conjugation to PE is not definitively known and levels of Atg8-PE/LC3-II can increase even in autophagy mutants that cannot form autophagosomes.<sup>262</sup> One method that can be used to examine LC3-II membrane association is differential extraction in Triton X-114, which can be used with mammalian cells,<sup>258</sup> or western blot analysis of total membrane fractions following solubilization with Triton X-100, which is helpful in plants.<sup>150,151</sup> Another approach is to examine colocalization of LC3 with ATG5 (or other ATG proteins); the ATG12-ATG5 conjugate does not typically remain associated with autophagosomes, meaning that colocalized structures would correspond to phagophores. Importantly, we stress again that numbers of GFP-LC3 puncta, similar to steady-state LC3-II levels, reflect only a snapshot of the numbers of autophagy-related structures (e.g., autophagosomes) in a cell, and not autophagic flux.

Finally, we offer a general note of caution with regard to using GFP. First, the GFP tag is large, in particular relative to the size of LC3; therefore, it is possible that a chimera may behave differently from the native protein in some respects. Second, GFP is not native to most systems, and as such it may be recognized as an aberrant protein and targeted for degradation, which has obvious implications when studying autophagy. Third, some forms of GFP tend to oligomerize, which may interfere with protein function and/or localization. Fourth, EGFP inhibits polyubiquitination<sup>263</sup> and may cause defects in other cellular processes. Fifth, not all LC3 puncta represent LC3-II and correspond to autophagosomes.<sup>137,264,265</sup> Accordingly it would be prudent to complement any assays that rely on GFP fusions (to Atg8/LC3 or any protein) with additional methods that

**Table 1.** Genetic and pharmacological regulation of autophagy<sup>†</sup>

Method	Comments
1. 3-methyladenine (3-MA)	A PtdIns3K inhibitor that effectively blocks an early stage of autophagy by inhibiting the class III PtdIns3K, but is not a specific autophagy inhibitor. 3-MA also inhibits the class I PtdIns3K and can thus, at suboptimal concentrations in long-term experiments, promote autophagy in some systems, as well as affect cell survival through AKT1 and other kinases.
2. 10-NCP	10-(4'-N-diethylamino)butyl)-2-chlorophenoxazine; an AKT1 inhibitor that induces autophagy in neurons. <sup>238</sup>
3. 17-AAG	An inhibitor of the HSP90-CDC37 chaperone complex, induces autophagy in certain systems (e.g., neurons), but impairs starvation-induced autophagy and mitophagy in others by promoting the turnover of ULK1. <sup>239</sup>
4. ATG4 <sup>C74A</sup>	An active site mutant of ATG4 that is defective for autophagy. <sup>240</sup>
5. Bafilomycin A <sub>1</sub> (or concanamycin A)	A V-ATPase inhibitor that causes an increase in lysosomal/vacuolar pH, and ultimately blocks fusion of autophagosomes with the vacuole.
6. Calcium	An autophagy activator that can be released from ER or lysosomal stores under stress conditions; however, calcium can also inhibit autophagy. <sup>241</sup>
7. Chloroquine, NH <sub>4</sub> Cl	Lysosomotropic compounds that elevate/neutralize the lysosomal/vacuolar pH.
8. Deletion	This method provides the most direct evidence for the role of an autophagic component; however, more than one gene involved in autophagy should be targeted to avoid indirect effects.
9. E-64d	A membrane-permeable cysteine protease inhibitor that can block the activity of a subset of lysosomal hydrolases; should be used in combination with pepstatin A for inhibiting lysosomal protein degradation.
10. Knockdown	This method provides relatively direct evidence for the role of an autophagic component. However, the efficiency of knockdown varies, as does the stability of the targeted protein. In addition, more than one gene involved in autophagy should be targeted to avoid indirect effects.
11. KU-0063794	An MTOR inhibitor that binds the catalytic site and activates autophagy. <sup>242,243</sup>
12. Leupeptin	An inhibitor of cysteine, serine and threonine proteases that can be used in combination with pepstatin A and/or E-64d to block lysosomal protein degradation. Leupeptin is not membrane permeable, so its effect on cathepsins may depend on endocytic activity.
13. microRNA	Can be used to reduce the levels of target mRNA(s) or block translation.
14. NAADP-AM	Activates the lysosomal two pore channel and induces autophagy. <sup>244</sup>
15. NED-19	Inhibits the lysosomal two-pore channel and NAADP-induced autophagy. <sup>244</sup>
16. NVP-BEZ235	A dual inhibitor of PIK3CD/p110 and the MTOR catalytic site that activates autophagy. <sup>245,246</sup>
17. Pathogen-derived	ICP34.5, vBCL2, vCFLAR/vFLIP, influenza M2, and HIV Nef autophagy inhibitor transfection.
18. Pepstatin A	An aspartyl protease inhibitor that can be used to partially block lysosomal degradation; should be used in combination with other inhibitors such as E-64d. Pepstatin A is not membrane permeable.
19. Protease inhibitors	These chemicals inhibit the degradation of autophagic substrates within the lysosome/vacuole lumen. A combination of inhibitors (e.g., leupeptin, pepstatin A and E-64d) is needed for complete blockage of degradation.
20. Rapamycin	Inhibits MTOR by binding to RPTOR, thus inducing autophagy, but only provides partial inhibition.
21. Resveratrol	A natural polyphenol that induces autophagy via activation of AMPK. <sup>247,248</sup>
22. RNAi	Can be used to inhibit gene expression.
23. RSVAs	Synthetic small-molecule analogs of resveratrol that potently activate AMPK and induce autophagy. <sup>249</sup>
24. Thapsigargin	An inhibitor of the sarcoplasmic/endoplasmic reticulum Ca <sup>2+</sup> ATPase (SERCA) that inhibits autophagic sequestration through the depletion of intracellular Ca <sup>2+</sup> stores; <sup>250</sup> however, thapsigargin may also block fusion of autophagosomes with endosomes by interfering with recruitment of RAB7, resulting in autophagosome accumulation. <sup>251</sup> Long-term thapsigargin treatment may induce ER stress and a secondary stimulation of autophagy.
25. Torin1	A catalytic MTOR inhibitor that induces autophagy and provides more complete inhibition than rapamycin (it inhibits all forms of MTOR). <sup>252</sup>
26. Trehalose	An inducer of autophagy that may be relevant for the treatment of different neurodegenerative diseases. <sup>253,254</sup>
27. Tunicamycin	A glycosylation inhibitor that induces autophagy due to ER stress. <sup>255</sup>
28. Vinblastine	A depolymerizer of both normal and acetylated microtubules that interferes with autophagosome-lysosome fusion. <sup>156</sup>
29. Wortmannin	An inhibitor of PtdIns 3-kinase that blocks autophagy, but is not a specific inhibitor (see 3-MA above).

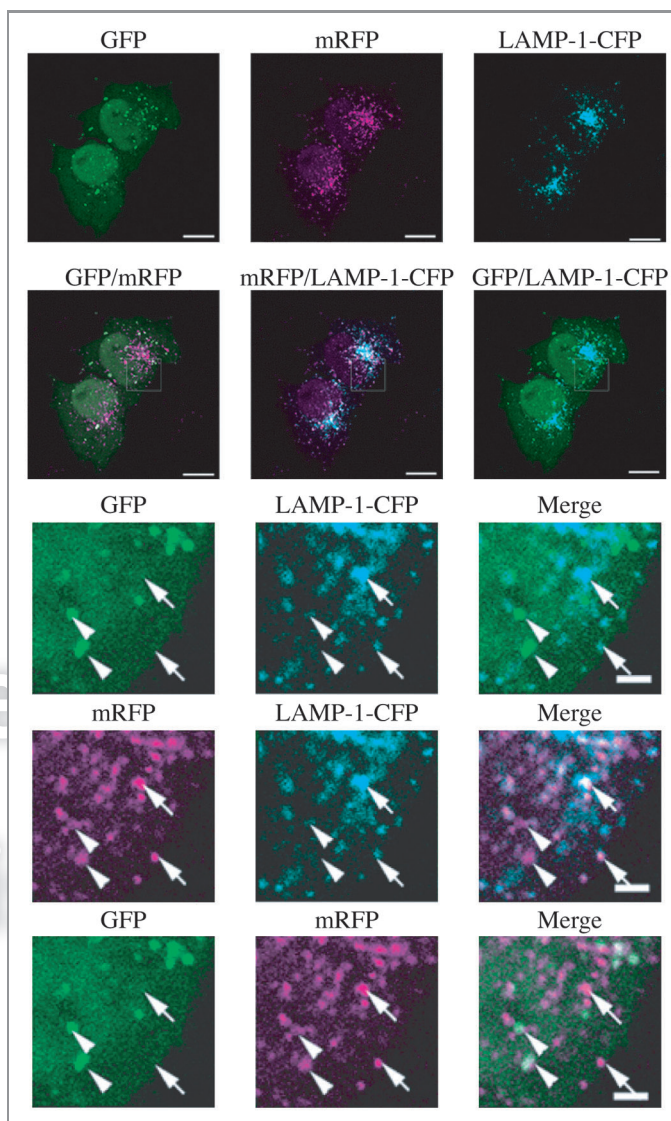
<sup>†</sup>This table is not meant to be complete, as there are many compounds and genetic methods that regulate autophagy, and new ones are being discovered routinely.

avoid the use of this fluorophore. Similarly, with the emergence of “super-resolution” microscopy methods such as photoactivated localization microscopy (PALM), new tags are being used (e.g., the EosFP green to red photoconvertible fluorescent protein, or the Dronpa GFP-like protein) that will need to be tested and validated.<sup>266</sup>

**Conclusion:** GFP-LC3 provides a marker that is relatively easy to use for monitoring autophagy induction (based on the appearance of puncta), or colocalization; however, it is not a preferred method for determining flux. In addition, it is recommended to use additional assays along with GFP-LC3 fluorescence microscopy to monitor autophagy.

*e. Tandem mRFP/mCherry-GFP fluorescence microscopy.* A fluorescence assay that is particularly designed to monitor flux relies on the use of a tandem monomeric RFP-GFP-tagged LC3 (tfLC3; Fig. 10).<sup>181</sup> The GFP signal is sensitive to the acidic and/or proteolytic conditions of the lysosome lumen, whereas mRFP is more stable. Therefore, colocalization of both GFP and mRFP fluorescence indicates a compartment that has not fused with a lysosome, such as the phagophore or an autophagosome. In contrast, an mRFP signal without GFP corresponds to an amphisome or autolysosome. Other fluorophores such as mCherry are also suitable instead of mRFP,<sup>229</sup> and an image-recognition algorithm has been developed to quantify flux of the reporter to acidified compartments.<sup>242,267</sup> One of the major advantages of the tandem mRFP/mCherry-GFP reporter method is that it enables simultaneous estimation of both the induction of autophagy and flux through autophagic compartments in essentially native conditions, without requiring any drug treatment. The use of more than one time point allows visualization of increased early autophagosomes followed by increases in late autophagosomes as an additional assurance that flux has been maintained.<sup>268</sup> In addition, this method can be used to monitor autophagy in high-throughput drug screening studies.<sup>242</sup> The quantification of “yellow only” and “red only” dots in a stable tandem-fluorescent LC3-reporter cell line can be automated by a Cellomics microscope that can be used to assess a huge population of cells (1,000 or more) over a large number of random fields of view. This can give rise to more accurate data than can be achieved by manual assessment of a few selected cells.<sup>167,269</sup>

An alternative dual fluorescence assay involves the Rosella biosensor. This assay monitors the uptake of material to the lysosome/vacuole and complements the use of the tandem mRFP/mCherry-GFP reporter. The assay is based upon the genetically encoded dual color-emission biosensor Rosella, a fusion between a relatively pH-stable fast-maturing RFP variant, and a pH-sensitive GFP variant. When targeted to specific cellular compartments or fused to an individual protein, the Rosella biosensor provides information about the identity of the cellular component being delivered to the vacuole/lysosome for degradation. Importantly, the pH-sensitive dual color fluorescence emission provides information about the environment of the biosensor during autophagy of various cellular components. In yeast, Rosella has been successfully used to monitor autophagy of cytosol, mitochondria (mitophagy) and the nucleus (nucleophagy).<sup>270,271</sup>



**Figure 10.** The GFP and mRFP signals of tandem fluorescent LC3 (tfLC3, mRFP-GFP-LC3) show different localization patterns. HeLa cells were cotransfected with plasmids expressing either tfLC3 or LAMP1-CFP. Twenty-four hours after the transfection, the cells were starved in Hanks’ solution for 2 h, fixed and analyzed by microscopy. The lower panels are a higher magnification of the upper panels. Bar, 10  $\mu$ m in the upper panels and 2  $\mu$ m in the lower panels. Arrows in the lower panels point to (or mark the location of) typical examples of colocalized signals of mRFP and LAMP1. Arrowheads point to (or mark the location of) typical examples of colocalized particles of GFP and mRFP signals. This figure was previously published in reference 181 and is reproduced by permission of Landes Bioscience, copyright 2007.

Furthermore, the Rosella biosensor can be used as a reporter under various conditions including nitrogen depletion-dependent induction of autophagy.<sup>270,271</sup> The Rosella biosensor can also be expressed in mammalian cells to follow nonselective autophagy (cytoplasmic turnover), or mitophagy.<sup>271</sup>

**Cautionary notes:** The use of tandem mRFP/mCherry-GFP-LC3/Atg8 reporters in live imaging experiments can be complicated by the motion of LC3/Atg8 puncta. As a consequence,

conventional confocal microscopy may not allow visualization of colocalized mRFP/mCherry-GFP puncta. In this case, GFP or colocalized puncta represent newly formed autophagic structures whereas mRFP/mCherry-only puncta are ambiguous. Spinning disk confocal microscopy, or rapid acquisition times may be required for imaging tandem mRFP/mCherry-GFP proteins, although these techniques require a brighter fluorescent signal associated with what may be undesirably higher levels of transgene expression. One solution is to use the mTagRFP-mWasabi-LC3 chimera,<sup>272a</sup> as mTagRFP is brighter than mRFP1 and mCherry, and mWasabi is brighter than EGFP.<sup>272b</sup> Another possibility is to use fixed cells; however, this presents an additional concern: The use of tandem mRFP/mCherry-GFP relies on the quenching of the GFP signal in the acidic autolysosome; however, fixation solutions are often neutral or weak bases, which will increase the pH of the entire cell. Accordingly, the GFP signal may be restored after fixation (Fig. 11), which would cause an underestimation of the amount of signal that corresponds only to RFP (i.e., in the autolysosome). Thus, the tissue or cell samples must be properly processed to avoid losing the acidic environment of the autolysosomes. In addition, there may be weak fluorescence of EGFP even in an acidic environment (pH between 4 and 5).<sup>180,258</sup> Therefore, it may be desirable to choose a monomeric green fluorescent protein that is more acid sensitive than EGFP for assaying autophagic flux.

Another caution in the interpretation of the tandem fluorescent marker is that colocalization of GFP and mRFP/mCherry might also be seen in the case of impaired proteolytic degradation within autolysosomes, or altered lysosomal pH. Finally, expression of tandem mRFP-GFP-LC3 is toxic to some cancer cell lines relative to GFP-LC3 or RFP-LC3 (Choi KS, personal communication). The cytotoxicity of DsRed and its variants such as mRFP1 is associated with downregulation of BCL2L1/Bcl-x<sub>L</sub>.<sup>273</sup> In contrast to mRFP-GFP-LC3, overexpression of mTagRFP-mWasabi-LC3 does not appear to be toxic to HeLa cells (Lin J, personal communication).

The Rosella assay has not been tested in a wide range of mammalian cell types. Accordingly, the sensitivity and the specificity of the assay must be verified independently until this method has been tested more extensively and used more widely.

Finally, it is ideal to capture the dynamic behavior of autophagy in real time, in order to generate data revealing the rate of formation and clearance of autophagosomes over time, rather than single data points. For example, by acquiring signals from two fluorescent constructs in real time, the rate of change in colocalization signal as a measure of the fusion rate and recycling rate between autophagosomes and lysosomes can be assessed. Importantly, due to the integral dynamic relationship of autophagic flux with the onset of apoptosis and necrosis it is advantageous to monitor cell death and autophagic flux parameters concomitantly over time, which FRET-based reporter constructs make possible.<sup>274</sup>

**Conclusion:** The use of tandem fluorescent constructs, which display different emission signals depending on the environment (in particular, GFP fluorescence is particularly sensitive to an acidic pH), provides a convenient way to monitor autophagy flux in many cell types.

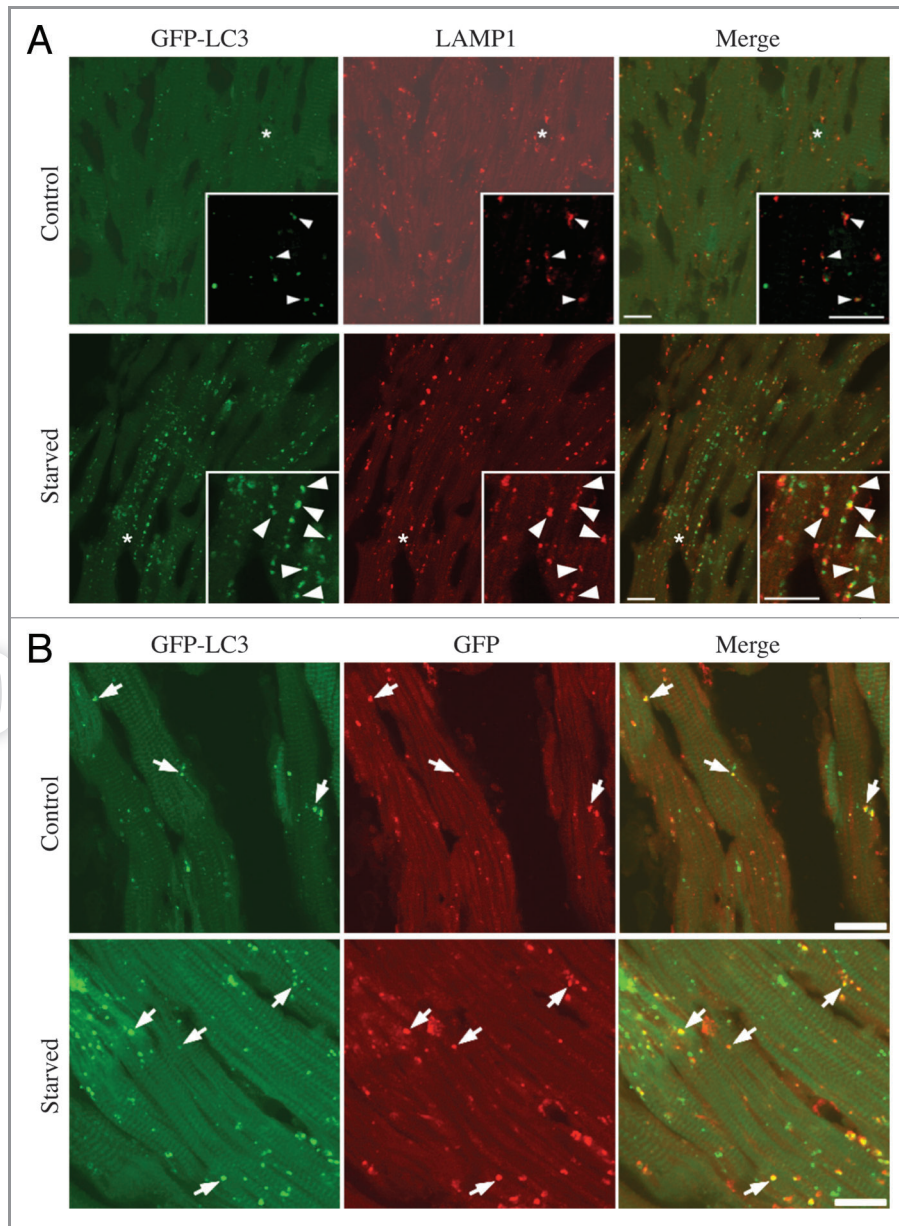
*f. Autophagic flux determination using flow and multispectral imaging cytometry.* Whereas fluorescence microscopy, in combination with novel autophagy probes, has permitted single cell analysis of autophagic flux, automation for allowing medium- to high-throughput analysis has been challenging. A number of methods have been developed that allow the determination of autophagic flux using the fluorescence-activated cell sorter (FACS),<sup>154,223,236,275-277</sup> and commercial kits are now available for monitoring autophagy by flow cytometry. These approaches make it possible to capture high-content images of cells in flow (up to 1,000 cells/sec), and are particularly useful for cells that grow in suspension. Optimization of image analysis permits the study of cells with heterogeneous LC3 puncta, thus making it possible to quantify autophagic flux accurately in situations that might perturb normal processes (e.g., microbial infection).<sup>277,278</sup> Since EGFP-LC3 is a substrate for autophagic degradation, total fluorescence intensity of EGFP-LC3 can be used to indicate levels of autophagy in living mammalian cells.<sup>275</sup> When autophagy is induced, the decrease in total cellular fluorescence can be precisely quantified in large numbers of cells to obtain robust data. In another approach, soluble EGFP-LC3-I can be depleted from the cell by a brief saponin extraction so that the total fluorescence of EGFP-LC3 then represents that of EGFP-LC3-II alone (Fig. 12A).<sup>235,236</sup> Since EGFP-LC3 transfection typically results in high relative levels of EGFP-LC3-I, this treatment significantly reduces the background fluorescence due to non-autophagosome-associated reporter protein. By comparing treatments in the presence or absence of lysosomal degradation inhibitors, subtle changes in the flux rate of the GFP-LC3 reporter construct can be detected. If it is not desirable to treat cells with lysosomal inhibitors to determine rates of autophagic flux, a tandem mRFP/mCherry-EGFP-LC3 (or similar) construct can also be used for autophagic flux measurements in FACS experiments (see *Tandem mRFP/mCherry-GFP fluorescence microscopy*).<sup>276</sup>

These methods, however, require the cells of interest to be transfected with reporter constructs. Since the saponin extraction method can also be combined with intracellular staining for endogenous LC3 protein, subtle changes in autophagic flux can be measured without the need for reporter transfections (Fig. 12B). This enables investigations of autophagic flux in a wide variety of cell types and tissues.

**Cautionary notes:** Care must be taken when applying flow cytometry measurements to adherent cells, particularly neurons and other cells with interdigitated processes, as the preparation of single cell suspensions entails significant levels of plasma membrane disruption and injury that can secondarily induce autophagy.

Users of the saponin extraction method should carefully titrate saponin concentrations and times of treatment in order to ensure specific extraction of LC3-I in their systems. Also, it has been observed in some cell types that saponin treatment can lead to non-autophagic aggregation of LC3,<sup>237</sup> which should be controlled for in these assays (see *GFP-Atg8/LC3 fluorescence microscopy*).

Cell membrane permeabilization with digitonin and extraction of the nonmembrane-bound form of LC3 allows combined

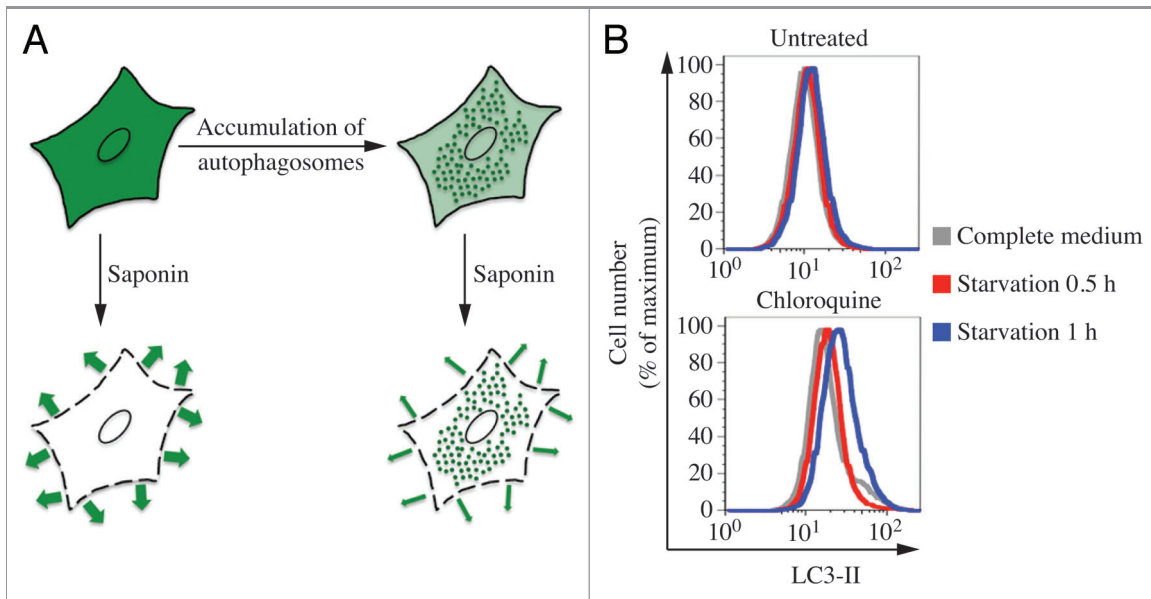


**Figure 11.** GFP fluorescence in the autolysosome can be recovered upon neutralization of the pH. (A) GFP-LC3 emits green fluorescence in the autolysosomes of post-mortem processed heart sections. Cryosections of 3.8% paraformaldehyde fixed ventricular myocardium from 3-week old GFP-LC3 transgenic mice at the baseline (control) or starved for 24 h (starved) were processed for immunostaining using a standard protocol (buffered at pH 7.4). Most of the GFP-LC3 puncta are positive for LAMP1, suggesting that the autolysosomes had recovered GFP fluorescence. (B) Colocalization between GFP-LC3 direct fluorescence (green) and indirect immunostaining for GFP (red). Sections processed as in (A) were immunostained for GFP using a red fluorescence-tagged secondary antibody, and the colocalization with GFP fluorescence was examined by confocal microscopy. Almost all of the red puncta emit green fluorescence. Images provided by Xuejun Wang.

staining of membrane-associated LC3-II protein and any markers for detection of autophagy in relation to other cellular events/processes. Based on this approach a method for monitoring autophagy in different stages of the cell cycle was developed.<sup>279</sup> Thus, the presence of basal or starvation-induced autophagy is detected in G<sub>1</sub>, S and G<sub>2</sub>/M phases of the cell cycle in MEFs with doxycycline-regulated ATG5 expression. In these experiments cells were gated based on their DNA content, and the relative intensity of GFP-LC3-II and LC3-II expression. This approach

might also be used for the detection of autophagic flux in different stages of the cell cycle or subG<sub>1</sub> apoptotic cell population by measuring accumulation of LC3-II in the presence or absence of lysosomal inhibitors.

Although GFP-LC3 can be used as a reporter for flow cytometry, it is more stable (which is not necessarily ideal for flux measurements) than GFP-SQSTM1 or GFP-NBR1 (NBR1 is a selective autophagic substrate with structural similarity to SQSTM1<sup>280</sup>); GFP-SQSTM1 displays the largest magnitude



**Figure 12.** Saponin extraction allows quantification of LC3-II fluorescence by FACS. (A) Schematic diagram of the effects of the saponin wash. Due to the reorganization of the EGFP-LC3 reporter protein, induction of autophagosome formation does not change the total levels of fluorescence in EGFP-LC3-transfected cells. However, extraction of EGFP-LC3-I with saponin results in a higher level of fluorescence in cells with proportionally higher levels of EGFP-LC3-II-containing autophagosomes. This figure was previously published in reference 236 (B) Saponin extraction can also be used to measure flux of endogenous LC3 protein. Human osteosarcoma cells were starved of amino acids and serum by incubation in EBSS, for the indicated times in the presence or absence of a 1 h chloroquine (50  $\mu$ M) treatment. Cells were then washed with PBS containing 0.05% saponin and processed for FACS analysis for endogenous LC3. These data are provided by K.E. Eng and G.M. McInerney.

change following the induction of autophagy by amino acid deprivation or rapamycin treatment, and may thus be a better marker for following autophagic flux by this method (confirmed in SH-SY5Y neuronal cell lines stably expressing GFP-SQSTM1; Valente EM, personal communication).<sup>281</sup> However, SQSTM1 changes can be cell type and context specific. In some cell types, there is no change in overall SQSTM1 levels despite strong levels of autophagy induction, verified by the tandem mRFP/mCherry-GFP-LC3 reporter as well as Atg7- and lysosome-dependent turnover of cargo proteins (Chu CT, personal observation). In other contexts, a robust loss of SQSTM1 does not correlate with increased autophagic flux as assessed by a luciferase-based measure of flux.<sup>166</sup> Thus, appropriate positive and negative controls, and assessment of SQSTM1 mRNA levels, may be needed prior to the use of SQSTM1 as a flux indicator in a particular cellular context.

**Conclusion:** Medium- to high-throughput analysis of autophagy is possible using flow and multispectral imaging cytometry (Fig. 13). The advantage of this approach is that larger numbers of cells can be analyzed with regard to GFP-LC3 puncta, cell morphology and/or autophagic flux, and concomitant detection of surface markers can be included, potentially providing more robust data than is achieved with other methods.

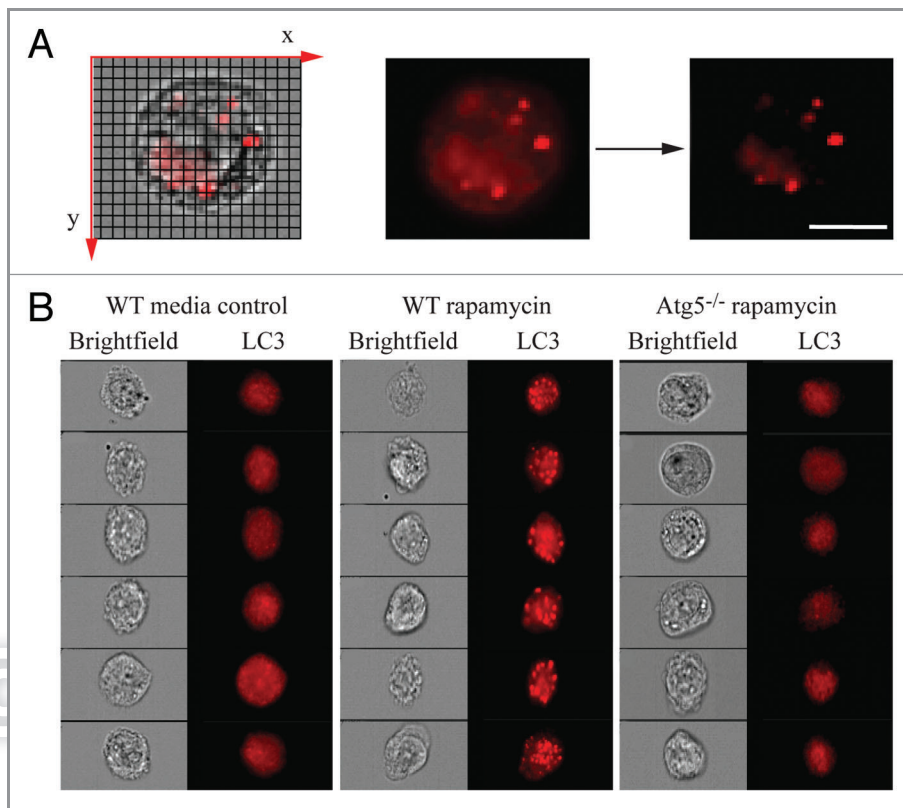
*g. Immunohistochemistry.* Immunodetection of ATG proteins (particularly LC3 and BECN1) has been reported as a prognostic factor in various human carcinomas, including lymphoma,<sup>140,282</sup> breast carcinoma,<sup>283</sup> endometrial adenocarcinoma,<sup>284,285</sup> head and neck squamous cell carcinoma,<sup>286,287</sup> hepatocellular carcinoma,<sup>288,289</sup> gliomas,<sup>290</sup> non-small cell lung carcinomas,<sup>291</sup> pancreatic<sup>292</sup> and colon adenocarcinomas,<sup>293-295</sup> as well as in cutaneous and uveal

melanomas.<sup>296,297</sup> Importantly, this kind of assay should be performed as recommended by the Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK).<sup>298</sup> As we identify new drugs for modulating autophagy in clinical applications, this type of information may prove useful in the identification of subgroups of patients for targeted therapy.<sup>299-301</sup>

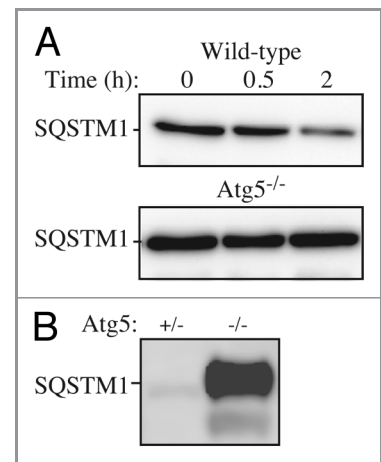
In mouse and rat tissues, endogenous LC3, ATG4B, and ATG9A have been detected by immunohistochemical analyses using both paraffin sections and cryosections.<sup>208,302-304</sup> When autophagosomes are absent, the localization pattern of LC3 in the cells of various tissues is diffuse and cytosolic. Moreover, intense fibrillary staining of LC3 is detectable along dendrites of intact neurons, whereas granular staining for LC3 appears mainly in the perikarya of neurons in cathepsin D- or cathepsins B- and L-deficient mouse brains.<sup>208</sup> In developing retinal tissue in chicken, BECN1 and AMBRA1 are detected by immunofluorescence.<sup>305,306</sup> Finally, in non-mammalian vertebrates, BECN1 was detected during follicular atresia in the ovary of three fish species using paraffin sections; a punctate immunostaining for BECN1 was scattered throughout the cytoplasm of the follicular cells when they were in intense phagocytic activity for yolk removal (Rizzo E, unpublished results).

**Cautionary notes:** One problem with immunohistochemistry for LC3 is that in some tissues this protein can be localized in structures other than autophagosomes. For example, in murine hepatocytes and cardiomyocytes under starved conditions, endogenous LC3 is detected not only in autophagosomes but also on lipid droplets.<sup>307</sup> In neurons in ATG7-deficient mice, LC3 is accumulated in ubiquitin- and SQSTM1-positive aggregates.<sup>308</sup>





**Figure 13.** Assessing autophagy with multispectral imaging cytometry. (A) Bright Detail Intensity (BDI) measures the foreground intensity of bright puncta (that are three pixels or less) within the cell image. For each cell, the local background around the spots is removed before intensity calculation. Thus, autophagic cells with puncta have higher BDI values. (B) Media control (untreated wild type), rapamycin-treated wild-type and *Atg5*<sup>-/-</sup> MEFs were gated based on BDI. Representative images of cells with high or low BDI values. Scale bar, 10  $\mu$ m. Images provided by M.L. Albert.



**Figure 14.** Regulation of the SQSTM1 protein during autophagy. (A) The level of SQSTM1 during starvation. *Atg5*<sup>+/+</sup> and *Atg5*<sup>-/-</sup> MEFs were cultured in DMEM without amino acids and serum for the indicated times, and then subjected to immunoblot analysis using anti-SQSTM1/p62 antibody (Progen Biotechnik). This figure was previously published in reference 25 and is reproduced by permission of Landes Bioscience, copyright 2007. (B) The level of SQSTM1 in the brain of neural cell-specific *Atg5* knockout mice. This image was generously provided by Dr. Taichi Hara (Tokyo Medical and Dental University).

Thus, immunodetection of LC3 in cytoplasmic granules is not sufficient to monitor autophagy in vivo.

**Conclusion:** It has not been clearly demonstrated that immunohistochemistry of ATG proteins in tissues corresponds to autophagy activity, and this area of research needs to be further explored before we can make specific recommendations.

3. *SQSTM1/p62 and related LC3 binding protein turnover assays.* In addition to LC3, SQSTM1/p62, or other receptors such as NBR1, can also be used as a protein marker, at least in certain settings.<sup>25,309</sup> The SQSTM1 protein serves as a link between LC3 and ubiquitinated substrates.<sup>98</sup> SQSTM1 and SQSTM1-bound polyubiquitinated proteins become incorporated into the completed autophagosome and are degraded in autolysosomes, thus serving as a readout of autophagic degradation (Fig. 14). Inhibition of autophagy correlates with increased levels of SQSTM1 in mammals and *Drosophila*, suggesting that steady-state levels of this protein reflect the autophagic status.<sup>304,310-315</sup> Similarly, decreased SQSTM1 levels are associated with autophagy activation. The phosphorylation of SQSTM1 at Ser403 appears to regulate its role in the autophagic clearance of ubiquitinated proteins, and anti-phospho-SQSTM1/p62 antibodies can be used to detect the modified form of the protein.<sup>234</sup>

**Cautionary notes:** SQSTM1 contains an LC3 interacting motif as well as a ubiquitin binding domain, and appears to act by linking ubiquitinated substrates with the autophagic machinery. Nonetheless, it would be prudent to keep in mind that SQSTM1 contains domains that interact with several signaling molecules,<sup>316</sup> and SQSTM1 may be part of the mechanistic target of rapamycin (MTOR) complex 1 (TORC1).<sup>317</sup> Thus, it may have additional functions that need to be considered with regard to its role in autophagy. In the context of autophagy as a stress response, the complexity of using SQSTM1 as an autophagy marker is underscored by its capacity to modulate the NFE2L2/NRF2 anti-oxidant response pathway through a KEAP1 binding domain.<sup>318</sup> In fact, SQSTM1 may, itself, be transcriptionally induced by NFE2L2.<sup>319</sup> Furthermore, it is necessary to examine endogenous SQSTM1 because overexpression of this protein leads to the formation of protein inclusions. In fact, even endogenous SQSTM1 becomes Triton X-100-insoluble in the presence of protein aggregates and when autophagic degradation is inhibited; thus, results with this protein are often context-dependent. In addition, SQSTM1 participates in proteasomal degradation, and its level may also increase when the proteasome is inhibited.<sup>320</sup> Accordingly, the SQSTM1 degradation rate should

be analyzed in the presence of an inhibitor such as epoxomicin or lactacystin to determine the contribution from the proteasome (see *Autophagy inhibitors and inducers* for potential problems with MG132).<sup>321</sup> SQSTM1 is also a substrate for CASP6/caspase 6 and CASP8/caspase 8 (as well as CAPN1/calpain 1), which may confound its use in examining cell death and autophagy.<sup>322</sup> Another issue is that some phosphatidylinositol 3-kinase (PtdIns3K) inhibitors such as LY294002, and to a lesser extent wortmannin (but apparently not 3-MA)<sup>256</sup> can inhibit protein synthesis;<sup>323</sup> this might in turn affect the turnover of SQSTM1 and LC3, which could influence conclusions that are drawn from the status of these proteins regarding autophagy flux or aggresome-like induced structures (ALIS) formation. Accordingly, it may be advisable to measure protein synthesis and proteasome activity along with autophagy under inhibitory or activating conditions. With regard to protein synthesis, it is worth noting that this can be monitored through a nonradioactive method.<sup>324</sup> Finally, *SQSTM1* may be transcriptionally upregulated under certain conditions,<sup>227,325</sup> further complicating the interpretation of results. For example, *SQSTM1* upregulation, and at least transient increases in the amount of SQSTM1, is seen in some situations where there is an increase in autophagic flux.<sup>326-328</sup> Of interest, SQSTM1 hyperexpression at both gene and protein levels can be observed in muscle atrophy induced by cancer, though not by glucocorticoids, suggesting that the stimulus inducing autophagy may also be relevant to the differential regulation of autophagy-related proteins (Penna F, Costelli P, unpublished observations). One solution to problems relating to variations in SQSTM1 synthesis is to use a HaloTag<sup>®</sup>-SQSTM1/p62 chimera.<sup>329</sup> The chimeric protein can be covalently labeled with HaloTag<sup>®</sup> ligands, and the loss of signal can then be monitored without interference by subsequent changes in protein synthesis. Similarly, a stable cell line expressing EGFP-tagged SQSTM1 under the control of an inducible promoter can be used to assess the rates of SQSTM1 degradation, taking into account the limitations outlined above (see *Autophagic flux determination using flow and multispectral imaging cytometry*).<sup>281</sup> Yet another solution is to employ a radioactive pulse-chase assay to measure the rates of SQSTM1 degradation.<sup>330</sup>

Western blot analysis using NP40 or Triton X-100 lysis in autophagic conditions typically shows a reduction in SQSTM1 levels. However, this does not necessarily indicate that SQSTM1 is degraded, because SQSTM1 aggregates are insoluble in these detergent lysis conditions.<sup>227,331</sup> Moreover, in some instances SQSTM1 levels do not change in the soluble fractions despite autophagic degradation, a finding that might be explained by simultaneous transcriptional induction of the gene encoding SQSTM1, since the soluble fraction accounts only for the diffuse or free form of SQSTM1. Accumulation of SQSTM1 in the Triton X-100-insoluble fraction can be observed when autophagy-mediated degradation is inhibited. Under conditions of higher autophagic flux, accumulation of SQSTM1 in Triton X-100-insoluble fractions may not be observed and SQSTM1 levels may be reduced or maintained. The simplest approach to circumvent many of these problems is by using lysis buffer that allows identification of the entire cellular pool of SQSTM1 (e.g.,

containing 1% SDS); however, additional assessment of both Triton X-100-soluble and -insoluble fractions will provide further information regarding the extent of SQSTM1 oligomerization.<sup>308</sup>

To conclusively establish SQSTM1 degradation by autophagy, SQSTM1 levels in both Triton X-100-soluble and -insoluble fractions need to be determined upon treatment with autophagy inducers in combination with autophagy inhibitors, such as those that inhibit the autolysosomal degradation steps (e.g., protease inhibitors, chloroquine or bafilomycin A<sub>1</sub>, or genetically by knocking out or knocking down *LAMP2*). Additionally, an alteration in the levels of SQSTM1 may not be immediately evident with changes observed in autophagic flux upon certain chemical perturbations (Sarkar S, personal communication). Whereas LC3 changes may be rapid, clearance of autophagy substrates may require a longer time. Therefore, if LC3 changes are assessed at 6 h or 24 h after a drug treatment, SQSTM1 levels can be tested not only at the same time points, but also at later time points (24 h or 48 h) for determining the maximal impact on substrate clearance. An alternative method is immunostaining for SQSTM1 with and without autophagy inhibitors, which will appear as either a diffuse or punctate pattern. Experiments with autophagy inducers and inhibitors, in combination with western blot and immunostaining analyses, best establish autophagic degradation based on SQSTM1 turnover. A final point, however, is that empirical evidence suggests that the species-specificity of antibodies for detecting SQSTM1 must be taken into account. For example, some commercial antibodies recognize both human and mouse SQSTM1, whereas others detect the human, but not the mouse protein (see the *Autophagy Forum* at <https://www.landesbioscience.com/journals/autophagy/forum/> for information pertaining to anti-SQSTM1/p62 antibodies).<sup>332</sup> Another issue with detecting SQSTM1 in the context of human diseases is that it can be mutated (e.g., in Paget disease of bone).<sup>333</sup> Thus, care should be taken to ensure that potential mutations are not affecting the epitopes that are recognized by anti-SQSTM1 antibodies when using western blotting to detect this protein.

**Conclusion:** There is not always a clear correlation between increases in LC3-II and decreases in SQSTM1. Thus, although analysis of SQSTM1 can assist in assessing the impairment of autophagy or autophagy flux, we recommend using SQSTM1 only in combination with other methods such as LC3-II turnover to monitor flux.

**4. TOR, AMPK and Atg1/ULK1.** Atg1/ULK1 are central components in autophagy that likely act at more than one stage of the process. There are multiple ULK isoforms in mammalian cells including ULK1, ULK2, ULK3, ULK4 and STK36.<sup>334</sup> ULK3 is a positive regulator of the Hedgehog signaling pathway,<sup>335</sup> and its overexpression induces both autophagy and senescence.<sup>336</sup> Along these lines, ectopic ULK3 displays a punctate pattern upon starvation-induced autophagy induction.<sup>336</sup> ULK3, ULK4 and STK36, however, lack the domain present on ULK1 and ULK2 that bind ATG13 and RB1CC1/FIP200.<sup>337</sup> Thus, ULK3 may play a role that is restricted to senescence, and that is independent of the core autophagy machinery. ULK2 has a higher degree of identity with ULK1 than any of the other homologs,

and they may have similar functions that are tissue specific; however, ULK1 may be the predominant isoform involved in autophagy, as knockdown of ULK2 does not affect movement of ATG9.<sup>338</sup> The stability and activation of ULK1, but not ULK2, is dependent on its interaction with the HSP90-CDC37 chaperone complex. Pharmacological or genetic inhibition of the chaperone complex increases proteasome-mediated turnover of ULK1, impairing its kinase activity and ability to promote both starvation-induced autophagy and mitophagy.<sup>239</sup>

AMP-activated protein kinase (AMPK) is a multimeric serine/threonine protein kinase comprising  $\alpha$ - (catalytic),  $\beta$ - (scaffold), and  $\gamma$ - (regulatory) subunits. The enzyme activity of AMPK is absolutely dependent on phosphorylation of the  $\alpha$ -subunit on Thr172,<sup>339,340</sup> and can, therefore, be conveniently monitored by western blotting with a phosphospecific antibody against this site. In some cells, Thr172 is phosphorylated by the  $\text{Ca}^{2+}$ -activated protein kinase CAMKK2/CaMKK $\beta$ ; in other cells by the constitutively active kinase STK11/LKB1, regulation of AMPK activity being performed primarily by Thr172-dephosphorylating protein phosphatases such as protein phosphatase 1 and PPP2CB/PP2A.<sup>341</sup> Thr172 dephosphorylation is modulated by adenine nucleotides that bind competitively to regulatory sites in the  $\gamma$ -subunit. AMP and ADP inhibit dephosphorylation and promote AMPK activity, whereas  $\text{Mg}^{2+}$ -ATP has the opposite effect.<sup>340</sup> AMPK thus acts as a fine-tuned sensor of the overall cellular energy charge that regulates cellular metabolism to maintain energy homeostasis. Activation of AMPK is also associated with the phosphorylation of downstream enzymes involved in ATP-consuming processes, such as fatty acid (acetyl-CoA carboxylase) and cholesterol (hydroxymethylglutaryl-CoA dehydrogenase) biosynthesis.

The role of AMPK in autophagy is complex, and highly dependent on both cell type and metabolic conditions. In liver cells, AMPK suppresses autophagy at the level of cargo sequestration as indicated by the rapid sequestration-inhibitory effects of a variety of AMPK activators, whereas it appears to stimulate autophagy in many other cell types, including fibroblasts, colon carcinoma cells and skeletal muscle.<sup>342-351</sup> Autophagy-promoting effects of AMPK are most evident in cells cultured in a complete medium with serum and amino acids, where cargo sequestration is otherwise largely suppressed.<sup>348</sup> Presumably, AMPK antagonizes the autophagy-inhibitory effect of amino acids (at the level of phagophore assembly) by phosphorylating proteins involved in TORC1 signaling, such as TSC2<sup>352</sup> and RPTOR/RAPTOR<sup>353</sup> as well the TORC1 target ULK1 (see below).<sup>354-356</sup>

Compound C is an effective and widely used inhibitor of activated (phosphorylated) AMPK.<sup>357</sup> However, being a non-specific inhibitor of oxidative phosphorylation,<sup>358,359</sup> this drug has been observed to *inhibit* autophagy under conditions where AMPK is already inactive or knocked out,<sup>360</sup> and has even been shown to *stimulate* autophagy by an AMP-independent mechanism.<sup>359,361</sup> Compound C thus cannot be used as a stand-alone indicator of AMPK involvement.

TORC1 is an autophagy-suppressive regulator that integrates growth factor, nutrient and energy signals. In most systems, inhibition of MTOR leads to induction of autophagy, and

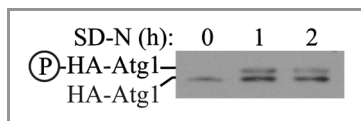
AMPK activity is generally antagonistic toward MTOR function. TORC1 mediates the autophagy-inhibitory effect of amino acids, which stimulate the MTOR protein kinase through a RRA/RAG GTPase dimer. Insulin and growth factors activate TORC1 through upstream kinases including AKT1 (protein kinase B), and extracellular signal regulated kinase (MAPK3-MAPK1, or ERK) when the energy supply is sufficient, whereas energy depletion may induce AMPK-mediated TORC1 inhibition and autophagy stimulation, for example, during glucose starvation. Amino acid starvation, on the other hand, can strongly induce autophagy even in cells completely lacking AMPK catalytic activity.<sup>362</sup>

AMPK and TORC1 regulate autophagy through coordinated phosphorylation of ULK1; under glucose starvation, AMPK promotes autophagy by directly activating ULK1 through phosphorylation, although the exact AMPK-mediated ULK1 phosphorylation site(s) remains unclear.<sup>351,354-356</sup> Under conditions of nutrient sufficiency, high TORC1 activity prevents ULK1 activation by phosphorylating alternate ULK1 residues and disrupting the interaction between ULK1 and AMPK. There are commercially available phospho-specific antibodies that recognize different forms of ULK1. For example, phosphorylation at Ser555, an AMPK site, is indicative of increased autophagy in response to nutrient stress, whereas Ser757 is targeted by MTOR to inhibit autophagy. Even the autophagy-suppressive effects of AMPK could, conceivably, be mediated through ULK1 phosphorylation, for example, at the inhibitory site Ser638.<sup>363</sup> AMPK inhibits MTOR by phosphorylating and activating TSC2.<sup>364</sup> Therefore, AMPK is involved in processes that synergize to activate autophagy, by directly activating ULK1, and indirectly impairing MTOR-dependent inhibition of ULK1. The identification of ULK1 as a direct target of TORC1 and AMPK represents a significant step toward the definition of new tools to monitor the induction of autophagy. However, further studies directed at identifying physiological substrates of ULK1 will be essential to understand how ULK1 activation results in initiation of the autophagy program. Along these lines, ULK1 phosphorylates AMBRA1,<sup>365</sup> and the MYLK/MLCK-like protein Sqa,<sup>366</sup> as well as ATG13 and RB1CC1/FIP200.<sup>367-370</sup> In addition, ULK1 binds to, and phosphorylates, RPTOR, leading to inhibition of TORC1.<sup>371</sup> Furthermore, ULK1 itself appears to be able to mediate inhibitory AMPK phosphorylation to generate a negative feedback loop.<sup>372</sup>

TORC1 activity can be monitored by following the phosphorylation of its substrates, such as EIF4EBP1 (4E-BP1/PHAS-I) and RPS6KB1 (p70S6 kinase) or the latter's downstream target, the ribosomal protein S6 (RPS6), for which good commercial antibodies are available.<sup>373-375</sup> In mammalian cells, the analysis should focus on the phosphorylation of RPS6KB1 at Thr389, and EIF4EBP1 at Thr37 and Thr46, which are directly phosphorylated by TORC1.<sup>376</sup> The TORC1-dependent phosphorylation of EIF4EBP1 can be detected as a molecular mass shift by western blot.<sup>375</sup> Examining the phosphorylation status of RPS6KB1 and EIF4EBP1 may be a better method for monitoring TORC1 activity than following the phosphorylation of proteins such as RPS6, because the latter is not a direct substrate of TORC1

(although RPS6 phosphorylation is a good readout for RPS6KB1/2 activities, which are directly dependent on MTOR), and it can also be phosphorylated by other kinases such as RPS6KA/RSK. Furthermore, the mechanisms that determine the selectivity as well as the sensitivity of TORC1 for its substrates seem to be dependent on the integrity and configuration of TORC1. For example, rapamycin strongly reduces RPS6KB1 phosphorylation, whereas its effect on EIF4EBP1 is more variable. In the case of rapamycin treatment, EIF4EBP1 can be phosphorylated by TORC1 until rapamycin disrupts TORC1 dimerization and its integrity, whereas RPS6KB1 phosphorylation is quickly reduced when rapamycin simply interacts with MTOR in TORC1 (see *Autophagy inhibitors and inducers* for information on catalytic MTOR inhibitors such as torin1).<sup>376</sup> Since it is likely that other inhibitors, stress, and stimuli may also affect the integrity of TORC1, a decrease or increase in the phosphorylation status of one TORC1 substrate does not necessarily correlate with changes in others, including ULK1. Therefore, reliable antiphospho-ULK1 antibodies should be used to directly examine the phosphorylation state of ULK1, along with additional experimental approaches to analyze the role of the MTOR complex in regulating autophagy.

Activation/assembly of the Atg1 complex in yeast (composed of at least Atg1-Atg13-Atg17-Atg29-Atg31) or the ULK1 complex in mammals (ULK1-RB1CC1-ATG13-C12orf44/ATG101) is one of the first steps of autophagy induction. Therefore, activation of this complex can be assessed to monitor autophagy induction. In yeast, dephosphorylation of Atg13 is associated with activation/assembly of the core complex, which can be followed by immunoprecipitation or western blotting.<sup>377-380</sup> In addition, the autophosphorylation of Atg1 at Thr226 is required for its kinase activity and for autophagy induction; this can be detected using phospho-specific antibodies, by immunoprecipitation or western blotting (Fig. 15).<sup>381,382</sup> In mammalian cells, the phosphorylation status of ULK1 at the activating sites (Ser317, 467, 555, 637, 777, or Thr574) or dephosphorylation at inactivating sites (Ser638, 757) can be determined using phospho-specific antibodies,<sup>355,356</sup> or by western blotting.<sup>383</sup> In general, the core complex is stable in mammalian cells, although, as noted above, upstream inhibitors (MTOR) or activators (AMPK) may interact dynamically with it, thereby determining the status of autophagy.



**Figure 15.** *S. cerevisiae* cells transformed with a plasmid encoding HA-Atg1 were cultured to mid-log phase and shifted to SD-N (minimal medium lacking nitrogen that induces a starvation response). Immunoblotting was done with anti-HA antibody. The upper band corresponds to autophosphorylation of Atg1. This figure was modified from data previously published in reference 381 and is reproduced by permission of the American Society for Cell Biology, copyright 2011.

One additional topic that bears on ULK1 concerns the process of LC3-associated phagocytosis. LAP is a type of phagocytosis in macrophages that involves the conjugation of LC3 to single-membrane pathogen-containing phagosomes, a process that promotes phagosome acidification and fusion with lysosomes.<sup>131</sup> Most of the core autophagy components are required for LAP, but the two processes can be distinguished by the presence or absence, respectively, of a double-membrane sequestering vesicle. ULK1 is not needed for LAP, which provides a more convenient means for distinguishing between the two processes.<sup>134</sup>

**Cautionary notes:** A decrease in TORC1 activity is a good measure for autophagy induction; however, TORC1 activity does not necessarily preclude autophagy induction because there are TOR-independent mechanisms that induce autophagy.<sup>384-387</sup> Along these lines, whereas in most systems inhibition of MTOR leads to the induction of autophagy, there are instances in commonly used cancer cell lines in which MTOR appears to be a positive effector.<sup>388</sup> Furthermore, in adult skeletal muscle, autophagy gene upregulation and autophagosome formation is independent of TORC1 but partially dependent on MTOR complex 2 (TORC2), as shown by the finding that autophagosome formation is increased by knockdown of RICTOR, a component of TORC2, but not TORC1.<sup>346</sup> In addition, TORC1 is downstream of AKT1; however, oxidative stress inhibits MTOR, thus allowing autophagy, despite the concomitant activation of AKT1.<sup>115</sup> Also, persistent autophagy induction can cause negative feedback that results in the reactivation of MTOR under conditions of ongoing starvation.<sup>389</sup> Thus, it is necessary to be cautious in deciding how to monitor the MTOR pathway, and to verify that the pathway being analyzed displays MTOR-dependent inhibition.

One problem in monitoring assembly of the ULK1 complex is the low abundance of endogenous ULK1 in many systems, which makes it difficult to detect phospho-ULK1 by western blot analysis. In addition, Atg1/ULK1 is phosphorylated by multiple kinases, and the amount of phosphorylation at different sites can increase or decrease during autophagy induction. Thus, although there is an increase in phosphorylation at the activating sites upon induction, the overall phosphorylation states of ULK1 and ATG13 are decreased under conditions that lead to induction of autophagy; therefore, monitoring changes in phosphorylation by following molecular mass shifts upon SDS-PAGE may not be informative. In addition, such phosphorylation/dephosphorylation events are expected to occur relatively early (1–2 h) in the signaling cascade of autophagy. Therefore, it is necessary to optimize treatment time conditions. Finally, in Arabidopsis and possibly other eukaryotes, the ATG1 and ATG13 proteins are targets of autophagy, which means that their levels may drop substantially under conditions that induce autophagic turnover.<sup>174</sup>

At present, the use of Atg1/ULK1 kinase activity as a tool to monitor autophagy is limited because only a few physiological substrates have been identified, and the importance of the Atg1/ULK1-dependent phosphorylation has not been determined. Nonetheless, Atg1/ULK1 kinase activity appears to increase when autophagy is induced, irrespective of the pathway leading to induction. As additional physiological substrates of Atg1/ULK1

are identified it will be possible to follow their phosphorylation in vivo as is done with analyses for MTOR. Nonetheless, it must be kept in mind that monitoring changes in the activity of Atg1/ULK1 is not a direct assay for autophagy, although such changes may correlate with autophagy activity. Furthermore, in some cells ULK1 has functions in addition to autophagy, such as in axonal transport and outgrowth, and its activity state may thus reflect its role in these processes.<sup>390-395</sup> Accordingly, other methods as described throughout these guidelines should also be used to follow autophagy directly.

Finally, there is not a complete consensus on the specific residues of ULK1 that are targeted by AMPK or MTOR. Similarly, apparently contradictory data have been published regarding the association of AMPK and MTOR with the ULK1 kinase complex under different conditions. Therefore, caution should be used in monitoring ULK1 phosphorylation or the status of ULK1 association with AMPK until these issues are resolved.

**Conclusion:** Assays for Atg1/ULK1 can provide detailed insight into the induction of autophagy, but are not a direct measurement of the process. Similarly, analysis of MTOR substrates such as RPS6KB1 and EIF4EBP1 are not recommended readouts for autophagy, and need to be combined with other assays that directly monitor autophagy activity.

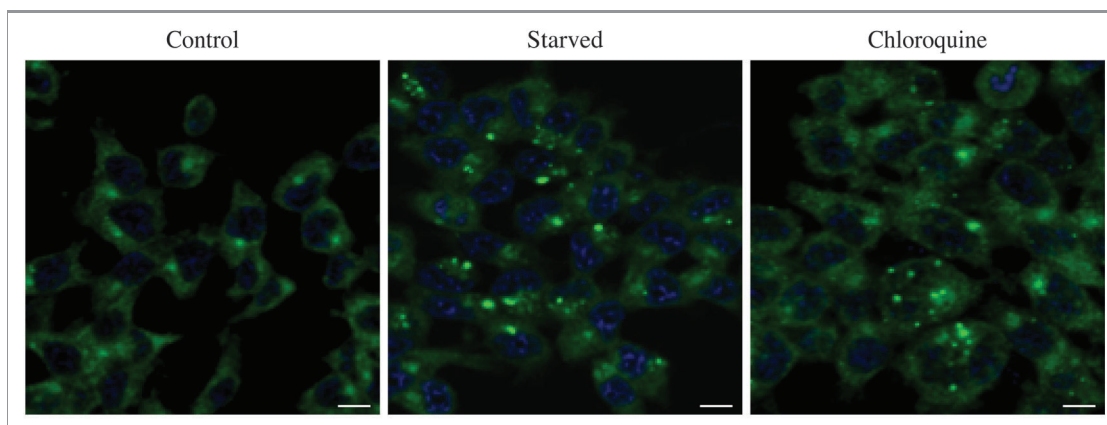
**5. Additional autophagy-related markers.** Although Atg8/LC3 has been the most extensively used protein for monitoring autophagy, other proteins can also be used for this purpose. Here, we discuss some of the more commonly used or better-characterized possibilities.

*a. ATG9.* ATG9/Atg9 is the only integral membrane ATG protein that is essential for autophagosome formation. ATG9 displays partial colocalization with GFP-LC3.<sup>396</sup> Perhaps the most unique feature of ATG9/Atg9, however, is that it localizes to multiple discrete puncta, whereas most Atg proteins are detected primarily in a single punctum or diffusely within the cytosol. Yeast Atg9 may cycle between the phagophore assembly site (PAS) and peripheral reservoirs;<sup>397</sup> the latter correspond to tubulovesicular clusters that are precursors to the phagophore.<sup>398</sup>

Anterograde movement to the PAS is dependent on Atg11, Atg23, Atg27 and actin. Retrograde movement requires Atg1-Atg13, Atg2-Atg18 and the PtdIns3K complex I.<sup>399</sup> Mutants such as *atg1Δ* accumulate Atg9 exclusively at the PAS, and this phenotype forms the basis of the transport of Atg9 after knocking out *ATG1* (TAKA) assay.<sup>63</sup> In brief, this is an epistasis analysis in which a double-mutant strain is constructed (one of the mutations being *atg1Δ*) that expresses Atg9-GFP. If the second mutated gene encodes a protein that is needed for Atg9 anterograde transport, the double mutant will display multiple Atg9-GFP puncta. In contrast, if the protein acts along with or after Atg1, all of the Atg9-GFP will be confined to the PAS. Monitoring the localization of ATG9 has not been used extensively in higher eukaryotes, but this protein displays the same type of dependence on Atg1/ULK1 for cycling as seen in yeast,<sup>396,399</sup> suggesting that it is possible to follow this protein as an indication of ULK1 and ATG13 function.<sup>370</sup>

*b. ATG12-ATG5.* ATG5, ATG12, and ATG16L1, associate with the phagophore and have been detected by fluorescence or immunofluorescence (Fig. 16).<sup>400,401</sup> Endogenous ATG5, ATG12 or ATG16L1 puncta formation can be followed to monitor autophagy upregulation. Under physiological conditions, the endogenous proteins are predominantly diffusely distributed throughout the cytoplasm. Upon induction of autophagy, for example during starvation, there is a marked increase in the proportion of cells with punctate ATG5, ATG12 and ATG16L1. Furthermore, inhibitors of autophagosome formation result in a block in this starvation-induced puncta formation, and this assay is very robust in mammalian cells.

ATG12-ATG5 conjugation has been used in some studies to measure autophagy. In Arabidopsis and some mammalian cells it appears that essentially all of the ATG5 and ATG12 proteins exist in the conjugated form and the expression levels do not change, at least during short-term starvation.<sup>150,400-402</sup> Therefore, monitoring ATG12-ATG5 conjugation per se may not be a useful method for following the induction of autophagy. It is worth noting, however, that in some cell lines free ATG5 can be detected,<sup>403</sup> suggesting that the amount of free ATG5 may be



**Figure 16.** Confocal microscopy image of HCT116 cells immunostained with human-specific antibody to ATG12. Cells were starved for 8 h or treated with chloroquine (50  $\mu$ M) for 3 h. Scale bar, 10  $\mu$ m. Image provided by M. Llanos Valero, M.A. de la Cruz and R. Sanchez-Prieto.

cell line-dependent. One final parameter that may be considered is that the total amount of the ATG12–ATG5 conjugate may increase following prolonged starvation as has been observed in hepatocytes and both mouse and human fibroblasts (Cuervo AM, personal communication; Sarkar S, personal communication).

*c. ATG14.* Yeast Atg14 is the autophagy-specific subunit of the Vps34 complex I,<sup>404</sup> and a human homolog, named ATG14 (ATG14L/BARKOR), has been identified.<sup>405–408</sup> ATG14 localizes primarily to phagophores. The C-terminal fragment of the protein is named the BATS domain (BARKOR/ATG14(L) autophagosome targeting sequence), and is able to direct GFP and BECN1 to autophagosomes in the context of a chimeric protein.<sup>409</sup> Currently, a good antibody that can be used to detect the endogenous ATG14 does not exist. ATG14-GFP or BATS-GFP detected by fluorescence microscopy or TEM can be used as a phagophore marker; however, ATG14 is not localized exclusively to phagophores, as it can also be detected on mature autophagosomes as well as the ER.<sup>409,410</sup> Accordingly, detection of ATG14 should be performed in combination with other phagophore and autophagosome markers.

*d. ATG16L1.* ATG16L1 has been used to monitor the movement of plasma membrane as a donor for autophagy, and thus an early step in the process; ATG16L1 is located on phagophores, but not on completed autophagosomes.<sup>269,411</sup> ATG16L1 can be detected by immuno-TEM, by immunostaining of Flag epitope-tagged ATG16L1, and/or by the use of GFP-tagged ATG16L1.

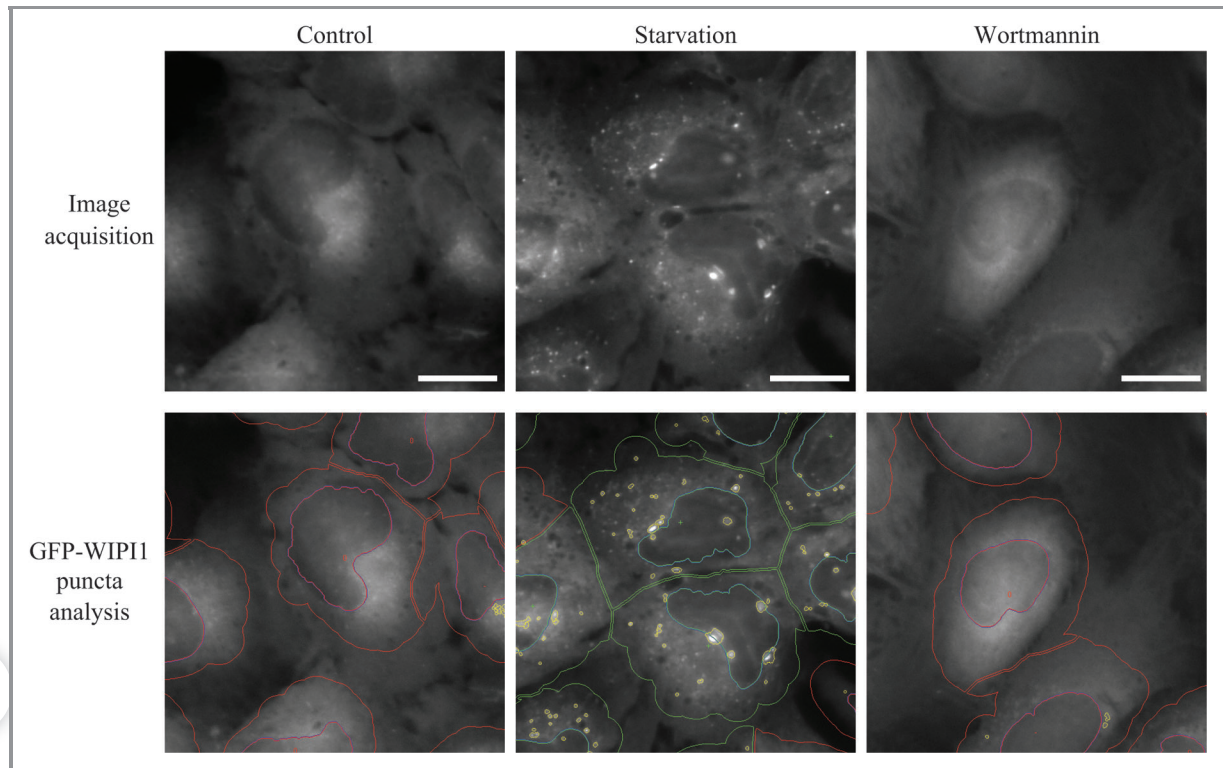
*e. Atg18/WIPI family.* Yeast Atg18<sup>412,413</sup> and Atg21<sup>262</sup> (or the mammalian WIPI homologs<sup>414</sup>) are required for both macroautophagy (i.e., nonspecific sequestration of cytoplasm) and autophagy-related processes (e.g., the Cvt pathway,<sup>415,416</sup> specific organelle degradation,<sup>74</sup> and autophagic elimination of invasive microbes<sup>77,78,80,81,417</sup>).<sup>412</sup> These proteins bind phosphatidylinositol 3-phosphate (PtdIns3P) that is present at the phagophore and autophagosome<sup>418,419</sup> and also PtdIns(3,5)P<sub>2</sub>. Human WIPI1 and WIPI2 function downstream of the phosphatidylinositol 3-kinase class III (PtdIns3KC3) complex I [PIK3C3, BECN1, PIK3R4/VPS15, ATG14] and upstream of both the ATG12 and LC3 ubiquitin-like conjugation systems.<sup>418,420,421</sup> Upon the initiation of the autophagic pathway, WIPI1 and WIPI2 bind PtdIns3P and accumulate at limiting membranes, such as those of the endoplasmic reticulum, where they participate in the formation of omegasomes and/or autophagosomes. On the basis of quantitative fluorescence microscopy, this specific WIPI protein localization has been used as an assay to monitor autophagy in human cells.<sup>419</sup> Using either endogenous WIPI1 or WIPI2, detected by indirect fluorescence microscopy or EM, or transiently or stably expressed tagged fusions of GFP to WIPI1 or WIPI2, basal autophagy can be detected in cells that display WIPI puncta at autophagosomal membranes. In circumstances of increased autophagic activity, such as nutrient starvation or rapamycin administration, the induction of autophagy is reflected by the elevated number of cells that display WIPI puncta when compared with the control setting. Also, in circumstances of reduced autophagic activity such as wortmannin treatment, the reduced number of WIPI puncta-positive cells reflects the

inhibition of autophagy. Basal, induced and inhibited formation of WIPI puncta closely correlate with both the protein level of LC3-II and the formation of GFP-LC3 puncta.<sup>419,421</sup> Accordingly, WIPI puncta can be assessed as an alternative to LC3. Automated imaging and analysis of fluorescent WIPI1 (Fig. 17) or WIPI2 puncta represents an efficient and reliable opportunity to combine the detection of WIPI proteins with other parameters. It should be noted that there are two isoforms of WIPI2 (2B and 2D),<sup>421</sup> and in *C. elegans* WIPI4 (EPG-6) has been identified as the WIPI homolog required for autophagy.<sup>422</sup> Thus, these proteins, along with the currently uncharacterized WDR45L/WIPI3, provide additional possibilities for monitoring phagophore and autophagosome formation.

**Cautionary notes:** With regard to detection of the WIPI proteins, endogenous WIPI1 puncta cannot be detected in many cell types,<sup>418</sup> and the level of transiently expressed GFP-WIPI1 puncta is cell context-dependent,<sup>418,419</sup> however, it has been used in human and mouse cell systems<sup>348,419</sup> and mCherry-Atg18 also works in transgenic *Drosophila* (Juhász G, personal communication), although one caution with regard to the latter is that GFP-Atg18 expression can induce autophagy in the fat body of fed larvae (Kiger A, unpublished observation). GFP-WIPI1 and GFP-WIPI2 have been detected on the completed (mature) autophagosome by freeze-fracture analysis,<sup>60</sup> but endogenous WIPI2 has not been detected on mRFP-LC3- or LAMP2-positive autophagosomes or autolysosomes using immunolabeling.<sup>418</sup> Accordingly, it may be possible to follow the formation and subsequent disappearance of WIPI puncta to monitor autophagy induction and flux using specific techniques. As with GFP-LC3, overexpression of WIPI1 or WIPI2 can lead to the formation of aggregates, which are stable in the presence of PtdIns3K inhibitors.

*f. BECN1/Atg6.* BECN1/Atg6 and PIK3C3/VPS34 are essential partners in the autophagy interactome that signals the onset of autophagy,<sup>404,423,424</sup> and many researchers use BECN1 as a way to monitor autophagy. BECN1 is inhibited by its binding to the anti-apoptotic protein BCL2.<sup>425</sup> Autophagy is induced by the release of BECN1 from BCL2 by pro-apoptotic BH3 proteins, phosphorylation of BECN1 by DAPK (at Thr119, located in the BH3 domain),<sup>426</sup> or phosphorylation of BCL2 by MAPK8/JNK1 (at Thr69, Ser70 and Ser87).<sup>427,428</sup> The relationship between BECN1 and BCL2 is more complex in developing cerebellar neurons as it appears that the cellular levels of BCL2 are, in turn, post-translationally regulated by an autophagic mechanism linked to a switch from immaturity to maturity.<sup>429,430</sup> It is important to be aware, however, that certain forms of macroautophagy are induced in a BECN1-independent manner, and are not blocked by PtdIns3K inhibitors.<sup>97,431</sup> Interestingly, caspase-mediated cleavage of BECN1 inactivates BECN1-induced autophagy and enhances apoptosis in several cell types,<sup>432</sup> emphasizing that the crosstalk between apoptosis and autophagy is complex.

Although a population of BECN1 may localize in proximity to the trans-Golgi network,<sup>433</sup> it is also present at the ER and mitochondria.<sup>425</sup> In keeping with these observations, in cerebellar organotypic cultures BECN1 co-immunoprecipitates with BCL2



**Figure 17.** Automated WIPI1 puncta image acquisition and analysis monitors the induction and inhibition of autophagy. Stable U2OS clones expressing GFP-WIPI1 were selected using 0.6  $\mu\text{g/ml}$  G418 and then cultured in 96-well plates. Cells were treated for 3 h with nutrient-rich medium (control), nutrient-free medium (EBSS), or with 233 nM wortmannin. Cells were fixed in 3.7% paraformaldehyde and stained with DAPI (5  $\mu\text{g/ml}$  in PBS). An automated imaging and analysis platform was used to determine the number of both GFP-WIPI1 puncta-positive cells and the number of GFP-WIPI1 puncta per individual cell.<sup>348</sup> Cells without GFP-WIPI1 puncta are highlighted in red (cell detection) and purple (nuclei detection), whereas GFP-WIPI1 puncta-positive cells are highlighted in yellow (GFP-WIPI1 puncta detection), green (cell detection) and blue (nuclei detection). Bars, 20  $\mu\text{m}$ . These images were provided by S. Pfisterer and T. Proikas-Cezanne.

that is primarily localized at the mitochondria and ER, and in a mouse model of neurodegeneration autophagic vacuoles in Purkinje neurons contain partially digested organelles that are immunoreactive for BCL2.<sup>430,434</sup> In addition, BECN1 and PIK3C3 can be present in multiple complexes, so caution must be exercised when monitoring localization. On induction of autophagy by various stimuli the presence of BECN1- and PIK3C3-positive macroaggregates can be detected in the region of the Golgi complex by immunofluorescence.<sup>115,435</sup> Thus, BECN1-GFP puncta detected by fluorescence microscopy or TEM may serve as an additional marker for autophagy induction;<sup>436</sup> however, as with any GFP chimeras there is a concern that the GFP moiety interferes with correct localization of BECN1. To demonstrate that BECN1 or PtdIns3K macroaggregates are an indirect indication of ongoing autophagy, it is mandatory to show their specific association with the process by including appropriate controls with inhibitors (e.g., 3-MA) or autophagy gene silencing. When a BECN1-independent autophagy pathway is induced, such aggregates are not formed regardless of the fact that the cell expresses BECN1 (e.g., as assessed by western blotting) (Isidoro C, personal communication).

*g. DRAM1.* DRAM1 (damage-regulated autophagic modulator 1) is a gene induced by activated TP53/p53 in response to

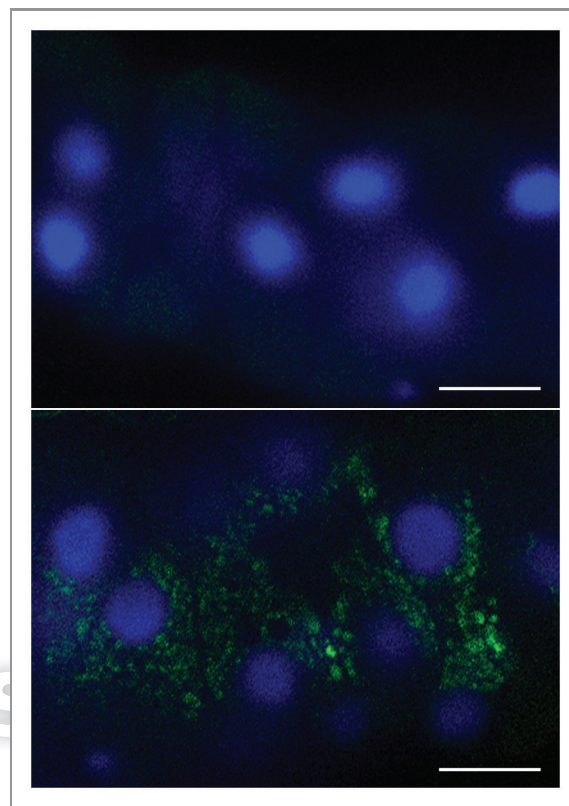
different types of cellular stress, including DNA damage.<sup>438,439</sup> DRAM1 is a small hydrophobic protein with six transmembrane domains. It is detected as a subpopulation in the Golgi and cis-Golgi, colocalizing with GOLGB1/giantin and GOLGA2/GM130, and also in early and late endosomes and lysosomes, colocalizing with EEA1 and LAMP2.<sup>439</sup> The elimination of DRAM1 by siRNA blocks autophagy,<sup>439,440</sup> as effectively as elimination of BECN1, indicating it is an essential component for this process, although its mechanism of action is not known. The time course of autophagy as a consequence of DRAM1 activation can be monitored following the disappearance of the VRK1 protein, a direct target of this process, by immunoblot.<sup>439</sup> Detection of *DRAM1* RNA is very easy by qRT-PCR during autophagy;<sup>438,439</sup> however, detection of the DRAM1 protein is very difficult because of its small size and hydrophobicity, which makes generation of specific antibodies a complicated process, and in general these have very low sensitivity.

*b. ZFYVE1/DFCP1.* ZFYVE1/DFCP1 (double FYVE-containing protein 1) binds PtdIns3P that localizes to the ER and Golgi. The ER population of ZFYVE1 is involved in formation of the omegasome.<sup>437</sup> Starvation induces the translocation of ZFYVE1 to punctate structures on the ER. ZFYVE1 partially colocalizes with WIPI1 upon nutrient starvation,<sup>421</sup> and also with WIPI2.<sup>418</sup>

**Conclusion:** Proteins other than Atg8/LC3 can be monitored to follow autophagy, and can be important tools to define specific steps of the process. For example, WIPI puncta formation can be used to monitor autophagy, but, similar to Atg8/LC3, should be examined in the presence and absence of inhibitors. Analysis of WIPI puncta should be combined with other assays because individual members of the WIPI family might also participate in additional, uncharacterized functions apart from their role in autophagy. At present, we caution against the use of changes in BECN1 localization as a marker of autophagy induction.

**6. Transcriptional and translational regulation.** The induction of autophagy in certain scenarios is accompanied by an increase in the mRNA levels of certain autophagy genes, such as *ATG8/Lc3*<sup>441,442</sup> and *Atg12*,<sup>443</sup> and an autophagy-dedicated microarray was developed as a high-throughput tool to simultaneously monitor the transcriptional regulation of all genes involved in, and related to, autophagy.<sup>444</sup> The gene that shows the greatest transcriptional regulation in the liver (in response to starvation and circadian signals) is *Ulk1*, but others also show more limited changes in mRNA levels including *Gabarapl1*, *Bnip3* and, to a minor extent, *Lc3B* (Lin JD, personal communication). In several mouse and human cancer cell lines, ER stress and hypoxia increase the transcription of *LC3*, *ATG5* and *ATG12* by a mechanism involving the unfolded protein response (UPR). Similarly, a stimulus-dependent increase in *LC3B* expression is detected in neutrophils undergoing autophagy induction.<sup>445</sup> Increased expression of *Atg5* in vivo after optic nerve axotomy in mice<sup>446</sup> and increased expression of *Atg7*, *Becn1* and *Lc3a* during neurogenesis at different embryonic stages in the mouse olfactory bulb are also seen.<sup>447</sup> *LC3* and *ATG5* are not required for the initiation of autophagy, but mediate phagophore expansion and autophagosome formation. In this regard, the transcriptional induction of *LC3* may be necessary to replenish the *LC3* protein that is turned over during extensive ER stress- and hypoxia-induced autophagy.<sup>443,448</sup> Thus, assessing the mRNA levels of *LC3* and other autophagy-related genes by northern blot or qRT-PCR may provide correlative data relating to the induction of autophagy. It is not clear if these changes are sufficient to induce autophagy, however, and therefore these are not direct measurements.

Of note, large changes in *Atg* gene transcription just prior to *Drosophila* salivary gland cell death (that is accompanied by an increase in autophagy) are detected in *Atg2*, *Atg4*, *Atg5* and *Atg7*, whereas there is no significant change in *Atg8a* or *Atg8b*.<sup>449,450</sup> Autophagy is critical for *Drosophila* midgut cell death, which is accompanied by transcriptional upregulation of all of the *Atg* genes tested, including *Atg8a* (Fig. 18).<sup>199,451</sup> Similarly, in the silkworm (*B. mori*) larval midgut the occurrence of autophagy is accompanied by an upregulation of *Atg5*, *Atg6* and *Atg8* mRNA levels.<sup>452</sup> Transcriptional upregulation of *Drosophila Atg8a* and *Atg8b* is also observed in the fat body following induction of autophagy at the end of larval development,<sup>453</sup> *Atg5*, *Atg6*, *Atg8a* and *Atg18* are upregulated in the ovary of starved flies,<sup>454</sup> and an increase in *Drosophila Atg8b* is observed in cultured *Drosophila l(2)mbn* cells following starvation (Gorski S, personal communication). An upregulation of plant *ATG8* may be needed during



**Figure 18.** pGFP-Atg8a can be used to monitor autophagy in *Drosophila melanogaster*. The autophagosome marker pGFP-Atg8a, results in expression of Atg8a fused to GFP from the endogenous *Atg8a* promoter.<sup>199</sup> Live imaging of gastric caeca from *Drosophila melanogaster* midgut; pGFP-Atg8a puncta (green) and Hoechst 33342 (blue). Midgut from early third instar larvae prior to the onset of cell death (top) and from dying midgut at 2 h after puparium formation (bottom). Bar, 25  $\mu$ m. Figure provided by D. Denton and S. Kumar.

the adaptation to reproductive growth; a T-DNA inserted mutation of rice *ATG8b* blocked the change from vegetative growth to reproductive growth in both homozygous and heterozygous plant lines (Zhang M-Y, unpublished results).

Similarly, the upregulation of autophagy-related genes (*Lc3*, *Gabarapl1*, *Bnip3*, *Atg4b*, *Atg12l*) has been documented at the transcriptional and translational level in several other species (e.g., mouse, rat, trout, Arabidopsis and maize) under conditions of ER stress,<sup>443</sup> and diverse types of prolonged (several days) catabolic situations including cancer cachexia, diabetes mellitus, uremia and fasting.<sup>151,346,455-457</sup> Along these lines, *ATG9* and *ATG16L1* are transcriptionally upregulated upon influenza virus infection (Khalil H, personal communication), and in *C. elegans*, the FOXA transcription factor PHA-4 regulates the expression of several autophagy-related genes.<sup>458</sup> Such prolonged induction of the expression of *ATG* genes has been thought to allow the replenishment of critical proteins (e.g., *LC3* and *GABARAP*) that are destroyed during autophagosome fusion with the lysosome.<sup>459</sup> The polyamine spermidine increases life span and induces autophagy in cultured yeast and mammalian cells, as well as in nematodes and flies; in aging yeast, spermidine treatment



triggers epigenetic deacetylation of histone H3 through inhibition of histone acetyltransferases, leading to significant upregulation of various autophagy-related transcripts.<sup>460</sup>

In addition to the *ATG* genes, transcriptional upregulation of *VMPI* (a protein that is involved in autophagy regulation and that remains associated with the completed autophagosome) can be detected in mammalian cells subjected to rapamycin treatment or starvation, and in tissues undergoing disease-induced autophagy.<sup>461</sup> *VMPI* is an essential autophagy gene that is conserved from Dictyostelium to mammals,<sup>232,462</sup> and the *VMPI* protein regulates early steps of the autophagic pathway.<sup>420</sup> *VMPI* is poorly expressed in mammalian cells under nutrient-normal conditions, but is highly upregulated in cells undergoing autophagy, and the expression of *VMPI* induces autophagosome formation.

A gene regulatory network, named CLEAR (coordinated lysosomal enhancement and regulation) that controls both lysosome and autophagosome biogenesis was identified using a systems-biology approach.<sup>463-465</sup> The basic helix-loop-helix transcription factor EB (TFEB) acts as a master gene of the CLEAR network and positively regulates the expression of both lysosomal and autophagy genes, thus linking the biogenesis of two distinct types of cellular compartments that cooperate in the autophagic pathway. TFEB activity is regulated by starvation,<sup>463</sup> and can thus serve as a new tool for monitoring transcriptional regulation connected with autophagy. Along these lines, the erythroid transcription factor GATA1 and its coregulator Friend of GATA1 induce the transcription of multiple genes encoding autophagy components; this developmentally regulated transcriptional response is coupled to increases in autophagosome number as well as the percent of cells that contain autophagosomes.<sup>466</sup> Finally, CEBPB/C/EBP $\beta$  is a transcription factor that regulates autophagy in response to the circadian cycle.<sup>467</sup>

Although less work has been done on post-transcriptional regulation, several studies implicate microRNAs in controlling the expression of proteins associated with autophagy.<sup>164,468-470</sup>

**Cautionary notes:** Most of the *ATG* genes do not show significant changes in mRNA levels when autophagy is induced. Even increases in *LC3* mRNA can be quite modest and are cell type- and organism-dependent.<sup>471</sup> In addition, it is generally better to follow protein levels because that is the ultimate readout that is significant with regard to the initiation and completion of autophagy, although *ATG* protein amounts do not always change significantly and the extent of increase is again cell type- and tissue-dependent. In some cases (e.g., yeast *ATG14*), increased transcription is not accompanied by increased protein levels, apparently due to changes in translation efficiency under starvation conditions (Abeliovich H, unpublished data). Finally, changes in autophagy protein levels are not sufficient evidence of autophagy induction, and must be accompanied by additional assays as described herein. Thus, monitoring changes in mRNA levels for either *ATG* genes or autophagy regulators may provide some evidence for autophagy induction, but should be used along with other methods.

Another general caution pertains to the fact that in any cell culture system different types of cells (for example, those undergoing autophagy or not) exist simultaneously. Therefore,

only an average level of protein or mRNA expression can be evaluated with most methods. This means that the results regarding specific changes in autophagic cells could be hidden due to the background of the average data. Along these lines, experiments using single-cell real-time PCR to examine gene expression in individual cardiomyocytes with and without signs of autophagy revealed that the transcription of *MTOR* markedly and significantly increased in autophagic cells in intact cultures (spontaneously undergoing autophagy) as well as in cultures treated with proteasome inhibitors to induce autophagy (Dosenko V, personal communication). Finally, researchers need to realize that mammalian cell lines may have mutations that alter autophagy signaling or execution; this problem can be avoided by using primary cells.

**Conclusion:** Although there are changes in *ATG* gene expression that coincide with, and may be needed for, autophagy, this has not been carefully studied experimentally. Therefore, at the present time we do not recommend the monitoring of *ATG* gene transcription as a general readout for autophagy unless there is clear documentation that the change(s) correlates with autophagy activity.

**7. Autophagic protein degradation.** Protein degradation assays represent a well-established methodology for measuring autophagic flux, and they allow good quantification. The general strategy is first to label cellular proteins by incorporation of a radioactive amino acid (e.g., [<sup>14</sup>C]-leucine, [<sup>14</sup>C]-valine or [<sup>35</sup>S]-methionine; although valine may be preferred over leucine due to the strong inhibitory effects of the latter on autophagy), preferably for a long time to achieve sufficient labeling of the long-lived proteins that best represent autophagic substrates, and then to follow this with a long cold-chase so that the assay starts well after labeled short-lived proteins are degraded (which occurs predominantly via the proteasome). Next, the time-dependent release of acid-soluble radioactivity from the labeled protein in intact cells or perfused organs is measured.<sup>3,472,473</sup> Note that the inclusion of the appropriate unlabeled amino acid (i.e., valine, leucine or methionine) in the starvation medium at a concentration equivalent to that of other amino acids in the chase medium is necessary; otherwise, the released [<sup>14</sup>C]-amino acid is effectively re-incorporated into cellular proteins, which results in a significant underestimation of protein degradation. The turnover of specific proteins can also be measured in a pulse-chase regimen using the Tet-ON/OFF system and subsequent western blot analysis.<sup>474,475</sup>

In this type of assay a considerable fraction of the measured degradation will be non-autophagic, and thus it is important to also measure, in parallel, cell samples treated with autophagy-suppressive concentrations of 3-MA or amino acids, or obtained from mutants missing central *ATG* components (however, the latter assumes that non-autophagic proteolytic activity remains unchanged, which is unlikely); these values are then subtracted from the total. The complementary approach of using compounds that block other degradative pathways, such as proteasome inhibitors, may cause unexpected results and should generally be avoided due to crosstalk among the degradative systems. For example, blocking proteasome function may activate

autophagy.<sup>476-479</sup> Thus, when using inhibitors it is critical to know whether the inhibitors being used alter autophagy in the particular cell type and context being examined. In addition, because 3-MA could have some autophagy-independent effects in particular settings it is advisable to verify that the 3-MA-sensitive degradation is also sensitive to general lysosomal inhibitors (such as NH<sub>4</sub>Cl or leupeptin).

The use of stable isotopes, such as <sup>13</sup>C and <sup>15</sup>N, in quantitative mass spectrometry-based proteomics allows the recording of degradation rates of thousands of proteins simultaneously. These assays may be applied to autophagy-related questions enabling researchers to investigate differential effects in global protein or even organelle degradation studies.<sup>480,481</sup> SILAC (stable isotope labeling with amino acids in cell culture) can also provide comparative information between different treatment conditions, or between a wild type and mutant.

Another assay that could be considered relies on the limited proteolysis of a betaine homocysteine methyltransferase (BHMT) fusion protein. The 44-kDa full-length BHMT protein is cleaved in hepatocyte amphisomes in the presence of leupeptin to generate 32-kDa and 10-kDa fragments.<sup>482-485</sup> Accumulation of these fragments is time-dependent and is blocked by treatment with autophagy inhibitors. A modified version of this marker, GST-BHMT, can be expressed in other cell lines where it behaves similar to the wild-type protein.<sup>486</sup> Other substrates may be considered for similar types of assays. For example, the neomycin phosphotransferase II-GFP (NeoR-GFP) fusion protein is a target of autophagy.<sup>487</sup> Transfection of lymphoblastoid cells with a plasmid encoding NeoR-GFP followed by incubation in the presence of 3-MA leads to an accumulation of the NeoR-GFP protein as measured by flow cytometry.<sup>488</sup>

A similar western blot assay is based on the degradation of a cytosolic protein fused to GFP. This method has been used in yeast and Dictyostelium cells using GFP-PgkA and GFP-Tkt-1 (phosphoglycerate kinase and transketolase, respectively). In this case the relative amount of the free GFP and the complete fusion protein is the relevant parameter for quantification; although it may not be possible to detect clear changes in the amount of the full-length chimera, especially under conditions of limited flux.<sup>29,36</sup> As described above for the marker GFP-Atg8/LC3, nonsaturating levels of lysosomal inhibitors are also needed in Dictyostelium cells to slow down the autophagic degradation, allowing the accumulation and detection of free GFP. It should be noted that this method monitors bulk autophagy since it relies on the passive transit of a cytoplasmic marker to the lysosome. Consequently, it is important to determine that the marker is distributed homogeneously in the cytoplasm.

One of the most useful methods for monitoring autophagy in *S. cerevisiae* is the Pho8Δ60 assay. *PHO8* encodes the vacuolar alkaline phosphatase, which is synthesized as a zymogen before finally being transported to and activated in the vacuole.<sup>489</sup> A molecular genetic modification that eliminates the first 60 amino acids prevents the mutant (Pho8Δ60) from entering the endoplasmic reticulum, leaving the zymogen in the cytosol. When autophagy is induced, the mutant zymogen is delivered to the vacuole nonselectively inside autophagosomes along with other

cytoplasmic material. The resulting activation of the zymogen can be easily measured by assays for alkaline phosphatase.<sup>178</sup> To minimize background activity, it is preferable to have the gene encoding cytosolic alkaline phosphatase (*PHO13*) additionally deleted (although this is not necessary when assaying certain substrates).

**Cautionary notes:** Measuring the degradation of long-lived proteins requires prior radiolabeling of the cells, and subsequent separation of acid-soluble from acid-insoluble radioactivity. The labeling can be done with relative ease both in cultured cells, and in live animals.<sup>3</sup> In cells, it is also possible to measure the release of an unlabeled amino acid by chromatographic methods, thereby obviating the need for prelabeling;<sup>490</sup> however, it is important to keep in mind that amino acid release is also regulated by protein synthesis, which in turn is modulated by many different factors. In either case, one potential problem is that the released amino acid may be further metabolized. For example, branched chain amino acids are good indicators of proteolysis in hepatocytes, but not in muscle cells where they are further oxidized (Meijer AJ, personal communication). In addition, the amino acid can be reincorporated into protein; for this reason, such experiments can be performed in the presence of cycloheximide, but this raises additional concerns (see *Turnover of autophagic compartments*). In the case of labeled amino acids, a nonlabeled chase is added where the tracer amino acid is present in excess (being cautious to avoid using an amino acid that inhibits autophagy), or by use of single pass perfused organs or superfused cells.<sup>491,492</sup> The perfused organ system also allows for testing the reversibility of effects on proteolysis and the use of autophagy-specific inhibitors in the same experimental preparation, which are crucial controls for proper assessment.

If the autophagic protein degradation is low (as it will be in cells in replete medium), it may be difficult to measure it reliably above the relatively high background of non-autophagic degradation. It should also be noted that the usual practice of incubating the cells under “degradation conditions,” that is, in a saline buffer, indicates the potential autophagic *capacity* (maximal attainable activity) of the cells rather than the autophagic *activity* that prevails in vivo or under rich culture conditions. Finally, inhibition of a particular degradative pathway is typically accompanied by an increase in a separate pathway as the cell attempts to compensate for the loss of degradative capacity.<sup>158,478,493</sup> This compensation might interfere with control measurements under conditions that attempt to inhibit macroautophagy; however, as the latter is the major degradative pathway, the contributions of other types of degradation over the course of this type of experiment are most often negligible.

The Pho8Δ60 assay requires standard positive and negative controls (such as an *atg1Δ* strain), and care must be taken to ensure the efficiency of cell lysis. Glass beads lysis works well in general, provided that the agitation speed of the instrument is adequate. Instruments designed for liquid mixing with lower speeds should be avoided. We also recommend against holding individual sample tubes on a vortex, as it is difficult to maintain reproducibility; devices or attachments are available to allow multiple tubes to be agitated simultaneously.

**Conclusion:** Measuring the turnover of long-lived proteins is a good method for determining autophagic flux.

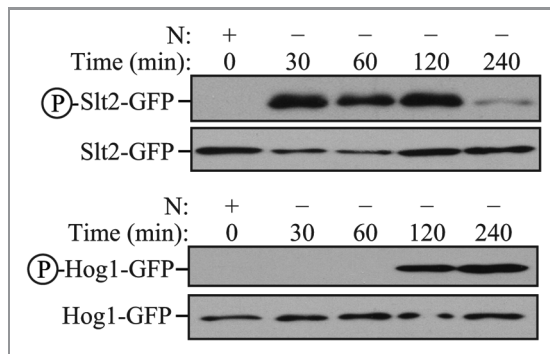
**8. Selective types of autophagy.** Although autophagy can be nonspecific, in particular during starvation, there are many examples of selective types of autophagy.

*a. The Cvt pathway, mitophagy, pexophagy and piecemeal microautophagy of the nucleus in yeast.* The precursor form of aminopeptidase I (prApe1) is the major cargo of the Cvt pathway in yeast, a biosynthetic autophagy-related pathway.<sup>82</sup> The pro-peptide of prApe1 is proteolytically cleaved upon vacuolar delivery, and the resulting shift in molecular mass can be monitored by western blot. Under starvation conditions, prApe1 can enter the vacuole through nonspecific autophagy, and thus has been used as a marker for both the Cvt pathway and autophagy. The yeast Cvt pathway is unique in that it is a biosynthetic route that utilizes the autophagy-related protein machinery, whereas other types of selective autophagy are degradative. The latter include pexophagy, mitophagy, reticulophagy, ribophagy and xenophagy, and each process has its own marker proteins, although these are typically variations of other assays used to monitor the Cvt pathway or autophagy. One common type of assay involves the processing of a GFP chimera similar to the GFP-Atg8/LC3 processing assay (see *GFP-Atg8/LC3 lysosomal delivery and proteolysis*). For example, yeast pexophagy utilizes the processing of Pex14-GFP and Pot1 (thiolase)-GFP,<sup>494,495</sup> whereas mitophagy can be monitored by the generation of free GFP from Om45-GFP, Idh1-GFP, Idp1-GFP or mito-DHFR-GFP.<sup>496-500</sup> Localization of these mitochondrially-targeted proteins (or specific MitoTracker dyes) or similar organelle markers such as those for the peroxisome [e.g., GFP-SKL with Ser-Lys-Leu at the C terminus that acts as a peroxisomal targeting signal, acyl-CoA oxidase 3 (Aox3-EYFP) that allows simultaneous observation of peroxisome-vacuole dynamics with the single FITC filter set, or GFP-catalase] can also be followed by fluorescence microscopy.<sup>412,495,501-503</sup> In addition, yeast mitophagy requires both the Slt2 and Hog1 signaling

pathways; the activation and phosphorylation of Slt2 and Hog1 can be monitored with commercially available phospho-specific antibodies (Fig. 19).<sup>381</sup> It is also possible to monitor pexophagy in yeasts by the disappearance of activities of specific peroxisome markers such as catalase, alcohol oxidase or amine oxidase in cell-free extracts,<sup>504</sup> or permeabilized cell suspensions. Catalase activity, however, is a useful marker only when peroxisomal catalases are the only such enzymes present. In *S. cerevisiae* there are two genes encoding catalase activity, and only one of these gene products is localized in peroxisomes. Plate assays for monitoring the activity of peroxisomal oxidases in yeast colonies are also available.<sup>501,505</sup> The decrease in the level of endogenous proteins such as alcohol oxidase or Pot1 can be followed by western blotting,<sup>412,506-509</sup> TEM,<sup>510</sup> fluorescence microscopy<sup>412,511,512</sup> or laser confocal scanning microscopy of GFP-labeled peroxisomes.<sup>513,514</sup>

In yeast, nonspecific autophagy can be induced by nitrogen starvation conditions, whereas degradative types of selective autophagy generally require a carbon source change or ER stress for efficient induction. For example, to induce a substantial level of mitophagy, cells need to be precultured in a nonfermentable carbon source such as lactate or glycerol to stimulate the proliferation of mitochondria. After sufficient mitochondria proliferation, shifting the cells back to a fermentable carbon source such as glucose will cause the autophagic degradation of superfluous mitochondria.<sup>497</sup> It should be noted that in addition to carbon source change, simultaneous nitrogen starvation is also required for efficient mitophagy induction. This is possibly because excessive mitochondria can be segregated into daughter cells by cell division if growth continues.<sup>478</sup> A similar carbon source change from oleic acid or methanol to ethanol or glucose (with or without nitrogen starvation) can be used to assay for pexophagy.<sup>515</sup> In addition, mitophagy can also be induced by culturing the cells in a nonfermentable carbon source to post-log phase. In this case, mitophagy may be induced because the energy demand is lower at post-log phase and the mitochondrial mass exceeds the cell's needs.<sup>75,516,517</sup> It has been suggested by several workers in the field that this type of mitophagy, also known as "stationary phase mitophagy," reflects a quality-control function that culls defective mitochondria that accumulate in nondividing, respiring cells.<sup>518</sup> Similar, pexophagy can be induced by culturing the cells in a peroxisome proliferation medium to post-log phase (Farré J-C, unpublished results). Along these lines, it should also be realized that selective types of autophagy continuously occur at a low level under noninducing conditions. Thus, organelles such as peroxisomes have a finite life span and are turned over at a slow rate by autophagy-related pathways.<sup>519</sup>

Piecemeal microautophagy of the nucleus (PMN, also micro-nucleophagy) is another selective autophagic subtype, which targets portions of the nucleus for degradation.<sup>520-522</sup> In *S. cerevisiae*, the nuclear outer membrane, which is continuous with the nuclear ER, forms contact sites with the vacuolar membrane. These nucleus-vacuole junctions (NVJs) are generated by interaction of the outer nuclear membrane protein Nvj1 with the vacuolar protein Vac8.<sup>523</sup> Nvj1 further recruits the ER-membrane protein Tsc13, which is involved in the synthesis of



**Figure 19.** *S. cerevisiae* cells were cultured to mid-log phase and shifted to SD-N for the indicated times. Samples were taken before (+) and at the indicated times after (-) nitrogen starvation. Immunoblotting was done with anti-phospho-Slt2 and anti-phospho-Hog1 antibody. This figure was modified from data previously published in reference 381 and is reproduced by permission of the American Society for Cell Biology, copyright 2011.

very-long-chain fatty acids (VLCFAs) and Osh1, a member of a family of oxysterol-binding proteins. Upon starvation the NVJs bulge into the vacuole and subsequently a PMN-vesicle pinches off into the vacuole. PMN vesicles thus contain nuclear material and are limited by three membranes with the outermost derived from the vacuole, and the two inner ones from the nuclear ER. It is not clear which nuclear components are removed by PMN, but since PMN is not a cell death mechanism per se, most likely superfluous material is recycled. During PMN the NVJs are selectively incorporated into the PMN vesicles and degraded. Accordingly, PMN can be monitored using the proteins that are associated with the NVJs as markers. To quantitatively follow PMN, an assay analogous to the above-described GFP-Atg8/LC3 processing assay has been established using either GFP-Osh1 or Nvj1-GFP. These GFP chimeras are, together with the PMN-vesicles, degraded in the vacuole. Thus, the formation of the relatively proteolysis-resistant GFP detected in western blots correlates with the PMN rate. In fluorescence microscopy, PMN can be visualized with the same constructs, and a chimera of mCherry fused to a nuclear localization signal (NLS-mCherry) can also be used. To assure that the measured PMN rate is indeed due to selective micronucleophagy, appropriate controls such as cells lacking Nvj1 or Vac8 should be included. Detailed protocols for the described assays are provided in reference 524.

*b. Reticulophagy and ribophagy.* Activation of the UPR in the ER in yeast induces a type of selective macroautophagy of the ER.<sup>525-527</sup> This process is termed reticulophagy to be consistent with the terms pexophagy and mitophagy.<sup>527</sup> Reticulophagy-associated autophagosomes are lamellar-membraned structures that contain ER proteins. In theory, since reticulophagy is selective, it should be able to sequester parts of the ER that are damaged, and eliminate protein aggregates that cannot be removed in other ways. Reticulophagy may also serve to limit the UPR, by reducing the ER to a normal level after a particular stress condition has ended. Some of the mutated dysferlin protein, LGMB2B/Miyoshi type muscle dystrophy, which accumulates in the ER, is degraded by ER-stress mediated reticulophagy.<sup>528</sup> In addition to activation of the UPR, PtdIns3P and its binding proteins could be good markers for reticulophagy.<sup>437</sup> Autophagy is also used for the selective removal of ribosomes.<sup>529</sup> This process can be monitored by western blot, following the generation of free GFP from Rpl25-GFP or the disappearance of ribosomal subunits such as Rps3. Vacuolar localization of Rpl25-GFP can also be seen by fluorescence microscopy.

**Cautionary notes:** The Cvt pathway has been demonstrated to occur only in yeast. In addition, the sequestration of prApe1 is specific, even under starvation conditions, as it involves the recognition of the propeptide by a receptor, Atg19, which in turn interacts with the scaffold protein Atg11.<sup>530,531</sup> Thus, unless the propeptide is removed, prApe1 is recognized as a selective substrate. Overexpression of prApe1 saturates import by the Cvt pathway, and the precursor form accumulates, but is rapidly matured upon autophagy induction.<sup>218</sup> In addition, mutants such as *vac8Δ* and *tlg2Δ* accumulate prApe1 under rich conditions, but not during autophagy.<sup>380,532</sup> Accordingly, it is possible to monitor the processing of prApe1 when overexpressed, or in

certain mutant strains to follow autophagy induction. However, even the latter conditions may be misleading, as they do not indicate the size of the autophagosome. The Cvt complex (prApe1 bound to Atg19) is smaller than typical peroxisomes or mitochondrial fragments that are subject to autophagic degradation. Accordingly, particular mutants may display complete maturation of prApe1 under autophagy-inducing conditions, but may still have a defect in other types of selective autophagy, as well as being unable to induce a normal level of nonspecific autophagy.<sup>63</sup> For this reason, it is good practice to evaluate autophagosome size and number by TEM. Actually, it is much simpler to monitor autophagic bodies (rather than autophagosomes) in yeast. First, the vacuole is easily identified, making the identification of autophagic bodies much simpler. Second, autophagic bodies can be accumulated within the vacuole, allowing for an increased sample size. It is best to use a strain background that is *pep4Δ vps4Δ* to prevent the breakdown of the autophagic bodies, and to eliminate confounding vesicles from the multivesicular body pathway. One caveat to the detection of autophagic bodies, however, is that they may coalesce in the vacuole lumen, making it difficult to obtain an accurate quantification.

In general, when working with yeast it is preferable to use strains that have the marker proteins integrated into the chromosome rather than relying on plasmid-based expression, because plasmid numbers can vary from cell to cell. The GFP-Atg8, or similar, processing assay is easy to perform and is suitable for analysis by microscopy as well as western blotting; however, particular care is needed to obtain quantitative data for GFP-Atg8, Pex14-GFP or Om45-GFP, etc. processing assays (see cautionary notes for *GFP-Atg8/LC3 lysosomal delivery and proteolysis*). An alternative is an organelle targeted Pho8Δ60 assay. For example, mitoPho8Δ60 can be used to quantitatively measure mitophagy.<sup>498</sup> In addition, for the GFP-Atg8 processing assay, 2 h of starvation is generally sufficient to detect a significant level of free (i.e., vacuolar) GFP by western blotting as a measure of nonselective autophagy. For selective types of autophagy, the length of induction needed for a clearly detectable free GFP band will vary depending on the rate of cargo delivery/degradation. Usually 6 h of mitophagy induction is needed to be able to detect free GFP (e.g., from Om45-GFP) by western blot under starvation conditions, whereas stationary phase mitophagy typically requires 3 d before a free GFP band is observed.

*c. Vacuole import and degradation pathway.* In yeast, gluconeogenic enzymes such as fructose-1,6-bisphosphatase (Fbp1, also called FB Pase), malate dehydrogenase (Mdh2), isocitrate lyase (Icl1) and phosphoenolpyruvate carboxykinase (Pck1) constitute the cargo of the vacuole import and degradation (Vid) pathway.<sup>533</sup> These enzymes are induced when yeast cells are glucose starved (grown in a medium containing 0.5% glucose and potassium acetate). Upon replenishing these cells with fresh glucose (a medium containing 2% glucose), these enzymes are degraded in either the proteasome<sup>534-536</sup> or the vacuole<sup>533,537</sup> depending on the duration of starvation. Following glucose replenishment after 3 d glucose starvation, the gluconeogenic enzymes are delivered to the vacuole for degradation.<sup>538</sup> These enzymes are sequestered in specialized 30- to 50-nm vesicles

called Vid vesicles.<sup>539</sup> Vid vesicles can be purified by fractionation and gradient centrifugation; western blotting analysis using antibodies against organelle markers and Fbp1, and the subsequent verification of fractions by EM facilitate their identification.<sup>539</sup> Furthermore, the amount of marker proteins in the cytosol compared with the Vid vesicles can be examined by differential centrifugation. In this case, yeast cells are lysed and subjected to differential centrifugation. The Vid vesicle-enriched pellet fraction and the cytosolic supernatant fraction are examined with antibodies against Vid24, Vid30, Sec28 and Fbp1.<sup>540-542</sup>

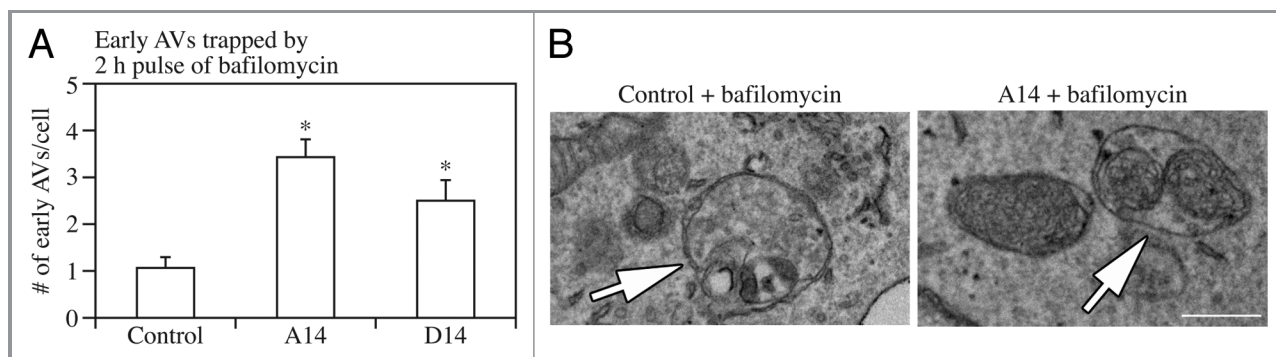
Vid/endosomes containing their cargo aggregate around endocytic vesicles forming on the plasma membrane and are released into the cytoplasm. The Vid/endosomes can be purified by fractionation and density gradient centrifugation.<sup>543</sup> The fractions containing purified Vid/endosomes can be identified by western blot analysis using antibodies against Vid24, Fbp1 and the endosomal marker Pep12. The distribution of Vid vesicles containing cargo destined for endosomes, and finally for the vacuole, can be examined using FM 4-64, a lipophilic dye that stains endocytic compartments and the vacuole limiting membrane.<sup>544</sup> In these experiments, starved yeast cells are replenished with fresh glucose and FM 4-64, and cells are collected at appropriate time points for examination by fluorescence microscopy.<sup>541</sup> The site of degradation of the cargo in the vacuole can be determined by studying the distribution of Fbp1-GFP, or other Vid cargo markers in wild-type and *pep4Δ* cells.<sup>545</sup> Cells can also be examined for the distribution of Fbp1 at the ultrastructural level by immuno-TEM.<sup>543</sup>

As actin patch polymerization is required for the delivery of cargo to the vacuole in the Vid pathway, distribution of Vid vesicles containing cargo and actin patches can be examined by actin staining (with phalloidin conjugated to rhodamine) using fluorescence microscopy.<sup>543</sup> The distribution of GFP tagged protein and actin is examined by fluorescence microscopy. GFP-Vid24, Vid30-GFP and Sec28-GFP colocalize with actin during prolonged glucose starvation and for up to 30 min following

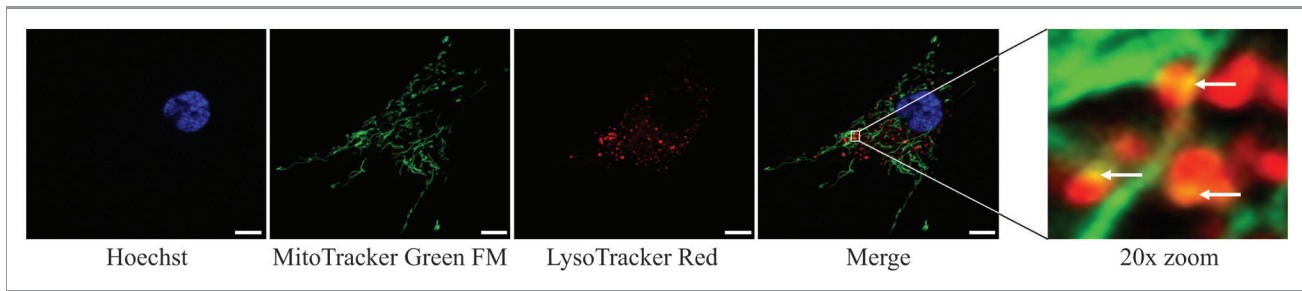
glucose replenishment in wild-type cells; however, colocalization is less obvious by the 60 min time point.<sup>540,543</sup>

*d. Mammalian mitophagy and peroxisome degradation.* There is no consensus at the present time with regard to the best method for monitoring mammalian mitophagy. As with any organelle-specific form of autophagy, it is necessary to demonstrate: (1) increased levels of autophagosomes containing mitochondria, (2) maturation of these autophagosomes to culminate with mitochondrial degradation, which can be blocked by specific inhibitors of autophagy or of lysosomal degradation, and (3) whether the changes are due to selective mitophagy or increased mitochondrial degradation during generalized autophagy. Techniques to address each of these points have been reviewed.<sup>41</sup>

Ultrastructural analysis at early time points can be used to establish selective mitophagy, although a maturation inhibitor may be needed to trap early autophagosomes with recognizable cargo (Fig. 20). Depending on the use of specific imaging techniques, dyes for living cells or antibodies for fixed cells have to be chosen. In any case, transfection of the autophagosomal marker GFP-LC3 and visualization of mitochondria (independent of their mitochondrial membrane potential) makes it possible to determine the association of these two cellular components. This may appear as fluorescence colocalization or as rings of GFP-LC3 surrounding mitochondria in higher resolution images. For live cell imaging microscopy, a method that marks mitochondria independently of mitochondrial membrane potential, such as MitoTracker<sup>®</sup> Green FM or transfection with a matrix-targeted fluorescent protein, should be used to detect mitochondrial structures. Antibodies that specifically recognize mitochondrial proteins such as VDAC1, TOMM20/TOM20 or complex IV subunit I may be used to visualize mitochondria in immunohistochemical experimental procedures.<sup>546,547</sup> Colocalization analyses of mitochondria and autophagosomes provide an indication of the degree of sequestration. TEM can be used to demonstrate the presence of mitochondria within autophagosomes (referred



**Figure 20.** Autophagosomes with recognizable cargo are rare in cells. (A) To assess relative rates of autophagosome formation, the fusion inhibitor bafilomycin A<sub>1</sub> (10 nM) was applied for 2 h prior to fixation with 2% glutaraldehyde in order to trap newly formed autophagosomes. Two different *PINK1* shRNA lines exhibit increased AV formation over 2 h compared with the control shRNA line. \**p* > 0.05 vs. Control. (B) Autophagosomes in bafilomycin A<sub>1</sub>-treated control cells contain a variety of cytoplasmic structures (left, arrow), while mitochondria comprise a prominent component of autophagosomes in A14 bafilomycin A<sub>1</sub>-treated (*PINK1* shRNA) cells (right, arrow). Scale bar, 500 nm. These data indicate induction of selective mitophagy in *PINK1*-deficient cells. This figure was modified from Figure 2 published in Chu CT. A pivotal role for *PINK1* and autophagy in mitochondrial quality control: implications for Parkinson disease. *Hum Mol Genet* 2010; 19:R28–37.



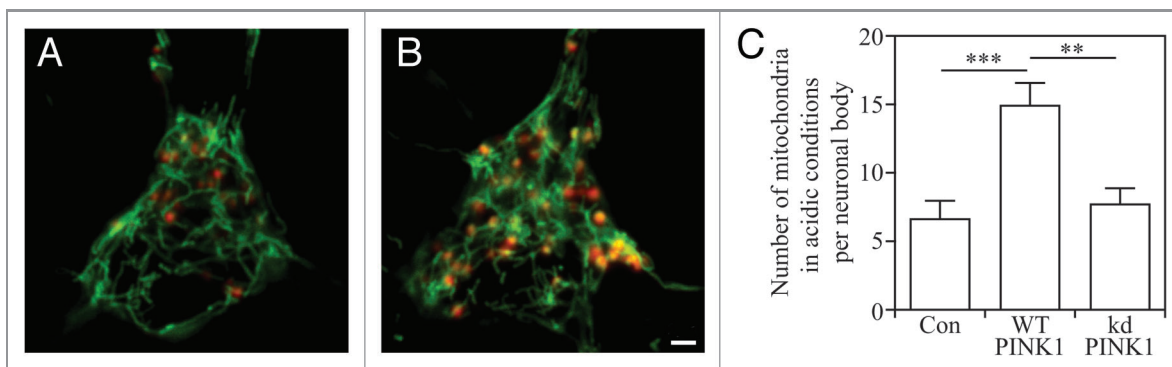
**Figure 21.** Human fibroblasts showing colocalization of mitochondria with lysosomes. The degree of colocalization of mitochondria with lysosomes in human fibroblasts was measured via live cell imaging microscopy at 37°C and 5% CO<sub>2</sub> atmosphere using the ApoTome<sup>®</sup> technique. LysoTracker<sup>®</sup> Red DND-99 staining was applied to mark lysosomal structures (red), and MitoTracker<sup>®</sup> Green FM to visualize mitochondria (green). Hoechst 33342 dye was used to stain nuclei (blue). A positive colocalization is indicated by yellow signals (merge) due to the overlap of LysoTracker<sup>®</sup> Red and MitoTracker<sup>®</sup> Green staining (white arrows). Scale bar, 10 μm. Statistical evaluation is performed by calculating the Pearson's coefficient for colocalizing pixels. Image provided by L. Burbulla and R. Krüger.

to as mitophagosomes during mitophagy), and this can be coupled with bafilomycin A<sub>1</sub> treatment to prevent fusion with the lysosome.<sup>41</sup>

The fusion process of mitophagosomes with hydrolase-containing lysosomes represents the next step in the degradation process. To monitor the amount of fused organelles via live cell imaging microscopy, MitoTracker<sup>®</sup> Green FM, as a mitochondrial marker without significant membrane potential dependence and LysoTracker<sup>®</sup> Red DND-99 may be used to visualize the fusion process (Fig. 21). Independent of the cell-type specific concentration used for both dyes, we recommend exchanging MitoTracker<sup>®</sup> Green FM with normal medium after incubation with the dye, whereas it is best to maintain the LysoTracker<sup>®</sup> Red staining in the incubation medium during the acquisition of images. For immunocytochemical experiments, antibodies specific for mitochondrial proteins and an antibody against the lysosomal-associated membrane protein 1 (LAMP1) can be used. Overlapping signals appear as a merged color and can be used as indicators for successful fusion of autophagosomes that contain mitochondria with lysosomal structures.<sup>548</sup> To measure the correlation between two variables by imaging techniques, such as the

colocalization of two different stainings, we recommend some form of correlation analysis to assess the value correlating with the strength of the association. This may use, for example, ImageJ software, or other colocalization scores that can be derived from consideration not only of pixel colocalization, but also from a determination that the structures have the appropriate shape. During live-cell imaging, the two structures should move together in more than one frame. Mitophagy can also be quantitatively monitored using a mitochondria-targeted version of the pH-dependent Keima protein.<sup>549</sup> The peak of the excitation spectrum of the protein shifts from 440 nm to 586 nm when mitochondria are delivered to acidic lysosomes, which allows easy quantification of mitophagy (Fig. 22). However, it should be noted that long exposure times of the specimen to intense laser light lead to a similar spectral change.

The third and last step of the degradation process is the monitoring of the amount of remaining mitochondria by analyzing the mitochondrial mass. This final step provides the opportunity to determine the efficiency of degradation of dysfunctional, aged or impaired mitochondria. Mitochondrial mass can either be measured by a FACS technique using MitoTracker<sup>®</sup>



**Figure 22.** Detection of mitophagy in primary cortical neurons using mitochondria-targeted Keima. Neurons transfected with mito-Keima were visualized using 458 nm (green, mitochondria at neutral pH) and 561 nm (red, mitochondria in acidic pH) laser lines and 575 nm band pass filter. Compared with the control (A) wild-type PINK1 overexpression (B) increases the number of the mitochondria exposed to acidic conditions. Scale bar, 2 μm. (C) Quantification of red dots suggests increased mitophagy in wild-type PINK1 but not in the kinase dead PINK1<sup>K219M</sup>-overexpressing neurons. Figure provided by V. Choubey and A. Kaasik.

Green FM or, on a single cell basis, by either live cell imaging or immunocytochemistry with use of MitoTracker<sup>®</sup> Green FM or MitoTracker<sup>®</sup> Red FM for the former, and antibodies specifically raised against different mitochondrial proteins for the latter. Immunoblot analysis of the levels of mitochondrial proteins from different mitochondrial subcompartments is valuable for validating the data from FACS or microscopy studies. EM can also be used to verify loss of entire mitochondria, and PCR (or fluorescence microscopy) to quantify mitochondrial DNA.

In addition to monitoring the steady-state levels of different steps of mitophagy, investigation of the mitophagic flux is needed to decipher which steps within this process potentially fail to result in efficient mitochondrial degradation. Therefore, appropriate treatment may be applied to prevent mitochondrial degradation at distinct steps of the process.

Certain cellular models require stress conditions to measure the mitochondrial degradation capacity, as basal levels are too low to reliably assess organelle clearance. Hence, it may be useful to pretreat the cells with uncoupling agents, such as CCCP that stimulate mitochondrial degradation and allow measurements of mitophagic activity; however, it should be kept in mind that, although helpful to stimulate mitochondrial degradation, this treatment is not physiological. Another method to induce mitophagy is by expressing and activating a mitochondrially-localized fluorescent protein photosensitizer such as Killer Red.<sup>550</sup> The excitation of Killer Red results in an acute increase of superoxide that causes mitochondrial damage resulting in mitophagy (Kim PK, unpublished results). The advantage of using a genetically encoded photosensitizer is that it allows for both spatial and temporal control in inducing mitophagy.

Relatively little work has been done in the area of mammalian peroxisome degradation by autophagy, and at present it is not known if this is a selective process. Typically, peroxisomes are induced by treatment with hypolipidemic drugs such as clofibrate or dioctyl phthalate, and degradation is induced by drug withdrawal, although starvation without prior proliferation can also be used. Loss of peroxisomes can be followed enzymatically or by immunoblot, monitoring enzymes such as fatty acyl-CoA oxidase (note that this enzyme is sometimes abbreviated “AOX,” but should not be confused with the enzyme alcohol oxidase that is frequently used in assays for yeast pexophagy) or catalase, and also by EM.<sup>551,552</sup> Finally, a HaloTag<sup>®</sup>-PTS1 marker that is targeted to peroxisomes has been used to fluorescently label the organelle.<sup>553</sup>

**Cautionary notes:** There are many assays that can be used to monitor specific types of autophagy, but caution must be used in choosing an appropriate marker(s). It is best to monitor more than one protein, and to include an inner membrane or matrix component in the analysis. In particular, it is not sufficient to follow a single mitochondrial outer membrane protein because these can be degraded independently of mitophagy. Although the localization of PARK2/PARKIN to mitochondria as monitored by fluorescence microscopy is associated with the early stages of protonophore uncoupler (CCCP)-driven mitochondria degradation,<sup>169</sup> this by itself cannot be used as a marker for mitophagy, as these events can be dissociated.<sup>554</sup> PARK2

translocates to damaged mitochondria and ubiquitinates a wide range of outer membrane proteins including VDAC1, MFN1/2 and TOMM20.<sup>547,555-557</sup> This results in the preferential degradation of mitochondrial outer membrane proteins by the proteasome, while inner membrane proteins and mitochondrial DNA<sup>558</sup> remain intact. Monitoring loss of a single protein such as TOMM20 by western blot or fluorescence microscopy to follow mitophagy may thus be misleading.<sup>555</sup> MitoTracker dyes are widely used to stain mitochondria and, when colocalized with GFP-LC3, they can function as a marker for mitophagy. However, staining with certain MitoTracker dyes depends on mitochondrial activity and membrane potential, so that damaged, or sequestered nonfunctional mitochondria may not be stained.

Although it is widely assumed that macroautophagy is the major mechanism for degradation of entire organelles, there are multiple mechanisms that may account for the disappearance of mitochondrial markers. These include proteasomal degradation of outer membrane proteins and/or proteins that fail to correctly translocate into the mitochondria, degradation due to proteases within the mitochondria, and reduced biosynthesis or import of proteins. In addition to mitophagy, mitochondria can be eliminated by extrusion from the cell (mitoptosis).<sup>559</sup> Thus, it is advisable to use a variety of complementary methods to monitor mitochondria loss including TEM, single cell analysis of LC3 fluorescent puncta, and western blot, in conjunction with flux inhibitors and specific inhibitors of autophagy induction compared with inhibitors of the other major degradation systems (see cautions in *Autophagy inhibitors and inducers*).

Likewise, although the mechanism(s) of peroxisome degradation in mammals awaits further elucidation, it can occur by both autophagic and proteasome-dependent mechanisms.<sup>560</sup> Thus, controls are needed to determine the extent of degradation that is due to the proteasome. Moreover, two additional degradation mechanisms have been suggested: the action of the peroxisome-specific Lon protease and the membrane disruption effect of 15-lipoxygenase.<sup>561</sup>

*e. Aggrephagy.* Aggrephagy is the selective removal of aggregates by macroautophagy.<sup>562</sup> This process can be followed in vitro (in cell culture) and in vivo (in mice) by monitoring the levels of an aggregate-prone protein such as an expanded polyglutamine (polyQ)-containing protein or mutant SNCA ( $\alpha$ -synuclein). Levels are quantified by immunofluorescence or traditional immunoblot. Similarly, fluorescently tagged aggregated proteins such as polyQ80-CFP can be monitored via immunoblot and immunofluorescence. A polyQ80-luciferase reporter, which forms aggregates, can also be used to follow aggrephagy.<sup>563</sup> A nonaggregating polyQ19-luciferase or untagged full-length luciferase serves as a control. The ratio of luciferase activity from these two constructs can be calculated to determine autophagic flux.

Autophagic degradation of endogenous aggregates such as lipofuscin can in some cell types be monitored by fluorescence microscopy, utilizing the autofluorescence of lipofuscin particles. The amount of lipofuscin in primary human adipocytes can be reduced by activation of autophagy, and the amount of lipofuscin is dramatically reduced in adipocytes from patients with type 2 diabetes and chronically enhanced autophagy.<sup>209</sup>

**Cautionary notes:** Caution must be used when performing immunoblots of aggregated proteins, as many protein aggregates fail to enter the resolving gel and are retained in the stacking gel. In addition, the polyQ80-luciferase in the aggregated state lacks luciferase activity whereas soluble polyQ80-luciferase retains activity. Therefore, caution must be used when interpreting results with these vectors, as treatments that increase aggregation or enhance protein aggregation can lead to a decrease in luciferase activity.<sup>564</sup> Finally, soluble polyQ reporters can be degraded by the proteasome; thus, changes in the ratio of polyQ19-luciferase:polyQ80-luciferase may also reflect proteasomal effects and not just changes in autophagic flux.

*f. Xenophagy.* The macroautophagy pathway has emerged as an important cellular factor in both innate and adaptive immunity. Many *in vitro* and *in vivo* studies have demonstrated that genes encoding macroautophagy components are required for host defense against infection by bacteria, parasites and viruses. Macroautophagy may be defective in human diseases such as inflammatory bowel disease, since genes encoding essential macroautophagy components have been linked to the disease. Xenophagy is often used as a term to describe autophagy of microbial pathogens, mediating their capture and delivery to lysosomes for degradation. Since xenophagy presents an immune defense, it is not surprising that microbial pathogens have evolved strategies to overcome it. The interactions of such pathogens with the autophagy system of host cells are complex and have been the subject of several excellent reviews.<sup>76-81,565-570</sup> Here we will make note of a few key considerations when studying interactions of microbial pathogens with the autophagy system.

LC3 is commonly used as a marker of macroautophagy. However, studies have established that LC3 can promote phagosome maturation independently of macroautophagy through LC3-associated phagocytosis (see cautionary notes in *Atg8/LC3 detection and quantification*). Other studies show that macroautophagy of *Salmonella Typhimurium* is dependent on ATG9, an essential macroautophagy protein, whereas LC3 recruitment to bacteria does not require ATG9.<sup>571</sup> In contrast, macroautophagy of these bacteria requires ubiquitination of target proteins (not yet identified) and recruitment of three ubiquitin-binding adaptor proteins, SQSTM1,<sup>572</sup> CALCOCO2/NDP52<sup>573</sup> and OPTN.<sup>574</sup> Therefore, the currently available criteria to differentiate LAP from macroautophagy include: (1) LAP involves LC3 recruitment to bacteria in a manner that requires reactive oxygen species production by an NADPH oxidase. It should be noted that most cells express at least one member of the NADPH oxidase (also known as NOX) family. Targeting expression of the common CYBA/p22<sup>phox</sup> subunit is an effective way to disrupt the NOX NADPH oxidases. Scavenging of reactive oxygen species by antioxidants such as resveratrol and  $\alpha$ -tocopherol is also an effective way to inhibit LAP. In contrast, N-acetylcysteine, which raises cellular glutathione levels, does not inhibit LAP.<sup>575</sup> (2) Macroautophagy of bacteria requires ATG9, while LAP apparently does not.<sup>571</sup> (3) LAP involves single-membrane structures. For LAP, CLEM (with LC3 as a marker) is expected to show single-membrane structures that are LC3<sup>+</sup> LAP.<sup>131</sup> In

contrast, macroautophagy is expected to generate double-membrane structures surrounding cargo (which may include single membrane phagosomes, giving rise to triple-membrane structures<sup>571</sup>). (4) Macroautophagy of bacteria requires protein ubiquitination and ubiquitin-binding adaptors (SQSTM1, CALCOCO2, OPTN, and possibly others), whereas LAP does not. It is anticipated that more specific markers of LAP will be identified as these phagosomes are further characterized.

Nonmotile *Listeria monocytogenes* can be targeted to double-membrane autophagosomes upon antibiotic treatment,<sup>576</sup> which indicates that macroautophagy serves as a cellular defense against microbes in the cytosol. However, subsequent studies have revealed that macroautophagy can also target pathogens within phagosomes, damaged phagosomes or the cytosol. Therefore, when studying microbial interactions by EM, many structures can be visualized, with any number of membranes encompassing microbes, all of which may be LC3<sup>+</sup>.<sup>577</sup> As discussed above, single-membrane structures that are LC3<sup>+</sup> may arise through LAP, and we cannot rule out the possibility that both LAP and macroautophagy may operate at the same time to target the same phagosome.

Viruses can also be targeted by autophagy, and in turn can act to inhibit autophagy. For example, infection of a cell by influenza and dengue viruses<sup>578</sup> or enforced expression of the hepatitis B virus C protein<sup>579</sup> have profound consequences for autophagy, as viral proteins such as NS4A stimulate autophagy and protect the infected cell against apoptosis, thus extending the time in which the virus can replicate. Conversely, the herpes simplex virus ICP34.5 protein inhibits autophagy by targeting BECN1.<sup>580</sup> While the impact of ICP34.5's targeting of BECN1 on virus replication in cultured permissive cells is minimal, it has a significant impact upon pathogenesis *in vivo*, most likely through interfering with activation of CD4<sup>+</sup> T cells.<sup>581,582</sup> Care must be taken in determining the role of autophagy in virus replication, as some viruses such as vaccinia use double-membrane structures that form independently of the autophagy machinery.<sup>583</sup> Similarly, dengue virus replication, which appears to involve a double-membrane compartment, requires the ER rather than autophagosomes,<sup>584</sup> whereas coronaviruses use a non-lipidated version of LC3 (see *Atg8/LC3 detection and quantification*).<sup>137</sup> Yet another type of variation is seen with hepatitis C virus, which requires BECN1, ATG4B, ATG5 and ATG12 for initiating replication, but does not require these proteins once an infection is established.<sup>585</sup>

Finally, it is important to realize that there may be other macroautophagy-like pathways that have yet to be characterized. For example, in response to cytotoxic stress (treatment with etoposide), autophagosomes are formed in an ATG5- and ATG7-independent manner.<sup>26</sup> While this does not rule out involvement of other macroautophagy regulators/components in the formation of these autophagosomes, it does establish that the canonical macroautophagy pathway involving LC3 conjugation is not involved. In contrast, RAB9 is required for this alternative pathway, potentially providing a useful marker for analysis of these structures. Returning to xenophagy, *Mycobacterium tuberculosis* can be targeted to autophagosomes in



an ATG5-independent manner.<sup>586</sup> Furthermore, up to 25% of intracellular *Salmonella typhimurium* are observed in multi-lamellar membrane structures resembling autophagosomes in *Atg5*<sup>-/-</sup> MEFs.<sup>572</sup> These findings indicate that an alternate macroautophagy pathway is relevant to host-pathogen interactions. Moreover, differences are observed that depend on the cell type being studied. *Yersinia pseudotuberculosis* is targeted to autophagosomes where they can replicate in bone marrow-derived macrophages,<sup>587</sup> whereas in RAW264.7 and J774 cells, bacteria are targeted both to autophagosomes, and LC3-negative, single-membrane vacuoles (Lafont F, personal communication).

*g. Lipophagy.* The specific macroautophagic degradation of lipid droplets represents another type of selective autophagy.<sup>588</sup> Lipophagy requires the core autophagic machinery and can be monitored by following triglyceride content, or total lipid levels using BODIPY 493/503 or HCS LipidTOX neutral lipid stains with fluorescence microscopy, cell staining with Oil Red O, or ideally label-free techniques such as CARS or SRS microscopy. TEM can also be used to monitor lipid droplet size and number.

Cautionary notes: With regard to changes in the cellular neutral lipid content, the presence and potential activation of cytoplasmic lipases that are unrelated to lysosomal degradation must be considered.

*h. Zymophagy.* Zymophagy is a protective mechanism induced during pancreatitis that results in the selective degradation of activated zymogen granules, which are deleterious for the pancreatic acinar cells.<sup>589</sup> This process can be monitored by TEM, identifying autophagosomes containing secretory granules, by following SQSTM1 degradation by western blot, and by examining the subcellular localization of VMP1-EGFP, which relocates to granular areas of the cell upon zymophagy induction. Colocalization of trypsinogen (which is packaged within zymogen granules) and LC3, or of GFP-ubiquitin (which is recruited to the activated granules) with RFP-LC3 can also be observed by indirect or direct immunofluorescence microscopy, respectively.

*i. Allophagy.* In metazoans, mitochondria, and hence mitochondrial DNA, from the sperm is eliminated by an autophagic process. This process of allogeneic (nonself) organelle autophagy is termed “allophagy.”<sup>590,591</sup> During allophagy in *C. elegans*, both paternal mitochondria and membranous organelles (a sperm-specific membrane compartment) are eliminated by the 16-cell stage (100–120 min post-fertilization).<sup>592,593</sup> The degradation process can be monitored in living embryos with GFP::ubiquitin, which appears in the vicinity of the sperm chromatin (labeled for example with mCherry-histone H2B) on the membranous organelles within 3 min after fertilization. GFP fusions and antibodies specific for LGG-1 and LGG-2 (Atg8/LC3 homologs), which appear next to the sperm DNA, membranous organelles and mitochondria (labeled with CMXRos or mitochondria-targeted GFP) within 15 to 30 min post-fertilization, can be used to verify the autophagic nature of the degradation. TEM can also be utilized to demonstrate the presence of mitochondria within autophagosomes in the early embryo.

**Conclusion:** There are many assays that can be used to monitor specific types of autophagy, but caution must be used in choosing

an appropriate marker(s). The potential role of other degradative pathways for any individual organelle or cargo marker should be considered, and it is advisable to use more than one marker or technique.

**9. Autophagic sequestration assays.** Autophagic activity can also be monitored by the sequestration of autophagic cargo, using either an (electro)injected, inert cytosolic marker such as [<sup>3</sup>H] raffinose,<sup>594</sup> or an endogenous cytosolic protein such as lactate dehydrogenase (LDH),<sup>595</sup> in the latter case along with treatment with a protease inhibitor (e.g., leupeptin) to prevent intralysosomal degradation of the protein marker. The assay simply measures the transfer of cargo from the soluble (cytosol) to the insoluble (sedimentable) cell fraction (which includes autophagic compartments), with no need for a sophisticated subcellular fractionation (a filtration assay would presumably work just as well as centrifugation, although it would be necessary to verify that the filtration membrane does not destroy the integrity of the postnuclear supernatant compartments). The cargo marker can be quantified by an enzymatic assay, or by western blotting. In principle, any intracellular component can be used as a cargo marker, but cytosolic enzymes having low sedimentable backgrounds are preferable. Membrane-associated markers are less suitable, and proteins such as LC3, which are part of the sequestering system itself, will have a much more complex relationship to the autophagic flux than a pure cargo marker such as LDH.

In yeast, sequestration assays are typically done by monitoring protease protection of an autophagosome marker or a cargo protein. For example, prApe1, and GFP-Atg8 have been used to follow completion of the autophagosome.<sup>596</sup> The relative resistance or sensitivity to an exogenous protease in the absence of detergent is an indication of whether the autophagosome (or other sequestering vesicle) is complete or incomplete, respectively. Thus, this method also distinguishes between a block in autophagosome formation vs. fusion with the vacuole. The critical issues to keep in mind involve the use of appropriate control strains and/or proteins, and deciding on the correct reporter protein. In addition to protease protection assays, sequestration can be monitored by fluorescence microscopy during pexophagy of methanol-induced peroxisomes, using GFP-Atg8 as a pexophagosome marker, and BFP-SKL to label the peroxisomes. The vacuolar sequestration process during micropexophagy can also be monitored by formation of the vacuolar sequestration membrane (VSM) stained with FM 4–64.<sup>501,507</sup>

Sequestration assays can be designed to measure flux through individual steps of the autophagy pathway. For example, intralysosomally degraded sequestration probes such as [<sup>14</sup>C]-lactate or LDH will mark prelysosomal compartments in the absence of degradation inhibitors. Hence, their accumulation in such compartments can be observed when fusion with lysosomes is suppressed, for example, by a microtubule inhibitor such as vinblastine.<sup>597</sup> Furthermore, lactate hydrolysis can be used to monitor the overall autophagic pathway (autophagic lactolysis).<sup>598</sup> One caveat, however, is that inhibitors may affect sequestration indirectly, for example, by modifying the uptake and metabolism (including protein

synthesis) of autophagy-suppressive amino acids (see *Autophagy inhibitors and inducers*).

A variation of this approach applicable to mammalian cells includes live cell imaging. Autophagy induction is monitored as the movement of cargo, such as mitochondria, to GFP-LC3-colocalizing compartments, and then fusion/flux is measured by delivery of cargo to lysosomal compartments.<sup>258,599</sup> In addition, sequestration of fluorescently tagged cytosolic proteins into membranous compartments can be measured, as fluorescent puncta become resistant to the detergent digitonin.<sup>600</sup> Use of multiple time points and monitoring colocalization of a particular cargo with GFP-LC3 and lysosomes can also be used to assess sequestration of cargo with autophagosomes as well as delivery to lysosomes.<sup>601</sup>

**Cautionary notes:** The electro-injection of radiolabeled probes is technically demanding, but the use of an endogenous cytosolic protein probe is very simple and requires no pretreatment of the cells other than with a protease inhibitor. Another concern with electro-injection is that it can affect cellular physiology, so it is necessary to verify that the cells behave properly under control situations such as amino acid deprivation. An alternate approach for incorporating exogenous proteins into mammalian cell cytosol is to use “scrape-loading,” a method that works for cells that are adherent to tissue culture plates.<sup>602</sup> Finally, these assays work well with hepatocytes but may be problematic with other cell types, and it can be difficult to load the cell while retaining the integrity of the compartments in the post-nuclear supernatant (Tooze S, unpublished results). General points of caution to be addressed with regard to live cell imaging relate to photobleaching of the fluorophore, cell injury due to repetitive imaging, autofluorescence in tissues containing lipofuscin, and the pH sensitivity of the fluorophore.

There are several issues to keep in mind when monitoring sequestration by the protease protection assay in yeast.<sup>596</sup> First, as discussed in *Selective types of autophagy*, prApe1 is not an accurate marker for nonspecific autophagy; import of prApe1 utilizes a receptor (Atg19) and a scaffold (Atg11) that make the process specific. In addition, vesicles that are substantially smaller than autophagosomes can effectively sequester the Cvt complex. Another problem is that prApe1 cannot be used as an autophagy reporter for mutants that are not defective in the Cvt pathway, although this can be bypassed by using a *vac8Δ* background.<sup>603</sup> At present, the prApe1 assay cannot be used in any system other than yeast. The GFP-Atg8 protease protection assay avoids these problems, but the signal-to-noise ratio is typically substantially higher. In theory, it should be possible to use this assay in other cell types, but at present no publications report its use other than in yeast.

**Conclusion:** Sequestration assays present another valid method for monitoring autophagy, and in particular for discriminating between conditions where the autophagosome is complete (but not fused with the lysosome/vacuole) or open (i.e., a phagophore). These assays can also be modified to measure autophagic flux.

**10. Turnover of autophagic compartments.** Inhibitors of autophagic sequestration (e.g., amino acids, 3-MA or wortmannin) can be used to monitor the disappearance of autophagic elements

(phagophores, autophagosomes, autolysosomes) to estimate their half-life by TEM morphometry/stereology. The turnover of the autophagosome or the autolysosome will be differentially affected if fusion or intralysosomal degradation is inhibited.<sup>14,16,24,604</sup> The duration of such experiments is usually only a few hours; therefore, long-term side effects or declining effectiveness of the inhibitors can be avoided. It should be noted that fluorescence microscopy has also been used to monitor the half-life of autophagosomes, monitoring GFP-LC3 in the presence and absence of bafilomycin A<sub>1</sub> or following GFP-LC3 after starvation and recovery in amino acid-rich medium (see *Atg8/LC3 detection and quantification*).<sup>18,605</sup>

**Cautionary notes:** The inhibitory effect must be strong and the efficiency of the inhibitor needs to be tested under the experimental conditions to be employed. Cycloheximide is sometimes used as an autophagy inhibitor, but this is problematic because of the many potential indirect effects. Cycloheximide inhibits translational elongation, and therefore protein synthesis. In addition, it decreases the efficiency of protein degradation in several cell types (Cuervo AM, personal communication) including hematopoietic cells (Edinger A, personal communication). Treatment with cycloheximide causes a potent increase in TORC1 activity, which can decrease autophagy, in part as a result of the increase in the amino acid pool resulting from suppressed protein synthesis (Shen H-M, personal communication).<sup>606,607</sup> In addition, at high concentrations (in the millimolar range) cycloheximide inhibits complex I of the mitochondrial respiratory chain,<sup>608,609</sup> but this is not a problem, at least in hepatocytes, at low concentrations (10–20 μM) that are sufficient to prevent protein synthesis (Meijer AJ, personal communication).

**Conclusion:** The turnover of autophagic compartments is a valid, but less preferred method, for monitoring flux, and cycloheximide must be used in these experiments with caution.

**11. Autophagosome-lysosome colocalization and dequenching assay.** Another method to demonstrate the convergence of the autophagic pathway with a functional degradative compartment is to incubate cells with the bovine serum albumin derivative dequenched (DQ)-BSA that has been labeled with the red-fluorescent BODIPY TR-X dye; this conjugate will accumulate in lysosomes. The labeling of DQ-BSA is so extensive that the fluorophore is self-quenched. Proteolysis of this compound results in dequenching and the release of brightly fluorescent fragments. Thus, DQ-BSA is useful for detecting intracellular proteolytic activity as a measure of a functional lysosome.<sup>610</sup>

Furthermore, DQ-BSA labeling can be combined with GFP-LC3 to monitor colocalization, and thus visualize the convergence, of amphisomes with a functional degradative compartment (DQ-BSA is internalized by endocytosis). This method can also be used to visualize fusion events in real-time experiments by confocal microscopy (live cell imaging). Along similar lines, other approaches for monitoring convergence are to follow the colocalization of RFP-LC3 and LysoSensor Green (Bains M, Heidenreich KA, personal communication), mCherry-LC3 and LysoSensor Blue,<sup>259</sup> or tagged versions of LC3 and LAMP1 (Macleod K, personal communication) or CD63<sup>258</sup> as a measure

of the fusion of autophagosomes with lysosomes. It is also possible to trace autophagic events by visualizing the pH-dependent excitation changes of the coral protein Keima.<sup>549</sup> This quantitative technique is capable of monitoring the fusion of autophagosomes with lysosomes, that is, the formation of an autolysosome, and the assay does not depend on the analysis of LC3.

**Cautionary notes:** Some experiments require the use of inhibitors (e.g., 3-MA or wortmannin) or overexpression of proteins (e.g., RAB7 dominant negative mutants) that may also affect the endocytic pathway or the delivery of DQ-BSA to lysosomes (e.g., wortmannin causes the swelling of late endosomes<sup>611</sup>). In this case, the lysosomal compartment can be labeled with DQ-BSA overnight before treating the cells with the drugs, or prior to the transfection.

**Conclusion:** DQ-BSA provides a relatively convenient means for monitoring lysosomal protease function, and to follow the fusion of autophagosomes with the lysosome. Colocalization of autophagosomes (fluorescently tagged LC3) with lysosomal proteins or dyes can also be monitored.

**12. Tissue fractionation.** The study of autophagy in the organs of larger animals, in large numbers of organisms with very similar characteristics, or in tissue culture cells provides an opportunity to use tissue fractionation techniques as has been possible with autophagy in rat liver.<sup>34,48,612-617</sup> Because of their sizes [smaller than nuclei but larger than membrane fragments (microsomes)], differential centrifugation can be used to obtain a subcellular fraction enriched in mitochondria and organelles of the autophagic-lysosomal system, which can then be subjected to density gradient centrifugation to enrich autophagosomes, amphisomes, autolysosomes and lysosomes.<sup>34,48,617-621</sup> Any part of such a fraction can be considered to be a representative sample of tissue constituents and used in quantitative biochemical, centrifugational and morphological studies of autophagic particle populations.

The simplest studies of the autophagic process take advantage of sequestered marker enzymes, changes in location of these enzymes, differences in particle/compartment size and differential sensitivity of particles of different sizes to mechanical and osmotic stress (for example, acid hydrolases are found primarily in membrane-bound compartments and their latent activities cannot be measured unless these membranes are lysed). Such a change in enzyme accessibility can be used to follow the time course of an exogenously induced, or naturally occurring, autophagic process.<sup>612,614,616</sup>

Quantitative localization of enzymatic activity (or any other marker) to specific cytoplasmic particle populations and changes in the location of such markers during autophagy can be performed using rate sedimentation ultracentrifugation (see *Autophagic sequestration assays*).<sup>618</sup> Similar results can be obtained with isopycnic centrifugation where particles enter a density gradient (sometimes made with sucrose but iso-osmotic media such as iodixanol, metrizamide and Nycodenz may be preferred as discussed below under *Cautionary notes*) and are centrifuged until they reach locations in the gradient where their densities are equal to those of the gradient.<sup>618</sup>

The fractionation of organelles can also be evaluated by protein-correlation-profiling (PCP), a quantitative mass spectrometry-based

proteomics approach. Similar to the biochemical assays described above, gradient profiles of marker proteins can be recorded and compared with proteins of interest.<sup>278</sup> Compared with classical biochemical approaches, PCP allows the proteome-wide recording of protein gradient profiles.

Particle populations in subcellular fractions evaluated with quantitative biochemical and centrifugational approaches can also be studied with quantitative morphological methods. Detailed morphological study of the particle populations involved in the autophagic process usually requires the use of EM. The thin sections required for such studies pose major sampling problems in both intact cells<sup>622</sup> and subcellular fractions.<sup>618</sup> With the latter, 2,000,000 sections can be obtained from each 0.1 ml of pellet volume, so any practical sample size is an infinitesimally small subsample of the total sample.<sup>618</sup> However, through homogenization and resuspension, complex and heterogeneous components of subcellular fractions become randomly distributed throughout the fraction volume. Therefore, any aliquot of that volume can be considered a random sample of the whole volume. What is necessary is to conserve this property of subcellular fractions in the generation of a specimen that can be examined with the electron microscope. This can be done with the use of a pressure filtration procedure.<sup>618,623</sup> Because of the thinness of the sections, multiple sections of individual particles are possible so morphometric/stereological methods<sup>622</sup> must be used to determine the volume occupied by a given class of particles, as well as the size distribution and average size of the particle class. From this information the number of particles in a specific particle class can be calculated.<sup>624</sup> Examination of individual profiles gives information on the contents of different types of particles and their degree of degradation, as well as their enclosing membranes.<sup>612,614</sup>

**Cautionary notes:** When isolating organelles from tissues and cells in culture it is essential to use disruption methods that do not alter the membrane of lysosomes and autophagosomes, compartments that are particularly sensitive to some of those procedures. For example teflon/glass motor homogenization is suitable for tissues with abundant connective tissue, such as liver, but for circulating cells or cells in culture, disruption by nitrogen cavitation is a good method to preserve lysosomal membrane stability;<sup>625</sup> however, this method is not suitable for small samples and may not be readily available. Other methods, including “Balch” or “Dounce” homogenizers also work well.<sup>626,627</sup> During the isolation procedure it is essential to always use iso-osmotic solutions to avoid hypotonic or hypertonic disruption of the organelles. In that respect, because lysosomes are able to take up sucrose if it is present at high concentrations, the use of sucrose gradients for the isolation of intact lysosome-related organelles is strongly discouraged.

As with the isolation of any other intracellular organelle, it is essential to assess the purity of each preparation, as there is often considerable variability from experiment to experiment due to the many steps involved in the process. Correction for purity can be done through calculation of recovery (percentage of the total activity present in the homogenate) and enrichment (dividing by the specific activity in the homogenate)

of enzymes or protein markers for those compartments (e.g.,  $\beta$ -hexosaminidase is routinely used to assess lysosomal purity, but enzymes such as CTSB/cathepsin B may also be used and may provide more accurate readouts).<sup>625</sup> Because of the time-consuming nature of quantitative morphological studies, such studies should not be performed until simpler biochemical procedures have established the circumstances most likely to give meaningful morphometric/stereological results.

Finally, it is worthwhile noting that not all lysosomes are alike. For example, there are differences among primary lysosomes, autolysosomes and telolysosomes. Furthermore, what we refer to as “lysosomes” are actually a very heterogeneous pool of organelles that simply fulfill five classical criteria, having a pH < 5.6, mature cathepsins, the presence of LAMP proteins, a single membrane, and the absence of endosomal and recycling compartment markers (e.g., the mannose-6-phosphate receptor or RAB5). But even applying those criteria we can separate lysosomes with clear differences in their proteome and other properties, and these distinct populations of lysosomes are likely to participate in different functions in the cell (see *Chaperone-mediated autophagy*).<sup>628</sup>

**Conclusion:** Considering the limited methods available for in vivo analysis of autophagy, tissue fractionation is a valid, although relatively laborious, method for monitoring this process. Care must be taken to ensure that sample analysis is representative.

**13. Analyses in vivo.** Monitoring autophagic flux in vivo or in organs is one of the least developed areas at present, and ideal methods relative to the techniques possible with cell culture may not exist. Importantly, the level of basal autophagy, time course of autophagic induction, and the bioavailability of autophagy-stimulating and -inhibiting drugs is likely tissue specific. Moreover, basal autophagy or sensitivity to autophagic induction may vary with animal age, sex or strain background. Therefore methods may need to be optimized for the tissue of interest. One method for in vivo studies is the analysis of GFP-LC3/Atg8 (see *GFP-Atg8/LC3 fluorescence microscopy* above). Autophagy can be monitored in tissue (e.g., skeletal muscle, liver and retina) in vivo in transgenic mice systemically expressing GFP-LC3,<sup>126,446</sup> or in other models by transfection with GFP-LC3 plasmids.<sup>346</sup> In addition, tissue-specific GFP-LC3 mice have been generated for monitoring cardiac myocytes.<sup>629,630</sup> In these settings, GFP fluorescent puncta are indicative of autophagic structures. In addition, cleavage of GFP-LC3 to generate free GFP can be evaluated. This has been successfully performed in mouse liver,<sup>172,631</sup> suggesting the GFP-LC3 cleavage assay may also be applied to in vivo studies. Alternatively, confocal laser scanning microscopy, which makes it possible to obtain numerous sections and substantial data about spatial localization features, can be a suitable system for studying autophagic structures (especially for whole mount embryo in vivo analysis).<sup>632</sup> In addition, this method can be used to obtain quantitative data through densitometric analysis of fluorescent signals.<sup>633</sup>

Another possibility is immunohistochemical staining, an important procedure that may be applicable to human studies as well, considering the role of autophagy in neurodegeneration, myopathies and cardiac disease where samples may be limited

to biopsy/autopsy tissue. Immunodetection of LC3 as definite puncta is possible in paraffin-embedded tissue sections and fresh frozen tissue, by either immunohistochemistry or immunofluorescence,<sup>140,634-637</sup> however, this methodology has not received extensive evaluation, and does not lend itself well to dynamic assays. Other autophagic substrates can be evaluated via immunohistochemistry and include SQSTM1, NBR1, ubiquitinated inclusions and protein aggregates. Similarly, autophagy can be evaluated by measuring levels of these autophagic substrates via traditional immunoblot; however, their presence or absence needs to be cautiously interpreted as some of these substrates can accumulate with either an increase or a decrease in autophagic flux (see *SQSTM1/p62 and related LC3 binding protein turnover assays*). Bone marrow transfer has been used to document in vivo the role of autophagy in the reverse cholesterol transport pathway from peripheral tissues or cells (e.g., macrophages) to the liver for secretion in bile and for excretion,<sup>638</sup> and a study shows that transglutaminase type 2 protein levels decrease in mouse liver in vivo upon starvation in an autophagy-dependent manner (and in human cell lines in vitro in response to various stimuli; Piacentini M, personal communication), presenting additional possible methods for following autophagy activity.

It is also possible to analyze tissues ex vivo, and these studies can be particularly helpful in assessing autophagic flux as they avoid the risks of toxicity and bioavailability of compounds such as bafilomycin A<sub>1</sub> or other autophagy inhibitors. Along these lines, autophagic flux can be determined by western blot in retinas placed in culture for 4 h with protease inhibitors. This method could be used in tissues that can remain “alive” for several hours in culture such as the retina (Boya P, unpublished observations) and brain slices (Desai S, unpublished observations).

Several studies have demonstrated the feasibility of monitoring autophagic flux in vivo in skeletal muscle. Starvation is one of the easiest and most rapid methods for stimulating the autophagic machinery in skeletal muscles. Twelve h of fasting in mice may be sufficient to trigger autophagy in muscle,<sup>639,640</sup> but the appropriate time should be determined empirically. Data about the autophagic flux can be obtained by treating mice with, for example, chloroquine,<sup>640</sup> leupeptin<sup>641</sup> or colchicine<sup>153</sup> and then monitoring the change in accumulation of LC3 (see cautionary notes). This type of analysis can also be done with liver, by comparing the LC3-II level in untreated liver (obtained by a partial hepatectomy) to that following subsequent exposure to chloroquine (Skop V, Papackova Z, Cahová M, personal communication). Additional reporter assays to monitor autophagy flux in vivo need to be developed, including tandem fluorescent-LC3 transgenic mice, or viral vectors to express this construct in vivo in localized areas.

Some biochemical assays may be used to at least provide indirect correlative data relating to autophagy, in particular when examining the role of autophagy in cell death. For example, cellular viability is related to high CTSB activity and low CTSD/cathepsin D activities.<sup>642</sup> Therefore, the appearance of the opposite levels of activities may be one indication of the initiation of autophagy (lysosome)-dependent cell death. The question of “high” vs. “low” activities can be determined by comparison to

the same tissue under control conditions, or to a different tissue in the same organism, depending on the specific question.

With regard to living mammals, a minimally invasive method that may be used even in humans is to measure the arterio-venous amino acid exchange rate in the peripheral tissues as a measure of postabsorptive protein catabolism. In humans, the insulin- and amino acid-sensitive postabsorptive (autophagic) net protein catabolism in the peripheral (mostly skeletal muscle) tissue can be measured by determining the amino acid exchange rate across the lower extremities, as defined by the difference between the plasma amino acid concentrations in the femoral artery and femoral vein multiplied by the blood flow.<sup>643-645</sup> Amino acid exchange studies show that the peripheral tissues take up amino acids during the post-prandial (fed) state and release amino acids in the postabsorptive (fasted) state, i.e., in a state with relatively low plasma insulin and amino acid levels. This post-absorptive release of amino acids is strongly inhibited by infusion of insulin or by exogenous supply of amino acids, suggesting that it is mainly mediated by a lysosomal/autophagic mechanism of protein catabolism.<sup>643-650</sup> However, the relative contribution of autophagy to the post-absorptive release of amino acids may be changed in disease states (see cautionary notes).

**Cautionary notes:** The major hurdle with *in vivo* analyses is the identification of autophagy-specific substrates and the ability to “block” autophagosome degradation with a compound such as bafilomycin A<sub>1</sub>. Regardless, it is still essential to adapt the same rigors for measuring autophagic flux *in vitro* to measurements made with *in vivo* systems. Moreover, as with cell culture, in order to substantiate a change in autophagic flux it is not adequate to rely solely on the analysis of static levels or changes in LC3-II protein levels on western blot using tissue samples. To truly measure *in vivo* autophagic flux using LC3-II as a biomarker, it is necessary to block lysosomal degradation of the protein. Several studies have successfully done this in select tissues *in vivo*. Certain general principles need to be kept in mind: (a) Any autophagic blocker, whether leupeptin, bafilomycin A<sub>1</sub>, chloroquine or microtubule depolarizing agents such as colchicine or vinblastine must significantly increase basal LC3-II levels. The turnover of LC3-II or rate of basal autophagic flux is not known for tissues *in vivo*, and therefore short treatments (e.g., 4 h) may not be as effective as blocking for longer times (e.g., 12 to 24 h). (b) The toxicity of the blocking agent needs to be considered (e.g., treating animals with bafilomycin A<sub>1</sub> for 2 h can be quite toxic) and food intake must be monitored. If long-term treatment is needed to see a change in LC3-II levels, then confirmation that the animals have not lost weight may be needed. Mice may lose a substantial portion of their body weight when deprived of food for 24 h, and starvation is a potent stimulus for the activation of autophagy. (c) The bioavailability of the agent needs to be considered. For example, many inhibitors such as bafilomycin A<sub>1</sub> or chloroquine have relatively poor bioavailability to the central nervous system. To overcome this problem, intracerebroventricular injection can be performed.

When analyzing autophagic flux *in vivo*, one major limitation is the variability between animals. Different animals do not always activate autophagy at the same time. To improve the statistical

relevance and avoid unclear results, these experiments should be repeated more than once and each experiment should include several animals. Induction of autophagy in a time-dependent manner by fasting mice for different times requires appropriate caution. Mice are nocturnal animals, so they preferentially move and eat during the night, while they mostly rest during daylight. Therefore, in such experiments it is better to start food deprivation early in the morning, in order to avoid the possibility that the animals have already been fasting for several hours. The use of chloroquine is technically easier, since it only needs one intraperitoneal injection per day, but the main concern is that chloroquine has some toxicity. Chloroquine suppresses the immunological response in a manner that is not due to its pH-dependent lysosomotropic accumulation (chloroquine interferes with lipopolysaccharide-induced TNF/TNF $\alpha$  gene expression by a nonlysosomotropic mechanism),<sup>651</sup> as well as through its pH-dependent inhibition of antigen presentation.<sup>652</sup> Therefore, chloroquine treatment should be used for short times and at doses that do not induce severe collateral effects, which may invalidate the measurement of the autophagic flux, and care must be exercised in using chloroquine for studies on autophagy that involve immunological aspects. It is also important to have time-matched controls for *in vivo* analyses. That is, having only a zero hour time point control is not sufficient because there may be substantial diurnal changes in basal autophagy.<sup>467</sup> For example, variations in basal flux in the liver associated with circadian rhythm may be several fold, which can equal or exceed the changes due to starvation. Along these lines, in order to allow comparisons of a single time-point it is important to specify what time of day the measurement is taken, and the lighting conditions under which the animals are housed. It is also important that the replicate experiments are conducted at the same time of day. Controlling for circadian effects can greatly reduce the mouse-to-mouse variability in autophagy markers and flux (Haspel JA, Choi AMK, personal communication).

The amino acid exchange rate, which has been suggested as a minimal invasive marker for measuring autophagic flux *in vivo* should be used with special caution. Postprandial suppression of amino acid release by peripheral tissues is strongly mediated by insulin and amino acids and is therefore thought to be mediated by autophagy (see above). However, the ubiquitin-proteasome system likely confounds this investigation, as this pathway can also be inhibited by insulin and amino acids.<sup>653</sup> In addition, multiple disease states have been associated with an altered activity of the ubiquitin-proteasome system, further complicating the use of the amino acid exchange rate as a marker of autophagy.<sup>654</sup>

When analyzing basal autophagic level *in vivo* using GFP-LC3 transgenic mice,<sup>126</sup> one pitfall is that GFP-LC3 expression is driven by the cytomegalovirus enhancer and  $\beta$ -actin (CAG) promoter, so that the intensity of the GFP signal may not always represent the actual autophagic activity, but rather the CAG promoter activity in individual cells. For example, GFP-LC3 transgenic mice exhibit prominent fluorescence in podocytes, but rarely in tubular epithelial cells in the kidney,<sup>126</sup> but a similar GFP pattern is observed in transgenic mice carrying CAG promoter-driven non-tagged GFP.<sup>655</sup> Furthermore, proximal

tubule-specific ATG5-deficient mice<sup>656</sup> display a degeneration phenotype earlier than podocyte-specific ATG5-deficient mice,<sup>657</sup> suggesting that autophagy, and hence LC3 levels, might actually be more prominent in the former.

One caution in using approaches that monitor ubiquitinated aggregates is that the accumulation of ubiquitin may indicate a block in autophagy, inhibition of proteasomal degradation, or may correspond to structural changes in the substrate proteins that hinder their degradation. In addition, only cytosolic and not nuclear ubiquitin is subject to autophagic degradation. It is helpful to analyze aggregate degradation in an autophagy-deficient control strain, such as an autophagy mutant mouse, whenever possible to determine whether an aggregate is being degraded by an autophagic mechanism. This type of control will be impractical for some tissues such as those of the central nervous system because the absence of autophagy leads to rapid degeneration. Accordingly, the use of *Atg16l1* hypomorphs or *Becn1*<sup>-/-</sup> heterozygotes may help circumvent this problem.

**Conclusion:** Although the techniques for analyzing autophagy *in vivo* are not as advanced as those for cell culture, it is still possible to follow this process (including flux) by monitoring, for example, GFP-LC3 by fluorescence microscopy, and SQSTM1 and NBR1 by immunohistochemistry and/or western blotting.

**14. Cell death.** In a limited number of cases, autophagy has been established as the cause of cell death;<sup>97,199,658-664</sup> although opposite results have been reported using analogous experimental settings.<sup>665</sup> Furthermore, many of the papers claiming a causative role of autophagy in cell death fail to provide adequate evidence.<sup>666</sup> Other papers suffer from ambiguous use of the term “autophagic cell death,” which was coined in the 1970s<sup>667</sup> in a purely morphological context to refer to cell death with autophagic features (especially the presence of numerous secondary lysosomes); this was sometimes taken to suggest a role of autophagy in the cell death mechanism, but death-mediation was not part of the definition.<sup>668</sup> Unfortunately, the term “autophagic cell death” is now used in at least three different ways: (a) autophagy-associated cell death (the original meaning); (b) autophagy-mediated cell death (which could involve a standard mechanism of cell death such as apoptosis, but triggered by autophagy); (c) a distinct mechanism of cell death, independent of apoptosis or necrosis. Clearly claim (b) is stronger than claim (a), and needs to be justified by proof that inhibiting autophagy, through either genetic or chemical means, prevents cell death.<sup>669</sup> Claim (c) is still stronger, because, even if the cell death is blocked by autophagy inhibition, proof needs to be provided that the cell death mechanism is not apoptosis or necrosis.<sup>670</sup> In view of the current confusion it may be preferable to replace the term “autophagic cell death” by other terms such as “autophagy-associated cell death” or “autophagy-mediated cell death,” unless the criteria in claim (c) above have been satisfied. Along these lines, it is preferable to use the term “autophagy-dependent cell death” instead of “autophagy-mediated cell death” when it is proven that autophagy is a prerequisite for the occurrence of cell death, but it is not proven that autophagy mechanistically mediates the switch to cell death. A special caution should also be taken when describing developmental programmed cell death in plants, which is in most

cases executed by the growing lytic vacuoles and therefore is referred to as “vacuolar cell death.”<sup>671</sup> Although the morphology of vacuolar cell death resembles a combination of macro- and microautophagy, there is no genetic evidence yet that this death requires the core autophagic machinery. Finally, the relationship between autophagy and cell death may significantly differ as a function of the model system being studied. For example, upon induction by starvation of multicellular development in the protist *Dictyostelium*, autophagy (or at least *Atg1*) is required to protect against starvation-induced cell death, allowing vacuolar developmental cell death to take place instead.<sup>672,673</sup> Autophagy may be involved not only in allowing this death to occur, but also in the vacuolization process itself.<sup>674</sup>

**Cautionary notes:** In brief, rigorous criteria must be met in order to establish a death-mediating role of autophagy, as this process typically promotes cell survival. These include a clear demonstration of autophagic flux as described in this article, as well as verification that inhibition of autophagy prevents cell death [claim (b) above; if using a knockdown approach, at least two *ATG* genes should be targeted], and that other mechanisms of cell death are not responsible [claim (c) above]. As part of this analysis, it is necessary to examine the effect of the specific treatment, conditions or mutation on cell viability using several methods.<sup>675</sup> In the case of postmitotic cells such as neurons or retinal cells, cell death—and cell rescue by autophagy inhibition—can usually be established *in vivo* by morphological analysis,<sup>676</sup> and in culture by cell counts and/or measurement of the release of an enzyme such as LDH into the medium at early and late time points; however, a substantial amount of neuronal cell death occurs during neurogenesis, making it problematic to carry out a correct analysis *in vivo* or *ex vivo*.<sup>677,678</sup> In populations of rapidly dividing cells, the problems may be greater. A commonly used method is the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, or a related assay using a similar, or a water-soluble, tetrazolium salt. The main concern with the MTT assay is that it measures mitochondrial activity, but does not allow a precise determination of cellular viability or cell death, whereas methods that show cell death directly (e.g., trypan blue exclusion) fail to establish the viability of the remaining cell population.<sup>679</sup> Accordingly, a preferred alternative is to accurately quantify cell death by appropriate cytofluorometric or microscopy assays.<sup>675</sup> Moreover, long-term clonogenic assays should be employed when possible to measure the effective functional survival of cells.

**Conclusion:** In most systems, ascribing death to autophagy based solely on morphological criteria is insufficient; autophagic cell death can only be demonstrated as death that is suppressed by the inhibition of autophagy, through either genetic or chemical means.<sup>669</sup> In addition, more than one assay should be used to measure cell death.

**15. Chaperone-mediated autophagy.** The primary characteristic that makes chaperone-mediated autophagy (CMA) different from the other autophagic variants described in these guidelines is that it does not require formation of intermediate vesicular compartments (autophagosomes or microvesicles) for the import of cargo into lysosomes.<sup>680,681</sup> Instead, the CMA substrates are

translocated across the lysosomal membrane through the action of HSPA8/HSC70 located in the cytosol and lysosome lumen, and the lysosome membrane protein LAMP2A. To date, CMA has only been identified in mammalian cells, and accordingly this section refers only to studies in mammals.

The following methods are commonly utilized to determine if a protein is a CMA substrate (see ref. 682 for experimental details): (a) Analysis of the amino acid sequence of the protein to identify the presence of a KFERQ-related motif that is an absolute requirement for all CMA substrates.<sup>683</sup> (b) Colocalization studies with lysosomal markers (typically LAMP2A and/or LysoTracker) to identify a fraction of the protein associated with lysosomes. The increase in association of the putative substrate under conditions that upregulate CMA (such as prolonged starvation) or upon blockage of lysosomal proteases (to prevent the degradation of the protein) helps support the hypothesis that the protein of interest is a CMA substrate. However, association with lysosomes is necessary but not sufficient to consider a protein an authentic CMA substrate, because proteins delivered by other pathways to lysosomes will also behave in a similar manner. A higher degree of confidence can be attained if the association is preferentially with the subset of lysosomes active for CMA (i.e., those containing HSPA8 in their lumen), which can be separated from other lysosomes following published procedures.<sup>628</sup> (c) Co-immunoprecipitation of the protein of interest with cytosolic HSPA8. Due to the large number of proteins that interact with this chaperone, it is usually better to perform affinity isolation with the protein of interest and then analyze the isolated proteins for the presence of HSPA8 rather than vice versa. (d) Co-immunoprecipitation of the protein of interest with LAMP2A.<sup>684</sup> Due to the fact that the only antibodies specific for the LAMP2A variant (the only one of the three LAMP2 variants involved in CMA<sup>105,685</sup>) are generated against the cytosolic tail of LAMP2A, where the substrate also binds, it is necessary to affinity isolate the protein of interest and then analyze for the presence of LAMP2A. Immunoblot for LAMP2A in the precipitate can only be done with the antibodies specific for LAMP2A and not just those that recognize the luminal portion of the protein that is identical in the other LAMP2 variants. If the protein of interest is abundant inside cells, co-immunoprecipitations with LAMP2A can be done in total cellular lysates, but for low abundance cellular proteins, preparation of a membrane fraction (enriched in lysosomes) by differential centrifugation may facilitate the detection of the population of the protein bound to LAMP2A. (e) Selective upregulation and blockage of CMA to demonstrate that degradation of the protein of interest changes with these manipulations. Selective chemical inhibitors for CMA are not currently available. Note that general inhibitors of lysosomal proteases (e.g., bafilomycin A<sub>1</sub>, NH<sub>4</sub>Cl, leupeptin) also block the degradation of proteins delivered to lysosomes by other autophagic and endosomal pathways. The most selective way to block CMA is by knockdown of LAMP2A, which causes this protein to become a limiting factor.<sup>105</sup> The other components involved in CMA, including HSPA8, HSP90, GFAP, and EEF1A1/eF1 $\alpha$  are all multifunctional cellular proteins, making it difficult to interpret the effects of knockdowns. Overexpression of

LAMP2A<sup>684</sup> is also a better approach to upregulate CMA than the use of chemical modulators. The two compounds demonstrated to affect degradation of long-lived proteins in lysosomes,<sup>686</sup> 6-aminonicotinamide and geldanamycin, lack selectivity, as they affect many other cellular processes. In addition, in the case of geldanamycin, the effect on CMA can be the opposite (inhibition rather than stimulation) depending on the cell type (this is due to the fact that the observed stimulation of CMA is actually a compensatory response to the blockage of HSP90 in lysosomes, and different cells activate different compensatory responses).<sup>687</sup> (f) The most conclusive way to prove that a protein is a CMA substrate is by reconstituting its direct translocation into lysosomes using a cell-free system.<sup>682</sup> This method is only possible when the protein of interest can be purified, and it requires the isolation of the population of lysosomes active for CMA. Internalization of the protein of interest inside lysosomes upon incubation with the isolated organelle can be monitored using protease protection assays (in which addition of an exogenous protease removes the protein bound to the cytosolic side of lysosomes, whereas it is inaccessible to the protein that has reached the lysosomal lumen; note that pre-incubation of lysosomes with lysosomal protease inhibitors before adding the substrate is required to prevent the degradation of the translocated substrate inside lysosomes).<sup>688</sup> The use of exogenous protease requires numerous controls (see ref. 682) to guarantee that the amount of protease is sufficient to remove all the substrate outside lysosomes, but will not penetrate inside the lysosomal lumen upon breaking the lysosomal membrane. The difficulties in the adjustment of the amount of protease, has led to the development of a second method that is more suitable for laboratories that have no previous experience with these procedures. In this case, the substrate is incubated with lysosomes untreated or previously incubated with inhibitors of lysosomal proteases, and uptake is determined as the difference of protein associated with lysosomes not incubated with inhibitors (in which the only remaining protein will be the one associated with the cytosolic side of the lysosomal membrane) and those incubated with the protease inhibitors (which contain both the protein bound to the membrane and that translocated into the lumen).<sup>689</sup> Confidence that the lysosomal internalization is by CMA increases if the uptake of the substrate can be competed with proteins previously identified as substrates for CMA (e.g., GAPDH/glyceraldehyde-3-phosphate dehydrogenase or ribonuclease A, both commercially available as purified proteins), but is not affected by the presence of similar amounts of nonsubstrate proteins (such as ovalbumin or PPIA/cyclophilin A). Blockage of uptake by pre-incubation of the lysosomes with antibodies against the cytosolic tail of LAMP2A also reinforces the hypothesis that the protein is a CMA substrate.

In other instances, rather than determining if a particular protein is a CMA substrate, the interest may be to analyze possible changes in CMA activity under different conditions or in response to different modifications. We enumerate here the methods, from lower to higher complexity that can be utilized to measure CMA in cultured cells and in tissues (see ref. 682 for detailed experimental procedures). (a) Measurement of changes

in the intracellular rates of degradation of long-lived proteins, when combined with inhibitors of other autophagic pathways, can provide a first demonstration in support of changes that are due to CMA. For example, CMA is defined as lysosomal degradation upregulated in response to serum removal but insensitive to PtdIns3K inhibitors. (b) Measurement of levels of CMA components is insufficient to conclude changes in CMA because this does not provide functional information, and changes in CMA components can also occur under other conditions. However, analysis of the levels of LAMP2A can be used to support changes in CMA detected by other procedures. Cytosolic levels of HSPA8 remain constant and are not limiting for CMA, thus providing no information about this pathway. Likewise, changes in total cellular levels of LAMP2A do not have an impact on this pathway unless they also affect their lysosomal levels (i.e., conditions in which LAMP2A is massively overexpressed lead to its targeting to the plasma membrane where it cannot function in CMA). It is advisable that changes in the levels of these two CMA components are confirmed to occur in lysosomes, either by colocalization with lysosomal markers when using image-based procedures or by performing immunoblot of a lysosomal enriched fraction (purification of this fraction does not require the large amounts of cells/tissue necessary for the isolation of the subset of lysosomes active for CMA). (c) Tracking changes in the subset of lysosomes active for CMA. This group of lysosomes is defined as those containing HSPA8 in their lumen (note that LAMP2A is present in both lysosomes that are active and inactive for CMA, and it is the presence of HSPA8 that confers CMA capability). Immunogold or immunofluorescence against these two proteins (LAMP2A and HSPA8) makes it possible to quantify changes in the levels of these lysosomes present at a given time, which correlates well with CMA activity.<sup>628</sup> (d) Analysis of lysosomal association of fluorescent artificial CMA substrates. Two different fluorescent probes have been generated to track changes in CMA activity in cultured cells using immunofluorescence or FACS analysis.<sup>628</sup> These probes contain the KFERQ and context sequences in frame with photoswitchable or photoactivated fluorescent proteins. Activation of CMA results in the mobilization of a fraction of the cytosolic probe to lysosomes and the subsequent change from a diffuse to a punctate pattern. CMA activity can be quantified as the number of fluorescent puncta per cell or as the decay in fluorescence activity over time because of degradation of the artificial substrate. Because the assay does not allow measuring accumulation of the substrate (which must unfold for translocation), it is advisable to perform a time-course analysis to determine gradual changes in CMA activity. Antibodies against the fluorescent protein in combination with inhibitors of lysosomal proteases can be used to monitor accumulation of the probe in lysosomes over a period of time, but both the photoswitchable and the unmodified probe will be detected by this procedure.<sup>690</sup> As for any other fluorescence probe based on analysis of intracellular “puncta” it is essential to include controls to confirm that the puncta are indeed lysosomes (colocalization with LysoTracker or LAMPs and lack of colocalization with markers of cytosolic aggregation such as ubiquitin), and do not reach the lysosomes through other autophagic

pathways (insensitivity to PtdIns3K inhibitors and sensitivity to LAMP2A knockdown are good controls in this respect). (e) Direct measurement of CMA using in vitro cell free assays. Although the introduction of the fluorescent probes should facilitate measurement of CMA in many instances, they are not applicable for tissue samples. In addition, because the probes measure binding of substrate to lysosomal membranes it is important to confirm that enhanced binding does not result from defective translocation. Lastly, the in vitro uptake assays are also the most efficient way to determine primary changes in CMA independently of changes in other proteolytic systems in the cells. These in vitro assays are the same ones described in the previous section on the identification of proteins as substrates of CMA, but are performed in this case with purified proteins previously characterized to be substrates for CMA. In this case the substrate protein is always the same and what changes is the source of lysosomes (from the different tissues or cells that are to be compared). As described in the previous section, binding and uptake can be analyzed separately using lysosomes previously treated or not with protease inhibitors. The analysis of the purity of the lysosomal fractions prior to performing functional analysis is essential to conclude that changes in the efficiency to take up the substrates results from changes in CMA rather than from different levels of lysosomes in the isolated fractions. Control of the integrity of the lysosomal membrane and sufficiency of the proteases are also essential to discard the possibility that degradation is occurring outside lysosomes because of leakage, or that accumulation of substrates inside lysosomes is due to enhanced uptake rather than to decreased degradation.

**Cautionary notes:** The discovery of a new selective form of protein degradation in mammals named endosomal-microautophagy (e-MI)<sup>691</sup> has made it necessary to reconsider some of the criteria that applied in the past for the definition of a protein as a CMA substrate. The KFERQ-like motif, previously considered to be exclusive for CMA, is also used to mediate selective targeting of cytosolic proteins to the surface of late endosomes. Once there, substrates can be internalized in microvesicles that form from the surface of these organelles in an ESCRT-dependent manner. HSPA8 has been identified as the chaperone that binds this subset of substrates and directly interacts with lipids in the late endosomal membrane, acting thus as a receptor for cytosolic substrates in this compartment. At a practical level, to determine if a KFERQ-containing protein is being degraded by CMA or e-MI the following criteria can be applied: (a) Inhibition of lysosomal proteolysis (for example with  $\text{NH}_4\text{Cl}$  and leupeptin) blocks degradation by both pathways. (b) Knockdown of LAMP2A inhibits CMA but not e-MI. (c) Knockdown of components of ESCRT I and II (e.g., VPS4 and TSG101) inhibits e-MI but not CMA. (d) Interfering with the capability to unfold the substrate protein blocks its degradation by CMA, but does not affect e-MI of the protein. In this respect soluble proteins, oligomers and protein aggregates can undergo e-MI, but only soluble proteins can be CMA substrates. (e) In vitro uptake of e-MI substrates can be reconstituted using isolated late endosomes whereas in vitro uptake of CMA substrates can only be reconstituted using lysosomes.



Another pathway that needs to be considered relative to CMA is chaperone-assisted selective autophagy (CASA).<sup>692</sup> CASA is dependent on HSPA8 and LAMP2 (although it is not yet known if it is dependent solely on the LAMP2A isoform). Thus, a requirement for these two proteins is not sufficient to conclude that a protein is degraded by CMA.

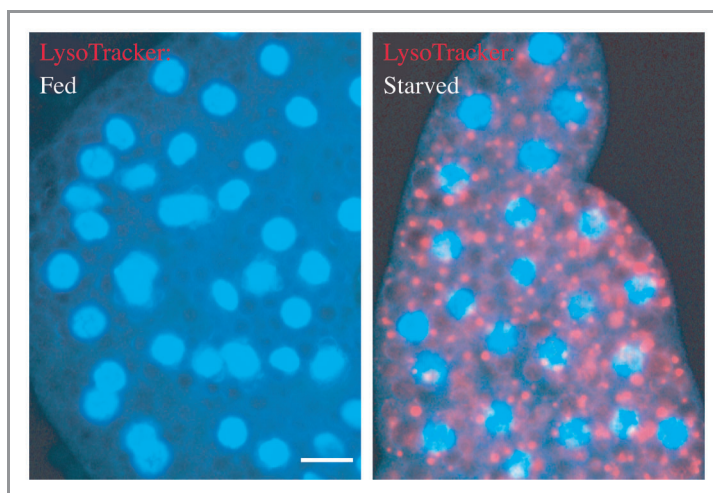
**Conclusion:** One of the key issues with the analysis of CMA is verifying that the protein of interest is an authentic substrate. Methods for monitoring CMA that utilize fluorescent probes are available that eliminate the need for the isolation of CMA-competent lysosomes, one of the most difficult aspects of assaying this process.

## B. Comments on Additional Methods

**1. Acidotropic dyes.** Among the older methods for following autophagy is staining with acidotropic dyes such as monodansylcadaverine (MDC),<sup>693</sup> acridine orange,<sup>694</sup> Neutral Red,<sup>632</sup> LysoSensor Blue<sup>695</sup> and LysoTracker Red.<sup>198,696</sup>

Cautionary notes: Although MDC was first described as a specific marker of autophagic vacuoles<sup>697</sup> subsequent studies have suggested that this, and other acidotropic dyes, are not specific markers for early autophagosomes,<sup>258</sup> but rather label later stages in the degradation process. For example, autophagosomes are not acidic, and MDC staining can be seen in autophagy-defective mutants<sup>401</sup> and in the absence of autophagy activation.<sup>698</sup> MDC may also show confounding levels of background labeling unless narrow bandpass filters are used. However, in the presence of vinblastine, which blocks fusion with lysosomes, MDC labeling increases, suggesting that under these conditions MDC can label late-stage autophagosomes.<sup>699</sup> Along these lines, cells that overexpress a dominant negative version of RAB7 (the T22N mutant) show colocalization of this protein with MDC; in this case fusion with lysosomes is also blocked<sup>700</sup> indicating that MDC does not just label lysosomes. Finally, MDC labeling could be considered to be an indicator of autophagy when the increased labeling of cellular compartments by this dye is prevented by treatment with autophagy inhibitors such as wortmannin or 3-MA.

Overall, staining with MDC or its derivative monodansylamylamine (MDH)<sup>693</sup> is not, by itself, a sufficient method for monitoring autophagy. Similarly, LysoTracker Red, Neutral Red and acridine orange are not ideal markers for autophagy because they primarily detect lysosomes. These markers are, however, useful for monitoring selective autophagy when used in conjunction with protein markers or other dyes. For example, increased colocalization of mitochondria with both GFP-LC3 and LysoTracker can be used as evidence of autophagic cargo delivery to lysosomes. Moreover, LysoTracker Red has been used to provide correlative data on autophagy in *Drosophila melanogaster* fat body cells (Fig. 23).<sup>197,198</sup> However, additional assays, such as GFP-Atg8/LC3 fluorescence or EM, should be used to substantiate results obtained with acidotropic dyes whenever possible. One important caution when co-imaging with LysoTracker Red and a green-fluorescing marker (e.g., GFP-LC3 or MitoTracker Green) is that it is necessary to control for rapid red-to-green



**Figure 23.** 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 Hoechst 33342 (blue) to stain the nucleus. Tissues were isolated from fed (left) or 3 h starved (right) animals. Bar, 25  $\mu$ m. This figure was modified from data presented in reference 198, Scott RC, Schuldiner O, Neufeld TP, Role and regulation of starvation-induced autophagy in the *Drosophila* fat body, *Dev Cell* 2004; 7:167–78, copyright 2004, with permission from Elsevier.

photoconversion of the LysoTracker, which can otherwise result in an incorrect interpretation of colocalization.<sup>701</sup>

Some of the confusion regarding the interpretation of results with these dyes stems in part from the nomenclature in this field. Indeed, the discussion of acidotropic dyes points out why it is advisable to differentiate between the terms “autophagosome” and “autophagic vacuole,” although they are occasionally, and incorrectly, used interchangeably. The autophagosome is the sequestering compartment generated by the phagophore. The fusion of an autophagosome with an endosome or a lysosome generates an amphisome or an autolysosome, respectively. The early autophagosome is not an acidic compartment, whereas amphisomes and autolysosomes are acidic. Earlier names for these compartments are “initial autophagic vacuole (AVi),” “intermediate autophagic vacuole (AVi/d)” and “degradative autophagic vacuole (AVd),” respectively. Thus, acidotropic dyes can stain late autophagic vacuoles (in particular autolysosomes), but not the initial autophagic vacuole, the early autophagosome. With the above caveats in mind, the combined use of early and late markers of autophagy is highly encouraged, and when quantifying mammalian lysosomes, it is important to keep in mind that increases in both lysosome size and number are frequently observed. Finally, in order to avoid confusion with the plant and fungal vacuole, the equivalent organelle to the lysosome, we recommend the use of the term “autophagosome” instead of “autophagic vacuole,” and the use of “autophagic compartment” when the specific nature of the structure is not known.

**Conclusion:** Given the development of better techniques that are indicators of autophagy, the use of acidotropic dyes to study this process is discouraged, and relying entirely on such dyes is not acceptable.

**2. Autophagy inhibitors and inducers.** In many situations it is important to demonstrate an effect resulting from inhibition or stimulation of autophagy (see ref. 702 for a partial listing of regulatory compounds), and a few words of caution are worthwhile in this regard. Most chemical inhibitors of autophagy are not entirely specific, and it is important to consider possible dose- and time-dependent effects. Accordingly, it is generally preferable to analyze specific loss-of-function *Atg* mutants. However, it must be kept in mind that some apparently specific *Atg* gene products may have autophagy-independent roles (e.g., ATG5 in cell death, and the PIK3C3-containing complexes—including BECN1—in apoptosis, endosomal function and protein trafficking).<sup>403,432,703-706</sup> Therefore, the experimental conditions of inhibitor application and their side effects must be carefully considered. In addition, it must be emphasized once again that autophagy, as a multistep process, can be inhibited at different stages. Sequestration inhibitors, including 3-MA, LY294002 and wortmannin, inhibit class I as well as class III PtdIns3Ks.<sup>134,257,707</sup> The class I enzymes generate products [PtdIns(3,4,5)P<sub>3</sub>] that inhibit autophagic sequestration, whereas the class III product (PtdIns3P) generally stimulates autophagic sequestration. The overall effect of these inhibitors is typically to block autophagy because the class III enzymes that are required to activate autophagy act downstream of the negative regulatory class I enzymes, although cell death may ensue in cell types that are dependent upon high levels of AKT1 for survival. The effect of 3-MA (but not that of wortmannin) is further complicated by the fact that it has different temporal patterns of inhibition, causing a long-term suppression of the class I PtdIns3K, but only a transient inhibition of the class III enzyme. In cells incubated in a complete medium for extended periods of time, 3-MA may, therefore (particularly at suboptimal concentrations), promote autophagy by inhibition of the class I enzyme.<sup>257</sup> Thus, wortmannin may be considered as an alternative to 3-MA for autophagy inhibition.<sup>257</sup> However, wortmannin can induce the formation of vacuoles that may have the appearance of autophagosomes, although they are swollen late endocytic compartments.<sup>611</sup> Furthermore, studies have demonstrated that inhibition of autophagy with 3-MA or wortmannin can have effects on cytokine transcription, processing and secretion, particularly IL1 family members,<sup>708-710</sup> but 3-MA also inhibits the secretion of some cytokines (e.g., TNF, IL6) in an autophagy-independent manner (Harris J, unpublished observations). Thus, in studies where the effect of autophagy inhibition on specific cellular processes is being investigated, it is important to confirm results using other methods, such as RNA silencing. Due to these issues, it is of great interest that inhibitors with specificity for the class III PtdIns3Ks, and their consequent effects on autophagy, have been described.<sup>165</sup>

Cycloheximide, a commonly used protein synthesis inhibitor in mammals, is also an inhibitor of sequestration *in vivo*,<sup>14-16,92,604,711-715</sup> and in various cell types *in vitro*,<sup>344,716</sup> and it has been utilized to investigate the dynamic nature of the regression of various autophagic elements.<sup>14-16,24,92,712,713</sup> The mechanism of action of cycloheximide in short-term experiments is not clear, but it has no direct relation to the inhibition of

protein synthesis.<sup>344</sup> This latter activity, however, may complicate certain types of analysis when using this drug.

A significant challenge for a more detailed analysis of the dynamic role of autophagy in physiological and pathophysiological processes, for instance with regard to cancer and cancer therapy, is to find more specific inhibitors of autophagy signaling which do not affect other signaling cascades. For example, in the context of cellular radiation responses it is well known that PtdIns3Ks (e.g., ATM, PRKDC/DNA-PKcs), in addition to signaling through the PtdIns3K-AKT1 pathway, have a major role in the regulation of DNA-damage repair.<sup>717</sup> However, 3-MA, which is a nonspecific inhibitor of class 3 PtdIns3Ks, can alter the function of other classes of this enzyme, which are involved in the DNA-damage repair response. This is of particular importance for investigations into the role of radiation-induced autophagy in cellular radiation sensitivity or resistance.<sup>718,719</sup>

Most other inhibitory drugs act at post-sequestration steps. These types of agents have been used in many experiments to both inhibit endogenous protein degradation and to increase the number of autophagic compartments. They cause the accumulation of sequestered material in either autophagosomes or autolysosomes, or both, because they allow autophagic sequestration to proceed. The main categories of these types of inhibitors include the vinca alkaloids (e.g., vinblastine) and other microtubule poisons that inhibit fusion, inhibitors of lysosomal enzymes (e.g., leupeptin, pepstatin A and E-64d), and compounds that elevate lysosomal pH (e.g., inhibitors of vacuolar-type ATPases such as bafilomycin A<sub>1</sub> and concanamycin A (another V-ATPase inhibitor), and weak base amines including methyl- or propylamine, chloroquine, and Neutral Red, some of which slow down fusion). Ammonia is a very useful agent for the elevation of lysosomal pH in short-term experiments, but has been reported to cause a stimulation of autophagy during long-term incubation of cells in a full medium,<sup>720</sup> under which conditions a good alternative might be methylamine or propylamine.<sup>721</sup> Along these lines, it should be noted that the half-life of glutamine in cell culture media is approximately two weeks due to chemical decomposition, which results in media with lowered glutamine and elevated ammonia concentrations that can affect the autophagic flux (either inhibiting or stimulating autophagy, depending on the concentration<sup>722</sup>). Thus, the use of freshly prepared cell culture media with glutamine is advised, to help reduce experimental variation. A special note of caution is also warranted in regard to chloroquine. Although this chemical is commonly used as an autophagy inhibitor, chloroquine may initially stimulate autophagy (Dorsey FC, personal communication; Franco R, personal communication).

Some data suggest that some nanomaterials may also be novel inhibitors of autophagy, by as yet unidentified mechanisms.<sup>723</sup> It is worth noting that lysosomal proteases fall into three general groups, cysteine, aspartic acid and serine proteases. Therefore, the fact that leupeptin, a serine and cysteine protease inhibitor, has little or no effect does not necessarily indicate that lysosomal degradation is not taking place; a combination of leupeptin, pepstatin A and E-64d may be a more effective treatment. However, it should also be pointed out that these protease

inhibitors can exert inhibitory effects not only on lysosomal proteases, but also on cytosolic proteases; that is, degradation of proteins might be blocked through inhibition of cytosolic instead of lysosomal proteases. Conversely, it should be noted that MG132 (Z-leu-leu-leu-al) and its related peptide aldehydes are commonly used as proteasomal inhibitors, but they can also inhibit certain lysosomal hydrolases such as cathepsins and calpains.<sup>724</sup> Thus, any positive results using MG132 do not rule out the possibility of involvement of the autophagy-lysosome system. Therefore, even if MG132 is effective, it is important to confirm the result using more specific proteasomal inhibitors such as lactacystin or epoxomicin. Finally, there are significant differences in cell permeability among protease inhibitors. For example, E-64d is membrane permeable, whereas leupeptin and pepstatin A are not (although there are derivatives that display greater permeability such as pepstatin A methyl ester).<sup>725</sup> Thus, when analyzing whether a protein is an autophagy substrate, caution should be taken in utilizing these protease inhibitors to block autophagy.

As with the PtdIns3K inhibitors, many autophagy-suppressive compounds are not specific. For example, okadaic acid<sup>726</sup> is a powerful general inhibitor of both type 1 (protein phosphatase 1) and type 2A (PPP2CB/PP2A) protein phosphatases.<sup>727</sup> Bafilomycin A<sub>1</sub> and other compounds that raise the lysosomal pH may have indirect effects on any acidified compartments. Moreover, treatment with bafilomycin A<sub>1</sub> for extended periods (18 h) can cause significant disruption of the mitochondrial network in cultured cells (Gegg ME, personal communication), and either bafilomycin A<sub>1</sub> or concanamycin A cause swelling of the Golgi in plants,<sup>728</sup> and increase cell death by apoptosis in cancer cells (Rao VA, personal communication). Bafilomycin A<sub>1</sub> is often used at a final concentration of 100 nM, but much lower concentrations such as 1 nM may be sufficient to inhibit autophagic-lysosomal degradation and are less likely to cause indirect effects;<sup>154,729,730</sup> however, appropriate inhibitory concentrations should be empirically determined for each cell type.<sup>6</sup> One final caution with regard to bafilomycin A<sub>1</sub> is that activation of lysosomally localized TORC1 depends on an active V-ATPase.<sup>364</sup> This means that treatment with bafilomycin A<sub>1</sub> could elevate the autophagic flux through MTOR inhibition, while simultaneously interfering with flux by inhibiting fusion. Accordingly, other compounds may be preferred for flux measurements. Thus, although these various agents can inhibit different steps of the autophagic pathway, their potential side effects must be considered in interpretation of the secondary consequences of autophagy inhibition, especially in long-term studies. For example, lysosomotropic compounds can increase the rate of autophagosome formation by inhibiting TORC1.<sup>731</sup> Along these lines, chloroquine treatment may cause an apparent increase in the formation of autophagosomes possibly by blocking fusion with the lysosome (Dorsey FC, Cleveland JL, personal communication). This conclusion is supported by the finding that chloroquine reduces the colocalization of LC3 and LysoTracker despite the presence of autophagosomes and lysosomes (Simon AK, personal communication). Concanamycin A blocks sorting of vacuolar proteins in plant cells in addition to inhibiting vacuolar

acidification.<sup>732</sup> Furthermore, in addition to causing the accumulation of autophagic compartments, many of these drugs seem to be stimulators of sequestration in many cell types, especially *in vivo*.<sup>93,235,604,712,716,733-737</sup> Although it is clear why these drugs cause the accumulation of autophagic compartments, it is not known why they stimulate sequestration. One possibility, at least for hepatocytes, is that the inhibition of protein degradation reduces the intracellular amino acid pool, which in turn upregulates sequestration. A time-course study of the changes in both the intra- and extracellular fractions may provide accurate information regarding amino acid metabolism. For these various reasons, it is important to include appropriate controls; along these lines, MTOR inhibitors such as rapamycin or amino acid deprivation can be utilized as positive controls for inducing autophagy. In many cell types, however, the induction of autophagy by rapamycin is relatively slow, or transient, allowing more time for indirect effects; thus, rapamycin may fail to activate autophagy in cultured primary neurons, despite its potent stimulation of autophagy in some cancer cell lines,<sup>238,738,739</sup> and, similarly, it does not induce autophagy in human neuroblastoma SH-SY5Y cells, which can differentiate into neuron-like cells (Diaz-Nido J, personal communication). Thus, glucose depletion may be much more efficient at inducing autophagy than rapamycin or amino acid starvation in neurons in culture (Germain M, Slack R, personal communication); although a number of compounds can also be quite efficient autophagy inducers in neurons including the calpain inhibitor calpeptin.<sup>238,740,741</sup> Several small molecule inhibitors, including torin1, PP242, KU-0063794, PtdIns-103 and NVP-BEZ235, have been developed that target the catalytic domain of MTOR in an ATP-competitive manner.<sup>154,252,742-745</sup> In comparison to rapamycin, these catalytic MTOR inhibitors are more potent, and hence are stronger autophagy agonists in most cell lines.<sup>242,252,746</sup> The use of these second-generation MTOR inhibitors may reveal that some reports of mTOR-independent autophagy may actually reflect the use of the relatively weak inhibitor rapamycin. Furthermore, the use of these compounds has revealed a role for TORC1 and TORC2 as independent regulators of autophagy.<sup>747</sup> Finally, a specialized class of compounds with  $\alpha,\beta$ -unsaturated ketone structure tends to induce autophagic cell death, accompanied by changes in mitochondrial morphology; since the cytotoxic action of these compounds is efficiently blocked by *N*-acetyl-L-cysteine, the  $\beta$ -position in the structure may interact with an SH group of the targeted molecules.<sup>748</sup> Due to the potential pleiotropic effects of various drug treatments, it is incumbent upon the researcher to demonstrate that autophagy is indeed inhibited, by using the methodologies described herein. Accordingly, it is critical to verify the effect of a particular biochemical treatment with regard to its effects on autophagy induction or inhibition when using a cell line that was previously uncharacterized for the chemical being used. Similarly, cytotoxicity of the relevant chemical should be assessed.

The use of gene deletions/inactivations (e.g., in primary or immortalized *Atg*<sup>-/-</sup> MEFs,<sup>401</sup> plant T-DNA or transposon insertion mutants,<sup>200,749</sup> or *in vivo* using transgenic knockout models<sup>750,751</sup> including Cre-lox based “conditional” knockouts<sup>230,231</sup>)

or functional knockdowns (e.g., with RNAi against *ATG* genes) is the preferred approach when possible because these methods allow a more direct assessment of the resulting phenotype; however, different floxed genes are deleted with varying efficiency, and the proportion deleted must be carefully quantified (Hwang S, Virgin IV HW, personal communication). Studies also suggest that microRNAs may be used for blocking gene expression.<sup>164,468,469,752</sup> In certain contexts, it is advisable when using a knockout or knockdown approach to examine multiple autophagy-related genes to exclude the possibility that the phenotype observed is due to effects on a non-autophagic function(s) of the corresponding protein, especially when examining the possibility of autophagic cell death (in contrast, if examining whether perturbation induces clearance of a substrate via autophagy, a single *ATG* gene knockout is probably sufficient). This is particularly the case in evaluating BECN1, which interacts with anti-apoptotic BCL2 family proteins,<sup>425</sup> or when low levels of a target protein are sufficient for maintaining autophagy as is the case with *ATG5*.<sup>171</sup> With regard to *ATG5*, a better approach may be to use a dominant negative (K130R) version.<sup>706,753,754</sup> Along these lines, and as stated above for the use of inhibitors, when employing a knockout or especially a knockdown approach, it is again incumbent upon the researcher to demonstrate that autophagy is actually inhibited, by using the methodologies described herein. Finally, we note that the long-term secondary consequences of gene knockouts or knockdowns are likely much more complex than the immediate effects of the actual autophagy inhibition. To overcome this concern, inducible knockout systems might be useful.<sup>171,315</sup> One additional caveat to knockdown experiments is that pathogen-associated molecular pattern (PAMP) recognition pathways can be triggered by double-stranded RNAs (dsRNA), like siRNA probes, or the viral vector systems that deliver shRNA.<sup>755</sup> Some of these, like TLR-mediated RNA recognition,<sup>756</sup> can influence autophagy by either masking any inhibitory effect or compromising autophagy independent of the knockdown probe. Therefore, nontargeting (scrambled) siRNA or shRNA controls should be used with the respective transfection or transduction methods in the experiments that employ *ATG* knockdown. Another strategy to specifically interfere with autophagy is to use dominant negative inhibitors. Delivery of these agents by transient transfection, adenovirus, or TAT-mediated protein transduction offers the possibility of their use in cell culture or in vivo.<sup>753</sup> However, since autophagy is an essential metabolic process for many cell types and tissues, loss of viability due to autophagy inhibition always has to be a concern when analyzing cell death-unrelated questions. In this respect it is noteworthy that some cell-types of the immune system such as dendritic cells<sup>260</sup> seem to tolerate loss of autophagy fairly well, whereas others such as T and B cells are compromised in their development and function after autophagy inhibition.<sup>757,758</sup>

In addition to pharmacological inhibition, RNA silencing, gene knockout and dominant negative RAB and *ATG* protein expression, pathogen-derived autophagy inhibitors can also be considered to manipulate autophagy. Along these lines ICP34.5,

viral BCL2 homologs and viral CFLAR/FLIP of herpesviruses block autophagosome formation,<sup>425,580,759</sup> whereas M2 of influenza virus and HIV Nef block autophagosome degradation.<sup>278,760</sup> However, as with other tools discussed in this section, transfection or transduction of viral autophagy inhibitors should be used in parallel with other means of autophagy manipulation, because these proteins are used for the regulation of usually more than one cellular pathway by the respective pathogens.

There are fewer compounds that act as inducers of autophagy, but the initial characterization of this process was due in large part to the inducing effects of glucagon, which appears to act through indirect inhibition of MTOR via the activation of STK11/LKB1-AMPK.<sup>615,616,761</sup> Currently, the most commonly used inducer of autophagy is rapamycin, an allosteric inhibitor of TORC1, although one caution is that MTOR is a major regulatory protein that is part of the insulin signaling pathway, and it controls processes other than autophagy, so that rapamycin will ultimately affect many metabolic pathways.<sup>379,762-764</sup> In particular, the strong effects of MTOR on protein synthesis may be a confounding factor when analyzing the effects of rapamycin. MTOR-independent regulation can be achieved through lithium, sodium valproate and carbamazepine, compounds that lower the myo-inositol-1,4,5-triphosphate levels.<sup>765</sup> In vivo treatment of embryos with cadmium results in an increase in autophagy, probably to counter the stress, allowing cell survival through the elimination/recycling of damaged structures.<sup>632</sup> Autophagy may also be regulated by the release of calcium from the endoplasmic reticulum under stress conditions;<sup>212,726,766</sup> however, additional calcium signals from other stores such as the mitochondria and lysosomes could also play an important role in autophagy induction. The activation of the lysosomal two pore channel (TPC), by nicotinic acid adenine dinucleotide phosphate (NAADP) induces autophagy, which can selectively be inhibited by the TPC blocker NED-19, or by pre-incubation with BAPTA, showing that lysosomal calcium also modulates autophagy.<sup>244</sup>

Another way to induce autophagy, both in cultured cells and in vivo, is through transcriptional control. For example, this can be achieved either through overexpression or post-translational activation of the gene encoding TFEB (see *Transcriptional and translational regulation*), a transcriptional regulator of the biogenesis of both lysosomes and autophagosomes.<sup>463,464</sup> Similarly, adenoviral-mediated expression of the transcription factor CEBPB/C/EBP $\beta$  induces autophagy in hepatocytes.<sup>467</sup>

Relatively little is known about direct regulation via the *ATG* proteins, but there is some indication that tamoxifen acts to induce autophagy by increasing the expression of BECN1 in MCF7 cells.<sup>767</sup> However, in U87MG cells treated with tamoxifen BECN1 does not appear to be upregulated, whereas the levels of LC3-II and SQSTM1 are increased, while LAMP2B is down-regulated and CTSD and CTSL1 activities are almost completely blocked (Choi KS, personal communication). Thus, the effect of tamoxifen may differ depending on the cell type. Other data suggest that tamoxifen acts by blocking cholesterol biosynthesis, and that the sterol balance may determine whether autophagy acts in a protective vs. cytotoxic manner.<sup>768,769</sup> Finally, screens have identified small molecules that induce autophagy independently

of rapamycin, and allow the removal of misfolded or aggregate-prone proteins,<sup>770,771</sup> suggesting that they may prove useful in therapeutic applications. However, caution should be taken because of the crosstalk between autophagy and the proteasomal system. For example, trehalose, an MTOR-independent autophagy inducer,<sup>253</sup> can compromise proteasomal activity in cultured primary neurons.<sup>739</sup>

**Conclusion:** Rapamycin is much less effective at inhibiting MTOR and inducing autophagy than catalytic inhibitors, and the latter should therefore be considered for use instead of rapamycin; however, it must be kept in mind that catalytic inhibitors also affect MTORC2. The main concern with pharmacological manipulations is pleiotropic effects of the compound being used. Accordingly, genetic confirmation is preferred whenever possible.

**3. Basal autophagy.** Basal levels of LC3-II or GFP-LC3 puncta may change according to the time after addition of fresh medium to cells, and this can lead to misinterpretations of what basal autophagy means. This is particularly important when comparing the levels of basal autophagy between different cell populations (such as knockout vs. wild-type clones). If cells are very sensitive to nutrient supply and display a high variability of basal autophagy, the best experimental condition is to monitor the levels of basal autophagy at different times after the addition of fresh medium. One example is the chicken lymphoma DT40 cells (see *Chicken B-lymphoid DT40 cells* below) and their knockout variant for all three inositol 1,4,5-trisphosphate receptor isoforms.<sup>241,772,773</sup> In these cells, no differences in basal levels of LC3-II can be observed up to 4 h after addition of fresh medium, but differences can be observed after longer times (Vicencio JM, Szabadkai G, personal communication). This concept should also be applied to experiments in which the effect of a drug upon autophagy is the subject of study. If the drugs are added after a time in which basal autophagy is already high, then the effects of the drug can be masked by the cell's basal autophagy, and wrong conclusions may be drawn. To avoid this, fresh medium should be added first in order to reduce and equilibrate basal autophagy in cells under all conditions, and then the drugs can be added. The basal autophagy levels of the cell under study must be identified beforehand in order to know the time needed to reduce basal autophagy.

A similar caution must be exercised with regard to cell culture density and hypoxia. When cells are grown in normoxic conditions at high cell density, HIF1A/HIF-1 $\alpha$  is stabilized at levels similar to that obtained with low-density cultures under hypoxic conditions.<sup>774</sup> This results in the induction of BNIP3 and BNIP3L/NIX and "hypoxia"-induced autophagy, even though the conditions are theoretically normoxic.<sup>775</sup> Therefore, researchers need to be careful about cell density in order to avoid accidental induction of autophagy.

It should be realized that also in yeast species, medium changes can trigger a higher "basal" level of autophagy in the cells. In the methylotrophic yeast species *Pichia pastoris* and *Hansenula polymorpha* a shift of cells grown in batch from glucose to methanol results in stimulation of autophagy.<sup>776,777</sup> A shift to a new medium can be considered a stress situation. Thus, it appears to be essential to cultivate the yeast cells for a number of hours to

stabilize the level of basal autophagy, before performing experiments intended to study levels of (selective) autophagy (e.g., pexophagy). Finally, plant root tips cultured in nutrient-sufficient medium display constitutive autophagic flux (i.e., a basal level), which is enhanced in nutrient-deprived medium.<sup>696,778,779</sup>

**Conclusion:** The levels of basal autophagy can vary substantially and can mask the effects of the experimental parameters being tested. Changes in media and growth conditions need to be examined empirically to determine effects on basal autophagy and the appropriate times for subsequent manipulations.

**4. Experimental systems.** Throughout these guidelines we have noted that it is not possible to state explicit rules that can be applied to all experimental systems. For example, some techniques may not work in particular cell types or organisms. In each case, the efficacy of autophagy promoters, inhibitors and measurement technique must be empirically determined, which is why it is important to include appropriate controls. Differences may also be seen between in vivo or perfused organ studies and cell culture analyses. For example, insulin has no effect on proteolysis in suspended rat hepatocytes, in contrast to the result with perfused rat liver. The insulin effect reappears, however, when isolated hepatocytes are incubated in stationary dishes<sup>780,781</sup> or are allowed to settle down on the matrix (Häussinger D, personal communication). The reason for this might be that autophagy regulation by insulin and some amino acids requires volume sensing via integrin-matrix interactions and also intact microtubules.<sup>782-784</sup> Along these lines, the use of whole embryos makes it possible to investigate autophagy in multipotent cells, which interact among themselves in their natural environment, bypassing the disadvantages of isolated cells that are deprived of their normal network of interactions.<sup>632</sup> In general, it is important to keep in mind that results from one particular system may not be generally applicable to others.

**Conclusion:** Although autophagy is conserved from yeast to human, there may be tremendous differences in the specific details among systems. Thus, results based on one system should not be assumed to be applicable to another.

**5. Nomenclature.** In order to minimize confusion regarding nomenclature, we make the following notes: In general, we follow the conventions established by the nomenclature committees for each model organism whenever appropriate guidelines are available, and briefly summarize the information here using "ATG1" as an example for yeast and mammals. The standard nomenclature of autophagy-related genes, mutants and proteins for yeast is *ATG1*, *atg1* (or *atg1 $\Delta$*  in the case of deletions) and *Atg1*, respectively, according to the guidelines adopted by the *Saccharomyces* Genome Database ([http://www.yeastgenome.org/gene\\_guidelines.shtml](http://www.yeastgenome.org/gene_guidelines.shtml)). For mammals we follow the recommendations of the International Committee on Standardized Genetic Nomenclature for Mice ([www.informatics.jax.org/mgihome/nomen/](http://www.informatics.jax.org/mgihome/nomen/)), which dictates the gene and protein designations *Atg1* and ATG1 (for all rodents), respectively, and the guidelines for human genes established by the HUGO Nomenclature Committee (<http://www.genenames.org/guidelines.html>), which states that human gene symbols are in the form *ULK1*, and recommends that proteins use the same designation without italics, as with ULK1.

## C. Methods and Challenges of Specialized Model Systems

There are now a large number of model systems being used to study autophagy. These guidelines cannot cover every detail, and this article is not meant to provide detailed protocols. Nonetheless, we think it is useful to briefly discuss what techniques can be used in these systems, and to highlight some of the specific concerns and/or challenges. We also refer readers to the three volumes of *Methods in Enzymology* that provide additional information for “nonstandard” model systems.<sup>38-40</sup>

**1. *C. elegans*.** *C. elegans* has a single ortholog of most yeast Atg proteins; however, two nematode homologs exist for Atg4 and Atg8.<sup>785</sup> Multiple studies have established *C. elegans* as a useful multicellular genetic model to delineate the autophagy pathway and associated functions (see for example refs. 189, 462, 592, 593 and 786). The LGG-1/Atg8/LC3 reporter is the most commonly used tool to detect autophagy in *C. elegans*. Similar to Atg8, which is incorporated into the double membrane of autophagic vesicles during autophagy,<sup>113,187,442</sup> the *C. elegans* LGG-1 localizes into cytoplasmic puncta under conditions known to induce autophagy. Fluorescent reporter fusions of LGG-1/Atg8 with GFP, DsRED or mCherry have been used to monitor autophagosome formation in vivo, in the nematode. These reporters can be expressed either in specific cells and tissues or throughout the animal.<sup>189,593,787,788</sup> LGG-2 is the second LC3 homolog and is also a convenient marker for autophagy either fused to GFP,<sup>789</sup> especially when expressed from an integrated transgene to prevent its germline silencing,<sup>592</sup> or using specific antibodies.<sup>592</sup> The exact function of LGG-1 vs. LGG-2 remains to be addressed.

For observing autophagy by GFP-LC3 fluorescence in *C. elegans*, it is best to use integrated versions of GFP-LC3<sup>592,593,790</sup> (GFP::LGG-1 and GFP::LGG-2; **Fig. 24**) rather than extrachromosomal transgenic strains<sup>189,789</sup> because the latter show variable expression among different animals or mosaic expression (Kang C, personal communication; Galy V, personal communication). It is also possible to carry out indirect immunofluorescence microscopy using antibodies against endogenous LGG-1,<sup>462,593</sup> or LGG-2.<sup>592</sup> In addition, with the integrated version, or with antibodies directed against endogenous LGG-1, it is possible to perform a western blot analysis for lipidation, at least in embryos,<sup>790</sup> and in the whole animal,<sup>593</sup> respectively. Finally, we point out the increasing availability of instruments that are capable of “super-resolution” fluorescence microscopy, which will further enhance the value and possibilities afforded by this technology.<sup>791,792</sup>

**2. Chicken B-lymphoid DT40 cells and retina.** The chicken B-lymphoid DT40 cell line represents a suitable tool for the analysis of autophagic processes in a nonmammalian vertebrate system. In DT40 cells, foreign DNA integrates with a very high frequency by homologous recombination compared with random integration. This makes the cell line a very valuable tool for the generation of cellular gene knockouts. Generally, the complete knockout of autophagy-regulatory proteins is preferable compared with RNAi-mediated knockdown, since in some cases these proteins function normally when expressed at reduced levels.<sup>171</sup>

Different Atg-deficient DT40 cell lines already exist, including *atg13*<sup>-/-</sup>, *ulk1*<sup>-/-</sup>, *ulk2*<sup>-/-</sup>, *ulk1/2*<sup>-/-</sup>,<sup>793</sup> *becn1*<sup>-/-</sup> and *rb1cc1/fip200*<sup>-/-</sup> (Stork B, personal communication). Many additional non-autophagy-related gene knockout DT40 cell lines have been generated and are commercially available.<sup>794</sup>

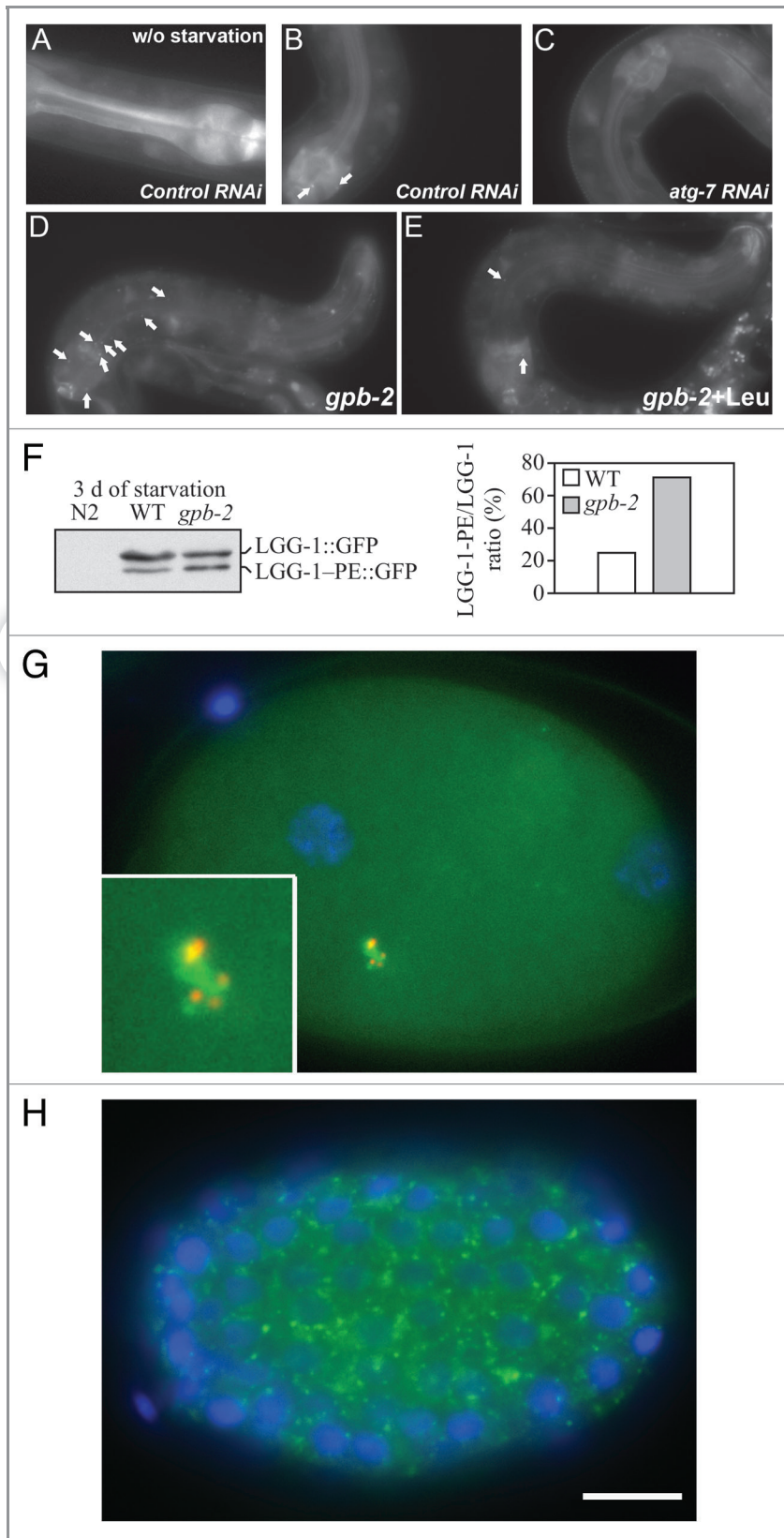
DT40 cells are highly proliferative (the generation time is approximately 10 h) and knockout cells can be easily reconstituted with cDNAs by retroviral gene transfer for the mutational analysis of signaling pathways. DT40 cells mount an autophagic response upon starvation in EBSS,<sup>793</sup> and autophagy can be analyzed by a variety of assays in this cell line. Steady-state methods that can be used include TEM, LC3 western blotting and fluorescence microscopy; flux measurements include monitoring LC3-II turnover and tandem mRFP/mCherry-GFP-LC3 fluorescence microscopy. Using *atg13*<sup>-/-</sup> and *ulk1/2*<sup>-/-</sup> DT40 cells, it was shown that ATG13 and its binding capacity for RB1CC1 are mandatory for both basal and starvation-induced autophagy in this cell line, whereas ULK1/2 and in vitro-mapped ULK1-dependent phosphorylation sites of ATG13 appear to be dispensable for these processes.<sup>793</sup>

Another useful system is chick retina, which can be used for monitoring autophagy at different stages of development. For example, lipidation of LC3 is observed during starvation, and can be blocked with a short-term incubation with 3-MA.<sup>305,306</sup> LEP-100 antibody is commercially available for the detection of this lysosomal protein.

**Cautionary notes:** Since the DT40 cell line derives from a chicken bursal lymphoma, not all ATG proteins and autophagy-regulatory proteins are detected by the commercially available antibodies produced against their mammalian orthologs. The chicken genome is almost completely assembled, which facilitates the design of targeting constructs. However, in the May 2006 chicken (*Gallus gallus*) v2.1 assembly, 5% of the sequence has not been anchored to specific chromosomes, and this might also include autophagy regulatory genes. It is possible that there is some divergence within the signaling pathways between mammalian and nonmammalian model systems. One example might be the role of ULK1/2 in starvation-induced autophagy described above. Additionally, neither rapamycin nor torin1 seem to be potent inducers of autophagy in DT40 cells, although MTOR activity is completely repressed as detected by phosphorylated RPS6KB western blotting.<sup>793</sup> Finally, DT40 cells represent a transformed cell line, being derived from an avian leukosis virus (ALV)-induced bursal lymphoma. Thus, DT40 cells release ALV into the medium, and the 3'-long-terminal repeat has integrated upstream of the c-myc gene, leading to an increased c-myc expression.<sup>795</sup> Both circumstances might influence basal and starvation-induced autophagy.

**3. Chlamydomonas.** It is possible to detect Atg8 modification as well as an increase in the amount of the protein by western blotting in response to autophagy activation.<sup>207</sup> Detection of Atg8 by immunofluorescence microscopy assays is also a reliable method to study autophagy, although it is recommended that this be combined with western blot analysis.

**4. Drosophila.** *Drosophila* provides an excellent system for in vivo analysis of autophagy. In this case, the problem of



**Figure 24.** GFP::LGG-1 and GFP::LGG-2 are autophagy markers in *C. elegans*. (A–F) Animals were generated that carry an integrated transgene expressing a GFP-tagged version of *Igg-1*, the *C. elegans* ortholog of mammalian *MAP1LC3*. Representative green fluorescence images in the pharyngeal muscles of (A) control RNAi animals without starvation, (B) control RNAi animals after 9 d of starvation, (C) *atg-7* RNAi animals after 9 d of starvation, (D) starvation-hypersensitive *gpb-2* mutants without leucine after 3 d of starvation, and (E) *gpb-2* mutants with leucine after 3 d of starvation. The arrows show representative GFP::LGG-1-positive punctate areas that label pre-autophagosomal and autophagosomal structures. (F) The relative levels of PE-conjugated and unconjugated GFP::LGG-1 were determined by western blotting. These figures were modified from data previously published in Kang C, You YJ, Avery L, Dual roles of autophagy in the survival of *Caenorhabditis elegans* during starvation. *Genes Dev* 2007, 21:2161–71, Copyright © 2007, *Genes & Development* by Cold Spring Harbor Laboratory Press and Kang C, Avery L, Systemic regulation of starvation response in *Caenorhabditis elegans*. *Genes Dev* 2009, 23:12–7, Copyright © 2011, *Genes & Development* by Cold Spring Harbor Laboratory Press, www.genesdev.org. (G and H) GFP::LGG-2 serves as a marker for autophagosomes in early *C. elegans* embryos. (G) GFP::LGG-2 expressed in the germline from an integrated transgene reveals the formation of autophagosomes (green) around sperm-inherited membranous organelles (red). DNA of the two pronuclei is stained (blue). (H) Later during development, GFP::LGG-2-positive structures are present in all cells of the embryo. Scale bar, 10  $\mu$ m. Images provided by V. Galy.

either overexpress the gene of interest, or silence the gene through expression of a transgenic RNA interference construct, or homozygous mutant cells are generated. These gain- or loss-of-function clones are surrounded by wild-type cells, which serve as an internal control for autophagy induction. In such an analysis, autophagy in these genetically distinct cells is always compared with neighboring cells of the same tissue, thus eliminating most of the variability and also ruling out potential non-cell-autonomous effects that may arise in mutant animals. Along these lines, clonal analysis should be an integral part of in vivo *Drosophila* studies when possible.

LC3-II western blotting using antibodies against mammalian proteins does not work in *Drosophila* (Baehrecke E, Denton D, Kumar S, Neufeld T, unpublished results). Western blotting has been used successfully in *Drosophila*

animal-to-animal variability can be circumvented by the use of clonal mutant cell analysis, a major advantage of this model system. In this scenario, somatic clones of cells are induced that by monitoring flies expressing human GFP-LC3<sup>101,197</sup> or using an antibody directed against the endogenous Atg8 protein.<sup>454,796</sup> In addition, cultured *Drosophila* (S2) cells can be stably transfected

with GFP fused to *Drosophila* Atg8a, which generates easily resolvable GFP-Atg8a and GFP-Atg8a-PE forms that respond to autophagic stimuli (Wilkinson S, personal communication). Similarly, cultured *Drosophila* cells (l(2)mbn or S2) stably transfected with EGFP-humanLC3B respond to autophagy stimuli (nutrient deprivation) and inhibitors (3-MA, bafilomycin A<sub>1</sub>) as expected, and can be used to quantify GFP-LC3 puncta, which works best using fixed cells with the aid of an anti-GFP antibody.<sup>797a</sup> However, in the *Drosophila* eye, overexpression of GFP-Atg8 results in a significant increase in Atg8-PE by western blot, and this occurs even in control flies in which punctate GFP-Atg8 is not detected by immunofluorescence (Fanto M, unpublished results), and in transfected *Drosophila* Kc167 cells, uninducible but persistent GFP-Atg8 puncta are detected (Kiger A, unpublished results). In contrast, expression of GFP-LC3 under the control of the *rh1* promoter in wild-type flies did not result in the formation of LC3-II detectable by western blot, nor the formation of punctate staining; however, increased GFP-LC3 puncta by immunofluorescence or LC3-II by western blot were observed upon activation of autophagy.<sup>797b</sup> Autophagy can also be monitored with mCherry-Atg18, which is displayed in punctate patterns that are very similar to mCherry-Atg8a (Juhász G, personal communication). Tandem fluorescence reporters have been established in *Drosophila* in vivo, where GFP-mCherry-Atg8a is expressed in the nurse cells of the developing egg chamber.<sup>662</sup> A *Drosophila* transgenic line (Ref(2)P-GFP) and a specific antibody against Ref(2)P, the *Drosophila* SQSTM1 homolog, are available to follow SQSTM1 expression and localization.<sup>312,368</sup>

**5. Filamentous fungi.** As in yeast, autophagy is involved in nutrient recycling during starvation.<sup>193,194,798-801</sup> In addition, macroautophagy seems to be involved in many normal developmental processes such as sexual and asexual reproduction, where there is a need for reallocation of nutrients from one part of the mycelium to another to supply the developing spores and spore-bearing structures.<sup>194,798,799,801,802</sup> Similarly, autophagy also affects conidial germination under nitrogen-limiting conditions.<sup>194</sup> In *Podospora anserina*, autophagy has been studied in relation to incompatibility reactions between mating strains where it seems to play a prosurvival role.<sup>192,802</sup> Of special interest to many researchers of autophagy in filamentous fungi has been the possible involvement of autophagy in plant pathogen infection and growth inside the host.<sup>193,798,799,803-805</sup> Autophagy also appears to be necessary for the development of aerial hyphae,<sup>194,798,802,804</sup> and for appressorium function in *Magnaporthe oryzae* and *Colletotrichum orbiculare*.<sup>193,803,804</sup> Some of these effects could be caused by the absence of autophagic processing of storage lipids (lipophagy) to generate glycerol for increasing turgor.<sup>798,804,805</sup>

Methods for functional analysis of autophagy have been covered in a review article.<sup>806</sup> Most studies on autophagy in filamentous fungi have involved deleting some of the key genes necessary for autophagy, followed by an investigation of what effects this has on the biology of the fungus. Most commonly, *ATG1* and/or *ATG8* has been deleted.<sup>193,798,799,801,802,804</sup> To confirm that the deletion(s) affects autophagy, the formation of autophagic bodies in the wild type and the mutant can be

compared. In filamentous fungi the presence of autophagic bodies can be detected using MDC staining,<sup>193,798</sup> TEM<sup>193,799</sup> or fluorescence microscopy to monitor Atg8 tagged with a fluorescent protein.<sup>194,801,802</sup> This type of analysis is most effective after increasing the number of autophagic bodies by starvation, in combination with decreasing the degradation of the autophagic bodies through the use of the protease inhibitor PMSF,<sup>193,799,801,802</sup> or alternatively by adding the autophagy-inducing drug rapamycin.<sup>194,798</sup> In filamentous fungi it might also be possible to detect the accumulation of autophagic bodies in the vacuoles using differential interference contrast (DIC) microscopy.<sup>801,802</sup> Additional information regarding the timing of autophagy induction can be gained by monitoring transcript accumulation of *ATG1* and/or *ATG8* using qRT-PCR.<sup>799</sup>

**6. Honeybee.** The reproductive system of bees, or insects with merostic polytrophic ovaries, with regard to the ovary developmental cycle can be a very useful tool to analyze and monitor physiological autophagy. Both queen and worker ovaries of Africanized *A. mellifera* display time-regulated features of cell death that are, however, linked to external stimuli.<sup>807</sup> Features of apoptosis and autophagy are frequently associated with the degeneration process in bee organs, but only more recently has the role of autophagy been highlighted in degenerating bee tissues. TEM is the primary method currently being used to monitor autophagy by following the formation of autophagosomes and autolysosomes. This can be combined with cytochemical and immunohistochemical detection of acid phosphatase as a marker for autolysosomes.<sup>808,809</sup> Acidotropic dyes can also be used to follow autophagy in bee organs, as long as the cautions noted in this article are followed. The honeybee genome has been sequenced, and differential gene expression has been used to monitor *Atg18* in bees parasitized by *Varroa destructor*.<sup>810</sup>

**7. Human.** Considering that much of the research conducted today is directed at understanding the functioning of the human body, in both normal and disease states, it is pertinent to include humans and primary human tissues and cells as important models for the investigation of autophagy. Although clinical studies are not readily amenable to these types of analyses, it should be kept in mind that the TORC1 inhibitor rapamycin is available as a clinically approved drug (e.g., sirolimus). Furthermore, fresh biopsies of some human tissues are possible to obtain. Blood, in particular, as well as samples of adipose and muscle tissues, can be obtained from needle biopsies or from elective surgery. For example, in a large study, adipocytes were isolated from pieces of adipose tissue (obtained during surgery) and examined for insulin signaling and autophagy. It was demonstrated that autophagy was strongly upregulated (based on LC3 flux, EM, and lipofuscin degradation) in adipocytes obtained from obese patients with type 2 diabetes compared with nondiabetic subjects.<sup>209</sup>

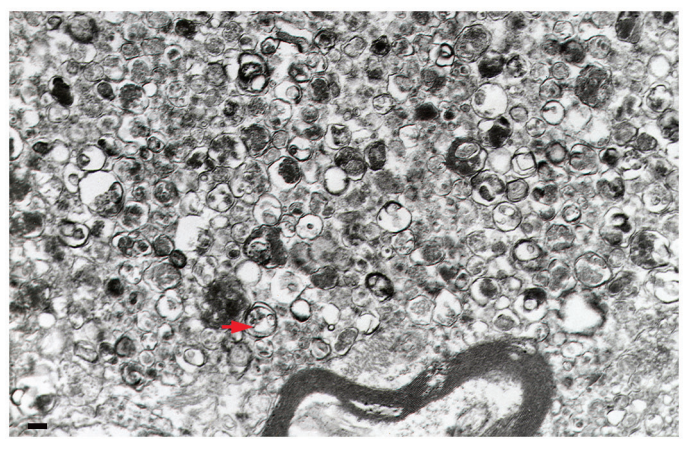
The major caveat of the work concerning autophagy on human tissue is the problem of postmortem times and fixation. Post-mortem times are typically longer in autopsy material than when surgical biopsies are obtained. For tumors, careful sampling to avoid necrosis, hemorrhagic areas and non-neoplastic tissue is required. The problem of fixation is that it can diminish the antibody binding capability; in addition, especially in autopsies, material is not



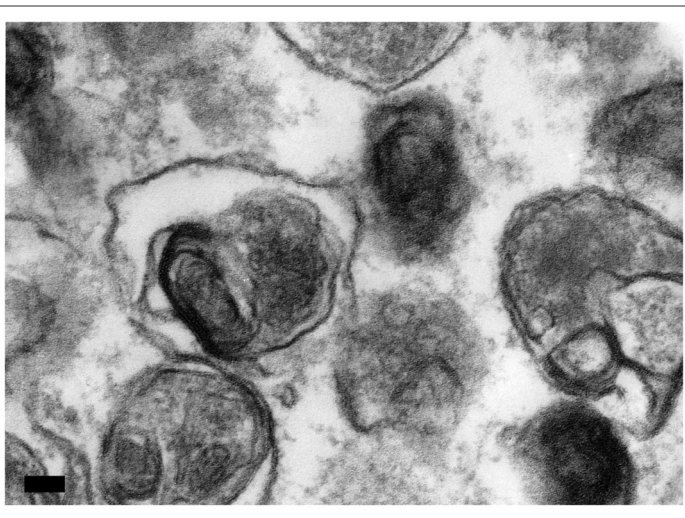
obtained immediately after death.<sup>811,812</sup> The possibilities of postmortem autolysis and fixation artifacts must always be taken into consideration when interpreting changes attributed to autophagy.<sup>813</sup> Analyses of these types of samples require not only special antigen retrieval techniques, but also histopathological experience to interpret autophagy studies by immunohistochemistry, immunofluorescence or TEM.

The situation is even worse with TEM, where postmortem delays can cause vacuolization. Researchers experienced in the analysis of TEM images corresponding to autophagy should be able to identify these potential artifacts because autophagic vacuoles should contain cytoplasm. While brain biopsies may be usable for high quality TEM (Figs. 25 and 26), this depends upon proper handling at the intraoperative consultation stage, and such biopsies are performed infrequently except for brain tumor diagnostic studies. An analysis that examined liver and skeletal muscle from critically ill patients utilized tissue biopsies that were taken within  $30 \pm 20$  min after death and were flash-frozen in liquid nitrogen followed by storage at  $-80^{\circ}\text{C}$ .<sup>815</sup> Samples could subsequently be used for EM and western blot analysis.

A major limitation of studying patient biopsies is that only static measurements can be performed. This limitation does not apply, however, for dynamic experiments on tissue biopsies or cells derived from biopsies, as described above.<sup>209</sup> Multiple measurements over time, especially when deep (vital) organs are involved, are impossible and ethically not justifiable. Hence, quantitative flux measurements are virtually impossible in patients. To overcome these problems to the extent possible and to gain a more robust picture of the autophagic status, observational studies need to include two different aspects. First, a static marker for phagophore or autophagosome formation needs to be measured. This can be done by assessing ultrastructural changes with TEM and/or on the molecular level by measuring LC3-II protein levels. Second, accumulation of autophagy substrates, such as SQSTM1 and (poly) ubiquitinated proteins can provide information on the overall efficacy of the pathway and can be a surrogate marker of the consequences of altered autophagic flux, especially when autophagy is insufficient, although these changes can also be affected by the ubiquitin-proteasome system as mentioned above. In addition, and even more so when problems with specific pathways are suspected (e.g., mitophagy), specific substrates of these pathways should be determined. Again, none of these measurements on its own provides enough information on (the efficacy of) autophagy, because other processes may confound every single parameter. However, the combination of multiple analyses should be informative. Although still in its infancy with regard to autophagy, it is worth pointing out that mathematical modeling has the power to bridge whole body *in vivo* data with *in vitro* data from tissues and cells. The usefulness of so-called hierarchical or multilevel modeling has thus been demonstrated when examining the relevance of insulin signaling to glucose uptake in primary human adipocytes compared with whole-body glucose homeostasis.<sup>816</sup>



**Figure 25.** A large dystrophic neurite from a brain biopsy of a patient with Gerstmann-Sträussler-Scheinker (GSS) disease not unlike those reported for Alzheimer disease.<sup>814</sup> This structure is filled with innumerable autophagic vacuoles, some of which are covered by a double membrane. Electron dense lysosomal-like structures are also visible. The red arrow points to a double-membrane autophagic compartment. Scale bar, 200 nm. Image provided by P. Liberski.



**Figure 26.** A high-power electron micrograph from a brain biopsy showing autophagic vacuoles in a case of ganglioglioma. Scale bar, 200 nm. Image provided by P. Liberski.

The amino acid exchange rate, which has been suggested as a minimal invasive marker for measuring autophagic flux *in vivo* should be used with special caution, as its utility is debatable in disease models (see *Analyses in vivo*).

Finally, a stepwise process can be proposed for linking changes in the autophagic pathway to changes in disease outcome. First, in an observational study, the changes in the autophagic pathway (see above) should be quantified and linked to changes in disease outcome. To prove causality, a subsequent autophagy-modifying intervention should be tested in a randomized study. Before an

intervention study is performed in human patients, the phenotype of (in)active autophagy contributing to poor outcome should be established in a validated animal model of the disease. For the validation of the hypothesis in an animal model, a similar two-step process is suggested, with the assessment of the phenotype in a first stage, followed by a proof-of-concept intervention study (see *Large animals*).

**8. Hydra.** Hydra is a freshwater cnidarian animal that provides a unique model system to test autophagy either in the context of nutrient deprivation, as these animals easily survive several weeks of starvation,<sup>817,818</sup> or in the context of regeneration, because in the absence of protease inhibitors, bisection of the animals leads to an uncontrolled wave of autophagy; in the latter case, an excess of autophagy in the regenerating tip immediately after amputation is deleterious.<sup>819-821</sup> Most components of the autophagy and MTOR pathways are evolutionarily conserved in Hydra.<sup>818</sup> For steady-state measurements, autophagy can be monitored by western blot for ATG8/LC3, by immunofluorescence (using antibodies to ATG8/LC3, LBPA or RSK), or with dyes such as MitoFluor Red 589 and LysoTracker Red. Flux measurements can be made by following ATG8/LC3 turnover using lysosomal protease inhibitors (leupeptin and pepstatin A), or in vivo labeling using LysoTracker Red. It is also possible to monitor MTOR activity with phosphospecific antibodies to RPS6KB kinase and EIF4EBP1, or to examine gene expression by semiquantitative RT-PCR, using primers that are designed for Hydra. Autophagy can be induced by RNAi-mediated knockdown of *Kazal1*,<sup>819,820</sup> or with rapamycin treatment, and can be inhibited with wortmannin or bafilomycin A<sub>1</sub>.<sup>817,818</sup>

**9. Large animals.** Assessment of autophagy (and, in particular, autophagic flux) in clinically relevant large animal models is critical in establishing its (patho)physiological role in multiple disease states. For example, evidence obtained in swine suggests that upregulation of autophagy may protect the heart against damage caused by acute myocardial infarction or “heart attack.”<sup>822</sup> Autophagy also plays an important role in the development and remodeling of the bovine mammary gland. In vitro studies with the use of a three-dimensional culture model of bovine mammary epithelial cells (MECs) have shown that this process is involved in the formation of fully developed alveoli-like structures.<sup>823</sup> Earlier studies show that intensified autophagy is observed in bovine MECs at the end of lactation and during the dry period, when there is a decrease in the levels of lactogenic hormones, increased expression of auto/paracrine apoptogenic peptides, increased influence of sex steroids and enhanced competition between the intensively developing fetus and the mother organism for nutritional and bioactive compounds.<sup>824,825</sup> These studies were based on some of the methods described elsewhere in these guidelines, including GFP-Atg8/LC3 fluorescence microscopy, TEM, and western blotting of LC3 and BECN1. Creation of a specific GFP-LC3 construct by insertion of cDNA encoding bovine LC3 into the pEGFP-C1 vector makes it possible to observe induction of autophagy in bovine MECs in a more specific manner than can be achieved by immunofluorescence techniques, in which the antibodies do not show specific reactivity to bovine cells and tissues.<sup>823,825</sup> However, it is important to remember that definitive confirmation

of cause-and-effect is challenging for studies on large animals, given the lack or poor availability of specific antibodies and other molecular tools, the frequent inability to utilize genetic approaches, and the often prohibitive costs of administering pharmacological inhibitors in these translational preparations.

In contrast with cell culture experiments, precise monitoring of autophagic flux is practically impossible in large animals. Theoretically, repetitive analyses of small tissue biopsies should be performed to study ultrastructural and molecular alterations over time in the presence or absence of an autophagy inhibitor (e.g., chloroquine). However, several practical problems impede applicability of this approach. First, repetitive sampling of small needle biopsies in the same animal (a major challenge by itself) could be assumed to induce artifacts following repetitive tissue destruction, especially when deep (vital) organs are involved. In addition, chemical inhibitors of autophagy have considerable side effects and toxicity, hampering their usage. Also, the general physical condition of an animal may confound results obtained with administration of a certain compound, for instance altered uptake of the compound when perfusion is worse.

Therefore, in contrast to cells, where it is more practical to accurately document autophagic flux, we suggest the use of a stepwise approach in animal models to provide a proof-of-concept with an initial evaluation of sequelae of (in)active autophagy and the relation to the outcome of interest.

First, prior to an intervention, the static ultrastructural and molecular changes in the autophagic pathway should be documented and linked to the outcome of interest (organ function, muscle mass or strength, survival, etc.). These changes can be evaluated by light microscopy, EM and/or by molecular markers such as LC3-II. In addition, the cellular content of specific substrates normally cleared by autophagy should be quantified, as such measurement, despite the static nature, could provide a clue about the results of altered autophagic flux in vivo. These autophagic substrates can include SQSTM1 and (poly) ubiquitinated substrates or aggregates, but also specific substrates such as damaged mitochondria. As noted above, measurement of these autophagic substrates is mainly informative when autophagic flux is prohibited/insufficient, and, individually, all have specific limitations for interpretation. As mentioned several times in these guidelines, no single measurement provides enough information on its own to reliably assess autophagy, and all measurements should be interpreted in view of the whole picture. In every case, both static measurements reflecting the number of autophagosomes (ultrastructural and/or molecular) and measurements of autophagic substrates as surrogate markers of autophagic flux need to be combined. Depending on the study hypothesis, essential molecular markers can further be studied to pinpoint at which stage of the process autophagy may be disrupted.

After having identified a potential role of autophagy in mediating an outcome in a clinically relevant animal model, an autophagy-modifying intervention should be tested. For this purpose, an adequately designed, randomized controlled study of sufficient size on the effect of a certain intervention on the phenotype and outcome can be performed in a large animal model. Alternatively, the effect of a genetic intervention can be

studied in a small animal model with clinical relevance to the studied disease.

As mentioned above, exact assessment of autophagic flux requires multiple time points, which cannot be done in the same animal. Alternatively, different animals can be studied for different periods of time. Due to the high variability between animals, however, it is important to include a sufficiently high number of animals per time point. This thus limits feasibility and the number of time points that can be investigated. The right approach to studying autophagy in large animals likely differs depending on the question that is being addressed. Several shortcomings regarding the methodology, inherent to working with large animals, can be overcome by an adequate study design. As for every study question, the use of an appropriate control group with a sufficient number of animals is crucial in this regard.

**10. Lepidoptera.** Some of the earliest work in the autophagy field was performed in the area of insect metamorphosis.<sup>667</sup> Microscopy and biochemical research revealed autophagy during the metamorphosis of American silkmoths and the tobacco hornworm, *Manduca sexta*, and included studies of the intersegmental muscles, but they did not include molecular analysis of autophagy. Overall, these tissues cannot be easily maintained in culture, and antibodies against mammalian proteins do not often work. Accordingly, these studies were confined to biochemical measurements and electron micrographs. During metamorphosis, the bulk of the larval tissue is removed by autophagy and other forms of proteolysis.<sup>826</sup> *Bombyx mori* is now used as a representative model among Lepidoptera, for studying not only the regulation of autophagy in a developmental setting, but also the relations between autophagy and apoptosis. The advantages of this model are the large amount of information gathered on its developmental biology, physiology and endocrinology, the availability of numerous genetic and molecular biology tools, and a completely sequenced genome.<sup>827</sup> The basic studies of *B. mori* autophagy have been performed in four main larval systems: the silk gland, the fat body, the midgut and the ovary. The methods used for these studies are comparatively similar, starting from EM, which is the most widely used method to follow the changes of various autophagic structures and other features of the cytosol and organelles that are degraded during autophagy.<sup>452,828-831</sup> Immuno-TEM also can be used, when specific antibodies for autophagic markers are available. Acidotropic dyes such as MDC and LysoTracker Red staining have been used as markers for autophagy in silk moth egg chambers combined always with additional assays.<sup>828,829</sup> Acidic phosphatase also can be used as a marker for autolysosomal participation in these tissues.<sup>452,830,832</sup> Systematic cloning and analysis revealed that homologs of most of the *Atg* genes identified in other insect species such as *Drosophila* are present in *B. mori*, and 11 *Atg* genes have now been identified in the silkworm genome, as well as other genes involved in the MTOR signal transduction pathway.<sup>833,834</sup> Variations in the expression of several of these genes have been monitored not only in silkworm larval organs, where autophagy is associated with development,<sup>452,833-835</sup> but also in the fat body of larvae undergoing starvation.<sup>833</sup>

In the IPLB-LdFB cell line, derived from the fat body of the caterpillar of the gypsy moth *Lymantria dispar*, indirect

immunofluorescence experiments have demonstrated an increased number of Atg8-positive dots in cells with increased autophagic activity; however, western blotting did not reveal the conversion of Atg8 into Atg8-PE. Instead, a single band with an approximate molecular mass of 42 kDa was observed that was independent of the percentage of cells displaying punctate Atg8 (Malagoli D, unpublished results). In contrast, with *B. mori* midgut, the use of an antibody specific for BmAtg8 makes it possible to monitor BmAtg8 processing to BmAtg8-PE by western blotting.<sup>452</sup> Thus, the utility of monitoring Atg8 in insects may depend on the particular organism and antibody.

**11. Neotropical teleosts.** In tropical environments, fish have developed different reproductive strategies, and many species have the potential for use as biological model in cell and molecular biology, especially for studying the mechanisms that regulate gametogenesis and embryo development. In these fish, the ovary is a suitable experimental model system for studying autophagy and its interplay with cell death programs due to the presence of postovulatory follicles (POFs) and atretic follicles (AFs), which follow different routes during ovarian remodeling after spawning.<sup>836</sup> In the fish reproductive biology, POFs are excellent morphological indicators of spawning, whereas AFs are relevant biomarkers of environmental stress. In addition, many freshwater teleosts of commercial value do not spawn spontaneously in captivity, providing a suitable model for studying the mechanisms of follicular atresia under controlled conditions.<sup>837</sup> When these species are subjected to induced spawning, the final oocyte maturation (resumption of meiosis) occurs, and POFs are formed and quickly reabsorbed in ovaries after spawning.<sup>838</sup> Assessment of autophagy in fish has been primarily made using TEM at different times of ovarian regression.<sup>839</sup> Due to the difficulty of obtaining antibodies specific for each fish species, immunodetection of ATG-proteins (mainly LC3 and BECN1) by immunohistochemistry associated with analyses by western blotting can be performed using antibodies that are commercially available for other vertebrates. Such studies suggest dual roles for autophagy in follicular cells;<sup>836</sup> however, evaluation of the autophagic flux in different conditions is critical for establishing its physiological role during follicular regression and ovarian remodeling after spawning. Given the ease of obtaining samples and monitoring them during development, embryos of these fish are also suitable models for studying autophagy that is activated in response to different environmental stressors, particularly in studies in vivo.

**12. Odontoblasts.** Odontoblasts are long-lived dentin-forming postmitotic cells, which evolved from neural crest cells early during vertebrate evolution. These cells are aligned at the periphery of the dental pulp and are maintained during the entire healthy life of a tooth. As opposed to other permanent postmitotic cells such as cardiac myocytes or central nervous system neurons, odontoblasts are significantly less protected from environmental insult, such as dental caries and trauma. Mature odontoblasts develop a well-characterized autophagic-lysosomal system, including a conspicuous autophagic vacuole that ensures turnover and degradation of cell components. Immunocytochemical and TEM studies make it possible to monitor age-related changes in autophagic activity in human odontoblasts.<sup>840</sup>

**13. Planarians.** Planarians are one of the favorite model systems in which to study regeneration and stem cell biology, and represent a unique model where it is possible to investigate autophagy in the context of regeneration, stem cells and growth. Currently the method used to detect autophagy is TEM. A detailed protocol adapted to planarians has been described.<sup>841,842</sup> However, complementary methods to detect autophagy are also needed, since TEM cannot easily distinguish between activation and blockage of autophagy, which would both be observed as an accumulation of autophagosomes. Detection of autophagy by other methods are being developed (González-Estévez C, personal communication), including immunohistochemistry and western blotting approaches for the planarian homolog of LC3. Several commercial antibodies against human LC3 have been tried for cross-reactivity without success (see the *Autophagy Forum* for details) and three planarian-specific antibodies have been generated. Some preliminary results show that LysoTracker Red can be a useful technique on whole-mount planarians. Interestingly, most of the components of the autophagy and MTOR signaling machinery are evolutionarily conserved in planarians, and an RNA interference screen is being performed. Whether autophagy genes vary at the mRNA level during starvation and after depletion of MTOR signaling components is also currently being investigated (González-Estévez C, personal communication).

**14. Plants.** The fluorophore of the red fluorescent protein shows a relatively high stability under acidic pH conditions. Thus, chimeric RFP fusion proteins that are sequestered within autophagosomes and delivered to the plant vacuole can be easily detected by fluorescence microscopy. Furthermore, fusion proteins with some versions of RFP tend to form intracellular aggregates, allowing the development of a visible autophagic assay for plant cells.<sup>843</sup> For example, fusion of cytochrome b5 and the original (tetrameric) RFP generate an aggregated cargo protein that displays cytosolic puncta of red fluorescence and, following vacuolar delivery, diffuse staining throughout the vacuolar lumen. This system allows autophagy to be monitored through fluorescence microscopy with minimum damage to intact plant cells. In addition, the size difference between the intact and processed cargo protein allows the quantification of autophagic degradation through the detection of RFP after separation of total protein by gel electrophoresis, similar to the GFP-Atg8/LC3 processing assay described for yeast and mammals.

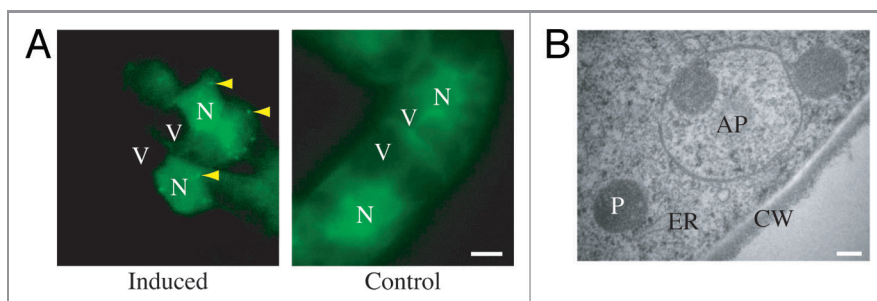
Arabidopsis cells can be stably transfected with GFP fused to plant ATG8, and the lipidated and nonlipidated forms can be separated by SDS-PAGE.<sup>150</sup> Furthermore, the GFP-ATG8 processing assay is particularly robust in Arabidopsis and can be observed not only by western blotting but also by a diffuse fluorescent haze that appears in vacuoles together with punctate GFP fluorescence, which is presumed to represent free soluble GFP and GFP-ATG8-PE still bound to autophagic

bodies, respectively.<sup>151,174</sup> Thus, as with other systems, autophagosome formation in plants can be monitored through the use of fluorescent protein fusions to ATG8, and by TEM (Fig. 27). A tandem fluorescence reporter system is also available in Arabidopsis.<sup>844</sup>

Furthermore, it is possible to use plant homologs of SQSTM1 and NBR1 (JOKA2 in tobacco<sup>845</sup> and NBR1 in Arabidopsis<sup>844</sup>) as markers for autophagy when constructed as fluorescent chimeras. It is worth noting that in some plant models including Arabidopsis<sup>200,402</sup> and somatic embryos of *Picea abies* (Norway spruce) (Minina A, Bozhkov PV, personal communication), the high turnover rate of Atg8-decorated autophagosomes precludes their detection in untreated cells using fluorescent markers (GFP/RFP-Atg8, MDC or LysoTracker) and necessitates pretreatment with concanamycin A. However, the latter treatment will increase vacuolar pH, which may subsequently interfere with its detection by MDC and LysoTracker.

In some systems, including fungi and plants, the size of the vacuole is sufficiently large such that fusion of the autophagosome results in the release of the inner vesicle into the organelle lumen; the resulting single-membrane vesicle is termed an autophagic body (Fig. 1).<sup>200,846</sup> The accumulation of autophagic bodies can be detected by light microscopy in cells that lack vacuolar hydrolase activity (e.g., the *pep4Δ* yeast mutant) or in the presence of inhibitors that interfere with hydrolase activity (e.g., PMSF or concanamycin A). Using Nomarski optics (differential interference contrast) it is easy to distinguish and quantify yeast vacuoles that lack autophagic bodies from those that have accumulated them, and the same is true for plants.

Other methods described throughout these guidelines can also be used in plants. For example, in tobacco cells cultured in sucrose starvation medium, the net degradation of cellular proteins can be measured by a standard protein assay; this degradation is inhibited by 3-MA and E-64c (an analog of E-64d), and is thus presumed to be due to autophagy.<sup>847</sup> In addition, the cytoplasm in tobacco cells that is present in transvacuolar strands that cross the large central vacuole can be seen by TEM to disappear during starvation-induced autophagy.<sup>847</sup>



**Figure 27.** Detection of macroautophagy in tobacco BY-2 cells. (A) Induction of autophagosomes in tobacco BY-2 cells expressing YFP-NtAtg8 (shown in green for ease of visualization) under conditions of nitrogen limitation (Induced). Arrowheads indicate autophagosomes that can be seen as a bright green dot. No such structure was found in cells grown in normal culture medium (Control). Bar, 10  $\mu$ m. N, nucleus; V, vacuole. (B) Ultrastructure of an autophagosome in a tobacco BY-2 cell cultured for 24 h without a nitrogen source. Bar, 200  $\mu$ m. AP, autophagosome; P, plastid; CW, cell wall. This image was provided by K. Toyooka (RIKEN Plant Science Center).

**Cautionary notes:** Although the detection of vacuolar RFP can be applied to both plant cell lines and to intact plants, it is not practical to measure RFP fluorescence in intact plant leaves, due to the very high red fluorescence of chloroplasts. Furthermore, different autophagic induction conditions cause differences in protein synthesis rates; thus, special care should be taken to monitor the efficiency of autophagy by quantifying the intact and processed cargo proteins. With regard to autophagic body accumulation, it is difficult to quantify their number and/or volume, although their presence or absence can be examined by light microscopy or TEM. In addition, the accumulation of autophagic bodies requires the inhibition of vacuolar hydrolase activity. Therefore, to demonstrate turnover, the assay must be performed either in the absence and presence of appropriate inhibitors, or in both a wild-type strain and a strain with a mutation/deletion in a gene encoding a vacuolar hydrolase(s) (see *Selective types of autophagy*). Otherwise, accumulation of autophagic bodies could instead indicate a defect in the lysis/degradation step of autophagy. Finally, this method is not well suited for systems other than plants or fungi because lysosomes are too small for detection by standard (i.e., nonfluorescence) light microscopy, and fusion with autophagosomes does not generate autophagic bodies (Fig. 1).

**15. Protists.** An essential role of autophagy during the differentiation of parasitic protists (formerly called protozoa) is clearly emerging. Only a few of the known *ATG* genes are present in these organisms, which raises the question about the minimal system that is necessary for the normal functioning of autophagy. The reduced complexity of the autophagic machinery in many protists provides a simplified model to investigate the core mechanisms of autophagosome formation necessary for selective proteolysis, and will open a completely new area in autophagy research. Some of the standard techniques used in other systems can be applied to protists including indirect immunofluorescence using antibodies generated against ATG8, the generation of stable lines expressing mCherry- or GFP-fused ATG8 for live microscopy, and immuno-TEM analyses. Extrachromosomal constructs of GFP-ATG8 also work well with lower eukaryotes,<sup>201-203</sup> as do other fluorescently-tagged ATG proteins including ATG5 and ATG12.

The unicellular amoeba *Dictyostelium discoideum* provides another useful system for monitoring autophagy.<sup>848</sup> The primary advantage of *Dictyostelium* is that it has a unique life cycle that involves a transition from a unicellular to a multicellular form. Upon starvation, up to 100,000 single cells aggregate by chemotaxis and form a multicellular structure that undergoes morphogenesis and cell-type differentiation. Development proceeds via the mound stage, the tipped aggregate and a motile slug and culminates with the formation of a fruiting body that is composed of a ball of spores supported by a thin, long stalk made of vacuolized dead cells. Development is dependent on autophagy and, at present, all of the generated mutants in *Dictyostelium* autophagy genes display developmental phenotypes of varying severity.<sup>848,849</sup> *D. discoideum* is also a versatile model to study infection with human pathogens and the role of autophagy in the infection process. The susceptibility of *D. discoideum* to microbial

infection and its strategies to counteract pathogens are similar to those in higher eukaryotes.<sup>850</sup> Along these lines, *Dictyostelium* utilizes some of the proteins involved in autophagy that are not present in *S. cerevisiae* including C12orf44/Atg101, Uvrag and Vmp1, in addition to the core Atg proteins. Autophagy can be monitored in *Dictyostelium* by fluorescence microscopy of GFP-Atg8 or GFP-Atg18, by TEM and by western blot to detect free GFP or the loss of SQSTM1.<sup>36,152,848</sup>

One cautionary note with regard to the use of GFP-ATG8 in protists is that these organisms display some “nonclassical” variations in their ATG proteins. For example, *Leishmania* contains many apparent ATG8-like proteins (the number varying per species; e.g., up to 25 in *L. major*) grouped in four families, but only one labels true autophagosomes even though the others form puncta,<sup>202</sup> and ATG12 requires truncation to provide the C-terminal glycine before it functions in the canonical way. Unusual variants in protein structures also exist in other protists, including the malaria parasite *Plasmodium falciparum*, which expresses ATG8 with a terminal glycine not requiring cleavage to become functional in autophagy.<sup>851</sup> Thus, in each case care needs to be applied and the use of the protein to monitor autophagy validated. In addition, due to possible divergence in the upstream signaling kinases, classical inhibitors such as 3-MA, or inducers like rapamycin, which are not as potent for trypanosomes<sup>852</sup> or apicomplexan parasites as in mammalian cells or yeast, must be used with caution (Coppens I, personal communication);<sup>201</sup> however, RNAi knockdown of TORC1 (e.g., TOR1 or RPTOR) is effective in inducing autophagy.

The scuticociliate *Philasterides dicentrarchi* has proven to be a good experimental organism for identifying autophagy-inducing drugs or for autophagy initiation by starvation-like conditions, since this process can be easily induced and visualized in this ciliate.<sup>853</sup> In scuticociliates, the presence of autophagic vacuoles can be detected by TEM, fluorescence microscopy or confocal laser scanning microscopy by using dyes such as MitoTracker Deep Red FM<sup>®</sup> and MDC.

Finally, a novel autophagy event has been found in *Tetrahymena thermophila*, which is a free-living ciliated protist. A remarkable, virtually unique feature of the ciliates is that they maintain spatially differentiated germline and somatic nuclear genomes within a single cell. The germline genome is housed in the micronucleus, while the somatic genome is housed in the macronucleus. These nuclei are produced during sexual reproduction (conjugation), which involves not only meiosis and mitosis of the micronucleus and its products, but also degradation of some of these nuclei as well as the parental old macronucleus. Hence, there should be a mechanism governing the degradation of these nuclei. The inhibition of PtdIns3Ks with wortmannin or LY294002 results in the accumulation of additional nuclei during conjugation.<sup>854</sup> During degradation of the parental old macronucleus, the envelope of the nucleus becomes MDC- and LysoTracker Red-stainable without sequestration of the nucleus by a double membrane and with the exposure of certain sugars and phosphatidylserine on the envelope.<sup>855</sup> Subsequently, lysosomes fuse only to the old parental macronucleus, but other co-existing nuclei such as developing new macro- and

micronuclei are unaffected.<sup>855</sup> This evidence suggests that selective autophagy may be involved in the regulation of the degradation of nuclei during conjugation, but its mechanism apparently differs from the classical pathway. Indeed, no *ATG* genes homologous to those in yeast are characterized in this organism to date.

**16. Rainbow trout.** Salmonids (e.g., salmon, rainbow trout) experience long periods of fasting often associated with seasonal reductions in water temperature and prey availability or spawning migrations. As such, they represent an interesting model system for studying and monitoring the long-term induction of autophagy. Moreover, the rainbow trout (*Oncorhynchus mykiss*) displays unusual metabolic features that may allow us to gain a better understanding of the nutritional regulation of this degradative system (i.e., a high dietary protein requirement, an important use of amino acids as energy sources, and an apparent inability to metabolize dietary carbohydrates). It is also probably one of the most deeply studied fish species with a long history of research performed in physiology, nutrition, ecology, genetics, pathology, carcinogenesis and toxicology.<sup>856</sup> Its relatively large size compared with model fish such as zebrafish or medaka, makes rainbow trout a particularly well-suited alternative model to carry out biochemical and molecular studies on specific tissues or cells that are impossible to decipher in small fish models. The genomic resources in rainbow trout are now being extensively developed; a high-throughput DNA sequencing program of EST has been initiated associated with numerous transcriptomics studies,<sup>857-860</sup> and the full genome sequence is now available.

Most components of the autophagy and associated signaling pathways (AKT1, TOR, AMPK, FOXO) are evolutionarily conserved in rainbow trout;<sup>457,861-863</sup> however, not all *ATG* proteins and autophagy-regulatory proteins are detected by the commercially available antibodies produced against their mammalian orthologs. Nonetheless, the expressed sequence transcript databases facilitate the design of targeting constructs. For steady-state measurement, autophagy can be monitored by western blot or by immunofluorescence using antibodies to *ATG8/LC3*.<sup>863</sup> Flux measurements can be made in a trout cell culture model (e.g., in primary culture of trout myocytes) by following *ATG8/LC3* turnover in the absence and presence of bafilomycin A<sub>1</sub>. It is also possible to monitor the mRNA levels of *ATG* genes by real-time PCR using primer sequences chosen from trout sequences available in the above-mentioned expressed sequence transcript database. A major challenge in the near future will be to develop for this model the use of RNAi-mediated gene silencing in order to analyze the role of some signaling proteins in the control of autophagy, and also the function of autophagy-related genes in this species.

**17. Sea urchin.** Sea urchin embryo is an appropriate model system for studying and monitoring autophagy and other defense mechanisms activated during physiological development and in response to stress.<sup>632</sup> This experimental model offers the possibility of detecting *LC3* through both protein gel blot and immunofluorescence in situ analysis. Furthermore, in vivo staining of autolysosomes with acidotropic dyes can also be performed. Studies on whole embryos make it possible to obtain qualitative and quantitative data for autophagy and also to get information

about spatial localization aspects in cells that interact among themselves in their natural environment. Cautionary notes include the standard recommendation that it is always preferable to combine molecular and morphological parameters to support the validity of the data.

**18. Ticks.** In the hard tick *Haemaphysalis longicornis*, endogenous autophagy-related proteins (*Atg6* and *Atg12*) can be detected by western blotting and/or by immunohistochemical analysis of midgut sections.<sup>864,865</sup> It is also possible to detect endogenous *Atg3* and *Atg8* by western blotting using antibodies produced against the *H. longicornis* proteins (Umemiya-Shirafuji R, unpublished results). Commercial antibodies against mammalian *ATG* orthologs (*ATG3*, *ATG5*, and *BECN1*) can also be used for western blotting; however, when the tick samples include blood of a host animal, the animal species immunized with autophagy-related proteins should be checked before use to avoid nonspecific background cross-reactivity. In addition to these methods, TEM is recommended to detect autophagosomes and autolysosomes. Although acidotropic dyes can be useful as a marker for autolysosomes in some animals, careful attention should be taken when using the dyes in ticks. Since the midgut epithelial cells contain acidic organelles (e.g., lysosomes) that are related to blood digestion during blood feeding, this method may cause confusion. It is difficult to distinguish between autophagy (autolysosomes) and blood digestion (lysosomes) with acidotropic dyes. Another available monitoring method is to assess the mRNA levels of tick *ATG* genes by real-time PCR.<sup>866</sup> However, this method should be used along with other approaches such as western blotting, immunostaining, and TEM as described in this article. Unlike model insects, such as *Drosophila*, powerful genetic tools to assess autophagy are still not established in ticks. However, RNAi-mediated gene silencing is currently being developed to analyze the function of autophagy-related genes in ticks during nonfeeding periods (Umemiya-Shirafuji R, unpublished results).

**19. Zebrafish.** Zebrafish have many characteristics that make them a valuable vertebrate model organism for the analysis of autophagy. For example, taking advantage of the transparency of embryos, autophagosome formation can be visualized in vivo during development using transgenic GFP-Lc3 and GFP-Gabarap fish. The addition of 1-phenyl-2-thiourea (PTU) to media inhibits melanogenesis allowing visualization of later-stage embryos. Lysosomes can also be readily detected in vivo by the addition of LysoTracker Red to fish media prior to visualization. Additionally, protocols have been developed to monitor *Lc3* protein levels and conjugation to PE by western blot analysis using commercially available *Lc3* antibodies.<sup>35,879</sup>

Because of their translucent character and external fertilization and development, this vertebrate has proven to be an exceptional choice for developmental research. In situ hybridization of whole embryos can be performed to determine expression patterns. Knockdown of gene function is performed by treatment with morpholinos; the core autophagy machinery protein *Gabarap*,<sup>880</sup> and regulatory proteins such as the phosphoinositide phosphatase *Mtmr14*,<sup>881</sup> *Rptor* and *Mtor*,<sup>882</sup> have all been successfully knocked down by morpholino treatment. However, research is

currently somewhat hampered by the deficiency of efficient methods for targeted gene mutations and deletions.

Zebrafish are ideal organisms for in vivo drug discovery and/or verification because of their relatively small size and aqueous habitat, and several chemicals have been identified that modulate zebrafish autophagy activity.<sup>879</sup> Many chemicals can be added to the media and are absorbed directly through the skin. Because of simple drug delivery and the onset of neurodegenerative disease phenotypes at the larval stage, zebrafish are a promising organism for the study of autophagy's role in neurodegenerative disease. Along these lines, a zebrafish model of Huntington disease has been developed.<sup>740</sup>

**20. Food biotechnology.** Required for yeast cell survival under a variety of stress conditions, autophagy has the potential to contribute to the outcome of many food fermentation processes. For example, autophagy induction is observed during the primary fermentation of synthetic grape must,<sup>867</sup> and during sparkling wine production (secondary fermentation).<sup>868</sup> A number of genome-wide studies have identified vacuolar functions and autophagy as relevant processes during primary wine fermentation or for ethanol tolerance, based on gene expression data or cell

viability of knockout yeast strains;<sup>867,869-873</sup> however, understanding the actual relevance of autophagy in yeast-driven food fermentation processes would require addressing this issue experimentally using some of the methods available for *S. cerevisiae* as described in these guidelines.

Autophagy is a target for some widespread food preservatives used to prevent yeast-dependent spoilage. For example, the effect of benzoic acid is exacerbated when concurrent with nitrogen starvation.<sup>874</sup> This opened the way to devise strategies to improve the usefulness of sorbic and benzoic acid, taking advantage of their combination with stress conditions that would require functional autophagy for yeast cell survival.<sup>875</sup> Practical application of these findings would also require extending this research to other relevant food spoilage yeast species, which would be of obvious practical interest.

In the food/health interface, the effect of some food bioactive compounds on autophagy in different human cell types has already attracted some attention.<sup>876,877</sup> Interpreting the results of this type of research, however, warrants two cautionary notes.<sup>878</sup> First, the relationship between health status and autophagic activity is obviously far from being direct. Second, experimental

**Table 2.** Recommended methods for monitoring autophagy

Method	Description
1. Electron microscopy	Quantitative electron microscopy, immuno-TEM; monitor autophagosome number, volume
2. Atg8/LC3 western blotting	Western blot. The analysis is performed in the absence and presence of lysosomal protease or fusion inhibitors to monitor flux; an increase in the LC3-II amount in the presence of the inhibitor is usually indicative of flux
3. GFP-Atg8/LC3 lysosomal delivery and proteolysis	Western blot ± lysosomal fusion or degradation inhibitors; the generation of free GFP indicates lysosomal/vacuolar delivery
4. GFP-Atg8/LC3 fluorescence microscopy	Fluorescence microscopy, FACS to monitor vacuolar/lysosomal localization. Also, increase in punctate GFP-Atg8/LC3 or Atg18/WIPI
5. Tandem mRFP/mCherry-GFP fluorescence microscopy, Rosella	Flux can be monitored as a decrease in green/red (yellow) fluorescence (phagophores, autophagosomes) and an increase in red fluorescence (autolysosomes)
6. SQSTM1/p62 and related LC3 binding protein turnover	The amount of SQSTM1 increases when autophagy is inhibited and decreases when autophagy is induced
7. MTOR, AMPK and Atg1/ULK1 kinase activity	Western blot, immunoprecipitation or kinase assays
8. WIPI fluorescence microscopy	Quantitative fluorescence analysis using endogenous WIPI proteins, or GFP- or Myc-tagged versions. Suitable for high-throughput imaging procedures.
9. Transcriptional and translational regulation	Northern blot, or qRT-PCR, autophagy-dedicated microarray
10. Autophagic protein degradation	Turnover of long-lived proteins to monitor flux
11. Pex14-GFP, GFP-Atg8, Om45-GFP, mitoPho8Δ60	A range of assays can be used to monitor selective types of autophagy. These typically involve proteolytic maturation of a resident enzyme or degradation of a chimera, which can be followed enzymatically or by western blot
12. Autophagic sequestration assays	Lysosomal accumulation by biochemical or multilabel fluorescence techniques, and TEM with a maturation inhibitor
13. Turnover of autophagic compartments	Electron microscopy with morphometry/stereology
14. Autophagosome-lysosome colocalization and dequenching assay	Fluorescence microscopy
15. Sequestration and processing assays in plants	Chimeric RFP fluorescence and processing, and light and electron microscopy
16. Tissue fractionation	Centrifugation, western blot and electron microscopy
17. Degradation of endogenous lipofuscin	Fluorescence microscopy

design in this field must take into account the actual levels of these molecules in the target organs after ingestion, as well as exposure time and their transformations in the human body. In addition, attention must be paid to the fact that several mechanisms might contribute to the observed biological effects. Thus, relevant conclusions about the actual involvement of autophagy on the health-related effect of food bioactive compounds would only be possible by assaying the correct molecules in the correct concentrations.

## Conclusions and Future Perspectives

There is no question that research on the topic of autophagy has expanded dramatically since the publication of the first set of guidelines.<sup>1</sup> To help keep track of the field we have published a glossary of autophagy-related molecules and processes,<sup>883,884</sup> and there are now databases that are specifically dedicated to autophagy including the Human Autophagy-dedicated Database (HADb; [www.autophagy.lu](http://www.autophagy.lu)) and the Autophagy Database (<http://tp-apg.genes.nig.ac.jp/autophagy/>).

With this continued influx of new researchers we think it is critical to try to define standards for the field. Accordingly, we have highlighted the uses and caveats of an expanding set of recommended methods for monitoring macroautophagy in a wide range of systems (Table 2). Importantly, investigators need to determine whether they are evaluating levels of early or late autophagic compartments, or autophagic flux. If the question being asked is whether a particular condition changes autophagic flux (i.e., the rate of delivery of autophagy substrates to lysosomes or the vacuole, followed by degradation), then assessment of steady-state levels of autophagosomes (e.g., by counting GFP-LC3 puncta, monitoring the amount of LC3-II without examining turnover, or by single time point electron micrographs) is not

sufficient as an isolated approach. In this case it is also necessary to directly measure the flux of autophagosomes and/or autophagy cargo (e.g., in wild-type cells compared with autophagy-deficient cells, the latter generated by treatment with an autophagy inhibitor or resulting from *ATG* gene knockdowns). Collectively, we strongly recommend the use of multiple assays whenever possible, rather than relying on the results from a single method.

As a final reminder, we stated at the beginning of this article that this set of guidelines is not meant to be a formulaic compilation of rules, because the appropriate assays depend in part on the question being asked and the system being used. Rather, these guidelines are presented primarily to emphasize key issues that need to be addressed such as the difference between measuring autophagy components, and flux or substrate clearance; they are not meant to constrain imaginative approaches to monitoring autophagy. Indeed, it is hoped that new methods for monitoring autophagy will continue to be developed, and new findings may alter our view of the current assays. Similar to the process of autophagy, this is a dynamic field, and we need to remain flexible in the standards we apply.

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## Glossary

For a more complete listing of autophagy-related terms and definitions we refer readers to Klionsky DJ, Baehrecke EH, Brumell JH, Chu CT, Codogno P, Cuervo AM, et al. A comprehensive glossary of autophagy-related molecules and processes (2nd edition). *Autophagy* 2011; 7:1273–94.

**Amphisome:** Intermediate compartment formed by the fusion of an autophagosome with an endosome; this compartment has not yet fused with a lysosome (also referred to as an acidic late autophagosome).

**Autolysosome:** A degradative compartment formed by the fusion of an autophagosome or amphisome with a primary lysosome or telolysosome. Upon completion of degradation, or when degradation has reached an end point, this compartment (again) becomes a telolysosome (also referred to as a residual body).

**Autophagosome:** A cytosolic membrane-bound compartment typically denoted by a limiting double membrane. The early autophagosome in particular contains cytoplasmic components and organelles that are morphologically unchanged because the compartment has not fused with a lysosome and lacks proteolytic enzymes.

**Autophagy:** A collection of processes typically involving degradative delivery of a portion of the cytoplasm to lysosomes or the plant or fungal vacuole that does not involve direct transport through the endocytic or vacuolar protein sorting, Vps, pathways.

**Chaperone-mediated autophagy (CMA):** Import and degradation of soluble cytosolic proteins by chaperone-dependent, direct translocation across the lysosomal membrane.

**Cytoplasm-to-vacuole targeting (Cvt):** A biosynthetic pathway in fungi that transports resident hydrolases to the vacuole through a selective autophagy-related process.

**Lysosome:** A degradative organelle in higher eukaryotes that compartmentalizes a range of hydrolytic enzymes and maintains a highly acidic pH. A primary lysosome is a relatively small compartment that has not yet participated in a degradation process, whereas secondary lysosomes are sites of present or past digestive activity. The secondary lysosomes include autolysosomes and telolysosomes. Autolysosomes/early secondary lysosomes are larger compartments actively engaged in digestion, whereas telolysosomes/late secondary lysosomes do not have significant digestive activity and contain residues of previous digestions. Both may contain material of either autophagic or heterophagic origin.

**Macroautophagy:** The largely nonspecific autophagic sequestration of cytoplasm into a double- or multiple-membrane-delimited compartment (an autophagosome) of nonlysosomal/vacuolar origin and its subsequent degradation by the lysosomal system. Note that certain proteins and organelles may be selectively degraded via a macroautophagy-related process, and conversely, some cytosolic components such as cytoskeletal elements are selectively excluded.

**Microautophagy:** Uptake and degradation of cytoplasm by protrusion, invagination or septation of the lysosome or vacuole membrane.

**Mitophagy:** The selective autophagic sequestration and degradation of mitochondria.

**Nucleophagy:** The selective autophagic degradation of the nucleus.

**Pexophagy:** A selective type of autophagy involving the sequestration and degradation of peroxisomes; can occur by a micro- or macroautophagic process.

**Phagophore:** Membrane cisterna that has been implicated in an initial event during formation of the autophagosome. Previously referred to as the “isolation membrane.”

**Phagophore assembly site (PAS):** A perivacuolar compartment or location that is involved in the formation of Cvt vesicles and autophagosomes in yeast. The PAS may supply membranes during the formation process or may be an organizing center where most of the autophagic machinery resides, at least transiently.

**Phosphatidylinositol 3-kinase (PtdIns3K):** A family of enzymes that add a phosphate group to the 3' hydroxyls on the inositol ring of phosphoinositides. The class III phosphatidylinositol 3-kinases are stimulatory for autophagy, whereas class I enzymes are inhibitory.

**Reticulophagy:** A type of selective macroautophagy of the ER. Autophagy counterbalances endoplasmic reticulum expansion during the unfolded protein response. Activation of the UPR in yeast induces reticulophagy.

**Ribophagy:** The selective autophagic sequestration and degradation of ribosomes.

**Vacuole:** The fungal and plant counterpart of the lysosome; this organelle also carries out storage and osmoregulatory functions. The plants' bona fide equivalent of the lysosome is the lytic vacuole.

**Xenophagy:** The selective degradation of microbes (e.g., bacteria, fungi, parasites and/or viruses) through an autophagy-related mechanism.

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