A New Fluorescence-Based Assay for Autophagy

Tassula Proikas-Cezanne[1,*] and Patrice Codogno[2]
[1]Autophagy Laboratory, Interfaculty Institute for Cell Biology, Eberhard Karls University Tuebingen, 72076 Tuebingen, Germany
[2]INSERM U984, University Paris-Sud 11, 92296 Châtenay-Malabry, France
*Correspondence: tassula.proikas-cezanne@uni-tuebingen.de
DOI 10.1016/j.chembiol.2011.08.001

Monitoring autophagy through fluorescence-based visualization procedures is an important challenge to understand vesicular pathways designated for lysosomal degradation. In this issue of *Chemistry & Biology*, Katayama et al. report a new approach to trace autophagic events by visualizing the pH-dependent excitation changes of the coral protein Keima.

Autophagy is an evolutionarily conserved survival mechanism that is compromised in a variety of human diseases (Meijer and Codogno, 2009). By both specific and unspecific degradation of cytoplasmic material via the lysosomal compartment, several types of autophagy secure eukaryotic cellular homeostasis. Macroautophagy is defined by the generation of autophagosomes, unique multimembrane vesicles that either specifically sequester recognized cargo or that engulf cytoplasmic bulk material. By fusing with endosomes, the generated autophagosomes acquire acidic properties before they finally deliver their cargo to the lysosomal compartment. These vesicles, fused from autophagosomes and lysosomes, are called autolysosomes, or autophagolysosomes (see Figure 1). In contrast, the two other described autophagic pathways, microautophagy and chaperone-mediated autophagy, are defined by a specific cargo direction into the lysosomal compartment, without the generation of autophagosomes (Mizushima et al., 2008). A variety of methods have been developed to assay macroautophagy in multicellular eukaryotes, but most studies employ LC3 (or Atg8) to track both the formation of autophagosomes and the quantity of degradable cargo that is transported from the autophagosome to the lysosomal compartment (i.e., the autophagic flux) (Mizushima et al., 2010; Tanida et al., 2005). LC3 is involved in the elongation of the initial autophagosomal template membrane (phagophore) by contributing to hemifusion events during the formation of complete autophagosomes (Nakatogawa et al., 2007). The LC3 protein is anchored through conjugation of its C terminus to the polar head of phosphatidylethanolamine (LC3 lipidation) to both the outer membrane and the inner membrane of the autophagosome. While most of the LC3 at the outer autophagosomal membrane is recycled to the cytosol, the fraction of LC3 that is bound to the inner membrane is transported to the lysosomal compartment where it is degraded (see Figure 1).

Mainly, the following three LC3-based methods are widely used to assess macroautophagy in quantitative terms: (1) GFP-LC3 for fluorescence-based visualization of autophagosomes, (2) RFP-GFP tandem-tagged LC3 for fluorescence-based measurement of autophagosome maturation, and (3) anti-LC3 western blotting to determine LC3 lipidation (Mizushima et al., 2010). Using all of the above LC3-based assays, the autophagic flux can be measured using lysosomal inhibitors, such as bafilomycin A1. Nevertheless, these LC3-based assays are limited in that LC3 also marks nonautophagosomal vesicles in the endosomal compartment (Reggiori et al., 2010). Recently, the phagophore marker proteins WIPI-1 and WIP1-2 that specifically bind phosphatidylinositol-3 phosphate (PtdIns(3)P) have been shown to also localize at the outer and inner membrane of autophagosomes (Proikas-Cezanne and Robenek, 2011), hence WIPI-1 is also employed to assess macroautophagy, e.g., with high throughput fluorescence-based systems (see Figure 1).

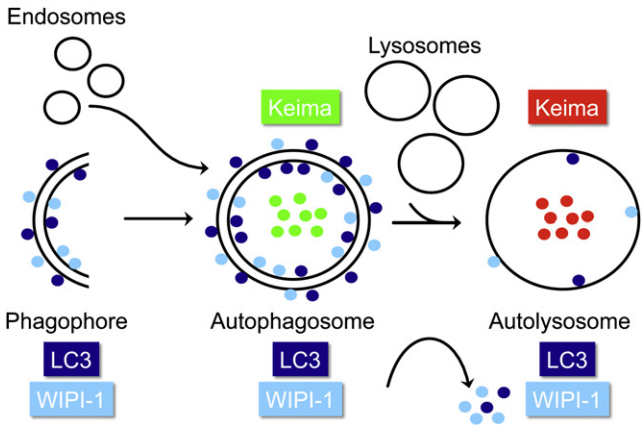

Figure 1. Overview over Macroautophagy
Illustrated here are the different steps involved in macroautophagy that can be followed with the use of the fluorescence-based marker proteins LC3 and WIPI. Keima is a novel probe that marks acidic compartments, including autolysosomes (Katayama et al., 2011).

In this issue, the Miyawaki group reports the characterization of Keima, a coral-derived fluorescent protein, as a novel probe to specifically measure autolysosome formation (Katayama et al., 2011). What makes this probe particularly interesting is that the protein has a large Stokes shift and a pH-dependent reversible bimodal excitation spectrum. In fluorescence microscopy, ionized Keima is detected as a red fluorescent signal at acidic pH (586 nm) and neutral Keima as a green fluorescent signal at higher pH (438 nm). Upon expression

in eukaryotic cells, the Keima protein appeared to be distributed throughout the cytoplasm and the nucleus (Katayama et al., 2011). Interestingly, upon nutrient starvation, Keima relocated to green and red punctate structures that did not form in the presence of either wortmannin or bafilomycin A1, and the authors attributed the observed increase in 550/438 nm excitation ratio (<pH 6.0) to autolysosome formation. Using Keima fused to the mitochondrial-targeting sequence of subunit VIII of the cytochrome c oxidase (COX VIII), the Miyawaki group induced mitochondrial impairment and visualized the delivery of dysfunctional mitochondria marked with the ubiquitin ligase Parkin to the lysosomal compartment. Here, the Keima-based assay might provide a vital tool to study mitophagy in higher eukaryotic cells, as selective mitophagy is generally attributed to macroautophagy. However, new results indicate that novel routes of microautophagy deliver specific cargo from the endosomal to the lysosomal compartment (Santambrogio and Cuervo, 2011) and, at least in yeast, degradation of mitochondria also occurs via selective microautophagy (Kissová et al., 2007). Especially in the field of autophagy and neurodegeneration, Keima might provide a vital tool to distinguish between different routes of delivering damaged mitochondria to the lysosomal compartment, either through selective micro- or macroautophagy. Further, the Keima probe can also be used to visualize autophagic events in conditions of impaired Atg (autophagy-related) protein function, as impressively demonstrated by the Miyawaki group here in providing evidence for the presence of autolysosomes in Atg5-deficient mouse embryonic fibroblasts in the absence of lipidated LC3 (Katayama et al., 2011). In future studies, it will become important to precisely distinguish between the subsets of Keima that are localized in late endosomes, autophagosomes, and autolysosomes. However, as the path of autophagy research is rocky and full of surprises—as is any other research field, it is vital to now have more than only LC3 available to quantitatively assess the complexity of this cellular process.

REFERENCES

Katayama, H., Kogure, T., Mizushima, N., Yoshimori, T., and Miyawaki, A. (2011). Chem. Biol. *18*, this issue, 1042–1052.

Kissová, I., Salin, B., Schaeffer, J., Bhatia, S., Manon, S., and Camougrand, N. (2007). Autophagy *3*, 329–336.

Meijer, A.J., and Codogno, P. (2009). Crit. Rev. Clin. Lab. Sci. *46*, 210–240.

Mizushima, N., Levine, B., Cuervo, A.M., and Klionsky, D.J. (2008). Nature *451*, 1069–1075.

Mizushima, N., Yoshimori, T., and Levine, B. (2010). Cell *140*, 313–326.

Nakatogawa, H., Ichimura, Y., and Ohsumi, Y. (2007). Cell *130*, 165–178.

Proikas-Cezanne, T., and Robenek, H. (2011). J. Cell. Mol. Med., in press. Published online May 12, 2011. 10.1111/j.1582-4934.2011.01339.x.

Reggiori, F., Monastyrska, I., Verheije, M.H., Calì, T., Ulasli, M., Bianchi, S., Bernasconi, R., de Haan, C.A., and Molinari, M. (2010). Cell Host Microbe *7*, 500–508.

Santambrogio, L., and Cuervo, A.M. (2011). Autophagy *7*, 652–654.

Tanida, I., Minematsu-Ikeguchi, N., Ueno, T., and Kominami, E. (2005). Autophagy *1*, 84–91.

