CHAPTER SIX

Assays to Assess Autophagy Induction and Fusion of Autophagic Vacuoles with a Degradative Compartment, Using Monodansylcadaverine (MDC) and DQ-BSA

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Abstract

In this chapter we describe the use of monodasylcadaverine (MDC) and DQ-BSA, two practical and convenient tools to study the autophagic pathway. MDC is a lysosomotropic compound useful for the identification of autophagic vesicles by fluorescence microscopy and, in addition, to assess autophagy induction via the accumulation of MDC-labeled vacuoles. However, the increase of

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Methods in Enzymology, Volume 452
ISSN 0076-6879, DOI: 10.1016/S0076-6879(08)03606-9

autophagosomes does not necessarily reflect autophagosome maturation and degradation of the sequestered materials, thus the use of DQ-BSA in conjunction with and autophagic marker is an appropriate technique to monitor the formation of the autolysosome, the degradative compartment. Therefore, here we discuss the advantages of the utilization of these two methods to characterize the autophagy pathway.

1. Overview

Several methods have been developed to study the autophagy pathway and the steps involved in the maturation of autophagosomes to autolysosomes, an acidic hydrolase rich organelle in which the sequestered material is degraded. Monodasylcadaverine (MDC) and DQ-BSA are useful tools for identification and visualization of the autophagic process by fluorescence microscopy. The lysosomotropic compound MDC was initially characterized as a selective marker of autolysosomes (Biederbick *et al.*, 1995). However, more recent studies indicate that MDC also works as a marker of earlier autophagic compartments, and the accumulation of MDC-positive vesicles responds to autophagy induction when this process is stimulated, both in cultured cells and in animals (Munafó and Colombo, 2001; Iwai-Kanai *et al.*, 2008). On the other hand, the use of the bovine serum albumin derivative (DQ-BSA) conjugated to a self-quenched fluorophore, in conjunction with an autophagic marker, is a helpful bona fide method for detecting the formation of an active degradative compartment, the autolysosome, allowing the visualization of the completion of the autophagic pathway. Here, we detail both methods and attempt to clarify some controversial points.

2. Assessing Autophagy Induction by Fluorescence Microscopy and Fluorometry

2.1. Labeling autophagic vacuoles with monodansylcadaverine (MDC) in cultured cells

Monodansylcadaverine (MDC) has autofluorescent properties with an excitation wavelength at 365 nm, due to a dansyl group conjugated to cadaverine, a diamine-pentane (Fig. 6.1). MDC accumulates in lipid-rich membranous compartments and gets trapped in adjacent membranes. Elsässer and collaborators initiated the studies with MDC and established that it accumulates as a selective fluorescent marker for autophagic vacuoles under *in vivo* conditions by interacting with membrane lipids that are highly concentrated in the autophagic compartments. In our laboratory, we

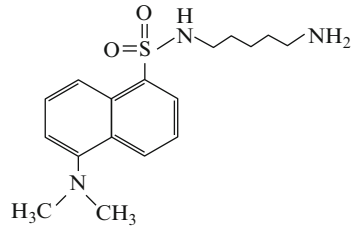

Figure 6.1 Formula of monodansylcadaverine (MDC). A dansyl group conjugated to a diamine-pentane is responsible for the autofluorescence.

characterized the labeling of cells with MDC and the incorporation of this compound by fluorometry (see subsequent sections) in cells subjected to different autophagy modulators (Munafó and Colombo, 2001).

MDC can be used to label autophagic vacuoles in several cell types (Biederbick *et al.*, 1995). These investigators have indicated that dose-response experiments with PaTu 8902 cells show that the optimal concentration for *in vivo* labeling is 0.05 to 0.1 m*M*, whereas cells detached and disintegrated when the MDC concentration exceeded 0.1 m*M*. When MDC is incorporated into cells, the accumulation of this fluorescent reagent is observed in spherical compartments at the perinuclear region, in spots distributed throughout the cytoplasm, or both, depending on the cell type used. Biederbick *et al.* also demonstrated by ultrastructural analysis of fractions obtained on sucrose density gradients that the labeled compartments correspond to autophagic vacuoles in different stages of development. The MDC-labeled autophagic vacuoles do not contain fluid-phase markers and are spatially separated from endosomal compartments, but they do include lysosomal enzymes.

Our group demonstrated that in cells subjected to a physiological or pharmacological stimulus of autophagy, such as amino acid deprivation or rapamycin treatment, the size and number of MDC-fluorescent vesicles markedly increases (Munafó and Colombo, 2001). In contrast, when cells are previously treated with well-known inhibitors of autophagy, such as 3-methyladenine (3-MA) and wortmannin (WM), the MDC incorporation is almost completely inhibited, indicating that MDC-labeled compartments respond to the dynamic changes in autophagy that occur in response to several modulators. In addition, a population of the MDC-labeled vesicles colocalizes with LC3, a specific autophagosome marker. When CHO cells overexpressing GFP-LC3 are subjected to autophagy induction and subsequently labeled with MDC, a good colocalization of LC3 and MDC is observed, correlating with autophagosome formation in response to the autophagy stimulus (Munafó and Colombo, 2001). It has been suggested that MDC is accumulated only in autophagic vacuoles that have already fused with lysosomes, an acidic compartment. However, vinblastine, a

microtubule despolymerizing agent that leads to the accumulation of autophagic vacuoles by blocking fusion with lysosomes alters the distribution and size of MDC-marked autophagosomes with an evident increase in the MDC labeling (Munafó and Colombo, 2001).

Because MDC is a basic compound and acts as a lysosomotropic agent, being concentrated into acidic compartments by an ion-trapping mechanism, a neutral derivative known as monodansylpentane (MDH) was synthesized (Niemann *et al.*, 2001). Because of the properties of this new compound, MDH staining of autophagic vacuoles is independent of the acidic pH, and thus of an ion-trapping mechanism, but shows the same preferences for autophagic membrane lipids as MDC (Niemann *et al.*, 2001). Furthermore, the authors indicate that under *in vivo* conditions MDH is a more stringent marker for autophagic vacuoles than MDC.

We and other colleagues have demonstrated that the GTPase Rab7 decorates autophagic vesicles and is required for the normal progression of the autophagy pathway (Gutierrez *et al.*, 2004; Jager *et al.*, 2004; Kimura *et al.*, 2007). In cells overexpressing wild-type GFP-Rab7 and GFP-Rab7Q67L (a Rab7 constitutively active mutant), MDC associates with the Rab7-decorated vesicles incubated in either full-nutrient or starvation conditions. On the other hand, cells transfected with the dominant negative mutant (Rab7T22N) present a diffused localization in the cytosol in cells incubated under control conditions; but when autophagy is induced, the Rab7T22N protein localizes on the MDC-labeled vesicles. It is important to mention that the expression of the Rab7T22N mutant hampers fusion between autophagosomes and lysosomes (Gutierrez *et al.*, 2004; Fader *et al.*, 2008), thus these MDC-labeled vesicles cannot account for autolysosomes. We also tested MDH in transfected cells with wild-type Rab7 and the Rab7-negative mutant. Our results show that MDH colocalizes with both wild-type Rab7 and T22N-decorated vesicles in nutrient-deprived cells. Therefore, similar to MDC, MDH can be used as a good marker of autophagic vesicles. A significant problem with this compound is that it is not commercially available and thus needs to be synthesized (Niemann *et al.*, 2001).

In a recent publication, Roberta Gottlieb and collaborators (Iwai-Kanai *et al.*, 2008) investigated the use of MDC and the lysosomotropic drug chloroquine to measure autophagic flux in transgenic mice expressing mCherry-LC3 (a full description of this method is also presented in this volume). Interestingly, the results show mCherry-LC3-positive dots colocalizing with MDC in myocardium from hearts of mice injected with both rapamycin and chloroquine. Thus, chloroquine administration, which inhibits lysosomal activity by neutralizing its pH, does not prevent the accumulation of MDC, implying that MDC accumulates independent of the acidic pH. Furthermore, chloroquine hampers the starvation-induced colocalization of the lysosomal marker LAMP-2 and GFP-LC3 as well as the colocalization of mitochondria with lysosomes, indicating that the formation of the autolysosome is blocked in the

presence of chloroquine (Boya *et al.*, 2005). Taken together, these data indicate that MDC-labeled vesicles are not exclusively autolysosomes.

The individual steps of *in vivo* MDC staining when autophagy is induced by amino acid deprivation in Chinese hamster ovary (CHO) cells are described subsequently:

1. Plate CHO cells at 80% of confluence in 2 ml of culture media (α-MEM containing 10% fetal bovine serum (FBS), 2 m*M* glutamine, 100 U/ml penicillin, and 100 g/ml streptomycin) on a 6-well tissue-culture dish containing a cover glass for each well.
2. Incubate the cells 24 h at 37 °C in an atmosphere of 95% air and 5% CO_2.
3. Wash the cells 3 times with PBS to remove the media and add 2 ml of Earle's balanced salts solution (EBSS, starvation media; Sigma) to induce autophagy, or complete tissue culture medium for control conditions. Incubate the cells 2 h at 37 °C.
4. Subsequently, control and starved cells are incubated with 0.05 μM MDC in PBS at 37 °C for 10 min.
5. After incubation, the cells are washed 4 times with PBS and immediately analyzed by fluorescence microscopy (see Fig. 6.2). The microscope should be equipped with the adequate filters (MDC has an autofluorescence at 365 and 525 nm wavelength, for excitation and emission, respectively).

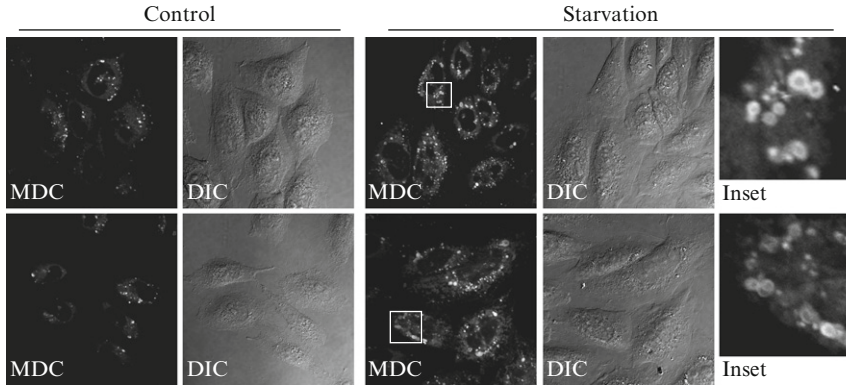

Figure 6.2 MDC-labeled vesicles are induced by starvation. CHO cells were incubated in α-MEM medium or in EBSS medium (starvation) at 37 °C for 2 h. Following this incubation period, both starved and control cells were incubated with 0.05 m*M* MDC for 10 min at 37 °C and then washed 4 times with PBS, pH 7.4. Cells were immediately analyzed by confocal microscopy. *Insets*: MDC-labeled vesicles with a clear ring-like structure, which is consistent with the preferential labeling of the double-membrane lipid-rich autophagic compartment.

Note: Even though MDC labeling can be visualized both in *in vivo* conditions and after fixation (Biederbick *et al.*, 1995), it is important to take into account that the staining intensity can be markedly decreased under certain fixation conditions. Although we recommend incubating the cells with 3% paraformaldehyde for 6 min, still the fluorescence intensity is very dim. Thus, it is preferable to visualize MDC in nonfixed cells.

2.2. Assessing autophagy induction by fluorometry in cells labeled with MDC

Another useful technique to determine autophagy induction is measuring intracellular MDC by fluorometry (Fig. 6.3).

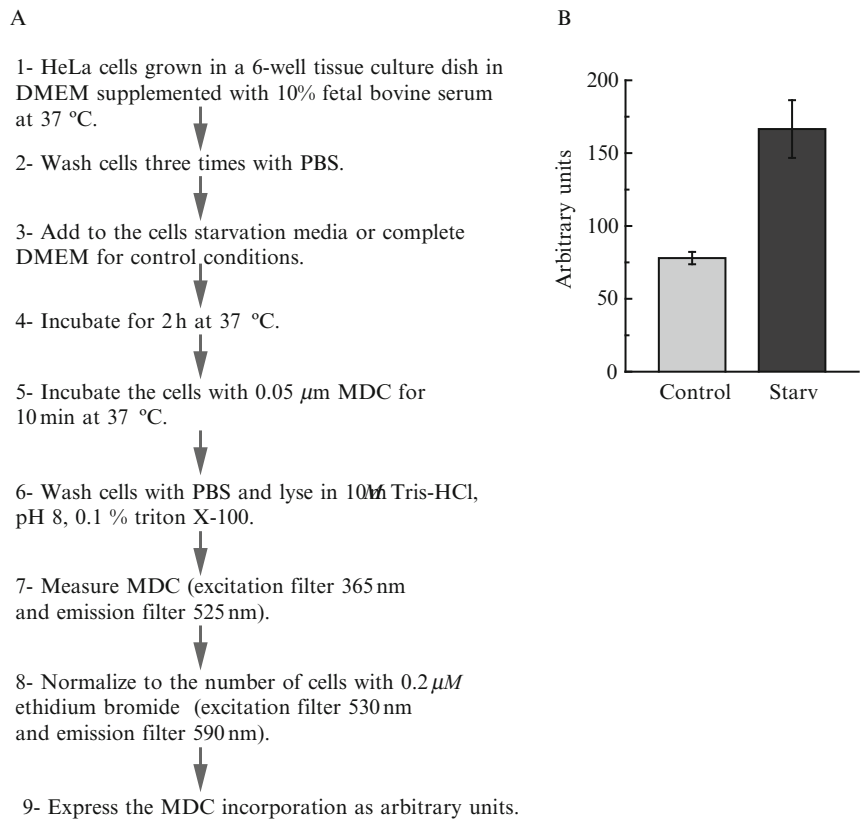

Figure 6.3 *Panel A*: Steps to incorporate and measure intracellular MDC by fluorescence photometry in HeLa cells. *Panel B*: Intracellular MDC was measured by fluorescence photometry in cells incubated in control or starvation media. The MDC uptake markedly increased when autophagy was induced by amino acid deprivation.

1. Cells are plated in 2 ml of culture medium (DMEM or α-MEM depending on the cell type used) on a 6-well tissue-culture dish to 80% of confluence.
2. Wash the cells 3 times with PBS to remove the media and add 2 ml of Earle's balanced salts solution (EBSS, starvation media; Sigma) to induce autophagy, or complete culture medium for control conditions. Incubate the cells 2 h at 37 °C.
3. Subsequently, the cells are incubated with 0.05 μM MDC in PBS for 10 min at 37 °C.
4. After incubation, the cells are washed 4 times with PBS pH 7.4, and then lysed in 10 m*M* Tris-HCl, pH 8, containing 0.1% Triton X-100.
5. Measure intracellular MDC by fluorescence photometry (excitation wavelength 365 nm, emission filter 525 nm) in a Packard Fluorocount microplate reader. To normalize for the amount of cells present in each well, a solution of ethidium bromide is added to a final concentration of 0.2 m*M* and the DNA fluorescence is measured with the adequate filters, 530 nm and 590 nm wavelength, for excitation and emission, respectively. The MDC incorporation is expressed as specific activity (arbitrary units).

In addition to the morphological analysis of the autophagy pathway by fluorescence microscopy, the biochemical assay described previously makes it possible to quantitatively monitor autophagy induction in a simple manner (Munafó and Colombo, 2001).

Special comments and clarifications: As indicated previously, there is evidence that MDC can interact with membrane lipids to function as a solvent polarity probe (Niemann*et al.*, 2005), and because one of the features of autophagic vacuoles is their high content of lipids, MDC has been widely used as an autophagic marker. However, one exhaustive study indicates that MDC labels only acidic compartments working just as a lysosomotropic compound (Bampton *et al.*, 2005). In addition, MDC dots can be detected in $Atg5^{-/-}$ mouse embryonic stem cells (Mizushima, 2004), indicating that MDC does not stain only autophagic compartments. Nevertheless, when the acidic pH is hampered by applying well-known inhibitors, the MDC fluorescence is reduced in some cell types (Niemann *et al.*, 2005; Bampton *et al.*, 2005); in others it is almost not affected (Iwai-Kanai *et al.*, 2008). Thus, the ion-trapping mechanism is not sufficient to explain the MDC labeling because only a certain fraction of MDC accumulation seems to depend on the acidic pH. In contrast, when the uptake of acridine orange is assessed under the same conditions (i.e., blocking the acidic pH) no labeling at all is observed, which is expected with a restrictive lysosomotropic compound. Furthermore, in paraformaldehyde-fixed cells, in which no proton gradients are maintained, the labeling of vesicles with MDC still occurs. Thus, MDC labeling in fixed cells, although weaker, is likely more specific as indicated in the work of Karla Kirkegaard (Jackson

et al., 2005). These differences suggest an additional mechanism for the MDC staining besides ion trapping (see also subsequent sections).

Furthermore, some studies suggest that MDC only marks autophagic compartments after fusion to acidic endo/lysosomes (Bampton *et al.*, 2005). However, we would like to point out that in the presence of vinblastine, which hampers fusion with lysosomes, a marked amount of enlarged MDC-labeled vesicles accumulate. Furthermore, in conditions that stimulate autophagy, several of the MDC-labeled vesicles appear as a ring-like structure (see Fig. 6.2; Munafó and Colombo, 2001), indicating that the drug is preferentially intercalating in the membranes more than just labeling a diffuse acidic compartment. These discrepancies, perhaps related to the cell type, have caused some concerns about the usefulness of MDC. Nevertheless, as indicated earlier, in the recent work by Gottlieb and collaborators (Iwai-Kanai *et al.*, 2008) in animals, MDC labeled LC3-marked vesicles even in the presence of the lysosomotropic agent chloroquine, which neutralizes the lysosomal pH. Although, it is likely that MDC can label autolysosomes, the observations described clearly suggest that MDC is able to label autophagic structures in spite of the disruption of their acidic pH.

3. Monitoring the Formation of an Autolysosome

3.1. Labeling lysosomes with DQ-BSA

It is well known that the autophagic and endocytic pathways join together to generate a vesicular compartment known as the amphisome (Klionsky, 2007). Subsequently, this compartment fuses with a lysosome, in which the trapped materials are degraded by lysosomal enzymes. Although amphisomes lack lysosomal proteases such as acid phosphatase and cathepsin, they are acidic (Stromhaug *et al.*, 1993). Thus, the use of an acidic pH marker such as LysoTracker does not allow the distinction between amphisomes and autolysosomes. Moreover, even the detection of lysosomal enzymes does not necessarily mean that this compartment is functional (i.e., degradative). To solve this problem, a red BODIPY dye conjugated to bovine serum albumin (DQ-BSA, Molecular Probes) can be used. This BSA derivative is so heavily labeled that the fluorophore is self-quenched. Proteolysis of this compound results in dequenching and release of brightly fluorescent fragments. Thus, the use of DQ-BSA is useful for the visualization of intracellular proteolytic activity (see Fig. 6.4).

To analyze the convergence between an autophagic compartment with a functional lysosome, the following protocol may be followed:

1. Cells are incubated for 1 h at 37 °C with DQ-BSA (10 μg/ml in complete tissue culture medium).

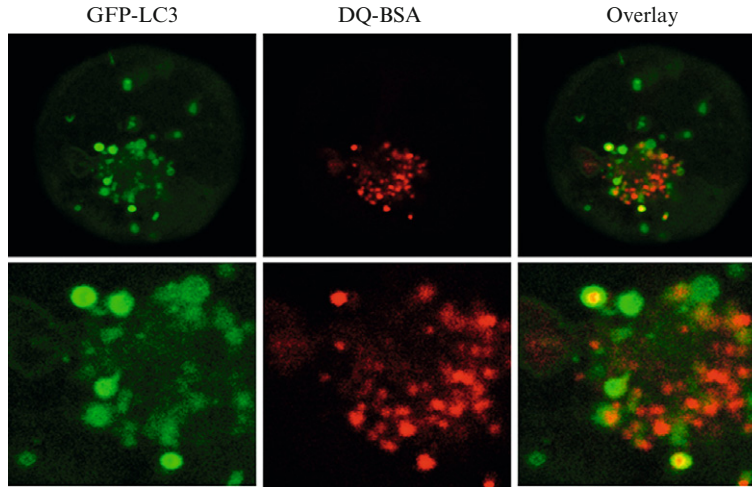

Figure 6.4 Autolysosomes labeled with DQ-BSA. Stably transfected K562 cells overexpressing pEGFP-LC3 were incubated with DQ-BSA (10μg/ml in RPMI + 10% fetal bovine serum) for 12 h at 37 °C to label the lysosomal compartment. Cells were then washed twice with PBS and incubated for 2 h at 37 °C in starvation media. Cells were mounted on coverslips and immediately analyzed by confocal microscopy. *Lower panels*: higher magnification of the upper panels (images from Fader and Colombo).

2. Cells are then washed twice with PBS to remove excess probe.
3. The cells are then incubated in starvation medium (EBSS, starvation media; Sigma) to induce autophagy or under different experimental conditions. Incubation for 2–3 h is usually sufficient to allow for detection of DQ-BSA fluorescence.
4. Autophagosomes can be visualized by detecting the autophagic protein LC3 by indirect immunofluorescence, or alternatively by using transfected cells overexpressing GFP-LC3 (see Fig. 6.4).

An important recommendation is that when using inhibitors (e.g., wortmannin) or overexpression of proteins (e.g., Rab7 dominant negative mutants), the internalization or the transport of DQ-BSA to the lysosomes may be hampered. 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 with the mutant proteins.

3.2. Monitoring fusion of an autophagosome/amphisome with a lysosomal compartment by real-time imaging

The degradation of the chromogenic self-quenched BSA can be also used to image the convergence of the autophagy and lysosomal pathways in real time. Cells overexpressing GFP-LC3 can be labeled with DQ-BSA as

indicated previously. The fusion between an autophagosome/amphisome (e.g., labeled in green) with a compartment containing the highly red fluorescent products generated by hydrolytic cleavage (i.e., the lysosome) can be monitored by confocal microscopy.

For this purpose coverslips with the labeled cells are placed in a temperature-controlled stage and analyzed by time-lapse confocal microscopy. A total of 30 slides can be acquired every 5 s with the proper green and red filter sets using a laser-scanning confocal unit attached to an upright fluorescence microscope.

4. Concluding Remarks

Here we have described basic protocols to assay autophagy induction by fluorescence microscopy and fluorometry and a useful assay to monitor the last step of the pathway, the fusion with a degradative lysosomal compartment. Labeling and incorporation of MDC is an easy and fast method that reflects starvation-induced autophagic activity, in both cultured cells and animals. On the other hand, detecting the colocalization of fluorescent-labeled fragments, generated by the lysosomal degradation of DQ-BSA in conjunction with an autophagic protein, is the most reliable method for monitoring fusion between an autophagic vacuole with an active proteolytic compartment. It is important to take into account that in many publications the presence of a lysosomal enzyme has been taken as an indication of fusion with a lysosome. However, it is essential to consider that the detection of an enzyme by immunofluorescence does not necessarily mean that the compartment is degradative, as the enzyme might be present but in an inactive form or the compartment might be proteolytically nonfunctional.

ACKNOWLEDGMENTS

Our work in this area is funded by grants from Agencia Nacional de Promoción Científica y Tecnológica (PICT2004 # 20711 and PICT 2005 # 38420), CONICET (PIP # 5943), and SECTyP (Universidad Nacional de Cuyo).

REFERENCES

Bampton, E. T., Goemans, C. G., Niranja, D., Mizushima, N., and Tolkovsky, A. M. (2005). The dynamics of autophagy visualized in live cells: From autophagosome formation to fusion with endo/lysosomes. *Autophagy* **1**(1), 23–36.

Biederbick, A., Kern, H. F., and Elsasser, H. P. (1995). Monodansylcadaverine (MDC) is a specific *in vivo* marker for autophagic vacuoles. *Eur. J. Cell Biol.* **66**(1), 3–14.

Boya, P., González-Polo, R. A., Casares, N., Perfettini, J. L., Dessen, P., Larochette, N., Métivier, D., Meley, D., Souquere, S., Yoshimori, T., Pierron, G., Codogno, P., *et al.* (2005). Inhibition of macroautophagy triggers apoptosis. *Mol. Cell Biol.* **25**(3), 1025–1040.

Fader, C. M., Sanchez, D., Furlan, M., and Colombo, M. I. (2008). Induction of autophagy promotes fusion of multivesicular bodies with autophagic vacuoles in k562 cells. *Traffic* **9**(2), 230–250.

Gutierrez, M. G., Munafó, D. B., Beron, W., and Colombo, M. I. (2004). Rab7 is required for the normal progression of the autophagic pathway in mammalian cells. *J. Cell Sci.* **117**(13), 2687–2697.

Iwai-Kanai, E., Yuan, H., Huang, C., Sayen, M. R., Perry-Garza, C. N., Kim, L., and Gottlieb, R. A. (2008). A method to measure cardiac autophagic flux *in vivo*. *Autophagy* **4**(3), 322–329.

Jackson, W. T., Gidding, T. H., Jr., Taylor, M. P., Mulinyawe, S., Rabinovitch, M., Kopito, R. R., and Kirkegaard, K. (2005). Subversion of cellular autophagosomal machinery by RNA viruses. *PLoS Biol.* **3**(5), e156.

Jager, S., Bucci, C., Tanida, I., Ueno, T., Kominami, E., Saftig, P., and Eskelinen, E.-L. (2004). Role for Rab7 in maturation of late autophagic vacuoles. *J. Cell Sci.* **117**(20), 4837–4848.

Kimura, S., Noda, T., and Yoshimori, T. (2007). Dissection of the autophagosome maturation process by a novel reporter protein, tandem fluorescent-tagged LC3. *Autophagy* **3**(5), 452–460.

Klionsky, D. J. (2007). Autophagy: From phenomenology to molecular understanding in less than a decade. *Nat. Rev. Mol. Cell Biol.* **8**(11), 931–937.

Mizushima, N. (2004). Methods for monitoring autophagy. *Int. J. Biochem. Cell Biol.* **36**(12), 2491–2502.

Munafó, D. B., and Colombo, M. I. (2001). A novel assay to study autophagy: regulation of autophagosome vacuole size by amino acid deprivation. *J. Cell Sci.* **114**(20), 3619–3629.

Niemann, A., Baltes, J., and Elsässer, H. P. (2001). Fluorescence properties and staining behavior of monodansylpentane, a structural homologue of the lysosomotropic agent monodansylcadaverine. *J. Histochem. Cytochem.* **49**(2), 177–185.

Niemann, A., Takatsuki, A., and Elsasser, H. P. (2005). The lysosomotropic agent monodansylcadaverine also acts as a solvent polarity probe. *J. Histochem. Cytochem.* **48**(2), 251–258.

Stromhaug, P. E., and Seglen, P. O. (1993). Evidence for acidity of prelysosomal autophagic/endocytic vacuoles (amphisomes). *Biochem. J.* **291**(1), 115–121.