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Analysis of Autophagosome Membrane Cycling by Fluorescence Microscopy

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Summary

Autophagy is a physiological process functionally linked to cellular dynamics during starvation, cardiomyopathies, neurodegeneration, cellular immunity, and certain cancers. Although nearly 30 autophagy-related (*ATG*) genes have been identified and characterized, the molecular mechanisms of this process are only partially understood. One aspect of the pathway that has been intensely studied is the identity of the membrane source for newly formed autophagosomes. Although it occurs at a basal level, autophagy is an inducible process. The process of autophagosome formation involves recruitment and delivery of membrane and recycling of Atg proteins. Despite continuing attempts to identify the source of the autophagosome membrane, we are only recently beginning to understand the nature of autophagosome formation and the role of membrane protein cycling in this process. There now exists an assay utilizing fluorescence microscopy to monitor the localization, and therefore the movement, of membrane-associated Atg proteins. We describe here a method that allows visualization of Atg membrane proteins in order to observe their potential source membranes and also to determine the temporal order of action of other Atg proteins with regard to their movement.

Key Words: Autophagy; autophagosome formation; membrane cycling; membrane transport; vacuole.

1. Introduction

Cell survival requires the ability to adapt to rapidly changing conditions. Autophagy is a cellular recycling mechanism involving either targeted or bulk degradation and reuse of intracellular components, which enables cells to survive periods of nutrient limitation, to combat viral and bacterial infection,

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and is implicated in preventing some types of neurodegenerative disease and cancer ***(1–3)***. Autophagy is a general term for a number of related processes and exists both as a typically nonspecific, inducible process called macroautophagy and as one of a number of pathways responsible for exclusive delivery of specific cargo to the lysosome (or the vacuole in yeast). Here we discuss a method that can be used to observe membrane protein recruitment during macroautophagy and the cytoplasm to vacuole targeting (Cvt) pathway, although the protocol can potentially be used to observe membrane protein cycling for other autophagy-related pathways.

In brief, macroautophagy involves the sequestration of bulk cytoplasm, including entire organelles, at the putative site of autophagosome formation, the phagophore assembly site (PAS). Fusion of the enclosing vesicle with the lysosome/vacuole follows the completion of sequestration and ultimately results in the degradation and recycling of the autophagosome cargo. Other autophagy-related pathways differ from macroautophagy only in the material sequestered and the site of enclosure. Current models have divided these pathways into a series of discrete steps: (1) induction; (2) cargo selection and packaging (for specific types of autophagy); (3) vesicle nucleation; (4) vesicle expansion and completion; (5) retrieval of Atg components; (6) targeting, docking and fusion with the lysosome/vacuole; (7) breakdown of the vesicle contents; and (8) recycling of the resulting macromolecules ***(4)***. At present, nearly 30 autophagy-related (*ATG*) genes have been identified in yeast ***(5–7)***. Current investigations seek to clarify the role of the corresponding gene products in these pathways.

The nature of membrane recruitment in autophagosome formation is not well defined. Various intracellular compartments have been proposed as the source of autophagosome membranes. Included in these potential membrane contributors are the endoplasmic reticulum (ER), Golgi complex, plasma membrane, and mitochondria (***8–12***; J. Legakis, W.-L. Yen, and D.J. Klionsky, unpublished observations). Recent studies have shed some light on the dynamics of this process, suggesting that membrane for the newly forming autophagosomes is recruited in part from mitochondria and the Golgi complex by specific Atg proteins (***11–13***; J. Legakis, W.-L. Yen, and D.J. Klionsky, unpublished observations). One of these is the transmembrane protein Atg9, which has been shown to cycle between the PAS and the mitochondria ***(11,13)***. The transport of Atg9 after knocking out *ATG1* (TAKA) assay is a fluorescence microscopy method to monitor movement of Atg9, thus following membrane recruitment to the PAS ***(13–15)***. The principle behind this assay relies upon the properties of Atg9 and its movement between the PAS and other subcellular compartments. Atg9 is one of two known integral membrane proteins involved in autophagosome formation ***(16)***. In wild-type cells, Atg9 resides in multiple

punctate structures within the cell, including the PAS, mitochondria, and other unidentified compartments, and cycles between these sites ***(11)***. In contrast, in cells with *ATG1* deleted, Atg9 is restricted to the PAS, unable to travel to the other subcellular locations ***(13)***.

Utilizing an epistasis approach, by introducing a second mutation (for example, *atgX*Δ) in addition to the *ATG1* deletion, a temporal function in Atg9 movement can be assigned to the protein of interest. Specifically, the protein can be categorized as either (1) required for anterograde movement of Atg9 to the PAS; (2) needed for retrograde Atg9 movement (from the PAS); or (3) not involved in Atg9 cycling. For example, Atg9-YFP is manifest as a single punctate structure in *atg13*Δ cells, and shows a similar phenotype in *atg1*Δ *atg13*Δ cells. Thus, Atg13 appears to act at the same time or after Atg1 in Atg9 cycling and is required for retrograde Atg9 transport **Fig. 1**). In contrast, Atg9-YFP is localized at multiple punctate structures in both *atg27*Δ and *atg1*Δ *atg27*Δ cells, suggesting that Atg27 acts before Atg1 in movement of Atg9 and is involved in anterograde Atg9 trafficking **Fig. 1**). A protein is not involved in Atg9 cycling if Atg9 is localized in multiple punctate structures (one of which must be shown to correspond with the PAS) in the single mutant, and in a single punctate structure in conjunction with *atg1*Δ. Moreover, real-time cycling of Atg9 can be observed using a temperature-sensitive allele of *ATG1* (*atg1ts*). By

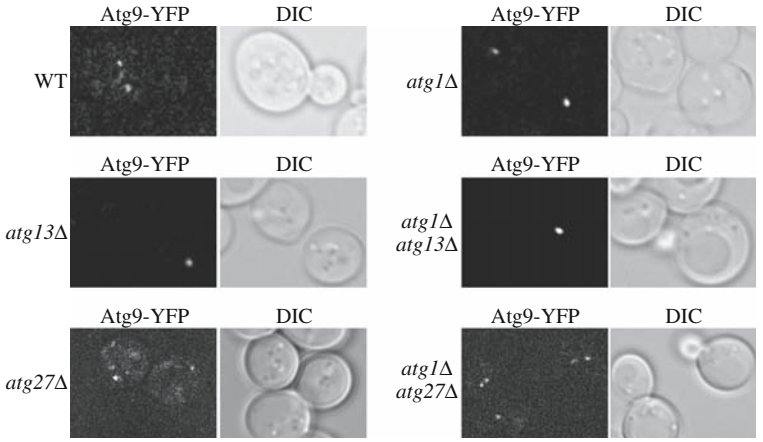

Fig. 1. Epistasis analysis of Atg9-YFP. Wild-type, *atg1*Δ, *atg13*Δ, *atg1*Δ, *atg13*Δ *atg27*Δ, and *atg1*Δ *atg27*Δ cells expressing the Atg9-YFP fusion protein were grown to mid-log phase, harvested and analyzed by fluorescence microscopy. Atg9-YFP in the *atg27*Δ and *atg1*Δ *atg27*Δ strains is not located at the PAS (data not shown) indicating that Atg27 is needed for anterograde movement of Atg9. DIC, differential interference contrast.

repeatedly shifting between the permissive and nonpermissive temperatures, the movement of Atg9-GFP can be followed from its wild-type localization at multiple punctate structures to concentrating at the PAS (anterograde transport) and redistributing back to the multiple punctate sites (retrograde movement). This assay also allows the identification of other factors (e.g., non-Atg proteins) required for Atg9 cycling.

In addition, the TAKA assay can be utilized to follow trafficking of two other membrane-associated Atg proteins, Atg23 and Atg27 (***12***; J. Legakis, W.-L. Yen, and D. J. Klionsky, unpublished observations). Atg23 is a peripheral membrane protein that cycles between the mitochondria and the PAS (***13,17***; J. Legakis, W.-L. Yen, and D. J. Klionsky, unpublished observations), and Atg27 is a transmembrane protein that has recently been shown to cycle from the mitochondria/Golgi to the PAS ***(12)***. Although these proteins, similar to Atg9, cycle between the PAS and other subcellular compartments, their movement is not entirely coincident with that of Atg9 (***14***; J. Legakis, W.-L. Yen, and D. J. Klionsky, unpublished observations). Continued use of the TAKA assay will likely lead to a more complete understanding of the subcellular compartments in which these three proteins reside, possibly to discovery of other proteins involved in the cycling process, and to a better overall understanding of membrane recruitment for autophagosome formation.

2. Materials

2.1. Cell Culture

1. Rich medium (YPD): 1% yeast extract, 2% peptone, 2% glucose (*see* **Notes 1** and **2**).
2. Synthetic minimal medium (SMD): 0.67% yeast nitrogen base, 2% glucose, amino acids, and vitamins, as needed.
3. Synthetic medium lacking nitrogen (SD-N): 0.17% yeast nitrogen base without amino acids, 2% glucose.

2.2. Yeast Stable Transformation

1. 50% PEG-4000: polyethylene glycol, molecular weight 3,350 g/mol, 50% w/v.
2 Lithium acetate solution: 1 *M* lithium acetate, pH 7.5 (adjusted with acetic acid). Dilute this solution 10-fold for 100 m*M* lithium acetate.
3. Denatured single-stranded DNA (ssDNA): 2 mg/mL heat-denatured (5 min boiling) salmon sperm DNA, sheared by sonication (approximately 3 min with a probe sonicator).
4. Transformation mix: 240 µL 50% PEG-4000, 36 µL lithium acetate solution, 50 µL ssDNA, 9 µL sterile double-distilled or Millipore filtered H_2O, 25 µL transforming DNA (PCR product).

2.3. Yeast Plasmid Transformation

1. 10X TE buffer: 100 m*M* Tris-HCl, pH 7.5.
2. 10X lithium acetate buffer: 1 *M* lithium acetate, pH 7.5 (adjusted with acetic acid).
3. TE/lithium acetate buffer: 1 mL 10X TE buffer (10 m*M* final), 1 mL 10X lithium acetate buffer (100 m*M* final), 8 mL sterile H_2O. This solution should be made fresh for each set of transformations.
4. 50% PEG-4000: polyethylene glycol, molecular weight 3350 g/mol, 50% w/v. This solution should be filter sterilized and made fresh every 3–4 weeks.
5. Denatured single-stranded DNA (ssDNA): 2 mg/mL heat-denatured (5 min boiling) salmon sperm DNA, sheared by sonication (approximately 3 min with a probe sonicator).
6. Transformation mix: 150 μL 50% PEG-4000, 10 μL ssDNA, 1–2 μL plasmid DNA (from a miniprep).

2.4. Fluorescence Microscopy

1. Fixation buffer: 50 m*M* KH_2PO_4, pH 6.5–8.0 (*see* **Note 3**), 1.5% formaldehyde, 1 μ*M* $MgCl_2$.
2. Wash buffer: 50 m*M* KH_2PO_4, pH 6.5–8.0 (same pH as the fixation buffer), 1 μ*M* $MgCl_2$.

3. Methods

The proteins monitored for this assay, Atg9, Atg23 and Atg27, are all dynamic proteins residing in multiple punctate structures. It is possible to visualize these proteins in live cells, but because they cycle rapidly between the compartments, for accurate colocalization studies with most microscopes, the cells must be fixed. Fixation also enhances the fluorescent signal, as the proteins are stationary. Furthermore, for accurate evaluation of their behavior, fluorescent chimeras of the proteins should be created by chromosomal tagging, thus allowing visualization of the proteins expressed at physiological levels. Other fluorescent organelle markers, for example, RFP-Atg8 for the PAS, Mito-BFP for the mitochondria, etc., can either be introduced by plasmid transformation, or the tag can be integrated into the chromosome, similar to Atg9-GFP. Furthermore, many subcellular structures can be stained using dyes such as MitoTracker Red (Invitrogen) to visualize mitochondria prior to fixation.

3.1. Cell Culture

1. All yeast colonies are maintained on agar plates containing rich or selective media. Colonies on plates can be stored for up to one month at 4°C. Liquid cultures are grown from a single colony.

2. Cells are first grown in liquid medium overnight, then diluted and re-grown to mid-log phase in rich (YPD), or selective (SMD), medium, as needed (*see* **Note 4**). For starvation conditions, the cells are grown in medium lacking nitrogen (SD-N) for one hour prior to viewing.

3.2. Yeast Stable Transformation: Strain Construction and Stable Transformations for Integration into the Chromosome

1. Fluorescent tagging of Atg9 with GFP (or RFP, BFP, etc.) is achieved by PCR-based integrations of the tag at the 3′ end of the gene. This allows expression of the fusion protein under the control of the native promoter. The template for integration of Atg9-GFP is pFA6-GFP-HIS3 *S.k.* (or -KAN; ***18***). Approximately 10 µg (generally 25 µL of a standard PCR reaction) of the appropriate PCR product is sufficient to generate transformants.
2. Cells are grown overnight at 30°C (*see* **Note 5**), diluted in 5 mL culture medium/transformation (referred to as one unit of competent cells) to OD600 $\leq$ 0.25, regrown to OD600 0.8–1.0, harvested by centrifugation, and washed once with 4 mL of H_2O per unit of competent cells. Resuspend in 100 µL of 100 m*M* lithium acetate per unit of competent cells and aliquot 100 µL of the competent cells into a separate microcentrifuge tube for each transformation (*see* **Note 6**).
3. Pellet the cells by centrifugation at 3000 rpm for 3 min, resuspend by pipetting in transformation mix totaling 360 µL and incubate at 30°C for 30 min to 3 h (*see* **Note 5**). A negative control should be included in which the cells are incubated in transformation mix lacking DNA.
4. Transfer the cells to 37–42°C (heat shock) and incubate 30 min to 1 h (*see* **Note 5**).
5. Pellet the cells by centrifugation at 4000 rpm for 2 min, aspirate the supernatant, resuspend each sample in 200 µL H_2O, and spread transformed cells on plates selective for the appropriate auxotrophic marker or antibiotic resistance factor (*see* **Note 7**).
6. Incubate plates 2–3 days at 30°C (*see* **Note 5**).
7. Individual colonies can be screened for Atg9-GFP expression by Western blot using anti-GFP antibodies (commercially available), or by PCR-based screening.

3.3. Yeast Plasmid Transformation

1. Cells are grown overnight at 30°C (*see* **Note 5**), diluted in 10 mL culture medium/6 transformations to OD600 0.5, regrown to OD600 0.8–1.0, harvested by centrifugation at 3000 rpm for 3 min and washed once with 1 mL of H_2O, followed by a wash in 1 mL TE/lithium acetate buffer. Cells are then resuspended in 150 µL lithium acetate, and are now referred to as competent cells.
2. Twenty-five µL of competent cells are aliquoted into microcentrifuge tubes, transformation mix is added to each condition, and incubated at 30°C for 30 min (*see* **Note 5**). A negative control should be included in which the cells are incubated in transformation mix lacking plasmid DNA.

3. Tubes are then transferred to 37–42°C for 15 min (*see* **Note 5**).
4. Cells are pelleted by centrifugation at 4000 rpm for 2 min, resuspended in 200 μL H_2O and spread onto selective agar plates (*see* **Note 7**).
5. Plates are grown at 30°C for 2–3 days (*see* **Note 5**), and colonies can be screened by Western blotting for expression of the protein encoded by the plasmid.

3.4. Fluorescence Microscopy with Fixation (Basic Protocol)

1. Cells expressing chromosomally tagged Atg9-GFP (and, if desired, plasmids encoding RFP-Atg8 and/or Mito-BFP) are first grown overnight at the appropriate temperature, followed by dilution of 5 mL of culture to OD600 ≤ 0.5 and regrowth to OD600 0.8–1.0.
2. Cells are harvested by centrifugation at 3000 rpm for 3 min in 15 mL conical tubes, resuspended by pipetting in 2.5 mL fixation buffer, and incubated with gentle mixing for 30 min at room temperature.
3. Wash cells once with 2.5 mL wash buffer, and resuspend in 200 μL wash buffer.
4. Drop 2 μL of cell solution onto a microscope slide and carefully place a cover slip over the top, avoiding the introduction of bubbles under the cover slip.
5. The cells are visualized with a fluorescence microscope with deconvolution software for image analysis (or a confocal microscope), first establishing the focus with differential interference or phase contrast microscopy. If using a deconvolution microscope, software must be used to deconvolve the images in order to evaluate a single focal plane of the cells. Typical localization patterns of Atg9-GFP, in both wild-type (colocalized with the PAS marker, RFP-Atg8 in **Fig. 2B**) and *atg1*Δ cells are shown in **Fig. 2**.

3.5. Fluorescence Microscopy with Fixation (Cycling Protocol)

1. Cells expressing chromosomally tagged Atg9-GFP, harboring an *ATG1* deletion and a plasmid-based, reversible temperature-sensitive allele of *ATG1* (*atg1ts*), are first grown overnight at the permissive temperature of 24°C, followed by dilution of 10 mL of cell culture to OD600 ≤0.5 and regrowth to OD600 0.8–1.0. Plasmids encoding organelle markers or other fluorescent proteins can be introduced as necessary. This experiment requires continuing the growth of cells in liquid culture for a number of hours; therefore it is recommended that the culture be periodically diluted to maintain it at mid-log growth phase. In order to accurately evaluate the movement of these proteins within a single population of cells, it is necessary to simultaneously prepare an aliquot of the cells for microscopy while maintaining the remainder of the liquid culture at the appropriate temperature for subsequent analysis. Therefore, the complete steps required for one condition (a) will be outlined, followed by the instructions for the next condition (b), and so on **Fig. 3**).

2a. One mL of cells is removed from the culture, pelleted in a microcentrifuge tube at 3000 rpm for 3 min, resuspended in 0.5 mL of fixation buffer, and incubated at room temperature, with gentle shaking for 30 min.

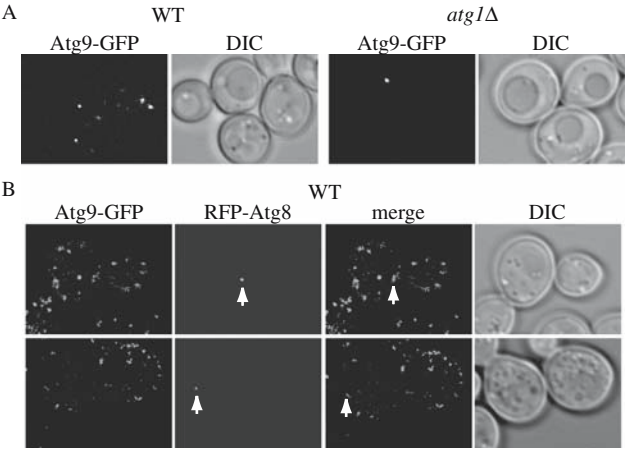

Fig. 2. Atg9-GFP localization in wild-type and *atg1*Δ cells. **(A)** Wild-type and *atg1*Δ cells expressing an Atg9-GFP chimera were grown to mid-log phase, harvested and viewed by fluorescence microscopy. **(B)** Wild-type cells harboring chromosomally tagged Atg9-GFP and transformed with a plasmid encoding RFP-Atg8 were grown to mid-log phase, subjected to mild fixation, and examined by fluorescence microscopy. A white arrow marks the position of RFP-Atg8. DIC, differential interference contrast.

3a. The aliquot of cells is pelleted as above, washed once in 0.5 mL of wash buffer, and resuspended in 50 µL of wash buffer, followed by evaluation by fluorescence microscopy, as outlined in **Subheading 3.4., step 5**.
2b. At the same time, the remaining cell culture is shifted to the nonpermissive temperature of 37°C, and incubated for 1 h.
3b. One mL of cells is removed from the culture, pelleted in a microcentrifuge tube as above, resuspended in 0.5 mL of fixation buffer, and incubated at room temperature, with gentle shaking for 30 min.
4b. The aliquot of cells is pelleted as above, washed once in 0.5 mL of wash buffer, resuspended in 50 µL of wash buffer, followed by evaluation by fluorescence microscopy, as outlined in **Subheading 3.4., step 5**.
2c. Meanwhile, the remaining cell culture is shifted to the permissive temperature of 24°C and incubated for 1 h.
3c. One mL of cells is removed from the culture, pelleted in a microcentrifuge tube as above, resuspended in 0.5 mL of fixation buffer, and incubated at room temperature, with gentle shaking for 30 min.
4c. The aliquot of cells is pelleted as above, washed once in 0.5 mL of wash buffer, resuspended in 50 µL of wash buffer, followed by evaluation by fluorescence microscopy, as outlined in **Subheading 3.4., step 5**. An example of Atg9-GFP cycling evaluated by a similar protocol is shown in **Fig. 4**.

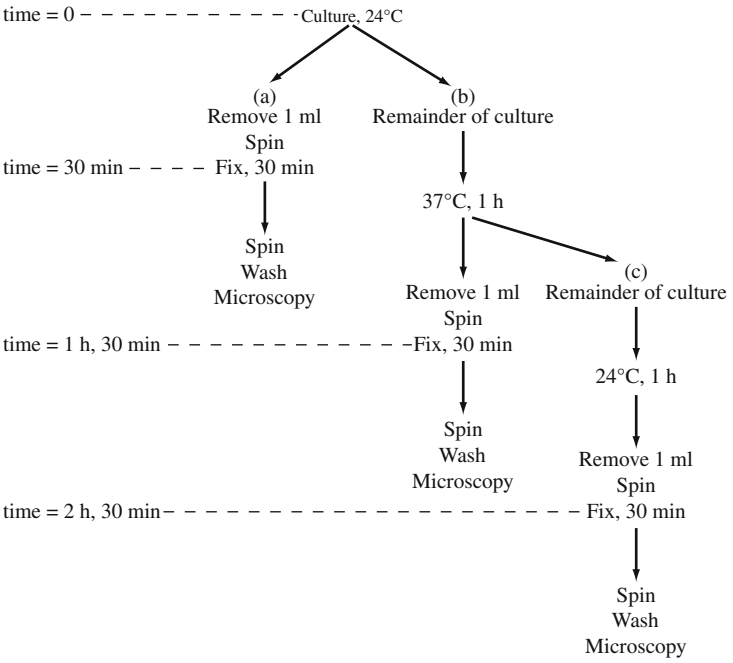

Fig. 3. Flow chart for sample preparation during TAKA assay with a temperature sensitive *atg1* mutant strain. See text for details.

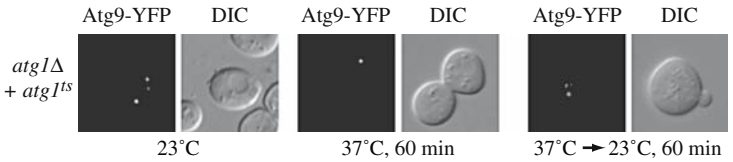

Fig. 4. Atg9 can cycle to and from the PAS. The *atg1*Δ strain expressing Atg9-YFP was transformed with a plasmid bearing *atg1ts*. Cells were grown in SMD selective medium at 24°C to OD600 0.8, shifted to 37°C for 60 min., then shifted back to 24°C for another 60 min. Before each temperature shift, cells were imaged by fluorescence microscopy. DIC, differential interference contrast. (Reproduced from **ref. *14*** with permission from Cell Press.)

4. Notes

1. All solutions should be prepared in water that has a resistance of 18.2 MΩ-cm (referred to throughout as H_2O).
2. All solutions for cell culture and transformations should be sterile. Furthermore, all of these procedures should be carried out using sterile conditions.

3. The pH of the fixation and wash buffers can be adjusted to optimize staining of other intracellular structures by pH-dependent dyes.
4. Yeast cells in culture can be diluted and regrown indefinitely, as long as they are evaluated at or near mid-log phase (OD600 = 0.8–1.0).
5. Temperature-sensitive mutant strains should be grown at the appropriate permissive temperature (usually 23–26°C) and the heat shock should be carried out at 30–37°C.
6. The cells can be stored overnight at 4°C in the 100 m*M* lithium acetate solution.
7. If using the *kan*^{r} marker (kanamycin resistance), pellet cells, resuspend in 1 mL YPD/transformation and incubate 30°C for 1 h (*see* **Note 5**) prior to spreading onto plates.

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