In Vitro Dissection of Autophagy

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Autophagy is an essential cellular process for bulk degradation of cytoplasmic components through the lysosome. Underlying this process is an intricate interaction between protein factors and the cell endomembrane system, leading to a gradual maturation of the autophagic membrane. This structure sequesters a portion of the cytoplasm by the formation of a double-membrane compartment called the autophagosome. The autophagosome then delivers the cargo to the lysosome to complete degradation. The molecular mechanism accounting for the generation of the autophagic membrane is a longstanding question. Here, a cell-free approach that has been established to understand the mechanism of early autophagic membrane generation is described. This system has provided insight into the membrane source of the autophagosome, the early protein-membrane associations, and the membrane remodeling that generates the autophagosomal precursors. The cell-free assay, in combination with other established approaches (e.g., cell imaging), will facilitate a deeper understanding of the mechanism of autophagy. © 2017 by John Wiley & Sons, Inc.

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INTRODUCTION

This unit describes an in vitro approach based on cell-free reconstitution to dissect the mechanism of autophagy (see Background Information for details). The cell-free reaction here refers to the creation of an in vitro reaction to mimic a certain cellular process by combining cytosol and cellular membranes from broken cells with nucleotides in a test tube. Because they are directly derived from a living cell, the active components of the cytosol and cellular membranes are native, in contrast to the use of purified proteins and synthetic liposomes. When properly designed, the cell-free assay is able to recapitulate the key aspects of different cellular events. Cell-free approaches have been successfully employed to make groundbreaking discoveries in multiple key cellular processes including protein synthesis and translocation, vesicular transport, transcription, and cell division. Some cell-free systems are so broadly employed that they have been commercialized. A good example is the rabbit reticulocyte lysate system for in vitro translation (Jagus & Beckler, 2003).

In cell-free reconstitution, the cytosol and membrane are derived from broken cells. Cytosol or membrane-fractionation approaches may be employed to isolate the active components and subsequently identify the factors by mass spectrometry. A biochemical approach is usually a good complement to genetic approaches, e.g., the combination of a cell assay with genetic screening, to fully dissect the mechanism of a cellular process. The

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merit of an in vitro biochemical approach is that it allows one to directly pinpoint the role of a factor. The drawback is that not all cellular processes can be easily reconstituted in vitro. A substantial optimization is usually necessary and different controls are required to validate the physiological relevance of the established cell-free reaction.

A set of biochemical methods to answer several of the following key questions in autophagosome biogenesis is described (Burman & Ktistakis, 2010; Ge, Baskaran, Schekman, & Hurley, 2014a; Lamb, Yoshimori, & Tooze, 2013; Mizushima, Levine, Cuervo, & Klionsky, 2008; Rubinsztein, Shpilka, & Elazar, 2012) (see Background Information for details): (1) What is the membrane origin of the autophagosome? (2) How are different autophagic factors activated during each step of autophagy? (3) How is the autophagosomal membrane precursor generated from the membrane source? The biochemical methods include: (1) a cell-free LC3 lipidation assay to determine an early step of autophagosome biogenesis (see Basic Protocol 1), (2) a three-step membrane fractionation method to identify the cellular membrane that triggers autophagosome biogenesis in the cell-free LC3 lipidation assay (see Support Protocol), (3) a cell-free membrane recruitment assay to analyze the activation of the indicated autophagic factors (see Basic Protocol 2), and (4) a cell-free small autophagosomal precursor formation assay to reconstitute the biogenesis of early autophagosomal precursors (see Basic Protocol 3).

BASIC PROTOCOL 1

CELL-FREE LC3 LIPIDATION

A cell-free LC3 lipidation assay that recapitulates several major regulatory landmarks of autophagosome biogenesis is described here (Ge, Melville, Zhang, & Schekman, 2013). The key components of the cell-free assay include the cytosol, cellular membrane, nucleotides, and LC3 probe. The cytosol provides the key regulatory components, such as different autophagy-related gene (ATG) proteins (see Background Information for details), for autophagosome biogenesis. The cellular membrane donates membranes to the autophagosome. The process is controlled by certain molecular determinants on the cellular membrane and active components of the cytosol. The nucleotides provide ATP for energy and GTP for activating certain GTP-binding proteins, which are usually required for membrane remodeling events in the cell. LC3 lipidation is the readout of autophagosome biogenesis in the cell-free reaction. Of the four aforementioned steps, the preparation of nucleotide is a standard protocol (Bednarek et al., 1995) and is not described in detail. Below, a protocol for cytosol and membrane preparation, LC3 protein purification, and the cell-free reaction (Fig. 11.23.1) is described.

Materials

Atg5 knockout (KO) mouse embryonic fibroblast (MEF) cells (Dr. Noboru Mizushima)
Dulbecco's modified Eagle medium (DMEM) plus 10% FBS (fetal bovine serum)
PBS (see recipe), room temperature and ice cold
0.05% trypsin-EDTA (1×; Gibco)
B88 lysis buffer (see recipe)
Trypan blue
Human HEK293T (human embryonic kidney) cells (Berkeley Tissue Culture Center)
E. coli BL21 cells (NEB)
pET28a-LC3 (Ge et al., 2013)
IPTG
Imidazole
Protease inhibitors cocktail (Roche)
Lysozyme
Triton X-100

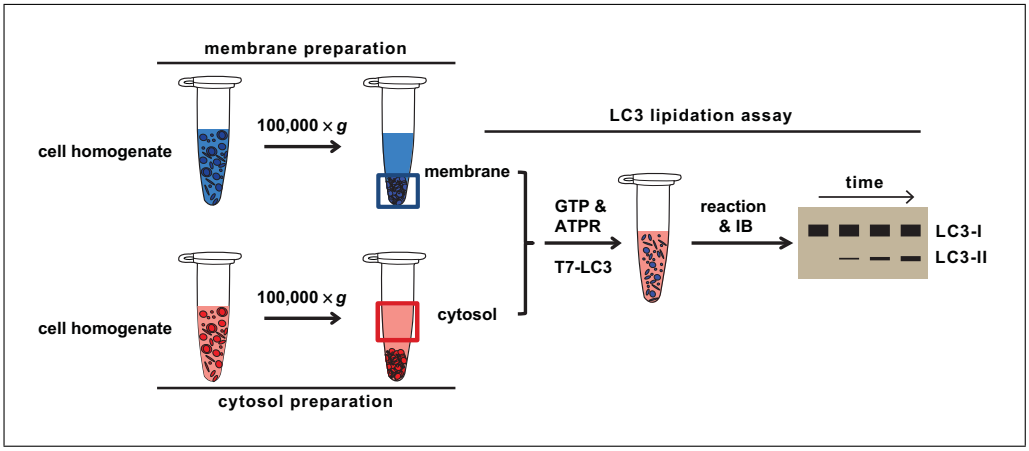

Figure 11.23.1 Cell-free LC3 lipidation assay (modified from Brier, Zhang, & Ge, 2016; Ge et al., 2013). Membrane preparation: Atg5 KO MEF cells are homogenized and ultracentrifuged at 100,000 × *g*. The membrane pellet is used as the membrane fraction. Cytosol preparation: wild-type MEF or HEK293T cells are homogenized and ultracentrifuged at 100,000 × *g*. The supernatant is used as the cytosol fraction. LC3 lipidation reaction: cytosol, membrane, nucleotides (GTP&ATPR), and LC3-I are incubated for different times. The samples are then analyzed by immunoblotting. The lipidated LC3 appears as a faster migrating band with increased intensity according to time. The LC3-I band shows little change even when more LC3 lipidation occurs in the longer time durations because the overall lipidation efficiency is usually <20%.

Ni-Sepharose (packed beads) (GE)
0.2% Tween-20
NAP-10 columns (GE)
Thrombin (Roche)
4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF, Sigma)
Coomassie blue
10× ATP regeneration system (see recipe)
10 mM GTP stock (10 µl/tube, store at least 2 years at −80°C)
B88 buffer (see recipe)
SDS-PAGE loading buffer
Earle's balanced salt solution (EBSS; Thermo)
Phospholipids C reagent (Wako)

10-cm cell culture dishes
Centrifuge tubes (1.5-, 15-, and 50-ml)
Refrigerated benchtop swinging bucket centrifuge (e.g., Sorvall ST 16R, Thermo)
22-G needles and syringes (1-, 3-, and 5-ml)
Glass slides and coverslips
Microscope
2.5-, 3.5-, and 5-ml ultracentrifuge tubes
Benchtop ultracentrifuges (>150,000 × *g*, e.g., Optima Max-XP, Beckman *or* e.g., Optima XE-90, Beckman)
Microplate spectrophotometer (e.g., xMark™ Microplate Absorbance Spectrophotometer, Bio-Rad)
37°C shaker
Spectrophotometer (with cuvettes, e.g., Spectronic Genesys 5, Thermo)
Centrifuge for large volumes (e.g., Sorvall RC6+)
Sonicator (e.g., Branson Sonifier 450)
Rotator, 4ºC
Protein purification columns
Centrifugal filter (10-kD cutoff)
30°C water bath

Additional reagents and equipment for the Bradford assay, SDS-PAGE

Prepare total cellular membrane

1. Culture Atg5 knockout (KO) mouse embryonic fibroblast (MEF) cells (Mizushima et al., 2001) in DMEM plus 10% FBS for 2 to 3 days to confluence in one to three 10-cm dishes in a standard 37°C cell culture incubator.

   Use cells with <20 passages. Membranes from cells with passages >20 are less active in triggering LC3 lipidation and are prone to fragmentation, making it difficult to isolate them during membrane fractionation as described in Support Protocol.

2. Remove medium and wash cells one time with 4 ml PBS per 10-cm dish. Trypsinize cells with 1 ml of 1× trypsin-EDTA per dish for 2 to 5 min at room temperature. Then add 5 ml DMEM plus 10% FBS to quench trypsin.

3. Transfer cell suspension into a 15-ml conical tube and centrifuge 5 min at 600 × *g*, 4°C, to collect cells. Remove supernatant and suspend cell pellet in 5 ml ice-cold PBS. Repeat centrifugation.

4. Discard supernatant, suspend cell pellet with B88 lysis buffer (use a volume about three times cell pellet volume), pipetting up and down ten times using a 1- or 3-ml syringe with a 22-G needle attached.

   Avoid generation of bubbles when passing through the needle.

5. Place 3 µl of lysate onto a glass slide and stain with trypan blue. Check under a microscope the cell fraction that is trypan blue positive. If 80% to 90% of cells are positive, proceed to the next step. If <80% of cells are positive, homogenize cells again, checking cell lysis every five strokes until 80% to 90% of cells are lysed.

   It is not recommended to obtain complete cell lysis because some active membrane structures will be damaged with excessive homogenization.

6. Transfer lysate to a 1.5-ml ultracentrifuge tube and ultracentrifuge 30 min at 100,000 × *g*, room temperature.

7. Discard supernatant and resuspend total membrane pellet in 50 to 100 µl B88 lysis buffer.

   The pellet in this step is hard and difficult to resuspend. Incubate pellet in B88 lysis buffer 30 min to 1 hr on ice, after which the pellet will soften. Do not vortex. Suspend pellet by pipetting up and down.

8. Remove 1 µl of membrane suspension to measure the phosphatidylcholine (PC) concentration using a microplate spectrophotometer. Adjust membrane concentration to 0.6 µg/µl PC using B88 lysis buffer.

   PC is the most abundant lipid in the membrane. Therefore, PC is used as a standard to normalize membrane amounts in the following steps.

Prepare cytosol

9. Culture human HEK293T (human embryonic kidney) cells in DMEM plus 10% FBS for 2 to 3 days to confluence (usually more than two 10-cm dishes). Remove medium and incubate cells with 5 ml per 10-cm dish DMEM plus 10% FBS (nutrient-rich) or EBSS (starve) for 1 hr in a 37°C cell culture incubator.

   In addition to starvation, cells can also be treated with drugs, such as Rapamycin and Torin-1, to activate the cytosolic components involved in autophagy. In addition to HEK293T cells, cytosol from COS7 and wild-type MEF cells are also active in triggering LC3 lipidation.

10. Scrape cells into the medium and transfer cell suspension to a 15-ml conical tube. Centrifuge 5 min at 600 × *g*, 4°C, to collect cells. Remove supernatant and resuspend cell pellet with 5 ml ice-cold PBS. Repeat centrifugation.

    When resuspending cell pellets with PBS, tap pellets or gently pipet up and down. Do not pipet vigorously or vortex because this will break cells and lead to low yield of cytosol.

11. Discard supernatant, resuspend cell pellet with an equal volume of B88 lysis buffer, and pipet up and down ten times using a 1- or 3-ml syringe with a 22-G needle attached.

12. Place 3 µl of lysate onto a glass slide and stain with trypan blue. Check the cell fraction that are trypan blue positive. If 80% to 90% of cells are positive, proceed to the next step. If <80% of cells are positive, homogenize cells again, checking cell lysis every five strokes until 80% to 90% of cells are lysed.

    Do not obtain a complete cell lysis because excessive homogenization reduces cytosol activity.

13. Transfer lysate to a 1.5- or 3.5-ml ultracentrifuge tube and ultracentrifuge 30 min at 100,000 × *g*, room temperature.

14. Collect supernatant and repeat step 13 if the supernatant is not clear.

    Usually, if a large number of cells are harvested, a lipid layer will appear on top after centrifugation. Avoid this lipid layer when collecting the supernatant. Collect the pure supernatant located between the cell-debris pellet and the lipid layer, sacrificing the cytosol that is near either the top or the bottom. If the lipid layer is too broad to avoid, collect the supernatant with some lipid contamination and repeat centrifugation of the cytosol several times until a clear supernatant of cytosol is obtained. The lipid layer usually becomes thinner after each round of centrifugation.

    The clear supernatant is the cytosol ready for use in the following reaction.

15. Measure protein concentration of the clear supernatant using the Bradford assay. Dispense into 200-µl aliquots and store up to 3 years at −80°C.

    Avoid repeated freeze/thaws.

Purify T7-LC3

Below is a standard His-tag protein purification protocol. T7-LC3 is easy to express and purify. The typical yield is ~20 mg/liters *E. coli*.

16. Culture 20 ml *E. coli* BL21 cells with pET28a-LC3 (aa1-120, human) on a shaker overnight at 37°C.

17. Expand total culture to 1 liter (1:50 expansion) and shake 1 to 2 hr at 37°C until OD_{600} reaches 0.5 to 0.8.

18. Induce protein expression with 100 µM IPTG for 5 hr at 23ºC and collect *E. coli* by centrifuging 10 min at 10,000 × *g*, room temperature.

19. Suspend *E.coli* pellet with 20 ml of 2× PBS plus 15 mM imidazole and protease inhibitors cocktail. Add lysozyme at 0.5 mg/ml and incubate 30 min on ice.

20. Add Triton X-100 to a concentration of 0.5%. Sonicate solution five to seven times with 15-sec bursts until the solution is not viscous.

21. Centrifuge lysate 30 min at 100,000 × *g*, room temperature, transfer supernatant to a clean 50-ml conical tube, and rotate with 1 ml Ni-Sepharose (packed beads) 2 hr at 4°C.

22. Load the mixture into an empty protein purification column (e.g., a Biorad gravity column of a suitable size) and drain the flow through.

23. Wash beads with 70 ml cold 2× PBS plus 25 mM imidazole and 0.2% Tween-20 followed by 50 ml cold 2× PBS plus 25 mM imidazole.

24. Elute the bound His-T7-LC3 with 10 ml of 2× PBS plus 250 mM imidazole, concentrate the eluate to 1 ml with a centrifugal filter (10-kD cutoff) and buffer exchange to PBS by running through a NAP-10 column.

25. Remove the His tag of His-T7-LC3 (usually ~4 mg/ml) with 1 U/ml of thrombin 1 hr at room temperature, then add 1 mg/ml AEBSF to deactivate thrombin. Store in 10- to 20-μl aliquots and up to 3 years at −80°C.

    The cleaved His tag remaining in the T7-LC3 does not affect lipidation; therefore, step 25 can be skipped.

26. Check protein quality and estimate concentration by running an SDS-PAGE and staining with Coomassie Blue.

Perform cell-free lipidation reaction

27. For each reaction, mix the following in a low-retention 1.5-ml microcentrifuge tube on ice:

    Cytosol (2 mg/ml final concentration)
    3 μl 10× ATP regeneration system
    GTP (0.15 mM final concentration)
    0.2 μg T7-LC3 membrane (0.2 mg/ml PC content final concentration)
    B88 buffer to bring total volume to 30 μl

28. Gently tap tube to mix and incubate 30 to 90 min at 30°C.

29. Quench reaction by adding SDS-PAGE loading buffer to make a total of 80 μl. Load 12 μl of sample for immunoblotting with a T7 antibody.

    A big band (unlipidated T7-LC3) migrating ~17 Kd and a faster migrating thinner band (lipidated T7-LC3) ~3 to 5 mm lower than the big band should be visible on the membrane. Run a 12.5%, 15%, or 8% to 16% gradient to better separate the lipidated LC3 from the unlipidated LC3.

    Sometimes the unlipidated band signal is so strong that it covers the lipidated signal. In this case, adding a 20,000 × g centrifugation step after step 28 is recommended. Then remove ¾ of the supernatant and add SDS-PAGE loading buffer to the pellet and remaining supernatant. The lipidated LC3 should be fully recovered in the pellet. By removing the supernatant, we only reduce the level of unlipidated LC3.

SUPPORT PROTOCOL

THREE-STEP MEMBRANE FRACTIONATION AND LIPIDATION REACTION

A longstanding quest in the autophagy field is to identify the membrane source of the autophagosome. The cell-free LC3 lipidation assay can be combined with membrane fractionation approaches to identify the membrane source of the autophagosome by determining the active membrane fraction that triggers LC3 lipidation.

This protocol describes a three-step fractionation method that has been established (Ge et al., 2013), including a differential centrifugation, a sucrose gradient, and an Optiprep gradient performed sequentially (Fig. 11.23.2). The purpose is to identify a membrane fraction that triggers LC3 lipidation. In general, after each step of membrane fractionation, LC3 lipidation is performed with the obtained fractions. The most active fraction is

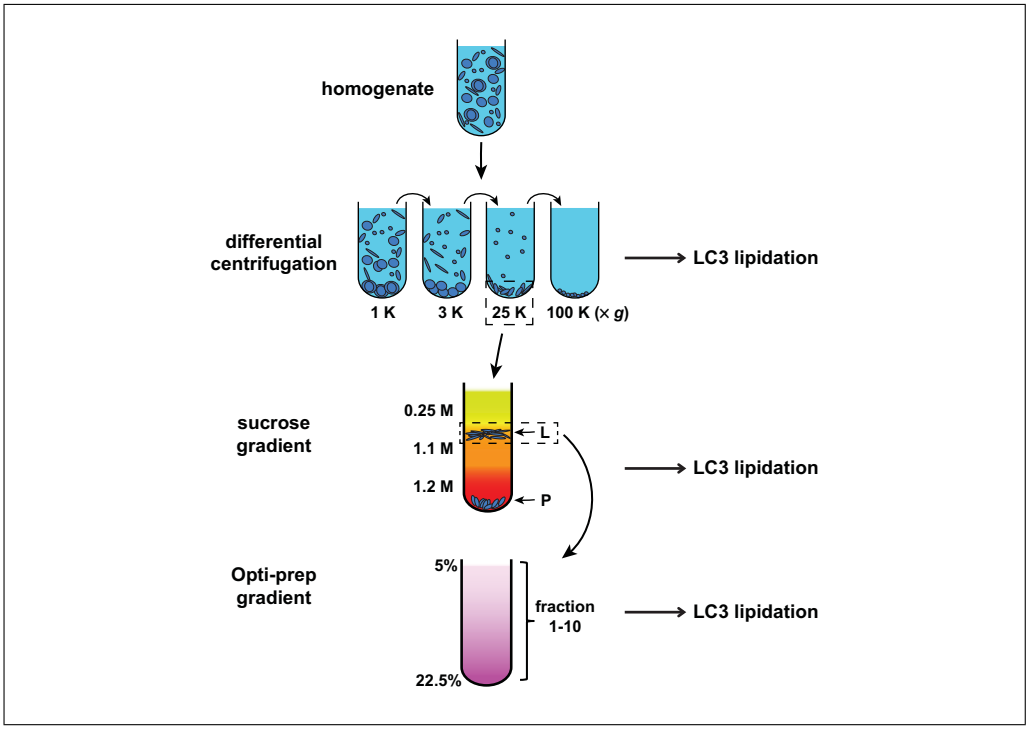

Figure 11.23.2 Three-step membrane fractionation (modified from Ge et al., 2013). Atg5 KO MEF cells are homogenized and subjected to differential centrifugation to separate the total membrane into four fractions. Membranes from these fractions are analyzed for their ability to trigger LC3 lipidation. The 25 K fraction, which is most active, is selected and further separated through a sucrose gradient ultracentrifugation. An L and P fraction are obtained and a similar LC3 lipidation assay is performed. The L fraction is selected and further resolved through a linear gradient Optiprep ultracentrifugation. Ten fractions are collected and LC3 lipidation is performed to determine the activity of each membrane fraction.

selected and further separated with subsequent procedures until the identity of the active membrane is known. In this case, the major activity resides in the 25,000 × *g* membrane after differential centrifugation. The 25,000 × *g* membrane in the sucrose gradient is further separated to obtain an L and a P fraction. The L fraction was found to be active and was further resolved in an Optiprep gradient. In the final step, the lipidation activity co-migrates with the distribution of the ER-Golgi intermediate compartment markers ERGIC53 and SEC22B (Appenzeller-Herzog & Hauri, 2006).

Additional Materials *(also see Basic Protocol 1)*

Atg5 KO MEF (Dr. Noboru Mizushima)
HB1 lysis buffer (see recipe)
1.25 M, 1.1 M, and 0.25 M sucrose buffers (Golgi isolation kit, Sigma)
HB1 buffer (see recipe)
50% Optiprep solution (see recipe)
Optiprep dilution buffer (see recipe)

Perform cell lysis

1. Culture Atg5 KO MEF (ten 15-cm dishes) in DMEM plus 10% FBS for 2 to 3 days to confluence at 37°C.

   Use cells with passages <20.

2. Remove medium and wash cells one time with PBS. Trypsinize cells with 1 ml of 1× trypsin per dish. Then add 5 ml DMEM plus 10% FBS to quench trypsin.

3. Transfer cell suspension to 15-ml conical tubes and centrifuge 5 min at 600 × *g*, 4°C, to collect cells. Remove supernatant and suspend cell pellet with 5 ml ice-cold PBS. Repeat centrifugation.

4. Discard supernatant, resuspend cell pellet with HB1 lysis buffer (use a volume about three times cell pellet volume), and pipet up and down then times using a syringe with a 22-G needle attached.

   Avoid any bubble generation.

5. Pipet 3 µl of lysate onto a glass slide and stain with trypan blue. Check the cell fraction that is trypan blue positive. If 80% to 90% of cells are positive, proceed to the next step. If <80% of cells are positive, homogenize cells again, checking cell lysis every five strokes until 80% to 90% of cells are lysed.

   Avoid >90% lysis.

Perform differential centrifugation

6. Centrifuge cell lysate 10 min at 1000 × *g*, 4°C, in a swinging bucket rotor. Transfer supernatant to a new 15-ml centrifuge tube. Suspend pellet with HB1 lysis buffer (use a volume about three times pellet volume) and centrifuge 10 min at 1000 × *g*, 4°C. Keep pellet (1 K membrane) and combine supernatant from the two centrifugations.

   The pellet after the 1000 × g spin is difficult to see because of turbidity of the supernatant caused by remaining membranes. Look at the pellet against a monochromatic background. Dark blue is usually a good choice. Carefully transfer the supernatant. If the pellet is not visible, increase centrifugation speed to 1200-1500 × g. To increase recovery, wash pellet with lysis buffer and repeat centrifugation.

7. Centrifuge supernatant 10 min at 3000 × *g*, 4°C, in a swinging bucket rotor. Keep pellet (3 K membrane) and transfer supernatant to a 13 × 51–mm polycarbonate ultracentrifuge tube.

8. Ultracentrifuge supernatant 20 min at 25,000 × *g*, 4°C, in a TLA100.3 rotor. Keep pellet (25 K membrane) and transfer supernatant to 13 × 51–mm polycarbonate ultracentrifuge tubes.

9. Ultracentrifuge supernatant 30 min at 100,000 × *g*, room temperature, in a TLA100.3 rotor. Keep pellet (100 K membrane) and discard supernatant.

   The cell-free lipidation assay was performed with the four membrane fractions. The 25 K membrane is the active membrane. Therefore, the 25 K membrane fraction was further separated with the sucrose gradient described below.

Prepare sucrose gradient

10. Suspend the 25 K membrane pellet with 0.75 ml of 1.25 M sucrose buffer.

11. Pass membrane suspension ten times through a syringe with a 22-G needle attached to fully disperse the membrane pellet.

12. Transfer suspension into an 11 × 34–mm clear ultracentrifuge tube. Layer 0.5 ml of 1.1 M sucrose buffer and 0.5 ml of 0.25 M sucrose on top of the membrane suspension.

13. Ultracentrifuge 2 hr at 120,000 × *g*, 4°C, in a TLS-55 rotor.

14. Collect the membrane layer located at the boundary of 0.25/1.1 M sucrose (L fraction) as well as the pellet (P fraction).

    Sometimes the L fraction layer is broad. If this is the case, collect all floating membranes.

15. Dilute L fraction three times with HB1 buffer in 13 × 51–mm polycarbonate ultracentrifuge tubes and ultracentrifuge 40 min at 100,000 × *g*, 4°C, in a TLA100.3 rotor.

    The cell-free lipidation assay has been performed with both of these membrane fractions. The L fraction is the active membrane. Therefore, the L fraction was further separated with the following Optiprep gradient.

Perform Optiprep gradient ultracentrifugation

16. Prepare 22.5%, 19%, 16%, 12%, 8%, and 5% Optiprep solutions by diluting the 50% Optiprep solution with Optiprep dilution buffer.

17. Suspend the L fraction membrane pellet with 1 ml of 19% Optiprep gradient by passing ten times through a syringe with a 22-G needle attached.

18. In a 13 × 51–mm clear ultracentrifuge tube, carefully layer Optiprep gradients of 0.5 ml of 22.5%, 1 ml of 19% (with the membrane from step 17), 0.9 ml of 16%, 0.9 ml of 12%, 1 ml of 8%, 0.5 ml of 5%, and 0.2 ml HB1 buffer.

    Carefully load the gradient and minimize disturbance.

19. Ultracentrifuge 3 hr at 150,000 × *g*, 4°C, in an SW-55 rotor.

    Multiple membrane layers should show up after this step.

20. Collect ten fractions, 0.5-ml each, from top to bottom. Dilute each fraction with 2 ml B88 buffer in a 13 × 51–mm polycarbonate centrifuge tube and ultracentrifuge 40 min at 100,000 × *g*, 4°C.

21. Keep membrane pellets (no. 1 through 10).

Perform lipidation reaction

22. Suspend all membrane pellets in three times pellet volume size with B88 buffer and centrifuge to collect the membrane (membranes from sucrose gradient or Optiprep, centrifuge 30 min at 100,000 × *g*; membranes from differential centrifugation, use the same speed for collecting the membrane).

    The purpose is to buffer exchange to B88 buffer because all reactions are performed in B88 buffer.

23. Suspend membrane pellets with a minimum amount of B88 lysis buffer, measure PC concentration, and adjust to 0.6 µg/µl PC.

24. Perform LC3 lipidation reaction with the membranes using Basic Protocol 1.

MEMBRANE RECRUITMENT ASSAY

BASIC PROTOCOL 2

Autophagosome biogenesis requires the assembly of cytosolic ATG proteins on special sites of the cellular membrane. These ATG proteins are recruited to the membrane in a hierarchical manner. Membrane targeting of these ATG proteins is key for their activation. A cell-free membrane recruitment assay to determine membrane targeting of certain ATG proteins to reflect the activation of different stages of autophagosome biogenesis (Ge et al., 2013) is described here.

The basic steps include a cell-free reaction that allows targeting of cytosolic ATG proteins to the membrane, and a membrane flotation assay that separates the membrane from the reaction to determine the membrane-associated ATG proteins after the reaction (Fig. 11.23.3).

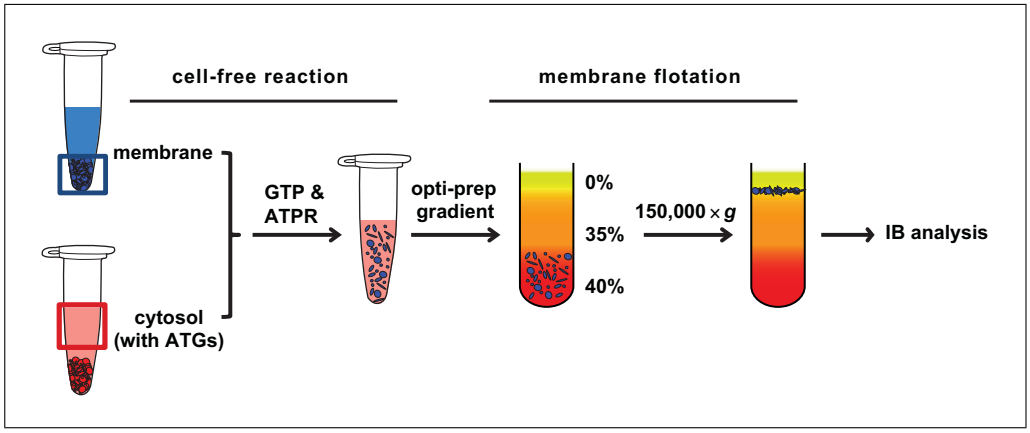

Figure 11.23.3 Membrane recruitment assay. The post-nuclear supernatant of Atg5 KO MEF membrane is collected. Cytosol from HEK293T cells with exogenously expressed ATG proteins is prepared. The membrane, cytosol, and nucleotides (GTP & ATPR) are incubated together, during which the ATG proteins in the cytosol are recruited to the membrane. After incubation, the reaction mixture is loaded into the bottom of an Opti-prep gradient and subjected to ultracentrifugation. The membrane fraction together with the bound ATG proteins will float to the top after centrifugation. The amount of the ATG proteins in the top fraction corresponds to their membrane recruitment.

Additional Materials *(also see Basic Protocol 1)*

X-tremeGene HP cell transfection reagent (Roche)
50% Optiprep in B88 buffer (see recipes)

Prepare cytosol

1. Transfect HEK293T cells with plasmids encoding ATG proteins that are going to be tested using X-tremeGene reagent following the manufacturer's instructions.
2. Prepare cytosol according to Basic Protocol 1, steps 1 through 8, steps 9 through 15.

Prepare membrane

3. Collect and lyse Atg5 KO MEF according to Basic Protocol 1.
4. Centrifuge lysate 10 min at 1000 × *g*, 4ºC, and collect supernatant.
5. Ultracentrifuge supernatant (post-nuclear supernatant) 40 min at 100,000 × *g*, room temperature, in a TLA100.3 rotor and collect pellet.
6. Resuspend pellet in B88 lysis buffer and dilute to 0.6 µg/µl of PC.

Perform cell-free reaction

7. In a low-retention 1.5-ml microcentrifuge tube, prepare a similar cell-free reaction containing cytosol (2 µg/µl), 1× ATP regeneration system, GTP (0.15 mM), and membrane (0.2 µg/µl PC). Add B88 buffer to bring the total volume to 50 µl.
8. Gently tap tube to mix and incubate mixture 1 hr at 30ºC.

Perform membrane flotation

9. After the 1-hr reaction, add 200 µl of 50% Optiprep in B88 buffer to adjust the final concentration of Optiprep to 40%.
10. Transfer to an 11 × 34–mm polycarbonate ultracentrifuge tube and layer with 200 µl of 35% Optiprep in B88 buffer, followed by 50 µl B88 buffer.
11. Ultracentrifuge 1.5 hr at 150,000 × *g*, room temperature, in a TLS-55 rotor.

There will be a membrane band between the B88 buffer and 35% Optiprep layers.

12. Collect seven fractions from top to bottom (70 µl) each.

13. Determine membrane recruitment of the relevant ATG proteins by immunoblot.

The first fraction contains ~70% to 80% of the membrane and the second fraction contains ~20%. The soluble components are in the last four fractions.

SMALL AUTOPHAGOSOMAL PRECURSOR FORMATION ASSAY

BASIC PROTOCOL 3

Autophagic membranes are derived from intracellular organelles. The membrane conversion process involves multiple membrane remodeling processes. Small membrane precursors of the autophagosome are generated during starvation. These precursors are active for LC3 lipidation. Therefore, the following cell-free assay was established to determine the generation of small autophagosomal precursors under different conditions (Ge, Zhang, & Schekman, 2014b).

This protocol includes two major steps: (1) the cell-free small membrane formation assay and (2) the cell-free lipidation assay. The former is adapted from the established COPII vesicle formation assay (Kim, Hamamoto, Ravazzola, Orci, & Schekman, 2005; Schindler & Schekman, 2009) to generate different kinds of small membranes (including the small autophagosomal precursors) in a test tube. Differential centrifugation is used to isolate the generated small vesicles. The latter step determines the generation of autophagosomal precursors in the small membrane fraction based on the fact that these autophagosomal precursors are active for LC3 lipidation (Fig. 11.23.4).

Additional Materials *(also see Basic Protocol 1 and Support Protocol)*

Refrigerated bench-top microcentrifuge (e.g., Eppendorf Centrifuge 5430R)

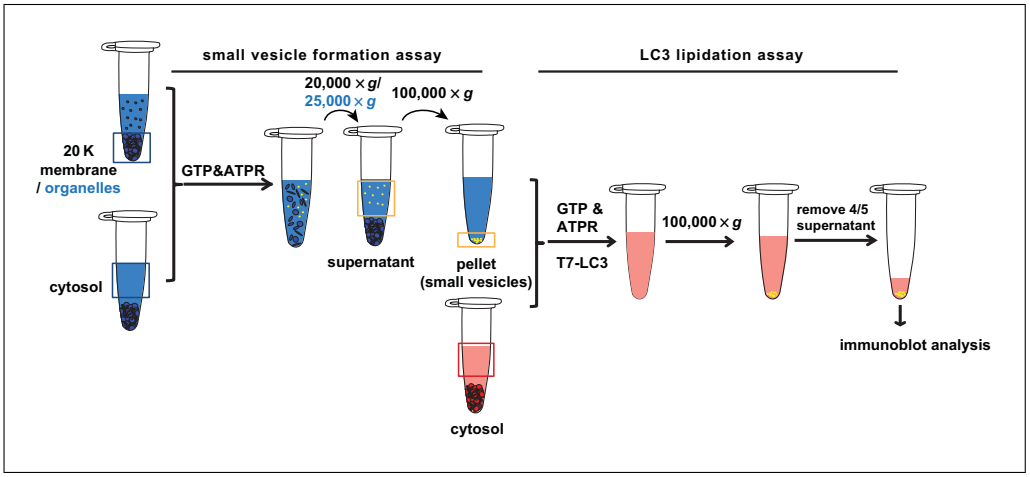

Figure 11.23.4 Cell-free autophagosomal precursor formation assay (modified from Ge et al., 2014b). Small vesicle formation assay: The donor membrane consists of the membrane fraction from the 20,000 × g centrifugation of the Atg5 KO MEF membrane after cell lysis, or organelle fractions obtained from the three-step membrane fractionation. The cytosol is from Atg5 KO MEF cells. The donor membrane, cytosol, and nucleotides (GTP & ATPR) are incubated together, during which the small vesicles are formed in the cell-free reaction. The mixture is centrifuged at 20,000 × *g* (25,000 × *g* for organelles) to remove the donor membrane. The supernatant is ultracentrifuged at 100,000 × *g* to enrich the small vesicles that may contain the small autophagosomal precursors. LC3 lipidation assay: To determine the amount of autophagosomal precursors in the small vesicle fraction, the LC3 lipidation assay is performed with these vesicles. After the reaction, the mixture is ultracentrifuged at 100,000 × *g* and 4/5 of the supernatant is carefully removed. Immunoblotting is performed with the remaining supernatant and pellet. The level of LC3 lipidation is a reflection of the amount of autophagosomal precursors generated in the above reaction.

Total donor membrane preparation

Usually, total donor membrane is used in most experiments

1. Collect and lyse Atg5 KO MEF in B88 lysis buffer according to Basic Protocol 1.
2. Microcentrifuge lysate 20 min at 20,000 × *g*, 4°C.

   In this case, the 100,000 × g small vesicle fraction is not needed.
3. Discard the supernatant and resuspend pellet with B88 buffer at three times pellet volume.
4. Repeat centrifugation and resuspend donor membrane pellet with a minimum volume of B88 lysis buffer (about one and a half times volume of pellet).
5. Adjust concentration to 1 μg/μl PC using B88 lysis buffer.

Prepare donor membrane from membrane fractionation

The purpose here is to find the membrane fraction active for generating autophagosomal precursors.

6. Collect and lyse Atg5 KO MEF in HB1 lysis buffer according to the Support Protocol.
7. Collect 3 K membrane fraction according to the Support Protocol.
8. Collect P membrane fraction according to the Support Protocol, except after resuspending the pellet with B88 buffer, centrifuge membrane 40 min at 25,000 × *g*, 4ºC. Keep the pellet and resuspend it in a minimum volume of B88 lysis buffer.
9. Collect four fractions from Optiprep gradient as follows: Perform the Optiprep gradient and collect ten fractions according to the Support Protocol. Combine all to four fractions: F1 (1 and 2), F2 (3 and 4), F3 (5 and 6), and F4 (7 through 10). Dilute the combined fractions with B88 buffer (at least three times the volume of the Optiprep fraction) and collect the membrane by centrifuging 40 min at 25,000 × *g*, 4°C. Suspend in B88 lysis buffer.
10. Adjust all membrane fractions to a concentration of 1 μg/μl PC.

Prepare cytosol

In Atg5 KO MEF, accumulation of small autophagosomal precursors has been observed. Therefore, cytosol from Atg5 KO MEF was used to reconstitute the formation of autophagosomal precursor in the cell-free assay.

11. Prepare Atg5 KO MEF cytosol and HEK293T cytosol according to Basic Protocol 1.

    Do not contaminate cytosol with any membrane.

Perform cell-free small vesicle formation assay

12. In a low-retention 1.5-ml microcentrifuge tube, prepare a cell-free reaction containing Atg5 KO MEF cytosol (2 μg/μl), 1× ATP regeneration system, GTP (0.15 mM), and membrane (0.2 μg/μl PC). Add B88 buffer to bring the total volume to 50 μl.
13. Gently tap tube to mix and incubate mixture 1 hr at 30°C.
14. Centrifuge reaction mixture 20 min at 20,000 × *g* (total donor membrane) or 20 min at 25,000 × *g*, 4°C (membrane fractions).

    The purpose of this step is to remove the donor membrane.

15. Transfer 35 µl supernatant to a new 1.5-ml microcentrifuge tube and ultracentrifuge 30 min at 100,000 × *g*, in a TLA100.3 rotor with adapters or a TLA-55 rotor.

16. Discard the supernatant and keep the pellet.

    The pellet consists of the small membranes generated in the test tube. Sometimes the pellet is difficult to see, but it is there.

Analyze generation of small autophagosomal precursors via LC3 lipidation

17. Prepare a master mix of HEK293T cytosol (2 µg/µl), 1× ATP regeneration system, GTP (0.15 mM), and T7-LC3 (10 ng/ml) in B88 buffer.

    Prepare a mastermix that is 10 to 20 µl more than the actual total volume used for all samples.

18. Suspend the pellet from step 16 with 15 µl master mix by pipetting.

19. Incubate mixture 1 hr at 30°C.

20. Ultracentrifuge mixture 30 min at 100,000 × *g*. Carefully remove 12 µl supernatant.

    Because there is not much membrane in the small membrane fraction, the lipidated LC3 level is low. By removing the supernatant, the amount of unlipidated LC3 is reduced, which prevents the strong signal of the unlipidated LC3 from obscuring the lipidated LC3 signal.

21. Add 60 µl of 1× SDS-PAGE loading buffer to the pellet plus 3 µl supernatant. Determine the LC3 lipidation level using a 12-µl sample on an immunoblot.

REAGENTS AND SOLUTIONS

Use deionized water in all recipes and protocol steps.

ATP regeneration system, 10×

400 mM creatine phosphate
2 mg/ml creatine phosphokinase
10 mM ATP in B88 buffer
Prepare 100-µl aliquots
Store for at least 2 years at −80°C

B88 buffer

20 mM HEPES-KOH, pH 7.2
250 mM sorbitol
150 mM potassium acetate
5 mM magnesium acetate
Store for at least 2 years at 4°C

The pH of the B88 buffer is crucial. Lower pH leads to low lipidation efficiency and higher pH leads to non-specific lipidation.

B88 lysis buffer

B88 buffer (see recipe) with protease inhibitors (Roche)
Phosphatase inhibitors (Roche)
0.3 mM DTT
Store for at least 2 years at −20ºC

HB1 buffer

20 mM HEPES-KOH, pH 7.2
400 mM sucrose
1 mM EDTA

Store for at least 2 years at 4°C

HB1 lysis buffer

HB1 buffer (see recipe)
Protease inhibitors (Roche)
Phosphatase inhibitors (Roche)
0.3 mM DTT
Store for at least two years at −20°C

Optiprep dilution buffer

20 mM Tricine-KOH, pH 7.4
250 mM sucrose
1 mM EDTA
Store for at least 2 years at 4°C

Optiprep solution, 50%

50% Optiprep (Sigma)
20 mM Tricine-KOH, pH 7.4
2 mM sucrose
1 mM EDTA
Store for at least 2 years at 4°C

PBS

137 mM sodium chloride
2.7 mM potassium chloride
10 mM disodium phosphate
1.8 mM monopotassium phosphate
Also may be purchased commercially (e.g., GIBCO)

COMMENTARY

Background Information

Autophagy is a catabolic cellular process broadly related to many physiological processes and pathological conditions, such as cancer, neurodegeneration, and metabolic syndromes (Levine & Kroemer, 2008; Mizushima et al., 2008). Autophagy is usually activated by stress conditions, such as starvation. During starvation-induced autophagy, a cascade of cytosolic signals induces the activation of the ATG proteins to act sequentially at special sites in the cell leading to formation of a double-membrane vesicle called the autophagosome. The autophagosome encapsulates part of the cytoplasm and transports the trapped components to the lysosome for degradation (Burman & Ktistakis, 2010; Feng, He, Yao, & Klionsky, 2014; Ge et al., 2014a).

Biogenesis of the autophagosome is a central step of autophagy. Since its discovery in the 1950s, a long-standing question has been how the autophagosome is generated in the cell. In the 1990s, a breakthrough was made by genetic studies that identified the key regulatory factors, namely the ATG genes, which are essential for autophagy. Subsequently, cell-imaging-based approaches characterized the phagophore, a cup-shaped membrane precursor of the autophagosome, and indicated hot spots of autophagic membrane assembly, called phagophore assembly sites (PAS). However, it is still unclear how these ATG proteins act together with the intracellular membrane system (which generates the autophagic membrane) to build the autophagosome (Levine & Klionsky, 2017; Ohsumi, 2014).

There have been two conundrums in the field. (1) What is the membrane origin of the autophagosome? (2) How is the source membrane converted into the autophagic membrane? In the early stages of autophagosome biogenesis, the membrane origin of the autophagosome is buried in the endomembrane system and the nascent autophagic membrane precursors lack a distinct morphology, such as a cup-shaped phagophore or a double-membrane autophagosome. These problems made it difficult to answer the two questions through traditional cell-imaging-based approaches.

To answer the first question, it was sought to establish a biochemical approach based on cell-free reconstitution to functionally determine the early stage of autophagosome biogenesis (Ge et al., 2013). The rationale is as follows. Through a successful cell-free reconstitution, it was directly determined which cellular membrane triggers autophagosome biogenesis by combining this assay with a membrane fractionation approach.

It has been well recognized that LC3 lipidation is a key step of autophagy. The process involves a covalent linkage of a cytosolic protein LC3 (LC3-I) to phosphatidylethanolamine (PE) located on the autophagic membrane precursors (Ichimura et al., 2000; Kabeya et al., 2000). Under physiological conditions, the lipidated LC3 (LC3-II) decorates different stages of the autophagic membrane. Therefore, the LC3 lipidation is a good marker for autophagosome biogenesis. Cytosolic catalysts of the lipidation reaction include the following ATG proteins: ATG12-5/16, ATG3, and ATG7. The process is carried out through a ubiquitin-like conjugation process (Feng et al., 2014). In vitro studies using purified lipidation catalysts, LC3, and synthetic liposomes with PE have reconstituted the pure enzymatic reaction of LC3 lipidation (Hanada et al., 2007; Oh-oka, Nakatogawa, & Ohsumi, 2008). Inspired by the in vitro assay and the importance of LC3 lipidation as a marker, a cell-free reaction based on LC3 lipidation to reconstitute an early step of autophagosome biogenesis was established (see Basic Protocol 1). LC3 lipidation in this system recapitulates many key regulatory landmarks of cellular autophagy regulation including starvation induction, MTORC1 regulation, and dependence on a series of key ATG proteins (Ge et al., 2013).

This cell-free assay was combined with a three-step membrane fractionation approach (see Support Protocol) to trace the active membrane fraction and identify the ERGIC as a key membrane origin of the autophagosome. In search of the interaction between the ERGIC and ATG proteins, the cell-free reaction was extended to a membrane flotation assay (see Basic Protocol 2) based on the fact that the majority of the ATG proteins need to associate with the membrane to perform their function in autophagic membrane assembly. With this approach, it was found that an autophagic phosphatidylinositol 3-kinase (PI3K) associates with the ERGIC and therefore determines the ERGIC as a membrane origin of the autophagosome (Ge et al., 2013).

To answer the second question, a small autophagosomal precursor generation assay was established (see Basic Protocol 3) because the accumulation of small autophagosomal precursors in Atg5 KO MEF was observed. With this assay, it was found that a double requirement of PI3K and COPII machinery for generating small autophagosomal precursors from the ERGIC was necessary. Through cooperative action, the ERGIC membrane is converted into the autophagic membrane as an essential step for supplying membranes for the autophagosome (Ge et al., 2014b).

This unit describes reconstituting autophagy during a cell-free system. In addition, another form of reconstitution based on purified protein and synthetic liposomes has also been developed in the field. Together, these in vitro approaches have set the basis for in vitro reconstitution to deepen the understanding of the mechanistic actions of autophagic factors and the membrane underlying biogenesis of the autophagosome.

Critical Parameters and Troubleshooting

Detailed critical parameters and troubleshooting have been indicated in each protocol.

Cell-free assay (Basic Protocol 1)

Preserve the activity of cytosol and membrane by adhering to the following: (1) The cell passages should not be >20. (2) Strictly follow the 80% to 90% lysis efficiency when collecting cytosol and membrane. (3) Make sure protease inhibitors, phosphatase inhibitors, and DTT are in the lysis buffer. (4) For membrane preparation, the membrane buffer must be exchanged to B88 by washing with B88 buffer once. (5) For cytosol preparation, the amount of lysis buffer added should not be more than two times that of the cell pellet volume in order to keep protein concentration high.

The pH of B88 buffer must be 7.2. The incubation temperature must be 30ºC. Keep consistency of the total composition of the buffer. For example, in comparing LC3 lipidation activity with different drugs, make sure to add equal amounts of solvents (e.g., DMSO) used to dissolve the drugs in all samples, including controls.

Membrane fractionation (Support Protocol)

Maintain membrane integrity by doing the following: (1) the cell passages should not be >20; (2) strictly follow the 80% to 90% lysis

Table 11.23.1 Time Considerations of Key Procedures

Procedure	Time (hr)
Cytosol preparation	6
Total membrane preparation	2
Lipidation reaction	1.5
LC3 protein purification	24
Three-step membrane fractionation	24
Membrane recruitment	1.5 + 1.5 (reaction + centrifugation)
Small autophagosome precursor formation	1.5 + 1.5 lipidation

efficiency when collecting cytosol and membrane; (3) ensure that the size of the needle is 22-G; (4) avoid excessive bubble formation when homogenizing the cells; (5) avoid vortexing.

Avoid fraction contamination and carefully collect each fraction. Ensure gradient consistency; pre-make sucrose and Optiprep gradients for 10 to 15 experiments and keep at 4°C.

Membrane recruitment assay (Basic Protocol 2)

Preserve cytosol and membrane activity and membrane integrity (see above).

Small autophagosome precursor generation assay (Basic Protocol 3)

Preserve cytosol and membrane activity and membrane integrity (see above).

Avoid donor membrane contamination. After the reaction and the medium-speed centrifugation, carefully transfer supernatant (2/3 of supernatant) and avoid any amount of pellet transfer.

Anticipated Results

Cell-free assay (Basic Protocol 1)

A time-dependent increase of LC3 lipidation starting from 30 min of incubation and peaking ~90 min of incubation will be observed. A two- to three-fold increase of LC3 lipidation with membrane and cytosol from starved cells versus non-starved cells occurs. LC3 lipidation inhibited by PI3K inhibitors in a dose-dependent manner will be seen. LC3 lipidation is compromised in Ulk1 KO cells and totally abolished in Atg5, Atg3, or Atg7 KO cells. LC3 lipidation is blocked using membranes from H89-treated cells (ERGIC-depleted).

Membrane fractionation (Support Protocol)

The following describes the content of each membrane fraction:

a. 1 K membrane fraction: enriched in nucleus, mitochondria, and ER;

b. 3 K membrane: enriched in mitochondria, ER, and lysosomes;

c. 25 K membrane: enriched in plasma membrane, endosomes, Golgi, ERGIC, ER, and peroxisomes;

d. 100 K membrane: enriched in Golgi, endosomes, and small ER fragments, vesicles;

e. L membrane: enriched in plasma membrane, endosomes, Golgi, ERGIC, some light membranes, and a small amount of ER and mitochondria;

f. P membrane: enriched in ER and light mitochondria;

g. Optiprep gradient no. 1-2: enriched in light membrane fractions;

h. Optiprep gradient no. 3-4: enriched in ERGIC;

i. Optiprep gradient no. 5-6: enriched in plasma membrane and endosomes;

j. Optiprep gradient no. 7-8: enriched in some ER and mitochondria;

k. Optiprep gradient no. 9-10: enriched in Golgi.

Membrane recruitment assay (Basic Protocol 2)

Starvation-enhanced fractionation of ATG14 and DFCP1 is found in the top membrane fraction. Depletion of the ERGIC membrane abolishes the fractionation of ATG14 and DFCP1 in the top membrane fraction.

Small autophagosome precursor generation assay (Basic Protocol 3)

Starvation-enhanced and PI3K-dependent generation of LC3 lipidation-active small membranes was found. The ERGIC is the donor membrane that generates the LC3 lipidation-active small membranes. COPII inhibitors block the generation of the small LC3 lipidation-active small membrane.

Time Considerations

See Table 11.23.1 for time considerations.

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