Selective autophagy analysis in yeast (mitophagy, pexophagy, ER-phagy/nucleophagy, the Cvt pathway)

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METHOD

For details on yeast culture and media, also see the protocol "Cultivation of Yeast Cells and Induction of Autophagy".

(1) Mitophagy

To monitor mitophagy, yeast strains expressing the mitochondria matrix protein Idh1 C-terminally fused with GFP are used (we usually tag genomically-encoded Idh1). Here, two major conditions that induce mitophagy are described.

(1-1) Respiratory conditions (Post-log phase in glycerol medium)

1. Culture yeast cells in SD/CA medium to mid-log phase (OD_{600}=~1.5) at 30°C as described in the protocol "Cultivation of Yeast Cells and Induction of Autophagy".

NOTE A mutant for the mitophagy-specific receptor Atg32 should be included as a control in order to judge whether Idh1-GFP degradation occurs through mitophagy under your experimental conditions.

2. Inoculate this culture to SG/CA respiratory medium at an OD_{600} of 0.01, and culture to post-log phase, in which GFP fragments generated by vacuolar degradation of Idh1-GFP become detected in immunoblotting analysis.

NOTE The culture usually reaches post-log phase in ~48 h under our experimental conditions. We usually take samples at 24, 48, and 72 h.

3. Take 1 ml of the culture, and harvest cells by centrifugation at 15,000 *g* for 1 min at 4°C using a centrifuge (MX-105, TOMY). Store the cell pellets at −30°C until all samples are obtained.

4. Prepare samples for SDS-PAGE and perform immunoblotting analysis using anti-GFP antibody as described in the protocol "GFP-Atg8 Cleavage Assay". The bands of Idh1-GFP and GFP fragments will appear near the molecular weight markers of 75 kD and 25 kD, respectively.

(1-2) Nitrogen starvation conditions

1. Culture yeast cells in YPD medium to mid-log phase (OD_{600}=~1.5) at 30°C as described in the protocol "Cultivation of Yeast Cells and Induction of Autophagy".

NOTE A mutant for the mitophagy-specific receptor Atg32 should be included as a control in order to judge whether Idh1-GFP degradation occurs through mitophagy under your experimental conditions.

2. Harvest the cells by centrifugation at 2,000 *g* for 1 min at room temperature using a centrifuge (LC-200, TOMY).

3. Remove the supernatant, and suspend the cell pellets in the same volume of YPL respiratory medium.

4. Centrifuge, remove the supernatant, and resuspend the cell pellets in the same volume of YPL medium.

5. Inoculate this suspension to YPL medium at an OD_{600} of 0.15, and culture to an OD_{600} of 1.5.

6. Take 1 ml of the culture, and harvest cells as samples before starvation by centrifugation at 15,000 *g* for 1 min at 4°C using a centrifuge (MX-105, TOMY). Store the cell pellets at −30°C until all samples are obtained.

7. Harvest the rest of the cells, and suspend them in the same volume of SD(-N) nitrogen starvation medium.

8. Centrifuge, and resuspend the cell pellets in the same volume of SD(-N) medium.

9. After incubation, take 1 ml of the culture, and harvest cells by centrifugation.

NOTE GFP fragments generated by vacuolar degradation of Idh1-GFP become detected in immunoblotting analysis after starvation for ~4 h under our experimental conditions. We usually take samples at 0, 4, and 8 h.

10. Prepare samples for SDS-PAGE and perform immunoblotting analysis using anti-GFP antibody as described in the protocol "GFP-Atg8 Cleavage Assay". The bands of Idh1-GFP and GFP fragments will appear near the molecular weight markers of 75 kD and 25 kD, respectively.

(2) Pexophagy

To monitor pexophagy, yeast strains expressing the peroxisomal membrane protein Pex11 C-terminally fused with GFP are used (we usually tag genomically-encoded Pex11). Here, two major conditions that induce pexophagy are described.

(2-1) Post-log phase in oleate medium

1. Culture yeast cells in SD/CA medium overnight at 30°C as described in the protocol "Cultivation of Yeast Cells and Induction of Autophagy".

NOTE A mutant for the pexophagy-specific receptor Atg36 should be included as a control in order to judge whether Pex11-GFP degradation occurs through pexophagy under your experimental conditions.

2. Inoculate a 1/10 volume of this culture into YM2 oleate medium, culture to post-log phase.

3. Take 1 ml of the culture, and harvest cells by centrifugation at 15,000 *g* for 1 min at 4°C using a centrifuge (MX-105, TOMY).

NOTE GFP fragments generated by vacuolar degradation of Pex11-GFP become detected in immunoblotting analysis after culture in YM2 medium for ~48 h under our experimental conditions. We usually take samples at 24, 48, and 72 h.

4. Prepare samples for SDS-PAGE and perform immunoblotting analysis using anti-GFP antibody as described in the protocol "GFP-Atg8 Cleavage Assay". The bands of Pex11-GFP and GFP fragments will appear near the molecular weight markers of 50 kD and 25 kD, respectively.

(2-2) Nitrogen starvation conditions

1. Culture yeast cells in SD/CA medium overnight at 30°C as described in the protocol "Cultivation of Yeast Cells and Induction of Autophagy".

NOTE A mutant for the pexophagy-specific receptor Atg36 should be included as a control in order to judge whether Pex11-GFP degradation occurs through pexophagy under your experimental conditions.

2. Inoculate a 1/10 volume of this culture into YM2 oleate medium, and culture for 18 h to proliferate peroxisomes.

3. Take 1 ml of the culture, and harvest cells as samples before starvation by centrifugation at 15,000 *g* for 1 min at 4°C using a centrifuge (MX-105, TOMY).

4. Harvest the rest of the cells by centrifugation at 2,000 *g* for 1 min at room temperature using a centrifuge (LC-200, TOMY).

5. Remove the supernatant, and suspend the cell pellets in the same volume of SD(-N) nitrogen starvation medium.

6. Centrifuge, and resuspend the cell pellets in the same volume of SD(-N) medium.

7. After incubation, take 1 ml of the culture, and harvest cells by centrifugation.
NOTE GFP fragments generated by vacuolar degradation of Pex11-GFP become detected in immunoblotting analysis after starvation for ~4 h under our experimental conditions. We usually take samples at 0, 4, and 8 h.
9. Prepare samples for SDS-PAGE and perform immunoblotting analysis using anti-GFP antibody as described in the protocol "GFP-Atg8 Cleavage Assay". The bands of Pex11-GFP and GFP fragments will appear near the molecular weight markers of 50 kD and 25 kD, respectively.

(3) ER-phagy/nucleophagy

To monitor ER-phagy, yeast strains expressing Sec63 C-terminally fused with GFP are used (This membrane protein localizes to all the three ER subdomains: the perinuclear, cytoplasmic, and cortical ER). To examine degradation of the perinuclear ER (nuclear envelope) and the cytoplasmic/cortical ER separately, cells expressing the perinuclear ER membrane protein (outer nuclear membrane protein) Hmg1 and the reticulon Rtn1 C-terminally fused with GFP are used, respectively. Yeast cells expressing the inner nuclear membrane protein Src1 or the nucleolar protein Nop1 C-terminally fused with GFP can be used to monitor degradation of these nuclear subdomains. We usually tag these proteins encoded on the genome.
1. Culture yeast cells to mid-log phase (OD_{600}=~1.5) at 30°C, and induce ER-phagy/nucleophagy by nitrogen starvation or rapamycin treatment as described in the protocol "Cultivation of Yeast Cells and Induction of Autophagy".
NOTE Mutants for the ER-phagy/nucleophagy-specific receptors Atg39 and Atg40 should be included as controls in order to judge whether degradation of GFP-fused ER/nuclear proteins occurs through ER-phagy or nucleophagy under your experimental conditions.
2. Take 1 ml of the culture, and harvest cells by centrifugation at 15,000 *g* for 1 min at 4°C using a centrifuge (MX-105, TOMY). Store the cell pellets at −30°C until all samples are obtained.
NOTE GFP fragments generated by vacuolar degradation of GFP-fused ER/nuclear proteins become detected in immunoblotting analysis after nitrogen starvation/rapamycin treatment for ~6 h under our experimental conditions. We

usually take samples at 6-24 h.

3. Prepare samples for SDS-PAGE and perform immunoblotting analysis using anti-GFP antibody as described in the protocol "GFP-Atg8 Cleavage Assay". The bands of Sec63-GFP, Hmg1-GFP, Rtn1-GFP, Src1-GFP, Nop1-GFP, and GFP fragments will appear near the molecular weight markers of 100 kD, 150 kD, 50 kD, 150 kD, 75 kD, and 25 kD, respectively.

(4) The Cvt pathway

A number of vacuolar enzymes are transported to the vacuole via the Cvt pathway under nutrient replete conditions. The aminopeptidase Ape1 is a major cargo of this pathway, and its propeptide at the N terminus is removed by a vacuolar processing enzyme to be an enzymatically-active mature form. This Ape1 processing is used to monitor activity of the Cvt pathway. In addition, if Ams1, another cargo of the Cvt pathway, is fused with GFP, the level of GFP fragments produced by its vacuolar cleavage within the linker region also indicate activity of this pathway. We usually tag genomically-encoded Ams1.

1. Culture yeast cells to mid- to late-log phase (OD_{600}=~2-6) at 30°C as described in the protocol "Cultivation of Yeast Cells and Induction of Autophagy".

NOTE A mutant for the Cvt pathway-specific receptor Atg19 should be included as a control in order to judge whether Ape1 maturation or Ams1-GFP cleavage occurs through the Cvt pathway under your experimental conditions.

2. Take 1 ml of the culture, and harvest cells by centrifugation at 15,000 *g* for 1 min at 4°C using a centrifuge (MX-105, TOMY). Store the cell pellets at −30°C until all samples are obtained.

NOTE Both Ape1 maturation and Ams1-GFP cleavage efficiently occur in cells grown to late-log phase (OD_{600}=~6) than earlier phase.

3. Prepare samples for SDS-PAGE and perform immunoblotting analysis using anti-Ape1 or anti-GFP antibodies as described in the protocol "GFP-Atg8 Cleavage Assay". The bands of mature Ape1, Ams1-GFP, and GFP fragments will appear near the molecular weight markers of 50 kD, 150 kD, and 25 kD, respectively.

MATERIALS

REAGENTS

- Bacto yeast extract (BD, 212750)
- Bacto peptone (BD, 211677)
- Glucose (Nacalai Yesque, 16806-54)
- Lactic acid (Wako, 128-00056)
- NaOH (Wako, 198-13765)
- Yeast nitrogen base w/o amino acids and ammonium sulfate (BD, 233520)
- Ammonium sulfate (Nacalai Tesque, 02620-04)
- Bacto casamino acids (BD, 223050)
- Glycerol (Nacalai Tesque, 17018-83)
- Adenine sulfate (Ade; Wako, 010-19612)
- L-Tryptophan (Trp; Wako, 204-03382)
- Uracil (Ura; Wako, 212-0062)
- Oleate (Nacalai Tesque, 25630-64)
- Tween 40 (Nacalai Tesque, 35701-82)
- Anti-Ape1 antibodies (rabbit polyclonal, our laboratory stock)

EQUIPMENT

- Centrifuge MX-105 (TOMY)
- Centrifuge LC-200 (TOMY)
- Vacuum filter system, 0.22-μm pore PES (Corning, 431098)

REAGENT PREPARATION

YPL medium

- 1% (w/v) yeast extract
- 2% (w/v) peptone
- 0.01% (w/v) glucose
- 2% (v/v) lactic acid

To prepare 1 L of this medium, dissolve 10 g of bacto yeast extract, 20 g of bacto peptone, and 100 mg of glucose in 980 ml of distilled water, and add 20 ml of lactic acid. Adjust the pH to 5.5 using 5 M NaOH. Sterilize using a vacuum filter system. Store at room temperature.

SG/CA medium

• 0.17% (w/v) yeast nitrogen base w/o amino acids and ammonium sulfate
• 0.5% (w/v) ammonium sulfate
• 0.5% (w/v) casamino acids
• 0.1% (w/v) glucose
• 3% (v/v) glycerol
• 0.002% (w/v) adenine sulfate
• 0.002% (w/v) tryptophan
• 0.002% (w/v) uracil

To prepare 1 L of this medium, dissolve 1.7 g of yeast nitrogen base w/o amino acids and ammonium sulfate, 5 g of ammonium sulfate, and 5 g of bacto casamino acids in 900 ml of distilled water. Dissolve 1 g of glucose in 50 ml of distilled water. Mix 30 ml of glycerol with 20 ml of distilled water. Autoclave at 121°C for 20 min. Mix these three solusions. Add 10 ml of 100× Ade, 100× Trp, and 100× Ura (see the protocol "Cultivation of Yeast Cells and Induction of Autophagy"). Store at room temperature.

YM2 medium

• 0.17% (w/v) yeast nitrogen base w/o amino acids and ammonium sulfate
• 0.5% (w/v) ammonium sulfate
• 1% (w/v) casamino acids
• 0.1% (w/v) glucose
• 0.1% (v/v) yeast extract
• 0.12% (v/v) oleate
• 0.2% (v/v) Tween-40
• 0.002% (w/v) adenine sulfate
• 0.002% (w/v) tryptophan
• 0.002% (w/v) uracil

To prepare 1 L of this medium, dissolve 1.7 g of yeast nitrogen base w/o amino acids and ammonium sulfate, 5 g of ammonium sulfate, 10 g of bacto casamino acids, 1 g of glucose, and 1 g of yeast extract in 1 L of distilled water. Mix with 2 ml of Tween-40, 1.2 ml of oleate, and 10 ml of 100× Ade, 100× Trp, and 100× Ura (see the protocol "Cultivation of Yeast Cells and Induction of Autophagy").

Sterilize using a vacuum filter system. Store at room temperature.