16

Microautophagy in the Yeast *Saccharomyces cerevisiae*

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Summary

Microautophagy involves direct invagination and fission of the vacuolar/lysosomal membrane under nutrient limitation. In *Saccharomyces cerevisiae* microautophagic uptake of soluble cytosolic proteins occurs via an autophagic tube, a highly specialized vacuolar membrane invagination. At the tip of an autophagic tube vesicles (autophagic bodies) pinch off into the vacuolar lumen for degradation. Formation of autophagic tubes is topologically equivalent to other budding processes directed away from the cytosolic environment, e.g., the invagination of multivesicular endosomes, retroviral budding, piecemeal microautophagy of the nucleus and micropexophagy. This clearly distinguishes microautophagy from other membrane fission events following budding toward the cytosol. Such processes are implicated in transport between organelles like the plasma membrane, the endoplasmic reticulum (ER), and the Golgi. Over many years microautophagy only could be characterized microscopically. Recent studies provided the possibility to study the process in vitro and have identified the first molecules that are involved in microautophagy.

Key Words: Calmodulin; microautophagy; vacuole; VTC (vacuolar transporter chaperone); yeast.

1. Introduction

When yeast cells sense nutrient (e.g., nitrogen) limitation, they stop dividing and enter the stationary phase. They adapt to the new environmental conditions by breaking down large quantities of cellular constituents by autophagy. The phenomenon enables cells to survive long periods of starvation. Autophagy is a highly conserved lysosomal transport and degradation pathway of all eukaryotic cells ***(1)***. In the yeast *Saccharomyces cerevisiae* the lysosomal

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compartment is called the "vacuole." It is the main compartment for the storage, recycling, and breakdown of cellular constituents. The organelle can easily be stained for in vivo fluorescence microscopy. Yeast vacuoles can be prepared in milligram amounts per day. This is why vacuoles represent an excellent tool for biochemical studies. The highly dynamic vacuolar organelle undergoes morphological changes dependent on the cell cycle and environmental changes such as nutrient availability **(Fig. 1)**. Concomitant with daughter cell emergence, vacuoles begin to fragment into small vesicles that are transported into the daughter cell. There, the vesicles fuse to build up a new vacuolar compartment.

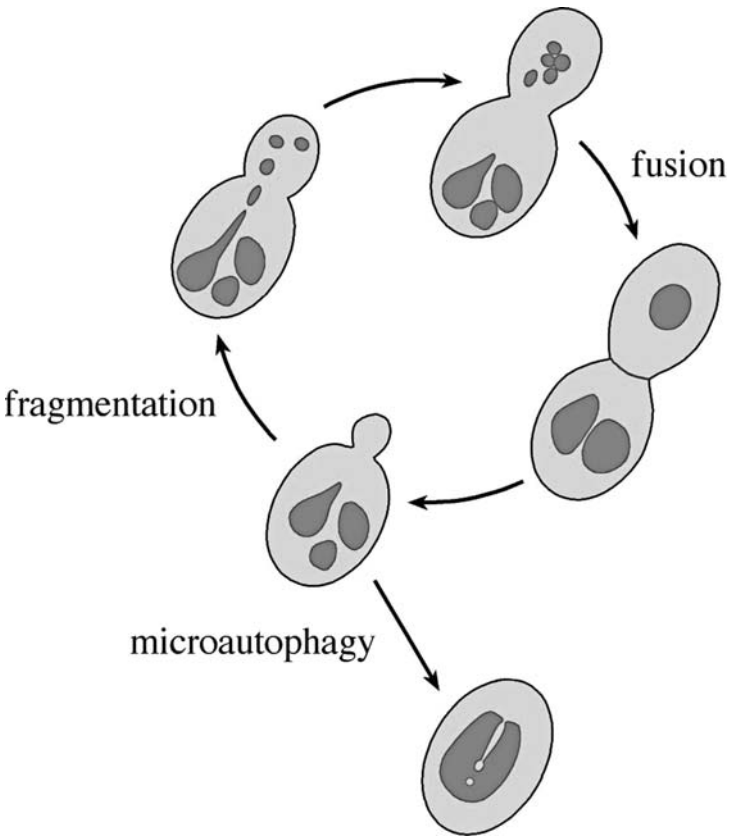

Fig. 1. Morphological changes of the yeast vacuole. When cells divide, vacuoles fragment into small vesicles that are transported into the daughter cell. There they fuse to build up new vacuoles. When cells are starved they stop dividing. Then, the cell compensates the influx of lipid and protein into vacuolar membranes that is caused by macroautophagy. This is achieved by means of microautophagy, a direct membrane invagination, and fission of autophagic bodies into the vacuolar lumen.

Upon entry into stationary phase, yeast cells develop a large single central vacuole, which then degrades large quantities of cytosol and/or organelles. There are two entry pathways for bulk cytosolic compounds: Macroautophagy is defined as the formation of double-layered vesicles (autophagosomes) that enclose cytosolic compounds. They target these compounds for degradation by fusing their outer membrane with vacuoles ***(2)***. Vacuolar fusion and macroautophagy have been studied intensively over the last decade. In contrast, little is known about microautophagy, a process comprising a direct invagination and budding of vesicles (autophagic bodies) into the vacuolar lumen. It has been characterized microscopically **(Fig. 2)** ***(3)*** and could be reconstituted in a cell-free system composed of purified vacuoles and cytosolic extracts. This in vitro system measures the uptake of a luciferase reporter substrate **(Fig. 3)** ***(4)***. Using pharmacological substances, the in vitro uptake reaction can be dissected into different kinetic stages ***(5)***. According to their ability to block the reaction at different kinetic stages, these inhibitors have been defined as early-acting class A inhibitors (nystatin, GTP?S, aristolochic acid) and late-acting class B inhibitors (W-7, valinomycin/FCCP, K252a and rapamycin) ***(5)***. Recent findings have identified the first molecular players acting during microautophagy. Both apocalmodulin (i.e., the calcium free conformation of calmodulin) and the vacuolar transporter chaperone (VTC) complex act late in the reaction ***(6,7)***.

Microautophagy of soluble cytosolic components is topologically equivalent to invaginations occurring during (1) multivesicular body (MVB) formation at the endosome ***(8,9)***, (2) retroviral budding at the endosome or the plasma membrane ***(10)***, (3) piecemeal microautophagy of the nucleus (PMN), which tranfers parts of the nucleus into vacuoles ***(11),*** and (4) micropexophagy in methylotrophic yeasts which leads to the degradation of peroxisomes ***(12–17)***. Although microautophagy of soluble components, like macroautophagy, is induced by nitrogen starvation and Rapamycin (a pharmacological agent inhibiting Tor kinase signaling) and although pexophagic vacuole invagination depends on Atg proteins ***(18–21)***, there is no evidence to date that Atg proteins are directly involved in either PMN ***(11)*** or microautophagic uptake. Macroautophagy seems to be a prerequisite for sustained microautophagy, however ***(4)***. Microautophagy is controlled by the TOR and EGO (composed of Ego1p, Gtr2p, and Ego3p) signaling complexes ***(22)***. It leads to direct uptake and degradation of the vacuolar boundary membrane.

The main functions of microautophagy may lie in the transition from starvation-induced growth arrest to logarithmic growth ***(22)*** and in maintenance of organellar size and membrane composition ***(3)***. Since microautophagy leads to uptake and degradation of the vacuolar boundary membrane, it could compensate the enormous influx of membrane caused by macroautophagy.

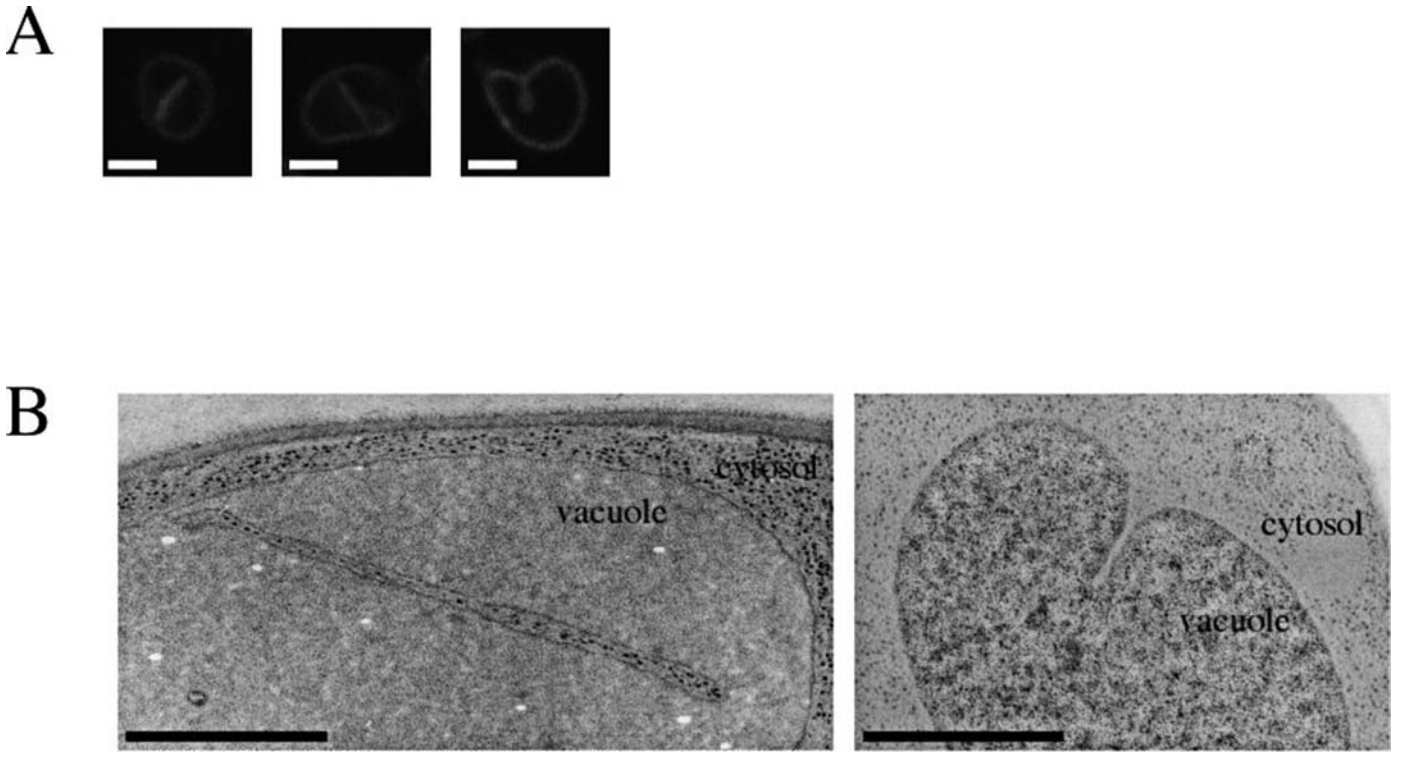

Fig. 2. Microautophagic invaginations in vivo as seen by **(A)** fluorescence microscopy using a strain expressing GFP-Vtc4p and **(B)** ultrathin sectioning electron microscopy. Bars correspond to 1 μm in **(A)** and **(B)**.

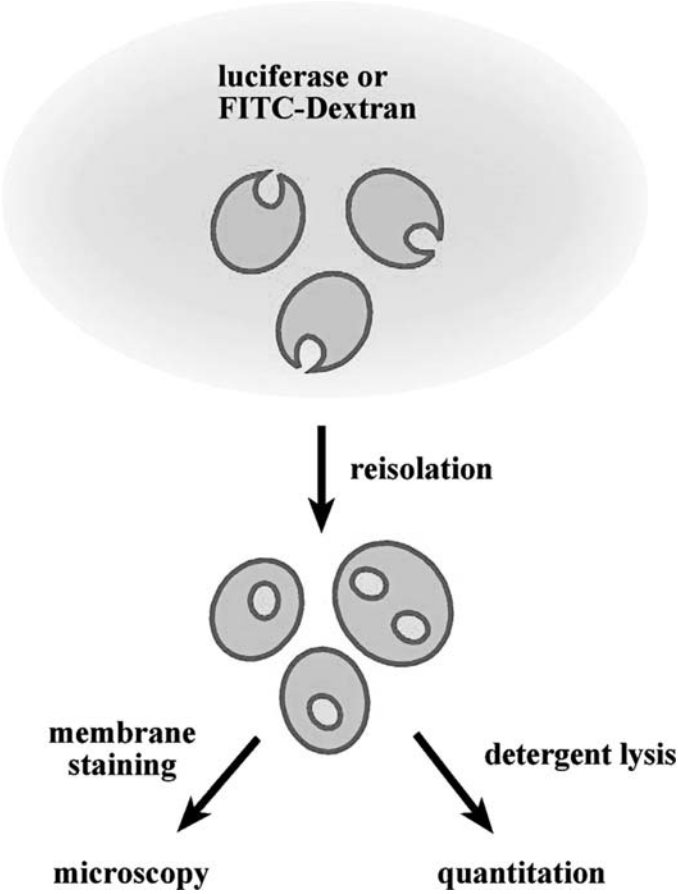

Fig. 3. Schematic presentation of reconstituted in vitro system for microautophagy. Isolated vacuoles are incubated with cytosol, buffer, salts, ATP-regenerating system, and the uptake substrate for 1 h at 4 or 27°C. After the uptake reaction, the vacuoles are washed thoroughly and processed for detection of the uptake substrate (firefly luciferase or FITC-dextran) ***(4)***.

Microautophagic vacuole invagination (with the exception of PMN and pexophagy) might hence be responsible for maintenance of organellar size and membrane composition rather than for cell survival under nutrient restriction. A function in organelle homeostasis should render microautophagic membrane invagination dependent on membrane influx via macroautophagy, which has been observed ***(4)***.

This hypothesis is also consistent with ultrastructural data. In freeze-fracture electron microscope nascent microautophagic vesicles that bud off from the tips

of autophagic tubes appear as virtually free from intramembranous particles, i.e., there appears to be lateral heterogeneity induced along the autophagic tube that prevents large membrane proteins from entering the microautophagic vesicle membrane. Microautophagic vesicles share this exceptional ultrastructural feature with nascent autophagosomes, which form in the cytosol by macroautophagy and are also are virtually free of intramembraneous particles. This suggests that microautophagy might compensate macroautophagic membrane influx in terms of both quantity and quality.

The yeast vacuole is a highly dynamic organelle that undergoes dramatic morphological changes **(Fig. 1)**. Therefore, the vacuole serves as an excellent model system to study membrane dynamic processes. The organelle can easily be stained for in vivo fluorescence microscopy. Besides, yeast vacuoles can be prepared in milligram amounts per day making vacuoles an excellent system for biochemical studies and in vitro assays. These assays allow one to reconstitute authentic reactions with a minimum of factors necessary to drive the process. These factors (e.g., vacuoles, cytosol, purified proteins, salts, chemicals) can be added independent of each other. In addition, they serve to render the process accessible to defined concentrations of pharmaceuticals and compounds (e.g., purified proteins or reporter molecules) which cannot easily penetrate whole cells due to their chemical nature or size. In vitro reactions can also be arrested at different stages by inhibitors to define kinetic phases. Often certain protein conformations or interactions accumulate in these phases and can then be further characterized. This allows one to not only identify new factors implicated in the process but also to explore their mechanisms of action in detail.

2. Materials

2.1. Buffers

1. PS buffer: 10 m*M* piperazine-*N*,*N*′-bis(2-ethanesulfonate) [PIPES]/KOH pH 6.8, 200 m*M* sorbitol.
2. Cytosol buffer: 40 m*M* PIPES/KOH, pH 6.8, 0.5m*M* $MgCl_2$, 150 m*M* KCl, 200 m*M* sorbitol, 1 m*M* dithiothreitol [DTT], 0.2 m*M* phenylmethylsulfonyl fluoride [PMSF], 0.1 m*M* pefabloc SC, 0.5 µg/mL pepstatin A, 50 µ*M* o-phenanthroline.
3. Spheroplasting buffer for vacuole preparation: 50 m*M* potassium phosphate, pH 7.5, 600 m*M* sorbitol in YPD with 0.2% glucose.
4. Washing buffer for lyticase preparation: 25 m*M* Tris-HCl, pH 7.4.

2.2. Media

1. YPD: 1% yeast extract, 2% Bacto peptone 2% glucose.
2. YPD containing 200 n*M* Rapamycin (Alexis, dissolve 100 X in DMSO).

3. SD(-N): 0.67% Difco yeast nitrogen base without amino acids and without ammonium sulfate, 2% glucose, for starvation.
4. LB medium: 2% tryptone, 1% yeast extract, 1% NaCl, pH 7.

2.3. Cytosol Preparation

Cytosol is usually prepared from yeast strain K91-1A. This strain is deficient for soluble phosphatates. This is advantageous when cytosol preparations are used for in vitro microautophagy assays, especially when values are normalized to vacuolar alkaline phosphatase activity.

2.4. Lyticase for Vacuole Preparation

β-1,3-Glucanase from Oerskovia xanthineolytica ***(23,24)*** is expressed in *E. coli* strain RSB 805. The protein is purified from the periplasmic space ***(25)***.

2.5. Fluorescence Microscopy

1. FM4-64 (Molecular Probes) is dissolved as a 100X stock solution (10 m*M*) in dimethyl sulfoxide (DMSO) or ethanol and stored at –20°C.
2. For immobilization of yeast cells use Seaplaque agarose in 10 m*M* PS buffer.

3. Methods

3.1. Yeast Cell Culture

Yeast cells are precultured in YPD for 6–8 h at 30°C and then diluted for logarithmic overnight growth (14–16 h, 30°C, 225 rpm) in 2-L Erlenmeyer flasks with 1 L of YPD medium. For starving cells, overnight cultures were harvested at an optical density (OD 600) of 2, centrifuged (4 min 3800*g*), washed with sterile water, resuspended in 1 L of SD(-N) starvation medium, and incubated (3 h, 30°C, 225 rpm) (*see* **Note 2**).

3.2. Cytosol Preparation

1. Harvest overnight yeast cultures at OD_{600} = 4.5, centrifuged (4 min, 3800*g*), wash with sterile water, harvest again, resuspend in 1 L of SD(-N), and incubate for 3–4 h at 30°C and 255 rpm. For preparation of cytosol from nonstarved cells, replace SD(-N) by YPD in this incubation.
2. Harvested cells and wash as described above, first with water, and then with one pellet volume of chilled cytosol buffer. After centrifugation (5 min, 3,800g 4°C), resuspend the pellet in a small volume of cytosol buffer so that a thick slurry results.

3. Freeze the suspension as little nuggets in liquid nitrogen and blend (6–8 times for 30 s) in a Waring blender filled with liquid nitrogen.
4. Thaw cells and centrifuge the lysate (10 min, 12,000*g*, 4°C). Ultracentrifuge the supernatant (20 min, 125,000*g*, 2°C), discard the fatty top fraction, and recover the clarified cytosol.
5. Adjust the protein concentration of the cytosolic fraction to 25–30 mg/mL with cytosol buffer (do not use preparations below 10 mg/mL for in vitro microautophagy assays). Freeze 50- to 300-µL aliquots in liquid nitrogen and store at –80°C.

3.3. Lyticase Preparation for Vacuole Preparation

1. Grow 10 l RSB 805 in LB medium with 100 µg/mL ampicillin to OD_{600} = 0.6.
2. Add 0.4 m*M* isopropyl thriogalactose (IPTG) and let cells grow for another 5 h at 30°C.
3. Harvest cells (10 min, room temperature, 4200*g*), wash with 50 mL washing buffer and centrifuge (10 min, room temperature, 4200*g*).
4. Resuspend pellets in 200 mL washing buffer, supplemented with 2 m*M* EDTA and an equal volume of 40% (w/v) sucrose in washing buffer and gently shake at room temperature for 20 min.
5. Harvest cells (10 min, room temperature, 4200*g*) and completely remove the supernatant. Chill flasks on ice.
6. Resuspend the cells from 1 L of culture in 20 mL ice cold 0.5 m*M* $MgSO_4$, shake gently (4°C, 20 min) and centrifuge (10 min, 4°C, 4200*g*).
7. The supernatant contains Lyticase and some *E. coli* periplasmic proteins. Adjust the protein concentration of the supernatant to 1 mg/mL. The lyticase solution can be stored at –20°C in 6-mL aliquots without further purification. It should be thawed directly before use for digestion of yeast cell walls (*see* **Note 3**).

3.4. Vacuole Preparation

1. Grow cells in YPD overnight to OD_{600} = 2.5-3.0, harvest (2 min, 3,800 g, 4°C), and resuspend them in 50 mL of 30 m*M* Tris-HCl, pH 8.9 with 10 m*M* DTT. Incubate cells (5 min, 30°C).
2. Centrifuge cells as above, resuspend in 12 mL of spheroplasting buffer, add 3 mL of a lyticase solution (concentration of stock solution: 1 mg/mL) and transfer into 30-mL Corex tubes. Incubate cells (25 min, 30°C) (*see* **Note 2**).
3. Reisolate spheroplasts (4°C, 1 min, 2500*g*) and resuspend in 2.5 mL 15% Ficoll 400 in PS buffer by gentle stirring with a plastic rod and/or gentle vortexing.
4. Add DEAE-dextran (300 µL) from a 0.4-mg/mL stock in 15% Ficoll 400 in PS buffer. Incubate spheroplasts (2 min at 0°C, then 90 s at 30°C), chill again, transfer to an SW41 ultracantrifugation tube, and overlay with 3 mL 8% Ficoll 400, 3 mL 4% Ficoll 400, and 1.5–2 mL 0% Ficoll 400 in PS buffer. After centrifugation (85 min, 154,000*g*, 2°C,), harvest vacuoles from the 0–4% interphase.

5. For storage of vacuoles, add glycerol (final concentration 10% w/v from a 50% stock) to a fresh vacuole suspension. Freeze the suspension as little nuggets in liquid nitrogen and store them at –80°C.

3.5. Fluorescence Microscopy

3.5.1. Staining of Vacuoles In Vivo

1. Grow cells logarithmically in 5 mL of YPD overnight. (20-mL tubes, 30°C, 225). Then, supplement 1 mL of the suspension with 10–20 µ*M* FM4-64 and incubate for 1 h.
2. Reisolate the cells (2 min, 1300*g*), wash twice with deionized water, reisolate again, and resuspend to an OD_{600} of 0.5-1 in YPD, YPD containing Rapamycin, or SD-(N) medium without stain.
3. Let cells grow in these media for 3–4 h. Centrifuge 400 µL of the cell suspension (1 min, 12,000 g) and resuspend the cells in 40 µL of the identical medium for concentration.
4. Transfer 5–8 µL of the concentrated cell suspension to a slide, cover immediately with a cover slip, and investigate immediately with a confocal microscope or a convential fluorescence microscope (*see* **Note 4**).

3.5.2. GFP-Vtc-Proteins

1. Grow cells logarithmically in 5 mL of YPD overnight. (20-mL tubes, 30°C, 225).
2. Reisolate the cells (2 min, 1300*g*), wash twice with deionized water, reisolate again, and resuspend to an OD_{600} of 0.5-1 in YPD, YPD containing Rapamycin, or SD-(N) medium.
3. Let cells grow in these media for 3–4 h. Centrifuge 400 µL of the cell suspension (2 min, 1300*g*) and resuspend the cells in 40 µL of the identical medium for concentration.
4. Transfer 5–8 µL of the concentrated cell suspension to a slide, cover immediately with a cover slip, and investigate immediately with a confocal microscope or a convential fluorescence microscope.

3.6. Electron Microscopy

3.6.1. Thin Sectioning for Ultrastructural Studies

To cryoimmobilize yeast cells by high-pressure freezing proceed as described previously ***(26)***:

1. Suck living specimen into cellulose microcapillaries of 200 µm diameter and transfer 2-mm-long capillary tube segments to aluminum platelets of 200 µm depth containing 1-hexadecene.

2. Sandwich the platelets with platelets without any cavity and then freeze with a high-pressure freezer (Bal-Tec HPM 010, Balzers, Liechtenstein). Remove extraneous hexadecene from the frozen capillary tubes under liquid nitrogen and transfer to 2 mL-microtubes with screw caps (Sarstedt #72.694) containing the substitution medium precooled to –90°C.
3. Keep samples for ultrastructural studies in a freeze-substitution unit (Balzers FSU 010, Bal-Tec, Balzers, Liechtenstein) in 2% osmium tetroxide in anhydrous acetone at –90°C for 32 h, warm up to –60°C within 3 h, keep at –60°C for 4 h, warm up to –40°C within 2 h, and keep there for 4 h.
4. Wash the samples with acetone and transfer them into an acetone-Epon mixture at –40°C, infiltrate at room temperature in Epon, and polymerize at 60°C for 48 h.
5. View ultrathin sections, stained with uranyl acetate and, if required, with lead citrate, in a Philips CM10 electron microscope at 60 kV.

3.6.2. Freeze-Fracture Analysis

1. Sandwich living yeast cells or isolated vacuoles between thin copper sheets (Bal-Tec; Balzers AG), mount on tweezers, an rapidly inject into melting propane (–185°C), as described before ***(27)***.
2. Insert the sandwiches under liquid nitrogen into a freeze-fracture unit Type BAF 300 (Balzers AG), fracture at –100°C, and replicate by 45° platinum-shadowing.
3. Transfer replicas into 2.5% sodium dodecyl sulfate (SDS) with 30 m*M* sucrose in 10 m*M* Tris-HCl buffer, pH 8.3. After vigorous shaking for 30 min, wash replicas several times with distilled water, and mount onto copper grids for routine electron microscopic analysis in an electron microscope (EM) 10 (Zeiss).

3.6.3. Thin Sectioning for Immunogold Labeling

1. Process samples as described in **Subheadings 1.** and **2.** for thin sectioning for ultrastructural studies.
2. Process samples for immuno/affinity labeling in 0.5% acrolein or uranyl acetate (depending) in anhydrous ethanol using the same temperature/time schedule for freeze-substitution.
3. After washing with ethanol, transfer the samples into an ethanol-Lowicryl K11M mixture, infiltrate with the polar methacrylate resin Lowicryl K11M (Polysciences, Eppelheim, Germany) and polymerize by UV irradition at –40°C for 48 h.
4. Immunolabeling can be done as described previously ***(28,29)*** using affinity purified antibodies (typically 0.5–3 µg/mL in 0.1% acetylated bovine serum albumen [BSA] or 0.2% gelatine and 0.5% BSA in phosphate=buffered saline [PBS]) to proteins or lipids of interest. Detect antibodies from rabbit or mouse with protein A labeled with 15-nm gold particles (the signal for antibody derived from goat should be enhanced with anti-goat antibody in between). View ultrathin sections, stained with uranyl acetate, in a Philips CM10 EM at 60 kV.

3.7. Reconstituted Microautophagic Assay In Vitro

Microautophagic activity can be reconstituted in a cell-free system composed of purified vacuoles and cytosolic extracts ***(4)***. Vacuoles internalize a reporter enzyme (firefly luciferase, or, if assay by microscopy is desired, fluorophores coupled to high MW carriers—e.g., FITC-dextranes) in an ATP-dependent fashion. After the uptake reaction, internalized luciferase reporter can be reisolated with the vacuoles: It is protected against proteinase K digestion by the vacuolar boundary membrane and by the membrane of the microautophagic vesicle. After proteolytically removing luciferase that has not been taken up, the vacuolar membranes can be lysed and reporter enzyme activity can be quantified by a chemoluminescent assay.

3.7.1. Standard Reaction

1. A standard reaction has a volume of 45 µL and contains: vacuoles (0.2 mg/mL, either freshly prepared or thawed from a –80°C stock), 3 g/mL cytosol from starved cells, 105 m*M* KCl, 7 m*M*$MgCl_2$, 2.2 m*M* ATP, 88 m*M* disodium creatine phosphate, 175 U/mL creatine kinase, 17 µg/mL luciferase, 100 µ*M* DTT, 0.1 m*M* pefabloc SC, 0.5 m*M* o-phenanthrolin, 0.5 µg/mL pepstatin A, 200 m*M* sorbitol, 10 m*M* PIPES/KOH pH 6.8 (*see* **Note 5**). Incubate this mixture for 1 h at 27°C.
2. For measuring luciferase uptake, chill the samples on ice, dilute with 300 µL 150 m*M* KCl in PS buffer, centrifuge (6500*g*, 3 min, 2°C), wash the pellet once more with 300 µL 150 m*M* KCl in PS buffer by gently pipetting three to four times up and down with a 1-mL pipet.
3. Centrifuge again and resuspended in 55 µL150 m*M* KCl in PS buffer by shaking on a shaker (1400 rpm, 8 min, 4°C).
4. Add Proteinase K (0.3 mg/mL from 18x stock) and incubate on ice for 23 min. Stop digestion by adding 55 µL 1 m*M* PMSF/150 m*M* KCl in PS buffer.
5. Determine luciferase activity using an assay kit according to the manufacturer's instruction (Berthold Detection Systems, Pforzheim, Germany): mix 25 µL sample with 25 µL lysis buffer and add 50 µL substrate mix ***(30)*** directly before counting light emission in a microplate luminometer (LB 96 V, Berthold Technologies, Bad Wildbad, Germany) (*see* **Note 1**).
6. If you run uptake reactions in the presence of pharmaceuticals or antibodies, we recommend determining alkaline phosphatase activity in a 25-µL aliquot as described previously ***(4)***. This serves as an internal reference for the quantity of pelleted vacuoles. Calculate uptake activity as the quotient of luciferase activity over alkaline phosphatase activity (counts per second/ OD_{405} per min) and normalize to a standard reaction run without any pharmaceuticals or antibodies (60 min, 27°C), which is set to 100%. When comparing different yeast strains, check the level of mature alkaline phosphatase in vacuolar preparations (e.g., by Western blotting or alkaline phosphatase assay). Protein levels can differ in

mutants in comparison to wild-type strains. In these cases uptake activity is not referred to alkaline phosphatase activity.

3.7.2. Kinetic Analysis

Using a pharmacological approach including low molecular weight inhibitors, the in vitro uptake reaction can be dissected into different kinetic stages ***(5)***. According to their ability to block the reaction at different kinetic stages, these inhibitors have been defined as early-acting class A inhibitors (nystatin, GTPγS, aristolochic acid) and late-acting class B inhibitors (W-7, valinomycin/FCCP, K252a, and rapamycin). For this kind of analysis, run standard uptake reactions and add inhibitors at different time points. Then, continue the incubation until the end of a standard 60-min reaction period. Inhibitors influencing very early steps of microautophagy (class A inhibitors) lose their activity if added late during the reaction, whereas inhibitors acting on late events of microautophagy (class B inhibitors) remain active throughout the reaction. As a control, transfer samples to ice, which also stops the reaction **(Fig. 4)**.

3.7.3. Rapid Uptake

Late-acting inhibitors in microautophagy can be further classified depending on their ability to inhibit rapid uptake ***(5)***. Rapid uptake of luciferase occurs after preincubation of vacuoles under standard conditions supporting microautophagic membrane invagination, but in the absence of the reporter enzyme. It is assumed that this allows the vacuoles to complete all preparatory reactions for uptake, e.g., to form an invagination, without producing a luciferase signal. The formation of vesicles can then be scored by adding luciferase for a short period of time. This allows only rapid uptake from preformed invaginations but is too short for the formation of new invaginations. Thus, this criterion can be used to distinguish the preparation for uptake (tube formation) from its completion (vesicle scission).

1. After 60 min of incubation without luciferase, add the reporter enzyme to an uptake reaction. Incubate the samples for another 5 min to permit reporter enzyme uptake.
2. Terminate uptake by chilling, diluting, and centrifuging the reactions and analyze pelleted vacuoles for luciferase uptake as described above.

4. Notes

1. Buffers. Do not add leupeptin to the lysis buffer for cytosol preparation because this inhibits the firefly luciferase reporter enzyme.

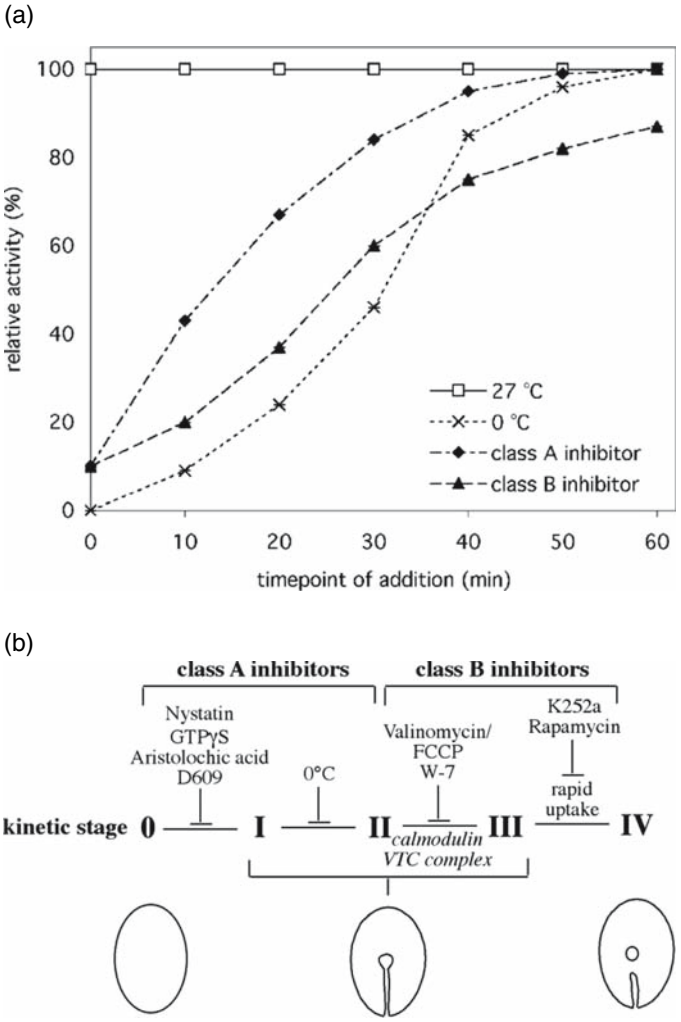

Fig. 4. Kinetic analysis of in vitro microautophagy. **(A)** Schematic example of a time course experiment with inhibitors. In vitro microautophagic reactions are started. At the indicated time points, the samples receive inhibitors, or control buffer, and are incubated further at 27°C until the end of the 60-min reaction period. For the ice curve, an aliquot is set on ice at the indicated time points and kept there until the end of the reaction period. Reactions performed without inhibitor at 27°C are set to 100% **(B)** Schematic overview of the kinetic steps. The in vitro assay can be inhibited at different stages by addition of inhibitors or by transfer to 0°C. GTPγS: guanosine 5′-O-(3-thiotriphosphate); FCCP: *p*-trifluoromethoxy carbonyl cyanide phenyl hydrazone; VTC: vacuolar transporter chaperone. (Adapted from **refs. *5*** and ***6***.).

2. Yeast cell culture. For temperature-sensitive yeast strains, all steps are carried out at 25°C as long as no temperature shock is desired. We usually carry out temperature shock at 37°C for the desired time. Note that heating of large volumes (e.g., 1 L) from 25 to 37°C will take a considerable time.
3. Vacuole preparation. The optimal OD_{600} for harvesting cells, the time for spheroplasting and the amount of DEAE-dextran added can vary for different yeast strains. If necessary, vacuoles can be prepared with up to 1 m*M* PMSF in the spheroplasting buffer to reduce protein degradation.
4. Fluorescence microscopy. To avoid fast movement of cells when investigated with a fluorescence microscope, cells can be immobilized the following way: 7 µL of the cell suspension is mixed with 7µL of 0.4% low-melting-point agarose in PS buffer (kept liquid at 35°C). Twelve µL of this mixture are transferred to a slide, covered immediately with a cover slip, and chilled at 4°C for 5 min to immobilize the cells. The intensity of illumination has to be minimized to avoid structural damage to the vacuoles that can occur at higher light intensities or after prolonged illumination of the same field.
5. Reconstituted microautophagic assay in vitro. Prepare all samples on ice. Thaw cytosol/vacuole nuggets directly before use and do not freeze again. Direct comparisons of activity should be performed only with vacuoles from the same batch of preparation because there can be variations in absolute activities from batch to batch. These are often related to changes the composition of media ingredients supplied. Our experience is that the quality of, e.g., peptones or yeast extracts varies considerably over the year, even when purchased from the same supplier. Addition of antibodies/pharmaceuticals to the in vitro reaction can induce formation of large clusters of vacuoles, which may be hard to resuspend. In theses cases you should prolong the resuspension steps carefully without destroying the vacuoles. Alternatively, prepare, e.g., F_{ab} fragments from antibodies.

Acknowledgments

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References

1. Reggiori, F., and Klionsky, D. J. (2002) *Eukaryot. Cell* **1,** 11–21.
2. Baba, M., Takeshige, K., Baba, N., and Ohsumi, Y. (1994) *J. Cell Biol.* **124,** 903–913.
3. Muller, O., Sattler, T., Flotenmeyer, M., Schwarz, H., Plattner, H., and Mayer, A. (2000) *J. Cell Biol.* **151,** 519–528.
4. Sattler, T., and Mayer, A. (2000) *J. Cell Biol.* **151,** 529–538.
5. Kunz, J. B., Schwarz, H., and Mayer, A. (2004) *J. Biol. Chem.* **279,** 9987–9996.

6. Uttenweiler, A., Schwarz, H., and Mayer, A. (2005) *J. Biol. Chem.* **280,** 33289–33297.
7. Uttenweiler, A., Schwarz, H., Neumann, H., and Mayer, A. (2007) *Mol. Biol. Cell* **18,** 166–175.
8. Gruenberg, J., and Stenmark, H. (2004) *Nat. Rev. Mol. Cell Biol*. **5,** 317–323.
9. Babst, M. (2005) *Traffic* **6,** 2–9.
10. Demirov, D. G., and Freed, E. O. (2004) *Virus Res*. **106,** 87–102.
11. Roberts, P., Moshitch-Moshkovitz, S., Kvam, E., O'Toole, E., Winey, M., and Goldfarb, D. S. (2003) *Mol. Biol. Cell* **14,** 129–141.
12. Veenhuis, M., Douma, A., Harder, W., and Osumi, M. (1983) *Arch. Microbiol*. **134,** 193–203.
13. Tuttle, D. L., Lewin, A. S., and Dunn, W. A., Jr. (1993) *Eur. J. Cell Biol*. **60,** 283–290.
14. Tuttle, D. L., and Dunn, W. A., Jr. (1995) *J, Cell Sci*, **108 (Pt 1),** 25–35.
15. Sakai, Y., Koller, A., Rangell, L. K., Keller, G. A., and Subramani, S. (1998) *J, Cell Biol*, **141,** 625–636.
16. Mukaiyama, H., Baba, M., Osumi, M., et al. (2004) *Mol. Biol. Cell* **15,** 58–70.
17. Mukaiyama, H., Oku, M., Baba, M., et al. (2002) *Genes Cells* **7,** 75–90.
18. Hutchins, M. U., Veenhuis, M., and Klionsky, D. J. (1999) *J. Cell Sci*. **112 (Pt 22),** 4079–4087.
19. Kim, J., Dalton, V. M., Eggerton, K. P., Scott, S. V., and Klionsky, D. J. (1999) *Mol. Biol. Cell* **10,** 1337–1351.
20. Yuan, W., Stromhaug, P. E., and Dunn, W. A., Jr. (1999) *Mol. Biol. Cell* **10,** 1353–1366.
21. Stromhaug, P. E., Bevan, A., and Dunn, W. A., Jr. (2001) *J. Biol. Chem* **276,** 42422–42435.
22. Dubouloz, F., Deloche, O., Wanke, V., Cameroni, E., and De Virgilio, C. (2005) *Mol. Cell* **19,** 15–26.
23. Scott, J. H., and Schekman, R. (1980) *J. Bacteriol*. **142,** 414–423.
24. Shen, S. H., Chretien, P., Bastien, L., and Slilaty, S. N. (1991) *J. Biol. Chem*. **266,** 1058–1063.
25. Reese, C., Heise, F., and Mayer, A. (2005) *Nature* **436,** 410–414.
26. Hohenberg, H., Mannweiler, K., and Muller, M. (1994) *J. Microsc*. **175 (Pt 1),** 34–43.
27. Gulik-Krzywicki, T., and Costello, M. J. (1978) *J. Microsc*.**112,** 103–113.
28. Tommassen, J., Leunissen, J., van Damme-Jongsten, M., and Overduin, P. (1985) *EMBO J*. **4,** 1041–1047.
29. van Bergen en Henegouwen, P. M., and Leunissen, J. L. (1986) *Histochemistry* **85,** 81–87.
30. Gaunitz, F., and Papke, M. (1998) *Methods Mol. Biol*. **107,** 361–370.