

# Autophagosome Formation and Molecular Mechanism of Autophagy

Isei Tanida

## Abstract

Autophagy (macroautophagy), or the degradation of large numbers of cytoplasmic components, is induced by extracellular and intracellular signals, including oxidative stress, ceramide, and endoplasmic reticulum stress. This dynamic process involves membrane formation and fusion, including autophagosome formation, autophagosome-lysosome fusion, and the degradation of intra-autophagosomal contents by lysosomal hydrolases. Autophagy is associated with tumorigenesis, neurodegenerative diseases, cardiomyopathy, Crohn's disease, fatty liver, type II diabetes, defense against intracellular pathogens, antigen presentation, and longevity. Among the proteins and multimolecular complexes that contribute to autophagosome formation are the PI(3)-binding proteins, the PI3-phosphatases, the Rab proteins, the Atg1/ULK1 protein-kinase complex, the Atg9•Atg2-Atg18 complex, the Vps34-Atg6/beclin1 class III PI3-kinase complex, and the Atg12 and Atg8/LC3 conjugation systems. Two ubiquitin-like modifications, the Atg12 and LC3 conjugations, are essential for membrane elongation and autophagosome formation. Recent findings have revealed that processes of selective autophagy, including pexophagy, mitophagy, ERphagy (reticulophagy), and the p62-dependent degradation of ubiquitin-positive aggregates, are physiologically important in various disease states, whereas "classical" autophagy is considered nonselective degradation. Processes of selective autophagy require specific Atg proteins in addition to the "core" Atg complexes. Finally, methods to monitor autophagic activity in mammalian cells are described. *Antioxid. Redox Signal.* 14, 2201–2214.

## Introduction

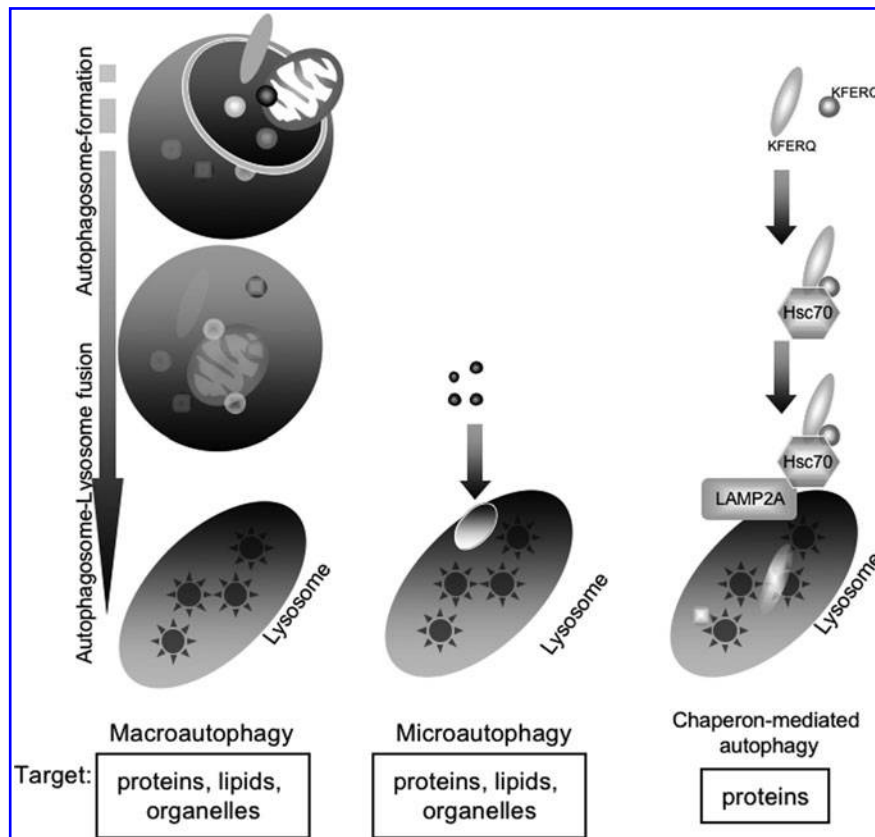
THE AUTOPHAGY-LYSOSOME SYSTEM and the ubiquitin-proteasome system are the two major protein degradation systems in eukaryotes, including yeasts, plants, and animals. Autophagy (macroautophagy) was first described as a response to starvation by the liver, with the term "autophagy" derived from the Latin words for "self" and "eating." Later biochemical and morphological studies of autophagy indicated its physiological importance, although its molecular mechanism was unclear because of the absence of molecular markers of autophagosomes and/or autophagy (100). To date, at least three types of autophagic pathways have been described, which differ in their routes to lysosomes: macroautophagy (also called simply "autophagy") (3), microautophagy (1), and chaperone-mediated autophagy (Fig. 1) (7). Macroautophagy is a degradation system that accompanies the dynamic process of autophagosome formation (75, 110), whereas microautophagy involves the sequestering by the lysosome itself of part of the cytoplasm. Both macroautophagy and microautophagy are basically the nonselective degradation of protein, lipids, and organelles. In contrast, chaperone-mediated autophagy involves the selective degradation of proteins with a specific-signal se-

quence called the "KFERQ" motif and is dependent on the molecular chaperone Hsc70. Moreover, chaperone-mediated autophagy does not involve the degradation of lipids or organelles. Lysosome-associated protein-2A functions as a receptor during chaperone-mediated autophagy.

About 30 years after the first description of autophagy, autophagic bodies, as intermediate compartments with intra-autophagosomal membranes, were first identified in yeast vacuoles under starvation conditions (100). Taking advantage of yeast genetics, autophagy-defective (*atg/apg/aut*) mutants of *Saccharomyces cerevisiae* were isolated as yeasts showing little or no accumulation of autophagic bodies under starvation conditions (75, 113). The *ATG* genes were later isolated and characterized (Table 1) (75, 113). Most *ATG* genes contribute to autophagosome formation, with many being well conserved from yeast to mammals. In this review, the word "autophagy" denotes macroautophagy unless otherwise indicated.

## Autophagy and Diseases

Autophagy is a process during which cytoplasmic components are degraded in bulk and includes the degradation of proteins, lipids, sugars, and organelles (mitochondria,



**FIG. 1. Macroautophagy, microautophagy, and chaperone-mediated autophagy.** Macroautophagy is classical "autophagy." During macroautophagy, dynamic membrane formation and fusion occur, including autophagosome formation and fusion. Most autophagy-related genes (Atg) contribute to autophagosome formation. Microautophagy is another type of autophagy, during which the lysosomal membrane is invaginated into the lysosome to engulf cytoplasmic components directly. Chaperone-mediated autophagy is specific for KFERQ motif-containing proteins. The latter proteins are recognized by Hsc70 and translocated into lysosome by LAMP2A, a lysosomal membrane protein. Macroautophagy and microautophagy degrade proteins, lipids, and organelles, whereas chaperone-mediated autophagy degrades proteins only. LAMP2A, lysosome-associated protein-2A.

endoplasmic reticulum [ER], and peroxisomes) (12, 59). Autophagy can be induced by extracellular and intracellular signals, including oxidative stress, growth factors, ceramide, ER stress, and glucose, amino acid, and serum starvation (Fig. 2) (12, 59). Autophagy contributes to cellular survival against nutrient deprivation and the turnover of damaged organelles and is related to tumorigenesis, neurodegenerative diseases (including Parkinson's, Alzheimer's, Huntington's, and Creutzfeldt-Jakob diseases), cardiomyopathy (e.g., Danon disease), Crohn's disease, fatty liver, type II diabetes, defenses against intracellular pathogens, antigen presentation, and longevity (12, 49, 63, 73).

#### Autophagic Flow in Mammalian Cells: From the Formation of Isolation Membranes to Autolysosomes

Following the induction of autophagy, the omegasome is formed from the ER, resulting in the formation of a cup-shaped structure, the isolation membrane, also called the preautophagosome or phagophore (Fig. 3, Initiation) (62, 67). The isolation membrane subsequently elongates to engulf cytoplasmic components, including organelles (Fig. 3, Elongation), and finally closes to form the autophagosome (initial autophagic vacuole [AVi]), a structure with a double membrane (Fig. 3, Maturation) (67, 95). The outer membrane of the autophagosome fuses with a lysosome to form an autolysosome (degradative autophagic vacuole [AVd]) (Fig. 3, Autophagosome-Lysosome fusion). Finally, lysosomal hydrolytic enzymes, including cathepsins (proteases) and lipases, degrade the intra-autophagosomal components and the inner membrane of the autophagosome (Fig. 3, Degrada-

tion) (10). In rat hepatocytes under starvation conditions, the entire process of autophagy occurs within 10–15 min.

#### "Core" Atg Complexes for Nucleation of Autophagosomes

The "core" Atg proteins are divided into five subgroups: the Atg1/ULK1 protein-kinase complex, the Atg9•Atg2-Atg18 complex, the Vps34-Atg6/beclin1 class III phosphoinositide 3-kinase (PI3-kinase) complex, the Atg12 conjugation system, and the Atg8/LC3 conjugation (lipidation) system (Table 1). In mammals, the Atg1/ULK1 protein-kinase complex is composed of ULK1 (a protein kinase), Atg13, FIP200, and Atg101 (19, 22, 23, 29, 57, 60). ULK1/Atg1 and Atg13 are downstream of the mTorC1 complex. FIP200 is important for the stability and phosphorylation of ULK1. Atg101 is a protein conserved in various eukaryotes (from *Schizosaccharomyces pombe* to mammals), but not in *S. cerevisiae*. Atg101 is important for the stability and basal phosphorylation of Atg13 and ULK1 (23, 57). Atg13 is responsible for Atg14 recruitment to the preautophagosomal structure in yeasts (80), and Atg14 is a subunit of the autophagy-specific Vps34 class III PI3-kinase complex in yeasts and mammals (25, 33, 37, 80), suggesting that the ULK1-Atg13-FIP200-Atg101 complex interacts with the Atg14-Vps34 class III PI3-kinase complex. Atg29 and Atg31 are Atg1-interacting proteins in yeasts, but have no mammalian homologs (30, 36).

The Atg9•Atg2-Atg18 complex is composed of Atg9/mAtg9, Atg2, and Atg18/WIPI-1. Atg9 is the only integral Atg membrane protein in yeasts (77, 97); its mammalian homologs are mAtg9/Atg9L1, which is expressed ubiquitously, and

Atg9L2, which is specific to the placenta and pituitary gland (120). Under nutrient-rich conditions, mAtg9 localizes to the trans-Golgi network and partial endosomes, whereas, under starvation conditions, mAtg9 localizes to autophagosomes in a process dependent on ULK1 (120). During autophagy, mAtg9 and the phosphatidylinositol 3-phosphate [PI(3)P]-binding protein WIPI-1/Atg18 localize to LC3-positive puncta, making LC3-II/Atg8-PE a promising autophagosomal marker (79, 84). Atg18 constitutively interacts with Atg2 in yeasts, and Atg9 interacts with the Atg2-Atg18 complex under starvation conditions (90). Atg27 is required for autophagy-dependent cycling of Atg9 in yeasts (118). To date, mammalian homologs of Atg2 and Atg27 have not been identified. According to the yeast Atg9 model, mAtg9 would interact with the mammalian Atg18-Atg2 complex during autophagy.

The subunit composition of the Vps34-Atg6/beclin1 class III PI3-kinase complex has been shown to change depending on its function (33, 37, 55, 97, 121). The Vps34-Vps15-beclin1/Atg6 complex is a core complex of class III PI3-kinase (92). In yeasts, the Atg14-Vps34-Vps15-Atg6 complex plays an indispensable role in autophagy, whereas the Vps38-Vps34-Vps15-Atg6 complex is essential for vacuolar protein sorting (37). In mammals, at least three types of class III PI3-kinase complexes contribute to autophagy (25, 50, 51, 55, 99, 121). The Atg14-Vps34-Vps15-beclin1 complex is essential for autophagosome formation, and the UVRAG/Vps38-Vps34-Vps15-beclin1 complex functions positively in autophagosome maturation and endocytic traffic (25, 51). In contrast, the Rubicon-UVRAG-Vps38-Vps34-Vps15-beclin1 complex negatively regulates autophagosome maturation and endocytic traffic (55). Ambra1, a protein containing a WD40 domain that activates beclin1-regulated autophagy, regulates autophagy, and has a crucial role in embryogenesis (14). In sensory neurons, Vps34-independent autophagosome formation and autophagy have been identified as a non-canonical autophagy pathway, requiring Atg7 (122) and suggesting the possibility of a mammalian-specific alternative autophagy pathway independent of Vps34.

### Two Ubiquitin-Like Conjugation Systems Are Essential for Autophagosome Formation

Two ubiquitin-like conjugation systems, the Atg12 and Atg8/LC3 (lipidation) conjugation systems, contribute directly to the elongation of isolation membranes and the maturation of autophagosomes (Fig. 4) (11, 81, 110). In the Atg12 conjugation system, Atg12 is activated by Atg7, an E1-like enzyme; transferred to Atg10, an E2-like enzyme, and conjugated to Atg5 to form Atg12-Atg5 conjugates (38, 65, 66, 89, 103). At present, no E3-like enzyme for Atg12 conjugation has been identified. Although the amino acid structures of Atg12 and ubiquitin are dissimilar, Atg12 possesses a ubiquitin-fold (65). As in ubiquitin, the carboxyl terminal Gly of Atg12 is essential for the formation of thio-ester bonds with the active site Cys residues of Atg7 and Atg10 and is also essential for the formation of amide bonds with the Lys residues in Atg5 (65, 70, 76, 89, 103, 107). Atg16 interacts with Atg5, forming a multimeric complex (47, 62, 64).

The second ubiquitin-like conjugation system, the Atg8/LC3 conjugation system, is unique, in that its target is a phospholipid, phosphatidylethanolamine (PE) (24, 31). Therefore,

the Atg8/LC3 conjugation system has been called Atg8/LC3 "lipidation." In yeasts, Atg8 is synthesized as proAtg8, which is cleaved by Atg4, a cysteine protease, to expose Gly at its carboxyl terminus. Atg8 is activated by the same E1-like enzyme, Atg7, transferred to Atg3, a second E2-like enzyme, and finally conjugated to PE to form Atg8-PE (106, 107). Atg8-PE localizes to autophagosomes, whereas Atg8 is present in the cytosol. Atg8-PE on the cytosolic surface of autophagosomes is cleaved to Atg8 by Atg4 to recycle Atg8, a recycling that has been shown essential for effective autophagy (24). Atg8 mediates membrane tethering and hemifusion (74). To date, at least four mammalian Atg8 homologs have been identified: microtubule-associated protein 1 light chain 3 (LC3/MAP1-LC3/LC3B), GABA<sub>A</sub> receptor-associated protein (GABARAP), Golgi-associated ATPase enhancer of 16 kDa (GATE-16), and mAtg8L. LC3 is the best characterized of these proteins and is regarded as a functional homolog of yeast Atg8 in autophagy (31). LC3 is synthesized as proLC3, which is cleaved by Atg4B to form LC3-I, with the carboxyl terminal Gly exposed (31). LC3-I is activated by Atg7, transferred to Atg3, and finally conjugated to PE (106, 107). The LC3-PE conjugate has been designated LC3-II (31). The carboxyl terminal Gly of LC3 is also essential for the formation of a thio-ester bond with the active site Cys residues of Atg7 and Atg3, as well as being essential for the formation of an amide bond with PE (94, 108). As LC3-I is localized in the cytosol and LC3-II is localized to autophagosomes (31), LC3-II (LC3-PE conjugate) is a promising autophagosomal marker in mammals. LC3-II on the cytoplasmic surface of autophagosomes is delipidated by Atg4B to recycle LC3-I for further autophagosome formation.

The other Atg8 homologs are also modified by Atg4B (Atg4A for GATE-16), Atg7, and Atg3 (32, 94, 101, 104). The carboxyl termini of the proforms of GABARAP, GATE-16, and mAtg8L are cleaved by Atg4B (or Atg4A for proGATE-16) to expose Gly, and the resulting GABARAP-I, GATE-16-I, and mAtg8L-I are modified to GABARAP-II, GATE-16-II, and mAtg8L-II, respectively (101, 104, 105). Membrane-localized GABARAP-II on the cytoplasmic surface is also delipidated by Atg4B. Little endogenous GATE-16-II (*i.e.*, the lipidated form) has been observed in rat and mouse tissues and cell lines (110). The physiological function of GATE-16-II is not yet known, whereas GATE-16 is an essential component of intra-Golgi transport and postmitotic Golgi reassembly independent of its lipidation (86). Although mAtg8L-II was not observed during starvation-induced autophagy, it was identified during the differentiation of C2C12 cells to myocytes (105).

Atg12 conjugation is closely interrelated to Atg8/LC3 lipidation. Atg5 deficiency results in a defect of Atg8/LC3 lipidation (67, 71). The yeast Atg12-Atg5 conjugate functions *in vitro* as an E3-like enzyme for Atg8 lipidation (18). Mammalian Atg16L determines the site of LC3-lipidation, that is, autophagosome formation (15). Therefore, the Atg16/Atg12-Atg5 complex may function as an E3-ligase complex to facilitate LC3-lipidation complex and may also act to determine the site of autophagy. Lack of Atg3 in mammals leads to a decrease in the Atg12-Atg5 conjugate as well as impairing LC3 lipidation (95). Further, loss of Atg3 has been associated with a defective process of autophagosome formation, including defects in the elongation and complete closure of the isolation membranes, resulting in malformed autophagosomes.

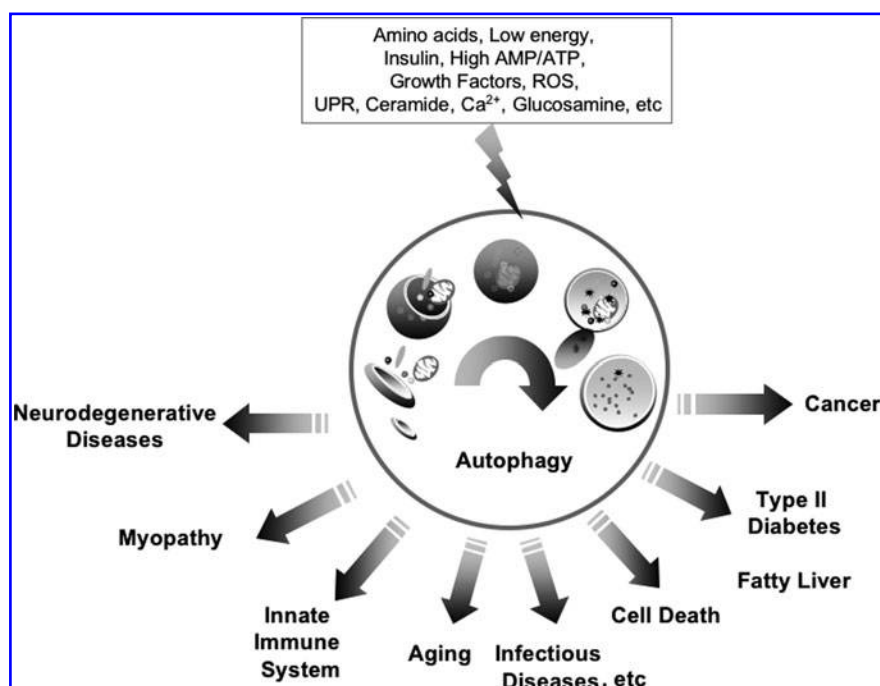
TABLE 1. AUTOPHAGY-RELATED GENES

Yeasts	Mammals	Comments	References
Atg1/ULK1 protein kinase complex ATG1/APG1/AUT3/CVT10 ATG13/APG13 ATG17	ULK1 ATG13 FIP200 ATG101	Protein kinase; target of mTor kinase Phosphorylated protein; target of mTor kinase Essential for both stability and phosphorylation of ULK1 Important for stability and basal phosphorylation of Atg13 and ULK1; conserved from <i>S. pombe</i> to mammals	(60) (22) (19) (23, 57)
ATG29 ATG31	? ?	Yeast specific? Yeast specific?	(36) (30)
Atg9•Atg2-Atg18 complex ATG9/APG9/AUT9/CVT7 ATG2/ATG2 ATG18/AUT10/CVT18 ATG27	ATG9A, ATG9B ? WIPI-1, 2, 3, 4 ?	Membrane protein Atg18-interacting protein PI(3)P-binding protein Protein required for autophagy-dependent cycling of Atg9	(77, 120) (90) (85, 90) (118)
Vps34-Atg6/Beclin1 class III PI3-kinase complex ATG6/VPS30/APG6 ATG14 VPS38	BECN1 ATG14 UVRAG Rubicon AMBRA1 PIK3C3/VPS34 PIK3R4/VPS15	Beclin1; one of the core subunits; bcl2-interacting protein Enhancer of autophagosome formation Enhancer of autophagosome-lysosome fusion and endocytic traffic Negative regulator of autophagosome-lysosome fusion and endocytic traffic WD40 domain containing a positive regulator of autophagy PI3-kinase that interacts with Rab5 and Rab7 Core activator of the Vps34 PI3-kinase complex	(33, 52) (25, 33) (50) (55, 121) (14) (37) (37)
VPS34 VPS15			
Atg12 conjugation ATG12/APG12 ATG5/APG5 ATG16 ATG7/APG7 ATG10/APG10	ATG12 ATG5 ATG16L1 ATG7 ATG10	Modifier conjugates with Atg5 Target of Atg12 localizing to isolated membranes Atg16L determines the site of LC3 conjugation E1-like enzyme for Atg12 and Atg8/LC3 conjugation E2-like enzyme for Atg12 conjugation	(65, 66) (65, 66) (62, 64) (65, 103, 107) (76, 89)

(continued)

TABLE 1. (CONTINUED)

<i>Yeasts</i>	<i>Mammals</i>	<i>Comments</i>	<i>References</i>
Atg8/LC3 conjugation ATG8/APG8/AUT7	MPA1/LC3B/LC3B	Modifier conjugating with PE and localizing to autophagosomes; LC3-A and LC3-C are isoforms	(31)
	GABARAP	Modifier; GABA <sub>A</sub> receptor-associating protein	(115)
	GATE-16	Modifier; Golgi-associated ATPase enhancer of 16 kDa	(86)
	GABARAPL1	Modifier; myotube differentiation specific?	(105)
ATG7/APG7	ATG7/APG7	E1-like enzyme for Atg12 and Atg8/LC3 conjugation	(103, 107)
ATG3/APG3	ATG3/APG3	E2-like enzyme for Atg12 and Atg8/LC3 conjugation	(24, 106)
ATG4/APG4/AUT2	ATG4A-D (autophagins 1–4)	Cytosolic cysteine protease for processing and recycling of Atg8/LC3	(24, 104)
PI(3)P-related proteins	WDFY3/ALFY	FYVE domain-containing protein associated with protein granules and autophagic membranes	(91)
	ZFYVE1/DFCP1	Double FYVE domain-containing protein 1; omegasome localization for autophagosome initiation	(4)
	FYCO1	Rab7 effector binding to LC3 and PI(3)P and mediating microtubule plus end-directed vesicle transport	(82)
	MTMR14	Jumpy; PI(3)P phosphatase; negative regulator of autophagosome initiation	(114)
	MTMR3	PI(3)P phosphatase; negative regulator of autophagosome initiation	(98)
Essential proteins for degradation of autophagic body ATG15/AUT5/CVT17	?	Lipase essential for degradation of autophagic bodies	(9, 112)
ATG22	?	Vacuolar protein important for degradation of autophagic bodies	(117)
Selective autophagy	SQSTM1	p62, LC3-, and ubiquitin-binding protein essential for selective autophagy of cytosolic aggregated proteins	(5, 45, 88)
	?	Mitophagy	(41)
UTH1	?	Mitophagy	(34)
ATG32	PARK2	Parkin; cytosolic ubiquitin E3-ligase essential for mitophagy	(16, 54)
	PINK1/PARK6	PTEN-induced putative kinase 1 essential for mitophagy	(16, 54)
ATG11	?	Cvt pathway specific adaptor protein for Cvt vesicle formation	(88)
ATG19	?	Cvt pathway-specific receptor of aminopeptidase I	(88)
ATG20	?	Cvt pathway-specific nexin family protein	(119)
ATG21	?	Cvt pathway-specific PI(3)P-binding protein	(48, 56, 96)
ATG24	?	Pexophagy	(2)
ATG25	?	Pexophagy	(72)
ATG26	?	Pexophagy	(116)
PEX14	PEX14	Pexophagy	(20)
Cvt, cytoplasm-to-vacuole targeting; PE, phosphatidylethanolamine; PI3-kinase, phosphoinositide 3-kinase; PI(3)P, phosphatidylinositol 3-phosphate.			



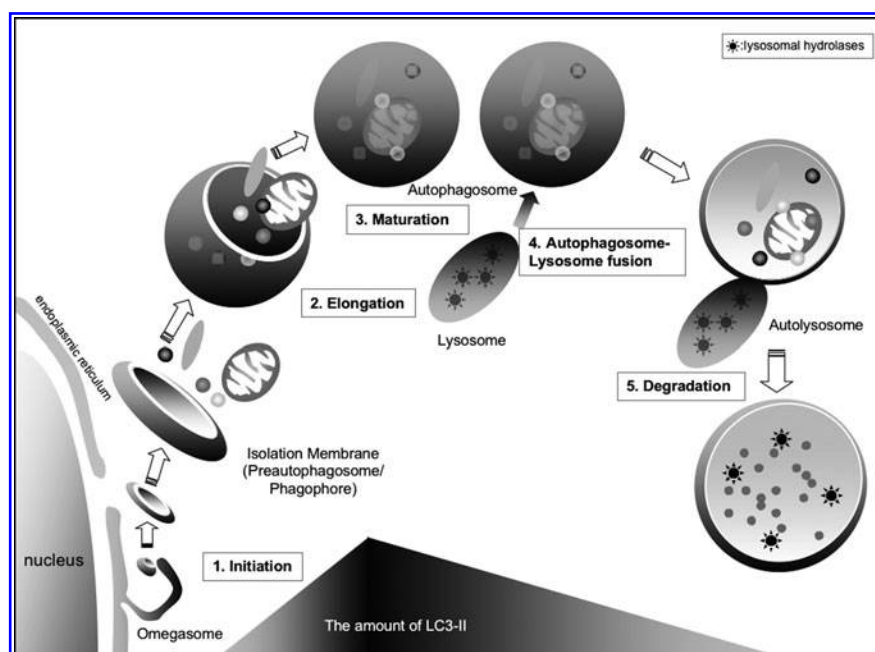
**FIG. 2. Physiological signals and diseases related to autophagy.** Autophagy is induced by many physiological signals and is related to many diseases and processes, including neurodegenerative diseases (e.g., Alzheimer's, Parkinson's, Huntington's, and Creutzfeldt-Jakob disease), cardiomyopathy (e.g., Danon disease), tumorigenesis, antigen presentation, infectious diseases, cell death, type II diabetes, and fatty liver.

In cells lacking Atg3, Atg16L and Atg5 are localized to elongated isolation membranes/incomplete autophagosomes, suggesting that elongation of the isolation membrane can occur in the absence of LC3 lipidation (95). Thus, in addition to its E3-like function, the Atg16/Atg12-Atg5 complex may function in the elongation of isolation membranes.

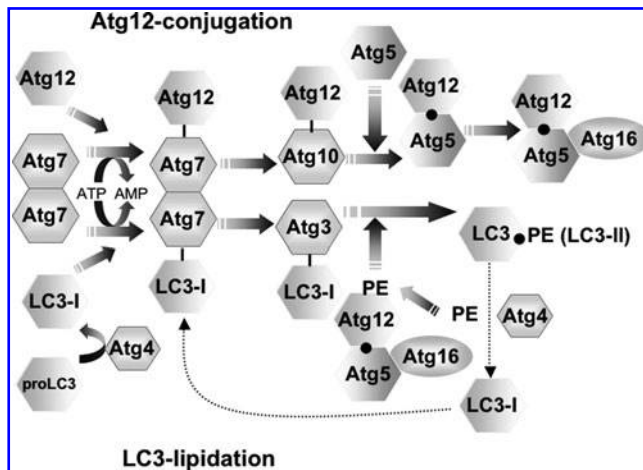
#### Initial Step of Autophagy: Omegasomes and the Initiation of Isolation Membranes

Autophagy accompanies dynamic membrane formation and membrane fusion (Fig. 3). During the first step of au-

tophagy, the ULK1-Atg13-FIP200-Atg101 kinase complex activates autophagic signaling *via* the mTor-signaling pathway. The omegasome, which is shaped like the Greek letter omega, is associated with the ER (Fig. 5) (4). A PI(3)P-binding protein, double FYVE domain-containing protein 1 (DFCP1), is localized to PI(3)P on the omegasome under starvation conditions, but localizes to the ER and Golgi under nutrient-rich conditions. The Atg14-Vps34-beclin1 PI3-kinase complex positively regulates the DFCP1-positive omegasome formation. Thereafter, the isolation membrane is formed inside the ring of the omegasome, and the Atg12-Atg5/Atg16 complex is localized to the isolation membrane (47, 62, 67). The protein



**FIG. 3. Schematic model of autophagy.** Following the induction of autophagy, an omegasome is formed from the endoplasmic reticulum (Initiation), followed by the formation of an isolation membrane, which elongates to engulf cytoplasmic components (Elongation). Finally, the isolation membrane is enclosed to form an autophagosome (Maturation). During these steps, the amount of LC3-II increases gradually. After autophagosome formation, the lysosome fuses with the autophagosome (Autophagosome-Lysosome fusion) to form an autolysosome. Intra-autophagosomal contents are degraded by lysosomal hydrolases (Degradation). All of these events occur within 10–15 min.

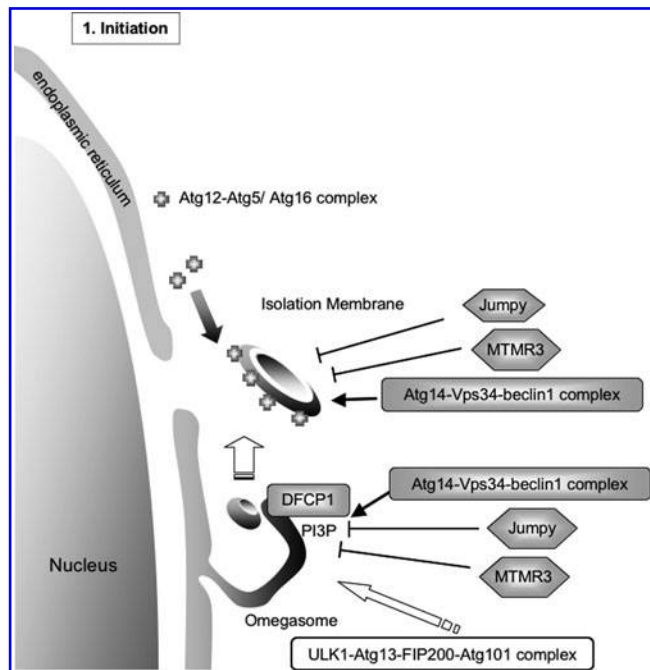


**FIG. 4. Atg proteins involved in Atg12 conjugation and LC3 lipidation during mammalian autophagy.** Atg12 is activated by Atg7 (an E1-like enzyme), transferred to Atg10 (an E2-like enzyme), and finally conjugated to Atg5. The carboxyterminal Gly of Atg12 is essential for these reactions. The Atg12-Atg5 conjugate forms a complex with Atg16L. Atg16L determines the site of the autophagosome. LC3 is synthesized as proLC3 in the cytosol. Soon after its translation, proLC3 is cleaved by Atg4B to expose the carboxy-terminal Gly of LC3 (LC3-I). LC3-I is activated by the same E1-like enzyme, Atg7, transferred to Atg3 (a second E2-like enzyme), and conjugated to PE. The LC3-PE conjugate is LC3-II, a membrane bound form of LC3. The Atg12-Atg5 conjugate functions as an E3-like ligase for LC3 lipidation. PE, phosphatidylethanolamine.

mAtg9, the ULK1-Atg13-FIP200-Atg101 kinase complex, and the Atg14-Vps34-beclin1 PI3-kinase complex also localize to the isolation membrane. DFCP1 itself, however, is likely not required for autophagosome formation. Two PI(3)P phosphatases (Jumpy/MTMR14 and MTMR3) negatively regulate the formation of omegasomes and isolation membranes by decreasing the level of PI(3)P (98, 114).

#### Elongation of the Isolation Membrane and Formation of Autophagosomes

Following the formation of the isolation membrane, it elongates to engulf cytoplasmic components (Fig. 6). The Atg12-Atg5/Atg16 complex is localized to the cup-shaped structure. In the later stages of isolation membrane elongation, the Atg12-Atg5/Atg16 complex progressively dissociates from the isolation membrane, whereas LC3-II gradually localizes to both sides of the isolation membrane (see Fig. 6, Cross-section 1) (67). During this process, LC3-I, a cytosolic form of LC3, is progressively lipidated by Atg7, Atg3, and the Atg12-Atg5/Atg16 complex to form LC3-II. Finally, the isolation membrane closes to form the autophagosome (Fig. 6, Maturation). Although LC3-II is localized to autophagosomes, most of the Atg12-Atg5/Atg16 complex dissociates from the autophagosome (67). The autophagosome is a double membrane structure, with LC3-II present on both the cytosolic and the intra-autophagosomal surfaces (Fig. 6, Cross-section 2). Therefore, LC3-II increases during autophagosome formation. Rab32 and Rab33 also contribute to the elongation of the isolation membrane (21, 26). Following



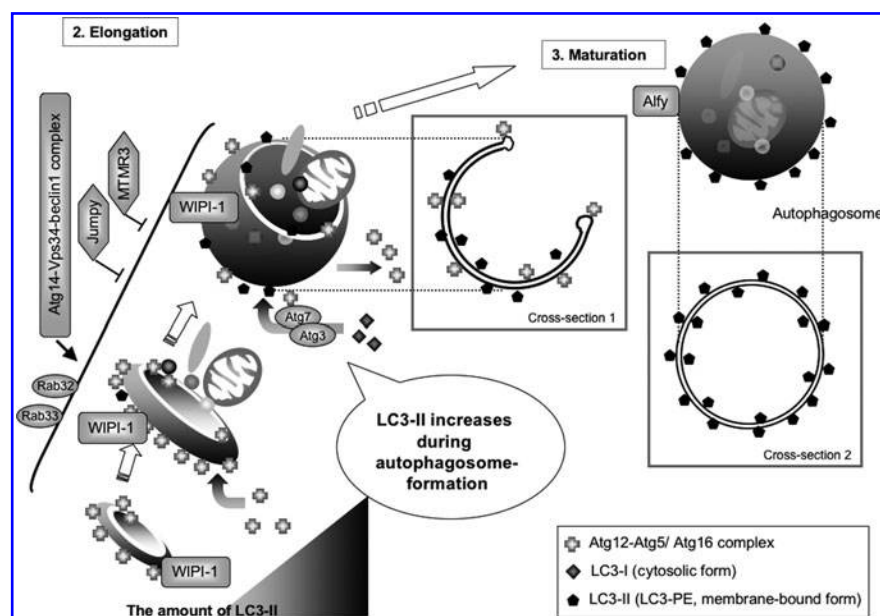
**FIG. 5. The initial step in the formation of the isolation membrane.** Following the induction of autophagy, the ULK1-Atg13-FIP200-Atg101 complex is activated *via* the mTor kinase signaling pathway. The omegasome is formed by association with DFCP1, a step involving the Atg14-Vps34-beclin1 PI3-kinase complex. Two phosphatases, Jumpy and MTMR3, are negative regulators of this process. Following omegasome formation, the isolation membrane is formed by association with the Atg5-Atg12/Atg16L complex. DFCP1, double FYVE domain-containing protein 1; PI3-kinase, phosphoinositide 3-kinase.

autolysosome formation, Atg4B delipidates LC3-II on the cytoplasmic surface to recycle LC3-I (Fig. 7).

#### Autophagosome-Lysosome Fusion and Degradation

Soon after autophagosome formation, its outer membrane fuses with lysosomes to form autolysosomes, a process requiring Rab7 (17, 28). A FYVE and coiled-to-coil domain-containing protein FYCO1 functions as a Rab7 effector, binding to LC3 and PI(3)P, and mediating microtubule plus end-directed vesicle transport (82). The fusion of autophagosomes and lysosomes is positively regulated by the UVRAG-Vps34-beclin1 PI3-kinase complex and negatively regulated by the Rubicon-UVRAG-Vps34-beclin1 PI3-kinase complex (25, 50, 55, 99, 121). At present, it is unclear how and what controls the composition of these Vps34-beclin1 complexes. Alf, a PI(3)P-binding FYVE domain-containing protein, has been found to localize with autophagosomes and protein granules (91). Functional multivesicular bodies are required for autophagic Alf-mediated clearance of protein aggregates (13).

Following autolysosome formation, the lysosomal hydrolases, including cathepsins and lipases, degrade the intra-autophagosomal contents, whereas cathepsins degrade LC3-II on the intra-autophagosomal surface (109, 111). In yeasts, Atg15, a vacuolar lipase, and Atg22, a vacuolar membrane protein, are indispensable for the specific



**FIG. 6. Elongation of the isolation membrane and maturation of the autophagosome.** During the elongation of the isolation membrane, the Atg5-Atg12/Atg16L complex and WIPI-1 localize to the membrane to form a cup-shaped structure. LC3-II localizes to the isolation membrane, while the Atg5-Atg12/Atg16L complex dissociates from it. The Atg14L-Vps34-beclin1 PI3-kinase complex, Rab32, and Rab33, are positive regulators, whereas Jumpy and MTMR3 are negative regulators of this process. The isolation membrane finally encloses to form the autophagosome. During these steps, LC3-II increases gradually. The insets show changes in LC3-II and the Atg5-Atg12/Atg16L complex.

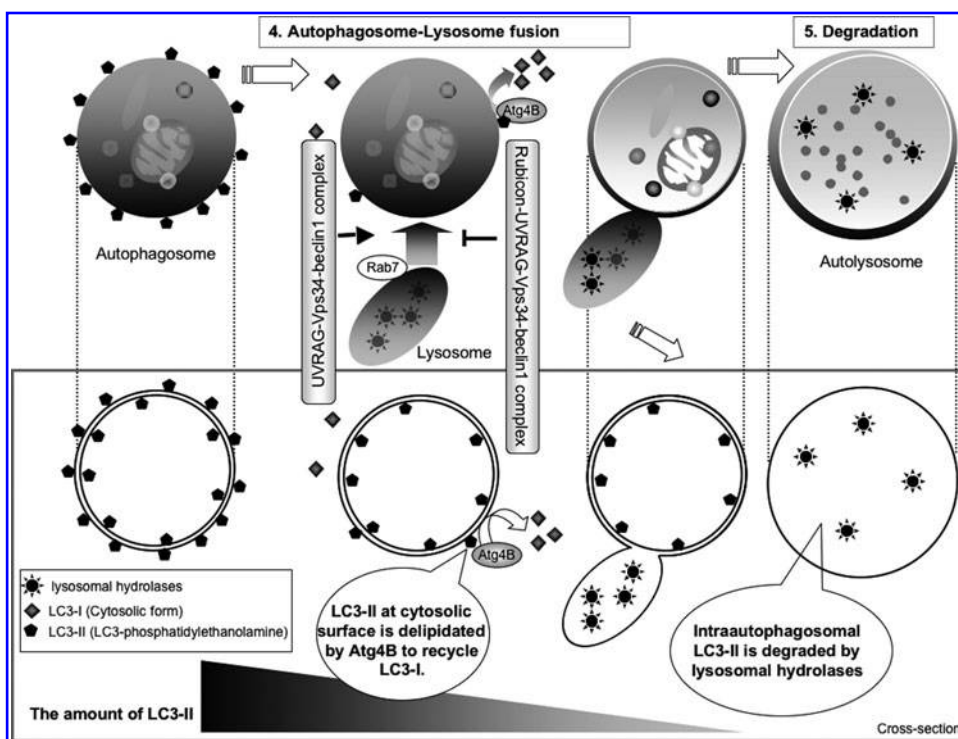
degradation of autophagic bodies (9, 112, 117). At present, mammalian homologs of yeast Atg15 and Atg22 have not been identified.

### Selective Autophagy

In addition to classical or nonselective autophagy, recent findings have shown that processes of selective or chaperone-mediated autophagy are physiologically important (Table 1). These processes are mediated by p62/SQSTM1, a ubiquitin- and LC3-binding protein (5, 45, 83) that also binds to LC3A (one of the other LC3 isoforms), GABARAP, GATE-16 (GA-

BARAPL2), and mAtg8L (GEC1/GABARAPL1) (83). Following their binding to p62, these ubiquitinated protein aggregates are sequestered into autophagosomes. Therefore, when autophagy is impaired, p62- and ubiquitin-positive aggregates accumulate in cells.

Mitophagy is a process of mitochondria-selective autophagy that functions in the clearance of damaged mitochondria (42). This process is mediated by the Parkin and PTEN-induced putative kinase 1 (PINK1) proteins, which are encoded by Parkinson disease-linked genes (8, 54). Parkin is a ubiquitin E3 ligase, and PINK1 contributes to the translocation of Parkin into damaged mitochondria, inducing turnover



**FIG. 7. Autophagosome-lysosome fusion and degradation of intra-autophagosomal contents by lysosomal hydrolases.** Soon after autophagosome formation, the lysosome fuses with the autophagosome to form the autolysosome. During this step, LC3-II on the outer membrane of the autophagosome is delipidated by Atg4B to recycle LC3-I for autophagosome formation. Autophagosome-lysosome fusion is positively regulated by Rab7 and the UVRAG-Vps34-beclin1 PI3-kinase complex, whereas it is negatively regulated by the Rubicon-UVRAG-Vps34-beclin1 complex. After the formation of the autolysosome, the lysosomal hydrolases degrade the intra-autophagosomal contents, including LC3-II. Therefore, the amount of LC3-II decreases during these steps. The inset shows the decrease in LC3-II.

of damaged mitochondria *via* autophagy. Parkin mediates the ubiquitylation of voltage-dependent anion channel 1 and mitofusin (16), and p62 contributes to Parkin/PINK1-mediated mitophagy. Uth1 and Atg32 have been identified as mitophagy-specific proteins in yeasts (34, 41), but their mammalian homologs have not been identified yet.

Pexophagy is a process of peroxisome-specific autophagy, which has been investigated in the methylotrophic yeast *Pichia pastoris* (87). In addition to most of the “core” Atg proteins, which are required for pexophagy, this process requires the proteins Atg24, Atg25, and Atg26, which are specific to and indispensable for pexophagy (2, 72, 116). In mammals, Atg7 deficiency leads to a defect in pexophagy (27), and Pex14 contributes to pexophagy (20).

The cytoplasm-to-vacuole targeting (Cvt) pathway is a unique protein transport system in yeasts (53). Aminopeptidase I and  $\alpha$ -mannosidase are synthesized in the cytosol and selectively packaged into Cvt vesicles during their formation. The Cvt vesicles fuse with the vacuoles, in which the transported proteins are processed to their active forms. Although the Cvt pathway is selective and nondegradative, it includes several Cvt pathway-specific Atg genes, including Atg11, Atg19, Atg20, and Atg21 (48, 56, 88, 96, 119). At present, there have been no reports of a Cvt pathway in mammals.

ER-specific autophagy is designated for ERphagy or reticulophagy and contributes to the protein quality control process in the ER (40, 46). In addition to the ubiquitin-proteasome-mediated ERAD process (ERAD I), there is a stress-induced ER response of autophagy, ERAD II. At present, the molecules contributing to ER-specific recognition of autophagy have not been identified.

### Estimation of Autophagic Activity

Because of the pathophysiological importance of autophagy, it is necessary to estimate autophagic activity accurately and quantitatively. However, as autophagy is a dynamic and complicated process, it must be analyzed correctly. Two classical but definitive methods, conventional morphometry of autophagosomes and autolysosomes by electron microscopy and measurement of autophagic flux by assessing long-lived protein degradation, are still useful. Among these conventional methods are the LC3-II turnover assay using lysosomal inhibitors to measure autophagic flux, measurement of the number of LC3-positive puncta (or green fluorescent protein [GFP]-LC3 puncta), and the tandem fluorescent protein-LC3 (tfLC3/monomeric red fluorescent protein [mRFP]-GFP-LC3) color change assay. Critical issues and guidelines for monitoring autophagy have been described (43, 69).

The LC3-II turnover assay is a measure of autophagic flux, in which LC3-II is assayed by immunoblotting with anti-LC3 antibody in the presence and absence of lysosomal inhibitors. During autophagosome formation, cytosolic LC3-I is conjugated to PE, with the resulting LC3-II localized to autophagosomes (31). These autophagosomes fuse with lysosomes to form autolysosomes, in which sequestered intra-autophagosomal components (including intra-autophagosomal LC3-II) are degraded. Therefore, LC3-II itself is used as a marker of autophagosome formation and its degradation to monitor late autophagic flux (102). A mixture of E64d (a membrane-permeable inhibitor of cathepsins B, H, and L) and pepstatin A (a membrane-permeable inhibitor of cathepsins D

and E) is used to inhibit lysosomal function (102). Treatment of cells with this inhibitor cocktail results in significant accumulation of autolysosomes (and LC3-II dots) because there is little degradation of their contents. Thus, the accumulation of LC3-II reflects the activity of the delivery process of LC3-II into lysosomes, that is, autophagic flux. Fluorescent microscopic assays of LC3-positive puncta in the presence and absence of the lysosomal inhibitor cocktail may also be useful. Methods for immunoblotting and immunostaining of endogenous LC3 have been described (109). Bafilomycin A1 (an inhibitor of V-ATPase) is also used to inhibit autophagy and to estimate the autophagic flux of LC3-II. As V-ATPase contributes to the acidification of other organelles, including the Golgi and endosomes, bafilomycin A1 may show multiple off-target effects (44, 93).

Cells with defective autophagy show increased concentrations of ubiquitin and p62 (45). A dominant negative mutant of Rab7, Rab7<sup>T22N</sup>, inhibits autophagosome-lysosome fusion (17, 28). Thus, overexpression of this Rab7 mutant under starvation conditions results in the augmented accumulation of autophagosomes. Increases in p62- and ubiquitin-positive inclusions in tissues may indicate insufficient autophagy.

Although LC3 fused to GFP (GFP-LC3) is useful for *in vivo* imaging of autophagosome formation (61, 68), caution must be exercised because of the limitations of GFP-LC3 (6, 35). GFP-LC3 tends to form puncta in cells independent of autophagy, and GFP fluorescence in lysosomes may occur even after degradation of the LC3 moiety. Therefore, this method tends to overestimate the number of autophagosomes. These problems may be avoided by using a mutant, GFP-LC3 $\Delta$ G, as a negative control. GFP-LC3 transgenic mice, however, can be used to study autophagy in many tissues outside the brain, where few GFP-LC3 puncta are observed (58, 68). As the brain is one of the organs most protected against starvation, most of the endogenous LC3 in the brain is in the cytosolic form, LC3-I.

The mRFP-GFP-LC3 color change assay is based on the difference in pH stability between GFP and mRFP. Autophagosomes have a pH similar to that of the cytosol, whereas autolysosomes have acidic pH. At acidic pH, the fluorescence of mRFP is stable, whereas that of GFP decreases. Therefore, the merged color of mRFP-GFP-LC3 in autophagosomes is yellow, whereas that in autolysosomes is red (39). This assay is suitable for real-time (and short-term) monitoring of autophagy, but care should be taken for use in long-term monitoring of autophagy. The fluorescence derived from GFP in the lysosomes has been observed even after the degradation of LC3 (35).

The enzyme-based pho8 $\Delta$ 60 assay system has been used for the quantitative estimation of autophagy in yeasts (78). At present, however, an enzyme-based quantitative assay for autophagy in mammals has not been established yet (69).

### Concluding Remarks

Research on autophagy is expanding because of findings of its increased pathophysiological importance in mammals. We have reviewed the “core” Atg complexes essential for autophagosome formation, their functional roles during autophagy, selective autophagy, and assays of autophagy. Most autophagy-related proteins are conserved from yeasts to mammals;

these proteins have been characterized and divided into five subgroups. Mammalian cells have mammalian-specific Atg proteins and more complicated mechanisms, probably because mammalian cells utilize autophagic machinery for tissue- and cell-specific functions in addition to self-defense mechanisms against intracellular and extracellular stresses. In addition to the dynamic and complicated processes of autophagy in mammals, mammalian-specific functions during autophagy or autophagic mechanisms may complicate the analysis of autophagy in mammals. Although LC3-II is a promising autophagosomal marker, it is necessary to carefully interpret the results of LC3-II immunoblotting and fluorescence analyses (69). Future pathophysiological and clinical applications based on autophagy will require the use of autophagy-specific inhibitors and/or activators. Atg4B, a cysteine protease, and autophagy-specific class III PI3-kinase complex are candidates for drug screening.

### Acknowledgments

This study was supported in part by grants-in-aid from the Ministry of Health, Labor, and Welfare of Japan and by Grants-in-Aid for Scientific Research on Priority Areas "Proteolysis in the Regulation of Biological Processes" from the Ministry of Education, Science, Sports, and Culture of Japan.

### References

- Ahlberg J, Marzella L, and Glaumann H. Uptake and degradation of proteins by isolated rat liver lysosomes. Suggestion of a microautophagic pathway of proteolysis. *Lab Invest* 47: 523–532, 1982.
- Ano Y, Hattori T, Oku M, Mukaiyama H, Baba M, Ohsumi Y, Kato N, and Sakai Y. A sorting nexin PpAtg24 regulates vacuolar membrane dynamics during pexophagy via binding to phosphatidylinositol-3-phosphate. *Mol Biol Cell* 16: 446–457, 2005.
- Arstila A and Trump B. Studies on cellular autophagocytosis. The formation of autophagic vacuoles in the liver after glucagon administration. *Am J Pathol* 53: 687–733, 1968.
- Axe EL, Walker SA, Manifava M, Chandra P, Roderick HL, Habermann A, Griffiths G, and Ktistakis NT. Autophagosome formation from membrane compartments enriched in phosphatidylinositol 3-phosphate and dynamically connected to the endoplasmic reticulum. *J Cell Biol* 182: 685–701, 2008.
- Bjorkoy G, Lamark T, Brech A, Outzen H, Perander M, Overvatn A, Stenmark H, and Johansen T. p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. *J Cell Biol* 171: 603–614, 2005.
- Ciechomska IA and Tolkovsky AM. Non-autophagic GFP-LC3 puncta induced by saponin and other detergents. *Autophagy* 3: 586–590, 2007.
- Cuervo A and Dice J. A receptor for the selective uptake and degradation of proteins by lysosomes. *Science* 273: 501–503, 1996.
- Dagda R, Cherra S, Kulich S, Tandon A, Park D, and Chu C. Loss of PINK1 function promotes mitophagy through effects on oxidative stress and mitochondrial fission. *J Biol Chem* 284: 13843–13855, 2009.
- Eppl UD, Suriapranata I, Eskelinen EL, and Thumm M. Aut5/Cvt17p, a putative lipase essential for disintegration of autophagic bodies inside the vacuole. *J Bacteriol* 183: 5942–5955, 2001.
- Eskelinen EL. Fine structure of the autophagosome. *Methods Mol Biol* 445: 11–28, 2008.
- Eskelinen EL. New insights into the mechanisms of macroautophagy in mammalian cells. *Int Rev Cell Mol Biol* 266: 207–247, 2008.
- Eskelinen EL and Saftig P. Autophagy: a lysosomal degradation pathway with a central role in health and disease. *Biochim Biophys Acta* 1793: 664–673, 2009.
- Filimonenko M, Stuffers S, Raiborg C, Yamamoto A, Malerod L, Fisher EM, Isaacs A, Brech A, Stenmark H, and Simonsen A. Functional multivesicular bodies are required for autophagic clearance of protein aggregates associated with neurodegenerative disease. *J Cell Biol* 179: 485–500, 2007.
- Fimia GM, Stoykova A, Romagnoli A, Giunta L, Di Bartolomeo S, Nardacci R, Corazzari M, Fuoco C, Ucar A, Schwartz P, Gruss P, Piacentini M, Chowdhury K, and Cecconi F. Ambra1 regulates autophagy and development of the nervous system. *Nature* 447: 1121–1125, 2007.
- Fujita N, Itoh T, Omori H, Fukuda M, Noda T, and Yoshimori T. The Atg16L complex specifies the site of LC3 lipidation for membrane biogenesis in autophagy. *Mol Biol Cell* 19: 2092–2100, 2008.
- Geisler S, Holmstrom KM, Skujat D, Fiesel FC, Rothfuss OC, Kahle PJ, and Springer W. PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1. *Nat Cell Biol* 12: 119–131, 2010.
- Gutierrez MG, Munafò DB, Beron W, and Colombo MI. Rab7 is required for the normal progression of the autophagic pathway in mammalian cells. *J Cell Sci* 117: 2687–2697, 2004.
- Hanada T, Noda NN, Satomi Y, Ichimura Y, Fujioka Y, Takao T, Inagaki F, and Ohsumi Y. The Atg12-Atg5 conjugate has a novel E3-like activity for protein lipidation in autophagy. *J Biol Chem* 282: 37298–37302, 2007.
- Hara T, Takamura A, Kishi C, Iemura S, Natsume T, Guan JL, and Mizushima N. FIP200, a ULK-interacting protein, is required for autophagosome formation in mammalian cells. *J Cell Biol* 181: 497–510, 2008.
- Hara-Kuge S and Fujiki Y. The peroxin Pex14p is involved in LC3-dependent degradation of mammalian peroxisomes. *Exp Cell Res* 314: 3531–3541, 2008.
- Hirota Y and Tanaka Y. A small GTPase, human Rab32, is required for the formation of autophagic vacuoles under basal conditions. *Cell Mol Life Sci* 66: 2913–2932, 2009.
- Hosokawa N, Hara T, Kaizuka T, Kishi C, Takamura A, Miura Y, Iemura S, Natsume T, Takehana K, Yamada N, Guan JL, Oshiro N, and Mizushima N. Nutrient-dependent mTORC1 association with the ULK1-Atg13-FIP200 complex required for autophagy. *Mol Biol Cell* 20: 1981–1991, 2009.
- Hosokawa N, Sasaki T, Iemura S, Natsume T, Hara T, and Mizushima N. Atg101, a novel mammalian autophagy protein interacting with Atg13. *Autophagy* 5: 973–979, 2009.
- Ichimura Y, Kirisako T, Takao T, Satomi Y, Shimonishi Y, Ishihara N, Mizushima N, Tanida I, Kominami E, Ohsumi M, Noda T, and Ohsumi Y. A ubiquitin-like system mediates protein lipidation. *Nature* 408: 488–492, 2000.
- Itakura E, Kishi C, Inoue K, and Mizushima N. Beclin 1 forms two distinct phosphatidylinositol 3-kinase complexes with mammalian Atg14 and UVRAG. *Mol Biol Cell* 19: 5360–5372, 2008.
- Itoh T, Fujita N, Kanno E, Yamamoto A, Yoshimori T, and Fukuda M. Golgi-resident small GTPase Rab33B interacts

- with Atg16L and modulates autophagosome formation. *Mol Biol Cell* 19: 2916–2925, 2008.
27. Iwata J, Ezaki J, Komatsu M, Yokota S, Ueno T, Tanida I, Chiba T, Tanaka K, and Kominami E. Excess peroxisomes are degraded by autophagic machinery in mammals. *J Biol Chem* 281: 4035–4041, 2006.
  28. Jager S, Bucci C, Tanida I, Ueno T, Kominami E, Saftig P, and Eskelinen EL. Role for Rab7 in maturation of late autophagic vacuoles. *J Cell Sci* 117: 4837–4848, 2004.
  29. Jung CH, Jun CB, Ro SH, Kim YM, Otto NM, Cao J, Kundu M, and Kim DH. ULK-Atg13-FIP200 complexes mediate mTOR signaling to the autophagy machinery. *Mol Biol Cell* 20: 1992–2003, 2009.
  30. Kabeya Y, Kawamata T, Suzuki K, and Ohsumi Y. Cts1/Atg31 is required for autophagosome formation in *Saccharomyces cerevisiae*. *Biochem Biophys Res Commun* 356: 405–410, 2007.
  31. Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, Kominami E, Ohsumi Y, and Yoshimori T. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosomal membranes after processing. *EMBO J* 19: 5720–5728, 2000.
  32. Kabeya Y, Mizushima N, Yamamoto A, Oshitani-Okamoto S, Ohsumi Y, and Yoshimori T. LC3, GABARAP and GATE16 localize to autophagosomal membrane depending on form-II formation. *J Cell Sci* 117: 2805–2812, 2004.
  33. Kametaka S, Okano T, Ohsumi M, and Ohsumi Y. Apg14p and Apg6/Vps30p form a protein complex essential for autophagy in the yeast, *Saccharomyces cerevisiae*. *J Biol Chem* 273: 22284–22291, 1998.
  34. Kanki T, Wang K, Cao Y, Baba M, and Klionsky DJ. Atg32 is a mitochondrial protein that confers selectivity during mitophagy. *Dev Cell* 17: 98–109, 2009.
  35. Katayama H, Yamamoto A, Mizushima N, Yoshimori T, and Miyawaki A. GFP-like proteins stably accumulate in lysosomes. *Cell Struct Funct* 33: 1–12, 2008.
  36. Kawamata T, Kamada Y, Suzuki K, Kuboshima N, Aki-matsu H, Ota S, Ohsumi M, and Ohsumi Y. Characterization of a novel autophagy-specific gene, ATG29. *Biochem Biophys Res Commun* 338: 1884–1889, 2005.
  37. Kihara A, Noda T, Ishihara N, and Ohsumi Y. Two distinct Vps34 phosphatidylinositol 3-kinase complexes function in autophagy and carboxypeptidase Y sorting in *Saccharomyces cerevisiae*. *J Cell Biol* 152: 519–530, 2001.
  38. Kim J, Dalton VM, Eggerton KP, Scott SV, and Klionsky DJ. Apg7p/Cvt2p is required for the cytoplasm-to-vacuole targeting, macroautophagy, and peroxisome degradation pathways. *Mol Biol Cell* 10: 1337–1351, 1999.
  39. Kimura S, Noda T, and Yoshimori T. Dissection of the autophagosome maturation process by a novel reporter protein, tandem fluorescent-tagged LC3. *Autophagy* 3: 452–460, 2007.
  40. Kincaid MM and Cooper AA. ERADicate ER stress or die trying. *Antioxid Redox Signal* 9: 2373–2387, 2007.
  41. Kissova I, Deffieu M, Manon S, and Camougrand N. Uth1p is involved in the autophagic degradation of mitochondria. *J Biol Chem* 279: 39068–39074, 2004.
  42. Kissova I, Plamondon LT, Brisson L, Priault M, Renouf V, Schaeffer J, Camougrand N, and Manon S. Evaluation of the roles of apoptosis, autophagy, and mitophagy in the loss of plating efficiency induced by Bax expression in yeast. *J Biol Chem* 281: 36187–36197, 2006.
  43. Klionsky DJ, Abeliovich H, Agostinis P, Agrawal DK, Aliev G, Askew DS, Baba M, Baehrecke EH, Bahr BA, Ballabio A, Bamber BA, Bassham DC, Bergamini E, Bi X, Biard-Piechaczyk M, Blum JS, Bredeisen DE, Brodsky JL, Brumell JH, Brunk UT, Bursch W, Camougrand N, Cebollero E, Cecconi F, Chen Y, Chin LS, Choi A, Chu CT, Chung J, Clarke PG, Clark RS, Clarke SG, Clave C, Cleveland JL, Codogno P, Colombo MI, Coto-Montes A, Cregg JM, Cuervo AM, Debnath J, Demarchi F, Dennis PB, Dennis PA, Deretic V, Devenish RJ, Di Sano F, Dice JF, Difiglia M, Dinesh-Kumar S, Distelhorst CW, Djavaheri-Mergny M, Dorsey FC, Droge W, Dron M, Dunn WA, Jr., Duszenko M, Eissa NT, Elazar Z, Esclatine A, Eskelinen EL, Fesus L, Finley KD, Fuentes JM, Fueyo J, Fujisaki K, Galliot B, Gao FB, Gewirtz DA, Gibson SB, Gohla A, Goldberg AL, Gonzalez R, Gonzalez-Estevéz C, Gorski S, Gottlieb RA, Haussinger D, He YW, Heidenreich K, Hill JA, Hoyer-Hansen M, Hu X, Huang WP, Iwasaki A, Jaattela M, Jackson WT, Jiang X, Jin S, Johansen T, Jung JU, Kadowaki M, Kang C, Kelekar A, Kessel DH, Kiel JA, Kim HP, Kimchi A, Kinsella TJ, Kiselyov K, Kitamoto K, Knecht E, et al. Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes. *Autophagy* 4: 151–175, 2008.
  44. Klionsky DJ, Elazar Z, Seglen PO, and Rubinsztein DC. Does bafilomycin A1 block the fusion of autophagosomes with lysosomes? *Autophagy* 4: 849–950, 2008.
  45. Komatsu M, Waguri S, Koike M, Sou YS, Ueno T, Hara T, Mizushima N, Iwata J, Ezaki J, Murata S, Hamazaki J, Nishito Y, Iemura S, Natsume T, Yanagawa T, Uwayama J, Warabi E, Yoshida H, Ishii T, Kobayashi A, Yamamoto M, Yue Z, Uchiyama Y, Kominami E, and Tanaka K. Homeostatic levels of p62 control cytoplasmic inclusion body formation in autophagy-deficient mice. *Cell* 131: 1149–1163, 2007.
  46. Kondratyev M, Avezov E, Shenkman M, Groisman B, and Lederkremer GZ. PERK-dependent compartmentalization of ERAD and unfolded protein response machineries during ER stress. *Exp Cell Res* 313: 3395–3407, 2007.
  47. Kuma A, Mizushima N, Ishihara N, and Ohsumi Y. Formation of the approximately 350-kDa Apg12-Apg5-Apg16 multimeric complex, mediated by Apg16 oligomerization, is essential for autophagy in yeast. *J Biol Chem* 277: 18619–18625, 2002.
  48. Leao-Helder AN, Krikken AM, Gellissen G, van der Klei IJ, Veenhuis M, and Kiel JA. Atg21p is essential for macrophagy and microautophagy in the yeast *Hansenula polymorpha*. *FEBS Lett* 577: 491–495, 2004.
  49. Levine B and Kroemer G. Autophagy in the pathogenesis of disease. *Cell* 132: 27–42, 2008.
  50. Liang C, Feng P, Ku B, Dotan I, Canaani D, Oh BH, and Jung JU. Autophagic and tumour suppressor activity of a novel Beclin1-binding protein UVRAG. *Nat Cell Biol* 8: 688–699, 2006.
  51. Liang C, Lee JS, Inn KS, Gack MU, Li Q, Roberts EA, Vergne I, Deretic V, Feng P, Akazawa C, and Jung JU. Beclin1-binding UVRAG targets the class C Vps complex to coordinate autophagosome maturation and endocytic trafficking. *Nat Cell Biol* 10: 776–787, 2008.
  52. Liang XH, Jackson S, Seaman M, Brown K, Kempkes B, Hibshoosh H, and Levine B. Induction of autophagy and inhibition of tumorigenesis by beclin 1. *Nature* 402: 672–676, 1999.
  53. Lynch-Day M and Klionsky D. The Cvt pathway as a model for selective autophagy. *FEBS Lett* 584: 1359–1366, 2010.

54. Matsuda N, Sato S, Shiba K, Okatsu K, Saisho K, Gautier CA, Sou YS, Saiki S, Kawajiri S, Sato F, Kimura M, Komatsu M, Hattori N, and Tanaka K. PINK1 stabilized by mitochondrial depolarization recruits Parkin to damaged mitochondria and activates latent Parkin for mitophagy. *J Cell Biol* 189: 211–221, 2010.
55. Matsunaga K, Saitoh T, Tabata K, Omori H, Satoh T, Kurotori N, Maejima I, Shirahama-Noda K, Ichimura T, Isobe T, Akira S, Noda T, and Yoshimori T. Two Beclin 1-binding proteins, Atg14L and Rubicon, reciprocally regulate autophagy at different stages. *Nat Cell Biol* 11: 385–396, 2009.
56. Meiling-Wesse K, Barth H, Voss C, Eskelinen EL, Epple UD, and Thumm M. Atg21 is required for effective recruitment of Atg8 to the preautophagosomal structure during the Cvt pathway. *J Biol Chem* 279: 37741–37750, 2004.
57. Mercer CA, Kaliappan A, and Dennis PB. A novel, human Atg13 binding protein, Atg101, interacts with ULK1 and is essential for macroautophagy. *Autophagy* 5: 649–662, 2009.
58. Mizushima N. Methods for monitoring autophagy. *Int J Biochem Cell Biol* 36: 2491–2502, 2004.
59. Mizushima N. Physiological functions of autophagy. *Curr Top Microbiol Immunol* 335: 71–84, 2009.
60. Mizushima N. The role of the Atg1/ULK1 complex in autophagy regulation. *Curr Opin Cell Biol* 22: 132–139, 2010.
61. Mizushima N and Kuma A. Autophagosomes in GFP-LC3 transgenic mice. *Methods Mol Biol* 445: 119–124, 2008.
62. Mizushima N, Kuma A, Kobayashi Y, Yamamoto A, Matsubae M, Takao T, Natsume T, Ohsumi Y, and Yoshimori T. Mouse Apg16L, a novel WD-repeat protein, targets to the autophagic isolation membrane with the Apg12-Apg5 conjugate. *J Cell Sci* 116: 1679–1688, 2003.
63. Mizushima N, Levine B, Cuervo AM, and Klionsky DJ. Autophagy fights disease through cellular self-digestion. *Nature* 451: 1069–1075, 2008.
64. Mizushima N, Noda T, and Ohsumi Y. Apg16p is required for the function of the Apg12p-Apg5p conjugate in the yeast autophagy pathway. *Embo J* 18: 3888–3896, 1999.
65. Mizushima N, Noda T, Yoshimori T, Tanaka Y, Ishii T, George MD, Klionsky DJ, Ohsumi M, and Ohsumi Y. A protein conjugation system essential for autophagy. *Nature* 395: 395–398, 1998.
66. Mizushima N, Sugita H, Yoshimori T, and Ohsumi Y. A new protein conjugation system in human. The counterpart of the yeast Apg12p conjugation system essential for autophagy. *J Biol Chem* 273: 33889–33892, 1998.
67. Mizushima N, Yamamoto A, Hatano M, Kobayashi Y, Kabeya Y, Suzuki K, Tokuhisa T, Ohsumi Y, and Yoshimori T. Dissection of autophagosome formation using Apg5-deficient mouse embryonic stem cells. *J Cell Biol* 152: 657–668, 2001.
68. Mizushima N, Yamamoto A, Matsui M, Yoshimori T, and Ohsumi Y. *In vivo* analysis of autophagy in response to nutrient starvation using transgenic mice expressing a fluorescent autophagosome marker. *Mol Biol Cell* 15: 1101–1111, 2004.
69. Mizushima N, Yoshimori T, and Levine B. Methods in mammalian autophagy research. *Cell* 140: 313–326, 2010.
70. Mizushima N, Yoshimori T, and Ohsumi Y. Mouse Apg10 as an Apg12-conjugating enzyme: analysis by the conjugation-mediated yeast two-hybrid method. *FEBS Lett* 532: 450–454, 2002.
71. Mizushima N, Yoshimori T, and Ohsumi Y. Role of the Apg12 conjugation system in mammalian autophagy. *Int J Biochem Cell Biol* 35: 553–561, 2003.
72. Monastyrskaya I, Kiel JA, Krikken AM, Komduur JA, Veenhuis M, and van der Klei IJ. The Hansenuia polymorpha ATG25 gene encodes a novel coiled-coil protein that is required for macropexophagy. *Autophagy* 1: 92–100, 2005.
73. Munz C. Enhancing immunity through autophagy. *Annu Rev Immunol* 27: 423–449, 2009.
74. Nakatogawa H, Ichimura Y, and Ohsumi Y. Atg8, a ubiquitin-like protein required for autophagosome formation, mediates membrane tethering and hemifusion. *Cell* 130: 165–178, 2007.
75. Nakatogawa H, Suzuki K, Kamada Y, and Ohsumi Y. Dynamics and diversity in autophagy mechanisms: lessons from yeast. *Nat Rev Mol Cell Biol* 10: 458–467, 2009.
76. Nemoto T, Tanida I, Tanida-Miyake E, Minematsu-Ikeguchi N, Yokota M, Ohsumi M, Ueno T, and Kominami E. The mouse APG10 homologue, an E2-like enzyme for Apg12p conjugation, facilitates MAP-LC3 modification. *J Biol Chem* 278: 39517–39526, 2003.
77. Noda T, Kim J, Huang WP, Baba M, Tokunaga C, Ohsumi Y, and Klionsky DJ. Apg9p/Cvt7p is an integral membrane protein required for transport vesicle formation in the Cvt and autophagy pathways. *J Cell Biol* 148: 465–480, 2000.
78. Noda T, Matsuura A, Wada Y, and Ohsumi Y. Novel system for monitoring autophagy in the yeast *Saccharomyces cerevisiae*. *Biochem Biophys Res Commun* 210: 126–132, 1995.
79. Obara K, Sekito T, Niimi K, and Ohsumi Y. The Atg18-Atg2 complex is recruited to autophagic membranes via phosphatidylinositol 3-phosphate and exerts an essential function. *J Biol Chem* 283: 23972–23980, 2008.
80. Obara K, Sekito T, and Ohsumi Y. Assortment of phosphatidylinositol 3-kinase complexes—Atg14p directs association of complex I to the pre-autophagosomal structure in *Saccharomyces cerevisiae*. *Mol Biol Cell* 17: 1527–1539, 2006.
81. Ohsumi Y. Molecular dissection of autophagy: two ubiquitin-like systems. *Nat Rev Mol Cell Biol* 2: 211–216, 2001.
82. Pankiv S, Alemu EA, Brech A, Bruun JA, Lamark T, Overvatn A, Bjorkoy G, and Johansen T. FYCO1 is a Rab7 effector that binds to LC3 and PI3P to mediate microtubule plus end-directed vesicle transport. *J Cell Biol* 188: 253–269, 2010.
83. Pankiv S, Clausen TH, Lamark T, Brech A, Bruun JA, Outzen H, Overvatn A, Bjorkoy G, and Johansen T. p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. *J Biol Chem* 282: 24131–24145, 2007.
84. Proikas-Cezanne T, Ruckerbauer S, Stierhof YD, Berg C, and Nordheim A. Human WIPI-1 puncta-formation: a novel assay to assess mammalian autophagy. *FEBS Lett* 581: 3396–3404, 2007.
85. Proikas-Cezanne T, Waddell S, Gaugel A, Frickey T, Lupas A, and Nordheim A. WIPI-1alpha (WIPI49), a member of the novel 7-bladed WIPI protein family, is aberrantly expressed in human cancer and is linked to starvation-induced autophagy. *Oncogene* 23: 9314–9325, 2004.
86. Sagiv Y, Legesse-Miller A, Porat A, and Elazar Z. GATE-16, a membrane transport modulator, interacts with NSF and the Golgi v-SNARE GOS-28. *EMBO J* 19: 1494–1504, 2000.
87. Sakai Y, Oku M, van der Klei IJ, and Kiel JA. Pexophagy: autophagic degradation of peroxisomes. *Biochim Biophys Acta* 1763: 1767–1775, 2006.

88. Shintani T and Klionsky DJ. Cargo proteins facilitate the formation of transport vesicles in the cytoplasm to vacuole targeting pathway. *J Biol Chem* 279: 29889–29894, 2004.
89. Shintani T, Mizushima N, Ogawa Y, Matsuura A, Noda T, and Ohsumi Y. Apg10p, a novel protein-conjugating enzyme essential for autophagy in yeast. *EMBO J* 18: 5234–5241, 1999.
90. Shintani T, Suzuki K, Kamada Y, Noda T, and Ohsumi Y. Apg2p functions in autophagosome formation on the perivacuolar structure. *J Biol Chem* 276: 30452–30460, 2001.
91. Simonsen A, Birkeland HC, Gillooly DJ, Mizushima N, Kuma A, Yoshimori T, Slagsvold T, Brech A, and Stenmark H. Alf, a novel FYVE-domain-containing protein associated with protein granules and autophagic membranes. *J Cell Sci* 117: 4239–4251, 2004.
92. Simonsen A and Tooze SA. Coordination of membrane events during autophagy by multiple class III PI3-kinase complexes. *J Cell Biol* 186: 773–782, 2009.
93. Sobota JA, Back N, Eipper BA, and Mains RE. Inhibitors of the V0 subunit of the vacuolar H<sup>+</sup>-ATPase prevent segregation of lysosomal- and secretory-pathway proteins. *J Cell Sci* 122: 3542–3553, 2009.
94. Sou YS, Tanida I, Komatsu M, Ueno T, and Kominami E. Phosphatidylserine in addition to phosphatidylethanolamine is an *in vitro* target of the mammalian Atg8 modifiers, LC3, GABARAP, and GATE-16. *J Biol Chem* 281: 3017–3024, 2006.
95. Sou YS, Waguri S, Iwata J, Ueno T, Fujimura T, Hara T, Sawada N, Yamada A, Mizushima N, Uchiyama Y, Kominami E, Tanaka K, and Komatsu M. The Atg8 conjugation system is indispensable for proper development of autophagic isolation membranes in mice. *Mol Biol Cell* 19: 4762–4775, 2008.
96. Stromhaug PE, Reggiori F, Guan J, Wang CW, and Klionsky DJ. Atg21 is a phosphoinositide binding protein required for efficient lipidation and localization of Atg8 during uptake of aminopeptidase I by selective autophagy. *Mol Biol Cell* 15: 3553–3566, 2004.
97. Suzuki K, Kirisako T, Kamada Y, Mizushima N, Noda T, and Ohsumi Y. The pre-autophagosomal structure organized by concerted functions of APG genes is essential for autophagosome formation. *EMBO J* 20: 5971–5981, 2001.
98. Taguchi-Atarashi N, Hamasaki M, Matsunaga K, Omori H, Kistakis NT, Yoshimori T, and Noda T. Modulation of local PtdIns3P levels by the PI phosphatase MTMR3 regulates constitutive autophagy. *Traffic* 11: 468–478, 2010.
99. Takahashi Y, Coppola D, Matsushita N, Cuaing HD, Sun M, Sato Y, Liang C, Jung JU, Cheng JQ, Mule JJ, Pledger WJ, and Wang HG. Bif-1 interacts with Beclin 1 through UV-RAG and regulates autophagy and tumorigenesis. *Nat Cell Biol* 9: 1142–1151, 2007.
100. Takeshige K, Baba M, Tsuboi S, Noda T, and Ohsumi Y. Autophagy in yeast demonstrated with proteinase-deficient mutants and conditions for its induction. *J Cell Biol* 119: 301–311, 1992.
101. Tanida I, Komatsu M, Ueno T, and Kominami E. GATE-16 and GABARAP are authentic modifiers mediated by Apg7 and Apg3. *Biochem Biophys Res Commun* 300: 637–644, 2003.
102. Tanida I, Minematsu-Ikeguchi N, Ueno T, and Kominami E. Lysosomal turnover, but not a cellular level, of endogenous LC3 is a marker for autophagy. *Autophagy* 1: 84–91, 2005.
103. Tanida I, Mizushima N, Kiyooka M, Ohsumi M, Ueno T, Ohsumi Y, and Kominami E. Apg7p/Cvt2p: a novel protein-activating enzyme essential for autophagy. *Mol Biol Cell* 10: 1367–1379, 1999.
104. Tanida I, Sou YS, Ezaki J, Minematsu-Ikeguchi N, Ueno T, and Kominami E. HsAtg4B/HsApg4B/autophagin-1 cleaves the carboxyl termini of three human Atg8 homologues and delipidates microtubule-associated protein light chain 3- and GABAA receptor-associated protein-phospholipid conjugates. *J Biol Chem* 279: 36268–36276, 2004.
105. Tanida I, Sou YS, Minematsu-Ikeguchi N, Ueno T, and Kominami E. Atg8L/Apg8L is the fourth mammalian modifier of mammalian Atg8 conjugation mediated by human Atg4B, Atg7 and Atg3. *FEBS J* 273: 2553–2562, 2006.
106. Tanida I, Tanida-Miyake E, Komatsu M, Ueno T, and Kominami E. Human Apg3p/Aut1p homologue is an authentic E2 enzyme for multiple substrates, GATE-16, GABARAP, and MAP-LC3, and facilitates the conjugation of hApg12p to hApg5p. *J Biol Chem* 277: 13739–13744, 2002.
107. Tanida I, Tanida-Miyake E, Ueno T, and Kominami E. The human homolog of *Saccharomyces cerevisiae* Apg7p is a Protein-activating enzyme for multiple substrates including human Apg12p, GATE-16, GABARAP, and MAP-LC3. *J Biol Chem* 276: 1701–1706, 2001.
108. Tanida I, Ueno T, and Kominami E. Human light chain 3/MAP1LC3B is cleaved at its carboxyl-terminal Met121 to expose Gly120 for lipidation and targeting to autophagosomal membranes. *J Biol Chem* 279: 47704–47710, 2004.
109. Tanida I, Ueno T, and Kominami E. LC3 and Autophagy. *Methods Mol Biol* 445: 77–88, 2008.
110. Tanida I, Ueno T, and Kominami E. LC3 conjugation system in mammalian autophagy. *Int J Biochem Cell Biol* 36: 2503–2518, 2004.
111. Tanida I, Wakabayashi M, Kanematsu T, Minematsu-Ikeguchi N, Sou YS, Hirata M, Ueno T, and Kominami E. Lysosomal turnover of GABARAP-phospholipid conjugate is activated during differentiation of C2C12 cells to myotubes without inactivation of the mTor kinase-signaling pathway. *Autophagy* 2: 264–271, 2006.
112. Teter SA, Eggerton KP, Scott SV, Kim J, Fischer AM, and Klionsky DJ. Degradation of lipid vesicles in the yeast vacuole requires function of Cvt17, a putative lipase. *J Biol Chem* 276: 2083–2087, 2001.
113. Tsukada M and Ohsumi Y. Isolation and characterization of autophagy-defective mutants of *Saccharomyces cerevisiae*. *FEBS Lett* 333: 169–174, 1993.
114. Vergne I, Roberts E, Elmaoued RA, Tosch V, Delgado MA, Proikas-Cezanne T, Laporte J, and Deretic V. Control of autophagy initiation by phosphoinositide 3-phosphatase jumpy. *EMBO J* 28: 2244–2258, 2009.
115. Wang H, Bedford FK, Brandon NJ, Moss SJ, and Olsen RW. GABA<sub>A</sub>-receptor-associated protein links GABA<sub>A</sub> receptors and the cytoskeleton. *Nature* 397: 69–72, 1999.
116. Yamashita S, Oku M, Wasada Y, Ano Y, and Sakai Y. PI4P-signaling pathway for the synthesis of a nascent membrane structure in selective autophagy. *J Cell Biol* 173: 709–717, 2006.
117. Yang Z, Huang J, Geng J, Nair U, and Klionsky DJ. Atg22 recycles amino acids to link the degradative and recycling functions of autophagy. *Mol Biol Cell* 17: 5094–5104, 2006.
118. Yen WL, Legakis JE, Nair U, and Klionsky DJ. Atg27 is required for autophagy-dependent cycling of Atg9. *Mol Biol Cell* 18: 581–593, 2007.
119. Yorimitsu T and Klionsky DJ. Atg11 links cargo to the vesicle-forming machinery in the cytoplasm to vacuole targeting pathway. *Mol Biol Cell* 16: 1593–1605, 2005.

120. Young AR, Chan EY, Hu XW, Kochl R, Crawshaw SG, High S, Hailey DW, Lippincott-Schwartz J, and Tooze SA. Starvation and ULK1-dependent cycling of mammalian Atg9 between the TGN and endosomes. *J Cell Sci* 119: 3888–3900, 2006.
121. Zhong Y, Wang QJ, Li X, Yan Y, Backer JM, Chait BT, Heintz N, and Yue Z. Distinct regulation of autophagic activity by Atg14L and Rubicon associated with Beclin 1-phosphatidylinositol-3-kinase complex. *Nat Cell Biol* 11: 468–476, 2009.
122. Zhou X, Wang L, Hasegawa H, Amin P, Han B, Kaneko S, He Y, and Wang F. Deletion of PIK3C3/Vps34 in sensory neurons causes rapid neurodegeneration by disrupting the endosomal but not the autophagic pathway. *Proc Natl Acad Sci USA* 107: 9424–9429, 2010.

Address correspondence to:

Dr. Isei Tanida

Department of Biochemistry and Cell Biology

National Institute of Infectious Diseases

1-23-1, Toyama

Shinjyuku 162-8640

Japan

E-mail: tanida@nih.go.jp

Date of first submission to ARS Central, July 19, 2010; date of acceptance, August 13, 2010.

### Abbreviations Used

Atg = autophagy-related genes and their products  
 beclin1 = autophagy-related bcl2-interacting Atg6 homolog  
 Cvt = cytoplasm-to-vacuole targeting  
 DFCEP1 = double FYVE domain-containing protein 1  
 ERAD = endoplasmic reticulum associated protein degradation  
 GABARAP = GABA<sub>A</sub> receptor-associated protein  
 GATE-16 = Golgi-associated ATPase enhancer of 16kDa  
 GFP = green fluorescent protein  
 LC3 = wild-type human microtubule-associated protein 1 light chain 3  
 LC3-I = soluble unlipidated form of LC3  
 LC3-II = LC3-phospholipid conjugate  
 mRFP = monomeric red fluorescent protein  
 MTMR = myotubularin-related protein  
 mTor = mammalian target of rapamycin  
 PE = phosphatidylethanolamine  
 PI3-kinase = phosphoinositide 3-kinase  
 PI(3)P = phosphatidylinositol 3-phosphate  
 PINK1 = PTEN-induced putative kinase 1  
 ULK1 = unc51-like kinase 1, mammalian homolog of Atg1  
 WIPI = WD-repeat protein interacting phosphoinositides

**This article has been cited by:**

1. Anthony P. Sinai, Paul D. Roepe. 2012. Autophagy in Apicomplexa: a life sustaining death mechanism?. *Trends in Parasitology* **28**:9, 358-364. [[CrossRef](#)]
2. Omar El Bounkari, Jürrgen Bernhagen. 2012. MIF and autophagy: a novel link beyond “eating”. *Cell Research* . [[CrossRef](#)]
3. Markus Islinger, Sandra Grille, H. Dariush Fahimi, Michael Schrader. 2012. The peroxisome: an update on mysteries. *Histochemistry and Cell Biology* . [[CrossRef](#)]
4. Saskia Schlossarek, Daniel R. Englmann, Karim R. Sultan, Markus Sauer, Thomas Eschenhagen, Lucie Carrier. 2012. Defective proteolytic systems in Mybpc3-targeted mice with cardiac hypertrophy. *Basic Research in Cardiology* **107**:1. . [[CrossRef](#)]
5. Alexander I. May, Rodney J. Devenish, Mark Prescott. 2012. The Many Faces of Mitochondrial Autophagy: Making Sense of Contrasting Observations in Recent Research. *International Journal of Cell Biology* **2012**, 1-18. [[CrossRef](#)]
6. Antero Salminen, Juha M. T. Hyttinen, Anu Kauppinen, Kai Kaarniranta. 2012. Context-Dependent Regulation of Autophagy by IKK-NF- $\kappa$ B Signaling: Impact on the Aging Process. *International Journal of Cell Biology* **2012**, 1-15. [[CrossRef](#)]
7. M. Isabel González Siso, M. Esperanza Cerdán. 2012. Kluyveromyces lactis: A Suitable Yeast Model to Study Cellular Defense Mechanisms against Hypoxia-Induced Oxidative Stress. *Oxidative Medicine and Cellular Longevity* **2012**, 1-14. [[CrossRef](#)]
8. Ellen Wirawan, Tom Vanden Berghe, Saskia Lippens, Patrizia Agostinis, Peter Vandenabeele. 2011. Autophagy: for better or for worse. *Cell Research* . [[CrossRef](#)]
9. Tamar Avin-Wittenberg, Arik Honig, Gad Galili. 2011. Variations on a theme: plant autophagy in comparison to yeast and mammals. *Protoplasma* . [[CrossRef](#)]
10. Dipak K. Das . 2011. Autophagy: A Story of Live or Let Die. *Antioxidants & Redox Signaling* **14**:11, 2163-2164. [[Citation](#)] [[Full Text HTML](#)] [[Full Text PDF](#)] [[Full Text PDF with Links](#)]
11. Sunandini Sridhar, Yair Botbol, Fernando Macian, Ana Maria Cuervo. 2011. Autophagy and disease: always two sides to a problem. *The Journal of Pathology* n/a-n/a. [[CrossRef](#)]