—Review—

Review Series: Frontiers of Model Animals for Human Diseases

Pathophysiological Role of Autophagy: Lesson from Autophagy-Deficient Mouse Models

Yoshinobu ICHIMURA and Masaaki KOMATSU

*Protein Metabolism Project, Tokyo Metropolitan Institute of Medical Science,
Setagaya-ku, Tokyo 156-8501, Japan*

Abstract: Autophagy is a cellular degradation system in which cytoplasmic components including organelles are sequestered by double membrane structures called autophagosomes and sequestered materials are degraded by lysosomal hydrolases for supply of amino acids and for cellular homeostasis. The autophagy induced in response to nutrient deprivation is executed in a nonselective fashion, and adaptation to nutrient-poor conditions is the main purpose of autophagy. On the other hand, recent studies have shed light on another indispensable role for starvation-independent or constitutive autophagy in cellular homeostasis, which is mediated by selective degradation of a specific substrate(s). Herein, we introduce pathophysiological roles of starvation-induced, constitutive, and selective autophagy (in particular, selective turnover of p62 through autophagy) disclosed by autophagy-deficient mouse models.

Key words: Atg5, Atg7, autophagy, p62, protein degradation

Introduction

Autophagy is an intracellular degradation system accompanied by dramatic membrane dynamics. When autophagy is initiated, the membrane sac appears in the cytosol and then elongates to enwrap cytoplasmic constituents including organelles. The elongating membrane called an isolation membrane is bent and subsequently closed by fusion of each edge, forming a double membrane structure termed an autophagosome (Fig. 1A–D). The outer membrane of the autophagosome fuses with lysosome, and finally the inner contents are degraded by lysosomal hydrolases [60] (Fig. 1A). The resulting amino acids and lipids are utilized for macromolecule

synthesis and as an energy source. Since discovery of a series of essential genes for autophagy in the yeast *Saccharomyces cerevisiae* by Ohsumi's group [69], various autophagy-defective models including mice have been generated and analyzed [49, 59]. Consequently, a variety of the physiological functions has been unveiled. Starvation adaptation is the most fundamental physiological role of autophagy. For instance, systemic autophagy-deficient mice exhibit a serious low amino acid symptom in the neonatal starvation period when nutrient supply from placenta is suddenly terminated [43]. Meanwhile, the capacity of autophagosome to sequester cytoplasm massively makes possible removal of degenerated and unneeded organelles and protein aggregates. Thus, loss

(Received 16 February 2011 / Accepted 10 March 2011)

Address corresponding: M. Komatsu, Protein Metabolism Project, Tokyo Metropolitan Institute of Medical Science, Setagaya-ku, Tokyo 156-8501, Japan

Fig. 1. Autophagy. (A) Schematic diagram of autophagy. The degradation system, including the membrane dynamics, is induced in response to cellular emergencies such as nutrient deprivation. The isolation membranes/phagophores engulf the cytoplasmic constituents including organelles, and the resulting autophagosomes fuse with lysosomes, resulting in complete degradation of the sequestered cytoplasmic components by lysosomal hydrolases. (B–D) Electron micrographs of primary mouse embryonic fibroblasts under nutrient-poor conditions. C and D indicate higher magnification views of an autophagosomal double membrane and isolation/phagophore, respectively. Arrows show inner and outer membranes of the autophagosome. Bar: 0.5 μm .

of autophagy in mouse tissues is always accompanied by accumulation of proteinous aggregates and disordered organelles even in feeding conditions, leading to life-threatening diseases [59]. The autophagosome is a double membranous globular organelle with a diameter of approximately 1 μm and a volume of $0.5 \times 10^{-18} \text{ m}^3$ that can entrap hundreds of thousands of proteins of average molecular weight (Fig. 1B and 1C). Therefore, autophagy has been generally considered a bulk protein degradation machinery that does not select the protein load. However, growing lines of evidence have shed light on other autophagy modes that selectively degrade aggregated proteins, unnecessary or damaged organelles, and invading bacterial cells [26, 35, 42].

Molecular Mechanism of Autophagy

The molecular mechanism for autophagosome formation has been mainly disclosed by studies of the yeast *S. cerevisiae*. To date, genetic studies of *S. cerevisiae* have revealed over 30 genes related to autophagy (*ATG*; Autophagy-related gene), and it is known that 18 genes among them are essential for autophagosome formation [69, 112]. Importantly, most *ATG* genes are well conserved in mammals, and they function in almost a similar mode to corresponding proteins in yeast, while some mammalian-specific proteins do exist. At present, it is known that Atg proteins are comprised of four subclasses and that these subclasses function sequentially and

Fig. 2. Four subclasses of Atg proteins. (A) ULK1-complex. (B) Phosphatidylinositol 3-kinase (PI3K)-complex. (C) Atg12 and LC3 conjugation systems. (D) Atg9-containing complex. At present, it is known that Atg proteins are comprised of four subclasses, and these subclasses function sequentially and cooperatively to regulate formation of autophagosomes.

cooperatively to regulate formation of autophagosomes (Fig. 2A–2D). Here, we introduce the molecular mechanism of mammalian autophagy.

ULK1 complex

At the individual level, the blood level of amino acids and/or insulin regulates autophagic activity, and thus mTORC1 (mammalian target of rapamycin complex 1), which is a downstream factor in insulin and/or amino acid signals, controls autophagy. A serine/threonine protein kinase, ULK1 (mammalian counterpart of yeast

autophagy protein Atg1), forms a complex including Atg13, Atg101, and FIP200 [14, 18, 19]. Under nutrient-rich conditions, mTORC1 is incorporated into the ULK1 complex and subsequently phosphorylates ULK1 and Atg13, which suppress autophagic activity (Fig. 2A) [10, 18, 27]. In response to starvation, mTORC1 dissociates from the ULK1 complex, and resulting dephosphorylated ULK1 phosphorylates FIP200 to initiate formation of autophagosomes (Fig. 2A) [10, 18, 27].

PI3K complex

Autophagy requires a phosphatidylinositol 3-kinase (PI3K) that generates phosphatidylinositol 3-phosphate (PI3P) on an isolation membrane and/or autophagosome. The class III PI3K complex consisting of Vps15, Vps34, Beclin 1, and Atg14 is involved in early autophagosome formation (Fig. 2B) [22, 103]. Another type of PI3K complex comprised of Vps15, Vps34, Beclin 1, and UVRAG (UV irradiation resistance-associated gene) has been identified. This type of PI3K complex facilitates autophagosome maturation as well as maturation of endosomes (Fig. 2B) [55, 119]. In contrast, when Rubicon (RUN domain, a cysteine-rich domain containing Beclin 1-interacting protein) associates with a subpopulation of UVRAG-containing PI3K complex, the complex localizes to the late endosome/lysosome and negatively regulates both autophagy at a later step and the endocytic pathway (Fig. 2B) [55, 119].

Atg conjugation systems

Remarkably, approximately half of Atg proteins related to autophagosome formation are comprised of Atg conjugation systems, in which the enzymatic reactions involved are, in principle, analogous to the ubiquitin conjugation system including E1 (activating enzyme) and E2 (conjugating enzyme) (Fig. 2C). Both ubiquitin-like modifiers, Atg12 and LC3 (mammalian Atg8p homologue), are activated by a common E1-like enzyme, Atg7, and transferred to two different E2-like enzymes, Atg10 and Atg3, respectively (Fig. 2C) [20, 61, 62, 73, 96, 104, 105]. Whereas Atg12 forms an isopeptide bond with Atg5, LC3 forms an amide bond with phosphatidylethanolamine (PE) dependent on Atg12-Atg5 conjugation (Fig. 2C). The Atg12p-Atg5p conjugate interacts with another gene product, Atg16p, to ultimately form a tetrameric complex in yeasts [44]. The mammalian Atg16p counterpart, Atg16L, also orchestrates a high molecular complex with Atg12-Atg5 [58]. While Atg12-Atg5 conjugation is indispensable for elongation of the isolation membrane, the PE-bound LC3 (LC3-II) is considered important for membrane biogenesis [29, 68] and/or closure of the isolation membrane [7, 100]. Indeed, the Atg12-Atg5-Atg16L complex is localized on the isolation membranes during their elongation and disassembled from them before completion of autophago-

somal formation [58]. In comparison, the LC3-II stays on the outer and inner membranes of the isolation membranes until completion of autophagosome formation. In the final stage, LC3-II on the inner membrane is degraded by fusion of autophagosomes to lysosomes, whereas LC3-II on the outer membrane is released from autophagosomes by a specific cysteine protease, Atg4 [33, 106]. Accordingly, LC3 is well utilized as an autophagosome marker [29, 64], and autophagic activity is estimated by measuring the turnover of LC3-II [32]. Importantly, the attachment of PE to LC3 is almost completely impaired in *Atg5* knockout cells, indicating that these two LC3- and Atg12-conjugation systems contribute to the formation of autophagosomes in a cooperative manner [63]. The Atg12-Atg5 conjugate seems to function as an E3-like enzyme for the LC3 lipidation reaction [8, 12].

Atg9 complex

Most of the Atg proteins are cytosolic and transiently associate with the PAS (pre autophagosomal structure/phagophore assembly site) by interacting with other Atg components and/or lipids. Atg9 is the only conserved integral membrane protein among Atg proteins. In yeast, Atg9p localizes on both dispersed peripheral membrane structures and PAS under nutrient rich conditions [113]. In response to starvation, Atg9p physically interacts with Atg17p (yeast homologue of FIP200) and dominantly localizes to the PAS [95]. Thereafter, Atg9p forms a complex with Atg2p and Atg18p (yeast homologue of WIPI2), which is dependent on Atg1p- and PI3-kinases [51, 88, 110]. And, it was reported that retrieval transport of Atg9p to the peripheral membrane pools occurs in an Atg27p- and Atg23p-dependent manner [88]. On the other hand, mammalian Atg9 cycles between the trans-Golgi network (TGN) and endosomes positive for Rab7 and Rab9 under normal conditions [114]. Upon starvation, Atg9 binds to p38IP, which increases the amount of dispersed Atg9 (Fig. 2D) [111]. The dispersal Atg9 subsequently localizes to phagophore, depending on p38IP, ULK1, Atg13, and PI3-kinase (Fig. 2D) [111]. Atg9 is returned to endosomes by p38 α that negatively regulates interaction of Atg9 with p38IP [111]. The aforementioned high motility of Atg9 in the assembly and disassembly at the autophagosome formation site

implies that Atg9-containing membrane structures supply a portion of the membrane source of the autophagosome.

Conventional Knockout Mice for *Atg* Genes

Upon nutrient deprivation, formation of autophagosomes is dramatically induced in cytoplasm, and then cytoplasmic components are nonselectively metabolized to amino acids and lipids, which is assumed to enhance cell viability under starvation conditions. This adaptive autophagy has been postulated as one of the major roles of autophagy. Indeed, *atg* mutants in *S. cerevisiae* are sensitive to the lethal effect of nitrogen deprivation, while they do not exhibit any phenotypes in nutrient-rich conditions [107]. Moreover, suppression of autophagy leads to abnormalities of physiological phenomena that accompany starvation such as fruiting body formation of slime mold [81], dauer formation of *Caenorhabditis elegans* [56], and sporulation of *S. cerevisiae* [107]. Mizushima's group uncovered that even in mammals, autophagy is important for mouse neonates. Embryos are constantly supplied nutrients from the mother via the placenta. After birth, such supply is suddenly terminated, and the neonates are forced to fast. In wild-type mice, autophagy is induced during such a neonate starvation period. The amino acid supply in this period through autophagy seems to be crucial because *Atg5*-deficient mice are born at a Mendelian ratio but exhibit a lower amino acid level in this period and die within 1 day after birth [43]. Conventional knockout mice for *Atg3*, *Atg7*, *Atg9*, and *Atg16L* showed similar phenotypes [40, 90, 91, 100]. The exact reason for neonatal death in those knockout mice is still controversial because they showed a suckling defect that was probably due to a neuronal defect. However, the above studies demonstrate unequivocally that amino acid supply through autophagy is important for mouse neonate survival. On the other hand, mice lacking *Beclin 1*, which is involved in both autophagy and endocytosis [16], exhibited severe developmental defect followed by death *in utero* [86, 115]. Targeted deletion of *FIP200* (a potential mammalian counterpart of yeast autophagy protein Atg17p) in the mouse led to embryonic death at mid/late gestation associated with heart failure and liver degeneration [9].

Though reasons for inconsistent phenotypes among different *Atg*-knockout mice remain unclear, some *Atg* proteins might have another function apart from autophagy.

Conditional Knockout Mice for *Atg* Genes

Because defective autophagy in mice results in neonatal lethality, the analysis of adult mice by systemic *Atg* knockout is impossible. To investigate the physiological roles of autophagy in mice, Mizushima's group and ours independently generated conditional knockout mice for autophagy-essential genes, *Atg5* and *Atg7*, respectively [13, 40], and a number of groups have generated and analyzed various tissue-specific autophagy-deficient mice (Table 1).

Global turnover of cellular components

Besides the essential roles of adaptive autophagy, it has been proposed that constitutive autophagy, which operates constitutively at a low rate even under a nutrient-rich environment, contributes to cellular homeostasis through global turnover of cellular proteins and organelles. Actually, loss of *Atg7* in the mouse liver (*Atg7^{ff}*, Alb-*Cre*, and *Atg7^{ff}*, Mx1-*Cre*) caused severe hepatomegaly accompanied by disordered structure of hepatic lobules and hypertrophy of hepatocytes, resulting in severe liver injury [40]. Mice lacking *Atg5* or *Atg7* in the central nervous system (*Atg5^{ff}*, nestin-*Cre*, and *Atg7^{ff}*, nestin-*Cre*) exhibited behavioral deficits, such as abnormal limb-clasping reflexes, locomotor ataxia, and lack of coordination, as well as massive neuronal loss in the cerebral and cerebellar cortices [13, 38]. *Atg5*-deletion in cardiomyocyte (*Atg5^{ff}*, MerCreMer) led to cardiac hypertrophy, left ventricular dilatation, and systolic dysfunction [67]. Skeletal muscle-specific *Atg5* (*Atg5^{ff}*, HSA-*Cre*) or *Atg7* knockout mice (*Atg7^{ff}*, ML-Cf-*Cre*) resulted in profound muscle atrophy and an age-dependent decrease in force [52, 87]. Pancreatic beta cell-specific *Atg7* knockout mice (*Atg7^{ff}*, Rip-*Cre*) showed degeneration of islets and impaired glucose tolerance with reduced insulin secretion [5, 28]. Mice lacking *Atg5* in podocytes (*Atg5^{ff}*, Podocin-*Cre*) exhibited a glomerulopathy with aging [15].

Electron microscopic analyses of the tissue-specific

Table 1. Phenotypes of *Atg*-knockout mice

Genotype	Tissue	Phenotype	Reference
<i>Atg3</i> ^{-/-} , <i>Atg5</i> ^{-/-} , <i>Atg7</i> ^{-/-} , <i>Atg9</i> ^{-/-} , and <i>Atg16L</i> ^{-/-}	Whole-body	Death within 1 day after birth and suckling defect.	[40, 43, 90, 91, 100]
<i>Beclin 1</i> ^{-/-}	Whole-body	Developmental defect and death <i>in utero</i> (E7.5d–E8.5d).	[86, 115]
<i>FIP200</i> ^{-/-}	Whole-body	Embryonic death at mid/late gestation associated with heart failure and liver degeneration.	[9]
<i>Atg7</i> ^{fl/fl} , Alb- <i>Cre</i> , <i>Atg7</i> ^{fl/fl} , Mx1- <i>Cre</i> and <i>Atg5</i> ^{fl/fl} , Mx1- <i>Cre</i>	Liver	Liver injury and accumulation of ub/p62-positive inclusions and degenerated mitochondria.	[13, 39, 40]
<i>Atg5</i> ^{fl/fl} , nestin- <i>Cre</i> and <i>Atg7</i> ^{fl/fl} , nestin- <i>Cre</i>	Brain	Neurodegeneration and accumulation of ub/p62-positive inclusions.	[13, 38]
<i>Atg5</i> ^{fl/fl} , Pcp2- <i>Cre</i> and <i>Atg7</i> ^{fl/fl} , Pcp2- <i>Cre</i>	Purkinje cells	Accumulation of concentric membranous structures in axon and the axonal degeneration.	[41, 75]
<i>Atg5</i> ^{fl/fl} , MerCreMer- <i>Cre</i>	Cardiomyocytes	Cardiac hypertrophy, left ventricular dilatation, systolic dysfunction, and accumulation of ub/p62-positive inclusions and mitochondria.	[67]
<i>Atg7</i> ^{fl/fl} , MLCf- <i>Cre</i>	Skeletal muscle	Muscle atrophy and age-dependent decrease in force and accumulation of ub/p62-positive inclusions and mitochondria.	[52]
<i>Atg5</i> ^{fl/fl} , HSA- <i>Cre</i>	Skeletal muscle	Atrophy of fast muscle fiber. Accumulation of ub/p62-positive inclusions.	[87]
<i>Atg7</i> ^{fl/fl} , Rip- <i>Cre</i>	Pancreatic beta cells	Degeneration of islets and impaired glucose tolerance with reduced insulin secretion. Accumulation of ub/p62-positive inclusions and mitochondria.	[5, 28]
<i>Atg5</i> ^{fl/fl} , Podocin- <i>Cre</i>	Podocytes	Glomerulopathy with aging. Accumulation of ub/p62-positive inclusions.	[15]
<i>Atg7</i> ^{fl/fl} , Vav- <i>Cre</i>	Hematopoietic stem cells	Anaemia as well as lymphopenia and accumulation of degenerated mitochondria.	[65]
<i>Atg5</i> ^{fl/fl} , Lck- <i>Cre</i> and <i>Atg7</i> ^{fl/fl} , Lck- <i>Cre</i>	T cells	Reduced number of T cells. Failure to reduce mitochondrial content as well as an imbalance in pro- and antiapoptotic protein expression.	[84, 85, 101]
<i>Atg5</i> ^{fl/fl} , CD19- <i>Cre</i>	B cells	Decrease in the number of B cells.	[57]
<i>Atg7</i> ^{fl/fl} , Ap2- <i>Cre</i>	Adipocytes	Decreased mass of white adipose tissue and enhanced insulin sensitivity. Multilocular lipid droplet and accumulated mitochondria.	[99, 117]
<i>Atg5</i> ^{fl/fl} , Zp3- <i>Cre</i>	Oocytes	Death at the 8-cell stage.	[108]

autophagy-deficient mice showed marked accumulation of swollen and deformed mitochondria in the mutant hepatocytes [40], pancreatic beta cells [5, 28], and cardiac [67] and skeletal myocytes [52], the appearance of concentric membranous structures consisting of an endoplasmic reticulum (ER) or sarcoplasmic reticulum in mutant hepatocytes [40], neuronal axons [41, 75] and skeletal myocytes [52], and an increased number of peroxisomes in mutant hepatocytes [40]. In addition to the accumulation of aberrant organelles, histological analyses of tissues with defective autophagy showed accumulation of polyubiquitinated proteins in almost all tissues, although the level varied from one region to another, forming inclusion bodies whose size and number increased with aging [13, 38, 39]. Importantly, the mutant

tissues, especially the autophagy-deficient liver and skeletal muscle, seemed to have functionally intact proteasomes [39, 52], whose impairment is generally known to cause abnormal ubiquitin-mediated proteolysis. Autophagy deficiency is considered to result in delays of global turnover of cytoplasmic components, resulting in accumulation of misfolded and/or unfolded proteins followed by the formation of inclusion bodies and deformed organelles. In other words, basal autophagy acts as a quality control machinery for cytoplasmic components and is crucial for homeostasis of various postmitotic cells, and therefore, constitutive autophagy fights diseases. Meanwhile, Johansen's group suggested that the autophagosome selectively uptakes the polyubiquitinated protein aggregates via autophagosomal protein,

LC3-, and ubiquitin-binding protein, p62 [1] (see below).

Cell remodeling

It has been postulated that removal of organelles through autophagy contributes to differentiation and development accompanied by intracellular remodelling such as hematopoiesis and adipogenesis. In mammalian red blood cells, expulsion of the nucleus followed by removal of other organelles, such as mitochondria, are necessary differentiation steps. Hematopoietic-specific *Atg7* knockout mice (*Atg7^{ff}*, *Vav-Cre*) exhibited severe anaemia as well as lymphopenia, and the mutant erythrocytes markedly accumulated degenerated mitochondria, but not other organelles, followed by cell death [65]. Mitochondrial content is regulated in developmental T cells, in which high mitochondrial content in thymocytes is shifted to lower mitochondrial content in mature T cells. *Atg5* or *Atg7*-deleted T cells (*Atg5^{ff}*, *Lck-Cre* or *Atg7^{ff}*, *Lck-Cre*) fail to reduce their mitochondrial content, resulting in increased reactive oxygen species (ROS) production as well as an imbalance in pro- and antiapoptotic protein expression [84, 85, 101]. These lines of evidence imply an essential role of mitochondria autophagy (mitophagy) in hematopoiesis. Unlike T cells, the content of mitochondria is not affected during development of B cells, but lack of *Atg5* in B cells (*Atg5^{ff}*, *CD19-Cre*) was associated with decrease in the number [57]. White adipocytes have a unique structure in which nearly the entire cell volume is occupied by one large lipid droplet, the molecular and cellular processes involved in the cytoplasmic remodeling necessary to create this structure have been poorly defined. Deletion of *Atg7* in adipose tissue (*Atg7^{ff}*, *Ap2-Cre*) was associated with decreased mass of the white adipose tissue and enhanced insulin sensitivity [99, 117]. The mutant white adipocytes were smaller and had a larger volume of cytosol, contained multilocular lipid droplets and accumulated mitochondria [117]. These studies indicate that autophagy in adipocytes is important for differentiation as well as lipid metabolism *in vivo*.

Early development

By deletion of *Atg5* in oogenesis (*Atg5^{ff}*, *Zp3-Cre*), Mizushima's group demonstrated that autophagy is es-

sential for very early development in mice [108]. They noticed that autophagy is induced after fertilization and that such induction continues until the 8-cell stage [108]. During this period, maternal proteins in fertilized eggs are substituted with embryonic proteins, and it is known that fertilized eggs in mammals do not have enough nutrients. Thus, they postulated that amino acid supply through autophagy during early development is important for synthesis of embryonic proteins. To prove this hypothesis, they developed oocyte-specific *Atg5*-deficient mice (*Atg5^{ff}*, *Zp3-Cre*) and showed that the mutant embryos died in the 8-cell stage, implying that supply of amino acids via autophagy is essential for early embryogenesis [108].

Selective Autophagy

Suppression of autophagy in mouse tissues is always accompanied by marked accumulation of ubiquitinated proteins and of organelles, in particular degenerated mitochondria, suggesting selectivity of autophagy.

Mitophagy

Concomitant with energy production through oxidative phosphorylation in the mitochondria, these organelles generate ROS, causing protein, lipid, and DNA oxidation and often inducing cell death. Therefore, quality control of mitochondria is essential for cellular homeostasis, and it is thought that such a process is achieved through autophagy. Recent studies described the molecular mechanism through which surplus mitochondria are selectively targeted for autophagy in *S. cerevisiae*. These studies identified Atg32p as a specific factor responsible for directing selective mitochondrial degradation through autophagy (mitophagy) [30, 78]. Atg32p is a single-pass mitochondria outer membrane protein, and its N-terminus is exposed to the cytoplasm. Like Atg19p in the cytoplasm to vacuole targeting (CVT) pathway [50], Atg32p restricts autophagosome formation to around the targeted mitochondrion by gathering Atg proteins around it [30, 78]. Expression of *ATG32* is induced in response to oxidative stress, and subsequently, Atg32p localizes to the damaged mitochondrion, though the detailed molecular mechanism remains unclear. Like Atg19p, Atg32p can associate directly with

Fig. 3. Mitophagy. (A) Atg32p-mediated mitophagy in the yeast *S. cerevisiae*. (B) Nix/Bnip3L- and PINK1-Parkin-mediated mitophagy in mammals.

Atg11p (an adaptor protein tethering Atg proteins essential for autophagosome formation to selective cargos) and Atg8p. Thus, autophagosome formation is induced around the Atg32p-localized mitochondrion followed by selective degradation of the damaged mitochondrion (Fig. 3A).

Although no homologue of yeast Atg32p has been identified in higher eukaryotes including those of humans and mice, Nix/Bnip3L might fulfill the function of Atg32p in higher organisms. Nix/Bnip3L, whose structure is similar to that of Atg32p, is also an outer mitochondrial membrane protein and plays an important role in mitophagy in erythroid differentiation [77, 92, 94]. Although autophagosome formation probably occurs in *Nix/Bnip3L*-deficient reticulocytes, mitochondrial elimination is impaired in these cells. Consequently, mutant reticulocytes are exposed to increased amounts of ROS

and die, resulting in severe anemia in *Nix/Bnip3L*-knock-out mice [92, 94]. Depolarization of the mitochondrial membrane potential of mutant reticulocytes by treatment with an uncoupling agent results in restoration of mitophagy in the cells, emphasizing the importance of Nix/Bnip3L for depolarization of mitochondria and implying that autophagy targets the uncoupled mitochondria (Fig. 3B) [92].

Meanwhile, Narendra *et al.* have recently uncovered other important players in mitophagy in higher eukaryotes, PINK1 and Parkin [71, 72]. PINK1 and Parkin are important players in autosomal recessive parkinsonism, as they have been genetically linked to a pathway that protects against progressive mitochondrial damage and dysfunction. PINK1, a mitochondrial kinase known to function upstream of Parkin, accumulates in the mitochondria upon treatment with an uncoupling agent, car-

bonyl cyanide *m*-chlorophenylhydrazone (CCCP), and this accumulation is both necessary and sufficient for Parkin recruitment to the mitochondria [54, 72, 109]. Once Parkin is translocated from the cytoplasm to the depolarized mitochondria, the mitochondria become surrounded by autophagosomes in a Parkin ubiquitin-ligase activity-dependent manner (Fig. 3B) [54, 72, 109]. Importantly, either PINK1 or Parkin with Parkinson's disease-related mutation is associated with impaired mitophagy upon CCCP treatment, suggesting a link between defective mitophagy and Parkinson's disease.

Selective turnover of p62

The p62 protein is a ubiquitously expressed cellular protein that is conserved in metazoa but not in plants and fungi, and it has been known that the mutations give rise to inherited Paget's disease [17, 48]. This protein functions as a signaling hub that could determine whether cells survive, by activating the TRAF6-NF- κ B pathway [93] or die by facilitating the aggregation of caspase-8 and the downstream effector caspases [25]. Thus, the quantity of p62 should be strictly controlled [36]. The p62 is localized at the autophagosome formation site [23] and directly interacts with LC3, an autophagosome localizing protein [21, 76, 82, 97]. Subsequently, the p62 is incorporated into the autophagosome and then degraded (Fig. 4A and 4B). Therefore, the impaired autophagy is accompanied by accumulation of p62 followed by formation of aggregates positive for p62 and ubiquitinated proteins because of the nature of both self-oligomerization and ubiquitin-binding of p62 [39, 74]. Notably, inclusion bodies positive for both ubiquitin and p62 have been identified in various neurodegenerative diseases (i.e., Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis) [45] and liver disorders (i.e., alcoholic hepatitis, steatohepatitis, and hepatocellular carcinoma) [102, 116]. Intriguingly, the p62-positive aggregates observed in hepatocytes and neurons of liver- and brain-specific *Atg7* deficient mice are completely dispersed by additional loss of *p62* [39], suggesting involvement of p62 in the formation of disease-related inclusion bodies.

Molecular basis for selective autophagy

Autophagic degradation of p62 is severely attenuated

by mutation of Phox and Bem1p (PB1) domain, which is responsible for self- and hetero-oligomerization [21, 46]. Moreover, p62 is targeted to the autophagosome formation site on the ER in a PB1-dependent manner [23]. Furthermore, Kopito's group proposed that protein oligomerization drives autophagic substrate selection [89]. These reports indicate that oligomerization of p62 is a key event that the p62 becomes an autophagic substrate. By a series of analyses of p62 mutants, we have identified that the LC3-interacting region consists of 11 amino acids of mouse p62, named LRS (LC3 recognition sequence) (Fig. 4A) [21]. LRS is almost identical to the previously reported LIR (LC3-interacting region) of human p62 [82]. This amino acid sequence showed a significant and conserved motif that is composed of an acidic cluster (DDD/DEE) and two hydrophobic amino acid motifs (WxxL/WxxV) [21]. Crystal structure analysis of the LC3-LRS complex revealed that the acidic cluster of Asp337-Asp339 in LRS interacts with basic amino acids in the N-terminal α 1 helix of LC3 and that the hydrophobic amino acids, Trp-340 and Leu-343 in LRS, interact with two hydrophobic pockets on the surface of the ubiquitin fold of LC3 [21]. Furthermore, it has been reported that the WxxL motif in Atg19p, a receptor for the CVT pathway, interacts with the hydrophobic pockets of Atg8p and that the interaction is necessary for vacuolar transport of amino peptidase I (Ape I) in the CVT pathway [76]. In agreement with these reports, recent studies revealed that Atg32p/Nix, essential for mitophagy, contain the WxxL motif for interaction with Atg8p/LC3 family proteins [77, 78]. These findings suggest that the cargo receptors (or cargos) interact with LC3 through their WxxL motifs and are subsequently recruited to vacuoles/lysosomes in selective autophagy.

The Physiological Meaning of Selective Autophagy of p62

p62 as a receptor for ubiquitinated proteins

Growing lines of evidence indicate that p62, together with ubiquitinated proteins, is transported into the autophagosomes, implying that p62 is a receptor for ubiquitinated proteins that is necessary for their degradation in lysosomes (Fig. 4B) [26]. However, the level of ubiq-

Fig. 4. Selective turnover of p62 by autophagy. (A) Domain structure of p62, Nbr1, and Alfy. PB1 can exhibit self-oligomerization and hetero-oligomerization with other proteins containing the PB1 domain such as Nbr1. LC3 recognizes p62 and Nbr1 through the LIR (LC3 interacting region)/LRS (LC3 recognition sequence). p62 interacts with Keap1 via the KIR (Keap1 interacting region). ZNF: Zinc finger domain. T6BD: TRAF6 binding domain. UBA: Ubiquitin associated domain. CC: Coiled-coil domain. BEACH: *Beige and Chediak-Higashi* domain. FYVE: PI3P-binding FYVE domain. NBR: NBR domain. (B) A model of p62 as a receptor for ubiquitinated proteins. The complex comprised of p62, Nbr1, and Alfy interacts with polyubiquitinated proteins via the UBA domain of p62 and/or Nbr1. The complex is selectively sequestered in the autophagosomes through the interaction of LIR/LRS-LC3. This process is achieved efficiently by self-oligomerization of p62 via the PB1 domains. (C) p62 functions as a signaling hub through oligomerization for apoptosis, NF- κ B, and stress response.

ubiquitinated protein in *p62*-knockout tissues was quite lower than in autophagy-deficient tissues [39]. A possible explanation for this discrepancy is the presence of another receptor(s) such as neighbor of BRCA1 gene 1 (Nbr1). The structure of Nbr1 is similar to that of p62, and Nbr1 can bind both LC3 and ubiquitinated proteins through the LIR and ubiquitin-associated (Uba) domain, respectively (Fig. 4A) [34]. Like p62, Nbr1 is sequestered into the autophagosome via LC3-interaction and/or p62-interaction and markedly accumulates in autophagy-deficient tissues [34], suggesting that Nbr1 might compensate the loss of p62. Since no proteins with structures similar to those of p62 and Nbr1 exist in mice, analysis of double knockout mice for *p62* and *Nbr1* is needed to clarify this issue. Another possibility is that because p62 functions to build the aggregates, ubiquitinated proteins that fail to form aggregation due to loss of p62 might be degraded through the ubiquitin-proteasome system. Further studies are needed to clarify how many cellular ubiquitinated proteins are transported into the autophagosomes through p62 and/or Nbr1. Moreover, it is a critical issue to specify a signal(s) that modulates selective degradation of ubiquitinated protein aggregates by autophagy. As a receptor, p62 also would contribute to autophagic degradation of Parkin-mediated ubiquitinated mitochondria [11], midbody ring [83], peroxisomes [31], and microbes [118]. On the other hand, very recently it was reported that elimination of mitochondria upon CCCP treatment (i.e., ubiquitinated mitochondria) occurs even in *p62*-deficient cells [70, 79], suggesting that the compensated mechanism might exist in cells.

Phosphatidylinositol-3-phosphate (PI3P)-binding protein Alf1 (Autophagy-linked FYVE protein) is also a prerequisite for selective degradation of aggregated proteins such as polyQ [2, 6, 98]. Though LIR/LRS in Alf1 has not yet been identified, Alf1 physically interacts with Atg5 and p62 (Fig. 4A) and functions as a scaffold for recruitment of their cargos to the isolation membrane [2, 6].

p62 as a signaling hub

Besides the function of p62 as a ubiquitin receptor, p62 is involved in several signal transduction pathways through its ability of self-oligomerization. The p62 is a scaffold protein for cell survival and death signaling

pathways such as the NF- κ B pathway, Wnt signaling, and apoptosis (Fig. 4C) [66], and it is plausible that excess accumulation of p62 leads to dysregulated activation of these signaling pathways. In agreement with this hypothesis, p62 has been identified as a component of inclusion bodies observed in various human diseases including hepatocellular carcinoma and malignant glioma [116]. Also, the loss of *p62* diminished Ras-induced lung adenocarcinoma [4]. Moreover, dysregulation of the NF- κ B signal in autophagy-incompetent cells was due, at least in part, to accumulation of p62, which subsequently enhanced tumorigenesis [53]. These lines of evidence suggest aberrant signals based on p62-accumulation lead to tumorigenesis. In addition, we found that constant activation of Nrf2 through excess accumulation of p62 (see below) also contributes to tumor development (unpublished data). Considering that the homeostatic level of p62 protein is strictly regulated by constitutive autophagy, selective autophagy regulates the NF- κ B pathway, apoptosis, and environmental stress response, and therefore a defect in autophagy might lead to various diseases. Indeed, loss of *p62* markedly attenuated liver injury resulting from autophagy deficiency [39]. This implies that impaired turnover of p62 is a major cause of the pathogenic changes seen in the livers of autophagy-deficient mice.

How does accumulation of p62 in the autophagy-deficient liver cause the disease onset? Recently, we and other groups independently found that p62 interacts with the Nrf2-binding site on Keap1, a component of Cullin3-type ubiquitin ligase for Nrf2 [3, 24, 37, 47]. Thus, overproduction of p62 or autophagy deficiency competes with the interaction between Nrf2 and Keap1, resulting in stabilization of Nrf2 and transcriptional activation of Nrf2 target genes including a battery of detoxifying enzymes and a series of antioxidant proteins (Fig. 5A) [3, 24, 37, 47]. Interestingly, this sustained activation of Nrf2 by p62 appears to be a major cause of toxicity in autophagy-impaired livers [37, 89]. Loss of *Atg7* in mouse livers resulted in severe p62 accumulation followed by Keap1 inactivation and Nrf2 activation (please refer to *Atg7*-KO in Fig. 5B). Such activation was suppressed by additional loss of *p62* or *Nrf2* (please refer to *p62 Atg7*-DKO and *Nrf2 Atg7*-DKO in Fig. 5B). The phenotypes observed in the *Atg7*-deficient liver, such as

Fig. 5. Genetic analyses of liver-specific *Atg7*-deficient mice. (A) Schematic representation of the Nrf2-Keap1 pathway. Canonical Nrf2-Keap1 pathway (left). Regulation of the Nrf2-Keap1 pathway by p62 (right). (B) Summary of phenotypes for *Atg7*-, *Keap1*-, *p62 Atg7*-, and *Nrf2 Atg7*-knockout mice.

hepatocytic hypertrophy, severe hepatomegaly, and liver injury, were ameliorated by concomitant loss of *p62* or *Nrf2*, demonstrating that persistent Nrf2 activation is a major pathogen in autophagy-deficient mouse

livers [37]. While both *Keap1*-deficient and *Atg7*-deficient mouse livers showed hyperactivation of Nrf2, liver-specific *Keap1*-knockout mice exhibited only mild liver injury (please refer to *Keap1*-KO in Fig. 5B) [80].

Therefore, it is evident that the sustained activation of Nrf2 under suppressive autophagy, but not the persistent activation of Nrf2 alone, leads to hepatocytic hypertrophy due to increased cellular protein volume followed by hepatomegaly and liver injury (please compare with *Atg7*-KO and *Keap1*-KO in Fig. 5B). Because numerous antioxidative and detoxification enzymes are induced strongly through the Nrf2 activation and occupy substantial cytoplasmic space, it is plausible that such Nrf2 target proteins should be removed expeditiously when they are not needed. Our results indicate that the impaired turnover of induced Nrf2-target proteins through autophagy has unfavorable effects for hepatocytes. For avoiding unnecessary expression of Nrf2-target proteins, higher animals might acquire a transient expression system (i.e., the Nrf2-Keap1 system) for antioxidant proteins and detoxification enzymes.

Acknowledgments

We thank Satoshi Waguri (Fukushima Medical University School of Medicine) for providing the electron micrographs used in the manuscript. We also thank Dr. Hiromichi Yonekawa of Tokyo Metropolitan Institute of Medical Science for giving us this opportunity to write this review. This work is supported by Grants-in-Aid for Scientific Research (to M.K. and Y.I.) from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and by the Japan Science and Technology Agency (M.K.).

References

1. Bjorkoy, G., Lamark, T., Brech, A., Outzen, H., Perander, M., Overvatn, A., Stenmark, H., and Johansen, T. 2005. p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. *J. Cell Biol.* 171: 603–614.
2. Clausen, T.H., Lamark, T., Isakson, P., Finley, K., Larsen, K.B., Brech, A., Overvatn, A., Stenmark, H., Bjorkoy, G., Simonsen, A., and Johansen, T. 2010. p62/SQSTM1 and ALFY interact to facilitate the formation of p62 bodies/ALIS and their degradation by autophagy. *Autophagy* 6: 330–344.
3. Copple, I.M., Lister, A., Obeng, A.D., Kitteringham, N.R., Jenkins, R.E., Layfield, R., Foster, B.J., Goldring, C.E., and Park, B.K. 2010. Physical and functional interaction of sequestosome 1 with Keap1 regulates the Keap1-Nrf2 cell defense pathway. *J. Biol. Chem.* 285: 16782–16788.
4. Duran, A., Linares, J.F., Galvez, A.S., Wikenheiser, K., Flores, J.M., Diaz-Meco, M.T., and Moscat, J. 2008. The signaling adaptor p62 is an important NF-kappaB mediator in tumorigenesis. *Cancer Cell* 13: 343–354.
5. Ebato, C., Uchida, T., Arakawa, M., Komatsu, M., Ueno, T., Komiya, K., Azuma, K., Hirose, T., Tanaka, K., Kominami, E., Kawamori, R., Fujitani, Y., and Watada, H. 2008. Autophagy is important in islet homeostasis and compensatory increase of beta cell mass in response to high-fat diet. *Cell Metab.* 8: 325–332.
6. Filimonenko, M., Isakson, P., Finley, K.D., Anderson, M., Jeong, H., Melia, T.J., Bartlett, B.J., Myers, K.M., Birkeland, H.C., Lamark, T., Krainc, D., Brech, A., Stenmark, H., Simonsen, A., and Yamamoto, A. 2010. The selective macroautophagic degradation of aggregated proteins requires the PI3P-binding protein Alf. *Mol. Cell* 38: 265–279.
7. Fujita, N., Hayashi-Nishino, M., Fukumoto, H., Omori, H., Yamamoto, A., Noda, T., and Yoshimori, T. 2008. An Atg4B mutant hampers the lipidation of LC3 paralogues and causes defects in autophagosome closure. *Mol. Biol. Cell* 19: 4651–4659.
8. Fujita, N., Itoh, T., Omori, H., Fukuda, M., Noda, T., and Yoshimori, T. 2008. The Atg16L complex specifies the site of LC3 lipidation for membrane biogenesis in autophagy. *Mol. Biol. Cell* 19: 2092–2100.
9. Gan, B., Peng, X., Nagy, T., Alcaraz, A., Gu, H., and Guan, J.L. 2006. Role of FIP200 in cardiac and liver development and its regulation of TNFalpha and TSC-mTOR signaling pathways. *J. Cell Biol.* 175: 121–133.
10. Ganley, I.G., Lam du, H., Wang, J., Ding, X., Chen, S., and Jiang, X. 2009. ULK1.ATG13.FIP200 complex mediates mTOR signaling and is essential for autophagy. *J. Biol. Chem.* 284: 12297–12305.
11. Geisler, S., Holmstrom, K.M., Skujat, D., Fiesel, F.C., Rothfuss, O.C., Kahle, P.J., and Springer, W. 2010. PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1. *Nat. Cell Biol.* 12: 119–131.
12. Hanada, T., Noda, N.N., Satomi, Y., Ichimura, Y., Fujioka, Y., Takao, T., Inagaki, F., and Ohsumi, Y. 2007. The Atg12-Atg5 conjugate has a novel E3-like activity for protein lipidation in autophagy. *J. Biol. Chem.* 282: 37298–37302.
13. Hara, T., Nakamura, K., Matsui, M., Yamamoto, A., Nakahara, Y., Suzuki-Migishima, R., Yokoyama, M., Mishima, K., Saito, I., Okano, H., and Mizushima, N. 2006. Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. *Nature* 441: 885–889.
14. Hara, T., Takamura, A., Kishi, C., Iemura, S., Natsume, T., Guan, J.L., and Mizushima, N. 2008. FIP200, a ULK-interacting protein, is required for autophagosome formation in mammalian cells. *J. Cell Biol.* 181: 497–510.
15. Hartleben, B., Godel, M., Meyer-Schwesinger, C., Liu, S., Ulrich, T., Kobler, S., Wiech, T., Grahmmer, F., Arnold, S. J., Lindenmeyer, M.T., Cohen, C.D., Pavenstadt, H., Kerjaschki, D., Mizushima, N., Shaw, A.S., Walz, G., and Huber, T.B. 2010. Autophagy influences glomerular disease susceptibility and maintains podocyte homeostasis in aging mice. *J. Clin. Invest.* 120: 1084–1096.
16. He, C. and Levine, B. 2010. The Beclin 1 interactome. *Curr.*

- Opin. Cell Biol.* 22: 140–149.
17. Hocking, L.J., Lucas, G.J., Daroszewska, A., Mangion, J., Olavesen, M., Cundy, T., Nicholson, G.C., Ward, L., Bennett, S.T., Wuyts, W., Van Hul, W., and Ralston, S.H. 2002. Domain-specific mutations in sequestosome 1 SQSTM1 cause familial and sporadic Paget's disease. *Hum. Mol. Genet.* 11: 2735–2739.
 18. Hosokawa, N., Hara, T., Kaizuka, T., Kishi, C., Takamura, A., Miura, Y., Iemura, S., Natsume, T., Takehana, K., Yamada, N., Guan, J.L., Oshiro, N., and Mizushima, N. 2009. Nutrient-dependent mTORC1 association with the ULK1-Atg13-FIP200 complex required for autophagy. *Mol. Biol. Cell* 20: 1981–1991.
 19. Hosokawa, N., Sasaki, T., Iemura, S., Natsume, T., Hara, T., and Mizushima, N. 2009. Atg101, a novel mammalian autophagy protein interacting with Atg13. *Autophagy* 5: 973–979.
 20. 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. 2000. A ubiquitin-like system mediates protein lipidation. *Nature* 408: 488–492.
 21. Ichimura, Y., Kumanomidou, T., Sou, Y.S., Mizushima, T., Ezaki, J., Ueno, T., Kominami, E., Yamane, T., Tanaka, K., and Komatsu, M. 2008. Structural basis for sorting mechanism of p62 in selective autophagy. *J. Biol. Chem.* 283: 22847–22857.
 22. Itakura, E., Kishi, C., Inoue, K., and Mizushima, N. 2008. Beclin 1 forms two distinct phosphatidylinositol 3-kinase complexes with mammalian Atg14 and UVRAG. *Mol. Biol. Cell* 19: 5360–5372.
 23. Itakura, E. and Mizushima, N. 2011. p62 targeting to the autophagosome formation site requires self-oligomerization but not LC3 binding. *J. Cell Biol.* 192: 17–27.
 24. Jain, A., Lamark, T., Sjøttem, E., Larsen, K.B., Awuh, J.A., Overvatn, A., McMahon, M., Hayes, J.D., and Johansen, T. 2011. p62/SQSTM1 is a target gene for transcription factor NRF2 and creates a positive feedback loop by inducing antioxidant response element-driven gene transcription. *J. Biol. Chem.* 285: 22576–22591.
 25. Jin, Z., Li, Y., Pitti, R., Lawrence, D., Pham, V.C., Lill, J.R., and Ashkenazi, A. 2009. Cullin3-based polyubiquitination and p62-dependent aggregation of caspase-8 mediate extrinsic apoptosis signaling. *Cell* 137: 721–735.
 26. Johansen, T. and Lamark, T. 2011. Selective autophagy mediated by autophagic adapter proteins. *Autophagy* 7: 279–296.
 27. Jung, C.H., Jun, C.B., Ro, S.H., Kim, Y.M., Otto, N.M., Cao, J., Kundu, M., and Kim, D.H. 2009. ULK-Atg13-FIP200 complexes mediate mTOR signaling to the autophagy machinery. *Mol. Biol. Cell* 20: 1992–2003.
 28. Jung, H.S., Chung, K.W., Won Kim, J., Kim, J., Komatsu, M., Tanaka, K., Nguyen, Y.H., Kang, T.M., Yoon, K.H., Kim, J.W., Jeong, Y.T., Han, M.S., Lee, M.K., Kim, K.W., Shin, J., and Lee, M.S. 2008. Loss of autophagy diminishes pancreatic beta cell mass and function with resultant hyperglycemia. *Cell Metab.* 8: 318–324.
 29. Kabeya, Y., Mizushima, N., Ueno, T., Yamamoto, A., Kirisako, T., Noda, T., Kominami, E., Ohsumi, Y., and Yoshimori, T. 2000. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *EMBO J.* 19: 5720–5728.
 30. Kanki, T., Wang, K., Cao, Y., Baba, M., and Klionsky, D.J. 2009. Atg32 is a mitochondrial protein that confers selectivity during mitophagy. *Dev. Cell* 17: 98–109.
 31. Kim, P.K., Hailey, D.W., Mullen, R.T., and Lippincott-Schwartz, J. 2008. Ubiquitin signals autophagic degradation of cytosolic proteins and peroxisomes. *Proc. Natl. Acad. Sci. U.S.A.* 105: 20567–20574.
 32. Kimura, S., Fujita, N., Noda, T., and Yoshimori, T. 2009. Monitoring autophagy in mammalian cultured cells through the dynamics of LC3. *Methods Enzymol.* 452: 1–12.
 33. Kirisako, T., Ichimura, Y., Okada, H., Kabeya, Y., Mizushima, N., Yoshimori, T., Ohsumi, M., Takao, T., Noda, T., and Ohsumi, Y. 2000. The reversible modification regulates the membrane-binding state of Apg8/Aut7 essential for autophagy and the cytoplasm to vacuole targeting pathway. *J. Cell Biol.* 151: 263–276.
 34. Kirkin, V., Lamark, T., Sou, Y.S., Bjorkoy, G., Nunn, J.L., Bruun, J.A., Shvets, E., McEwan, D.G., Clausen, T.H., Wild, P., Bilusic, I., Theurillat, J.P., Overvatn, A., Ishii, T., Elazar, Z., Komatsu, M., Dikic, I., and Johansen, T. 2009. A role for NBR1 in autophagosomal degradation of ubiquitinated substrates. *Mol. Cell* 33: 505–516.
 35. Kirkin, V., McEwan, D.G., Novak, I., and Dikic, I. 2009. A role for ubiquitin in selective autophagy. *Mol. Cell* 34: 259–269.
 36. Komatsu, M. and Ichimura, Y. 2010. Physiological significance of selective degradation of p62 by autophagy. *FEBS Lett.* 584: 1374–1378.
 37. Komatsu, M., Kurokawa, H., Waguri, S., Taguchi, K., Kobayashi, A., Ichimura, Y., Sou, Y.S., Ueno, I., Sakamoto, A., Tong, K.I., Kim, M., Nishito, Y., Iemura, S., Natsume, T., Ueno, T., Kominami, E., Motohashi, H., Tanaka, K., and Yamamoto, M. 2010. The selective autophagy substrate p62 activates the stress responsive transcription factor Nrf2 through inactivation of Keap1. *Nat. Cell Biol.* 12: 213–223.
 38. Komatsu, M., Waguri, S., Chiba, T., Murata, S., Iwata, J., Tanida, I., Ueno, T., Koike, M., Uchiyama, Y., Kominami, E., and Tanaka, K. 2006. Loss of autophagy in the central nervous system causes neurodegeneration in mice. *Nature* 441: 880–884.
 39. Komatsu, M., Waguri, S., Koike, M., Sou, Y.S., 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. 2007. Homeostatic levels of p62 control cytoplasmic inclusion body formation in autophagy-deficient mice. *Cell* 131: 1149–1163.
 40. Komatsu, M., Waguri, S., Ueno, T., Iwata, J., Murata, S., Tanida, I., Ezaki, J., Mizushima, N., Ohsumi, Y., Uchiyama, Y., Kominami, E., Tanaka, K., and Chiba, T. 2005. Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice. *J. Cell Biol.* 169: 425–434.
 41. Komatsu, M., Wang, Q.J., Holstein, G.R., Friedrich, V.L. Jr., Iwata, J., Kominami, E., Chait, B.T., Tanaka, K., and

- Yue, Z. 2007. Essential role for autophagy protein Atg7 in the maintenance of axonal homeostasis and the prevention of axonal degeneration. *Proc. Natl. Acad. Sci. U.S.A.* 104: 14489–14494.
42. Kraft, C., Peter, M., and Hofmann, K. 2010. Selective autophagy: ubiquitin-mediated recognition and beyond. *Nat. Cell Biol.* 12: 836–841.
43. Kuma, A., Hatano, M., Matsui, M., Yamamoto, A., Nakaya, H., Yoshimori, T., Ohsumi, Y., Tokuhisa, T., and Mizushima, N. 2004. The role of autophagy during the early neonatal starvation period. *Nature* 432: 1032–1036.
44. Kuma, A., Mizushima, N., Ishihara, N., and Ohsumi, Y. 2002. 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.
45. Kuusisto, E., Salminen, A., and Alafuzoff, I. 2001. Ubiquitin-binding protein p62 is present in neuronal and glial inclusions in human tauopathies and synucleinopathies. *Neuroreport* 12: 2085–2090.
46. Lamark, T., Perander, M., Outzen, H., Kristiansen, K., Overvatn, A., Michaelsen, E., Bjorkoy, G., and Johansen, T. 2003. Interaction codes within the family of mammalian Phox and Bem1p domain-containing proteins. *J. Biol. Chem.* 278: 34568–34581.
47. Lau, A., Wang, X.J., Zhao, F., Villeneuve, N.F., Wu, T., Jiang, T., Sun, Z., White, E., and Zhang, D.D. 2011. A noncanonical mechanism of Nrf2 activation by autophagy deficiency: direct interaction between Keap1 and p62. *Mol. Cell Biol.* 30: 3275–3285.
48. Laurin, N., Brown, J.P., Morissette, J., and Raymond, V. 2002. Recurrent mutation of the gene encoding sequestosome 1 SQSTM1/p62 in Paget disease of bone. *Am. J. Hum. Genet.* 70: 1582–1588.
49. Levine, B. and Klionsky, D.J. 2004. Development by self-digestion: molecular mechanisms and biological functions of autophagy. *Dev. Cell* 6: 463–477.
50. Lynch-Day, M.A. and Klionsky, D.J. 2010. The Cvt pathway as a model for selective autophagy. *FEBS Lett.* 584: 1359–1366.
51. Mari, M., Griffith, J., Rieter, E., Krishnappa, L., Klionsky, D.J., and Reggiori, F. 2010. An Atg9-containing compartment that functions in the early steps of autophagosome biogenesis. *J. Cell Biol.* 190: 1005–1022.
52. Masiero, E., Agatea, L., Mammucari, C., Blaauw, B., Loro, E., Komatsu, M., Metzger, D., Reggiani, C., Schiaffino, S., and Sandri, M. 2009. Autophagy is required to maintain muscle mass. *Cell Metab.* 10: 507–515.
53. Mathew, R., Karp, C.M., Beaudoin, B., Vuong, N., Chen, G., Chen, H.Y., Bray, K., Reddy, A., Bhanot, G., Gelinas, C., Dipaola, R.S., Karantza-Wadsworth, V., and White, E. 2009. Autophagy suppresses tumorigenesis through elimination of p62. *Cell* 137: 1062–1075.
54. Matsuda, N., Sato, S., Shiba, K., Okatsu, K., Saisho, K., Gautier, C.A., Sou, Y.S., Saiki, S., Kawajiri, S., Sato, F., Kimura, M., Komatsu, M., Hattori, N., and Tanaka, K. 2010. PINK1 stabilized by mitochondrial depolarization recruits Parkin to damaged mitochondria and activates latent Parkin for mitophagy. *J. Cell Biol.* 189: 211–221.
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. 2009. Two Beclin 1-binding proteins, Atg14L and Rubicon, reciprocally regulate autophagy at different stages. *Nat. Cell Biol.* 11: 385–396.
56. Melendez, A., Tallozy, Z., Seaman, M., Eskelinen, E.L., Hall, D.H., and Levine, B. 2003. Autophagy genes are essential for dauer development and life-span extension in *C. elegans*. *Science* 301: 1387–1391.
57. Miller, B.C., Zhao, Z., Stephenson, L.M., Cadwell, K., Pua, H.H., Lee, H.K., Mizushima, N., Iwasaki, A., He, Y.W., Swat, W., and Virgin, H.W. 4th. 2008. The autophagy gene ATG5 plays an essential role in B lymphocyte development. *Autophagy* 4: 309–314.
58. Mizushima, N., Kuma, A., Kobayashi, Y., Yamamoto, A., Matsubae, M., Takao, T., Natsume, T., Ohsumi, Y., and Yoshimori, T. 2003. Mouse Apg16L, a novel WD-repeat protein, targets to the autophagic isolation membrane with the Apg12-Apg5 conjugate. *J. Cell Sci.* 116: 1679–1688.
59. Mizushima, N. and Levine, B. 2010. Autophagy in mammalian development and differentiation. *Nat. Cell Biol.* 12: 823–830.
60. Mizushima, N., Levine, B., Cuervo, A.M., and Klionsky, D.J. 2008. Autophagy fights disease through cellular self-digestion. *Nature* 451: 1069–1075.
61. Mizushima, N., Noda, T., Yoshimori, T., Tanaka, Y., Ishii, T., George, M.D., Klionsky, D.J., Ohsumi, M., and Ohsumi, Y. 1998. A protein conjugation system essential for autophagy. *Nature* 395: 395–398.
62. Mizushima, N., Sugita, H., Yoshimori, T., and Ohsumi, Y. 1998. A new protein conjugation system in human. The counterpart of the yeast Apg12p conjugation system essential for autophagy. *J. Biol. Chem.* 273: 33889–33892.
63. Mizushima, N., Yamamoto, A., Hatano, M., Kobayashi, Y., Kabeya, Y., Suzuki, K., Tokuhisa, T., Ohsumi, Y., and Yoshimori, T. 2001. Dissection of autophagosome formation using Apg5-deficient mouse embryonic stem cells. *J. Cell Biol.* 152: 657–668.
64. Mizushima, N., Yamamoto, A., Matsui, M., Yoshimori, T., and Ohsumi, Y. 2004. In vivo analysis of autophagy in response to nutrient starvation using transgenic mice expressing a fluorescent autophagosome marker. *Mol. Biol. Cell* 15: 1101–1111.
65. Mortensen, M., Ferguson, D.J., Edelmann, M., Kessler, B., Morten, K.J., Komatsu, M., and Simon, A.K. 2010. Loss of autophagy in erythroid cells leads to defective removal of mitochondria and severe anemia in vivo. *Proc. Natl. Acad. Sci. U.S.A.* 107: 832–837.
66. Moscat, J. and Diaz-Meco, M.T. 2009. p62 at the crossroads of autophagy, apoptosis, and cancer. *Cell* 137: 1001–1004.
67. Nakai, A., Yamaguchi, O., Takeda, T., Higuchi, Y., Hikoso, S., Taniike, M., Omiya, S., Mizote, I., Matsumura, Y., Asahi, M., Nishida, K., Hori, M., Mizushima, N., and Otsu, K. 2007. The role of autophagy in cardiomyocytes in the basal state and in response to hemodynamic stress. *Nat. Med.* 13: 619–624.

68. Nakatogawa, H., Ichimura, Y., and Ohsumi, Y. 2007. Atg8, a ubiquitin-like protein required for autophagosome formation, mediates membrane tethering and hemifusion. *Cell* 130: 165–178.
69. Nakatogawa, H., Suzuki, K., Kamada, Y., and Ohsumi, Y. 2009. Dynamics and diversity in autophagy mechanisms: lessons from yeast. *Nat. Rev. Mol. Cell Biol.* 10: 458–467.
70. Narendra, D., Kane, L.A., Hauser, D.N., Fearnley, I.M., and Youle, R.J. 2010. p62/SQSTM1 is required for Parkin-induced mitochondrial clustering but not mitophagy; VDAC1 is dispensable for both. *Autophagy* 6: 1090–1106.
71. Narendra, D., Tanaka, A., Suen, D.F., and Youle, R.J. 2008. Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. *J. Cell Biol.* 183: 795–803.
72. Narendra, D.P., Jin, S.M., Tanaka, A., Suen, D.F., Gautier, C.A., Shen, J., Cookson, M.R., and Youle, R.J. 2010. PINK1 is selectively stabilized on impaired mitochondria to activate Parkin. *PLoS Biol.* 8: e1000298.
73. Nemoto, T., Tanida, I., Tanida-Miyake, E., Minematsu-Ikeguchi, N., Yokota, M., Ohsumi, M., Ueno, T., and Kominami, E. 2003. The mouse APG10 homologue, an E2-like enzyme for Apg12p conjugation, facilitates MAP-LC3 modification. *J. Biol. Chem.* 278: 39517–39526.
74. Nezis, I.P., Simonsen, A., Sagana, A.P., Finley, K., Gaumer, S., Contamine, D., Rusten, T.E., Stenmark, H., and Brech, A. 2008. Ref2P, the *Drosophila melanogaster* homologue of mammalian p62, is required for the formation of protein aggregates in adult brain. *J. Cell Biol.* 180: 1065–1071.
75. Nishiyama, J., Miura, E., Mizushima, N., Watanabe, M., and Yuzaki, M. 2007. Aberrant membranes and double-membrane structures accumulate in the axons of Atg5-null Purkinje cells before neuronal death. *Autophagy* 3: 591–596.
76. Noda, N.N., Kumeta, H., Nakatogawa, H., Satoo, K., Adachi, W., Ishii, J., Fujioka, Y., Ohsumi, Y., and Inagaki, F. 2008. Structural basis of target recognition by Atg8/LC3 during selective autophagy. *Genes Cells* 13: 1211–1218.
77. Novak, I., Kirkin, V., McEwan, D.G., Zhang, J., Wild, P., Rozenknop, A., Rogov, V., Lohr, F., Popovic, D., Occhipinti, A., Reichert, A.S., Terzic, J., Dotsch, V., Ney, P.A., and Dikic, I. 2010. Nix is a selective autophagy receptor for mitochondrial clearance. *EMBO Rep.* 11: 45–51.
78. Okamoto, K., Kondo-Okamoto, N., and Ohsumi, Y. 2009. Mitochondria-anchored receptor Atg32 mediates degradation of mitochondria via selective autophagy. *Dev. Cell* 17: 87–97.
79. Okatsu, K., Saisho, K., Shimanuki, M., Nakada, K., Shitara, H., Sou, Y.S., Kimura, M., Sato, S., Hattori, N., Komatsu, M., Tanaka, K., and Matsuda, N. 2010. p62/SQSTM1 cooperates with Parkin for perinuclear clustering of depolarized mitochondria. *Genes Cells* 15: 887–900.
80. Okawa, H., Motohashi, H., Kobayashi, A., Aburatani, H., Kensler, T.W., and Yamamoto, M. 2006. Hepatocyte-specific deletion of the *keap1* gene activates Nrf2 and confers potent resistance against acute drug toxicity. *Biochem. Biophys. Res. Commun.* 339: 79–88.
81. Otto, G.P., Wu, M.Y., Kazgan, N., Anderson, O.R., and Kessin, R.H. 2003. Macroautophagy is required for multicellular development of the social amoeba *Dictyostelium discoideum*. *J. Biol. Chem.* 278: 17636–17645.
82. Pankiv, S., Clausen, T.H., Lamark, T., Brech, A., Bruun, J.A., Outzen, H., Overvatn, A., Bjorkoy, G., and Johansen, T. 2007. p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. *J. Biol. Chem.* 282: 24131–24145.
83. Pohl, C. and Jentsch, S. 2009. Midbody ring disposal by autophagy is a post-abscission event of cytokinesis. *Nat. Cell Biol.* 11: 65–70.
84. Pua, H.H., Dzhagalov, I., Chuck, M., Mizushima, N., and He, Y.W. 2007. A critical role for the autophagy gene Atg5 in T cell survival and proliferation. *J. Exp. Med.* 204: 25–31.
85. Pua, H.H., Guo, J., Komatsu, M., and He, Y.W. 2009. Autophagy is essential for mitochondrial clearance in mature T lymphocytes. *J. Immunol.* 182: 4046–4055.
86. Qu, X., Yu, J., Bhagat, G., Furuya, N., Hibshoosh, H., Troxel, A., Rosen, J., Eskelinen, E.L., Mizushima, N., Ohsumi, Y., Cattoretti, G., and Levine, B. 2003. Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. *J. Clin. Invest.* 112: 1809–1820.
87. Raben, N., Hill, V., Shea, L., Takikita, S., Baum, R., Mizushima, N., Ralston, E., and Plotz, P. 2008. Suppression of autophagy in skeletal muscle uncovers the accumulation of ubiquitinated proteins and their potential role in muscle damage in Pompe disease. *Hum. Mol. Genet.* 17: 3897–3908.
88. Reggiori, F., Tucker, K.A., Stromhaug, P.E., and Klionsky, D.J. 2004. The Atg1-Atg13 complex regulates Atg9 and Atg23 retrieval transport from the pre-autophagosomal structure. *Dev. Cell* 6: 79–90.
89. Riley, B.E., Kaiser, S.E., Shaler, T.A., Ng, A.C., Hara, T., Hipp, M.S., Lage, K., Xavier, R.J., Ryu, K.Y., Taguchi, K., Yamamoto, M., Tanaka, K., Mizushima, N., Komatsu, M., and Kopito, R.R. 2010. Ubiquitin accumulation in autophagy-deficient mice is dependent on the Nrf2-mediated stress response pathway: a potential role for protein aggregation in autophagic substrate selection. *J. Cell Biol.* 191: 537–552.
90. Saitoh, T., Fujita, N., Hayashi, T., Takahara, K., Satoh, T., Lee, H., Matsunaga, K., Kageyama, S., Omori, H., Noda, T., Yamamoto, N., Kawai, T., Ishii, K., Takeuchi, O., Yoshimori, T., and Akira, S. 2009. Atg9a controls dsDNA-driven dynamic translocation of STING and the innate immune response. *Proc. Natl. Acad. Sci. U. S. A.* 106: 20842–20846.
91. Saitoh, T., Fujita, N., Jang, M.H., Uematsu, S., Yang, B.G., Satoh, T., Omori, H., Noda, T., Yamamoto, N., Komatsu, M., Tanaka, K., Kawai, T., Tsujimura, T., Takeuchi, O., Yoshimori, T., and Akira, S. 2008. Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1 β production. *Nature* 456: 264–268.
92. Sandoval, H., Thiagarajan, P., Dasgupta, S.K., Schumacher, A., Prechal, J.T., Chen, M., and Wang, J. 2008. Essential role for Nix in autophagic maturation of erythroid cells. *Nature* 454: 232–235.
93. Sanz, L., Diaz-Meco, M.T., Nakano, H., and Moscat, J. 2000. The atypical PKC-interacting protein p62 channels NF- κ B activation by the IL-1-TRAF6 pathway. *EMBO J.* 19: 1576–1586.

94. Schweers, R.L., Zhang, J., Randall, M.S., Loyd, M.R., Li, W., Dorsey, F.C., Kundu, M., Opferman, J.T., Cleveland, J.L., Miller, J.L., and Ney, P.A. 2007. NIX is required for programmed mitochondrial clearance during reticulocyte maturation. *Proc. Natl. Acad. Sci. U.S.A.* 104: 19500–19505.
95. Sekito, T., Kawamata, T., Ichikawa, R., Suzuki, K., and Ohsumi, Y. 2009. Atg17 recruits Atg9 to organize the pre-autophagosomal structure. *Genes Cells* 14: 525–538.
96. Shintani, T., Mizushima, N., Ogawa, Y., Matsuura, A., Noda, T., and Ohsumi, Y. 1999. Apg10p, a novel protein-conjugating enzyme essential for autophagy in yeast. *EMBO J.* 18: 5234–5241.
97. Shvets, E., Fass, E., Scherz-Shouval, R., and Elazar, Z. 2008. The N-terminus and Phe52 residue of LC3 recruit p62/SQSTM1 into autophagosomes. *J. Cell Sci.* 121: 2685–2695.
98. Simonsen, A., Birkeland, H.C., Gillooly, D.J., Mizushima, N., Kuma, A., Yoshimori, T., Slagsvold, T., Brech, A., and Stenmark, H. 2004. Alfz, a novel FYVE-domain-containing protein associated with protein granules and autophagic membranes. *J. Cell Sci.* 117: 4239–4251.
99. Singh, R., Xiang, Y., Wang, Y., Baikati, K., Cuervo, A.M., Luu, Y.K., Tang, Y., Pessin, J.E., Schwartz, G.J., and Czaja, M.J. 2009. Autophagy regulates adipose mass and differentiation in mice. *J. Clin. Invest.* 119: 3329–3339.
100. Sou, Y.S., 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. 2008. The Atg8 conjugation system is indispensable for proper development of autophagic isolation membranes in mice. *Mol. Biol. Cell* 19: 4762–4775.
101. Stephenson, L.M., Miller, B.C., Ng, A., Eisenberg, J., Zhao, Z., Cadwell, K., Graham, D.B., Mizushima, N., Xavier, R., Virgin, H.W. 4th., and Swat, W. 2009. Identification of Atg5-dependent transcriptional changes and increases in mitochondrial mass in Atg5-deficient T lymphocytes. *Autophagy* 5: 625–635.
102. Stumptner, C., Fuchsichler, A., Heid, H., Zatloukal, K., and Denk, H. 2002. Mallory body--a disease-associated type of sequestosome. *Hepatology* 35: 1053–1062.
103. Sun, Q., Fan, W., Chen, K., Ding, X., Chen, S., and Zhong, Q. 2008. Identification of Barkor as a mammalian autophagy-specific factor for Beclin 1 and class III phosphatidylinositol 3-kinase. *Proc. Natl. Acad. Sci. U.S.A.* 105: 19211–19216.
104. Tanida, I., Mizushima, N., Kiyooka, M., Ohsumi, M., Ueno, T., Ohsumi, Y., and Kominami, E. 1999. Apg7p/Cvt2p: a novel protein-activating enzyme essential for autophagy. *Mol. Biol. Cell* 10: 1367–1379.
105. Tanida, I., Tanida-Miyake, E., Komatsu, M., Ueno, T., and Kominami, E. 2002. 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.
106. Tanida, I., Ueno, T., and Kominami, E. 2004. 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.
107. Tsukada, M. and Ohsumi, Y. 1993. Isolation and characterization of autophagy-defective mutants of *Saccharomyces cerevisiae*. *FEBS Lett.* 333: 169–174.
108. Tsukamoto, S., Kuma, A., Murakami, M., Kishi, C., Yamamoto, A., and Mizushima, N. 2008. Autophagy is essential for preimplantation development of mouse embryos. *Science* 321: 117–120.
109. Vives-Bauza, C., Zhou, C., Huang, Y., Cui, M., de Vries, R. L., Kim, J., May, J., Tocilescu, M.A., Liu, W., Ko, H.S., Magrane, J., Moore, D.J., Dawson, V.L., Grailhe, R., Dawson, T.M., Li, C., Tieu, K., and Przedborski, S. 2010. PINK1-dependent recruitment of Parkin to mitochondria in mitophagy. *Proc. Natl. Acad. Sci. U.S.A.* 107: 378–383.
110. Wang, C.W., Kim, J., Huang, W.P., Abeliovich, H., Stromhaug, P.E., Dunn, W.A. Jr., and Klionsky, D.J. 2001. Apg2 is a novel protein required for the cytoplasm to vacuole targeting, autophagy, and pexophagy pathways. *J. Biol. Chem.* 276: 30442–30451.
111. Webber, J.L. and Tooze, S.A. 2010. New insights into the function of Atg9. *FEBS Lett.* 584: 1319–1326.
112. Xie, Z. and Klionsky, D.J. 2007. Autophagosome formation: core machinery and adaptations. *Nat. Cell Biol.* 9: 1102–1109.
113. Yen, W.L., Legakis, J.E., Nair, U., and Klionsky, D.J. 2007. Atg27 is required for autophagy-dependent cycling of Atg9. *Mol. Biol. Cell* 18: 581–593.
114. Young, A.R., Chan, E.Y., Hu, X.W., Kochl, R., Crawshaw, S.G., High, S., Hailey, D.W., Lippincott-Schwartz, J., and Tooze, S.A. 2006. Starvation and ULK1-dependent cycling of mammalian Atg9 between the TGN and endosomes. *J. Cell Sci.* 119: 3888–3900.
115. Yue, Z., Jin, S., Yang, C., Levine, A.J., and Heintz, N. 2003. Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor. *Proc. Natl. Acad. Sci. U.S.A.* 100: 15077–15082.
116. Zatloukal, K., Stumptner, C., Fuchsichler, A., Heid, H., Schnoelzer, M., Kenner, L., Kleinert, R., Prinz, M., Aguzzi, A., and Denk, H. 2002. p62 Is a common component of cytoplasmic inclusions in protein aggregation diseases. *Am. J. Pathol.* 160: 255–263.
117. Zhang, Y., Goldman, S., Baerga, R., Zhao, Y., Komatsu, M., and Jin, S. 2009. Adipose-specific deletion of autophagy-related gene 7 atg7 in mice reveals a role in adipogenesis. *Proc. Natl. Acad. Sci. U.S.A.* 106: 19860–19865.
118. Zheng, Y.T., Shahnazari, S., Brech, A., Lamark, T., Johansen, T., and Brumell, J.H. 2009. The adaptor protein p62/SQSTM1 targets invading bacteria to the autophagy pathway. *J. Immunol.* 183: 5909–5916.
119. Zhong, Y., Wang, Q.J., Li, X., Yan, Y., Backer, J.M., Chait, B.T., Heintz, N., and Yue, Z. 2009. Distinct regulation of autophagic activity by Atg14L and Rubicon associated with Beclin 1-phosphatidylinositol-3-kinase complex. *Nat. Cell Biol.* 11: 468–476.