Molecular Cell
Review

A Role for Ubiquitin in Selective Autophagy

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DOI 10.1016/j.molcel.2009.04.026

Ubiquitination is the hallmark of protein degradation by the 26S proteasome. However, the proteasome is limited in its capacity to degrade oligomeric and aggregated proteins. Removal of harmful protein aggregates is mediated by autophagy, a mechanism by which the cell sequesters cytosolic cargo and delivers it for degradation by the lysosome. Identification of autophagy receptors, such as p62/SQSTM1 and NBR1, which simultaneously bind both ubiquitin and autophagy-specific ubiquitin-like modifiers, LC3/GABARAP, has provided a molecular link between ubiquitination and autophagy. This review explores the hypothesis that ubiquitin represents a selective degradation signal suitable for targeting various types of cargo, ranging from protein aggregates to membrane-bound organelles and microbes.

Introduction

Ubiquitin (Ub) is a small protein highly conserved from yeast to man. It was first described in the context of protein degradation, but later shown to participate in regulation of other cellular processes, such as endocytosis, signal transduction, and DNA repair (Kirkin and Dikic, 2007; Welchman et al., 2005) (Table 1). Conjugation of Ub is a complex reaction that requires E1, E2, and E3 enzymes and leads to the formation of an isopeptide bond between the C-terminal Gly of Ub and the ε-amino group of a Lys residue of the substrate protein (Hershko and Ciechanover, 1998). Due to the presence of internal Lys residues, Ub can repeatedly be attached to itself, forming chains with various topologies and functions (Ikeda and Dikic, 2008). Modification of a protein with Ub chains (polyubiquitination), in which Ub is linked via Lys48, targets the substrate to the proteasomal pathway, while attachment of a single Ub moiety (monoubiquitination) and/or oligomeric Lys63-linked Ub chains, i.e., during the process of endocytosis, marks substrate proteins for degradation in lysosomes (Welchman et al., 2005). Recent experimental data have provided evidence for the involvement of Ub in yet another fundamental lysosome-dependent degradation system, autophagy. This catabolic pathway, capable of targeting individual proteins, larger macromolecular complexes, and complete organelles, is of great importance for cellular homeostasis and survival, while its deregulation has been linked to pathological conditions, such as neurodegeneration and cancer (Ohsumi, 2001; Xie and Klionsky, 2007; Levine and Kroemer, 2008).

The generic term "autophagy" comprises several processes by which the lysosome acquires cytosolic cargo, with three types of autophagy being discerned in the literature: (1) macroautophagy, characterized by the formation of a crescent-shaped structure (the phagophore) that expands to form the double-membrane autophagosome, capable of fusion with the lysosome; (2) microautophagy, in which lysosomes invaginate and directly sequester cytosolic components; and (3) chaperone-mediated autophagy (CMA), which involves translocation of unfolded proteins across the lysosomal membrane (Mizushima et al., 2008). As the main focus of this review is on the process of macroautophagy, we will refer to it throughout the text as simply autophagy.

While autophagosomes can sequester cytosolic material nonspecifically, for example, as a response to starvation, there is ample evidence for selective autophagic degradation of various cellular structures, including protein aggregates, mitochondria, and microbes (Xie and Klionsky, 2007). The mechanism of selective autophagy is not well understood; however, the involvement of Ub in this process is evident: analogous to the proteasome, where ubiquitinated cargo is delivered by Ub receptors (Elsasser and Finley, 2005; Husnjak et al., 2008), autophagic clearance of protein aggregates requires Ub-binding receptors p62 and NBR1 (Kirkin et al., 2009; Komatsu et al., 2007; Pankiv et al., 2007) (Figure 1 and 2). It is envisaged that by simultaneous binding to both Ub and the autophagosome-associated Ub-like (UBL) proteins (i.e., LC3/GABARAP proteins), these molecules can mediate docking of ubiquitinated protein aggregates to the autophagosome, thereby ensuring their selective degradation. Here, we summarize available evidence for the role of Ub in autophagy and postulate that attachment of Ub moieties to various cellular cargos constitutes a universal degradation signal recognized by two major intracellular proteolytic systems: the proteasome and the lysosome.

Ubiquitin in Autophagic Degradation of Protein Substrates

Protein Misfolding and Aggregation

Protein misfolding, which results in the exposure of hydrophobic residues normally confined within the folded proteins, accompanies normal protein synthesis but can be dramatically enhanced by numerous stimuli, including endoplasmatic reticulum and oxidative stress, starvation, mutation, and heat shock (Goldberg, 2003). Misfolded polypeptides are recognized by molecular chaperones of the heat shock protein (HSP) family, which bind to and shield exposed hydrophobic surfaces from the cytosol while promoting protein refolding (Goldberg, 2003). In addition, HSPs interact with Ub E3 ligases, such as CHIP and Parkin (Imai et al., 2002), which

Table 1. Properties of Yeast and Human Autophagy-Specific UBLs in Comparison to Ub

UBL		Precursor Processing Enzymes	Activating Enzyme	Conjugating Enzyme(s)	Ligating Enzymes	Conjugation Substrate	Deconjugation Enzymes	Known Functions
Yeast and human	Ub	DUBs	E1 enzyme	E2 enzymes	E3 ligases	various proteins	DUBs	acts as a signal for proteasomal and lysosomal degradation; provides a scaffold for signal transduction and DNA repair machineries
Yeast	ATG8	ATG4	ATG7	ATG3	ATG5-ATG12	PE	ATG4	(1) mediates expansion of the phagophore via membrane tethering and hemifusion; (2) acts as a ligand for autophagic receptors: ATG19 (Cvt pathway in yeast) and p62 and NBR1 (in selective autophagy in higher organisms)
Human	MAP1LC3A (LC3A) MAP1LC3B (LC3B) MAP1LC3C (LC3C) GABARAP GABARAPL-1 GABARAPL-2/GATE-16 GABARAPL-3	ATG4A ATG4B ATG4C ATG4D	ATG7	ATG3	ATG5-ATG12	PE	ATG4A ATG4B ATG4C ATG4D	
Yeast	ATG12	-	ATG7	ATG10	-	ATG5	-	as a conjugate with ATG5, has an E3-like activity toward ATG8 family proteins
Human	ATG12	-	ATG7	ATG10	-	ATG5	-	
Yeast	ATG5	-	-	-	-	-	-	(1) in complex with ATG16, acts as a scaffold for autophagosome formation; (2) as a conjugate with ATG12, has an E3-like activity toward ATG8 family proteins
Human	ATG5	-	-	-	-	-	-	

DUB, deubiquitinating enzymes; ATG, autophagy-related protein; PE, phosphatidylethanolamine; MAP1LC3, microtubule-associated proteins 1A/1B light chain 3; GABARAP, Gamma-amino-butyric acid receptor-associated protein; GABARAPL, Gamma-aminobutyric acid receptor-associated protein-like; GATE-16, Golgi-associated ATPase Enhancer of 16 kDa.

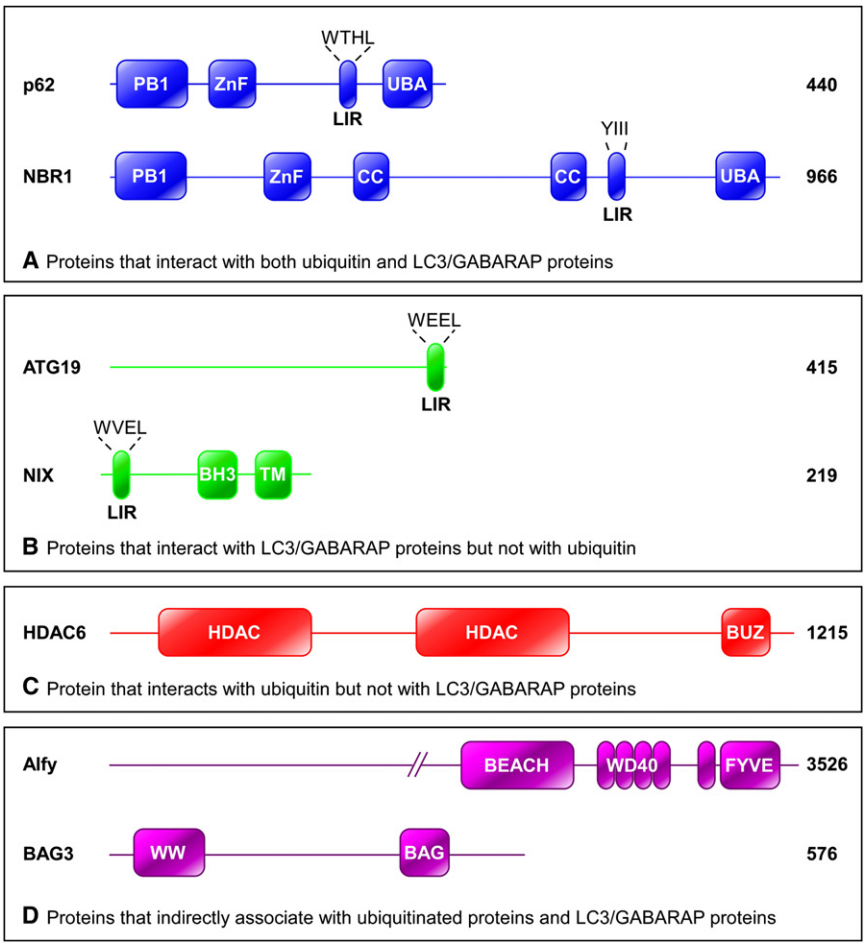

Figure 1. Proteins Involved in Selective Autophagy and Their Domain Organization.
(A) p62 and NBR1 are autophagy receptors that interact with both ubiquitin conjugated to the target and LC3/GABARAP on the autophagosome, thereby promoting autophagy of ubiquitinated targets.
(B) ATG19 is a receptor protein that interacts with both preApe1 aggregates and ATG8, thereby promoting delivery of preApe1 to the vacuole by the Cvt pathway in yeast. NIX is a mitochondrial protein that interacts with LC3/GABARAP and might participate in mitophagy.
(C) HDAC6 is a Ub-binding protein that plays a role in aggresome formation but also affects selective autophagy of ubiquitinated misfolded proteins; its precise role in autophagy is, however, not clear.
(D) ALFY is a large protein associated with both ubiquitinated proteins and autophagosomal markers; it interacts with PtdIns(3)P via the FYVE domain. See text for BAG3 description. Numbers indicate length of human proteins in amino acids with the exception of ATG19, which is a yeast protein. *BAG*, Bcl-2-associated athanogene 1 domain; BEACH, BEACH domain; BH3, Bcl-2 homology 3 domain; BUZ, ubiquitin-binding zinc finger; CC, coiled-coil domain; FYVE, Fab1, YOTB/ZK632.12, Vac1, and EEA1 domain; HDAC, histone deacetylase domain; LIR, LC3-interacting region; PB1, Phox and Bem1p domain; TM, transmembrane domain; UBA, Ub-associated domain; WD40, WD40 repeats; WW, WW domain; ZnF, Zinc finger domain.

promote substrate polyubiquitination and, thus, prime terminally misfolded polypeptides for degradation by the proteasome.

Protein aggregation occurs when protein misfolding is left unresolved by the chaperone-assisted refolding or proteasomal degradation. Polymerization of misfolded proteins is mediated by nonspecific hydrophobic interactions of partially unfolded polypeptide chains that can lead to the formation of microscopically visible structures known as inclusion bodies and aggresomes (Kopito, 2000) (Figure 2). For simplicity, inclusion bodies can be viewed as multiple intracellular foci into which misfolded protein oligomers are sequestered, whereas the aggresome is a structure formed via the retrograde transport of aggregated proteins on microtubules and is usually found at the microtubule organizing center (MTOC) (Kopito, 2000) (Figure 2). The biological role of protein inclusions is not entirely clear. They may play protective role by sequestering toxic misfolded protein species and providing the cell with an opportunity of delayed protein degradation (Bjorkoy et al., 2005; Kopito, 2000). Alternatively, they may inactivate the proteasome and mediate cytotoxicity (Bence et al., 2001). The dual role of inclusions highlights the vital importance of alternative degradation pathways that would be amenable to degradation of bulky substrates. Thus, inhibition of the proteasome potently induces autophagy, which serves as a compensatory mechanism for degradation of accumulating polyubiquitinated misfolded proteins.

The Autophagosome: The Door to Purgatory

The autophagosome can engulf bulky substrates, including membrane-bound organelles. Genetic analyses of yeast mutants with autophagic defects have identified more than 30 autophagy-related (ATG) proteins that govern generation and maturation of the autophagosome as well as its degradation in the vacuole (Suzuki and Ohsumi, 2007). The most important functional groups of ATG proteins, as illustrated by an extensive homology with mammalian genes, include: (1) ATG1 kinase complex (ATG1, ATG13 and ATG17); (2) PI3K complex (Vps34 and Atg6); and (3) two UBL systems (ATG12-ATG5 and ATG8 [Table 1]) (Suzuki and Ohsumi, 2007).

The autophagy-specific UBL proteins are of particular interest due to their similarity with Ub (Kirkin and Dikic, 2007) (Table 1). Thus, although ATG12 and ATG8 family proteins (known as LC3 and GABARAP proteins in mammalian cells) do not share any primary sequence homology with Ub, they structurally resemble Ub (Paz et al., 2000; Sugawara et al., 2004; Suzuki et al., 2005). They are also conjugated to their substrates, ATG5 and phosphatidylethanolamine (PE), respectively, via a C-terminal Gly residue (Ohsumi, 2001). Both ATG12 and ATG8 conjugation systems are required for autophagosome maturation. However, upon completion of this process, the ATG12-ATG5 complex dissociates from the autophagosomal membrane, while intravesicular ATG8 remains attached to the membrane through the fusion of the autophagosome with the lysosome (Ohsumi, 2001). This latter property has allowed the use of ATG8 and LC3 as

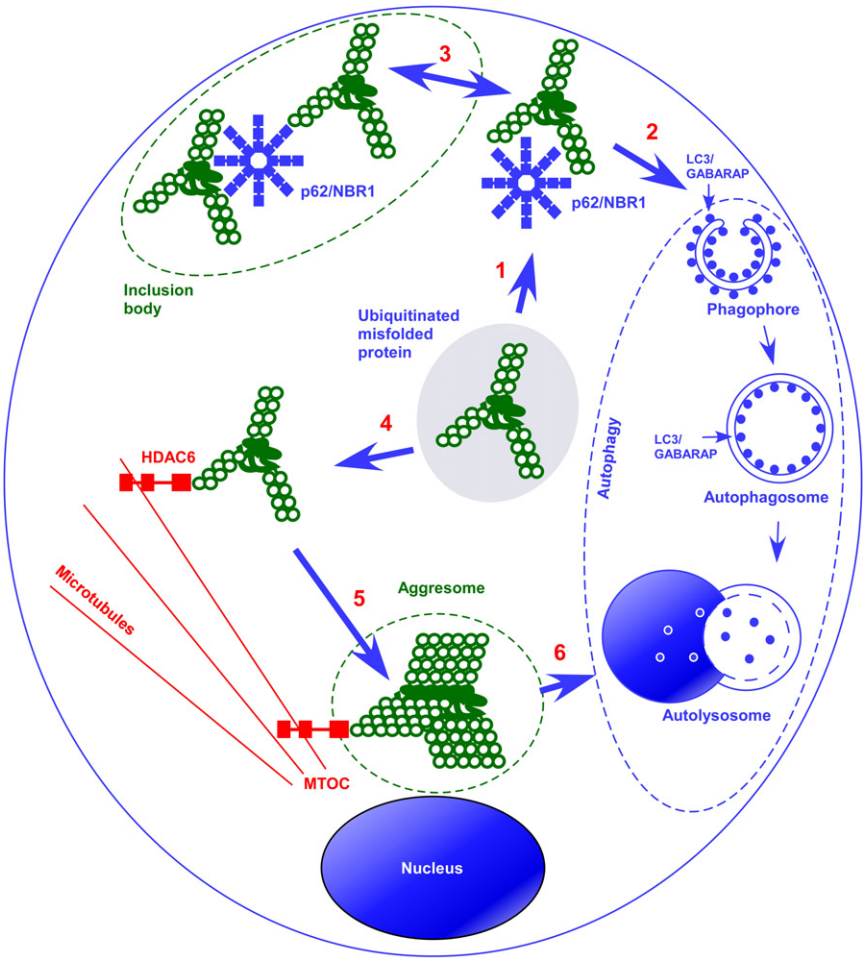

Figure 2. A Model for the Function of p62, NBR1, and HDAC6 Proteins in Selective Autophagy of Ubiquitinated Misfolded Proteins

Oligomerized misfolded proteins are ubiquitinated and recognized by the Ub-binding domain of oligomeric p62 and NBR1 proteins, drawn as spokes of a wheel (although polyUb chains are depicted, it is possible that monoubiquitination is sufficient for target recognition) (1), which target them for selective degradation by autophagy (2). Oligomeric p62 and NBR1 also mediate formation of proteinacious inclusion bodies (3). Binding of HDAC6 to ubiquitinated proteins ensures their transport along the microtubules toward the MTOC (4), where excess misfolded proteins can be organized into an aggresome (5). Inclusion bodies (3) and aggresomes (6) may allow autophagic degradation of stored misfolded proteins. Closed boxes, Ub-binding domains; empty circles, Ub; filled circles, conjugated LC3/GABARAP proteins.

autophagosomal markers (Klionsky et al., 2008). In the conjugated form, ATG8 can mediate tethering and hemifusion of lipid membranes (Nakatogawa et al., 2007). Therefore, ATG8 might constitute the driving force for the autophagosome expansion by tethering delivered lipid membrane components and facilitating their incorporation into the expanding phagophore.

Intimate involvement of UBLs in the autophagosome formation points at an interesting aspect of autophagosomal biology. The Ub fold is a widespread structural element in proteins involved in cell signaling, and the list of Ub-like proteins, existing both as small protein modifiers (classical UBLs) and as an integral part (domain) of larger proteins (Ub-like domain proteins, UDPs), has grown rapidly (Jentsch and Pyrowolakis, 2000; Kirkin and Dikic, 2007). Conjugated Ub moieties are recognized by Ub-binding domain (UBD)-containing proteins (Hicke et al., 2005), and similarly, we expect that autophagy-specific UBLs, via noncovalent interactions, may recruit components of signaling complexes to the nascent autophagosomes and/or provide a means of selectivity for certain types of autophagic cargo. Support for this hypothesis came from the identification of proteins that bind ATG8/LC3/GABARAP and facilitate selective degradation of misfolded and ubiquitinated proteins, p62 and NBR1 (Bjorkoy et al., 2005; Kirkin et al., 2009; Komatsu et al., 2007; Pankiv et al., 2007) (Figure 2).

Ubiquitin-Binding Proteins in Selective Autophagy

p62/SQSTM1—The Prototype Autophagic Receptor. p62/SQSTM1 is a multifunctional adaptor protein that has been implicated in cell signaling and differentiation (Moscat et al., 2007). It was originally characterized as an interaction partner of atypical protein kinase C (aPKC) (Puls et al., 1997; Sanchez et al., 1998), and this interaction was shown to be mediated by the conserved module found at the very N-terminus of p62, known as Phox and Bem1p (PB1) domain, which also mediates oligomerization of p62. p62 also possesses a zinc-finger domain and a C-terminal Ub-binding UBA domain (Figure 1). The UBA domain of p62 can bind both K48-linked and K63-linked Ub chains but with a higher affinity for K63 chains (Long et al., 2008; Tan et al., 2007; Wooten et al., 2008). The ability of p62 to interact with Ub has placed it into the focus of intense research. Thus, mutations in the UBA domain of p62 have been linked to the etiology of Paget's disease characterized by abnormal osteoclastogenic activity (Cavey et al., 2005). It is generally believed that the Ub-binding activity of p62 is essential for RANKL-NF-κB signaling that regulates osteoclastogenesis. However, the exact mechanism for the involvement of the p62-Ub interaction in the disease progression remains unclear. Further complicating the situation, p62 has been shown to affect NF-κB activation in an Ub/UBA-binding-independent manner (Moscat et al., 2007).

Several lines of evidence point at another important biological role of p62. Ub-positive inclusion bodies have been found positive for p62 (Nagaoka et al., 2004; Zatloukal et al., 2002). This prompted the Johansen group to hypothesize the involvement of p62 in autophagic clearance of aggregation-prone proteins with the idea that p62 might represent the missing link between the protein aggregation and autophagy. They were able to show that p62 was itself degraded by autophagy and formed

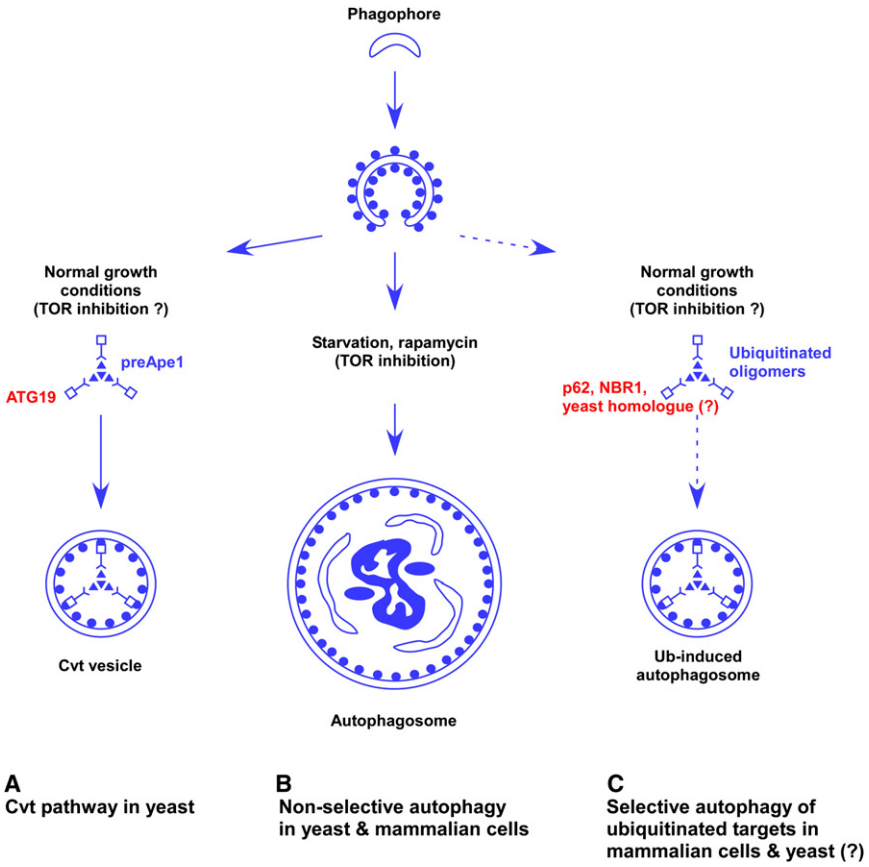

Figure 3. Comparison of the Yeast-Specific Cytosole-to-Vacuole (Cvt) Pathway with the Process of Nonselective Autophagy and the Selective Autophagy of Ubiquitinated Targets Mediated by p62 and NBR1

(A) Constitutive formation of smaller autophagosomes (Cvt vesicles) mediates delivery of cytosolic precursors of resident vacuolar hydrolyses, such as preApe1, and represents a form of selective autophagy in yeast cells. ATG19 interacts with both aggregated preApe1 and ATG8 on the autophagosome (filled circles).

(B) In contrast, larger autophagosomes are formed in yeast during starvation response, a nonselective process regulated by TOR kinase signaling.

(C) Similar to the Cvt pathway, ubiquitinated aggregates are recognized by specific receptors (like p62 and NBR1) and targeted to autophagosomes via their interaction with LC3/GABARAP proteins conjugated to the lipid membrane of the autophagosome (filled circles). The role of TOR signaling in selective autophagy has not been clarified. The broken arrows indicate the fact that this process has not formally been demonstrated in yeast cells—it, therefore, remains hypothetic.

a shell-like structure around experimentally-induced Ub-positive protein aggregates, which were also positive for LC3. This process was proposed to be dependent on the PB1 and UBA domains of p62 and, hence, on its ability to interact with itself and Ub (Bjorkoy et al., 2005). Subsequent experiments showed the physical interaction between p62 and LC3 (Komatsu et al., 2007; Pankiv et al., 2007) and LC3-Interacting Region (LIR) in human p62 was defined as a short linear peptide of 22 amino acids characterized by an acidic cluster (Asp-335, Asp-336, and Asp-337) and a conserved aromatic residue (Trp-340) (Pankiv et al., 2007).

Structural studies on the complex formed by the LC3 and the LIR of p62 showed that the WTHL tetrapeptide of the p62-LIR adopts a β-conformation and forms an intermolecular parallel β sheet with the β2-strand of LC3 (Ichimura et al., 2008; Noda et al., 2008). Importantly, the side chain of the conserved Trp in the WTHL motif binds deeply in the hydrophobic pocket formed at the interface between the N-terminal domain (α2) and the UBL domain of LC3. In contrast, the conserved Leu is engaged in the interaction with a hydrophobic pocket on the UBL domain itself (Ichimura et al., 2008; Noda et al., 2008). A similar motif (WEEL) was shown to be responsible for the interaction between ATG19 (Figure 1) and ATG8 in the Cvt pathway in yeast (Figure 3) (Noda et al., 2008), suggesting that the WxxL motif may define the LIR consensus sequence in otherwise unrelated ATG8/LC3-interacting proteins. This binding mode is reminiscent of the interaction between the SUMO-Interacting Motif (SIM) and SUMO involved in the regulation of transcription and nuclear protein import (Hecker et al., 2006).

LIR-deficient forms of p62 produce Ub-positive and LC3-negative aggregates in the cytoplasm of cells lacking endogenous p62. These protein aggregates also depend on the functional PB1 domain of p62, which underscores the relevance of the p62 oligomerization potential (Ichimura et al., 2008; Pankiv et al., 2007).

p62 is an LC3 interaction partner in vivo (Komatsu et al., 2007; Pankiv et al., 2007) and is constantly degraded by autophagy (Bjorkoy et al., 2005), establishing p62 as a useful marker for autophagic vesicle turnover (Klionsky et al., 2008). In support of this notion, mice with tissue-specific autophagy deficiency (*Atg7* knockout mice) demonstrate accumulation of p62 in Ub-positive protein inclusions in neurons and hepatocytes (Komatsu et al., 2007). Curiously, the autophagy inhibition-associated toxicity observed in the liver cells differs from that seen in the brain with respect to p62 dependence: whereas removal of *p62* alleviated liver impairment in $Atg7^{-/-}/p62^{-/-}$ mice, these mice still suffered from neuronal death (Komatsu et al., 2007). As in both cell types removal of p62 led to dramatic reduction in microscopically visible protein aggregates, there are likely to be cell-type-specific functions that contribute to p62-associated toxicity in autophagy-deficient tissues. Similar to autophagy-deficient mice, fruit flies deficient in autophagy develop inclusions in neurons, which disappear when *Drosophila*'s *p62* homolog *Ref(2)P* is deleted (Nezis et al., 2008), highlighting the evolutionarily conserved role of p62 in protein inclusion formation.

The aggregate-promoting properties of p62 may explain why autophagy-proficient *p62* knockout mice do not show strong accumulation of Ub-positive protein inclusions, as might be anticipated from the proposed role of p62 in mediating autophagic degradation of ubiquitinated proteins (Komatsu et al., 2007).

Yet, an age-dependent buildup of Ub in neurons of $p62^{-/-}$ and associated neurodegeneration were reported (Wooten et al., 2008). One explanation for the delayed onset of the apparent neuron-specific autophagy defects in *p62* knockout mice (in comparison to those seen in the *Atg7*- and *Atg5*-deficient mice [Hara et al., 2006; Komatsu et al., 2006]) may be the existence of functional homologs of p62 in higher organisms.

NBR1—The New Autophagy Receptor. NBR1 (Neighbor of BRCA1 gene 1) was originally cloned as a candidate gene for the ovarian cancer antigen CA125 (Campbell et al., 1994). Given the remarkable similarity in the domain organization (Figure 1) and direct interaction with p62, NBR1 has subsequently been studied in cell signaling and differentiation: in muscle cells, NBR1 is a signaling mediator downstream of the giant kinase titin (Lange et al., 2005). More recently, NBR1 was shown to be involved in autophagic degradation of ubiquitinated targets (Kirkin et al., 2009).

NBR1 was identified as a direct binding partner of the autophagosome-specific ATG8/LC3/GABARAP modifiers both in vitro and in vivo. Mutational analysis and peptide mapping studies indicated the presence of a LIR at the C terminus of NBR1, which comprises a modified WxxL motif (i.e., YIII, aa 732–735 in human NBR1) (Figure 1). The presence of the LIR ensures that, even in the absence of p62, NBR1 interacts with the autophagosome-associated UBLs and is degraded by autophagy. Inhibition of autophagy or deletion of the LIR domain leads to accumulation of NBR1 in vivo (Kirkin et al., 2009).

NBR1 binds Ub via its UBA domain with a bias toward the K63-linked polyUb chains (Kirkin et al., 2009). Importantly, recruitment of Ub-positive cargo into lysosomes is dependent on both p62 and NBR1. Like p62, NBR1 plays a role in cross-linking of ubiquitinated misfolded proteins and is necessary for puromycin-induced inclusion body formation and protein aggregation following a block in autophagy. NBR1 performs this function via self-association mediated by the coiled-coiled domain (Figure 1) and also via interaction with oligomeric p62 and ubiquitinated substrates (Figure 2). Interestingly, NBR1 is a component of the Mallory bodies, protein inclusions found in the liver of patients with alcoholic and nonalcoholic steatohepatitis (Kirkin et al., 2009).

HDAC6—A Ubiquitin- and Dynein-Binding Adaptor. Sequestration of misfolded, ubiquitinated proteins into aggregates may provide a protection mechanism, leading to reduced cytotoxicity and enhanced autophagic clearance of reactive protein species. However, when the rate of formation exceeds that of clearance, aggregates are delivered to MTOC-associated aggresomes. Therefore, aggresomes are thought to constitute a long-term repository of misfolded proteins en route for autophagic degradation (Figure 2). Since aggresomes frequently consist of ubiquitinated proteins, Ub-binding proteins may mediate their formation. The noncanonical histone deacetylase HDAC6 (Figure 1) has been implicated in this process (Matthias et al., 2008).

Experimentally induced Ub-positive aggresomes contained HDAC6, and siRNA-mediated depletion of HDAC6 compromised the aggresome formation, which could be rescued only by a Ub binding-competent form of HDAC6 (Kawaguchi et al., 2003). Curiously, this situation is reminiscent of p62 and NBR1, which are both recruited to Ub-positive aggregates and necessary for their formation. HDAC6 binds Ub via the C-terminal BUZ domain (Figure 1) that shows preference for K63-linked Ub chains (Olzmann et al., 2007). However, unlike p62 and NBR1, HDAC6 possesses no LIR. It interacts directly with dynein motors, which are necessary for transportation of aggregated misfolded proteins to the MTOC (Kawaguchi et al., 2003). The process of the active transport along the microtubules is essential for the aggresome formation, as microtubule-depolymerizing drugs efficiently inhibit this process (Rodriguez-Gonzalez et al., 2008). Importantly, lysosomes are enriched at the MTOC, and mature autophagosomes, like unwrapped protein aggregates, are transported along the microtubules for their fusion with lysosomes (Fass et al., 2006). Thus, aggresomes might allow concentration of all the necessary components for efficient autophagic clearance of aggregated proteins.

HDAC6 was required for autophagic degradation of mutant proteins during proteasomal inhibition. Here, HDAC6 influenced both the recruitment of autophagy-specific proteins to the aggregates and the lysosomal dynamics (Iwata et al., 2005; Pandey et al., 2007). In addition, aggresome formation and its autophagic degradation was dependent on deacetylase activity of HDAC6, which influenced microtubule dynamics (Iwata et al., 2005; Kawaguchi et al., 2003; Matthias et al., 2008; Pandey et al., 2007). Curiously, a very recent report defines acetylation of mutant huntingtin as a prerequisite for its autophagic degradation (Jeong et al., 2009). More work is necessary to understand the role of acetylation in protein degradation.

Other Proteins Involved in Selective Autophagy of Ubiquitinated Proteins. Besides proteins that directly bind Ub, there are several players that lack discernable UBDs but have been found associated with both ubiquitinated proteins and autophagosomal markers. One such protein is Alfy (Figure 1), a 400 kDa protein that contains the PtdIns(3)P-binding FYVE domain. Via this domain, Alfy may interact with autophagosomal membranes, as PtdIns(3)P is important in endosomal and autophagosomal membrane traffic (Simonsen et al., 2004). It is presently unknown how Alfy is recruited to ubiquitinated cargo.

Further, a senescence-activated Hsc/Hsp70 cochaperone BAG3 (Figure 1) has been proposed to mediate autophagic degradation of ubiquitinated proteins in aging cells (Gamerdinger et al., 2009). BAG3 colocalizes with p62-positive protein aggregates and autophagic markers and can stimulate autophagic flux. Intriguingly, although interacting with p62, BAG3 itself is not degraded by autophagy. In younger cells, BAG3 is poorly expressed, whereas the related cochaperone BAG1 plays a much more prominent role. In contrast to BAG3, BAG1 mediates proteasomal degradation of ubiquitinated proteins. It is thought that changes in the ratio between BAG1/BAG3 proteins might provide a mechanism for autophagic pathway activation during execution of the senescence program (Gamerdinger et al., 2009).

Ubiquitination at the Crossroads of the Autophagy and Proteasomal Pathways

Ubiquitinated cytosolic proteins undergo degradation via one of the two major routes: the proteasome or the lysosome. What determines whether a given Ub-labeled protein substrate will enter one or the other pathway? Classically, conjugation with K48-linked polyUb chains allows recognition of the proteolytic

substrate by UBD-containing proteasomal receptors, whereas the K63-linked chains have been associated with nonproteolytic functions of Ub (Welchman et al., 2005). However, more recently, K63-linkage has been implicated in proteolytic degradation of misfolded and aggregated proteins (Olzmann et al., 2007; Tan et al., 2008; Wooten et al., 2008). Given the reported preference of the known Ub-binding autophagy receptors for K63-linked Ub chains, K63 Ub chain-marked cargo may be preferentially targeted to the autophagy/lysosomal degradation pathway in vivo.

On the other hand, p62 has been shown to compete for ubiquitinated cargo with the classical proteasomal receptors. Inhibition of autophagy and accumulation of p62 slowed down degradation of regular (short-lived) proteasomal substrates, most likely due to the excessive interaction between stabilized oligomeric p62 and K48 Ub-conjugated substrates (Korolchuk et al., 2009). Intriguingly, p62 has also been suggested to directly interact with the proteasome (Seibenhener et al., 2004), which raises the question whether autophagy receptors may have a more direct role in proteasomal degradation of ubiquitinated proteins.

Although polyUb chains are most frequently associated with proteolytic degradation, monoubiquitination may be sufficient as a signal for selective autophagy. While monoUb-driven autophagy depends on p62 (Kim et al., 2008), low affinity of the p62 UBA domain toward monoUb (Kirkin et al., 2009; Long et al., 2008) suggests that other factors, such as substrate/receptor oligomerization, will play a role in selective degradation of monoubiquitinated cargo by autophagy.

Deubiquitination is an important step in the proteasomal degradation of polyubiquitinated proteins. Ub moieties are necessarily removed from the substrate protein prior to its insertion into the narrow opening of the proteasome. In contrast, the autophagosome is able to take up bulky substrates, ranging from protein aggregates to membrane-bound organelles, and deliver them for degradation in the lysosome. Yet, deubiquitination may be involved in autophagy to reduce bulkiness of a complex substrate and/or to allow Ub recycling. For instance, p62-dependent autophagic degradation of ubiquitinated midbody ring structure, which physically separates two daughter cells during cytokinesis, involves gradual loss of Ub signal (Pohl and Jentsch, 2009). The latter phenomenon may, however, be explained by slow degradation kinetic of this large structure, which might have allowed observation of its integral components long after Ub-specific signal had been degraded.

Mitophagy, Pexophagy, Ribophagy, Xenophagy: Many Names, One Mechanism?

The autophagosomal membrane is envisaged to enwrap structures of varying size and geometry. This property of the autophagosome ensures that highly complex cytosolic cargo, including ribosomes and mitochondria, is efficiently degraded by autophagy, for instance, during a starvation response (Klionsky et al., 2008). While forming autophagosomes can engulf substrate rather stochastically, mounting evidence suggests that, similar to ubiquitinated proteins, ribosomes, mitochondria, peroxisomes, and intracellular bacteria may all be selectively targeted for autophagic degradation.

Ubiquitin in Selective Degradation of Mitochondria

Mitochondria provide an important example of selective autophagy of organelles. Damage and loss of mitochondrial potential are proposed to lead to targeted autophagic degradation of this organelle, aptly named mitophagy (Elmore et al., 2001). Spectacular enveloping of fluorescently labeled mitochondria with GFP-LC3-positive autophagosomes during the starvation-induced mitophagy in hepatocytes derived from GFP-*LC3* transgenic mice provide compelling evidence for the existence of this process (Kim et al., 2007). Opening of the mitochondrial permeability transition (MPT) pore and subsequent rupture of the outer mitochondrial membrane is likely to cause release of as yet unknown autophagy-promoting factors. Several proteins found in yeast to be associated with mitochondrial membranes, such as Uth1p (Kissova et al., 2004) and Aup1 (Tal et al., 2007), are reported to be indispensable for the process of mitophagy. In mammals, mitochondria-localized BH3 domain-only protein BNIP3L/NIX (Figure 1) was shown to be critically involved in the entrapment of mitochondria by autophagosomes during the programmed elimination of mitochondria in maturing reticulocytes (Sandoval et al., 2008; Schweers et al., 2007).

In the absence of published evidence for a clearly defined signal for autophagic targeting of superfluous or damaged mitochondria, we consider the possibility that induced conjugation of monoUb/Ub chains to an exposed mitochondrial protein may provide the missing link between the mitochondrion and the autophagosome (Figure 4). E3 ligases, residing in or at the mitochondrial membranes (Chu et al., 2009; Li et al., 2008), may regulate this process. Alternatively, induced recruitment of cytosolic E3 ligases can also be envisaged. Recently, Parkin has been shown to be recruited to depolarized mitochondria and mediate their autophagic degradation (Narendra et al., 2008). It remains to be determined what the ubiquitinated substrates on the mitochondrial membranes are and whether the known autophagic receptors, p62 and NBR1, could provide the mechanistic link between mitochondrial depolarization, ubiquitination, and mitophagy.

Interestingly, early studies on mitochondrial degradation in reticulocytes suggested that the process was Ub dependent (Rapoport et al., 1985). Supportive evidence also comes from the reports that describe ubiquitination of sperm mitochondria during their selective destruction after fertilization in mammals (Sutovsky et al., 1999, 2000). More work is necessary to unequivocally demonstrate the role of mitochondrial ubiquitination in mitophagy.

Ubiquitination of Peroxisomes and Selective Autophagy

Peroxisomes constitute another type of organelle that undergoes regulated autophagic degradation, commonly referred to as pexophagy. In methylotrophic yeast, both micropexophagy (direct engulfment of the organelle by the vacuole) and macropexophagy (autophagosome-mediated delivery of peroxosomes to the vacuole) can be experimentally induced during yeast adaptation to an alternative carbon and energy source, such as glucose or ethanol (Dunn et al., 2005). Several peroxisomal proteins have been shown to be essential for both forms of pexophagy in yeast, with ATG30 being proposed to bind both the proteins found on the peroxisome (Pex3 and Pex14) and those involved in autophagosome formation

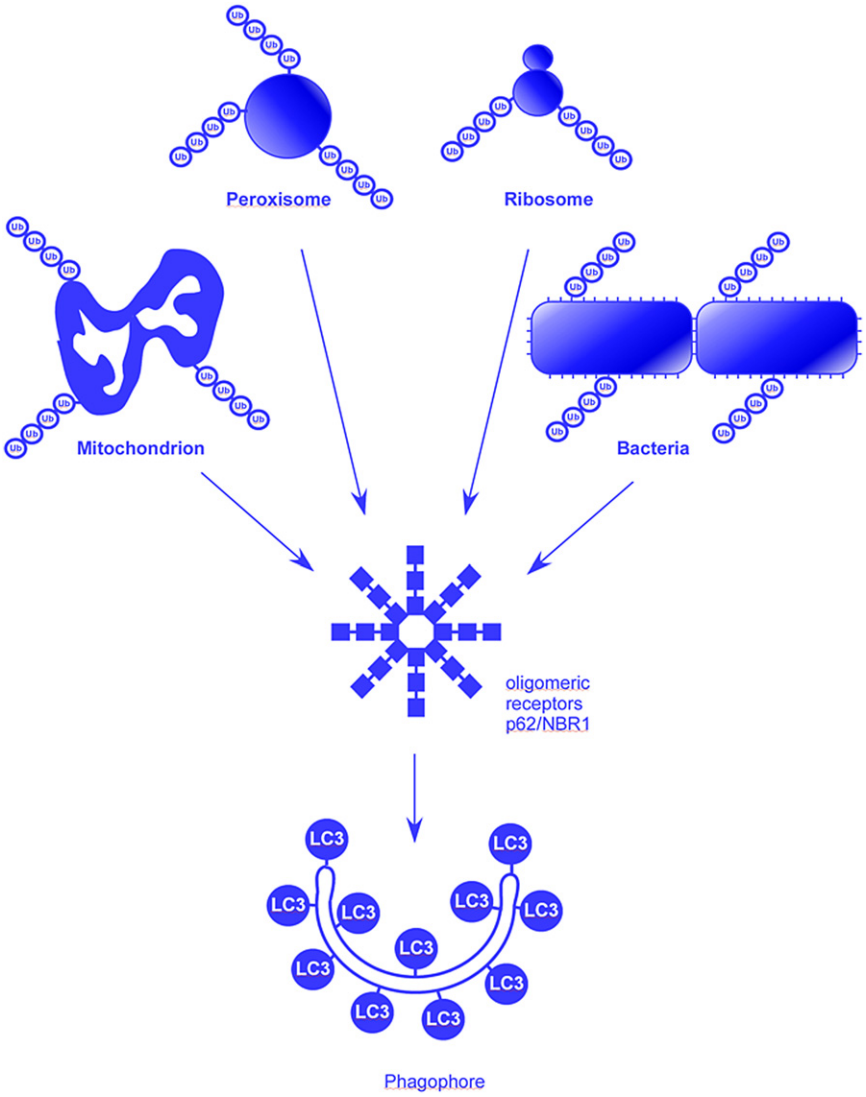

Figure 4. Proposed Role of Ubiquitination and Autophagy Receptors in Mitophagy, Pexophagy, Ribophagy and Xenophagy

Conjugation of monoUb (data not shown) or Ub chains (depicted) to the ribosomal proteins or those residing in the limiting membranes of organelles, such as mitochondria and peroxisomes, may constitute a signal that directs targeted autophagosome formation. Intracellular bacteria are also associated with Ub; however, the nature of this association is less clear. p62 and NBR1 form oligomers and bind both Ub and ATG8/LC3 on the phagophore and, thereby, might mechanistically link ubiquitination to selective autophagy.

(ATG11 and ATG17) (Farre et al., 2008). Yet, the signal present on peroxisomes that initiates selective degradation of these organelles has not been defined.

It seems plausible that Ub is involved in pexophagy (Figure 4). Several peroxisomal targeting signal (PTS) receptors (Pex5, Pex18, and Pex20) are subject to both mono- and polyubiquitination by cytosolic and peroxisomal Ub-conjugating machineries (Platta and Erdmann, 2007). While polyubiquitination of Pex5 is thought to promote its degradation, receptor monoubiquitination reportedly mediates extraction of the PTS receptor from the peroxisomal membrane to ensure its recycling and availability for the next round of importing of peroxisomal resident proteins (Platta et al., 2007). It is tempting to speculate that pexophagy might be triggered or promoted by prolonged retention of ubiquitinated proteins in the peroxisomal membrane. Strikingly, experimental fusion of a single Ub moiety to an integral membrane peroxisomal protein, PMP34, is sufficient to induce autophagic degradation of peroxisomes in mammalian cells. This form of pexophagy is partially dependent on the functional p62, suggesting involvement of p62 in selective autophagy of peroxisomes (Kim et al., 2008).

Ubiquitination in Selective Elimination of Ribosomes

Very recently, a novel mechanism for selective removal of ribosomes, i.e., ribophagy, in yeast was proposed. By labeling ribosomal proteins with GFP, Kraft et al. demonstrated that ribosomes are specifically targeted to the vacuole upon starvation, the process dependent on functional autophagic machinery. Importantly, genetic screens identified a Ub-specific protease, Ubp3, and an associated factor, Bre5, as proteins essential for ribophagy, but not for more general substrate degradation during starvation-induced autophagy. Moreover, ubiquitination of several ribosome-associated proteins was strongly increased in yeast deficient for Ubp3 (Kraft et al., 2008). While the authors propose that deubiquitination may assist in packaging of ribosomal components in the autophagosome, it is equally possible that, prior to the deubiquitinating step, Ub chains may serve as a signal for targeted elimination of ribosomes. More work is necessary to understand what triggers ubiquitination of ribosomal proteins during starvation and whether ubiquitination per se is a prerequisite for selective degradation of ribosomes by autophagy (Figure 4).

Ubiquitination as Antibacterial Mechanism

Autophagy emerges as an important mechanism for clearing intracelullar pathogens and, in this context, is referred to as xenophagy. Several medically relevant bacteria, including *Mycobacterium tubercuolosis*, *Salmonella enterica*, and *Listeria monocytogenes* are degraded by autophagy in vitro. Importantly, coevolution of the pathogens with the host's innate immune response system has allowed some intracellular bacteria to develop mechanisms that ensure evasion or even hijacking of the autophagic machinery for their own benefit (Levine and Deretic, 2007). Similar to the selective degradation of organelles, the specific signal that mediates recognition of intracellular pathogens by the autophagosome has not been defined

thus far. However, a potential role for Ub signaling in xenophagy is emerging. For example, Ub was recognized as a vital factor in plant resistance to bacterial pathogens (Nishimura and Somerville, 2002), and association with Ub chains was demonstrated for both gram-negative and gram-positive intracellular bacteria (Perrin et al., 2004). Intriguingly, only free cytosolic bacteria, but not those found within acidic vacuoles, were positive for Ub. As proteasomes were recruited to ubiquitinated *Salmonella typhimurium*, authors proposed that bacteria-associated Ub might signal local degradation of bacterial proteins, thereby restricting their growth and stimulating presentation of bacterial antigens via MHC class I molecules (Perrin et al., 2004). We hypothesize that Ub associated with intracellular pathogens, similar to ubiquitination of protein aggregates, promotes targeted autophagosome formation and pathogen destruction. It remains to be seen whether autophagy receptors, such as p62 and NBR1, can link bacterial ubiquitination with the autophagic machinery and, thereby, mediate xenophagy (Figure 4).

Conclusions and Perspectives

Autophagy has generally been considered as a ''nonselective'' process. Yet, a growing body of evidence suggests that autophagy can be selective in targeting protein complexes, organelles, and microbes. Characterization of the molecular mechanisms, which govern the specificity, is still in its infancy but has been made possible by the identification of ATG proteins that regulate nucleation and maturation of the autophagosome in yeast. The central question of selective autophagy is how a particular substrate can engage the autophagosomal machinery to mediate phagophore assembly at a specific cellular location. The Cvt pathway in yeast may provide an answer to this question (Figure 3): in this prototypic pathway, preApe1 aggregates are recognized by a receptor protein (ATG19) that recruits and activates components of the autophagic machinery (Suzuki and Ohsumi, 2007). It is tempting to speculate that accumulation of ubiquitinated cargo might mimic this process via receptor-like proteins leading to targeted phagophore nucleation (Figure 3). Discovery of p62 and NBR1 as autophagy receptors, which interact with both Ub and UBLs of the ATG8 protein family, is an important step in exploring this hypothesis. Presence of p62/NBR1 homologs with identical functions in lower organisms demonstrates conservation of the mechanisms of Ub-mediated autophagy during the course of evolution (Nezis et al., 2008). It will now be essential to show whether ubiquitination is also involved in selective autophagy in yeast and to identify functional p62/NBR1 orthologs to allow dissection of the process in this established model system for selective autophagy.

Targeted degradation of misfolded proteins, damaged organelles, and microbes by autophagy is an important avenue of research that holds promise of new therapeutic approaches in treatment of cancer, neurodegenerative diseases, and infectious diseases. We propose that induced ubiquitination on the cargo surface provides a signal that leads to autophagosome formation. This phenomenon may provide an exciting opportunity to modulate levels of selective autophagy in different pathological conditions as illustrated by the recent progress in therapeutic targeting of the Ub system in cancer. However, while ubiquitination of peroxisomes, mitochondria, and pathogens has been formally demonstrated, much work is required to prove Ub as a universal degradation signal valid also for these targets.

By analogy to other Ub-regulated processes, ubiquitination of autophagic substrates has to be tightly regulated. Some of the key questions in this respect are what is the upstream signaling that results in the recruitment of dedicated E3 ligases to the autophagy substrate? Which E3 ligases are specific for selective autophagy of the respective substrates? What is the nature of the Ub signal as primary proautophagic event—is it monoUb or Ub chains of a specific topology? Furthermore, it is unclear what the role of TOR signaling in Ub-mediated autophagy is and how Ub-binding autophagic receptors (via interaction with ATG8 family proteins) are able to translate the Ub signal into the autophagosome formation process. Proteomic analysis of molecular complexes in vivo will likely identify the mechanistic links between ubiquitination and autophagy, which will require extensive genetic and functional characterization.

ACKNOWLEDGMENTS

We would like to thank Terje Johansen and Trond Lamark for critically reading the manuscript. We apologize to those investigators whose contributions are not described here because of space limitations. I.N. is supported by a long term EMBO postdoctoral fellowship. I.D. acknowledges support from the Deutsche Forschungsgemeinschaft and the Cluster of Excellence ''Macromolecular Complexes'' of the Goethe University Frankfurt (EXC115).

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