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Posttranslational modification of autophagy-related proteins in macroautophagy

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Abbreviations: AMPK, AMP-activated protein kinase; ATG, autophagy-related; MTORC1, mechanistic target of rapamycin complex 1; PE, phosphatidylethanolamine; PTM, posttranslational modification; Ub, ubiquitin; Ubl, ubiquitin like.

Macroautophagy is an intracellular catabolic process involved in the formation of multiple membrane structures ranging from phagophores to autophagosomes and autolysosomes. Dysfunction of macroautophagy is implicated in both physiological and pathological conditions. To date, 38 autophagy-related (*ATG*) genes have been identified as controlling these complicated membrane dynamics during macroautophagy in yeast; approximately half of these genes are clearly conserved up to human, and there are additional genes whose products function in autophagy in higher eukaryotes that are not found in yeast. The function of the ATG proteins, in particular their ability to interact with a number of macroautophagic regulators, is modulated by posttranslational modifications (PTMs) such as phosphorylation, glycosylation, ubiquitination, acetylation, lipidation, and proteolysis. In this review, we summarize our current knowledge of the role of ATG protein PTMs and their functional relevance in macroautophagy. Unraveling how these PTMs regulate ATG protein function during macroautophagy will not only reveal fundamental mechanistic insights into the regulatory process, but also provide new therapeutic targets for the treatment of autophagy-associated diseases.

Introduction

Autophagy, discovered more than half a century ago via electron microscopy,[1-3] has been implicated in several physiological and pathological cellular processes including development, differentiation, metabolism, inflammation, myopathies, immunity, and cell death.[4-7] Autophagy occurs at a constitutive basal level, but it is upregulated in response to various types of stress.[8] Autophagy plays a critical role in maintaining cellular homeostasis and it is considered to be a double-edged sword, primarily acting in a cytoprotective manner, for example in the prevention of certain neurodegenerative diseases, but as a result also promoting survival in some types of cancer.[9-11] In addition, although autophagy is critical to maintaining normal cell physiology, dysregulated autophagy can result in compromised cell function and even death.

At least 3 recognized types of autophagy have been identified: chaperone-mediated autophagy, which uses HSPA8/HSC70 (heat shock 70kDa protein 8) to sequester cargo proteins containing a KFERQ motif; microautophagy, which is a direct lysosomal uptake and degradative process; and the best studied, macroautophagy (hereafter referred to as autophagy). As a housekeeping process, autophagy starts with the engulfment of intracellular cargo by the phagophore that then expands through the acquisition of lipids, and ultimately seals to generate a completed double-membrane structure termed the autophagosome. The autophagosome subsequently fuses with the lysosome to form the autolysosome, which has the ability to degrade substrates to produce amino acids and presumably other metabolites such as fatty acids for recycling and reuse (**Fig. 1**).[12] Substrates of autophagy are both selective and nonselective and include unused macromolecules (e.g., nucleic acids, proteins, carbohydrates, and lipids), damaged or superfluous organelles (e.g., endoplasmic reticulum, peroxisomes, ribosomes, mitochondria, and the nucleus), and invasive microbes.[13] Multiple membrane sources (including the plasma membrane, endoplasmic reticulum, endosome, mitochondria, and Golgi apparatus) are identified as contributing to the supply of membranes needed for phagophore nucleation and/or expansion, and autophagosome formation,[14] indicating that the regulation of the autophagic network is complex.[15]

Autophagy is a highly conserved process across eukaryotes.[16] The study of the molecular basis of autophagy started with the discovery of Apg5 (now Atg5),[16b] Aut1 (now Atg3),[17] Apg13 (now Atg13),[18] and Apg1 (now Atg1; whose mammalian orthologs are ULK1 and ULK2 [unc-51 like autophagy activating kinases 1 and 2])[19] in *Saccharomyces cerevisiae* in 1996-7. Currently, 38 *ATG* genes have been identified as controlling the induction and complicated membrane dynamics that occur during autophagy in yeasts;[20] approximately half of these proteins have clear

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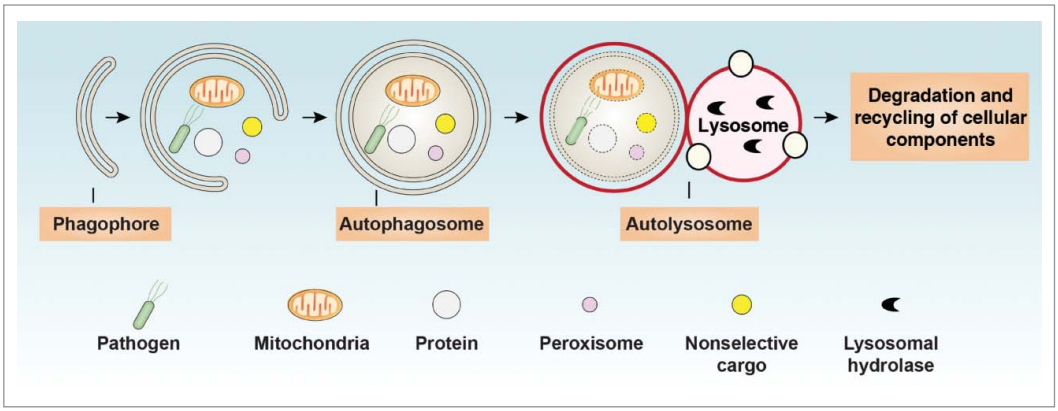

Figure 1. Overview of the autophagy process. Autophagy is a lysosome-mediated degradation and recycling pathway that involves the formation of multiple membrane structures ranging from phagophores to autophagosomes and autolysosomes.

homologs, orthologs, or paralogs in mammalian cells and plants.[15,16] The Atg proteins can form several functional complexes associated with other regulators involved in autophagy initiation and execution at different stages of the process as outlined below.

1. **Induction.** In yeast, induction of autophagosome formation is regulated by the Atg1-Atg13-Atg17-Atg31-Atg29 kinase complex.[16] In mammals, this complex contains the core proteins ULK1 (or ULK2; hereafter we only refer to ULK1), ATG13, and RB1CC1/FIP200 (RB1-inducible coiled-coil 1; the functional ortholog of yeast Atg17), which is stable and required for induction of autophagosome formation.[21,22] In mammals, ATG101 is also part of the complex; this protein, which binds to ATG13, has no known yeast ortholog,[23,24] but has an ortholog in *C. elegans*, EPG-9.[25] The mechanistic target of rapamycin complex 1 (MTORC1) and AMP-activated protein kinase (AMPK) are 2 key regulators of the ULK1 kinase complex and will be discussed in detail below. AMPK is active when cells suffer from energy starvation, thus representing a mechanism to switch on autophagy to catabolize cellular components to generate ATP. When nutrients are insufficient, MTORC1 is turned off and autophagy is again turned on, in this case providing a mechanism to restore amino acid levels through the breakdown of proteins. Thus, the regulation of autophagy induction depends, in part, on the balance between external energy and nutrient supply. Other types of stress including hypoxia, and excess reactive oxygen species can also induce autophagy.
2. **Nucleation.** During the nucleation of the phagophore, proteins and lipids are recruited for the process that ultimately leads to autophagosome formation. In both yeast and mammals, the class III phosphatidylinositol 3-kinase (PtdIns3K) complex plays an important role in the nucleation of the phagophore. The PtdIns3K complex contains the core proteins PIK3C3/VPS34 (phosphatidylinositol 3-kinase, catalytic subunit type 3), PIK3R4/p150 (phosphoinositide-3-kinase, regulatory subunit 4; the yeast ortholog is Vps15), BECN1 (Beclin 1, autophagy-related; the yeast ortholog is Vps30/Atg6) and ATG14 (also Atg14 in yeast).[26-29] One of the key functions of the PtdIns3K complex is the generation of phosphatidylinositol-3-phosphate (PtdIns3P), a phosphoinositide that serves as a landmark on the membrane to recruit other factors involved in the process of autophagosome formation. Regulation of the PtdIns3K complex occurs largely through host cofactors (e.g., ATG14, UVRAG, SH3GLB1/BIF-1, KIAA0226/RUBICON, AMBRA1, HMGB1, and PINK1) that interact with BECN1,[30] which is important for the crosstalk between autophagy and apoptosis.[31]
3. **Elongation**. Subsequent to nucleation, the phagophore expands by membrane addition, which is accomplished by 2 ubiquitin-like (Ubl) conjugation systems.[32] In yeast, the first of these systems covalently conjugates the Ubl Atg12 to Atg5 by the E2-like enzyme Atg10, whereas the second system conjugates the Ubl Atg8 to phosphatidylethanolamine (PE) by the E2-like enzyme Atg3, after Atg8 has been processed by the cysteine protease Atg4.[33,34] Both the Atg8 and Atg12 proteins are activated by the E1-like enzyme Atg7.[35] The Atg12–Atg5 conjugate forms a complex with Atg16, which in turn promotes Atg8–PE conjugation in an E3-like manner,[36] although it is not essential for this process to occur.[37] Mammalian orthologs and paralogs of this system have been identified and function as in yeast. The ATG12–ATG5 conjugation system containing the core proteins ATG5, ATG12, ATG7, ATG10, and ATG16L1 (the ortholog of yeast Atg16); and the Ubl LC3 conjugation system containing the core proteins MAP1LC3/LC3 (microtubule-associated protein 1 light chain 3, a major ortholog family, among the orthologs and paralogs of yeast Atg8), ATG3, ATG4, and ATG7. Unlike yeast, mammals have 4 isoforms of ATG4 (ATG4A to ATG4D) and 3 of LC3 (LC3A to LC3C). In addition to the LC3 subfamily, the GABARAP proteins (including GABARAP, GABARAPL1, and GABARAPL2/GATE-16), another subfamily of Atg8 paralogs in mammals, may regulate a later step of autophagosome maturation.[38] As with Atg8 in yeast, LC3 lipidation requires an initial cleavage at the extreme C terminus of LC3 by the ATG4B protease. Notably, the lipidated LC3 and GABARAP paralog protein families of yeast Atg8 specifically associate with phagophore membranes, and the Atg8/LC3/GABARAP proteins determine the size of the autophagosome;[39,40] this protein also participates in cargo

recruitment and membrane protein complex assembly/disassembly.

In addition to Ubl conjugation systems, Atg9/ATG9-mediated cycling systems containing the core proteins Atg9/ATG9, Atg2/ATG2, and Atg18/WIPI1/2 contribute to the elongation of the phagophore. Atg9/ATG9 is thought to participate in membrane delivery from donor sources to the expanding phagophore in both yeast and mammals, although the precise role of this protein is not known.[41,42] In yeast, the movement of Atg9 during phagophore elongation depends on the Atg11, Atg23, and Atg27 proteins, and multimerization of Atg9, whereas the release of Atg9 from the phagophore assembly site (PAS; the site of phagophore nucleation) involves Atg2-Atg18 and Atg1-Atg13.[42,43] In mammals, the movement of ATG9 from the trans-Golgi network or late endosomes to the phagophore is regulated by the activity of ULK1, PtdIns3K, and MAPK14/p38 (mitogen-activated protein kinase 14).[41,44]

4. **Completion and fusion**. Once autophagosome formation is complete, mammalian autophagosomes move along microtubules in a dynein motor-dependent manner and cluster close to the microtubule-organizing center near the nucleus, where they fuse with lysosomes.[45] In axons, this movement requires MAPK8IP1/JIP1 binding to LC3.[46] In addition to microtubules, HSPB1 (heat shock 27kDa protein 1)-mediated actin filament dynamics also facilitate the movement of autophagosomes to lysosomes.[47] Recent studies have suggested that the SNARE (soluble NSF [N-ethyl-maleimide-sensitive fusion protein] attachment protein receptor) family proteins (e.g., VAMP7, STX17/syntaxin 17, Tlg2, Vam3, and Ykt6) are required for autophagosome fusion with endosomes or lysosomes/vacuoles in mammals and yeast.[48-50] The SNARE family consists of more than 30 members in mammalian cells and plays a central role in intracellular membrane trafficking events. Thus, the cytoskeleton along with SNARE proteins participates in autophagosome completion and fusion, reflecting the complexity of the interplay between autophagic and endocytic vesicle trafficking. However, the role of Atg/ATG proteins at this stage of the process remains unknown.
5. **Degradation and efflux.** The vacuole/lysosome is a degradative organelle, and in general the autophagic process is not complete until the sequestered cargo is broken down and the resulting macromolecules are released back into the cytosol. This part of autophagy relies on the many hydrolases present within the lumen, and on integral membrane transporters that are needed for efflux.[51]

Collectively, autophagy consists of several sequential steps and is a very complex process that involves Atg/ATG proteins and other components to assemble the required machinery.[52] Accumulating evidence indicates that posttranslational modifications of Atg/ATG proteins afford significant flexibility for the regulation of autophagic and nonautophagic pathways.[53,54] Here, we highlight emerging evidence for the PTM-mediated regulation of Atg/ATG proteins and its functional relevance in autophagy.

Posttranslational Modifications and Atg/ATG Proteins

PTMs play a critical role in the regulation of protein activity involved in almost all aspects of cell structure and function.[55] They generally are biochemical processes involving chemical modification of a protein after its translation, resulting in inhibition or enhancement of the protein's activity.[56] The major types of PTMs include phosphorylation, glycosylation, ubiquitination, methylation, acetylation, lipidation, and proteolysis. A protein can be modified multiple times by different PTMs or by the same PTM at different residues. PTMs can also occur in a separate or sequential manner on a single protein site.[55] In addition, PTMs can be either reversible or irreversible depending on the nature of the modification. Below, we discuss several common types of PTMs that occur on Atg/ATG proteins as well as non-ATG proteins involved in the regulation of autophagy.[57-59]

Phosphorylation of Atg/ATG Proteins

Phosphorylation is a ubiquitous regulatory mechanism in protein modifications, during which a phosphate (or more than one in many cases) is added. Protein kinases and phosphatases are responsible for catalyzing the addition or removal of a phosphate from their substrates, respectively. Phosphorylation can occur on several amino acids such as serine ("Ser"), threonine ("Thr"), tyrosine ("Tyr"), histidine, and aspartate ("Asp"). In eukaryotes, phosphorylation on serine is the most common, followed by threonine and tyrosine. Protein phosphorylation could act by introducing a negative charge at a specific site that influences a protein's structural conformation, enzymatic activities, molecular association, and/or subcellular localization in signaling pathways.

Atg1/ULK1 complex

Phosphorylation events play vital roles in the initiation phase of autophagy via regulating Atg1/ULK1 complex assembly and activity in yeast and mammalian cells (**Fig. 2**). Different laboratories have observed that various protein kinases are responsible for phosphorylation of Atg1/ULK1 at different amino acid sites during autophagy (**Table 1**). In yeast, the Atg1 complex is composed of several proteins such as Atg1 and Atg13 that are essential for PAS formation as well as retrieval of Atg9 from the PAS to the cytoplasmic pool.[42,60] In yeast, the Atg1-Atg13 complex is thought to act downstream of TORC1, a protein complex that functions as a nutrient sensor and controls protein synthesis.[61] Atg1 and Atg13 are normally phosphorylated through association with TORC1 under nutrient-rich conditions.[62-64] Under starvation conditions or treatment with the TORC1 inhibitor rapamycin, inhibition of TORC1 activities can lead to rapid dephosphorylation of Atg13 at Ser348, Ser437, Ser438, Ser496, Ser535, Ser541, Ser646, and Ser649 and concomitant dephosphorylation of Atg1, which leads to the induction of autophagy.[64,65] PKA (protein kinase A) is a second messenger-dependent enzyme that is involved in the regulation of various cellular processes, including autophagy.[66,67] Inactivation of the

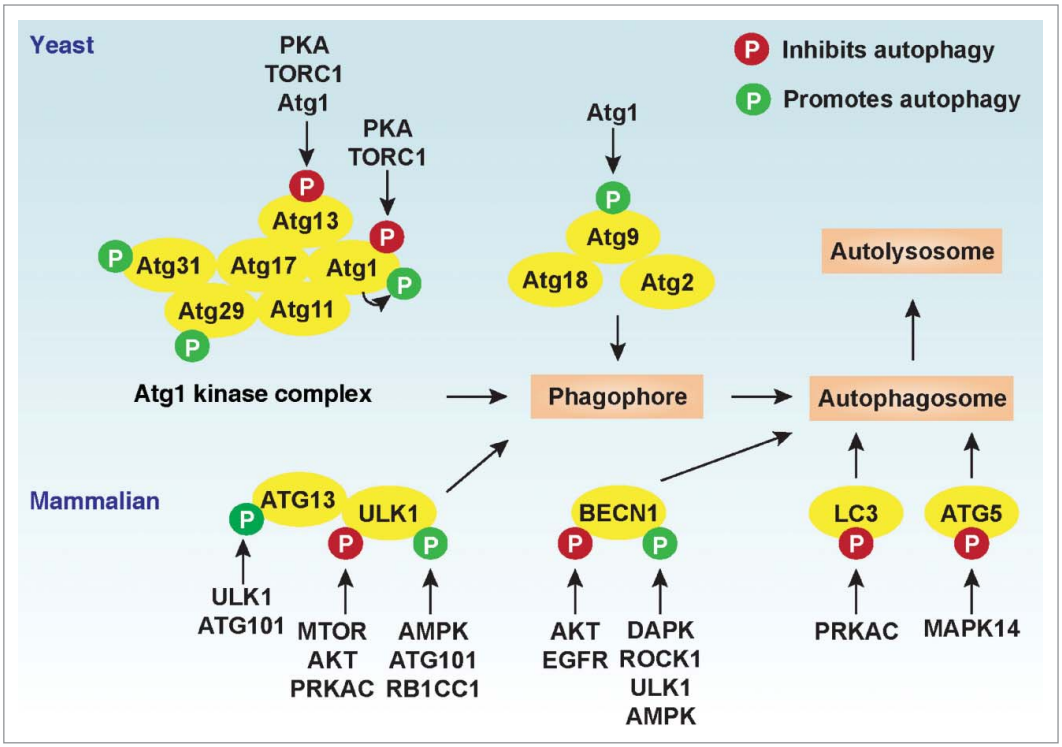

Figure 2. Regulation of Atg/ATG proteins by phosphorylation. In yeast, the initiation of autophagy involves the inhibition of TORC1- and PKA-mediated phosphorylation of Atg1 and Atg13 under starvation conditions. Atg1 is then activated via autophosphorylation, which is responsible for Atg9 localization to the phagophore assembly site. In addition, phosphorylation of Atg29 stimulates autophagy by activating the Atg17-Atg31-Atg29 complex, which is required for subsequent interaction with Atg11. In mammalian cells, phosphorylation of ULK1 and BECN1 by the indicated kinases has dual roles in the regulation of the autophagic response. Phosphorylation of LC3 and ATG5 by PRKAC and MAPK14, respectively, inhibits autophagy. In addition, ULK1- and ATG101-mediated ATG13 phosphorylation promotes selective autophagy.

PKA signaling pathway is sufficient to induce autophagy in yeast through dephosphorylation of Atg13 at Ser344, Ser347, and Ser581, and Atg1 at Ser508 and Ser515, which regulates their localization at the PAS.[65,68] Furthermore, dephosphorylation of Atg13 may facilitate its interaction with Atg1 or Atg17 and the induction of autophagy.[61,65]

In addition to yeast and mammals, Atg13 phosphorylation levels are influenced by TORC1 and Atg1 kinases in *Drosophila*.[60,69] Atg1 facilitates hyperphosphorylation of Atg13 and autophagy induction in response to starvation in *Drosophila*.[69] Collectively, these findings suggest that dephosphorylation/phosphorylation of Atg13 acts as an initiating trigger for autophagy through inactivation of TORC1 and PKA (PRKAC in mammals) signals or activation of Atg1/ULK1 kinase depending on the context and species.

Autophosphorylation at Thr226 and Ser230 within the Atg1 kinase-activation loop is required for sustaining Atg1 kinase activity during autophagy induction following treatment with the TORC1 inhibitor rapamycin in yeast.[62,70] The presence of Atg13 and Atg17 is required for Atg1 autophosphorylation, although the mechanism remains unknown.[70] Besides Atg1 and Atg13, the Atg1 kinase complex in yeast also contains a complex composed of Atg17-Atg31-Atg29. This complex is constitutively formed and activated by the phosphorylation of Atg29 at multiple residues such as Ser197, Ser199, and Ser201 in the C terminus (amino acids 101 to 213) under nitrogen starvation conditions, but the kinases that are responsible remain unknown.[71] Phosphorylation of Atg29 likely promotes a conformational change, which is required for the induction of autophagy and its interaction with phosphorylated Atg11, a scaffold protein for binding multiple Atg proteins (e.g., Atg9, Atg19, Atg20, Atg24, *Pichia pastoris* Atg30, Atg32, and Atg36) in currently identified selective types of autophagy including the cytoplasm-to-vacuole targeting (Cvt) pathway, mitophagy, and pexophagy.[71,72] The series of phosphorylation events controlling these interactions can be quite complex. For example, phosphorylation of Atg19 by Hrr25 and of Atg32 by casein kinase 2 is required for their interaction with Atg11.[73,74]

Phosphorylation plays dual roles in regulating the scaffolding and kinase functions of ULK1 to coordinate the autophagic response in mammalian cells.[75] In human embryonic kidney (HEK)-293 cells, autophosphorylation at Thr180 within the ULK1 kinase-activation loop is required for sustaining ULK1 kinase activity and its intracellular localization to phagophores upon starvation.[76] In mammalian cells, MTORC1 contains 5 components: MTOR, the catalytic subunit of the complex; RPTOR/Raptor (regulatory associated protein of MTOR, complex 1); MLST8/GβL (MTOR associated protein, LST8 homolog [*S. cerevisiae*]); AKT1S1/PRAS40 (AKT1 substrate [proline-rich]); and DEPTOR (DEP domain containing MTOR-interacting protein).[77] In human HEK-293 cells, MTORC1 complex components (e.g., RPTOR and MTOR) interact with ULK1/2, which causes ULK1/2 phosphorylation and subsequent ATG13 phosphorylation under nutrient-rich conditions.[21,22] Moreover, MTORC1-mediated phosphorylation of ATG13 and ULK1/2 has negative effects on ULK activity.[21] ULK1/2 can directly phosphorylate ATG13 and RB1CC1 to regulate autophagosome formation.[21] Interestingly, RB1CC1 and ULK1 are functionally connected, and RB1CC1 is also required for the stability and phosphorylation of ULK1 in mouse cells.[78]

Table 1. Regulation of Atg/ATG proteins by PTMs in autophagy

Yeast	Mammal	PTM	Site	Activated by	PTM Functions	References
Atg1	ULK1	Phosphorylation	Thr180 (human)	Autophosphorylation	Maintains ULK1 kinase activity	76
		Phosphorylation	Thr226 (yeast)	Autophosphorylation	Maintains Atg1 kinase activity	70
		Phosphorylation	Ser230 (yeast)	Autophosphorylation	Maintains Atg1 kinase activity	62
		Phosphorylation	Ser317 (human) Ser777 (human)	AMPK	Inhibits MTORC1 and promotes autophagy under glucose starvation	80
		Phosphorylation	Ser467 (mouse) Ser555 (mouse) Thr574 (mouse) Ser637 (mouse)	AMPK	Promotes mitophagy	195
		Phosphorylation	Ser508 (yeast) Ser515 (yeast)	PKA	Prevents its association with the PAS and inhibits autophagy	68
		Phosphorylation	Ser555 (human)	AMPK	Promotes ULK1 binding to YWHA/14-3-3	76
		Phosphorylation	Ser555 (human) Ser637 (human) Thr659 (human)	AMPK	Promotes localization of ATG9 to perinuclear clusters	84
		Phosphorylation	Ser638 (human) Ser758 (human)	MTORC1	Inhibits autophagy upon starvation	79
		Phosphorylation	Ser774 (human)	AKT	Inhibits autophagy in response to insulin	76
		Phosphorylation	Ser757 (mouse)	MTORC1	Inhibits ULK1 binding to AMPK and subsequent autophagy	80
		Phosphorylation	Ser1043 (mouse)	PRKAC/PKA	Inhibits autophagy	75
		Phosphorylation	Ser1047 (mouse)	Autophosphorylation	Promotes the closed clamp conformation of the ULK1 complex	75
		Phosphorylation	NA (human)	ATG101	ATG101 lacks kinase activity. Binds to ATG13 and promotes autophagy	23
		Phosphorylation	NA (mouse)	RB1CC1/FIP200	RB1CC1 lacks kinase activity. Increases ULK1 stability and autophagy	78
		Acetylation	Lys162 (human) Lys606 (human)	KAT5/TIP60	Stimulates starvation-induced autophagy	165
		Ubiquitination	ND (human)	TRAF6 AMBRA1	Promotes ULK1 self-association and activity during autophagy	134
Atg3	ATG3	Acetylation	Lys19 (yeast) Lys48 (yeast)	Esa1	Increases interaction between Atg3 and Atg8	161
		Acetylation	Lys183 (yeast)	Esa1	Increases Atg3-mediated Atg8 lipidation	161
		Deacetylation	Lys19 (yeast) Lys48 (yeast)	Rpd3	Inhibits autophagy	161
		Proteolysis	Asp169 (human)	CASP8	Inhibits autophagy and promotes apoptosis	185
		Sulfation	Cysteine (yeast)	Sulfate ion	Increases Atg3-mediated Atg8 lipidation	193
Atg4	ATG4A/B/C/D	Proteolysis	C-terminal Asp63 (human)	CASP3	Increases both autophagy and apoptosis	187
Atg5	ATG5	Phosphorylation	Thr75 (mouse)	MAPK14/p38	Inhibits autophagy	121
		Acetylation	ND (human)	EP300/p300	Inhibits autophagy	163
		Deacetylation	ND (human and mouse)	SIRT1	Stimulates starvation-induced autophagy	164
		Proteolysis	ND (human)	CASP3, 6, 8, 9, 10	Increases apoptosis and inhibits autophagy	188
		Proteolysis	C-terminal Thr193 (human)	CAPN/calpain	Increases apoptosis and inhibits autophagy	183

Atg6	BECN1	Phosphorylation	Ser14 (human)	ULK	Increases PtdIns3K complex activity in response to amino acid withdrawal	92
		Phosphorylation	Ser91 (human) Ser94 (human)	AMPK	Increases PtdIns3K complex activity and membrane association in autophagy or mitophagy	104,115
		Phosphorylation	Thr119 (human)	DAPK or ROCK1	Dissociates BECN1 from BCL2	108,109
		Phosphorylation	Tyr229 (human) Tyr233 (human) Tyr352 (human)	EGFR	Inhibits PtdIns3K complex activity	114
		Phosphorylation	Ser234 (human) Ser295 (human)	AKT	Inhibits PtdIns3K complex activity	113
		Ubiquitination	Lys117 (mouse)	TRAF6	Dissociates BECN1 from BCL2 and promotes autophagy	130
		Ubiquitination	Lys349 (human)	NEDD4	Promotes BECN1 degradation	132
		Ubiquitination	Lys437 (human)	AMBRA1	Increases PtdIns3K complex activity and autophagy	131
		Deubiquitination	ND (human and mouse)	USP10 USP13	Increases PtdIns3K complex activity	133
		Proteolysis	C-terminal Asp149 (mouse and human) Asp146 (human) Asp133 (mouse and human)	CASP3, 6, 8, 9, 10	Increases apoptosis and inhibits autophagy	184,188-192
Atg7	ATG7	Acetylation	ND (human)	EP300/p300	Inhibits autophagy	163
		Deacetylation	ND (human and mouse)	SIRT1	Stimulates starvation-induced autophagy	164
Atg8	LC3A/B/C, GABARAP, GABARAPL1/2	Phosphorylation	Thr6 Thr29	PRKC/PKC	Has no impact on autophagy	123
		Phosphorylation	Ser12 (human LC3)	PRKAC/PKA	Inhibits LC3 recruitment to autophagosome	122
		Lipidation	Gly116 (yeast), C-terminal Gly in human and mouse LC3, GABARAP, GABARAPL1/2)	Atg3/ATG3 Atg7/ATG7	Converts to phagophore-associating form, LC3-II	34,196-198
		Proteolysis	Lys65- Gln77 (human LC3)	20S proteasome	Degrades LC3	181
		Proteolysis	Arg68 (human LC3)	ATG4B	Affects C-terminal cleavage of LC3B	176
		Proteolysis	Gly116 (yeast), C-terminal Gly in human and mouse, LC3, GABARAP, GABARAPL1/2)	Atg4/ATG4A/B	Promotes processing and deconjugation of Atg8/LC3/GABARAP	40,167,177
		Proteolysis	ND (mouse LC3)	CTSE/cathepsin E	Degrades autophagosomal membrane components	180
		Acetylation	ND (human LC3)	EP300/p300	Inhibits autophagy	163
		Deacetylation	ND (human and mouse LC3)	SIRT1	Stimulates starvation-induced autophagy	164
Atg9	ATG9A/B	Phosphorylation	Multiple Ser residues (yeast)	Atg1	Recruits Atg8 and Atg18 to the site of autophagosome formation and expands the phagophore membrane	89
		Glycosylation	Asn99 (human ATG9A)	ND	Coordinates membrane transport from donor sources to site of autophagosome formation	41
Atg11	NA	Phosphorylation	ND (yeast)	ND	Scaffold protein in selecting autophagy for PAS organization	72
Atg12	ATG12	Acetylation (human)	ND	EP300/p300	Inhibits autophagy	163
		Deacetylation (human and mouse)	ND	SIRT1	Stimulates starvation-induced autophagy	164

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Table 1. Regulation of Atg/ATG proteins by PTMs in autophagy *(Continued)*

Yeast	Mammal	PTM	Site	Activated by	PTM Functions	References
Atg13	ATG13	Phosphorylation	Ser48 (human) Thr170 (human) Thr331 (human) Thr428 (human) Thr478 (human)	ULK1	Has no impact on starvation-induced autophagy	91
		Phosphorylation	Ser318 (human)	ULK1	Promotes mitophagy	90
		Phosphorylation	Ser344 (yeast) Ser437 (yeast) Ser581 (yeast)	PKA	Regulates the association of Atg13 with the PAS, and inhibits autophagy	65
		Phosphorylation	S348 (yeast) S437 (yeast) S438 (yeast) S496(yeast) S535 (yeast), S541(yeast) S646 (yeast) and S649 (yeast)	TORC1	Regulates the interaction between Atg13 and Atg1, and inhibits autophagy	64
		Phosphorylation	ND (*Drosophila*)	Atg1	Promotes Atg13 activity and autophagy	69
		Phosphorylation	N/A (human)	ATG101	Binds to ATG13 and promotes autophagy	23
Atg16	ATG16L1	Proteolysis	C-terminal Thr300 (human)	CASP3	Inhibits autophagy and promotes apoptosis	186
Atg18	WIPI1/2	Phosphorylation	ND (yeast)	PKA	Regulates the lipid binding capacity	68
Atg29	N/A	Phosphorylation	Multiple Ser and Thr residues such as Ser197, Ser199, and Ser201 (yeast)	ND	Binds to Atg11 and initiates PAS assembly	71
Atg30	N/A	Phosphorylation	Ser112 (yeast) Ser71 (yeast)	ND	Binds to Atg11 or Atg8, and promotes pexophagy	116,117
Atg32	N/A	Phosphorylation	Ser114 (yeast)	MAPK	Binds to Atg11 or Atg8, and promotes mitophagy	116,118-120
Atg36	N/A	Phosphorylation	Ser31 (yeast) Ser97 (yeast)	ND	Binds to Atg11 or Atg8, and promotes pexophagy	116

NA, not applicable; ND, not determined.

The interactions between MTORC1 and ULK1/2 significantly decrease during starvation, which leads to dephosphorylation of ULK1/2 and ATG13, increased kinase activity of ULK, and subsequent induction of autophagy.[21,22] Several MTORC1-mediated phosphorylation sites of ULK1 have been recently mapped. For example, MTORC1 is required for phosphorylation of ULK1 at Ser638 and Ser758 in human cancer cells (e.g., HeLa and U2OS) under nutrient-rich conditions and these sites are dramatically dephosphorylated upon starvation.[79] Phosphorylation of ULK1 at Ser757 by MTORC1 inhibits the ULK1-AMPK interaction and subsequent autophagy in mouse cells.[80] In contrast, MTORC1-mediated phosphorylation of ULK1 at Ser758 in human U2OS cell results in a reassociation between ULK1 and AMPK and subsequent inhibition of autophagy.[79] These studies have revealed a model for ULK1 regulation and autophagy induction involving MTORC1-dependent signaling by which MTORC1 can downregulate autophagy through inactivation and destabilization of ULK1.

The serine/threonine family of kinases termed AKT (v-akt murine thymoma viral and oncogene homolog) regulates multiple biological processes including cell survival, proliferation, and autophagy. AKT is required for phosphorylation of ULK1 at Ser774 in human HEK-293 cells, which limits the autophagic response following insulin treatment.[76] In addition to MTORC1 and AKT, PRKAC/PKA (protein kinase A, cAMP-activated) negatively regulates autophagy by direct phosphorylation of ULK1 at Ser1043 in mouse cells, preventing ULK1 activation; ULK1 autophosphorylation at Ser1047 promotes phosphorylation at the Ser1043 site.[75]

In contrast to the aforementioned negative regulators, AMPK is generally a positive regulator of ULK1 activity. When cellular energy level drops, AMPK is activated and MTORC1 activity becomes inhibited by AMPK-mediated phosphorylation of TSC2 (tuberous sclerosis 2) at Thr1271 and Ser1387, and by phosphorylation of RPTOR at Ser722 and Ser792, which leads to the induction of autophagy.[81] In addition to inhibition of MTORC1 activity by AMPK, ULK1 is conversely activated by phosphorylation at multiple sites by AMPK in response to glucose starvation[82] and cyclic dinucleotides;[83] phosphorylation at Ser317 and Ser777 initiates autophagy in human HEK-293 cells.[80] Another study indicates that ULK1 contains 4 AMPK substrate motif sites at Ser467, Ser555, Thr574, and Ser637. These sites are conserved in higher eukaryotes, and as far back as *Caenorhabditis elegans* (Ser555 and Ser574).[82] Importantly, AMPK-mediated ULK1 phosphorylation at Ser467, Ser555, Thr574, and Ser637 is required all or in part for mitophagy induction,[82] ATG9 localization,[84] TMEM173/STING (transmembrane protein 173)-mediated type I interferon immune response pathway activation,[83] and ULK1 interaction with YWHA/14-3-3 (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein).[76,84,85]

Furthermore, ATG101 was discovered as a binding protein for ATG13 that is important for the stability and basal phosphorylation of both ATG13 and ULK1.[23,24] However, it is unlikely that ATG13 and ULK1 are direct subtracts of ATG101, because currently ATG101 is not known to have kinase activity. ULK1 can phosphorylate AMPK and MTORC1 subunits such as RPTOR.[86-88] Further studies show that the components of the autophagy machinery such as yeast Atg9 (multiple Ser residues),[89] ATG13 (Ser318, Ser48, and multiple Thr residues),[90,91] RB1CC1, AMBRA1 (autophagy/beclin-1 regulator 1), BECN1 (Ser14), and DAPK3/ZIPK (death-associated protein kinase 3) are direct phosphorylation targets of Atg1/ULK1 kinase, and these modifications result in complicated negative or positive feedback loop effects.[80,89,92-95] Together, these findings suggest that the Atg1/ULK1 serine-threonine kinase can serve as a convergence point for multiple signals that control autophagy and other cellular processes through its alterations in phosphorylation status as well as kinase activity.

Vps34/PIK3C3 and the PtdIns3K complex

Autophagosome formation relies on the generation of PtdIns3P at the PAS and/or on the phagophore membrane by a protein complex containing the PtdIns3K, whose catalytic subunit is termed Vps34/PIK3C3. In yeast, Vps34 forms 2 distinct PtdIns3K complexes; complex I contains Vps34-Vps15-Vps30/Atg6-Atg14-Atg38 and complex II is comprised of Vps34-Vps15-Vps30/Atg6-Vps38. Complex I is involved in generating autophagic membranes, whereas complex II is involved in the vacuolar protein sorting pathway. Vps15 is a serine/threonine protein kinase that mediates phosphorylation of Vps34, a PTM that is required to form the PtdIns3K complexes.[96] Vps34 is the sole enzyme in yeast that can phosphorylate phosphatidylinositol to form PtdIns3P. The cellular level and activity of PtdIns3P is also controlled by PtdIns3P phosphatases that belong to the myotubularin family.[97] In yeast, the PtdIns3P phosphatase Ymr1 is recruited to the PAS at an early stage of autophagy; subsequent dephosphorylation of PtdIns3P by Ymr1 is required for Atg protein disassembly during autophagosome completion.[98] In contrast, the PtdIns3P phosphatase MTMR14/Jumpy (myotubularin related protein 14), and PTPRD/PTPσ (protein tyrosine phosphatase, receptor type, D) act as negative regulators of autophagy by dephosphorylating PtdIns3P or inhibiting PtdIns3P expression in *C. elegans* and/or mammalian cells.[99-102] In addition, depletion of PtdIns3P phosphatases MTMR6 and MTMR7 increase LC3-II in mammalian cells suggesting they are also negative regulators of autophagy, although the mechanism is unknown.[100] These findings suggest that phosphorylation/dephosphorylation of PtdIns3P can either positively or negatively regulate autophagic initiation depending on the PtdIns3P kinases and phosphatases involved.

The human core complex of the PtdIns3K complex consists of 3 major components including PIK3C3/VPS34, PIK3R4/p150, and BECN1. Additional components contribute to the formation of at least 3 distinct PtdIns3K complexes that are either stimulatory or inhibitory for autophagy. Human ATG14/ATG14L/BARKOR and UVRAG are likely functional orthologs of yeast Atg14 and Vps38, respectively, and along with AMBRA1 function in stimulatory complexes; KIAA0226/Rubicon is a component of the inhibitory complex. Phosphorylation of PIK3C3 at Thr159 and Thr668 by CDK1 (cyclin-dependent kinase 1), CDK5, or AMPK inhibits autophagy,[103,104] whereas

phosphorylation of PIK3C3 at multiple sites by PRKD/PKD (protein kinase D) promotes autophagy.[105] BECN1, a mammalian ortholog of Vps30/Atg6, is considered to function in part as a scaffold/platform, which allows multiple signals to converge.[31]

Following starvation or MTOR inhibition, activated ULK1 directly phosphorylates BECN1 on Ser14 to increase PtdIns3K complex activation, and participates in autophagy initiation by activation of ATG14-bound PIK3C3 in human HEK-293 cells.[92] There are 2 general classes of BCL2 (B-cell CLL/lymphoma 2) proteins; antiapoptotic proteins, which include BCL2, BCL2L1/BCL-xL, BCL2L2/BCL-W, and MCL1 that inhibit autophagy, and proapoptotic BH3-only proteins such as BNIP3, BAD, BIK, PMAIP1/NOXA, BBC3/PUMA, and BCL2L11/BIMEL that induce autophagy.[106] Interaction of BCL2 with BECN1 prevents the association of BECN1 with PIK3C3 and this process is negatively regulated by phosphorylation.[107] Phosphorylation of BECN1 at Thr119 by DAPK or ROCK1 (Rho-associated, coiled-coil containing protein kinase 1) triggers BCL2L1 or BCL2 dissociation from BECN1 in human HEK-293 or HeLa cells, therefore activating the PtdIns3K complex and autophagy upon nutrient deprivation.[108,109] Moreover, MAPK8/JNK1 (mitogen-activated protein kinase 8) and MAPK1/ERK2 mediate phosphorylation of BCL2 and promote autophagy by facilitating the association of BECN1 with PIK3C3 or HMGB1 (high mobility group box 1), respectively.[110,111] HMGB1 is a nuclear protein that translocates to the cytoplasm to induce autophagy through binding BECN1[111] although HMGB1-independent autophagy exists.[112] In contrast, AKT-mediated BECN1 phosphorylation at Ser295 and Ser234 limits autophagy in human cancer cells through promoting YWHA/14-3-3-dependent BECN1 sequestration to the cytoskeletal protein VIM/vimentin, which contributes to a number of cancer hallmarks ranging from cell proliferation and survival to angiogenesis and increased metabolism.[113] The oncogenic receptor tyrosine kinase EGFR (epidermal growth factor receptor)-mediated BECN1 phosphorylation at Tyr229, Tyr233 and Tyr352 decreases PtdIns3K complex activation and autophagy in lung cancer cells, which may contribute to tumor progression and chemoresistance.[114] Moreover, AMPK activates autophagy and mitophagy in response to starvation or valinomycin by phosphorylation of BECN1 at Ser91 and Ser94, and this process depends on ATG14.[104,115] Thus, Vps34/PIK3C3-PtdIns3K complex activity in autophagy is tightly controlled, either negatively or positively, by phosphorylation of its components.

Other

Besides Atg1/ULK1 and the Vps34/PIK3C3-containing PtdIns3K complex, phosphorylation can fine-tune the function of other Atg proteins (e.g., Atg30, Atg32 and Atg36) involved in pexophagy and mitophagy in yeast[116-120] and the activity of Ubl systems in mammalian cells (**Table 1**). In mammalian cells, ATG5 is a component of the ATG12–ATG5 conjugation system, acting as an E3-like ubiquitin ligase for LC3 conversion to the PE-conjugated LC3-II form as indicated above. This conversion is critical for autophagosome formation. Phosphorylation of ATG5 at Thr75 by MAPK14 inhibits starvation- and lipopolysaccharide-induced autophagy, which indicates a negative regulatory role of MAPK14 in autophagosome maturation.[121] LC3 conversion to the lipidated form and its localization to the phagophore or autophagosome membrane serve as a marker for monitoring autophagy.[40] PRKAC/PKA-mediated LC3 phosphorylation at Ser12 in the human SH-SY5Y neuroblastoma cell line prevents LC3 turnover and recruitment to the phagophore in response to rapamycin and parkinsonian neurotoxin (e.g., 1-methyl-4-phenylpyridinium).[122] In contrast, PRKC/PKC inhibits autophagy in an LC3 phosphorylation-independent manner, although PRKC mediates LC3 phosphorylation at Thr6 and Thr29 following phorbo-12-myristate-13-acetate/PMA or calyculin A treatment in HEK-293 cells.[123] Further systematic analyses will facilitate our understanding of the relationship between LC3 phosphorylation and autophagy induction in response to different stressors.

Glycosylation of ATG Proteins

Glycosylation is one of the major PTMs that have a significant effect in the biosynthetic-secretory pathways involving the endoplasmic reticulum and Golgi apparatus. Approximately half of all proteins undergo this modification in which sugar moieties are added to specific amino acids; glycosylation can be critical to achieve proper protein folding, distribution, stability, and activity.

ATG9/Atg9 is a multispanning membrane protein that functions in the delivery of lipid that is used for the expansion of the phagophore at the early stage of autophagy.[42,124,125] Self-interaction and aggregates of Atg9 are required for its trafficking and function at the step of phagophore expansion in yeast.[43] There are 2 isoforms of ATG9 in mammalian cells; ATG9A is ubiquitously detected in adult tissues, while ATG9B is highly expressed in the placenta (trophoblast cells) and pituitary gland.[126] ATG9 is the only transmembrane ATG protein that is absolutely required for autophagosome formation. ATG9A is normally localized in the trans-Golgi network and late endosomes, whereas treatment with the MTORC1 inhibitor rapamycin or amino acid starvation can cause its redistribution to LC3-positive autophagic vesicles.[41] ATG9A has 4 possible N-glycosylation sites (N99, N129, N224, and N507), and only Asn99 is known to be glycosylated in human HEK-293 cells.[41] In part due to its glycosylation, ATG9A appears to function in coordinating membrane transport from donor sources to the autophagosome formation site.[41,127]

Ubiquitination of ATG Proteins

Ubiquitination is one of the crucial mechanisms for the destruction of cellular proteins after their synthesis to implement a continual state of protein flux. Proteins that are tagged by attachment of the 8-kDa, 76 amino acid polypeptide ubiquitin (Ub) are then recognized by the 26S proteasome to catalyze the degradation of the ubiquitinated protein. The completion of the reaction cascade requires the Ub activating enzyme (E1), a Ub conjugating enzyme (E2), a Ub ligase (E3), and, in some cases, a

Ub chain elongation factor (E4).[128] Ubiquitination of targeted proteins leads to either their degradation or alteration of signaling capabilities.

Ubiquitination plays multiple roles in the regulation of autophagy in organisms ranging from yeast to mammals, although the mechanism may be distinct from the ubiquitin proteasome pathway.[16,129] Ubiquitination events are considered to be involved in complex assembly and translocation through the regulation of ATG protein levels as well as their interactions with other proteins. Aside from the 2 Ubl conjugation systems (ATG12–ATG5 and LC3-II) that are responsible for phagophore expansion during autophagy as discussed in the Introduction (**Fig. 3A**),[33,34] actual ubiquitination occurs on ATG proteins such as BECN1 and ULK1 (**Fig. 3B**). BCL2 binding to BECN1 prevents PtdIns3K complex activity, suggesting that dissociation between BECN1 and BCL2 is required for autophagy induction.[107] In addition to BECN1 phosphorylation,[108] Lys63-linked ubiquitination of BECN1 at Lys117 by TRAF6 (TNF receptor-associated factor 6, E3 ubiquitin protein ligase)[130] or at Lys437 by AMBRA1[131] regulate its binding to BCL2 as well as subsequent PtdIns3K complex activity. TRAF6-mediaed ubiquitination of BECN1 amplifies lipopolysaccharide-, IFNG/interferon γ- and amino acid starvation-induced autophagy in mouse macrophages.[130] WASH (WAS protein family homolog/Wiskott–Aldrich syndrome protein [WASP] and SCAR homolog), a member of the WASP family with a role in endosomal sorting, inhibits AMBRA1-mediated ubiquitination of BECN1 and starvation-induced autophagy in human HeLa cancer cells.[131] Lys11- and Lys63-linked ubiquitination of BECN1 by NEDD4 (neural precursor cell expressed, developmentally down-regulated 4, E3 ubiquitin protein ligase) promote BECN1 proteasomal degradation in the absence of PIK3C3 in human HeLa cancer cells.[132] Moreover, deubiquitinating enzymes such as USP10 (ubiquitin specific peptidase 10) and USP13 (ubiquitin specific peptidase 13 [isopeptidase T-3]) are involved in the regulation of BECN1 ubiquitination in human and mouse cells, which promotes autophagy.[133] In contrast, USP10 and USP13 are inhibited by spautin-1, a novel inhibitor of autophagy, which causes increased ubiquitination and degradation of BECN1 in cancer cells.[133] Additionally, AMBRA1 interacting with TRAF6 promotes Lys63-linked ubiquitination of ULK1 and its subsequent stabilization and activity during autophagy induction, whereas MTORC1-mediated AMBRA1 phosphorylation at Ser52 inhibits its role in ULK1 modification in human HEK-293 cells.[134] Interestingly, a study on the deubiquitinating enzyme interactome indicates that ULK1 is a potential target of USP10 in human HEK-293 cells, although the functional significance of this finding remains unknown.[135]

Ubiquitination is also essential for cargo recognition and selective autophagy processes.[136-140] Several targets of selective autophagy have been described, including mitochondria (mitophagy),[141] peroxisomes (pexophagy),[142] lysosomes (lysophagy),[143] lipid droplets (lipophagy),[144] secretory granules (zymophagy),[145] the endoplasmic reticulum (reticulophagy),[146] nucleus (nucleophagy),[147] RNA (RNautophagy),[148] pathogens (xenophagy),[149] ribosomes (ribophagy),[150] aggregate-prone proteins (aggrephagy),[151] and ferritin (ferritinophagy).[152] In addition to cargo degradation, selective autophagy also contributes to the secretion of immune molecules such as cytokines (e.g., IL1) and damage-associated molecular pattern molecules (e.g., HMGB1).[153] The selective recruitment of these targets requires selective receptors (e.g., SQSTM1/p62, BNIP3L, NBR1, CALCOCO2, and OPTN) and scaffold proteins, which usually contain a Ub-binding

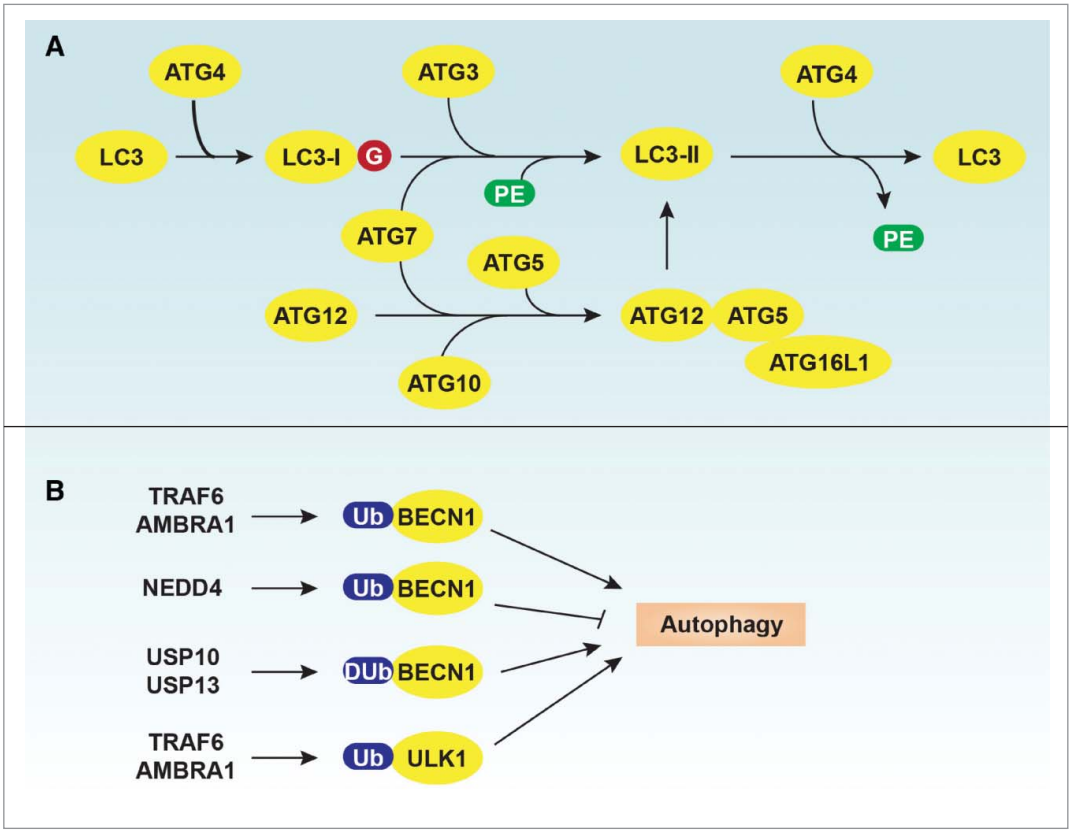

Figure 3. Regulation of ATG proteins by ubiquitination. (**A**) Two Ubl conjugation systems in mammalian cells. (**B**) Ubiquitination or deubiquitination of BECN1 and ULK1 regulate the autophagic response.

domain and Atg8/LC3/GABARAP-interacting region. Thus, selective autophagy uses Ub as a degradation signal and Ub-binding proteins as specific receptors to transport cargo to the lysosome for degradation or secretion.[136-140]

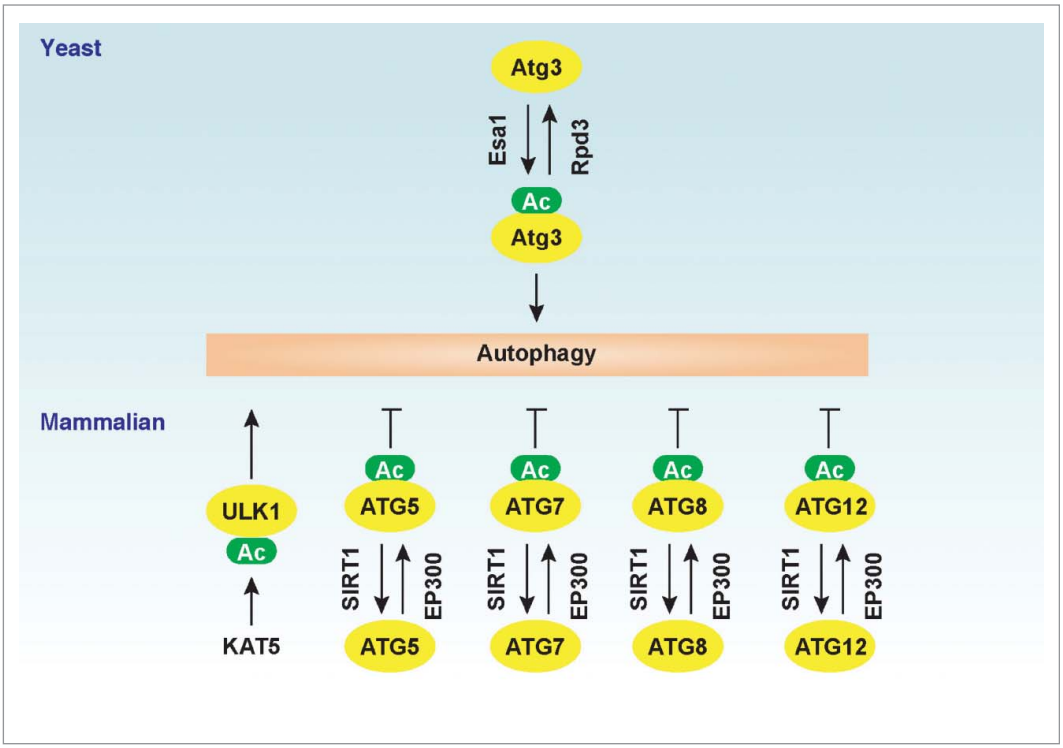

Figure 4. Regulation of Atg/ATG proteins by acetylation. In yeast, acetylation of Atg3 by Esa1 increases autophagy, whereas deacetylation of Atg3 by Rpd3 decreases autophagy. In mammalian cells, acetylation of ATGs (ATG5, ATG7, ATG8, and ATG12) by EP300 decreases autophagy, whereas deacetylation of these ATGs by SIRT1 increases autophagy. In addition, KAT5-mediated ULK1 acetylation promotes autophagy.

Acetylation of Atg/ATG Proteins

Acetylation is described as a reaction that transfers an acetyl group onto a chemical compound. The posttranslational acetylation of histone and nonhistone proteins has been reported to play an important role in cell biology and associated regulatory processes.

In addition to histones[154-156] and transcription factors (e.g., FOXO1 [forkhead box O1] and FOXO3),[157,158] ATG proteins are regulated through acetylation/deacetylation modifications that influence the outcome of autophagy (**Fig. 4**).[58,59] The NAD-dependent deacetylase SIRT1 (sirtuin 1)-mediated deacetylation of FOXO3 is essential for autophagy induction and cell survival, and the mechanism by which acetylated FOXO3 promotes the transcription of *Lc3* and *Bnip3* in mouse tissues.[157,159] Different from FOXO3-mediated transcriptional control of *ATG* gene expression, FOXO1 promotes oxidative stress- or starvation-induced autophagy independent of its transcriptional activity.[158] FOXO1 shuttles between the cytoplasm and the nucleus. In response to stress, FOXO1 is acetylated after dissociation from SIRT2 (sirtuin 2), which leads to the translocation of FOXO1 from the nucleus to the cytoplasm, where acetylated FOXO1 binds to ATG7 to promote autophagy in human cancer cells.[158] FOXO1 can also be deacetylated by SIRT1, which leads to upregulation of RAB7, a small GTPase that controls membrane trafficking and the maturation of phagosomes and autophagic vacuoles.[160] This process increases starvation-induced autophagic flux in cardiac myocytes.[160]

As noted above, Atg3 is a ubiquitin-conjugating enzyme (E2) analog that conjugates Atg8 to PE after Atg4-dependent processing and subsequent activation of the C-terminal residue by Atg7 in yeast.[34] In yeast, Atg3 acetylation levels regulate autophagic processes through different mechanisms.[161] For example, upregulation of Atg3 acetylation at Lys19 and Lys48 by the Esa1 acetyltransferase promotes interaction between Atg3 and Atg8, whereas downregulation of Atg3 acetylation at these sites by the histone deacetylase Rpd3 limits autophagy.[161] Moreover, upregulation of Atg3 acetylation at Lys183 by Esa1 increases the Atg3 lipid-conjugating activity.[161] In addition to LC3, ATG3 can also be conjugated to ATG12 in mammals or higher eukaryotes, which increases mitochondrial mass, improves bioenergetic function, and inhibits cell death.[162] It is unknown whether ATG3 acetylation regulates this process in higher eukaryotes.

Knockdown of the EP300/p300 acetyltransferase inhibits acetylation of ATG5, ATG7, ATG8, and ATG12, which in turn promotes autophagy in human HeLa cells under starvation conditions.[163] In contrast, overexpressed SIRT1 in human (e.g., HeLa) and mouse (e.g., embryonic fibroblast) cells can directly deacetylate ATG5, ATG7, LC3, and ATG12, which in turn initiates autophagy during starvation, whereas *sirt1*$^{-/-}$ cells and mice exhibit the accumulation of damaged organelles and autophagy deficiency.[164] These findings suggest a dual role of acetylation in the regulation of ATG function in autophagy induction. Furthermore, interplay between acetylation and other PTMs (e.g., phosphorylation and lipidation) also affect autophagic processes through multi-PTMs of ATG proteins.[161,165] For example, under growth factor deprivation conditions, activation of GSK3 (glycogen synthase kinase 3) leads to phosphorylation of KAT5/TIP60 (K [lysine] acetyltransferase 5) at Ser86, which directly acetylates ULK1 at Lys162 and Lys606 in human cancer

cells.[165] Acetylation of ULK1 by KAT5 increases both kinase activity and the function of ULK1 at the stage of autophagy induction.[165] These observations indicate that cooperation between acetyltransferases and deacetylases provides a molecular basis for the fine-tuning of autophagy.

Lipidation of Atg/ATG Proteins

Lipidation is a modification that gives proteins distinct membrane affinities. Membrane localization of these proteins depends on their covalent modifications by specific lipids, resulting in communication with additional proteins associated with the intracellular face of the membrane.

Members of the Atg8/LC3/GABARAP family of proteins are covalently attached to phagophore membranes, functioning in phagophore elongation and cargo recognition, and the lipid conjugation of Atg8/LC3/GABARAP is essential for autophagosome formation.[16,34,166] In addition, the lipidated form of Atg8/LC3/GABARAP is associated with the phagophore and autophagosome membrane, and therefore is extensively used to examine autophagic activity.[40,167] During the LC3 lipidation cascade in mammalian cells, nascent LC3 is proteolytically processed to expose a C-terminal glycine (equivalent to the removal of the arginine residue of yeast Atg8). The resulting LC3-I is first activated by ATG7 via a thioester bond that is formed between a catalytic cysteine residue of ATG7 and the C-terminal glycine of LC3-I. The LC3–ATG7 intermediate exchanges with ATG3 in forming a second thioester intermediate, LC3–ATG3. Finally, the ATG12–ATG5-ATG16L1 E3 complex functions as a scaffold and promotes the lipid conjugation of LC3 through recruiting the E2 enzyme ATG3 to PE.[168] This process converts the soluble form, LC3-I, into the lipidated form, LC3-II, which can attach to the phagophore membrane (**Fig. 3**). A structural and mutational study found that both ATG12 and ATG5 are directly involved in E3 activity through residues that are assembled into a continuous surface patch upon conjugation.[169] In addition to binding to ATG5, ATG12 has another surface (at the side opposite from the interface with ATG5), which functions as the site for interaction with ATG3. This interaction is critical for E2-E3 complex formation.[169,170] In addition, the N-terminal 20 amino acids of mouse and human ATG3 form a membrane curvature-sensing amphipathic helix that allows LC3/GABARAP lipidation to proceed efficiently.[171] Finally, the N-terminal domain of ATG16L1 is responsible for the localization of the ATG12–ATG5 conjugate to membranes during autophagosome formation.[168,170]

Proteolysis of Atg/ATG Proteins

Some proteins are activated by proteolysis following translation because they are synthesized as inactive precursors, and proteolysis is involved in several different stages of autophagy. In the initiation phase of autophagy, Atg8 and its orthologs are synthesized as inactive precursors, which are then activated through removal of a C-terminal polypeptide segment. In yeast, Atg8 is cleaved by the cysteine protease Atg4 following translation, removing its C-terminal arginine residue and leaving the glycine 116 residue at the C terminus. This C-terminal glycine is required for interaction with the conjugation system and subsequent Atg8 lipidation.[34] Atg4 is also required for a deconjugation step in which PE is removed from Atg8 on the surface of the autophagosome.[172] The deconjugation step is required for disassembly of Atg proteins and the completion of autophagosome formation.[173] Thus, Atg4 functions in 2 stages of autophagy, phagophore expansion and subsequent autophagosome completion. The single Atg4 in yeast has 4 mammalian orthologs, ATG4A, ATG4B, ATG4C, and ATG4D. Whereas ATG4A and ATG4B have potent enzymatic activities, ATG4C and ATG4D seem to have only minor effects on LC3 processing.[174] Like Atg4 in yeast, ATG4B plays a role in the processing and deconjugation of LC3B in mammalian cells.[167,175] Arginine 68 in human LC3B is a specific residue facilitating the interaction between LC3B and ATG4B by forming a salt bridge.[176] As discussed above, the proteolytically processed LC3 (LC3-I) is subjected to conjugation with PE catalyzed by ATG7 and ATG3, yielding the phagophore membrane-associated form, PE-conjugated LC3 (LC3-II).[40,167,177] Consequently, starvation-stimulated formation of reactive oxygen species, specifically H_2O_2, in mitochondria can lead to oxidation of the catalytic site of ATG4A/B (Cys81).[178] Oxidized ATG4A/B loses deconjugation activity resulting in an increase in membrane-associated LC3-II (**Fig. 3**).[178] Thus, ATG4 activity in the autophagic process may be redox-regulated.

In the execution of autophagy, lysosomal hydrolases/cathepsins are involved in the degradation of autophagosomal membrane components.[179] As the association of LC3 with the autophagosome membrane is a reversible process, the deconjugated LC3 is recycled and participates in a new conjugation reaction (as mentioned above), whereas LC3-II trapped inside the autophagosome is degraded following fusion with the lysosome. Macrophages derived from CTSE/cathepsin E-deficient mice exhibit abnormal membrane trafficking and delayed degradation of autophagosomal membrane proteins.[180] The ATP- and ubiquitin-independent 20S proteasome can completely degrade LC3.[181] This process requires the N-terminal helices of LC3, and can be inhibited by SQSTM1.[181] SQSTM1 can bind LC3-II and bring SQSTM1-containing protein aggregates to the autophagosome for degradation.[182] These findings suggest a negative or competitive regulation mechanism between the proteasome and autophagy.[181]

CAPN/calpains and CASP/caspases can selectively cleave ATGs, which regulates the balance and switch between autophagy to apoptosis in response to various stimuli (**Fig. 5**).[183-192] For example, ATG5 is cleaved at Thr193 by CAPN1 and CAPN2, but not CASP3, CASP6, CASP7, CASP8, or CASP9, in an apoptotic human cervix adenocarcinoma cell line (HeLa) and a human T lymphocyte cell line (Jurkat).[183] Truncated ATG5 lacks autophagy-inducing capacity, whereas it can induce apoptosis through binding to BCL2L1 at the mitochondria and promoting the release of mitochondrial prodeath factors such as CYCS/cytochrome c.[183] Interestingly, the combination of

TNFSF10/TRAIL (tumor necrosis factor [ligand] superfamily, member 10) and arginine deprivation induces ATG5 cleavage through CASP6, CASP9, and CASP10, but not CASP8 activation in human melanoma cell lines (A375 and A2058), which contributes to apoptosis;[188] however, the CASP cleavage sites of ATG5 remain unknown.

Cleavage of BECN1 has been observed in multiple apoptotic cells, and this cleavage is mediated by CASP3, CASP6, CASP8, CASP9, and CASP10.[184,188,189,191,192] The cleaved C-terminal BECN1 cannot induce autophagy, but induces apoptosis once it translocates into the mitochondria and cooperates with other proapoptotic BCL2 members.[184] Asp149,[184,190] Asp133,[184,191] and Asp146[191] at the C terminus of BECN1 have been reported as CASP cleavage sites in human or mouse BECN1, which depends on the cell type and treatment. In addition to caspases, calpains might be responsible for BECN1 cleavage, although the putative cleavage site(s) remains unidentified.[184] Moreover, cleavage of ATG3 at Asp169 by CASP8[185] and cleavage of ATG16L1 at Thr300 by CASP3[186] impairs autophagy during receptor-activated cell death or bacterial infection. In contrast to the above discussion of cleaved ATG proteins lacking autophagy activity, cleavage of ATG4D at Asp163 by CASP3 enhances the priming and delipidation of GABARAPL1, but not GABARAPL2 and LC3 in human cancer cells.[187] These findings represent the molecular crosstalk between apoptosis and autophagy, although it is clearly complex.[5]

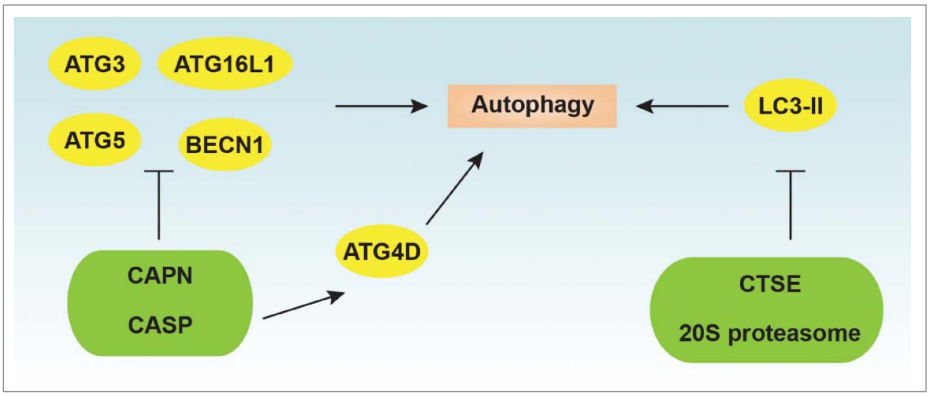

Figure 5. Regulation of ATG proteins by proteolysis. CAPN/calpain and CASP can inhibit autophagy by degrading or cleaving ATGs such as ATG3, ATG5, ATG16L1, and BECN1. In contrast, CASP-mediated ATG4D cleavage promotes autophagy. In addition, CTSE/cathepsin E and the 20S proteasome are involved in the degradation of the phagophore and autophagosome membrane component LC3-II.

Sulfation of Atg Proteins

Sulfation occurs in the trans-Golgi network and is mediated by a membrane-bound protein with its active site on the lumenal side. A sulfate ion is bound near the catalytic cysteine of Atg3 in yeast.[193] The sulfate ion of Atg3 may mimic the phosphate group of PE and bind to the PE-binding site.[193] In addition, 3 basic residues (Lys183, His232, Lys235), which surround the sulfate ion, might recognize the phosphate moiety of PE.[193] This may provide a molecular basis for understanding the mechanism of the Atg3-mediated lipidation reaction.

Conclusions

In the 1960s, Christian de Duve, a Nobel Prize-winning Belgian cytologist and biochemist, coined and described "autophagy," which literally means "self-eating." During the past 15 years, significant progress has been made toward understanding the morphology, mechanism, and regulation of autophagy.[52,194] Currently, autophagy has become a hot field in translational medicine. The most important regulators for autophagy are autophagy-related genes/proteins and the first members were identified in yeast in 1996-7. As we have discussed in this review, multiple PTMs exist on ATG proteins during the complicated membrane reshuffling processes in yeast and mammalian cells that are part of autophagy (**Table 1**). We are surely at a very stimulating and exciting stage in the study of ATG protein modifications. Usually, the patterns of modification across a single ATG protein are combinatorial. Different PTMs can exhibit distinct downstream responses in autophagy. Although these PTMs play a critical role in the regulation of ATG protein functions in different stage of autophagy, many specific questions remain to be answered: How do different PTM "codes" communicate to fine-tune the process of autophagy? What are the essential enzymes responsible for specific PTMs in different ATG proteins? What are the effects of PTMs on structural modifications of ATG proteins? How do PTMs qualitatively and quantitatively sense different forms of autophagic stimulus in different species? How should PTMs in autophagy be systematically measured and monitored? Of course, the mechanistic basis of PTM encoding presents an urgent scientific and technological challenge. The complexity of crosstalk and cellular signaling may help explain the PTM "code." More work is needed for a complete description of the process, mechanism, and function of PTMs in the regulation of autophagy. This will not only reveal fundamental mechanistic insights into the regulatory process, but also provide new therapeutic targets for the treatment of autophagy-associated diseases. In particular, dissection of the upstream pathways that regulate the PTM will be crucial for developing pharmaceutics to target autophagy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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