Emerging Mechanisms in Initiating and Terminating Autophagy

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Autophagy is a major degradative process activated in a rapid and transient manner to cope with stress conditions. Whether autophagy is beneficial or detrimental depends upon the rate of induction and the appropriateness of the duration. Alterations in both autophagy initiation and termination predispose the cell to death, and affect the execution of other inducible processes such as inflammation. In this review we discuss how stress signaling pathways dynamically control the activity of the autophagy machinery by mediating post-translational modifications and regulatory protein interactions. In particular, we highlight the emerging role of TRIM and CULLIN families of ubiquitin ligases which play opposite roles in the autophagy response by promoting or inhibiting, respectively, the activity of the autophagy initiation complex.

Autophagy – A Tightly Regulated Response To Stress
Macroautophagy, hereafter referred as autophagy (see Glossary), protects cells from stress-induced damage by engulfing non-functional cellular components into double-membrane vesicles called autophagosomes and delivering them to the lysosome for degradation [1]. By this mechanism, it also maintains an appropriate amount of ‘building elements’ to be recycled for macromolecular synthesis and energy production when external nutrient supply is limited [1].

Dysregulated autophagy is associated with several pathological conditions [2]. On the one hand, defective autophagy leads to the accumulation of protein aggregates or dysfunctional organelles that predispose to neurodegeneration, muscular dystrophy, and metabolic syndromes [1,3]. Impairments in autophagy are also associated with increased inflammation and the development of autoimmune pathologies such as Crohn’s disease [4]. This is often coupled with accumulation of reactive oxygen species (ROS), which can also cause genomic alterations that lead to tumorigenesis [1]. On the other hand, an unrestrained response turns autophagy into a cell death process as a result of excessive self-digestion. Damage induced by excessive autophagy is observed in myocardial and cerebral ischemia [5].

A crucial feature of autophagy is that it is a highly dynamic process able to sense intracellular stress within minutes and rapidly mount a response to cope with the damage. This is made possible by the ability of a multitude of unrelated cellular pathways to converge on the autophagy machinery to signal a diversity of stressors [6]. A series of signal transduction kinases were initially identified as crucial switches of autophagy protein activity [6]. It is now emerging that the autophagy response to stress also requires the participation of other post-translational modification enzymes, with a major role played by E3 ubiquitin ligases in controlling autophagy.

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*Trends
Autophagy is a catabolic process essential for the basal turnover of cellular components. In response to stress stimuli, autophagy is rapidly induced to ensure the restoration of cellular homeostasis. The duration of autophagy response is also tightly regulated to avoid uncontrolled cell digestion.

Autophagy is mainly regulated at the post-translational level. Phosphorylation and ubiquitination are the best-characterized post-translational modifications responsible for the regulation of the autophagy core machinery both at the initiation and termination stages.

Recently, two large families of E3 ubiquitin ligases, TRIM and CULLIN, have been identified as crucial regulators of the autophagy response by acting, respectively, as platforms for the activation of the core machinery and driving its proteasomal degradation to terminate the process.

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complex stability [6]. In this review we provide an integrated view of how the functional interaction between stress signaling pathways and the core autophagy proteins results in the proper initiation and termination of the autophagy response.

The Core Autophagy Proteins

The autophagy process consists of a sequence of molecular events that lead to the formation of the autophagosome which enwraps intracellular material and eventually fuses to the lysosome to degrade its contents [1,7]. Each step is under the control of specific autophagy complexes whose activity is directly or indirectly regulated by stress signaling pathways [6].

The ULK complex is composed of protein kinases ULK1 and ULK2, in association with the FIP200 scaffold protein and the regulatory subunits ATG13 and ATG101, which are thought to activate ULK proteins by inducing conformational changes [8]. This complex is essential for transmitting stress signals to the site where the autophagosome will be formed, mainly in nutrient- or energy-deprived conditions, by both activating activating phosphorylation of downstream autophagy proteins and playing non-catalytic, scaffolding roles [8,9].

The VPS34 complex I is composed of the class III phosphatidylinositol-3-kinase VPS34, an enzyme that also plays a role in endocytosis, together with the autophagy-specific cofactors BECLIN-1 and ATG14, as well as the VPS15 scaffold protein which is required for the assembly of the catalytic and regulatory subunits [10,11]. This complex allows the formation of phosphatidylinositol-3-phosphate (PI3P), a key signal for the recruitment of autophagy factors required for the nucleation of the autophagosome precursor membrane (termed the isolation membrane or phagophore) [7]. PI3P is mainly produced in specialized structures of the endoplasmic reticulum (ER), as described in a recent review [12], that are preferentially located in the contact sites of ER with other organelles, such as the ER–mitochondria junction [13] and the ER–Golgi intermediate compartment [14].

The phagophore membrane undergoes expansion by a mechanism that involves the recruitment of ATG9 and a large family of mammalian paralogs of yeast Atg8 (mATG8s) that includes the LC3 and GABARAP subfamilies [7]. Vesicles containing the transmembrane protein ATG9 shuttle between the trans-Golgi network, the endosomal compartment, and the phagophore [7,15]. Although its exact function remains unclear, ATG9 is thought to deliver lipids to autophagosome precursors. mATG8s are inserted in the phagophore by covalent attachment to the lipid phosphatidyethanolamine [16]. The modification of LC3s, the best-characterized mATG8s, occurs through an ubiquitination-like cascade regulated by the LC3 protease ATG4, the E1 activating enzyme ATG7, the E2 conjugating enzyme ATG3, and the E3 ligase complex comprising ATG5/ATG12/ATG16L1 [16]. The LC3 membrane insertion site is determined by the PI3P interacting protein WIPI2, which binds to ATG16L1 and recruits the E3 ligase to the isolation membrane [17]. During autophagosome biogenesis, LC3 carries out membrane tethering and hemifusion activities which are thought to control the size of autophagosomes [18]. Moreover, LC3 interacts with cargo receptors, such as the sequestosome 1/p62-like family proteins, which confer selectivity to autophagic degradation [19].

Finally, the fusion of the autophagosome with the lysosome is regulated by proteins that are largely shared with the endocytic pathway. They include the VPS34 complex II, in which BECLIN-1, VPS34, and VPS15 are present, while UVRAG replaces ATG14 [7,10], which acts in cooperation with the small GTPase protein RAB7 [20], the RAB7 effector protein PLEKHM1 [21], the SNARE protein syntaxin 17 [22], and the homotypic fusion and vacuole protein sorting complex (HOPS) [23]. Both autophagosomes and endosome fusion with the lysosomes are negatively regulated by RUBICON, which binds to the VPS34 complex II in a mTORC1-dependent manner and interferes with the activity of RAB7 and HOPS [24].

Glossary

Autophagy: lysosomal degradation of intracellular components which is achieved by three different mechanisms, defined as macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA). In macroautophagy, autophagosomes sequester portions of cytoplasm and deliver them to lysosomes for degradation. In microautophagy, components are directly sequestered through invaginations of the endolysosomal membrane. CMA selectively degrades proteins containing a KFERQ motif, which is recognized by cytosolic chaperones and delivered to lysosomes via LAMP2A.

CULLINs: the largest family of E3 ubiquitin ligases promoting ubiquitination and degradation of proteins involved in a variety of cellular processes. CULLINs are protein complexes composed by four elements: the catalytic core (RING-finger proteins 1 and 2), the scaffolds (CUL1, 2, 3, 4A, 4B, 5, 7, 9), the adaptors (SKP1, elongin B/C, and DDB1) and the substrate receptors (>400 proteins classified on the basis of structural domains: F-BOX, SOCS, BTB and WD40).

Mechanistic target of rapamycin (mTOR): a Ser/Thr kinase that regulates cell growth and metabolism in response to extracellular signals. mTOR is part of two distinct complexes, TORC1 and TORC2, which differ in the accessory proteins they contain, such as RAPTOR and RICTOR, respectively. mTORC1 controls protein synthesis, ribosomal biogenesis, transcription, and autophagy. mTORC2 mainly regulates endocrine signaling, such as insulin, IGF-1, and leptin.

Tumor necrosis factor receptor-associated factor 6 (TRAF6): an E3 ubiquitin ligase acting downstream from multiple receptor families, such as the TNFR superfamily, interleukin-1/Toll-like receptor family, tumor growth factor-β receptors, and the T cell receptor. In this context, TRAF6 activates the transcription factor NF-κB by catalyzing the non-degradative (Lys63-linked) ubiquitination of various target proteins, including the TAK1/TAB2/TAB3 complex, subsequently activating IκKs and MAPKs.
Regulation of Autophagy Initiation

The autophagy machinery is constitutively expressed in all mammalian cells, suggesting that regulation of autophagy by stress stimuli occurs mainly at the post-translational level. In this scenario, a coordinated cascade of phosphorylation and ubiquitination has emerged as a major switch to activate the autophagy machinery (Figure 1 provides an overview of the currently known post-translational modifications of core autophagy proteins, as discussed below).

Regulation by Signal Transduction Kinases

ULK1, mTORC1, and Growth Factor-Regulated Kinases

The best-characterized stimulus that induces autophagy is nutrient deprivation. Under fed conditions, intracellular amino acids and growth factors activate mTORC1, a mechanistic target of rapamycin (mTOR) kinase complex that positively regulates anabolic processes and represses catabolic ones, including autophagy. mTORC1 inhibits the ability of ULK complex to activate downstream ATG proteins by phosphorylating ULK1 at Ser638/758 and its partner ATG13 at Ser258 [25,26]. Interestingly, mTORC1 may redirect rather than fully inhibit ULK1 activity. Indeed, it has been observed that type I interferons require ULK1 to activate p38 MAPK and induce the expression of interferon-responsive genes. In this context, mTORC1 stimulates ULK1 activity through its phosphorylation at Ser758 [27].

In nutrient starvation, inactive mTORC1 is released from ULK1, allowing ULK1 to phosphorylate its own complex components and downstream autophagy targets [25]. ULK1 stimulates PI3P synthesis by phosphorylating members of the VPS34 complex I, including BECLIN-1 (Ser15/30/96/279), VPS34 (Ser249), and ATG14 (Ser29) [28–30]. Moreover, ULK1 activates AMBRA1, a WD40 protein required for VPS34 complex I stability, by releasing it from different inhibitors such as the dynein motor [31] and the E3 ubiquitin ligase CULLIN-4 [32]. ULK1-dependent phosphorylation sites on AMBRA1 have been identified (Ser465/635), although their role has yet to be assessed [29]. Conversely, AMBRA1 activity is suppressed by mTORC1 phosphorylation of Ser52 [33].

In addition to activating mTORC1, growth factors also inhibit BECLIN-1 activity through AKT-mediated phosphorylation at Ser234/295, which mediates BECLIN-1 interaction with 14-3-3 proteins and its consequent sequestration to the cytoskeleton [34]. Moreover, the epidermal growth factor receptor (EGFR) suppresses autophagy by phosphorylating BECLIN-1 at Tyr229/233/352 [35]. These modifications increase BECLIN-1 binding to the negative regulators BCL-2 and RUBICON, and decrease its association with VPS34. EGFR has a more complex role in autophagy regulation. In fact, unstimulated EGFR acts as a positive regulator of autophagy by forming a complex with LAPTM4b in the endosomal compartment, which is able to displace RUBICON from BECLIN-1, setting the latter free to initiate autophagy [36].

AMP Kinase (AMPK)

Energy limitation, either from excessive energy consumption or shortage of energy stores, is another relevant autophagy stimulus. A high AMP/ATP ratio activates the LKB1/AMPK pathway, which stimulates catabolic processes to provide elements for ATP synthesis [37]. In particular, AMPK activates autophagy by phosphorylating ULK1 at multiple sites, including Ser317/555, and BECLIN-1 at Ser91/Ser94 [38–40]. Moreover, AMPK is able to differentially control the endocytic and autophagic pools of VPS34. In fact, AMPK inhibits endosomal VPS34 by phosphorylating it at Thr163/Ser165, thereby shutting down this process when external energy sources become limited. Conversely, the pro-autophagic VPS34 is not phosphorylated by AMPK because of the protective role of ATG14, which instead favors an activating phosphorylation of BECLIN-1 at Ser91/Ser94 by AMPK [41]. AMPK also phosphorylates the scaffold protein RACK1 to induce its binding to BECLIN-1, VPS15, and ATG14, promoting the assembly of VPS34 complex I [40].
AMPK indirectly activates autophagy by repressing mTOR activity through inhibition of the mTORC1 component RAPTOR and activation of the mTORC1 inhibitor TSC2[37]. However, ULK1 activation by AMPK can occur in the absence of mTORC1 inhibition, such as in muscle cells during physical exercise[42].

Importantly, many other stimuli converge on AMPK to activate autophagy. For example, calcium-mobilizing pathways stimulate autophagy via CAMKK2-mediated phosphorylation of AMPK[43].

Stress Kinases Targeting the BECLIN-1/BCL-2 Interaction

Under non-stress conditions, the autophagosome-forming activity of BECLIN-1 is inhibited by the interaction of with specific members of the BCL-2 family: the anti-apoptotic proteins BCL-2, BCL-xL, MCL-1 at the ER [44], and the pro-apoptotic protein BIM at microtubules [45].

Autophagy induction requires the dissociation of these proteins from BECLIN-1, and this is achieved through either signaling kinases or protein–protein displacement (Box 1). JNK was the first identified kinase able to trigger BECLIN-1 activity by releasing BCL-2 binding upon phosphorylation [46]. JNK is activated in nutrient starvation by its recruitment to acetylated tubulin through the JIP1/kinesin complex [47]. The kinase ROCK1 also contributes to this
activation by both phosphorylating BECLIN-1 at Thr119 in the BH3 domain and by stimulating JNK via JIP1 phosphorylation [48]. Several other stimuli induce autophagy via JNK, such as those inducing ER stress via the IRE1/TRAF2/ASK1 pathway [49]. Relevant examples of physiological processes in which autophagy is regulated by JNK are the degradation of unfolded type II collagen in growth plate chondrocytes during bone formation [50], and the breakdown of myelin in Schwann cells after nerve injury [51].

DAPK, a stress kinase activated in response to oxidative and ER stress, is also able to release BCL-2 from BECLIN-1. In this case, binding is disrupted by phosphorylating the BH3 domain of BECLIN-1 (Thr119) [52]. DAPK also activates VPS34 indirectly by phosphorylating protein kinase D, which in turn binds to and phosphorylates VPS34 [53].

Of note, phosphorylation of BECLIN-1 at a different site in the BH3 domain can instead strengthen the BCL-2/BECHIN-1 interaction, as shown for phosphorylation of Thr108 by the pro-apoptotic kinase MST1 [54]. The relevance of this kinase in autophagy regulation has been confirmed analyzing Mst1 transgenic mice, which show cardiac dysfunction associated, on the one hand, with the accumulation of protein aggregates as a consequence of excessive autophagy inhibition by BCL-2 and, on the other hand, on the reduced amount of BCL-2 available to block the pro-apoptotic BH3-only protein BAX [54].

Taken together, these data underline how the BECLIN-1/BCL-2 association is a crucial target of both pro- and anti-autophagic stress kinases. What remains less well characterized is the mechanism by which the BECLIN-1 activity is inhibited by BCL-2. A recent report provided evidence that the inhibitory activity of BCL-2 may reside in its ability to prevent activating phosphorylation of BECLIN-1 at Ser90, which is mediated by the stress kinases MAPKAPK2/MAPKAPK3 and DAPK3 [55]. Whether this is also the case for other activating modifications of BECLIN-1 and VPS34 remains to be verified.

**Box 1. Regulation of BECLIN-1 Function Through Its BH3 Domain**

The BH3 domain of BECLIN-1 mediates the inhibitory interaction with the anti-apoptotic members of BCL-2 family. The crucial role of these proteins in the control of autophagy is also underlined by the presence of several viral BCL-2-like proteins which hijack the autophagy inhibitory function of their cellular homologs [91]. For example, alphaherpesvirus-1 ICP54.5, human cytomegalovirus TRS1, Kaposi’s sarcoma herpesvirus vBCL-2, and murine gamma herpesvirus 68 M11 inhibit the formation of autophagosomes by binding to BECLIN-1 in infected cells.

In addition to phosphorylation, the BCL-2/BECLIN-1 interaction is disrupted by competitive binding to a series of proteins. Stress-induced expression of BH3-only proteins, such as tBID, BAD, PUMA, NOXA, NIX, and BNIP3, frees BECLIN-1 from BCL-2 family proteins and induces autophagy [92]. AMBRA1 is also able to bind to BCL-2 through a BH3-like domain. Under non-stress conditions, BCL-2 inhibits the pool of AMBRA1 at mitochondria. When autophagy is induced, AMBRA1 dissociates from BCL-2 by an as-yet unknown mechanism. Active AMBRA1 can migrate to the ER where it activates BECLIN-1 by reducing its interaction with BCL-2, or contributes to mitophagy by binding to both the E3 ligase Parkin and LC3 on damaged mitochondria [93,94].

Non-BH3 only proteins may also disrupt BECLIN-1/BCL-2 interaction to induce autophagy, as shown for VMP1, a stress-inducible ER/Golgi transmembrane protein [95], and HMGB1, a chromatin-associated protein that interacts with BECLIN-1 upon nucleus-to-cytosol translocation in a ROS-dependent manner [96].

Irrespective of the nature of the initiating stimulus, increasing evidence shows that a series of major stress-response signaling pathways establish a strict crosstalk with autophagy to restore homeostasis [56–58]. Emerging examples are represented by cGAS- and TAK1-regulated pathways that transduce signals from innate and adaptive immune receptors.

The TAK1 kinase and its partners TAB2/TAB3, which are known to control NF-κB transcriptional activity [59], regulate BECLIN-1 activity through direct interactions. In the absence of autophagy
stimuli, TAB2/TAB3 bind to and inhibit BECLIN-1 [56]. Upon autophagy induction, BECLIN-1 is released from TAB2/TAB3 and engages a stimulatory interaction with TAK1. Activation of TAK1 contributes to autophagy by acting on the AMPK/mTORC1 axis. The important role of TAK1 in autophagy was recently confirmed using liver-specific knockout mice that show severe autophagy inhibition associated with the development of hepatosteatosis and hepatocarcinoma when challenged with a high-fat diet [60]. Notably, treatment with the mTORC1 inhibitor rapamycin reduced lipid accumulation and hepatocarcinoma by restoring autophagy in the liver of TAK1 knockout mice.

The cytosolic DNA sensor cGAS, which activates the IRF3-mediated expression of type I interferons in response to viral and bacterial infection [61], regulates both ULK1 and BECLIN-1 activity. The direct interaction between cGAS and BECLIN-1 enhances the autophagy response to cytosolic pathogen DNA [57]. In particular, this association leads to the release of RUBICON from BECLIN-1, which allows activation of pathogen-selective autophagy. Moreover, induction of autophagy by cGAS has a crucial role in self-limiting its own pathway to avoid excessive inflammation. In fact, BECLIN-1 interacts with cGAS and inhibits its activity, while ULK1, also activated by cGAS, mediates inhibitory phosphorylation of STING, a downstream effector of cGAS [58].

Regulation by E3 Ubiquitin Ligases and Deubiquitinases
In addition to phosphorylation, autophagy initiation is controlled by ubiquitination of ULK and VPS34 complexes. This post-translational modification, mediated by E3 ubiquitin ligases and reversed by deubiquitinases, regulates autophagy by modulating protein activity (non-degradative ubiquitination) or leading to protein degradation (degradative ubiquitination). These different effects depend on the type of poly-ubiquitination, with Lys48-linked chains usually leading to proteasomal degradation and Lys63-linked chains modifying protein interaction properties.

Non-Degradative Ubiquitination
The role of non-degradative ubiquitination in stress signaling pathways was first characterized in the regulation of innate immune response, where, together with phosphorylation, it is a crucial covalent modification leading to the activation of the transcription factor NF-κB [62]. Tumor necrosis factor receptor-associated factor 6 (TRAF6), a central E3 ubiquitin ligase of NF-κB signaling pathways [63], also participates in autophagy stimulation by mediating Lys63-linked polyubiquitination of ULK1 [33]. The specific induction of autophagy by TRAF6 is conferred by AMBRA1, which mediates the interaction between TRAF6 and ULK1. Non-degradative ubiquitination by TRAF6 stimulates ULK1 self-association, a prerequisite for its kinase activity. AMBRA1 also indirectly promotes ULK1 activity by stabilizing the mTOR inhibitor DEPTOR [32]. AMBRA1 blocks DEPTOR degradation by interfering with the E3 ligase CULLIN-5. Because AMBRA1-dependent inhibition of CULLIN-5 requires ULK1 activity, this mechanism acts as a positive amplification loop triggered by ULK1 to expedite the decline of mTORC1 activity and allow rapid induction of the autophagy response.

TRAF6 also triggers Lys63-linked ubiquitination of BECLIN-1, which is counteracted by the deubiquitinase A20 [64]. This modification, mapping to the BH3 domain (Lys117) of BECLIN-1, is required for autophagy induction by blocking interaction with BCL-2 and favoring protein oligomerization through an ubiquitin-binding domain of BECLIN-1. TRAF6 has a well-established role in the regulation of both TAK1 and JNK signaling [59], although whether TRAF6 also controls autophagy through these kinases remains largely unexplored.

While the role of AMBRA1 in Lys117-ubiquitination of BECLIN-1 remains unassessed, AMBRA1 has been shown to stimulate K63-linked ubiquitination at Lys437, which promotes
BECLIN-1/VPS34 association [65]. CULLIN-4 has been proposed as an E3 ligase for Lys437-ubiquitination of BECLIN-1; however, the observed dissociation of CULLIN-4 from AMBRA1 in the early steps of the autophagy response suggests that other E3 ligases may play a direct role in this process. AMBRA1-mediated ubiquitination of BECLIN-1 is negatively regulated by WASH, a factor whose deficiency in mice causes embryonic lethality associated with excessive autophagy [65].

Degradative Ubiquitination

A further level of regulation of autophagy initiation is represented by the modulation of the protein levels of VPS34 complex I by the ubiquitin–proteasome system. Various types of degradative ubiquitination negatively regulate BECLIN-1 stability. Blocking the BECLIN-1/VPS34 interaction causes Lys11-linked ubiquitination of BECLIN-1 at Lys437 by the E3 ligase NEDD4, which induces its degradation [66]. Moreover, degradation of BECLIN-1 is observed during bacterial infection, and this is mediated by the E3 ligase RNF216 through Lys48-linked ubiquitination [67].

The level of ATG14 is also controlled by ubiquitin-dependent degradation. The E3 ligase responsible for this regulation is the CULLIN-3 complex containing ZBTB16 as the substrate receptor [68]. Upon serum starvation, stability of ATG14 is increased by the kinase GSK3β, which phosphorylates ZBTB16 inducing its self-degradation. Conversely, ATG14 is destabilized by G protein-coupled receptors signaling through GSK3β repression. Importantly, induction of autophagy by pharmacological inhibition of these receptors reduces the accumulation of misfolded proteins in mouse models of Huntington’s disease.

Degradative ubiquitination of BECLIN-1 is counteracted by various deubiquitinases, including USP10 and USP13, which are the targets of the autophagy inhibitor spautin [69]. Moreover, BECLIN-1 stability is increased by USP19, which counteracts Lys11-linked ubiquitination at lysine 437 [70]. USP19-mediated BECLIN-1 stabilization regulates not only the autophagy response to nutrient starvation but also the ability of BECLIN-1 to restrict innate immunity by inhibiting the RIG-1/MAVS/TBK1/IRF3 pathway.

Interestingly, deubiquitinases play a role in the repression of BECLIN-1 by BCL-2 family proteins. Indeed, MCL-1 induces BECLIN-1 degradation by displacing it from the deubiquitinase USP9X, as observed in tumor cells that highly express MCL-1, such as melanoma [71].

The Autophagy Initiation Complex

Recent evidence points to the association of ULK1 and BECLIN-1 in a common complex as a key requirement for autophagy induction. The assembly of this ‘autophagy initiation complex’ is promoted by specific scaffold proteins whose gathering activity is under the control of signaling kinases and E3 ubiquitin ligases (Figure 2).

Regulation by the Exocyst Complex

The first evidence of the presence of an ULK1/BECLIN-1 common complex came from studies on Exocyst, a protein complex originally identified for its role in tethering exocytic vesicles to plasma membrane [72]. More recently, Exocyst has been found to be involved in various physiological processes including inflammation, the cell cycle, cell migration, and tumor invasion [72].

The Exocyst complex is composed of eight subunits: SEC3, SEC5, SEC6, SEC8, SEC10, SEC15, EXO70, and EXO84. Two-hybrid screening assays identified direct interaction between Exocyst proteins and the autophagy factors FIP200, ATG14, and RUBICON [73]. Coimmunoprecipitation and colocalization assays then revealed a dynamic interaction of ULK1, BECLIN-1, and ATG5/ATG12 complexes with distinct Exocyst subcomplexes, depending on nutrient availability. In unstressed conditions, inactive ULK1 and BECLIN-1 associate with a SEC5-positive/EXO84-negative complex of 500 kDa at the Golgi compartment (Figure 2A). This
complex is also involved in the regulation of both mTORC1 and innate immune TBK1–IRF3 signaling pathways. Nutrient starvation and pathogen infection both induce the formation of an EXO84-positive/SEC5-negative complex of >700 kDa, characterized by active ULK1 and BECLIN-1 as well as LC3 positivity (Figure 2A). The formation of the ‘pro-autophagic’ EXO84 complex depends on the presence of the GTPase RALB. However, RALB is not exclusive to EXO84 activation because it also stimulates innate immunity through the SEC5 subcomplex [74]. Notably, subcomplex activation is determined by the non-degradative ubiquitination of RALB. Ubiquitinated RALB at Lys47 localizes at the SEC5 subcomplex, while deubiquitination is a prerequisite for RALB interaction with the EXO84 complex. This switch is regulated by the deubiquitinase USP33, while specific E3 ubiquitin ligases targeting RALB remain unknown (Figure 2A).

To date, two signaling kinases have been described as converging on the Exocyst complex to induce autophagy [74]. The EGFR/LAPTM4B complex associates with SEC5 to promote autophagy by releasing RUBICON from BECLIN-1 [36] (Figure 2A). The pro-apoptotic kinase STK38 stimulates the binding of EXO84 with BECLIN-1 and RALB [75] (Figure 2B). In addition, interaction with the Exocyst complex determines whether STK38 acts as a pro-autophagic or

Figure 2. Regulation of the Autophagy Initiation Complex. Three distinct systems promote the interaction between ULK and VPS34 complexes. (A) Exocyst. In high-nutrient conditions, ULK and VPS34 complexes associate with a Sec5-positive Exocyst complex. This complex is also involved in the regulation of mTORC1 activity and the innate immune response. Under nutrient-deprivation conditions, ULK1 and BECLIN-1 associate with an Exo84-positive/Sec5-negative Exocyst complex, which also interacts with ATG5/ATG12 and LC3. Ubiquitination levels of RalB, regulated by USP33, determine which Exocyst complex RALB associates with. The interaction of unstimulated EGFR with LAPTM4B contributes to autophagy induction by binding to the Sec5-positive Exocyst complex and dissociating RUBICON from VPS34 complex I. (B) TRIMosome. A subset of TRIM family proteins (see text) act as a platform to assemble ULK1 and BECLIN-1 into a common complex to promote autophagosome formation. These TRIMs also bind to ATG16L1, mATG8s, and specific cargos to allow target recognition. Specific TRIM proteins are involved in the regulation of autophagy in basal conditions, nutrient deprivation, IFN-γ stimulation, or HIV infection (see text) (C) IRGM. Pathogen intracellular sensors activate IRGM through TRAF6-dependent ubiquitination. Activated IRGM interacts with ULK and VPS34 complexes, together with ATG16L1, recruiting them into a single complex. IRGM pro-autophagic activity is also mediated by AMPK.
pro-apoptotic factor. In fact, loss of Exocyst activity by RALB inhibition causes STK38 hyper-activation upon autophagy induction, which leads to induction of cell death.

**Regulation by the TRIMosome**

*Tripartite motif proteins* (TRIMs) proteins are a large family of E3 ubiquitin ligases that have recently been described to regulate the formation of the autophagy initiation complex [76]. Various members of this family participate in autophagosome formation, as shown by the effect of TRIM silencing on both basal and mTORC1-dependent autophagy. Importantly, the majority of the autophagic TRIMs (TRIM5α, TRIM6, TRIM17, TRIM20, TRIM21, TRIM22, TRIM49) bind to both active ULK1 and BECLIN-1, and are required to assemble them into a common complex. In addition, TRIMs contribute to BECLIN-1 activation by favoring the dissociation from the negative regulators BCL-2 and TAB2 [76]. Specific TRIMs also bind to ATG16L1 (TRIM20) and mATG8s (TRIM5α, TRIM20, TRIM21) [76,77]. Overall, these data indicate that TRIMs act as platforms (TRIMosomes) to allow autophagosome formation (Figure 2B). Evidence that EXO84 interacts with TRIM17, but not with TRIM5 [76], suggests that Exocyst and TRIMosome cooperate in some circumstances to assemble the initiation complex.

The role of TRIMs in autophagy is not limited to the induction of the process, and they directly contribute to selective recognition of autophagy cargos by cooperating with mATG8s and p62 family members. Identified targets are HIV ENV protein through TRIM5α [76], IFN-inducing transcription factor IRF3 through TRIM21 [77], and various components of the inflammasome such as NLRP3, NLRP1, and pro-caspase 1 through TRIM20 [77], as well as AIM2 through TRIM11 [78]. Importantly, the early and late autophagic functions of TRIMs appear to be interdependent because TRIM5α mutants unable to bind LC3 are also defective in activating ULK1 [76].

Finally, the recent identification of TRIM31 as an inducer of autophagy in intestinal epithelial cells independently of BECLIN-1, ATG5, and ATG7 suggests that TRIM may also be involved in the regulation of non-canonical forms of autophagy [79].

**Regulation by IRGM**

IRGM is a member of a large family of GTPases dedicated to a cell-autonomous defense response to infections [80]. The crucial role of IRGM in immunity is suggested by the association between IRGM mutations and susceptibility to tuberculosis and Crohn’s disease [80]. IRGM activates xenophagy, a selective form of autophagy for pathogens, when stimulated by several pathogen intracellular sensors including NOD2, NOD1, RIG-I, and TLR3 [81] (Figure 2C). IRGM interacts with ULK1 and BECLIN-1, and favors their assembly in a common complex together with ATG16L1 (Figure 2C). Notably, the E3 ligase TRAF6 ubiquitinates IRGM, and promotes its oligomerization and consequent formation of the autophagy initiation complex (Figure 2C). Moreover, in the absence of IRGM, the proteins ULK1, ATG14, AMBRA1, and ATG16L1 undergo proteasomal degradation, suggesting a role for IRGM in controlling degradative ubiquitination. IRGM also stimulates autophagy via AMPK, and this is presumably related to the ability of IRGM to modulate mitochondrial function [82].

Taken together, these studies highlight the presence of a higher-order assembly of autophagy proteins during autophagy initiation. Importantly, this level of regulation relies on ‘hub’ proteins shared by multiple inducible processes, indicating that different cellular activities may be directly coordinated in response to stress.

**Regulation Of Autophagy Termination**

Although it has long been known that autophagy is self-inhibited to avoid death by unrestrained cell digestion, the mechanisms that regulate autophagy termination have begun to be elucidated only recently (Figure 3).
Regulation by mTORC1
The expected termination signal is resolution of the stress that triggered autophagy. Consistent with this assumption, mTORC1 localizes to the lysosome surface where it senses the increased availability of amino acids following lysosomal degradation [83]. Stimulation of mTORC1 by amino acids requires an interaction with Rag GTPases, a family of Ras-related GTP-binding proteins whose activity is regulated by a series of amino acid binding proteins such as lysosomal permeases, aminoacyl-tRNA synthetases, and sestrins. mTORC1 reactivation may thus contribute to shutting down autophagy when nutrients are replenished [84] (Figure 3). Moreover, mTORC1 reactivation plays a crucial role in the regeneration of ‘ready-to-use’ lysosomes from ‘end-point’ autolysosomes which takes place via membrane tubulation [84].

Regulation by CULLINs
It is now evident that, in addition to mTORC1 reactivation, autophagy is self-inhibited independently of stress resolution because prolonged autophagy in persistent stress conditions can be more detrimental than the causative stress. The main mechanism determining the duration of the autophagy response is the proteasomal degradation of ULK and VPS34 complex members, which occurs shortly after their activation.

CULLINs are the main E3 ubiquitin ligases responsible for triggering degradation of autophagy proteins to terminate the process (Figure 3). AMBRA1 was the first autophagy protein to be

Figure 3. Temporal Regulation of Autophagy Response in Nutrient Starvation. Nutrient starvation leads to mTORC1 inactivation and dissociation from lysosomal membrane, which promotes the activation of ULK and VPS34 complexes via a multitude of post-translational modifications (autophagy initiation). Once activated, ULK and VPS34 complexes allow the execution of the autophagy response. Autophagy termination is subsequently triggered by degradative ubiquitination (Ub) of active ULK and BECLIN-1 complexes performed by the indicated E3 ubiquitin ligases. In addition, amino acids produced by lysosomal digestion reactivate mTORC1, which in turn inhibits autophagy. Abbreviation: AA, amino acid levels.
identified as a target for CULLINs [32]. In particular, AMBRA1 stability is regulated by CULLIN-4 in a time-dependent manner. In nutrient-rich conditions, CULLIN-4 association limits AMBRA1 abundance. ULK1 activation by nutrient deprivation causes a rapid release of AMBRA1 from CULLIN-4 and consequent AMBRA1 protein stabilization. Several hours later, CULLIN-4 reassociates with AMBRA1 and triggers its degradation, initiating autophagy termination. Expression of a WD40-domain mutant of AMBRA1, which cannot bind to CULLIN-4, results in a prolonged autophagy response. The E3 ligase RNF2 has also been reported to induce AMBRA1 degradation to terminate autophagy [85]. Whether or not CULLIN-4 and RNF2 work together remains to be determined.

Recent research has highlighted how other members of the ULK and VPS34 complexes are targeted by CULLINs for proteasomal degradation at the end of the autophagy response [86] (Figure 3). Specifically, ULK1 autophosphorylation at Ser1042/Thr1046 triggers its interaction with CULLIN-3 via the receptor KLHL20 and consequent Lys48-linked ubiquitination. KLHL20 also binds to and ubiquitinates active BECLIN-1 and VPS34. Other members of the ULK and VPS34 complexes, such as ATG13 and ATG14, are degraded during starvation in a KLHL20-dependent but indirect manner because no direct ubiquitination by KLHL20 was observed. Interestingly, KLHL20 also controls the stability of the pro-autophagic kinase DAPK when activated by type I IFN signaling [87], suggesting that BECLIN-1 kinases could also be degraded to terminate autophagy.

Importantly, preventing autophagy termination during nutrient starvation by blocking either CULLIN-3- or CULLIN-4-mediated degradation of autophagy regulators increases susceptibility to cell death stimuli. This observation confirms that, for the pro-survival role of autophagy, the timely switching off of the autophagy response is as crucial as its rapid induction [32,86].

What remains poorly characterized is whether the identified mechanisms of autophagy termination upon nutrient starvation also apply to other type of stress stimuli. Recent evidence shows that CULLIN-1 is responsible for terminating autophagy after induction by DNA damaging agents [88]. In particular, CULLIN-1 targets VPS34 for proteasomal degradation through binding to its receptor FBXL20. Two regulatory events are required for this degradation: (i) VPS34 phosphorylation by CDK1 at Thr159, which occurs during mitotic arrest induced by DNA damage agents, and (ii) transcriptional induction of FBXL20 by the tumor-suppressor protein p53. Importantly, in this system degradation of VPS34 by DNA damage does not exclusively affect autophagy and also affects the activity of VPS34 in endocytosis [88].

Finally, degradation of autophagy proteins by the caspase and calpain proteases has been shown to be responsible for autophagy termination by apoptotic stimuli (Box 2).

**Box 2. Autophagy Termination by Apoptosis**

An alternative and irreversible form of autophagy termination is executed by the apoptotic machinery [92,94]. Autophagy represents the primary cellular strategy to adapt to and cope with stress. However, when stress exceeds a crucial threshold and duration, apoptosis needs to be activated to allow damaged cells to die in a controlled manner to avoid harming the surrounding healthy cells. At the point of no return, the autophagy machinery is rapidly degraded by the apoptotic executioner proteases, such as caspases and calpains, as shown for ATG5, BECLIN-1, AMBRA1, ATG3, ATG4D, and ATG16L1 [8]. Notably, a Thr300Ala substitution in ATG16L1, which is associated with increased risk of developing Crohn’s disease, leads to a reduced ATG16L1 protein level as a result of enhanced degradation by CASPASE-3 upon death-receptor activation or starvation [97].

Interestingly, apoptotic cleavage products of ATG5, ATG4D, BECLIN-1, and AMBRA1 acquire a pro-apoptotic function by translocating to mitochondria and interfering with BCL-2/BCL-X[L] [8,99]. During inflammation, BECLIN-1 and ATG5 cleavage by calpains can be prevented by the interaction with HMGB1. This protective binding has been shown to contribute to the autophagy-dependent resistance of different tumor cells to chemotherapy [99].
Acetylation has more recently emerged as an important post-translational modification that controls autophagy by targeting autophagy regulators in either direct or indirect mechanisms [100].

ULK1 is positively regulated by acetylation at Lys162/606, which is mediated by the acetyltransferase TIP60 under conditions of serum starvation. TIP60 is activated by GSK3β-mediated phosphorylation at Ser86 [101]. However, the details on how ULK1 is activated by acetylation remain to be determined.

By contrast, BECLIN-1 and ATG proteins involved in autophagosome expansion are negatively regulated by acetylation. The protein deacetylases sirtuins act as important players in this context by modulating autophagy in transcriptional-dependent and -independent manner [100]. SIRT1 directly activates BECLIN-1 by reversing inhibitory acetylation at Lys430/437 carried out by the autophagy transducer p300 [102]. SIRT1-mediated deacetylation is stimulated by casein kinase 1-mediated phosphorylation of BECLIN-1 at Ser409. Acetylation impairs BECLIN-1 activity by promoting interaction with the autophagy inhibitor RUBICON.

SIRT1 also interacts and positively regulates the activity of ATG5, ATG7, and LC3. In particular, it has been shown that, in unstressed conditions, a consistent fraction of LC3 has a nuclear localization dependent on acetylation at Lys49 and Lys51 [103]. Autophagy induction stimulates SIRT1-mediated deacetylation of LC3, which allows its nucleus-to-cytoplasm translocation and binding to ATG7 that is required for lipidation. Interestingly, under conditions of glucose starvation, AMPK drives SIRT1 activation by phosphorylating GAPDH, which translocates to the nucleus and displaces the SIRT1 repressor DBC1 [104]. It must be emphasized that p300 has both pro- or anti-autophagic activities depending on its subcellular localization. Indeed, BAG6/BAT3-regulated translocation of p300 from cytoplasm to nucleus impedes negative acetylation of autophagy proteins, while it stimulates autophagy gene transcription in a p33-dependent manner [105].

Concluding Remarks and Future Perspectives

A major challenge in the autophagy field is understanding how the autophagy machinery is promptly activated in response to stress. Transducing stress signals to the autophagy process appears as an intricate puzzle in which the activity of autophagy protein complexes is mainly regulated through a combination of post-translational modifications that control the process in a dynamic manner [6]. At first, these modifications allow autophagy complexes to be rapidly activated and to assemble at the site of autophagosome formation [12]. Active complexes are eventually inhibited to self-limit the response, mainly by mechanisms involving proteasomal degradation [89]. Although essential modifications of autophagy proteins, mainly represented by phosphorylation, ubiquitination, and more recently acetylation (Box 3), have been individually characterized, how they are integrated and influence each other remains largely unknown. The recent structural characterization of ULK and VPS34 complexes by electron cryo-microscopy (cryo-EM) may represent a powerful tool for elucidating how multiple signals impact on this process [90].

Available information on the “On-Off” mechanisms of autophagy has been mainly obtained in nutrient- and energy-restricted conditions. The recent characterization of several types of selective autophagy [19], in addition to providing insights on how specific damaged components are recognized, opens up new questions on how specific stresses may signal to the autophagy machinery (see Outstanding Questions). The large families of TRIM and CULLIN E3 ubiquitin ligases are highly promising candidates for satisfying the requirements of multiple target recognition and signal transduction activation that are essential to regulate autophagy in response to specific types of damage and stimuli.

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