Macroautophagy Signaling and Regulation

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Abstract Macroautophagy is a vacuolar degradation pathway that terminates in the lysosomal compartment. Macroautophagy is a multistep process involving: (1) signaling events that occur upstream of the molecular machinery of autophagy; (2) molecular machinery involved in the formation of the autophagosome, the initial multimembrane-bound compartment formed in the autophagic pathway; and (3) maturation of autophagosomes, which acquire acidic and degradative capacities. In this chapter we summarize what is known about the regulation of the different steps involved in autophagy, and we also discuss how macroautophagy can be manipulated using drugs or genetic approaches that affect macroautophagy signaling, and the subsequent formation and maturation of the autophagosomes. Modulating

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autophagy offers a promising new therapeutic approach to human diseases that involve macroautophagy.

Abbreviations

3-MA	3-Methyladenine
4E-BP1	Eukaryotic translational initiation factor 4E-binding protein 1
AMPK	AMP-activated protein kinase
ATG	Autophagy-related
DAP kinase	Death-associated protein kinase
DRAM	Damage-regulated autophagy modulator
DRP-1	Death-associated related protein kinase 1
eIF2α	Eukaryotic initiation factor 2 alpha
ERK	Extracellular signal-regulated protein kinase
FDA	Food and drug administration
JNK	c-Jun N-terminal kinase
LC3	Light chain 3
MAPK	Mitogen-activated protein kinase
(m)TOR	(Mammalian) target of rapamycin
PE	Phosphatidyl ethanolamine
PERK	Protein kinase R-like endoplasmic reticulum kinase
PI3K	Phosphatidylinositol 3-phosphate kinase
PKR	Double-stranded RNA-activated protein kinase
Rheb	Ras homolog enriched in brain
ROS	Reactive oxygen species
SNARE	Soluble NSF attachment protein receptors
TSC	Tuberous sclerosis complex

1 Introduction

Macroautophagy (referred to as "autophagy" below) is a mechanism conserved among eukaryotic cells that starts with the formation of a multimembrane-bound vacuole, known as an autophagosome, which ultimately fuses with the lysosomal compartment to degrade the sequestered material (Klionsky 2007; Levine and Klionsky 2004; Mizushima et al. 2008; Ohsumi 2001). The seminal discovery of *ATG* (autophagy-related) genes, originally in yeast and then in multicellular organisms, constituted an important breakthrough in our understanding of how autophagosomes are formed, and of the importance of autophagy in cell physiology and in human diseases (see the chapters by Yang and Klionsky and by Mizushima in this volume). However, it is absolutely essential to find out how autophagy is regulated if we are to grasp how it changes in response to stress, including infection. The intricacy of

the regulation of autophagy and apoptosis suggests that there is a subtle dialog between these two processes that determines the fate of the cell (Codogno and Meijer 2005; Gozuacik and Kimchi 2007; Levine and Yuan 2005; Maiuri et al. 2007c).

In this chapter, we divide the regulation of autophagy into three successive levels (Fig. 1), even though the boundaries between them are not clear-cut. The first level of regulation is defined as the signaling pathways that terminate upstream of the Atg machinery. Archetypal examples of this first level of regulation are signaling pathways that terminate at mTOR (Meijer and Codogno 2006), but other signaling pathways with targets within the molecular machinery that have not yet been identified also fall into this category. Many growth factors and cytokines modulate autophagy at this level (Deretic 2006; Lum et al. 2005). The immunosuppressor rapamycin triggers autophagy by interfering with mTOR activity (Meijer and Codogno 2006). The second level of regulation involves modulating the Atg machinery by protein-protein interactions or modulating Atg activity by signaling molecules (Maiuri et al. 2007a; Pattingre and Levine 2006; Scherz-Shouval and Elazar 2007). The Beclin 1/Bcl-2 interaction (Erlich et al. 2007; Maiuri et al. 2007b; Pattingre et al. 2005) and the modification of Atg4 by reactive oxygen species fall within this category (Scherz-Shouval et al. 2007). The third level of regulation involves the late stage of autophagy (maturation and fusion with the lysosomal compartment) (Eskelinen 2005). Blockade during the late stage of autophagy is a hallmark of Alzheimer's disease (Yu et al. 2005), and of a rare cardiomyopathy known as Danon disease (Nishino et al. 2000; Tanaka et al. 2000). A given autophagy modulator may target more than one levels of autophagy; for example, starvation triggers autophagy by targeting both levels 1 and 2 (Pattingre et al. 2008). We do not yet know whether starvation can also modulate level 3.

As will be discussed in greater detail in subsequent chapters, pathogens are also able to interfere with all levels of autophagy. Herpes simplex virus 1 (HSV-1) blocks autophagy by interfering with levels 1 and 2 (Orvedahl and Levine 2008). *Listeria monocytogenes*, *Shigella,* and some other bacteria evade sequestration by manipulating level 1 and/or level 2 (Birmingham et al. 2008; Levine and Deretic 2007; Ogawa et al. 2005). Some bacteria, such as *Legionella pneumophila* and *Coxiella burnetii,* block autophagy at level 3, allowing them to avoid being trapped in the lysosome and escape degradation (Romano et al. 2007; Swanson 2006). Poliovirus probably manipulates both levels 2 and 3 to stimulate autophagy to its own benefit (Taylor and Kirkegaard 2008).

The division of autophagy into the three levels that we propose in this chapter is also important from a functional point of view. Autophagy performs two nonexclusive tasks: it sequesters cytoplasmic material and it degrades it (Mizushima 2005). In some contexts, the sequestration function is the most important, for example when harmful compounds are segregated from the cytoplasm (Komatsu et al. 2007; Rubinsztein 2006). The second purpose of autophagy is to degrade materials sequestered in the lysosomal compartment. The importance of this second function is illustrated by starvation-induced autophagy, which provides amino acids and fatty acids to maintain metabolism and ATP levels when extracellular nutrients are in short supply (Kuma et al. 2004; Lum et al. 2005). These two functions imply

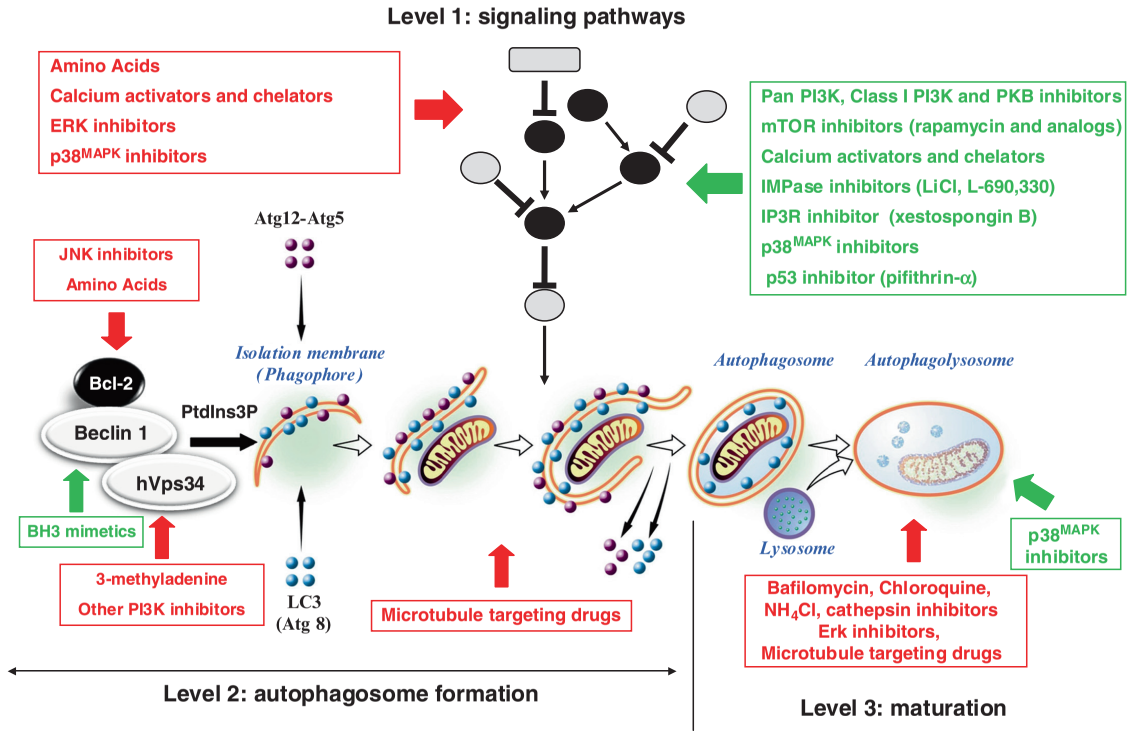

Fig. 1 Different levels of autophagy regulation. The regulation of autophagy can be divided into three levels. The first level is defined by the signaling pathways that terminate upstream of the Atg machinery involved in autophagosome formation. The second level of regulation is the Atg machinery involved in the formation of autophagosomes. The third level of regulation corresponds to the late stage of autophagy (maturation and fusion with the lysosomal compartment). Various drugs that activate (*green*) or inhibit (*red*) autophagy at the various different levels of regulation are indicated in the figure

critical regulations at levels 2 and 3, respectively. It is clear that a dysfunction at level 3 will have a greater adverse effect when ATP levels and metabolism are compromised. Level 1 is important in situations in which autophagy is stimulated, but basal autophagy is probably also finely regulated by signaling pathways, although the mechanisms involved in this are poorly defined.

In this chapter, we will describe the three levels of regulation of the autophagic pathway as shown in Fig. 1, and then discuss possible ways of manipulating autophagy at these different levels of regulation.

2 Signaling Pathways

Autophagy is known to be induced by a wide variety of stimuli (e.g., nutrient and growth factor depletion, hypoxia, drug and radiation treatment). Many signaling pathways and second messengers have been shown to regulate the activity of the Atg machinery involved in the formation of autophagosomes. This constitutes level 1 of autophagy regulation. Many of these pathways converge on the evolutionarily conserved kinase TOR (target of rapamycin). However, other autophagy signaling pathways act independently of TOR, especially in mammalian cells (e.g., the inositol phosphate pathway). Moreover, in mammalian cells, it is not clear how these signaling pathways impinge on the molecular machinery of autophagy. The aim of this section is to give an overview of the autophagy signaling that regulates the biogenesis of autophagosomes. Readers can consult recent reviews dedicated to the signaling of autophagy to obtain a more detailed description of this aspect of autophagy (Codogno and Meijer 2005; Gozuacik and Kimchi 2007).

2.1 TOR-Dependent Signaling Pathways

The kinase TOR is a major evolutionarily conserved sensor in the autophagy signaling pathway in eukaryotes, but it also regulates many other aspects of cell function, including transcription, translation, cell size and cytoskeletal organization (Schmelzle and Hall 2000). In mammals, mTOR can be included in two different complexes (Schmelzle and Hall 2000), mTORC1 and mTORC2. Although these two TOR complexes share common components, they display distinct cellular functions and phosphorylate different downstream substrates (Jacinto et al. 2004; Loewith et al. 2002). The activity of mTORC1 is regulated via the integration of many signals, including growth factors, insulin, nutrients, energy availability, and cell stressors such as hypoxia, osmotic stress, reactive oxygen species and viral infection (Corradetti and Guan 2006). mTORC1 is the only known target of the drug rapamycin, and is required for signaling to S6K and 4E-BP1. mTORC1 has recently been shown to consist of four proteins: mTOR, mLST8 (also known as GβL), proline-rich PKB/Akt substrate 40-kDa (PRAS40), and raptor (regulatory

associated protein of mTOR), and it plays a major role in controlling translation and cell growth in response to nutrients. The adaptor protein mLST8 is common to both mTOR complexes. Raptor binds mTOR, S6K and 4EBP1 and facilitates mTOR phosphorylation of these molecules; but whether raptor enhances or represses mTOR kinase activity remains unclear (Hara et al. 2002; Kim et al. 2002). Unlike mTORC1, mTORC2 has some functions that cannot be inhibited by rapamycin, including the control of actin cytoskeleton dynamics (Jacinto et al. 2004; Loewith et al. 2002). The mTORC2 complex consists of mTOR, mLST8, mammalian stress-activated protein kinase-interacting protein 1 (mSin1), and rictor (for rapamycin-insensitive companion of mTOR) (Sarbassov et al. 2004).

2.1.1 mTORC1

To date, the signaling pathway including Class I PI3K/PKB and mTORC1 is the autophagy-regulating pathway that has undergone the most investigation. Class I PI3K enzymes phosphorylate PtdIns4P and PtdIns(4,5)P_2 to produce PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, which bind to Akt (also known as protein kinase B: PKB) (Brazil and Hemmings 2001). PKB is responsible for activating mTOR via tuberous sclerosis complex 1/2 (TSC1/2), GTPase-activating proteins, and Rheb, a GTPase protein (Fig. 2). When this pathway is activated by challenging receptors that recruit class I PI3K or by expressing a constitutive active form of PKB, this has an inhibitory effect on autophagy (Arico et al. 2001). The phosphatase PTEN, which hydrolyzes PtdIns(3,4,5)P_3, has a stimulatory effect on autophagy by abolishing class I PI3K/PKB inhibition (Meijer and Codogno 2004). The mTORC1 complex is also inhibited by AMP-activated kinase (AMPK), which reflects the energy status of the cell (see Sect. 2.1.3.).

Growth factors activate the class I PI3K/Akt/mTOR pathway and consequently inhibit autophagy. Historically, awareness that macroautophagic sequestration is controlled via transduction pathways emerged from pioneering studies of the effect of insulin, glucagon, and glucocorticosteroids on liver proteolysis (Deter and De Duve 1967; Hopgood et al. 1981; Mortimore and Ward 1976). In addition to the negative effect of insulin on liver protein degradation, various growth promoting factors and serum suppress autophagic proteolysis in many mammalian cell types (Blommaart et al. 1997b). In the absence of growth factors, cells are unable to take up nutrients from the extracellular medium. In this context, mTOR is inhibited and autophagy has been shown to rescue cells from death by maintaining ATP levels in starved cells (Lum et al. 2005). However, this autophagy-mediated survival mechanism is self-limiting, and persistent growth factor deprivation leads to cell death within a few weeks. This is probably due to severe degradation of essential organelles and macromolecules induced by the prolonged stimulation of autophagy.

Amino acids are the final products of the autophagic pathway, and so it is not surprising that they are able to negatively regulate autophagy and that this capacity is conserved from yeast to humans. Conversely, it has long been known that amino acid deprivation stimulates autophagy (Poso et al. 1982). However, the mechanism

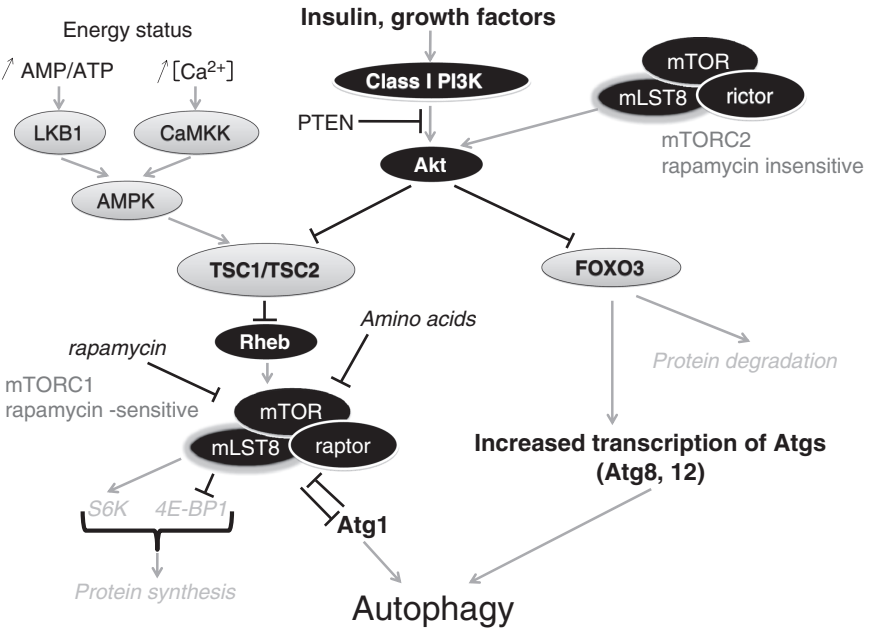

Fig. 2 Roles of the two mTOR complexes in the regulation of autophagy. mTOR exists in two different complexes, mTORC1 and mTORC2. Mammalian TORC1 (mTORC1) is composed of mLST8 (GβL) and raptor. It is sensitive to rapamycin, and immediately regulates autophagy in response to cellular stress. The mTOR signaling pathway can receive input from growth factors via class I PI3K, which activates Akt. In turn, Akt activates mTORC1 via the inhibition of TSC1/TSC2. The energy sensor AMPK (AMP-activated kinase), which is activated when the AMP/ATP ratio increases, inactivates mTORC1 by activating TSC2. Calcium signaling can also inhibit mTORC1 by activating AMPK. Atg1 Ser/Thr protein kinase functions downstream of mTORC1, but it also exercises negative feedback control over mTOR. The rapamycin-insensitive complex mTORC2 is composed of mTOR, mLST8 (GβL) and rictor; it seems to be implicated in the long-term regulation of autophagy by activating Akt, which inhibits FOXO3. FOXO3 is known to increase the transcription of several Atg genes, such as *ATG8/LC3* or *ATG12*. Molecules shown in *gray* correspond to autophagy-activating molecules, and those shown in *black* to autophagy-inhibiting molecules

by which amino acids control mTOR and inhibit autophagy is still a matter of debate (Gulati and Thomas 2007; Meijer and Codogno 2006). It has been proposed that the GTPase Rheb, which has GTPase activity controlled by the GTPase-activating protein TSC2, may integrate amino acid signaling upstream of mTOR (Sarbassov et al. 2005). Amino acids may also act at the level of the mTORC1 complex by controlling the stability of the mTOR/raptor complex (Hara et al. 2002; Kim et al. 2002). The stability of this complex is increased in cells starved of amino acids, and is correlated with the inhibition of mTOR-dependent signaling. In mammals, amino acids have recently been shown to mediate mTOR/raptor-dependent signaling by activating class III PI3K (Byfield et al. 2005; Nobukuni et al. 2005).

This signaling pathway is dependent on the increase in intracellular Ca^{2+} and the direct binding of Ca^{2+}/calmodulin to the class III PI3K/mTORC1 complex (Gulati et al. 2008). Nevertheless, in *Drosophila*, class III PI3K does not act upstream of TOR, suggesting that we must be cautious regarding the impact of amino acids in activating the different regulating complexes of autophagy (Juhasz et al. 2008).

2.1.2 mTORC2

Much less is known about the upstream regulation and function of TORC2 than for TORC1. The rictor protein directs the specificity of mTORC2 towards Akt and the FoxO3 transcription factor, and away from S6K and 4EBP1 (Guertin et al. 2006; Sarbassov et al. 2005). Akt/PKB activation blocks FoxO3 activation and autophagy, and this effect is not prevented by rapamycin (Mammucari et al. 2007). It therefore seems likely that decreased PI3K/Akt signaling activates autophagy not only through mTORC1 but also, albeit more slowly, via a transcription-dependent mechanism involving FoxO3 (Zhao et al. 2007). Indeed, in vivo in skeletal muscle, activated FoxO3 increases the transcription of two autophagy-related genes, *LC3* and *Bnip3* (a BH3-only protein), leading to the induction of autophagosome formation (Mammucari et al. 2007; Mammucari et al. 2008). Inhibition of rictor causes the translocation of FoxO3 to the nucleus and induces autophagy. In fact, FoxO3 regulates both the ubiquitin proteasome and autophagy systems during muscle atrophy.

2.1.3 AMPK

Apart from being an autophagy sensor, mTOR can also sense changes in the cellular energy via AMPK. Activation of AMPK inhibits mTOR-dependent signaling by interfering with the activity of GTPase Rheb, and with protein synthesis (Meijer and Codogno 2004). This is consistent with the switching off of ATP-dependent processes (Hardie 2004) during periods of energy crisis. In starved cells, when the AMP/ATP ratio increases, the binding of AMP to AMPK promotes its activation by the AMPK kinase LKB1 (Corradetti et al. 2004; Shaw et al. 2004). Moreover, Ca^{2+}/calmodulin-dependent kinase kinase β (CaMKK-β) has been identified as being an AMPK kinase (Hawley et al. 2005; Woods et al. 2005). The activity of AMPK is required for autophagy to be induced in response to starvation in mammalian cells (Meley et al. 2006) and in yeast (Wang et al. 2001) in a TORC1-dependent manner. Moreover, autophagy induction is also dependent on the inhibition of mTORC1 by AMPK in non starved cells in response to an increase in free cytosolic Ca^{2+} (Hoyer-Hansen et al. 2007). In this setting, the activation of AMPK and stimulation of autophagy are dependent on CaMKK-β. The induction of autophagy through AMPK activation probably also occurs in other settings, such as hypoxia (Degenhardt et al. 2006; Laderoute et al. 2006). AMPK is probably a general regulator of autophagy upstream of mTOR (Hoyer-Hansen and Jaattela 2007; Meijer

and Codogno 2007). Another potential candidate of autophagy regulation downstream of AMPK is elongation factor-2 kinase (eEF-2 kinase), which controls the rate of peptide elongation (Hait et al. 2006). Activation of eEF-2 kinase increases autophagy and slows protein translation (Wu et al. 2006). The activity of eEF-2 kinase is regulated by mTOR, S6K, and AMPK (Browne et al. 2004; Browne and Proud 2002). During periods of ATP depletion, AMPK is activated and eEF-2 kinase is phosphorylated (Browne et al. 2004), leading to a balance between the inhibition of peptide elongation and the induction of autophagy. How eEF-2 kinase impinges on the molecular machinery of autophagy remains to be elucidated. Autophagy is activated by AMPK in a p53-dependent manner (Feng et al. 2005). However, the cytoplasmic form of p53 has been shown to have an inhibitory effect on autophagy (Tasdemir et al. 2008), suggesting that activation of autophagy by p53 depends on its transactivating effect on genes such as DRAM (see Sect. 4.2.5) (Crighton et al. 2006).

2.1.4 Downstream Targets of mTOR

The transcriptional factor FoxO3 acts downstream of mTORC2, as discussed above. The major downstream targets of mTORC1 are the Atg1 Ser/Thr protein kinase complex, S6 kinase (S6K) and 4E-BP1. The 4E-BP1 protein is an inhibitor of protein translation, but it is not related to autophagy. The Atg1 complex regulates various steps in autophagosome formation, but its physiological target remains to be identified (Nair and Klionsky 2005; Stephan and Herman 2006). It has been suggested that S6K, a kinase that phosphorylates ribosomal protein S6, may regulate autophagy. In *Drosophila*, S6K has been shown to contribute to stimulating autophagy, although it is not mandatory for its initiation (Scott et al. 2004). It has been suggested that in mammalian cells, S6K may contribute to the basal activity of autophagy via its feedback inhibition of the class I PI3K-dependent insulin signaling pathway (Klionsky et al. 2005). However, a recent study has shown that the rate of autophagy was not altered in the striated muscles in S6K-deficient mice (Mieulet et al. 2007), and so further studies are needed to clarify the role of S6K in autophagy.

2.2 mTOR-Independent Pathways

After LiCl treatment, autophagy is induced via the inhibition of inositol monophosphatase independently of mTOR inhibition (Sarkar et al. 2005). The depletion of free inositol and reduced levels of myo-inositol-1,4,5-phosphate (IP_3) stimulate autophagy. According to these findings, inhibition of the endoplasmic reticulum (ER) IP_3 receptor stimulates autophagy (Criollo et al. 2007). Interestingly, enhancing the level of IP_3 inhibits the autophagy induced by nutrient depletion. IP_3 is a Ca^{2+}-mobilizing second messenger that releases Ca^{2+} from the ER, and in this way may control autophagy as described above (Hoyer-Hansen et al. 2007). These findings

suggest that inositol and IP_3 may regulate autophagy either via a signaling pathway parallel to mTOR, or by impinging on the molecular machinery of autophagy downstream of mTOR.

2.3 Other Pathways

In this section, we will summarize some of the other signaling pathways that have been shown to regulate autophagy in mammalian cells. Some of these signaling pathways engage in crosstalk with mTOR signaling, such as eIF2α kinases, sphingolipids or NF-κB, whereas the final target of other pathways remains to be identified or are independent of mTOR.

2.3.1 eIF2α Kinases

A relationship between autophagy and eIF2α phosphorylation has been shown during starvation in *Saccharomyces cerevisiae* and during starvation and viral infection in mammalian cells (Tallóczy et al. 2002). The eIF2α kinases are a family of evolutionarily conserved serine/threonine kinases that regulate stress-induced translational arrest. In yeast, GCN2, the yeast eIF2α kinase, targets the eIF2α-regulated transcriptional transactivator, GCN4, and induces autophagy in response to starvation (Tallóczy et al. 2002). In mammals, there are four distinct eIF2α kinases, GCN2, PKR, PERK, and HRI, which are activated by amino acid starvation, viral infection, ER stress, and heme depletion, respectively (Garcia et al. 2007). Thus, it is possible that various stress conditions, including ER stress and viral infection, that activate eIF2α kinases may have the ability to induce autophagy in mammalian cells.

2.3.1.1 PERK

Accumulation of misfolded proteins in the ER activates PERK (protein kinase R-like endoplasmic reticulum kinase), which phosphorylates eIF2α. During aggregate-prone protein accumulation, PERK/eIF2α phosphorylation stimulates autophagy by upregulating Atg12, and probably also by activating the Atg5–Atg12–Atg16 complex (Kouroku et al. 2007). The accumulation of misfolded proteins by autophagy default leads to neurodegenerative disorders in mouse models (Hara et al. 2006; Komatsu et al. 2006). In contrast with protein aggregate-induced autophagy, rapamycin-induced autophagy and starvation-induced autophagy are not mediated by the PERK/eIF2α pathway. This shows that eIF2α phosphorylation does not modulate all types of stress-induced autophagy (Yorimitsu and Klionsky 2007). More recently, PERK-dependent regulation has been shown to induce autophagy in human glioblastoma cells, and to lead to cell death via c-Jun N-terminal kinase (JNK) activation (Park et al. 2008). However, treatment of PERK-deficient cells with thapsigargin, an ER stressor, induces the activation of autophagy in a manner

similar to that produced in wild-type cells (Ogata et al. 2006). Therefore, it is not clearly established which signaling pathway from the ER is involved in autophagy induced by ER stress.

2.3.1.2 PKR

Double-stranded RNA-dependent protein kinase (PKR), whose gene expression is upregulated by type I IFNs, is a key player in the antiviral action of interferon. When activated, PKR phosphorylates eIF2α, which blocks translation, leading to the shutoff of protein synthesis, and thereby inhibits viral replication. Not surprisingly, a wide variety of viruses have evolved strategies to counteract this (Kirkegaard et al. 2004). PKR has been shown to promote autophagy during both viral infection and starvation, although the mechanism is still unclear (Tallóczy et al. 2002; Tallóczy et al. 2006). Herpes simplex virus is unable to trigger autophagy in $PKR^{-/-}$ and Ser-51 nonphosphorylable mutant eIF2α murine embryonic fibroblasts. Furthermore, PKR and eIF2α Ser-51-dependent autophagy processes are both antagonized by the herpes simplex virus neurovirulence protein, ICP34.5. Thus, autophagy is a novel evolutionarily conserved function of the PKR pathway targeted by viral virulence gene products.

2.3.1.3 GCN2

In yeast, GCN2-dependent phosphorylation of eIF2α is necessary for the effective translation of mRNAs encoding Gcn4, a transcriptional activator of several autophagy genes, which is stimulated by nutrient starvation (Natarajan et al. 2001). Starvation-induced autophagy depends on the activity of Gcn2, which can be rescued by PKR in GCN2-disrupted yeast (Tallóczy et al. 2002). Interestingly, in GCN2-disrupted yeast, it is possible to induce autophagy by adding rapamycin, suggesting that GCN2 is not a downstream target of mTORC1.

2.3.2 MAP Kinases

The mitogen-activated (MAP) kinases are involved both in the induction of autophagy (level 1) and in the maturation of the autophagosome (level 3). In this section, we will discuss the relationship between MAP kinases and the induction of autophagy. We will define the role of MAP kinases in autophagosome maturation in Sect. 4.3.

2.3.2.1 $p38^{MAPK}$

The involvement of $p38^{MAPK}$ in the control of autophagy seems to be cell-type dependent. In cultured rat hepatocytes and in flow-through perfused rat liver, amino acid-induced cell swelling caused by Na^+-dependent concentrative transport of certain amino acids

inhibits autophagy independently of mTOR by activating $p38^{MAPK}$ (Haussinger et al. 1999). Similarly, blockade of $p38^{MAPK}$ signaling induces an autophagic response in colorectal cancer cells (Comes et al. 2007). In contrast, the accumulation of glial fibrillary acidic protein (GFAP) aggregates induces the activation of p38 and subsequently mTOR-dependent autophagy in Alexander disease, a rare, fatal neurological disorder (Tang et al. 2008). Finally, in murine myotubes, $p38^{MAPK}$ is not involved in the regulation of autophagy by amino acids (Tassa et al. 2003).

2.3.2.2 Extracellular Signal-Regulated Kinases

Activation of extracellular signal-regulated kinases (ERK1/2) has been shown to activate autophagy in different cell types. In response to neurotoxins, the activation of ERK stimulates autophagy in neurons (Zhu et al. 2003). In human colon cancer cells, amino acid starvation activates the ERK1/2 signaling pathway by promoting the phosphorylation of Raf-1, which reduces its kinase activity towards MEK1/2, the upstream kinase activators of ERK1/2, and thereby triggers autophagy (Pattingre et al. 2003a). Accordingly, soyasaponins induce ERK1/2-dependent autophagy in the same cell types, suggesting that the regulation of autophagy by ERK1/2 is not limited to that which occurs during nutrient starvation in intestinal cells. In MCF7 breast cancer cells, TNFα causes an increase of ERK1/2 activity, and subsequent induction of autophagy (Sivaprasad and Basu 2008).

2.3.2.3 c-Jun N-Terminal Kinases

In mouse fibroblasts, autophagy and cell death are dependent on the activation of JNK, and on the transcriptional activity of c-Jun (Yu et al. 2004). Whether the transcriptional activity of c-Jun is required to regulate autophagy remains to be carefully investigated. Indeed, the cytoplasmic target of JNK has been shown to control autophagy independently of the transcriptional activity of c-Jun (see Sect. 3).

2.3.3 Protein Kinases C

Protein kinases C (PKC) comprise a family of serine/threonine kinases that are involved in the transduction of signals for cell proliferation, differentiation, apoptosis, and angiogenesis. Two members of the PKC family have been recently involved in autophagy regulation. PKCδ constitutively suppresses autophagy through the induction of tissue transglutaminase (TG2) in pancreatic cancer cells (Akar et al. 2007). Conversely, inhibition of Ca^{2+}-dependent PKCτ prevents ER stress-induced autophagy (Sakaki et al. 2008). It seems that PKCτ activation is necessary for autophagy in response to ER stress, but not in response to amino acid starvation (Sakaki et al. 2008).

2.3.4 DAP Kinases

The death-associated protein kinase (DAPk) family contains three closely related serine/threonine kinases, known as DAPk, ZIPk, and DRP-1 (Gozuacik and Kimchi 2006). These three kinases may form multiprotein complexes that are able to induce apoptotic or autophagic cell death in response to various cellular stresses. It seems that the most studied member of the family, DAPk, is able to induce an autophagic cell death that is involved in its tumor suppressor activity. Interestingly, DRP-1 modulates both starvation-induced and IFN-induced autophagy (Inbal et al. 2002), whereas DAPk does not (Gozuacik and Kimchi 2007). Furthermore, DRP-1 is anchored in the autophagosome membrane, and may be involved in the formation of this vacuole (Inbal et al. 2002).

2.3.5 Heterotrimeric G Proteins

Previous studies have shown that a cytoplasmic heterotrimeric G_{i3} protein regulates autophagy in the human colon cancer HT-29 cell line (Ogier-Denis et al. 1995). Autophagy is stimulated when GDP is bound to the $G_{\alpha i3}$ protein (Ogier-Denis et al. 1996). It is not surprising that G_{α}-interacting protein (GAIP), a regulator of G proteins that activates the hydrolysis of GTP by the $G_{\alpha i3}$ protein, has been shown to increase the rate of autophagy (De Vries et al. 2000b; Ogier-Denis et al. 1997b). The phosphorylation of GAIP, which stimulates its GTPase activity and therefore the autophagic pathway, is dependent upon the activity of ERK1/2 (Pattingre et al. 2003a). Another G-protein regulator, AGS3 (Activator of G protein Signaling 3), which has been shown to interact with the GDP-bound form of $G_{\alpha i3}$ (De Vries et al. 2000a; Takesono et al. 1999) is involved in the control over an early step in autophagy prior to the formation of the autophagosome (Pattingre et al. 2003b).

Interestingly, $G_{\alpha i3}$ is also crucial to the antiautophagic action of insulin in mouse hepatocytes (Gohla et al. 2007). $G_{\alpha i3}$ is associated with the autophagosomal membrane in starvation-induced autophagy, and relocates to the plasma membrane in response to insulin stimulation (Gohla et al. 2007). G proteins are also implicated in autophagy and, at least in this cellular model, they have an antiautophagic role.

2.3.6 NF-κB

In cells without activated NF-κB, TNFα upregulates the expression of Beclin 1 and induces autophagy. These processes are dependent on reactive oxygen species (ROS) (Djavaheri-Mergny et al. 2006). NF-κB is an antiautophagic factor and protects cells against the action of TNFα. It has also been shown that autophagy degrades I-κB, the inhibitor of NF-κB (Xiao 2007). NF-κB is then activated and inhibits autophagy. This seems to be a regulatory feedback mechanism that prevents a burst of autophagy and autophagic cell death (Xiao 2007). We will discuss the role of ROS during autophagosome formation in Sect. 3.3.1.

2.3.7 Sphingolipids

Sphingolipids, such as ceramide and sphingosine-1-phosphate, are involved in various cellular processes, and particularly in apoptosis (Ogretmen and Hannun 2004; Spiegel and Milstien 2003). Ceramide is a pro-apoptotic signal, and sphingosine-1-phosphate modulates the apoptotic effect of ceramide. Both ceramide and sphingosine-1-phosphate are able to stimulate autophagy (Daido et al. 2004; Lavieu et al. 2007). However, their mechanisms of action are not well known. Ceramide-induced autophagy is characterized by the inhibition of Akt/PKB upstream of mTOR (Scarlatti et al. 2004). Sphingosine kinase 1 overexpression has no effect on Akt/PKB, but sphingosine-1-phosphate does seem to inhibit mTOR independently of class I PI3K (Lavieu et al. 2006).

3 Autophagosome Formation

Autophagy begins with the formation of a pre-autophagosomal sequestering cistern that subsequently gives rise to an isolation membrane or phagophore (Fengsrud et al. 2004; Suzuki and Ohsumi 2007). A class III PI3K complex containing Atg6/Beclin 1 mediates the initial nucleation of the isolation membrane. The precise origin of the membrane is still not fully understood. Atg5 has been shown to localize to the membranes of nascent phagophores, where its conjugation to Atg12 is involved in the expansion of the isolation membrane. This Atg5/Atg12 complex is required for the binding to the phagophore of Atg8/LC3 after it has been conjugated with phosphatidylethanolamine. This membrane then elongates to form the autophagosome, which is a double membrane-bound structure in the 0.5–1.5 μm range in mammalian cells. So far, 31 Atg proteins have been identified and characterized. Eighteen of these Atg proteins, Atg1–Atg10, Atg12–Atg14, Atg16–Atg18, Atg29, and Atg31, play roles in autophagosome formation (Suzuki and Ohsumi 2007). In this section, we will not attempt to provide a detailed description of the functions of all Atg proteins; instead, we will focus on the steps that can be regulated, and on the different regulatory mechanisms (protein-protein interactions, post-translational modifications, transcriptional regulation, and cytoskeleton). Readers can consult recent comprehensive reviews of the role of Atg proteins in the formation of autophagosomes (Suzuki and Ohsumi 2007; Xie and Klionsky 2007; Yoshimori and Noda 2008), as well as the chapter by Yang and Klionsky in this volume.

3.1 Atg1 and Its Partners

In yeast, autophagosomes are generated at a specific site near the vacuolar membrane, known as the preautophagosomal structure (PAS), where the Atg1 complex is recruited (Suzuki and Ohsumi 2007; Xie and Klionsky 2007). The Atg1 Ser/Thr protein

kinase is contained in a dynamic protein complex with Atg17, Atg11/Cvt9, Atg13, and Vac8, the composition of which depends upon the phosphorylation status of Atg1 and Atg13 (Pattingre et al. 2008). The Atg1 complex functions downstream of TOR (see Sect. 2), and TOR signaling controls the Atg1–Atg13 interaction via the phosphorylation level of Atg13 (Klionsky 2005). Under nutrient-rich conditions, in which Atg1 and Atg13 are highly phosphorylated, Atg1 interacts with Atg17 and Atg11/Cvt9, and Atg13 is associated with Vac8. In response to starvation, dephosphorylated Atg13 interacts with Atg1 and with other partners, resulting in Atg17-dependent autophagy. Similarly, rapamycin promotes the dephosphorylation of Atg13 and the activation of Atg1. In addition, other Atgs have been found to interact with the Atg1 complex at the PAS (Suzuki and Ohsumi 2007).

Homologs of Atg1 have been found in multicellular organisms and plants, and their function in autophagy is conserved (Klionsky et al. 2003). However, the precise role of the Atg1 complex during the formation of the autophagosome in metazoans is not known, in part because several components of the Atg1 complex are not evolutionarily conserved (Atg11, Atg13, Atg17), and in part because the physiological target of the kinase activity of Atg1 remains to be identified. In mammals, two Atg1 homologs, Ulk1 and Ulk2, localize on the elongating isolation membrane under starvation conditions, and the kinase activity of Ulk1 and 2 is required to stimulate autophagy (Chan et al. 2007; Hara et al. 2008). FIP200 (a 200-kDa focal adhesion kinase family interacting protein) is a recently discovered mammalian autophagic factor which interacts with Ulk1 and 2, and is required for their phosphorylation (Hara et al. 2008). Interestingly, it has been proposed that FIP200 could be a counterpart of Atg17 in yeast (Hara et al. 2008). Ulk1 and 2 are downstream of mTORC1, and negatively feedback to it. A recent study in *Drosophila* demonstrates that dAtg1 is required to stimulate autophagy, and that it exercises negative feedback control on dTOR (Scott et al. 2007). Interestingly, cells with a high level of Atg1-dependent autophagy are eliminated by apoptosis, suggesting that the Atg1-dependent regulation of autophagy is important in order to keep autophagy in a range compatible with cell survival.

3.2 Atg6/Beclin 1

In yeast, Atg6 forms two complexes with the class III phosphatidylinositol 3-kinase (Vps34) and its regulatory factor Vps15. Complex I includes Atg14, whereas complex II contains Vps38, and both these proteins act as connectors between Atg6 and VPs34/Vps15. Complex I regulates autophagy, and complex II is required for vacuolar protein sorting of carboxypeptidase Y (Kihara et al. 2001b). Among the Atg proteins, Atg6 is relatively unique in that it is not "autophagy-specific." Beclin 1, the mammalian ortholog of Atg6, shares 24.4% identity with Atg6 in yeast. The interaction between Beclin 1 and Vps34 is conserved in mammals, and the Beclin 1/hVps34 complex is also able to bind to different partners (see below). Mammalian homologs of Atg14 and Vps38 have been recently identified (reviewed in Longetti

and Tooze 2009). Interestingly, in Atg6-defective yeast, Beclin 1 is only able to restore the autophagy function of this mutant, suggesting that Beclin 1 does not regulate other lysosomal trafficking pathways (Furuya et al. 2005).

Beclin 1 was discovered in a two-hybrid screen as a Bcl-2-interacting protein (Liang et al. 1998). The Beclin 1/hVps34 complex contributes to autophagosome formation by allowing other Atg proteins to relocate to the pre-autophagosomal structure. Endogenous Beclin 1 localizes to the TGN (*trans*-Golgi network), the mitochondria, the perinuclear membrane and the endoplasmic reticulum (ER) (Kihara et al. 2001a; Pattingre et al. 2005). The Beclin 1/hVps34 interaction (and, as a result, autophagic levels) can be modulated. Beclin 1 is part of a multimolecular complex and acts as a platform, recruiting activators or repressors of Beclin 1/hVps34-dependent autophagy. Beclin 1 also has tumor-suppressive activity in breast cancer cells. Sequence and structural studies indicate that Beclin 1 has a Bcl-2-binding domain (BBD), a central coiled-coil domain (CCD), an evolutionarily conserved domain (ECD), as well as a BH3-only domain and a nuclear export signal (Furuya et al. 2005; see Fig. 3). This last domain is responsible for transporting Beclin 1 from the nucleus to the cytosol, and it is only the cytosolic form that regulates autophagy (Liang et al. 2001). The ECD is essential for Vps34 binding (Furuya et al. 2005). It has been shown recently that Beclin 1 forms a large homo-oligomer, which may contribute to its own regulation (Ku et al. 2008).

3.2.1 Negative Regulators

In addition to their key role in the regulation of apoptosis, the Bcl-2 family proteins have recently been shown to be negative regulators of autophagy (Liang et al. 1998; Pattingre et al. 2005; Shimizu et al. 2004). The Bcl-2 family proteins, Bcl-2, Bcl-xL, and Bcl-w, and to a lesser extent Mcl-1, interact with Beclin 1 and interfere with the complex formation between Vps34 and Beclin 1 (Erlich et al. 2007; Liang et al. 1998). However, Beclin 1 does not interact with the pro-apoptotic proteins of the same family, such as Bax (Liang et al. 1998). Bcl-2 proteins do not directly compete with Vps34 for binding to Beclin 1, since they bind to the Bcl-2 binding domain of Beclin 1, whereas Vps34 is thought to bind to its EC domain. JNK has recently been identified as the kinase responsible for the phosphorylation of Bcl-2 during nutrient starvation (Wei et al, 2008) or ceramide treatment (Pattingre et al., 2009). Phosphorylation of Bcl-2 occurs in the ER and leads to decreased interaction between Bcl-2 and Beclin 1, which in turn stimulates autophagy. Conversely, under nutrient-rich conditions, when autophagy is inhibited, Bcl-2 is not phosphorylated and it interacts strongly with Beclin 1. Recently, a BH3 domain that forms an amphipathic helix was identified in the Beclin 1 sequence from amino acids 108 to 127 (Oberstein et al. 2007). BH3-only proteins, including Bad and Bim, disrupt the Beclin 1-Bcl-2 (or Bcl-xL) complex and stimulate autophagy (Maiuri et al. 2007b; Oberstein et al. 2007).

Several viral proteins can also block the Beclin 1/Vps34 interaction. γ-Herpesviruses, including murine γ-herpesvirus 68, Kaposi's sarcoma-associated herpesvirus,

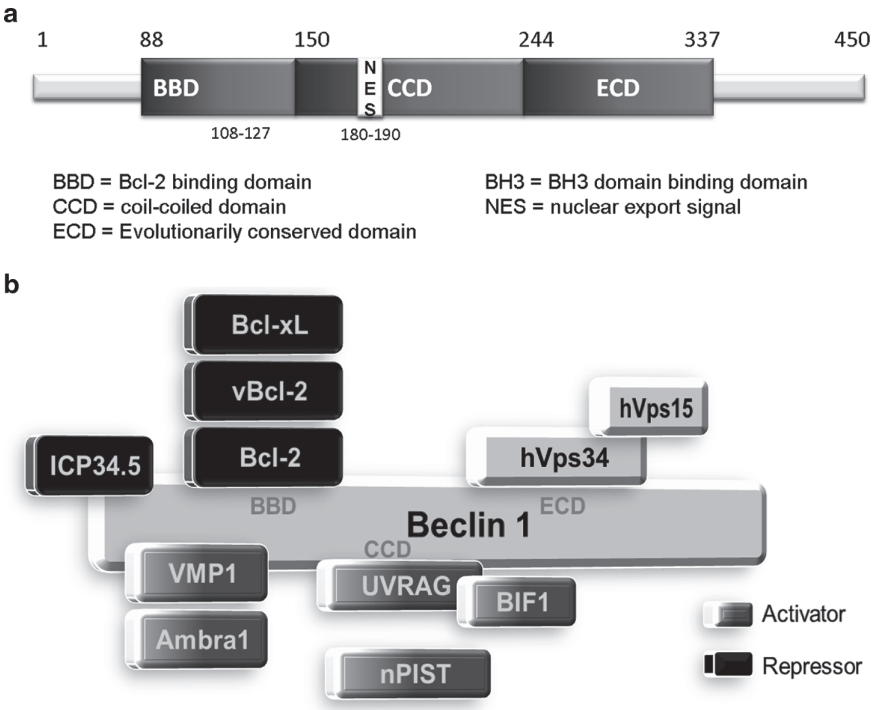

Fig. 3 Beclin 1 and its partners. **A** Schematic representation of the domains of human Beclin 1. Beclin 1 has a Bcl-2 binding domain (BBD, amino acids 88–150), a coiled coil domain (CCD, amino acids 150–244), and an evolutionarily conserved domain (ECD, amino acids 244-337). A BH3-only domain (BH3, amino acids 108–127) is present within the BBD, and a nuclear export signal (NES) is located between amino acids 180 and 190. **B** Beclin 1 functions as a platform for the formation of the complex. Bcl-2 proteins interact with the BBD of Beclin 1, UVRAG and nPIST with the CCD, and Vps34 with the ECD. The interaction between Beclin 1 and Vps34 is reinforced by UVRAG, associated with Bif-1 (activators), which upregulates autophagy. AMBRA, VMP1 and nPIST interact with Beclin 1 to stimulate autophagy (activators), whereas Beclin 1 is inhibited by Bcl-2, Bcl-xL, v-Bcl-2 and ICP34.5, which are all inhibitors of autophagy (repressors)

herpesvirus saimiri and rhesus rhadinovirus, encode viral homologs of Bcl-2 (v-Bcl-2) (Liang et al. 2008). These homologs can bind to Beclin 1 with much higher affinity than cellular Bcl-2, and the binding affinity seems to correlate directly with antiautophagic activity. It is interesting to note that these v-Bcl-2 do not have the phosphorylation sites of Bcl-2. This means that their binding to Beclin 1 cannot be modulated, and they constitutively block autophagy. ICP34.5 is a herpes simplex virus type 1 neurovirulence protein that is also able to bind to Beclin 1 and to inhibit autophagy, but it has no homology with Bcl-2 and does not bind to Beclin 1 through its Bcl-2-binding domain (Orvedahl et al. 2007). A mutant virus containing a Beclin 1-binding-deficient form of ICP34.5 fails to inhibit autophagy in neurons, and is highly neuroattenuated in mice (Orvedahl et al. 2007). Thus, Beclin 1 is targeted by several viruses to downregulate autophagy and confer pathogenicity.

3.2.2 Positive Regulators

Several proteins have been recently discovered to be active components of the pro-autophagic multimolecular complex. The UV irradiation resistance-associated gene protein (UVRAG) is a positive regulator of the Beclin 1–Vps34 complex (Liang et al. 2006, 2007). UVRAG and Beclin 1 interact directly through their coiled-coil domain by forming an α-helical bundled structure. The coiled-coil domain of Beclin 1 interacts with another protein, nPIST, which also positively regulates autophagy (Yue et al. 2002). Bax-interacting factor 1 (Bif-1) interacts with Beclin 1 through UVRAG via its SH3 domain and increases autophagosome biogenesis (Takahashi et al. 2007). During nutrient deprivation, Bif-1 accumulates in autophagosomes, where it colocalizes with LC3, Atg5, and Atg9. The activating molecule in Beclin 1-regulated autophagy (AMBRA1) positively regulates autophagy and inhibits cell proliferation (Fimia et al. 2007). Downregulation of AMBRA1 by small interfering RNA (siRNA) reduces Beclin 1-mediated autophagy levels in a manner consistent with a decrease in the association of Vps34 with Beclin 1. AMBRA1 is unique to vertebrates, and is mainly expressed in the brain, where it plays an essential role during development (Cecconi et al. 2007). Vacuole membrane protein 1 (VMP1) is a recently discovered transmembrane protein that triggers autophagosome formation in mammalian cells (Ropolo et al. 2007). VMP1 interacts with Beclin 1 through its hydrophilic C-terminal region, named the Atg domain. VMP1 also colocalizes with LC3. It has recently been shown that the small GTPase Rab5, previously known to be a regulator of early endocytosis, also interacts with and activates Vps34 in the Beclin 1–Vps34 complex, and thereby positively regulates autophagosome formation (Ravikumar et al. 2008). Rab5, Beclin 1 or Vps34 inhibition leads to decreased Atg5/Atg12 conjugation, suggesting that Rab5 acts at the autophagosome precursor stage (Ravikumar et al. 2008).

3.3 Post-translational Modifications of Atg Proteins

Post-translational protein modifications can regulate the activity of Atg proteins in the autophagic pathway as described below. The ubiquitin-like conjugations of Atg12 to Atg5 and of Atg8/LC3 to the polar head of PE are fundamental to the formation of autophagosomes (Ohsumi 2001). The oxidation of a cysteine residue near the catalytic site of Atg4 is important in regulating its effect on Atg8/LC3 (Scherz-Shouval et al. 2007). Other post-translational modifications that modulate the activity of Atg proteins, such as acetylation or ubiquitination, are now emerging (Baxter et al. 2005; Lee et al. 2008). Moreover, proteolytic cleavage may regulate the function of Atg proteins in the autophagic pathway, but also may unmask new functions of these proteins (Codogno and Meijer 2006). The discovery that the calpain-dependent cleavage of Atg5 generates an amino-terminal pro-apoptotic fragment is a promising lead in this new field (Yousefi et al. 2006).

3.3.1 Ubiquitin-Like Conjugated Systems

Two ubiquitin-like systems act sequentially in the expansion and completion of autophagosome formation. The first involves the conjugation of Atg12 to Atg5, which occurs constitutively soon after the individual proteins have been synthesized. Atg12 is activated by Atg7, a homolog of E1 enzyme, and then conjugated to Atg5 by Atg10 (E2-like enzyme). The second system involves the conjugation of Atg8/LC3 to phosphatidyl ethanolamine (PE), a component of the phospholipid bilayer, in a reaction that requires both Atg7 (E1-like) and Atg3 (E2-like). Atg5/Atg12 interact with Atg16L, which is a coiled-coil protein, to form an approximately 800 kDa complex through the homo-oligomerization of Atg16L (Mizushima et al. 2003). This complex is specifically present on isolation membranes, and is never present on mature autophagosomes. The membrane localization of Atg16L complex determines the site of LC3 lipidation. The Atg16L complex is a new type of E3-like enzyme that functions as a scaffold for LC3 lipidation on the isolation membrane (Fujita et al. 2008). The Rab small GTPases Rab33A and Rab33B, initially localized in the Golgi, specifically interact with Atg16L in a GTP-dependent manner without affecting the integrity of the Atg5–12/Atg16L complex (Itoh et al. 2008). Moreover, Rab33B also modulates autophagosome formation. One possible function of Rab33 may be to recruit the Atg5–12/Atg16L complex to the surfaces of membrane structures.

3.3.2 Atg4 and ROS

Reactive oxygen species (ROS) have been shown to regulate starvation-induced autophagy by regulating the activity of Atg4 (Scherz-Shouval et al. 2007). The protein kinase Atg4 cleaves the C terminus of Atg8/LC3 as a prerequisite for its conjugation to PE on the autophagosomal membrane. Atg4 also cleaves conjugated Atg8 and removes it from the mature autophagosome for recycling. ROS released from the mitochondria inhibit Atg4 by oxidation. Indeed, one cysteine residue located near the catalytic site of Atg4 is redox regulated. Once Atg4 has been inactivated, its substrate Atg8 can be conjugated to autophagosomes. Because ROS are short-lived molecules, it has been hypothesized that oxidation occurs only close to the mitochondria (Scherz-Shouval and Elazar 2007). Further away from the mitochondria, Atg4 will be active and therefore cleave Atg8 from the autophagosomal membrane for recycling.

3.3.3 Atg Acetylation

It has been shown recently that Sirt1, a mammalian deacetylase belonging to the sirtuin family, is necessary for autophagy (Lee et al. 2008). Sirt1 interacts directly with several Atg proteins, such as Atg5, Atg7, and Atg8, and deacetylates them. These proteins were acetylated under normal conditions, and acetylation levels were reduced by Sirt1 during starvation. A lack of Sirt1 inhibits autophagosome

formation during starvation and leads to increased levels of p62. In *Drosophila melanogaster,* the activity of a second deacetylase, histone deacetylase 6 (HDAC6), a microtubule-associated deacetylase, has been linked to compensatory autophagy induced when the proteasome has been impaired (Pandey et al. 2007).

3.4 Transcriptional Regulation of Atg Proteins

Forced expression of Atg1 leads to excessive autophagy and triggers apoptosis (Scott et al. 2007). Interestingly, activation of the transcription factor E2F1, which is frequently required for apoptosis, is also involved in the expression of some genes related to the autophagic pathway (Polager et al. 2008). Activation of E2F1 by 4-hydroxytamoxifen upregulates the expression of LC3, Atg1, Atg5, and DRAM in U2OS cells containing an inducible E2F1. Moreover, E2F1 has been shown to be linked to the promoter of Beclin 1, even if the effect of E2F1 on Beclin 1 is not still clear (Weinmann et al. 2001). However, this finding supports the proposal that autophagy may be regulated at the transcriptional level. The expression of Atg5 has been shown to be increased during autophagic cell death in *bax/bak*$^{-/-}$ MEFs (Shimizu et al. 2004). Recent studies have provided evidence that the expression of some Atg proteins and of proteins related to autophagy is increased during muscle wasting (see Sect. 2.1.2) (Mammucari et al. 2007). This emerging aspect of the regulation of autophagy will probably yield important information about the impact of autophagy on cell homeostasis.

3.5 The Cytoskeleton

Cytoskeletal elements are involved at both levels 2 and 3 of autophagy regulation. Previous studies based on the inhibition of autophagy following the disruption of the network of cytokeratin intermediate filaments by okadaic acid (Blankson et al. 1995) suggested that this class of filaments could be involved in the formation of autophagosomes.

Recent studies have reported that disruption of microtubules not only slows down the maturation of autophagosomes (see Sect. 4) but also decreases the formation of autophagosomes (Fass et al. 2006; Kochl et al. 2006). These results suggest that microtubules may be implicated in the biogenesis of autophagosomes. One intriguing possibility is that the microtubule network may form a boundary preventing the undesirable fusion between phagophores and autophagosomes (Fass et al. 2006).

Cytochalasin B and D, which induce the depolymerization of microfilaments, were reported to reduce the formation of autophagosomes (Aplin et al. 1992). More recently, upregulation of F-actin polymerization has been reported during TRAIL-mediated autophagy (Han et al. 2008). Along with these observations, some elements

of the autophagy machinery have been shown to interact with the actin cytoskeleton (Monastyrska et al. 2008).

4 Maturation Step

The maturation of autophagosomes is important in situations in which cells need to degrade their autophagic cargo to avoid nutrient and ATP depletion (e.g., during starvation-induced autophagy), to circumvent parasitic effects (accumulation of bacteria in the autophagosome for persistent infection), and also to prevent the accumulation of toxic compounds in autophagic compartments (generation of Aβ peptide in Alzheimer's disease). Blockade of the flux or excessive flux could both have adverse consequences for cell homeostasis. It is commonly assumed that excessive flux could be associated with self-destruction of the cell by self-digestion, but we can also envisage that excessive flux could buffer the lysosomal pH as a result of massive fusion with autophagosomes that have a neutral lumen pH. In any case, regulation of the maturation of autophagosomes (i.e., of the flux into the lysosomal compartment) is an important decision for cells in response to stress situations.

4.1 Morphology and Definition of Late Stages of Autophagy

Autophagosomes in yeast fuse with the vacuole to deliver autophagic bodies into the vacuolar lumen. Autophagic cargos become accessible to vacuolar hydrolysis after the breakdown of the membranes of the autophagic bodies by the lipase Atg15 (Epple et al. 2001; Teter et al. 2001). This scenario exists in mammalian cells, but the autophagosome can also merge with endocytic compartments (both early and late endosomes are competent at fusing with autophagosomes). Seglen has coined the evocative term "amphisome"—from the Greek *amphi* (both) plus *soma* (body)—for this unique structure (reviewed in Fengsrud et al. 2004) that has acquired acidic and degradative properties. Amphisomes correspond to intermediate autophagic vacuoles or AVi/d (AVi initial autophagic vacuoles are autophagosomes, and AVd degradative autophagic vacuoles are lysosomes; see Eskelinen 2005; Fengsrud et al. 2004 for an insightful discussion of the terminology of autophagic compartments). Fusion with lysosomes is the final fate of amphisomes.

4.2 Regulation of the Maturation Events

The maturation of autophagosomes depends on molecules that allow the autophagosomes to fuse with the vesicular compartments of the vacuolar system (endosomes, lysosomes). The late stage of autophagy is also dependent on molecules that regulate

the acidification of the autophagic compartments and molecules that are involved in recycling of degraded material from the lysosomal compartment.

4.2.1 SNAREs

SNAREs (soluble NSF attachment protein receptors) are basic elements required for intracellular membrane fusion (Gurkan et al. 2007; Rothman and Wieland 1996). Depending on their role in vesicular transport, SNAREs are divided into two groups: vesicle (v-SNAREs) and target SNAREs (t-SNARES). In yeast, the vacuolar t-SNAREs Vam3 (Darsow et al. 1997) and Vti1 (Ishihara et al. 2001), are needed to complete the fusion between the autophagosome and the vacuole. The mammalian homolog of i: Vti1, Vti1b, may be involved in the late stages of autophagy, because the maturation of autophagic vacuoles is delayed in hepatocytes isolated from mice in which Vti1b has been deleted (Atlashkin et al. 2003).

4.2.2 Rab Proteins

Rab proteins are a family of monomeric GTPases necessary for vesicular transport along the exo/endocytic pathway (see for review Zerial and McBride 2001). The most compelling evidence that a Rab protein plays a role in the autophagic pathway is with Rab7. In yeast, the fusion of the autophagosome with the vacuole is dependent upon a Rab7 homolog, Ypt7 (Kirisako et al. 1999). In mammalian cells, Colombo and coworkers (Gutierrez et al. 2004), and Eskelinen and coworkers (Jager et al. 2004) have shown that Rab7 is required for the maturation of autophagosomes. However, a functional Rab7 is not mandatory for the fusion with endocytic compartments to occur. Interestingly, a functional Rab11 is required for the fusion of autophagosome and multivesicular bodies during starvation-induced autophagy in erythroleukemic cells (Fader et al. 2008). These findings suggest that fusion of specific membrane-bound compartments during the maturation of autophagosomes engage different sets of Rab proteins, and possibly associated cohort proteins. Other Rab proteins, such as Rab22 and Rab24, have a subcellular localization compatible with having a role in autophagy (Egami et al. 2005; Mesa et al. 2001; Olkkonen et al. 1993).

4.2.3 ATPases

4.2.3.1 v-ATPases

Vacuolar ATPases (v-ATPases) are ubiquitous proteins located in acidic compartments (Forgac 2007). Inhibiting the activity of v-ATPase by bafilomycin A1 or concanamycin A blocks the lysosomal pumping of H^+ and consequently inhibits lysosomal enzymes which are active at low pH. It has been proposed that bafilomycin A1 may block the late stage of autophagy by interfering with the fusion of autophagosomes with endosomes and lysosomes (Yamamoto et al. 1998). However, recent

studies show that what bafilomycin A1 blocks is not fusion events in the autophagic pathway but the degradation step in lysosomes (Fass et al. 2006; Mousavi et al. 2001). Overall, the resulting effect of v-ATPase inhibition is an interruption of autophagic flux.

4.2.3.2 AAA ATPases

ATPases associated with various cellular activities proteins (AAA ATPases) are a family of proteins broadly engaged in intracellular membrane fusion (White and Lauring 2007). NSF is an AAA ATPase that binds to SNARE complexes and utilizes ATP hydrolysis to disassemble them, thus facilitating SNARE recycling. In yeast mutants lacking Sec18 (the yeast homolog of NSF), autophagosomes are formed but do not fuse with the vacuole (Ishihara et al. 2001). However it is not known whether the ATPase activity of NSF is involved in the later stages of autophagy in mammalian cells. Nevertheless, we do know that NSF activity is attenuated during starvation, which could account for the slow fusion between autophagosomes and lysosomes observed when autophagy is induced by starvation (Fass et al. 2006). SKD1 (Vps4), another AAA ATPase protein, is required for the maturation of autophagosomes (Nara et al. 2002) in mammalian cells. Vps4/Csc1, which controls the assembly of ESCRT complexes on multivesicular membrane (see below), is involved in autophagosome maturation (Rusten et al. 2007) in *Drosophila*, and in autophagosome fusion with the vacuole in yeast (Shirahama et al. 1997).

4.2.4 ESCRT and Hrs

Endosomal sorting complex required for transport (ESCRT) mediates the biogenenesis of multivesicular bodies and the sorting of proteins in the endocytic pathway (Raiborg et al. 2003). It has been recently demonstrated that the multisubunit complex ESCRT III is required for autophagosomes to fuse with multivesicular bodies to generate amphisomes, and is also involved in the fusion of autophagosomes with lysosomes (Rusten et al. 2007). ESCRT III dysfunction associated with the autophagic pathway may have important implications for understanding some neurodegenerative diseases (such as frontotemporal dementia linked to chromosome 3 and amyotropic lateral sclerosis) (Filimonenko et al. 2007; Lee et al. 2007). Hrs protein plays a major role in endosomal sorting upstream of ESCRT complexes (Raiborg et al. 2003a). Hrs contains a FYVE domain that binds specifically to PtdIns3P. It has recently been shown that Hrs facilitates the maturation of autophagosome (Tamai et al. 2007), which raises the intriguing possibility that PtdIns3P may be required for autophagosome formation via the Beclin 1 complex and its maturation via Hrs. It is interesting to note that the endosomal PtdIns(3)P 5-kinase Fab1, which uses PtdIns3P to produce PtdIns(3,5)P2, is required in *Drosophila* for amphisomes to fuse with lysosomes (Rusten et al. 2007). Since the inactivation of Fab1 in yeast causes a marked enlargement of the vacuole, which

fails to acidify correctly (Yamamoto et al. 1995), the production of PtdIns(3,5)P2 may play an important part in maintaining organelle homeostasis in the autophagic pathway. The role of PIKfyve (the mammalian ortholog of Fab1) in autophagy has not been yet investigated.

4.2.5 Endo/lysosomal Proteins

4.2.5.1 LAMP-2

LAMPs (lysosomal associated membrane proteins) are a family of heavily glycosylated transmembrane endo/lysosomal proteins (Eskelinen et al. 2003). Autophagic degradation has been shown to be impaired in hepatocytes isolated from LAMP-2-deficient mice (Tanaka et al. 2000). In LAMP-2-deficient mice that reproduce a human cardiomyopathy (Danon disease) (Nishino et al. 2000), the fusion of autophagosomes with the lysosomal compartment seems to be impaired, whereas their fusion with multivesicular bodies is not. However, no defect in autophagy was observed in LAMP-2-deficient mouse fibroblasts (Eskelinen et al. 2004). Blockade in the later stage of autophagy only occurs in fibroblasts deficient in both LAMP-1 and LAMP-2. Differences in autophagic activity observed between hepatocytes and fibroblasts may be responsible for the cell-type-specific effect of LAMP-1 and -2 depletion (Eskelinen 2005).

4.2.5.2 DRAM

Damage-regulated autophagy modulator (DRAM), which encodes a 238-amino acid protein, is generally conserved through evolution but has no ortholog in yeast (Crighton et al. 2006). DRAM is a direct target of p53. The protein is a multispanning transmembrane protein present in the lysosome. DRAM may regulate the late stage of autophagy, but surprisingly it also controls autophagosome formation (Crighton et al. 2006). This suggests a possible new paradigm in which feedback signals from the lysosomes control the early stages of autophagy.

4.2.5.3 Recycling Molecules

Two categories of lysosomal recycling molecules can be distinguished. The first category consists of the lysosomal proteins that recycle entities needed for the ongoing autophagic pathway. Proteins such as DRAM and Fab1, which were discussed in preceding sections, may fall into this category, although this has not been conclusively demonstrated. The second category includes lysosomal transporters that recycle nutrients generated by the lysosomal degradation of macromolecules. Several transporters in the lysosomal membrane have been shown to recycle amino acids, monosaccharides, or lipids (reviewed in Lloyd 1996). Atg22 was recently

identified as an amino acid transporter in the vacuole membrane of *S. cerevisiae* (Yang et al. 2006). Atg22, which regulates the final stage of autophagy (i.e., recycling from the lysosomal/vacuolar compartment), is crucial for maintaining cell survival during nutrient starvation (Yang et al. 2006).

4.2.6 Microtubules

As discussed in Sect. 3, microtubules are involved in the formation of autophagosomes. Originally, the involvement of microtubules was demonstrated in the later steps of the autophagic pathway. The destabilization of microtubules by either vinblastine (Hoyvik et al. 1991) or nocodazole (Aplin et al. 1992) blocks the maturation of autophagosomes, whereas their stabilization by taxol increases the fusion between autophagic vacuoles and lysosomes (Yu and Marzella 1986). Subsequent findings have confirmed the role played by microtubules in fusion with the acidic compartment (Jahreiss et al. 2008; Kochl et al. 2006; Webb et al. 2004). Autophagosomes move bidirectionally along microtubules, and their centripetal movement is dependent on the motor protein dynein (Jahreiss et al. 2008; Ravikumar et al. 2005; Webb et al. 2004). Two types of fusion have been documented (Jahreiss et al. 2008), including (1) complete fusion of the autophagosome with the lysosome; and (2) transfer of material from the autophagosome to the lysosomal compartment following a kiss-and-run fusion process in which two separate vesicles are maintained. However, it has been reported that autophagosome fusion with lysosomes is microtubule-independent during starvation-induced autophagy (Fass et al. 2006). Under these conditions, autophagosomes are formed in the vicinity of lysosomes and the fusion of vesicles may be independent of microtubules.

4.3 Signaling and Maturation of Autophagosomes

4.3.1 MAPKs

Protein kinases of the MAPK kinase family (JNK, p38, ERK1/2) have been shown to regulate autophagy at both level 1 and level 2. However, p38 and ERK1/2 are probably also involved in regulating the late stage of autophagy. A recent report shows that the activation of ERK1/2 promotes the formation of large autolysosomes (Corcelle et al. 2006). Thus, activating ERK1/2 activates both the formation and maturation of autophagosomes. A protein involved in regulating the activity of trimeric G_{i3} protein has been shown to act downstream of ERK1/2, and to regulate the early stages of autophagy (Pattingre et al. 2003a). However, the target of ERK1/2 in the regulation of the late stage of autophagy remains to be identified. As in its inhibitory effect on the early stage of autophagy in hepatocytes (Haussinger et al. 1999), p38 has also been shown to have an inhibitory effect on the maturation of autophagosomes (Corcelle et al. 2007). Here too, the targets of p38 in the early and late stages of autophagy are not yet identified.

5 How Autophagy Can Be Manipulated

Understanding how autophagy can be manipulated is important for potential therapeutic applications of autophagy. In this section we will focus on drugs that act at different stages of autophagy (Rubinsztein et al. 2007). Of course, autophagy can be manipulated by genetic approaches. Atg-knockout mice and Atg knockdown by RNA interference-based methods are of fundamental importance in identifying the function of autophagy in various physiological and pathophysiological situations (Levine and Kroemer 2008). Genetic approaches are important not only for investigating the autophagic machinery (level 2 of regulation), but also for investigating the signaling of autophagy (regulation level 1). For example, knockdown of mTOR partners (raptor and rictor) led to the discovery that mTOR complex 2, but not mTOR complex 1, is involved in the regulation of autophagy during the atrophy of skeletal muscle (Mammucari et al. 2007).

One of the drugs most often used to stimulate autophagy is the immunosuppressive agent rapamycin (Meijer and Codogno 2006). Rapamycin targets the kinase TOR by binding to the 12 kDa immunopilin FKBP12. The rapamycin-FKBP12 complex inhibits mTORC1. It should be noted that chronic treatment with rapamycin has an inhibitory effect on the supposedly "rapamycin-insensitive" mTORC2 (Rubinsztein et al. 2007). The role of autophagy in the effects observed with analogs of rapamycin (CCI-779, RAD001, and AP23573), which are currently used in clinical trials, remains to be carefully investigated (Faivre et al. 2006). Drugs that act on signaling elements upstream of mTOR are also useful for manipulating autophagy (Meijer and Codogno 2006). However, caution is called for with regard to the specificity of some of these drugs when investigating their effect on autophagy. For example, AICAR, an AMPK activator, has an inhibitory effect on autophagy that seems to be independent of AMPK (Meley et al. 2006; Samari and Seglen 1998).

Autophagy can be manipulated independently of mTOR using drugs that act on the myo- inositol phosphate metabolism (Sarkar et al. 2005). Lithium chloride, sodium valproate and carbamazepine, which lower the levels of myo-inositol-1,4,5-triphosphate (IP_3), induce autophagy. Accordingly, xestospongin B, an inhibitor of the IP_3 receptor, is a potent inducer of autophagy (Criollo et al. 2007).

Recently, screens for drugs regulating autophagy have been undertaken. The first strategy employed was to identify compounds that enhance the growth-inhibitory effects of rapamycin in yeast (these compounds were named SMERs, for small-molecule enhancers) (Sarkar et al. 2007). Three of these SMERs were shown to induce autophagy independently of rapamycin in mammalian cells. One of the major points of interest of these SMERs is that they do not have the immunosuppressive effect of rapamycin. Other strategies were based on screening a library of compounds (Williams et al. 2008; Zhang et al. 2007). One of these screens identified seven drugs that had already received FDA approval for the treatment of human diseases (schizophrenia, cardiovascular disorders) (Zhang et al. 2007). Interestingly, some of these drugs are known to act on Ca^{2+} channels and the Ca^{2+} current. The screening of FDA-approved drug libraries also identified minoxidil (a K^+ ATP channel opener) and clonidine (a G_i signaling activator) as activators of autophagy (Williams et al. 2008; Zhang et al. 2007).

One of the drugs most often used to inhibit autophagy is 3-methyladenine (3-MA) (Seglen and Gordon 1982). 3-MA inhibits the formation of autophagosomes by interfering with the activity of hVps34 in the Beclin 1 complex (Petiot et al. 2000). Thus, 3-MA inhibits autophagy by acting at level 2 of autophagy regulation. In fact, 3-MA is a PI3K inhibitor and a similar effect on autophagy has also been observed with two other PI3K inhibitors, wortmannin and LY294002 (Blommaart et al. 1997a). However, these inhibitors also interfere with the activity of class I PI3K and other kinases. For example, 3-MA inhibits the activity of p38MAPK and JNK (Tolkovsky et al. 2002). The design of specific inhibitors for the different classes of PI3K would be important to enable us to target the various different enzymes in this family more specifically. These enzymes sometimes have opposite effects on the regulation of autophagy (Petiot et al. 2000). Interestingly, the activity of the Beclin 1 complex in autophagy can also be manipulated via the Beclin 1/Bcl-2 interaction. Pharmacological BH3-mimetic compounds, such as ABT737, stimulate autophagy by competitively disrupting the interaction between the BH3-domain of Beclin 1 and Bcl-2 (or Bcl-xL) (Maiuri et al. 2007b).

Specific modulation of level 3 of autophagy regulation requires the targeting of entities that only control this part of the autophagic pathway. This would exclude microtubules (which are also involved at level 2, unless the target is dynein, a microtubule motor that is not involved in level 2). It also rules out targeting the signaling pathways identified so far at level 3, because p38 and ERK1/2 can also be involved at level 1 in some cell types. Some endo/lysosomal proteins are not likely good candidates, because DRAM is probably also involved at level 2. So far the most specific targets identified in level 3 are inhibitors of v-ATPase (bafilomycin A1, concanamycin A) and the dominant-negative form of Rab7. Brefeldin A, which blocks several membrane fusion events in the exocytic and endocytic pathways by interfering with the exchange of GDP for GTP on Arf, does not interrupt the autophagic pathway (Ogier-Denis et al. 1997a; Purhonen et al. 1997). This suggests that guanine nucleotide exchange factors containing a Sec7 domain, a target of brefeldin A, are not involved in membrane fusion events in the autophagic pathway. Although this is not discussed in this chapter, it should be pointed out that inhibiting lysosomal activity by cathepsin inhibitors or lysosomotropic agents such as chloroquine is also a valuable tool for blocking the late stage of autophagy (Amaravadi et al. 2007; Boya et al. 2005). It has been suggested that chloroquine treatment may block cytoprotective autophagy in tumor cells that resist chemotherapy (Amaravadi and Thompson 2007). Combining an autophagy inhibitor such as chloroquine with apoptosis-inducing chemotherapies may lead to improved tumor regression and reduced tumor recurrence.

6 Conclusions

Some progress has been made in understanding how autophagy is regulated. There are many ways to regulate the formation of autophagosomes. Besides the long-known (although still not completely defined) mTOR complex 1, which occurs upstream of the Atg1/Ulk1 and Ulk2 complexes, it has now been established that the Beclin 1 complex (which can also be designated PI3K complex I by analogy with the two

PI3K complexes in yeast) and Atg4 are also possible sites for regulation by signaling molecules. We cannot exclude the possibility that there are other possible points within the molecular machinery at which autophagy can be regulated. The machinery responsible for the maturation of autophagosomes and their fusion with acidic compartments is also regulated by several entities, including ATPase and GTPase activities, SNARE, MAPK signaling, and cytoskeletal motor proteins. However, more investigation is required to understand the roles of these different regulation systems in the late stages of autophagy. The tight regulation of the different stages of autophagy is a safety procedure that allows cells to retain control over a self-eating process that ends up in a "suicide bag," to use Christian de Duve's vivid description of lysosomes (De Duve and Wattiaux 1966).

The use of drugs, RNA interference and gene invalidation provide various ways to manipulate the autophagic pathway in order to study its role in different physiological and pathophysiological situations. The recent development of screening for drugs that modulate autophagy offers new perspectives for therapeutic interventions in human disease (Sarkar et al. 2007; Zhang et al. 2007), and is revealing new regulatory circuits during level 1 of the autophagic pathway (Williams et al. 2008). Moreover, drugs that interfere with level 3 provide a new way to modulate autophagy in the context of cancer therapy (Amaravadi and Thompson 2007). A future challenge will be to design drugs that specifically target level 2 of autophagy regulation.

The dialog between pathogens and autophagy, which will be discussed elsewhere in this issue of *Current Topics in Microbiology and Immunology*, depends at least partially on the ability of these microorganisms to exploit the vast repertoire of autophagy-regulating mechanisms to introduce "flats and sharps" in order to modulate the full musical score of autophagy.

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