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Autophagy regulation by nutrient signaling

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The ability of cells to respond to changes in nutrient availability is essential for the maintenance of metabolic homeostasis and viability. One of the key cellular responses to nutrient withdrawal is the upregulation of autophagy. Recently, there has been a rapid expansion in our knowledge of the molecular mechanisms involved in the regulation of mammalian autophagy induction in response to depletion of key nutrients. Intracellular amino acids, ATP, and oxygen levels are intimately tied to the cellular balance of anabolic and catabolic processes. Signaling from key nutrient-sensitive kinases mTORC1 and AMP-activated protein kinase (AMPK) is essential for the nutrient sensing of the autophagy pathway. Recent advances have shown that the nutrient status of the cell is largely passed on to the autophagic machinery through the coordinated regulation of the ULK and VPS34 kinase complexes. Identification of extensive crosstalk and feedback loops converging on the regulation of ULK and VPS34 can be attributed to the importance of these kinases in autophagy induction and maintaining cellular homeostasis.

Keywords: autophagy; ULK1; AMPK; VPS34; amino acids; oxygen; mTORC1

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Introduction

Macroautophagy, referred to hereafter simply as autophagy, is the primary catabolic program activated by cellular stressors including nutrient and energy starvation [1]. Autophagy begins by the *de novo* production of the autophagosome, a double membraned vesicle that expands to engulf neighbouring cytoplasmic components and organelles [2]. Autophagosome formation is driven by the concerted action of a suite of proteins designated as ATG or ‘autophagy-related’ proteins [3]. The mature autophagosome then becomes acidified after fusion with the lysosome, forming the autolysosome [3]. Lysosome fusion with the autophagosome provides luminal acid hydrolases that degrade the captured proteins, lipids, carbohydrates, nucleic acids, and organelles to provide nutrients that are then secreted back into the cytoplasm by lysosomal permeases for the cell’s use under stress conditions. Autophagy can also be induced by damaged organelles, protein aggregates, and infected pathogens to maintain cell integrity or exert defense response. This review will primarily focus on recent advances in the

mechanisms regulating autophagy in response to nutrients (amino acids, glucose, and oxygen).

The core autophagy proteins

In order to explain autophagy regulation, we will first describe the autophagy machinery in this section. ATG proteins are often listed in six functional groups that cooperate to perform key processes in autophagosome formation [3]: first, UNC-51-like kinase 1 (ULK1, a yeast Atg1 homolog) kinase complex comprised of ULK1, FIP200 (also known as RB1CC1), ATG13L, and ATG101 [4-9]; second, the VPS34 kinase complex (a class III phosphatidylinositol (PtdIns) 3-kinase) comprised of VPS34 (also known as PIK3C3), VPS15 (also known as PIK3R4), Beclin-1, and ATG14 or UVRAG (these proteins bind Beclin-1 mutually exclusively) [10-21]; third, PtdIns 3-phosphate (PtdIns(3)P) binding proteins including WD-repeat-interacting phosphoinositide proteins and zinc finger FYVE domain-containing protein 1 (also known as DFCEP1) [22-25]; fourth, the ATG5-12 ubiquitin-like conjugation system including the E3-ligase-like complex comprised of ATG12-ATG5-ATG16L (in which there is an isopeptide bond between ATG12 and ATG5) [26, 27]; fifth, the microtubule-associated protein 1-light chain 3 (LC3) phosphatidylethanolamine conjugation

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system (in which phosphatidylethanolamine is conjugated to LC3 by the ATG12-ATG5-ATG16L complex) [27, 28]; and sixth, ATG9a (a multi-spanning transmembrane protein), the only transmembrane protein among the ATG proteins [29]. The last group also includes the transmembrane protein vacuole membrane protein 1, which is not an ATG protein but is required for autophagy in mammals [30, 31]. The ATG proteins in this list have been ranked hierarchically and temporally in mammals [30, 31].

Autophagy and the ubiquitin-proteasome system constitute the major degradative processes in the cell. While increasing evidence suggests that there is significant crosstalk between autophagy and the ubiquitin systems, we would like to highlight two important distinctions. First, autophagy generates energy in its degradation of macromolecules, while the proteasome system consumes ATP in the degradation process. Second, autophagy is virtually unlimited in the size of the hydrolysis targets (i.e., protein, lipid, carbohydrate, etc.) that it can break down. Accordingly, entire organelles, viruses, and large protein aggregates are selectively broken down by the autolysosome (reviewed in [32-34]). Because of these differences, autophagy is the degradative force upregulated in response to nutrient starvation, mitochondrial depolarization, pathogen infection, and toxic protein aggregates.

The requirement for autophagy in maintaining cellular nutrient homeostasis is dramatically seen in ATG5- or ATG7-null neonatal mouse. Born with little physical defects and in predicted Mendelian ratios, these autophagy-defective mice die within a 24-h period after birth [35, 36]. Force-feeding can prolong survival, indicating a metabolic facet to the premature death. Analysis of key metabolites confirms that the autophagy-defective neonates suffer from a systemic amino acid deficiency and decreased glucose levels [35, 36]. Interestingly, in cultured normal hepatocytes the rate of protein degradation increases by a stunning 3% of total protein/h upon starvation. Nearly all of this increase is attributed to autophagy [35, 37]. Material recycling by autophagy is an evolutionally conserved mechanism required for the consumption of cytoplasmic ingredients under times of nutrient restriction [35, 36, 38]. Therefore, under periods of acute starvation, autophagy acts as an indispensable stress-responsive process capable of temporarily restoring cellular nutrient and energy balance.

Autophagy initiation

In mammals, the site of origin for autophagosome formation is the phagophore. The organelles that contribute

lipids to the phagophore remain an active topic of debate and competing models are reviewed in detail elsewhere [2]. Currently, there is compelling evidence that the endoplasmic reticulum-mitochondrial interface plays an important role in the genesis of starvation-induced autophagosomes [39, 40], while a significant portion of autophagosomes have also been described as containing lipids from the Golgi and plasma membranes [41-43]. The recruitment of ATG proteins to the phagophore along with the acquisition of lipids expands the membrane to form a cup-shaped precursor of the autophagosome termed the omegasome [44]. The step-wise progression of autophagosome formation is largely characterized by the recruitment and detachment of autophagosomal proteins to the maturing organelle [2, 3, 45].

ATG protein recruitment to the phagophore initiates autophagy

One of the earliest detectable events in autophagy initiation is the formation of ULK1 puncta [30] (Figure 1). In mammals, ULK1 and ULK2 (hereafter ULK kinase will be used to refer to ULK1 and ULK2) are the only serine/threonine kinases in the dedicated autophagy machinery and are homologous to yeast ATG1 [29, 46]. Genetic evidence suggests that ULK/ATG1 lies upstream of the recruitment of other ATG proteins [30]. The activity of ULK kinase is required for the recruitment of VPS34 to the phagophore [30, 31]. VPS34 is the catalytic component of multiple protein complexes, some of which are implicated in autophagy-independent mechanisms, while others function in distinct stages of autophagy. Of these complexes, VPS34 complex containing VPS15, Beclin-1, and ATG14 is specifically recruited to the phagophore to phosphorylate PtdIns, producing PtdIns(3)P (Figure 1) [15, 20, 30, 31]. PtdIns(3)P is essential for recruitment of a class of phospholipid-binding proteins whose exact functions in autophagy initiation remain enigmatic; however, in mammals and yeast they have been shown to play a role in autophagy [22, 23, 25, 30]. Additionally, the production of PtdIns(3)P has recently been shown to stabilize ULK1 at the omegasome [47]. The recruitment of oligomers of ATG12-conjugated ATG5 bound to ATG16L also coincides with ULK1 puncta formation [48, 49]. The formation of the ATG12-ATG5-ATG16L complex requires the ubiquitin-like conjugation system involving ATG7 and ATG10 (reviewed in [50]) and optimal ULK1 puncta formation upon amino-acid withdrawal requires the direct binding of FIP200 to ATG16L (Figure 1) [48, 49]. Functionally, ATG12-5-ATG16L is required for the conjugation of LC3 to phosphatidylethanolamine [28]. LC3B is a mammalian homolog of yeast ATG8, and is

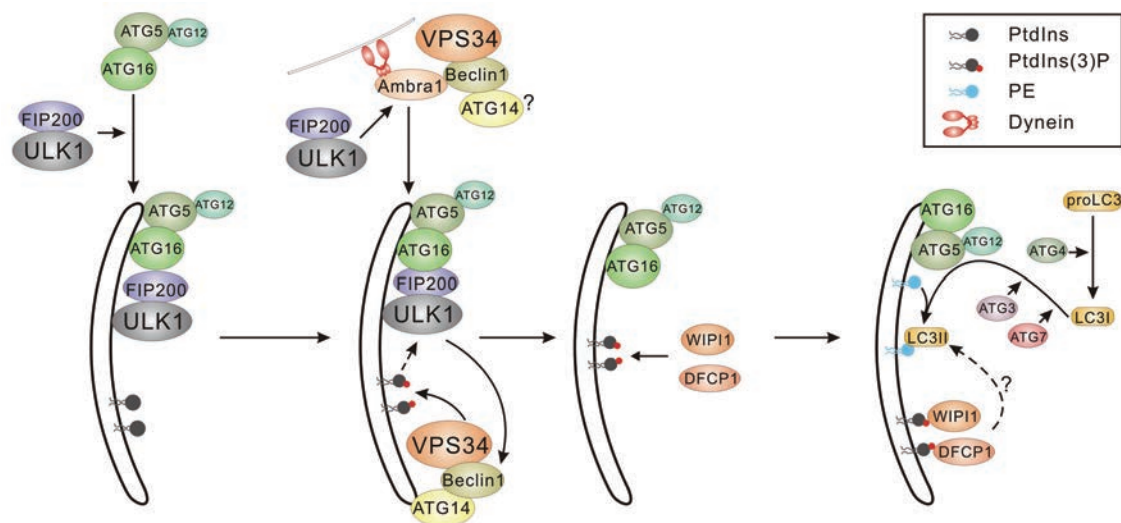


Figure 1 ATG protein recruitment in mammalian autophagosome formation. Temporal and functional relationship between ATG-protein complexes in autophagosome formation is depicted. These relationships were assembled from multiple independent studies to generate a working model with details summarized in the text. The core of VPS34 complexes, containing VPS34 and VPS15, is depicted as VPS34.

the most important and best characterized LC3 paralog from the family containing LC3 A, B, C for the induction of autophagy [28, 51]. The conjugation of LC3-phosphatidylethanolamine is thought to be required for the closure of the expanding autophagosomal membrane [52] (Figure 1). Finally, the phagophore contains two trans-membrane proteins ATG9 and vacuole membrane protein 1 that are required for generation of the autophagosome and they retain punctate localization under nutrient-rich conditions [30, 53].

The formation of the phagophore instigated by recruitment of ATG proteins is potently increased by withdrawal of nutrients, such as amino acids and glucose, so it is perhaps unsurprising that the kinases that sense these metabolites have recently been described to regulate autophagy initiation in response to changing energy and nutrient levels.

Amino acid signaling to mTORC1

The knowledge that autophagy is responsive to fluctuations in amino acids predates the identification and cloning of the ATG genes. In 1977, Schworer and colleagues showed that perfusion of rat livers in the absence of amino acids rapidly induced autophagosome number [54]. It was subsequently shown that branched chain-amino acids, in particular leucine, were responsible for the repression of protein turnover and autophagy [55, 56]. One of the key downstream effectors of amino acid-mediated autophagy repression is mammalian target of

rapamycin or mechanistic TOR (mTOR) [57, 58].

mTOR is a highly conserved serine/threonine kinase that is capable of integrating signals from many stimuli including amino acids, energy levels, oxygen, growth factors, and stress to coordinate cell growth and maintain metabolic homeostasis [59]. mTOR forms two functionally distinct complexes in mammals, mTORC1 (mTOR complex 1) and mTORC2 (mTOR complex 2). It is mTORC1 that is sensitive to both growth factors and nutrients, and the presence of amino acids has been shown to be essential for activation of the mTORC1 kinase [60]. Proteins including Ste-20-related kinase MAP4K3 and VPS34 have been described to play a role in amino acid signaling possibly through regulation of phosphatases and endocytic trafficking upstream of mTORC1 [12, 61-64]. Nevertheless, the clearest mechanism for mTORC1 activation by amino acids came from identification of the Rag GTPase complexes that tether mTORC1 to the lysosome [65, 66] (Figure 2). The Rag family proteins are members of the Ras family of GTPases, comprised of four members (RagA-D) that form heterodimers. A Rag dimer, comprised of an A/B subunit with a C/D subunit, binds mTORC1 in the presence of amino acids at the lysosome [65, 66]. Amino acid stimulation promotes Rag activation where Rag A/B is GTP-bound and Rag C/D is GDP-bound. Rag complexes are themselves not membrane-bound but are tethered to the lysosome through a complex called the Ragulator complex, which recruits Rag to lysosome and also functions as a guanine nucleotide exchange factor to stimulate Rag activation in

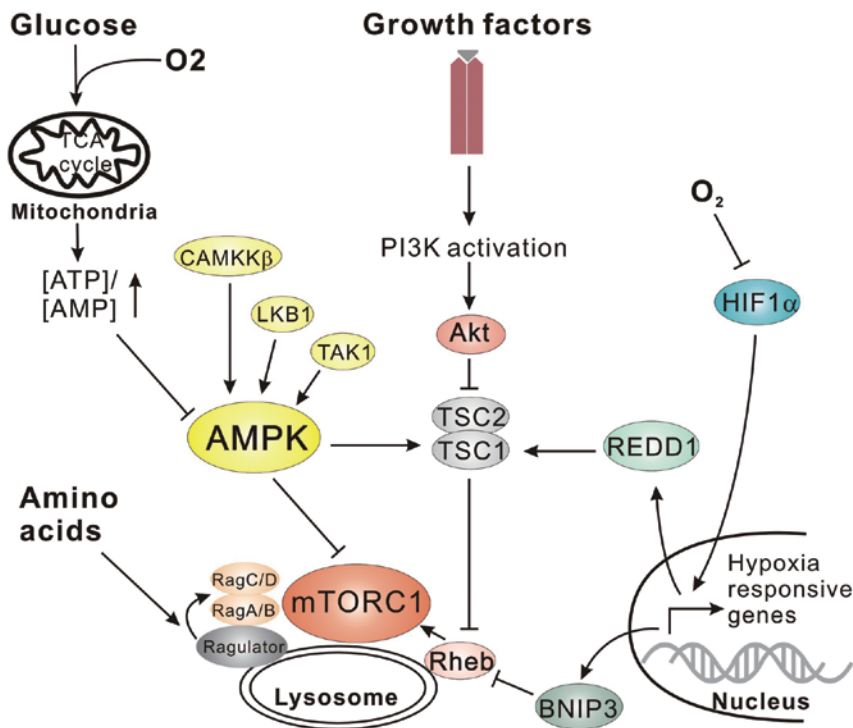


Figure 2 Upstream nutrient signaling to mTORC1 and AMPK. Nutrient starvation results in the inactivation of mTORC1. Oxygen or nutrient deficiency can activate AMPK through ADP:AMP accumulation, negatively regulating mTORC1 through either AMPK-mediated phosphorylation of mTORC1 or activation of the upstream repressor TSC. Limited oxygen also upregulates hypoxia-responsive genes, which are capable of suppressing mTORC1 signaling through the activation of TSC or inhibition of Rheb. Amino-acid withdrawal or inactivation of the PI3K pathway inhibits mTORC1 signaling through negatively regulating the activation of mTORC1 at the lysosome by Rag GTPases and Rheb.

response to amino acid sufficiency [67] (Figure 2). The recruitment of mTORC1 to the lysosome brings it into proximity with another small GTPase Rheb that is absolutely required for mTORC1 activation [68-70]. Rheb itself is negatively regulated by the tuberous sclerosis complex (TSC1/2), which acts as a GTPase activating protein for Rheb [68, 70-74] (Figure 2). In the presence of growth factors, the TSC complex is inactivated by the PI3K pathway through multiple mechanisms including direct repression of TSC by AKT-mediated (alternatively referred to as protein kinase B) phosphorylation [72, 75] (Figure 2). Therefore, full activation of mTORC1 can only be achieved in the presence of both amino acids and growth factors.

Downstream targets of mTORC1 in autophagy

mTORC1 is established as a potent repressor of autophagy in eukaryotes (TORC1 in yeast). Importantly, inhibition of mTORC1 is sufficient to induce autophagy in the presence of nutrients in yeast or mammalian cells [76-78], establishing mTORC1 as a conserved and critical repressor of autophagy. Direct repression of ATG1/ULK1 kinase by TORC1 is conserved across eukaryotes; however, the mechanisms of repression differ considerably. In mammalian cells, the ULK-ATG13L-FIP200 trimeric complex is stable regardless of the nutrient

status [1]. mTORC1 can interact with the ULK1 kinase complex and directly phosphorylates the ATG13L and ULK1 subunits to repress ULK1 kinase activity, although most sites have not been mapped or characterized [6-8] (Figure 3). Recently, mTORC1 was shown to phosphorylate Ser757 on ULK1, a site now verified by multiple groups [79-82]. Phosphorylation of Ser757 is important for mTORC1 to repress autophagy induction. When mTORC1 is inhibited, ULK1 undergoes autophosphorylation and trans-phosphorylation of binding partners ATG13L and FIP200, leading to an activation of the kinase complex under starvation conditions.

ULK regulation by mTORC1 in response to nutrients is functionally conserved across eukaryotes. Treatment of *S. cerevisiae* with rapamycin is sufficient to induce autophagy in the presence of nutrients [83]. TORC1-mediated repression of autophagy in yeast is accomplished through regulation of the ATG1 (homologue of mammalian ULK) kinase complex [83]. While the functional repression of ATG1 kinase complex by TORC1 is conserved, the proposed mechanisms differ considerably. In yeast, ATG1 forms an active kinase complex through an interaction with ATG13 and ATG17 (a functional homologue of mammalian FIP200) [3, 4]. Under times of nutrient sufficiency, TORC1 phosphorylates ATG13 on multiple sites thereby preventing its association with ATG1 [83-85]. TORC1 inhibition by nutrient starvation

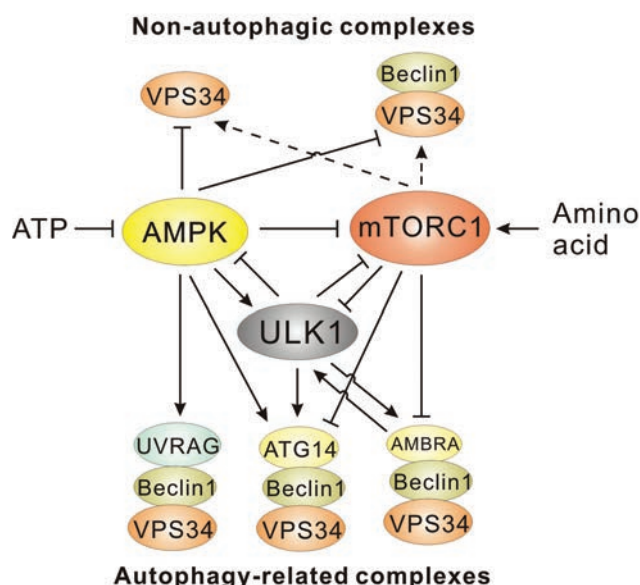


Figure 3 Regulation of ULK1 and VPS34 complexes by nutrients and upstream kinases. Nutrient starvation activates ULK1 through AMPK-mediated phosphorylation or loss of mTORC1-mediated repression. Activation of ULK1 has been described to initiate a positive-feedback loop through the phosphorylation of the mTORC1 complex and a negative-feedback loop through the phosphorylation of AMPK. Activities of the core VPS34 complexes, containing VPS34 and VPS15 (depicted as VPS34 in all complexes), and Beclin-1-bound VPS34 are inhibited under starvation. AMPK-mediated repression of these complexes is caused by direct phosphorylation of the VPS34 catalytic subunit. Amino acid-induced activation of these complexes is mTORC1-dependent but not direct and does not involve ULK1 kinase. ATG14-containing VPS34 complexes are activated by AMPK or ULK1 through phosphorylation of Beclin-1 or can be inhibited by mTORC1-mediated phosphorylation of ATG14. UVRAG-containing VPS34 complexes are activated by AMPK-mediated phosphorylation of Beclin-1 in response to starvation. ULK1 phosphorylates AMBRA1, freeing VPS34 from the cytoskeleton to act at the phagophore. AMBRA1 acts in a positive-feedback loop with TRAF6 to promote ULK1 activation.

or rapamycin treatment relieves the repression of ATG13 allowing the formation of an active ATG1-ATG13-ATG17 complex and induction of autophagy. However, it has recently been proposed that stability of the trimeric ATG1 kinase complex is not regulated by TORC1 or nutrient status in yeast, raising the possibility of alternative mechanism(s) in the regulation of the yeast ATG1 complex [86]. In mammalian cells, mTORC1 does not appear to regulate the formation of the ULK kinase complex [79]. Thus, TORC1-mediated phosphorylation of ATG13 is proposed to inhibit ATG1 kinase activity via phosphorylation of the kinase complex, as it does in fly

and mammals [5-8, 87, 88]. Moreover, mTORC1 also inhibits ULK1 activation by phosphorylating ULK and interfering with its interaction with the upstream activating kinase AMPK [79].

In yeast, ATG1 has been proposed to be downstream of Snf1 (AMPK homologue); however, the underlying mechanism remains to be determined [89]. Curiously, the yeast TORC1 has been described to inhibit Snf1, which is opposite to the AMPK-mediated repression of mTORC1 seen in mammals [90]. Together, these studies indicate that autophagy induction in eukaryotes is intimately tied to cellular energy status and nutrient availability through the direct regulation of the ATG1/ULK kinase complex by TORC1 and AMPK.

Interestingly, another facet of mTORC1-mediated autophagy repression has recently emerged. Under nutrient sufficiency, mTORC1 directly phosphorylates and inhibits ATG14-containing VPS34 complexes through its ATG14 subunit [91] (Figure 3). Upon withdrawal of amino acids, ATG14-containing VPS34 complexes are dramatically activated. Abrogation of the 5 identified mTORC1 phosphorylation sites (Ser3, Ser223, Thr233, Ser383, and Ser440) resulted in an increased activity of ATG14-containing VPS34 kinase under nutrient rich conditions, although not to the same level as nutrient starvation [91]. Stable reconstitution with a mutant ATG14 resistant to mTORC1-mediated phosphorylation also increased autophagy under nutrient rich conditions [91]. The mTORC1-mediated direct repression of both ULK1 and pro-autophagic VPS34 complexes provides important mechanistic insights into how intracellular amino acids repress the initiation of mammalian autophagy.

mTORC1 also indirectly regulates autophagy by controlling lysosome biogenesis through direct regulation of transcription factor EB (TFEB) [92, 93]. TFEB is responsible for driving the transcription of several lysosomal and autophagy-specific genes. mTORC1 and TFEB colocalize to the lysosomal membrane where mTORC1-mediated TFEB phosphorylation promotes YWHA (a 14-3-3 family member) binding to TFEB, leading to its cytoplasmic sequestration [92]. Under amino-acid withdrawal or inactivation of amino acid secretion from the lysosome, mTORC1 is inactivated and the unphosphorylated TFEB translocates to the nucleus. Artificial activation of mTORC1 by transfection of constitutively active Rag GTPase mutants results in a constitutive localization of TFEB in the cytoplasm and deletion of TFEB results in a decreased autophagy response to nutrient withdrawal and reduction in the cellular lysosome compartment [93]. Through the repression of TFEB, ULK kinase complexes, and VPS34-kinase complexes, mTORC1 is able to

negatively regulate both the initiation and maturation of the autophagosome.

Paradoxically, under prolonged starvation the role of mTORC1 in autophagy flips from a repressor to a promoter of autophagy [94]. Under times of severe nutrient deprivation, autophagy is rapidly induced and a large portion of cellular lysosomes are used to form autolysosomes. The restoration of a normal complement of lysosomes requires recycling of the autolysosomal membrane. For membrane recycling to occur, mTORC1 must be activated by the secreted amino acids from the mature autolysosome, which allows for the formation of an empty tubule that protrudes from the autolysosome [94]. These tubules eventually mature into lysosomes, restoring cellular lysosome numbers. The multiple levels at which mTORC1 can regulate and be regulated by autophagy are uniquely illustrated in the lysosomal storage disease mucopolidosis type IV (MLIV) where mTORC1 reactivation by the mature autolysosome is inhibited (see Box 1).

Recent studies have greatly advanced our understanding of the complex crosstalk between autophagy and mTORC1 signaling, and it will be exciting to see what new connections will be uncovered between these two key processes in maintaining nutrient/energy homeostasis.

Energetic stress and AMPK signaling

In order to maintain metabolic homeostasis, the cell must strictly match the generation and consumption of ATP. The intracellular ratio of ATP:ADP:AMP is an important indicator of cellular energy levels. Increased levels of ADP and AMP signal to the cell that it must curtail energy-intensive processes. These nucleotides are directly sensed by the AMPK. AMPK is a trimeric serine/threonine kinase essential for an appropriate response to energetic stress (reviewed in [98]). The catalytic α subunit of AMPK is phosphorylated by upstream regulatory kinases LKB1, calcium/calmodulin-dependent protein

kinase kinase- β , and TAK1 [99-101] (Figure 2). Phosphorylation of AMPK α within the activation loop (T172) by upstream kinases is required for activity [102-104]. The β subunit acts as a linker between α and γ subunits and may have additional regulatory function(s), such as glycogen-binding. AMPK can be allosterically activated through the binding of AMP to one of four Bateman domains in the γ subunit, resulting in allosteric activation of the associated α subunit. More importantly, AMP and ADP activate AMPK by preventing dephosphorylation of T172 in the AMPK α subunit [105, 106, 107]. The binding of ADP does not elicit allosteric activation but does promote stabilization of the activation loop [102, 108]. Reduction in cellular ATP levels, caused by glucose withdrawal or other stressors such as mitochondrial dysfunction initiates a cellular metabolic response through AMPK targets that seek to generate energy by increasing glucose uptake and glycolysis and stimulating lipid catabolism (for detailed review, see [109]).

Downstream targets of AMPK in autophagy

Activation of autophagy in response to energetic stress is an essential mechanism to maintain metabolic homeostasis and cell viability. AMPK has recently been shown to be an essential mediator of autophagy induction in response to glucose withdrawal and essential for cytoprotection under these conditions [79, 110]. There are several mechanisms by which AMPK can promote autophagy. Importantly, AMPK is an established negative regulator of the mTOR signaling cascade [74, 111]. This can be accomplished by AMPK-mediated phosphorylation of the TSC complex which is a negative regulator of mTORC1 activation at the lysosome (Figure 2). Alternatively, AMPK can directly phosphorylate the Raptor subunit of the mTORC1 complex, which induces 14-3-3 binding and inhibits mTORC1 target phosphorylation [112] (Figure 2). Through both these mechanisms, AMPK is able to relieve mTOR-mediated autophagy repression.

Box1 mTOR signaling and autophagy in MLIV

MLIV is caused by a deficiency in the cation channel encoded by MCOLN1. MCOLN1 is required for the fusion of autophagosomes to lysosomes. When MCOLN1 function is disrupted, there is a buildup of autophagosomes that are bound to lysosomes but unable to fuse [95, 96]. The resulting defect in autophagic flux causes decreased mTORC1 activity, which in turn causes a de-repression of lysosomal biogenesis, with TFEB likely playing a role. The end result is a drastic increase in acidic vesicles and defective autolysosome precursors. Remarkably, in the *Drosophila* model of MLIV, activation of *Drosophila* TORC1 by introduction of a protein-rich diet was sufficient to reverse the MLIV phenotype [97]. This study shows that not only is *Drosophila* TORC1 involved in the pathology of MLIV, but also that amino acids generated by autophagy are an important source for *Drosophila* TORC1 activation.

AMPK is also capable of directly phosphorylating and activating ULK1 kinase [79, 113]. Work from our lab found that Ser317 and Ser777 (in the mouse ULK1 protein) phosphorylation of ULK1 by AMPK is required for ULK1 activation and proper induction of autophagy upon glucose starvation [79] (Figure 3). Moreover, the interaction between ULK1 and AMPK was antagonized by mTORC1-mediated Ser757 phosphorylation of ULK1, indicating a tight control of ULK1 activity in response to nutrient and energy levels. Several additional phosphorylation sites were found (Ser467, Ser556, Thr575, and Ser638) to be important for mitophagy [110] and Ser556 phosphorylation was shown to be required for 14-3-3 binding to ULK1 [113]. Interestingly, another study also found many overlapping AMPK and mTORC1-dependent phosphorylation events on ULK1 with some details conflicting with previous reports, possibly due to different starvation conditions used in these reports [81]. In total, these studies clearly demonstrate that AMPK and mTORC1 both tightly control ULK1 function through protein phosphorylation.

AMPK has also recently been shown to regulate multiple VPS34 complexes upon glucose withdrawal. Under starvation, AMPK inhibits VPS34 complexes that do not contain pro-autophagic adaptors, such as UVRAG and ATG14 (see Beclin-1 binding partners in Table 1). These VPS34 complexes are not involved in autophagy but rather are involved in cellular vesicle trafficking. Inhibition was shown to be mediated through direct phosphorylation of VPS34 on Thr163 and Ser165 by AMPK [114] (Figure 3). Concomitantly, AMPK enhances VPS34 kinase activity in complexes containing UVRAG or ATG14 by phosphorylation of Beclin-1 on

Ser91 and Ser94 (Figure 3). The ATG14- or UVRAG-containing VPS34 complexes are involved in autophagy initiation. Activation of ATG14-containing VPS34 complexes through Beclin-1 phosphorylation was shown to be required for the induction of autophagy upon glucose withdrawal [114]. Interestingly, inhibitory phosphorylation of VPS34 was shown to be important for survival in response to glucose withdrawal; however, it did not affect autophagy induction. Further studies will be required to shed light on how repression of total PtdIns(3)P levels promotes survival under energetic stress.

Oxygen availability

Oxygen is an essential nutrient that is required for key metabolic processes within the cell. Perhaps one of the most important functions of molecular oxygen within the cell is in oxidative respiration. Oxygen along with the electron transport chain in the mitochondria is necessary for generating ATP through oxidative phosphorylation [115]. Hypoxia results in a reduction in ATP levels, at least transiently, which activates AMPK and inactivates mTOR [116-118] (Figure 2). The activation of AMPK and inactivation of mTOR are adaptive responses as the cell shifts its metabolic priorities, producing energy in other ways such as increased glycolysis as well as reducing energy-consuming processes.

One of the best-characterized events of the hypoxic response is stabilization of the HIF1 α transcription factor [115, 119]. In the absence of oxygen, HIF1 α escapes proteasomal degradation by the von Hippel-Lindau tumor suppressor and accumulates in the nucleus where it activates the transcription of a wide array of genes that are

Table 1 Beclin-1 interacting proteins implicated in starvation-induced autophagy

Protein	Interaction and function	Reference
<i>Positive regulators of autophagy</i>		
VPS34	catalytic subunit of phosphatidylinositol 3-kinase complexes	[11, 155]
VPS15	cofactor of VPS34 required for production of PtdIns(3)P	[17, 151]
UVRAG	promotes autophagy, present in late endosomes	[11, 21, 156]
ATG14	promotes autophagy, essential for localization of VPS34 to phagophore	[11, 21]
AMBRA1	promotes autophagy, nutrient-dependent localization of Beclin-1	[131, 157]
HMGB1	promotes autophagy, increases VPS34 activity	[158]
Bif-1	promotes autophagy, promotes UVRAG-containing VPS34 complexes	[159]
<i>Negative regulators of autophagy</i>		
Rubicon	inhibits autophagy, antagonizes UVRAG-containing VPS34 complexes	[16, 19]
Bcl-2	inhibits autophagy, inhibits Beclin-1-containing VPS34 complexes	[142]
Bcl-xL	inhibits autophagy, binds Beclin-1 complexes at the ER	[145]
IP3R	inhibits autophagy, binds Beclin-1 complexes at the ER	[160]

necessary for metabolic adaptation to reduced oxygen levels [120]. Two hypoxia responsive genes, BNIP3 and BNIP3L, aid in balancing ATP consumption by increasing mitochondrial autophagy under low oxygen conditions [121]. Moreover, BNIP3 has been described to negatively regulate mTORC1 activation possibly through binding of the small GTPase Rheb [122] (Figure 2). Interestingly, another hypoxia responsive gene REDD1 has also been implicated in negatively regulating mTORC1 through activation of the TSC complex [123-125] (Figure 2). Additionally, some HIF-responsive genes have been described to affect VPS34 complex formation (discussed below). Together these studies show that oxygen depletion in the cell is intimately tied to the upstream regulation of autophagy by AMPK and mTORC1.

The autophagy initiating kinase ULK

ULK is the most upstream ATG protein regulating autophagy initiation in response to inductive signals. ULK1 was identified as the mammalian homolog of *Caenorhabditis elegans* Unc-51, which was originally characterized as being essential for neuronal axon guidance [126]. In mammals, the *ULK1*-knockout mouse has a very mild phenotype showing defects in reticulocyte development and mitochondrial clearance in these cells [127]. This is likely due to the functional redundancy with ULK2 that has been described for autophagy induction [128, 129]. ULK directly interacts with ATG13L and FIP200 through the C-terminal domain and both interactions can stabilize and activate ULK-kinase [5-8]. The ULK-kinase complex is under tight regulation in response to nutrients, energy, and growth factors as described in previous sections. The original phospho-mapping of murine ULK1 identified 16 phosphorylation sites, although the kinases responsible for several of these phosphorylation events remain unknown [80]. Additional studies have increased the number of phosphorylation sites to over 40 residues on ULK1 including a critical site on the activation loop T180, that is required for autophosphorylation [113]. In addition to autophosphorylation, ULK can phosphorylate both ATG13L and FIP200, and the intact kinase complex is required for ULK localization to the phagophore and autophagy induction [4-6, 8].

Downstream targets of ULK

Despite ULK's pivotal role in conveying nutrient signal to the autophagy cascade, the mechanisms and downstream targets responsible were until recently enigmatic. Three direct targets of ULK1 have recently been identified as well as two feedback loops to mTORC1 and

AMPK. Recent work from our lab found that ULK1 and ULK2 directly phosphorylate Beclin-1 on S15 (murine S14) and this phosphorylation is required for activation of ATG14-containing VPS34 complexes [130] (Figure 3). The ability of Beclin-1 and ULK1 to bind *in vivo* was promoted by ATG14, which was proposed to act as an adaptor in Beclin-1 binding to ULK. Interestingly, the ability of ATG14 to promote Beclin-1 phosphorylation was abolished in mutants that could not localize to the phagophore, indicating that the activation of ATG14-containing VPS34 complexes may occur specifically at the phagophore (Figure 1). The conserved phosphorylation site on Beclin-1 was shown to be required for proper induction of autophagy in mammals and autophagy during *C. elegans* embryogenesis [130].

A Beclin-1 binding partner, activating molecule in Beclin1-regulated autophagy 1 (AMBRA1), has also been identified as a target for ULK1-mediated phosphorylation [131] (Figure 3). Under nutrient-rich conditions, AMBRA1 binds Beclin-1 and VPS34 at the cytoskeleton through an interaction with dynein. Upon starvation, ULK1 phosphorylates AMBRA1, and Beclin-1 then translocates to the endoplasmic reticulum, allowing VPS34 to act at the phagophore [131] (Figure 1). This model is in agreement with previous findings that ATG14-containing VPS34 complexes require ULK-kinase to localize to the phagophore [15, 20, 30]. However, it is currently unclear if Beclin-1 binds ATG14 and AMBRA1 in the same complex at the site of the phagophore. Interestingly, AMBRA1 was shown to act in an mTORC1-sensitive positive-feedback loop to promote K63-linked ubiquitination of ULK1 through recruitment of the E3-ubiquitin ligase TRAF6 [132] (Figure 3).

ULK1 has also been described to phosphorylate zipper interacting protein kinase, also known as DAPK3 [133]. It was reported that ULK-induced zipper interacting protein kinase phosphorylation plays a role in the redistribution of the transmembrane protein ATG9a from the transgolgi network to peripheral endosomes that are capable of being incorporated into the autophagosome [133], which has been described to be nutrient sensitive [5, 29]. However, it was recently reported by multiple groups that the localization of ATG9a to the autophagosomal membrane is ULK-independent and that it was the recycling of ATG9a that is ULK-sensitive [53, 134]. In an alternative ULK-independent model, ATG9a is bound and inhibited by p38-interacting protein and then released after starvation-induced phosphorylation of p38-interacting protein by MAPK p38 [135]. However, ULK clearly regulates some ATG9a-related processes [29, 133, 136]. Additional studies will be needed to clarify the role of zipper interacting protein kinase and ULK kinase in ATG9a localization to the autophagosomal membrane.

ULK1 feedback loops

ULK1 has recently been described to initiate two feedback loops. Two groups have independently identified ULK1 as a negative regulator of mTORC1-signaling through phosphorylation of the raptor subunit [137, 138]. The proposed model is that ULK-mediated phosphorylation of raptor results in a reduction in the ability of mTORC1 to bind substrate. This would represent a positive-feedback loop that may be important for ramping up the signaling in the earlier stages of autophagy, while amino acids that are secreted from the autolysosome would then re-activate mTOR in later stages of autophagy. ULK1 was also described to bind and phosphorylate its upstream regulator AMPK on all three subunits, though surprisingly this regulation was also inhibitory [139]. This would represent a negative-feedback loop in response to AMPK-mediated ULK activation. Clearly, there are several interdependencies between AMPK-mTOR-ULK kinases, some of which may seem counterintuitive in regulating the activation of autophagy in response to nutrient stress. It is possible that under the fluctuations of nutrient/energy levels that occur physiologically *in vivo* some of these signals may act dichotomously. Alternatively, different feedback loops could be activated under different stress conditions or act temporally.

Regulation of VPS34-kinase complexes in response to nutrients

Generation of PtdIns(3)P at the phagophore is necessary for the expansion of the membrane. Production of PtdIns(3)P at the phagophore is controlled by at least three known mechanisms: (1) localization of the VPS34 kinase complex, regulated by Beclin-1:ATG14/AMBRA binding and controlled by ULK-kinase activity [16, 19-21, 30, 131]; (2) activation of VPS34 kinase activity, controlled by ULK1, mTOR, and AMPK in response to nutrients [91, 114, 130] (activity is also affected by binding to Beclin-1 and ATG14 [114]); and (3) regulation of VPS34 complex formation through the Beclin-1 interaction [140-142].

The core VPS34 complex that is comprised of VPS34 and the regulator VPS15 likely does not directly act in promoting autophagosome formation [114]. VPS34-VPS15 complexes are likely the predominant form in the cell as quantitative immuno-depletion revealed that the majority of VPS34-VPS15 is not bound to Beclin-1, although the relative abundance of different VPS34 complexes is cell type-dependent [114]. VPS34 complexes

that have a role in promoting autophagy contain Beclin-1 [142]. However, it appears that for VPS34 to produce PtdIns(3)P at the correct site and stage of autophagy, additional components are needed. Beclin-1 acts as an adaptor for pro-autophagic VPS34 complexes to recruit additional regulatory subunits such as ATG14 and UVRAG [11, 15, 16, 19-21]. ATG14 or UVRAG binding to the VPS34 complex potentially increases the PI3 kinase activity of VPS34. Moreover, the dynamics of VPS34-Beclin-1 interaction has been described to regulate autophagy in a nutrient-sensitive manner [140, 142, 143]. A list of Beclin-1 interactors with known functions has been summarized (see Table 1); however, this section will focus on changes in VPS34 complex composition that are sensitive to alteration of nutrients.

The ability of VPS34 complexes containing Beclin-1 to promote autophagy can be negatively regulated by Bcl-2 as well as family members Bcl-xl and viral Bcl-2 [142, 144-146]. Bcl-2 binding to the BH3 domain in Beclin-1 at the endoplasmic reticulum and not the mitochondria seems to be important for the negative regulation of autophagy, and Bcl-2-mediated repression of autophagy has been described in several studies [140, 142, 143, 145, 147, 148]. The nutrient-deprivation autophagy factor-1 was identified as a Bcl-2 binding partner that specifically binds Bcl-2 at the ER to antagonize starvation-induced autophagy [149]. There are two proposed models for the ability of Bcl-2 to inhibit VPS34 activity. In the predominant model, Bcl-2 binding to Beclin-1 disrupts VPS34-Beclin-1 interaction resulting in the inhibition of autophagy [140, 142] (Figure 4). Alternatively, Bcl-2 has been proposed to inhibit pro-autophagic VPS34 through the stabilization of dimerized Beclin-1 [14, 150] (Figure 4). It remains to be seen if the switch from Beclin-1 homo-dimers to UVRAG/ATG14-containing heterodimers is a physiologically relevant mode of VPS34 regulation. Given the number of studies that see stable interactions under starvation between VPS34 and Beclin-1 [62, 91, 114, 130, 143, 151] and those that see a disruption [140, 142], it is quite likely that multiple mechanisms exist to regulate VPS34 complexes containing Beclin-1. It may be noteworthy that studies that do not see changes in the VPS34-Beclin-1 interaction tend to use shorter time points (~1 h amino acid starvation), while studies that see disruption tend to use longer time points (~4 h). If the differences cannot be explained by media composition or cell type, it would be interesting to determine if Bcl-2 is inhibiting VPS34 through Beclin-1 dimerization at shorter time points, or if the negative regulation of VPS34-Beclin-1 complexes by Bcl-2 happens with a temporal delay upon nutrient deprivation.

The ability of Bcl-2 to bind Beclin-1 is also regulated

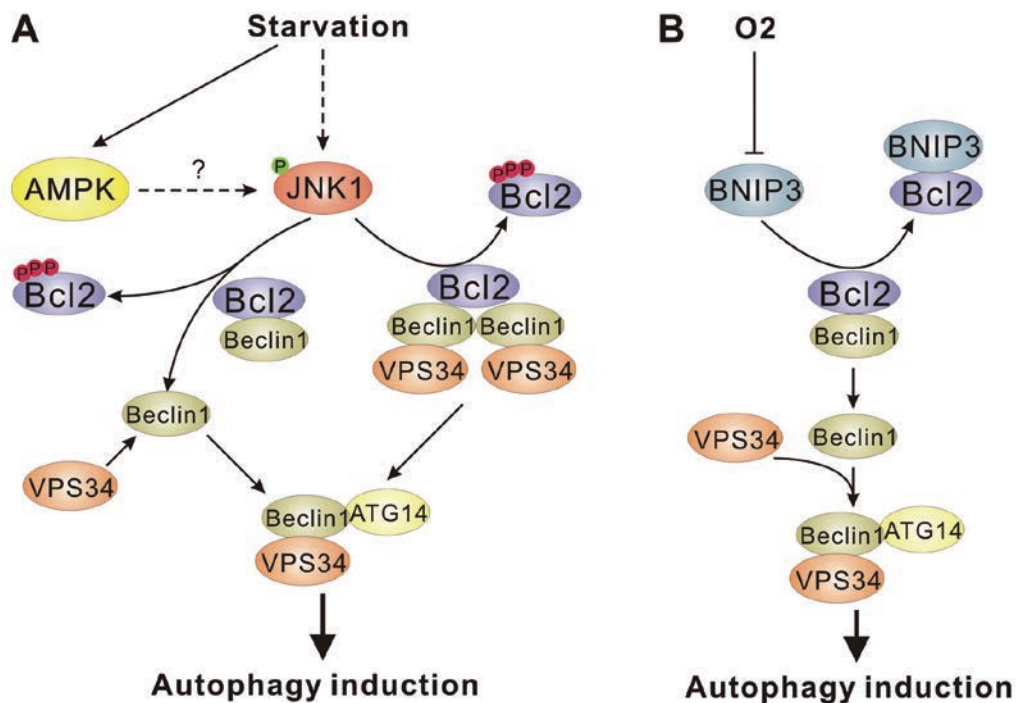


Figure 4 Regulation of VPS34 complex formation in response to nutrients. **(A)** Starvation activates JNK1 kinase, possibly through direct phosphorylation by AMPK. JNK1 phosphorylates Bcl-2, relieving Bcl-2-mediated repression of Beclin-1-VPS34 complexes. Bcl-2 may inhibit VPS34 complexes by disrupting Beclin-1-VPS34 interaction (left arrow) or by stabilizing an inactive Beclin-1 homodimeric complex (right arrow). **(B)** Hypoxia upregulates BNIP3 expression, which can bind Bcl-2, thereby relieving Bcl-2-mediated repression of Beclin-1-VPS34 complexes.

by phosphorylation. Levine and colleagues have shown that starvation-induced autophagy requires c-Jun N-terminal protein kinase 1 (JNK1)-mediated phosphorylation of Bcl-2 [140]. JNK1 but not JNK2 phosphorylates Bcl-2 on three residues (Thr69, Ser70, and Ser87) resulting in the dissociation of Bcl-2 from Beclin-1 (Figure 4). Interestingly, mutants of Bcl-2 containing phospho-mimetic residues at JNK1 phosphorylation sites led to increased autophagy levels indicating that activation of JNK1 is essential for relieving Bcl-2-mediated suppression of autophagy [140]. A potential mechanism for JNK1 activation upon starvation has recently been proposed. He *et al.* [143] showed that AMPK activation can promote JNK1 signaling to Bcl-2 and increase autophagy. Moreover, they showed that AMPK can phosphorylate JNK1 *in vitro* and AMPK-JNK1 interaction is increased *in vivo* upon AMPK activation by metformin (Figure 4A). However, this observation is very surprising because the activation loop sites in JNK do not fit the AMPK consensus and AMPK is not known to have tyrosine kinase activity. Further studies are needed to confirm a direct activation of JNK1 by AMPK. Nevertheless, this study presents a potential mechanism linking the decrease in cellular

energy to the Bcl-2-mediated regulation of autophagy. Lowered oxygen level has also been described to disrupt the Bcl-2-Beclin-1 interaction. Under hypoxia, HIF1 α target genes BNIP3 and BNIP3L have been described as having a role in driving autophagy by displacing Bcl-2 from Beclin-1 [152, 153]. The BH3 domain of BNIP3 was described to bind and sequester Bcl-2, thus relieving its inhibition of Beclin-1 (Figure 4B). Taken together, these studies clearly indicate an inhibitory role for Bcl-2 on Beclin-1 in autophagy. It is quite likely that additional insights into this regulatory mechanism will be forthcoming.

Our understanding of the mechanisms regulating VPS34 complexes in response to nutrient deprivation has rapidly advanced in recent years. However, the identification of parallel pathways, such as ULK- and AMPK-mediated activation of ATG14-containing VPS34 complexes, has also raised questions of which regulatory pathways are relevant in response to different starvation stimuli (i.e., glucose vs amino-acid withdrawal) and whether there is crosstalk between the regulatory pathways that converge upon VPS34 complexes. Answering these questions will undoubtedly shed light on nuances

of autophagy induction in mammals that have previously been unappreciated.

Conclusion

The ability of both mTORC1 and AMPK to regulate autophagy induction through ULK and VPS34 kinases has raised important questions. e.g., is there interplay between mTORC1- and AMPK-mediated phosphorylation of the ATG14-containing VPS34 complexes? The PI3K pathway has been described to regulate autophagy via mTORC1-dependent and independent mechanisms. The relationship between these two pathways in autophagy induction remains an open question. Moreover, characterization of signals that intersect to provide the cell-type specificity of autophagic induction *in vivo* has been described, but for the most part the underlying mechanisms remains to be revealed [154].

The formation of ULK1 puncta is an early marker for autophagy induction. However, the mechanism regulating ULK1 translocation to the phagophore is poorly understood. The identity of membrane-bound ULK-receptors as well as upstream signals essential for regulating ULK localization remain unknown and are important outstanding questions. To date, only a handful of ULK targets have been identified and no consensus motif for the kinase has been described. The identification and characterization of additional ULK targets will undoubtedly shed light on the mechanisms of ULK-dependent autophagic processes that remain elusive.

As described above, the relationship between mTORC1-, AMPK-, and ULK-mediated regulation of the VPS34 complexes remains to be determined. Moreover, the regulation of VPS34 kinase activity by complex formation and phosphorylation is poorly understood and would benefit from studies providing structural insights. Additionally, the physiological significance of reducing total PtdIns(3)P levels under starvation is not entirely clear. It may be simply that running the endocytic pathway is an energy intensive endeavor, or perhaps membrane cycling or cell signaling from the endosomes is important in times of starvation. Finally, the exact role of PtdIns(3)P-binding proteins in promoting autophagy remains to be determined. Given the potential redundancy of these proteins, it remains a difficult question to tackle. Overall, the field has made great progress in understanding how nutrient information is transmitted to the autophagy pathway and like any good discovery, this has left us with as many questions as answers.

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