On signals controlling autophagy

It's time to eat yourself healthy

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Macroautophagy, for simplicity frequently called autophagy, is a mechanism used by cells to survive periods of starvation by degrading cytoplasmic components and releasing much-needed metabolites and energy. In so doing, autophagy also achieves another feat: removal of dysfunctional and toxic proteins and protein aggregates, as well as entire organelles, such as functionally impaired mitochondria. Therefore up-regulation of autophagy is now considered to be of potential therapeutic benefit in numerous diseases, including neurodegenerative conditions resulting from accumulation of misfolded, intracytoplasmic, aggregate-prone proteins. This article discusses a complex network of signalling pathways upstream of autophagy and potential targets that may allow precise and efficient control of autophagy for therapeutic purposes.

The phenomenon where a cell degrades portions of its own cytoplasm by transporting them to lysosomes was discovered nearly half a century ago by Christian de Duve, who also gave the process its current name, autophagy. Despite the early discovery of autophagy, its cellular status was overshadowed for a long time by its younger cousin, another major degradative pathway called the ubiquitin–proteasome system (UPS). This delayed appreciation of the importance of autophagy resulted from the lack of knowledge of its underlying molecular mechanisms and the consequent inability to specifically test its biological relevance in many settings. Following the discovery of the key machinery in yeast in the early 1990s, however, autophagy research rapidly intensified and, in recent years, this intracellular protein trafficking and degradative pathway has become a hot topic in cellular biology and physiology. One reason for the interest among scientists and the public alike is because of its roles in cellular and organismal health and longevity. Indeed, autophagy perturbations have been recognized as a causative factor in a number of human pathologies, including neurodegeneration, heart diseases, diabetes and cancer[1]. Moreover, the autophagic pathway may have an impact on aging, which is the most important risk factor in the development of many human diseases. Thus, many of the treatments prolonging lifespan in model organisms from yeast to mice are doing so in autophagy-dependent manners, indicating that efficient protein degradation by autophagy may be a major determinant of lifespan extension[2]. As a result, treatments enhancing (or, sometimes, inhibiting) autophagy are now widely believed to be of promising therapeutic potential. How does a basic cellular process have such a wide range of important roles? To answer this question, we need to have a closer look at the pathway itself.

Autophagy at a glance

As with any other intracellular vesicular trafficking pathway, autophagy starts with the formation of a vesicle. The autophagosome is a double-membraned structure formed around portions of cytoplasm containing cellular components that are destined for degradation. It is transported along microtubules and its life ends when it fuses to lysosomes, where autophagic

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Abbreviations: AC, adenylate cyclase; AD, Alzheimer's disease; Atg, AuTophaGy; HD, Huntington's disease; Jnk-1, c-Jun N-terminal kinase 1; MTOC, microtubule-organizing centre; mTOR, mammalian target of rapamycin; mTORC, mTOR complex; OPMD, oculopharyngeal muscular dystrophy; PABPN1, poly(A)-binding protein, nuclear 1; PD, Parkinson's disease; PI3K, phosphoinositide 3-kinase; PLC, phospholipase C; TOR, target of rapamycin; TSC, tuberous sclerosis complex; UPS, ubiquitin–proteasome system; Vps, vacuolar protein sorting.

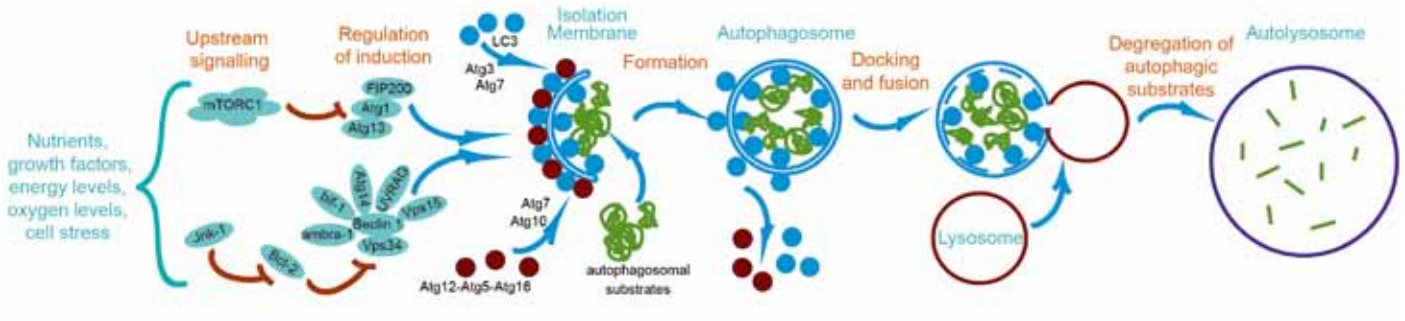

Figure 1. Sequential events of the autophagic pathway. Autophagy is activated in response to various stress conditions, such as starvation, low energy or low oxygen levels. Phosphorylation events within signalling pathways, such as mTOR and Jnk-1, regulate autophagosome formation. Autophagosomes eventually fuse with lysosomes to form hybrid organelles called autolysosomes, where their substrates ae degraded. Ambra-1, activating molecule in Beclin 1-regulate autophagy; Bif-1, Bax-interacting factor 1; FIP200, focal adhesion kinase family-interacting protein of 200 kDa; UVRAG, UV radiation resistance-associated gene.

substrates are degraded. The process of autophagosome formation and maturation is under tight control and is orchestrated by a set of dedicated proteins, many of which have an Atg number (which stands for AuTophaGy, as originally designated in yeast). These are organized into several functional complexes[1] (Figure 1). The early stages of autophagosome formation require the class III phosphoinositide 3-kinase (PI3K), vacuolar protein sorting (Vps) 34, as a part of the macromolecular complex with Beclin 1/Atg6, Atg14, Vps15, Ambra-1, UV radiation resistance-associated gene (UVRAG) and Bax-interacting factor 1 (Bif-1). This complex is activated when inhibition by Bcl-2 is released, following its phosphorylation by c-Jun N-terminal kinase 1 (Jnk-1). Initiation of autophagosome formation also depends on another complex consisting of Atg1, Atg13 and focal adhesion kinase family-interacting protein of 200 kDa (FIP200), which is regulated by mammalian target of rapamycin (mTOR) kinase activity (see below). Following the formation of the pre-autophagosomal structures, these subsequently elongate to form mature double-membraned vesicles, a process requiring two ubiquitin-like conjugation reactions. In one reaction, Atg5 is modified by a small ubiquitin-like protein Atg12 aided by Atg7 (E1-like) and Atg10 (E2-like) enzymes. The Atg5–Atg12 conjugate then becomes covalently attached to Atg16, forming a supramolecular complex that is essential for autophagosome formation, which dissociates from the vesicle once it is fully formed. In contrast, phosphatidylethanolamine-conjugated Atg8/LC3-II, the result of another ubiquitin-like reaction involving Atg7 and Atg3 (E2-like enzyme), remains attached to the vesicle throughout its lifespan and it is nowadays the most commonly used autophagy marker, allowing researchers to monitor both the formation and degradation of autophagosomes.

Autophagy substrates can be recruited into autophagosomes either non-selectively or with a degree of selectivity, often towards bulkier substrates. It is the capacity of the pathway to dispose of large intracellular entities, from oligomeric protein complexes to entire organelles, which makes it stand out from other degradative pathways, including the UPS. This provides the pathway with an ability to regulate cellular homoeostasis by clearing damaged and dysfunctional cellular components and thus prevent cellular damage. One way that autophagy could protect cellular and organismal health was first realized a decade ago when we demonstrated that it influenced the degradation of pathogenic intracytoplasmic neurodegenerative disease-causing proteins[3].

Autophagy as a therapeutic target in proteinopathies

The most common neurodegenerative conditions, the 'sporadic' forms of Alzheimer's disease (AD) and Parkinson's disease (PD), are classified as proteinopathies (or protein conformational disorders) caused by misfolded and aggregated proteins. These diseases are characterised by complex aetiologies with multiple genetic and environmental components. Instead, Huntington's disease (HD) and oculopharyngeal muscular dystrophy (OPMD) are monogenic (resulting from a mutation in a single gene) proteinopathies and are more amenable to genetic modelling. The paper by Ravikumar et al.[3] took advantage of this fact by investigating cellular pathways capable of degrading two mutant proteins. One was a fragment of huntingtin with the expanded polyglutamine repeat mutation that causes HD. The second was enhanced green fluorescent protein tagged to a polyalanine stretch, as a model of the polyalanine expansions in the poly(A)-binding protein, nuclear 1 (PABPN1), that causes OPMD. Both the mutant huntingtin fragment and the polyalanine-expanded

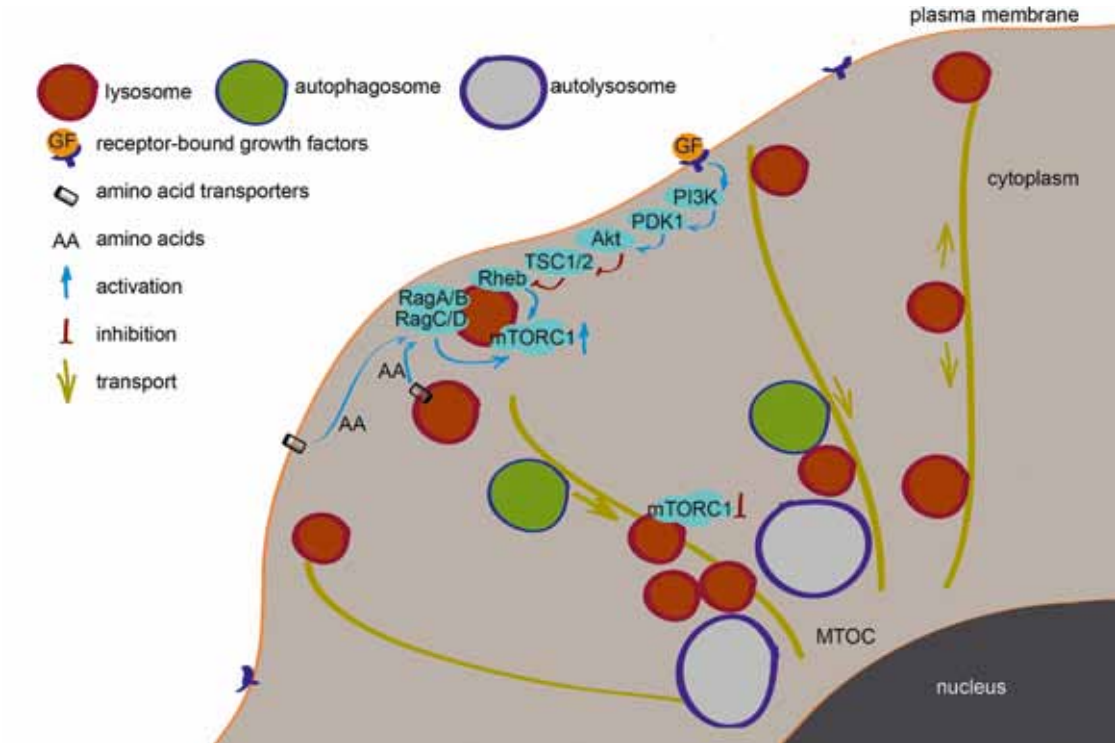

Figure 2. mTOR is a chief signalling pathway suppressing autophagy in the presence of nutrients. mTORC1 is activated on the cytoplasmic surface of lysosomes by signals from growth factors and amino acids. Activation of mTORC1 is facilitated by the transport of mTORC1-positive lysosomes towards the plasma membrane, where signal transduction cascades originate. Instead, transport of lysosomes towards the MTOC during periods of starvation facilitates autophagosome–lysosome fusion events. PDK1, phosphoinositide-dependent kinase 1.

enhanced green fluorescent protein were found to be degraded by autophagy (in addition to the UPS, which was the only previously known catabolic pathway involved)[3]. This provided the first hint that autophagy may be a new pathway that could be exploited in the treatment of proteinopathies.

Since that initial discovery, it has become clear that most other intracytoplasmic pathological neurodegenerative disease-related proteins, including tau (the cause of tauopathies such as AD and frontotemporal dementia), mutant forms of α-synuclein (which cause forms of PD), several ataxin proteins (mutated in different spinocerebellar ataxias), and polyglutamine-expanded androgen receptor (Kennedy's disease) are autophagy substrates in tissue culture models[4]. At the same time, the UPS was found to be less equipped for the degradation of aggregation-prone proteins once they have started to oligomerise. Part of the reason for this is thought to be the inability of bulkier substrates, such as oligomeric proteins, to access the narrow opening of the proteasome. The proteasome also appears to be unable to cleave between successive residues in a polyglutamine tract, which may result in the release of naked stretches of polyglutamine, which have even higher toxicity compared with the original protein[4].

Interestingly, in many cases, only the mutant, but not wild-type, forms of disease-related proteins have a strong dependence on autophagy for their degradation. This is the case, for example, with wild-type huntingtin and ataxin-3, which are degraded efficiently by alternative pathways, such as the UPS[5]. This suggests a degree of selectivity within the autophagic pathway towards certain substrates, where various modifications, such as protein oligomerization, ubiquitylation or acetylation, have been proposed to enhance their incorporation into autophagosomes. Similarly, autophagy is capable of degrading entire organelles such as mitochondria (a process termed mitophagy) discriminating against 'healthy' organelles and preferentially degrading the ones with impaired membrane potential. This feature of mitophagy is of particular relevance to neurodegenerative diseases where cytotoxicity is often thought to be caused by reactive oxygen species (ROS) generated by faulty mitochondria. This link is strengthened further by the findings that certain proteins mutated in forms of PD, such as Parkin and Pink1, or proteins accumulating in

neurons in various neurodegenerative diseases, such as p62, have been implicated in mediating selective recruitment of mitochondria to autophagosomes[6]. Although the research into the mechanisms of selectivity within the pathway is still in its infancy, it may provide potential points of application to further stimulate selective degradation of pathological substrates in various diseases.

Drugs such as rapamycin or lithium that stimulate autophagy and induce degradation of pathogenic proteins were tested in *in vivo* models of neurodegenerative diseases, including fruitflies, zebrafish and mice. Importantly, these drugs increased degradation of various pathogenic proteins (such as mutant huntingtin and the protein causing spinocerebellar ataxia type 3), which correlated with an improvement of pathological, neurological and behavioural markers of the diseases, suggesting that enhanced degradation of the pathogenic protein is a promising disease-treatment strategy. One potential problem, however, was that drugs such as rapamycin have side-effects in humans, which may reduce compliance, especially if one plans to give drugs to healthy individuals to delay disease onset. This desire to identify 'cleaner' or safer drugs has been one of the driving forces behind the intensive studies aiming to elucidate the signalling pathways regulating autophagy.

TOR controls self-eating

Rapamycin activates autophagy by inhibiting the activity of a large protein kinase TOR (target of rapamycin) as part of a multisubunit complex that, in mammals, is called mTOR complex (mTORC) 1. (Another complex, mTORC2, is not subject to direct rapamycin inhibition, its role in autophagy is less clear and therefore it is not discussed further.) mTORC1 is positioned at the bottom of the PI3K/Akt (also known as protein kinase B) signalling axis that receives signals from different inputs, including growth factor receptors at the plasma membrane (Figure 2). In addition, mTORC1 integrates other upstream signals, such as the presence of amino acids, glucose, metabolites and intracellular energy status (detected by AMP-dependent protein kinase, AMPK)[7]. The PI3K/Akt/mTORC1 pathway converts all of these signals into downstream messages to promote cell growth and proliferation. This is achieved by activation of anabolic processes and by suppression of autophagy. TOR inhibits autophagy by phosphorylating Atg1 and Atg13, leading to inactivation of their kinase activities and inhibition of the Atg1–Atg13–FIP200 autophagosome initiation complex[8]. Several key processes regulating mTORC1 signalling are described briefly next.

Regulation by growth factors

Being at the heart of the nutrient sensing system, TOR is subject to multiple layers of control, many of which were discovered only recently, and the whole area remains under intense scientific investigation[7]. The signals from growth factors are transmitted via their respective receptors on the plasma membrane, leading to activation of PI3K, producing phosphatidylinositol 3,4,5-trisphosphate on the cytoplasmic side of the membrane. Activated in the presence of this lipid, phosphoinositide-dependent kinase 1 (PDK1) then phosphorylates Akt, which phosphorylates and inhibits tuberin, a component of heterodimeric tuberous sclerosis complex (TSC1/2). The function of TSC1/2 is to inhibit the small GTPase Rheb, a GTPase-activating protein (GAP). In the absence of active TSC1/2, Rheb is free to interact with and to activate mTORC1[7,9].

Regulation by amino acids

mTORC1 activity responds to alterations in amino acid levels, and, although some components of this pathway were described recently, the process is still poorly understood. Several amino-acid-sensing pathways have been proposed, the best-described process involving activation of mTORC1 by Rag GTPases[10,11]. The heterodimeric Rag complex resides on the surface of late endocytic vesicles (late endosomes and lysosomes) via its interaction with the multimeric Ragulator complex. The presence of amino acids affects nucleotide loading of the Rag complex, resulting in its interaction with regulatory associated protein of mTOR (raptor), a component of the mTORC1 complex. This enables recruitment of mTORC1 to the lysosomal membrane where it becomes activated by lysosome-resident Rheb. As Rheb is also involved in activation of mTORC1 by growth factor signalling, growth factor sensing requires amino-acid-mediated lysosomal recruitment of mTORC1. Several components of this signalling pathway are GTPases, which are potentially druggable, therefore making this process an attractive target for future drug interventions.[9–11]

Regulation by intracellular pH

We recently uncovered yet another layer of control over mTORC1 activity[12] (Figure 2). In cells exposed to moderate starvation, lysosomes, together with their associated mTORC1, relocate from being close to the plasma membrane to the perinuclear microtubule-organizing centre (MTOC) area. These changes in

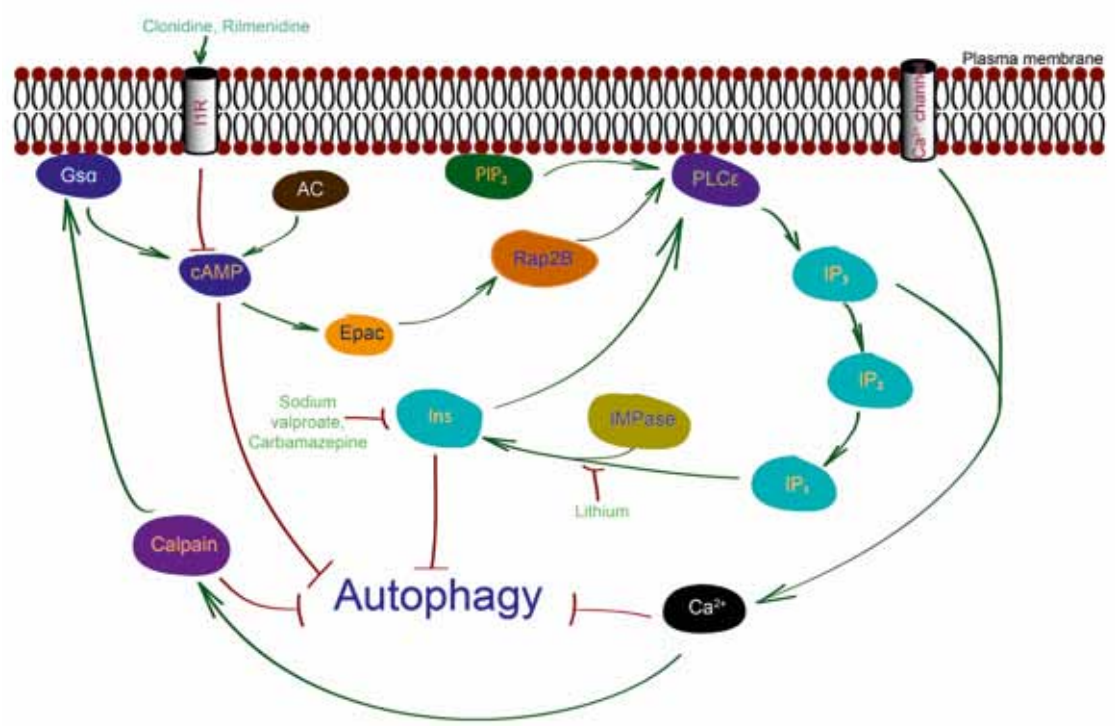

Figure 3. mTOR-independent signalling events regulating autophagy. Some of the drugs found to activate autophagy by interfering with this pathway are shown in green. Gsα, α-subunit of heterotrimeric G-proteins; I1R, imidazoline receptor I(1)R; IMPase, inositol monophosphatase; Ins, inositol; IP_1, inositol 1-phosphate; IP_2, inositol 1,4-bisphosphate; IP_3, inositol 1,4,5-trisphosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate.

nutrient-dependent repositioning regulate mTORC1 activity as well as autophagy: bringing lysosomal mTORC1 close to the plasma membrane increases the exposure of mTORC1 to its upstream signals when nutrients are replenished, whereas retrieving lysosomes to the MTOC facilitates autophagosome–lysosome fusion, providing acceptor sites for autophagosomes, which are transported towards the perinuclear area. The changes in lysosomal positioning in response to nutrients are mediated by intracellular pH, which we found to be increased during starvation. This potentially allows regulation of mTORC1 activity independently of nutrients and therefore offers an opportunity to mimic the effect of dietary restriction through pharmaceutical means. Indeed, mTORC1 activity can be inhibited by an increase in intracellular pH even in the presence of nutrients[12].

Other signals to eat yourself

Several additional pathways controlling autophagy independently of TOR have been identified in recent years. The first example of such regulation comes from an observation that inositol and inositol 1,4,5-trisphosphate inhibit autophagy independent of TOR activity[13]. Inositol 1,4,5-trisphosphate is generated by phospholipase C (PLC) from phosphatidylinositol 4,5-bisphosphate, which can be dephosphorylated in a stepwise fashion into free inositol. The autophagy-inducing effect of lithium was attributed to its ability to inhibit phosphomonoesterase enzymes involved in the latter stage of this inositol phosphate metabolic pathway, resulting in depletion of the inositol pool. Several other drugs in clinical use, such as carbamazepine and valproate, are also capable of activating autophagy by interfering with this pathway.

More recently, the metabolism of inositol phosphates has been incorporated into a larger cyclic signalling pathway regulating autophagy[5] (Figure 3). It has been shown that the effect of inositol 1,4,5-trisphosphate on autophagy is mediated by cytosolic Ca^{2+} released from intra- and extra-cellular stores by activation of inositol 1,4,5-trisphosphate-dependent Ca^{2+} receptors on the endoplasmic reticulum. Elevated levels of Ca^{2+}activate cytoplasmic proteases of the calpain family. These cleave the α-subunit of heterotrimeric G-proteins ($G_s\alpha$), which increases the activity of these proteins and, in turn,

stimulates adenylate cyclase (AC). cAMP generated by AC inhibits autophagy by initiating a cascade of signalling events. Here, stimulated by cAMP, the guanine-nucleotide-exchange factor exchange protein directly activated by cAMP (Epac) activates a small GTPase Rap2B, which stimulates PLC, which finally completes the potential cycle by producing inositol 1,4,5-trisphosphate. An exciting outcome of this pathway is that it provides an entirely new set of drug targets that can be used to regulate autophagy. Many such drugs are already available and approved for long-term use in humans. Several of these, including the imidazoline-1 receptor agonists rilmenidine and clonidine, have been tested in animal models of HD and are awaiting use in clinical trials[5,14].

Perspectives

Until recently, the only known way to activate autophagy was by reducing nutrients (dietary restriction), interfering with their uptake or by direct inhibition of nutrient signalling pathway by drugs, such as rapamycin. The recent identification of entirely new cellular mechanisms regulating autophagy is very exciting as these may provide safer, and possibly more specific, up-regulation. Such findings are of importance as they may offer new ways to control age-related diseases and, potentially, even the process of aging itself. This, however, should not preclude further studies of autophagy-activating signalling events, as it is likely that only the very first clues have been uncovered, with many more layers of complexity still awaiting our attention. ■

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