

# Autophagy response: manipulating the mTOR-controlled machinery by amino acids and pathogens

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**Abstract** Macroautophagy is a self-degradative process that normally maintains cellular homeostasis via a lysosomal pathway. It is induced by different stress signals, including nutrients and growth factors' restriction as well as pathogen invasions. These stimuli are modulated by the serine/threonine protein kinase mammalian target of rapamycin (mTOR) which control not only autophagy but also protein translation and gene expression. This review focuses on the important role of mTOR as a master regulator of cell growth and the autophagy pathway. Here, we have discussed the role of intracellular amino acid availability and intracellular pH in the redistribution of autophagic structures, which may contribute to mammalian target of rapamycin complex 1 (mTORC1) activity regulation. We have also discussed that mTORC1 complex and components of the autophagy machinery are localized at the lysosomal surface, representing a fascinating mechanism to control the metabolism, cellular clearance and also to restrain invading intracellular pathogens.

**Keywords** Autophagy · LC3 · Pathogens · Amino acids · mTOR

## Introduction

Autophagy is an essential cell process in charge of degrading mainly intracellular components and also materials

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incorporated from the extracellular environment such as pathogenic microorganisms (Boya et al. 2013). This process has been involved in both physiological and pathological cell biology activities such as metabolic balance, development, aging, neurodegeneration, cancer, infection and immunity, among others (Mehrpour et al. 2010). One of the major autophagic stimuli is nutrients' restriction which rapidly activates autophagy with the concomitant inhibition of protein synthesis (Boya et al. 2013). When amino acid availability is scarce, an evolutionary conserved protein complex mTORC1 (mammalian target of rapamycin complex 1) is modulated and an intricate signaling network is generated to control not only autophagy but also protein translation and gene expression (see below). Thus, mTORC1 functions as a master controller that integrates several inputs to balance both anabolic and catabolic cellular mechanisms. Indeed, when the amino acid supply is not sufficiently maintained by amino acid transporters, mTORC1 signaling switch off leads to autophagy activation causing the breakdown of internal protein reservoirs to keep appropriate cellular amino acid levels. It has been shown that one of the master regulators of mTORC1 is the amino acid leucine (reviewed by Suryawan and Davis 2011) which also represses proteosomal degradation (Nakashima et al. 2005). Thus, the two major intracellular degradation processes are shut down.

In this review, current knowledge in amino acid signaling and the regulation of autophagy is discussed, with particular emphasis on the role of mTORC1 which is not only regulated by physiological conditions such as the extracellular amino acid supply but also by invading intracellular pathogens.

## mTORC1, a master regulator of key cellular functions

The process of autophagosome formation involves: I) deformation of a membrane to form the phagophore, II)

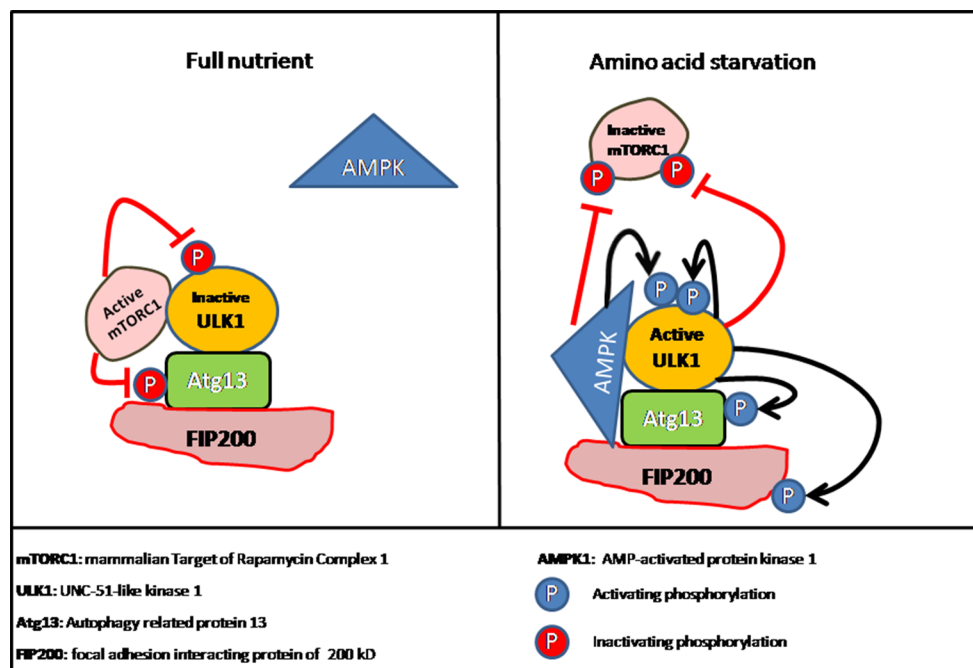
elongation, III) fusion of the extremes to generate a double membrane vesicle (i.e., autophagosome) and IV) maturation of this compartment through interactions with late endosomes and lysosomes (Hamasaki and Yoshimori 2010; Weidberg et al. 2011). Several of these steps depend on or are tightly regulated by a group of proteins called ATG (autophagy-related proteins). Up today, at least 30 ATG proteins have been characterized in yeast (Suzuki et al. 2007) and most of them have a homolog or ortholog in mammalian cell (Longatti and Tooze 2009; Xie and Klionsky 2007). The essential ATG proteins usually are grouped into six functional categories (Mizushima et al. 2011). First, a yeast ATG1 homolog UNC-51-like kinase 1 (ULK1) kinase complex comprised of ULK1, FIP200 (also known as RB1CC1), ATG13L, and ATG101; Second, the VPS34 lipid kinase complex (a class III phosphatidylinositol (PtdIns) 3-kinase) comprised of VPS34, VPS15, Beclin-1 (BECN1), and ATG14 or UVRAG (which bind BECN1 mutually exclusively); third, phosphatidyl inositol 3 phosphate (PtdIns(3)P)-binding proteins including WD-repeat-interacting phosphoinositide proteins (WIPI) and zinc finger FYVE domain-containing protein 1 (also known as DFCP1); fourth, the ATG5-12 ubiquitin-like conjugation system comprised of ATG12-ATG5-ATG16L; fifth, the microtubule-associated protein 1-light chain 3 (LC3) phosphatidylethanolamine conjugation system (in which phosphatidylethanolamine is conjugated to LC3 by the ATG12-ATG5-ATG16L complex); and sixth, ATG9a (a multi-spanning transmembrane protein), the only transmembrane protein among the ATG proteins. The transmembrane protein vacuole membrane protein 1 (VMP1), which is not an ATG protein but is required for autophagy in mammals, is considered part of the last group (Mizushima et al. 2011).

In the regulation of autophagy, the key complex is ULK1, which converges different signals through AMPK that senses the energy status and mTORC1 that senses amino acid availability. mTOR is an evolutionarily conserved serine–threonine kinase that belongs to the superfamily of phosphatidylinositol-3 kinase-related kinases (PI3 KK). In mammals, TOR forms two different multiprotein complexes, mTORC1 and mTORC2, which have different protein partners and also diverse kinase specificity (Zoncu et al. 2011b). The catalytic mTOR subunit DEPTOR, Tti1–Tel2 complex and mLST8 are common in both mTOR complexes. In contrast, the regulatory-associated protein of mTOR (also called Raptor), DEP domain-containing mTOR-interacting protein (Deptor) and mTOR-associated protein LST8 homolog (also known as GβL) are specific to mTORC1. On the other hand, the mTORC2 is associated with Sin1, GβL, Deptor and RAPTOR-independent companion of mTOR (called Rictor) (Betz and Hall 2013). mTORC1 is able to regulate numerous cellular

processes, such as cell growth, proliferation, cell cycle, and autophagy.

The ULK1 complex in mammals is formed by ULK1/2, FIP200, ATG13L and ATG101 and in contrast to ATG1 complex in yeast, the ULK1 complex is constitutively assembled (Wu et al. 2014), being regulated by differential phosphorylation. mTORC1 regulates this complex through inhibition of kinase activity. In nutrient-rich conditions, mTORC1 is associated with the ULK1-ATG13-FIP200 complex and phosphorylates ULK1 and ATG13 suppressing the kinase activity of the ULK1 complex (Hara and Mizushima 2009). Interestingly, this phosphorylation in ULK1 prevents the association of AMPK1, being a point of contact between the regulation from amino acids and glucose pathways (Ganley et al. 2009; Hosokawa et al. 2009; Jung et al. 2009). During the starvation stimuli, mTORC1 is released from the complex and ULK1 and ATG13 are dephosphorylated and, as a consequence, the ULK1 kinase activity is activated. When ULK1 is active, it undergoes an autophosphorylation event and also phosphorylates ATG13L and FIP200, which, in turn, these proteins enhance the kinase activity of ULK1 (Kim et al. 2011). An interestingly data that enhance the complexity of the regulation of the pathway is that the active ULK1 complex may directly phosphorylate mTORC1 keeping this complex inactive (Ganley et al. 2009; Jung et al. 2009). Another point of regulation is at the level of the small GTPase RhoA, a molecule that regulates actin dynamics at ATG14L nucleation sites (Chang et al. 2009; Jung et al. 2011; Kao Tseng-Ting et al. 2010). Recently, it has been demonstrated that RhoA could modulate mTORC1 activity through repression of TSC2 phosphorylation (Aguilera et al. 2012).

One of the first events in the process of autophagosome formation is the recruitment of the ULK1 complex and it is known that this recruitment is necessary for the subsequent recruitment and activation of the VPS34 lipid kinase complex (Gordon et al. 2014). Mammalian cells possess two types of PI3 Ks that regulate autophagy, class I and class III. The class III complexes are constituted by VPS34, VPS15, and BECN1 (Itakura and Mizushima 2010). These core components are part of at least three different complexes: the ATG14L-containing complex (Backer 2008) that participates in autophagy and is regulated by binding of BECN1 to AMBRA 1 or BCL2 (Itakura et al. 2008; Sun et al. 2008), a second complex that participates both in autophagy and endocytosis in which ATG14L and AMBRA 1 are replaced by UVRAG (Salminen et al. 2013) and its positive regulator SH3GLB1, and a third complex where SH3GLB1 is replaced by KIAA0226/Rubicon, which inhibits UVRAG; this third complex is believed to negatively regulate autophagy (Itakura et al. 2008).



**Fig. 1** The complexity of ULK1 complex regulation. In a full nutrient condition, active mTORC1 binds to the ULK1 complex leading to its inactivation by phosphorylation of both ULK1 and ATG13 subunits. In addition, mTORC1 binding prevents AMPK interaction with the ULK1 complex. When cells are under starvation condition, mTORC1 is inactivated and released from the ULK1 complex. ULK1 and ATG13 are dephosphorylated and, as a consequence, the

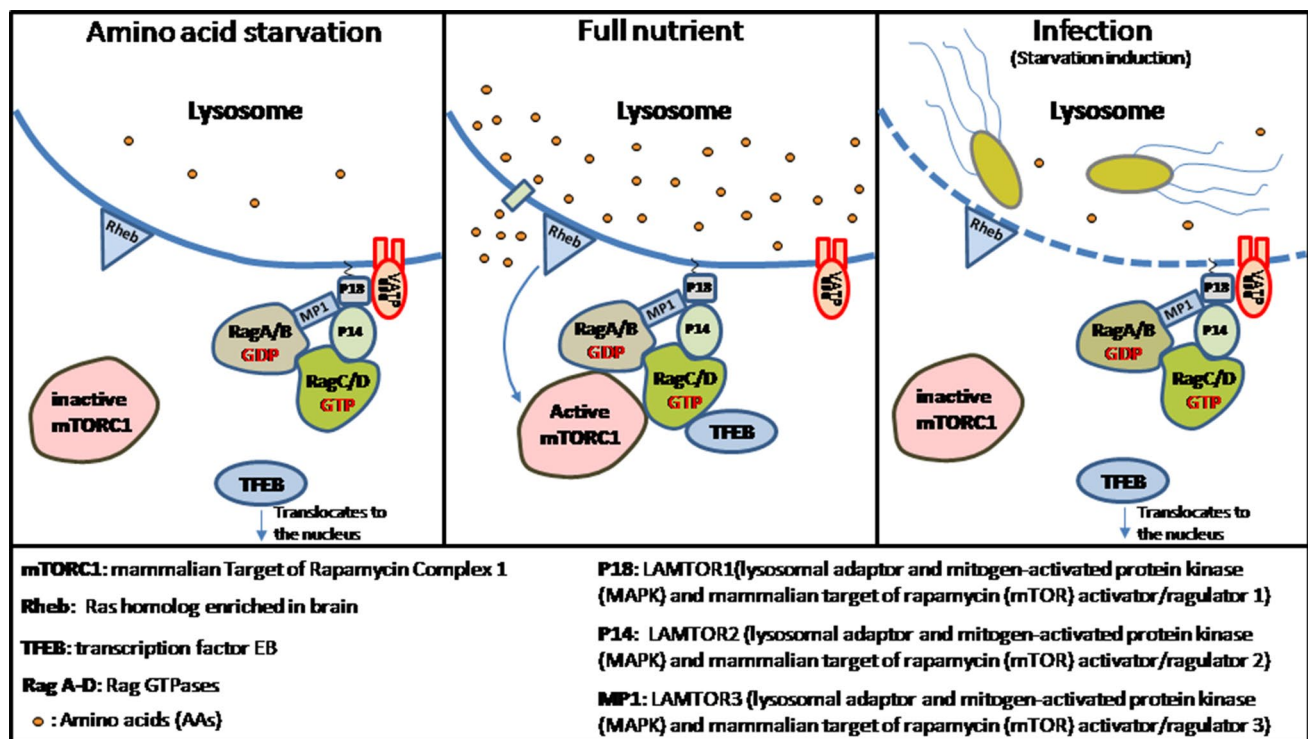
ULK1 kinase activity is turned on. The dephosphorylation also allows the interaction of AMPK with the complex, leading to a phosphorylation that helps with ULK1 activation. Active ULK1 undergoes an autophosphorylation event phosphorylating also ATG13L and FIP200. In nutrient deprivation, both ULK1 and AMPK1 contribute to keep mTORC1 in an inactive state

It has been shown that activated ULK1 phosphorylates BECN1 in Ser 14, which enhances the activity of the BECN1-VPS34-ATG14L complex (Matsunaga et al. 2009; Zhong et al. 2009), increasing PtdIns(3)P production at the phagophore. ATG14L was proposed to act as an adaptor in BECN1 binding to ULK. Interestingly, the ability of ATG14L to promote BECN1 phosphorylation was abolished in mutants that could not localize to the phagophore, indicating that the activation of VPS34 lipid kinase complex may occur specifically at the phagophore formation site (Russell et al. 2013). The generated PtdIns(3)P is subsequently bound by different effectors that are necessary for the progression of autophagosome formation like WIPI and DFCP1 (Russell et al. 2014). Recently, it has been observed that ATG13 could bind PtdIns(3)P, generating a positive loop where ULK1 complex activates the PI3 K complex generating PtdIns(3)P which, in turn, stabilizes the ULK1 complex enhancing the response (Hamasaki and Yoshimori 2010). Interestingly, in nutrient-rich conditions, mTORC1 also regulates the VPS34 kinase complex by direct phosphorylation of the ATG14L subunits and inhibits the lipid kinase activity. It has been shown that overexpression of an ATG14L mutant resistant to mTORC1 phosphorylation is

sufficient to activate autophagy in nutrient-rich conditions (Karanasios et al. 2013).

The evident complexity of autophagy regulation needs plasticity of the pathway to respond to the different stimulus that requires autophagy activation using the same molecular machinery. One example is the role of autophagy to protect cells in a situation of oxidative stress (Tang et al. 2013). In cells that have dysfunctional mitochondria the level of ATP decreases and an elevated amount of ROS is produced (Wu et al. 2014). The low ATP levels activate AMPK stimulating autophagy, whereas the high levels of ROS regulate the pathway directly by oxidation of Atg4 (Scherz-Shouval et al. 2007) and indirectly through the redox sensor HMBG1. In turn, HMBG1 releases Beclin1 from the complex Bcl2-Beclin1 (Tang et al. 2010) and stimulates the activity of the Vps34 lipid kinase complex, a key complex in the initiation of the autophagy.

Based on all the data described, it is evident that the regulation of autophagy activation comprises a high complexity (Figs. 1, 2) due to a crosstalk of several pathways, and further studies are necessary to fully understand the regulation of this pathway.



**Fig. 2** Rags and the Ragulator complex are required to activate mTORC1 at the surface of acidic intracellular compartments. Ragulator (LAMTOR 1-3)-Rag complex and the v-ATPase (vacuolar H<sup>+</sup>-ATPase) are associated with the lysosomal surface. Amino acids (AAs) regulate both the recruitment and activation of mTORC1 to the lysosomal membrane. In the presence of a low concentration of AAs, v-ATPase-Ragulator-Rag complex is unable to recruit mTORC1, which maintains a cytosolic distribution. In contrast, AAs transported

outside the lysosome are sensed by the v-ATPase-Ragulator complex, which finally switches the Rag nucleotide state generating the active form of the protein. Activated Rag recruits mTORC1 to the lysosomal surface whereas Rheb stimulates the kinase activity of mTORC1. Some pathogens are capable to generate a starvation-like condition due to the piercing of the lysosomal membrane, leading to the inactivation of mTORC1

### mTOR Localization at membrane surfaces

#### Intracellular machinery for amino acid sensing

Diverse signals regulate the mTORC1 pathway, including insulin, hypoxia, mitochondrial function, glucose and amino acid availability. Several of these signals are integrated by the Tuberous Sclerosis Complex (TSC1-TSC2) tumor suppressor, which acts as an important regulator of mTORC1 function. TSC1-TSC2 is a GTPase-activating protein (GAP) for Rheb, a small guanosine triphosphate (GTP)-binding protein that interacts with mTORC1 and promotes its kinase activity (Yuan et al. 2013). In contrast, proline-rich Akt substrate 40 kDa (PRAS40), which is phosphorylated by AKT, induces the mTORC1 inhibition. PRAS40 is able to bind to Raptor preventing mTOR activation by Rheb (Manning and Cantley 2007).

Rag GTPases belong to the Ras superfamily of proteins and are represented by four small GTPases named Rag A-D. Based on homology analysis, RagA/B and RagC/D are homologous to the Gtr1p and Gtr2p proteins of *S.*

*cerevisiae*, respectively. The Rag proteins function as heterodimers in which the active complex consists of GTP-bound RagA or B complexed with GDP-bound Rag C or D (Fonseca et al. 2007; Oshiro et al. 2007; Sancak et al. 2007; Vander Haar et al. 2007). Studies using GTP-bound or GDP-bound Rag mutants have demonstrated that the binding of Rag GTPases to mTORC1 is regulated by amino acids and they control the nucleotide state of the Rag (Gao and Kaiser 2006; Sekiguchi et al. 2001). The presence of amino acids induces generation of GTP-RagA/B, leading to raptor binding and the assembly of an activated mTORC1 complex (Kim et al. 2008; Sancak et al. 2008). On the other hand, the inactive conformation of Rags (GDP-bound RagA/B and GTP-bound RagC/D) is observed in the absence of amino acids and as a consequence mTORC1 is inactivated. Consistent with these data, co-purification assays have demonstrated that Rictor did not interact with any Rag heterodimer. Unlike Rheb, the kinase activity of mTORC1 is not directly stimulated by Rag proteins (Sancak et al. 2008). Interestingly, the small GTPase Rags are able to control the subcellular localization of mTORC1.



Several groups have demonstrated that the intracellular localization of mTORC1 changes dramatically in an amino acid-dependent manner. It has been reported that during starvation, mTORC1 acquires a diffused intracellular distribution whereas in the presence of amino acids an intracellular puncta pattern is observed. This distribution change has also been reported from different groups by the overexpression of Rag-GTP, Rag-GDP mutants or Rag knock-down (Sancak et al. 2008). Therefore, these results suggest that it is possible to reproduce the activity of mTORC1 in the presence or absence of amino acids when the proper Rag mutants are overexpressed.

Rags and mTOR proteins are harbored at the lysosome surface

Several studies have demonstrated that mTORC1 localizes at the lysosome and late endosome membranes in the presence of amino acid or active Rags (Flinn et al. 2010; Kalender et al. 2010; Korolchuk et al. 2011; Narita et al. 2011; Yoon et al. 2011). This is consistent with the fact that mTORC1 has been localized at the perinuclear region and colocalizes with Rab7 (Sancak et al. 2010a; Zoncu et al. 2011a). Likewise, mTORC1 has been also detected in Rab7-positive compartments in cells overexpressing the active form of Rags, even in the absence of amino acids.

As indicated above, mTORC1 is activated by the small GTPase Rheb in response to amino acid stimulation, allowing the recruitment on mTORC1 to the lysosome surface (Sancak et al. 2008). This mTORC1 translocation requires Rag GTPases and Ragulator (a pentameric protein complex that contain HBXIP, MP, p14, p18 and C7orf59) (Saucedo et al. 2003; Stocker et al. 2003). Therefore, amino acids stimulate the active form of RagsA/B, inducing the binding to Raptor and its association to activated mTORC1 complex (Bar-Peled et al. 2012; Sancak et al. 2008, 2010a). It is important to note that conditions which inhibit late endosome formation or deflect the Regulator complex to a different intracellular compartment alter mTORC1 activation (Sancak et al. 2008), indicating lysosome or late endosome surface is necessary for mTOR activation.

Genetic screening assays in flies have identified members of the proton-assisted amino acid transporter (PAT) family, which might function as regulators of TORC in *Drosophila* (Efeyan et al. 2012; Flinn et al. 2010; Sancak et al. 2010a). Later studies have indicated that PAT plays an important role as an amino acid sensor protein at the lysosomal surface (Goberdhan et al. 2005). This suggests that PATs not only act as transporters, but also are part of the “amino acid-sensing system” for mTORC1 localized at the lysosomal membrane.

In a very recent paper, it has been proposed a new role for Rags in controlling activation of transcription factor

EB (TFEB), a master regulator of autophagic and lysosomal gene expression. Interaction of TFEB with Rag heterodimers induces the recruitment of the transcription factor EB to the lysosome in an amino acid-dependent manner. Therefore, the retention of TFEB promotes the repression of genes involved both in autophagy and lysosomal functions (Goberdhan 2010). Moreover, some authors have postulated that the tumor suppressor protein, folliculin (FNCL), is recruited to the cytoplasmic surface of lysosomes when amino acids are depleted. Interestingly, folliculin is required for the recruitment of mTORC1 to the lysosomes, a process stimulated by amino acids in a Rag-dependent fashion. It has been recently demonstrated that folliculin has a critical role in this process via its direct interaction with the GTPase RagA. Indeed, folliculin preferentially interacts with inactive RagA/B mutants and this interaction is through the GTPase domain. Depletion of folliculin hampers the recruitment of mTORC1 to the lysosomal membrane and also alters the lysosomal pH. In addition, folliculin is required to sequester the transcription factor EB (TFEB) from the nucleus, a key regulator of lysosome biogenesis (Martina and Puertollano 2013). Thus, folliculin is a critical molecule that modulates lysosomal function in response to amino acid availability.

Sensing amino acids at the lysosomal compartment

Lysosomes are dynamic organelles and their localization within the cell has frequently been noticed to respond to a variety of treatments. Previous reports have shown that cells incubated in starvation conditions present an increased proportion of perinuclear lysosomes, suggesting that the change in lysosomal positioning could have an impact due to the stimulation of the autophagic flux (Sardiello et al. 2009). Interestingly, Dr. Rubinsztein and collaborators have speculated that intracellular pH and lysosomal movement affect the binding of KIF2 protein to lysosomes or microtubules, modifying the levels of this protein in lysosomal fractions (Korolchuk et al. 2011). Furthermore, recent results from our laboratory have indicated that a population of ATP-loaded autophagic vacuoles, but not autolysosome, is redistributed to the cell periphery under starvation conditions (Korolchuk et al. 2011). In addition, we have also demonstrated that the motor protein KIF5 is necessary for the transport of these autophagic vacuoles to the cell edge. These give an important role for nutrient levels and the intracellular pH in the autophagic vacuoles redistribution, which may contribute to mTORC1 activity regulation.

As mentioned before, the small GTPase Rag is localized to the lysosome surface, but this protein lacks lipid motifs necessary for membrane anchoring. Thus, Rag proteins are unable to bind to the lysosomal surface when Ragulator is genetically deleted, losing its function and acquiring a

cytosolic distribution (Fader et al. 2012). This indicates that in response to amino acids, Ragulator is a crucial complex that recruits mTORC1 to the lysosome. Some authors have proposed that the “starting point” for the kinase activity of mTORC1 is due to the Rag–Regulator complex which allows the amino acid-dependent binding of mTORC1 to Rheb (Sancak et al. 2010a). This is in agreement with the fact that in mammalian cells the small GTPase Rheb is observed in lysosomal membrane (Efeyan et al. 2012). During starvation, the autophagic pathway concludes when the intracellular molecules trapped within the autophagosome are degraded in the lysosome. This process generates new amino acids which recycle back to the cytoplasm, leading to the recruitment of mTORC1 to the surface of autophagolysosomes and reactivation of mTORC1 (Buerger et al. 2006; Saito et al. 2005; Sancak et al. 2010a). Interestingly, this observation suggests that the lysosome is not the end point but the beginning of amino acid mTORC1 signaling (Yu et al. 2010). An interesting assay was performed using a cell-free system to determine the role of the lysosomes as an amino acid sensor. In this experiment, incubation of purified lysosomes with amino acids and purified mTORC1 was enough to catalyze the mTORC1 binding to the lysosome. This indicates that the lysosome itself is the amino acid sensor compartment which activates the small GTPase Rag (Efeyan et al. 2012). Some authors have suggested that amino acids are sensed within the lysosomal lumen, leading to Rag activation. Interestingly, Rag GTPases also bind to the lysosomal vacuolar-type H<sup>+</sup>-ATPase which is a way for Rags to sense the amino acid within the lysosome (Zoncu et al. 2011b). It has been proposed that the v-ATPase has an essential role in amino acid signaling to mTOR. Indeed, v-ATPase allows the amino acid transport from the inside to outside the lysosome, generating a proton gradient. This gradient allows sensing the amino acid amount by a direct interaction with Regulator and the Rags (Zoncu et al. 2011b). Recent reports have shown the presence of a mechanism for detecting leucine levels in the cytoplasm (Efeyan et al. 2012). Interestingly, in mammalian cells, leucyl-tRNA synthetase (LRS) was proposed to interact with GTP-bound RagD in a leucine-dependent manner, and as a consequence promoting its conversion to the inactive form, stimulating the pathway (Bonfils et al. 2012; Han et al. 2012).

Regarding autophagy, several reports have demonstrated that mTORC1 has a central relevance when this complex is localized at the lysosomal surface. As mentioned, the active form of mTORC1 hampers phagophore formation via inhibition of the kinase ULK1/ATG1 and its interaction with ATG13 (Han et al. 2012). An interesting assay performed by Kim and collaborators has demonstrated that under starvation, overexpression of the active form of Rags inhibits autophagosome formation whereas overexpression

of inhibitory Rags mutants restores the autophagy pathway (Hosokawa et al. 2009; Kim et al. 2011). The coexistence of both the mTORC1 complex and the autophagy machinery at the surface of the lysosomal compartment shows a new synchronized mechanism for metabolism and cellular clearance.

### Amino acids and pathogens

Cumulative evidence indicates that autophagy is a key degradative process that controls cellular infection caused by intracellular microorganisms. We and others (E. Kim et al. 2008) have previously demonstrated that induction of autophagy acts as a defense mechanism against invading bacterial pathogens, a process known as xenophagy. It is well known that pathogenic mycobacteria, as a survival mechanism, hamper the maturation of the phagosome preventing their acidification and avoiding fusion with the lysosomes (for a review see (Gutierrez et al. 2004; Nakagawa et al. 2004). In our studies, we found that in infected macrophages incubated under full nutrient control conditions, *Mycobacterium bovis* BCG remained in a phagosomal compartment mostly devoid of lysosomal enzymes (Rohde et al. 2007). However, when autophagy was activated by amino acid starvation or by other means (i.e., rapamycin treatment), BCG-containing phagosomes recruited the protein LC3 and colocalized with other autophagy markers as well as with markers of lysosomal compartments (Gutierrez et al. 2004). Thus, autophagy induction overrides the maturation block imposed by *Mycobacterium* leading to pathogen destruction.

Following those initial studies with the attenuated *M. bovis* BCG we studied in detail the interaction between the autophagic pathway and *M. marinum*, a pathogenic mycobacterium that has striking similarities to *M. tuberculosis*. Interestingly, we found that a substantial fraction of *M. marinum*-containing phagosomes actively recruited the autophagic protein LC3, regardless of the nutritional conditions of the culture media (Gutierrez et al. 2004). However, these LC3-positive compartments have features of late endocytic compartments (i.e., Rab7 and LAMP1 labeling) they do not acquire lysosomal enzymes or degradative properties. Therefore, *M. marinum* is able to hamper both autophagosomal and phagosomal maturation, even in cells in which the autophagic pathway has been activated by starvation.

The fact that *M. marinum*-containing phagosomes recruited the autophagic protein LC3 even in metabolically replete cells (i.e., incubated in the presence of amino acids) was a surprising observation. Interestingly, the group of Stephen Girardin found that infection of epithelial cells with *Salmonella* or *Shigella* triggers an autophagic

response as a consequence of an acute amino acid starvation state due to damage of the pathogen-containing compartments (Lerena and Colombo 2011). *Shigella flexneri* is a pathogen that initially damages phagosomal membrane before escaping toward the cytoplasm (Tattoli et al. 2012). On the other hand, *Salmonella enterica* serovar Typhimurium invades cells using a Type III secretion system (TTSS), subsequently residing in a specific self-tailored endosomal compartment called Salmonella containing vacuole (SCV) (Paetzold et al. 2007). The authors demonstrate that in *Shigella*-infected cells, the intracellular amino acid sensor GCN2 was rapidly phosphorylated indicating a rapid starvation signal. GCN2 (general control, non-derepressible 2), one of the four kinases that in mammals phosphorylate the eIF2 $\alpha$ -kinase, together with HRI, PEK and PERK, functions as a nutrient sensor and it is activated by uncharged tRNAs. As all eIF2 $\alpha$ -related protein kinases, GCN2 exerts its function via phosphorylation of a specific amino acid in eIF2 $\alpha$  (Ser-51 in yeast and mammals) (Steele-Mortimer 2008). This phosphorylation causes a global translational arrest and this event has been related to starvation-mediated autophagy (Baird and Wek 2012). Although the molecular mechanisms of autophagy induction via eIF2- $\alpha$  phosphorylation are not fully understood, crosstalk with the mTORC1 pathway has been demonstrated (Tallóczy et al. 2002). This response connection seems to be an archaic defense mechanism, because it is also activated in response to a membrane damage caused by several pore-forming toxins (PTFs) like *S. aureus*  $\alpha$ -toxin, *Vibrio cholerae* cytolysin, streptolysin O and *E. coli* hemolysin (Valbuena et al. 2012). Indeed, activation by membrane damage is due to efflux of potassium, and the loss of this cation impairs the internalization of certain amino acids like leucine, generating an amino acid starvation signal (Kloft et al. 2010). Other bacterial exoproducts are also able to activate autophagy through eIF2- $\alpha$ , such as the case of [(S)-3-oxo-C12-HSL], a quorum-sensing hormone produced by *Pseudomonas aeruginosa*. In contrast to PFTs, (S)-3-oxo-C12-HSL did not cause amino acid starvation and eIF2- $\alpha$  is phosphorylated by PKR instead of GCN2 (Kloft et al. 2010). Interestingly the GCN2-eIF2- $\alpha$  pathway is also involved in immune modulation participating in the innate immune response, T cell differentiation and protection against from autoimmune encephalomyelitis (Von Hoven et al. 2012).

Indeed, *Shigella* leads to a fast and sustained reduction in the levels of the amino acids, leucine and isoleucine, in the cytosol of infected cells. In contrast, in *Salmonella*-infected cells the cytosolic levels of these two amino acids resulted in a progressive normalization after 4 h (Castilho et al. 2014). It has been shown that once the bacteria has damaged the containing compartment or it is free in the cytoplasm, it recruits the protein ubiquitin (Tattoli et al.

2012). This ubiquitin-coated bacteria are recognized by NDP52 (nuclear dot protein 52 kDa), an adaptor protein that binds both ubiquitin and LC3, which in turn, targets this pathogen for autophagy as part of an innate defense mechanism (Randow 2011). Using the recruitment of the autophagy adaptor NDP52 as a marker of vacuolar disruption, a sustained recruitment to endomembranes was observed in *Shigella*-infected cells, whereas in the case of *Salmonella*, NDP52 was only transiently recruited to the SCV and its distribution was normalized a few hours after infection (Thurston et al. 2009). Thus, it is likely that the fast repair of the damaged membranes in *Salmonella*-infected cells accounts for the rapid normalization in amino acid levels.

In coincidence with the results of Girardin and collaborators we demonstrated that *M. marinum* LC3-recruitment to the pathogen-containing compartment depended on bacterial viability and a functional ESX-1 secretion system (Tattoli et al. 2012). This secretion system is encoded by the Region of Difference 1 (RD1), a virulence gene cluster present in *M. marinum* as well as in *M. tuberculosis* but absent in the attenuated strain *M. bovis* BCG (Lerena and Colombo 2011). The ESX-1 secretion system is responsible for the secretion of the proteins CFP-10 and ESAT-6 which has hemolytic properties (Lewis et al. 2003; Pym et al. 2002). ESAT-6 has been specifically pointed out as a factor responsible for the damage of the phagosomal membrane and escape of *M. marinum* toward the cytoplasm (Cosma et al. 2008; Gao et al. 2004; Smith et al. 2008). Thus, *M. marinum* lacking the RD1 locus does not have a functional ESX-1 secretion system and hence, the  $\Delta$ RD1 mutant is not able to escape from its containing phagosome (Smith et al. 2008). We demonstrated that *M. marinum*  $\Delta$ RD1, unlike the wt strain, was unable to recruit LC3 to the phagosomal compartment in cells incubated in a full nutrient condition. In addition, we have also found that Galectin 3 a sensor of damaged membranes is recruited to *M. marinum*-containing phagosomes in an ESX-1 dependent manner (Recalde and Colombo, unpublished results). Thus, our results are in agreement with the idea that membrane piercing acts as an upstream signal that triggers an autophagic response likely by the generation of a starvation state in the infected cells. In summary, Girardin and collaborators uncovered a novel mechanism involving amino acid starvation signaling and mTOR redistribution as key players to modulate the host xenophagy pathway.

On the other hand, *Legionella pneumophila* has been used as a model to study signaling pathways of the innate immune system (Smith et al. 2008). Some publications have presented evidence suggesting that mTOR is involved in immunoregulation (Vance 2010). Recent results from Dr. Craig Roy have demonstrated that macrophage infection by pathogenic *L. pneumophila* resulted in ubiquitination

of PI(3)K, Akt and mTOR, leading to diminished mTOR activity (Ivanov and Roy 2013; Powell et al. 2012). This reduced activity due to Akt-dependent regulation of TSC2, suggests that this response is independent of nutrient sensing (Ivanov and Roy 2013). Moreover, a very recent report has demonstrated that *L. pneumophila* triggers a nutritional remodeling of the host cell, generating an increased intracellular level of amino acids to ensure pathogen proliferation (Smith et al. 2005).

#### Salmonella and Shigella: two pathogens that modulate mTOR recruitment to endomembranes

As described above, mTORC1 activity is tightly controlled by the cellular nutrient availability and is recruited to the surface of endo/lysosomal compartments in response to intracellular amino acid levels. On the other hand, *Salmonella* resides in an endosomal/phagosomal vacuole (SCV) that is remodeled by pathogen-secreted factors to generate its replicative niche, acquiring markers of early and late endosomal compartments. In non-infected cells, mTOR localizes to late endosome/lysosomal compartments labeled with LAMP2 (Bruckert et al. 2014). However, in *Salmonella*-infected cells, during the first 2 h of infection, the endomembrane localization of mTOR was markedly affected changing mostly to a cytosolic disperse distribution. This change was concomitant with a marked reduction in amino acid levels as mentioned above. In contrast, at later infection times, mTORC1 progressively relocated to maturing SCVs (Sancak et al. 2008; Tattoli et al. 2012). Thus, SCVs is a novel bacteria-modified compartment, which is able to recruit mTORC1 complexes. Similarly, *Shigella* also caused the relocation of mTORC1 in infected cells, but in this case, the cytosolic redistribution of the complex was sustained in time.

As indicated, the recruitment of mTOR to lysosomal membranes depends on the binding of Raptor to the Ragulator/Rag GTPases complex (Tattoli et al. 2012); (Sancak et al. 2008, 2010a). In *Shigella*-infected cells, the recruitment of Raptor was hampered and its dispersion was due to a reduced activity of the Rag GTPases (Sancak et al. 2008, 2010b). Likewise, mTORC1 localization to the SCV was Raptor dependent and also required Rag/Ragulator targeting to the SCV. Interestingly, this relocation and activation of mTORC1 on the SCV vacuole seems to contribute to *Salmonella* escape from autophagy.

An interesting finding is that the initial redistribution and inactivation of mTOR signaling in *Salmonella*-infected cells depends on factor(s) secreted by the bacteria itself, encoded by the SPI-1 (pathogenicity island 1). In contrast, the normalization of the amino acid levels and recruitment of mTOR to the SCV at later infection times seems to be independent of bacterial secreted effectors (Tattoli et al.

2012) but it depends on the active transport of amino acids by the host transporter systems. Noteworthy, it appears that amino acids are transported from an intracellular vesicular compartment rather than from the plasma membrane. Further studies are necessary to unequivocally identify the source involved in the replenishment of amino acid levels at later *Salmonella* infection times. In addition, it would be exciting to determine if other pathogens also modulates amino acid availability and mTORC1 activity after infecting the host cells.

#### Autophagy offers lodge and nutrients for intracellular pathogens

In contrast to the situations mentioned above, some bacteria and viruses are able to use autophagic structures as a replicative niche (Deretic and Levine 2009). However, how autophagy contributes to the proliferation of these heterogeneous groups of pathogens remains unknown.

Several pathogens such as *Coxiella burnetii* (Gutierrez et al. 2005), *Anaplasma phagocytophilum* (Niu et al. 2008), *Yersinia pestis* or *pseudotuberculosis* (Moreau et al. 2010; Pujol et al. 2009) and *Staphylococcus aureus* (Mestre et al. 2010; Schnaith et al. 2007) replicate inside the cell assisted by macroautophagy (Mestre et al. 2010; Schnaith et al. 2007; Pujol et al. 2009). Previous data have shown that *Y. pestis* increases its proliferation in conditions that stimulate autophagy whereas these species fail to grow in atg5 <sup>-/-</sup> cells. Similarly, *C. burnetii* is capable to replicate in a LC3-positive vacuole and autophagy induction by starvation or by other means favor this process (Gutierrez et al. 2005). In addition, it has been shown that autophagic proteins such as LC3 and Beclin 1 have an essential role in the formation, maturation and maintenance of the *Coxiella*-replicative vacuole (Gutierrez et al. 2005; Vázquez and Colombo 2010).

Ultra structural analysis of poliovirus-infected cells demonstrated an accumulation of autophagic structures (Dales et al. 1965). Likewise, perinuclear autophagosomes were observed in cells infected with picornavirus where the virus assembles its replication machinery. These data suggest that poliovirus is able to use the autophagy machinery for its own replication. Other works have demonstrated that autophagy is necessary to generate vesicular structures, involved in virus exocytosis (Jackson et al. 2005).

Dengue virus also stimulates autophagy and increases the fatty acids levels, favoring efficient replication (Heaton and Randall 2010). Similarly, Steele and collaborators have demonstrated that *Francisella tularensis* needs autophagy for growing (Steele et al. 2013). Interestingly, this pathogen is able to grow in autophagy-deficient cells incubated with an excess of pyruvate or amino acids. These data suggest that autophagy is necessary to increase the levels of



cytosolic nutrients, favoring bacteria replication (Steele et al. 2013).

Previous studies have suggested that activation of AMPK is beneficial for intracellular pathogens because it stimulates glucose utilization and autophagy, generating energy and nutrients for pathogen growth. This is the case of the parasitic form of *Leishmania* which produces mTOR inactivation and AMPK activation, supporting parasites replication (Jaramillo et al. 2011). On the other hand, studies from our laboratory have demonstrated that autophagy induction favors *Trypanosoma cruzi* invasion, likely by recruiting autophagosomes to the entry site (Romano et al. 2009; Romano et al. 2012). While avoidance of autophagy by several pathogens is observed, clearly other pathogens can manipulate the autophagic pathway to provide intracellular nutrients, supporting pathogens replication.

### Concluding remarks

In this review, we have compiled evidences that shown the role of mTOR as a master regulator of cell growth and autophagy from yeast to human. We have discussed, with particular accent, the role of intracellular amino acid levels and the intracellular pH in the autophagic vacuoles redistribution, which may contribute to mTORC1 activity regulation. The coexistence of both the autophagy machinery and mTORC1 complex at the lysosomal surface displays an interesting mechanism that controls the metabolism, cellular clearance and even the fate of invading intracellular pathogens. As indicated, an important issue is how the levels of amino acids are sensed to transmit specific nutrient availability signals to the master regulator mTOR. Recent information points to leucyl-tRNA synthetase as a novel molecule of this nutritional pathway by directly modulating RagD, a component of the Rag GTPases that bind and modulate mTORC1. It is likely that future research will be devoted to identify other molecules that may participate either directly (i.e., interacting proteins) or indirectly (via a signaling cascade) in the fine tuning of the mTOR pathway.

**Conflict of interest** The authors declare that there are no conflicts of interest.

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