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Review

Regulation of autophagy by phosphatidylinositol 3-phosphate

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ABSTRACT

The simple phosphoinositide phosphatidylinositol 3-phosphate (PI(3)P) has been known to have important functions in endocytic and phagocytic traffic, and to be required for the autophagic pathway. In all of these settings, PI(3)P appears to create platforms that serve to recruit specific effectors for membrane trafficking events. In autophagy, PI(3)P may form the platform for autophagosome biogenesis.

1. Formation and consumption of PI(3)P

Phosphoinositides (PI's) are formed by the phosphorylation of phosphatidylinositol (PI) on its inositol ring. With the exception of the 2′ and 6′ positions all free OH groups of the inositol ring can be phosphorylated. The unique functional role of each type of PI within the cell can be attributed to the arrangement of phosphate groups around the inositol ring. Phosphoinositide 3-kinases (PI3 kinases) are the enzymes responsible for phosphorylating the 3′OH-position of the inositol ring of PI, and three classes of these enzymes exist in cells [1,2] Class I PI3 kinases most commonly phosphorylate PI(4,5)P_2 at its 3′OH group to produce PI(3,4,5)P_2 (often referred to as PIP3). Class III PI3 kinases are the orthologues of the yeast vesicular protein-sorting protein Vps34, and these enzymes can only utilise PI as a substrate (Fig. 1). Class II PI3 Kinase can use PI, PI(4)P and PI(4,5)P_2 as substrates, with a strong preference for PI. It is likely that the type III enzyme is responsible for the majority of PI(3)P synthesis within cells, whereas the type II enzymes appear to be involved in specialised signal-dependent settings [3]. PI(3)P can be further phosphorylated by a 4′-kinase at its 4′-position to generate PI(3,4)P_2 (Fig. 1). Additionally, PI(3)P can be phosphorylated by a PI(3)P 5-kinase known as PIKfyve in mammals (Fab1p in yeast) to form PI(3,5)P_2 [4]. 3′ Phosphatases that can act on PI(3)P include PTEN (phosphatase and tensin homologue) [5], myotubularin proteins such as Jumpy [6,7], and the yeast phosphatase, Sac1p [8].

2. Recognition of PI(3)P by specific protein domains

The generation of PI's by PI3 kinases has been found to be responsible for a diverse array of cellular signalling events, often mediated by distinct proteins that can bind to PI's. Lipid binding can affect the localization, conformation and/or activity of PI-binding proteins, and two types of PI(3)P-binding domains have been reported. One is the FYVE domain (whose name reflects the first four proteins found to contain it: Fab1p,YOTB, Vac1p and Early Endosome Antigen 1) [9,10]. At present 37 FYVE domain containing proteins have been identified in humans. These domains are known to bind to PI(3)P with high affinity (K_d = 50 nM) and they usually consist of approximately 65 amino acids with eight conserved cysteine residues. A hydrophobic loop in the FYVE domain initially interacts with the membrane in a non-specific manner. This leads to the subsequent exposure of a characteristic basic motif [(R/K) (R/K)HHCR] in the FYVE domain, which surrounds the third conserved cysteine residue, and this can bind to the inositol head group of PI(3)P [2,11]. PI(3)P can also bind to the 120 amino acid Phox homology (PX) domains. PX domains have been found in NADPH oxidase subunits, a PI3 kinase, sorting nexins, a SNARE, as well as some phospholipases and protein kinases. PI(3)P binding to PX domains is thought to occur via a pair of highly conserved basic motifs consisting of [RR(Y/F)] [11].

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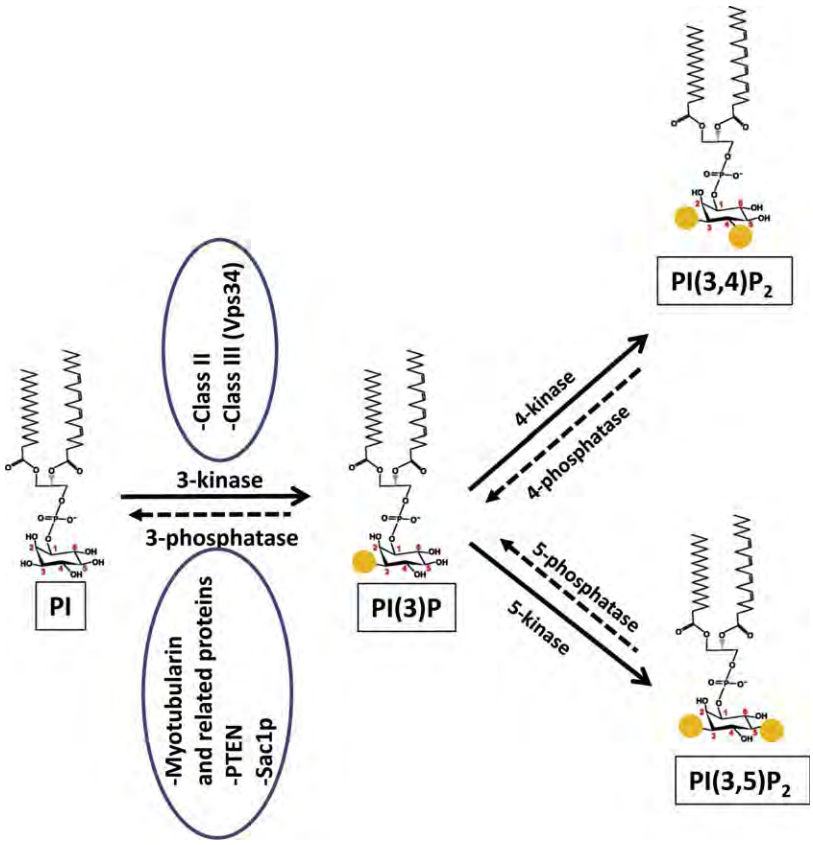

Fig. 1. Pathways for PI(3)P synthesis and conversion into PI, PI(3,4)P_2 and PI(3,5)P_2. PI(3)P can be synthesised from PI by class II and class III PI3-kinases (circled in blue). 3-Phosphatases (circled in blue) can convert PI(3)P back into PI. PI(3)P can also be converted into PI(3,4)P_2 and PI(3,5)P_2 by 4-kinases and 5-kinases, respectively.

3. Localization and role of PI(3)P within endocytic and phagocytic compartments

PI(3)P is maintained at a cellular concentration of approximately 200 μM, and is thought to be produced mainly by Vps34. Genetic and biochemical data have shown that PI(3)P regulates endocytic trafficking, and, in support of this, studies using a double FYVE domain as a probe have found that PI(3)P is enriched on early endosomes, in the internal vesicles of multivesicular endosomes and in yeast vacuoles [12]. Moreover, proteins that bind to PI(3)P are known to play a role in membrane trafficking events, directing traffic from endosomes and Golgi bodies to lysosomes (Fig. 2). Many of these proteins have FYVE domains such as EEA1, which regulates the fusion of endocytic membranes, and is recruited to early endosomes by Rab5-GTP and PI(3)P [13]. EEA1 recruitment to endosomal membranes acts to regulate proper membrane fusion by SNARE's (Soluble *N*-ethylmaleimide-sensitive factor Attachment protein Receptor's) [11]. Specifically, EEA1 has been shown to interact with at least two SNARE proteins, syntaxin6 and syntaxin13 [14]. Another FYVE domain containing protein, Rabenosyn-5, an orthologue of yeast Vac1p, is also required for early endosome fusion, and can bind to both PI(3)P and Rab5-GTP. When bound to PI(3)P Rabenosyn-5 can modulate SNARE complex formation with Rab5 and Sec1 (Vps45) proteins [15]. An adaptor protein SARA (Smad Anchor for Receptor Activation), also contains a FYVE domain which localizes it to endosomes. In endosomes SARA then acts to recruit the transcription factors Smad2 and Smad3 to the transforming growth factor β receptor [11]. Additionally, PX domain containing proteins have also been found to bind to PI(3)P and play a role in early endosomal trafficking events. For example, sorting nexins play a role in trafficking activated growth factor receptors, and sorting nexin 3 is targeted to endosomal PI(3)P pools via its PX domain [16]. The motility of endosomes along microtubules and actin filaments has also been shown to be dependent on both Rab5 and Vps34 (class III PI3 Kinase), and a kinesin motor, KIF16B, which can bind to PI(3)P via a PX domain has been identified [14].

FYVE domain containing proteins have also been found to play a role in directing target proteins to lysosomes via the formation of multivesicular endosomes (Fig. 2). For example, Fab1p, the yeast orthologue of PIKfyve, is a PI(3)P 5-kinase that also contains FYVE domains, and has been suggested to target cell surface receptors for degradation in lysosomes. Hrs (mammalian orthologue of yeast Vps27), contains multiple FYVE domains, and is also known to play a role in endosomal maturation and multivesicular endosome formation [17]. It is thought that Hrs regulates SNARE complex formation, as well as recognising ubiquitylated cargo and selecting it for degradation. Hrs is required for the formation of the ESCRT (endosomal sorting complex required for transport) complexes on endosomal membranes [14]. ESCRT complexes in turn are required for the sorting and recognition of ubiquitylated cargo protein into the internal vesicles of multivesicular endosomes. PX domain containing proteins have also been found to play a role in trafficking target proteins for degradation. For example, the yeast SNARE Vam7 is targeted to the vacuolar membrane via PI(3)P binding to its PX domain [18], here it is known to play a role in trafficking proteins to the vacuole [19].

Retrograde trafficking from endosomes to Golgi is important for the recycling of sorting receptors, such as yeast Vps10, and mammalian mannose-6 phosphate receptor. Two accessory proteins required for Vps34 activity in yeast, Vps30 and Vps38 (see below), have been shown to be essential for endosome to Golgi trafficking

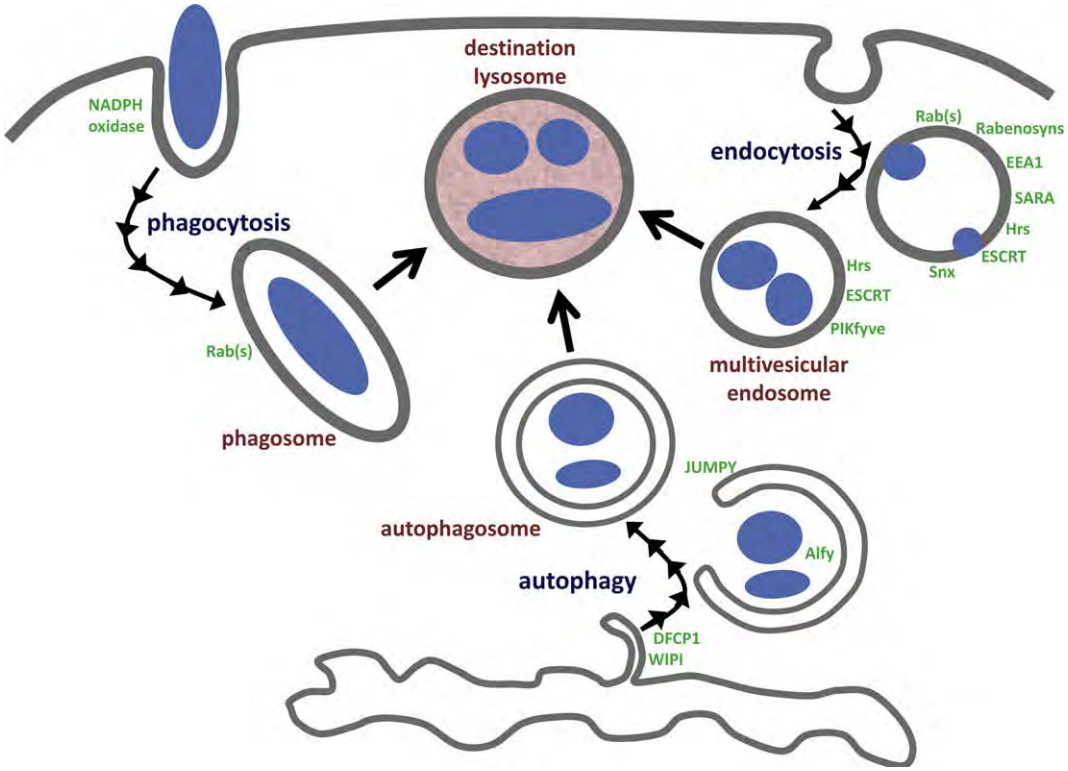

Fig. 2. PI(3)P has been found to play a role in endocytosis, phagocytosis as well as autophagy. FYVE and PX domain containing effector proteins such as EEA1, Rabenosyn-5, sorting nexins, Hrs, and SNARE's are thought to mediate the role played by PI(3)P in endocytosis. The $p40^{phox}$ NADPH oxidase subunit contains a PX domain which can bind membrane PI(3)P, once at the plasma membrane this subunit can then play a role in phagocytosis. PI(3)P is also required for autophagy, possible PI(3)P effectors for autophagy include the WIPI proteins, DFCP1, Alfy and the PI(3)P phosphatase Jumpy.

of Vps10, which suggests a requirement of Vps34 in this trafficking step. Studies in yeast have also identified a multi-protein 'retromer' complex as an important player in the endosome to Golgi trafficking of Vps10. Interestingly, two proteins in this complex, Vps5 and Vps17, both have PX domains and can specifically bind to PI(3)P [14]. The retromer complex is evolutionarily conserved, and has been shown to be involved in the retrograde trafficking of mannose-6 phosphate receptors. The mammalian retromer complex contains Snx1 and Snx2, which can bind to both PI(3)P and $PI(3,5)P_2$ [14].

In addition to its role in endocytosis, PI(3)P is also known to regulate phagocytosis (Fig. 2). Both Rab5 and Vps34 have been shown to be involved in phagosome maturation, and there is evidence that EEA1 and Hrs are the effectors that mediate this process [14]. Phagocytes utilise the NADPH oxidase system to destroy microorganisms by generating reactive oxygen species. Some of these oxidase subunits are cytosolic until an activation event causes them to translocate to the plasma membrane where catalytic subunits reside. Specifically, the $p40^{phox}$ subunit contains a PX domain which can bind membrane PI(3)P [20] whereas the PX domain of $p47^{phox}$ has been found to interact preferentially with $PI(3,4)P_2$, but also with PI3P, $PI(3,5)P_2$ and PIP3 [21].

4. PI(3)P involvement in autophagy

Autophagy is a fundamental intracellular trafficking pathway conserved from yeast to man. Macroautophagy involves the degradation of large portions of the cytoplasm, including organelles, whereas other types of autophagy with a more restricted target set have been described and are currently explored [22]. During macroautophagy (herein referred to as autophagy) portions of the cytoplasm are sequestered into specialised double membrane vesicles called autophagosomes and delivered to lysosomes for degradation (Figs. 2 and 3). During nutrient withdrawal cells can initiate autophagy to allow the breakdown of proteins into amino acids, which can subsequently be used for new protein synthesis or ATP generation [22–25]. In addition, autophagy is also required for the removal of damaged or unneeded organelles, including mitochondria (mitophagy) and peroxisomes (pexophagy) [26,27]. During initiation of autophagy a damaged organelle or portion of the cytosol is surrounded by a flat membrane sheet known as the isolation membrane or phagophore [28] (Fig. 3). The isolation membrane elongates and seals to itself to form an autophagosome. It is thought that the autophagosome engages with normal endocytic traffic in order to mature into a late autophagosome before fusing with lysosomes to form an autolysosome [29]. To date, 31 genes that are involved in autophagy have been identified by genetic screens in yeast [22,30], and these have been termed autophagy-related genes (Atg) genes. The core Atg genes can be subdivided into four subgroups: Atg1/unc-51-like kinase (ULK) and their regulators; Vps34 complex I; the Atg9 cycling complex; and conjugation pathways involving the ubiquitin-like proteins Atg12 and Atg8/LC3 [30–33].

One requirement common for all organisms during autophagy is the need for synthesis of PI(3)P, and this is based on very clear-cut genetic and biochemical data. Elimination of the gene coding for Vps34 in yeast inhibits autophagy, and replacement of the wild type allele with a mutant in the lipid kinase domain also blocks the pathway [31,32,34]. Similarly, starvation-induced autophagy was severely inhibited in *Drosophila* Vps34 loss of function and kinase-dead mutants [35]. Although a mouse knockout in the gene coding for Vps34 has not been reported, several independent groups have used siRNA against mammalian Vps34 to show that the protein is required for autophagy [36–39]. In terms of bio-

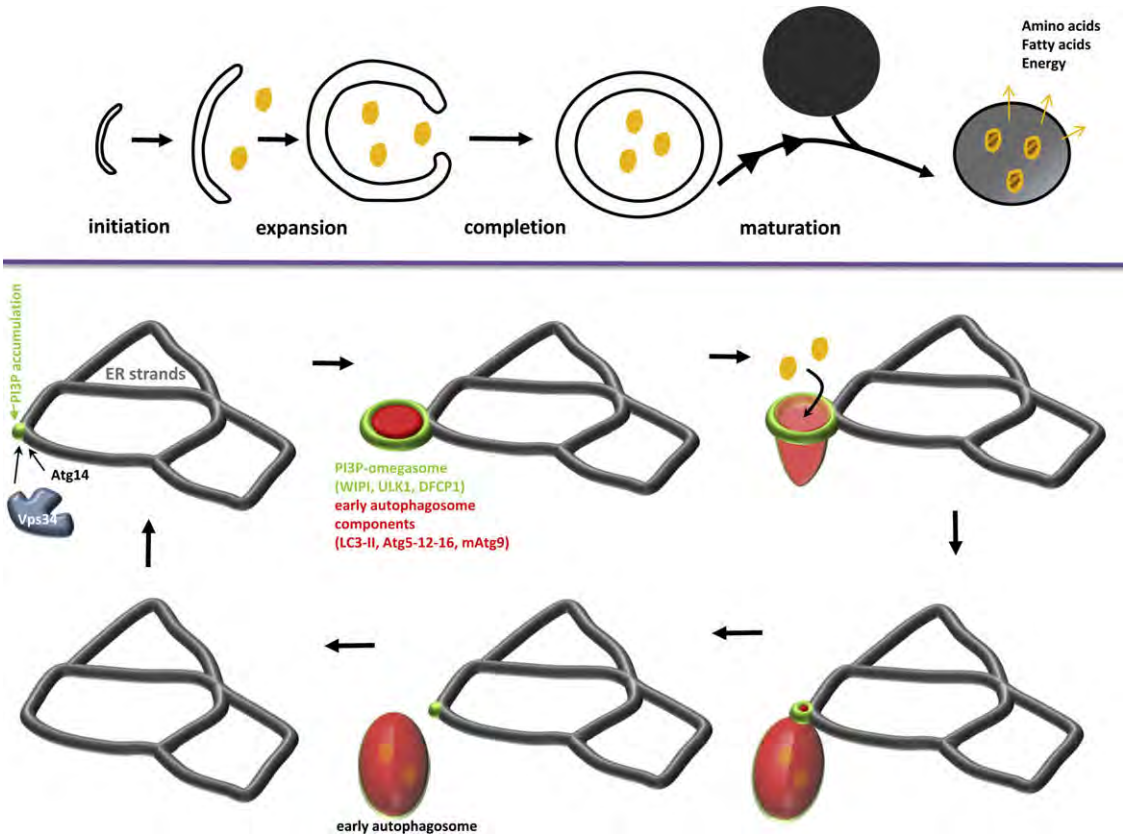

Fig. 3. Pathways of autophagy in mammalian cells. Upper panel: During autophagy portions of the cytosol are sequestered in autophagosomes, which are delivered to lysosomes. The autophagic pathway has four stages: initiation, expansion, completion and maturation. During initiation a portion of the cytosol is surrounded by a flat membrane sheet known as the isolation membrane. During the expansion phase the isolation membrane elongates and seals to itself to form an autophagosome. During the maturation step the autophagosome fuses with lysosomes, to form autolysosomes. The sequestered content, including proteins is broken down to amino acids which are then recycled back to the cytosol. Lower panel: Atg14 is thought to recruit Vps34 to the site of autophagosome synthesis. In starved cells PI(3)P localizes to punctate structures known as omegasomes, which are likely to originate from the ER. Omegasomes co-localize with early autophagic markers including LC3 and Atg5, and autophagosomes are thought to form within omegasomes. Eventually autophagosomes exit from omegasomes.

chemical approaches, definitive work is somewhat hampered by the paucity of an inhibitor of Vps34 that would not inhibit other classes of PI 3-kinases (see also [14]. Nevertheless, it has been shown that treatment with wortmannin or 3-methyladenine, both of which inhibit Vps34 activity, blocks autophagy in a variety of cell types [14,38,40–43].

Given the importance of PI(3)P for the induction of autophagy, where is it localized? Some studies have used a double FYVE domain probe to show that autophagosomes stain with this lipid [12,35,36]. In other studies, PI(3)P was shown to be transported to the vacuole via autophagosomes [31,32,44]. Since autophagosomes engage with the endosomal membrane system during their maturation, and because PI(3)P is known to regulate several maturation events, it is expected that PI(3)P on autophagosomes may facilitate this progression.

Work in our laboratory found that PI(3)P generation is also a key early event in autophagosome biogenesis (Fig. 3). A novel protein termed double FYVE domain-containing protein 1 (DFCP1), which was shown to bind to PI(3)P was utilised. It was found that upon starvation of stably expressing HEK-293 cells, GFP-DFCP1 translocates to a punctate compartment that partially co-localizes with the autophagic markers LC3 and Atg5 [36,45]. The translocation of DFCP1 was inhibited by the PI3-kinase inhibitors wortmannin and 3-methyladenine, and also by siRNA against Vps34 and Beclin1 (Vps30), a protein known to bind to and regulate Vps34. Unexpectedly for a PI(3)P binding protein, DFCP1 localizes to the ER and Golgi in nutrient rich conditions, and one interpretation of these data is that DFCP1 translocates from the ER or the Golgi to this autophagy-induced punctate compartment. Several other PI(3)P-binding probes tethered to the ER confirmed that the membranes of this punctate compartment are in dynamic equilibrium with the ER. Because these membranes were frequently seen to form an Ω-like shape in association with the ER we termed them ‘omegasomes’. From live imaging studies it was also apparent that autophagosomes form within omegasomes (Fig. 3). Therefore, omegasomes may provide a site for the biogenesis of autophagosomes. The DFCP1 positive omegasome appeared to be a donut-shaped ring which enclosed the LC3 positive autophagosome. The autophagosome eventually exited the omegasome, via either a smooth movement or the omegasome seemed to zip along the autophagosome. Internal reflection fluorescence microscopy (TIR-FM), confocal live imaging and immuno-EM were used to show that DFCP1 labelled omegasomes were in close contiguity with the ER. Finally, the localization of RFP-Vps34 was tracked in GFP-DFCP1 expressing HEK-293 cells. Vps34 was found to be localized to the cytosol and in vesicles thought to correspond to late endosomes/lysosomes, and it did not translocate upon starvation (Fig. 3). The Vps34 vesicles were always in close proximity to the ER, and were frequently seen to move along ER strands. Importantly, during starvation omegasomes formed in close association to the Vps34-containing vesicles, hence the Vps34 in these vesicles is likely to synthesise the PI(3)P found in omegasomes.

Subsequent to this work, other investigators were able to show how autophagosomes are connected to the ER using EM tomogra-

phy [46,47]. Although the localization of PI(3)P was not reported in these studies, it is reasonable to conclude at this point that a modified region of the ER provides a platform for the formation of autophagosomes.

5. Several Vps34 complexes exist in a cell

It is clear that PI(3)P, and hence its synthesising enzyme Vps34, play very important roles in autophagy initiation and progression. In addition, as described earlier, Vps34 and PI(3)P are also involved in several aspects of endocytic/phagocytic traffic. Given the spatial and mechanistic differences between these pathways, it is perhaps not surprising that Vps34 has been found to be present on several distinct complexes (Fig. 4) (for a recent review see also [33]). We will discuss these complexes separately for yeast and for higher eukaryotes. For the former, the situation is pretty clear at this point. For the latter, a plethora of putative components of these complexes are being identified and the easiest way to present the data is to focus on the different proteins individually.

5.1. Vps34 complexes in yeast

Autophagy has been extensively studied in yeast, and both Vps34 and its regulatory protein Vps15 have been shown to be essential for this process [34]. Vps34 and Vps15 were originally identified in yeast [48,49], and both have homologues in higher organisms, including mammals. Vps15 is a putative serine/threonine kinase that is known to be required for Vps34 activity, and therefore is indispensable for the role played by Vps34 in both endocytic trafficking and autophagy. Unexpectedly, it was found that Vps15 does not phosphorylate Vps34 [50], but Vps34 activity is abolished in Δ*vps15* yeast or yeast expressing kinase dead Vps15 [51].

Studies in yeast have identified two Vps34 containing complexes [34]. Complex I is composed of Vps34 and Vps15, as well as two accessory proteins, Vps30 and Atg14, this complex has proved to be essential for autophagy. Vps34, Vps15, and Vps30 are also present in Complex II, but Atg14 is replaced by Vps38, this complex is thought to be essential for the endosomal Vps pathway. These two complexes are characterized by the mutually exclusive expression of Atg14 or Vps38. Consistent with their presence in both complexes, deletion of Vps30, Vps15 or Vps34 resulted in both an inhibition of the sorting of the lysosomal hydrolase CPY (carboxypeptidase Y), as well an inhibition of autophagy [34]. Atg14 was shown to be essential for autophagy in yeast, as autophagy was found to be severely disrupted in Δ*atg14* yeast. Vps38 is essential for vacuolar protein sorting and retrograde transport, as shown by defective CPY sorting [34] and mislocalization of Vps10 [52] in Vps38-deficient yeast. In addition, co-immunoprecipitation of Vps30 with Vps15–Vps34 was decreased in a Δ*vps38* strain, indicating that Vps38 could mediate the interaction between Vps30 and Vps15–Vps34 [34].

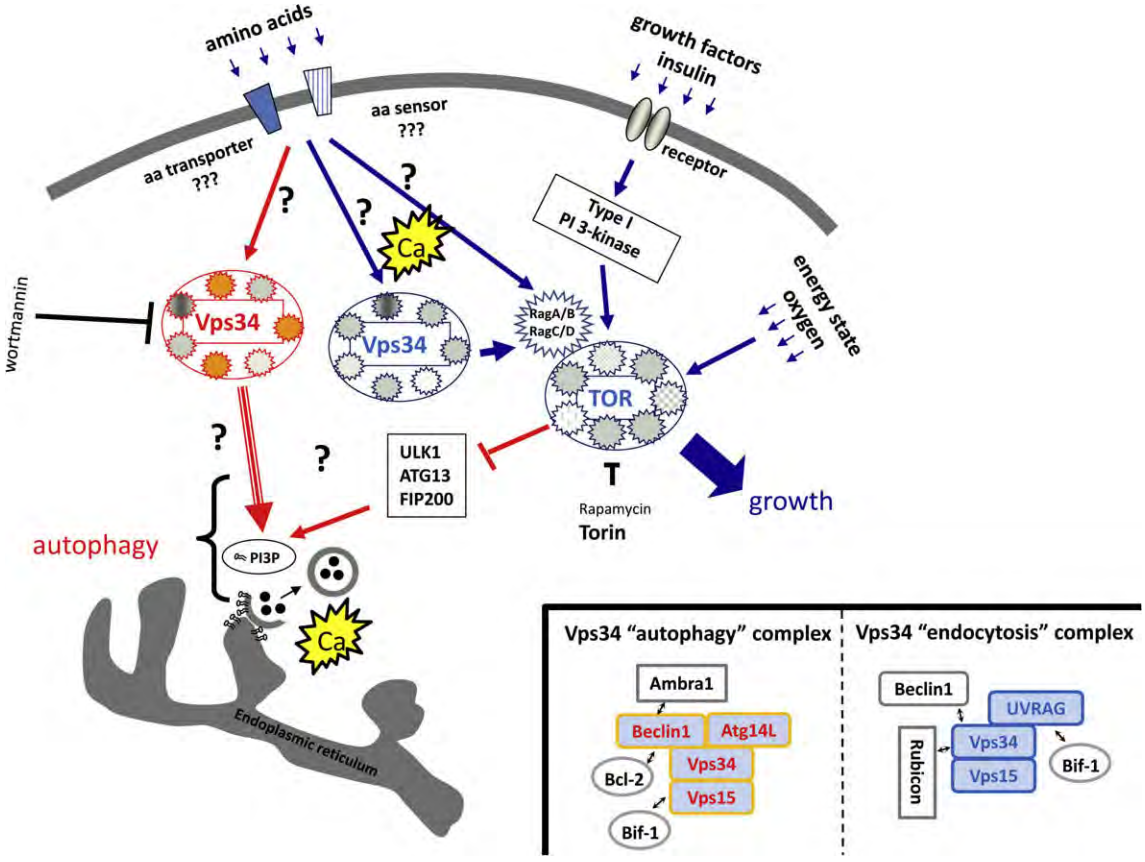

Fig. 4. TOR inactivation is a central event in autophagy. Amino acids, growth factors, and ATP and oxygen levels can regulate TOR activation. Class I PI3-kinases can signal from growth factor receptors to stimulate TOR activity. The Rag proteins are thought to signal from amino acids to TOR activation in a pathway involving the TOR activator Rheb. Vps34 can signal to activate TOR in amino-acid rich conditions, which leads to inhibition of autophagy (blue scheme), or it can signal to activate autophagy following TOR inactivation in amino acid poor conditions (red scheme). It is likely that spatially distinct Vps34-containing complexes mediate these effects. TOR acts to inhibit autophagy by associating with the ULK1–Atg13–FIP200 complex and phosphorylating ULK1 and Atg13 to block their activity and translocation to sites of autophagosome formation. Boxed area: Vps34 is present on two distinct complexes, one which mediates autophagy and one which mediates endocytosis. These are characterised by the mutually exclusive expression Atg14 or UVRAG.

5.2. Vps34 complexes in higher organisms

5.2.1. Vps15

Homologues of yeast Vps15, Vps30 (Beclin1 in mammals), Vps34, Vps38 (UVRAG in mammals) and Atg14 exist in higher organisms, including mammals. Mammalian Vps34 has been shown to be essential for autophagy (see above). Moreover, optimal Vps34 activity also requires Vps15 in mammalian cells [53]. Studies using *Drosophila* have also shown that autophagy is severely reduced in Δ*vps15* flies [54]. It is known that membrane targeting of Vps34 requires Vps15, and that the two proteins form a membrane-associated complex. Co-immunoprecipitation experiments in mammalian cells also revealed that Vps15 enhances the ability of the accessory proteins Beclin1 and UVRAG to interact with Vps34 (Fig. 4), and these proteins could only enhance the activity of Vps34 in the presence of Vps15 [53]. This is despite the fact that Beclin1 is known to directly bind to Vps34 [55]. The same study also found that expression of Beclin1/UVRAG also enhanced Vps34–Vps15 binding. Vps15 can also bind to both Rab5 and Rab7, and it is though that this binding helps localize Vps34 to both early and late endosomes [56].

5.2.2. Beclin1

Beclin1 is the mammalian homologue of yeast Vps30, and was first identified as a Bcl2 interacting protein [57]. Co-immunoprecipitation of Vps34 and Beclin1 has been observed in mammalian cells [55] and it is estimated that 50 percent of mammalian Vps34 is in a complex with Beclin1 (Fig. 4). Unlike yeast, in mammalian cells the processing of cathepsin D in the lysosome is normal in cells that express little Beclin1 [55] and in Beclin1 knockdown cells [58]. However, Beclin1 was found to be essential for autophagy in mammalian cells [58,59]. These studies suggest that in mammals Beclin1 is essential for the engagement of Vps34 in autophagy, but dispensable for the role played by Vps34 in endocytic trafficking and lysosomal sorting. Several proteins have been shown to interact with Beclin1 in mammalian cells, including Bcl2, UVRAG, Atg14, Rubicon, Bif1, Ambra1 and Rab5 (Fig. 4).

Mammalian Beclin1 has been found to also exist in two complexes that along with Vps34 and Vps15 contain either Atg14 or UVRAG (the mammalian orthologue of yeast Vps38) [37] (Fig. 4). As in yeast, the complex containing mammalian Atg14 is essential for autophagy, whereas the complex containing UVRAG is essential for endocytosis. UVRAG and Atg14 have been found to compete for binding to the N-terminal C2 domain of human Vps34. Human Vps34 has been found to be present almost entirely on UVRAG positive punctate in non-starvation conditions, and upon starvation 30% of the Vps34-GFP dots co-localized with Atg14 [37].

5.2.3. Bcl2

Bcl2 and $BclX_L$ can bind to Beclin1 to act as inhibitors of autophagy (Fig. 4). It is thought that the Bcl2/$BclX_L$-Beclin1-Vps34 complex has decreased Vps34 activity compared to complexes lacking Bcl2/$BclX_L$ [60–62]. Upon nutrient starvation the amount of Beclin1 that co-immunoprecipitates with Bcl2/$BclX_L$ decreases, which leads to autophagy. It is known that the BH3 (Bcl2 homology) domain of Beclin1 binds to Bcl2/$BclX_L$. Another BH3 domain containing protein Bad is activated upon nutrient starvation. Bad can compete with Beclin1 for binding to Bcl2/Bcl_{XL}, and increased co-immunoprecipitation of Bad with Bcl2/$BclX_L$ occurs upon nutrient starvation. In fact, over expression of Bad can induce autophagy in non-starved conditions [60,61]. Nutrient starvation also leads to the activation of c-Jun N-terminal Kinase 1 (JNK1), and it has been shown that JNK1 can phosphorylate Bcl2 at serine and threonine residues. Phosphorylation of Bcl2 by JNK1 is essential for autophagy, as when phosphorylated by JNK1 Bcl2 can no longer co-immunoprecipitate with Beclin1 [63]. It has been demonstrated that autophagy is inhibited when JNK1 is inhibited or with a Bcl2 mutant that is resistant to phosphorylation by JNK1. Conversely, constitutively active JNK1 can stimulate Bcl2 phosphorylation and autophagy in non-starved cells.

5.2.4. UVRAG

UVRAG (UV radiation resistance-associated gene) has been identified as a Beclin1 binding protein that can increase Beclin1-Vps34 interaction as well as Vps34 activity [64]. UVRAG is thought to be a mammalian orthologue of yeast Vps38 [37,65]. Early studies suggested that UVRAG participated in the formation of autophagosomes in a Beclin1-dependent manner [64]. However, further studies showed that treatment of cells with siRNA against UVRAG had no effect on autophagy [37]. Specifically, UVRAG was found to be localized on early and late endosomes, and co-expression of GFP-Vps34 with HA-UVRAG localized Vps34 to UVRAG positive structures (possibly endosomes), but UVRAG did not localize with any markers of autophagy [37]. Interestingly, in several studies UVRAG co-immunoprecipitated with Vps34 and Beclin1, indicating that these proteins exist in a complex that is similar to yeast complex II [37,66,67]. Hence, evidence suggests that Beclin1 is present in an endocytosis complex with UVRAG although another report has shown that Beclin1 is not essential for mammalian endocytosis [58]. It is thought that autophagosome maturation occurs by fusion of the autophagosome with different endosomal populations [29]. Therefore, since UVRAG is thought to play a role in endocytosis, it is possible that it could also regulate maturation of autophagosomes.

5.4.5. Atg14

As in yeast, mammalian Atg14 is present in a complex with Vps34, Vps15 and Beclin1, and this is essential for autophagy [37] (Fig. 4). Atg14 is thought to be involved early on in autophagogenesis, and localizes to the isolation membrane. Indeed, human Atg14 showed a high degree of co-localization on the isolation membrane with both Atg5 and Atg16L1 [37]. The same study found that Atg14 was present on starvation-induced punctate, these only partially co-localized with LC3, and Atg14 was not observed on the larger LC3 dots. Therefore, Atg14 may not be present on complete autophagosomes. It is known that Vps34 functions upstream of the Atg12–Atg5–Atg16L1 complex, the localization of Atg14 on punctate is not affected by the Vps34 inhibitor wortmannin, hence Atg14 localizes to autophagic membranes earlier than the Atg12–Atg5–Atg16L1 complex [37]. Interestingly, a mutant version of Atg14 that was unable to bind to Vps34 and Beclin1, could still accumulate to Atg16L1 and LC3 positive structures following starvation, indicating that Atg14 can localize to the isolation membrane, or possibly a pre-isolation membrane structure, independently of Vps34 or Beclin1. Moreover, Atg14 was found to be essential for autophagy, since autophagic flux was decreased in cells transfected with siRNA against Atg14 [37]. Additional studies also found that Atg14 was required for autophagy in mammalian cells [66,67]. Tracking of the subcellular localization of GFP-LC3 and electron microscopy of cells treated with Atg14 siRNA, revealed that GFP-LC3 remained in the nucleus and that autophagosomes were absent [37]. Thus Atgt14 is thought to be required for autophagosome formation rather than autophagosome maturation. Further experiments found that a block in autophagy caused by Atg14 depletion could be rescued by expression of wild-type Atg14 but not by the Atg14 mutant unable to bind Vps34 and Beclin1 [37]. Hence the role played by Atg14 in autophagy requires it to be in a complex with both Vps34 and Beclin1. Furthermore, Atg14 has been shown to increase Vps34 kinase activity, and this was dependent on both Beclin1 and Vps15 [67]. Therefore, Atg14 may act to recruit Vps34 to the isolation membrane, where it can stimulate its activity.

5.2.6. Rubicon

Recently, two studies identified Beclin1 binding proteins [66,67]. These included Vps15, Vps34, UVRAG, Atg14 (called Atg14L for Atg14-like in these studies) and a newly identified protein called Rubicon (RUN domain and cysteine-rich domain containing, Beclin1-interacting protein). Three types of Beclin–Vps34–Vps15 complex were found to exist: an Atg14 containing complex, an UVRAG complex and a Rubicon–UVRAG complex [66]. Knockdown of Rubicon expression was found to promote autophagic activity. Conversely, over expression of Rubicon inhibited autophagy [66,67] it also caused an abnormal morphology of late endosome/lysosome, and inhibited the transport of internalised EGFR to the lysosome [66]. It is thought Rubicon negatively regulates the maturation steps of the endocytic pathway and autophagy [66,67]. Over expression of Rubicon was also found to inhibit Vps34 kinase activity, but this did not require Beclin1. Rubicon was found to be expressed partially on early endosomes and multivesicular endosomes, but primarily on late endosomes/lysosomes [66,67]. This would suggest that the Rubicon–UVRAG–Beclin1–Vps34–Vps15 complex suppresses endocytosis and autophagosome maturation, whereas the UVRAG complex that does not contain Rubicon is required for endocytosis. Although Rubicon was found not to bind to PI(3)P, Rubicon-associated structures were enriched with PI(3)P. These structures were unaffected by wortmannin, suggesting that the maintenance of these structures was not dependent on PI(3)P [67].

5.2.7. Bif-1

Bif-1 (Bax-interacting factor 1) is a member of the endophilin protein family. Bif-1 can interact with Beclin1 indirectly via UVRAG (Fig. 4), to regulate the activity of Vps34 and induction of autophagy in mammalian cells [68]. This interaction with UVRAG could explain the participation of UVRAG in autophagy observed by some investigators [64]. However, convincing evidence also shows that UVRAG does not play a role in autophagy [37]. Knockdown of Bif-1 in HeLa cells resulted in a significant reduction of Vps34 activity [69]. Loss of Bif-1 has also been shown to suppress the starvation-induced activation of Bax [70]. Therefore, Bif-1 could induce autophagy by promoting Bax binding to Bcl2, thereby releasing Beclin1 from negative regulation by Bcl2. Starvation-induced Bif-1 foci co-localize with both Atg5 and LC3 [69]. During starvation Atg9 translocates from the Golgi to peripheral sites including endosomes, and Atg9 positive vesicles play a role in the synthesis and expansion of autophagosomal membranes [71]. Following nutrient starvation Bif-1 positive vesicles fuse with Atg9 positive compartments [68]. Like all endophilins, Bif-1 contains an N-terminal N-BAR (Bin-Amphiphysin-Rvs) domain, and these can bind to lipid bilayers to induce membrane curvature [72]. This feature of Bif-1 could prove to be important for membrane expansion and curvature of autophagosomes.

5.2.8. Ambra1

Ambra1 (activating molecule in Beclin1-regulated autophagy) is another protein that interacts with Beclin1, and Ambra1–Beclin1 complexes can also co-immunoprecipitate with Vps34 (Fig. 4). Both in cultured cells and embryos knockdown of Ambra1 expression can inhibit autophagy. Moreover, in cultured cells it was also found that knockdown of Ambra1 decreases Beclin1-Vps34 association [73].

5.2.9. Rab5

Finally, Rab5 has also been shown to be present in a complex with both Vps34 and Beclin1 [42]. Specifically, Rab5 was shown immunoprecipitate with Beclin1, but only in the presence of Vps34. Earlier studies have shown that Rab5 can interact with Vps15 [74]. Over expression of dominant negative Rab5 in COS-7 cells lead to a decrease in the proportion of cells with over 20 LC3 positive autophagosomes [42]. Conversely, over expression of wild-type or constitutively active Rab5 had the opposite effect. Inhibition of Vps34 by 3-methyladenine led to the appearance of Atg5 positive structures which co-localized with myc-FYVE and Beclin1, but not with LC3, indicating that they were early autophagic structures. Inhibition of Rab5 resulted in the appearance of similar Atg5 positive structures. Furthermore, Rab5 co-localized with the Atg5 positive structures that were induced by treatment with 3-methyladenine. Hence, Rab5 may play a role early on in autophagy, possibly via activation of Vps34. Specifically, it was found that both dominant negative Rab5 and 3-methyladenine resulted in decreased Atg5–Atg12 conjugation, a process known to be important for autophagosome formation. Interestingly, it has also been shown that in *Caenorhabditis elegans* Rab5 is necessary for the formation of peripheral ER tubules [75]. Hence, one could speculate that Rab5 could recruit Vps34 to omegasomes, which are in close contiguity to the ER (see above).

6. Effectors of PI(3)P during autophagy

Most PI(3)P binding proteins (effectors) are localized to endosomes, and have been found to play a role in endocytic trafficking (see above). However, a few PI(3)P binding proteins that are thought to play a role in autophagy have also been identified.

6.1. Yeast

In yeast Atg18, Atg21 and Ygr223c make up a family of PI binding proteins. All three proteins were found to localize to endosomes, and PI(3)P produced by complex II was essential for this localization [76]. Atg18 was also found on the pre-autophagosomal structure (PAS), and this localization was dependent on Atg14. Atg18 was also found to be required for efficient progression of both autophagy and the Cvt pathway, and both of these required PI(3)P binding to Atg18. Moreover, PI(3)P binding to Atg18 is essential for the association of an Atg18–Atg2 conjugate at the PAS [31]. It was found that Atg21 was required for the yeast Cvt pathway, but not for autophagy [77]. Ygr223c was found to play a role in a specific kind of autophagy whereby portions of the nucleus are sequestered and degraded (micronucleophagy) [76].

Several additional proteins in yeast, involved in selective autophagy pathways, were shown to bind to PI(3)P. Cvt13 and Cvt20, both of which contain PX domains that bind to PI(3)P [78] are essential for the Cvt pathway, but not for autophagy. Yeast PpAtg24 is thought to be a member of the sorting nexin family, and has also been found to bind to PI(3)P via a PX domain [79]. Deletion of PpAtg24 was found to disrupt both macro and micropexophagy, and it is thought that PpAtg24 is involved in pexophagosome vacuole fusion.

6.2. Higher organisms

WIPI-1 is the mammalian orthologue of yeast Atg18 [80,81]. It is a member of the WIPI family of four proteins, all of which contain WD40 repeats. WIPI-1 (also known as WIPI49) was found to bind to 3-phosphorylated PI's [80] and upon starvation human WIPI-1 was found to partially co-localize with LC3 on autophagosomes [81]. Recently, WIPI-1 was also found to be present on isolation membranes [7].

In humans Alfy, a PI(3)P binding protein that contains a FYVE domain has been identified, with putative orthologues in flies and worms [82]. Induction of autophagy by starvation caused Alfy to translocate from the nucleus to punctate cytoplasmic structures. Specifically, Alfy was found to co-localize with both Atg5 and LC3

on autophagic membranes. However, treatment with wortmannin and 3-methyladenine had no effect on the number of Alfy punctate, suggesting that Alfy is recruited to autophagic membranes following starvation independent of PI(3)P production. It was also found that inhibiting proteasomal degradation caused an increase in the number of Alfy punctate, and that Alfy co-localized with ubiquitin. Moreover, electron microscopy could detect similar structures inside autophagosomes. This led the authors to hypothesise that Alfy might recognise protein aggregates targeted for degradation and act as a scaffold for components of the autophagic machinery.

DFCP1 is another effector of PI(3)P during autophagy [36] as discussed above. It is a protein highly conserved in mammals, in other organisms such as chicken, xenopus, zebrafish, honeybee mosquito and several species of Drosophila, but not in *Drosophila melanogaster* or in yeast. Based on the absence from yeast and *D melanogaster*, we would suggest that its – as yet unknown – function must be not essential.

7. PI(3)P signal termination

Most pathways regulated by phosphoinositides depend not only on the generation but also on the consumption of the lipid signal. Conditions where the signal persists beyond a physiologically appropriate duration frequently result in diseases. Very recent data suggest a similar situation for the PI(3)P signal that regulates the induction of autophagy. A PI(3)P phosphatase called Jumpy has recently been identified in humans [7]. Jumpy is thought to inhibit autophagy, since knockdown of Jumpy expression caused an increase in basal levels of autophagy in several different cell types. Furthermore, Jumpy knock down stimulated both autophagosome formation and maturation. Jumpy was found to co-localize with Atg16, Atg12 and LC3 on isolation membranes, but also with LC3 on autophagosomes. Interestingly, knock down of Jumpy was found to increase the number of starvation-induced WIPI-1 punctate. Therefore, Jumpy can act to prevent PI(3)P-dependent WIPI-1 recruitment to autophagic membranes. A catalytically inactive Jumpy mutant that has lost the ability to negatively regulate autophagy is found in a congenital disease known as cetronuclear myopathy. Given the possible requirements for PI(3)P during autophagosome maturation it is likely that additional PI(3)P phosphatases will be shown to be involved in autophagy at stages later than induction.

8. Signalling during autophagy induction: Vps34 and TOR at centre stage

Autophagy appears to be regulated tightly at the induction stage whereas subsequent steps leading to autophagosome maturation and fusion with the lysosomal membrane system are mechanistically complicated but less likely to require signalling inputs.

The master regulator in autophagy is the serine/threonine protein kinase TOR (target of rapamycin) (Fig. 4). TOR exists in at least two complexes, TORC1 and TORC2, but only TORC1 is regulated by nutrients such as glucose and amino acids [83].

Activation of TORC1 has been found to positively regulate cell growth via ribosome biogenesis, increasing protein synthesis and inhibition of autophagy whereas inactivation of TOR is a strong inducer of autophagy in all organisms [23,84]. Earlier studies suggested a dichotomy between yeast and higher eukaryotes in the sensitivity of autophagy to TOR inactivation: rapamycin – a TOR inhibitor – was a strong inducer of autophagy in yeast but a relatively poor one in mammalian cells. More recently, the use of novel ATP-competitive TOR inhibitors such as a compound called Torin1, has shown an equally robust stimulation of autophagy in mammalian cells [85,86]. Therefore, it is very likely that TOR inactivation is a central critical event in autophagy, although the targets of TOR (see below) or the explanation of the different effects of Torin1 vs rapamycin on autophagy are still debated [86].

Three types of signals regulate TOR activation (Fig. 4): extracellular bioactive molecules such as amino acids and growth factors, energy availability especially ATP, and oxygen levels. Interference with any of these has been shown to affect TOR and consequently autophagy, and much attention is currently being focused on the molecular aspects of this regulation. We will focus our discussion on the regulation of TOR by amino acids downstream of the initial signal, since this pathway and its cross-talk with Vps34 are major inducers of autophagy. For recent reviews and a more extended discussion on how amino acids regulate TOR (including on the important question on whether the sensor(s) is intracellular or extracellular) see also [87,88]

It has been known for many years that amino acid availability in the extracellular medium regulates the phosphorylation state of p70 S6 kinase and elF-4E BP1, two downstream targets of TOR [89,90]. De-phosphorylation of these two targets by amino acid withdrawal is very fast, and it correlates very tightly with the induction of autophagy. However, the mechanism by which TOR senses amino acid levels had remained obscure until very recently when two independent groups identified the Rag (Ras-related GTPases) proteins as important intracellular mediators of amino acid sensing by TOR [91,92]. In subsequent work it was reported that amino acid sensing/TOR activation in yeast may also depend on the Rag homologues, although with subtle differences [93]. In the model proposed, GTP/GDP loading on the Rag hetero-tetramer is affected by amino acids, and this in turn affects the localization of TOR to perinuclear membranes containing one of its activators, the small GTPase activating protein Rheb. Thus, amino acids ultimately provide a signal for bringing TOR in proximity to its activator. Interestingly, the perinuclear membranes on which TOR meets its activator are related to late endosomes (they contain Rab7), and this brings into the picture Vps34.

Two lines of investigations connect Vps34 with TOR. On the one hand, the late endosomal compartment that appears to be important for amino acid sensing by TOR is regulated by Vps34 and its lipid product PI(3)P [14,94,95]. The Vps34 protein itself has also been shown to be present in this compartment (or interact with components of the compartment) by several groups [36,44,96]. On the other hand, signalling to TOR following amino acid stimulation is stimulated by Vps34 and inhibited by conditions that inactivate the protein or sequester its lipid product [97–99]. An attractive hypothesis may be that Vps34 and PI(3)P are involved in the co-localization of TOR with Rheb and in this way stimulate TOR signalling in response to amino acids. It should be noted however that this scheme is not seen in all organisms: genetic inactivation of Vps34 in Drosophila affects endocytosis but not TOR signalling [35].

If Vps34 is a positive regulator of TOR following amino acid stimulation how can it also be a positive regulator of autophagy following amino acid withdrawal and TOR inactivation? The answer to this important question is not known with any certainty. It is possible that the different Vps34 complexes (as discussed above) respond differently to amino acid levels; the endocytic complex may be positively regulated whereas the autophagic complex may be negatively regulated. If this is the case, specific components of the two complexes that would respond differently to amino acids are likely to be identified. Another possibility is that activation/inactivation of Vps34 are spatially controlled. For example ‘‘autophagic” Vps34 may be active only near the ER whereas ‘‘endocytic” Vps34 may be active on endosomes. Finally, it is conceivable that the differential specificity is not a property of the Vps34 complex itself but of an upstream regulator. In this context we wish to highlight the possible role of calcium in amino acid-

dependent TOR activation or autophagy. Seglen and colleagues were the first to report that autophagy depends on cytosolic calcium levels [100] and more recent work has extended these observations (discussed in [101–103]). Although the consensus on the role of calcium is still not apparent, two papers from independent groups have suggested that, at least for a subset of experimental models, elevation of cytosolic calcium by various agents induces autophagy [104] whereas chelation of intracellular calcium is inhibitory [105]. Our unpublished work also suggests that chelation of intracellular calcium inhibits the translocation of DFCP1 to omegasomes during starvation, i.e., calcium is an early requirement for the autophagic response (Chandra and Ktistakis, in preparation). Somewhat complementary to these studies, a recent study has also suggested that amino acids induce a rise in intracellular calcium which may be responsible for the subsequent activation of Vps34 ([106], but see also [53] for a suggestion that this may not be a direct effect). Interestingly, the calcium that is elevated following amino acid addition appears to come from the extracellular medium whereas the calcium that is required for autophagy after amino acid withdrawal comes from internal stores. Given that cells have the capacity to generate and interpret a huge variety of calcium signals [107], it is perhaps worth investigating further whether the shape and location of calcium signals may explain the dual effects of Vps34 in normal growth and autophagy.

In addition to the important open questions concerning the cross-talk between TOR and Vps34, equally important and open questions exist about signalling downstream of TOR leading to the formation of PI(3)P and the induction of autophagy. In yeast, TOR signals to a complex containing Atg1 (a conserved serine/threonine kinase that regulates autophagy), Atg13 (a scaffold protein) and Atg17 (another protein required for the stability of the complex) [108,109]. Under normal growth conditions, active TOR phosphorylates Atg13, de-stabilizing the Atg1–Atg13–Atg17 complex with the concomitant inactivation of the kinase activity of Atg1. This state is inhibitory for autophagy. Upon TOR inactivation, a complex of these three proteins is formed which results in translocation to pre-autophagosomal structures, activation of Atg1 and induction of autophagy. Mammalian cells maintain the basic structure of this system but with important differences. ULK1 (a mammalian homologue of Atg1) is one member in a family that contains several other related proteins with functions in autophagy but also in other pathways [110]. The equivalent proteins to Atg13 and Atg17 were recently identified by several groups [111–114] and they were shown to exist in complex with ULK1. Importantly, formation of this complex is not sensitive to amino acid levels, unlike in yeast. However interaction of TOR with the complex leading to phosphorylation of ULK1 and Atg13 and subsequent inhibition of translocation to autophagosomal structures appear to be dependent on nutrients. Therefore, in higher eukaryotes, the signal for the induction of autophagy is not the formation of the Atg1–Atg13–Atg17 complex, but the interaction of the complex with TOR. The mechanism for this – is it a spatial or a post-translational signal or a combination of the two – is of obvious importance but currently unknown. Other unanswered questions relate to the phosphorylation targets of ULK1, the mechanisms that localize the complex to autophagosomal structures and ultimately the pathway leading from interaction of the complex with TOR to Vps34 activation.

9. Concluding remarks

The induction of autophagy must be a very tightly-controlled decision since it involves sacrificing cellular material for energy generation. Even in cases where autophagy involves elimination of unneeded cellular components, it must be induced as a definitive step. The discovery that PI(3)P is an important requirement for autophagy, and the recent description of how it may be used in the induction step provide us with the basis for future investigations. It will be important to describe and characterize in molecular terms all of the steps leading from omegasome formation/expansion to autophagosome disengagement from the ER, inward budding and sealing off. Given the fast and spectacular nature of these movements during live imaging, it is possible to expect a significant requirement of energy, and a very complicated set of membrane alterations. Another important set of questions on autophagy induction concerns the nature of the early signals (upstream of PI(3)P), and the cross talk between TOR and Vps34. Here, it is very likely that the yeast and the mammalian systems will diverge somewhat in the sophistication of the circuitry used, and this will necessitate novel approaches that will not rely on yeast genetics. Such approaches include reverse genetic screens using genome-wide interfering RNAs and it is safe to say that several such screens are currently in the pipeline.

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