The regulation and function of Class III PI3Ks: novel roles for Vps34

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INTRODUCTION

Vps34 (vacuolar protein sorting 34) is a member of the PI3K (phosphoinositide 3-kinase) family of lipid kinases, all of which phosphorylate the 3′ hydroxy position of the phosphatidylinositol ring. In the accepted nomenclature for PI3Ks [1,2], Vps34 is classified as the sole Class III enzyme, whose substrate specificity is limited to phosphatidylinositol. Thus its product in cells is PtdIns3P. This distinguishes it from the more numerous and better studied Class I and Class II enzymes, which can produce PtdIns(3,4)P2, PtdIns(3,4,5)P3, or PtdIns(3,4,5)P5, depending on the isoform [1,2].

The first known functions of Vps34 were in the regulation of vesicular trafficking in the endosomal/lysosomal system, where it is involved in the recruitment of proteins containing PtdIns3P-binding domains to intracellular membranes [3,4]. However, Vps34 has also been implicated in other signalling processes, including nutrient sensing in the mTOR [mammalian target of rapamycin] pathway in mammalian cells, trimeric G-protein signalling to MAPK (mitogen-activated protein kinase) in yeast, and autophagy in both yeast and higher organisms [5–9]. Given the widening interest in Vps34, the present review will provide a focused discussion of its biochemistry, regulation and function in eukaryotic cells.

STRUCTURE AND CATALYTIC ACTIVITY OF Vps34

Vps34 was first discovered and cloned by Emr and co-workers in 1990, as part of a screen for vps mutants in Saccharomyces cerevisiae [10]. The sequence of Vps34p did not give a clue as to its function until the cloning of the mammalian IA PI3K, p110α, revealed it to be a lipid kinase [11]. The homology between VPS34 and p110α led to the direct demonstration that Vps34p has PI3K activity [12]. Vps34 homologues have been identified in unicellular organisms (Schizosaccharomyces pombe, Candida albicans, Dictyostelium discoideum), plants, Caenorhabditis elegans, Drosophila melanogaster, as well as vertebrates [13–19]. In mammals, hVps34 (mammalian Vps34 homologue) is ubiquitously expressed [19].

PI3Ks are classified by substrate specificity and subunit organization [1,2]. Class I enzymes, which produce PtdIns(3,4,5)P3, in vivo, all contain homologous 110 kDa catalytic subunits (p110α, β, δ and γ). The regulatory subunits for Class IA enzymes (p85α, β, p55γ and p55γ) and Class IB enzymes (p101) are not structurally related. Class II PI3Ks do not produce PtdIns(3,4,5)P3, but appear to produce both PtdIns(3,4)P2 and PtdIns3P in vivo [20,21]. The three isoforms of Class II PI3K (commonly called PI3K C2α, β and γ) are monomeric and are notable for the presence of a C-terminal C2 domain not found in other PI3Ks. Finally, the sole Class III PI3K, Vps34, only produces PtdIns3P. The enzyme is closely associated with a protein kinase, Vps15, which has sometimes been described as a Vps34 regulatory subunit; the functional relationship between these two proteins is discussed below.

Vps34 exhibits considerable homology with the catalytic subunits of other PI3Ks, particularly at the level of domain sequence. However, Class III PI3Ks are also required for the induction of autophagy during nutrient deprivation. Finally, mammalian hVps34 is itself regulated by nutrients. Thus Class III PI3Ks are implicated in the regulation of both autophagy and, through the mTOR pathway, protein synthesis, and thus contribute to the integration of cellular responses to changing nutritional status.

Key words: hVps34, hVps15, phosphoinositide, phosphoinositide 3-kinase (PI3K), target of rapamycin (TOR), vacular protein sorting 34 (Vps34).

Abbreviations used: AICAR, 5-amino-4-imidazolecarboxamide riboside; AMPK, AMP-activated kinase; CPY, carboxypeptidase Y; CSF-1, colony-stimulating factor 1; Cvt, cytosol-to-vacuole; ECD, evolutionarily conserved domain; 4E-BP1, eukaryotic initiation factor 4E-binding protein 1; EEA1, early endosomal antigen 1; EGF, epidermal growth factor; eGFP, enhanced green fluorescent protein; ERK, extracellular signal-regulated kinase; PAK, p21-activated protein kinase; PAM, p55α-myristoylated alanine-rich C-kinase substrate; PIPK, phosphoinositide kinase; PX, phox homology; siRNA, small interfering RNA; SH3, Src homology 3; SNARE, soluble N-ethylmaleimide-sensitive fusion protein-attachment protein receptor; TOR, target of rapamycin; TORC1/2, TOR complex 1/2; TSC, tuberous sclerosis complex; UVRAG, UV radiation resistance-associated gene; Vps, vacular protein sorting; hVps, mammalian Vps homologue.

To avoid confusion, species-independent references to Class III PI3K use the term Vps34. Specific references to the Class III PI3K from yeast use the term Vps34p. The mammalian Class III PI3K was first cloned from human cells [19], and has been previously referred to as hVps34. Although this term is inaccurate, we have retained the usage in the present review in the interest of continuity with the pre-existing literature. A similar nomenclature is used for yeast Vps15p and mammalian hVps15 (formerly called p150 [29]).

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The domain structure of Vps34-associated proteins is shown. All domain borders are taken from the mammalian enzymes, with the exception of Atg14p and Vps38p, which occur only in yeast. BH, Bcl2-homology; C/C, coiled-coil.

organization. Currently, the only existing structure of a PI3K catalytic subunit is the Class I PI3Kγ structure solved by Williams and co-workers [22]. As compared with PI3Kγ, Vps34 has a structurally uncharacterized N-terminal region of approx. 50 amino acids, followed by C2, helical and kinase domains that are approx. 30% homologous with PI3Kγ (Figure 1). The C2 domain fold in the PI3Ks is related to that of PLCγ (phospholipase Cγ), and is likely to be involved with interactions with acidic phospholipids rather than Ca2+[22]. The helical domain has an important regulatory role in Class IA PI3K catalytic domains, where it mediates inhibitory contacts with the Class IA regulatory subunit p85 [23]. A parallel role for the Vps34 helical domain has not been studied. The kinase domain shows the classic two lobed structure characteristic of protein kinases. Budovskaya et al. [24] showed that the extreme C-terminal 11 residues of yeast Vps34p are required for lipid kinase activity, independently of any effects on binding to Vps15p (the putative serine kinase that associates with Vps34p, discussed below). Consistent with this finding, antibodies targeted to the C-terminus of mammalian hVps34 are potent inhibitors of the enzyme [25].

Vps34 enzymes are unique among PI3Ks in that they will only utilize phosphatidylinositol as a substrate. The reason for this selectivity is thought to lie in the make-up of the substrate recognition loop which, in Class I or II PI3K, contains multiple basic residues that accommodate the negative charges in PtdIns4P and PtdIns(4,5)P2 [26]. In contrast, this region of Vps34 is relatively uncharged, limiting Vps34 substrates to phosphatidylinositol itself. Vps34 is also distinct from Class I enzymes in that it is more active in the presence of Mn2+ than Mg2+[19]. Mammalian hVps34 will also utilize Ca2+-ATP, and reaches approx. 50% of maximal activity with this cation (M.R. Lau and M.J. Fry, personal communication). In this regard, it is similar to the C2β isoform of Class II PI3K, which is also active in the presence of Ca2+-ATP [27].

In addition to lipid kinase activity, PI3Ks also possess activity towards protein substrates. Yeast Vps34p is known to undergo autophosphorylation [28], although the sites of phosphorylation have not been identified. Panaretou et al. [29] showed that a complex of mammalian hVps34 with mammalian hVps15 showed significant kinase activity towards generic substrates such as myelin basic protein, but the relative contributions of the two proteins was not determined [29]. Although several potential substrates have been identified for Class I enzymes [30–32], authentic in vivo substrates for Vps34 enzymes have not yet been identified. A functional role for Vps34 protein kinase activity was suggested by differences in the phenotypes of C. albicans strains that were Vps34-null compared with strains expressing a lipid-kinase-deficient/protein-kinase-active mutant Vps34 [33], although these results could also reflect scaffolding functions of Vps34.

METHODS FOR STUDYING Vps34

Studies of hVps34 have been hampered by the lack of specific inhibitors. Interestingly, both yeast and human Vps34 share a lysine residue that, in human Class I PI3Ks, is the site of covalent modification by the pan-PI3K inhibitor wortmannin (Lys833 in PI3Kγ) [34,35]. However, whereas Class I enzymes and mammalian hVps34 are extremely sensitive to wortmannin (IC50 = 5–15 nM), yeast Vps34p is relatively resistant (IC50 = 3 μM) [28]. The reason for this disparity in inhibitor sensitivity is not understood. Yeast Vps34p is also inhibited by LY294002 (IC50 = 50 μM) in a range similar to that for other PI3Ks and PI3K-related protein kinases such as mTOR [28]. At the present time, specific small molecule inhibitors of hVps34 have not been identified, although a recent upsurge in pharmaceutical company interest in hVps34 as a potential drug target should alleviate this problem. Although
3-methyladenine has been suggested to be a specific inhibitor of hVps34 [9], it in fact inhibits Class I and II PI3Ks as well [36].

Courtean and co-workers demonstrated that antibodies to the C-terminus of Class IA PI3Ks are specific inhibitors of these enzymes [37,38]. This approach has been successfully applied to mammalian hVps34 [25], and microinjection of inhibitory anti-hVps34 antibodies has been a useful tool for the identification of hVps34-dependent functions in mammalian cells (see below) [5,39–46].

Methods for measuring Vps34 activity in cells and in vitro are similar to methods used for other PI3K isoforms, and share some of the same limitations; this area has been comprehensively reviewed recently [47]. PtdIns3P, the product of Vps34, can be extracted from [32P]orthophosphate- or [3H]myo-inositol-labelled cells, and the deacylated lipids can be separated by ion-exchange HPLC [48]. This method does not, of course, provide information as to the spatial localization of the lipids. MS-based methods are also being developed for analysis of phosphoinositide abundance in cells and tissues [47]. A blotting method, in which lipid extracts from cells are probed with recombinant FYVE domains from SARA (Smad anchor for receptor activation), has been used to measure PtdIns3P in intact cells [49].

A widely used alternative method, analogous to the use of GFP (green fluorescent protein)–PH (pleckstrin homology) domains for detection of PtdIns(3,4,5)P3 and PtdIns(3,4)P2, is the expression of GFP-linked FYVE or PX (phox homology) domains, which are specific for PtdIns3P; tandem domains are sometimes used to increase binding affinity [50,51]. Although these constructs do provide information about spatial localization of PtdIns3P, they cannot be used to accurately quantify the net production of PtdIns3P in cells. The threshold for detection of cytosolic eGFP (enhanced GFP) by fluorescence microscopy is approx. 200 nM [52], meaning that small vesicles labelled with an eGFP reporter may not be visible above background autofluorescence. Thus a change in the size of eGFP–FYVE-labelled membranous structures (e.g. through the fusion or aggregation of pre-existing eGFP-labelled vesicles) will cause an increase in the fluorescence signal independently of any change in PtdIns3P production. This is a particular problem in the quantification of PtdIns3P in autophagosomal structures, which tend to be detected as large cytosolic vesicular aggregates. In contrast, detection of eGFP-labelled probes in the plasma membrane can be accurately quantified using TIRF (total internal reflection fluorescence) microscopy (reviewed in [53]).

Even as a probe of the sites of PtdIns3P production, there are a number of caveats involved in the use of FYVE and PX domain constructs. First, as is the case with PH-domain probes [54], FYVE-domain probes tend to identify sites of PtdIns3P abundance, such as endosomes, but may fail to detect PtdIns3P in membranes with lower, but still physiologically important, concentrations of the lipid. Secondly, lipid-binding domains with similar lipid specificities may localize differently in cells [54]. For example, TAF11 (tandem FYVE fingers 1) contains two PtdIns3P-specific FYVE domains yet localizes to the Golgi, rather than to endosomes [56]. A third problem is that FYVE-domain probes can perturb PtdIns3P-dependent processes. Thus overexpression of a 2X-FYVE construct blocks endosomal fusion and EGF (epidermal growth factor) receptor sorting [57]. As an alternative to FYVE-domain-based probes, antibodies specific for PtdIns3P are commercially available, but they have not been as well characterized as antibodies for PtdIns(4,5)P2 and PtdIns(3,4,5)P3 [58–61].

Except in yeast, where Vps34p is the sole PI3K, methods for evaluation of intracellular PtdIns3P levels are not necessarily indicative of Vps34 activity. For example, previous studies have shown that Class II PI3Ks also produce PtdIns3P in vivo [21,62], although this seems to be primarily in the plasma membrane. In mammalian cells, the dephosphorylation of PtdIns(3,4,5)P3 by endosomal PtdIns-5- and 4-phosphatases provides another source of endosomal PtdIns3P [63]. Alternatively, the Type Ia inositol polyphosphate 4-phosphatase localizes to endosomes, where it produces PtdIns3P through the dephosphorylation of PtdIns(3,4)P2 [64]. The existence of Vps34-independent sources of PtdIns3P in vivo was directly demonstrated in C. elegans, where the phenotype of a Vps34 null mutant (let-512) is rescued by siRNA (small interfering RNA) knockdown of MTM (myotubularin) 1 and MTM6 [65]. Myotubularins are PtdIns3P-specific lipid phosphatases (see below), and their ability to rescue a null allele of Vps34 means that additional sources of PtdIns3P must exist in C. elegans. Consistent with this, siRNA knockdown of the C. elegans Class II PI3K, F39B1.1, reduces the rescue of let-512 mutants by the myotubulin knockdown.

The enzymatic activity of recombinant Vps34 is readily assayed in vitro [19], and the activity of endogenous Vps34 can be measured in immunoprecipitates of Vps34 or its binding partners [5,66]. As antibodies to Vps34 can be inhibitory, care must be taken to test antibodies for inhibition using recombinant Vps34, and to elute Vps34 from the antibody prior to assay if necessary [5]. Although lipid kinase activity can be measured in immunoprecipitates from cells expressing epitope-tagged Vps34, we have found that the specific activity of overexpressed mammalian hVps34 is in fact approx. 5% of that of endogenous hVps34 (Y. Yan and J.M. Backer, unpublished work). This presumably reflects the requirement for hVps15 and other hVps34-associated proteins for full activity (see below). Thus studies on the regulation of hVps34 that depend on its overexpression in the absence of hVps15, and perhaps other binding partners, may not accurately reflect the physiological regulation of the enzyme.

The hVps34 gene has not yet been disrupted in mice. Genetic approaches to studying Vps34 in metazoans has been useful in C. elegans, where mutations of the let-512 gene lead to L3/L4 embryonic lethality, disruption of endocytic uptake and an expansion of the outer nuclear membrane [17]. In Drosophila, mutants in ird1 (the Drosophila homologue of the Vps34-related kinase Vps15; see below), which would presumably also inhibit Drosophila Vps34, lead to a loss of starvation-induced induction of antimicrobial peptides, suggesting a link between Vps34 and innate immunity [67]. However, ird1 mutants also show constitutive activation of the Toll pathway, in contrast with results in RAW264.7 macrophages, where expression of kinase-dead hVps34 blocks TLR9 (Toll-like receptor 9)-mediated responses [68]. These could reflect differences in flies compared with mammals, or differences between the phenotype of inhibition of Vps34 compared with its presumed upstream regulator Vps15 (see below).

REGULATION OF Vps34 BY Vps15

Like Vps34p, Vps15p was cloned from S. cerevisiae by the Emr laboratory [69]. Although Vps15 has been described as a Vps34 regulatory protein, the sequence of Vps15 suggests that it functions as a protein kinase. Homologues have been described in mammals and Drosophila [29,67], and a share similar domain organization (Figure 1). Vps15 contains an N-terminal myristoylation consensus sequence, and the yeast and mammalian enzymes are myristoylated [29,70]. Mutation of the Glu2 acceptor site has no effect on partitioning of Vps15p into particulate fractions, but does partially reduce Vps15p phosphorylation and is additive with other Vps15p mutations for temperature-sensitive
growth [70]. Vps15 consists of a predicted protein kinase domain followed by a central region containing multiple HEAT repeats and a series of C-terminal WD40 domains. It was suggested by Murray et al. [71] that these WD40 repeats might form a β-propeller-like structure, similar to the C-terminus of Gβ subunits [71]; in fact recent studies from Dohlman and co-workers suggest that Vps15p binds to yeast Gβ subunits (see below) [7].

The relationship between the Vps34 and Vps15 proteins was first suggested by the similar phenotypes caused by their deletion in S. cerevisiae, which do not clearly fall into any of the five classes (A–E) of vps mutants defined by Stevens, Emr and their co-workers [72–74]. Δvps34 and Δvps15 stains resemble Class D vps mutants [72], with defects in the sorting of newly synthesized hydrolases to the yeast vacuole and in vacuolar segregation during mitosis, but with a relatively normal vacuole morphology. However, Δvps15 and Δvps34 strains also show sensitivity to growth at 37°C and to osmotic stress, and have abnormal cytoplasmic membranous structures, which are more characteristic of Class C mutants [10,73,74]. Thus Vps15p and Vps34p appear to form a distinct subset of vps gene products [75].

The similarities between the Vps34p and Vps15p phenotypes led to experiments showing that overexpression of Vps34p complements for growth at elevated temperatures in Vps15p-mutant strains containing a single mutation in the Vps15p kinase domain, but not Vps15p-null strains or strains containing triple mutants in the kinase domain [76]. Production of the Vps34p product, PtdIns3P, is abolished in Δvps15 strains or in strains expressing predicted kinase-dead mutants of Vps15p, and strains expressing a temperature-sensitive allele of Vps15p are temperature-sensitive for PtdIns3P production [77]. These results clearly show that a functional Vps15p with an intact kinase domain is required for Vps34p activity in vivo. Vps15p and Vps34p are both membrane-associated in yeast [76]. They also co-immunoprecipitate, and the deletion of Vps15p leads to a loss of Vps34p membrane association [76]. Interestingly, a native kinase domain of Vps15p is also required for its binding to Vps34p, point mutations at residues predicted to abolish kinase activity, based on homology with other serine/threonine kinases, led to a marked inhibition of Vps15p–Vps34p co-immunoprecipitation [77]. More recent analysis of the Vps34p–Vps15p interaction identified residues 837–864 in the C-terminus of Vps34p as sufficient to bind to the kinase and HEAT domains of Vps15p [24].

Although Vps15p is a protein kinase that binds Vps34p and whose putative kinase activity is required for Vps34p activity, Vps15p does not appear to be a Vps34p kinase. Vps15p is phosphorylated in yeast, but the preponderance of this activity is minimally affected by partial dephosphorylation near its putative kinase activity, Vps15p does not appear to be a Vps34p kinase. Vps34p co-immunoprecipitation [77]. The authors note that PtdIns3P levels are higher in the kinase-dead vps15 strain than in the Δvps15 strain, and suggest that the different phenotypes may reflect a threshold level of PtdIns3P required for sorting. Alternatively, these results may suggest that the critical role of Vps15p involves targeting of Vps34p as opposed to its activation.

Indeed, the in vitro lipid kinase activity of Vps34p extracted from wild-type and Δvps15 yeast has not been compared, and the activity of mammalian hVps34 was increased only 2-fold by complex formation with hVps15 [29]. On the other hand, siRNA knockdown of hVps15 in mammalian cells leads to a decrease in hVps34 protein levels (Y. Yan and J.M. Backer, unpublished work), suggesting that hVps15 may be required for hVps34 stability. The precise role of Vps15 in the regulation of Vps34 is still uncertain.

It is not clear why the same mutations that disrupt the protein kinase activity of Vps15p (as measured by its autophosphorylation) also weaken or abolish its binding to Vps34p [77]. It is possible that binding of Vps15p and Vps34p in yeast requires Vps15p-mediated phosphorylation of an as yet unidentified accessory protein. Vps34p and Vps15p do exist in complexes with other proteins (discussed below), but the deletion of these accessory proteins does not disrupt the Vps34p–Vps15p association in vivo [78]. Furthermore, direct Vps34–Vps15 binding was detected in a two-hybrid assay [24]. Similarly, recombinant mammalian hVps34 and hVps15 do directly associate in vitro [29], although the requirement for an intact hVps15 kinase domain for this interaction has not been tested.

Interestingly, Vps15p has not been formally proven to possess protein kinase activity. A comparison of the hVps15 sequence with that of other protein kinases shows significant differences; hVps15 lacks, for example, the canonical GXGXXG motif involved in ATP binding [79] (the sequence is GSTRFF in hVps15). The major evidence that Vps15p is a protein kinase comes from experiments in which point mutations predicted to disrupt kinase function also abolish Vps15p autophosphorylation [28,69]. However, since the Vps15p mutants were immunoprecipitated from yeast, and given that the same point mutations are known to abolish interactions between Vps15p and other proteins (e.g. Vps34p [77]), it remains possible that in vitro phosphorylation of Vps15p is in fact due to a co-purifying kinase. This kinase would presumably be present in sub-stoichiometric amounts, and might not have been detected in parallel immunoprecipitations from [35S]methionine-labelled yeast [69]. Similarly, the kinase activity of yeast Vps15p towards exogenous substrates has never been demonstrated, and the kinase activity of mammalian hVps15 has only been assayed in a complex with hVps34 [29]. Like yeast Vps34p, mammalian
hVps34 has protein as well as lipid kinase activity [19], so the identity of the active kinase(s) in this latter experiment is not clear.

**SIGNALLING BY Vps34: PtdIns3P-BINDING DOMAINS**

Vps34 signalling is presumed to be mediated by the membrane recruitment of proteins containing modular domains that bind to PtdIns3P. These domains included FYVE domains, zinc-finger domains named for the first four proteins known to contain the domain [Fab1p, YOTB, Vac1p, EEA1 (early endosomal antigen 1)] [80] and PX domains, named for the Phox homology domain of the p47phox and p40phox subunits of the phagocyte NADPH oxidase [81]. FYVE and PX domain proteins have been extensively reviewed [82–86] and will not be discussed at length here. FYE-domain containing proteins that regulate scaffolding and/or sorting steps in the endosomal system include EEA1 [87–89] and Hrs (hepatocyte-growth-factor-regulated tyrosine kinase substrate)/Vps27 [90–92]. The PtdIns3P 5-kinase Fab1/PIKfyve, which contains a FYVE domain, is involved in endosome/Golgi sorting and late-endosomal trafficking [93–98]. Whereas most of these proteins have been localized to endosome-related structures, Ally, a FYVE-domain-containing protein involved in autophagy, is localized in the nuclear envelope rather than in endocytic vesicles [99]. This may be related to the observation that disruption of the Vps34 gene in C. elegans (let-512) alters the morphology of the nuclear membrane [17]. PX-domain-containing proteins regulated by PtdIns3P include the phagocyte NADPH oxidase, via its p47phox and p40phox subunits [100–102], the sorting nexins SNX2, SNX3, SNX4 and SNX16 [103–106], the t- (target) SNARE (soluble N-ethylmaleimide-sensitive fusion protein-attachment protein receptor) Van7 [107], the CISK (cytokine-independent survival protein kinase) protein kinase, which is related to the Akt kinase [108,109], and the PX-domain-containing RGS (regulator of G-protein signalling) protein, RGS-PX1, a Gαs-specific GAP (GTPase-activating protein) that inhibits EGF receptor degradation when overexpressed [110].

**Vps34 IN THE ENDOCYTIC SYSTEM**

**Sites of Vps34 action in the endosomal system**

The discovery that yeast Vps34p is a PtdIns3-specific PI3K [11,12] led to numerous papers showing inhibition of vesicular trafficking in mammalian cells by PI3K inhibitors such as wortmannin (reviewed in [111]). However, it was not clear from these studies which PI3K isofrom was responsible. The late 1990s saw the identification of mammalian hVps34 and hVps15 to enlarged endocytic vesicles [87], and the identification of FYVE domains [57] will also inhibit signalling by Class II PI3Ks, which produce PtdIns3P in the plasma membrane [21,62]. Specific targeting of MTM1 to the early endosome [117] may be less affected by this problem. Inhibition of PtdIns3P production using hVps34 inhibitors (antibodies, kinase-dead mutants or drugs, when available) and hVps34 knockdown will block both PtdIns3P signalling as well as any signalling by the protein kinase activity of hVps34. The knockdown strategy also alters the composition of multiprotein complexes containing beclin-1 and hVps15 (see below), which could have additional effects on hVps34-independent functions of these proteins. An additional complication is that PI3K inhibitors increase the activation level of Rab5 in phagosomes and only block a subset of Rab5-dependent events [118]. Secondary effects of hVps34 inhibition due to enhanced Rab5 signalling in the early endosome might be expected to spare early endosomal trafficking events relative to events in the late endosome or multivesicular body.

Finally, it should be noted that some effects of hVps34 inhibition might be due to a decrease in the availability of PtdIns3P as a substrate for PIKfyve/Fab1, a PtdIns3P-5-kinease [94,119,120]. PIKfyve/Fab1 has been independently implicated in the maintenance of endosome and lysosome/vacuole morphology [119,121,122], the regulation of endosome-to-Golgi trafficking [95] and delivery of ubiquitinated cargo to the lysosome/vacuole lumen [98,119,123,124].

**Mechanism of Vps34 action in the endosomal system**

A model for the regulation of Vps34 in the early endosome came from the identification of mammalian hVps34 and hVps15 in the eluate from a RAB5–GTP affinity column [39]. In vitro experiments showed direct GTP-dependent binding of Rab5 to human hVps15 and hVps15–hVps34 dimers, but not to hVps34 itself. Rab5 binding requires the HEAT and WD40 domains of human hVps15 [71]. These experiments suggest that hVps34 is recruited to Rab-positive early endosomes through Rab5–Vps15 interactions. This model was validated in cell culture, where constitutively active Rab5 leads to the recruitment of endogenous or overexpressed hVps34 and hVps15 to enlarged EEA1-positive endosomes in HeLa cells [71]. The finding that overexpressed hVps34 is recruited to endosomes without co-expression of hVps15 was explained by the fact that hVps15...
is in excess compared with hVps34 in HeLa cells. Interestingly, a net movement of hVps34 or hVps15 from cytosolic to membrane compartments was not observed in cell fractionation studies [71], suggesting that the Rab5 induces a redistribution of hVps34–hVps15 between membrane compartments, rather than a direct recruitment from cytosol to membrane.

Overall, the model provides an attractive explanation for the specificity of Rab5 signalling in the early endosome, since Rab5 effectors such as EEA1 are recruited both directly, by binding to GTP–Rab5, and indirectly, by recruitment of hVps34–hVps15 and subsequent production of PtdIns3P [39,71,89] (Figure 2). Two other early endosomal regulatory proteins, Rabenosyn-5 and Rabankyrin-5, share the ability to bivalently interact with both Rab5 and, via FYVE domains, with PtdIns3P [125,126]. The assembly of these Rab- and PtdIns3P-binding proteins is thought to regulate early endosomal docking and fusion. Thus EEA1 interacts with syntaxin 6 and syntaxin 13, endosomal SNARE proteins that regulate vesicle docking/priming [127,128]. Rabenosyn-5 binds to hVps45 [126], which is related to Sec1 proteins that are negative regulators of SNARE pairing [129]. The endosomal role of Rabankyrin-5 is not clear, and it may play a more important role in the regulation of pinocytosis [125].

hVps34 and hVps15 also interact with Rab7 in late endosomes [130]. Unlike Rab5, interactions with Rab7 are strongest with wild-type or nucleotide-free (N125I) forms, as opposed to activated (Q76L) or inhibitory (T22N) forms. Accumulation of PtdIns3P in late endosomes, based on GFP–FYVE staining, is decreased in cells expressing dominant-negative Rab7 mutants, suggesting a role for Rab7 in maintaining hVps34 activity in the late endosome.

The last few years have seen rapid progress in dissecting the role of PtdIns3P signalling in ubiquitin-dependent sorting of internalized membrane proteins in the multivesicular body; this work has been extensively reviewed (see for example [131]) and will be briefly summarized here. The importance of ubiquitination during endocytic sorting was first discovered in yeast [132,133], and the ubiquitin-dependent endocytic sorting of the EGF, CSF-1 (colony-stimulating factor 1) and other receptors have been reported in mammalian cells (reviewed in [134–136]). The degradative sorting of internalized ubiquitinated cargo requires the sequential engagement of the ESCRT (endosomal sorting complex required for transport) complexes I, II and III [4,137,138], and the eventual invagination of the ubiquitinated membrane proteins into an intraluminal vesicle. A key regulator of this process is Vps27/Hrs, which contains both a FYVE domain and an UIM (ubiquitin-interacting motif) and is itself ubiquitinated [139]. Hrs/Vps27 interacts with endosomal PtdIns3P, with ubiquitinated cargo, and with ESCRT I, thereby initiating the assembly of the ESCRT complex on to endosomal membranes [92,140,141]. In addition, one of the components of ESCRT III, Vps24, binds to PtdIns(3,5)P2 [142]. Thus Vps34 plays a key role in the recruitment of ubiquitin to ESCRT, and consequently to the ubiquitinated cargo.

Finally, Vps34 is required for retrograde endosome-to-Golgi transport, a process that requires the assembly of Vps34p, Vps29p, Vps26p, Vps17p and Vps30p into what is termed the retromer complex [143]. This pathway is required for the retrieval from endosomes to the Golgi of Vps10p, the yeast homologue of the mammalian mannose 6-phosphate receptor [144]. Another protein required for Vps10p retrieval is Vps30p [145], which was subsequently shown to be present in a Vps34p–Vps15p–Vps30p–Vps38p complex involved in vesicular trafficking ([18]; discussed below). This led to the finding that the Vps34p–Vps15p–Vps30p–Vps38p complex was required for retromer assembly, presumably by the PtdIns3P-mediated recruitment of the PX-domain-containing proteins Vps5p and Vps17p [146]. Results from mammalian cells suggests that hVps34 might also influence the endosome-to-Golgi sorting by providing substrate for PIKfyve/Fab1, a PtdIns3P 5-kinase [94,119,120], which has been shown to regulate this pathway [95].

**Vps34 AND AUTOPHAGY**

Vps34 is essential for macroautophagy, a process by which cells degrade cytosolic content by the formation of a double-walled vesicular structure that eventually fuses with lysosomes [147,148]. Macroautophagy is a physiological response to nutrient deprivation, and has also been implicated in innate immunity, development, tumour suppression and clearance of neuronal aggregates [149–151]. Whereas autophagy is activated by nutrient deficiency, it is inhibited by nutrient sufficiency. In yeast, this is mediated by the nutrient-stimulated activation of the TOR protein kinase, which leads to the phosphorylation and inactivation of components of the autophagy pathway [147,148]. TOR is also an important regulator of cell growth, via its effects on transcription, protein synthesis and ribosome biogenesis (discussed below).

Autophagy has been most intensively studied in yeast, where mutants of autophagy-related Atg genes have provided important experimental tools [152]. A number of yeast vps genes are also required for autophagy, including VPS34 and VPS15. In
involved in pheromone signalling may also include Atg14p, which would function as a G
of Atg14p, presumably due to decreased stability.

deletion of any of these proteins resulted in decreased expression
Atg14p interacts with both Vps34p–Vps34p and Vps30p, since
of Vps30p with Vps34p–Vps15p. However, it was proposed that
interacts with both Vps34p–Vps34p and Vps30p, since
deletion of any of these proteins resulted in decreased expression

mammalian cells, hVps34 is also involved in nutrient signalling
to mTOR [5,6]; this is discussed below.

Tetrameric Vps34 complexes in yeast

A major advance in Vps34 signalling was the identification
by Ohsumi and co-workers of two distinct Vps34p–Vps15p-
containing complexes in yeast [8]. Both contain Vps34p, Vps15p
and a third protein, Vps30p/Atg6p, which had previously been
identified as being required for autophagy [153]. The complexes
were in fact identified by MS analysis of proteins isolated from
an anti-Vps30p antibody column. Additional analysis revealed
the presence of two other proteins, Vps38p and Atg14p; these
proteins are present in mutually exclusive complexes, based on
their ability to co-immunoprecipitate with Vps34p, Vps15p and
Vps30p but not with each other. Interestingly, the complexes are
functionally distinct. Deletion of VPS38 inhibits sorting of the
lysosomal hydrolase CPY (carboxypeptidase Y), but has no effect
on starvation-induced autophagy, whereas deletion of ATG14
inhibits autophagy, but has no effect on CPY sorting. Deletion
of VPS30 causes a loss of both CPY sorting and autophagy, but
does not affect the processing of newly synthesized protease
A or protease B (which occurs upon lysosomal delivery). In contrast, deletion of VPS34 or VPS15 blocks autophagy, CPY
and protease A/B processing, and also leads to decreased growth at
37°C. These results were the first indication of multiple Vps34p-
containing complexes that have distinct intracellular functions,
presumably by the differential regulation and/or targeting of
Vps34p (Figure 3).

The organization of the complex was deduced by co-immuno-
precipitation experiments in deletion strains lacking each com-
ponent [8]. Co-immunoprecipitation of Vps30p with Vps34p–Vps15p is markedly decreased in a Δvps38 strain, whereas co-
immunoprecipitation of Vps30p with Vps38p is unaffected by
deletion of VPS34 or VPS15. These results suggest that Vps38p
serves as a bridge between Vps34p–Vps15p and Vps30p. The
reciprocal experiment with Atg14p was less informative, as Atg14p
is expressed at low levels relative to Vps38p, making it difficult
to detect the effects of its deletion on co-immunoprecipitation
of Vps30p with Vps34p–Vps15p. However, it was proposed that
Atg14p interacts with both Vps34p–Vps34p and Vps30p, since
deletion of any of these proteins resulted in decreased expression
of Atg14p, presumably due to decreased stability.

In yeast, deletion of either ATG6/VPS30 or ATG14 blocks the
recruitment of Atg5p and Atg8p to a PAS (pre-autophagosomal
structure) that also contains Atg1p, Atg2p and Atg16p [154].
The presumed mechanism of this inhibition is either a defect in
vesicular trafficking leading to the missorting of PAS components
or a loss of PtdIns3P production in the nascent PAS, with
subsequent defects in the recruitment of later autophagic effector
proteins. These may include Tfr1, which is involved in the Cvt
(cytosol-to-vacuole) degradative pathway in yeast [155]; Ef1a
lacks FYVE or PX domains yet interacts with PtdIns3P via a basic
motif. Two additional components of the Cvt pathway, Cvt13 and
Cvt20, contain PtdIns3P-binding PX domains that are required
for their association with the pre-autophagosome [156]. However, the
mechanisms by which Atg6/Vps30–Vps34 complexes and the
production of PtdIns3 regulate autophagy are still incompletely understood.

Figure 3 Vps34p signalling complexes in yeast

Tetrameric complexes containing Vps15p, Vps34p, Vps30p/Atg6p and either Atg14p or Vps38p have been identified in yeast, and regulate vacuolar protein sorting and autophagy. Deletion of VPS34 or VPS15 causes additional trafficking phenotypes not seen with deletion of VPS30, ATG14 or VPS38, suggesting the existence of additional complexes. The Vps34p–Vps15p–Gpa1 complex involved in pheromone signalling may also include Atg14p, which would function as a Gγ protein.

Regulation of hVps34 in mammalian autophagy: beclin, Bcl2 and
UVRAG

Of the Vps34p–Vps15p-associated proteins discovered in yeast,
mammalian homologues have not been identified for either
Atg14p or Vps38p. The two proteins contain coiled-coil regions
but do not contain other structurally defined domains, and it is not
clear whether these proteins are needed for hVps34-dependent
functions in higher eukaryotes.

The mammalian homologue of Atg6p/Vps30p, beclin-1,
was first isolated as a Bcl2-interacting tumour suppressor in
mammalian cells [157,158]. The 450-amino-acid mammalian
protein contains a nuclear export signal [159], distinct Bcl2-
binding and coiled-coil domains, and a structurally unchar-
acterized region [the ECD (evolutionarily conserved domain)]
that is highly conserved among beclin zoologues (Figure 1)
[66]. In cells overexpressing both epitope-tagged beclin-1 and
mammalian hVps34, co-immunoprecipitation of the two species
is readily observed [66]. The overexpressed proteins greatly
exceed the amount of endogenous hVps15 or any potential
Vps38p homologue; these results suggest that, unlike yeast
Vps30p/Atg6p, beclin-1 can bind directly to mammalian hVps34.
This binding is mediated by the coiled-coil domain and ECD of
beclin, binding to the C2 domain of hVps34 [66,161]. Cross-
linking studies have suggested that 50% of mammalian hVps34

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is bound to beclin-1, as compared with 20% of Vps34p bound to Vps30p/Atg6p in yeast [162].

As mentioned above, Atg6p/Vps30p plays distinct roles in vesicular trafficking compared with autophagy in yeast [8]. Beclin-1 is also required for normal vesicular trafficking in C. elegans, where its knockout leads to a loss of PtdIns3P-rich vesicular structures and inhibition of GFP–vitellogenin uptake by oocytes [17]. Although beclin-1 and hVps34 were shown to co-localize in the trans-Golgi network in HeLa cells [162], the role of beclin-1 in vesicular trafficking in mammalian cells may be limited. Recent studies suggest that processing of newly synthesized cathepsin D in the mammalian lysosome is normal in cell lines that express little beclin, or in beclin-knockdown cells [66, 164]. Fluid-phase endocytosis, EGF receptor endocytosis and degradation, and maintenance of normal Golgi and endosome/lysosome morphology are also normal in beclin-1-knockdown cells [164]. In contrast, stable knockdown of hVps34 inhibits EGF receptor trafficking and cathepsin D processing [116]. Thus the trafficking functions of Vps30p/Atg6p/Beclin-1 in yeast and C. elegans may not be retained in higher organisms.

In contrast, beclin-1 is required for autophagy in both C. elegans and mammals [165–168]. Beclin-dependent autophagy is inhibited by overexpression of Bcl2, which also causes a decrease in beclin-1–hVps34 binding [169]. Thus the inhibition of autophagy by Bcl2 may be in part via a disruption of hVps34–beclin-1 interactions. The authors propose that the relative balance of beclin–Bcl2 compared with beclin–hVps34 complexes defines a continuum between apoptotic cell death, survival with normal levels of autophagy, and cell death due to hyperautophagy [169]. Bcl2 may also be involved in the regulation of autophagy by nutrients, as Bcl2–beclin-1 binding is inhibited by starvation [169]. A decrease in Bcl2–beclin-1 binding in starved cells would presumably lead to an increase in beclin-1–hVps34 binding, thereby promoting autophagy. Liang et al. [161] identified the UVRAG (UV radiation resistance-associated gene) tumour suppressor as another beclin-1-binding partner that regulates hVps34 [161]. UVRAG is a 699-amino-acid protein that is frequently mutated in human colon cancer and contains an N-terminal proline-rich domain as well as C2 and coiled-coil domains [170–173] (Figure 1). Cross-immunoprecipitation experiments demonstrated the presence of beclin-1–Bcl2–hVps34–UVRAG complexes; expression of isolated domains were used to show that the coiled-coil domains of beclin-1 and UVRAG mediate their binding, independently of beclin-1 binding to Bcl2. Expression of the isolated coiled-coil domain of UVRAG inhibits autophagy, presumably by disrupting beclin-1–UVRAG complexes. Similarly, autophagy is reduced by UVRAG knockdown.

Whereas Bcl2 binding to beclin is decreased in nutrient-starved cells [169], UVRAG binding to beclin is unaffected by starvation [161]. Moreover, increased UVRAG expression leads to an increase in the amount of beclin-1–associated hVps34, and also leads to an increase in the lipid kinase activity of overexpressed hVps34 in cells co-transfected with beclin-1. This activation requires UVRAG–beclin binding, as it is not observed with a binding-defective UVRAG mutant. These findings led to a model in which beclin-1–associated hVps34 is negatively regulated by Bcl2, and positively regulated by UVRAG. Under nutrient-replete conditions, Bcl2 binding would balance the effects of UVRAG and reduce the amount of beclin-associated hVps34 activity. Under conditions of nutrient starvation, dissociation of Bcl2 would cause a loss of its inhibitory input on beclin-1–associated hVps34 activity. In this case, the continued tonic positive input from UVRAG would result in an increase in beclin-1–associated hVps34 activity. It is not yet clear whether Bcl2 acts primarily to inhibit hVps34 binding to beclin-1 [169] or to inhibit the activity of the hVps34–beclin-1–UVRAG complex [161].

Recently, several other UVRAG-binding proteins have been implicated as regulators of autophagy that modulate the hVps34–beclin-1–UVRAG complex (Figure 1). The 1300-amino-acid protein Ambra1, originally identified as a gene involved in neural development, interacts with beclin-1 in a two-hybrid screen, and overexpressed Ambra1 binds to overexpressed beclin-1 via a structurally undefined central domain [174]. Moreover, Ambra1–beclin-1 complexes can be immunoprecipitated with endogenous hVps34. Embryos with homozygous defects in Ambra1 expression show decreased autophagy, and Ambra1 knockdown in cultured cells inhibits autophagy and decreases the amount of endogenous beclin-1–associated hVps34. Thus Ambra1 may regulate autophagy at least in part through its effects on hVps34–beclin-1 binding. In contrast with Ambra1, which interacts directly with Beclin-1, the endophilin family member Bif-1 binds directly to UVRAG [175]. The interaction was demonstrated with endogenous proteins, and subsequent analysis showed that the Bif-1 SH3 (Src homology 3) domain binds to the N-terminal proline-rich domain of UVRAG. Autophagy is inhibited in Bif-1−/− mouse embryonic fibroblasts, and isolated SH3 domains from Bif-1 inhibit autophagy, presumably by competitively inhibiting the formation of endogenous Bif-1–UVRAG complexes. Bif-1 knockdown also reduces the activity of overexpressed hVps34 in both fed and starved cells, and overexpression of the BIF-1 SH3 domain inhibits the activity of hVps34 in starved cells. These results suggest that the role of Bif-1 in autophagy is related in part to an enhancement of hVps34 activity in beclin–UVRAG complexes. Bif-1 may also act on the formation of the PAS by inducing membrane curvature through its BAR (Bin/amphiphysins/Rvs) domain [176].

The identification of UVRAG–Beclin-1–hVps34 complexes is significant, and the results clearly show that these complexes play important roles in autophagy (Figure 4). Taken together [161, 169, 174, 175], the studies suggest a general model in which rates of autophagy can be controlled by the regulated assembly of a UVRAG–Beclin-1–hVps34 complex, whose production of PtdIns3P is critical for formation of the PAS in starved cells. However, with regard to the regulation of hVps34, several aspects of the model have not been tested. First, the model predicts a net increase in beclin-associated hVps34 in nutrient-starved cells. This has been examined in MCF-7 cells overexpressing wild-type beclin, where starvation-induced changes in beclin–hVps34 binding were not observed [5]; the effect of starvation on endogenous hVps34–beclin-1 binding has not been examined. Similarly, this model predicts an increase in the activity of beclin-associated hVps34 activity in nutrient-starved cells. This has been addressed in two studies. Byfield et al. [5] showed a decrease in the specific activity of hVps34 in FLAG–beclin immunoprecipitates from starved MCF-7 cells, consistent with a decrease in total hVps34 activity; the decrease was apparent within 15 min, and reached approx. 50% after 4 h of starvation. In contrast, Tassa et al. [177] concluded that beclin-associated hVps34 activity increases transiently in starved C2C12 cells. However, the specificity of the lipid kinase assay used in this study is unclear, as the beclin-associated activity was insensitive to 50 nM wortmannin, a concentration of inhibitor that fully inhibits hVps34 [19] and blocks starvation-induced proteolysis [177].

To fully evaluate the UVRAG model, it will be necessary to determine what proportion of cellular beclin-1 and hVps34–beclin-1 complexes are bound to UVRAG. The differential regulation of a subset of beclin–hVps34 complexes through their association with UVRAG could be critical for autophagy, and the regulation of hVps34 activity in UVRAG–beclin-1 complexes

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The regulation and function of Class III PI3Ks

**Figure 4** hVps34 signalling complexes in mammalian cells

A summary of mammalian complexes involving hVps34; the intracellular locations of these complexes have not been determined and are likely to be distinct. Complexes of hVps34 with beclin-1, UVRAG and Bif-1 or hVps34, beclin-1 and Ambra1 have been observed; it is not yet clear whether these are distinct complexes or variants of the UVRAG–beclin-1 complex. Bcl2 binding to beclin-1 is nutrient-regulated and may modulate the binding of hVps34 to the beclin-1–UVRAG complex. The presence of hVps15 in these complexes has not yet been confirmed. It is not yet known whether signalling by hVps34–hVps15 to mTOR requires additional binding partners. Binding of hVps34–hVps15 to Rab proteins and MTM1 in endosomes is mutually exclusive.

**REGULATION OF Vps34 BY NUTRIENTS AND ITS ROLE IN mTOR SIGNALLING**

The TOR protein kinase is a central regulator of protein synthesis and cell growth in eukaryotes [178,179]. In both yeast and mammalian cells, TOR is present in two functionally distinct multiprotein complexes [180–182]. In mammalian cells, TORC1 (TOR complex 1) is sensitive to cellular nutritional state and regulates protein synthesis via the activation of a downstream kinase, S6K1 (S6 kinase 1), and the inhibition of an inhibitor of cap-dependent translation, 4E-BP1 (eukaryotic initiation factor 4E-binding protein 1) [183–187]. TORC2 (TOR complex 2) does not respond to changes in nutritional conditions, and has been implicated in cytoskeletal regulation as well as the regulatory phosphorylation of Akt at the Ser473 site [188,189]. The TORC1 is regulated by insulin and by nutrients, including glucose and amino acids, as well as a variety of cellular stresses [179,185,190,191]. The insulin-responsive inputs to mTOR include Akt-mediated inhibition of the TSC1–TSC2 dimer (where TSC is tuberous sclerosis complex), which is a GAP for the Rheb GTPase [190,192–195]. Rheb is required for activation of mTOR by insulin and directly binds to mTOR [185]. Nutrient inputs to mTOR include AMPK (AMP-activated kinase), whose activation by decreasing ATP/AMP ratios in glucose-starved cells leads to inhibition of mTOR [195–197]. The mechanism of amino acid regulation of mTOR is not known.

Recent studies have shown that hVps34 contributes to the regulation of mTOR by nutrients (Figure 5). Inhibition of hVps34 by microinjection of inhibitory antibodies, overexpression of FYVE domains to sequester PtdIns3P or siRNA-mediated knockdown of hVps34 expression blocks insulin-stimulated phosphorylation of both S6K1 and 4E-BP1 [5,6]. hVps34 knockdown also blocks amino acid stimulation of S6K1 [6]. Conversely, overexpression of hVps34 activates S6K1 in the absence of insulin stimulation. Given that hVps34 is not inhibited by the TORC1 inhibitor rapamycin [5], these results suggest that hVps34 is upstream of mTOR.

If hVps34 is upstream of mTOR, which of the inputs to mTOR does it regulate? hVps34 activity is not regulated by insulin, and knockdown of hVps34 does not inhibit Akt or block insulin-stimulated phosphorylation of TSC2 [5,6]. Consistent with a role for hVps34 in the nutrient input to mTOR, Byfield et al. [5] showed that mammalian hVps34 is inhibited by amino-acid deprivation. Conversely, addition of amino acids to starved cells leads to an increase in hVps34 activity in an immune complex assay, and an increase in intracellular PtdIns3P as detected by anti-PtdIns3P staining [6]. These studies are in disagreement with earlier study that measured total hVps34 activity in starved C2C12 cells [177]. However, the methods used to measure hVps34 in this study were indirect, and included (i) assays of total lipid kinase activity
hVps34 is inhibited by glucose and amino acid starvation or by AMPK activation, and is required for insulin-stimulated mTOR activation under nutrient-replete conditions. These results suggest that hVps34–hVps15 is upstream of mTOR and inhibited by AMPK. These results lead to the hVps34 paradox, shown in blue: hVps34 plays opposing roles in nutrient sensing, as a positive effector of autophagy, and as a positive effector of mTOR, which inhibits autophagy.

remaining in p85/p110-depleted lysates, despite the fact that such lysates still contain Class II PI3Ks and highly abundant PI4K, and (ii) in vitro lipid kinase activity of hVps34 after purification with an inhibitory antibody, but without elution of the enzyme prior to assay.

Studies in GRC LR+73 cells [a CHO (Chinese-hamster ovary) cell derivative [198]] also showed a decrease in hVps34 activity after incubation in glucose-free medium, even in the presence of amino acids [5]. Glucose starvation is known to activate AMPK [196,197], and treatment of cells with pharmacological activators of AMPK [the energy poison oligomycin and the AMP analogue AICAR (5-amino-4-imidazolecarboxamide riboside)] inhibit hVps34 activity [5]. This latter finding has been questioned by Meley et al. [199] in a study showing that the previously described inhibition of autophagy by AICAR [200] was probably due to off-target effects of the drug; the authors suggest a similar explanation for the effects of AICAR on hVps34. However, preliminary results suggest that the inhibition of hVps34 activity in glucose-starved cells is blocked by infection with a dominant-negative AMPK adenovirus (M. Byfield and J.M. Backer, unpublished work), suggesting that AMPK is in fact a negative regulator of hVps34.

Interestingly, the phenotypes of hVps34 siRNA knockdown as opposed to mTOR inhibition by rapamycin are not identical with regard to S6K1. Whereas Thr<sup>389</sup> phosphorylation is blocked in hVps34 siRNA-treated cells, other insulin-stimulated phosphorylation sites are unaffected, as measured by a gel-shift (slower electrophoretic migration) or by immunoblotting with anti-(phospho-Thr<sup>389</sup>/Ser<sup>392</sup>) antibodies [5]. In contrast, this selectivity for the Thr<sup>389</sup> site is seen after brief treatment of cells with rapamycin, but longer treatment leads to a complete loss of the insulin-stimulated gel shift and loss of other phosphorylation sites [5,201]. Thus inhibition of mTOR has more pleiotropic effects on S6K1 phosphorylation than does inhibition of hVps34. Finally, it should be noted that to date the published results use the phosphorylation of mTOR substrates as a readout for mTOR activity [5,6]; a direct effect of hVps34 on mTOR kinase activity has not been demonstrated.

The finding that hVps34 is a positive regulator of mTOR signalling that is inhibited by starvation presents a paradox (Figure 5). Vps34p and hVps34 are required for starvation-induced autophagy in yeast and mammalian cells [9,148,177,202], whereas TOR is an inhibitor of autophagy in yeast and mammals [203,204]. These results would predict that hVps34 activity should be activated by starvation, and that its activity would be inversely correlated with that of mTOR. In fact, hVps34 is inhibited by starvation, and its activity is positively correlated with mTOR activity [5,6]. However, it is important to note that studies in Drosophila show that dS6K (Drosophila S6K) plays a similarly paradoxical role with regard to autophagy and mTOR signalling: dS6K is positively regulated by mTOR, yet autophagy is blocked in dS6K<sup>−/−</sup> larvae [205]. Thus two distinct proteins in the mTOR pathway are required for autophagy, yet are inhibited by starvation and activated under conditions that activate TOR and inhibit autophagy. Neufeld and co-workers suggest that inhibition of dS6K by starvation might serve to limit cellular damage due to excessive autophagy [205]. Additional studies will be required to determine if this rationale also applies to hVps34.

**hVps34 AND PHAGOCYTOSIS**

hVps34 plays an important role in the regulation of phagocytosis. Studies from the groups of Grinstein [41] and Deretic [45] showed that hVps34 is not required for the engulfment of opsonized particles by macrophages, which in fact requires Class IA PI3Ks [206], but is required for phagosomal maturation and fusion with late endosomes/lysosomes. The accumulation of PtdIns3P in nascent phagosomes is transient, beginning after closure of the phagocytic cup and declining within 5–10 min; additional waves of PtdIns3P accumulation are seen at later
times [41,207]. Nascent phagosomes also contain activated Rab5, which may mediate the recruitment of hVps34 to these structures immediately after closure [118]. In contrast, the later waves of PtdIns3P accumulation are blocked by inhibitors of calmodulin and calmodulin kinase II (see below) [207]. It is not yet clear whether these increases in PtdIns3P are caused by a translocation of hVps34 as opposed to an increase in hVps34 specific activity.

*Mycobacterium tuberculosis* evades destruction by macrophages by inhibiting recruitment of EEA1 to phagosomes, thereby inhibiting phagosome maturation and fusion with lysosomes. [45]. Infection of macrophages with *M. tuberculosis* also blocks increases in cytosolic calcium that normally accompany phagocytosis of opsonized particles [208]; this inhibition has been attributed to either the production of a toxin, LAM (lipoarabinomannan) [209], or inhibition of sphingosine kinase [210]. Interestingly, Deretic and co-workers suggested that increases in cytosolic calcium might also regulate hVps34, since hVps34–hVps15 complexes bind to Ca\(^{2+}\)/calmodulin beads, and treatment of macrophages with the calmodulin inhibitor W7 blocks phagosomal accumulation of EEA1 and PtdIns3P [209]. However, later studies showed that *M. tuberculosis* secrete a lipid phosphatase, SapM, that hydrolyses PtdIns3P in the phagosome and may account for the loss of EEA1 binding in infected macrophages [211].

The effects of Ca\(^{2+}\)/calmodulin inhibitors on PtdIns3P production in macrophages may be due to modulation of hVps34 targeting rather than hVps34 activity, since treatment of macrophages with W7 has no effect on hVps34 activity measured in immunoprecipitates (M. Byfield and J.M. Backer, unpublished work). These effects are also apparently cell-type-specific [207], since calmodulin inhibitors have no effect on endosomal PtdIns3P levels or hVps34 activity in COS-7 cells, but do inhibit EEA1 association with endosomes in *vivo* and EEA1 binding to PtdIns3P-containing membranes *in vitro* [49]. This latter study proposed that Ca\(^{2+}\)/calmodulin helps to stabilize the structure of the EEA1 C-terminal FYVE domain, presumably via interactions with the IQ domain of EEA1 [212]. hVps34 does in fact contain two predicted non-IQ calmodulin-binding sites [207], since calmodulin inhibitors have no effect on EEA1 binding to PtdIns3P-containing membranes *in vitro* [49]. The physiological significance of calmodulin–hVps34 interactions in cells other than macrophages is not currently clear.

**Vps34 AND MAPK SIGNALLING: REGULATION BY TRIMERIC G-PROTEINS**

A number of studies suggest that mammalian hVps34 may be regulated by interactions with trimeric G-proteins. In HT-29 colon cancer cells, autophagy is inhibited by overexpression of an activated mutated form of G\(_{\alpha}\), but is restored by treatment of cells with synthetic PtdIns3P [9]. In RBL-2H3 basophilic leukaemia cells, antigen-stimulated degranulation is mediated by Fce-receptor signalling to Class I PI3Ks. However, in cells overexpressing the M\(_{\alpha}\), muscarinic receptor, carbachol-stimulated degranulation is blocked by inhibitory antibodies to hVps34 [46], suggesting a link between hVps34 and G\(_{\alpha}\)-coupled receptors.

Experiments in *S. cerevisiae* have provided a direct link between trimeric G-proteins and Vps34p–Vps15p. Dohlmam and co-workers identified both VPS34 and VPS15 in a screen for suppressors of GPA1–mediated transcriptional responses involved in pheromone signalling [7]. Deletion of either VPS34 or VPS15 inhibits \(\alpha\)-factor stimulation of transcriptional responses and MAPK activation, and reduces mating efficiency. Importantly, deletions of VPS30 or VPS38 failed to disrupt GPA1 signalling, nor do other vps mutants, suggesting that Vps34p and Vps15p have signalling functions independent of their role in trafficking and autophagy. Both proteins were shown to bind to Gpa1p. Vps34p bound in the manner of a Gpa1p effector: it exhibited preferential binding to GTPase-deficient mutants (Gpa1pQ323L), and expression of active Gpa1p increased endogenous production of PtdIns3P and caused the recruitment of a PX–GFP fusion protein to endosomes. In contrast, Vps15p bound preferentially to GDP-loaded Gpa1p. The authors propose that Vps15p functions as a G\(_{\beta}\) protein, binding to the G\(_{\alpha}\) Gpa1 through its C-terminal WD40 domains, which are predicted to form a \(\beta\)-propeller [7,71]. The known Vps34p–associated protein Atg14p is proposed to serve as a G\(_{\beta}\) protein, based on sequence homology with known G\(_{\beta}\) proteins, and Atg14p does in fact bind to Gpa1p in a GDP-dependent manner (H. Dohlmam, personal communication). Of note, a G\(_{\beta}\) pair composed of Vps15p–Atg14p would be somewhat atypical, in that membrane targeting is achieved via a myristoylated Vps15p (instead of a prenylated G\(_{\alpha}\)). Furthermore, Atg14p is not required for Vps15p stability (unlike traditional G\(_{\beta}\) pairs), although Vps15p does stabilize Atg14p [8].

**ANTAGONISTS OF VPS34 SIGNALLING: LIPID PHOSPHATASES**

hVps34 signalling is terminated by degradation of PtdIns3P. Two major pathways for PtdIns3P turnover have been elucidated. The first involves the sequestration of PtdIns3P in the internal vesicles of multivesicular bodies, where the lipids are presumably degraded by subsequent fusion of the multivesicular bodies with lysosomes [214]. However, hVps34 signalling is also antagonized by the myotubularin family of lipid phosphatases, whose mutation results in severe genetic diseases affecting skeletal muscle and the nervous system [215,216]. Myotubularin family members resemble tyrosine phosphatases but show activity toward PtdIns3P and PtdIns(3,5)P\(_2\) [217–220]. Interestingly, six out of 14 family members are catalytically inactive; the finding that mutations in the inactive MTM5 and MTM13 cause human disease [221,222] presumably involves the heterodimerization of inactive with active myotubularin isoforms. All myotubularins contain protein tyrosine phosphatase and PH-GRAM (PH glucosyltransferase, Rab-like GTPase activators and myotubularins) domains, and most contain coiled-coil domains. Some isoforms also contain SID (SET-interacting domain), PH and FYVE domains [216,224].

Interestingly, hVps34–hVps15 is found in a complex with MTM1 [225]. Binding is mediated by the WD40 domain of hVps15, which also binds to Rab5 and Rab7 [71,130]. Furthermore, co-immunoprecipitation experiments suggest that hVps15 binding to Rab5 or Rab7 and MTM1 is mutually exclusive. The binding of hVps15 to both activators (Rab5 and Rab7) and inhibitors (MTM1) of hVps34 signalling suggest that fine-tuning the magnitude or duration of endosomal PtdIns3P levels is important for endosomal function.

**hVps34 AND HUMAN DISEASE**

With the exception of two studies suggesting a linkage between mutations in the hVps34 promoter and schizophrenia [226,227], disease-related mutations or changes in hVps34 expression have not been identified in humans. In contrast, mutations in the myotubularin family of PtdIns3P phosphatases are involved
in myotubular myopathy and Charcot–Marie–Tooth neuropathy [215,217], presumably due to a deregulation of PtdIns3P synthesis. Although it is not yet known what cellular functions are disrupted in patients with these disorders, pharmacological inhibition of hVps34 might be useful in restoring normal levels of PtdIns3P.

Nonetheless, hVps34 is strongly implicated in a number of cellular processes that are involved in human disease, which could make hVps34 a candidate target for pharmacological modulation. hVps34 is a key regulator of autophagy, whose role in the immune system, in the clearance of pathological protein aggregations in neurodegenerative disease and in tumour suppression is increasingly clear [228]. For example, up-regulation of autophagy by inhibition of mTOR reduces toxicity in a Huntington’s disease model [229], whereas inhibition of autophagy by ablation of Atg7 in Purkinje cells leads to neurodegeneration [230]. Similarly, knockdown of either beclin-1 or hVps34 blocked the IGF-1 (insulin-like growth factor 1)-stimulated clearance of mutant huntingtin aggregates in HeLa cells [230a]. If pharmacological up-regulation of hVps34 leads to enhanced autophagy, then this might provide an alternative or adjunct approach to the treatment of neurodegenerative disorders. Alternatively, hyperactivation of the mTOR pathway by nutrient excess has been implicated in the development of insulin resistance [231] through the phosphorylation of IRS-1 (insulin receptor substrate 1) [232,233]. hVps34 is required for signalling through the mTOR pathway [5,6], and suppression of hVps34 activity might be useful in the management of insulin resistance in obesity. It is a significant problem that treatment of neurodegeneration compared with obesity/insulin resistance would not be effective in treatment of neurodegeneration [230].

Secondly, recent research has identified functions for Vps34 that extend beyond its well-established role in endocytic trafficking. These include nutrient sensing in the mTOR pathway, and GPCR (G-protein-coupled receptor)-mediated regulation of the MAPK pathways [5–7]. Thus a lingering question is whether these new functions are related to the old ones. That is to say, are the novel functions secondary to Vps34-mediated vesicular trafficking, or do they represent independent Vps34 activities? The results are not yet clear. There are multiple systems in which the endocytosis of signalling receptors plays a role in signalling [237], either by down-regulation of a signalling input (for example, in the CSF-1 receptor system [238]), or in some cases by moving receptors to a compartment that contains a signalling co-factor (for example, the role of the endosomal SARA protein in TGFβ (transforming growth factor β) signalling [239]).

UNANSWERED QUESTIONS IN Vps34 SIGNALLING

The present review has attempted to highlight aspects of Vps34 biology that remain unclear or controversial. First, at the biochemical level, we know surprisingly little about Vps34, and even less about Vps15. Does hVps34 activity in mammalian cells require hVps15 as it does in yeast? Is the Vps34–Vps15 interaction regulated, and if so by what? Is Vps15 a protein kinase, and, if so, what are its substrates? How many Vps34 complexes are there? Current evidence suggests at least three in S. cerevisiae (Vps34p–Vps15p–Vps30p–Atg14p, Vps34p–Vps15p–Vps30p–Vps38p and Vps34p–Vps15p–Gpa1p) [7,8]. Do these complexes also exist in mammalian cells, and are there mammalian homologues of ATG14 and VPS35? Are they related to the UVRAG–beclin-1–hVps34–Vps15 (presumably)–Bif1–Ambral1 complexes [66,161,174,175]? How do distinct Vps34 complexes confer differential signalling: by regulation of Vps34 activity or by differential targeting of Vps34? Granting that there has been disagreement as to whether subsets of Vps34 show increases or decreases in activity during starvation [5,6,177], what is the mechanism of the nutrient regulation of hVps34 activity and hVps34 binding to beclin-1?

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The regulation and function of Class III PI3Ks

Received 17 October 2007/18 December 2007; accepted 18 December 2007
Published on the Internet 29 January 2008, doi:10.1042/BJ20071427

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