Physiological role of Vps34 phosphatidylinositol 3-kinase in mammalian cells

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Doctor of Philosophy in Medical Sciences

Physiological Role of Vps34 Phosphatidylinositol 3-Kinase in Mammalian Cells

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In partial fulfillment of the requirements for the degree of
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PHYSIOLOGICAL ROLE OF VPS34 PHOSPHATIDYLINOSITOL 3-KINASE IN MAMMALIAN CELLS

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MEDICAL COLLEGE OF OHIO

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INTRODUCTION

Mammalian cells contain three distinct classes of phosphoinositide 3-kinases (PI3Ks) (Fruman et al. 1998, Vanhaesebroek et al. 2001). The most widely studied are the Class I enzymes, which play important roles in a variety of receptor-mediated signal transduction pathways (Katso et al. 2001). The Class I enzymes are heterodimers composed of p110 catalytic subunits and p85 or p55 regulatory subunits. Although they can utilize phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PI4P) or phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂) as substrates in cell-free assays, it is generally thought that PI4P and PI(4,5)P₂ are the preferred substrates in vivo (Vanhaesebroeck et al. 2001). The resulting products, phosphatidylinositol 3,4-bisphosphate (PI(3,4)P₂) and phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P₃), function as second messengers and activators of protein kinases containing phosphoinositide-binding domains (Leevers et al. 1999).

The Class II PI3Ks are larger than the Class I enzymes due to the presence of an additional regulatory domain (i.e., C2) (MacDougall et al. 1995, Virbasius et al. 1996). They lack p85-binding domains, and specific adapter subunits have not yet been defined. The Class II PI3Ks preferentially phosphorylate PI and PI4P over PI(4,5)P₂ in vitro (Arcaro et al. 1998). The physiological function of the Class II enzymes remains poorly understood. However, there is some evidence that they localize to clathrin coated vesicles and can be activated by growth factors and insulin (Gaidorov et al. 2001, Brown et al. 1999, Arcaro et al. 2000).
The Class III PI3Ks are unique in that they catalyze the phosphorylation of only PI to generate phosphatidylinositol 3-phosphate (PI3P) (Fruman et al. 1998, Odorizzi et al. 2000). The prototype for this class of enzyme, Vps34, was first identified in *Saccharomyces cerevisiae*, where it is one of several gene products required for delivery of soluble proteins to the vacuole and for autophagic sequestration of cytoplasmic proteins during starvation (Herman and Emr 1990, Herman et al. 1992, Schu et al. 1993, Kihara et al. 2001a). Under both circumstances, association of Vps34 with cellular membranes depends on a myristoylated regulatory subunit, Vps15 (Stack et al. 1993). In mammalian cells, Vps34 interacts with p150, a regulatory subunit similar to Vps15 (Volinia et al. 1995, Panaretou et al. 1997).

Phosphatidylinositol 3-phosphate is required for membrane recruitment of a number of proteins implicated in the regulation of vesicular transport and intracellular protein sorting (Corvera 2001, Gillooly et al. 2001). Some of these proteins contain a structural motif termed the FYVE domain which binds to PI3P with high affinity (Corvera et al. 1999, Fruman et al. 1999, Wurmser et al. 1999). Specific FYVE-domain proteins (e.g., EEA1, Rabenosyn-5, Rabip4) interact with Rab GTPases that control vesicle docking and fusion in the endocytic pathway (Simonsen et al. 1998, Kauppi et al. 2002, Nielsen et al. 2000, Cormont et al. 2001). Interestingly, some Rabs that function in the early (Rab5) and late (Rab7) steps of the endocytic pathway also interact with the p150 subunit of the mammalian Vps34 complex, suggesting that the synthesis of PI3P and the recruitment of FYVE-domain proteins may be coordinately regulated (Christoforidis et al. 1999b, Stein et al. 2003).
Early evidence that PI3Ks are important for protein trafficking in mammalian cells came from studies in which PI3K inhibitors, such as wortmannin, were found to impair targeting of pro-cathepsin D from the trans-Golgi network (TGN) to the lysosomal compartment (Brown et al. 1995, Davidson 1995). Subsequent work using a kinase-deficient dominant-negative form of mammalian Vps34 suggested that the block in cathepsin D maturation was related to a requirement for the Class III PI3K (Row et al. 2001). Separate lines of evidence have suggested that mammalian Vps34 may also be required for receptor sorting in the early endocytic pathway. For example, Siddhanta et al. (1998) reported that microinjection of an inhibitory antibody against Vps34 interfered with ligand-stimulated translocation of the platelet derived growth factor (PDGF) receptor between peripheral early endosomal and perinuclear late endosomal compartments. Further investigations using inhibitory antibodies or wortmannin have suggested that Vps34 may play an essential role in the formation of internal vesicles within multivesicular bodies (MVBs), a key late endosomal sorting compartment between early endosomes and lysosomes (Futter et al. 2001, Gruenberg and Maxfield 1995). Finally, in accord with studies in yeast, the mammalian Vps34 appears to play an important role in the process of macroautophagy in human cells subjected to nutrient deprivation (Petiot et al. 2000).

Although the aforementioned experimental approaches have provided important insights into the functions of Vps34 in mammalian cells, each has particular limitations. For example, wortmannin can simultaneously inhibit multiple classes of PI3Ks and at least one type of phosphatidylinositol 4-kinase, making it difficult to attribute
physiological effects to a specific enzyme (Vanhaesebroek et al. 2001, Meyers and Cantley 1997). Likewise, overexpression of interfering Vps34 mutants may tie up key effectors or docking proteins that normally interact with more than one distinct kinase. Finally, antibody microinjection studies, although precise, can be applied only to small populations of cells, precluding most biochemical analyses of protein trafficking pathways. In recent years the rapid development of methods for stable gene silencing by RNA-interference (RNAi) has provided a powerful new option for defining the functions of specific proteins in mammalian cells (Sui et al. 2002, Paul et al. 2002, Brummelkamp et al. 2002). I propose that utilizing RNAi technology will allow for the role of human Vps34 (hVps34) in protein trafficking to be more narrowly defined. Specifically, it is my hypothesis that suppression of hVps34 expression will impair only the trafficking pathways that absolutely require PI3P generated by hVps34 for normal function.

In the present study, RNAi technology has been utilized to inhibit the expression of hVps34 in cultured U-251 glioblastoma cells. Suppression of hVps34 caused extreme swelling of late endosomes, consistent with defective membrane internalization into MVBs without concomitant reduction of incoming membrane traffic from the TGN and early endosomes. However, in contrast to some earlier findings based on PI3K inhibitors, specific silencing of hVps34 expression did not impair: a) the exit of pro-cathepsin D from the TGN, b) the endocytic uptake of fluid-phase markers, c) the endocytic internalization and recycling of cell surface receptors, or d) the association of a FYVE-domain protein (EEA1) with early endosomes. These studies indicate that the role of hVps34 in producing PI3P for membrane trafficking is limited mainly to the late
endosome compartment, and that other mechanisms may exist to produce PI3P required for vesicular trafficking in the early endosomes and TGN.

In addition to the effects on the late endosome compartment, the hVps34 knockdown (KD) cells exhibited a marked reduction in cell growth over time. This reduced growth rate was accompanied by a considerable block in DNA synthesis. These findings are consistent with a previous study which demonstrated that the introduction of an inhibitory antibody against Vps34 inhibits insulin-stimulated DNA synthesis in GRC-LR+73 cells (Siddhanta et al. 1998). Combined, these results suggest that Vps34 may have a role in DNA synthesis and/or transcription.

Unlike the Class I PI3Ks, mammalian Vps34 has no known function in mitogenic signal transduction. Previous studies have indicated that the levels of PI(3,4)P$_2$ and PI(3,4,5)P$_3$ rise sharply in response to mitogenic stimuli while the levels of PI3P remain constant (Auger et al. 1989, Kapeller et al. 1991). Since the mammalian Vps34 is limited to the production of PI3P, it is surprising that Vps34 activity would be required for mitogenic signaling and DNA synthesis (Siddhanta et al. 1998). However, a recent study in plant cells indicating that Vps34 localizes to active sites of transcription suggests a direct link between Vps34 and nuclear function (Bunney et al. 2000). Based on these findings, it also is my hypothesis that mammalian Vps34 localizes to the nucleus and acutely regulates nuclear PI3P levels that are not detectable when whole cell lipid production is measured.

In order to address the possibility that hVps34 functions in the nucleus as well as in the endocytic pathway, the localization of hVps34 in cultured HEP-2 laryngeal carcinoma
cells was examined. The use of subcellular fractionation and detergent extraction techniques, determined that there are cytosolic, membrane, and nuclear pools of hVps34 in HEp-2 cells. Consistent with this, immunofluorescent localization of hVps34 revealed not only a diffuse endocytic staining pattern, but also a distinct punctate distribution in the nucleus. Further characterization revealed that the nuclear localization of hVps34 is sensitive to RNase extraction and inhibition of transcription with α-amanitin and actinomycin D. Combined, these findings suggest that there is a link between hVps34 function and transcription.

Although Bunney et al. (2000) found that Vps34 localized to active sites of transcription in plant cells, the present study is the first to provide evidence for the nuclear localization of Vps34 in mammalian cells. This localization may provide an explanation for the existence of a nuclear pool of PI3P and the effects of inhibiting hVps34 on DNA synthesis (Gillooly et al. 2000, Siddhanta et al. 1998); however, the exact function of hVps34 in the nucleus remains unclear.
LITERATURE

Phosphoinositides

Phosphatidylinositol, which consists of a 1D-\textit{myo}-inositol phosphate group linked to a diacylglycerol membrane anchor, serves as the fundamental building block for intracellular inositol lipids in eukaryotic cells. The D-\textit{myo}-inositol head group contains five free hydroxyl groups that serve as potential sites for phosphorylation. To date, it is known that PI can be reversibly phosphorylated at the 3, 4, and 5 positions (Fig. 1). Either single or multiple phosphorylation events at these positions can generate up to eight unique PI derivatives, collectively known as phosphoinositides (Vanhaesebroeck et al. 2001).

D3 Phosphorylated Phosphoinositides

Phosphoinositide function is regulated by various enzymes that generate and degrade these phosphorylated lipids (Fruman et al. 1998, Vanhaesebroeck et al. 2001). The PI3Ks represent a family of such enzymes. Specifically, PI3Ks phosphorylate the 3-hydroxyl position of the D-\textit{myo}-inositol head group of three different substrates including PI, PI4P, and PI(4,5)P$_2$ to generate PI3P, PI(3,4)P$_2$ and PI(3,4,5)P$_3$, respectively (Katso et al. 2001). The generation of 3-phosphorylated phosphoinositides influences a diverse array of cellular processes including membrane trafficking, cell growth, apoptosis, signal transduction, and cytoskeletal organization (Toker and Cantley 1997). Phosphatidylinositol 3-phosphate has been implicated in several membrane trafficking events and the levels of this phospholipid remain constant following mitogenic
Phosphatidylinositol can be reversibly phosphorylated at the 3, 4, and 5 positions of its inositol head group.
stimulation (Katso et al. 2001, Vanhaesebroeck et al. 2001). Therefore, the net rate of PI3P synthesis is equal to the net rate of turnover. This reflects the need for PI3P in a continuously active process such as membrane trafficking (Wurms et al. 1999). In contrast, the levels of PI(3,4)P\textsubscript{2} and PI(3,4,5)P\textsubscript{3} rise sharply in response to mitogenic stimuli. This is indicative of their described roles as second messengers in cell signaling cascades (Leevers et al. 1999, Katso et al. 2001, Vanhaesebroeck et al. 2001). Although 3-phosphorylated phosphoinositides function in different cellular processes, common themes of localized signal generation and recruitment of effector proteins appear to underlie their mechanism of action (Martin 1998).

The Family of PI3Ks

Differences in structure, substrate specificity, regulation, and function divide the PI3Ks into 3 separate classes (Vanhaesebroeck et al. 1997a).

Class I PI3Ks

The Class I PI3Ks can phosphorylate PI, PI4P, and PI(4,5)P\textsubscript{2} in vitro. However, agonists that stimulate these enzymes in vivo increase only the levels of PI(3,4)P\textsubscript{2} and PI(3,4,5)P\textsubscript{3}, indicating that PI4P and PI(4,5)P\textsubscript{2} are the preferred substrates (Vanhaesebroeck et al. 2001, Katso et al. 2001). All the PI3Ks from this class form heterodimers consisting of a 110 kDa catalytic subunit (p110) and an adaptor/regulatory subunit (Fruman et al. 1998). The Class I PI3Ks are subdivided into two classes (Class IA and Class IB) based primarily on functional differences associated with the binding of

**Class IA PI3Ks**

Although no Class IA PI3K family members have been found in yeast or plants, a variety of other organisms including *Caenorhabditis elegans*, *Drosophila melanogaster*, *Dictyostelium discoideum*, and mammals possess at least one Class IA PI3K gene (Morris et al. 1996, Leevers et al. 1996, Zhou et al. 1995, Hiles et al. 1992). The mammalian Class IA catalytic subunits include three p110 isoforms (p110α, p110β, and p110δ) which are encoded by three separate genes (Vanhaesebroeck et al. 2001). All of these isoforms contain an N-terminal adapter binding domain, a ras binding domain, and a C-terminal catalytic domain. While p110α and p110β are ubiquitously expressed, p110δ expression is limited to leukocytes (Vanhaesebroeck et al. 1997b). Despite differences in tissue distribution, all p110 catalytic subunits can associate with at least seven known adaptor proteins generated from the expression and alternative splicing of three different genes including p85α, p85β, and p55γ (Fruman et al. 1998, Katso et al. 2001). All of these Class IA adaptor proteins contain two src homology 2 (SH2) domains, which bind specifically to phosphorylated tyrosine residues in receptor proteins (Katso et al. 2001). This phosphotyrosine binding stimulates the p110 catalytic activity and allows the adaptor subunits to recruit cytosolic p110 to its substrates located in the plasma membrane (Vanhaesebroeck et al. 2001). Interaction of p110 with the small G protein ras at the plasma membrane also serves a similar function (Fruman et al. 1998).
Class $I_B$ PI3Ks

The Class $I_B$ PI3K, found only in mammals, is comprised of a $p_{110\gamma}$ catalytic subunit complexed with a 101 kDa adaptor subunit ($p_{101}$) (Stephens et al. 1997, Fruman et al. 1998). This enzyme shows a narrow tissue distribution with expression limited primarily to white blood cells (Vanhaesebroeck et al. 2001). Like the Class $I_A$ PI3K, $p_{110\gamma}$ contains a ras binding domain and associates with ras in vitro (Rubio et al. 1997). However, in contrast to the Class $I_A$ PI3K, $p_{110\gamma}/p_{101}$ activity is unaffected by phosphotyrosine domains. Instead, it has been shown that the Class $I_B$ enzyme is activated by the $\beta\gamma$ subunits of heterotrimeric G proteins (Stephens et al. 1994, Stoyanov et al. 1995, Lopez-Ilasaca et al. 1997, Tang and Downes 1997). Interestingly, Stoyanov et al. (1995) demonstrated that $G\beta\gamma$ subunits could directly activate $p_{110\gamma}$. In contrast, Stephens et al. (1997) reported that the $p_{101}$ adapter binds the $G\beta\gamma$ subunits and this binding is required for optimal $p_{110\gamma}$ activation. Under the latter circumstances, $p_{101}$ plays a role similar to Class $I_A$ adapters in the recruitment of $p_{110\gamma}$ to cellular membranes where its substrates reside (Fruman et al. 1998).

Class $II$ PI3Ks

Class II PI3K genes have been cloned from a variety of organisms including $D. melanogaster$, $C. elegans$, mice, and humans (MacDougall et al. 1995, Molz et al. 1996, Virbasius et al. 1996, Domin et al. 1997). Like the Class I PI3Ks, no Class II family members have been found in yeast or plants (Fruman et al. 1998). Although the Class II enzymes have been shown to preferentially phosphorylate PI and PI4P in vitro, the in vivo substrates have not yet been determined (Arcaro et al. 1998, Domin et al. 1997).
Mammals express three Class II isoforms (PI3KC2α, PI3KC2β, and PI3KC2γ) encoded by three separate genes (Katso et al. 2001). Although the α and β isoforms are ubiquitously expressed, γ expression is limited to the liver (Vanhaesebroeck et al. 2001). The Class II enzymes are large compared to other PI3Ks with molecular weights ranging from 170-210 kDa (Fruman et al. 1998). The defining feature of the Class II enzymes is the presence of a C-terminal C2 domain (Fruman et al. 1998, Vanhaesebroeck et al. 2001). Functionally, C2 domains have been implicated in mediating Ca\(^{2+}\)-dependent binding of proteins to lipid moieties. However, the C2 domain of the Class II PI3Ks lacks critical aspartic acid residues important for coordinating Ca\(^{2+}\) binding. Subsequently, the Class II PI3Ks have been shown to bind lipids in a Ca\(^{2+}\)-independent fashion (MacDougall et al. 1995, Arcaro et al. 1998).

Unlike the Class I PI3Ks which are mainly cytosolic and require an adaptor for membrane recruitment, the Class II enzymes are found predominantly in membrane fractions (Arcaro et al. 1998, Domin et al. 2000). Although no specific adapter subunits have been defined for the Class II enzymes, the membrane distribution may be mediated through the ability to bind clathrin (Gaidarov et al. 2001). The mechanism by which the Class II enzymes are regulated remains unclear. However, some studies have shown that stimulation of cells with epidermal growth factor (EGF), PDGF, or insulin can stimulate the lipid kinase activity in Class II PI3K immunoprecipitates (Arcaro et al. 2000, Brown et al. 1999). Therefore, it is possible that the Class II PI3Ks contribute to the increase in PI(3,4)P\(_2\) following mitogenic stimulation.
**Class III PI3Ks**

The Class III PI3K, Vps34, was first identified in yeast as a protein required for transport of newly synthesized hydrolases from the late Golgi to the vacuole (Herman and Emr 1990). Homologues have since been discovered in *D. discoideum, D. melanogaster*, and mammals (Zhou et al. 1995, Linassier et al. 1997, Volinia et al. 1995). To date only a single, ubiquitously expressed, Vps34 isoform has been identified in humans (Volinia et al. 1995). Vps34 can utilize only PI as a substrate. Therefore, it is postulated that Vps34 is responsible for the generation of most of the PI3P in cells (Fruman et al. 1998). In both yeast and mammals, Vps34 exists in a complex with an N-terminal myristoylated adapter subunit (Vps15 in yeast, p150 in mammals) (Stack et al. 1993, Volinia et al. 1995). This lipid modification serves to target Vps34 to cellular membranes where PI resides (Stack et al. 1993). Since the cellular levels of PI3P remain constant, Vps34 functions primarily to induce local increases in PI3P that regulate agonist-independent membrane trafficking events (Fruman et al. 1998).

**Vps34 is a PI-Specific PI3K Required for Membrane Trafficking in *Saccharomyces cerevisiae***

**Vacuolar Enzyme Sorting**

The secretory pathway has been shown to mediate the modification and delivery of proteins destined for a wide variety of intracellular and extracellular compartments. All proteins competent for entry into the ER, but lacking any additional sorting information, transit to the Golgi and are passively delivered to the cell surface via
membrane-enclosed transport vesicles (Pfeffer and Rothman 1987). Proteins destined for other intracellular compartments require specific sorting or retention signals in order to escape transport to the cell surface (Pfeffer and Rothman 1987). The yeast hydrolases, Proteinase A (PrA), Proteinase B (PrB) and Carboxypeptidase Y (CPY), are soluble enzymes that are segregated away from proteins destined for secretion and are targeted to the yeast lysosome-like vacuole (Mechler et al. 1982, Hasilik and Tanner 1978, Hemmings et al. 1981, Stevens et al. 1982). These enzymes are synthesized in the ER as inactive precursors. The precursors then transit through the Golgi and are targeted to the vacuole where they are processed into active or mature enzymes (Reviewed in Bryant and Stevens 1998).

In order to identify the components required for the specific segregation, packaging, and delivery of such enzymes to the yeast vacuole, various selection schemes were utilized to isolate yeast mutants that missorted inactive vacuolar hydrolases to the cell surface (Bankaitis et al. 1986, Robinson et al. 1988, Rothman and Stephens 1986). This allowed for the identification of a large number of vps (vacuolar protein sorting defective) mutants assigned to at least 47 unique complementation groups. One of these groups consisted of eight independent mutant alleles of the \textit{VPS34} locus (Robinson et al. 1988). Characteristics of yeast within this group include sensitivity to extreme temperatures as well as osmotic stress. Furthermore, yeast possessing a \textit{VPS34} mutation exhibited severe defects in the sorting of PrA, PrB, and CPY to the vacuole without effects on the secretion of other proteins (Robinson et al. 1988). However, the vacuoles within these mutants were morphologically indistinct from those of wild type yeast.
(Banta et al. 1988). Since the VPS34 mutants were competent for vacuole assembly, the VPS34 gene product was implicated in the sorting and delivery of soluble vacuolar hydrolases rather than being a structural component of the vacuole itself (Banta et al. 1988, Robinson et al. 1988, Herman and Emr 1990).

Following the identification of the VPS34 locus, Herman and Emr (1990) successfully cloned the VPS34 gene and began to characterize its protein product. Immunoprecipitation of S. cerevisiae extracts revealed that the VPS34 gene encodes a 95 kDa protein with a half-life of 90 min. Vps34 is considered to be rare in that it was found to account for only 0.01% of the total protein in logarithmically growing yeast. Initial sequencing determined that the protein lacks any apparent N-terminal sorting signal or membrane-spanning domains, suggesting that Vps34 does not itself enter the secretory pathway. Subcellular fractionation showed that 50% of the total cellular Vps34 is associated with membrane fractions. This membrane association could not be disrupted with detergent. However, treatment with 2 M urea readily solubilized the Vps34 from the particulate fraction, implying that Vps34 membrane association is mediated through interaction with other proteins (Herman and Emr 1990).

When Vps34 was initially described, it showed no similarity to any known proteins (Herman and Emr 1990). However, in 1992, identification of the p110 PI3K catalytic subunit from bovine brain revealed a significant homology to Vps34 (Hiles et al. 1992). Formation of PI3P is readily detectable in wild type yeast cell extracts with 90% of total PI3K activity present in membrane fractions (Stack et al. 1993, Schu et al. 1993). However, yeast strains carrying a VPS34 gene deletion lack any detectable PI3K activity,
indicating that Vps34 is the sole source of PI3P in yeast (Schu et al. 1993). Further characterization also revealed that the lipid kinase activity of Vps34 is specific to only PI and is enhanced by membrane association (Schu et al. 1993, Stack and Emr 1994). Finally, generation of PI3P by Vps34 is required for vacuolar protein sorting (Herman et al. 1992, Schu et al. 1993).

It was also noted that yeast with mutations in the VPS15 gene were phenotypically identical to Vps34 mutants, suggesting that the two proteins act at the same step in the vacuolar protein sorting pathway (Robinson et al. 1988, Herman et al. 1991). Herman et al. (1991) determined that the VPS15 gene encodes a 170 kDa protein with a half-life of 60 min. The N-terminus of this protein possesses an acceptor site for the attachment of myristic acid. Biochemical labeling of yeast with tritiated myristic acid indicated that Vps15 is in fact myristoylated in vivo. The presence of this lipid modification accounts for the association of more than 90% of the total cellular Vps15 with membrane fractions, specifically fractions containing Golgi markers (Herman et al. 1991). Further characterization showed that this protein shares significant sequence homology to protein kinases and can auto-phosphorylate. Mutations within the Vps15 protein kinase domain disrupt the localization of soluble vacuolar hydrolases, indicating that protein phosphorylation is important for this sorting process (Herman et al. 1991).

Direct evidence for a functional interaction between Vps34 and Vps15 was provided when Stack et al. (1993) noted that overexpression of Vps34 will suppress the sorting defects associated with Vps15 kinase-deficient mutants. Moreover, Vps15 kinase-deficient mutants lack appreciable PI3K activity. In conjunction with this,
chemical cross-linking and native immunoprecipitation experiments demonstrated not only a functional, but also a physical interaction between the two proteins. This interaction is required for the membrane localization and activation of Vps34 (Stack et al. 1993).

Although Vps34 exists as a phospho-protein in vivo and Vps15 protein kinase activity is required for PI3P synthesis, Vps15 does not act to directly phosphorylate Vps34 (Stack and Emr 1994). Rather, it has been shown that Vps34 can auto-phosphorylate, indicating that Vps34 is able to function as both a lipid and a protein kinase (Stack and Emr 1994). Interestingly, unlike the binding to Vps15, the auto-phosphorylation by Vps34 has no effect on its PI3K activity (Stack and Emr 1994). However, the possibility remains that Vps34 phosphorylates other proteins and this transphosphorylation in turn regulates PI3K activity. Despite this possibility, to date no other protein substrates are known to be phosphorylated by Vps34. Even though the precise role of Vps34 phosphorylation has not been determined, the aforementioned studies of vacuolar hydrolase transport in yeast have provided valuable insight into the mechanism whereby Vps15 recruits Vps34 to the cytoplasmic face of Golgi membranes and stimulates the local generation of PI3P (Fig. 2).

**Autophagy**

Since the characterization of Vps34 as a key regulator in the transport of soluble hydrolases from the Golgi to the vacuole, PI3K has been implicated in other yeast membrane trafficking pathways including autophagy (Kihara et al. 2001a). Autophagy is a general process by which bulk cytoplasmic components are targeted to the vacuole for
In yeast, soluble hydrolases are sorted away from the secretory pathway and are targeted to the vacuole. The recruitment of the Vps34 catalytic subunit to the cytoplasmic face of the late Golgi by the membrane bound Vps15 adaptor subunit stimulates the local generation of PI3P required for this process.
degradation (Klionsky and Emr 2000). In response to starvation, double membrane structures known as autophagosomes non-specifically engulf a portion of the cytoplasm that may include resident organelles. Subsequent fusion of the autophagosome with the vacuole then results in the degradation of the autophagosomal contents (Klionsky and Emr 2000) (Fig. 3). Kihara et al. (2001a) demonstrated that yeast with a VPS34 gene deletion are defective not only in the transport of soluble hydrolases to the vacuole but also in the formation of autophagosomes in response to nitrogen starvation.

Immunoprecipitation of yeast extracts revealed the presence of two distinct Vps34/Vps15 containing complexes. Both complexes share Vps34/Vps15 and Vps30 (also known as Apg6) as core components. However, one complex contains Apg14, a protein required for autophagy, while the other complex contains Vps38, another protein required for vacuolar hydrolase delivery (Kihara et al. 2001a). This revealed that the ability of Vps34/Vps15 to form different complexes, containing pathway-specific regulatory proteins, allows the PI3K to simultaneously participate in multiple vesicle-mediated trafficking events in yeast (Kihara et al. 2001a) (Fig. 4).

Identification of a Mammalian PI3K Complex Related to the Yeast Vps34/Vps15 Vacuolar Protein Sorting Complex

In mammalian cells, a mechanism similar to the yeast vacuolar sorting pathway exists. This pathway is responsible for segregating lysosomal enzymes away from proteins destined for secretion (Chapman 1994). The discovery of the Vps34/Vps15 complex and elucidation of its role in yeast, prompted the search for a homologous
Autophagy is a starvation-induced process whereby a non-specific portion of cytoplasm is engulfed by a double membrane structure known as an autophagosome. The fusion of the autophagosome with the vacuole results in the degradation of the contents of the autophagosome.
In yeast, the Vps34 PI3K immunoprecipitates with two distinct complexes. Both complexes contain a core of Vps34/Vps15 and Vps30. However, the complex required for sorting of vacuolar hydrolases contains Vps38 while the complex required for autophagy contains Apg14. The ability to form different complexes with specific regulatory proteins allows the Class III PI3K to participate in multiple trafficking pathways.
complex in mammalian cells that could function in lysosomal enzyme sorting. The use of primers based on conserved amino acid sequences in the kinase domain of yeast Vps34 and bovine p110α led to the identification of a human homologue of the yeast Vps34 protein (Volinia et al. 1995). The cDNA for hVps34 encodes a 100.7 kDa protein that exhibits a high degree of amino acid homology with yeast Vps34 (37% identity and 58% similarity). Northern blot analysis revealed that hVps34 mRNA is ubiquitously expressed with highest levels found in skeletal muscle (Volinia et al. 1995). Like the yeast PI3K, the substrate specificity of hVps34 is restricted only to PI. Furthermore, hVps34 can be immunoprecipitated with a 150 kDa protein (p150) that is homologous to yeast Vps15 (Volinia et al. 1995). Similar to Vps15, p150 is a myristoylated protein that serves to stimulate the PI3K activity of hVps34 (Panaretou et al. 1997).

The major biochemical difference between the yeast and human Vps34 appears to be sensitivity to wortmannin (Volinia et al. 1995). Wortmannin is a cell permeable, fungal metabolite that inhibits the lipid kinase activity of all the classes of PI3Ks (Fruman et al. 1998). Irreversible inhibition occurs via wortmannin’s ability to covalently react with a conserved lysine residue required for all PI3K catalytic activity (Wymann et al. 1996). The mammalian Class I and Class III enzymes exhibit a similar sensitivity to wortmannin with a 50% inhibitory concentration between 1 and 10 nM. However, the Class II enzyme is at least 10-fold less sensitive to treatment with wortmannin (Volinia et al. 1995, Virbasius et al. 1996, Domin et al. 1997). Similar to the mammalian Class II PI3K, yeast Vps34 PI3K activity is relatively insensitive to treatment with wortmannin, requiring micromolar concentrations for complete inhibition (Stack and Emr 1994).
PI3P is a Key Regulator of Vesicle-Mediated Protein Trafficking in Mammalian Cells

Lysosomal Enzyme (Cathepsin D) Sorting

In mammalian cells newly synthesized lysosomal enzymes are diverted from the secretory pathway by a receptor-mediated mannose-6-phosphate (M6P)-dependent mechanism. Soluble lysosomal enzymes modified with N-linked oligosaccharides bearing M6P residues bind to the M6P receptor in the TGN. These enzyme-receptor complexes are then selectively packaged into clathrin-coated vesicles and are transported to a pre-lysosomal/late endosomal compartment (Brown et al. 1986, Griffiths et al. 1988, Geuze et al. 1988). The acidic pH of this compartment facilitates the dissociation of the enzyme from the receptor allowing the unbound receptor to recycle back to the TGN for additional rounds of transport (Brown et al. 1986, Griffiths et al. 1988, Geuze et al. 1988, Goda and Pfeffer 1988). Following dissociation, the soluble enzymes are delivered to the lysosome (Mullock et al. 1994).

Several similarities exist between the pathways required for delivery of soluble proteins to the mammalian lysosome and the yeast vacuole. First, it has been shown that CPY exits the late Golgi and transits to a pre-vacuolar compartment (PVC) similar to the late endosome. Secondly, sorting to the PVC also occurs in a receptor (Vps10) dependent fashion (Graham and Emr 1991, Vida et al. 1993, Marcusson et al. 1994). Finally, as in the mammalian system, this sorting of CPY requires clathrin (Seeger and Payne 1992). The functional similarities between these two pathways, combined with the
fact that Vp34 is required for CPY sorting, led to the investigation of the role of PI3P in delivery of enzymes to the mammalian lysosome.

Cathepsin D is a mammalian aspartyl protease that is transported to the lysosome in a M6P dependent manner. This enzyme is initially synthesized as a ~53 kDa proenzyme which associates with the M6P receptor in the TGN. After M6P receptor-mediated delivery to the late endosome, cathepsin D is proteolytically processed into a ~47 kDa intermediate. Upon delivery to the lysosome, the intermediate is cleaved to yield the mature form of the enzyme which consists of a ~30 kDa polypeptide non-covalently linked to a 14 kDa polypeptide (Rijnboutt et al. 1992, Delbruck et al. 1994). The ability to trace the formation of the various forms of cathepsin D based simply on size has allowed this protein to serve as the major marker for the delivery of enzymes to the lysosome (Fig. 5).

Initial investigations into the role of PI3P in delivery of cathepsin D to the lysosome relied heavily on the use of wortmannin. Brown et al. (1995) demonstrated that treatment of cultured cells with wortmannin results in a rapid decrease in the cellular level of PI3P. A complete block in the intracellular maturation of cathepsin D into the 30 kDa form accompanies this loss of PI3P. Moreover, this defect in processing resulted from the diversion of pro-cathepsin D out of the M6P delivery system and into the constitutive secretory pathway (Brown et al. 1995, Davidson 1995). Davidson (1995) further demonstrated that the M6P recognition signal of cathepsin D was intact and thus
Pro-cathepsin D exists in the Golgi as a 53 kDa proenzyme. Following M6P receptor-mediated delivery to the endosome, cathepsin D is proteolytically processed into a 47 kDa intermediate form. Upon fusion of the late endosome with the lysosome, cathepsin D undergoes a final cleavage to yield a 30 kDa polypeptide non-covalently linked to a 14 kDa polypeptide. Transport from the Golgi to the lysosome can be assessed based on the proteolytic processing of cathepsin D.
the secretion arose from a deficiency in the sorting pathway rather than a loss of the M6P modification.

Although the use of wortmannin was able to establish a role for PI3P in cathepsin D transport, its broad PI3K specificity precluded the identification of the exact PI3K isoform required for this process. However, the parallels to the yeast CPY sorting system led investigators to postulate that mammalian Vps34 functions in cathepsin D sorting at the TGN in a fashion similar to the yeast Vps34/Vps15 complex. In 2001, Row et al. provided evidence that mammalian Vps34 is involved in cathepsin D processing. They successfully developed a kinase-deficient, dominant-negative form of the rat Vps34 homologue. This mutant was competent for interaction with p150, but lacked the ability to generate PI3P. Transfection of HeLa cells with the dominant-negative Vps34 resulted in a defect in cathepsin D processing. However, in contrast to wortmannin treatment, cells expressing dominant-negative Vps34 did not missort cathepsin D into the secretory pathway. Instead, it was determined that the defect in cathepsin D processing resulted from a kinetic delay in the formation of the 47 kDa and 30 kDa forms rather than hypersecretion of the proenzyme. In fact, secretion of pro-cathepsin D in cells expressing the Vps34 mutant was significantly lower than the basal secretion observed in non-transfected cells (Row et al. 2001).

Combination of the data derived from the wortmannin studies and the experiments with the dominant-negative Vps34 demonstrate that PI3P is important in the transport of cathepsin D to the lysosome (Brown et al. 1995, Davidson 1995, Row et al. 2001). However, unlike treatment with wortmannin, the loss of Vps34 function did not
result in the hypersecretion of pro-cathepsin D (Row et al. 2001). This difference strongly suggests that at least one other wortmannin sensitive enzyme distinct from Vps34 functions in the targeting of lysosomal enzymes (Row et al. 2001). Although the study by Row et al. (2001) helped to discern a role for Vps34 in cathepsin D processing, the underlying mechanism of coordinate PI3K function at the TGN remains elusive.

*The Endocytic Pathway*

In general, the endocytic pathway is characterized by the continuous and regulated formation of vacuoles from the plasma membrane. Receptor-ligand complexes and fluid phase cargo accumulate in clathrin coated pits which bud off into the cytosol to form clathrin coated vesicles. These vesicles rapidly lose this protein coat and fuse with a compartment known as the early endosome. The early endosome compartment represents a diverse array of vesicles and tubules located throughout the perinuclear region and the cell periphery. The presence of an active proton pump allows the early endosome to maintain a slightly acidic pH. This low pH facilitates the dissociation of many receptor-ligand complexes. Following dissociation, some receptors (e.g., transferrin receptor) accumulate in tubular extensions of the early endosome which bud off and recycle back to the plasma membrane. Other receptors (e.g., activated EGF and PDGF receptors) or fluid phase cargo destined for degradation traverse to the late endosome. This organelle tends to have a more spherical appearance and is found primarily in the perinuclear region. The defining characteristic of the late endosome is the ability to inwardly vesiculate and accumulate many internal vesicles. Therefore, late endosomes are often referred to as multivesicular bodies (MVBs) and these names are
used interchangeably. The sorting of proteins into the inner vesicles of MVBs is required for several cellular processes, most notably the down-regulation of activated signaling receptors. The late endosome also represents a point of convergence between the endocytic pathway and the lysosomal enzyme targeting pathway. Clathrin coated vesicles originating from the TGN also fuse with late endosome in order to deliver newly synthesized enzymes to the lysosome. Ultimately, the fusion of the late endosome with the lysosome results in the degradation of its lumenal contents (Reviewed in Mellman 1996) (Fig. 6).

**Endosome Morphology**

In addition to the missorting of cathepsin D, Brown et al. (1995) also showed that wortmannin treatment induced significant morphological changes in NRK cells. Within 15 min of incubation with wortmannin, these cells accumulated large acidic cytoplasmic vacuoles that labeled heavily with the M6P receptor (Brown et al. 1995). Over time, the M6P receptor recycled from this aberrant vacuolar compartment back to the TGN (Brown et al. 1995). Using the same cell line, Reaves et al. (1996) also detected the presence of lysosomal type I integral membrane proteins, found specifically in late endosomes and lysosomes, in the membranes of the wortmannin-induced swollen vacuoles. The recycling of the M6P receptor along with presence of late endosomal membrane markers suggested that the swollen vacuoles were derived from the late endosome compartment. In both cases, wortmannin did not affect the morphology of the TGN, lysosomes, or early endocytic compartments (Brown et al. 1995, Reaves et al. 1996).
Endocytic organelles coordinate vesicle-mediated protein transport between the trans-Golgi network, the plasma membrane, and the lysosome.
Further investigation into the wortmannin-induced swelling of the late endosome compartment has sparked debate as to the cause of this phenomenon. In contrast to the observations of Brown et al. (1995), Kundra and Kornfeld (1998) reported that wortmannin prevents the recycling of the M6P receptor from the late endosome back to the TGN and this failure to recycle results in swelling of the late endosomal compartment. Another study suggested that the late endosome swelling resulted from an inhibition of inward vesiculation (Fernandez-Borja et al. 1999). However, Bright et al. (2001) found that prolonged treatment of NRK cells with wortmannin did not prevent the accumulation of internal vesicles within the late endosome. Instead, as reported by Kundra and Kornfeld (1998), wortmannin inhibited membrane recycling from this compartment (Bright et al. 2001).

In order to directly address this debate, Futter et al. (2001) developed a lysosomal cross-linking technique that allows the development of MVBs to be followed in the absence of MVB-lysosome fusion. Using this method, it was determined that wortmannin treatment resulted in a five-fold reduction in the accumulation of internal vesicles within the MVB while recycling was only modestly inhibited (Futter et al. 2001). This supports the finding by Fernandez-Borja et al. (1999) that the swelling of the late endosome induced by wortmannin results primarily from the failure to inwardly vesiculate.

As in the studies with cathepsin D, the use of wortmannin does not allow for the identification of the PI3K isoform responsible for generating the PI3P needed for inward vesiculation of the late endosome. Initial experiments showed that the loss of Vps34
function did not result in the swelling of the late endosome compartment. Both the
overexpression of a dominant-negative Vps34 and microinjection of inhibitory anti-
Vps34 antibodies did not produce any gross effects on cellular morphology, suggesting
involvement of another PI3K isoform (Row et al. 2001, Siddhanta et al. 1998). Therefore, Futter et al. (2001) combined the use of specific inhibitory antibodies towards
p110α, p110β, and hVps34 with their lysosome cross-linking method, in order to further
assess the role of individual PI3Ks in MVB formation. Microinjection of p110α antibodies did not affect the morphology of the MVB at any dose (Futter et al. 2001).
While microinjection of p110β at low doses had no effects on MVBs, higher doses
resulted in the accumulation of unusually small MVBs with fewer internal vesicles
(Futter et al. 2001). This suggests that p110β may be involved in early events in the
endocytic pathway involving transport to the late endosome rather than inward
vesiculation. Only microinjection of the anti-hVps34 antibody closely mimicked the
effects of wortmannin on MVB formation in the absence of MVB-lysosome fusion
(Futter et al. 2001). This lead to the conclusion that hVps34 is the primary PI3K required
for inward vesiculation of the late endosome (Futter et al. 2001). However, this
conclusion creates a major unresolved discrepancy in the fact that previous studies
examining the loss of Vps34 function through dominant-negative or inhibitory antibody
approaches revealed no significant effects on late endosome morphology (Row et al.

Interestingly, Shpetner et al. (1996) reported that HepG2 and Cos-7 cells treated
with wortmannin did not accumulate cytoplasmic vacuoles. Instead, these cell lines

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developed extensive tubulation of endosomal compartments identified by labeling with transferrin or a lysosomally directed fluid phase marker (Shpetner et al. 1996). Although wortmannin appears to effect specific cell lines differently and the role of Vps34 remains unsettled, these reports implicate PI3P as an important regulator of endosome morphology.

Post-Endocytic Sorting

**PDGF Receptor (Lysosomal Targeting)**

The studies on the morphological effects of wortmannin treatment emphasize the significance of PI3P in regulating the structure of endocytic compartments containing lysosomal directed and recycling molecules. A functional role for a PI3K in endocytic trafficking was first shown using PDGF receptor mutants that were unable to bind the Class I PI3K. Following stimulation with PDGF, wild type receptors concentrated in juxtanuclear vesicular compartments and degraded rapidly (Joly et al. 1994). In contrast, the mutant receptors remained dispersed at the cell periphery and exhibited a much slower rate of degradation (Joly et al. 1994). Further analysis showed that the mutant receptors were effectively internalized but failed to target to the lysosome, suggesting that PI3Ks are important in post-endocytic sorting events (Joly et al. 1995). Shpetner et al. (1996) used wortmannin to further test the finding that PDGF receptor trafficking requires PI3K activity. Similar to the results obtained with PDGF receptor mutants, wortmannin inhibited the transport of the PDGF receptor from the cell periphery to perinuclear compartments following PDGF treatment. However, in the same study, transport of a fluid phase marker to the lysosome was unimpeded by the presence of
wortmannin (Shpetner et al. 1996). This indicates that wortmannin did not prevent endosome-lysosome fusion, but rather it inhibited the targeting of PDGF receptors to that pathway (Shpetner et al. 1996).

Although the initial experiments with PDGF receptor mutants implicated the Class I PI3K in PDGF receptor trafficking, more recent studies suggest Vps34 may also be involved. Microinjection of specific inhibitory antibodies against p110α surprisingly had no effect on the perinuclear accumulation of PDGF receptors following stimulation (Siddhanta et al. 1998). In contrast, microinjection of anti-Vps34 antibodies resulted in the retention of PDGF receptors in smaller vesicles that were localized to the cell periphery (Siddhanta et al. 1998). Even though both the Class I and the Class II PI3K have been implicated in PDGF trafficking to the lysosome, the extent of involvement of each of these enzymes in the sorting process remains unclear.

*EGF Receptor (Targeting to MVBs and the Lysosome)*

Analysis of the effects of wortmannin on trafficking of the EGF receptor yielded different results than the PDGF receptor experiments (Futter et al. 2001). It has been shown that following endocytic uptake, EGF receptors are sequestered into the internal vesicles of MVBs (Futter and Hopkins 1989, Hopkins et al. 1990, Futter et al. 1996). This internalization serves to down-regulate signaling by sequestering activated EGF receptors from downstream targets. Ultimately, EGF receptors are delivered to the lysosome on internal vesicles following MVB-lysosome fusion (van Deurs et al. 1995, Futter et al. 1996, Mullock et al. 1998). Using electron microscopy to trace EGF receptor localization, Futter et al. (2001) determined that over 80% of activated receptors localized
to lysosomes despite the presence of wortmannin. Although the EGF receptors effectively reached the lysosomes, a defect in sorting did occur at the late endosome (Futter et al. 2001). In wortmannin treated cells, activated EGF receptors were never sequestered into the internal vesicles of MVBs and were instead delivered to the lysosome on the surface of the late endosome. This resulted in only a slight decrease in the rate of receptor degradation (Futter et al. 2001). Microinjection of specific inhibitory antibodies revealed that loss of Vps34 activity results in the retention of activated EGF receptors on the surface of late endosome (Futter et al. 2001). Neither p110\(\alpha\) nor p110\(\beta\) antibodies had any effect on EGF receptor sorting, suggesting that Vps34 is responsible for the targeting of the EGF receptor to the internal vesicles of the MVB (Futter et al. 2001).

Transferrin Receptor (Recycling)

Unlike the PDGF and EGF receptors, endocytic uptake of the transferrin receptor does not result in trafficking to the lysosome (Mayor et al. 1993, Hopkins et al. 1994). Instead, following dissociation of iron from its ligand, transferring, in the early endosome, the transferrin receptor-transferrin complex is sorted out of the degradative pathway and recycles back to the plasma membrane (Mayor et al. 1993, Hopkins et al. 1994). Several studies have determined that wortmannin alters the transferrin receptor recycling pathway similarly in various different cell lines (Martys et al. 1996, Spiro et al. 1996, Shpetner et al. 1996). Essentially, treatment with wortmannin causes a decrease in the steady state number of cell surface transferrin receptors. This loss of receptor from the plasma membrane is the consequence of two separate effects on the sorting pathway.
First, wortmannin induces an approximately 60% increase in the internalization rate of the transferrin receptor. Secondly, wortmannin decreases the rate of recycling from the early endosome back to the plasma membrane by approximately 40% (Martys et al. 1996, Spiro et al. 1996, Shpetner et al. 1996). Combined, these effects cause a redistribution of the receptor from the plasma membrane to early endosomes. Interestingly, microinjection experiments showed that both anti-p110α and anti-Vps34 antibodies inhibit the recycling of the transferrin receptor (Siddhanta et al. 1998). However, the delay in efflux of transferrin from anti-p110α injected cells was much greater than in the anti-Vps34 injected cells, suggesting that p110α is the primary wortmannin-sensitive enzyme involved in regulation of transferrin recycling (Siddhanta et al. 1998).

Experiments analyzing the ability of wortmannin treated cells to effectively internalize, sort/recycle, and degrade receptor and fluid phase cargo from the plasma membrane definitively implicate PI3P as a major regulator of these processes. However, it is apparent that multiple PI3Ks can participate, to various extents, at any given step in the endocytic pathway. Consequently, the exact role that the individual PI3Ks play in endocytic sorting is still unclear.

Autophagy

Autophagy, a process responsible for the accelerated degradation of cellular proteins during starvation is conserved from yeast to mammals. Similar to the yeast process, mammalian autophagy involves the sequestration of cytoplasmic material into double membrane-bound autophagosomes, fusion of these vesicles with lysosomes, and degradation of the sequestered material (Reviewed in Klionsky 2005). Yeast with a
VPS34 gene deletion are defective in the formation of starvation-induced autophagosomes, indicating the importance of PI3P in this process (Kihara et al. 2001a).

Through the use of wortmannin, PI3K activity also was found to be an important regulator of mammalian autophagy. Blommaart et al. (1997) determined that wortmannin prevents autophagic sequestration in isolated rat hepatocytes. A similar effect also was characterized in human HT-29 colon cancer cells (Petiot et al. 2000). Furthermore, the introduction of anti-sense oligonucleotides specific for hVps34 inhibited both the synthesis of PI3P and autophagic protein degradation (Petiot et al. 2000). In conjunction with this, the overexpression of p150 in HT-29 cells results in coordinated increases in PI3P levels and autophagic protein degradation (Petiot et al. 2000). The introduction of exogenous PI3P is also an effective stimulus for the autophagic pathway. However, the introduction of PI(3,4,5)P3, the primary product of the Class I PI3K, inhibits autophagic protein degradation (Petiot et al. 2000). The stimulation of endogenous Class I PI3K using cytokines also produces the same effect (Petiot et al. 2000). This provides evidence that distinct classes of PI3Ks control the autophagic pathway, but in opposite directions (Petiot et al. 2000).

The discovery of an interaction between Vps34 and a mammalian homologue (Beclin) to the yeast Vps30/Apg6 protein provided further evidence for the involvement of Vps34 in autophagy (Kihara et al. 2001b). Liang et al. (1999) determined that like Vps34, Beclin is required for autophagy in mammalian cells. Through immunoprecipitation and chemical cross-linking, it was shown that all of the cellular Beclin associates with approximately 50% of the total Vps34 (Kihara et al. 2001b).
Immunofluorescent analyses revealed that both Beclin and Vps34 are colocalized in the TGN while the remaining Vps34 localized to punctate endosomal structures (Kihara et al. 2001b). This suggests that Beclin plays an important role in PI3P production as a subunit of a PI3K complex at the TGN, which in turn modulates the autophagic pathway. The fact that 50% Vps34 is not complexed to Beclin implies that, as in yeast, mammalian Vps34 may associate with multiple complexes in order to simultaneously regulate different trafficking events (Kihara et al. 2001b).

**Mechanism Underlying the Role of PI3P in Vesicle-Mediated Protein Trafficking**

The use of wortmannin combined with microinjection of inhibitory antibodies, overexpression of dominant-negative mutants, and introduction of antisense oligonucleotides has established a role for PI3Ks in vesicle-mediated protein trafficking. Experiments aimed at discovering downstream effectors of PI3Ks provided the first insight into the mechanism by which PI3P functions in these pathways. In order to identify potential PI3P targets, Patki et al. (1997) devised a method to isolate peripheral membrane proteins that localized in a wortmannin sensitive manner. A ~170 kDa protein (p170) was found to be greatly diminished in the membrane fractions of wortmannin treated cells when compared to untreated controls (Patki et al. 1997). Sequencing analysis determined the identity of p170 to be a protein previously identified as early endosome autoantigen 1 (EEA1) (Mu et al. 1995, Stenmark et al. 1996). At the time, EEA1 association with early endosomes was known to be mediated through its C-terminal FYVE domain (Stenmark et al. 1996). The FYVE domain, conserved in yeast
and mammals, is a cysteine-rich zinc finger, found in a diverse array of proteins (Gillooly et al. 2001). Many of these proteins, including the first four proteins known to possess this domain (Fab1p, YOTB, Vac1p, EEA1) have been implicated in vesicle mediated protein trafficking (Gillooly et al. 2001, Corvera 2001). Further characterization of the EEA1 FYVE domain revealed that it binds directly and specifically to PI3P, thus accounting for its wortmannin sensitive localization (Patki et al. 1998, Gaullier et al. 2000). Therefore, PI3P serves as a signal that selectively recruits proteins with PI3P binding domains to restricted membrane areas where they can perform a specific function.

More recent studies have shown that EEA1 localization depends not only on PI3P, but also on the small GTPase Rab5. In the active GTP-bound state, Rab5 coordinates the docking and fusion of early endosomes by recruiting protein complexes required for appropriate vesicle targeting (Takai et al. 2001). Using affinity chromatography with GTP-bound Rab5, Christoforidis et al. (1999a) determined that activated Rab5 interacts with at least 20 distinct proteins, including EEA1. Interestingly, a relationship between Rab5 and PI3P was first described by in vitro experiments that showed an activated Rab5 mutant can rescue endosome fusion blocked by wortmannin (Li et al. 1995). The nature of this relationship was subsequently clarified through the discovery that EEA1 is a critical effector of both Rab5 and PI3P (Simonsen et al. 1998, Christoforidis et al. 1999a).

The further characterization of Rab5 binding proteins lead to the discovery that PI3Ks are also Rab5 effectors (Christoforidis et al. 1999b). PI3K activity was markedly
increased in eluates from Rab5-GTP columns versus Rab5-GDP columns (Christoforidis et al. 1999b). In addition, this activity was almost completely inhibited by the presence of wortmannin (Christoforidis et al. 1999b). Identification of the source of the PI3K activity lead to the discovery that Rab5-GTP interacts with both the Class I and the Class III PI3Ks (Christoforidis et al. 1999b). Unlike the p110β subunit, which interacts directly with Rab5-GTP, the interaction with Vps34 is mediated through its adaptor protein p150 (Christoforidis et al. 1999b).

In order to determine which PI3K participated in Rab5 mediated early endosome fusion, Christoforidis et al. (1999b) used isoform specific antibodies in an in vitro endosome fusion assay. In this study, anti-Vps34 antibodies produced a major block in endosome fusion while anti-p110β antibodies had only a minor inhibitory effect (Christoforidis et al. 1999b). This lead to the conclusion that Vps34 is the primary PI3K required for endosome fusion. Consistent with this, only anti-Vps34 antibodies caused a substantial decrease in the amount of EEA1 in endosome fractions (Christoforidis et al 1999b).

Interestingly, the interaction of Rab5 with Vps34 does not modulate PI3K enzymatic activity (Christoforidis et al. 1999b). Instead the more likely function of this interaction is to localize the p150-Vps34 complex to the appropriate site in the endosome membrane. In turn, the production of PI3P by Vps34 is confined to a membrane microdomain, where the function of EEA1 in endosome fusion can be coordinated with other Rab5 effectors (Christoforidis et al. 1999b). To date, no function has been ascribed to the interaction between Rab5 and the Class I PI3K (Fig. 7).
Both the Class I and the Class III PI3K are effectors of Rab5. Rab5 serves to recruit Vps34/p150 to the membrane of early endosomes which regulates the local generation of PI3P. The localization of EEA1 is coordinated through an interaction with Rab5 and through binding of its FYVE domain to PI3P. This in turn facilitates proper docking and fusion of early endosomes. To date, no function has been ascribed to the interaction between Rab5 and the p110β, Class I PI3K.
Characterization of the interaction between Rab5 and Vps34 provided insight into the mechanism whereby Vps34 is recruited to early endosomal membranes for the local production of PI3P. Current studies have determined that the regulation of Vps34 by Rab proteins is not restricted to the early endosome. Stein et al. (2003) demonstrated that Vps34 is also an effector of Rab7, which localizes to the late endosome compartment. Moreover, this interaction is required for the transport of endocytosed proteins from early endosomes to late endosomes and lysosomes (Stein et al. 2003). However, unlike the interaction with Rab5, it appears as though Rab7-GTP acts to stimulate Vps34 PI3K activity (Stein et al. 2003).

The function of this interaction between Rab7 and Vps34 on the late endosome is still obscure. To date, no proteins have been clearly established as downstream effectors of Vps34 on the late endosome. However, another FYVE domain containing protein, PIKfyve (phosphinositide kinase for five position containing a fyve finger) has been shown to localize to late endosomes (Sbrissa et al. 2002). As evident by its name, PIKfyve is a lipid kinase that phosphorylates PI and PI3P at the five position to generate PI5P and PI(3,5)P2, respectively (Sbrissa et al. 1999). The generation of PI(3,5)P2 is required for inward vesiculation of the late endosome (Ikonomov et al. 2001). Overexpression of a dominant-negative form of PIKfyve induces swelling of the late endosome compartment that is rescued by the introduction of exogenous PI(3,5)P2 (Ikonomov et al. 2001).

Insight into the mechanism of PI(3,5)P2 function at the late endosome was gained through the discovery that PI(3,5)P2 mediates the interaction of “Class E” proteins with
late endosomal membranes (Whitley et al. 2003). The Class E proteins also were isolated in the screening for vacuolar protein sorting mutants in yeast (Conibear and Stevens 1998). Yeast with mutations in Class E proteins are distinguished by the presence of a swollen multilamellar organelle (Class E compartment) (Rieder et al. 1996). The Class E compartment is thought to represent a late endosome that is unable to form intraluminal vesicles, demonstrating a role for the Class E proteins in MVB formation (Rieder et al. 1996). Mammalian Class E homologues also have been shown to regulate the inward vesiculation of the late endosome (Babst et al. 2002, Bishop et al. 2002). Specifically, a complex comprised of four Class E proteins (Vps24, Vps2, Snf7, and Vps20) named the endosomal sorting complex required for transport -III (ESCRT-III) must assemble at the late endosome in order for inward vesiculation to occur (Katzmann et al. 2002). Whitley et al. (2003) demonstrated that Vps24 is a PI(3,5)P₂ binding protein. Therefore, PI3P serves not only as a localization signal for PIKfyve, but also as a substrate for the generation of PI(3,5)P₂ required for ESCRT-III assembly at the late endosome.

Similar to other endocytic compartments, the source of the PI3P required for PIKfyve localization remains ambiguous. Although Vps34 and PIKfyve have been shown to localize to the late endosome, Sbrissa et al. (2001) identified an association between PIKfyve and the Class I PI3K in both resting and insulin-stimulated 3T3-L1 adipocytes. Immunoprecipitation experiments revealed that PIKfyve exists in a complex with both the p85 and p110 subunits of the Class I PI3K (Sbrissa et al. 2001). Stimulation with insulin results in an increase in the PI3K activity associated with this complex (Sbrissa et al. 2001). Although no function has been attributed to this
interaction, it is possible that the Class I PI3K contributes to the pool of PI3P required for the formation of PI(3,5)P$_2$ and inward vesiculation of the late endosome (Fig. 8).

Based on the studies by Christoforidis (1999b) and Stein (2003), it is evident that the ability of PI3Ks to function in various trafficking events is coordinately regulated by Rab GTPases. However, the ability of Rab5 to interact with multiple PI3Ks raises doubt concerning the source of PI3P required for early endosome function (Christoforidis et al. 1999b). Further complexity arises at the late endosome. The generation of PI(3,5)P$_2$ is required for the inward vesiculation of the late endosome (Ikonomov et al. 2001). Through interaction with Rab7, Vps34 localizes to the late endosome (Stein et al. 2003). However, PIKfyve, the enzyme that generates PI(3,5)P$_2$, co-immunoprecipitates with the Class I PI3K (Sbrissa et al. 2001). This again raises questions regarding which PI3K is required for late endosome function. Currently, the absolute role of the different classes of PI3Ks at both the early and late endosomal compartments remains unclear.

**Functions of Vps34 Outside of Vesicle-Mediated Protein Trafficking**

**PI3Ks and DNA Synthesis**

It has been well established that PI3K activity is required for various mitogens and oncoproteins to stimulate DNA synthesis (Vanhaesebroek et al. 2001). Both Fantl et al. (1992) and Valius and Kazlauskas (1993) demonstrated that cells possessing mutant PDGF receptors unable to activate the Class I PI3K fail to display an increase in DNA synthesis following PDGF stimulation. Similarly, treatment of cells with wortmannin
Vps34 is an effector of Rab7 which is localized to the late endosome. PIKfyve is a FYVE domain containing protein that generates PI(3,5)P$_2$ from PI3P. The generation of PI(3,5)P$_2$ by PIKfyve is required for association of the ESCRT-III complex and inward vesiculation of the late endosome. PIKfyve associates with the Class I PI3K in adipocytes. Therefore, both the Class I and the Class III could potentially serve as the source of PI3P required for MVB formation.
also abrogates mitogen-induced DNA synthesis (Roche et al. 1998). Various studies have demonstrated that the levels of PI(3,4)P\textsubscript{2} and PI(3,4,5)P\textsubscript{3} rise sharply in response to mitogens such as insulin, whereas the levels of PI3P remain constant (Auger et al. 1989, Kapeller et al. 1991). This suggests that the Class I enzymes are the primary PI3Ks responsible for mitogen induced DNA synthesis. However, Siddhanta et al. (1998) demonstrated a requirement for both the Class I and Class III PI3Ks in insulin-stimulated DNA synthesis. Microinjection of both anti-p110\textalpha and anti-Vps34 antibodies in GRC-LR+73 cells prior to insulin stimulation resulted in a 70% decrease in the associated increase in DNA synthesis (Siddhanta et al. 1998). Further confirmation for the unexpected requirement of Vps34 in mitogenic signaling was obtained through the use of antisense oligonucleotides (Siddhanta et al. 1998). Microinjection of Vps34-specific antisense oligonucleotides resulted in an 80-90% inhibition of insulin-stimulated DNA synthesis (Siddhanta et al. 1998). Although both the Class I and the Class III PI3K are involved in insulin-induced DNA synthesis, the temporal requirement for the two enzymes is different (Siddhanta et al. 1998). The ability of anti-Vps34 antibodies to block DNA synthesis was greatly reduced after 6 h of insulin stimulation. In contrast the requirement of p110\textalpha lasted through 9 h of insulin treatment (Siddhanta et al. 1998). Although it is apparent that Vps34 is required at an early stage in the G1-S transition stimulated by insulin, the function of PI3P in mitogen-induced DNA synthesis remains unknown.
**Link Between Vps34 and Transcription in Plants**

Using primers based on conserved sequences in the bovine p110α and the yeast Vps34 PI3K domain, both Hong and Verma (1994) and Welters et al. (1994) were able to isolate the plant homologue of Vps34. Since its discovery, plant Vps34 has been implicated in several membrane trafficking events including membrane proliferation associated with root nodule formation and sorting to the plant vacuole (Hong and Verma 1994, Matsuoka et al. 1995). However, Dove et al. (1994) demonstrated that a substantial portion of the PI3K activity in plant cells is associated with a detergent-resistant nucleocytoskeletal compartment. Further characterization of the subcellular distribution of plant Vps34 using immunofluorescence microscopy revealed a punctate staining pattern throughout the nucleoplasm and within nucleoli (Bunney et al. 2000). Labeling of cells with bromo-UTP demonstrated that the nuclear Vps34 co-localized to active sites of transcription. Although Vps34 has not been localized to the nucleus of other cell types, these experiments with plant cells suggest that Vps34 may play a novel role in the transcriptional process.

**Perspective**

The discovery that Vps34 is a PI3K required for vacuolar enzyme sorting in yeast provided the first evidence for the importance of PI3P in vesicle-mediated protein trafficking (Stack et al. 1993, Schu et al. 1993). Clarifying the nature of PI3P involvement in protein trafficking in mammalian cells has relied heavily on the use of PI3K inhibitors such as wortmannin. The use of wortmannin has demonstrated a role for
PI3P in lysosomal enzyme sorting, receptor sorting in the early endocytic pathway, inward vesiculation of the late endosome, and autophagy (Brown et al. 1995, Davidson et al. 1995, Shpetner et al. 1996, Futter et al. 2001, Petiot et al. 2000). However, attempts to identify the role of specific PI3K isoforms in these pathways has proven to be very complex. For example, wortmannin induces the hypersecretion of pro-cathepsin D while overexpression of a kinase deficient Vps34 mutant results in retention of pro-cathepsin D in the Golgi (Brown et al. 1995, Davidson et al. 1995, Row et al. 2001). This suggests that at least one other wortmannin sensitive enzyme other that Vps34 functions in lysosomal enzyme sorting. Wortmannin also induces the swelling of the late endosome compartment (Brown et al. 1995, Davidson et al. 1995, Reaves et al. 1996). The use of inhibitory antibodies suggested that Vps34 was the PI3K responsible for this effect (Futter et al. 2001). However, the fact that overexpression of a kinase deficient Vps34 has no effect on endosome morphology contradicts the inhibitory antibody results (Row et al. 2001). Finally, both the Class I and the Class III PI3Ks have been shown to be effectors of regulatory proteins at both the early and late endocytic compartments (Christoforidis et al. 1999b, Sbrissa et al. 2001, Stein et al. 2003). Subsequently, the ability to define the exact role for each of these enzymes in early and late endosomal function has been limited.

Recent advances in RNAi technology have provided a powerful tool for defining the function of specific proteins in mammalian cells. This method also provides several advantages when compared to dominant-negative and inhibitory antibody approaches. For example, RNAi eliminates artifacts associated with overexpression which may result
in sequestering key regulatory proteins that interact with multiple PI3Ks. Second, unlike microinjection of antibodies, RNAi allows for the selection of a stable population of cells that can be subjected to biochemical analyses of protein trafficking. The present study aims to utilize this technology to suppress the expression of hVps34 in order to determine the specific trafficking pathways requiring PI3P generated by hVps34 for function.

Several studies also have implicated Vps34 in functions outside of vesicle-mediated protein trafficking. For example, Siddhanta et al. 1998 demonstrated a role for hVps34 in insulin-stimulated DNA synthesis. Furthermore, Bunney et al. (2000) showed that Vps34 localized to active sites of transcription in plant cells. To date, there have been no reports of the nuclear localization of Vps34 in mammalian cells. The present study aims to characterize the localization of hVps34 in order to gain insight into a possible nuclear function for this protein.
MATERIALS AND METHODS

Cell Lines

Human 293T embryonic kidney and HEp-2 laryngeal carcinoma cell lines were obtained from the American Type Culture Collection (Manassas, VA) and grown in Dulbecco’s Modified Essential Media (DMEM) supplemented with 10% fetal bovine serum (FBS). U-251 human glioblastoma cells were obtained from the National Cancer Institute Frederick Cancer DCT Tumor Repository (Frederick, MD) and were maintained in DMEM, supplemented with 10% FBS. The 293 GPG packaging cells (Ory et al. 1996) were maintained in DMEM containing 10% heat-inactivated FBS with 1 µg/ml puromycin, 300 µg/ml G418, and 2 µg/ml doxycycline. All cell lines were grown as monolayer cultures at 37°C in a 5% CO₂ atmosphere.

Plasmids

A pMT2 plasmid containing hVps34 cDNA was obtained from the lab of Dr. Michael Waterfield. An epitope tag was added by subcloning the hVps34 cDNA into the pcDNA3.1/HisA vector (Invitrogen, Carlsbad, CA). The pSUPER.retro.puro vector was obtained from OligoEngine (Seattle, WA).

hVps34 Short Interfering RNA (siRNA) Oligonucleotide Design and Cloning into pSUPER.retro.puro

Potential siRNA target sequences were obtained using a siRNA Target Finder and Design Tool provided by Ambion Inc. (Austin, TX) (http://www.ambion.com).
Approximately 200 potential 19 base target sequences were obtained using this method. Four of these 200 met the following criteria: (1) were preceded by a AA dinucleotide sequence, (2) had a 30-50% GC content, (3) contained no stretches of greater than four Ts, and (5) were not homologous to any other known sequences in the NCBI database. Oligonucleotides designed to generate short hairpin structures consisted of a BglII restriction site, the 19-base target sequence, a loop (TTCAAGAGA), the reverse compliment of the 19-base target, a polyT termination signal, and a HindIII restriction site. A one base mismatch was introduced into two of the 19 base target sequences in order to serve as negative controls. These negative control sequences did not match any known sequences in the NCBI database. Finally, the oligonucleotides were cloned into the pSUPER.retro.puro vector using the BglII and HindIII restriction sites.

**Knockdown of Overexpressed His<sub>6</sub>-hVps34**

Control or Knockdown (KD) pSUPER.retro.puro constructs were co-transfected into 293T cells with His<sub>6</sub>-hVps34-pcDNA3.1 at a ratio of 5:1, using Lipofectamine-Plus reagent (Invitrogen, Carlsbad, CA). Twenty-four and 48 h after transfection, cells were harvested in SDS sample buffer (62.5 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, and 5% β-mercaptoethanol). Equal amounts of protein, determined using a colorimetric protein assay (Bio-Rad, Hercules, CA), were subjected to SDS-PAGE. Proteins were electro-transferred to polyvinylidene fluoride (PVDF) membranes in buffer containing 10 mM NaHCO<sub>3</sub>, 3.0 mM Na<sub>2</sub>CO<sub>3</sub>, pH 9.5 at 0.1 Amp constant current for 16 h. The membranes were blocked in PBS containing 5% dry milk and 0.2% Tween20 (PBS-TM)
for 1 h at room temperature. Membranes then were probed with a monoclonal antibody against the His\textsubscript{6} tag (Sigma, St. Louis, MO) in PBS-TM for 1 h at room temperature. Subsequent to three PBS-TM washes, membranes were incubated in PBS-TM containing secondary HRP-conjugated goat anti-mouse IgG antibody (BD Biosciences, San Diego, CA) for 1 h at room temperature. Membranes were washed three times with PBS-TM and three times with PBS alone. Washed membranes were incubated with ECL chemiluminescent detection reagent (Amersham-Pharmacia Biotech, Arlington Heights, IL) for 1 min at room temperature. Finally, membranes were exposed to Fuji SuperRX x-ray film (Fujii Photo Film Co., LTD, Tokyo, Japan).

**Knockdown of Endogenous hVps34 Expression in U-251 Cells**

The 293 GPG packaging cells were seeded at 1.2x10\textsuperscript{7} cells/dish in 100 mm dishes in DMEM containing 10% heat inactivated FBS. Twenty-four hours later, the 293 GPG cells were transfected with pSUPER.retro.puro constructs using Lipofectamine-Plus reagent. Forty-eight and 72 h after transfection, the virus-enriched medium was collected and passed through a 0.22 \( \mu \text{m} \) filter. Infections of the U-251 cells were performed on three sequential days in the presence of 4.0 \( \mu \text{g/ml} \) hexadimethrine bromide (Sigma, St. Louis, MO). Twenty-four hours after the third infection, cells were trypsinized and replated in selection medium containing 1 \( \mu \text{g/ml} \) puromycin.
Assessment of hVps34 Knockdown

For each experiment, cells infected with control or hVps34 KD vectors were harvested in order to verify the decrease in hVps34 expression. Briefly, equal amounts of protein were subjected to SDS-PAGE and immunoblot analysis as described above, using a polyclonal antibody against hVps34 (Zymed Laboratories, San Francisco, CA) followed by HRP-conjugated goat anti-rabbit IgG (BD Biosciences, San Diego, CA). The ECL signals were quantified using a Kodak 440CF ImageStation. Phase contrast images of control and KD cells were obtained using an Olympus IX70 inverted microscope equipped with a SPOT RT Slider digital and SPOT Advanced imaging software (Diagnostic Instruments, Sterling Heights, MI).

Ultrastructure Analysis

For transmission electron microscopy (TEM), 5 d post-selection, cells infected with the hVps34 KD and control vectors were pelleted by centrifugation at 390xg for 5 min at 4°C and fixed with 3% glutaraldehyde buffered in 0.2 M sodium cacodylate pH 7.4 for 1 h at room temperature. Following three 10 min sodium cacodylate washes, pellets were subjected to secondary fixation with 1% osmium tetroxide in s-collidine buffer pH 7.4 for 2 h at room temperature. Pellets were washed three times for 10 min each in s-collidine buffer. Pellets were subjected to tertiary fixation in an aqueous saturated uranyl acetate solution pH 3.3 for 1 h at room temperature. Dehydration was carried out via two 10 min incubations in a graded series of chilled ethanol solutions (30%, 50%, 70%, 90%, 100%) and two final 10 min washes with 100% acetone. Pellets
were infiltrated overnight in a 50% acetone-50% Spurr’s resin (Electron Microscope Sciences, Fort Washington, PA) solution. Following infiltration, pellets were embedded in 100% Spurr’s resin which polymerized at 80°C overnight. Polymerized cell blocks were ultra-thin sectioned with a Reichert Om U3 ultramicrotome (Leica Microsystems, Wien, Austria). Sections were collected on copper 300 mesh support grids and post-section stained with uranyl acetate followed by lead citrate. They were examined using a Philips CM 10 transmission electron microscope.

**Immunofluorescent Identification of Cellular Organelles**

After 5 d of selection, cells infected with the hVps34 KD and control vectors were seeded on laminin-coated glass coverslips in 35mm dishes at 100,000 cells per dish. Twenty-four hours later, cells were washed with PBS, fixed and permeabilized with ice-cold methanol for 10 min, and blocked with 10% goat serum in PBS for 30 min. The following primary antibodies were applied for 1 h in 10% goat serum in PBS: anti-LAMP1 (University of Iowa Developmental Studies Hybridoma Bank, Iowa City, IA), anti-LGP85 (gift from Dr. Yoshitaka Tanaka), anti-M6P receptor, anti-calreticulin (Affinity Bioreagents, Golden, CO), or anti-GM130 (BD Biosciences, San Diego, CA). For detection of LBPA, cells were fixed with ice-cold 3% paraformaldehyde in PBS pH 7.2 for 15 min at room temperature and then quenched with 50 mM NH₄Cl in PBS (5 min). Anti-LBPA (gift from Dr. Toshihide Kobayashi) was applied overnight in PBS with 0.05% saponin at 4°C. Cells were washed three times with 10% goat serum in PBS for 3 min and incubated for 1 h with Alexa Fluor ® 568 goat anti-mouse IgG (Molecular
Probes, Eugene, OR) or Alexa Fluor® 488 goat anti-rabbit IgG (Molecular Probes, Eugene, OR) in 10% goat serum in PBS. Cells were washed three times with 10% goat serum in PBS for 3 min and three times in PBS alone for 3 min. Coverslips were dipped into distilled water, blotted dry, and then mounted onto glass slides with fluorescent mounting medium (DAKO Corporation, Carpinteria, CA). Photomicrographs were taken with a Nikon Eclipse 800 fluorescence microscope equipped with a Sensys digital camera. Images processed using ImagePro software (Media Cybernetics, Silver Spring, MD).

**Immunofluorescent Labeling of Acidic Compartments**

After 5 d of selection, cells infected with the hVps34 knockdown and control vectors were seeded on laminin-coated glass coverslips in 35mm dishes at 100,000 cells per dish. Twenty-four hours later, cells were washed three times in serum-free, phenol red-free DMEM (PRF-DMEM) and incubated with 2.5 µg/ml acridine orange (Molecular Probes, Eugene, OR) in serum-free, PRF-DMEM for 30 min at 37°C. The live cells were then washed twice with serum-free, PRF-DMEM and inverted onto a drop of serum-free, PRF-DMEM containing 20% glycerol and immediately examined as described above.

**Immunofluorescent Labeling of Endosomal and Lysosomal Compartments**

After 5 d of selection, cells infected with the hVps34 KD and control vectors were seeded on laminin-coated glass coverslips in 35 mm dishes at 100,000 cells per dish. Twenty-four hours later, cells were washed three times with PRF-DMEM containing
10% FBS and incubated with a fluid-phase tracer, Texas red (TxR)-dextran (10,000MW, 500 µg/ml; Molecular Probes, Eugene, OR) in PRF-DMEM plus 10% FBS for 16 h at 37°C. Cells were washed three times with PRF-DMEM plus 10% FBS and incubated for 2 h in dextran-free DMEM plus 10% FBS. Cells were processed for immunofluorescent LAMP1 localization and examined as described above.

**EEA1 Localization**

After 5 d of selection, cells infected with the hVps34 KD and control vectors were seeded in 100 mm dishes at 1x10^6 cells per dish and on laminin-coated glass coverslips in 35mm dishes at 100,000 cells per dish and grown for 24 h. Cells infected with the control vector and treated with 1 µM wortmannin (Sigma, St. Louis, MO) for 1 h served as a positive control. For subcellular fractionation, cells were trypsinized and collected by centrifugation at 390xg for 5 min at 4°C. The cell pellets were washed three times with ice-cold PBS and re-suspended in a hypotonic buffer containing 10 mM HEPES pH 7.5, 1.5 mM MgCl₂, 10 mM KCl, and 1 mM DTT supplemented with complete mini protease inhibitors (Roche, Indianapolis, IN). The cells were incubated on ice for 15 min and then homogenized by 15 strokes of a Teflon homogenizer. Sucrose was added to the cell homogenates to a final concentration of 0.25 M. Soluble and particulate fractions were obtained by centrifugation at 100,000xg for 1 h at 4°C. Equal percentages of each fraction were subjected to SDS-PAGE. Proteins were electro-transferred to PVDF membranes in buffer containing 25 mM Tris, 192 mM glycine, and 40% methanol at 100 v for 2 h. Subsequent immunoblotting was carried out as described above using a
monoclonal antibody against EEA1 (BD Biosciences, San Diego, CA). ECL signals were quantified as described earlier.

For immunofluorescent localization of EEA1, cells were washed three times with PBS, fixed with ice-cold 3% paraformaldehyde in PBS pH 7.2 for 15 min at room temperature, quenched with 50 mM NH₄Cl in PBS (5 min), permeabilized with 50 µg/ml digitonin in PBS (5 min), and blocked with 10% goat serum in PBS for 30 min. The anti-EEA1 antibody was applied for 1 h in 10% goat serum in PBS. After three washes with 10% goat serum in PBS, cells were incubated with Alexa Fluor ® 568 goat anti-mouse IgG in 10% goat serum in PBS and processed as described above.

**Endocytosis of Horseradish Peroxidase (HRP)**

After 3 d of selection, cells infected with the hVps34 KD and control vectors were seeded in 35mm dishes at 75,000 cells per dish and grown for 48 h. Cells were washed one time with serum-free DMEM and incubated at 37°C with 2 mg/ml HRP (Sigma, St. Louis, MO) in DMEM containing 1% BSA. At the indicated time points, cells were placed on ice, washed three times with ice-cold PBS plus 1% BSA and one time with PBS alone. Cells were scraped into PBS, collected by centrifugation at 390xg for 5 min, washed once more with PBS, and disrupted in PBS containing 0.5% Triton X-100. Cell lysates were clarified by centrifugation at 10,000xg for 5 min at 4°C. Finally, 7 µl aliquots of the supernatants were incubated with 100 µl 1-Step Turbo TMB enzyme-linked immunosorbent assay kit (Pierce Chemical, Rockford, IL) at room temperature for 7 min. The reaction was stopped by adding 100 µl 2 M sulfuric acid and the volume was
increased to 1.0 ml by addition of 800 µl distilled water. The absorbance at 450 nm was measured using a Beckman DU 640 spectrophotometer (Beckman-Coulter Corporation, Fullerton, CA) and results were normalized to protein content, determined by the BCA protein assay kit (Pierce Chemical, Rockford, IL).

Transferrin Recycling

After 3 d of selection, cells infected with the hVps34 KD and control vectors were seeded in 35mm dishes at 75,000 cells per dish and grown for 48 h. Following serum starvation for 1 h, cells were incubated at 37°C with 50 µg/ml biotinylated transferrin (Sigma, St. Louis, MO) in serum free DMEM for 30 min. Cells were place on ice, washed two times with ice-cold serum free DMEM and one time with buffer containing 150 mM NaCl, 10 mM acetic acid pH 3.5 for 1 min to remove residual surface bound biotinylated transferrin. Cells were chased for the indicated time points in medium containing 500 µg/ml unlabeled transferrin and 100 µM deferoxamine mesylate (Sigma, St. Louis, MO). The chase medium was collected and cells were lysed in buffer containing 50 mM HEPES pH 7.4, 150 mM NaCl, 10 mM EDTA, 2 mM EGTA, 1.0% TritonX-100, and 0.1% SDS. Samples were mixed with 5X SDS sample buffer and subjected to SDS-PAGE. Proteins were electro-transferred to PVDF membranes in buffer containing 25 mM Tris, 192 mM glycine, and 40% methanol at 100 v for 2 h. The membrane was blocked for 1 h at 4°C in TBST (50 mM Tris pH 7.4, 100 mM NaCl, 0.5% Tween) buffer containing 1 M glucose, 10% glycerol, 1.0% dry milk, and 3.0% BSA. Membranes were washed three times with TBST and incubated for 1 h at 4°C with
streptavidin-HRP in TBST to detect the biotinylated transferrin. The ECL signals were quantified as described earlier.

**EGF Receptor Internalization and Degradation**

After 5 d of selection, cells infected with the hVps34 KD and control vectors were seeded at equal density in 60 mm dishes and on laminin-coated glass coverslips in 35 mm dishes and grown for 24 h. The EGF receptor was allowed to accumulate on the cell surface by serum-starving the cells for 16 h in DMEM. Receptor internalization and degradation was stimulated by addition of 100 ng/ml EGF (Upstate Biotechnology, Lake Placid, NY) in serum-free DMEM. At the indicated time points, cells were washed twice with ice-cold PBS and harvested in SDS sample buffer. Equal amounts of protein were subjected to SDS-PAGE. Proteins were electro-transferred to PVDF membranes in buffer containing 25 mM Tris, 192 mM glycine, and 40% methanol at 100 v for 2 h. Subsequent immunoblotting was carried out as described above using antibodies against phospho-EGF receptor (Cell Signaling Technology, Beverly, MA), total EGF receptor (BD Biosciences, San Diego, CA), phospho-Erk and total Erk (Cell Signaling Technology, Beverly, MA). The ECL signals were quantified as described previously.

For IF analysis, at the indicated time points, the coverslips were processed according to the methanol fixation procedure described above. Cells were co-stained using antibodies against EGF receptor and LGP85. Images were obtained as described above.
Immunofluorescent Localization, Metabolic Labeling, and Steady State Levels of Cathepsin D

For immunofluorescent localization of cathepsin D, after 5 d of selection, cells infected with the hVps34 KD and control vectors were seeded on laminin-coated glass coverslips in 35 mm dishes at 100,000 cells per dish. Coverslips were processed according to the methanol fixation procedure described above using a polyclonal antibody against cathepsin D (Biodesign International, Saco, ME).

For metabolic labeling and immunoprecipitation of cathepsin D, after 5 d of selection, cells infected with the hVps34 KD and control vectors were seeded at equal density in 100 mm dishes and grown for 24 h. Cells were washed once and incubated for 30 min at 37°C in methionine-free DMEM containing 10% FBS. Cells were metabolically labeled for 30 min at 37°C with 100 µCi/ml 35S-methionine/cysteine (Easy Tag™ EXPRESS protein labeling mix, 1.175 µCi/µmol, NEN/PerkinElmer, Boston, MA) in methionine-free DMEM plus 10% FBS, washed once with PBS, and chased in complete medium supplemented with 200 µM methionine and 200 µM cysteine at 37°C for 4 h. The addition of 15 mM NH₄Cl to the chase medium served as a positive control for disruption of lysosomal trafficking. At the end of the chase, cells were washed twice, scraped into PBS, and collected by centrifugation at 400xg for 5 min at 4°C. Cell lysates were prepared in 200 µl RIPA buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1.0% Nonidet P40, 0.5% deoxycholate, 0.1% SDS, 2 mM EDTA) supplemented with complete mini protease inhibitors. Soluble and particulate fractions were obtained by centrifuging the cell lysates at 100,000xg for 1 h at 4°C. The soluble fractions were pre-incubated with
protein A sepharose beads for 30 min at 4°C. The beads were removed by centrifugation and the cleared fractions were incubated with a polyclonal antibody against cathepsin D for 2 h at 4°C. Immune complexes were collected by a second 1 h incubation with protein A sepharose beads. The SDS-PAGE and fluorographic analysis of the immunoprecipitated proteins was performed as described (Wilson et al. 1998).

For steady state levels of cathepsin D, 15 µl of the whole cell RIPA lysates prepared above were subjected to SDS-PAGE. Subsequent immunoblotting was carried out as described above using a polyclonal antibody against cathepsin D (Santa Cruz Biotechnology, Santa Cruz, CA).

**Peroxidase Staining and Immunogold Labeling of Lysosomal Compartments**

For peroxidase staining, after 5 d of selection, cells infected with the hVps34 KD and control vectors were pelleted by centrifugation at 390xg for 5 min at 4°C and fixed with 3% glutaraldehyde buffered in 0.2 M sodium cacodylate pH 7.4 for 1 h at room temperature. Pellets were washed three times with sodium cacodylate for 10 min each and then resuspended in DAB reaction buffer (0.5 M Tris pH 7.6, 1 mg/ml 3,3-diaminobenzidine-4HCl (Electron Microscopy Sciences, Fort Washington, PA) 0.01% hydrogen peroxide) overnight. Pellets were washed 3 times with cacodylate buffer and processed for routine TEM as described above with the exclusion of any steps requiring uranyl acetate.

For cathepsin D immunogold staining, 5 d post-selection, cells infected with the hVps34 KD and control vectors were pelleted and fixed in 1% glutaraldehyde buffered in
0.2 M sodium cacodylate pH 7.4 for 30 min at room temperature. Fixed cell pellets were washed twice with sodium cacodylate buffer for 10 min each and twice with 0.5 M NH₄Cl for 20 min each. Pellets were dehydrated via a graded series of chilled ethanol solutions (30%, 50%, 70%, 90%, 100%). Pellets were infiltrated overnight in a 50% Ethanol-50% LR White® Embedding media (London Resin Co., Ltd., Berkshire, England) solution. Following infiltration, pellets were embedded in 100% in LR White® Embedding media overnight at 55°C under pressure. Polymerized cell blocks were ultra-thin sectioned with a Reichert Om U3 ultramicrotome. Sections were collected on gold 200-mesh electron microscopy support grids and baked under a light for 15 min. Sections then were washed with PBS for 5 min and blocked in a solution of 1.0% fish gelatin in PBS for 1 h. Reaction with a goat anti-cathepsin D antibody was carried out for 2 h at room temperature, followed by six washes with PBS. Sections were incubated for 1 h with donkey anti-goat IgG conjugated with 6 nm colloidal gold (Jackson ImmunoResearch Labs, West Grove, PA), followed by three changes of PBS, a high salt wash (2.5M NaCl in PBS), and three more PBS washes. The samples were post-fixed with 1% glutaraldehyde, washed 10 times with distilled water, and stained with uranyl acetate and lead citrate. They were blot-dried and examined with a Philips CM 10 transmission electron microscope for localization of the colloidal gold probe.

**Cell Growth and DNA Synthesis**

To assess cell growth, control and KD cells that had been selected for 2 d in 1 μg/ml puromycin were seeded in 35mm dishes at 50,000 cells per dish. At the indicated
time points, the cells were trypsinized and counted using a Coulter Z-series particle counter (Beckman-Coulter Corporation, Fullerton, CA). To measure DNA synthesis, cells that had been selected for 2 d were seeded in 25cm² flasks at 150,000 cells per flask. At the indicated time points, cells were incubated for 5 h in medium containing 1.0 µCi/ml [methyl-³H]-thymidine (5 Ci/mmol, Amersham Biosciences, Piscataway, NJ). Incorporation of radioactivity into trichloroacetic acid (TCA)-precipitable material was determined as described previously (Maltese et al. 1981). Briefly, cells were pelleted and incubated in cold distilled water for 10 min at 4°C. Ten percent TCA was added for 20 min at 4°C. The precipitate was collected, washed three times with 10% TCA, and dissolved in 0.5N KOH. The samples were neutralized with HCl and mixed with EconoSafe™ counting cocktail (RPI, Mount Prospect, Illinois). Incorporated radioactivity was measured using a Beckman LS 3801 liquid scintillation counter (Beckman-Coulter Corporation, Fullerton, CA) and normalized to precipitated protein, determined with a colorimetric protein assay (Bio-Rad, Hercules, CA).

Detection of Apoptosis

For assessment of apoptotic cell death by the TdT-mediated dUTP nick end labeling (TUNEL) assay, after 5 d of selection, cells infected with the hVps34 KD and control vectors were seeded on laminin-coated glass coverslips in 35 mm dishes at 100,000 cells per dish. Forty-eight hours later, cells were fixed with ice-cold 3% paraformaldehyde in PBS pH 7.2 for 15 min at room temperature, quenched with 50 mM NH₄Cl in PBS for 5 min, permeabilized with 0.1% TritonX-100 in PBS for 2 min, and washed three times
PBS. The TUNEL assay was carried out as described in the protocol for the Apoptosis Detection System Kit (Promega Corporation, Madison, WI). Control cells treated with DNase (Sigma, St. Louis, MO) for 5 min served as a positive control. Briefly, fixed and permeabilized cells were equilibrated in a buffer containing 200 mM potassium cacodylate pH 6.6, 25 mM Tris pH 6.6, 0.2 mM DTT, 0.25 mg/ml BSA, and 2.5 mM cobalt chloride for 15 min. Cells were incubated with fluorescein-12-dUTP and TdT enzyme for 1 h at 37°C. Cells were washed twice with 2X SSC (300 mM sodium chloride and 30 mM sodium citrate pH 7) and three times with PBS. Coverslips were dipped into distilled water, blotted dry, mounted and examined as described previously.

For assessment of apoptotic cell death by annexin staining, control and KD cells that had been selected for 2 d were seeded in 60 mm dishes at 200,000 cells per dish. Control cells treated with 10 ng/ml TNFα (Calbiochem, San Diego, CA) plus 2.5 µg/ml cyclohexamide (Sigma, St. Louis, MO) overnight served as a positive control. At the indicated time points, floating and adherent cells were collected and stained with phycoerythrin (PE)-tagged annexin-V and 7-amino-actinomycin D as described in the protocol for the Guava Nexin™ kit (Guava Technologies Inc., Hayward, CA). Briefly, floating cells were obtained by collecting the medium from each dish. Adherent cells were trypsinized and resuspended in the previously collected media containing the floating cells. The combined cells were counted and 120,000 cells per sample were subjected to the following conditions. Each sample was washed one time with and resuspended in ice-cold 1x Nexin buffer. Cells were labeled with a Nexin-binding
solution containing annexin-V-PE and 7-amino-actinomycin D on ice and in the dark for
20 min. The staining was then quantified using a Guava personal cytometer.

**Immunofluorescent Localization of hVps34**

For immunofluorescent studies, HEp-2 cells were seeded on laminin-coated glass
coverslips in 35mm dishes at 100,000 cells per dish. Twenty-four hours later, cells were
fixed and permeabilized according to the methanol and PFA procedures described
previously, with the exception that cells fixed with PFA were permeabilized with 0.05%
saponin (10 min) or 0.1% TritonX-100 in PBS (5 min). All coverslips were blocked with
10% goat serum in PBS for 30 min. The anti-hVps34 polyclonal antibody was applied
for 1 h in 10% goat serum in PBS. After three 3 min washes with 10% goat serum in
PBS, cells were incubated with Alexa Fluor ® 488 goat anti-rabbit IgG in 10% goat
serum in PBS and then processed as described above. For antibody specificity, the anti-
hVps34 was incubated with a 10-fold molar excess of the specific peptide (pep A) used to
make the antibody or a non-specific hVps34 peptide (pep B) for 30 min prior to addition
to cells.

**Subcellular Fractionation Using a Discontinuous Sucrose Gradient**

In order to determine the subcellular distribution of hVps34, HEp-2 cells were
grown to 80% confluence in 15 100 mm dishes. Cells were trypsinized, pooled, and
collected by centrifugation at 390xg for 5 min at 4⁰C. The cell pellet was washed three
times with ice-cold PBS and re-suspended in a hypotonic buffer containing 10 mM
HEPES pH 7.9, 1.5 mM MgCl$_2$, 10 mM KCl, and 1 mM DTT supplemented with complete mini protease inhibitors. The cells were incubated on ice for 15 min and then disrupted by 15 passes through a 27 gauge needle. Sucrose was added to the cell homogenate to a final concentration of 0.25 M. The homogenate (H) was centrifuged at 2,500xg for 10 min at 4°C to produce a supernatant (S1) and pellet (P1). The P1 was resuspended in hypotonic buffer containing 0.25 M sucrose, layered onto a discontinuous sucrose gradient (0.25 M, 1.2 M, 1.6 M, 2.1 M in hypotonic buffer), and centrifuged at 130,000xg for 1h. The fractions at the interfaces between the 0.25 M/1.2 M and 1.2 M/1.6 M sucrose layers were collected, pooled, and labeled gradient 1 (G1). The fraction at the interface between the 1.6 M/2.1 M sucrose layers was collected and labeled gradient 2 (G2). Finally, the pellet was collected and labeled gradient 3 (G3).

The S1 was centrifuged at 150,000xg for 1 h at 4°C resulting in supernatant (S2) and pellet (P2) fractions. The P2 was resuspended in hypotonic buffer containing 0.25 M sucrose, layered onto a sucrose cushion (1.6 M in hypotonic buffer), and centrifuged at 150,000xg for 40 min at 4°C. The material at the 0.25 M/1.6 M interface was collected and labeled microsomes (M). The pellet was collected and combined with the G2 fraction. The total protein in each fraction was determined using a colorimetric protein assay (Bio-Rad, Hercules, CA) prior to the addition of SDS sample buffer. The fractions were subjected to SDS-PAGE. Subsequent immunoblotting was carried out as described above using antibodies, against hVps34, nuclear Lamin A/B (Zymed Laboratories, San Francisco, CA), LAMP1, EEA1, EGF receptor, Cytochrome c, and Syntaxin 8 (BD Biosciences, San Diego, CA).
**Isolation of Nuclei**

In order to isolate nuclei free of membrane contamination, HEp-2 cells were grown to 80% confluence in five 100mm dishes. Cells were trypsinized, pooled, and collected by centrifugation at 390xg for 5 min at 4°C. The cell pellet was washed three times with ice-cold PBS and re-suspended in a hypotonic buffer containing 10 mM HEPES pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, and 1 mM DTT supplemented with complete mini protease inhibitors. The cells were incubated on ice for 15 min and disrupted by 25 strokes with a Dounce homogenizer. The homogenate was centrifuged at 700xg for 10 min at 4°C to yield a supernatant (S1) and a “pre-stripped nuclear pellet.” Hypotonic buffer containing 0.8% TritonX-100 was added to the nuclear pellet. Following a 10 min incubation on ice, centrifugation at 700xg for 10 min at 4°C yielded a TritonX-100 extract (Ext1) and “stripped nuclear pellet 1.” The stripped nuclear pellet 1 was subjected to a second round of TritonX-100 extraction to yield a TritonX-100 extract (Ext 2) and a “stripped nuclear pellet 2.” Finally, the stripped nuclear pellet 2 was washed three times with hypotonic buffer containing 25% glycerol and labeled “nuclei final.” All of the samples were mixed with SDS sample buffer and subjected to SDS-PAGE. Immunoblotting was carried out as described above using antibodies against hVps34, nuclear Lamin A/B, LAMP1, EEA1 and Rab6 (Santa Cruz Biotechnology, Santa Cruz, CA). In order to confirm that hVps34 remained in the nucleus following TritonX-100 extraction, this procedure was repeated (excluding the final nuclear wash with 25% glycerol in hypotonic buffer). The fractions were subjected to immunoblot analysis as described above using a hVps34 antibody from an independent source (gift from Dr.
Jonathan Backer) and antibodies against nuclear Lamin A/B, Rab1b (Zymed Laboratories, San Francisco, CA), LDH (Sigma, St. Louis, MO), and Rab6.

**Differential Extraction of Cells on Coverslips**

In order to further characterize the nuclear localization of hVps34, HEp-2 cells were seeded on laminin-coated glass coverslips in 35 mm dishes at 75,000 cells per dish. Twenty-four hours later, cells were placed on ice and incubated sequentially in the following buffers for 10 min each with two PBS washes in between, (1): 10 mM PIPES pH 6.8, 100 mM NaCl, 3 mM MgCl$_2$, 300 mM sucrose, 0.5% TritonX-100 and (2): 10 mM PIPES pH 6.8, 3 mM MgCl$_2$, 300 mM sucrose, 0.5% TritonX-100, 0.5% deoxycholate, 1.0% Tween20. Cells were removed from ice and incubated in buffer (3): 10 mM PIPES pH 6.8, 50 mM NaCl, 3 mM MgCl$_2$, 300 mM sucrose, 0.5% TritonX-100, 100 µg/ml RNase (Roche, Indianapolis, IN) for 20 min at room temperature. Cells were fixed with ice-cold methanol for 10 min followed by ice-cold 3% paraformaldehyde in PBS pH 7.2 for 15 min. Free baldheads were quenched with 50 mM 50 mM NH$_4$Cl in PBS for 5 min at room temperature. Subsequent immunofluorescent localization of hVps34 was carried out as described above. Following the final washes in distilled water, cells were incubated with 300 nM diamidino-phenylindole (DAPI) in water for 5 min to stain DNA. Cells were washed three times with water, mounted and examined as described above.
Differential Extraction of Cells in Suspension

HEp-2 cells were grown to 80% confluence in six 100 mm dishes. Cells were trypsinized, pooled, and collected by centrifugation at 390xg for 5 min at 4°C. The cell pellet was washed three times with ice-cold PBS and subjected to two rounds of Triton X-100 extraction in buffer containing 10 mM PIPES pH 6.8, 100 mM NaCl, 3 mM MgCl₂, 300 mM sucrose, 0.5% TritonX-100. The TritonX-100 extracted nuclei were incubated for 15 min on ice in buffer containing 10 mM PIPES pH 6.8, 3 mM MgCl₂, 300 mM sucrose, 0.5% TritonX-100, 0.5% deoxycholate, 1.0% Tween20 (TDT buffer) and centrifuged at 2500xg for 5 min at 4°C. The supernatant was removed and labeled “TDT extract 1.” The pellet was subjected to another TDT extraction yielding a “TDT extract 2” and a TDT extracted nuclear pellet. This pellet was incubated for 20 min at room temperature in buffer containing 10 mM PIPES pH 6.8, 50 mM NaCl, 3 mM MgCl₂, 300 mM sucrose, 0.5% TritonX-100, 100 µg/ml RNase (Roche, Indianapolis, IN) and centrifuged at 10,000xg for 5 min at 4°C. The supernatant was removed and labeled “RNase extract.” The pellet was labeled “nuclei final.” All of the samples were mixed with SDS sample buffer and subjected to SDS-PAGE. Subsequent immunoblotting was carried out as described above using antibodies, against hVps34 and nuclear Lamin B₂ (Zymed Laboratories, San Francisco, CA).

Nuclear Localization of hVps34 Following Inhibition of Transcription

For immunofluorescent localization of hVps34, HEp-2 cells were seeded on laminin-coated glass coverslips in 60 mm dishes at 133,000 cells per dish. Forty-eight
hours later, to inhibit transcription, cells were incubated for 4 h with 2.5 µg/ml actinomycin or two different concentrations (10 µg/ml, 20 µg/ml) of α-amanitin (Sigma, St. Louis, MO) in DMEM supplemented with 10% FBS. Immunofluorescent localization of hVps34 was carried out according to the methanol fixation procedure described previously.
RESULTS

Hypothesis I: Suppression of hVps34 Expression will Impair Only the Trafficking Pathways that Absolutely Require PI3P Generated by hVps34 for Normal Function

The pSUPER.retro.puro Constructs Effectively Knockdown Overexpressed His$_6$-tagged-hVps34 in a Transient System

The pSUPER.retro.puro vectors were engineered to contain sequences matching a unique region of the hVPS34 mRNA, or “control” sequences that harbored a one base mismatch and did not match any other known sequences in the NCBI database (Fig. 9A). In order to test the ability to specifically target hVps34, the pSUPER.retro.puro vectors were co-transfected with His$_6$-hVps34-pcDNA3.1 in human 293T cells. Twenty-four hours post-transfection, cells were harvested and subjected to immunoblot analysis to assess the level of His$_6$-hVps34 expression. As illustrated in Fig. 9B, both knockdown constructs targeting hVps34 effectively reduced the expression of His$_6$-hVps34. On the other hand, the mismatch controls exhibited expression levels similar to cells co-transfected with His$_6$-hVps34-pcDNA and empty pSUPER.retro.puro. Based on these results, the “knockdown 2” (KD) and “control 1” (control) constructs were used in all the following experiments to assess the consequences of knocking down endogenous hVps34.
Fig. 9. The pSUPER.retro.puro Knockdown Constructs are Specific to hVps34

(A) siRNA target sequences designed for suppression of hVps34 expression and negative controls possessing a one base mismatch. The numbers represent the position within the 2664bp hVps34 cDNA sequence. (B) 293T cells co-transfected with His$_6$-hVps34-pcDNA3.1 and pSUPER.retro.puro constructs were harvested 24 h post-transfection and subjected to immunoblot analysis with monoclonal anti-His$_6$ IgG.
Reduction of hVps34 Expression by siRNA-Mediated Gene Silencing Causes Accumulation of Cytoplasmic Vacuoles

The 293 GPG packaging cells were transfected with the pSUPER.retro.puro control or KD vectors in order to generate retroviral particles harboring the hVps34 control or KD siRNA sequences and the gene for puromycin resistance. In preliminary tests with several cell lines infected with a GFP reporter construct, the human U-251 glioblastoma cell line exhibited high infection efficiency. Therefore, U-251 cells were infected with the control or KD virus and selected with puromycin in order to obtain a cell population in which expression of hVps34 was specifically and uniformly suppressed. As illustrated by the immunoblots in Fig. 10A, expression of hVps34 was almost undetectable in puromycin-resistant cells that received the hVps34 KD virus, compared with cells that were infected with the control virus. In all of the experiments described in this study, similar immunoblot results were obtained, verifying that expression of hVps34 was decreased by at least 90% relative to the parallel control cultures. Expression levels of several unrelated proteins (e.g., lactate dehydrogenase, calreticulin, lamin B2) were comparable in the control and KD cells, indicating that the loss of hVps34 expression was not due to a general effect of the siRNA on protein synthesis.

Examination of the cultures by phase contrast microscopy revealed striking morphological differences between the hVps34 KD cells and the matched controls (Fig. 10B). Specifically, the KD cells were filled with numerous large phase-lucent spherical cytoplasmic vacuoles ranging in size from 1 to 8 µm in diameter.
Fig. 10. siRNA-Mediated Suppression of hVps34 Expression in U-251 Glioma Cells

Induces Large Phase-Lucent Vacuoles

(A) U-251 cells infected with retrovirus carrying the control or hVps34 KD siRNA sequences surviving after 6 d of puromycin selection were subjected to immunoblot analysis with a polyclonal anti-hVps34 IgG as described in the Materials and Methods. Nuclear lamin B2 served as a loading control. (B) Phase contrast image of the live cells. Bar represents 10 µm.
Vacuoles in the hVps34 KD Cells are Electron-Lucent and Bound by a Single Membrane

Comparison of the control and hVps34 KD cells using electron microscopy revealed that the vacuoles in the KD cells were generally electron-lucent (Fig. 11A). However, these vacuoles contained some sparsely distributed electron-dense material along with a few internal vesicles and membrane whorls (Fig. 11B, arrows). The vacuoles also were circumscribed by a single membrane, indicating that they did not represent enlarged autophagosomes, which are double membrane-bound structures (Fig. 11B, arrowhead).

Vacuoles in hVps34 KD Cells are Derived from Late Endosomes

To begin to assess the origin of the vacuoles in the hVps34 KD cells, indirect immunofluorescence microscopy was performed using a series of antibodies against proteins localized in specific organelles (Fig. 12). The limiting membranes of the vacuoles were clearly outlined by antibodies against LAMP1 and another late endosome/lysosome glycoprotein, LGP85 (Gough et al. 1999, Kuronita et al. 2002). The enlarged vacuoles in the hVps34 KD cells did not react with an antibody against lysobisphosphatidic acid (LBPA), a phospholipid that is normally concentrated in the internal membranes of MVBs (Kobayashi et al. 1998). In the control cells, LAMP1, LGP85 and LBPA were all localized to clusters of punctuate structures adjacent to the nucleus, typical of the late endosome and/or lysosome distribution of these markers seen in many types of cells.
Fig. 11. Vacuoles in the hVps34 KD Cells are Membrane-Bound Structures with Occasional Internal Vesicles and Electron-Dense Material

Control and hVps34 KD cells were examined by electron microscopy. (A) Control and KD cells magnified 3,900X. Bar represents 5 µm. (B) Highlighted region of KD cell from panel A at 21,000X. The bar represents 1 µm. Labeled structures: mitochondria (m), nucleus (n), vacuole (v). The arrowhead points to single membrane surrounding the vacuole. Arrows point to occasional internal vesicles and electron-dense material.
Control and hVps34 KD cells were seeded at 100,000 cells/dish in 35 mm dishes. Twenty-four h later cells were examined by immunofluorescence microscopy using the primary antibodies indicated at the left of the figure. The bar represents 10 µm.
Immunofluorescent staining with antibodies to proteins associated with membranes of endoplasmic reticulum or Golgi apparatus confirmed that the vacuoles did not arise directly from these compartments in the hVps34 KD cells (Fig. 12). Calreticulin exhibited a diffuse pattern throughout the cytoplasmic regions not occupied by the vacuoles, and GM130 exhibited a compact juxtanuclear distribution typical of Golgi localization. The cation-independent M6P receptor did not accumulate in the limiting membranes of the vacuoles. Instead, the M6P receptor was detected mainly in a compact region adjacent to the nucleus, similar to the GM130 Golgi marker (Fig. 12). The M6P receptor is involved in the delivery of newly synthesized lysosomal hydrolases from the TGN to the MVB (Le Borgne and Hoflack 1998, Ghosh et al. 2003, Press et al. 1998). The distribution of M6P receptor in the control and hVps34 KD cells is consistent with previous reports indicating that at steady state most of the M6P receptor is localized in the TGN (Kobayashi et al. 1998, Hirst et al. 1998).

To further characterize the cytoplasmic vacuoles, supravital staining of the cells with the lysosomotropic agent, acridine orange (AO) was performed (Fig. 13A). The non-protonated monomeric form of AO emits green fluorescence in the cytoplasm. However, when the dye enters an acidic compartment (e.g., lysosomes or late endosomes), the protonated form becomes trapped in aggregates that fluoresce bright red or orange (Traganos and Darzynkiewicz 1994, Paglin et al. 2001, Kanzawa et al. 2003). The images in Fig. 13A demonstrate that the numerous vacuoles observed in the hVps34 KD cells are acidic vesicular organelles that sequester AO.
Fig. 13. Vacuoles in the hVps34 KD Cells are Acidic and Receive Traffic from the Endocytic Compartment

(A) Control and hVps34 KD cells were incubated with 2.5 µg/ml acridine orange for 30 min. Live cells were examined by fluorescence microscopy using green (excitation wavelength: 500 nm, emission wavelength: 520 nm) and red (excitation wavelength: 488 nm, emission wavelength 655 nm) filters. Red fluorescence emanates from AO in acidic compartments. The scale bar represents 10 µm. (B) Control and KD cells were incubated with 500 µg/ml TxR-dextran for 16 h. Following a 2 h incubation, cells were fixed and co-stained with a monoclonal antibody against LAMP1. Arrows indicate vacuoles containing both LAMP1 and TxR-dextran. Arrowheads point to vacuoles lacking TxR-dextran. The bar represents 10 µm.
Based on the presence of lysosomal membrane markers and their ability to sequester AO, I hypothesized that the vacuoles in the hVps34 KD cells were derived from late endosomes or lysosomes. To further test this hypothesis, the endosomal system was labeled by adding a fluid phase tracer, TxB-dextran, to the culture medium for 16 h (Fig. 13B). The distribution of TxB-dextran was compared with that of LAMP1, a membrane glycoprotein restricted to lysosomes and late endosomes (Gough et al. 1999). In the control cells, the TxB-dextran was concentrated in a cluster of small vesicles adjacent to the nucleus. Consistent with the expected uptake of TxB-dextran into endosomes and lysosomes, the tracer showed extensive co-localization with LAMP1-positive compartments (Fig. 13B). Examination of the hVps34 KD cells revealed that the fluid phase tracer was incorporated into some of the large LAMP1-positive cytoplasmic vacuoles (Fig. 13B, arrows) as well as numerous smaller vesicular structures. However, the persistence of some LAMP1-positive vacuoles that did not incorporate TxB-dextran after prolonged incubation (Fig. 13B, arrowheads) suggests that a subpopulation of these structures may be functionally disengaged from the fluid-phase endocytic pathway.

**Suppression of hVps34 Expression Does Not Disrupt the PI3P-Dependent Localization of the Early Endosome Protein, EEA1**

In an attempt to assess the integrity of the early endosomes, the subcellular distribution of EEA1, a FYVE-domain protein that is recruited to the early endosome membrane in a PI3P- and Rab5-dependent manner, was examined (Simonsen et al. 1998, Christoforidis et al. 1999a). There was no clear association of EEA1 with the membranes
of the numerous large vacuoles in the hVps34 KD cells (Fig. 14A). Instead, the protein was detected predominantly in a population of smaller vesicles with a pattern similar to the control cells. In careful comparisons of the EEA1-positive compartments in control versus hVps34 KD cells, ring-like structures that could represent swollen early endosomes in the KD cells were occasionally observed (Fig. 14A, arrow). However, these structures were distinct from the more abundant and much larger phase-lucent vacuoles. In contrast to the bright punctate fluorescence pattern of EEA1 in the control and hVps34 KD cells, cells treated with 1 µM wortmannin for 1 h showed only a diffuse reticular staining pattern, consistent with the release of EEA1 from endosomal membranes reported to occur in cells treated with PI3K inhibitors (Simonsen et al. 1998, Petiot et al. 2003).

To further examine the subcellular distribution of EEA1, control and KD cells were fractionated into cytosolic and particulate components and the partitioning of EEA1 between these components was determined by immunoblot analysis (Fig. 14B). The results revealed a very similar distribution of EEA1 in the control and hVps34 KD cells, with approximately 21-24% of the total EEA1 in the particulate fraction. In contrast, treatment with 1 µM wortmannin for 1 h resulted in an easily detected decline in the proportion of EEA1 associated with the particulate fraction (12%). When considered together with the immunofluorescence localization studies in Fig. 14A, these results indicate that EEA1 is able to associate with endosomal membranes in U-251 cells under conditions where expression of hVps34 is almost completely suppressed.
Fig. 14. Suppression of hVps34 Expression Does Not Prevent Membrane Association of the Early Endosome Marker, EEA1

(A) Control and hVps34 KD cells were seeded at 100,000 cells/dish in 35 mm dishes. Twenty-four hours later cells were examined by immunofluorescence microscopy using an anti-EEA1 antibody. Control cells treated with 1 µM wortmannin for 1 h served as a positive control for release of EEA1. (B) Cytosolic (S100) and particulate (P100) fractions prepared from control and KD cells as described in the Materials and Methods were subjected to immunoblot analysis with an antibody against EEA1. Control cells treated with 1 µM wortmannin for 1 h served as a positive control. The percentage of EEA1 in each fraction was determined using a Kodak 440CF Image Station.
Endocytic Internalization of HRP, Recycling of Transferrin, and Degradation of the EGF Receptor are Not Blocked in hVps34 KD Cells

The preceding studies of EEA1, coupled with the earlier observation that a fluid-phase tracer, TxR-dextran, was incorporated into LAMP1-positive compartments in the hVps34 KD cells (Fig. 13B) suggested that production of PI3P by hVps34 was not essential for delivery of early endosome cargo to late endosomes in U-251 cells. Previous studies have shown that when vesicular transport from early endosomes to late endosomes is inhibited, there is a reduction in cellular uptake of the soluble endocytic tracer, HRP (Li and Stahl 1993, Mayran et al. 2003). Thus, to further explore the integrity of the early endocytic pathway in the hVps34 KD cells, the rate of HRP uptake was determined. As shown in Fig. 15A, the rate of HRP uptake was not reduced in the KD cells. On the contrary, HRP uptake was significantly increased compared with the controls.

An increased accumulation of fluid phase markers has been linked to defects in the endocytic recycling pathway (Sonnichsen et al. 2000). The internalization of the transferrin receptor results in targeting to the recycling pathway. Following dissociation of iron from its ligand, transferrin, in the early endosome, the transferrin receptor-transferrin complex recycles back to the plasma membrane (Mayor et al. 1993, Hopkins et al. 1994). To explore the possibility that suppression of hVps34 expression causes a defect in endosome recycling, the fate of internalized transferrin was examined. As shown in Fig. 15B, hVps34 KD cells release internalized transferrin at a rate identical to
Fig. 15. Suppression of hVps34 Expression Does Not Impair Endocytosis of the Fluid Phase Marker, HRP or Recycling of Transferrin

(A) Control (-▲-) and hVps34 KD (-■-) cells were incubated for the indicated time periods with 2 mg/ml HRP in DMEM + 1% BSA. Washed cells were lysed and HRP activity was determined as described in the Materials and Methods. Each point represents the mean ± S.E. from triplicate dishes of each cell line. (B) Control (-▲-) and hVps34 KD (-■-) cells were incubated with 50 µg/ml of biotinylated transferrin for 30 min. and chased for the indicated time points in medium with an excess of unlabeled transferrin. The amount of biotinylated transferrin in the cells and the chase medium was determined as described in the Materials and Methods. Recycled transferrin is represented as the percentage of the total biotinylated transferrin that was released into the chase medium. Each point represents the mean ± S.E. from triplicate dishes of each cell line.
controls, indicating that the recycling pathway is not impaired by suppression hVps34 expression. Therefore, a defect in the endocytic recycling pathway does not account for the increase in HRP uptake in hVps34 KD cells. Although the basis for this increase remains to be determined, the HRP uptake and transferrin recycling results clearly argue against hVps34 being required for early steps in the endocytic internalization or recycling pathways.

As a final means to evaluate protein trafficking in the endocytic pathway, the fate of the activated EGF receptor was examined. In serum-deprived cells grown in the absence of EGF, degradation of the EGF receptor is minimal which results in accumulation on the cell surface. However, upon addition of EGF, the receptor is rapidly activated by tyrosine phosphorylation in the C-terminal cytoplasmic domain and the EGF-EGF receptor complexes are internalized into clathrin-coated early endosomes. Down-regulation of activated receptors depends upon their delivery to late endosomes, where receptor complexes are sorted into internal vesicles that are ultimately degraded when MVBs fuse with lysosomes (reviewed in Katzmann et al. 2002). When the EGF receptor was localized by immunofluorescence 30 min after addition of EGF to hVps34 KD cells, most of the receptors were found in small internal vesicles that were distinct from the large vacuoles labeled by the late endosome/lysosome marker, LGP85 (Fig. 16A). However, after 60 min, some EGF receptor could be detected in the limiting membranes surrounding the large LGP85-positive vacuoles (Fig. 16A, arrows). This suggested that delivery of EGF receptor from early endosomes to the surface membranes
Fig. 16. Suppression of hVps34 Expression Does Not Impede Receptor Internalization and Degradation

Control and hVps34 KD cells were stimulated with EGF after overnight incubation in serum-free medium. (A) Cells were fixed and co-stained with EGF receptor (EGFR) and LGP85 antibodies at 30 min or 60 min after addition of EGF. Arrows indicate vacuoles positive for both the EGF receptor and LGP85. The scale bar represents 10 µm. (B) For EGF receptor degradation, the control and KD cells were harvested at the indicated times after the addition of EGF and subjected to immunoblot analysis for total EGF receptor. The bar graph illustrates the data generated from Kodak Imager scans of blots from triplicate cultures of each cell line.
of the enlarged late endosomal structures was not impaired in the hVps34 KD cells. To examine the rate of degradation, immunoblot analysis of total EGF receptor was performed at intervals after addition of EGF. The results indicate that overall degradation of the receptor was similar in the hVps34 KD cells compared with the controls (Fig. 16B).

To assess the amount of activated receptor at intervals after EGF stimulation, the blots were re-probed with an antibody that specifically recognizes phosphorylated Y1068 in the C-terminal domain of the receptor. The C-terminal domain faces the cytoplasm when the EGF receptor is in the limiting membrane of the endosome, but it is incorporated into the lumen of vesicles that invaginate to the interior of the MVB (Katzmann et al. 2002). As shown in Fig. 17A, nearly all of the EGF receptor was in the non-phosphorylated state at the starting point, but within 30 min of adding EGF, both control and hVps34 KD cells exhibited a dramatic increase in the proportion of phospho-EGF receptor relative to total receptor. Interestingly, although there was little difference in the overall degradation of the EGF receptor (Fig. 16B), the ratio of phospho-EGF receptor to total EGF receptor in the hVps34 KD cells was approximately double that observed in the control cells (Fig. 17A). Thus, it appears that prior to being exposed to lysosomal proteases, a higher percentage of the EGF receptor remains phosphorylated in the hVps34 KD cells. In accord with this concept, activation of the ERK (p44/42 MAP kinase) signaling pathway, as measured by the ratio of phospho-ERK1/2 to total ERK1/2, was augmented in the hVps34 KD cells compared with the controls (Fig. 17B).
Control and hVps34 KD cells were stimulated with EGF after overnight incubation with serum-free medium. For EGF receptor (EGFR) phosphorylation, and signaling, the control and KD cells were harvested at the indicated times after the addition of EGF and subjected to immunoblot analysis for, (A) total EGF receptor (Fig. 16B), phospho-EGF receptor, and (B) phospho-ERK1/2 and total ERK1/2. The bar graphs illustrate the data generated from Kodak Imager scans of blots from triplicate cultures of each cell line.
Silencing of hVps34 Expression Allows Transport of Pro-cathepsin D from the TGN to Endosomes, but Slows Proteolytic Processing of the Endosomal Intermediate

To determine if the enlarged endosomal structures in the hVps34 KD cells were capable of accepting cargo normally delivered to late endosomes from the TGN, the processing of the lysosomal enzyme, cathepsin D was examined (Pohlmann et al. 1995). Newly synthesized pro-cathepsin D (51-53 kDa) associates with the cation-independent M6P receptor in the TGN and is delivered to the endosomal compartment, where it is activated by removal of the pro-peptide to generate an intermediate that migrates at 46-48 kDa on SDS gels. The final step in cathepsin D processing is completed in the lysosomes, where the intermediate is cleaved to the mature form, which contains two non-covalently linked chains of 30 kDa and 14 kDa (Rijnboutt et al. 1992, Delbruck et al. 1994).

Initial assessment of the immunofluorescent localization of cathepsin D revealed a striking accumulation of the enzyme in hVps34 KD cells, suggesting a defect in cathepsin D processing (Fig. 18). Diment et al. (1988) determined that the intermediate form of cathepsin D is associated with endosomal membranes. The final cleavage of cathepsin D into the mature form results in the release of the enzyme into the lumen of the lysosome (Diment et al. 1988). Although the antibody used for the immunofluorescent localization in the present study does not discriminate between the three forms of the enzyme, the concentration of cathepsin D around the limiting membrane of the enlarged vacuoles suggests there is an accumulation of the intermediate form of the enzyme (Fig. 18, arrows).
Control and hVps34 KD cells were seeded at 100,000 cells/dish in 35 mm dishes. Twenty-four hours later cells were examined by immunofluorescence microscopy using a polyclonal antibody against cathepsin D. The bar represents 10 µm.
To evaluate the processing of newly synthesized procathepsin D, a pulse-chase analysis was performed (Fig. 19). When $^{35}$S-methionine-labeled cathepsin D was immunoprecipitated after a 30 min pulse, nearly all of the radiolabeled protein was in the 53 kDa form in both control and KD cells. After a 4 h chase, the control cells converted most of the pro-cathepsin D to the mature form, with some intermediate form still detected. There was very little residual radiolabeled pro-cathepsin D in the hVps34 KD cell after a 4 h chase, suggesting that delivery of pro-cathepsin D to the late endosome compartment was not substantially altered in the absence of hVps34 (Fig. 19A). However, compared with the control, there was a 50% decrease in the relative amount of the mature 30 kDa cathepsin D and a corresponding increase in the 47 kDa intermediate (Fig. 19A). In contrast, a complete block instituted by raising the endosomal and lysosomal pH with NH$_4$Cl is manifested by the absence of any radiolabeled 30 kDa cathepsin D after a 4 h chase (Fig. 19A). These results are indicative of a kinetic block at the late endosome to lysosome transition, resulting in slower lysosomal processing of the cathepsin D intermediate.

This interpretation is reinforced by the immunoblot depicted in Fig. 19B, which shows that the steady-state level of the 47 kDa cathepsin D intermediate was markedly elevated in the hVps34 KD cells. On the other hand, in agreement with the pulse-chase study, there was no accumulation of the 53 kDa pro-cathepsin D that could indicate a problem with trafficking from the TGN. Likewise, analysis of pro-cathepsin D released into the medium showed no indication that an increased amount of pro-cathepsin D was being diverted into the secretory pathway in the hVps34 KD cultures (Fig. 19C).
Fig. 19. Silencing of hVps34 Expression Slows Proteolytic Processing of the Endosomal Intermediate of Cathepsin D

(A) Control and hVps34 KD cells were labeled with 100 µCi/ml $^{35}$S-methionine, then harvested after 30 min or chased in medium with unlabeled methionine for 4 h. A separate control culture was incubated with 15 mM NH$_4$Cl during the 4 h chase. Cathepsin D was immunoprecipitated and subjected to SDS-PAGE and fluorography. (B) Immunoblot analysis of endogenous cathepsin D in whole cell lysates from control and KD cells. (C) Control and hVps34 KD cells were labeled and chased as described above. At the indicated time points, Cathepsin D was immunoprecipitated from the chase media and then subjected to SDS-PAGE and fluorography.
The similar levels of mature cathepsin D detected in the immunoblot analysis of the control and KD cells might seem puzzling at first, given the slower intermediate processing observed in Fig. 19A. However, this is most likely related to the fact that lysosomal enzymes have long half-lives compared with their precursors so that, over time, a reduced rate of intermediate processing might have an imperceptible impact on the accumulated pool of end product detected by immunoblot assay (Hentze et al. 1984, Reilly et al. 1989, Nissler et al. 1999).

hVps34 KD-Induced Vacuoles Retain the Capacity to Fuse with Lysosomes

The finding that final proteolytic processing of the 47 kDa cathepsin D intermediate was slowed but not completely blocked in the hVps34 KD cells, suggested that the enlarged endosomes might be able to acquire lysosomal characteristics by limited fusion with pre-existing lysosomes. To investigate this possibility, hVps34 KD cells were examined by electron microscopy after staining them for endogenous lysosomal peroxidase activity with 3,3-diaminobenazidine (DAB) (Fig. 20A). Distinct spherical DAB-positive lysosomes were observed throughout the cytoplasmic compartment, contrasting with the larger DAB-negative vacuolar structures (Fig. 20A). In many instances the DAB-positive lysosomes were in direct contact with the enlarged endosomes (Fig. 20A, arrows), and in some cases these structures appeared to have merged, releasing peroxidase-positive material into the lumen of the vacuoles (Fig. 20A, arrowheads). The hVps34 KD cells also were subjected immunogold electron microscopy, using a primary antibody against cathepsin D to identify lysosomes (Fig. 92).
Fig. 20. Swollen Late Endosomes in hVps34KD Cells can Fuse with Lysosomes

(A) hVps34 KD cells were stained for endogenous peroxidase activity with DAB and then examined using electron microscopy. The arrow indicates a point of contact between smaller DAB-positive lysosome and a larger DAB-negative vacuole. The arrowheads indicate remnants of lysosomes that appear to have merged with the vacuoles. The bar represents 1 μm. (B) The KD cells were subjected to immunogold labeling with an antibody against cathepsin D. The left panel depicts a lysosome heavily labeled for cathepsin D (arrow) adjacent to a larger electron lucent vacuole. The right panel shows cathepsin D delivered to the lumen of an enlarged vacuole (arrowhead). The bar represents 0.5 μm.
20B). Smaller electron-dense structures heavily labeled with gold particles in close proximity to the larger electron-lucent vacuoles were frequently observed (Fig. 20B, arrow). In some cases it appeared that cathepsin D was being delivered to the lumen of the vacuole after fusion with the structure (Fig. 20B, arrowhead). These findings indicate that the enlarged endosome-derived vacuoles in the hVps34 KD cells retain the capacity to fuse with lysosomal compartments.

**Suppression of hVps34 Expression Inhibits Cell Proliferation**

The cells in the hVps34 KD cultures were approximately 2-3 times larger than those in the controls, and they did not reach a comparable density when maintained for several days after the initial puromycin selection. This prompted us to ask whether suppressing the expression of hVps34 might affect the growth of the U-251 cells. As shown in Fig. 21A, the growth rate of the KD cells was markedly reduced compared to the matched control cell line. A decreased rate of cell proliferation was confirmed by comparing the incorporation of $^3$H-thymidine into DNA in control versus KD cells at points where the control cells were at low density (day-2) or near confluence (day-6). As illustrated in Fig. 21B, the suppression of hVps34 expression results in a marked reduction in DNA synthesis when compared to control cells.

**Suppression of hVps34 Expression does not Induce Apoptosis**

The hVps34 KD cells exhibited a decrease in cell number over time that was accompanied by a block in DNA synthesis (Fig. 21). However, this does not exclude the
Fig. 21. hVps34 KD Cell Exhibit a Marked Reduction in Growth Rate

(A) Following 2 days of selection, control (▲-) and hVps34 KD (■-) cells were seeded in 35 mm dishes at an equal density of 50,000 cells/dish. At the indicated time points, triplicate dishes from each cell line were harvested and counted with a Coulter Z1 particle counter (mean ± S.E.) (B) control (■) and KD (■) cells were seeded in 25 cm² flasks at 150,000 cells/flask. On the indicated days triplicate flasks of each cell line were incubated with ³H-thymidine (1.0 µCi/ml) for 5 h. Radioactivity incorporated into TCA-precipitable material was counted and normalized to total cellular protein (mean ± S.E.).
possibility that apoptosis also contributes to the inability of the hVps34 KD cells to proliferate. In order to assess apoptotic cell death, control and hVps34 KD cells were subjected to TUNEL staining. This allows for the detection of DNA fragmentation that occurs in the later stages of apoptosis (Strasser et al. 2000). As seen in Fig. 22A, the nuclei of extremely vacuolated hVps34 KD cells were intact and TUNEL negative. Only non-specific staining of nucleoli (Fig. 22A arrows), common to the TUNEL stain, was observed (Pulera et al. 1998).

The TUNEL stain does not allow for detection of cells in the early stages of apoptosis. In order to ensure that the hVps34 KD cells were not in a stage of apoptosis prior to DNA fragmentation, an annexin-V binding assay was performed. In non-apoptotic cells, most phosphatidylserine (PS) molecules are localized to the inner leaflet of the plasma membrane. Soon after the induction of apoptosis, PS redistributes to the outer leaflet and becomes exposed to the extracellular environment. This allows for the binding of annexin-V, which has a strong, specific affinity for PS (Strasser et al. 2000). In order to assess the number of cells in the early stages of apoptotic cell death, control and hVps34 KD cells were labeled with PE-tagged annexin-V. The percentage of annexin-V positive cells in each population was determined at both early and late time points following selection, using a Guava personal cytometer. Figure 22B demonstrates that the suppression of hVps34 expression does not result in an increase in the percentage of annexin-V positive cells, even though U-251 cells were capable of a robust apoptotic response when treated with TNFα.
Fig. 22. hVps34 KD Cells are Non-Apoptotic

(A) After 5 days of selection, control and KD cells were seeded in 35 mm dishes at 100,000 cells/dish. Twenty-four hours later, DNA fragmentation was assayed using the TUNEL stain. Phase image of KD cell demonstrates extreme vacuolization of the cytoplasm. Arrows indicate background staining of nucleoli. Control cells treated with DNase served as a positive control. (B) Control (■) and KD (■) cells were seeded in 60 mm dishes at 200,000 cells/dish. On the indicated days, duplicate dishes from each cell line were harvested and stained with PE-tagged annexin-V. Annexin-positive cells were counted using a Guava personal cytometer. Cells treated overnight with TNFα (□) served as a positive control for apoptosis.
**Hypothesis II: Mammalian Vps34 Localizes to the Nucleus and Acutely Regulates Nuclear PI3P Levels That are Not Detectable When Whole Cell Lipid Production is Measured**

**Permeabilization of the Nuclear Envelope Allows for Detection of hVps34 in the Nucleus**

Consistent with a role in vesicle-mediated protein trafficking, various studies have demonstrated that hVps34 exhibits cytosolic, Golgi, early endosome and late endosome localization (Kihara et al. 2001b, Murray et al. 2002, Stein et al. 2003). However, recent reports suggest that hVps34 may function outside of protein trafficking. Siddhanta et al. (1998) demonstrated that hVps34 is required for insulin-stimulated DNA synthesis. Consistent with this finding, the studies in the preceding section indicate that the loss of hVps34 function through siRNA-mediated gene silencing inhibits DNA synthesis in the absence of a mitogenic stimulus. Furthermore, Bunney et al. (2000) showed that Vps34 localizes to nuclear transcription sites in plant cells. Combined, these findings led me to postulate that mammalian cells also possess a nuclear pool of Vps34 that functions in DNA synthesis and/or transcription. To investigate this possibility, indirect immunofluorescent microscopy was performed using several permeabilization techniques and an antibody against hVps34. In cells permeabilized with saponin, which does not disrupt the nuclear envelope, hVps34 exhibited a punctate staining pattern characteristic of endocytic compartments (Fig. 23). However, in cells permeabilized with
HEp-2 cells were seeded at 100,000 cells/dish in 35 mm dishes. Twenty-four hours later, cells were prepared for immunofluorescence microscopy using the fixation and permeabilization techniques indicated at the left of the figure. Cells were incubated with an anti-hVps34 antibody alone or antibody that had been pre-absorbed with a specific (pep A) or non-specific (pep B) hVps34 peptide.
TritonX-100 or methanol, which perforate the nuclear envelope, hVps34 exhibited a distinct nuclear localization (Fig. 23). Pre-absorption of the hVps34 antibody with the antibody-specific peptide eliminated the hVps34 signal while a non-specific peptide had no effect. This indicates that accessibility of the hVps34 antibody to the nucleus allows for the specific detection of a nuclear pool of hVps34.

**hVps34 Cofractionates with Nuclei and is Resistant to TritonX-100 Extraction**

In order to confirm the nuclear localization of hVps34, HEp-2 cells were fractionated over a discontinuous sucrose gradient (Fig. 24A). The resulting fractions were subjected to immunoblot analysis with organelle-specific antibodies. Consistent with previous findings, hVps34 is present in fractions containing cytosolic, Golgi, and endosomal markers (Kihara et al. 2001b, Murray et al. 2002, Stein et al. 2003). However, hVps34 is also readily detectable in the isolated nuclear fraction despite the fact that only 2 μg of total protein from this fraction could be analyzed (Fig. 24B, G3). Although the isolated nuclear fraction is free of most membrane contaminants, there is a detectable level of the lysosomal marker LAMP1 (Fig. 24B, G3). In order to ensure that the presence of hVps34 in the nuclear fraction was not the result of a lysosomal membrane contaminant, nuclei were purified by detergent extraction. As illustrated in Fig. 25A (nuclei final), the use of TritonX-100 allowed for the isolation of nuclei completely devoid of any membrane contaminants. However, approximately 10% of the total cellular hVps34 remained in the purified nuclear fraction, verifying that hVps34 stably
Fig. 24. Vps34 Fractionates with Nuclei

(A) HEp-2 cells were fractionated over a sucrose gradient. The fractions indicated in bold were collected and subjected to SDS-PAGE. (B) Immunoblot analysis of the fractions using the primary antibodies indicated at the right of the figure. The respective organelles are labeled to the left of the figure. Note: hVps34 fractionates with the nuclear matrix protein Lamin A/B (*G3).
HEp-2 cells were subjected to TritonX-100 extraction in order to remove any membrane contamination of the nuclear fraction. (A) Immunoblot analysis of the fractions with the antibodies indicated at the right of the figure. The respective organelles are labeled to the left of the figure (B) The TritonX-100 extraction was repeated and immunoblotted with two independent hVps34 antibodies and the marker antibodies indicated at the right of the figure.
associates with the nucleus (Fig. 25A). A similar result was obtained using a polyclonal hVps34 antibody generated against a different peptide epitope from an independent source (Dr. Jonathan Backer) (Fig. 25B).

**hVps34 in the Nucleus is Sensitive to RNase Extraction**

The nuclear matrix comprises the major structural component of the nucleus. It is operationally defined as the components that remain insoluble after sequential treatment of the nucleus with strong detergents, nucleases, and high ionic strength buffers (Ledeen and Wu 2004). The resistance of hVps34 to TritonX-100 extraction suggested that it may be associated with the nuclear matrix. In order to further characterize this association, HEp-2 cells were subjected to differential extraction with strong detergent followed by RNase treatment. As illustrated in Fig. 26A, extraction with strong detergent effectively eliminates the cytoplasmic fluorescence of hVps34 with limited effect on the nuclear signal. However, the addition of 100 µg/ml RNase greatly reduces the nuclear fluorescence, indicating the release of a substantial portion of the nuclear hVps34. This release can also be traced through immunoblot analysis of the detergent and RNase extracts. As shown in Fig. 26B, the hVps34 remaining in the nucleus following extraction with TritonX-100 alone is resistant to further detergent extractions. However, treatment with RNase results in the release of approximately half of the detergent-resistant hVps34 from the nuclei (Fig. 26B). Since the nuclear association of hVps34 is sensitive to RNase extraction, it cannot be considered part of the nuclear matrix.
Fig. 26. hVps34 Nuclear Association is Sensitive to RNase Extraction

(A) HEp-2 cells were seeded at 75,000 cells/dish in 35 mm dishes. Twenty-four hours later, cells were subjected to differential extraction with detergent followed by RNase. Cells were examined by immunofluorescence microscopy using an anti-hVps34 antibody and DAPI to visualize the nuclei. (B) The differential extracts were subjected to immunoblot analysis with antibodies against hVps34 and Lamin B₂.
Inhibition of Transcription Effects the Nuclear Distribution of hVps34

Since the nuclear association of hVps34 is RNA-dependent, I postulated that, as in plant cells, hVps34 localizes to active sites of transcription (Bunney et al. 2000). In order to demonstrate a relationship between hVps34 and transcription, the nuclear distribution of hVps34 in HEp-2 cells treated with actinomycin-D and α-amanitin was examined. Actinomycin-D intercalates into DNA and inhibits the progression of RNA polymerases (Perry and Kelley 1970, Sobell 1985). α-Amanitin binds to RNA polymerases and thus directly inhibits their enzymatic activity (de Mercoyrol et al. 1989). In either case, the transcriptional machinery is disrupted and the incorporation of ribonucleotides into nascent RNAs is blocked. Treatment of HEp-2 cells with actinomycin-D (Fig 27A) or α-amanitin (Fig. 27B) dramatically alters the nuclear distribution of hVps34. When transcription is blocked, the hVps34 fluorescence becomes less intense. This suggests that hVps34 nuclear localization depends on transcriptional activity.
HEp-2 cells were seeded at 133,000 cells/dish in 60 mm dishes. Forty-eight hours later, cells were incubated for 4 h with (A) 2.5 μg/ml actinomycin-D or (B) the indicated concentrations of α-amanitin. Cells were examined by immunofluorescence microscopy using an anti-hVps34 antibody.
DISCUSSION

The present study is the first to use the highly specific method of siRNA-mediated gene silencing to explore the function of the human Class III PI3K, hVps34. These observations indicate that silencing of hVps34 expression mainly affects membrane-sorting events within the late endosome compartment with surprisingly little or no effect on either the export of proteins from the TGN to endosomes or vesicular trafficking through the early part of the endocytic pathway. The acidic characteristics of the vacuoles in the hVps34 KD cells, coupled with the lack of internal membrane structures and the presence of LAMP1 and LGP85 in their limiting membranes, indicates that they represent enlarged late endosomes. Interestingly, despite their distorted size, these structures seem to retain a substantial capacity to merge with primary lysosomes, allowing for normal degradation of the EGF receptor and only partial impairment of the processing of the intermediate form of cathepsin D to the mature form.

One of the novel findings emerging from the present studies is the absence of any effect of blocking hVps34 expression on the first step in pro-cathepsin D processing, which depends on transport of the proenzyme from the TGN to the late endosome (Rijnboutt et al. 1992, Delbruck et al. 1994). Previous reports have indicated that hVps34 is localized predominantly in Golgi membranes and that inhibition of PI3K activity with wortmannin causes intracellular accumulation of unprocessed pro-cathepsin D, which then enters the secretory pathway (Kihara et al. 2001b, Brown et al. 1995, Davidson 1995). Follow-up studies by Row et al. (2001) implicated Vps34 as at least one probable wortmannin target by showing that overexpression of a kinase-deficient form of rat
Vps34 produced an impairment in pro-cathepsin D conversion to the 47 kDa endosomal intermediate. The discrepancy between these earlier results and the present findings based on siRNA-mediated silencing of hVps34 could be related to differences in the mechanisms used for interference with hVps34 function. For instance, siRNA is expected to eliminate endogenous hVps34 and any PI3P normally produced by this enzyme at specific subcellular sites. On the other hand, overexpression of a kinase-deficient form of Vps34 may act by competing with endogenous hVps34 for binding to the p150 adapter or other interacting proteins like Rab5 or Rab7 (Panaretou et al. 1997, Christoforidis et al. 1999b, Stein et al. 2003). In turn, this might cause perturbations of protein trafficking pathways due to titration of Vps34 partners, but not necessarily to loss of PI3P production by endogenous hVps34.

If hVps34 is not specifically responsible for producing PI3P needed for vesicular trafficking out of the TGN, it is conceivable that other closely related PI3Ks may fulfill this role. In this regard, it is worth noting that two novel PI3K activities have been described in the TGN. Both appear to be required for genesis of constitutive transport vesicles. The first is associated with TGN38 and a regulatory complex termed p62cplx (Jones et al. 1998). The second was found to associate with TGN46 (Hickinson et al. 1997). Neither of these PI3Ks has yet been characterized in sufficient detail to know how closely they might be related to hVps34 or whether they might be required for endosome and lysosome-directed trafficking of proenzymes associated with the M6P receptor.

The finding that hVps34 knockdown had no effect on the subcellular distribution of the early endosome protein, EEA1, was unexpected since previous studies using
FYVE-domain probes have demonstrated that PI3P is concentrated in microdomains in the membranes surrounding early endosomes, as well as in the internal vesicles of MVBs (Gillooly et al. 2000, 2003). Moreover, as mentioned earlier, inhibition of PI3K activity with wortmannin has been reported to cause the release of EEA1 from early endosomes (Simonsen et al. 1998, Petiot et al. 2003). Thus, the present results raise the intriguing possibility that the role of hVps34 is restricted to producing PI3P for specific compartments such as the MVB, while other kinases or phosphatases can generate PI3P in early endosomes. Although I can only speculate about the nature of such alternative pathways, several possibilities are worth considering. One possibility is that the Class I or Class II PI3Ks can contribute to maintaining the pool of PI3P in early endosomes. Class I PI3Ks are heterodimers composed of p110 catalytic subunits and p85 or p55 regulatory subunits (Vanhaesebroeck et al. 2001). Although the large increase in cellular PI(3,4,5)P_3 typically observed upon stimulation of Class I enzymes suggests that PI(4,5)P_2 is the preferred substrate in vivo, these enzymes can phosphorylate the 3-position of PI in cell-free systems (Fruman et al. 1998, Leevers et al. 1999). Thus, the possibility that Class I PI3Ks can produce sufficient PI3P for vesicular transport in some compartments cannot be completely ruled out. The physiological function of the Class II enzymes remains poorly understood, but there is some evidence that they are localized in the TGN and clathrin-coated vesicles (Prior and Clague 1999). It is indeed quite likely that the Class II PI3Ks can produce PI3P in mammalian cells, since these enzymes preferentially phosphorylate PI over PI4P and PI(4,5)P_3 in vitro (Fruman et al. 1998, Arcaro et al. 1998). A final scenario worth considering is the possibility that PI3P can be
generated in specific subcellular compartments through the sequential actions of inositol polyphosphate 5-phosphatase and inositol polyphosphate 4-phosphatase, which are capable of removing the 5-phosphate and the 4-phosphate from PI(3,4,5)P3 and PI(3,4)P2, respectively (Kisseleva et al. 2000, Norris et al. 1995, 1997). Such a mechanism might be particularly relevant for preserving the PI3P pool in the early endosomes derived from regions of the plasma membrane where PI(3,4,5)P3 appears to be generated (Prasad et al. 2002).

There have been conflicting reports regarding the importance of PI3K for post-endocytic sorting of activated receptors that enter the cell via clathrin-coated pits. In one study, wortmannin inhibited the formation of internal vesicles in the MVBs of HEp-2 cells, but the EGF receptor was still able to reach the perimeter membrane of lysosomes, exposing the N-terminal ligand-binding domain to a degradative environment (Futter et al. 2001). However, in another study with BHK-21 cells, wortmannin treatment prevented the translocation of ligand-stimulated EGF receptor from early endosomes to late endosomes (Petiot et al. 2003).

The present results support the conclusion that hVps34 is not absolutely essential for translocation of activated EGF receptor to late endosomes (Fig. 16). Within 60 min after EGF stimulation, the EGF receptor in the hVps34 KD cells reached the limiting membranes of enlarged LGP85-positive vacuoles and was degraded to an extent similar to that seen in the control cells. Since Futter et al. (1996) determined that the fusion of MVBs with lysosomes is required for EGF receptor degradation, the present results indicate that the enlarged vacuoles generated in the absence of hVps34 retain the capacity
for fusion with pre-existing lysosomes. This conclusion is further supported by ultrastructural evidence, where some vacuoles appear to be merging with DAB-positive structures containing endogenous peroxidase activity or electron-dense structures that contain cathepsin D (Fig. 20). Interestingly, although overall degradation of the EGF receptor was not substantially different between control and hVps34 KD cells, a much higher proportion of the receptor remained in an active phosphorylated state capable of activating the ERK pathway (Fig. 17). One mechanism for dephosphorylation of the EGF receptor involves protein tyrosine phosphatase 1B on the surface of the endoplasmic reticulum (Haj et al. 2002). However, the possibility that tyrosine dephosphorylation may also occur in conjunction with internalization of the EGF receptor into the MVBs has been suggested (Gill et al. 2002). It is well established that the phosphorylated C-terminal domain of the EGF receptor on the surface of endosomes is capable of signaling to the ERK pathway (Burke et al. 2001, Wiley and Burke 2001). The present results demonstrate that in the absence of PI3P generated by hVps34, an increased proportion of the EGF receptor is retained on the surface of the enlarged late endosomes in an active phosphorylated state prior to eventual degradation of the N-terminal domain by lysosomal proteases entering the lumen of the vacuole.

The morphological phenotype of the hVps34 KD cells resembles the “Class E” vacuolar phenotype that can be triggered in mammalian cells by interfering with the assembly of the ESCRT-III on the late endosome, which is required for internal vesicle formation (Babst et al. 2002, Bishop et al. 2002). This suggests a molecular mechanism whereby suppression of hVps34 might cause a failure of inward vesiculation of MVBs.
Specifically, PI(3,5)P$_2$ has been identified as an important phospholipid for membrane recruitment of hVps24, a key component of ESCRT-III (Whitley et al. 2003). Sbrissa et al. (1999) determined that the kinase responsible for generating PI(3,5)P$_3$ is in fact a PI3P-binding FYVE-domain protein termed PIKfyve. Therefore, the silencing hVps34 and localized depletion of PI3P might prevent membrane recruitment of PIKfyve and the subsequent PI(3,5)P$_3$-dependent assembly of ESCRT-III. Further investigation of this possibility will have to await the development of antibodies that can detect endogenous PIKfyve, which is expressed at very low levels in most cells.

In addition to the striking morphological changes affecting the late endosome compartment, hVps34 KD cells also exhibited a markedly reduced growth rate. In an earlier study, Siddhanta et al. (1998) found that a neutralizing antibody against hVps34 could block the insulin-stimulated increase in DNA synthesis in GRC-LR+73 cells when it was microinjected during a defined temporal window of the G1 phase of the cell cycle. This raises the intriguing possibility that, in addition to its role in membrane sorting in the late endosome, hVps34 might play a specific role in the control of DNA replication and/or transcription. Although studies using plant cells have found that Vps34 associates with discrete nuclear and nucleolar transcription sites, the present study is the first to demonstrate the RNA-dependent nuclear localization of mammalian Vps34 (Fig. 26).

Even though the subcellular distribution of the Class III PI3K has been previously examined using immunofluorescence microscopy, there have been no reports indicating that hVps34 localizes to the nucleus (Kihara et al. 2001b, Murray et al. 2002, Stein et al. 2003). To overcome discrepancies in fixation and permeabilization and to rule out
potential artifacts generated by the specific polyclonal antibody used in the present study, the nuclear localization of hVps34 was confirmed using subcellular fractionation and TritonX-100 techniques. Using these methods, it was determined that hVps34 fractionates with nuclear markers and remains associated with the nucleus following TritonX-100 extraction. Immunoblot analysis of the nuclear fraction subsequent to TritonX-100 extraction with a hVps34 antibody from an independent source (gift from Dr. Jonathan Backer) demonstrated that hVps34 invariably associates with the nucleus (Fig. 25B). Furthermore, it was determined that the nuclear hVps34 was resistant to extraction with harsh detergent, but was readily released upon treatment with RNase. Finally, the nuclear localization of hVps34 also was disrupted with transcriptional inhibitors (Fig. 27). These findings suggest that hVps34 may participate directly in nuclear functions at active sites of transcription.

Although the mechanism for the nuclear-cytoplasmic shuttling of hVps34 is unknown, it is interesting to note that the hVps34 binding protein, Beclin, also has been found to associate with the nucleus (Liang et al. 2001). Characterization of Beclin revealed that it contains a short leucine-rich sequence that resembles a nuclear export signal (NES) (Liang et al. 2001). Overexpression of wild type Beclin in human MCF7 breast cancer cells revealed that the protein exhibits both cytoplasmic and nuclear localization (Liang et al. 2001). However, mutations within the NES of Beclin cause the protein to be retained in the nucleus thus eliminating the cytoplasmic distribution (Liang et al. 2001). The efficient transport between the nucleus and the cytoplasm is required for Beclin to function in autophagy (Liang et al. 2001). Therefore, Beclin serves as a
potentially interesting target for future investigation into the mechanism for the nuclear-cytoplasmic shuttling as well as the nuclear function of hVps34.

It is also worth noting that a nuclear system for generating phosphoinositides exists in mammalian cells. Various methods have allowed for the detection of 3-phosphorylated phosphoinositides in the nucleus. For example, the presence of the PI(3,4,5)P₃ in the nucleus has been demonstrated through in vivo labeling and lipid extraction approaches (Neri et al. 1999, 2002, Tanaka et al. 1999). The development of a monoclonal antibody specific for PI(3,4)P₂ has allowed for the localization of this phosphoinositide at the nuclear membrane (Yokogawa et al. 2000). Moreover, Gillooly et al. (2000) exploited the ability of the FYVE domain to bind specifically to PI3P in order to determine the localization of PI3P. When overexpressed in both BHK and human fibroblasts, the FYVE domain probe was partly localized to the nucleolus by immunoelectron microscopy (Gillooley et al. 2000). Investigations into the enzymes responsible for generating nuclear 3-phosphorylated phosphoinositides revealed that both the Class I and the Class II PI3Ks localize to the nucleus (Zini et al. 1996, Kim 1998, Metjian et al. 1999, Neri et al. 2002, Sindic et al. 2001, Didichenko and Thelen 2001). The present study indicates the nuclear localization of the Class III PI3K as well.

Several studies have shown that the Class I PI3K translocates to the nucleus following mitogenic stimulation (Zini et al. 1996, Kim et al. 1998, Metjian et al. 1999, Neri et al. 2002). However, the mechanism governing the nuclear-cytoplasmic shuttling of this enzyme is currently unknown. Once in the nucleus, the Class I PI3K serves to generate a local pool of PI(3,4,5)P₃ that recruits protein kinase C (PKC)ζ to the nucleus
At least two nuclear proteins (nucleolin and ribonucleoprotein A1) involved in several aspects of RNA metabolism have been shown to be downstream targets of PKCζ (Ginisty et al. 1999, Zhou et al. 1997, Municio et al. 1995).

A nuclear pool of the Class II PI3K also has been linked to RNA processing (Sindic et al. 2001, Didichenko and Thelen 2001). Unlike the Class I PI3K, the Class II PI3K is constitutively associated with the nucleus (Didichenko and Thelen 2001). Characterization of the Class II PI3K revealed the presence of a short stretch of highly basic amino acids within the C2 domain that serve as a nuclear localization signal (Didichenko and Thelen 2001). Furthermore, the Class II PI3K was shown to localize to specific structures known as nuclear speckles (Didichenko and Thelen 2001). Speckles are dynamic structures containing elements of transcriptional and pre-mRNA processing machinery including RNA polymerase II, the SC-35 splicing factor, and small nuclear ribonucleoproteins (snRNPs) (Jackson et al. 2000). Combined, these studies and the present study suggest that PI3Ks participate in pathways that connect the generation of 3-phosphoinositides to nuclear regulatory events at the level of RNA processing and transport.

Currently, the mechanisms underlying the role of 3-phosphoinositides in regulation of transcription are mostly unknown. However, work with another family of phosphoinositide kinases, specifically the PI4P-5-kinases (PIPKs) has provided some insight into the role of phosphoinositides at active sites of transcription. At least two isoforms of PIPKs, along with their product PI(4,5)P₂, have been localized to nuclear speckle domains (Boronenkov et al. 1998). The local generation of PI(4,5)P₂ mediates the
function of the BAF protein complex which is required for chromatin remodeling and transcriptional activation (Zhao et al. 1998, Rando et al. 2002). This suggests a general mechanism by which phosphoinositides recruit complexes involved in chromatin arrangement and modulate accessibility to transcription factors. Future identification of 3-phosphoinositide-regulated proteins in the nucleus will be critical to our understanding of the role of PI3Ks in nuclear function.
CONCLUSIONS

1. The PI3P generated by hVps34 is required for the inward vesiculation of the late endosome compartment.

2. The inhibition of inward vesiculation induced by suppression of hVps34 expression results in the swelling of the late endosome compartment and the cytoplasmic accumulation of large vacuoles.

3. Late endosomes that have lost the ability to inwardly vesiculate retain the capacity to fuse with lysosomes.

4. The PI3P generated by hVps34 is not required for the early stages of the endocytic pathway.

5. The PI3P generated by hVps34 is not required for vesicular trafficking from the trans-Golgi network to the endosome.

6. Some of the Vps34 in mammalian cells localizes to the nucleus in an RNA-dependent manner.
SUMMARY

In this work, siRNA-mediated gene silencing was employed to define the role of hVps34 in generating PI3P required for vesicle-mediated protein trafficking in human U-251 glioblastoma cells. The suppression of hVps34 expression resulted in the cytoplasmic accumulation of large, acidic phase-lucent vacuoles. Morphological analysis revealed that these vacuoles were surrounded by a single membrane containing late endosomal/lysosomal markers. Interestingly, the suppression of hVps34 expression did not impair trafficking of cathepsin D from the TGN to late endosomes, endocytic uptake of fluid-phase markers, recycling of transferrin receptors, degradation of activated EGF receptors, or association of a PI3P-binding protein, EEA1, with early endosomes. This suggests that the PI3P generated by hVps34 is not required for trafficking in the early stages of the endocytic pathway or in trafficking from the TGN to the late endosome. However, EGF receptor phosphorylation and signaling were enhanced in the absence of hVps34, consistent with the retention of the EGF receptor on the limiting membranes of the enlarged late endosomes prior to degradation. These findings indicate that hVps34 plays a major role in generating PI3P for internal vesicle formation in late endosomes. The failure of the late endosome to inwardly vesiculate results in the swelling of this compartment and the cytoplasmic accumulation of large vacuoles.

This work also indicates that hVps34 may function not only at the late endosome, but also in the nucleus. Suppression of hVp34 expression inhibits DNA synthesis, implying a direct link between hVps34 and nuclear function. Immunofluorescent localization and subcellular fractionation of human HEp-2 laryngeal carcinoma cells
revealed that there is a distinct pool of hVps34 located in the nucleus. Furthermore, the nuclear hVps34 is sensitive to treatment with RNase. Although the exact function of hVps34 in the nucleus remains to be determined, the present findings suggest a role for hVps34 in nuclear regulatory events at the level of RNA processing and/or transport.
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ABSTRACT

The human Class III phosphatidylinositol 3-kinase (PI3K), hVps34, converts phosphatidylinositol (PI) to phosphatidylinositol 3-phosphate (PI3P). Previous studies using PI3K inhibitors have indicated that production of PI3P is important for vesicle-mediated trafficking events, including endocytosis, sorting of receptors in multivesicular bodies (MVBs), transport of lysosomal enzymes from the trans-Golgi network (TGN), and autophagy. This study utilizes siRNA-mediated gene silencing to define the specific trafficking pathways in which hVps34 functions in human U-251 glioblastoma cells. Suppression of hVps34 expression caused the accumulation of large, acidic phase-lucent vacuoles that contain lysosomal membrane proteins. Analysis of these structures by electron microscopy suggests that they represent swollen late endosomes that have lost the capacity for inward vesiculation but retain the capacity to fuse with lysosomes. In contrast to the effects on late endosomes, suppression of hVps34 expression did not inhibit trafficking of cathepsin D from the TGN to late endosomes, endocytic uptake of fluid-phase markers, recycling of transferrin receptors, degradation of activated epidermal growth factor (EGF) receptors, or association of a PI3P-binding protein, EEA1, with early endosomes. Nevertheless, EGF receptor phosphorylation and signaling were enhanced in the absence of hVps34, consistent with the retention of the EGF receptor on the limiting membranes of the enlarged late endosomes prior to degradation. These findings indicate that hVps34 plays a major role in generating PI3P required for internal vesicle formation in late endosomes and that, unexpectedly, other mechanisms may exist to generate the PI3P required for vesicular trafficking in the early endocytic
pathway or the TGN. Additionally, suppression of hVps34 expression in U-251 cells resulted in a marked reduction in cell growth accompanied by a block in DNA synthesis. Furthermore, investigation of the subcellular distribution of hVps34 in human HEp-2 laryngeal carcinoma cells revealed that hVps34 localizes to the nucleus. The nuclear pool of hVp34 is resistant to detergent extraction but can be partially released upon treatment with RNase. Overall, this suggests a role for hVps34 in nuclear regulatory events at the level of RNA processing and/or transport.