The mammalian phosphatidylinositol 3-phosphate 5-kinase (PIKfyve) regulates endosome-to-TGN retrograde transport

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Summary

The yeast gene *fab1* and its mammalian orthologue *Pip5k3* encode the phosphatidylinositol 3-phosphate [PtdIns(3)*P*] 5-kinases Fab1p and PIKfyve, respectively, enzymes that generates phosphatidylinositol 3,5-bisphosphate [PtdIns(3,5)P_2]. A shared feature of *fab1Δ* yeast cells and mammalian cells overexpressing a kinase-dead PIKfyve mutant is the formation of a swollen vacuolar phenotype: a phenotype that is suggestive of a conserved function for these enzymes and their product, PtdIns(3,5)P_2, in the regulation of endomembrane homeostasis. In the current study, fixed and live cell imaging has established that, when overexpressed at low levels in HeLa cells, PIKfyve is predominantly associated with dynamic tubular and vesicular elements of the early endosomal compartment. Moreover, through the use of small interfering RNA, it has been shown that suppression of PIKfyve induces the formation of swollen endosomal structures that maintain their early and late endosomal identity. Although internalisation, recycling and degradative sorting of receptors for epidermal growth factor and transferrin was unperturbed in PIKfyve suppressed cells, a clear defect in endosome to *trans*-Golgi-network (TGN) retrograde traffic was observed. These data argue that PIKfyve is predominantly associated with the early endosome, from where it regulates retrograde membrane trafficking to the TGN. It follows that the swollen endosomal phenotype observed in PIKfyve-suppressed cells results primarily from a reduction in retrograde membrane fission rather than a defect in multivesicular body biogenesis.

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Introduction

The endosomal system functions as an intracellular sorting network that serves to determine the fate of internalised cargo (Lemmon and Traub, 2000; Miaczynska and Zerial, 2002). From the plasma membrane, internalised cargo is delivered to sorting endosomes, where a number of trafficking decisions can be made. Nutrient receptors, such as low-density lipoprotein (LDL) or transferrin receptors release their ligands in the mildly acidic endosomal lumen and are returned to the plasma membrane via tubular intermediates (Maxfield and McGraw, 2004). This recycling pathway appears to be devoid of specific cargo-recognition motifs and is thought to occur by default via iterative, tubular-based fractionation that preferentially removes membrane-bound receptors through a process of geometric sorting (Dunn et al., 1989; Mayor et al., 1993). Actively signalling receptors, such as the epidermal growth factor (EGF) receptor, are prevented from using these default return pathways. Instead they are directed from the sorting endosome to the lysosome in a mechanism requiring ubiquitin-dependent retention in specialised endosomal regions. These regions are able to undergo inward budding into the endosomal lumen to form a multivesicular body (MVB) (Katzmann et al., 2002; Gruenberg and Stenmark, 2004), which is competent to fuse with the hydrolytic lysosome. It is also clear that the sorting endosome is able to receive biosynthetic traffic from the Golgi (Dahms et al., 1989; Waguri et al., 2003; Lin et al., 2004). Mannose 6-phosphate (M6P) receptors deliver their cargo of newly synthesised lysosomal hydrolases to the endosomal system in a manner dependent upon GGA proteins and the clathrin adaptor AP-1 (Bonifacino, 2004). Retrieval mechanisms also exist to return a number of *trans*-Golgi network (TGN)-resident proteins, such as TGN38/46 (Ghosh et al., 2003) or apo-hydrolase receptors to the TGN, where these receptors receive newly synthesised cargo for subsequent rounds of endosomal delivery (Lin et al., 2004). Thus the potential exists for transport along a variety of complex endosomal itineraries. It is thought that receptor fate is governed by the ability to interact with, or to avoid, a number of complex and, as yet ill-defined, endosomal sorting machineries.

A crucial component in endosomal sorting are 3-phosphoinositides, including phosphatidylinositol (3)-

monophosphate [PtdIns(3)*P*] (Gillooly et al., 2000; Petiot et al., 2003). Elegant studies have demonstrated that this lipid plays an essential role in sorting events, such as the inclusion of activated EGF receptors within MVBs (Petiot et al., 2003), rather than bulk transport of cargo through the endosomal system. In addition, there is evidence of a role for its 5′-phosphorylation product phosphatidylinositol (3,5)-bisphosphate [PtdIns(3,5)P_2] in regulating both endosomal sorting and endomembrane homeostasis (Michell et al., 2006). PtdIns(3,5)P_2 was originally identified in yeast (Dove et al., 1997) where its synthesis requires the product of the *FAB1* gene (Yamamoto et al., 1995) – which encodes for a PtdIns(3)*P* 5-kinase (Dove et al., 1997; Gary et al., 1998; Cooke et al., 1998; McEwen et al., 1999). *fab1Δ* cells, which are completely devoid of PtdIns(3,5)P_2, display multiple phenotypes that include a dramatically swollen vacuole, defective vacuolar acidification and vacuole inheritance, a reduced ability to grow at high temperatures, and a partial perturbation in MVB sorting (Yamamoto et al., 1995; Odorizzi et al., 1998; Gary et al., 1998; Cooke et al., 1998; Dove et al., 2002) (reviewed in Efe et al., 2005).

One outstanding question in Fab1p biology concerns the mechanism by which the swollen vacuole phenotype is generated in *fab1Δ* cells. As MVBs from *fab1Δ* cells contained fewer intraluminal vesicles (Gary et al., 1998) it has been argued that the swollen vacuolar phenotype may arise from a defect in inward invagination of the limiting membrane of the late endosome. Having said this, a defect in inward budding of the late endosome may not be the predominant mechanism inducing vacuolar swelling. In *fab1Δ* cells, sorting of endocytic cargo into MVBs is unperturbed and although sorting of biosynthetic cargo is blocked, this can be overcome by irreversible ubiquitylation of the specific cargo (Odorizzi et al., 1998; Dove et al., 2002; Katzmann et al., 2004). These data suggest that, rather than regulating the actual process of inward budding, Fab1p controls the sorting of a subset of MVB proteins. Consistent with this, a number of studies have identified various proteins known to be involved in MVB sorting, including mVps24, Ent3p and Ent5p, as potential PtdIns(3,5)P_2 effectors (Friant et al., 2003; Whitley et al., 2003; Eugster et al., 2004; Michell et al., 2006).

In addition to a role in MVB sorting, PtdIns(3,5)P_2 may also control retrograde traffic from the vacuole back to the endosome and/or Golgi; the vacuole is constantly receiving membrane from the endocytic and biosynthetic pathways, so a defect in membrane retrieval would lead to a vacuolar enlargement. Vac7p and Vac14p are upstream activators of Fab1p, whose genetic ablation induces a swollen vacuole morphology and defective synthesis of PtdIns(3,5)P_2 (Bryant and Stevens, 1998; Gary et al., 1998; Bonangelino et al., 2002; Dove et al., 2002; Rudge et al., 2004). Whereas Vac14p is required for proper degradative traffic (Dove et al., 2002), Vac7p is required for correct retrograde vacuole-to-TGN traffic (Bonangelino et al., 1997), suggesting that Vac7p-dependent PtdIns(3,5)P_2 production is required for this retrieval step. Further evidence in favour of a role for PtdIns(3,5)P_2 in vacuole-to-Golgi retrieval has stemmed from the characterisation of Svp1p (Atg18p) (Dove et al., 2004). This protein binds with high affinity PtdIns(3,5)P_2, and *svp1Δ* cells exhibit swollen vacuoles that arise through a defect in vesicle recycling from the limiting membrane of the yeast vacuole (Dove et al., 2004).

The mammalian equivalent of Fab1p is PIKfyve (Shisheva et al., 1999; Sbrissa et al., 1999), an enzyme that is associated with what have been presumed to be late endosomes in a manner dependent on a PtdIns(3)*P*-binding FYVE domain (Shisheva et al., 2001). Like Fab1p, PIKfyve is also essential for endomembrane homeostasis. Overexpression of a kinase-inactive mutant induces vacuolation of the endosomal system that can be rescued by microinjection of PtdIns(3,5)P_2 (Ikonomov et al., 2001; Ikonomov et al., 2002). Whereas this vacuolation induces swollen MVBs with fewer intraluminal vesicles and impairs soluble uptake of horseradish peroxidase, it does not affect trafficking of the transferrin receptor or the degradation of internalised EGF receptor (Ikonomov et al., 2003a). Additionally, PIKfyve has been shown to be associated with p40, a Rab9 effector required for retrieval of the cation-independent mannose 6-phosphate receptor (CI-MPR) from endosome to TGN, leading to the suggestion that PIKfyve regulates this pathway (Ikonomov et al., 2003b). Supporting this, WIPI49, a mammalian orthologue of Svp1p (Dove et al., 2004), controls endosome-to-TGN retrieval of the CI-MPR (Jeffries et al., 2004).

In the current study, we have re-examined the subcellular localisation of PIKfyve and, by using small interfering RNAs (siRNAs), we have examined the role of endogenous PIKfyve in endosomal sorting and membrane homeostasis. We present data consistent with a role for this enzyme in regulating early-endosome-to-TGN retrograde trafficking within HeLa cells.

Results

GFP-PIKfyve is enriched on early, rather than late endosomes

Previous studies have established that transiently transfected PIKfyve is localised to cytosolic punctae through an ability of its FYVE domain to associate with PtdIns(3)*P* (Shisheva et al., 2001; Ikonomov et al., 2001; Sbrissa et al., 2002). In an attempt to examine the subcellular localisation of endogenous PIKfyve, we generated anti-PIKfyve serum (Cabezas et al., 2006). Unfortunately, although this serum was able to detect endogenous protein by western blotting (see below), it failed to detect endogenous PIKfyve by immunofluorescence (data not shown). To overcome this, we transiently transfected HeLa cells with a green fluorescent protein (GFP)-tagged PIKfyve chimera (GFP-PIKfyve). In transfectants expressing low levels of GFP-PIKfyve, a punctate distribution was observed throughout the cytosol (Fig. 1). Consistent with previous data (Sbrissa et al., 2002), association with these punctae was dependent upon phosphoinositide 3-kinase (PI 3-kinase) activity, because incubation with wortmannin (100 nM for 10 minutes) resulted in dissociation of GFP-PIKfyve into the cytosol (supplementary material Fig. S1).

To define the nature of the PIKfyve-positive compartment, we co-stained HeLa cells expressing low levels of GFP-PIKfyve with antibodies against a variety of endocytic markers and quantified by confocal microscopy the level of overlapping signals by visual inspection (see Materials and Methods). In contrast to other studies, GFP-PIKfyve showed substantial but not complete colocalisation with markers of the early endosome, including sorting nexin-1 (SNX1) and early endosomal antigen-1 (EEA1) (Fig. 1A,B and Table 1). Consistent with PIKfyve being enriched on early endosomes, internalised EGF receptor was observed to be transported

through the PIKfyve-labelled compartment with maximal colocalisation observed 10 minutes after ligand addition (Fig. 1C, Table 1, and data not shown). Substantially reduced colocalisation was observed between the GFP-PIKfyve-labelled compartment and the late endosomal and/or lysosomal markers LAMP1/lgp120 and CD63 (Fig. 1D,E and Table 1). Under these conditions, GFP-PIKfyve appears enriched on membranes of the early rather than the late endosome (Cabezas et al., 2006). Such a localisation is entirely consistent with the enrichment of PtdIns(3)*P* on the early endosome and the requirement for a PtdIns(3)*P*-binding FYVE domain for PIKfyve membrane association (Gillooly et al., 2000). These data contrast with the reported association of overexpressed PIKfyve with a compartment that appears to correspond to late endosomes and MVBs (Ikonomov et al., 2003a; Sbrissa et al., 2002).

Table 1. Quantification of the degree of colocalisation between the punctate GFP-PIKfyve-positive compartment and various endocytic markers

Marker	Colocalisation (*n*=10)
EEA1	71±6
SNX1	80±7
EGFR (10-minute internalisation)	75±5
CD63	4±1
LAMP1	2±1

HeLa cells expressing low levels of GFP-PIKfyve were stained with antibodies against a variety of endocytic markers, and the level of overlapping signals was quantified by visual inspection (see Materials and Methods).

The PIKfyve-labelled early endosome is a highly dynamic tubulo-vesicular compartment

To examine the dynamics of the PIKfyve-positive endosome, we imaged HeLa cells expressing low levels of GFP-PIKfyve using live cell confocal microscopy. Cells were imaged at one frame per second over a 10-minute period. The vesicular PIKfyve-labelled compartment was observed to be highly motile, having an averaged velocity of 2.1±0.1 micrometers per second (*n*=40 vesicles) (Fig. 2A; supplementary material Movie 1). Analysis of directionality revealed that the majority of PIKfyve-labelled vesicles moved towards the perinuclear and/or TGN region (Fig. 2B,C). In addition, PIKfyve-labelled vesicles were observed to undergo homotypic fusion (Fig. 2D; supplementary material Movie 2) and to generate PIKfyve-positive tubular profiles (Fig. 2E, supplementary material Movie 3). Such data showing the dynamic tubulo-vesicular nature of the PIKfyve-labelled compartment are entirely consistent with it being of endosomal origin.

To visualise the cargo sorting within the PIKfyve-labelled endosome, we examined trafficking of ligands for the EGF and transferrin receptors using dual-wavelength live-cell confocal microscopy (Fig. 3). To follow degradative trafficking of the EGF receptor, we stimulated serum-starved HeLa cells expressing low levels of GFP-PIKfyve with Texas-Red-labelled EGF (TxR-EGF). After 15 minutes of

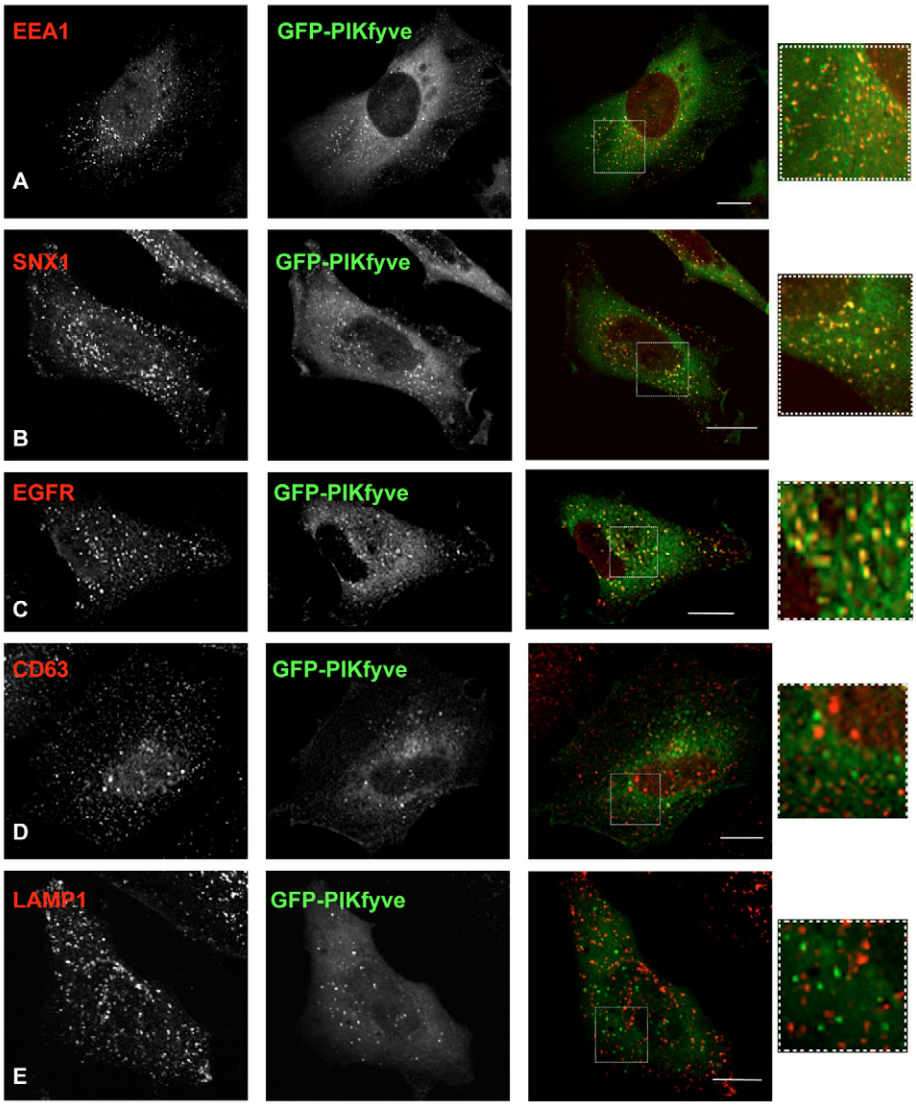

Fig. 1. At low-level expression GFP-tagged PIKfyve is predominantly associated with the early endosome. (A-E) HeLa cells were transiently transfected with GFP-PIKfyve and, after 24 hours, cells were fixed and stained for the early endosomal markers EEA1 (A) and SNX1 (B), internalised EGF receptor 10 minutes after EGF addition (C), and the late endosomal markers CD63 (D) and LAMP1 (E). Images are single, 0.1 μm confocal sections and are representative of more than ten imaged cells in each case. Bars, 10 μm. Boxed area in A-E is shown enlarged on the right.

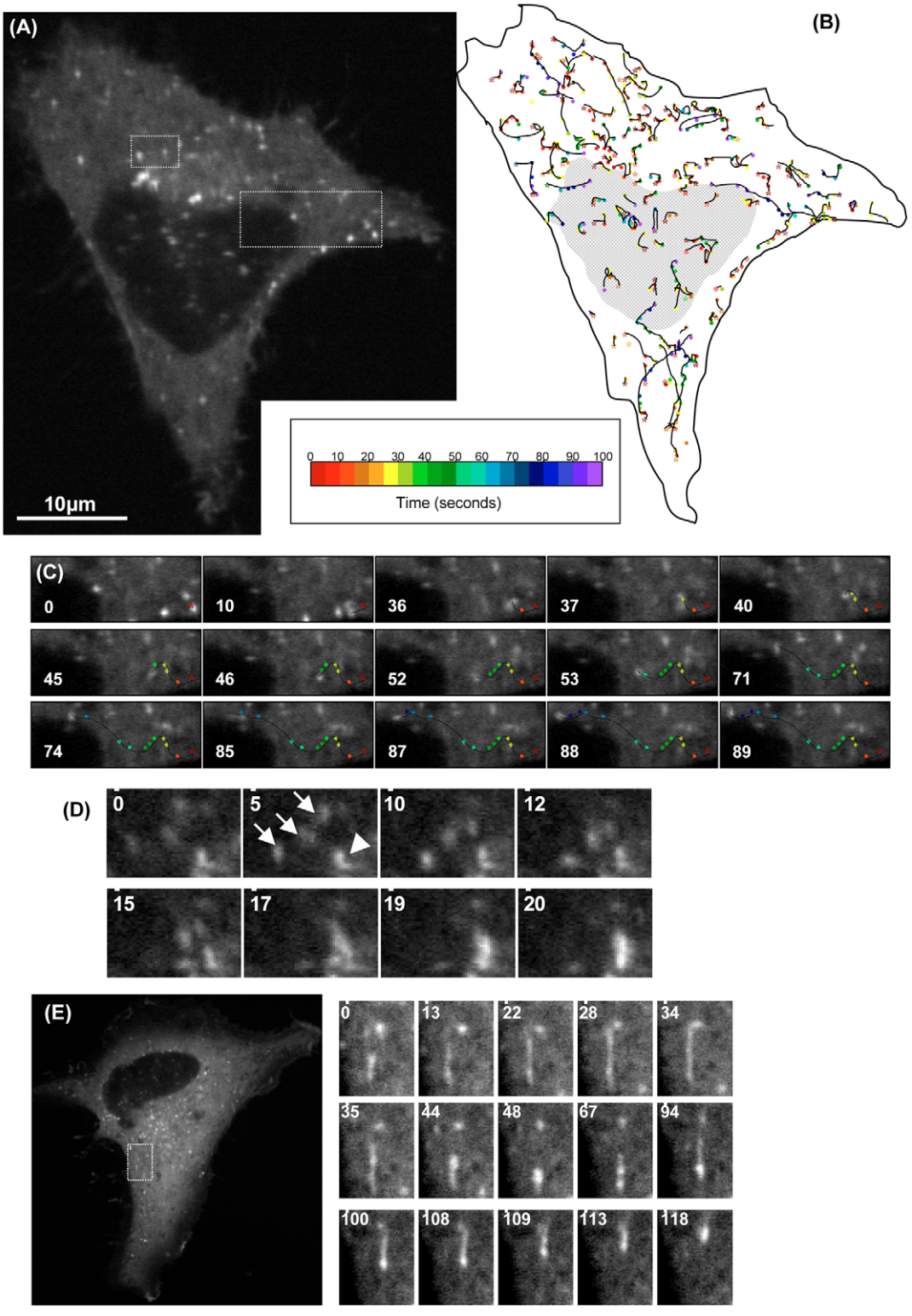

Fig. 2. The GFP-PIKfyve-positive early endosome is a highly dynamic tubular-vesicular compartment. (A,B) One HeLa cell expressing low levels of GFP-PIKfyve was imaged at one frame per second for a period of 10 minutes (A). Boxed areas indicate regions of interest detailed in C and D. Subsequently, data from a 100-second period were selected and each individual image was analysed (see Materials and Methods) to obtain a map of spatial temporal behaviour of the PIKfyve-labelled early endosome (B). Coloured asterisks are used to indicate the time at which the chosen vesicle first appeared in the image plane. Subsequent movements of >0.4 μm were followed over time. As indicated in the timescale bar, each colour corresponds to a specific 5-second period during the image window. The resulting 2D image gives an overview of both, the speed and directionality of the PIKfyve-positive punctae. The trafficking map revealed a directional trend, with several PIKfyve-positive vesicles tracking towards the perinuclear and/or TGN region. (C) The progress of one such vesicle is highlighted, with the first frame being denominated time point zero. (D,E) Live cell imaging also revealed that (D) PIKfyve-labelled vesicles (arrows) were able to undergo homotypic fusion (arrowhead), and that (E) the PIKfyve-labelled tubules were able to emminate from the vesicular elements of the PIKfyve-labelled compartment.

stimulation, TxR-EGF reached vesicular elements of the GFP-PIKfyve-labelled endosome (Fig. 3A). In all cells imaged, we did not observe trafficking of TxR-EGF along GFP-PIKfyve decorated tubules. We observed, however, the retention of TxR-EGF within the body of the endosome while GFP-PIKfyve-labelled membrane tubules exited this organelle (Fig. 3B, supplementary material Movie 4).

Given the tubular nature of the GFP-PIKfyve endosome, we also examined the sorting of recycling cargo. Serum-starved HeLa cells expressing low levels of GFP-PIKfyve were incubated with Alexa Fluor-568-labelled transferrin (Alexa^{568}-Tf). After 5 minutes of incubation, Alexa^{568}-Tf entered the vesicular element of the PIKfyve-labelled endosome and was observed to exit this compartment through tubular profiles that

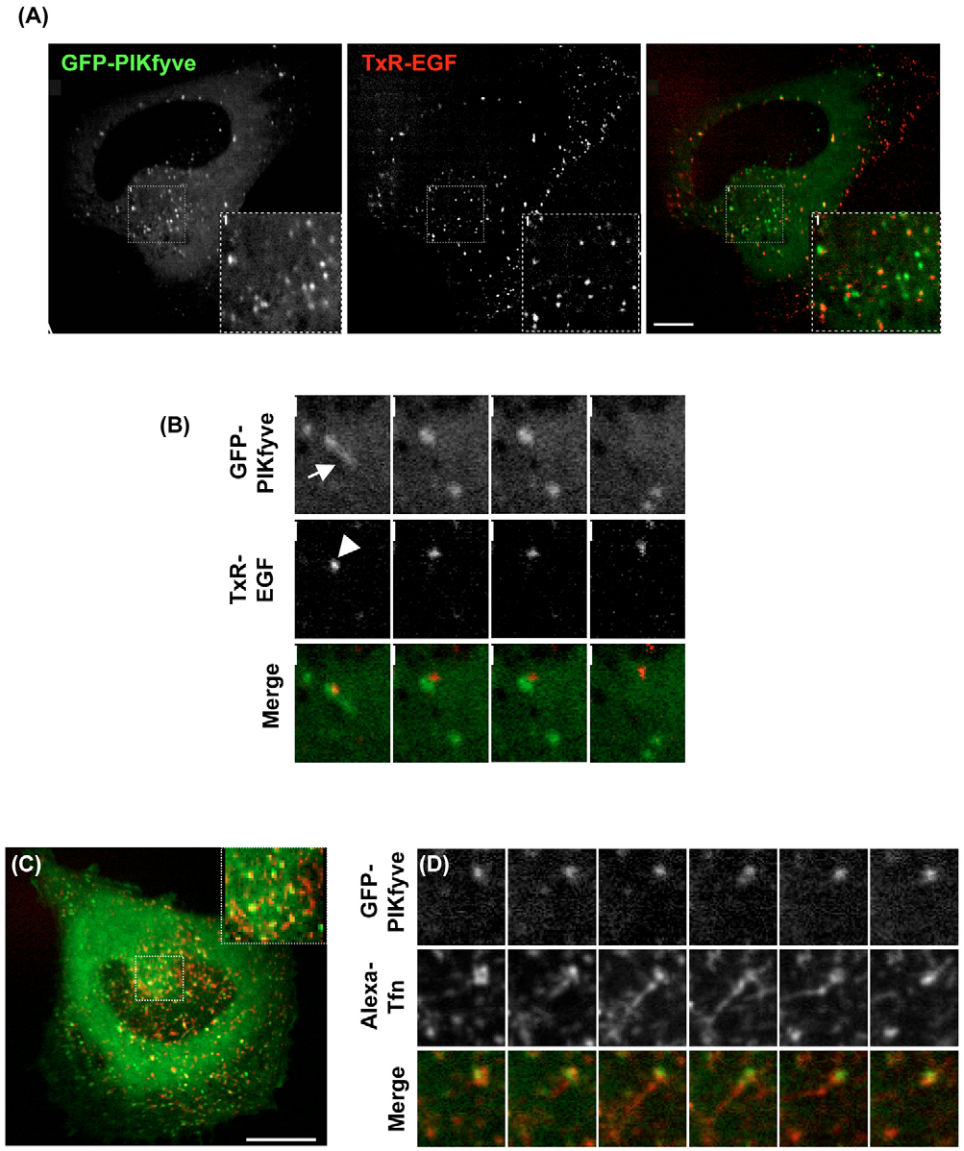

Fig. 3. Internalised Alexa^{568}-Tf and TxR-EGF are transported through the GFP-PIKfyve-labelled early endosome. (A-D) HeLa cells were transiently transfected with pEGFPC1-PIKfyve for 22 hours and serum-starved for 3 hours prior to live cell imaging. Cells were incubated with either (A,B) 200 ng/ml TxR-EGF or (C,D) 20 μg/ml Alexa^{568}-Tf. TxR-EGF reached the GFP-PIKfyve-positive compartment after 15 minutes, and is not transported along GFP-PIKfyve-positive tubules but remains in the endosomal body. Series of frames (B) depicting a GFP-PIKfyve-positive tubule (arrow) exiting an endosome positive for GFP-PIKfyve and TxR-EGF (arrowhead); data are also available in supplementary material as Movie 4. (C,D) Alexa^{568}-Tf also enters the GFP-PIKfyve-positive compartment after 5 minutes, and was observed to exit the GFP-PIKfyve-positive early endosome through tubular elements. Series of frames (D) depicting Alexa^{568}-transferrin-positive tubules exiting the GFP-PIKfyve early endsome; data are also available in supplementary material as Movie 5. Similar data were obtained in more than ten imaged cells for each case. Boxed areas in A and C indicate regions enlarged in the corresponding insets. Bars, 10 μm.

lacked detectable GFP-PIKfyve (Fig. 3C, supplementary material Movie 5). Overall, these data further confirm the nature of the PIKfyve-labelled compartment as an early endosome, and highlight that within this compartment cargo sorting appears to occur.

siRNA-mediated suppression of endogenous PIKfyve

The majority of studies examining the role of PIKfyve in endosomal function have relied upon overexpression of either wild-type PIKfyve or a kinase-dead mutant (PIKfyve^{K1831E}) that has been proposed to function as a dominant-negative kinase (Ikonomov et al., 2001). Given the inherent limitations in interpreting data that rely upon overexpression, we chose to employ siRNA technology to probe the function of endogenous PIKfyve. Five siRNA duplexes (I-V) were generated, each targeting a distinct region of the PIKfyve mRNA (supplementary material Table S1). To establish the efficiency of suppression, we raised an anti-peptide antibody targeting human PIKfyve (Fig. 4A). This revealed that, when used individually, siRNA duplex II gave a 47.5±12.4% reduction in PIKfyve levels (Fig. 4A). A similar level of suppression was observed with siRNA duplex V (data not shown), whereas the remaining siRNAs gave much lower levels of suppression (Fig. 4A). In experiments using dual transient transfection with siRNA duplexes II and V, the level of PIKfyve expression was dramatically decreased relative to the conditions using individual siRNA (Fig. 4A; 84.4±12.6% reduction in protein). Thus, by using siRNA duplexes, individually or in combination, we were able to generate various levels of PIKfyve suppression.

Suppression of PIKfyve leads to swelling of endosomal compartments

A characteristic phenotype of cells overexpressing PIKfyve^{K1831E} is the presence of a swollen endosomal compartment (Ikonomov et al., 2001). In HeLa cells suppressed for endogenous PIKfyve, swollen vesicles were observed when cells were viewed under phase-contrast (Fig. 4B,C). To quantify this phenotype, cells were imaged and scored as displaying the swollen-vacuole phenotype if two or

more swollen vacuoles were clearly present in an individual cell. This revealed that, when siRNA duplex II was used individually, such a phenotype was rare, occurring in only approximately 7% of cells (Fig. 4C; similar data were also observed when using siRNA duplex V individually; data not shown). By contrast, in cells treated with a combination of these siRNA duplexes, the swollen vesicular phenotype became much more pronounced, occurring in approximately 42% of cells (Fig. 4C).

To define the nature of the swollen vesicular compartment, we performed a series of colocalisation studies using markers that define compartments along the endocytic route (Fig. 5A). In initial experiments, we suppressed PIKfyve using siRNA duplex II. This revealed that, even in cells in which by phase contrast we failed to observe a swollen vacuole phenotype, the morphology of the early endosome was perturbed, appearing to be swollen in nature (Fig. 5A). This was best observed when analysing the early endosome upon transferrin receptor internalisation. At early time points, the transferrin receptor was found on the limiting membrane of early endosomes that appeared to be vacuolated (Fig. 5A). The late endosomal compartment appeared unaltered under these conditions of suppression (data not shown).

In cells in which phase contrast revealed a swollen vacuolar phenotype, we observed a wide range of swollen vesicles that varied in their diameter. Immunofluorescence analysis using early and late endosomal markers revealed a correlation between the size of the swollen endosomes and their relative early or late endosomal characteristics. Thus, the majority of large vacuoles were positive for the late endosomal markers LAMP1 and CD63 (Fig. 5B,C). However, such vacuoles did not appear enriched in the early endosomal markers EEA1 or SNX1 (Fig. 5D,E). Such data suggests that the majority of large, swollen vesicles are late endosomal in origin, whereas the smaller-diameter vacuoles appear to have early endosomal characteristics.

Overall, these data suggest that PIKfyve suppression alters normal endosomal homeostasis, such that although early and late endosomal characteristics are maintained, membrane trafficking is perturbed in such a way as to leading to early and late endosomal swelling.

Suppression of endogenous PIKfyve does not affect endosomal sorting of receptors for EGF or transferrin

To establish the role of PIKfyve in endosomal sorting, we examined the kinetics of internalisation, recycling and degradation of the EGF receptor. This cargo undergoes an ubiquitin-mediated sorting into the lumenal vesicles of the late endosome and/or MVB prior to its degradation within the lysosomal compartment; thus, a defect in MVB formation should result in a perturbation in EGF receptor degradation. To examine this, HeLa cells were treated with either individual or combined PIKfyve-specific siRNAs prior to incubation with ^{125}I-labelled EGF. Subsequent analysis of ^{125}I-EGF

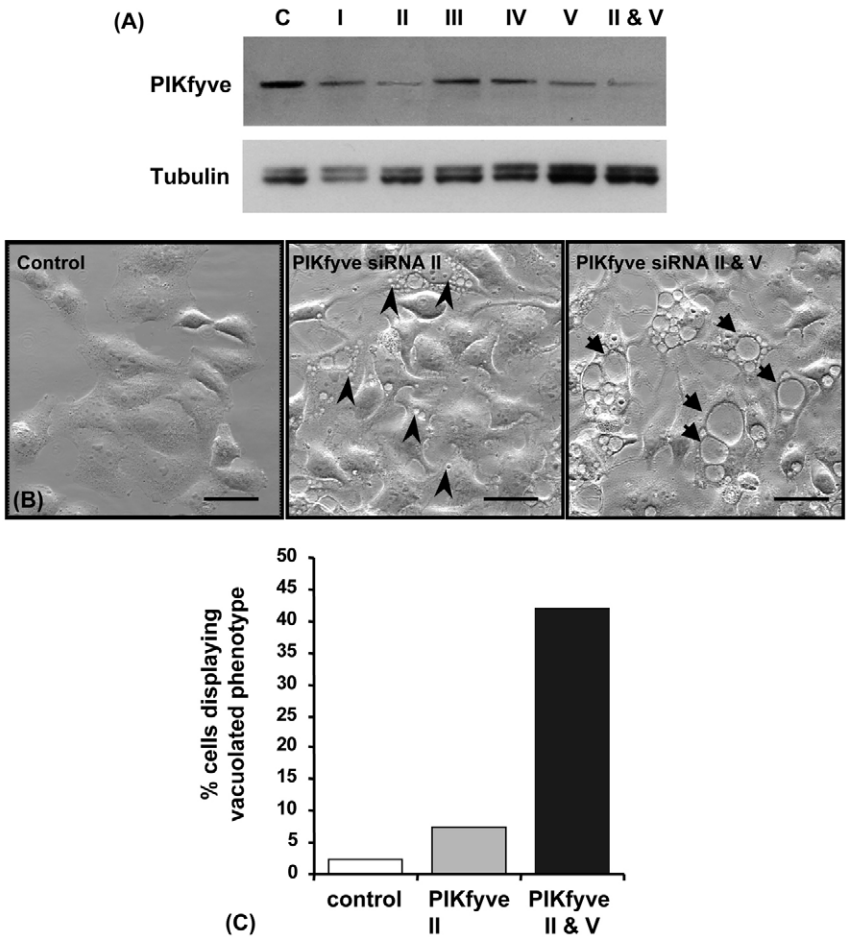

Fig. 4. siRNA-mediated suppression of endogenous PIKfyve leads to formation of enlarged, swollen cytoplasmic vacuoles. Five siRNA duplexes (I-V) were designed, each targeting a distinct region of human PIKfyve (see supplementary material Table 1). (A) HeLa cells were transiently transfected with each individual siRNA or a combination of siRNA duplexes II and V, for 72 hours prior to determining the level of PIKfyve using an antibody against human PIKfyve. Tubulin was used as a loading control. Depending on the siRNA used, varying levels of suppression were achieved, with the highest suppression routinely obtained when using a combination of siRNAs II and V (ratio 1:1). Data are representative of one series of experiments typical of at least three other independent experiments. (B) HeLa cells were transiently transfected with either control siRNA, PIKfyve-specific siRNA duplex II or a combination of PIKfyve-specific siRNA duplexes II and V. After 72 hours, cells were fixed and analysed as phase-contrast images. Each image is of a random field of view. Arrowheads and arrows indicate small and large vacuoles, respectively. Bars, 45 μm. (C) Quantification of the percentage of cells displaying a vacuolar phenotype was determined by visual inspection. A cell was scored as having such a phenotype if two or more swollen vacuoles were visible. Data are from 100 cells imaged for each condition.

internalisation, recycling and degradation revealed no significant effect on the kinetics of these pathways in cells suppressed for PIKfyve when compared with control cells (Fig. 6A). To confirm these results, we also followed the degradation of the receptor itself using western blotting (Fig. 6B). Again, in cells suppressed using either individual or combined siRNA duplexes, no significant effect on the rate of lysosomal-mediated EGF-receptor breakdown was observed (Fig. 6B). These data, therefore, suggest that early to late endosomal trafficking of the EGF receptor and its ongoing sorting into lumenal vesicles of MVBs is not significantly perturbed in HeLa cells whose endogenous PIKfyve has been suppressed.

To further these analyses, we also examined endosomal sorting of the transferrin receptor: a receptor that is recycled from the early endosome back to the plasma membrane by a default pathway. Here, similar results were obtained using ^{125}I-labelled transferrin – again, no significant perturbation in receptor internalisation, degradation or recycling was observed in cells treated with either individual or combined PIKfyve-targeting siRNA (supplementary material Fig. S2). Overall, when suppressed to these levels, endogenous PIKfyve does not appear to play a significant role in the endosomal sorting of receptors for EGF or transferrin. Such data is consistent with the observation – and indeed extends the evidence – that, overexpression of PIKfyve^{K1831E} does not perturb endosomal trafficking of these receptors (Ikonomov et al., 2003b).

In PIKfyve-suppressed cells early-endosome-to-TGN retrieval is perturbed

Given the evidence that PIKfyve associates with the Rab9 effector p40 (Ikonomov et al., 2003b), we next examined the role of endogenous PIKfyve in endosome-to-TGN retrieval of the CI-MPR. In initial experiments, we examined the steady-state distribution of this receptor in PIKfyve-suppressed HeLa cells (Fig. 7A). Compared with its normal TGN distribution, the CI-MPR was dispersed into peripheral cytosolic punctae in PIKfyve-suppressed cells (Fig. 7A). The peripheral CI-MPR-positive punctae were early-endosomal in nature because there was signficiant colocalisation with EEA1 (Fig. 7A). Such data is consistent with PIKfyve playing a role in early-endosome-to-TGN retrograde transport.

To verify this biochemically, we next examined the degradation of the CI-MPR in PIKfyve-suppressed cells. Previous studies have shown that inhibition of early-endosome-to-TGN retrieval of the CI-MPR increases the rate of receptor degradation (Arighi et al., 2004; Carlton et al., 2004). This appears to result from the dispersal of the receptor into early endosomes, from where it undergoes missorting into the lysosomal degradative pathway. In serum-starved PIKfyve-suppressed cells, we observed a significant reduction in the level of endogenous CI-MPR (Fig. 7B,C). This increased degradation was specific for the CI-MPR because no effect was observed on the level of receptors for either EGF or transferrin

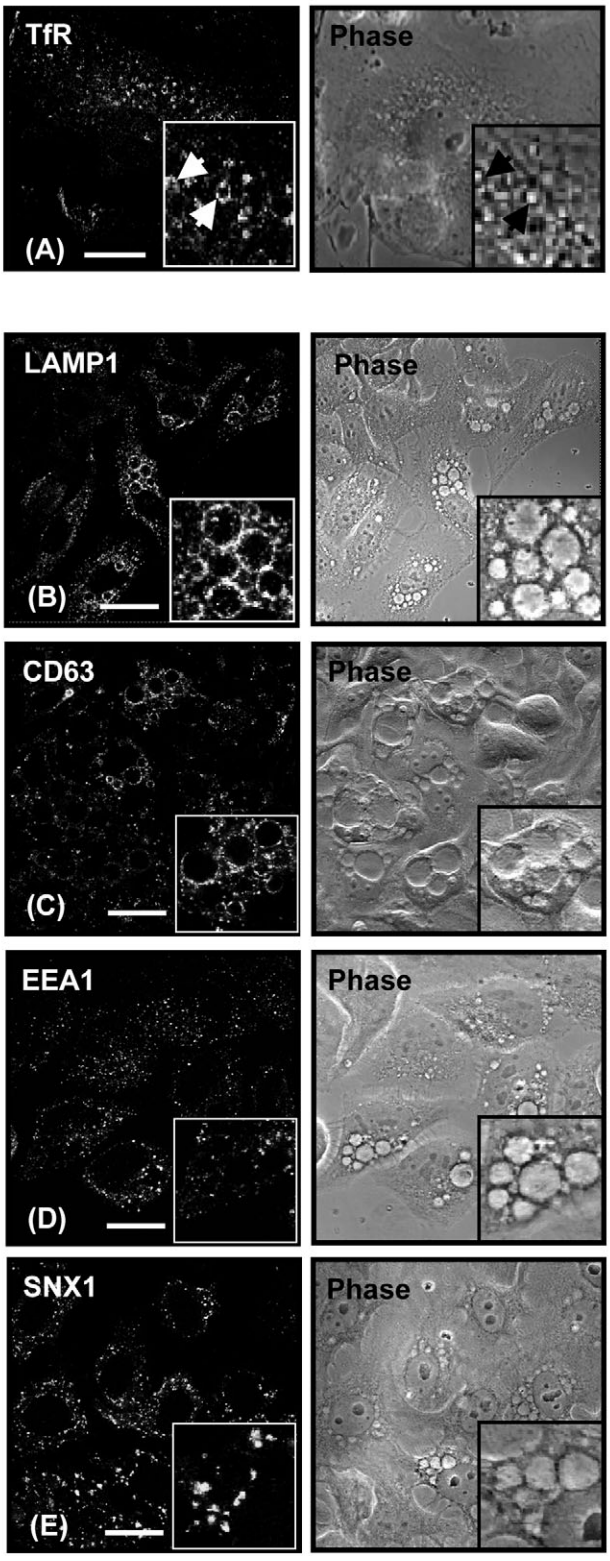

Fig. 5. PIKfyve suppression results in the swelling of both early and late endosomal compartments. (A-E) HeLa cells were transiently transfected with (A) PIKfyve-specific siRNA duplex II or (B-E) a combination of PIKfyve-specific siRNA duplexes II and V; left column, EM images; right column, phase-contrast images. Boxed insets show magnified regions. After 72 hours, cells were fixed and stained for (A) internalised transferrin receptor, 10 minutes after the addition of ligand (TfR), (B) LAMP1, (C) CD63, (D) SNX1 and (E) EEA1. For each condition similar images were observed in three independent experiments in which at least five randomly chosen fields of view were analysed. The arrows in (A) highlight the small vacuoles labelled with the internalised transferrin receptor. Bars, 10 μm (A), 25 μm (B-E).

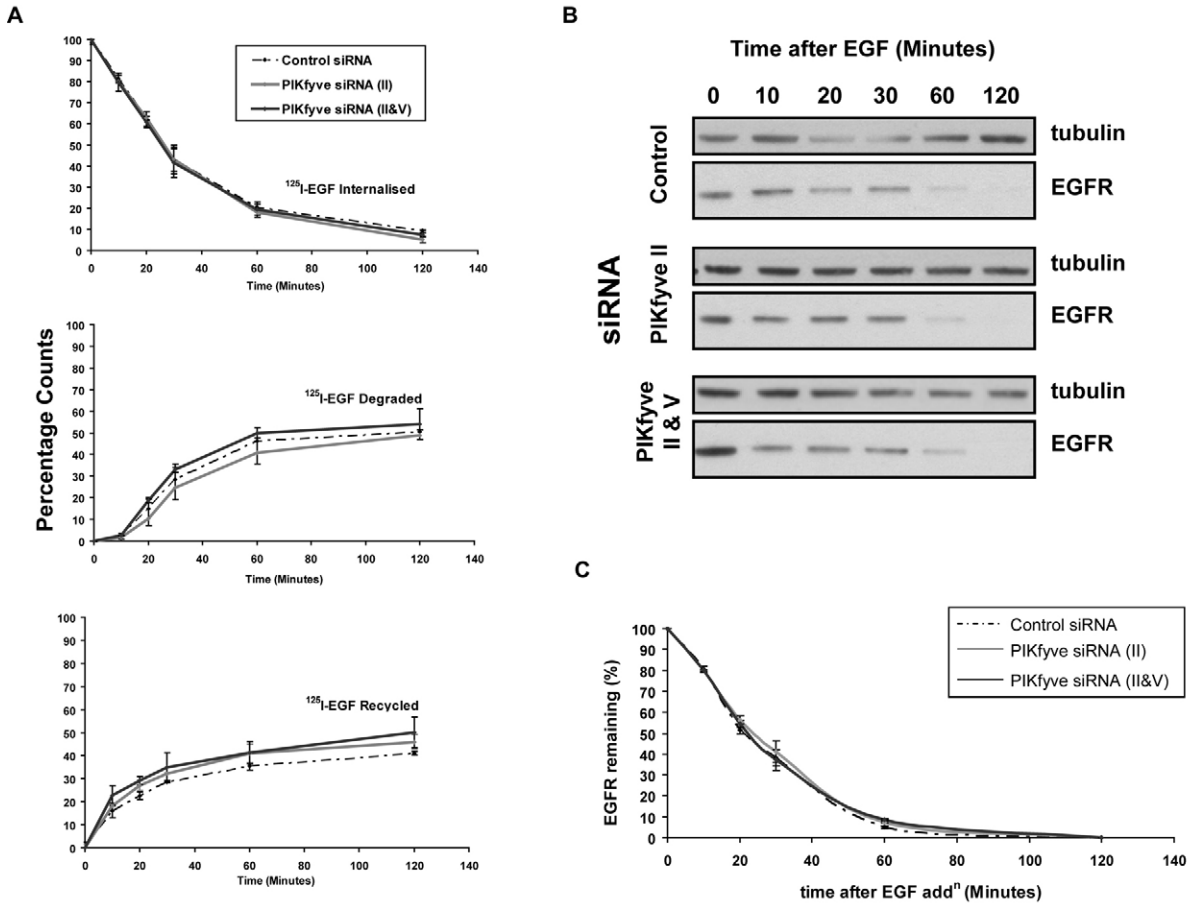

Fig. 6. Suppression of PIKfyve does not affect endosomal sorting of the EGF receptor. HeLa cells were treated with either control, PIKfyve specific siRNA duplex II or a combination of PIKfyve specific siRNA duplexes II and V for 72 hours. (A) Cells were serum-starved for 3 hours, labelled with ^{125}I-EGF at 1 kBq per well for 1 hour at 4°C, allowed to internalise surface-bound ^{125}I-EGF for 5 minutes at 37°C and were then returned to 4°C. Cells were chased using medium containing 100 ng/ml unlabelled EGF for various times at 37°C. Recycled, degraded and internalised fractions were subjected to γ-radiation counting. (B) Serum-starved cells were stimulated with 100 ng/ml EGF for 0, 10, 20, 30, 60 and 120 minutes, and western blotted with antibodies against EGF receptor or tubulin (loading control). (C) Quantification of data from three independent experiments.

(Fig. 7B,C). Overall, these data are consistent with PIKfyve controlling early-endosome-to-TGN retrograde transport.

To further this analysis, we examined whether PIKfyve-suppression was restricted to trafficking of the CI-MPR, or whether it constituted a more generic defect in endosome-to-TGN retrograde transport. To achieve this, we made use of various HeLaM cells lines stably expressing chimeras of CD8 and either CI-MPR, sortilin or furin cargo that undergo endosome-to-TGN transport (Seaman, 2004). At steady-state, in control cells, each cargo showed a marked enrichment at the TGN (Fig. 8). Consistent with the data described in Fig. 7, the steady-state distribution of CD8–CI-MPR was significantly perturbed in PIKfyve-suppressed cells, being redistributed to peripheral punctae that showed significant overlap with EEA1 (Fig. 8A). Interestingly, a similar redistribution was also observed for sortilin and furin (Fig. 8B,C). This argues that, the defect in endosome-to-TGN retrograde transport observed in PIKfyve-suppressed cells is not restricted to the CI-MPR but is a more generic defect affecting a number of distinct cargoes.

The kinetics of endosome-to-TGN transport is significantly perturbed in PIKfyve-suppressed cells

To examine the kinetics of endosome-to-TGN transport, we performed a series of antibody-uptake experiments using the CD8–CI-MPR and CD8-sortilin stably expressed in HeLaM cells (Seaman, 2004; Carlton et al., 2004) (for technical reasons we were unable to generate quantitative kinetics data on the transport of CD8-furin). Here, cells were labelled with the anti-CD8 antibody at 4°C to label surface protein. After a series of washes, cells were warmed to 37°C and the antibody was chased for various time points before the cells were washed and fixed (Fig. 9). Compared with control cells, suppression of PIKfyve led to a 77.2±13.3% reduction in the delivery of CD8–CI-MPR to the TGN46-labelled TGN after 32 minutes of uptake (Fig. 9A,B). Similar data were also obtained using CD8-sortilin, where delivery of anti-CD8 antibody to the TGN was reduced by 54.6±19.6% after 56 minutes of uptake (Fig. 9C,D). These observations are consistent with a reduced rate of endosome-to-TGN transport in PIKfyve-suppressed cells.

Discussion

It is clear that phosphoinositides play an important role in determining the identity and function of various compartments within the endocytic pathway. In this study, we have examined the localisation and function of the PtdIns(3)*P* 5-kinase PIKfyve in endosomal biology.

Using live and fixed cell confocal imaging, we have presented evidence that in HeLa cells expressing low levels of a GFP-tagged PIKfyve chimera, the enzyme is localised to dynamic cytosolic punctae that are enriched in early, rather than late endosomal markers. As the early endosome is enriched in PtdIns(3)*P*, our data is entirely consistent with previously published studies that have documented that the endosomal association of PIKfyve requires a wortmannin-sensitive PI 3-kinase and the ability of the PIKfyve FYVE domain to bind PtdIns(3)*P* (Shisheva et al., 2001). Having said this, other studies have shown that overexpressed PIKfyve associates most strongly with late endosomes and MVBs (Shisheva et al., 2001; Ikonomov et al., 2001). One explanation for these differences may arise from the lack of available antibodies capable of detecting endogenous PIKfyve by immunofluorescence. Although in our study we imaged cells overexpressing low levels of PIKfyve, other studies have used more prolonged overexpression (Shisheva et al., 2001; Ikonomov et al., 2001). It is therefore possible that the actual process of overexpressing PIKfyve has an effect on endosomal dynamics, such that it perturbs its normal endosomal localisation. Indeed, recent evidence has been presented supporting such a conclusion (Ikonomov et al., 2006).

In yeast, genetic analysis of *fab1Δ* cells has implicated Fab1p in a number of cellular functions, including retrograde transport from the vacuole, MVB formation and vacuole acidification (reviewed in Efe et al., 2005; Michell et al., 2006). Morphologically, *fab1Δ* cells are characterised by the appearance of an enlarged, swollen vacuole (Efe et al., 2005; Michell et al., 2006). Consistent with a certain level of conserved function, studies employing overexpression of a dominant-negative, catalytically dead version of PIKfyve (the PIKfyve^{K1831E} mutant), have reported the formation of enlarged endosome-like structures in a variety

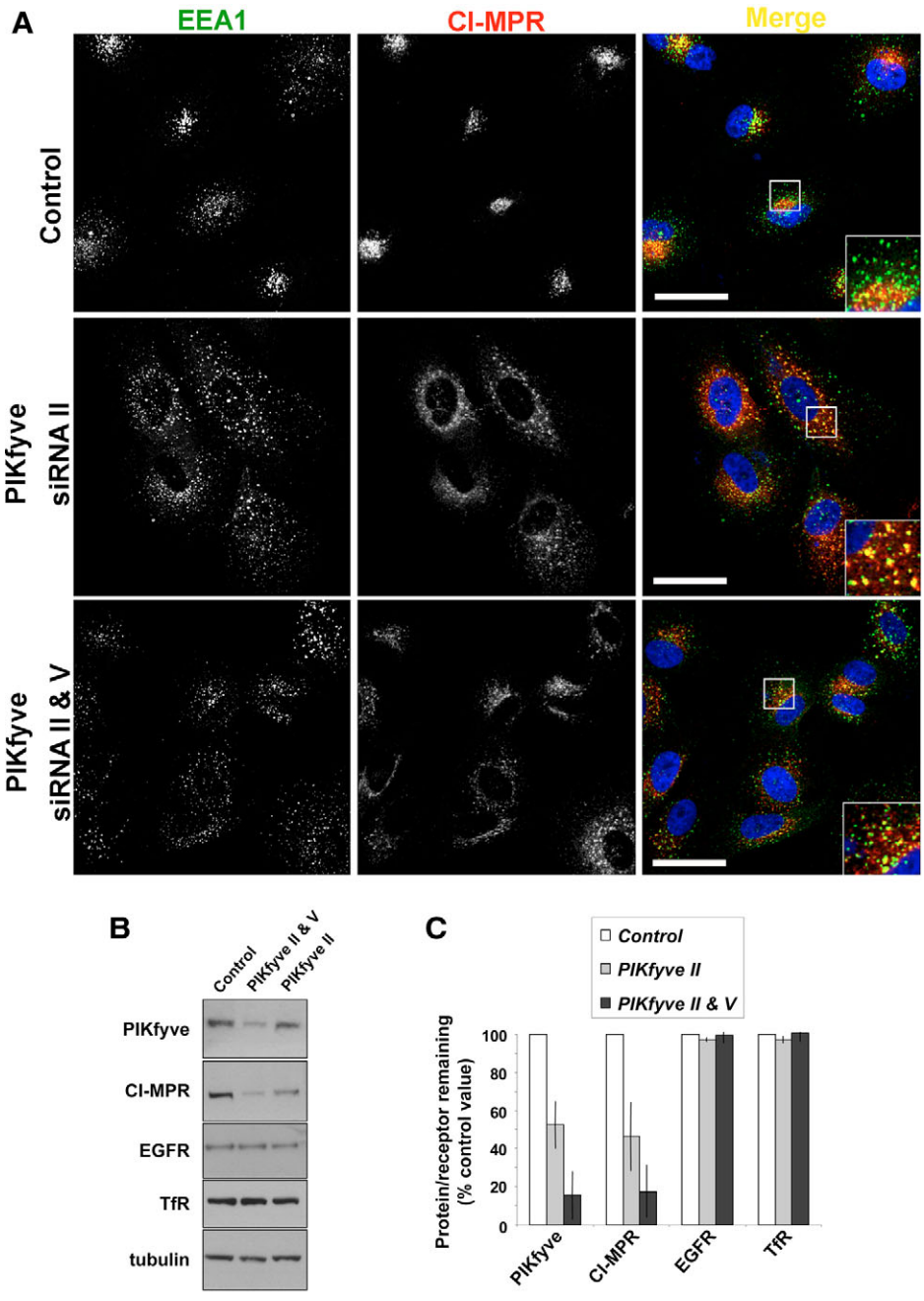

Fig. 7. Suppression of PIKfyve alters the steady-state distribution of the CI-MPR and enhances receptor degradation. (A) HeLa cells were treated for 72 hours with control siRNA, PIKfyve-specific siRNA duplex II or a combination of siRNA duplexes II and V. Cells were fixed and stained against CI-MPR and EEA1 prior to imaging. Suppression of PIKfyve results in a redistribution of the CI-MPR from the TGN to peripheral punctae that show considerable overlap with the EEA1-labelled compartment. Boxed areas are magnified in right corner to show degree of overlap. Bars, 20 μm. (B) HeLa cells were treated for 72 hours with either control, PIKfyve-specific siRNA duplex II or a combination of PIKfyve-specific siRNA duplexes II and V. Cells were lysed and lysates blotted for PIKfyve, EGF receptor, transferrin and CI-MPR, and tubulin (loading control). Representative results are shown. (C) Quantification of data from four independent experiments.

of mammalian cell types (Ikonomov et al., 2001). We have now extended these studies by observing the formation of large, swollen endosomal structures in PIKfyve-suppressed HeLa cells. In particular, we have observed a correlation between the level of PIKfyve suppression and the extent of endosomal swelling. Thus, in cells treated with a combination of siRNA

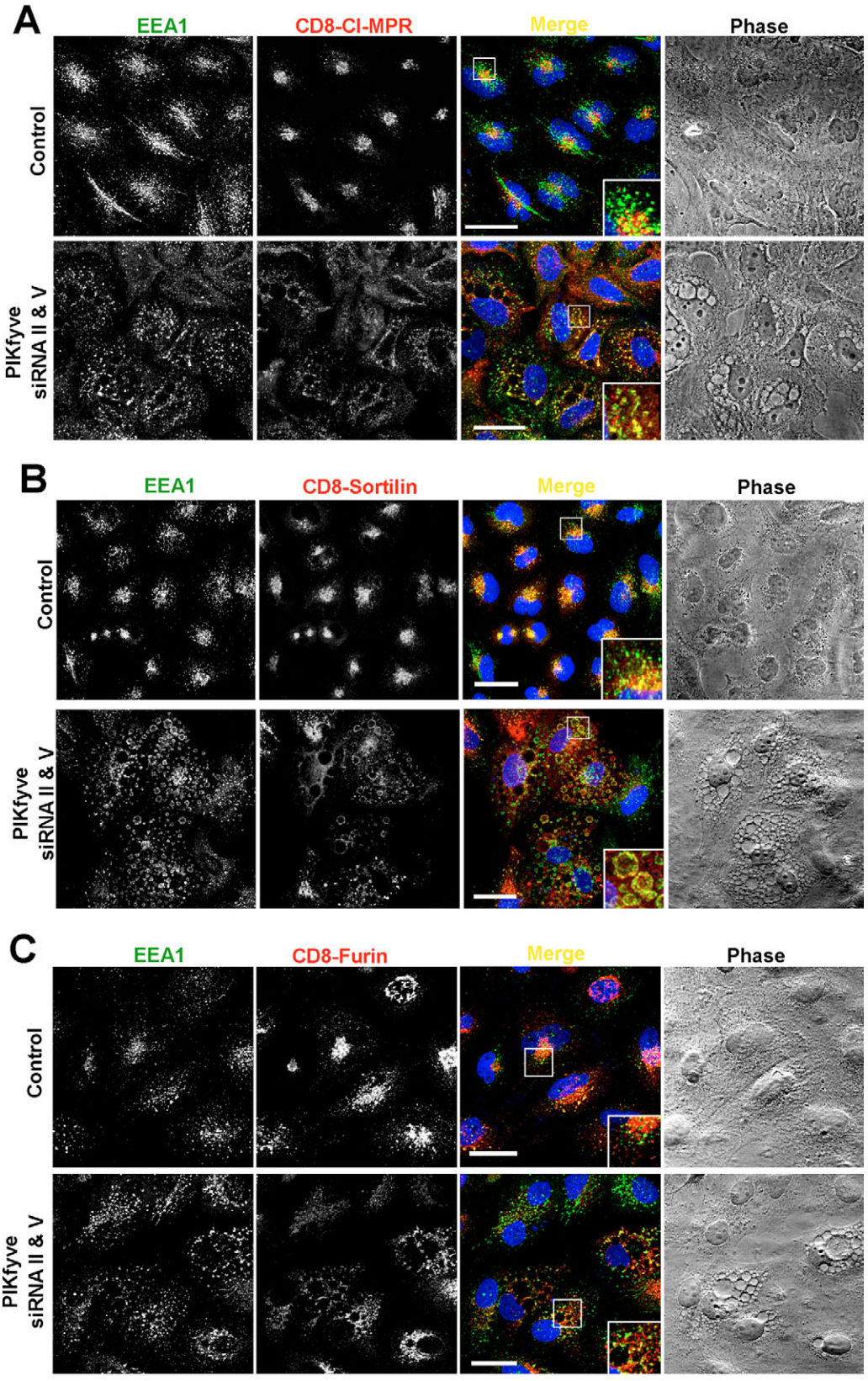

Fig. 8. PIKfyve suppression alters the steady-state distribution of not only CI-MPR but also sortilin and furin. (A-C) HeLaM cells stably expressing either (A) CD8-CI-MPR, (B) CD8-sortilin or (C) CD8-furin, were treated with control siRNA or a combination of PIKfyve duplexes II and V as described in Materials and Methods. Cells were then fixed and co-stained for CD8 and EEA1 prior to confocal imaging. Images are projections of at least eight confocal sections and are representative of over 40 cells imaged for each condition. Bars, 40 μm.

duplexes that lead to a strong suppression of PIKfyve, approximately 42% of cells showed the presence of two or more large, swollen endosomal structures. By contrast, treatment with single duplexes, which resulted in a weaker suppression, led to only 7% of cells displayed such a phenotype. Such data argue that, in HeLa cells endogenous PIKfyve plays an important role in controlling endosomal morphology and homeostasis.

The mechanism(s) by which PIKfyve controls endosomal homeostasis and, hence, why its suppression leads to the formation of swollen endosomal structures, still remains to be defined (reviewed in Michell et al., 2006). Under our conditions of PIKfyve suppression, we can distinguish at least two populations of swollen endosomal structures. The first of these are the large, swollen structures that are easily visible under phase contrast. These structures have a limiting membrane positive for late endocytic markers such as LAMP1 and CD63, but lack characteristics of the early compartment, such as the presence of EEA1, SNX1 and internalised transferrin receptor. The second vesicular population is smaller in diameter and more difficult to observe under phase contrast. Although not containing late endocytic markers, these structures contain internalised transferrin receptors and, hence, appear early endosomal in origin. Together, our data suggest that the large, swollen late endosomal compartment comprises an end-point morphological phenotype and that, prior to its formation, defects in early

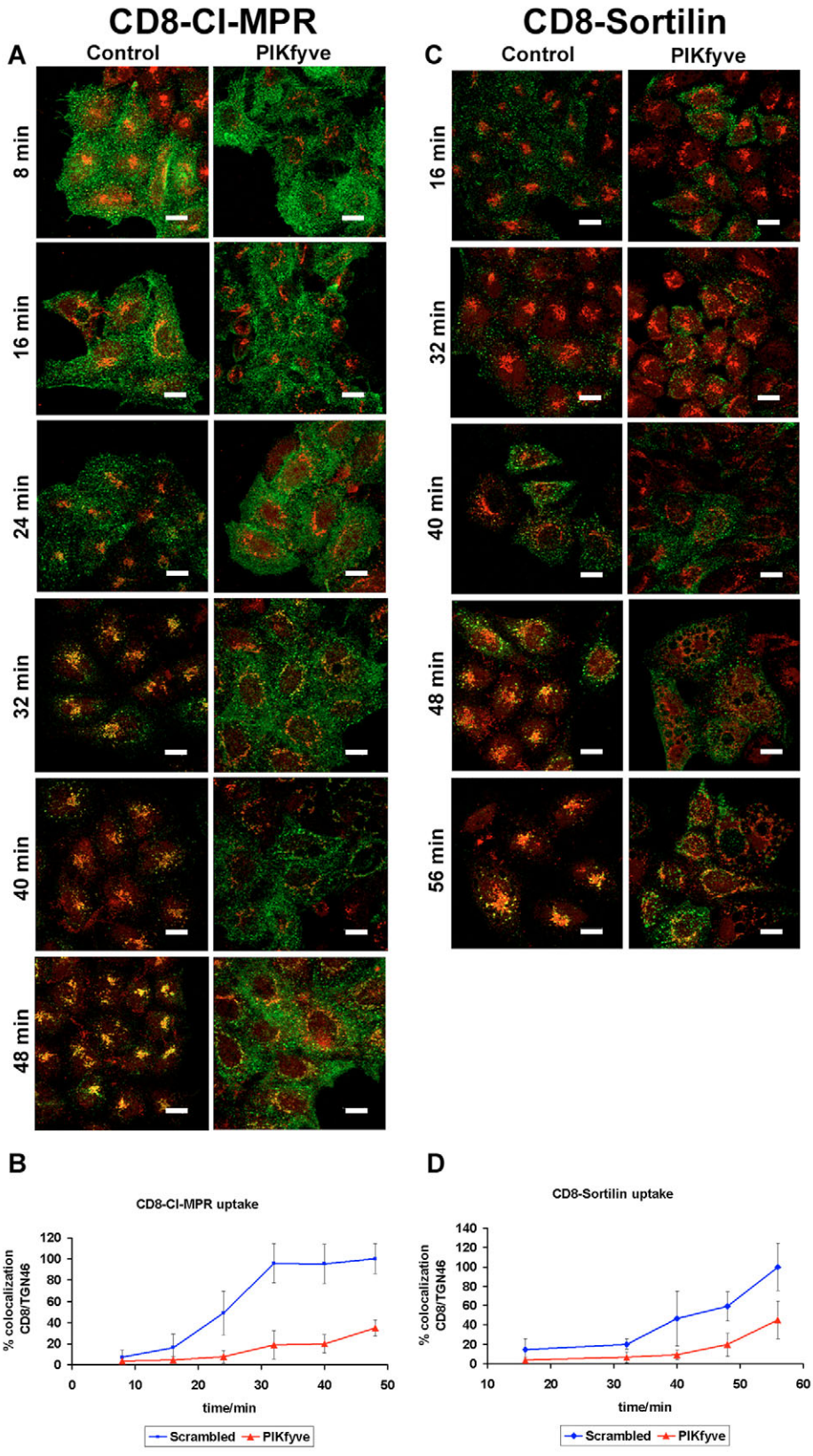

Fig. 9. Kinetics of endosome-to-TGN retrieval of CI-MPR and sortilin is perturbed in PIKfyve-suppressed cells. (A,C) Images depicting the kinetics of delivery of a cell-surface-labelled (A) CD8-CI-MPR chimera or (C) CD8-sortillin chimera (both in green) to the TGN46-labelled TGN (red) in control HeLaM cells or in HeLaM cells whose PIKfyve was suppressed by a combination of duplex II and V. Images are projections of eight confocal sections and are representative of more than 40 cells images for each condition. Bars, 20 μm. (B,D) Quantification of the kinetics of cell-surface-labelled (B) CD8-CI-MPR chimera or (D) CD8-sortilin chimera transport to the TGN46-labelled TGN in HeLaM cells, expressed as percent normalised colocalisation between the two labels. Data were obtained as outlined in Materials and Methods from $n \geqslant 40$ cells for each time point, and are expressed as the mean ± s.e.m.

endosomal structure and function (*e.g.* retrograde early-endosome-to-TGN transport) can be observed. Moreover, these data suggest that the functional identity of early and late endosomes and their maturation does not appear to be significantly perturbed in PIKfyve-suppressed cells. Supporting such a conclusion, conditions of PIKfyve suppression that induce the formation of large, swollen late endosomal structures, do not result in a detectable defect in the endosomal sorting and degradation of internalised EGF receptor or the endosomal recycling of transferrin receptor. Such data lead to the conclusion that PIKfyve does not play a significant role in recycling from the early endosome or in degradative sorting through the endo-lysosomal network. This, therefore, implies that PIKfyve does not play a significant role in ESCRT-mediated sorting of the EGF receptor and suggest that a defect in inward-budding of the limiting endosomal membrane is not the major factor behind the swollen vacuolar phenotype.

Although the delivery of membrane to the vacuole from the endocytic and biosynthetic pathways does not appear to be perturbed in *fab1Δ* cells (Odorizzi et al., 1998; Gary et al., 1998; Dove et al., 2002; Rudge et al., 2004), there is a defect in retrograde traffic from the vacuolar membrane back to the Golgi (Bonangelino et al., 2002). Indeed, the PtdIns(3,5)P_2 receptor Svp1p may well constitute one of the effectors that regulate this pathway (Dove et al., 2004; Reggiori et al., 2004). Such a defect in retrograde transport would induce swelling of the vacuole through an imbalance in membrane fusion versus fission. A similar mechanism might well explain the swollen endosomal morphology observed in PIKfyve-suppressed HeLa cells (see Fig. 10).

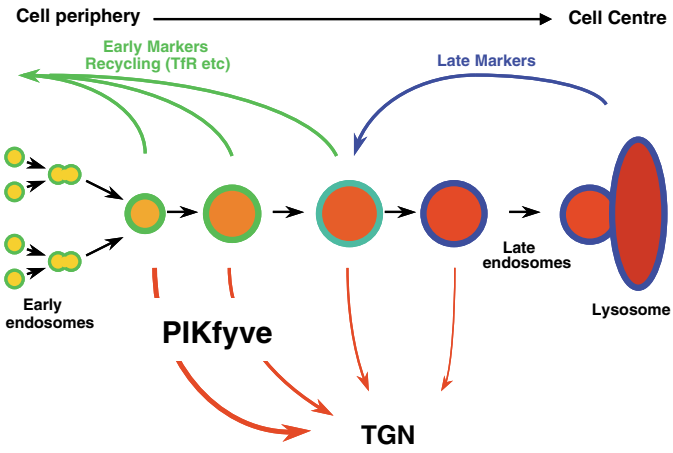

Fig. 10. Model depicting the proposed role of PIKfyve in regulating endosome-to-TGN retrograde transport. Our model is adapted from the model proposed by Rink and colleagues, describing the process of early to late endosomal progression (Rink et al., 2005). In their model, early endosomes are viewed as forming a temporally dynamic network of compartments that through fusion and fission events are constantly generated and renewed in the cell periphery. Progression to late endosomes occurs through repeated fusion events that enrich degradative cargo (e.g. the EGF receptor shown in red) in increasingly fewer and larger endosomes that are localised in the centre of the cell. During this progression, early and late endosomes maintain their identity through the loss of early markers, such as transferrin receptor (depicted in green), and the acquisition of late markers shown in blue (Rink et al., 2005). Our data support a model whereby PIKfyve modulates endosome-to-TGN retrograde transport primarily, but perhaps not solely, from an early endosomal compartment. A loss of, or reduction in, the activity of PIKfyve leads to a decrease in this membrane flux, which in turn induces a progressive swelling of the endosomal compartments.

Zerial and colleagues have recently proposed a model (Rink et al., 2005), which appears to reconcile details of both the maturation and vesicle-transport models of early to late endosome progression (Gruenberg et al., 1989; Griffiths and Gruenberg, 1991; Murphy, 1991). In their model, early endosomes are viewed as forming a temporally dynamic network of structures that, through fusion and fission events, are constantly generated and renewed in the cell periphery. Progression to late endosomes occurs through repeated fusion events that enrich degradative cargo (e.g. the EGF receptor) in increasingly fewer and larger endosomes localised in the centre of the cell. During this progression, early and late endosomes maintain their identity through the loss of early markers and the acquisition of late markers (Rink et al., 2005). In the present study, we have shown that, although early to late endosomal progression appears unperturbed in PIKfyve-suppressed cells – as determined by the degradation of EGF receptor and recycling of transferrin receptor – a defect in retrograde endosome-to-TGN transport can be observed. Thus, in PIKfyve-suppressed cells, rather than maintaining their steady-state TGN distribution, cargo such as CI-MPR, sortilin and furin, which normally cycle between the early endosome and the TGN (Lin et al., 2004; Seaman, 2004), is redistributed to peripheral early endosomes. Such a reduction in retrograde fission would thereby result in a progressive swelling of endosomal structures. Further support for a role of PIKfyve in endosome-to-TGN retrograde transport is the evidence that PIKfyve interacts with p40, a Rab9 effector implicated in retrograde traffic from the late endosome (Ikonomov et al., 2003b). Moreover, the identification of a number of potential mammalian PtdIns(3,5)P_2 effectors that lie on this pathway, including the mammalian proteins sorting nexin-1 (Cozier et al., 2002; Carlton et al., 2004) and the Svp1p orthologue WIPI49 (Jeffries et al., 2004), is entirely consistent with a role for PIKfyve and its lipid product PtdIns(3,5)P_2 in endosome-to-TGN transport.

In summary, these data suggest that in mammalian cells PIKfyve is predominantly associated with the PtdIns(3)*P*-enriched early endosome, from were it regulates retrograde membrane trafficking to the TGN. With the recent identification of PIKfyve mutations in patients with Francois-Neetens mouchetée fleck corneal dystrophy (Li et al., 2005) – here corneal flecks form possibly through generation of abnormal keratocytes that are swollen as a result of enlarged cytosolic vesicles (Nicholson et al., 1977) – our data suggest that part of the underlying cause of this disease may be a defect in retrograde endosome-to-TGN trafficking.

Materials and Methods

Antibodies and ligands

Anti-SNX1 and anti-EEA1 monoclonal antibodies and goat polyclonal antibodies were from Becton Dickinson and Santa Cruz, respectively. The anti-EGF receptor monoclonal antibody used for immunofluorescence was a kind gift from Peter Parker (CRUK, London, UK), whereas that used for western blotting (number 2232) was from Cell Signaling Technologies. Anti-CI-MPR rabbit polyclonal antibody was a kind gift from Paul Luzio (CIMR, Cambridge, UK), and the anti-transferrin

receptor antibody (number 13-6800) was from Zymed. Secondary antibodies conjugated to fluorophores were from Jackson ImmunoResearch. Fluorescent ligands were from Molecular Probes. The CD8 antibody used for decorating the CD8-chimeras was the mouse monoclonal UCHT4 (Ancell, Bayport, Minnesota), it was illuminated by an Alexa Fluor 488-conjugated anti-mouse antibody (Invitrogen, Paisley, UK). The *trans*-Golgi network was stained using a sheep anti-TGN46 antibody (Serotec, Oxford, UK), followed by staining with an Alexa Fluor 568-conjugated anti-sheep antibody (Invitrogen).

Generation of anti-PIKfyve antibody

Antiserum against human PIKfyve (see Cabezas et al., 2006) was obtained by immunising a rabbit with the peptide corresponding to the 15 N-terminal residues of human PIKfyve (MATDDKTSPTLDSANC) conjugated via a cysteine to *Limulus polyphemus* hemocyanine (BioGenes GmbH, Berlin, Germany). The antibody was affinity purified on a Sulfo-Link gel (Pierce, Rockford, IL) with covalently coupled antigenic peptide.

Transient transfection, cell imaging and quantification of overlapping signals

HeLa cells were cultured as previously described (Cozier et al., 2002), plated on glass coverslips and transfected with vector DNA at 50% confluency using Genejuice (Novagen) at a concentration of 0.2 μg vector DNA/μl cationic lipid. After 22 hours expression, cells were fixed using paraformaldehyde (4% w/v) for 15 minutes at room temperature and mounted on microscope slides using Mowiol. Fixed and live cell imaging was performed on a Leica AOBS confocal microscope and a Perkin Elmer UltraVIEW LCI respectively. Quantification of overlapping signals was achieved by visual inspection as described in Carlton et al. (Carlton et al., 2005).

siRNA transfection

siRNA duplexes designed against human PIKfyve or control duplex (see supplementary material Table 1) were purchased from Dharmacon. For transient transfection, HeLa cells were seeded in 6-well plates at a density of 7×10^4 cells per well prior to transfection using oligofectamine (Invitrogen) and 200 nM of the relevant siRNA duplex. Cells were incubated for a further 72 hours. In some experiments, a double-transfection protocol was used, whereby cells were again transfected 48 hours after the first transfection, Cells were then incubated for an additional 48 hours. Western blotting was performed using the ECL western blotting system (Amersham Pharmacia Biotech), and developed films were quantified by volume integration using ImageQuant software (Molecular Dynamics).

^{125}I-EGF trafficking assays

HeLa cells were transfected with siRNA duplexes for 72 hours as described above. For ^{125}I-EGF trafficking assays, cells were washed into DMEM containing 25 mM Hepes, 0.2% fatty-acid-free BSA (DHB) and incubated at 4°C with ^{125}I-EGF at 1 kBq per well for 1 hour. ^{125}I-bound to the cell surface was internalised by warming the cells to 37°C for 5 minutes. Cells were returned on ice and ^{125}I-EGF remaining at the cell surface was removed by a mild-acid strip (0.2 M acetic acid, 0.5 M NaCl, pH 4.5) and washed extensively with ice-cold PBS. Remaining cell surface receptors were saturated with 100 ng/ml EGF in DHB for 30 minutes at 4°C, at which point cells were returned to 37°C for the chase time indicated. At the end of the chase, medium was removed and a 2-minute acid strip with 0.2 M acetic acid, 0.5 M NaCl, pH 2.8, removed ^{125}I-EGF bound to receptors at the cell surface. Cells were solubilised with 1 M NaOH at room temperature for 30 minutes. Counts present in each fraction were determined by counting γ-radiation. Acid-precipitable material (recycled counts) was determined by summing recycled counts and counts removed by the acetic acid strip. Degraded counts were deemed the acid-soluble counts and counts released upon solubilisation of the cell monolayer were deemed internalised counts.

^{125}I-transferrin trafficking assays

For ^{125}I-transferrin trafficking, cells were washed into DHB and incubated at 37°C for 60 minutes with ^{125}I-transferrin at 1 kBq per well to equilibriate the endosomal system. Cells were placed on ice and cell-surface bound ^{125}I-transferrin was stripped off using ice-cold 0.2 M acetic acid, 0.5 M NaCl, pH 4.5 for 2 minutes, then washed extensively with ice-cold PBS. Cells were chased into DHB containing 50 μg/ml unlabelled transferrin for the indicated times. At the end of each time point, medium was removed and separated into acid-precipitable material (recycled counts) and acid-soluble material (degraded counts) by incubation with 3% trichloroacetic acid, 0.3% phosphotungstic acid for 30 minutes at 4°C followed by high-speed centrifugation. A 2-minute acid strip with 0.2 M acetic acid, 0.5 M NaCl, pH 2.8, removed transferrin bound to receptors at the cell surface. Cells were solubilised with 1 M NaOH at room temperature for 30 minutes. Counts present in each fraction were determined by counting γ-radiation. Recycled counts were determined by summing acid-precipitable counts and counts removed by the acetic acid strip.

Anti-CD8 uptake experiments

The uptake experiments were performed as previously described (Carlton et al., 2005). Samples were analysed using a TCS-NT confocal microscope (Leica, Wetzlar, Germany), at least four individual situations per specimen in eight stacks were recorded and analysed. Degree of colocalisation was measured using Metamorph software (Molecular Devices, Sunnyvale, CA) by acquiring the area of CD8- and TGN46-staining and measuring the percentage of CD8-positive area included in the TGN46-positive area.

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