PtdIns4KIIα generates endosomal PtdIns(4)P and is required for receptor sorting at early endosomes

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ABSTRACT **Phosphatidylinositol 4-kinase IIα (PtdIns4KIIα) localizes to the *trans*-Golgi network and endosomal compartments and has been implicated in the regulation of endosomal traffic, but the roles of both its enzymatic activity and the site of its action have not been elucidated. This study shows that PtdIns4KIIα is required for production of endosomal phosphatidylinositol 4-phosphate (PtdIns(4)P) on early endosomes and for the sorting of transferrin and epidermal growth factor receptor into recycling and degradative pathways. Depletion of PtdIns4KIIα with small interfering RNA significantly reduced the amount of vesicular PtdIns(4)P on early endosomes but not on Golgi membranes. Cells depleted of PtdIns4KIIα had an impaired ability to sort molecules destined for recycling from early endosomes. We further identify the Eps15 homology domain–containing protein 3 (EHD3) as a possible endosomal effector of PtdIns4KIIα. Tubular endosomes containing EHD3 were shortened and became more vesicular in PtdIns4KIIα-depleted cells. Endosomal PtdIns(4,5)P_2 was also significantly reduced in PtdIns4KIIα-depleted cells. These results show that PtdIns4KIIα regulates receptor sorting at early endosomes through a PtdIns(4)P-dependent pathway and contributes substrate for the synthesis of endosomal PtdIns(4,5)P_2.**

Monitoring Editor
Akihiko Nakano
RIKEN

Received: Aug 13, 2015
Revised: Jan 14, 2016
Accepted: Jan 19, 2016

INTRODUCTION

Intracellular membrane trafficking regulates protein and lipid localization, signal transduction, and defense against various infectious diseases (Gruenberg, 2009; Sorkin and von Zastrow, 2009). Two major forms of transport carriers can be distinguished morphologically: transport vesicles and tubular membrane carriers (Mayor *et al.*, 1993; Bard and Malhotra, 2006). These morphological features can be observed together on early endosomes, which represent stations for sorting cargoes destined for lysosomal degradation or recycling to the plasma membrane (Peden *et al.*, 2004; Rink *et al.*, 2005; Traer *et al.*, 2007; van Weering and Cullen, 2014). The formation of these transport intermediates is highly regulated by both protein factors and lipid metabolism, but the molecular mechanisms that underpin this are not fully understood.

Phosphoinositides (PIs) direct membrane traffic by providing temporal and spatial regulation of endosomal membranes through the recruitment of effector molecules (Simonsen *et al.*, 2001; van Meer and Sprong, 2004). The prevailing view is that different PI species are found on specific intracellular organelles (Di Paolo and de Camilli, 2006). For example, phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)P_2) is predominantly located on the cytoplasmic leaflet of the plasma membrane, whereas phosphatidylinositol 3-phosphate (PtdIns(3)P) and phosphatidylinositol

This article was published online ahead of print in MBoC in Press (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E15-08-0564) on January 28, 2016.

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Abbreviations used: EEA1, early endosome antigen 1; EGFR, epidermal growth factor receptor; FYVE, Fab1/YOTB/Vac1/EEA1; Hrs, hepatocyte growth factor–regulated tyrosine kinase substrate; OSBP, oxysterol-binding protein; PH, pleckstrin homology domain; PI, phosphoinositide; PtdIns(3)P, phosphatidylinositol 3-phosphate; PtdIns4K, phosphatidylinositol 4-kinase; PtdIns(4)P, phosphatidylinositol 4-phosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; siRNA, small interfering RNA; Tfn, transferrin; TGN, *trans*-Golgi network.

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(3,5)-bisphosphate (PtdIns(3,5)P_2) are located on early and late endosomes, respectively. Phosphatidylinositol 4-phosphate (PtdIns(4)P) is located primarily at the *trans*-Golgi network (TGN) but a significant pool has also been detected at the plasma membrane (PM; Hammond *et al.*, 2009; Hammond and Fischer, 2012). In addition to serving as signaling precursors, these organelle-specific PIs recruit effector proteins (Simonsen *et al.*, 1998; Wang *et al.*, 2003; Höning *et al.*, 2005) to regulate membrane traffic.

PtdIns(3)P is the best characterized of the endosomal PIs and plays central roles in endosomal fusion and sorting of molecules destined for degradation. PtdIns(3)P also acts as a decisive component of a regulatory switch at the early endocytic compartment (Simonsen *et al.*, 1998; Christoforidis *et al.*, 1999a; Lloyd *et al.*, 2002; Petiot *et al.*, 2003; Zoncu *et al.*, 2009). PtdIns(3)P is produced at early endosomes by class III PtdIns(3)P kinase (hVps34-p150), which is activated by Rab5 GTPase. PtdIns(3)P then recruits early endosome autoantigen 1 (EEA1) and hepatocyte growth factor–regulated tyrosine kinase substrate (Hrs), molecules that regulate endosomal fusion and maturation (Simonsen *et al.*, 1998; Christoforidis *et al.*, 1999b; Komada and Soriano, 1999; Raiborg *et al.*, 2002; Pons *et al.*, 2008).

Although PtdIns(4)P is a well-known signaling intermediate in agonist-dependent PtdIns(4,5)P_2 hydrolysis, it can also function as to recruit numerous binding proteins to the Golgi complex and is considered a key factor maintaining the structure and proteolipid identity of the organelle (Graham and Burd, 2011). PtdIns(4)P is increasingly recognized as a component of membranes of the PM (Hammond and Fischer, 2012; Nakatsu *et al.*, 2012) and endosomal system (Jović *et al.*, 2009). Indeed, a PtdIns(4,5)P_2 cycle involving PtdIns(4)P5KI and OCRL has been described that controls recycling and degradation of numerous cargoes (Vicinanza *et al.*, 2011). In addition, numerous endosomal proteins have been identified with PtdIns(4)P-binding specificity (Wang *et al.*, 2003; Godi *et al.*, 2004; Tóth *et al.*, 2006; D'Angelo *et al.*, 2007; Dippold *et al.*, 2009).

Although early endosomal membranes are enriched in PtdIns(3)P, the type IIα and type IIβ isoforms of phosphatidylinositol 4-kinase (PtdIns4KIIα and PtdIns4KIIβ, respectively) were also found on endosomes (Balla *et al.*, 2002; Waugh *et al.*, 2003; Minogue *et al.*, 2006). PtdIns4KIIα interacts with adapter protein 3 (AP-3; Craige *et al.*, 2008), which localizes to an endosomal sorting compartment (Peden *et al.*, 2004). PtdIns4KIIα is required for correct endolysosomal traffic of the epidermal growth factor receptor (EGFR; Minogue *et al.*, 2006). However, it remains to be determined whether PtdIns(4)P, the enzymatic product of PtdIns4KIIα, is generated on endosomes and how this lipid controls endosomal traffic. PtdIns4KIIβ contains an N-terminal AP-1–binding motif and functions in TGN–endosomal traffic (Wieffer *et al.*, 2013).

This study shows that both PtdIns(4)P and PtdIns4KIIα can be detected on early endosomes at sites distinct from PtdIns(3)P-positive membrane domains. It also demonstrates that depletion of PtdIns4KIIα reduces the level of endosomal PtdIns(4)P and PtdIns(4,5)P_2. The dynamin-like Eps15 homology domain–containing protein EHD3, which localizes on PtdIns(4)P positive membranes, is identified as a candidate effector for PtdIns4KIIα. Sorting of cargo into recycling and degradative pathways at the early endosome is also impaired, indicating that PtdIns4KIIα and PtdIns(4)P have indispensable roles in transport from early endosomes.

RESULTS

Both PtdIns4KIIα and PtdIns(4)P localize to early endosomes

PtdIns4KIIα localizes to the TGN and early and late endosomal compartments (Balla *et al.*, 2002; Waugh *et al.*, 2003; Minogue *et al.*, 2006) but has no demonstrated role in early endosomal sorting. Both endogenous PtdIns4KIIα and green fluorescent protein (GFP)–tagged proteins localize to EEA1-positive early endosomes (EEs; Figure 1A; Balla *et al.*, 2002; Waugh *et al.*, 2003), and depletion of PtdIns4KIIα causes scattering of EGF-positive endosomes (Minogue *et al.*, 2006), resembling the pharmacological blockage of EE transport with a dynamin inhibitor (Mesaki, Tanabe, *et al.*, 2011). We previously showed that PtdIns4KIIα small interfering RNA (siRNA) or inhibition with an monoclonal antibody impaired endosomal traffic and degradation of the EGFR (Minogue *et al.*, 2006). However, this study did not address the function of the enzymatic product PtdIns(4)P or its locus of action. PtdIns(4)P and PtdIns(4,5)P_2 are normally viewed as defining components of the TGN and plasma membrane, but significant pools of these PI species have been detected on intracellular vesicles (Watt *et al.*, 2002; Balla *et al.*, 2005; Hammond *et al.*, 2009; Jović *et al.*, 2009), and there is evidence for PtdIns(4)P and PtdIns(4,5)P_2 on endosomal sorting compartments (Jović *et al.*, 2009; Vicinanza *et al.*, 2011). Together these results point to the existence of endosomal pools of PtdIns(4)P and PtdIns(4,5)P_2. We therefore focused our studies on PtdIns4KIIα and its lipid product PtdIns(4)P by employing well-characterized and widely used techniques, including pleckstrin homology domain (PH) domain probes, to specifically detect endosomal PtdIns(4)P.

Several probes for PtdIns(4)P are available, including FAPP1-PH, oxysterol-binding protein (OSBP)–PH, and OSH2-PH domains (Varnai and Balla, 2008). Only GFP-OSBP-PH was able to detect PtdIns(4)P signals colocalizing with the early endosomal marker EEA1 (Supplemental Figure S1A). In contrast, GFP-FAPP1-PH mainly detected Golgi-localized PtdIns(4)P (Supplemental Figure S1B), whereas GFP-OSH2-PH localizes to the nucleus and plasma membrane as reported previously (Balla *et al.*, 2008). GFP-OSBP-PH detected signals on cytoplasmic vesicles and tubules, the plasma membrane, and the Golgi complex (Balla *et al.*, 2005). This indicates that these PH probes report distinct PtdIns(4)P pools. Furthermore, the observation that a vesicular pool remains after siRNA-mediated depletion of individual isoforms or specific pharmacologic inhibition of type III PtdIns4Ks (Balla *et al.*, 2005) suggests the existence of a hitherto-unexplained PtdIns(4)P pool. The existence of such a pool would be consistent with observed effects of PtdIns4KIIα knockdown on EGF trafficking (Minogue *et al.*, 2006).

Immunocytochemical techniques have been described for reporting intracellular PtdIns(4)P pools using bacterially expressed recombinant proteins and with anti-phosphoinositide antibodies as unbiased probes (Hammond *et al.*, 2009). Using these methods, an anti-PtdIns(4)P antibody detected an intracellular pool of PtdIns(4)P but not when the antibody was preabsorbed against PtdIns(4)P-containing liposomes (Supplemental Figure S1C). This antibody decorated intracellular membranes, partially overlapping with GFP-FAPP1-PH (in the juxtanuclear region) or GFP-OSBP-PH (both in the juxtanuclear location and on vesicles) but not with GFP-OSH2-PH (Supplemental Figure S1D), although GFP-FAPP1-PH showed the best correlation between GFP signals and anti-PtdIns(4)P signals in entire cells (Supplemental Figure S1E). There was a possibility that the postfix method (Hammond *et al.*, 2009) affected recognition of the endosomal vesicular pool, which was already bound by endogenous PI-binding proteins before fixation. To compare the effects, we compared postfixed and pre-fixed samples, using GFP-Hrs Fab1/YOTB/Vac1/EEA1 (FYVE; pre-fix) and glutathione *S*-transferase (GST)–HrsFYVE (postfix), both of which detect PtdIns(3)P pools. As shown in Supplemental Figure S1D, these probes significantly

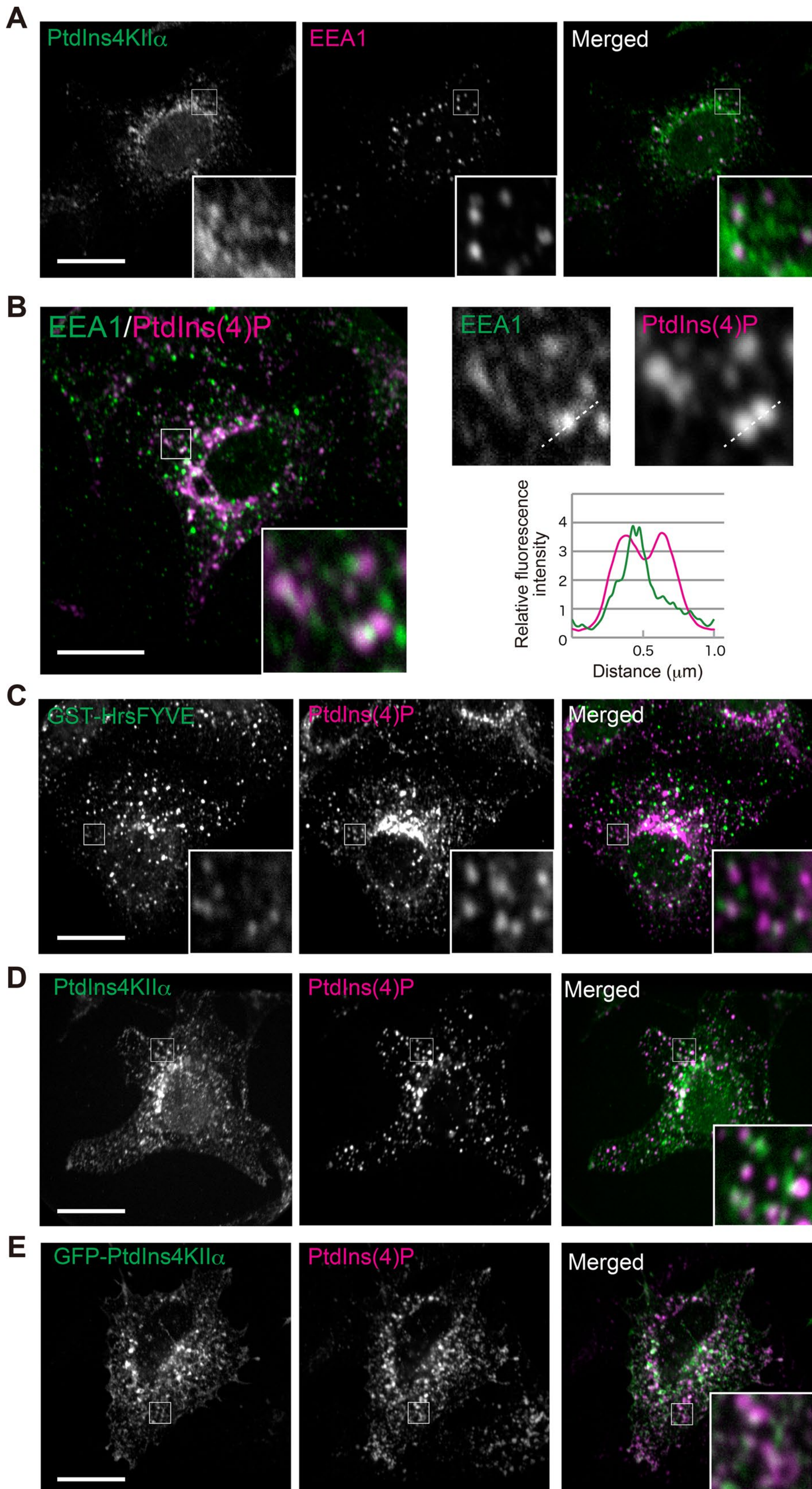

FIGURE 1: Both PtdIns4KIIα and PtdIns(4)P localize to early endosomes. HeLa cells were fixed and immunostained using (A) anti-PtdIns4KIIα (green) and anti-EEA1 (magenta), (B) anti-EEA1 (green) and PtdIns(4)P (magenta) or GST-HrsFYVE domain to detect PtdIns(3)P, and (C) further immunostained using anti-GST (green) and anti-PtdIns(4)P (magenta). In B, areas of colocalization and the segregation markers into domains are shown by intensity profiles. (D) HeLa cells were fixed and immunostained using anti-PtdIns4KIIα (green) to detect endogenous PtdIns4KIIα and anti-PtdIns(4)P (magenta) or (E) cells were transfected with GFP-PtdIns4KIIα and counterstained using anti-PtdIns(4)P (magenta).

overlapped, suggesting that the difference between the PtdIns(4)P antibody and PH probes does not result from processing for fixation; instead, these probes detect different pools of intracellular PtdIns(4)P.

We next performed coimmunostaining using anti-PtdIns(4)P and anti-EEA1. As shown in Figure 1B, PtdIns(4)P was found mainly in the juxtanuclear Golgi region, but there were distinct regions where PtdIns(4)P colocalized with EEA1 staining. Of interest, although PtdIns(4)P and EEA1 were observed on the same punctae, the staining only partially colocalized. Areas of colocalization of PtdIns(4)P and EEA1 are shown by line intensity profiles in Figure 1B. As shown in Figure 1C, similar results were obtained with GST-HrsFYVE, which specifically binds to PtdIns(3)P (Raiborg *et al.*, 2006; Hammond *et al.*, 2009). Given that EEA1 also binds to PtdIns(3)P and is recruited onto PtdIns(3)P-positive membrane domains (Simonsen *et al.*, 1998), these results indicate that some PtdIns(4)P is localized on early endosomes but at sites distinct from PtdIns(3)P-positive membrane domains. The latter are required for fusion with endocytic vesicles and the sorting of molecules to degradative compartments (Gruenberg, 2001; Miaczynska and Zerial, 2002). The polarization of these markers on early endosomes is significant because such domains have been extensively described in endosomal sorting compartments (de Renzis *et al.*, 2002; Peden *et al.*, 2004; Cullen and Korswagen, 2011).

PtdIns4KIIα partly localized to early endosomes (Figure 1A), as reported previously (Balla *et al.*, 2002; Waugh *et al.*, 2003; Minogue *et al.*, 2006). Next we investigated whether PtdIns4KIIα colocalizes with PtdIns(4)P on endosomes. As shown in Figure 1D, endogenous PtdIns4KIIα colocalized with endosomal PtdIns(4)P on distinct punctate structures. Again colocalization was partial, an observation that can be explained by the fact that much cellular PtdIns4KIIα is in a low-activity state (Waugh *et al.*, 2011), and so the enzyme would not be expected to localize completely with its product. Ectopically expressed GFP-PtdIns4KIIα also colocalized with endosomal

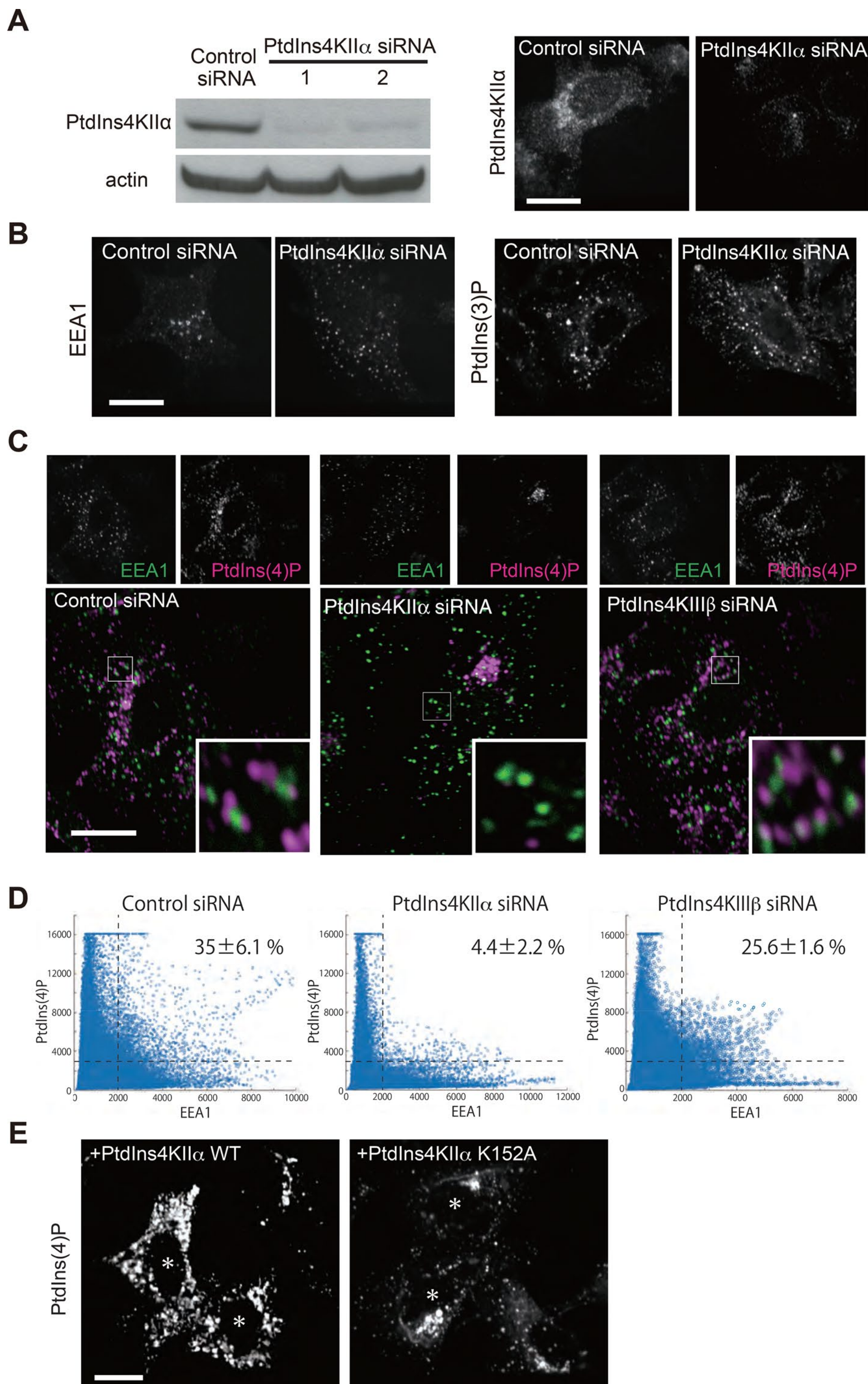

FIGURE 2: PtdIns4KIIα produces endosomal PtdIns(4)P. HeLa cells were transfected with control or PtdIns4KIIα-specific siRNAs. After 48 h, cells were lysed and processed for Western blot analysis. Alternatively, they were fixed and stained with (A) anti-PtdIns4KIIα antibody or (B) anti-EEA1 or anti-PtdIns(3)P. (C) To observe the effect on the distribution of PtdIns(4)P, cells transfected with control, PtdIns4KIIα, or PtdIns4KIIIβ siRNA were stained with anti-EEA1 and anti-PtdIns(4)P. (D) The intensity of signals is shown in a scatterplot measured from the average and SD from three to five cells. (E) siRNA-treated cells were transfected with siRNA-resistant GFP-PtdIns4KIIα WT or K152A, fixed, and stained with anti-PtdIns(4)P antibody. Asterisks indicate cells with GFP signals.

PtdIns(4)P (Figure 1E). Together these results clearly indicate that early endosomes contain PtdIns(4)P and suggest that PtdIns4KIIα is a candidate for the production of endosomal PtdIns(4)P.

PtdIns4KIIα is responsible for the production of PtdIns(4)P on early endosomes

We investigated whether PtdIns4KIIα is responsible for the synthesis of PtdIns(4)P on early endosomes. PtdIns4KIIα was depleted by siRNAs, and the reduction in protein level was confirmed by both Western blotting and immunofluorescence staining with an anti-PtdIns4KIIα antibody (Figure 2A). As reported previously (Minogue *et al.*, 2006; Craige *et al.*, 2008), early endosomes were dispersed around the cytoplasm in PtdIns4KIIα-depleted cells, as judged by EEA1 staining (Figure 2B). A similar effect was observed on the distribution of PtdIns(3)P-positive punctae, and no significant reduction in fluorescence signal was observed. Of interest, Golgi structure was slightly affected, suggesting that microtubule-dependent transport was impaired in PtdIns4KIIα-depleted cells (Supplemental Figure S8). However, microtubules in PtdIns4KIIα siRNA cells maintained their characteristic radial structure. These data suggest that some motor proteins on microtubules, such as dynein, may depend on PtdIns(4)P (Niu *et al.*, 2013).

Next we investigated the localization of endosomal PtdIns(4)P by coimmunostaining with anti-PtdIns(4)P and anti-EEA1 antibodies. As shown in Figure 2C, endosomal PtdIns(4)P was dramatically reduced in PtdIns4KIIα-depleted cells. Of interest, the PtdIns(4)P signals in the juxtanuclear Golgi region were still present in the PtdIns4KIIα-depleted cells, confirming that the bulk of Golgi-localized PtdIns(4)P was produced by a different PtdIns4K isoform, such as PtdIns4KIIIβ (Balla *et al.*, 2005). Indeed, in PtdIns4KIIIβ-depleted cells, the Golgi PtdIns(4)P signal was lost, whereas endosomal PtdIns(4)P was unaffected (Figure 2C and Supplemental Figure S2A). Quantitative analysis clearly shows that the signals having both EEA1 and PtdIns(4)P were specifically reduced in PtdIns4KIIα-depleted cells but not in PtdIns4KIIIβ-depleted cells (Figure 2D), whereas the PtdIns(4)P signals without EEA1 were reduced in PtdIns4KIIIβ. These results suggest that PtdIns(4)P signals located on early endosomes depend on PtdIns4KIIα. These results clearly demonstrate that PtdIns4KIIα depletion specifically affects endosomal PtdIns(4)P but not the major Golgi PtdIns(4)P signal, which requires the exclusively Golgi-localized PtdIns4KIIIβ isoform.

We also investigated AP-1, a clathrin adaptor whose localization depends on PtdIns(4)P (Wang *et al.*, 2003). In control cells, AP-1 displayed characteristic juxtanuclear staining corresponding to the TGN (Supplemental Figure S2B). This

staining was completely absent in PtdIns4KIIIβ-depleted cells; however, in PtdIns4KIIα-depleted cells, strong AP-1 staining was observed, indicating that the localization of the adaptor was unaffected. Although partial fragmentation was observed in some PtdIns4KIIα-depleted cells, these results indicate that the PtdIns(4)P production required for AP-1 recruitment at the TGN depends on PtdIns4KIIIβ but not PtdIns4KIIα. On the other hand, PtdIns(4)P production on endosomes depends solely on PtdIns4KIIα and not PtdIns4KIIIβ.

To confirm that PtdIns4KIIα directly produces PtdIns(4)P via its enzymatic activity, we expressed exogenous GFP-PtdIns4KIIα in PtdIns4KIIα-depleted cells. As shown in Figure 2E, expression of wild-type GFP-PtdIns4KIIα resulted in massive accumulation of vesicular PtdIns(4)P. This accumulation of PtdIns(4)P depended on its kinase activity, as the kinase-dead mutant (K152A) did not elevate levels of PtdIns(4)P. Of interest, these increased signals were not detected as efficiently by GST-PH domain–based PtdIns(4)P probes (only OSBP-PH detected a slight increase in endosomal PtdIns(4)P; Supplemental Figure S2C). This result underscores our finding that PH domain–based PtdIns(4)P probes can detect only subsets of PtdIns(4)P and that this anti-PtdIns(4)P antibody recognizes different pools in an unbiased manner, permitting the detection of PI(4)P derived from endosomal PtdIns4KIIα.

PtdIns4KIIα is required for sorting at early endosomes

Early endosomes sort molecules that are destined for recycling or degradation (Scott *et al.*, 2014). Impairment of sorting using several inhibitors results in altered distribution and motility of early endosomes (Mesaki, Tanabe, *et al.*, 2011; Ohashi, Tanabe, *et al.*, 2011). PtdIns4KIIα-depleted cells display both mislocalization of EGF-containing endosomes and impaired EGFR degradation (Minogue *et al.*, 2006). Therefore, based on this prior work and the data presented here earlier, we asked whether PtdIns(4)P and PtdIns4KIIα were indispensable for receptor sorting at early endosomes.

First, we investigated the efficiency of sorting from early endosomes in PtdIns4KIIα-knockdown cells. Transferrin (Tfn) and EGF were internalized into the siRNA-treated cells, and after 10 min, no significant alteration was observed among control, PtdIns4KIIα siRNA, and PtdIns4KIIIβ siRNA cells (Figure 3, A and B), indicating that PtdIns4KIIα is dispensable for endocytic uptake of the ligands. At 60 min, control and PtdIns4KIIIβ siRNA-treated cells showed distinct segregation of Tfn and EGF, indicating that normal endosomal sorting had occurred (Figure 3, A and B). On the other hand, PtdIns4KIIα-depleted cells still displayed significant colocalization of Tfn and EGF on endosomes at 30 min after internalization. Tfn also colocalized with EEA1 at 30 and 60 min, indicating that the sorting and exit from early endosomes was impaired (Figure 3C). These results indicate that PtdIns4KIIα is required for proper receptor sorting at early endosomes. We reported that spatial segregation of molecules into distinct subdomains of endosomal membranes is required for the motility of endosomes (Mesaki, Tanabe, *et al.*, 2011). Thus PtdIns4KIIα and its product PtdIns(4)P might be required for the segregation of molecules within early endosomal membranes and, subsequently, for endosomal motility toward the perinuclear region (Minogue *et al.*, 2006). Of interest, other marker/ligands transported from early endosomes, dextran and cholera toxin B subunit, were not affected by PtdIns4KIIα siRNA (Supplementary Figure S3), indicating that PtdIns4KIIα and PtdIns(4)P would be required for specific transport from early endosomes.

Next we analyzed the localization of PtdIns(4)P in cells containing internalized transferrin and found that PtdIns(4)P partially colocalized with transferrin-containing tubules (Figure 3D). Moreover, GFP-PtdIns4KIIα was observed on transferrin-containing tubules, using live-cell imaging (Figure 3E and Supplemental Movie S1). Of interest, in live time-series experiments, transferrin-containing tubules became severed at GFP-PtdIns4KIIα–positive domains (Figure 3E, bottom, and Supplemental Movie S1, white boxes). This strongly supports the hypothesis that PtdIns(4)P and PtdIns4KIIα are associated with receptor sorting at early endosomes.

Endosomal tubules require PtdIns4KIIα

These data suggest that both PtdIns(4)P and PtdIns4KIIα play an important role in receptor sorting at early endosomes. PIs often function as a platform for the recruitment of membrane trafficking machinery; for example, PtdIns(4,5)P_2 at the plasma membrane recruits AP-2, dynamin, and other proteins (Balla, 2013). We therefore considered that endosomal PtdIns(4)P may recruit effectors for membrane traffic to early endosomes. The EHD proteins comprise a family of dynamin-like, PtdIns(4)P-binding proteins known to regulate endocytic events (Naslavsky and Caplan, 2011). We therefore focused on EHDs as potential PtdIns(4)P effectors operating downstream of PtdIns4KIIα activity in early endosomal sorting. EHDs contain four members: EHD1 localizes to recycling endosomes, EHD2 on plasma membrane, and EHD3 and EHD4 on early endosomes (Naslavsky and Caplan, 2011). Of these, EHD1 and EHD4 were previously localized to PtdIns(4)P-containing membranes (Jović *et al.*, 2009). It has been reported that EHD3 is required for traffic from early endosomes (Naslavsky *et al.*, 2006). EHDs have the ability to generate lipid tubules in vitro and are therefore good candidate PtdIns(4)P effectors that may play a role in the formation of a vesicular and tubular sorting network at early endosomes. Indeed, when we compared their localization with PtdIns(4)P or PtdIns4KIIα, EHD1, EHD3, and EHD4 showed partial but significant colocalization (Figure 4, A and B, and Supplemental Figure S4, A and B). Of these, we focused on EHD3, as their localization was affected by the depletion of PtdIns4KIIα, as described later.

GFP-EHD3 showed extensive tubular localization in control cells, consistent with a previous study (Galperin *et al.*, 2002), and also colocalized with PtdIns(4)P and PtdIns4KIIα. We then investigated the effect of PtdIns4KIIα depletion on EHD3-containing tubules. In PtdIns4KIIα-depleted cells, EHD3-containing tubules were rarely seen, and the frequency of short tubules was increased (Figure 4, C and E). These effects were not observed upon depletion of PtdIns4KIIIβ (Figure 4C, right). The results imply that tubulation of EHD3-containing structures is under the control of PtdIns4KIIα, probably through the generation of endosomal PtdIns(4)P. Next we investigated whether tubule formation depended on the kinase activity of PtdIns4KIIα by reexpressing wild-type or mutant forms of the protein in siRNA-treated cells. Surprisingly, however, these tubules were rescued by the expression of kinase-dead mutant of PtdIns4KIIα (K152A; Figure 4D), whereas expression of wild-type PtdIns4KIIα induced large, globular, EHD3-containing vesicles. These results indicate that PtdIns4KIIα plays a role in the formation of EHD3-containing tubules independently of its kinase activity. Alternatively, the increased PtdIns(4)P produced by overexpressed PtdIns4KIIα (Figure 2E) may lead to vesicularization, perhaps through increased EHD3 tubule-severing activity. The increased colocalization of PtdIns4KIIα K152A and EHD3 also supports this hypothesis (Figure 4D). As reported earlier, depletion of EHD3 by siRNA resulted in inhibition of endosomal transport (Galperin *et al.*, 2002; George *et al.*, 2011), although significant inhibition was not observed under our experimental conditions (Supplemental Figure S5). It remains a possibility that other EHDs, such as EHD1 and EHD4, may be able to compensate for the loss of EHD3.

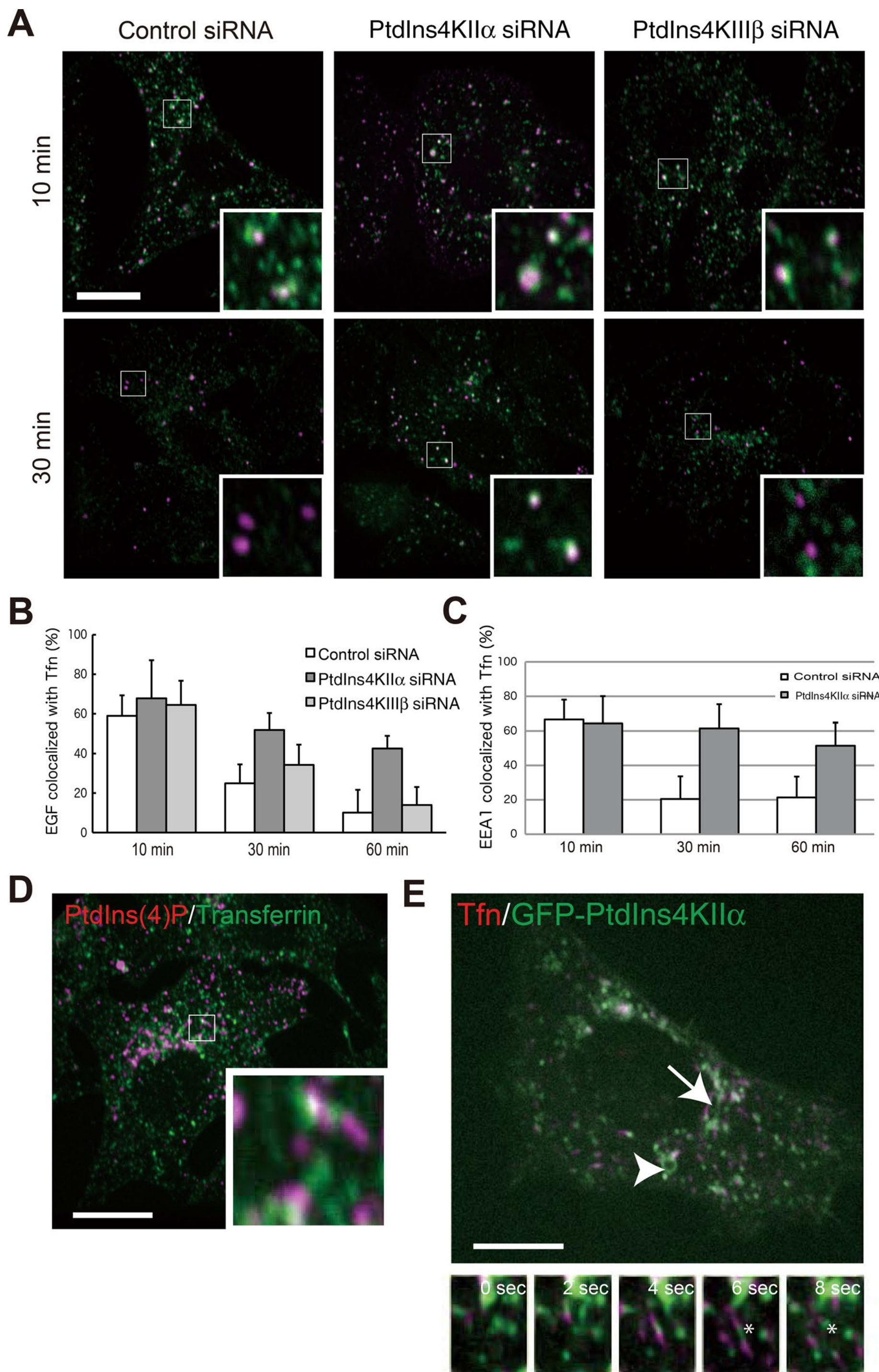

FIGURE 3: PtdIns4KIIα is required for receptor sorting at early endosomes. HeLa cells were transfected with control or PtdIns4KIIα-specific siRNAs. (A) Cells were labeled with Alexa Fluor 488–Tfn (green) and Alexa Fluor 555–EGF (magenta) on ice then washed and incubated at 37°C for 10 or 60 min. Colocalization of (B) EGF and Tfn or (C) Tfn and EEA1 was measured and calculated from 16 cells from two independent experiments. (D) Cells were labeled with Alexa Fluor 488–Tfn (green) for 15 min as in A then fixed and stained with anti-PtdIns(4)P antibody (magenta). (E) GFP-PtdIns4KIIα–transfected cells were labeled with Alexa Fluor 555–Tfn (magenta) for 10 min as in A and live images captured every 2 s. Arrow indicates endosomal tubules, which were visualized by Alexa Fluor 555–Tfn. Note that this tubule also contains GFP-PtdIns4KIIα. Arrowhead indicates large vacuolar structure, probably induced by overexpression of PtdIns4KIIα (Minogue *et al.*, 2006). Sequential images show the extension of tubular endosomes, followed by severing at GFP-PtdIns4KIIα–positive domain (indicated by asterisk).

Endosomal PtdIns(4,5)P_2 also requires PtdIns4KIIα

Another possible nonexclusive role of PtdIns(4)P in membrane traffic is as a substrate for production of other phospholipids. It has been reported that PtdIns(4)P acts at the plasma membrane and that PtdIns(4,5)P_2 is supplied via another source, such as endosomes (Hammond and Fischer, 2012). PtdIns(4,5)P_2 has been described on endosomal membranes, where it plays important roles in recycling pathways such as endosomal tubulation (Shinozaki-Narikawa *et al.*, 2006; Jović *et al.*, 2009; Vicinanza *et al.*, 2011). We therefore investigated whether PtdIns(4)P derived from PtdIns4KIIα contributes to the synthesis of endosomal PtdIns(4,5)P_2.

We used indirect immunofluorescence to detect endosomal PtdIns(4,5)P_2 signals (Supplemental Figure S6A), finding that endosomal PtdIns(4,5)P_2 did not colocalize with early endosomal markers EEA1 or PtdIns(3)P (Figure 5A), consistent with these being different endosomal compartments. Both EHD1 and EHD4 localized on PtdIns(4,5)P_2-positive membrane tubules (Supplemental Figure S6B), in line with a previous report (Jović *et al.*, 2009). Endosomal PtdIns(4,5)P_2 was enriched in domains adjacent to PtdIns(4)P staining and colocalized with the transferrin receptor (Figure 5, B and C). The close juxtaposition of PtdIns(4)P and PtdIns(4,5)P_2 suggested that endosomal PtdIns(4,5)P_2 might be the product of the sequential action of PtdIns4KIIα and a PtdIns(4)P5K. We therefore investigated whether the depletion of PtdIns4KIIα affected levels of vesicular PtdIns(4,5)P_2. The intensity of this punctate PtdIns(4,5)P_2 staining was significantly reduced in PtdIns4KIIα siRNA cells (Figure 5, D and E), indicating that endosomal PtdIns(4,5)P_2 is derived from endosomal PtdIns(4)P produced by PtdIns4KIIα and that the pools are metabolically linked.

Endosomal PtdIns(4,5)P_2 regulates traffic in the endosomal pathway (Vicinanza *et al.*, 2011). To assess the contribution by endosomal PtdIns(4,5)P_2 on transferrin recycling, we used 1-butanol as an indirect inhibitor of PtdIns(4)P5K acting through phosphatidic acid (Godi *et al.*, 1999). As shown in Supplemental Figure S7, A and B, 0.3% 1-butanol effectively reduced PtdIns(4,5)P_2 but not PtdIns(4)P. This experiment allowed us to specifically assess the role of PtdIns(4,5)P_2, but not of PtdIns(4)P, on endosomal traffic. We then examined the transport of transferrin from

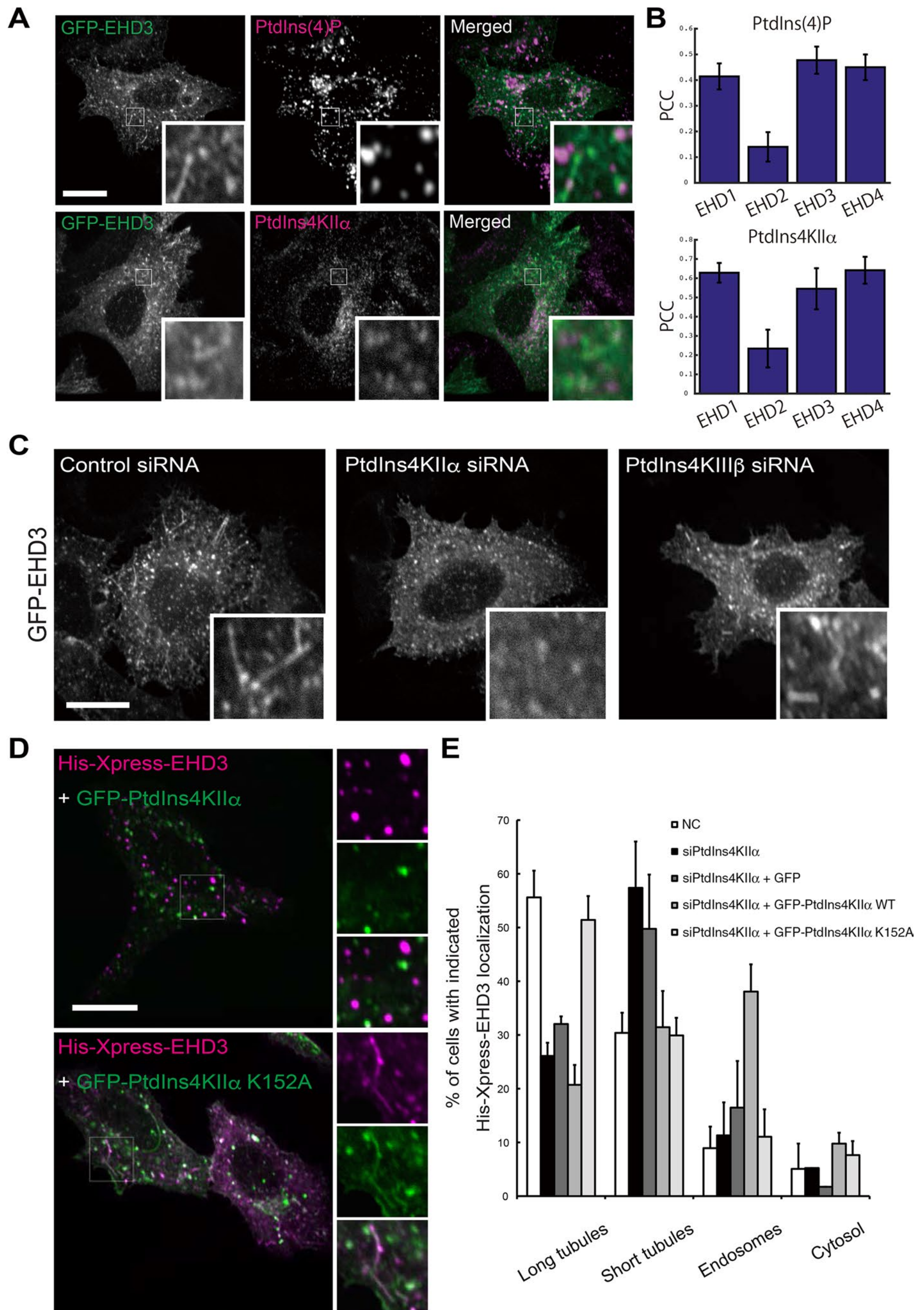

FIGURE 4: EHD3-containing endosomal tubules depend on PtdIns4KIIα. (A) HeLa cells were transfected with GFP-EHD3 (green), fixed, and immunostained with anti-PtdIns(4)P or anti-PtdIns4KIIα (magenta). (B) Pearson's correlation coefficients (PCCs) between GFP-EHDs and PtdIns(4)P or PtdIns4KIIα were calculated from four to seven cells. (C) siRNA-treated cells were transfected with GFP-EHD3 at 48 h after siRNA treatment, fixed, and observed 24 h later. Note that GFP-EHD3–positive endosomal tubules were observed in control and PtdIns4KIIIβ-siRNA cells but not in PtdIns4KIIα-depleted cells. (D) Cells were treated as in C, except that GFP-PtdIns4KIIα or its kinase-dead mutant (K152A) was cotransfected with His-Xpress-EHD3. (E) Approximately 50 cells were classified as long tubules (>2 µm), short tubules (<2 µm), endosomes (no tubules but punctate staining), or cytosol (no tubule/punctate staining). Counted from two independent experiments.

early endosomes and found that 0.3% 1-butanol has no effect on transferrin or EGF trafficking (Supplemental Figure S7C). This result indicates that PtdIns(4)P, but not PtdIns(4,5)P_2, is responsible for the impaired endosomal trafficking of EGF and transferrin cargo in PtdIns4KIIα siRNA cells.

DISCUSSION

This study demonstrates that PtdIns4KIIα produces PtdIns(4)P on early endosomes at a site distinct from the PtdIns(3)P-positive membrane domains. The depletion of PtdIns4KIIα by siRNA resulted in the impairment of receptor sorting from early endosomes, probably due to a lack of PtdIns(4)P produced on endosomal membranes.

PtdIns(4)P is primarily found at the TGN, where it is used by multiple pathways carrying cargo to and from the TGN, to endosomes, and to the PM. In the Golgi complex, PtdIns(4)P recruits numerous binding proteins to regulate membrane dynamics. These include AP-1, OSBP, phosphatidylinositol-four-P adaptor protein, and ceramide transporter (Graham and Burd, 2011). Outside of the Golgi, there is evidence for the existence of a quantitatively minor early endosomal pool of PtdIns(4,5)P_2 that contain the Tf receptor and other recycling markers (Vicinanza *et al.*, 2011). This barely detectable endosomal pool of PtdIns(4,5)P_2 is regulated by the PtdIns(4,5)P_2 5-phosphatase OCRL, which produces PtdIns(4)P, thereby implying a dynamic PtdIns(4)P/PtdIns(4,5)P_2 cycle on early endosomes. Such endosomal pools often elude detection using coincidence sensors biased toward recognition of Golgi pools of the lipid (Varnai and Balla, 2006). The present study demonstrates that these pools are functionally involved in receptor sorting at early endosomes.

How do PtdIns4KIIα and the PtdIns(4)P derived from its activity control endosomal sorting? Numerous endosomal trafficking events are dependent on PtdIns(4)P in the Golgi complex, but actin dynamics is also required for receptor sorting at early endosomes (Ohashi, Tanabe, *et al.*, 2011), suggesting that phosphoinositide metabolism could be required in a similar process during the formation and fission of endosomal trafficking intermediates. Sorting nexin-6 has been reported to bind to PtdIns(4)P and regulate retrograde delivery of cargo from endosomes to the TGN through dissociation of a dynein/dynactin motor complex (Niu *et al.*, 2013). Consistent with this, PtdIns4KIIα siRNA cells showed tat endosomes/Golgi complex dispersion without microtubule disorganization (Figure 2B and Supplemental Figure S8), suggesting that regulation by PtdIns(4)P may be a common mechanism of movement along microtubules. In addition, a recent study indicates that PtdIns4KIIα regulates endosomal traffic of transferrin through the interaction with VAMP3 (Jović *et al.*, 2014).

Another possibility is that PtdIns(4)P could be converted into PtdIns(4,5)P_2 at endosomes. Although our results show that 0.3%

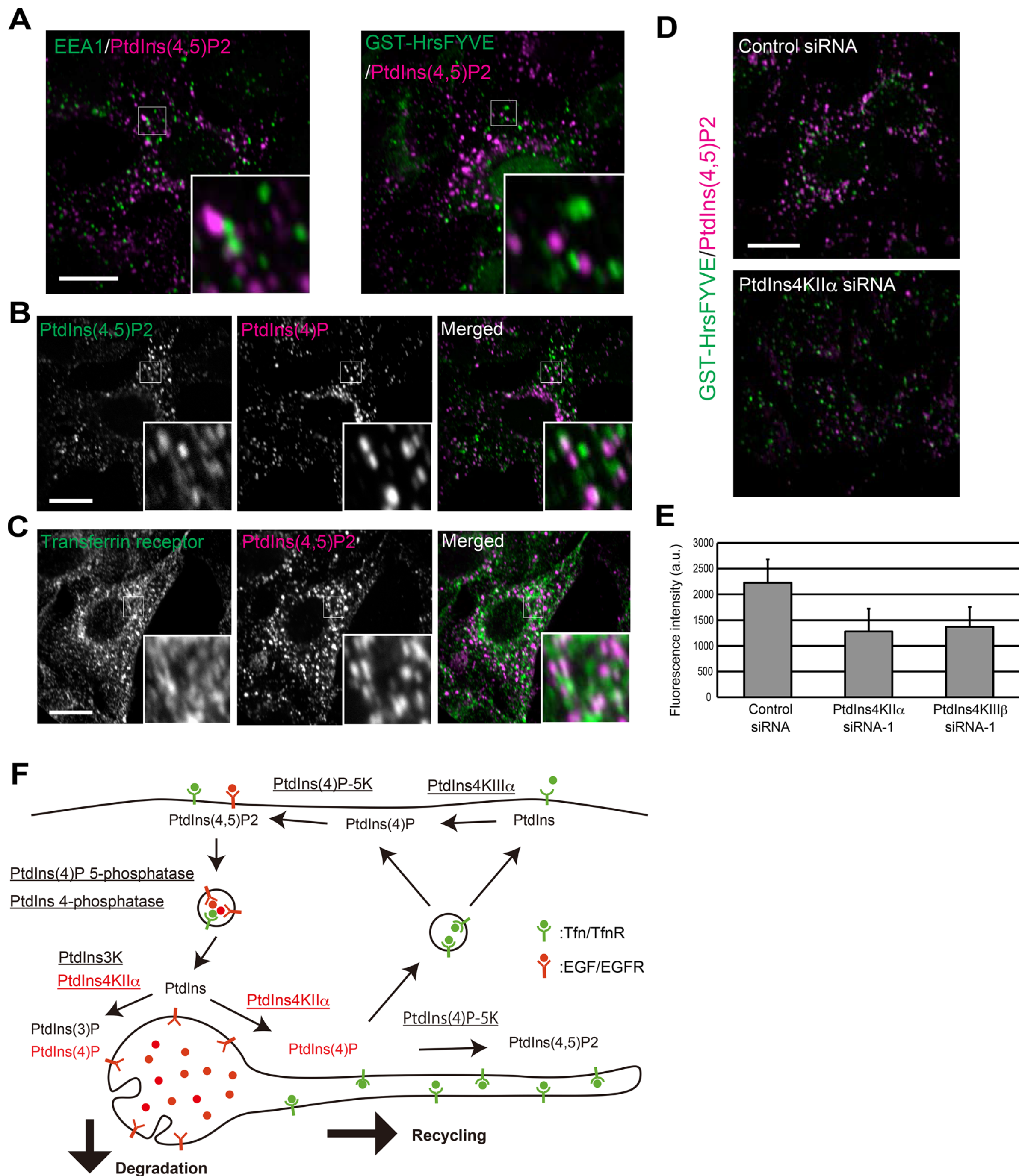

FIGURE 5: Endosomal PtdIns(4,5)P_2 requires endosomal PtdIns(4)P. (A) HeLa cells were immunostained with anti-EEA1 or GST-HrsFYVE, followed by anti-GST (green) and anti-PtdIns(4,5)P_2 (magenta). Cells were also immunostained with (B) anti-PtdIns(4,5)P_2 (green) and anti-PtdIns(4)P (magenta) or (C) anti-transferrin receptor (green) and anti-PtdIns(4,5)P_2 (magenta). (D) siRNA-treated cells were immunostained by GST-HrsFYVE, followed by anti-GST (green) and anti-PtdIns(4,5)P_2 (magenta). (E) The intensity of PtdIns(4,5)P_2 signals was measured from two independent experiments. (F) Compartmentalization of PtdIns(4)P metabolism and the proposed endosomal role of PtdIns4KIIα. PtdIns(4,5)P_2 required for receptor internalization at the plasma membrane is generated by PtdIns4KIIIα and type I PtdIns(4)P5K. PtdIns(4,5)P_2 is sequentially dephosphorylated by PtdIns(4)P 5-phosphatase and PtdIns 4-phosphatases. PtdIns-3-kinase phosphorylates endosomal PtdIns to generate PtdIns(3)P, which is required for the sorting of cargo into the degradative pathway. PtdIns4KIIα generates endosomal PtdIns(4)P (this study), which is potentially a substrate for endosomal PtdIns(4)P-5K activity. This PtdIns(4)P may therefore contribute to both recycling and degradative pathways through independent mechanisms or to indirect inhibition through inhibition of the recycling pathway (Mesaki, Tanabe, *et al.*, 2011).

1-butanol treatment, which depletes PtdIns(4,5)P_2, did not have a significant effect on transferrin recycling (Supplemental Figure S8), PtdIns(4,5)P_2 plays an essential role in early endosomal trafficking (Shinozaki-Narikawa *et al.*, 2006; Jović *et al.*, 2009). In support of this, evidence shows that PtdIns(4)P5KIγ is required for lysosomal sorting of EGFR (Sun *et al.*, 2013), and PtdIns(4)P produced by PtdIns4KIIα might be a substrate for this pathway. Excess production of endosomal PtdIns(4,5)P_2 impairs early endosomal transport (Vicinanza *et al.*, 2011), indicating that precise regulation of PtdIns(4,5)P_2 content is required for proper trafficking of endocytic recycling. Of course, there is a possibility that PtdIns(4)P or PtdIns(4,5)P_2 also regulates EHD3 activity through their direct interaction, as suggested in Figure 4B. The lipid regulation of EHD3 activity is under investigation in our laboratory. Here the normal tubular distribution of GFP-EHD3 was restored in siRNA-treated cells by the expression of a kinase-dead mutant of PtdIns4KIIα, indicating that kinase activity was not sufficient for rescue. Of interest, in a previous study of AP-3–dependent trafficking, inactive PtdIns4KIIα was unable to rescue endosomal trafficking defects, indicating that the enzyme contains noncatalytic determinants of endosomal traffic (Craige *et al.*, 2008). The vesicularization of GFP-EHD3–positive structures suggests that EHD3 localization is sensitive to the elevated levels of endosomal PtdIns(4)P that occur on overexpression of PtdIns4KIIα (Figure 2E). However, we could not link EHD3 tubulation to PtdIns4KIIα and PtdIns(4)P in this study, and further experiments will be needed to clarify the relationship between PtdIns4KIIα/PtdIns(4)P and EHD3.

Considerable effort has been directed toward the question of how the four PtdIns4K enzymes each contribute to the major subcellular PtdIns(4)P and PtdIns(4,5)P_2 pools and the extent to which these pools are metabolically separate from each other (Nakatsu *et al.*, 2012). Results are complicated by the finding that loss of PtdIns(4)P in one compartment can be rescued by membrane transport from another, suggesting redundancy at the level of the lipid rather than the activities that produce them. This model is supported by studies using acute organelle-specific depletion by PI-phosphatase recruitment (Szentpetery *et al.*, 2010; Niu *et al.*, 2013). Inhibition of type III isoforms with 10 µM wortmannin, phenylarsine oxide, or PIK93 can lead to the loss of up to 80% of total PtdIns(4)P, but under these conditions, a fraction of cytoplasmic vesicular PtdIns(4)P remains (Balla *et al.*, 2008). Together with work describing the existence of endosomal PtdIns(4)P and a PI cycle involving PtdInsP5KI and OCRL, the data here point to PI pools that function in cargo sorting from the early endosome into the degradative and recycling pathways. Consistent with this, we previously published evidence that PtdIns4KIIα is in a high-activity state in an endosomal sorting membrane that cofractionates with markers of the endosomal recycling compartment and TGN (Waugh *et al.*, 2011).

PtdIns4KIIα accounts for the majority of PtdIns(4)P-synthesizing activity in the brain (Guo *et al.*, 2003) and A431 cells (Waugh *et al.*, 2003). The present results demonstrate that depletion of PtdIns4KIIα did not inhibit the clathrin-mediated uptake of transferrin, indicating that PtdIns4KIIα does not contribute to the PtdIns(4,5)P_2 necessary for early endocytic traffic. The efficiency of EGF and transferrin uptake was not investigated here, but it is likely that other PtdIns4K isoforms may have contributed PtdIns(4)P necessary for PtdIns(4,5)P_2 synthesis at the plasma membrane (Hammond and Fischer, 2012; Nakatsu *et al.*, 2012). Our findings provides further evidence for a PtdIns4KIIα-derived pool of endosomal PtdIns(4)P, and our data support the idea that PtdIns(4)P derived from PtdIns4KIIα may be a nonredundant substrate for endosomal PtdIns(4,5)P_2. The role of PtdIns4KIIα in EGF and transferrin sorting is shown schematically in Figure 5F. During preparation of this article, two groups reported Sac2 as a specific PtdIns(4)P phosphatase located in endosomes that contributes to down-regulation of internalized PtdIns(4,5)P_2 (Hsu *et al.*, 2015; Nakatsu *et al.*, 2015). This dephosphorylation by Sac2 and phosphorylation by PtdIns4KIIα during endocytic traffic would be the next important question to be addressed. Our strategy in this study was based on a PtdIns(4)P-specific antibody and siRNA techniques, but recent reported tools such as novel PtdIns(4)P probes (Hammond *et al.*, 2014) and inhibitors (Bojjireddy *et al.*, 2014; Waring *et al.*, 2014) may provide further insight into the role of PtdIns4Ks and PtdIns(4)P.

MATERIALS AND METHODS

Cells, reagents, and antibodies

HeLa cells were obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). The cells were maintained in DMEM containing 10% fetal bovine serum (FBS) at 37°C under 5% CO_2. Mouse monoclonal anti-PtdIns(4)P antibody and mouse monoclonal anti-PtdIns(4,5)P_2 antibody were purchased from Echelon (Salt Lake City, UT). Mouse monoclonal anti-EEA1 antibody was purchased from BD Transduction Laboratories (San Diego, CA). Mouse monoclonal anti–transferrin receptor antibody was from Zymed (South San Francisco, CA), and rabbit monoclonal anti-EEA1 antibody was from Cell Signaling Technologies (Beverly, MA). Goat anti-GST antibody and rabbit anti-GST antibody were from Amersham (Piscataway, NJ) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Mouse monoclonal anti-PtdIns4KIIα antibody (1C4) has been previously described (Banerji *et al.*, 2010). Mouse anti-Xpress antibody, Alexa Fluor 488–conjugated Tfn, Alexa Fluor 555–conjugated EGF, Alexa Fluor 488–conjugated donkey anti-mouse immunoglobulin G (IgG), Rhodamine Red X–conjugated goat anti-mouse IgG, Alexa Fluor 555–conjugated goat anti-mouse IgM, Alexa Fluor 488–conjugated donkey anti-goat IgG, and Alexa Fluor 488–conjugated donkey anti-rabbit IgG were purchased from Invitrogen (Carlsbad, CA).

Plasmids

GFP-PtdIns4KIIα and its kinase-dead mutant, K152A, were previously described (Minogue *et al.*, 2006). The silent mutation in GFP-PtdIns4KIIα was generated with the QuikChange Lightning Site-Directed Mutagenesis Kits (Agilent, Santa Cruz, CA) using mutagenic primer (5′-CCTTCCAAAGATCAGCGACCCTAACTTCG-3′, 5′-CGAAGTTAGGGTCGCTGATCTTTGGAAGG-3′). GFP-OSBP-PH and GFP-FAPP1-PH were generous gifts from M. Satake (Tohoku University). eGFP-OSH2 PH was a generous gift from Gerry Hammond (National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD). pGEX-HrsFYVE and pVenus-HrsFYVE were generous gifts from T. Itoh (Kobe University, Kobe, Japan; Furutani *et al.*, 2006). HrsFYVE was subcloned into the pEGFP-C1 vector (Invitrogen). DNA was purified for transfection experiments by using a Plasmid Maxi Kit from Qiagen (Hilden, Germany).

Human EHD1, 2, and 4 and murine EHD3 cDNAs were obtained from the American Type Culture Collection (Manassas, VA), and EHD3 mutants were created by QuikChange Lightning Site-Directed Mutagenesis Kits using mutation primers (T94A: 5′-GATTGGGCCTGAGCCGACCGCTGATTCCTTCATAGCAGTG-3′, 5′-CACTGCTATGAAGGAATCAGCGGTCGGCTCAGGCCCAATC-3′; I157Q: 5′-GTGATCGACACACCGGGGCAGCTCTCTGGGGAGAAGCAG-3′, 5′-CTGCTTCTCCCCAGAGAGCTGCCCCGGTGTGTCGATCAC-3′;

K327D: 5′-GGGAAGGACACCGACAAGAAAGAACTGG-3′, 5′-CCAGTTCTTTCTTGTCGGTGTCCTTCCC-3′; and W485A: 5′-GCTGGGCAAGATCGCCAAGCTAGCCGAC-3′, 5′-GTCGGCTAGCTTGGCGATCTTGCCCAGC-3′). These cDNAs were subcloned into pEGFP-C1 and pcDNA6/His (Invitrogen).

Recombinant proteins

pGEX-HrsFYVE was transformed into BL21 (DE3), and recombinant protein was induced using 0.1 mM isopropyl-β-D-thiogalactoside for 1 h at 37°C. Cells were lysed by B-PER bacterial protein extraction reagent (Pierce, Rockford, IL) containing Complete protease inhibitor cocktail (Roche, Indianapolis, IN) for 30 min at 4°C. Cells were clarified by centrifugation (1 min at 15,000 × *g*) and the supernatants rocked with glutathione–Sepharose 4B (Amersham) for 1 h. Beads were collected by centrifugation (5 min at 600 × *g*) and then washed three times with B-PER. GST-HrsFYVE was eluted from the beads with B-PER containing 20 mM glutathione. Eluted proteins were frozen and used for experiments.

Preparation of liposomes and immunoabsorption

Lipids were purchased from Avanti Polar Lipids (Alabaster, AL). Sonicated liposomes were prepared as described in Blume *et al*. (2007). Briefly, liposomes were prepared from 70% phosphatidylcholine, 10% phosphatidylserine, 10% cholesterol, and 10% indicated phosphoinositides. These lipid mixtures were dried under nitrogen gas, hydrated in liposome buffer (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.5, 150 mM NaCl, 1 mM $MgCl_2$) to a final concentration of 1 mg/ml, mixed by vortexing for 1 min, and then sonicated. For immunoabsorption, anti-PtdIns(4)P or anti-PtdIns(4,5)P_2 was preincubated with 10 µg/ml liposomes containing PtdIns, PtdIns(3)P, PtdIns(4)P, or PtdIns(4,5)P_2 for 30 min. Liposomes were removed by ultracentrifugation at 108,000 × *g* for 30 min, and supernatants were processed for immunofluorescence study.

RNA interference

PtdIns4KIIα siRNAs (siRNA PtdIns4KIIα-1: 5′-CCAAAGAUAUCGGACCCUAtt-3′; and siRNA PtdIns4KIIα-2: 5′-GCAUCGGGCUACCACCAAAtt-3′) and negative control siRNA were purchased from Ambion (Austin, TX). HeLa cells plated in 35-mm-diameter dishes were transfected with each duplex siRNA (100 pmol) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The cells were analyzed 48–72 h after transfection.

Tfn and EGF internalization and immunofluorescence

HeLa cells cultured on coverslips were starved with serum-free medium containing 0.1% bovine serum albumin (BSA)/DMEM for 1 h. For Tfn and EGF internalization, the medium was replaced with serum-free DMEM containing Alexa Fluor 488– or Alexa Fluor 555–conjugated Tfn and/or Alexa Fluor 555–conjugated EGF (Invitrogen). Cells were incubated for 1 h on ice, washed with cold phosphate-buffered saline (PBS), and incubated with 10% FBS/DMEM at 37°C. The treated cells were then fixed with 3.7% formaldehyde for 15 min at room temperature, and immunofluorescence was performed as described previously (Tanabe and Takei, 2009). In the case of the anti-PtdIns4KIIα antibody, cells were permeabilized with 0.05% Triton X-100 for 10 min on ice, followed by standard immunostaining procedures. Staining of PtdIns(4)P, PtdIns(3)P, and PtdIns(4,5)P_2 was performed using anti-PtdIns(4)P antibody, GST-HrsFYVE, and anti-PtdIns(4,5)P_2 antibody, respectively, according to the Golgi staining method (Hammond *et al*., 2009) with slight modification. Cells were fixed with 2% paraformaldehyde for 15 min at room temperature, washed twice with PBS, and permeabilized with 20 µM digitonin for 5 min at room temperature. Cells were then washed once with PBS and blocked by incubating with 1% BSA in PBS (blocking buffer) for 1 h at room temperature. When staining for PtdIns(3)P, 0.5 µg/ml GST-HrsFYVE was added in the blocking buffer. After removal of GST-HrsFYVE by washing twice with PBS, cells were incubated with primary and secondary antibodies, followed by fixation with 2% paraformaldehyde for 2 min at room temperature. Cells were washed three times with PBS and mounted using Prolong Gold (Invitrogen). Image analysis was performed using ImageJ (National Institutes of Health, Bethesda, MD) and Matlab (MathWorks, Natick, MA). Pearson's *r* was calculated from the area of GFP-expressing cells using ImageJ coloc2 plug-in and plotted using Matlab.

Live imaging

Live imaging was performed as previously described (Mesaki, Tanabe, *et al*., 2011; Ohashi, Tanabe, *et al*., 2011). HeLa cells were plated on 35-mm-diameter dishes with glass bases (IWAKI, Tokyo, Japan). Tfn and EGF were internalized into the cells as described for the immunofluorescence experiments. Time-lapse images were taken using a confocal microscope as previously described (Tanabe and Takei, 2009) and acquired with pinholes set to one arbitrary unit every 2 s.

ACKNOWLEDGMENTS

We thank M. Satake and S. Kon (both of Tohoku University, Sendai, Japan) and T. Itoh (Kobe University, Kobe, Japan) for providing materials. This work was supported by grants from the Ministry of Education, Science, Sports, Culture and Technology of Japan (23770148 and 23113721) to K.T., JSPS Research Fellowships for Young Scientists to Y.H., and Biotechnology and Biological Sciences Research Council grants (BB/G021163/1 and BB/1007806/1) to S.M. Y. H. is a Research Fellow of the Japan Society for the Promotion of Science.

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