Eps15 Homology Domain 1-associated Tubules Contain Phosphatidylinositol-4-Phosphate and Phosphatidylinositol-(4,5)-Bisphosphate and Are Required for Efficient Recycling

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The C-terminal Eps15 homology domain (EHD) 1/receptor-mediated endocytosis-1 protein regulates recycling of proteins and lipids from the recycling compartment to the plasma membrane. Recent studies have provided insight into the mode by which EHD1-associated tubular membranes are generated and the mechanisms by which EHD1 functions. Despite these advances, the physiological function of these striking EHD1-associated tubular membranes remains unknown. Nuclear magnetic resonance spectroscopy demonstrated that the Eps15 homology (EH) domain of EHD1 binds to phosphoinositides, including phosphatidylinositol-4-phosphate. Herein, we identify phosphatidylinositol-4-phosphate as an essential component of EHD1-associated tubules *in vivo*. Indeed, an EHD1 EH domain mutant (K483E) that associates exclusively with punctate membranes displayed decreased binding to phosphatidylinositol-4-phosphate and other phosphoinositides. Moreover, we provide evidence that although the tubular membranes to which EHD1 associates may be stabilized and/or enhanced by EHD1 expression, these membranes are, at least in part, pre-existing structures. Finally, to underscore the function of EHD1-containing tubules *in vivo*, we used a small interfering RNA (siRNA)/rescue assay. On transfection, wild-type, tubule-associated, siRNA-resistant EHD1 rescued transferrin and β 1 integrin recycling defects observed in EHD1-depleted cells, whereas expression of the EHD1 K483E mutant did not. We propose that phosphatidylinositol-4-phosphate is an essential component of EHD1-associated tubules that also contain phosphatidylinositol-(4,5)-bisphosphate and that these structures are required for efficient recycling to the plasma membrane.

INTRODUCTION

Internalization of proteins and lipids at the eukaryotic cell surface is a highly regulated event essential to numerous cellular processes (Conner and Schmid, 2003). Plasma membrane proteins may be internalized via clathrin-coated pits or independently of clathrin. Internalization of surface proteins or lipids marks their entry into the endocytic pathway, where they may undergo several potential fates. Although some internalized proteins are destined for degradation via the lysosomal pathway, many proteins are destined for delivery back to the plasma membrane through one of the endocytic recycling pathways (Maxfield and McGraw, 2004). Once internalized, proteins and lipids are delivered to a sorting compartment referred to as the early endosome (EE). Subsequently, some proteins are trafficked out of the EE directly back to the plasma membrane in a “fast” or “bulk” recycling pathway, whereas other proteins destined for the

plasma membrane recycle in a highly regulated manner through a transitory endocytic recycling compartment (ERC) in a process known as “slow recycling” (Gruenberg and Maxfield, 1995; Maxfield and McGraw, 2004). The ERC is a morphologically and functionally distinct perinuclear compartment characterized by a collection of tubular membrane structures radiating from the microtubule-organizing center. Tubulation of endocytic membranes at various compartments, including the ERC, is an efficient mechanism of achieving a high ratio of membrane surface to luminal volume. Effectively, this may serve to concentrate cargo on the recycling membranes.

Coordinated and efficient transport of recycling proteins along the recycling pathway necessitates a high level of regulation. The Rab guanosine triphosphate (GTP)-binding proteins, particularly Rab4 and Rab11, play a critical role in this regulation (van der Sluijs *et al.*, 1992; Ullrich *et al.*, 1996). Although the role of Rab4 has been attributed primarily to the fast-recycling events initiated at the EE (Daro *et al.*, 1996), Rab11 mediates the exit of slow-recycling cargo out of the ERC (Ullrich *et al.*, 1996; Sheff *et al.*, 1999; Sonnichsen *et al.*, 2000). Recent studies have begun to focus on the function of Rab effector proteins, which (in most cases) bind to membrane-associated Rabs and further coordinate Rab activities with additional endocytic regulatory proteins (Hales *et al.*, 2001).

The C-terminal Eps15 homology domain (EHD)-containing proteins are a family of proteins that also carry out endocytic regulatory activity at the EE and the ERC (re-

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Abbreviations used: EE, early endosome; EHD, Eps15 homology domain; ERC, endocytic recycling compartment; PtdIns4P, phosphatidylinositol-4-phosphate; PtdIns(4,5)P₂, phosphatidylinositol-(4,5)-bisphosphate; Tf, transferrin.

viewed in Naslavsky and Caplan, 2005; Grant and Caplan, 2008). Mammalian cells express four EHD paralogs: EHD1, EHD2, EHD3, and EHD4. A genetic screen of *Caenorhabditis elegans* endocytic mutants initially identified receptor-mediated endocytosis (RME)-1, the only EHD worm orthologue, as an important regulator of yolk receptor recycling (Grant *et al.*, 2001). Similarly, EHD1, the best characterized human EHD protein, has been implicated in mediating recycling of various molecules, including the transferrin (Tf) receptor (Lin *et al.*, 2001), major histocompatibility complex (MHC) class I (Caplan *et al.*, 2002), the insulin responsive-glucose transporter GLUT4 (Guilherme *et al.*, 2004), cystic fibrosis transmembrane conductance regulator (Picciano *et al.*, 2003), α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid long-term potentiation receptor (Park *et al.*, 2004), and β 1 integrin receptors (Jovic *et al.*, 2007).

EHD proteins contain an N-terminal G domain involved in ATP binding (Lee *et al.*, 2005; Naslavsky *et al.*, 2006; Daumke *et al.*, 2007) and a central helical domain that generates a lipid binding surface (Daumke *et al.*, 2007). The C-terminal region is characterized by an EH domain, composed of a pair of EF-Hand helix-loop-helix motifs linked by a short antiparallel β -sheet (Daumke *et al.*, 2007; Kieken *et al.*, 2007). EH domains contain a hydrophobic pocket that mediates binding to proteins containing an asparagine-proline-phenylalanine (NPF) tripeptide motif (de Beer *et al.*, 1998; Daumke *et al.*, 2007; Kieken *et al.*, 2007). Recent studies have demonstrated that EHD proteins associate indirectly with Rab proteins, via EH domain interactions with Rab effectors containing NPF motifs and that these interactions facilitate the recycling of internalized receptors (Naslavsky *et al.*, 2004, 2006).

One of the hallmarks of EHD1 is its distinctive localization to the cytosolic face of tubular and vesicular structures emanating from the perinuclear ERC (Caplan *et al.*, 2002). Previous immunoelectron microscopy studies characterized these tubules as membrane-bound organelles of up to 200 nm in diameter and up to 10 μ m in length (Caplan *et al.*, 2002). Among the requirements for the generation/maintenance of EHD1-associated tubular membranes are an intact microtubule system (Caplan *et al.*, 2002), ATPase activity (Lee *et al.*, 2005; Daumke *et al.*, 2007), and EHD oligomerization (Naslavsky *et al.*, 2006). Additionally, we have shown that truncation of the C-terminal EH domain leads to a redistribution of EHD1 from tubular to vesicular membranes (Caplan *et al.*, 2002). Although the rationale for altered EHD1 localization has proved difficult to understand, our recent finding that EH domains are capable of interacting with phosphoinositides has provided an important clue to this puzzle, suggesting that the EHD1 EH domain mediates binding to specific phosphoinositides required for the generation and/or maintenance of EHD1-associated tubular membranes. Indeed, our recent nuclear magnetic resonance (NMR) solution structure of the EHD1 EH domain allowed us to postulate that lysine 483 serves as an important residue in phosphoinositide association (Naslavsky *et al.*, 2007). In support of this, a charge-reversal mutation of this residue to glutamate (K483E) caused the conversion of EHD1-associated tubular membranes to vesicular structures, similar to the effect of truncating the entire 100 amino acid EH domain (Naslavsky *et al.*, 2007).

Despite the uniqueness of the EHD1-associated tubular membranes, it has been extremely difficult to determine their physiological significance in mammalian cells. Indeed, although we have provided evidence for EH domain interactions with a variety of phosphoinositides, these studies

were performed in vitro, and little is known about the phosphoinositide components of EHD1-tubules in vivo.

In this study, we now provide the first in vivo evidence detailing the phosphoinositide constitution of EHD1-associated tubular membranes, and we identify phosphatidylinositol-4-phosphate (PtdIns4P) as a key constituent that along with phosphatidylinositol-(4,5)-bisphosphate (PtdIns(4,5)P₂) comprises these structures. By taking advantage of the previously solved EHD1 EH domain structure (Kieken *et al.*, 2007), we have used the EHD1 K483E point mutant that exclusively localizes to vesicular membranes. We demonstrate that this mutation in the EH domain decreases the affinity of its interaction with PtdIns4P and other phosphoinositides. Finally, we show that expression of this mutant is insufficient to rescue the impaired Tf and β 1 integrin recycling phenotypes observed when EHD1 is depleted from cells. Our studies provide the first evidence for a specific function of EHD1-associated tubules in endocytic recycling.

MATERIALS AND METHODS

Recombinant DNA Constructs

Cloning of the full-length wild-type Myc-EHD1, Myc-EHD1 K483E, Myc-EHD2, and HA-EHD4 (HA) and green fluorescent protein (GFP)-EHD1 have been described previously (Caplan *et al.*, 2002; Naslavsky *et al.*, 2006; Sharma *et al.*, 2008). Silent-GFP-EHD1 and Silent-GFP-EHD1 K483E were created using the QuikChange kit (Stratagene, La Jolla, CA), generating cDNA mutations in the oligonucleotide binding region (base pairs 942–963). SacI was cloned from a human brain library (Marathon-Ready; BD Biosciences, San Jose, CA), sequenced and subcloned with 5' KpnI and 3' BamHI restriction sites into EGFP-C1 vector. Based on alignments with other phosphatases, we predicted and designed a mutation to create a catalytically inactive SacI phosphatase mutant by introducing a substitution at R395A by using the QuikChange kit. GFP-oxysterol-binding protein (OSBP)-pleckstrin homology (PH) and GFP-phospholipase C (PLC) δ 1-PH domain constructs were generously provided by Dr. T. Balla (National Institutes of Health, Bethesda, MD). Hemagglutinin-tagged OSBP-PH domain was created by subcloning into the pHA-cytomegalovirus vector (Clontech, Palo Alto, CA). Myc-type I phosphatidylinositol-4-phosphate 5-kinase (PIP5KI) γ , GFP-H-Ras (GFP fused to the double palmitoylated and farnesylated carboxy-terminal tail of H-Ras), HA-Arf6, HA-Arf6-Q67L, and Myc-Synaptojanin2 were kindly provided by Dr. J. Donaldson (National Institutes of Health), FLAG-phosphatase and tensin homologue deleted on chromosome 10 (PTEN) was a gift from Dr. G. Taylor (University of Nebraska Medical Center, Omaha, NE) and GFP-Oculocerebrorenal Syndrome of Lowe protein (OCRL) was kindly provided by Dr. R. C. Aguilar (Purdue University, West Lafayette, IN). Cherry-Rab8a was a gift from Dr. J. Goldenring (Vanderbilt University, Nashville, TN).

Gene Knockdown by RNA Interference

Oligonucleotide duplexes targeting human EHD1 (Dharmacon RNA Technologies, Lafayette, CO) were transfected using Oligofectamine (Invitrogen, Carlsbad, CA) as described previously (Naslavsky *et al.*, 2004).

Antibodies and Reagents

Affinity-purified rabbit polyclonal antibodies directed against human EHD1 and EHD4 were described previously (Sharma *et al.*, 2008). The following antibodies were also used: mouse anti-Myc 9E10 and anti-HA epitope monoclonal antibodies (Covance Research Products, Princeton, NJ), mouse anti- α tubulin antibody (Invitrogen), mouse monoclonal immunoglobulin (Ig) M antibody against PtdIns(4)P (Echelon Bioscience, Salt Lake City, UT), mouse anti- β 1 integrin antibody (Serotec, Oxford, United Kingdom), rabbit anti-Giantin (Abcam, Cambridge, MA), mouse anti-early endosome antigen (EEA) 1 (BD Biosciences, San Jose, CA), mouse anti-lysosomal-associated membrane protein 1 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), Cy3-conjugated anti-mouse and anti-rabbit IgG, Alexa Fluor 488-conjugated antibody to mouse and rabbit IgG, and transferrin-Alexa Fluor 568 (Invitrogen). Goat anti-mouse horseradish peroxidase (HRP) and donkey anti-rabbit HRP were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). D-myo-Phosphatidylinositol-4-phosphate [PtdIns(4)P] diC4, PtdIns(4,5)P₂ diC4, and PtdIns(3,5)P₂ diC8 were purchased from Echelon Bioscience and dissolved at a concentration of 1.8 mM in a 20 mM Tris-HCl solution as described in the methods for NMR Spectroscopy below.

Immunofluorescence and Uptake Assays

HeLa cells were grown on coverglasses, transfected with FuGENE-6 (Roche Diagnostics, Indianapolis, IN), and fixed with 4% (vol/vol) paraformaldehyde.

hyde in phosphate-buffered saline (PBS) as described previously (Caplan *et al.*, 2002). Fixed cells were incubated with appropriate primary antibodies prepared in the staining solution (0.2% saponin, wt/vol and 0.5%, wt/vol bovine serum albumin in PBS). After the washes in PBS, cells were incubated with the appropriate fluorochrome-conjugated secondary antibody mixture dissolved in the staining solution for 30 min at room temperature. Images were acquired using an LSM 5 Pascal confocal microscope (Carl Zeiss, Thornwood, NY) with a 63X 1.4 numerical aperture objective with appropriate filters. Tf uptake was studied by first starving the cells in DMEM lacking serum (but containing 0.5% bovine serum albumin) for 30 min and then applying a 5-min pulse of 1 μ g/ml transferrin-Alexa Fluor 568 (Tf-568; Invitrogen). Cells were either fixed and mounted as described above for image analysis or subjected to a 20-min chase in complete media (containing 10% fetal bovine serum [FBS]) at 37°C followed by fixation. β 1 Integrin uptake was performed by starving the cells for 1 h at 37°C in DMEM without serum, followed by a 1-h pulse with 5 μ g/ml antibody to human β 1 integrin at 37°C. Surface antibodies were then removed by an acid rinse (0.5% acetic acid and 0.5 M NaCl, pH 3.0) for 1 min. The chase was performed in complete media (containing 10% FBS) at 37°C for 2 h, with a subsequent additional acid rinse and fixation. Cells were then incubated with the appropriate primary and secondary antibodies before mounting.

Protein Expression and Purification

The EH domain of human EHD1 (residues 436–534) was subcloned into the bacterial expression vector pGEX-6P-2 (GE Healthcare, Chalfont St. Giles, Buckinghamshire, United Kingdom). After the transformation into BL21 competent bacterial cells (Promega, Madison, WI), glutathione transferase (GST)-EH domain fusion protein was purified by standard methods. 15 N labeling was performed as described previously (Duffy *et al.*, 2002), followed by GST cleavage from the EH domains with PreScission Protease (GE Healthcare) and concentration of the samples using a Centrplus YM-10 column (Millipore, Billerica, MA).

NMR Spectroscopy

15 N-labeled EH domains and PtdIns4P were dissolved in 20 mM perdeuterated Tris-HCl, pH 7.0 (Sigma-Aldrich, St. Louis, MO), 100 mM KCl, and 2 mM CaCl_2 . The concentration of 15 N-labeled EH domains was maintained at 50 μ M during the titration with PtdIns4P. Gradient-enhanced two-dimensional $^1\text{H}/^{15}\text{N}$ -heteronuclear single quantum correlation (HSQC) experiments were acquired at 25°C using an INOVA 600 MHz spectrometer (Varian, Palo Alto, CA) fitted with a cold probe. All of the NMR spectra were processed using NMRPipe (Delaglio *et al.*, 1995) and analyzed with NMRView (Johnson and Bevinis, 1994). Binding curves from $^1\text{H}/^{15}\text{N}$ -HSQC titration experiment were best fitted to a nonlinear regression of a one-site binding model using Prism software (GraphPad Software, San Diego, CA).

RESULTS

Association of EHD1 with Tubular Membranes Is Phosphoinositide Specific

Our previous studies have demonstrated that EHD1 localizes to tubular and punctate membranes emanating from the ERC (Caplan *et al.*, 2002). Recent *in vitro* studies suggest that EHD1 may be involved in membrane tubulation *in vivo* (Daumke *et al.*, 2007). Additionally, it was demonstrated that EHD1 forges a functional relationship with the small GTP-binding protein Arf6 (Caplan *et al.*, 2002). Indeed, the cycling of Arf6 between its GTP- and guanosine diphosphate-bound forms impacts the localization of EHD1 with tubular membranes, although the reason for this has remained enigmatic. As we have shown previously (Caplan *et al.*, 2002), coexpression of the GTP-locked Arf6-Q67L mutant with Myc-EHD1 led to altered EHD1 localization, loss of EHD1-associated tubular structures, and recruitment of EHD1 to enlarged Arf6 endosomes (Figure 1, A and B; compare double-transfected cells marked with stars to cells transfected with only EHD1 [dashed border]). Because activated Arf6 directly activates PIP5K1 γ , a kinase responsible for generation of PtdIns(4,5) P_2 by phosphorylation of PtdIns4P, we tested directly whether enhanced expression of PIP5K1 γ affected EHD1-associated tubular membranes. Indeed, similar to Arf6-Q67L, coexpression of Myc-PIP5K1 γ with GFP-EHD1 abolished the localization of EHD1 to tubular membranes (Figure 1, C and D; compare double-transfected cells designated with stars to cells

transfected with only EHD1 [dashed boundaries]). Furthermore, *endogenous* EHD1-associated tubules were rarely observed upon overexpression of Myc-PIP5K1 γ for 24 h or longer (Figure 1, E and F; cells with stars express Myc-PIP5K1 γ , whereas cells with dashed boundaries are untransfected). It is noteworthy that upon shorter expression times of the exogenous Myc-PIP5K1 γ (Figure 1, G–N), the kinase could be observed in partial colocalization with remaining EHD1 tubular membranes (Figure 1, G–J; see arrows). However, between 16 and 24 h of Myc-PIP5K1 γ expression, the EHD1 tubules were almost entirely disrupted (Figure 1, K–N and E and F). We therefore hypothesized that PtdIns4P might be a critical component of the EHD1-associated tubular structures.

PtdIns4P Is Required for the Maintenance and/or Generation of EHD1-associated Tubules

To determine whether the depletion of PtdIns4P negatively impacts the localization of EHD1 to tubular membranes, we overexpressed Sac1, a phosphatase that specifically hydrolyzes PtdIns4P to phosphatidylinositol (PtdIns), thus reducing its levels in membranes. Coexpression of GFP-Sac1 with Myc-EHD1 dramatically altered EHD1 localization and almost abolished EHD1-associated tubular membranes, although EHD1 remained associated with aberrant vesicular structures (Figure 2, A and B; compare double-transfected cells marked with stars to cells transfected with only EHD1 [dashed border]). To confirm the enzymatic activity of Sac1 *in vivo*, we cotransfected GFP-Sac1 with a known marker of PtdIns4P, the PH domain of the oxysterol-binding protein (HA-OSBP-PH) (see Supplemental Figure 1, A–C). Depletion of PtdIns4P by Sac1 overexpression induced the dissociation of HA-OSBP-PH from Golgi membranes normally enriched with PtdIns4P, causing its displacement to the cytosol (Supplemental Figure 1, A–C; cell with star). Moreover, decreased PtdIns4P levels could be observed with anti-PtdIns4P antibodies in cells overexpressing GFP-Sac1 (Supplemental Figure 1, G and H) as described previously (Blagoveshchenskaya *et al.*, 2008). As a control, we designed a catalytically inactive Sac1 phosphatase with a critical arginine residue mutated (GFP-Sac1 R395A). This mutant was incapable of hydrolyzing PtdIns4P and therefore did not displace coexpressed HA-OSBP-PH from its primarily Golgi localization (see Supplemental Figure 1, D–F; cells with stars denote double-transfected cells). When coexpressed with EHD1, the GFP-Sac1-R395A phosphatase-dead mutant had no effect on the localization of EHD1 to tubular structures (Figure 2, C and D).

The effect of Sac1-based PtdIns4P reduction on EHD1 association with tubules seemed to be specific. Although GFP-Sac1 overexpression led to altered Golgi morphology consistent with the findings of Blagoveshchenskaya *et al.* (2008) (Supplemental Figure 3, A and B), other endocytic organelles, including lysosomes (Supplemental Figure 3, C and D), early endosomes (Supplemental Figure 3, E and F), the actin cytoskeleton (Supplemental Figure 4, A and B), and microtubules (Supplemental Figure 4, C and D), either displayed no detectable differences or only minor changes (i.e., EEA1 occurred in slightly enlarged endosomes). Furthermore, expression of OCRL, an inositol 5-phosphatase that hydrolyzes PtdIns(4,5) P_2 to PtdIns4P (Suchy *et al.*, 1995), had no observable effect on EHD1-containing tubular membranes (Supplemental Figure 5, A–D) and did not seem to induce recruitment of EHD1 to the plasma membrane (unpublished observations). Synaptojanin2, an additional phosphatase capable of hydrolyzing PtdIns(4,5) P_2 (to PtdIns) (McPherson *et al.*, 1996; Nemoto *et al.*, 1997; Malecz *et al.*,

Figure 1. PIP5KI γ overexpression induces the loss of EHD1-associated tubules. (A and B) HeLa cells were transiently cotransfected with GTP-locked HA-Arf6-Q67L and Myc-EHD1 (stars denote cotransfected cells). Cells were fixed, permeabilized, and incubated with rabbit anti-HA antibody and mouse monoclonal antibody (mAb) to the Myc-epitope. Cells were then incubated with the appropriate Alexa Fluor 568-conjugated anti-mouse IgG and Alexa Fluor 488-conjugated anti-rabbit antibodies. (C–N) HeLa cells were transfected with both Myc-PIP5KI γ and GFP-EHD1 (C and D) or with only Myc-PIP5KI γ (E–N). Cells were fixed after 8 h (G and H), 12 h (I and J), 16 h (K and L), 20 h (M and N), or 24 h (C–F), permeabilized, and incubated with a mouse mAb to the Myc-epitope (C–N) and with a rabbit polyclonal antibody against the endogenous EHD1 (E–N). Primary antibodies were detected using the Alexa Fluor 568-conjugated anti-mouse antibody alone (C and D) or in coincidence with Alexa Fluor 488-conjugated anti-rabbit antibody (E–N). Arf6 Q67L- and Myc-EHD1-expressing cells (A and B), PIP5KI γ - and GFP-EHD1-expressing cells (C and D), and PIP5KI γ -expressing cells (E and F) are denoted by stars. Dashed boundaries depict cells that express Myc-EHD1 but not HA-Arf6-Q67L (A and B), GFP-EHD1 but not PIP5KI γ (C and D), or endogenous EHD1 but not PIP5KI γ (E and F). Arrows mark partial colocalization of Myc-PIP5KI γ with endogenous EHD1. Bars, 10 μ m.

2000), had no significant effect on the subcellular distribution of EHD1 (Supplemental Figure 5, E and F) and had little or no impact on the Golgi, lysosomes, and early endosomes (Supplemental Figure 6, A–F). Because the PIP5KI γ activity generates significant amounts of PtdIns(4,5)P₂, we have also assessed the potential role of this phosphoinositide as a negative regulator of EHD1 tubular localization. To generate high levels of PtdIns(4,5)P₂ without directly altering the PtdIns4P concentration we have expressed PTEN, a PtdIns(3,4,5)P₃ phosphatase acting at the 3-position of the inositol ring (Mae-hama and Dixon, 1998). Increased levels of PtdIns(4,5)P₂ did not affect the tubular localization of EHD1 (Supplemental

Figure 5, G–J). Overall, these results support a critical role for PtdIns4P in the existence of EHD1-associated tubular membranes.

We next examined whether depletion of PtdIns4P affected the localization of *endogenous* EHD1 to tubular membranes. HeLa cells transfected with GFP-Sac1 exhibited a loss of endogenous EHD1-associated tubular membranes (Figure 2, E and F; star represents Sac1-transfected cells). To quantitatively evaluate the degree to which localization of EHD1 to tubules relies upon PtdIns4P levels, we compared wild-type Sac1-transfected cells with Sac1-R395A-transfected cells. As demonstrated in Figure 2I, upon transfection of the phos-

Figure 2. Depletion of PtdIns4P results in diminished EHD1- and EHD4-containing tubular membranes. (A–D) Sac1 phosphatase overexpression specifically interferes with the localization of EHD1 to tubular membranes. HeLa cells were transiently cotransfected for 24 h with constructs coding for Myc-EHD1 and either the wild-type GFP-Sac1 (A and B) or the phosphatase-dead mutant GFP-Sac1 R395A (C and D). Cells were fixed, permeabilized, and incubated with an mAb to the Myc-epitope that was detected using Alexa Fluor 568-conjugated anti-mouse secondary antibody. Stars depict cells expressing Myc-EHD1 and either GFP-Sac1 or the GFP-Sac1 R395A mutant, whereas dashed boundaries denote cells expressing only Myc-EHD1. (E–H) HeLa cells were transfected with a plasmid coding for the wild-type GFP-Sac1 and fixed after 24 h. After permeabilization, the cells were stained with rabbit anti-EHD1 (E and F) or anti-EHD4 (G and H) followed by Alexa Fluor 568-conjugated donkey anti-rabbit antibody. Stars mark cells expressing GFP-Sac1, whereas dashed boundaries indicate cells with no exogenous Sac1 expression. (I) Quantification of the percentage of cells showing complete loss of endogenous EHD1-associated tubules from the experiment depicted in E and F. Error bars represent SE of the mean from three independent normalized experiments. Bars, 10 μ m.

phatase-dead GFP-Sac1-R395A mutant, <20% of the cells exhibited no visible endogenous EHD1-associated tubular membranes, similar to our observations for untransfected cells. In contrast, in almost 70% of the cells depleted of PtdIns4P (at least in part) by wild-type Sac1, no endogenous EHD1-associated tubular membranes were detected. Another member of the EHD family of proteins that interacts with EHD1 is EHD4 (Sharma *et al.*, 2008). Endogenous EHD4 partially localizes to tubular membranes, and its subcellular distribution was disrupted by overexpression of GFP-Sac1 (Figure 2, G and H). Transfected HA-EHD4 was similarly disrupted by the overexpression of GFP-Sac1 (Supplemental Figure 2, A and B). However, EHD2, a more distantly related EHD paralogue that localizes to the plasma membrane and can be found on tubular structures when overexpressed, remained unaffected by Sac1-induced reduction of PtdIns4P levels (Supplemental Figure 2, C and D). These data implicate PtdIns4P as a major phosphoinositide involved in the generation and/or maintenance of EHD1-associated tubular membranes.

Phosphoinositide Composition of the EHD1-associated Tubular Membranes

Having identified PtdIns4P as an essential component of EHD1-associated tubules *in vivo*, we next sought to determine the identity of other phosphoinositides that comprise these tubular membranes. Initially, we used the OSBP-PH

domain as a marker of PtdIns4P (Figure 3D) and the Fab1, YOTB/ZK632.12, Vac1, and EEA1 (FYVE) domain of EEA1 to mark phosphatidylinositol-3-phosphate (PtdIns3P) (Figure 3A). Localization of phosphoinositide markers to the EHD1-associated tubular membranes was assessed in HeLa cells upon coexpression of Myc-EHD1 with either the FYVE domain of EEA1 (GFP-EEA1-FYVE) (Figure 3, B and C), OSBP-PH domain (GFP-OSBP-PH) (Figure 3, E and F), or PLC δ 1-PH domain as a marker of PtdIns(4,5) P_2 (unpublished observations). Although the EEA1 FYVE domain did not exhibit localization to EHD1-associated tubular structures, detectable levels of OSBP (as well as PLC δ 1; unpublished observations)-PH domains were found associated with these tubular membranes (compare Figure 3, B, C, E, and F; see insets). Note that a nuclear stain for GFP-OSBP-PH has been reported previously (Balla *et al.*, 2005). One possibility in these experiments is that the transfected PH domains are altering the lipid composition of the EHD1-containing tubules. To provide further support for the presence of PtdIns4P and PtdIns(4,5) P_2 on EHD1-associated tubular membranes, we took advantage of recently characterized commercial antibodies that recognize PtdIns4P (Blagoveshchenskaya *et al.*, 2008) and our own specific anti-EHD1 antibodies. As demonstrated in Figure 3, G and H, antibodies to PtdIns4P clearly aligned and partially colocalized with endogenous EHD1-containing tubules (see insets). Although antibodies to PtdIns(4,5) P_2 were not sufficiently specific for

Figure 3. EHD1 tubules contain PtdIns4P and PtdIns(4,5)P₂ but are largely devoid of PtdIns3P. (A–F) Constructs coding for the GFP-EEA1 FYVE domain and the GFP-OSBP-PH domain were either expressed alone in HeLa cells (A and D) or coexpressed together with Myc-EHD1 (B, C, E, and F). Cells were fixed, permeabilized, and incubated with a mouse mAb antibody to the Myc-epitope, followed by Alexa Fluor 568-conjugated anti-mouse secondary antibody. Images were obtained by confocal microscopy. Arrows (see insets in E and F) depict EHD1-associated tubular structures to which the PtdIns4P marker GFP-OSBP-PH (E and F) is localized. (G–J) Untransfected HeLa cells (G and H) or cells transfected with a plasmid coding for GFP-PLCδ1 PH domain (I and J) were fixed, permeabilized, and incubated with only a rabbit polyclonal antibody against the endogenous EHD1 (I and J) or together with a mouse monoclonal IgM antibody to PtdIns4P (G and H). Cells were then incubated with Alexa Fluor 568-conjugated anti-rabbit antibody (I and J) or with Alexa Fluor 488-conjugated anti-rabbit antibody together with the Alexa Fluor 568-conjugated anti-mouse IgM antibody (G and H). Bar, 10 μ m.

this type of experiment, *endogenous* EHD1 could be observed on some tubules colocalizing with the PtdIns(4,5)P₂ marker PLCδ1-PH domain (Figure 3, I and J). Collectively, these results suggest that EHD1 is localized to tubular membranes that require PtdIns4P and contain levels of PtdIns(4,5)P₂.

The EH Domain of EHD1 Interacts with PtdIns4P

We have shown previously that the EH domain of EHD1 (EH-1) can bind to phosphoinositides (Naslavsky *et al.*,

2007). Initially, filter paper binding assays with full-length EHD1 suggested that phosphoinositides containing a phosphorylation at the 3' position of the inositol ring may display slightly increased binding in vitro, although PtdIns4P also displayed significant binding by this assay (Naslavsky *et al.*, 2007). Recent studies have demonstrated that full-length EHD proteins show a broad range of phosphoinositide binding to liposomes in vitro (Blume *et al.*, 2007; Daumke *et al.*, 2007). In addition, the crystal structure of mouse EHD2

dimers has clearly identified a major lipid binding interface that does not require the EH domain for binding (Daumke *et al.*, 2007). Moreover, most *in vitro* binding/sedimentation assays cannot be performed with isolated EH domains, given the necessity for oligomerization to bind lipids (Lee *et al.*, 2005; Naslavsky *et al.*, 2006).

In this study, identification of the lipid components of EHD1-containing tubules was primarily done by *in vivo* experimentation. However, because NMR is sufficiently sensitive to detect even very low affinity interactions, we next addressed the ability of the EH-1 to bind PtdIns4P. Because this phosphoinositide is essential for EHD1 association with tubules *in vivo* (Figure 4A, inset), our intent was to identify key residues involved in the phosphoinositide binding by using NMR spectroscopy. In two-dimensional NMR $^1\text{H}^{15}\text{N}$ -HSQC experiments, we observed the resonances of amide protons of all EH-1 residues (with the exception of proline), in the presence and absence of titrated PtdIns4P. The spectrum of the wild-type EH-1 domain alone was superimposed with the spectrum of EH-1 in the presence of PtdIns4P. Each shift of the resonances was correlated to structural changes resulting from the lipid binding. We observed a number of residues that exhibited significant shifts in the presence of PtdIns4P (Figure 4A; black peaks denote the EH-1 alone, green peaks represent EH-1 in the presence of PtdIns4P). Additional NMR studies detected that the same residues exhibit similar chemical shifts in the presence of PtdIns(4,5)P₂ and PtdIns(3,5)P₂ as well (Supplemental Figure 7). These findings, combined with our recently solved EH-1 peak assignment and solution structure, allowed us to identify key shifting residues involved in PtdIns4P and other phosphoinositide binding (depicted in the molecular surface model Figure 4D; the degree of spectral shift is denoted by a color code in which red > orange > yellow). Interestingly, one of the residues with a significant degree of chemical shift was lysine 483 (residue outlined in black). Our previous studies have shown that a charge-reversal mutation of lysine to glutamate within the EH domain (EHD1 K483E) resulted in a dramatic redistribution of EHD1. Similar to truncation of the entire EHD1 EH domain, the K483E point mutation abolished EHD1-associated tubular membranes, resulting in its exclusive distribution to vesicular membranes (Figure 4B, inset) (Naslavsky *et al.*, 2007). Considering the proximity of lysine 483 to the hydrophobic NPF binding pocket, its potential to form salt bridges with negatively charged oxygen atoms on the phosphate groups of phosphoinositides, and this mutant's dramatic effect upon EHD1 localization, we hypothesized that this charge reversal may decrease the affinity of EH-1 for PtdIns4P and potentially other phosphoinositides. Accordingly, the EH domain K483E mutant was subjected to $^1\text{H}^{15}\text{N}$ -HSQC analysis in the presence and absence of PtdIns4P (Figure 4B). An overlay of the two $^1\text{H}^{15}\text{N}$ -HSQC spectra corresponding to wild-type and K483E EH-1 alone first confirmed that the two domains have identical folds, indicating that the domain structure was not disrupted by this mutation (Supplemental Figure 8). However, when assayed for PtdIns4P binding, this mutant displayed a significant decrease in its affinity for PtdIns4P (Figure 4B; red peaks denote EH-1 K483E domain alone, green peaks represent EH-1 K483E in the presence of PtdIns4P). The degree to which the K483E mutation decreased the affinity of phosphoinositide binding was quantified by measuring chemical shift changes of single key EH domain residues contributing to the PtdIns4P interaction. In addition to lysine 483, these included glycine 464, lysine 469, glutamate 470, valine 472, and glycine 482 (Figure 4C). Based on the chemical shifts of these residues in titration experi-

ments using incremental concentrations of PtdIns4P, we estimated theoretical dissociation constants (K_d) for the wild-type EH-1 at 2.0 ± 0.3 mM and above 6 mM for mutant EH-1 K483E (Figure 4C). It is noteworthy that the newly introduced glutamate residue at K483E also exhibited a detectable degree of chemical shift, indicating that although this mutation does significantly disrupt the phosphoinositide binding, it does not abolish it. Nevertheless, the dramatic loss of EHD1 K483E association with tubular membranes renders this mutant an excellent tool to analyze the function of these EHD1-containing structures.

EHD1 Associates with Pre-existing Tubular Membranes

One of the most challenging questions with regard to EHD1-containing tubular membranes is whether EHD1 is responsible for their generation or whether EHD1 associates with pre-existing membranes. Recent studies have provided solid evidence that EHD proteins are ATPases with homology to the dynamin family of GTPases (Caplan *et al.*, 2002; Lee *et al.*, 2005; Naslavsky *et al.*, 2006; Daumke *et al.*, 2007) and are intrinsically capable of tubulating membranes *in vitro* (Daumke *et al.*, 2007). *In vivo*, however, the interaction of EHD proteins with multiple partners, including Bin/amphiphysin/Rvs (BAR) domain-containing proteins such as the syndapins (Xu *et al.*, 2004; Braun *et al.*, 2005), suggests that the situation may be more complex.

To determine whether the tubular EHD1-containing membranes are present even in the absence of EHD1 from these structures, we used two additional well characterized components of tubular membranes: GFP fused to the double palmitoylated and farnesylated carboxy-terminal tail of H-Ras (GFP-H-Ras), which associates with tubular membranes of the clathrin-independent endocytic pathway (Porat-Shliom *et al.*, 2008); and Rab8a (Cherry-Rab8) (Roland *et al.*, 2007), which localizes to EHD1-containing tubular membranes. As demonstrated in Figure 5, GFP-H-Ras localizes to an array of tubular membranes that show significant overlap with tubules containing Myc-EHD1 (Figure 5, C and D, insets). This is not surprising, because GFP-H-Ras colocalizes with Arf6 and internalized MHC class I (Porat-Shliom *et al.*, 2008), both of which can also be observed colocalizing with EHD1-associated tubules (Caplan *et al.*, 2002). However, the tubular localization of GFP-H-Ras and Cherry-Rab8 is independent of EHD1, as revealed by the siRNA-mediated depletion of EHD1 (Figure 5, A and B). In addition, upon transfection of the nontubule-associated EHD1 K483E mutant, which localizes exclusively to vesicular membranes, the GFP-H-Ras displayed little or no colocalization with the EHD1 mutant but retained its distribution to existing tubular membranes (Figure 5, E and F). Furthermore, although Myc-EHD1 and Cherry-Rab8 displayed a high level of colocalization to tubular membranes (Figure 5, G and H; Roland *et al.*, 2007), in the presence of the nontubular EHD1 K483E mutant, Rab8 remained associated with tubular membranes (Figure 5, I and J). Similar results were observed using other cytosolic and nontubular EHD1 mutants (unpublished observations). These results suggest that whereas *in vitro* EHD proteins are intrinsically capable of promoting tubule generation, *in vivo*, at least in part, they associate with pre-existing tubular membranes.

EHD1-associated Tubules Are Required for Efficient Recycling to the Plasma Membrane

We next addressed the physiological role of these unique tubular structures. Previous studies have established that the reduction of EHD1 levels either by siRNA or in mouse embryonic fibroblast cells derived from EHD1 knockout

Figure 4. In vitro binding of the EHD1 EH domain (EH-1) to PtdIns4P is stabilized by lysine 483. (A and B) $^1\text{H}^{15}\text{N}$ -HSQC spectra of the wild-type EHD1 EH domain (A) and the nontubule-associated EHD1 K483E mutant EH domain (B) were acquired in the presence of 1.8 mM PtdIns4P. Chemical shifts upon the addition of PtdIns4P were displayed as peak shifts represented in green in comparison to either the spectra of the wild-type EHD1 EH domain alone shown in black (see oval boundaries) (A) or the EHD1 K483E EH domain alone displayed as red peaks (B). (C) Dissociation constants (K_d) were estimated for the individual EH domain residues: glycine 464, lysine 469, glutamate 470, valine 472, and glycine 482 for wild-type EH domain (black) and K483E mutant EH domain (red) by a nonlinear regression of a one-site binding model plotted for each residue. (D) Surface model of the EH domain residues participating in PtdIns4P binding based on the chemical shift variation during the titration with PtdIns4P (red represents residues with $^1\text{H}^{15}\text{N}$ chemical shift variation $\Delta\sigma > 0.2$; orange, $\Delta\sigma: 0.1\text{--}0.2$; yellow, $\Delta\sigma: 0.05\text{--}0.1$). Outlined residue (in black) represents lysine 483. Insets (A and B) depict the localization of full-length wild-type (A) and full-length mutant (B) EHD1.

Figure 5. EHD1 is recruited onto pre-existing tubular membranes. (A and B) HeLa cells were depleted of EHD1 by using siRNA and transfected with either GFP-H-Ras (A) or Cherry-Rab8 (B). Efficacy of EHD1 depletion was confirmed as described in Figure 6. (C–F) HeLa cells were transiently cotransfected with GFP-H-Ras and either wild-type Myc-EHD1 (C and D) or Myc-EHD1 K483E (E and F). Cells were fixed, permeabilized, and incubated with a mouse monoclonal antibody (mAb) to the Myc-epitope that was detected by Alexa Fluor 568-conjugated anti-mouse secondary antibody. (G–J) Cells cotransfected with Cherry-Rab8 and either Myc-EHD1 (G and H) or Myc-EHD1 K483E (I and J) were fixed and permeabilized. After the immunostaining with a mouse mAb to the Myc-epitope, cells were incubated with the Alexa Fluor 488-conjugated anti-

mouse secondary antibody. Insets and arrows show that loss of tubular localization of the EHD1 K483E mutant does not abolish the existence of these tubular membranes (see insets). Bars, 10 μ m.

mice results in delayed recycling of internalized receptors and their subsequent accumulation at the ERC. To specifically assess the role of EHD1-associated tubules in the process of recycling, we performed functional “rescue” assays. HeLa cells were either mock treated or treated with EHD1-siRNA. As depicted, EHD1 depletion efficacy ranged from 80 to 90% (Figure 6G). To delineate the functional significance of EHD1’s localization to tubular membranes, EHD1-siRNA-treated cells were transfected with an siRNA-resistant “silent” wild-type EHD1 mutant (Silent-GFP-EHD1; Figure 6, C and D) or an siRNA-resistant silent EHD1 K483E mutant (Silent-GFP-EHD1 K483E; Figure 6, E and F) that localizes exclusively to vesicular membranes. Cells were then pulsed with fluorescently labeled Tf, followed by a 20-min chase. As we have shown previously, compared with control cells (Figure 6A), in EHD1-depleted HeLa cells (Figure 6B) internalized Tf and its receptor displayed a delay in recycling and accumulated at the ERC (Figure 6, compare B and A). By reintroducing the siRNA-resistant version of wild-type tubule-associated EHD1 into these cells, we observed that the majority of cells resumed efficient Tf recycling out of the ERC (see representative micrographs in Figure 6, C and D). However, overexpression of the nontubule-associated EHD1 mutant Silent-GFP-EHD1 K483E did not significantly overcome the defect in Tf recycling, result-

ing in prolonged accumulation of labeled Tf in the compact perinuclear ERC (Figure 6, E and F; see cell with dashed border). Quantification of five independent experiments demonstrated that transfection with wild-type Silent-EHD1 rescued Tf recycling in 58% cells, compared with the rescue of only 24% of cells transfected with Silent-EHD1-K483E (Figure 6H).

We have also demonstrated previously that receptors internalized via clathrin-independent pathways are regulated by EHD1 (Caplan *et al.*, 2002; Jovic *et al.*, 2007). We now sought to determine whether tubule-associated EHD1 is required for the recycling of such receptors. To this aim, we followed the trafficking of β 1 integrin receptors (Figure 6, I–O). In these experiments, β 1 integrins are monitored by incubating cells for 1 h with anti- β 1 integrin antibodies that are then internalized as antibody-bound receptor complexes. Leftover noninternalized antibody on the cell surface is removed by a brief acid wash, and the cells are then “chased” for 2 h in complete media to allow recycling of the β 1 integrin/antibody complexes. An additional acid wash after the chase removes recycled complexes, and thus the remaining signal in the cell represents the level of nonrecycled β 1 integrins. As demonstrated, and as we have shown previously (Jovic *et al.*, 2007), EHD1 depletion causes a severe delay in the rate of β 1 integrin recycling and an

Figure 6. EHD1-associated tubular membranes are required for efficient recycling to the plasma membrane. (A–H) HeLa cells were either mock treated (A) or treated with EHD1-siRNA oligonucleotides (B–F) for 48 h. EHD1 knockdown efficacy was determined after calibration for protein content, and immunoblotting for EHD1, whereas actin was used as a control (G). Cells were pulsed with transferrin–Alexa-Fluor-568 (Tf-568) for 5 min, washed, and then chased in complete media for 20 min. Cells were then fixed and analyzed by confocal microscopy. (A and B) Functional efficacy of EHD1 depletion was confirmed by comparing the levels of accumulated Tf at the ERC in EHD1-depleted cells (B) compared with mock-treated cells (A). (C–F) HeLa cells treated with EHD1-siRNA for 24 h were transfected with plasmids encoding for an siRNA-resistant silent wild-type EHD1 (Silent-GFP-EHD1) or an siRNA-resistant silent EHD1 K483E (Silent-GFP-EHD1 K483E) mutant for an additional 24 h in the presence of the siRNA oligonucleotides. Cells were then pulsed with Tf-568 for 5 min, followed by a 20-min chase in complete media and subsequent fixation. (H) Quantitative analysis comparing the ability of wild-type EHD1 or the EHD1 K483E mutant to rescue Tf recycling in cells depleted of endogenous EHD1. HeLa cells treated with EHD1-siRNA and transfected with either Silent-GFP-EHD1 or Silent-GFP-EHD1 K483E (C–F) were evaluated for their ability to rescue the recycling defect incurred upon EHD1 depletion. Approximately 350 cells from five independent experiments were analyzed based on their distribution of internalized Tf. Cells exhibiting loss of Tf localization to the perinuclear ERC area (similar to mock cells in A) were scored as rescued. (I–N) HeLa cells were either mock treated (I) or treated with EHD1-siRNA oligonucleotides (J–N) for 24 h before transfection with the Silent-GFP-EHD1 (K and L) or Silent-GFP-EHD1 K483E (M and N) for an additional 24 h in the presence of the siRNA oligonucleotides. Cells were pulsed for 1 h with mouse anti- $\beta 1$ integrin antibodies, acid rinsed (stripped), washed, and chased in complete media for 2 h, followed by a second acid rinse. Internalized $\beta 1$ integrin receptors (bound by the antibodies) were then detected by Alexa Fluor 568-conjugated anti-mouse secondary antibodies. (O) Quantitative analysis of rescued $\beta 1$ integrin receptor recycling in the EHD1 knockdown cells transfected with wild-type EHD1 or the EHD1 K483E mutant (performed as outlined in H). One hundred cells each from three independent experiments were analyzed based on their distribution of internalized $\beta 1$ integrin receptor-antibody complex. Bars, 10 μ m.

accumulation of integrins in the ERC (compare mock-treated cells in Figure 6I with EHD1-siRNA in Figure 6J). As predicted, by reintroducing wild-type tubular EHD1 to the cells (by using the Silent-GFP-EHD1 siRNA-resistant mutant), we observed a recovery of the $\beta 1$ integrin recycling rates close to normal levels (Figure 6, K and L [see the outlined cell], and quantified in O). In contrast, when the nontubular EHD1 K483E mutant was reintroduced instead of the wild-type tubular EHD1, $\beta 1$ integrin did not increase its rate of recycling and remained accumulated at the ERC (Figure 6, M and N [see the outlined cell], and quantified in O). Although these results are consistent with the previously ascribed role for EHD1 in receptor recycling, to our knowledge this is the first study to directly attribute functional significance to EHD1-associated tubules, indicating that they are necessary for efficient recycling to the plasma membrane.

DISCUSSION

A characteristic hallmark of EHD1 is its localization to an array of tubular and vesicular structures emanating from the pericentriolar endocytic recycling (Caplan *et al.*, 2002). These tubular membranes range from very short structures to membranes that extend as long as 10 μm . Although the tubules do not align very well with either actin microfilaments or microtubules, they require intact microtubules for their preservation (Caplan *et al.*, 2002). Consistent with the high degree of homology between C-terminal EHD proteins, all four paralogs are capable of localizing to tubular membranes when overexpressed (Blume *et al.*, 2007; George *et al.*, 2007). However, only endogenous EHD1 and EHD4 have been observed on these structures (Sharma *et al.*, 2008).

Membrane curvature is a critical event that occurs during many important cellular processes, including receptor-mediated endocytosis (Farsad *et al.*, 2001; McMahon and Gallop, 2005; Zimmerberg and Kozlov, 2006). Recent studies have focused on a variety of membrane-bound proteins that induce membrane curvature, including epsins, amphiphysins, endophilins, and dynamin. Endophilins and amphiphysins contain BAR domains that deform lipid bilayers into tubules, and epsins cause curvature by inserting an amphipathic α helix into one leaflet of the lipid bilayer, causing an imbalance between the two bilayer leaflets and leading to membrane bending and tubulation (reviewed in Itoh and De Camilli, 2006). In contrast, dynamin forms rings that constrain lipid bilayers into tubular membranes of ~ 20 nm in diameter (Hinshaw and Schmid, 1995).

Recent studies have provided new evidence that C-terminal EHD proteins are capable of inducing membrane curvature (Daumke *et al.*, 2007). In support of this possibility, it has been demonstrated that all four C-terminal EHD proteins can bind to various phosphoinositides *in vitro* (Blume *et al.*, 2007; Daumke *et al.*, 2007; Naslavsky *et al.*, 2007). The crystal structure of mouse EHD2 has provided valuable new insight, identifying a primary membrane binding site localized to a polybasic cluster within the $\alpha 9$ helices of EHD2 dimers (Daumke *et al.*, 2007). The tips of these dimerized helices form a banana-shaped lipid binding surface, and further oligomerization of EHD2 dimers leads to the formation of ring-like structures around the neck of lipid tubules. These findings, combined with the ability of purified EHD2 to tubulate membranes *in vitro* (Daumke *et al.*, 2007), have provided compelling evidence for an analogy between EHD2 and dynamin function. Given the high level of identity between EHD2 and the other EHD paralogs, including EHD1, this implies EHD proteins may be involved in the regulation of tubulation or the detachment of budding ves-

icles/tubules from endosomal membranes. In contrast, we also examined the localization of two other markers of EHD1-associated tubules (GFP fused to the double palmitoylated and farnesylated carboxy-terminal tail of H-Ras and GFP-Rab8a). On removal of EHD1 from these structures we found that the localization of either tubule marker remained unaltered (Figure 5). These experiments suggest that *in vivo* the regulation and coordination of EHD1-associated membrane tubules may be more complex than originally envisioned and require the activity of other proteins.

Although progress in understanding the function of EHD proteins and their structure has grown exponentially in recent years, it has been extremely difficult to discern the actual functional role of these unique tubular structures that EHD1 associates with *in vivo*. Indirect evidence from our laboratory had previously hinted at a potential role for PtdIns(4,5) P_2 in the generation/maintenance of EHD1-associated tubular membranes through activation of Arf6 (Caplan *et al.*, 2002), which leads to PtdIns(4,5) P_2 generation (Honda *et al.*, 1999). These clues were provided from studies showing that either the Arf6 nucleotide status or direct overexpression of the Arf6 GTP-exchange factor (GEF) EFA6 or the Arf6 GTPase-activating protein ACAP1 led to a loss of EHD1-associated tubular membranes (Caplan *et al.*, 2002).

We and others have demonstrated that *in vitro*, EHD proteins can interact with an array of different phosphoinositides (Blume *et al.*, 2007; Daumke *et al.*, 2007; Naslavsky *et al.*, 2007). Indeed, full-length EHD proteins and EH domains (by sensitive NMR studies) display broad specificity for phosphoinositides. This phosphoinositide-EH domain binding has low affinity *in vitro*, probably because oligomerization is required for optimal membrane association (Lee *et al.*, 2005). To overcome the difficulties in understanding which phosphoinositides comprise EHD1-containing tubules *in vivo*, we have devised a strategy that involves the manipulation of phosphoinositide levels in cells. By these methods, we have identified PtdIns4P as a critical phosphoinositide component that together with PtdIns(4,5) P_2 is a constituent of EHD1-associated tubular membranes *in vivo*. Somewhat surprisingly, reduction of PtdIns4P levels either by its conversion to PtdIns(4,5) P_2 upon enhanced PIP5K1 γ expression or by Sac1-induced dephosphorylation of PtdIns4P to PtdIns, caused dramatic reduction of EHD1-associated tubular membranes. Although most studies suggest a primary role for PtdIns4P at the Golgi (Balla *et al.*, 2005; reviewed in De Matteis *et al.*, 2005), it has been demonstrated that phosphatidylinositol 4-kinase (PI4K) III β , a kinase that elevates PtdIns4P levels through the phosphorylation of PtdIns, partially localizes to recycling endosomes and can bind directly to Rab11 (de Graaf *et al.*, 2004). Indeed, we have observed a low level of recruitment of endogenous PI4K III β to EHD1-associated tubular membranes (upon EHD1 overexpression), suggesting that this kinase may regulate levels of PtdIns4P necessary for the generation/maintenance of these tubular membranes (unpublished observations). In contrast, siRNA depletion of PI4K III β did not affect the EHD1-associated tubular membranes, probably due to compensation by other PtdIns4P kinases (unpublished observations).

Despite the effect of expressing either an active GTP-locked Arf6 mutant (Arf6 Q67L) or expression of its GEF EFA6 on EHD1-associated tubules (Caplan *et al.*, 2002), the requirement for PtdIns(4,5) P_2 in the generation/maintenance of these membrane structures is not as straightforward as that of PtdIns4P. First, decreased PtdIns(4,5) P_2 levels induced by overexpression of Synaptojanin2, a phosphatase that dephosphorylates PtdIns(4,5) P_2 directly to Pt-

dIns (McPherson *et al.*, 1996), did not interfere with these tubular membranes. However, because anti-PtdIns(4,5)P₂ antibodies did not prove highly specific, we used a PtdIns(4,5)P₂ binding GFP-PLC δ 1-PH to assess colocalization with endogenous EHD1. PtdIns(4,5)P₂, which is highly concentrated on the plasma membrane, was detectable on several of EHD1-containing tubular membranes. Our data suggest that PtdIns(4,5)P₂ is likely a component of these tubular membranes; however, unlike PtdIns4P whose presence seems to be crucial for the association of EHD1 to these membranes, by itself PtdIns(4,5)P₂ does not seem to be strictly required.

A recent study has proposed that enhanced expression of PIP5K1 γ induces the formation of intracellular tubules (Shinozaki-Narikawa *et al.*, 2006). These results differ somewhat from our findings that overexpressing the active Arf6, which leads to generation of PtdIns(4,5)P₂ and concurrent reduction of PtdIns4P, causes a dramatic loss of EHD1 tubules (Caplan *et al.*, 2002; Jovic *et al.*, 2007) as well as Arf6 tubules (Brown *et al.*, 2001). One possible reason for these differences could result from a combination of the different cell lines, transfection times, and type of tubules monitored. For example, Shinozaki-Narikawa and coworkers used COS7 cells and for the most part followed tubules containing overexpressed PIP5K1 γ or other cotransfected proteins (including EHD1). In our study, we used HeLa cells but monitored the localization of endogenous (as well as transfected) EHD1 to tubular membranes. Moreover, we observed time-dependent differences in the localization of transgenic PIP5K1 γ and endogenous EHD1: within 8–12 h of transfection, a significant proportion of PIP5K1 γ was observed on tubular membranes, in partial colocalization with endogenous EHD1 (Figure 1, G–J). However, within 16–24 h of transfection, PIP5K1 γ lost all tubulation and began to exhibit its typical distribution pattern as described previously (Brown *et al.*, 2001). Over this time frame, endogenous EHD1 clearly was no longer associated with tubular membranes (Figure 1, K–N and E and F). Accordingly, our data suggests that the balance between PtdIns4P and PtdIns(4,5)P₂ might be essential for the association of EHD1 with tubular membranes.

Although loss of PtdIns(4,5)P₂ does not decrease the association of EHD1 with tubules, one possibility was that generation of high levels of this phosphoinositide may serve to negatively regulate this interaction. To assess this possibility, we used overexpression of PTEN, a PtdIns(3,4,5)P₃ phosphatase that increases PtdIns(4,5)P₂ levels. Although we cannot rule out a negative regulatory role for PtdIns(4,5)P₂, these data suggest that loss of PtdIns4P, rather than increased PtdIns(4,5)P₂ levels, is key to regulation of EHD1-associated tubular membranes.

In vivo, a large body of evidence supports a primary role for EHD1 in the regulation of endocytic recycling (reviewed in Grant and Caplan, 2008). Much of this evidence is based on knockdown of EHD1 in *C. elegans* (Grant *et al.*, 2001; Shi *et al.*, 2007), siRNA depletion of EHD1 in mammalian cells (Naslavsky *et al.*, 2004, 2006), and studies with embryonic fibroblasts derived from mice knocked down for EHD1 (Rapaport *et al.*, 2006; Jovic *et al.*, 2007). However, elucidating the role of EHD1-associated tubules by discerning the specific impact of reducing these tubular membranes (as opposed to the effect observed upon overall EHD1 depletion) has remained extremely challenging.

To overcome this obstacle, we have designed a novel approach. We have taken advantage of our recent findings derived from the NMR solution structure of the EHD1 EH domain demonstrating that a single point mutation of lysine 483 to glutamate (K483E) causes EHD1 to localize exclu-

sively to vesicular membranes (Naslavsky *et al.*, 2007). As we predicted, titration experiments using NMR show that although the EH domain K483E mutant folds in a similar manner to the wild-type domain, this mutation causes a decrease in the binding affinity to PtdIns4P and other phosphoinositides. Thus, despite the low affinity in vitro for EH domain/phosphoinositide binding, the decreased binding to PtdIns4P (and potentially other phosphoinositides) was sufficient to dramatically alter the subcellular localization of EHD1, highlighting the significance of EH-phosphoinositide binding. Armed with this mutant, which was introduced into EHD1-depleted cells as an siRNA-resistant protein, EHD1 K483E was compared with wild-type EHD1 in its ability to rescue the recycling delay observed (and well characterized) for Tf receptor and β 1 integrins in EHD1-depleted cells. The results, exhibited and quantified in Figure 6, provide evidence that EHD1-associated tubular membranes are required for efficient recycling of proteins internalized through either clathrin-dependent or clathrin-independent pathways.

Overall, in this study we have characterized the phosphoinositide requirements for EHD1-associated tubular membranes, and we found that PtdIns4P is a critical component of these structures. PtdIns(4,5)P₂ is also a constituent of these structures in vivo but decreased or increased levels of this phosphoinositide do not affect the localization of EHD1 to these membranes. We also provide evidence that although EHD proteins are capable of generating tubules in vitro, it seems that in vivo EHD1 associates, at least in part, with pre-existing tubular structures. Finally, based on our “recycling rescue” assays, we provide the first evidence for the function of EHD1-associated tubular membranes and show that conversion of EHD1 to an exclusively vesicular (and nontubular) localization pattern results in impaired Tf and β 1 integrin receptor recycling to the plasma membrane. Although a complete characterization of EHD1-associated tubular membranes will require the development of methods to purify and/or immunoprecipitate intact tubules for the purpose of comprehensive phospholipidomic analysis, our data indicate that levels of PtdIns4P are required for the generation and/or maintenance of these tubules and that their absence prevents efficient recycling.

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