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RESEARCH ARTICLE

Isoform 5 of PIPKIγ regulates the endosomal trafficking and degradation of E-cadherin

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ABSTRACT

Phosphatidylinositol phosphate kinases (PIPKs) have distinct cellular targeting, allowing for site-specific synthesis of phosphatidylinositol 4,5-bisphosphate [PI(4,5)P$_2$] to activate specific signaling cascades required for cellular processes. Several C-terminal splice variants of PIPKIγ (also known as PIP5K1C) exist, and have been implicated in a multitude of cellular roles. PI(4,5)P$_2$ serves as a fundamental regulator of E-cadherin transport, and PI(4,5)P$_2$-generating enzymes are important signaling relays in these pathways. We present evidence that the isoform 5 splice variant of PIPKIγ (PIPKIγi5) associates with E-cadherin and promotes its lysosomal degradation. Additionally, we show that the endosomal trafficking proteins SNX5 and SNX6 associate with PIPKIγi5 and inhibit PIPKIγi5-mediated E-cadherin degradation. Following HGF stimulation, activated Src directly phosphorylates PIPKIγi5. Phosphorylation of the PIPKIγi5 C-terminus regulates its association with SNX5 and, consequently, E-cadherin degradation. Additionally, this PIPKIγi5-mediated pathway requires Rab7 to promote degradation of internalized E-cadherin. Taken together, the data indicate that PIPKIγi5 and SNX5 are crucial regulators of E-cadherin sorting and degradation. PIPKIγi5, SNX and phosphoinositide regulation of lysosomal sorting represent a novel area of PI(4,5)P$_2$ signaling and research. PIPKIγi5 regulation of E-cadherin sorting for degradation might have broad implications in development and tissue maintenance, and enhanced PIPKIγi5 function might have pathogenic consequences due to downregulation of E-cadherin.

KEY WORDS: PIPKIγ, E-cadherin, Degradation, SNX5

INTRODUCTION

Type-1 phosphatidylinositol 4-phosphate 5-kinases (PIPKIs) are a family of enzymes that synthesize phosphatidylinositol 4,5-bisphosphate [PI(4,5)P$_2$]. The unique intracellular targeting of each member allows for the spatial and temporal control of the synthesis of PI(4,5)P$_2$, thereby regulating specific processes, such as endocytosis, actin assembly, formation of cell–cell contacts and adhesion to the extracellular matrix (Doughman et al., 2003; Heck et al., 2007; Ling et al., 2006; Schill and Anderson, 2009a). PI(4,5)P$_2$ influences physiological processes by binding to proteins containing domains such as the pleckstrin-homology (PH) domain, phox-homology (PX) domain, band 4.1 ezrin radixin moesin homology (FERM) domain or the Bin/Amphiphysin/Rvs (BAR) domain to modulate their activities (Betson et al., 2002; Harlan et al., 1994; Lemmon et al., 2002; Toker, 2002; Yoon et al., 2012). In particular, PI(4,5)P$_2$ regulates various components of the endocytic and endosomal trafficking pathways, including epsin, AP180, dynamin, sorting nexins (SNXs), ARFs and clathrin adaptor protein complexes (Carlton et al., 2005; Carlton and Cullen, 2005; Martin, 2001; Schill and Anderson, 2009a; Seet and Hong, 2006). Collectively, PI(4,5)P$_2$ is a potent regulatory molecule in diverse cellular signaling pathways, with broad effects on cellular function.

The three PIPKI isoforms (α, β and γ) have a high level of sequence divergence at the C-terminus, which allows for their distinct localization and function (Heck et al., 2007). Additionally, several C-terminal splice variants of PIPKIγ (PIPKIγi1, i2, i4 and i5) have been identified in mammalian cells, and these splice variants have specific functions (Bairstow et al., 2006; Di Paolo et al., 2002; Giudici et al., 2004; Ling et al., 2007; Ling et al., 2002; Schill and Anderson, 2009b; Sun et al., 2007; Xia et al., 2011). PIPKIγi1 consists of 640 amino acids and is localized to the plasma membrane and cytoplasm. The i1 N-terminal sequence is conserved in PIPKIγi2, i4 and i5. These three isoforms each contain a unique peptide sequence at the C-terminal end, which mediates specific localization and function of each isoform (Schill and Anderson, 2009b). With the recent discovery of PIPKIγi1–i6 in mammalian cells, here, we use the HUGO nomenclature for splice variants (PIPKIγ87 or PIPKIγ640 is referred to as PIPKIγi1, PIPKIγ90 or PIPKIγ668 is referred to as PIPKIγi2) (Schill and Anderson, 2009b; Xia et al., 2011). Specific isoforms have distinct functions. For example, PIPKIγi2 regulates focal adhesion dynamics and vesicle trafficking (Di Paolo et al., 2002; Kahlfeldt et al., 2010; Ling et al., 2002). In epithelial cells, PIPKIγi2 regulates the formation of cell–cell contacts through its association with the adhesion molecule E-cadherin and a specific interaction with the epithelial-specific AP1B clathrin adaptor complex (Ling et al., 2007). Additionally, PIPKIγi2 functionally links N-cadherin cell–cell junctions to regulated actin assembly (El Sayegh et al., 2007). These findings position PIPKIγ as a crucial regulator of the assembly of cell–cell contacts and the intracellular transport of components of these complexes. Recently, PIPKIγi5 was found to localize to endosomes and interact with the PI(4,5)P$_2$ effector SNX5 to regulate the lysosomal degradation of the epidermal growth factor receptor (EGFR) (Schill and Anderson, 2009b; Sun et al., 2013). Because E-cadherin binds to the conserved region that is present in PIPKIγi1, i2, i4 and i5, here, we examined the role of PIPKIγi5 in the lysosomal degradation of E-cadherin.

E-cadherin assembles into adherens junctions that maintain cell–cell adhesion (Guillemot et al., 2008; Hartsock and Nelson, 2008). The trafficking of E-cadherin regulates the formation,

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stability and disassembly of these complexes. PI(4,5)P$_2$ controls multiple aspects of E-cadherin transport; thus, PI(4,5)P$_2$-generating enzymes are essential signaling relays in these pathways (Schill and Anderson, 2009a). Recently, much work has focused on defining the cellular pathways that remove E-cadherin from the cell surface and promote its degradation in the lysosome, as this process is a direct contributor to the loss of cellular polarization observed during the epithelial-to-mesenchymal transition (EMT) (Giehl and Menke, 2008; Guarino et al., 2007). Multiple trafficking pathways control E-cadherin levels at the cell surface, including clathrin-dependent endocytosis (Ivanov et al., 2004) and macropinocytosis (Bryant et al., 2007). However, signals from the cellular microenvironment influence whether internalized E-cadherin is recycled or degraded (Giehl and Menke, 2008). The E-cadherin degradation pathway requires many components, including the ubiquitin ligase Hakai (also known as CBLL1) (Fujita et al., 2002; Pece and Gutkind, 2002), Rab GTPases, Hrs (also known as HGS) (Palacios et al., 2005; Toyoshima et al., 2007), growth factor receptors (Delva and Kowalczyk, 2009; Fujita et al., 2002; Orian-Rousseau and Ponta, 2008; Toyoshima et al., 2007) and Src kinase (Palacios et al., 2005; Shen et al., 2008). However, much remains to be discovered about the signals that sort E-cadherin for degradation.

SNXs are membrane-associated cytoplasmic proteins involved in multiple trafficking pathways. At early endosomes, SNXs often sort proteins for recycling to the cell surface and trans-Golgi network or for trafficking to the lysosome (Worby and Dixon, 2002). All SNXs contain a PX domain, and some also contain a BAR domain (Lemmon, 2003; Worby and Dixon, 2002). SNX5 directly interacts with PIPKIγi5 through its PX domain and binds to phosphoinositides, including PI(4,5)P$_2$, through its PX and BAR domains (Koharudin et al., 2009; Sun et al., 2013). At least one SNX is involved in the regulation of E-cadherin trafficking,

as SNX1 was found to promote the recycling of E-cadherin upon EGF-induced E-cadherin internalization (Bryant et al., 2007).

Here, we present evidence that PIPKIγi5 associates with E-cadherin and promotes E-cadherin lysosomal degradation. However, SNX5 acts antagonistically to PIPKIγi5, preventing E-cadherin degradation. Importantly, PIPKIγi5 appears to function as part of a defined E-cadherin degradation pathway. Furthermore, tyrosine phosphorylation of PIPKIγi5 regulates its interaction with SNX5 and its function in E-cadherin degradation, suggesting that these proteins might regulate one another to control the fate of E-cadherin.

RESULTS
PIPKIγi5 associates with E-cadherin *in vivo* and promotes E-cadherin degradation

PIPKIγi2 regulates E-cadherin trafficking *in vivo* (Akiyama et al., 2005; Ling et al., 2007). Because E-cadherin associates with the conserved kinase domain of PIPKIγ, this potentially allows for multiple PIPKIγ variants to regulate E-cadherin biology. To explore this, endogenous E-cadherin immunoprecipitates were western blotted with antibodies against specific PIPKIγ splice variants. PIPKIγi2 and PIPKIγi5, but not PIPKIγi4, were detected in E-cadherin immunoprecipitates from MCF10A mammary epithelial cells (Fig. 1A), T47D mammary ductal carcinoma cells and Mardin-Darby canine kidney (MDCK) cells (data not shown). To determine whether PIPKIγi5 colocalized with E-cadherin, HA–PIPKIγi5 was inducibly expressed in stably transfected MDCK cell lines, and the cells were processed for immunofluorescence microscopy. As shown in Fig. 1B, PIPKIγi5 colocalized with E-cadherin at cell–cell contacts and intracellular compartments. The association and localization of PIPKIγi5 with E-cadherin suggested that it might regulate E-cadherin biology.

Previously, PIPKIγi5 was shown to regulate the lysosomal degradation of EGFR (Sun et al., 2013). To determine whether
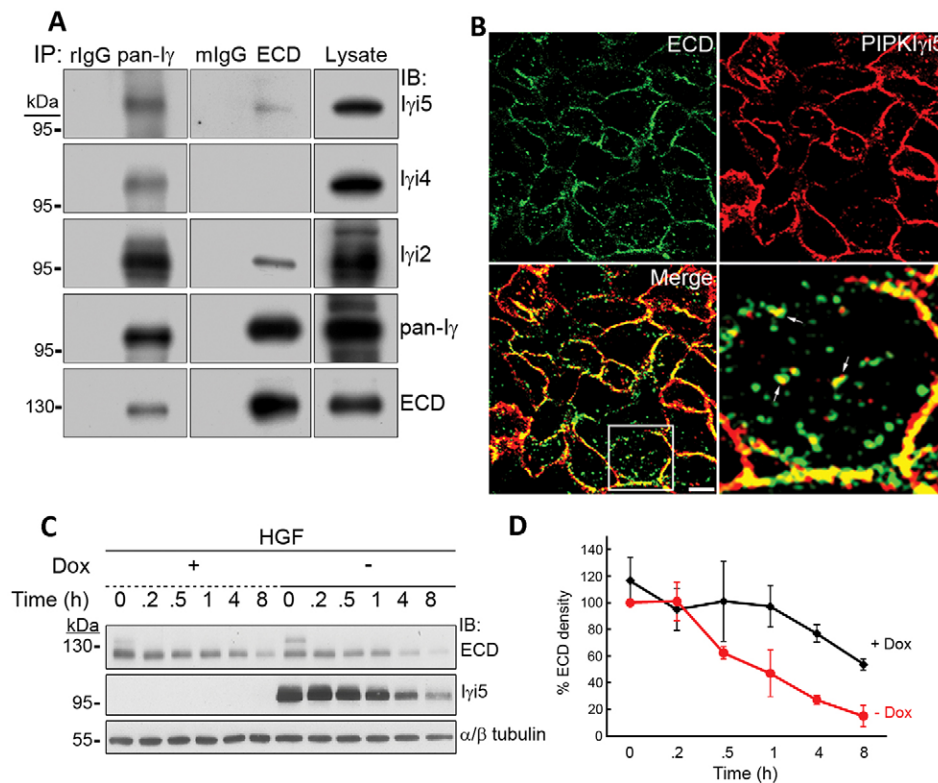
Fig. 1. Multiple PIPKIγ splice variants associate with E-cadherin. (A) Endogenous E-cadherin (ECD) and PIPKIγ (pan-Iγ) were immunoprecipitated (IP) from MCF10A cell lysates, and the immunocomplexes and cell lysates were western blotted (IB) with antibodies against each PIPKIγ splice variant or E-cadherin. Non-specific mouse or rabbit IgG was incubated with the cell lysate as a control. (B) TET-inducible MDCK cells were grown on coverslips and allowed to express HA–PIPKIγi5 for 72 h. Cells were then fixed and stained for HA–PIPKIγi5 (red) and E-cadherin (green), and analyzed as described in Materials and Methods. Colocalization of the two proteins in the overlay is indicated in yellow. Inset panel is 300% magnification of the outlined area. Arrows, colocalization of E-cadherin with HA–PIPKIγi5 within an intracellular compartment. Scale bar: 10 μm. (C) TET-inducible MDCK cells were grown for 72 h with (+) or without (−) doxycycline. After serum starvation, cells were treated with 50 ng/ml HGF for intervals up to 8 h, and E-cadherin levels were examined by western blotting. The western blot is representative of three independent experiments. (D) Quantification of E-cadherin levels from C. Data show the mean±s.e.m.

PIPKIγi5 controls the lysosomal sorting of E-cadherin, MDCKs grown in the presence or absence of doxycycline (to control PIPKIγi5 expression) were treated with hepatocyte growth factor (HGF), which induces the disassembly of adherens junctions and the lysosomal degradation of E-cadherin. E-cadherin protein content was measured by western blotting. Interestingly, cells with induced expression of PIPKIγi5 displayed an enhanced rate of E-cadherin degradation in response to HGF treatment (Fig. 1C,D). Furthermore, the expression of PIPKIγi1, PIPKIγi2 or a kinase-dead D316A mutant of PIPKIγi5 did not affect E-cadherin degradation (supplementary material Fig. S1A–C). MDCK cells treated with HGF were also examined by immunofluorescence microscopy. In the absence of HGF, E-cadherin was present at cell–cell contacts, where it colocalized with PIPKIγi5 (supplementary material Fig. S1D). After HGF stimulation in doxycycline-treated cells, the majority of E-cadherin was observed near the cell–cell contacts, with a small amount of E-cadherin detectable at late endosomes or lysosomes, as indicated by its colocalization with LysoTracker (supplementary material Fig. S1D). Following HGF treatment of PIPKIγi5-expressing cells, E-cadherin was observed at cell–cell contacts, but there was increased intracellular staining for E-cadherin, both at late endosomes and with PIPKIγi5 at distinct intracellular compartments and enlarged vesicles. These data suggest that PIPKIγi5 might enhance the targeting of E-cadherin to intracellular compartments upon stimulation with HGF and that E-cadherin might be sorted through PIPKIγi5-positive compartments prior to its degradation.

PIPKIγi5 and SNX5 play opposing roles in E-cadherin stability

SNX5 and PIPKIγi5 colocalize at endosomes and both are required for EGFR degradation (Sun et al., 2013). Therefore, further studies focused on how these two proteins might regulate the sorting of E-cadherin for degradation. In polarized epithelial cells, the majority of E-cadherin localizes at cell–cell contacts, with the exception of the fraction of E-cadherin that is actively undergoing intracellular trafficking (Bryant et al., 2007; Bryant and Stow, 2004; D'Souza-Schorey, 2005; Ling et al., 2007; Schill and Anderson, 2009a; Yap et al., 2007). To focus our study on E-cadherin that was undergoing trafficking, rather than the total cellular complement thereof, we chose HeLa cells, which do not normally express E-cadherin and do not form polarized monolayers. This model has been used previously to characterize E-cadherin trafficking and degradation (Houghton et al., 2012; Lock and Stow, 2005; Yang et al., 2006).

Using this system, E-cadherin was expressed with PIPKIγi5 or SNX5, and the E-cadherin protein content in cells was quantified. In these experiments, PIPKIγi5 expression correlated with a reduction of ~40% in the amount of E-cadherin (Fig. 2A,B). By contrast, the expression of SNX5 increased E-cadherin content by ~80% (Fig. 2A,B). However, coexpression of E-cadherin, SNX5 and PIPKIγi5 reduced E-cadherin content by 25% compared with control cells, indicating that PIPKIγi5 counteracts the increase in the amount of E-cadherin observed upon SNX5 expression. To confirm that the loss of E-cadherin observed upon PIPKIγi5 expression was due to lysosomal degradation, HeLa cells expressing E-cadherin and PIPKIγi5 were treated for 16 hours with increasing concentrations of chloroquine, which inhibits lysosomal degradation. As shown in Fig. 2A,B and supplementary material Fig. S2A, chloroquine effectively inhibited the degradation of E-cadherin in a dose-dependent manner, and the level of E-cadherin in chloroquine-treated cells expressing PIPKIγi5 and SNX5 was similar to the levels observed when PIPKIγi5 was

absent (Fig. 2A,B). Furthermore, the levels of endogenous N-cadherin and transferrin receptor (TfnR) were unaffected by the expression of either SNX5 or PIPKIγi5, indicating that a general change in protein degradation was not occurring in these cells. To determine whether these observations were due to changes in E-cadherin transcription, E-cadherin mRNA content was analyzed by quantitative (q)PCR (supplementary material Fig. S2B). The data indicated that there was no significant difference in E-cadherin mRNA levels upon expression of PIPKIγi5 or SNX5.

To determine whether the generation of $PI(4,5)P_2$ was required for PIPKIγi5 to destabilize E-cadherin, wild-type or kinase-dead PIPKIγi5 were expressed and the cellular levels of E-cadherin were assayed. As shown in Fig. 2C,D, kinase-dead PIPKIγi5 has a decreased effect on E-cadherin compared with the wild-type form, reducing E-cadherin levels by ~20% as compared with control cells. A similar trend was observed when SNX5 was coexpressed with kinase-dead PIPKIγi5, which was less effective at counteracting the E-cadherin-stabilizing effect of SNX5 (Fig. 2C,E). This is consistent with our observations that kinase-inactive PIPKIγi5 is unable to regulate EGFR degradation or to associate as strongly with SNX5 (Sun et al., 2013), and it suggests that either the generation of $PI(4,5)P_2$ by PIPKIγi5 or the binding of PIPKIγi5 to SNX5 inhibits the effect of the latter on E-cadherin stability.

SNX5 and SNX6 regulate E-cadherin turnover

Based on the amino acid sequences of SNX proteins, SNX5 is most similar to SNX6 (Worby and Dixon, 2002). The functional redundancy of SNX5 and SNX6 in endosomal trafficking is not well characterized, although both proteins function as part of the mammalian retromer (Wassmer et al., 2007). Immunoprecipitation experiments indicated that PIPKIγi5, but not other PIPKIγ splice variants, associate with SNX6 (supplementary material Fig. S2C) and that this interaction is direct (supplementary material Fig. S2D). Owing to the homology between SNX5 and SNX6 and the direct association of SNX6 with PIPKIγi5, it is likely that SNX6 is subject to regulation by PIPKIγi5, similar to SNX5. Expression of SNX6 also significantly enhanced E-cadherin stability by ~1.4-fold (Fig. 2F,G). SNX1 regulates the recycling of E-cadherin (Bryant et al., 2007). In contrast to SNX5 and SNX6, the expression of SNX1 had no effect on E-cadherin, TfnR or N-cadherin levels (Fig. 2F,G), suggesting that SNX1 functions in E-cadherin recycling through a distinct pathway.

To determine whether the expression of SNX6 or SNX1 could counteract PIPKIγi5-induced E-cadherin degradation, we coexpressed PIPKIγi5 with E-cadherin and the indicated SNX proteins. As shown in Fig. 2F,H, both SNX5 and SNX6 partially offset the loss of E-cadherin that was observed upon expression of PIPKIγi5, again suggesting that SNX5 and SNX6 promote E-cadherin stability, which is counteracted by PIPKIγi5. However, coexpression of PIPKIγi5 and SNX1 resulted in a ~25% decrease in E-cadherin levels. As the expression of SNX1 alone did not appear to affect E-cadherin, the combined results suggest that PIPKIγi5 might indirectly regulate SNX1 activity. As SNX1 has been shown to promote the recycling of internalized E-cadherin (Bryant et al., 2007), PIPKIγi5 potentially indirectly inhibits SNX1-mediated recycling of E-cadherin.

PIPKIγi5 promotes the targeting of E-cadherin to the lysosome

Both intra- and extracellular signals can initiate the dissolution of cell–cell contacts in epithelial cells, resulting in E-cadherin internalization (D'Souza-Schorey, 2005; Schill and Anderson,
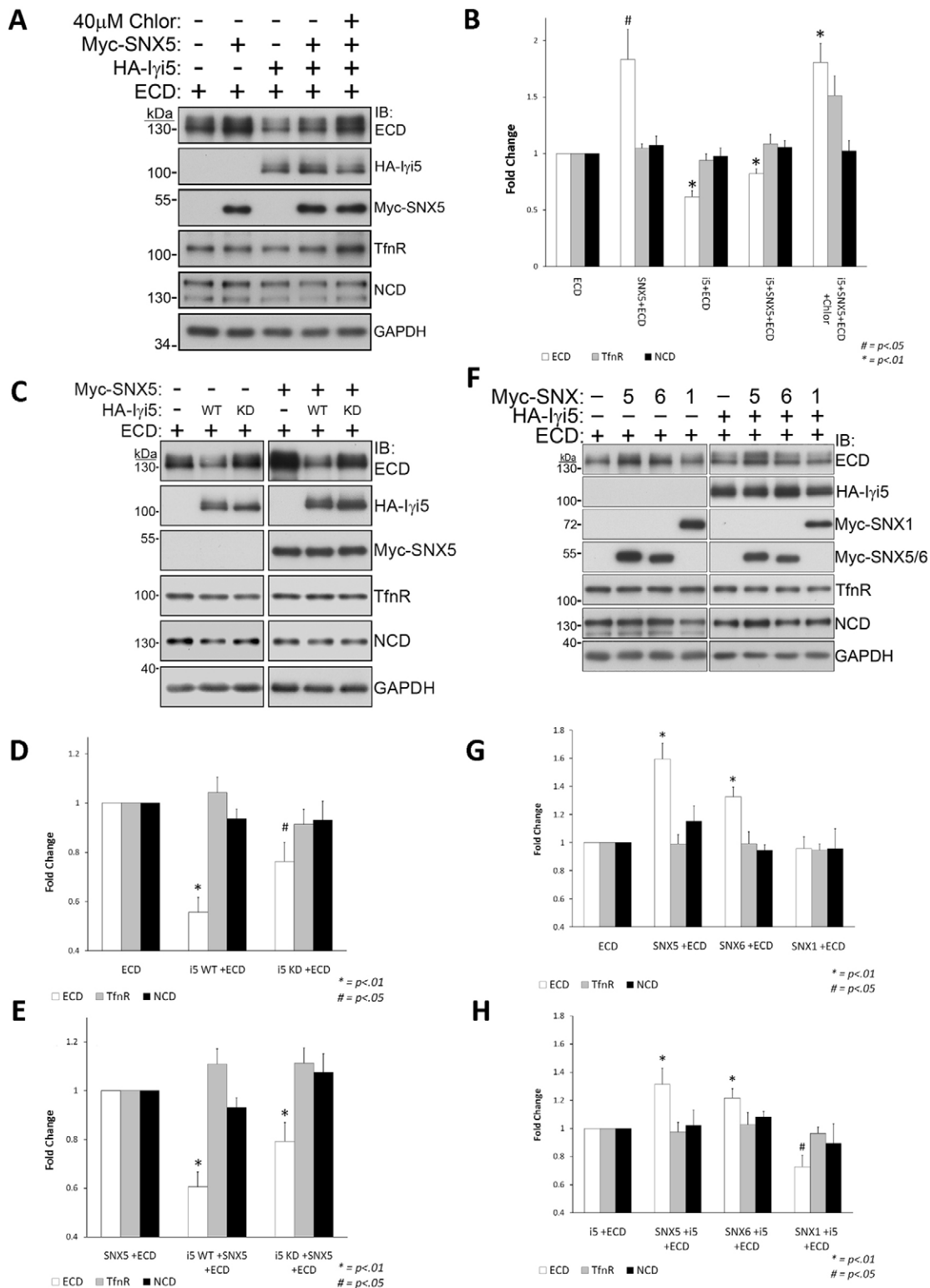
Fig. 2. PIPKIγi5 and SNX5 play opposing roles in E-cadherin stability. (A) HA–PIPKIγi5 (Iγi5), Myc–SNX5 and/or E-cadherin (ECD) were expressed in HeLa cells for 16 h and total cell lysates were western blotted (IB) for the indicated proteins. Cellular levels of transferrin receptor (TfnR), N-cadherin (NCD) and GAPDH were assayed as controls. '+ Chlor' cells were treated for 16 h with 40 µM chloroquine to inhibit lysosomal function. The western blot is representative of eight independent experiments. (B) Quantification of the western blot results depicted in A. (C) Wild-type HA–PIPKIγi5 (WT) or kinase-dead HA–PIPKIγi5 (KD, D316A) was coexpressed with Myc–SNX5 and/or ECD as indicated, and total cell lysates were analyzed by western blotting. The western blot is representative of eight independent experiments. (D,E) Quantification of the western blot depicted in C. (F) HA–PIPKIγi5, Myc–SNX5, SNX6 or SNX1, and ECD were expressed in HeLa cells for 16 h, and total cell lysates were western blotted for the indicated proteins. The western blot is representative of eight independent experiments. (G,H) Quantification of western blotting results depicted in F. Data show the mean±s.e.m.

2009a; Yap et al., 2007). Subsequently, E-cadherin is trafficked into sorting endosomes, where its fate is determined by signaling pathways. Once targeted for degradation, E-cadherin proceeds to the lysosome through a trafficking pathway involving Rab GTPases, ubiquitin ligases and the ubiquitin adaptor protein Hrs (Fujita et al., 2002; Palacios et al., 2005). There is also evidence that E-cadherin might be targeted for degradation at the

proteasome (Yang et al., 2006). Immunofluorescence microscopy was used to investigate the localization of intracellular E-cadherin. HA–PIPKIγi5 and Myc–SNX5 were coexpressed in HeLa cells, and the cells were labeled for EEA1 (a marker of early endosomes) and LAMP-1 (a marker of lysosomes). When expressed alone, E-cadherin was localized throughout the cell in a vesicular-like staining pattern that overlapped with that of EEA1 (Fig. 3A). A
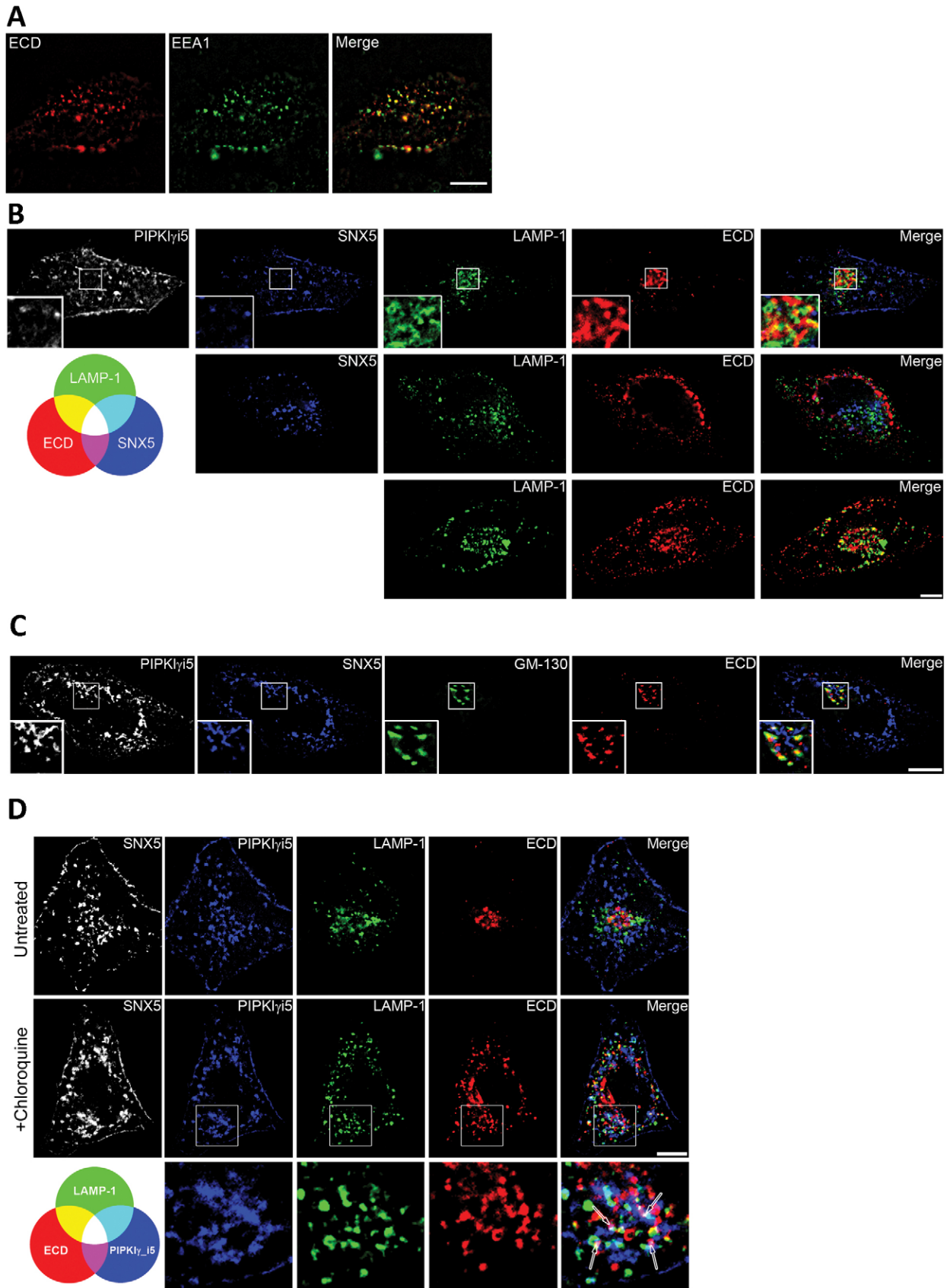
Fig. 3. See next page for legend.

Fig. 3. PIPKIγi5 promotes the lysosomal targeting of E-cadherin.
(A) HeLa cells plated on glass coverslips were transfected with E-cadherin
(ECD) for 16 h before fixation. Coverslips were then stained with antibodies
against EEA1 (green) and ECD (red). (B) HeLa cells plated on glass
coverslips were transfected with E-cadherin, Myc–SNX5 and/or PIPKIγi5 for
16 h before fixation. Coverslips were then stained with antibodies against
HA–PIPKIγi5, Myc–SNX5 (blue), LAMP-1 (green) and ECD (red). Inset
panel, 200% enlargement of the outlined area. (C) HeLa cells plated on glass
coverslips were transfected with E-cadherin, Myc–SNX5 and PIPKIγi5 for
16 h before fixation. Coverslips were then stained with antibodies against
HA–PIPKIγi5, Myc–SNX5 (blue), GM-130 (green) and ECD (red). Inset,
200% enlargement of the outlined area. (D) HeLa cells were transfected in
the absence or presence of 40 µM chloroquine and processed for
immunofluorescence as described in B. Inset, 200% enlargement of the
outlined area. Arrows, regions of colocalization between PIPKIγi5, E-
cadherin and LAMP-1. Scale bars: 10 µm.

subset of E-cadherin was localized to punctate compartments in
proximity to the plasma membrane. However, some E-cadherin
colocalized with LAMP-1, suggesting that active degradation of E-
cadherin occurs in these cells (Fig. 3B). Upon coexpression of E-
cadherin and SNX5, there was no apparent colocalization of
E-cadherin with LAMP-1 and minimal colocalization of SNX5
with E-cadherin (Fig. 3B). By contrast, upon expression of
PIPKIγi5, E-cadherin staining was mainly limited to the
perinuclear region, where it partially colocalized with LAMP-1
(Fig. 3B). Collectively, the data suggest that the E-cadherin
loss observed upon PIPKIγi5 expression is due to enhanced
targeting of E-cadherin to lysosomes after it enters the endosomal
system. By contrast, SNX5 appears to promote the trafficking of E-
cadherin away from the lysosome, but this activity can be
counteracted by PIPKIγi5. The compact perinuclear E-cadherin
distribution observed when PIPKIγi5 was expressed also partially
overlapped with that of the cis-Golgi marker GM130, which
suggests that this pool might be newly synthesized E-cadherin or
E-cadherin targeted for recycling (Fig. 3C). Additionally, in
chloroquine-treated cells, E-cadherin was found in endosomal-
like structures that partially colocalized with both LAMP-1 and
PIPKIγi5 (Fig. 3D).

We also tested whether E-cadherin degradation could be
mediated by the proteasome. Cells expressing PIPKIγi5, SNX5
and E-cadherin were treated with the proteasome inhibitor MG-
132 or the protease inhibitor leupeptin, and then analyzed for
E-cadherin expression (supplementary material Fig. S3A).
Treatment of cells with leupeptin appeared to have no effect on
E-cadherin loss. By contrast, treatment of cells with MG-132
prevented E-cadherin loss. As these results were conflicting, we
further examined MG-132-treated cells by immunofluorescence.
MG-132 treatment resulted in aberrant localization of PIPKIγi5
and SNX5 into large cytoplasmic clusters (supplementary
material Fig. S3B), suggesting that MG-132 interferes with
PIPKIγi5 targeting. Therefore, the lack of PIPKIγi5-mediated E-
cadherin degradation might be an artifact due to the mis-targeting
of PIPKIγi5 rather than inhibition of the proteasomal degradation
of E-cadherin. Given this evidence, it is likely that the majority of
E-cadherin loss in cells expressing PIPKIγi5 is mediated by the
lysosome.

PIPKIγi5 interacts with SNX5
Our previous work identified a specific interaction between the
unique C-terminal sequence of PIPKIγi5 and SNX5, which
required lipid kinase activity for a strong interaction (Sun et al.,
2013). Endogenous SNX5 immunoprecipitates from HeLa cells
were western blotted with anti-PIPKIγi5 antibody. As shown in

Fig. 4A, PIPKIγi5 was detected in the SNX5 immunoprecipitates.
To confirm that SNX5 associates with the unique portion of the
PIPKIγi5 C-terminus, an HA-tagged version of each PIPKIγ
splice variant was coexpressed with Myc–SNX5 in HeLa cells,
and Myc–SNX5 was immunoprecipitated from cell lysates. As
shown in Fig. 4B, only PIPKIγi5 associated with Myc–SNX5. To
determine the region within the PIPKIγi5 C-terminus that
associates with SNX5, C-terminal truncation mutants of
PIPKIγi5 were created and coexpressed with Myc–SNX5 in
HeLa cells (Fig. 4C,D). Although deletion of residues 675–707
(Δ675) of PIPKIγi5 did not inhibit its association with SNX5,
truncation at residue 659 (Δ659) abolished binding to SNX5
(Fig. 4D). The combined data confirms that the C-terminus of
PIPKIγi5 mediates the association with SNX5, and narrows the
binding region to residues 640–675 of PIPKIγi5.

**Tyrosine phosphorylation regulates PIPKIγi5 and its
association with SNX5**
The tyrosine kinase Src is known to phosphorylate PIPKIγi2 to
regulate its association with talin (Bairstow et al., 2005; de Pereda
et al., 2005; Lee et al., 2005; Ling et al., 2003). PIPKIγi2 is also
regulated by tyrosine phosphorylation downstream of growth factor
receptors (Sun et al., 2007). The unique C-terminus of PIPKIγi5
contains a tyrosine motif similar to the Src phosphorylation site in
PIPKIγi2 (Schill and Anderson, 2009b) (Fig. 4C). Therefore, we
investigated whether PIPKIγi5 is phosphorylated downstream of
Src and, consequently, whether the association of PIPKIγi5 and
SNX5 could be regulated by tyrosine phosphorylation. To
determine whether PIPKIγi5 was tyrosine phosphorylated, the
phosphorylation status of PIPKIγ splice variants in HeLa cells
cultured in 10% FBS was examined by immunoprecipitation of
overexpressed PIPKIγ and western blotting with an anti-
phosphotyrosine antibody. Fig. 5A illustrates that the PIPKIγ
splice variants are tyrosine phosphorylated to varying degrees.
Interestingly, these results also suggest that PIPKIγi5 is more
strongly phosphorylated than other PIPKIγ splice variants under
these conditions. Moreover, when MDCK cells expressing
PIPKIγi5 were stimulated with HGF, tyrosine phosphorylation of
PIPKIγi5 was enhanced (Fig. 5B). These data suggest that PIPKIγi5
is also regulated by phosphorylation downstream of HGF signaling.

Because Src is commonly activated downstream of growth factor
signaling, we investigated whether Src activity induces tyrosine
phosphorylation of PIPKIγi5. To explore this, PIPKIγi5 was
coexpressed with c-Src or a vector control, immunoprecipitated
and assayed for phosphorylation and association with Src. As shown
in Fig. 5C, wild-type but not kinase-dead PIPKIγi5 was robustly
phosphorylated upon c-Src coexpression. Kinase-inactive PIPKIγi5
might be a poor substrate for Src, or the activity of the protein
tyrosine phosphatase SHP-1 (also known as PTPN6) that associates
with PIPKIγ might be inhibited by PI(4,5)P$_2$ (Bairstow et al., 2005).
When overexpressed PIPKIγi5 is immunoprecipitated, c-Src
physically associates with PIPKIγi5 (Fig. 5C). To confirm the
association of PIPKIγi5 with Src, endogenous Src was
immunoprecipitated from HeLa cells expressing Myc–PIPKIγi5
and assayed by western blotting. The results in Fig. 5D illustrate that
endogenous Src associates with PIPKIγi5. To determine whether
Src activity regulates the association between PIPKIγi5 and SNX5,
c-Src was coexpressed with HA–PIPKIγi5 and Myc–SNX5 in HeLa
cells and HA–PIPKIγi5 was immunoprecipitated from cell lysates.
Expression of wild-type c-Src corresponded with a substantial
increase in PIPKIγi5 tyrosine phosphorylation and coincident loss of
SNX5 association, whereas kinase-dead Src did not affect PIPKIγi5
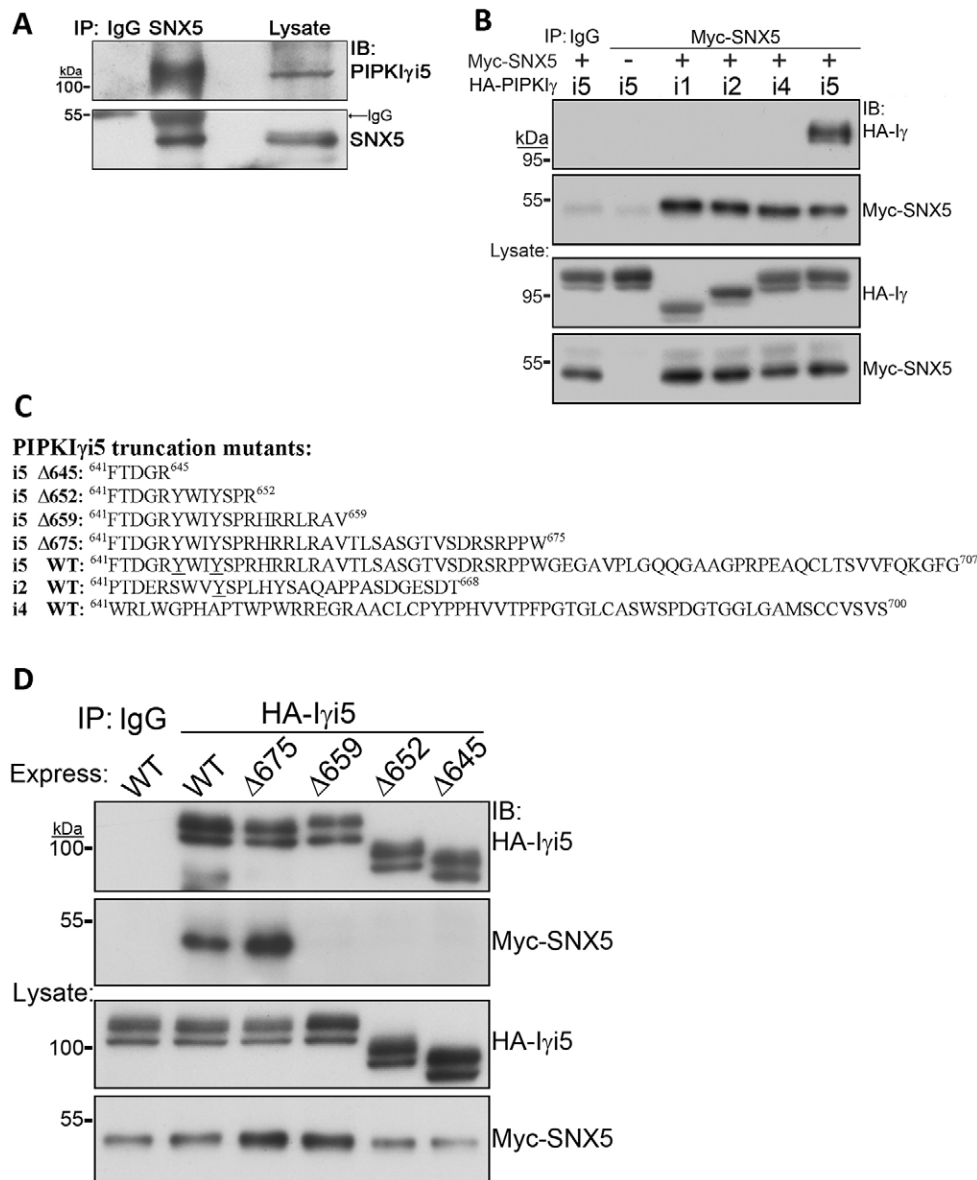
Fig. 4. PIPKγi5 associates with SNX5 *in vivo* through its unique C-terminus.
(A) HeLa cells were lysed and subjected to immunoprecipitation (IP) with an anti-SNX5 polyclonal antibody or rabbit IgG. The immunocomplexes were then western blotted (IB) with anti-SNX5 or anti-PIPKIγi5 polyclonal antibodies. (B) HA–PIPKIγ splice variants and Myc–SNX5 were coexpressed in HeLa cells, and Myc–SNX5 was immunoprecipitated from cell lysates. The immunocomplexes and their corresponding total cell lysate controls were then western blotted with anti-HA or anti-Myc antibodies. (C) Amino acid sequence of the unique C-terminus of PIPKIγi2, i4, i5 and i5 truncation mutants. Tyrosine 646 in PIPKIγi5 and tyrosine 649 in PIPKIγi2 and i5 are underlined. WT, wild type. (D) PIPKIγi5 C-terminal truncation mutants were coexpressed with Myc–SNX5 in HeLa cells and immunoprecipitated. The HA immunocomplexes and corresponding cell lysates were western blotted with anti-HA and anti-Myc antibodies.

C

PIPKIγi5 truncation mutants:

i5 Δ645: [641]FTDGR[645]
i5 Δ652: [641]FTDGRYWIYSPR[652]
i5 Δ659: [641]FTDGRYWIYSPRHRRLRAV[659]
i5 Δ675: [641]FTDGRYWIYSPRHRRLRAVTLSASGTVSDRSRPPW[675]
i5 WT: [641]FTDGRYWIYSPRHRRLRAVTLSASGTVSDRSRPPWGEGAVPLGQQGAAGPRPEAQCLTSVVFQKGFG[707]
i2 WT: [641]PTDERSWVYSPLHYSAQAPPASDGESDT[668]
i4 WT: [641]WRLWGPHAPTWPWRREGRAACLCPYPPHVVTPFPGTGLCASWSPDGTGGLGAMSCCVSVS[700]

phosphorylation or inhibit SNX5 co-immunoprecipitation (Fig. 5E). Thus, tyrosine phosphorylation likely serves as a negative regulator of the PIPKIγi5–SNX5 complex. Interestingly, kinase-dead PIPKIγi5 has a weaker interaction with SNX5 than wild-type PIPKIγi5, as shown by co-immunoprecipitation (supplementary material Fig. S4A) (Sun et al., 2013). Because kinase-dead PIPKIγi5 is weakly phosphorylated downstream of Src, it is unlikely that the reduced association of SNX5 with kinase-dead PIPKIγi5 is due to inhibition of the association by phosphorylation.

Tyrosine phosphorylation of SNX5 was not detected upon expression of wild-type c-Src, indicating that SNX5 is not a regulatory target of Src (supplementary material Fig. S4B). Additionally, wild-type and kinase-dead Src associated with PIPKIγi5 equally (supplementary material Fig. S4C), suggesting that expression of wild-type Src inhibits the PIPKIγi5–SNX5 association by phosphorylation and not by the physical displacement of SNX5 from PIPKIγi5 by Src binding. Taken together, the data indicate that tyrosine phosphorylation of PIPKIγi5 is a negative regulator of the PIPKIγi5–SNX5 interaction.

There are three tyrosine residues in the C-terminus of PIPKIγi5 within the proposed SNX5-binding region. To determine whether these residues are targets of Src phosphorylation, the tyrosines present in the PIPKIγi5 C-terminus were each mutated to phenylalanine and subjected to an *in vitro* Src kinase assay. As shown in Fig. 5F,G, the Y646F mutation of PIPKIγi5 diminished Src phosphorylation of the C-terminus, whereas Y639F and Y649F mutations showed no change in phosphorylation by Src. Next, we investigated whether these mutations affected the PIPKIγi5–SNX5 interaction. These mutants were expressed from an HA–PIPKIγi5 construct in cells and were assayed for their ability to immunoprecipitate SNX5. The Y646F mutation, which prevents phosphorylation by Src, increased the association of PIPKIγi5 with SNX5, whereas the Y649F mutation decreased this interaction (Fig. 5H). Mutation of both Y646F and Y649F also resulted in a decrease in SNX5 association with PIPKIγi5. These data suggest that phosphorylation of Y646 inhibits the association with SNX5. Alternatively, Y649 is not regulated by Src phosphorylation, but is partially required for the PIPKIγi5–SNX5 interaction.
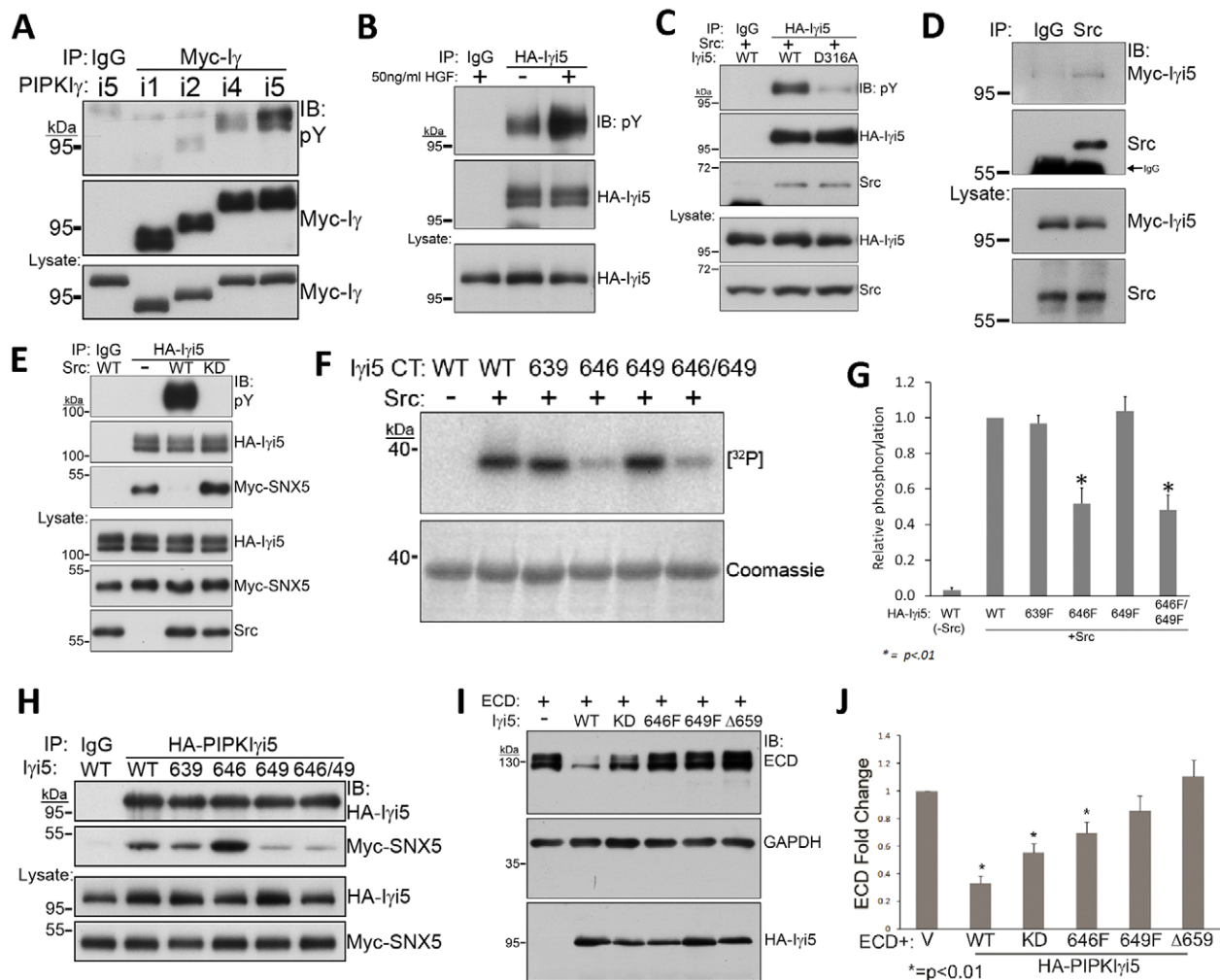
Fig. 5. The association between PIPKIγi5 and SNX5 is regulated downstream of Src. (A) Myc–PIPKIγ splice variants were immunoprecipitated (IP) from HeLa cells cultured in DMEM plus 10% FBS, and their phosphorylation status was assayed by western blotting (IB) with an anti-phosphotyrosine (pY) antibody. Total cell lysates were western blotted for Myc–PIPKIγ as a control. (B) MDCK cells were induced to express HA–PIPKIγi5 for 48 h and were then plated in DMEM plus 0.1% FBS for 16 h. Cells were incubated with (+) or without (−) 50 ng/ml HGF for 5 min, collected and lysed for immunoprecipitation with anti-HA antibody or mouse IgG. The immune complexes and cell lysate controls were then western blotted with the indicated antibodies. (C) Wild-type (WT) or kinase-dead (KD, D316A) PIPKIγi5 was coexpressed with c-Src, immunoprecipitated from HeLa cells as before, and western blotted to assay for tyrosine phosphorylation. Cell lysates were western blotted with the indicated antibodies as controls. (D) Myc–PIPKIγi5 was expressed in HeLa cells, and endogenous Src was immunoprecipitated from cell lysates. The immune complexes and control lysates were then western blotted with anti-Myc and anti-Src antibodies. (E) HA–PIPKIγi5 was coexpressed with Myc–SNX5 and either wild-type or kinase-dead c-Src, and HA–PIPKIγi5 was immunoprecipitated from the cell lysates. Western blotting of the immune complexes and their corresponding lysate controls was then performed with the indicated antibodies. (F) Wild-type or mutant 6His–PIPKIγi5 C-terminus (CT) was incubated with recombinant Src and γ-[^{32}P]-ATP for 15 min at 30°C, and reactions were separated by SDS-PAGE and exposed to autoradiography film for ~12 h. Equivalent amounts of PIPKIγi5 C-terminus substrate proteins were run on a second SDS-PAGE in parallel and stained with Coomassie Blue as an input control. (G) Quantification of the phosphorylation panel shown in F. Autoradiography images were quantified as described in Materials and Methods. Data from four independent experiments was normalized to wild-type PIPKIγi5 C-terminus+Src. (H) HA–PIPKIγi5 point mutants were coexpressed with Myc–SNX5 in HeLa cells. Immunoprecipitation using an anti-HA antibody was then performed as described above. Western blotting of the immunocomplexes and lysates was performed with the indicated antibodies. (I) HA–PIPKIγi5 (Iγi5) point mutants and/or E-cadherin (ECD) were expressed in HeLa cells for 16 h, and total cell lysates were western blotted for the indicated proteins as in Fig. 2. The western blot is representative of 14 independent experiments. (J) Quantification of the western blot depicted in I. Data show the mean±s.e.m.

Src phosphorylation of PIPKIγi5 might affect E-cadherin protein content. Coexpression of PIPKIγi5 with Src did not further enhance E-cadherin loss (supplementary material Fig. S4D,E). This might be due to high basal levels of PIPKIγi5 phosphorylation, and the amount of free PIPKIγi5 in this system might demonstrate a maximal effect. However, coexpression of Src with SNX5 reduced the magnitude of the increase in E-cadherin (supplementary material Fig. S4F,G). One explanation for this observation might be that the PIPKIγi5-mediated degradation

pathway is constantly activated in cells ectopically expressing Src. In this scenario, endogenous PIPKIγi5 maintains phosphorylation at Y646, which would inhibit its association with SNX5, promoting E-cadherin loss.

To examine how the identified truncation and point mutations of PIPKIγi5 affect E-cadherin degradation, E-cadherin was expressed with PIPKIγi5 Y646F, Y649F and Δ659 mutations. As shown in Fig. 5I,J, the Y646F, Y649F and Δ659 mutations had a diminished ability to induce E-cadherin degradation

compared with wild-type PIPKIγi5, and were less effective than kinase-dead PIPKIγi5. In addition to affecting phosphorylation and interaction with SNX5, these mutations might have additional consequences on PIPKIγi5 targeting, kinase activity or protein–protein interactions that impact on the regulation of E-cadherin. It is doubtful that these mutations affect the interaction of PIPKIγi5 with E-cadherin, because the conserved kinase domain in PIPKIγ mediates this interaction (Ling et al., 2007).

PIPKIγi5 and SNX5 function within the defined E-cadherin lysosomal-targeting pathway

Multiple studies have examined the degradation of E-cadherin, as reduced E-cadherin expression correlates with cancer progression (Fujita et al., 2002; Janda et al., 2006; Miyashita and Ozawa, 2007; Palacios et al., 2005; Shen et al., 2008; Toyoshima et al., 2007; Yang et al., 2006). Previously, the trafficking of EGFR from early to late endosomes was found to require PIPKIγi5 prior to lysosomal degradation of the receptor. Therefore, to assess the step in which PIPKIγi5 functions to promote E-cadherin loss, an siRNA screen of known components required for E-cadherin degradation was performed. As E-cadherin in subconfluent cells was found at early endosomes (Fig. 3A), the screen focused on

post-endocytic trafficking pathways. The Rab GTPases regulate specific trafficking steps, with Rab5 functioning in endocytosis and early endosomal trafficking, whereas Rab7 regulates trafficking to late endosomes and plays a role in sorting intraluminal vesicles to the lysosome for degradation (Huotari and Helenius, 2011; Hutagalung and Novick, 2011; Vanlandingham and Ceresa, 2009). E-cadherin that has been targeted for degradation is transported through Rab5- and Rab7-positive compartments, and the expression of Rab5 or Rab7 mutants delays E-cadherin degradation (Palacios et al., 2005). To assess the role of Rab GTPases in PIPKIγi5 function, siRNAs were used to knock down Rab5a or Rab7 protein expression in HeLa cells. The cells were then transfected with E-cadherin with or without PIPKIγi5. When E-cadherin was expressed alone in control, Rab5a- or Rab7-knockdown cells, there was no significant change in E-cadherin expression. Expression of PIPKIγi5 in control or Rab5a-knockdown cells resulted in a reduction in E-cadherin protein levels (Fig. 6A,B). However, knockdown of Rab7 inhibited the effect of PIPKIγi5 on E-cadherin expression. Rab7 appears to be required to mediate E-cadherin loss. This is not surprising, as Rab7 is known to regulate lysosomal trafficking of transmembrane proteins (Palacios et al., 2005). To further assess changes in
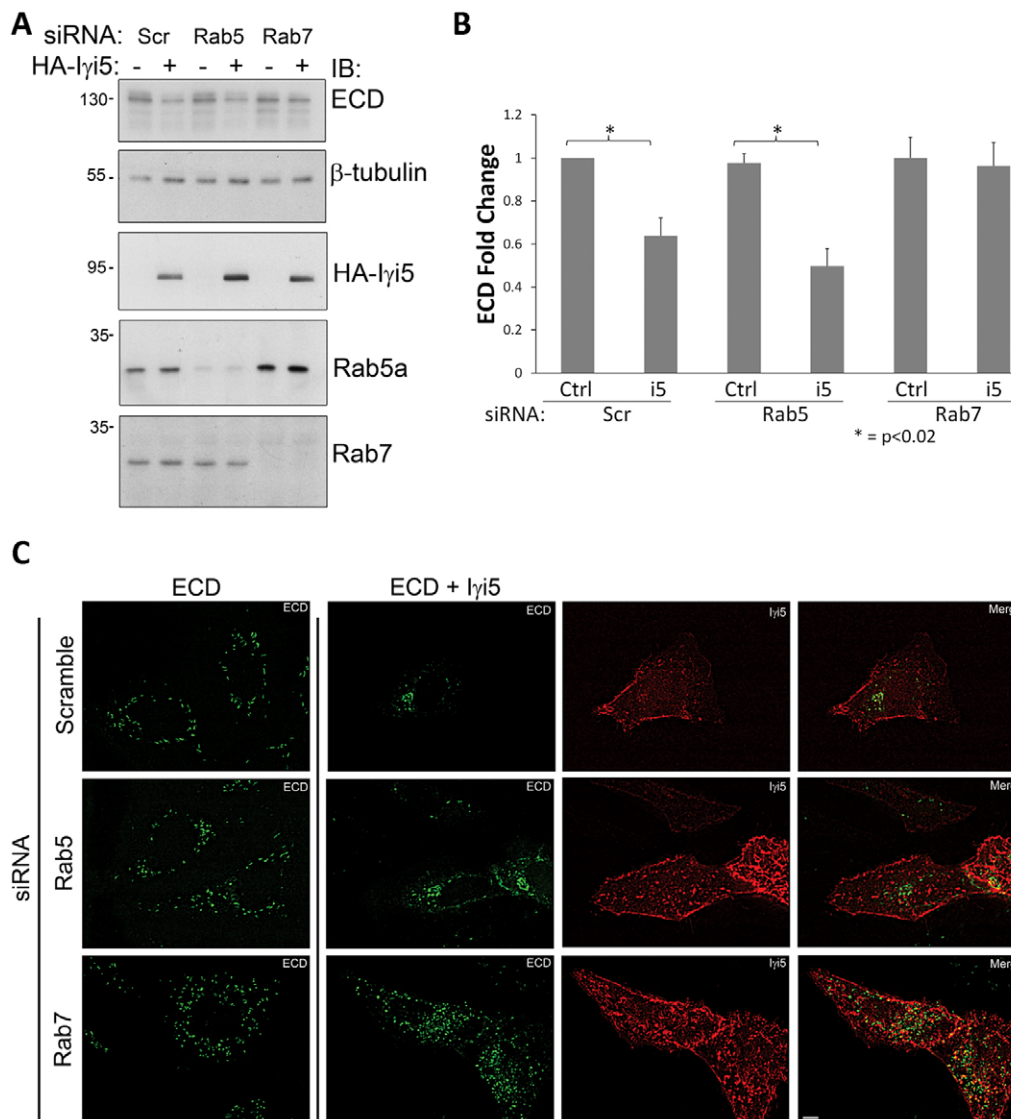
Fig. 6. PIPKIγi5-mediated E-cadherin degradation is Rab7 dependent. (A) HeLa cells were transfected with siRNA pools targeting Rab5a or Rab7 for 48 h, and were then transfected a second time with E-cadherin and HA–PIPKIγi5 constructs for 16 h. Non-targeting siRNA (Scr) was also transfected as a control. Cells were lysed directly into 5× protein loading buffer, separated by SDS-PAGE and western blotted (IB) with the indicated antibodies. β-tubulin was used as a loading control. The western blot is representative of five independent experiments. (B) Quantification of the blot shown in A. Error bars show the mean±s.e.m. (C) Following Rab5a or Rab7 knockdown as in A, HeLa cells were transfected with E-cadherin alone or with the HA–PIPKIγi5 construct. Coverslips were prepared as in Fig. 3, and stained with the indicated antibodies. Scale bar: 10 μm.

phenotype, the Rab5a- and Rab7-knockdown cells were also examined by immunofluorescence microscopy to examine whether E-cadherin localization was affected. When E-cadherin was expressed alone in control, Rab5a- or Rab7-knockdown cells, it maintained a punctate localization throughout the cell (Fig. 6C). However, following expression of PIPKIγi5, E-cadherin shifted to a compact perinuclear localization in control and Rab5a-knockdown cells, whereas expression of PIPKIγi5 in Rab7-knockdown cells results in punctate cytoplasmic localization for E-cadherin, similar to when E-cadherin is expressed alone. This further suggests that PIPKIγi5 requires a Rab7-dependent pathway for E-cadherin trafficking and degradation.

In addition to endosomal trafficking, the sorting of E-cadherin to the lysosome is dependent on its ubiquitylation (Fujita et al., 2002; Palacios et al., 2005). During endosome-to-lysosome trafficking, ubiquitylated E-cadherin is eventually sorted into intraluminal vesicles for degradation. This process is initiated by the ESCRT-0 complex protein Hrs, which interacts with ubiquitylated cargo and recruits additional ESCRT proteins to mediate intraluminal sorting (Henne et al., 2011). Hrs is an important regulator of E-cadherin degradation and is also required for PIPKIγi5-mediated degradation of EGFR (Palacios et al., 2005; Sun et al., 2013; Toyoshima et al., 2007). To assess the role of Hrs in PIPKIγi5-mediated lysosomal degradation of E-cadherin, siRNA was used to knock down endogenous Hrs. The knockdown of Hrs greatly enhanced the expression of E-cadherin. However, PIPKIγi5 expression reduced E-cadherin levels in Hrs-knockdown cells, although not as completely as in control cells (Fig. 7A,B). PIPKIγi5 appears to mediate a rate-limiting step under basal conditions, and its overexpression greatly enhances the sorting of E-cadherin for degradation. Additionally, PIPKIγi5 can directly interact with E-cadherin and this might allow for the degradation of E-cadherin protein in the absence of Hrs.

Of the known functions of SNX5, the best characterized is in the mammalian retromer complex, which regulates the recycling of transmembrane proteins between endosomes and the trans-Golgi network (TGN) (Wassmer et al., 2007; Wassmer et al., 2009), and the retromer has been shown to regulate E-cadherin trafficking to the TGN (Lohia et al., 2012). This pathway consists of the Vps heterotrimer and a SNX dimer (Bonifacino and Hurley, 2008). The knockdown of any component affects trafficking between the TGN and endosomes. As Vps35 is the main subunit of the retromer, siRNA was used to knock down Vps35 to assess its function in the regulation of E-cadherin. Surprisingly, knockdown of Vps35 resulted in a significant increase in E-cadherin protein content. This might be due to defects in the trafficking of lysosomal enzymes or other proteins. However, PIPKIγi5 expression in Vps35-knockdown cells reduced E-cadherin content to a level similar to that of control cells, suggesting enhanced function of this complex or a strong role for PIPKIγi5 in promoting E-cadherin loss, which is independent of Vps35 (Fig. 7C,D). The overexpression of the retromer components SNX5 and SNX6 might affect protein–protein interactions that are necessary for retromer function. To further assess how the retromer, PIPKIγi5, SNX5 and SNX6 might function together to regulate E-cadherin degradation, siRNA studies were also performed to knock down SNX5 and SNX6 individually or together. Although overexpression of these molecules resulted in a significant increase in E-cadherin expression, their knockdown appeared to have no effect on E-cadherin protein levels compared with levels in control cells (Fig. 7E,F). There are multiple possible explanations for this

observation. SNX5 and 6 could function in stabilizing E-cadherin by sequestering PIPKIγi5 or otherwise rendering it unable to push E-cadherin towards a degradation pathway. In support of this hypothesis, PIPKIγi5 overexpression continues to promote E-cadherin degradation after knockdown of SNX5 and SNX6 (Fig. 7E,F). The ability of PIPKIγi5 to promote E-cadherin degradation in the absence of Vps35, SNX5 and SNX6 indicates a retromer-independent pathway that is regulated by PIPKIγi5. However, PIPKIγi5 counteracts the stabilizing effect of SNX5 and SNX6 on E-cadherin.

DISCUSSION

The eventual loss of cell-surface E-cadherin and its subsequent degradation is a principal aspect of EMT. Here, we have identified PIPKIγi5 and SNX5 as regulators of E-cadherin. In this pathway, SNX5 inhibits the lysosomal targeting of E-cadherin. By contrast, PIPKIγi5 promotes E-cadherin degradation within the known lysosomal-degradation pathway, because chloroquine treatment or knockdown of Rab7 can inhibit PIPKIγi5 in this process. Additionally, knockdown of Hrs limits the effect of PIPKIγi5 on E-cadherin, which might be compensated for by additional ESCRT components to promote E-cadherin degradation in the absence of Hrs. These findings add a layer of complexity to the regulation of E-cadherin degradation through PIPKIγi5-generated PI(4,5)P$_2$ and phosphorylation of PIPKIγi5. These points have been summarized as a model depicted in Fig. 8. Generally, SNX5 and PIPKIγi5 associate at endosomal compartments. Stimulation with HGF results in the disassembly of adherens junctions and E-cadherin endocytosis. Src activation downstream of HGF phosphorylates PIPKIγi5, inhibiting the interaction of SNX5 with PIPKIγi5. When SNX5 is absent from the PIPKIγi5–E-cadherin complex, E-cadherin is transported to the lysosome. In this model, PIPKIγi5 promotes E-cadherin degradation, which would facilitate EMT, whereas SNX5 inhibits PIPKIγi5. However, the specific functions of SNX5 alone or in complex with PIPKIγi5 have not been thoroughly examined. There is the potential for SNX5 to regulate E-cadherin recycling while in complex with PIPKIγi5, which could regulate recycling and the biosynthetic trafficking of E-cadherin to the plasma membrane in a manner similar to the interaction between PIPKIγi2 and AP1B (Ling et al., 2007). Consequently, signaling pathways that regulate the association between PIPKIγi5 and SNX5 would have serious implications in tumorigenesis and the metastasis of tumor cells.

Tyrosine phosphorylation of PIPKIγ is a well-documented mechanism to modulate both PIPKIγ activity and protein–protein interactions (Bairstow et al., 2005; Itoh et al., 2000; Lee et al., 2005; Ling et al., 2003; Park et al., 2001; Sun et al., 2007). HGF is known to induce Src activation and E-cadherin endocytosis, ubiquitylation and degradation, leading to the characteristic depolarization and scattering of epithelial cells that is indicative of EMT (Cutrupi et al., 2000; Fujita et al., 2002; Giehl and Menke, 2008; Guarino et al., 2007; Matteucci et al., 2006; Palacios et al., 2005; Shelly and Herrera, 2002; Shen et al., 2008). Our data indicate that Y646 and Y649 in PIPKIγi5 are crucial for regulating its association with SNX5. Mutation of either Y646F or Y649F increased or decreased the association of PIPKIγi5 with SNX5, respectively. Surprisingly, both mutations inhibited the effect of PIPKIγi5 on E-cadherin degradation. This might be due to changes in PIPKIγi5 localization, post-translational modifications or protein–protein interactions. This result confounds the exact mechanism that regulates the association between PIPKIγi5 and SNX5, and further characterization of the PIPKIγi5–SNX5
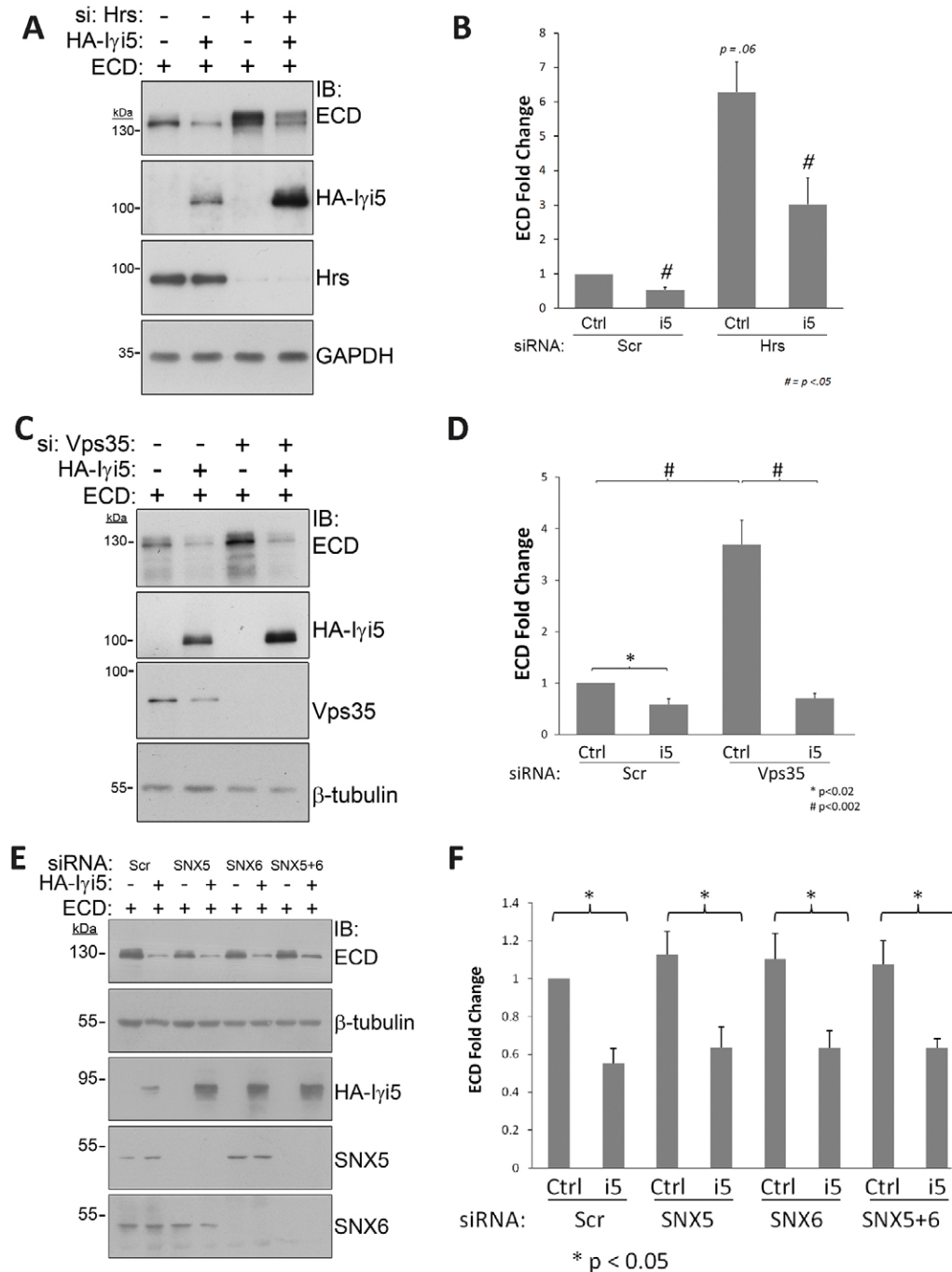
Fig. 7. PIPKIγi5 depends on known degradation pathways, but is retromer independent. (A) HeLa cells were transfected with siRNA pools (si) targeting Hrs for 48 h, and were then transfected a second time with E-cadherin and HA–PIPKIγi5 constructs for 16 h. Non-targeting siRNA (Scr) was also transfected as a control. Cells were lysed directly into 5× protein loading buffer, separated by SDS-PAGE and western blotted (IB) with the indicated antibodies. β-tubulin or GAPDH were used as loading controls. The western blot is representative of four independent experiments. (B) Quantification of the blot shown in A. (C) HeLa cells were prepared as in A, using siRNA to knockdown Vps35. Lysates were then analyzed by western blotting. The western blot is representative of four independent experiments. (D) Quantification of the blot shown in C. (E) HeLa cells were prepared as in A, using siRNA to knock down SNX5 or SNX6, individually and together. Lysates were then analyzed by western blotting. The western blot is representative of four independent experiments. (F) Quantification of the blot shown in E. Data show the mean±s.e.m.

interaction is required to fully understand the regulatory mechanisms at work in this pathway.

Both PIPKIγi2 and PIPKIγi5 interact with E-cadherin and synthesize PI(4,5)P$_2$, but these isoforms have contrasting effects on E-cadherin function. PIPKIγi2 regulates the trafficking and assembly of E-cadherin, whereas PIPKIγi5 regulates the lysosomal degradation of E-cadherin. This distinct effect is likely due to the unique protein–protein interactions and intracellular targeting for each splice variant. PIPKIγi2 and PIPKIγi5 are expressed in the majority of epithelial and non-epithelial cell lines, where they have unique functions. PIPKIγi2 regulates the trafficking of several proteins to and from the plasma membrane (Bairstow et al., 2006; Kahlfeldt et al., 2010; Ling et al.,

2007). The function of PIPKIγi5 is still emerging. We determined previously that PIPKIγi5 regulates the endosomal and intraluminal sorting of EGFR for degradation, through a complex containing Hrs and SNX5 (Sun et al., 2013). Here, we present additional data suggesting that PIPKIγi5 can sort E-cadherin for degradation. However, it should be noted that PIPKIγi5 is relatively low in abundance compared with total PIPKIγ expression; thus, using an overexpression system to examine PIPKIγi5 function is a current limitation for examining endogenous PIPKIγi5 function. It will be interesting to investigate the effects of PIPKIγi5 knockdown, and the development of antibodies that can detect the endogenous protein in epithelial cells will further our understanding of the endogenous localization and function of PIPKIγi5 in E-cadherin
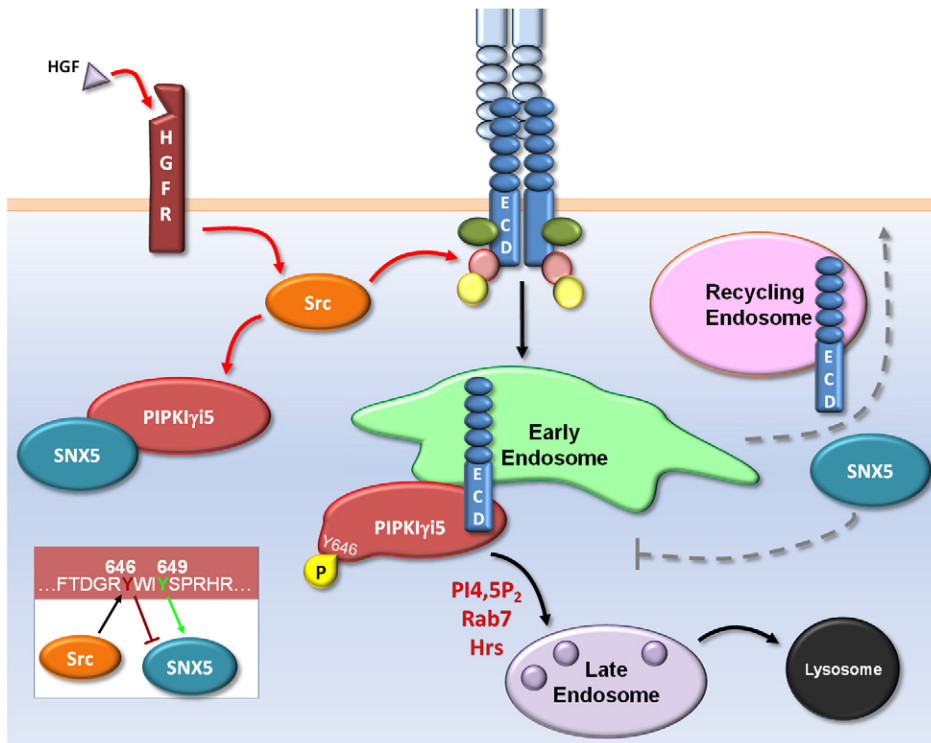
Fig. 8. PIPKIγi5 and SNX5 regulate E-cadherin targeting. A model depicting the proposed functions of PIPKIγi5 and SNX5 in regulating the lysosomal targeting of E-cadherin. HGF stimulation initiates the disassembly of adherens junctions and eventual degradation of E-cadherin. Additionally, HGF activation might also promote Src phosphorylation of PIPKIγi5 on Y646. Following phosphorylation, the interaction of PIPKIγi5 with SNX5 is inhibited, and PIPKIγi5 functions in a pathway to promote the degradation of E-cadherin in a Rab7-dependent manner. Hrs also appears to function in this pathway, but is not an absolute requirement for PIPKIγi5-mediated E-cadherin degradation. This E-cadherin degradation process also appears to require the lipid kinase activity of PIPKIγi5. The role of SNX5 in this pathway appears to be to inhibit the lysosomal targeting of E-cadherin and is potentially mediated by direct inhibition of PIPKIγi5 and other proteins necessary for degradation. Alternatively, it is possible that SNX5, alone or in complex with PIPKIγi5 and E-cadherin, could enhance E-cadherin recycling. However, additional studies are required to define the exact roles for SNX5 in E-cadherin trafficking.

trafficking. The development of improved siRNA and antibodies will be crucial to future studies of this pathway.

We also investigated the role of SNX function in this E-cadherin degradation pathway. Expression of SNX5, or the similar SNX6, resulted in an increase in E-cadherin stability, whereas SNX5 or SNX6 knockdown had no clear effect on E-cadherin and could not prevent PIPKIγi5-mediated E-cadherin loss. The potential for SNX1 regulation by PIPKIγi5 is illustrated by the fact that coexpression of PIPKIγ and SNX1, but not expression of SNX1 alone, resulted in a decrease in E-cadherin levels. As the phosphoinositide-binding preference of SNX1 is for phosphatidylinositol 3-phosphate and phosphatidylinositol 3,5-bisphosphate, it is likely that PIPKIγi5 indirectly regulates SNX1 activity through its association with SNX5 and SNX6. SNX1 was previously shown to promote the recycling of internalized E-cadherin (Bryant et al., 2007); PIPKIγi5 could potentially indirectly inhibit SNX1-mediated recycling of E-cadherin in polarized cells.

PIPKIγi5 regulates both EGFR and E-cadherin lysosomal sorting, with some specificity for these proteins (Sun et al., 2013). Many of the proteins required for endocytosis and lysosomal sorting are shared between EGFR and E-cadherin. At the plasma membrane, EGFR and E-cadherin interact, and this inhibits EGFR activity (Hoschuetzky et al., 1994; Mateus et al., 2007; Pece and Gutkind, 2000; Takahashi and Suzuki, 1996). Following stimulation, both are endocytosed and are found together at intracellular compartments (Bryant et al., 2007; Schill and Anderson, 2009a; Sorkin and Goh, 2008). At endosomes, both proteins are sorted through Rab5- and Rab7-positive compartments, until their eventual intraluminal sorting and lysosomal degradation (Chen et al., 2009; Dinneen and Ceresa, 2004; Palacios et al., 2005; Spinosa et al., 2008; Vanlandingham and Ceresa, 2009). E-cadherin and EGFR are ubiquitylated (Fujita et al., 2002; Levkowitz et al., 1998), and this signal is recognized by the ESCRT proteins (with Hrs recognizing ubiquitylated E-cadherin or EGFR) and is essential for their

degradation (Chin et al., 2001; Lloyd et al., 2002; Palacios et al., 2005; Raiborg et al., 2001). Therefore, any of these shared steps might be regulated by PIPKIγi5. siRNA-mediated knockdown of Rab7, but not Rab5, inhibited PIPKIγi5-induced E-cadherin degradation, suggesting that Rab7 is essential for the sorting of E-cadherin from endosomes to lysosomes in response to PIPKIγi5. In addition, Hrs knockdown substantially increased E-cadherin levels, but PIPKIγi5-induced degradation was only marginally inhibited by Hrs knockdown. This indicates that Hrs might not be required for PIPKIγi5-regulated E-cadherin degradation, due to the presence of alternative ubiquitin adaptors or the recruitment of additional sorting machinery by PIPKIγi5. There might be several effectors for $PI(4,5)P_2$ that contribute to this process. The best characterized appears to be SNX5, which can interact with PIPKIγi5 and the ubiquitin adaptor Hrs. Because Hrs is not absolutely required for PIPKIγi5 to exert its degradative effects on E-cadherin, there might be additional ESCRT- and $PI(4,5)P_2$-binding proteins present at endosomes that assist in this process. Additionally, $PI(4,5)P_2$ has been detected at endosomes, where proteins like clathrin, actin and Rab GTPases (such as RAB10) have all been implicated in endosomal $PI(4,5)P_2$ signaling pathways (Arneson et al., 1999; Rong et al., 2012; Shi et al., 2012; Vicinanza et al., 2008; Vicinanza et al., 2011; Watt et al., 2002).

One key difference between EGFR and E-cadherin is the interaction of these proteins with PIPKIγi5 and SNX5. PIPKIγi5 can directly interact with E-cadherin through its kinase domain, which might allow for direct PIPKIγi5-mediated control of the lysosomal degradation of E-cadherin. By contrast, EGFR degradation requires both PIPKIγi5 and SNX5 (Sun et al., 2013). Therefore, PIPKIγi5 might regulate the function of SNX5 through the synthesis of $PI(4,5)P_2$ to promote the lysosomal sorting of EGFR. Further study is necessary to identify the components in these sorting pathways that are specifically required for EGFR and E-cadherin.

E-cadherin is well characterized as a tumor suppressor, and loss of E-cadherin protein is associated with cancer initiation and

progression (Mosesson et al., 2008; Wijnhoven et al., 2000). There are also roles for PIPKIγ and SNX5 in cancer biology. Expression of PIPKIγ in breast tumors was found to correlate with poor patient prognosis (Sun et al., 2010), whereas variations in SNX5 and SNX6 mRNA levels were observed in some cancers (Liu et al., 2007; Shipitsin et al., 2007). Here, we have uncovered new players in the governance of E-cadherin degradation. The association between PIPKIγi5 and SNX5 represents a decision point, with SNX5 promoting the retention of E-cadherin, and PIPKIγi5 promoting its loss. Upsetting the balance of this signaling pathway by mutation or disease could theoretically lead to degradation of the E-cadherin pool that is needed to maintain adhesion. Because E-cadherin loss has implications in human disease, this signaling pathway might prove to be a target for therapeutic intervention. The expression of PIPKIγi5 might have a broad influence on cellular function by controlling the level of certain proteins, such as E-cadherin and EGFR.

MATERIALS AND METHODS

Expression constructs

Human PIPKIγ splice variants, SNX5, SNX6 and SNX1 were amplified by PCR for insertion into the pCMV-Myc and pCMV-HA vectors (Clontech). PIPKIγ point mutations and truncation mutants were generated using PCR primer overlap extension with primers containing the desired mutations. The E-cadherin expression vector was generated by PCR amplification of the human E-cadherin coding region and insertion into the pcDNA 3 vector (Invitrogen). c-Src expression vectors were created as described previously (Ling et al., 2003). Validated siRNA pools targeting SNX5, SNX6, Hrs, Vps35, Rab5a and Rab7, and non-targeting control oligo were obtained from Santa Cruz Biotechnology.

Antibodies

Polyclonal antibodies towards PIPKIγ splice variants were created as described previously (Ling et al., 2002; Schill and Anderson, 2009b). Commercially available antibodies were obtained as follows: mouse and rabbit IgG, anti-phosphotyrosine (4G10), anti-Myc (4A6), anti-Src, anti-β-tubulin and anti-HA polyclonal antibody were from Upstate; unconjugated and FITC- or Alexa-Fluor-647-conjugated anti-E-cadherin monoclonal antibody (mAb), anti-EEA1, anti-GM-130, anti-N-cadherin and anti-TfnR were from BD Biosciences; anti-SNX5, anti-SNX6, anti-Hrs, anti-SNX1, anti-Rab5a and anti-Rab7 were from Santa Cruz Biotechnology; anti-HA (HA.11) was from Covance; anti-LAMP-1 was from Abcam; Alexa-Fluor-350-, Alexa-Fluor-488-, Alexa-Fluor-555-, Alexa-Fluor-647- and Pacific-Blue-conjugated secondary antibodies were from Molecular Probes; and HRP-conjugated secondary antibodies for western blotting were from Jackson Immunoresearch.

Cell culture and transfection

PIPKIγi5 stable MDCK-TetOff cell lines (Clontech) were constructed and cultured as described previously (Ling et al., 2007). Expression was induced by a 72 h withdrawal of doxycycline. HeLa cells were plated at 5.5×10^5 cells per 60 mm plate, 2×10^5 per six-well plate or 0.6×10^5 per 12-well plate in DMEM supplemented with 10% FBS overnight before transfection with Lipofectamine 2000 or Oligofectamine (Invitrogen). For immunofluorescence and E-cadherin degradation assays in HeLa cells, cells were harvested for analysis ~16 h post-transfection. For siRNA-mediated knockdown of SNX5, SNX6, Vps35, Rab5a, Rab7 and Hrs, cells were incubated for 72 h before analysis.

Indirect immunofluorescence microscopy

MDCK and HeLa cells were grown on glass coverslips placed inside six-well plates until analysis. Coverslips were washed twice with PBS, fixed with 4% paraformaldehyde, then permeabilized with 0.5% Triton X-100 in PBS. Next, cells were blocked with 3% bovine serum albumin (BSA). Cells were treated with 1 µM LysoTracker Red DND-99 (Invitrogen) in DMEM for 30 min prior to fixation. Incubation with the primary

antibody was performed at 37℃ for 1 h or at 4℃ overnight. Incubation with fluorophore-conjugated secondary antibodies was performed at 37℃ for 30–45 min. Cells were washed between incubation steps with 0.1% Triton X-100 in PBS. Indirect immunofluorescence microscopy was performed using a 60× plan-fluor objective on a Nikon Eclipse TE2000U with a Photometrics CoolSNAP ES CCD camera. Images were captured using MetaMorph v6.3 (Molecular Devices) and processed with the '2D deconvolution' application. Images were exported to Photoshop CS2 (Adobe) for final processing and assembly.

Immunoprecipitation and immunoblotting

For immunoprecipitation of endogenous E-cadherin and PIPKIγ, cells were lysed in a solution containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% NP-40, 1 mM EDTA and 10% glycerol, supplemented with Roche Complete Mini Protease Inhibitor tablets. Analysis of the immunoprecipitates were performed as described previously (Ling et al., 2007). Immunoprecipitation of PIPKIγi5 with SNX5 was performed as described previously (Towler et al., 2004), with modifications. Briefly, cells were lysed in a solution containing 100 mM NaCl, 50 mM HEPES, 5 mM $MgCl_2$, 0.5% NP-40 and 2 mM Na_3VO_4, supplemented with protease inhibitors. Protein concentrations were calculated using the bicinchoninic acid (BCA) assay (Bio-Rad Laboratories), according to the manufacturer's instructions. PageRuler Prestained Protein Ladder (Fermentas) or Benchmark Prestained Protein Ladder (Invitrogen) was used as the molecular mass standard for western blotting. All immunoprecipitations were performed at least three times, and the results shown are representative of all experiments.

Phosphorylation studies

For steady-state phosphorylation, HeLa cells were transfected with the indicated constructs for 5 h in DMEM supplemented with 10% FBS, then the medium was changed to fresh DMEM with 10% FBS for incubation overnight. For growth factor stimulation studies, HeLa cells were transfected for 5 h in DMEM with 10% FBS, then were washed in PBS and replated with 0.1% FBS in DMEM overnight. For HGF stimulation, doxycycline was withdrawn from the MDCK-PIPKIγi5 stable cell lines for 48 h, then the cells were serum starved overnight. 50 ng/ml HGF (Sigma-Aldrich) was added for the indicated times prior to cell lysis. Cells were scraped into a solution containing 50 mM Tris-HCl pH 7.2, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 50 mM NaCl, 2 mM EDTA, 2 mM Na_3VO_4 and protease inhibitors, sonicated briefly and lysed for 30 min at 4℃. Myc- or HA-tagged PIPKIγ was immunoprecipitated with 3 µg of anti-HA or anti-Myc mAb for 16 h at 4℃. Lysates were then incubated for 1 h with Protein-G–Sepharose-4B (Amersham) to extract the immunocomplexes. The immunoprecipitates were then western blotted with 0.1 µg/ml anti-phosphotyrosine (4G10) mAb, as per the manufacturer's instructions.

For the *in vitro* Src phosphorylation studies, 7 µg of wild-type or mutant 6His-tagged PIPKIγi5 C-terminus (residues 485–707) was combined with 10 µCi of γ-[^{32}P]-ATP and 1 unit of human recombinant Src (Millipore) in 25 mM Tris-HCl pH 7.2, 30 mM $MgCl_2$, 5 mM $MnCl_2$, 500 µM EGTA, 500 µM DTT and 100 µM ATP for 15 min at 30℃. Reactions were terminated by the addition of 2× protein loading buffer (giving a final concentration of 12 mM Tris-HCl pH 6.8, 5% glycerol, 0.4% SDS and 1.25% β-mercaptoethanol) and separated by SDS-PAGE. [^{32}P] signal intensity was quantified using Quantity One (Bio-Rad), and statistical analysis of the data was performed in Microsoft Excel. Error bars indicate the standard error of the mean (s.e.m.) of each data set. Significance was calculated using Student's two-tailed *t*-test. All phosphorylation experiments were performed at least three times, and the results shown are representative of all experiments.

Quantification of E-cadherin degradation

HeLa cells were plated at 0.6×10^5 cells per well of a 12-well plate and were transfected with Lipofectamine 2000 and DNA expression vectors at ~32 h after plating. Cell confluency was ~60% at the time of transfection. 40 µM chloroquine was included in the medium where indicated. After 16 h, cells were lysed directly in 2× protein loading

buffer, and cell lysates were then quantified by using a modified Bradford absorbance assay. Protein samples were western blotted with the indicated antibodies. After developing western blots, the film was scanned using a transmitted-light scanner. Protein bands were quantified using ImageJ, and statistical analysis of the data was performed in Microsoft Excel. E-cadherin expression was normalized to that of β-tubulin or GAPDH. Error bars indicate the s.e.m. of each data set. Significance was calculated using Student's two-tailed t-test.

qPCR analysis of E-cadherin mRNA

HeLa cells were plated at 2×10^5 per well of a six-well plate and transfected as described above. Total cellular RNA was extracted using the RNeasy Mini extraction kit (Qiagen), according to the manufacturer's directions. 1 μg of RNA was reverse transcribed using Transcriptor (Roche), and the resulting cDNA was used for qPCR analysis on a Bio-Rad iQ5 system. Primers for E-cadherin and GAPDH were as follows: ECD R1 Fwd, 5′-AGGCCAAGCAGCAGTACATT-3′; ECD R1 Rev, 5′-AGGCCAAGCAGCAGTACATT-3′; ECD R2 Fwd, 5′-TGAGTGTCC-CCCGGTATCTTC-3′; ECD R2 Rev, 5′-CAGTATCAGCCGCTTTC-AGATTTT-3′; GAPDH Fwd, 5′-GAAGGTCGGAGTCAACGGATTT-3′; GAPDH Rev, 5′-GAATTTGCCATGGGTGGAAT-3′. Data were analyzed by the Pfaffl method, using GAPDH as the reference gene. The data shown are the result of five independent experiments, with internal duplicates for each experiment. Error bars indicate the s.e.m. of each data set. Significance was calculated using Student's two-tailed t-test.

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