

RESEARCH ARTICLE

Isoform 5 of PIPKI γ 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)P₂] to activate specific signaling cascades required for cellular processes. Several C-terminal splice variants of PIPKI γ (also known as PIP5K1C) exist, and have been implicated in a multitude of cellular roles. PI(4,5)P₂ serves as a fundamental regulator of E-cadherin transport, and PI(4,5)P₂-generating enzymes are important signaling relays in these pathways. We present evidence that the isoform 5 splice variant of PIPKI γ (PIPKI γ i5) associates with E-cadherin and promotes its lysosomal degradation. Additionally, we show that the endosomal trafficking proteins SNX5 and SNX6 associate with PIPKI γ i5 and inhibit PIPKI γ i5-mediated E-cadherin degradation. Following HGF stimulation, activated Src directly phosphorylates PIPKI γ i5. Phosphorylation of the PIPKI γ i5 C-terminus regulates its association with SNX5 and, consequently, E-cadherin degradation. Additionally, this PIPKI γ i5-mediated pathway requires Rab7 to promote degradation of internalized E-cadherin. Taken together, the data indicate that PIPKI γ i5 and SNX5 are crucial regulators of E-cadherin sorting and degradation. PIPKI γ i5, SNX and phosphoinositide regulation of lysosomal sorting represent a novel area of PI(4,5)P₂ signaling and research. PIPKI γ i5 regulation of E-cadherin sorting for degradation might have broad implications in development and tissue maintenance, and enhanced PIPKI γ i5 function might have pathogenic consequences due to downregulation of E-cadherin.

KEY WORDS: PIPKI γ , 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)P₂]. The unique intracellular targeting of each member allows for the spatial and temporal control of the synthesis of PI(4,5)P₂, 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)P₂ 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)P₂ 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)P₂ 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 PIPKI γ (PIPKI γ i1, 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). PIPKI γ i1 consists of 640 amino acids and is localized to the plasma membrane and cytoplasm. The i1 N-terminal sequence is conserved in PIPKI γ i2, 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 PIPKI γ i1–i6 in mammalian cells, here, we use the HUGO nomenclature for splice variants (PIPKI γ 87 or PIPKI γ 640 is referred to as PIPKI γ i1, PIPKI γ 90 or PIPKI γ 668 is referred to as PIPKI γ i2) (Schill and Anderson, 2009b; Xia et al., 2011). Specific isoforms have distinct functions. For example, PIPKI γ i2 regulates focal adhesion dynamics and vesicle trafficking (Di Paolo et al., 2002; Kahlfeldt et al., 2010; Ling et al., 2002). In epithelial cells, PIPKI γ i2 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, PIPKI γ i2 functionally links N-cadherin cell–cell junctions to regulated actin assembly (El Sayegh et al., 2007). These findings position PIPKI γ as a crucial regulator of the assembly of cell–cell contacts and the intracellular transport of components of these complexes. Recently, PIPKI γ i5 was found to localize to endosomes and interact with the PI(4,5)P₂ 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 PIPKI γ i1, i2, i4 and i5, here, we examined the role of PIPKI γ i5 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)P₂ controls multiple aspects of E-cadherin transport; thus, PI(4,5)P₂-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 CBL1) (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 PIPKIγ5 through its PX domain and binds to phosphoinositides, including PI(4,5)P₂, 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 PIPKIγ5 associates with E-cadherin and promotes E-cadherin lysosomal degradation. However, SNX5 acts antagonistically to PIPKIγ5, preventing E-cadherin degradation. Importantly, PIPKIγ5 appears to function as part of a defined E-cadherin degradation pathway. Furthermore, tyrosine phosphorylation of PIPKIγ5 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

PIPKIγ5 associates with E-cadherin *in vivo* and promotes E-cadherin degradation

PIPKIγ2 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. PIPKIγ2 and PIPKIγ5, but not PIPKIγ4, 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 PIPKIγ5 colocalized with E-cadherin, HA-PIPKIγ5 was inducibly expressed in stably transfected MDCK cell lines, and the cells were processed for immunofluorescence microscopy. As shown in Fig. 1B, PIPKIγ5 colocalized with E-cadherin at cell–cell contacts and intracellular compartments. The association and localization of PIPKIγ5 with E-cadherin suggested that it might regulate E-cadherin biology.

Previously, PIPKIγ5 was shown to regulate the lysosomal degradation of EGFR (Sun et al., 2013). To determine whether

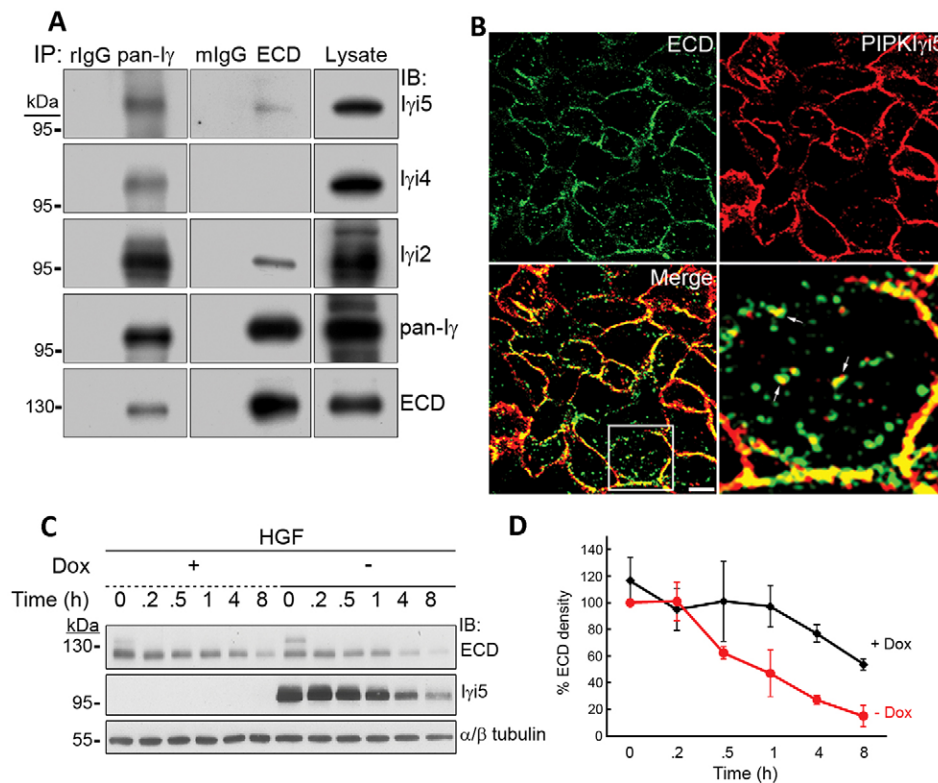


Fig. 1. Multiple PIPKIγ splice variants associate with E-cadherin. (A) Endogenous E-cadherin (ECD) and PIPKIγ (pan-γ) were immunoprecipitated (IP) from MCF10A cell lysates, and the immunocomplexes and cell lysates were western blotted (IB) with antibodies against each PIPKIγ splice variant or E-cadherin. 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-PIPKIγ5 for 72 h. Cells were then fixed and stained for HA-PIPKIγ5 (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-PIPKIγ5 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.

PIPKI γ 5 controls the lysosomal sorting of E-cadherin, MDCKs grown in the presence or absence of doxycycline (to control PIPKI γ 5 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 PIPKI γ 5 displayed an enhanced rate of E-cadherin degradation in response to HGF treatment (Fig. 1C,D). Furthermore, the expression of PIPKI γ 1, PIPKI γ 2 or a kinase-dead D316A mutant of PIPKI γ 5 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 PIPKI γ 5 (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 PIPKI γ 5-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 PIPKI γ 5 at distinct intracellular compartments and enlarged vesicles. These data suggest that PIPKI γ 5 might enhance the targeting of E-cadherin to intracellular compartments upon stimulation with HGF and that E-cadherin might be sorted through PIPKI γ 5-positive compartments prior to its degradation.

PIPKI γ 5 and SNX5 play opposing roles in E-cadherin stability

SNX5 and PIPKI γ 5 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 PIPKI γ 5 or SNX5, and the E-cadherin protein content in cells was quantified. In these experiments, PIPKI γ 5 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 PIPKI γ 5 reduced E-cadherin content by 25% compared with control cells, indicating that PIPKI γ 5 counteracts the increase in the amount of E-cadherin observed upon SNX5 expression. To confirm that the loss of E-cadherin observed upon PIPKI γ 5 expression was due to lysosomal degradation, HeLa cells expressing E-cadherin and PIPKI γ 5 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 PIPKI γ 5 and SNX5 was similar to the levels observed when PIPKI γ 5 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 PIPKI γ 5, 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 PIPKI γ 5 or SNX5.

To determine whether the generation of PI(4,5)P₂ was required for PIPKI γ 5 to destabilize E-cadherin, wild-type or kinase-dead PIPKI γ 5 were expressed and the cellular levels of E-cadherin were assayed. As shown in Fig. 2C,D, kinase-dead PIPKI γ 5 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 PIPKI γ 5, 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 PIPKI γ 5 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)P₂ by PIPKI γ 5 or the binding of PIPKI γ 5 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 PIPKI γ 5, but not other PIPKI γ 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 PIPKI γ 5, it is likely that SNX6 is subject to regulation by PIPKI γ 5, 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 PIPKI γ 5-induced E-cadherin degradation, we coexpressed PIPKI γ 5 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 PIPKI γ 5, again suggesting that SNX5 and SNX6 promote E-cadherin stability, which is counteracted by PIPKI γ 5. However, coexpression of PIPKI γ 5 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 PIPKI γ 5 might indirectly regulate SNX1 activity. As SNX1 has been shown to promote the recycling of internalized E-cadherin (Bryant et al., 2007), PIPKI γ 5 potentially indirectly inhibits SNX1-mediated recycling of E-cadherin.

PIPKI γ 5 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,

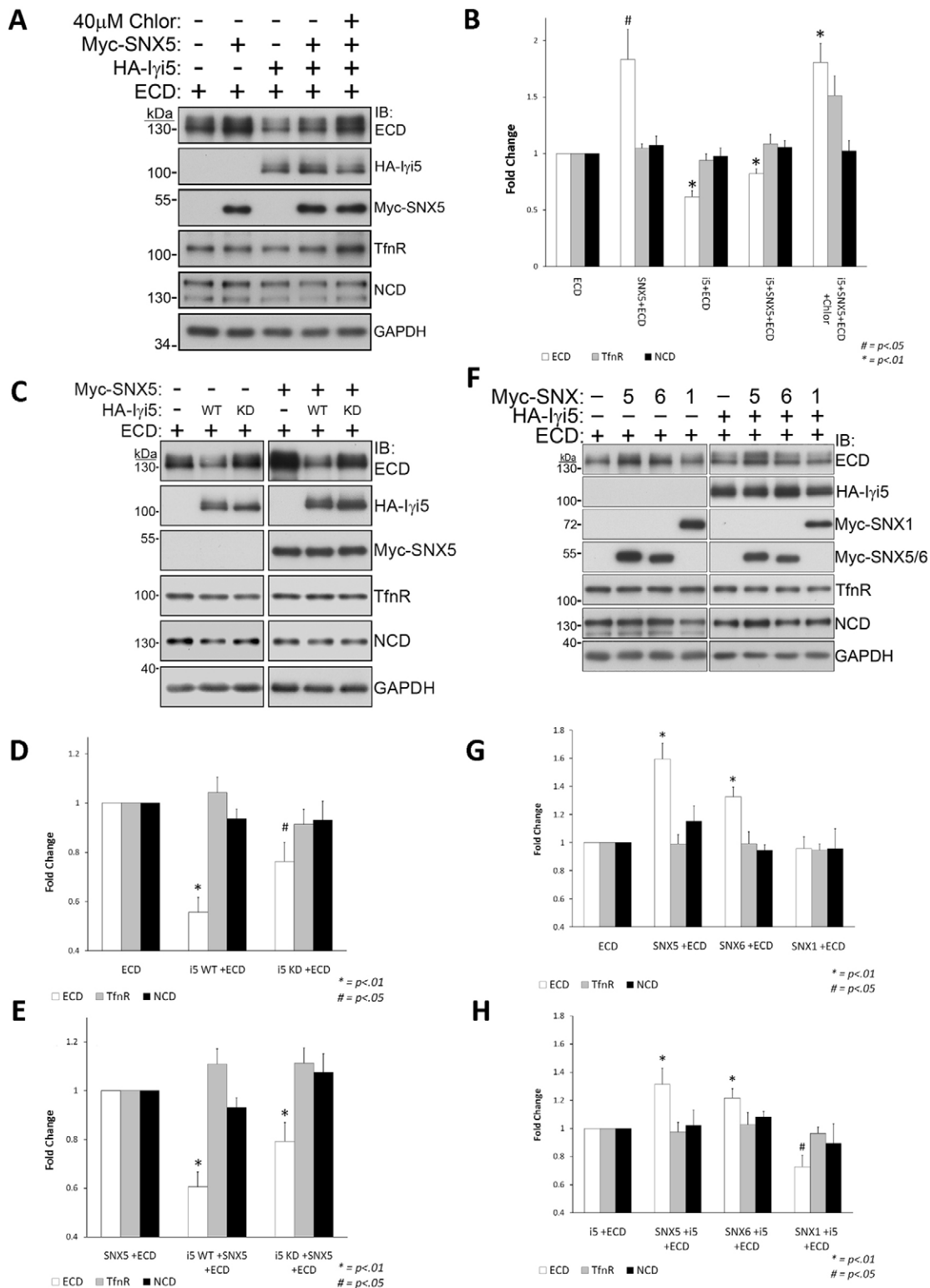


Fig. 2. PIPKIγi5 and SNX5 play opposing roles in E-cadherin stability. (A) HA-PIPKIγi5 (Iγi5), Myc-SNX5 and/or E-cadherin (E-cadherin) were expressed in HeLa cells for 16 h and total cell lysates were western blotted (IB) for the indicated proteins. Cellular levels of transferrin receptor (TfR), N-cadherin (N-cadherin) 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 E-cadherin 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 E-cadherin 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

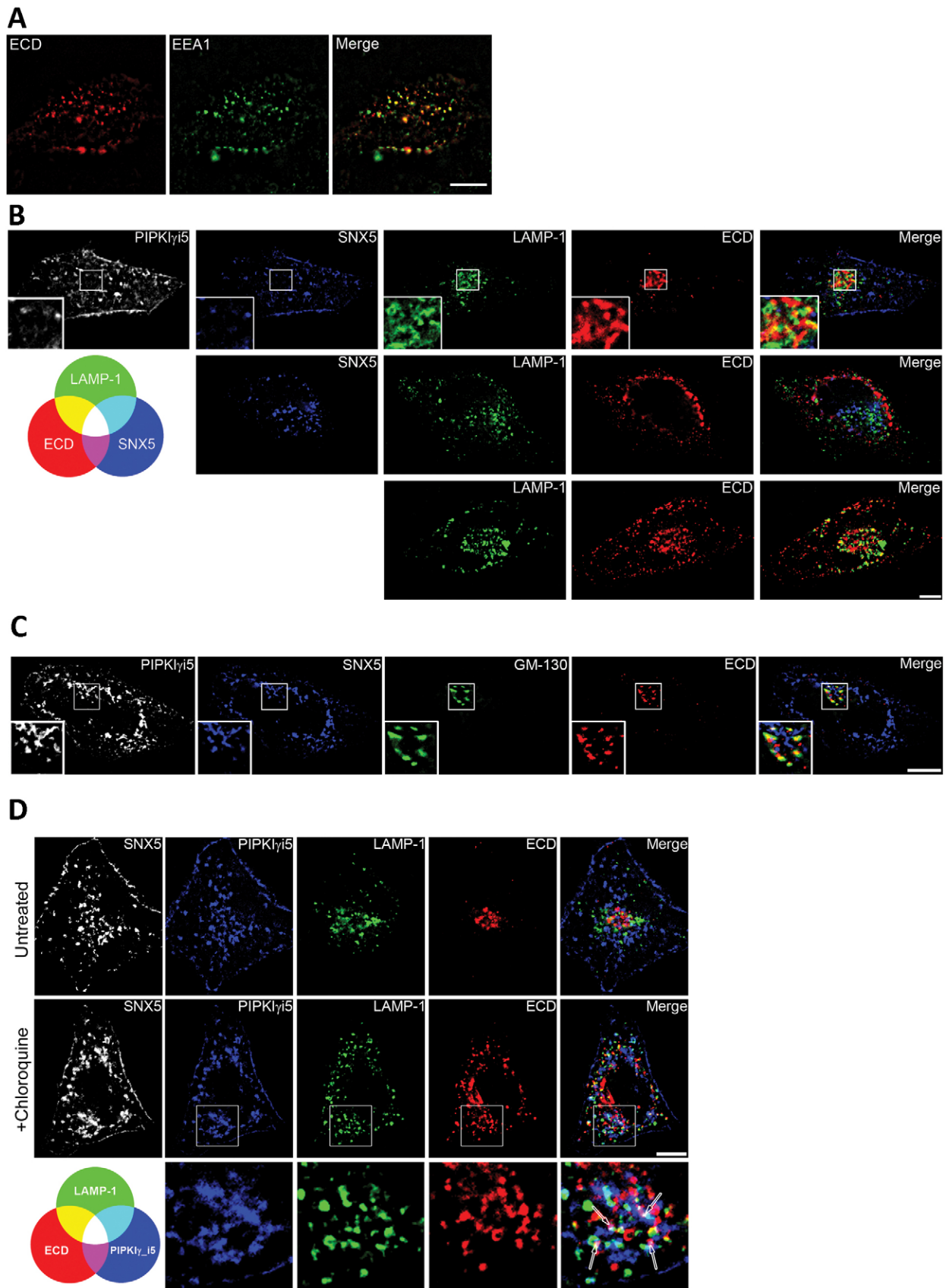


Fig. 3. See next page for legend.

Fig. 3. PIPKI γ 5 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 PIPKI γ 5 for 16 h before fixation. Coverslips were then stained with antibodies against HA-PIP KI γ 5, 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 PIPKI γ 5 for 16 h before fixation. Coverslips were then stained with antibodies against HA-PIP KI γ 5, 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 μ M chloroquine and processed for immunofluorescence as described in B. Inset, 200% enlargement of the outlined area. Arrows, regions of colocalization between PIPKI γ 5, E-cadherin and LAMP-1. Scale bars: 10 μ m.

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 PIPKI γ 5, 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 PIPKI γ 5 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 PIPKI γ 5. The compact perinuclear E-cadherin distribution observed when PIPKI γ 5 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 PIPKI γ 5 (Fig. 3D).

We also tested whether E-cadherin degradation could be mediated by the proteasome. Cells expressing PIPKI γ 5, 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 PIPKI γ 5 and SNX5 into large cytoplasmic clusters (supplementary material Fig. S3B), suggesting that MG-132 interferes with PIPKI γ 5 targeting. Therefore, the lack of PIPKI γ 5-mediated E-cadherin degradation might be an artifact due to the mis-targeting of PIPKI γ 5 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 PIPKI γ 5 is mediated by the lysosome.

PIP KI γ 5 interacts with SNX5

Our previous work identified a specific interaction between the unique C-terminal sequence of PIPKI γ 5 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-PIP KI γ 5 antibody. As shown in

Fig. 4A, PIPKI γ 5 was detected in the SNX5 immunoprecipitates. To confirm that SNX5 associates with the unique portion of the PIPKI γ 5 C-terminus, an HA-tagged version of each PIPKI γ splice variant was coexpressed with Myc-SNX5 in HeLa cells, and Myc-SNX5 was immunoprecipitated from cell lysates. As shown in Fig. 4B, only PIPKI γ 5 associated with Myc-SNX5. To determine the region within the PIPKI γ 5 C-terminus that associates with SNX5, C-terminal truncation mutants of PIPKI γ 5 were created and coexpressed with Myc-SNX5 in HeLa cells (Fig. 4C,D). Although deletion of residues 675–707 (Δ 675) of PIPKI γ 5 did not inhibit its association with SNX5, truncation at residue 659 (Δ 659) abolished binding to SNX5 (Fig. 4D). The combined data confirms that the C-terminus of PIPKI γ 5 mediates the association with SNX5, and narrows the binding region to residues 640–675 of PIPKI γ 5.

Tyrosine phosphorylation regulates PIP KI γ 5 and its association with SNX5

The tyrosine kinase Src is known to phosphorylate PIPKI γ 2 to regulate its association with talin (Bairstow et al., 2005; de Pereda et al., 2005; Lee et al., 2005; Ling et al., 2003). PIPKI γ 2 is also regulated by tyrosine phosphorylation downstream of growth factor receptors (Sun et al., 2007). The unique C-terminus of PIPKI γ 5 contains a tyrosine motif similar to the Src phosphorylation site in PIPKI γ 2 (Schill and Anderson, 2009b) (Fig. 4C). Therefore, we investigated whether PIPKI γ 5 is phosphorylated downstream of Src and, consequently, whether the association of PIPKI γ 5 and SNX5 could be regulated by tyrosine phosphorylation. To determine whether PIPKI γ 5 was tyrosine phosphorylated, the phosphorylation status of PIPKI γ splice variants in HeLa cells cultured in 10% FBS was examined by immunoprecipitation of overexpressed PIPKI γ and western blotting with an anti-phosphotyrosine antibody. Fig. 5A illustrates that the PIPKI γ splice variants are tyrosine phosphorylated to varying degrees. Interestingly, these results also suggest that PIPKI γ 5 is more strongly phosphorylated than other PIPKI γ splice variants under these conditions. Moreover, when MDCK cells expressing PIPKI γ 5 were stimulated with HGF, tyrosine phosphorylation of PIPKI γ 5 was enhanced (Fig. 5B). These data suggest that PIPKI γ 5 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 PIPKI γ 5. To explore this, PIPKI γ 5 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 PIPKI γ 5 was robustly phosphorylated upon c-Src coexpression. Kinase-inactive PIPKI γ 5 might be a poor substrate for Src, or the activity of the protein tyrosine phosphatase SHP-1 (also known as PTPN6) that associates with PIPKI γ might be inhibited by PI(4,5)P₂ (Bairstow et al., 2005). When overexpressed PIPKI γ 5 is immunoprecipitated, c-Src physically associates with PIPKI γ 5 (Fig. 5C). To confirm the association of PIPKI γ 5 with Src, endogenous Src was immunoprecipitated from HeLa cells expressing Myc-PIP KI γ 5 and assayed by western blotting. The results in Fig. 5D illustrate that endogenous Src associates with PIPKI γ 5. To determine whether Src activity regulates the association between PIPKI γ 5 and SNX5, c-Src was coexpressed with HA-PIP KI γ 5 and Myc-SNX5 in HeLa cells and HA-PIP KI γ 5 was immunoprecipitated from cell lysates. Expression of wild-type c-Src corresponded with a substantial increase in PIPKI γ 5 tyrosine phosphorylation and coincident loss of SNX5 association, whereas kinase-dead Src did not affect PIPKI γ 5

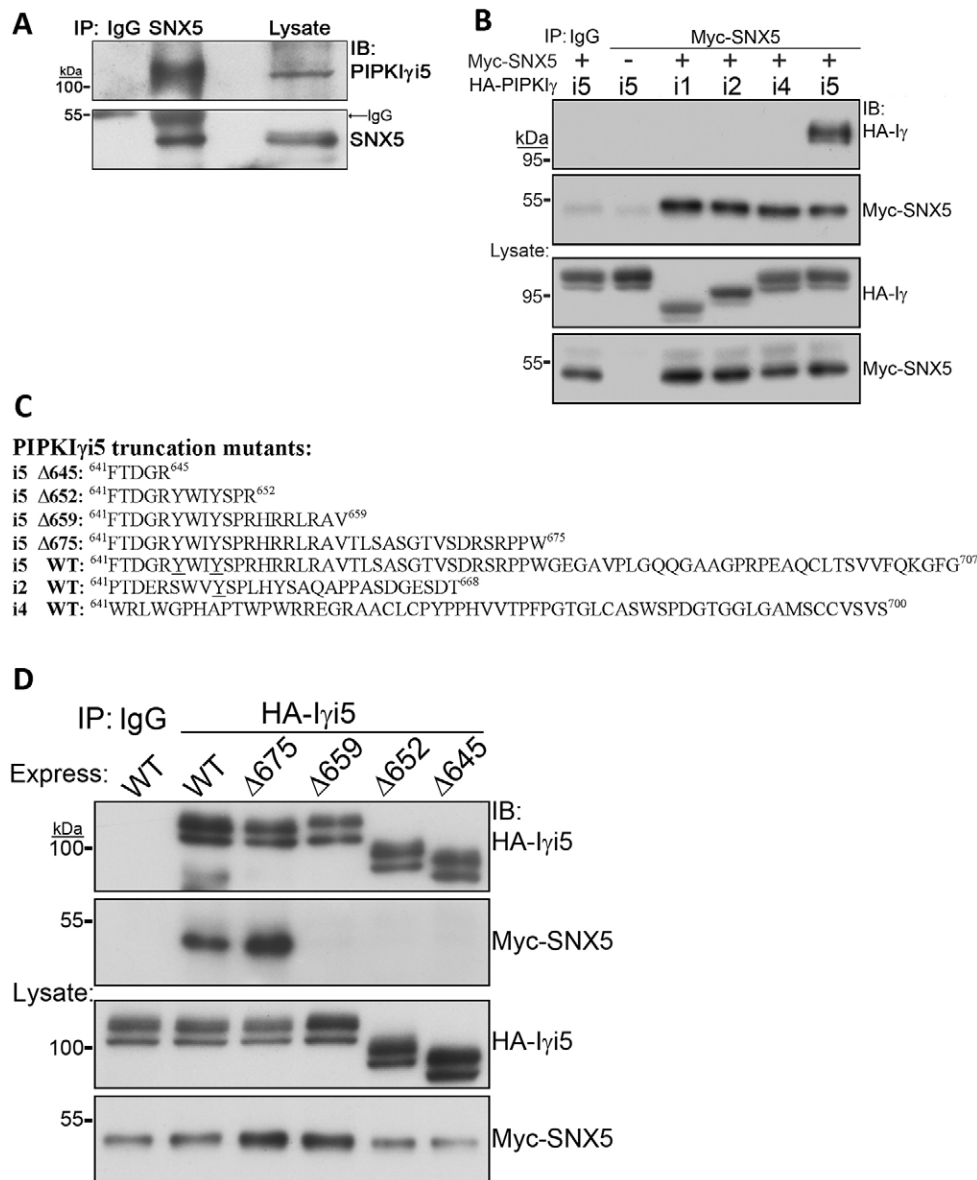


Fig. 4. PIPKI γ i5 associates with SNX5 *in vivo* through its unique C-terminus.

(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-PIPKI γ i5 polyclonal antibodies. (B) HA-PIPKI γ 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 PIPKI γ i2, i4, i5 and i5 truncation mutants. Tyrosine 646 in PIPKI γ i5 and tyrosine 649 in PIPKI γ i2 and i5 are underlined. WT, wild type. (D) PIPKI γ i5 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.

phosphorylation or inhibit SNX5 co-immunoprecipitation (Fig. 5E). Thus, tyrosine phosphorylation likely serves as a negative regulator of the PIPKI γ i5–SNX5 complex. Interestingly, kinase-dead PIPKI γ i5 has a weaker interaction with SNX5 than wild-type PIPKI γ i5, as shown by co-immunoprecipitation (supplementary material Fig. S4A) (Sun et al., 2013). Because kinase-dead PIPKI γ i5 is weakly phosphorylated downstream of Src, it is unlikely that the reduced association of SNX5 with kinase-dead PIPKI γ i5 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 PIPKI γ i5 equally (supplementary material Fig. S4C), suggesting that expression of wild-type Src inhibits the PIPKI γ i5–SNX5 association by phosphorylation and not by the physical displacement of SNX5 from PIPKI γ i5 by Src binding. Taken together, the data indicate that tyrosine phosphorylation of PIPKI γ i5 is a negative regulator of the PIPKI γ i5–SNX5 interaction.

There are three tyrosine residues in the C-terminus of PIPKI γ i5 within the proposed SNX5-binding region. To determine whether these residues are targets of Src phosphorylation, the tyrosines present in the PIPKI γ i5 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 PIPKI γ i5 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 PIPKI γ i5–SNX5 interaction. These mutants were expressed from an HA-PIPKI γ i5 construct in cells and were assayed for their ability to immunoprecipitate SNX5. The Y646F mutation, which prevents phosphorylation by Src, increased the association of PIPKI γ i5 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 PIPKI γ i5. 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 PIPKI γ i5–SNX5 interaction.

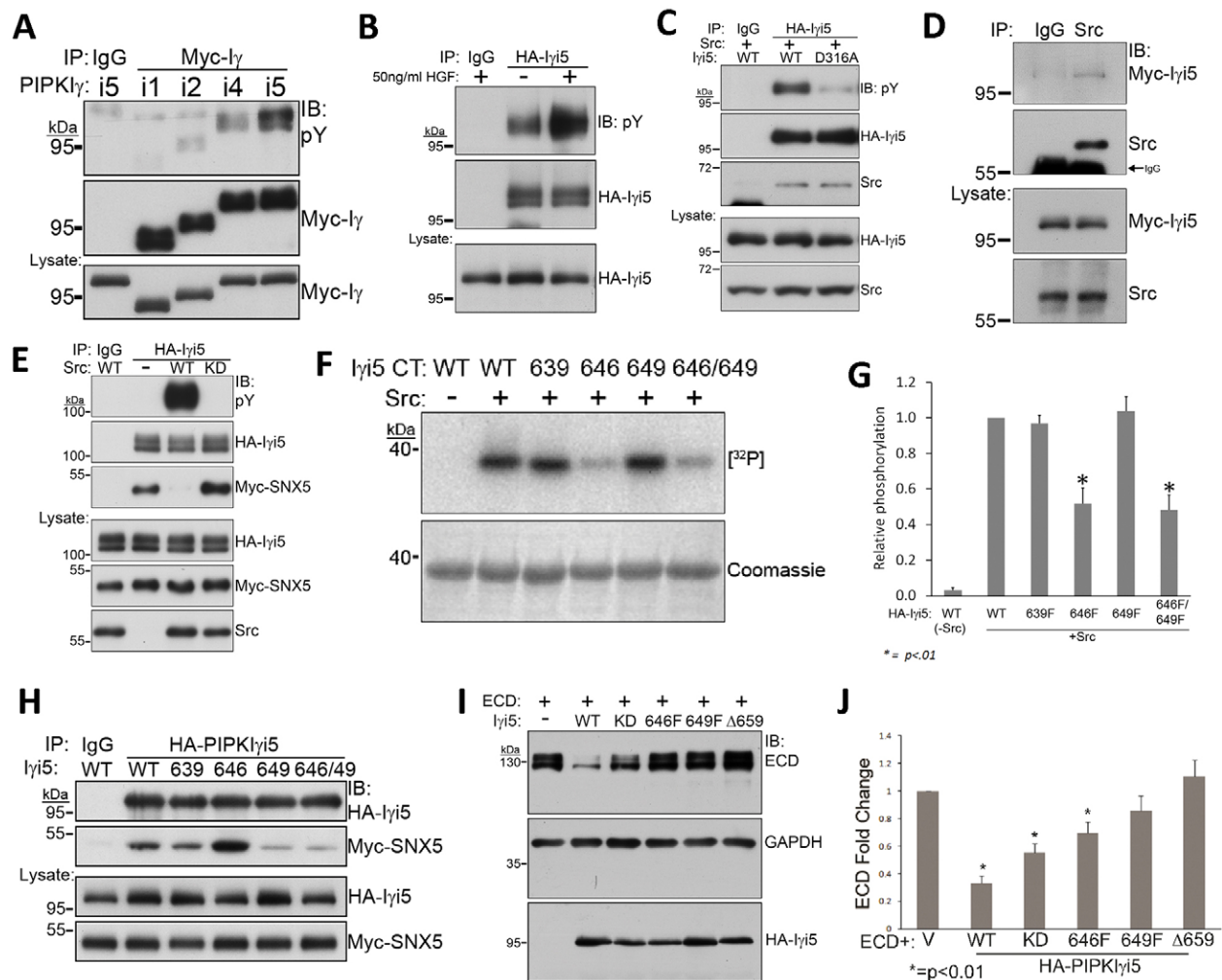


Fig. 5. The association between PIPKI γ 5 and SNX5 is regulated downstream of Src. (A) Myc-PIPKI γ splice variants were immunoprecipitated (IP) from HeLa cells cultured in DMEM plus 10% FBS, and their phosphorylation status was assayed by western blotting (IB) with an anti-phosphotyrosine (pY) antibody. Total cell lysates were western blotted for Myc-PIPKI γ as a control. (B) MDCK cells were induced to express HA-PIPKI γ 5 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) PIPKI γ 5 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-PIPKI γ 5 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-PIPKI γ 5 was coexpressed with Myc-SNX5 and either wild-type or kinase-dead c-Src, and HA-PIPKI γ 5 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-PIPKI γ 5 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 PIPKI γ 5 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 PIPKI γ 5 C-terminus+Src. (H) HA-PIPKI γ 5 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-PIPKI γ 5 (lyi5) 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 \pm s.e.m.

Src phosphorylation of PIPKI γ 5 might affect E-cadherin protein content. Coexpression of PIPKI γ 5 with Src did not further enhance E-cadherin loss (supplementary material Fig. S4D,E). This might be due to high basal levels of PIPKI γ 5 phosphorylation, and the amount of free PIPKI γ 5 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 PIPKI γ 5-mediated degradation

pathway is constantly activated in cells ectopically expressing Src. In this scenario, endogenous PIPKI γ 5 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 PIPKI γ 5 affect E-cadherin degradation, E-cadherin was expressed with PIPKI γ 5 Y646F, Y649F and Δ 659 mutations. As shown in Fig. 5I,J, the Y646F, Y649F and Δ 659 mutations had a diminished ability to induce E-cadherin degradation

compared with wild-type PIPKI γ 5, and were less effective than kinase-dead PIPKI γ 5. In addition to affecting phosphorylation and interaction with SNX5, these mutations might have additional consequences on PIPKI γ 5 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 PIPKI γ 5 with E-cadherin, because the conserved kinase domain in PIPKI γ 5 mediates this interaction (Ling et al., 2007).

PIPKI γ 5 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 PIPKI γ 5 prior to lysosomal degradation of the receptor. Therefore, to assess the step in which PIPKI γ 5 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 PIPKI γ 5 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 PIPKI γ 5. When E-cadherin was expressed alone in control, Rab5a- or Rab7-knockdown cells, there was no significant change in E-cadherin expression. Expression of PIPKI γ 5 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 PIPKI γ 5 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

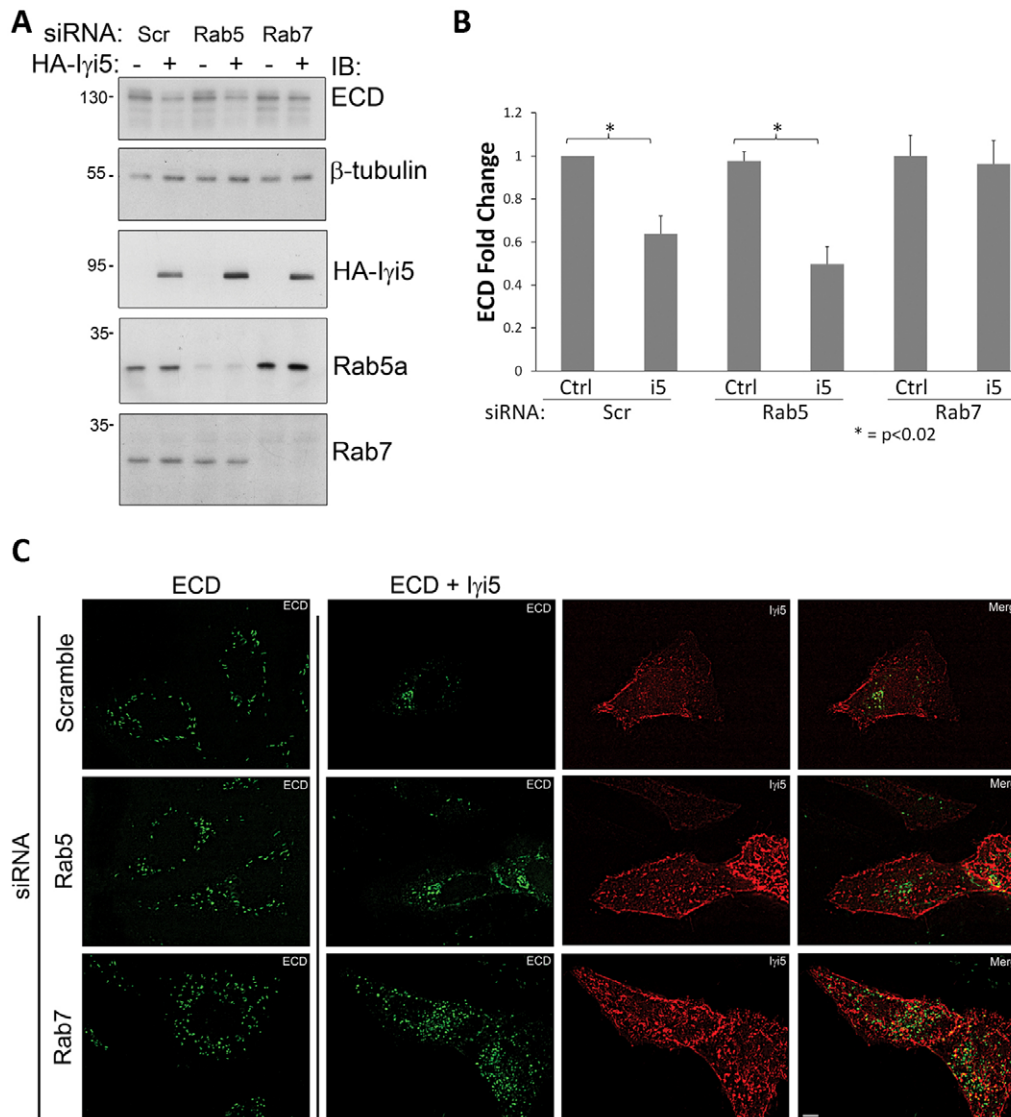


Fig. 6. PIPKI γ 5-mediated E-cadherin degradation is Rab7 dependent. (A) HeLa cells were transfected with siRNA pools targeting Rab5a or Rab7 for 48 h, and were then transfected a second time with E-cadherin and HA-PIP KI γ 5 constructs for 16 h. Non-targeting siRNA (Scr) was also transfected as a control. Cells were lysed directly into 5 \times 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 \pm s.e.m. (C) Following Rab5a or Rab7 knockdown as in A, HeLa cells were transfected with E-cadherin alone or with the HA-PIP KI γ 5 construct. Coverslips were prepared as in Fig. 3, and stained with the indicated antibodies. Scale bar: 10 μ m.

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 PIPKI γ 5, E-cadherin shifted to a compact perinuclear localization in control and Rab5a-knockdown cells, whereas expression of PIPKI γ 5 in Rab7-knockdown cells results in punctate cytoplasmic localization for E-cadherin, similar to when E-cadherin is expressed alone. This further suggests that PIPKI γ 5 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 PIPKI γ 5-mediated degradation of EGFR (Palacios et al., 2005; Sun et al., 2013; Toyoshima et al., 2007). To assess the role of Hrs in PIPKI γ 5-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, PIPKI γ 5 expression reduced E-cadherin levels in Hrs-knockdown cells, although not as completely as in control cells (Fig. 7A,B). PIPKI γ 5 appears to mediate a rate-limiting step under basal conditions, and its overexpression greatly enhances the sorting of E-cadherin for degradation. Additionally, PIPKI γ 5 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, PIPKI γ 5 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 PIPKI γ 5 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, PIPKI γ 5, 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 PIPKI γ 5 or otherwise rendering it unable to push E-cadherin towards a degradation pathway. In support of this hypothesis, PIPKI γ 5 overexpression continues to promote E-cadherin degradation after knockdown of SNX5 and SNX6 (Fig. 7E,F). The ability of PIPKI γ 5 to promote E-cadherin degradation in the absence of Vps35, SNX5 and SNX6 indicates a retromer-independent pathway that is regulated by PIPKI γ 5. However, PIPKI γ 5 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 PIPKI γ 5 and SNX5 as regulators of E-cadherin. In this pathway, SNX5 inhibits the lysosomal targeting of E-cadherin. By contrast, PIPKI γ 5 promotes E-cadherin degradation within the known lysosomal-degradation pathway, because chloroquine treatment or knockdown of Rab7 can inhibit PIPKI γ 5 in this process. Additionally, knockdown of Hrs limits the effect of PIPKI γ 5 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 PIPKI γ 5-generated PI(4,5)P₂ and phosphorylation of PIPKI γ 5. These points have been summarized as a model depicted in Fig. 8. Generally, SNX5 and PIPKI γ 5 associate at endosomal compartments. Stimulation with HGF results in the disassembly of adherens junctions and E-cadherin endocytosis. Src activation downstream of HGF phosphorylates PIPKI γ 5, inhibiting the interaction of SNX5 with PIPKI γ 5. When SNX5 is absent from the PIPKI γ 5–E-cadherin complex, E-cadherin is transported to the lysosome. In this model, PIPKI γ 5 promotes E-cadherin degradation, which would facilitate EMT, whereas SNX5 inhibits PIPKI γ 5. However, the specific functions of SNX5 alone or in complex with PIPKI γ 5 have not been thoroughly examined. There is the potential for SNX5 to regulate E-cadherin recycling while in complex with PIPKI γ 5, which could regulate recycling and the biosynthetic trafficking of E-cadherin to the plasma membrane in a manner similar to the interaction between PIPKI γ 2 and AP1B (Ling et al., 2007). Consequently, signaling pathways that regulate the association between PIPKI γ 5 and SNX5 would have serious implications in tumorigenesis and the metastasis of tumor cells.

Tyrosine phosphorylation of PIPKI γ is a well-documented mechanism to modulate both PIPKI γ 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 PIPKI γ 5 are crucial for regulating its association with SNX5. Mutation of either Y646F or Y649F increased or decreased the association of PIPKI γ 5 with SNX5, respectively. Surprisingly, both mutations inhibited the effect of PIPKI γ 5 on E-cadherin degradation. This might be due to changes in PIPKI γ 5 localization, post-translational modifications or protein–protein interactions. This result confounds the exact mechanism that regulates the association between PIPKI γ 5 and SNX5, and further characterization of the PIPKI γ 5–SNX5

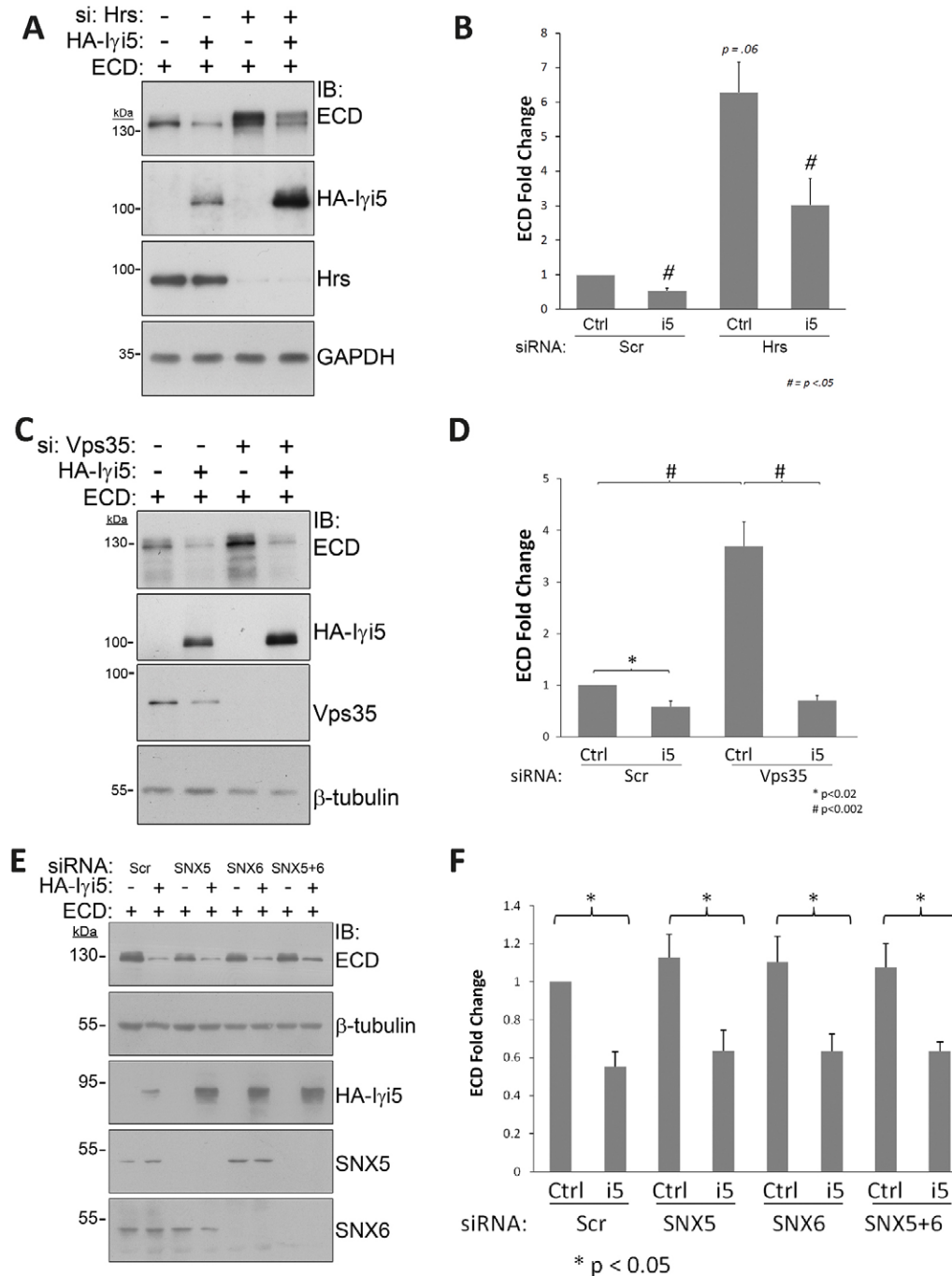


Fig. 7. PIPKI γ 5 depends on known degradation pathways, but is retromer independent.

(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-PIP KI γ 5 constructs for 16 h. Non-targeting siRNA (Scr) was also transfected as a control. Cells were lysed directly into 5 \times 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 \pm s.e.m.

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

Both PIPKI γ 2 and PIPKI γ 5 interact with E-cadherin and synthesize PI(4,5)P₂, but these isoforms have contrasting effects on E-cadherin function. PIPKI γ 2 regulates the trafficking and assembly of E-cadherin, whereas PIPKI γ 5 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. PIPKI γ 2 and PIPKI γ 5 are expressed in the majority of epithelial and non-epithelial cell lines, where they have unique functions. PIPKI γ 2 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 PIPKI γ 5 is still emerging. We determined previously that PIPKI γ 5 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 PIPKI γ 5 can sort E-cadherin for degradation. However, it should be noted that PIPKI γ 5 is relatively low in abundance compared with total PIPKI γ expression; thus, using an overexpression system to examine PIPKI γ 5 function is a current limitation for examining endogenous PIPKI γ 5 function. It will be interesting to investigate the effects of PIPKI γ 5 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 PIPKI γ 5 in E-cadherin

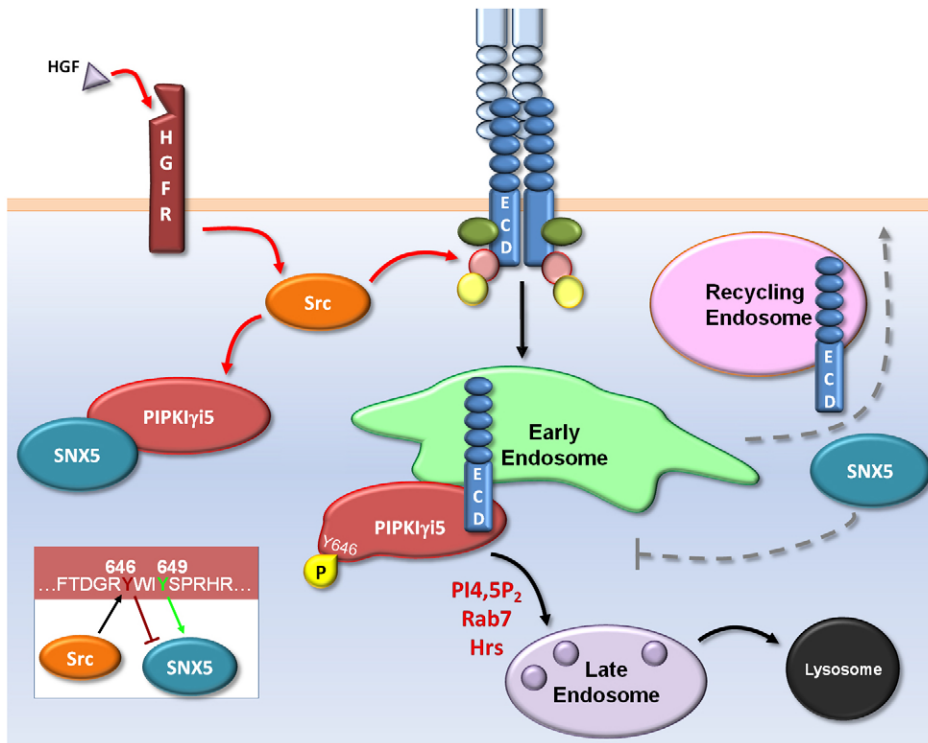


Fig. 8. PIPKIγi5 and SNX5 regulate E-cadherin targeting. A model depicting the proposed functions of PIPKIγi5 and SNX5 in regulating the lysosomal targeting of E-cadherin. HGF stimulation initiates the disassembly of adherens junctions and eventual degradation of E-cadherin. Additionally, HGF activation 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 ubiquitinated (Fujita et al., 2002; Levkowitz et al., 1998), and this signal is recognized by the ESCRT proteins (with Hrs recognizing ubiquitinated 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)P₂ 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)P₂-binding proteins present at endosomes that assist in this process. Additionally, PI(4,5)P₂ 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)P₂ 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)P₂ 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 PIPKI γ and SNX5 in cancer biology. Expression of PIPKI γ 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 PIPKI γ 5 and SNX5 represents a decision point, with SNX5 promoting the retention of E-cadherin, and PIPKI γ 5 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 PIPKI γ 5 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 PIPKI γ splice variants, SNX5, SNX6 and SNX1 were amplified by PCR for insertion into the pCMV-Myc and pCMV-HA vectors (Clontech). PIPKI γ 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 PIPKI γ 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

PIPKI γ 5 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 μ M LysoTracker Red DND-99 (Invitrogen) in DMEM for 30 min prior to fixation. Incubation with the primary

antibody was performed at 37°C for 1 h or at 4°C overnight. Incubation with fluorophore-conjugated secondary antibodies was performed at 37°C 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 \times 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 PIPKI γ , 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 PIPKI γ 5 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 MgCl₂, 0.5% NP-40 and 2 mM Na₃VO₄, 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-PIPKI γ 5 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 Na₃VO₄ and protease inhibitors, sonicated briefly and lysed for 30 min at 4°C. Myc- or HA-tagged PIPKI γ was immunoprecipitated with 3 μ g of anti-HA or anti-Myc mAb for 16 h at 4°C. 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 μ g/ml anti-phosphotyrosine (4G10) mAb, as per the manufacturer's instructions.

For the *in vitro* Src phosphorylation studies, 7 μ g of wild-type or mutant 6His-tagged PIPKI γ 5 C-terminus (residues 485–707) was combined with 10 μ Ci of γ -[³²P]-ATP and 1 unit of human recombinant Src (Millipore) in 25 mM Tris-HCl pH 7.2, 30 mM MgCl₂, 5 mM MnCl₂, 500 μ M EGTA, 500 μ M DTT and 100 μ M ATP for 15 min at 30°C. Reactions were terminated by the addition of 2 \times 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. [³²P] 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 μ M chloroquine was included in the medium where indicated. After 16 h, cells were lysed directly in 2 \times 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 μ g 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'-AGCCAAGCAGCAGTACATT-3'; ECD R1 Rev, 5'-AGGCCAAGCAGCAGTACATT-3'; ECD R2 Fwd, 5'-TGAGTGTCC-CCCGGTATCTTC-3'; ECD R2 Rev, 5'-CAGTATCAGCCGCTTTC-AGATTTT-3'; GAPDH Fwd, 5'-GAAGTCCGAGTCAACGGATTT-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.

Competing interests

The authors declare no competing interests.

Author contributions

N.J.S. performed immunoprecipitations, microscopy, E-cadherin degradation experiments and PIPKI γ 5 phosphorylation, and prepared the manuscript. A.C.H. performed E-cadherin degradation experiments and microscopy, and prepared the manuscript. S.C. performed HGF stimulation of MDCK cells and *in vitro* phosphorylation. R.A.A. provided scientific rationale and project guidance.

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Supplementary material

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Fig. S1: Active PIPKI γ 5 specifically promotes E-cadherin loss

(A) Inducible MDCK cells expressing HA tagged PIPKI γ 1, i5 WT or i5 KD were treated with HGF, and then examined for E-cadherin degradation by Western blot.

(B) Inducible MDCK cells expressing HA tagged PIPKI γ 2 or PIPKI γ 5 were treated with HGF for 4 hours, and then examined for E-cadherin degradation by Western blot. Antibodies to PIPKI γ (all splice variants) or PIPKI γ 5 demonstrate the level of overexpression in these cell lines. GAPDH was Western blotted as a loading control. The Western blot panel is representative of 4 independent experiments. Significance was calculated using the Student's 2-tailed T-test to $p < 0.05$ (*) where indicated.

(C) Quantification of (B). E-cadherin protein content was measured and normalized to GAPDH loading control.

(D) Inducible MDCK cell lines were grown as in (B). Cells were treated with lysotracker (red) 30 minutes prior to fixation. Cells were then fixed and stained for HA-PIPKI γ 5 (blue) and E-cadherin (green) and analyzed as described in the materials and methods. Scale = 10 μ m.

Fig. S2: Examination of chloroquine treatment, E-cadherin mRNA and PIPKI γ 5 interactions with SNX6

(A) HeLa cells were transfected with E-cadherin (ECD) and PIPKI γ 5 in the absence or presence of increasing concentrations of the lysosomal inhibitor chloroquine (Chlor). After 16 h, the cells were lysed directly into 5X protein sample buffer and subjected to Western blotting with the indicated antibodies. Blots were also probed with anti- β -tubulin antibody as a loading control.

(B) qPCR analysis of E-cadherin mRNA content from HeLa cells transfected as described in Figure 2 (A). “ECD R1” and “ECD R2” indicate two different sets of primers targeting ECD mRNA. Statistical analysis of qPCR data was performed as listed in the materials and methods (N = 5 independent experiments with internal duplicates). Error bars indicate the s.e.m. of each data set. Significance was calculated using the Student’s 2-tailed T-test.

(C) HA-PIPKI γ 1 or γ 5 was co-expressed with Myc-SNX6 in HeLa cells, and HA-PIPKI γ 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.

(D) Recombinant GST-SNX6 and 6His-PIPKI γ 5 were expressed and purified from *E. coli*. Binding was examined by an *in vitro* binding assay and samples analyzed by Western blot.

Fig. S3: MG-132 induces mis-localization of PIPKI γ 5 and SNX5

(A) HeLa cells were transfected with E-cadherin (ECD), SNX5 and PIPKI γ 5 in the absence or presence of the protease inhibitor leupeptin (100 μ g/ml) or the proteasome inhibitor MG-132 (200 μ M). After 16 h, the cells were lysed directly into 5X protein sample buffer and subjected to Western blotting with the indicated antibodies. Blots were also probed with anti-GAPDH antibody as a loading control.

(B) HeLa cells grown on coverslips were transfected with E-cadherin (ECD) and PIPKI γ 5 in the absence or presence of the proteasome inhibitor MG-132. Slides were prepared as in Figure 3, and stained with the indicated antibodies. Scale = 10 μ m.

Fig. S4: Src interacts with PIPKI γ 5 and regulates E-cadherin degradation

(A) Myc-SNX5 was co-expressed with HA-PIPKI γ 5, WT or KD mutant forms (D253A, D316A, or DDAA – (D253A + D316A)) and PIPKI γ 5 was immunoprecipitated. Total cell lysates were blotted for HA-PIPKI γ 5 and Myc-SNX5 as a control.

(B) Myc-SNX5 was co-expressed with vector, WT or KD c-Src and SNX5 was immunoprecipitated from HeLa cells cultured in 10% FBS/DMEM. SNX5 phosphorylation status was assayed via Western blot with an anti-phosphotyrosine (pY) antibody. Total cell lysates were Western blotted for Myc-SNX5 as a control.

(C) WT or KD c-Src was co-expressed with Myc-PIPKI γ 5 in HeLa cells. PIPKI γ 5 was immunoprecipitated, and Western blotted to assay for binding to c-Src. Cell lysates were Western blotted with the indicated antibodies as a control.

(D) E-cadherin was co-expressed with either HA-PIPKI γ 5 and/or c-Src. Cells were lysed directly into 5X protein loading buffer, separated by SDS-PAGE, and Western blotted with the indicated antibodies. GAPDH was Western blotted as a loading control. The Western blot panel is representative of 4 independent experiments.

(E) Quantification of (C). Error bars indicate the s.e.m. of each data set. Significance was calculated using the Student's 2-tailed T-test to $p < 0.03$ (*) where indicated.

(F) E-cadherin was co-expressed with either Myc-SNX5 and/or c-Src. Cells were lysed directly into 5X protein loading buffer, separated by SDS-PAGE, and Western blotted with the indicated antibodies. GAPDH was Western blotted as a loading control. The Western blot panel is representative of 9 independent experiments.

(G) Quantification of (E). Error bars indicate the s.e.m. of each data set. Significance was calculated using the Student's 2-tailed T-test to $p < 0.02$ (*) or $p < 0.052$ (#) where indicated.

FIGURE S1

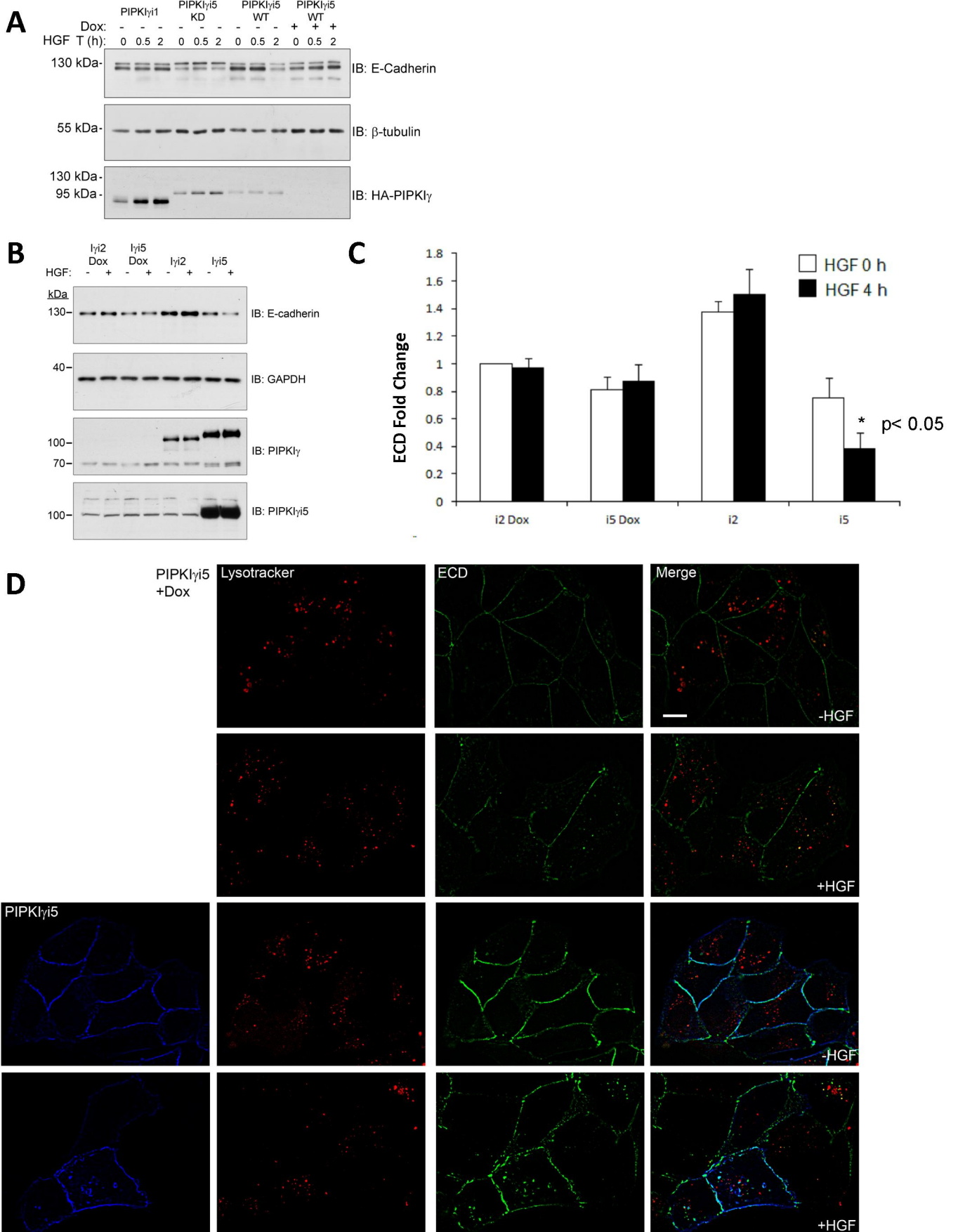


FIGURE S2

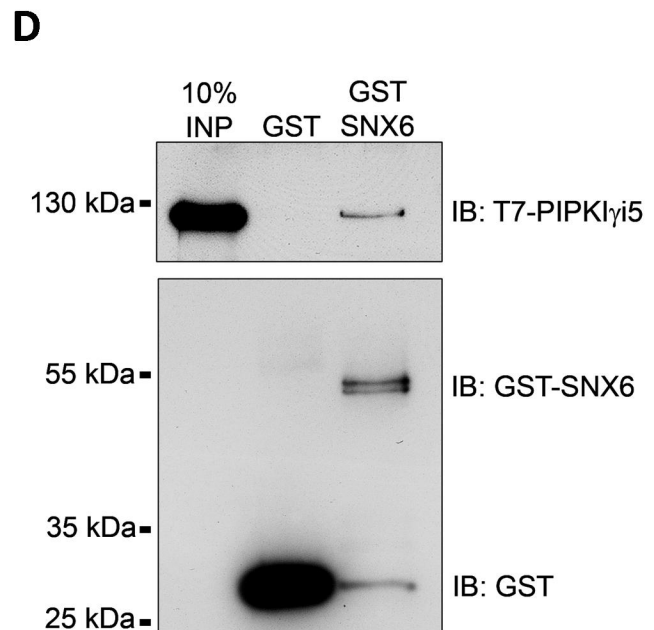
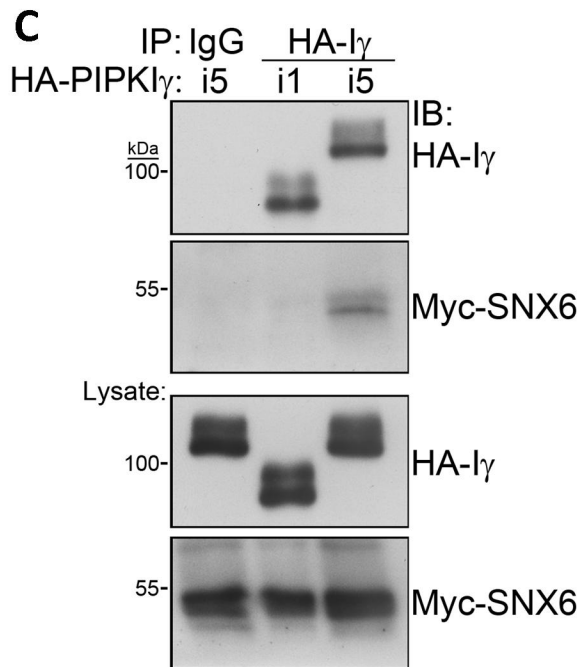
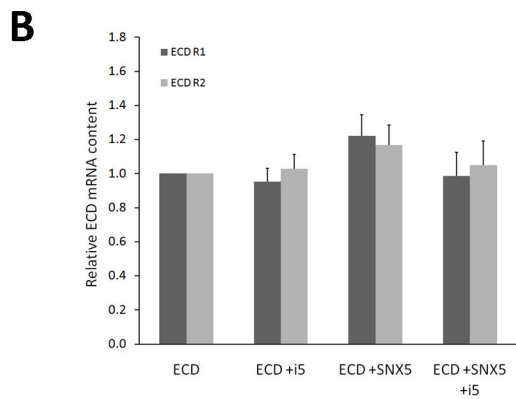
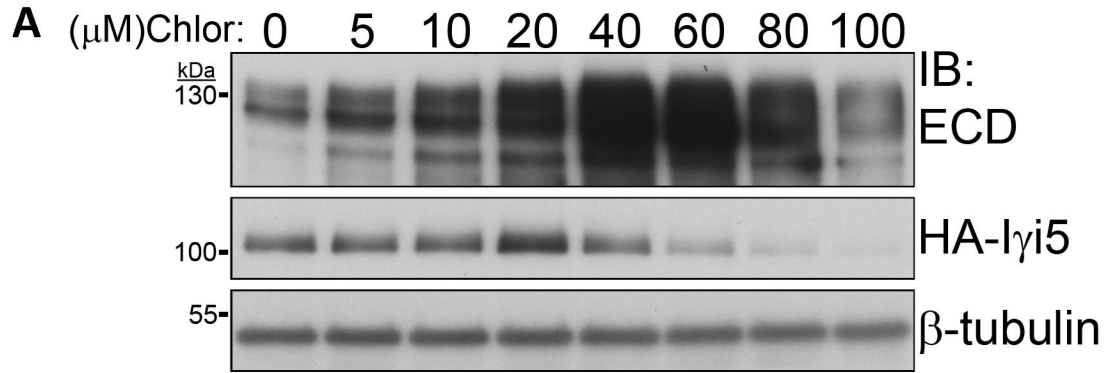


FIGURE S3

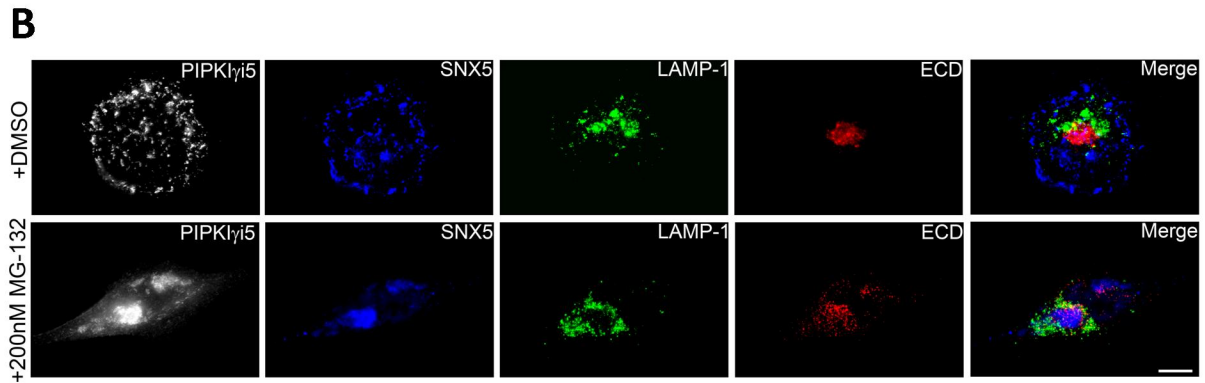
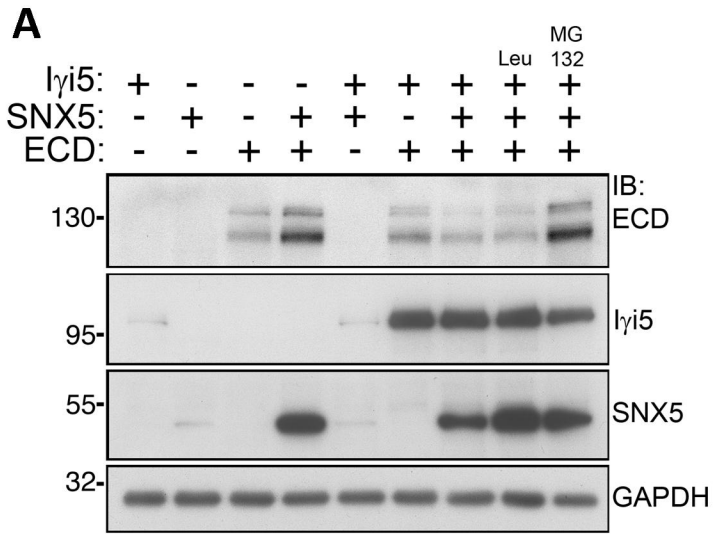


FIGURE S4

