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Endosomal Type Iγ PIP 5-Kinase Controls EGF Receptor Lysosomal Sorting

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SUMMARY

Endosomal trafficking and degradation of epidermal growth factor receptor (EGFR) play an essential role in the control of its signaling. Phosphatidylinositol-4,5-bisphosphate (PtdIns4,5P_2) is an established regulator of endocytosis, whereas PtdIns3P modulates endosomal trafficking. However, we demonstrate here that type I gamma phosphatidylinositol phosphate 5-kinase i5 (PIPKIγi5), an enzyme that synthesizes PtdIns4,5P_2, controls endosome-to-lysosome sorting of EGFR. In this pathway, PIPKIγi5 interacts with sorting nexin 5 (SNX5), a protein that binds PtdIns4,5P_2 and other phosphoinositides. PIPKIγi5 and SNX5 localize to endosomes, and loss of either protein blocks EGFR sorting into intraluminal vesicles (ILVs) of the multivesicular body. Loss of ILV sorting greatly enhances and prolongs EGFR signaling. PIPKIγi5 and SNX5 prevent Hrs ubiquitination, and this facilitates the Hrs association with EGFR that is required for ILV sorting. These findings reveal that PIPKIγi5 and SNX5 form a signaling nexus that controls EGFR endosomal sorting, degradation, and signaling.

INTRODUCTION

Epidermal growth factor receptor (EGFR) is a critical component of signaling pathways that govern cell growth and differentiation during embryogenesis and adult homeostasis (Schlessinger, 2002). The regulated activation of EGFR is essential for normal signaling, and loss of EGFR or its overactivation leads to multiple diseases (Casalini et al., 2004; Hynes and MacDonald, 2009). Following epidermal growth factor (EGF) stimulation, EGFR signaling is regulated by endocytic trafficking, where activated EGFR is internalized, and trafficking determines the fate of internalized EGFR, including recycling back to the plasma membrane, translocation to the nucleus, or trafficking to the lysosome for degradation (Carpenter and Liao, 2009; Sorkin and Goh, 2009). Internalized EGFR continues to signal from endosomal compartments until the agonist is separated from the receptor or the agonist-receptor complex is sorted into intraluminal vesicles (ILVs) of the multivesicular body (MVB) (McLaughlin et al., 2002; Sorkin and von Zastrow, 2009). Sorting and lysosomal degradation of activated EGFR are essential mechanisms to control EGFR signaling (Sorkin and von Zastrow, 2009).

Phosphoinositides play fundamental roles in membrane receptor endocytosis and endosomal sorting. PtdIns4,5P_2 is predominantly at the plasma membrane, where it modulates the formation of clathrin-coated pits and receptor endocytosis (Barbieri et al., 2001; Jost et al., 1998). At endosomes, PtdIns3P and PtdIns3,5P_2 are synthesized and are key lipid messengers for endosomal trafficking (Clague et al., 2009). Although PtdIns4,5P_2 is also synthesized on endosomal and lysosomal membranes, a role for PtdIns4,5P_2 in endosomal trafficking has not been defined (Arneson et al., 1999; Watt et al., 2002).

Type I gamma phosphatidylinositol phosphate kinase (PIPKIγ) is an enzyme that synthesizes PtdIns4,5P_2 by phosphorylation of PtdIns4P (Heck et al., 2007; Schill and Anderson, 2009b). The PIPKIγ gene is alternatively spliced, resulting in protein variants that contain unique extensions at the C terminus (Schill and Anderson, 2009b; Xia et al., 2011). The individual PIPKIγ extensions mediate interactions with unique binding partners, often PtdIns4,5P_2 effectors, which target each PIPKIγ splice variant to distinct subcellular compartments necessary for the specificity in PtdIns4,5P_2 signaling (Barlow et al., 2010; Heck et al., 2007). Six PIPKIγ variants have been identified in humans, known as PIPKIγi1, i2, i3, i4, i5, and i6 (Schill and Anderson, 2009b; Xia et al., 2011). PIPKIγi1 is the shortest splicing variant and is a major contributor to the PtdIns4,5P_2 pool that supports G-protein-coupled receptor-mediated inositol 1,4,5-trisphosphate generation and plays a critical role in Ca^{2+} flux (Wang et al., 2004). PIPKIγi2 has a 28 amino acid C-terminal extension that binds to the talin FERM domain (Di Paolo et al., 2002; Ling et al., 2002) and regulates talin assembly, adhesion dynamics, and migration (Sun et al., 2007). PIPKIγi2 also regulates protein trafficking and cell polarity through interactions with the clathrin adaptor protein complexes (AP) and the exocyst complex (Bairstow et al., 2006; Ling et al., 2007; Thapa et al., 2012). Recently, PIPKIγi4 and PIPKIγi5 were identified and found to distinctively localize to the nucleus and endosomes, respectively, but their biological functions are not defined (Schill and Anderson, 2009b).

Here, we show that PIPKIγi5 interacts with sorting nexin 5 (SNX5), a phosphoinositide binding protein. Loss of PIPKIγi5 or SNX5 results in a block of EGFR sorting into ILVs of the MVB and in prolonged and enhanced EGFR signaling. The data

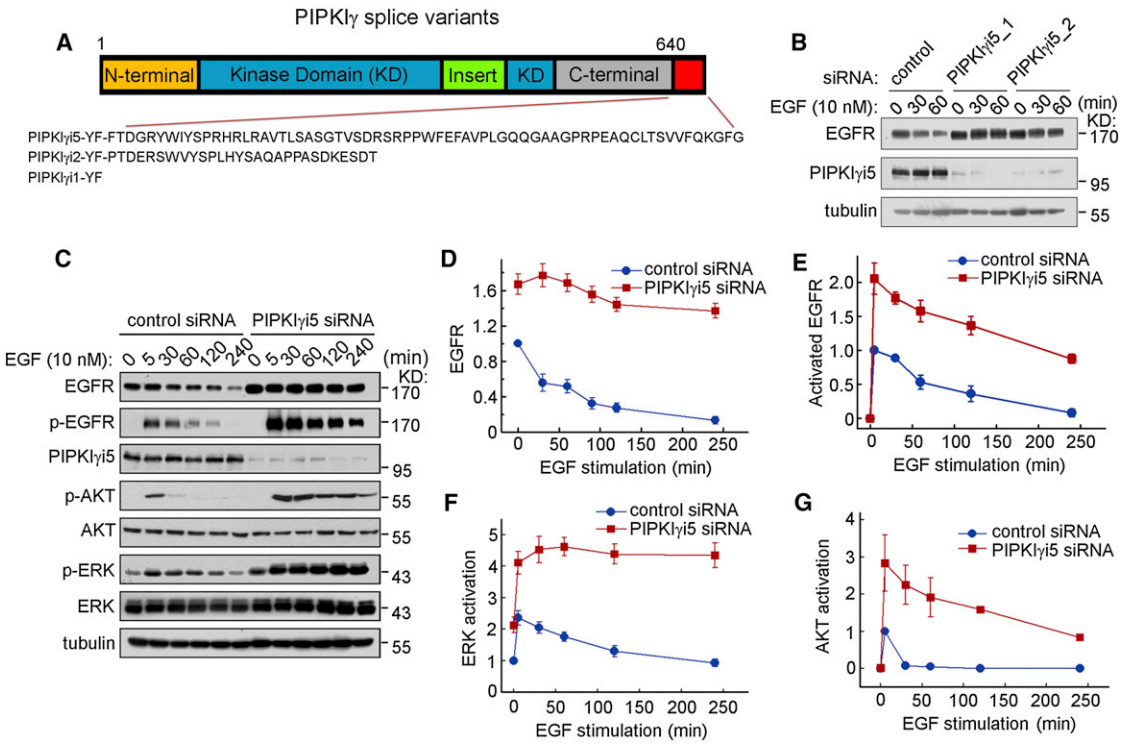

Figure 1. PIPKIγi5 Controls EGFR Downregulation and Signaling

(A) The domain structure and sequence of the C termini of PIPKIγi1, i2 and i5.

(B) Two different siRNAs specific for PIPKIγi5 similarly blocked EGF-induced (10 nM) EGFR downregulation in MDA-MB-231 cells. The PIPKIγi5_1 siRNA was used in further experiments.

(C–G) Control or PIPKIγi5-knockdown cells were treated with EGF (10 nM) for the times indicated (C). The EGFR protein level, EGFR activation, ERK activation, and AKT activation were detected. The following were quantified: EGFR protein level (D), EGFR activation detected by phospho-tyr1068 antibody (E), ERK activation (F), and AKT activation (G). Quantification of EGFR protein level and EGFR activation was normalized with tubulin level. Quantification of ERK or AKT activation was normalized with total ERK or AKT level. The values shown on graphs represent the mean ± SEM from three independent experiments.

See also Figure S1.

uncover a signaling nexus formed by PIPKIγi5, SNX5, and phosphoinositide generation that controls EGFR endosomal signaling, sorting, and degradation.

RESULTS

PIPKIγi5 Controls EGFR Degradation and Signaling

The C-terminal extensions of PIPKIγi1, i2, and i5 are shown in Figure 1A (Schill and Anderson, 2009b). PIPKIγi2 targets to adhesions and plays key roles in EGFR-mediated cell migration (Sun et al., 2007). To compare the roles of PIPKIγi5 and PIPKIγi2 in EGFR signaling, each variant was knocked down using isoform-specific small interfering RNAs (siRNAs). Strikingly, loss of PIPKIγi5 blocked EGF-induced EGFR degradation (Figures 1B–1D). This was specific for PIPKIγi5 as loss of PIPKIγi2 (Figures S1A and S1B available online) or other variants (not shown) had no impact on EGFR degradation. To rule out siRNA off-target effects, two different PIPKIγi5 siRNAs, PIPKIγi5_1 and PIPKIγi5_2, were used, and both knocked down PIPKIγi5 and blocked EGFR downregulation (Figure 1B). Loss of PIPKIγi5 in MDA-MB-231, A431, and SKBR3 cells blocked EGFR loss (Figures S1C–S1F), indicating that this is not a cell-type-specific role for PIPKIγi5. To determine the impact of PIPKIγi5 knockdown on EGFR activation, the autophosphorylation of EGFR on tyrosine 1068 was quantified. In cells lacking PIPKIγi5, the activation of EGFR was enhanced and prolonged (Figures 1C and 1E). Consistent with prolonged EGFR activation, both ERK and AKT activation were enhanced and prolonged (Figures 1C, 1F, and 1G) in PIPKIγi5-knockdown cells. There was no significant change in EGFR messenger RNA levels between control and PIPKIγi5-knockdown cells (Figure S1G), signifying a role for PIPKIγi5 in EGFR degradation. To determine if the role of PIPKIγi5 is dependent on the level of EGFR stimulation, cells were stimulated with a low EGF concentration (0.2 nM). Low EGF induced EGFR degradation in control cells (Figure S1H). In PIPKIγi5-knockdown cells, the degradation of EGFR induced by low EGF was also blocked and EGFR activation and downstream AKT signaling were enhanced and prolonged (Figure S1H).

To determine if PIPKIγi5 lipid kinase activity was required for EGFR downregulation, a knockdown-rescue approach was developed. Here, siRNA was used to knock down endogenous PIPKIγi5, and then wild-type PIPKIγi5 or kinase dead mutant (PIPKIγi5KD) vectors containing siRNA-resistant silent mutations were re-expressed using lentivirus-mediated infection. Expression of wild-type PIPKIγi5 but not PIPKIγi5KD rescued EGFR degradation in PIPKIγi5-knockdown cells (Figures S1I

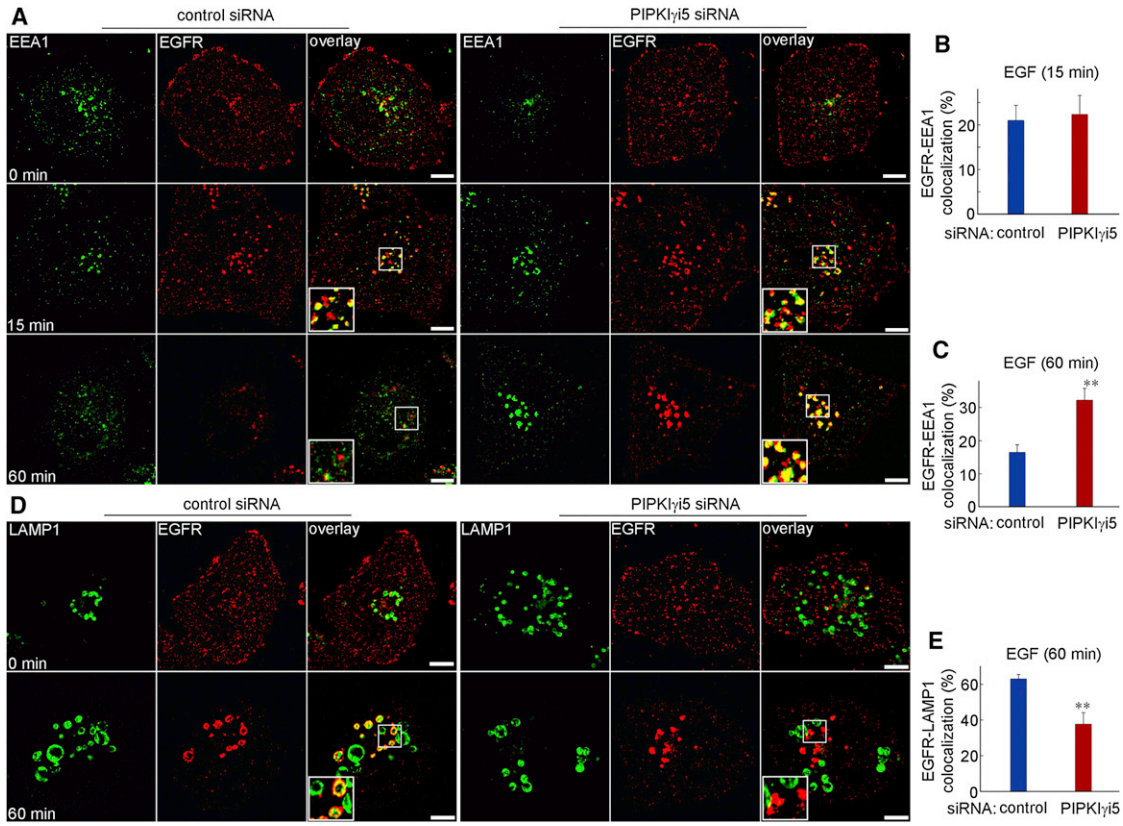

Figure 2. PIPKIγi5 Controls EGFR Endosomal Trafficking

MDA-MB-231 cells were transfected with control siRNA or PIPKIγi5 siRNA separately and then stimulated with EGF (10 nM) for the times indicated.

(A) Immunofluorescence staining with EGFR and EEA1 antibodies.

(B and C) Quantification of EGFR-EEA1 colocalization 15 min (B) or 60 min (C) after EGF stimulation.

(D) Cells were pretreated with the lysosome inhibitor chloroquine (50 μM) for 2 hr to prevent the rapid degradation of EGFR, stimulated with EGF (10 nM) for 60 min, and then stained with EGFR and LAMP1 antibodies.

(E) Quantification of EGFR-LAMP1 colocalization 60 min after EGF stimulation. Error bars indicate mean ± SEM (n = 150 cells from three independent experiments). Scale bar represents 10 μm. **$p < 0.01$.

See also Figure S2.

and S1J). These results confirm the role of PIPKIγi5 in EGFR degradation and indicate that kinase activity is required for PIPKIγi5 control of EGFR downregulation.

PIPKIγi5 Controls EGFR Lysosomal Sorting

To clarify the trafficking step that requires PIPKIγi5 for EGFR degradation, the uptake of Alexa Fluor 488-labeled EGF (10 nM) was quantified by flow cytometry to track the internalization of EGFR. Loss of PIPKIγi5 did not block EGFR internalization (Figures S2A and S2B). After 5 min of EGF stimulation, the amount of internalized EGF in PIPKIγi5-knockdown cells was ~1.5-fold that in control cells (Figure S2B), which is consistent with higher EGFR levels in PIPKIγi5-knockdown cells (Figure 1). Low EGF (≤2 ng/ml) treatment largely induces clathrin-mediated endocytosis (CME) of EGFR, while high EGF also induces nonclathrin endocytosis (Sigismund et al., 2008). CME is dependent on PtdIns4,5P_2 (Jost et al., 1998). To assess a role for PIPKIγi5 in CME, the endocytosis of transferrin receptor, which mainly undergoes CME, was studied. Knockdown of PIPKIγi5 did not affect transferrin receptor endocytosis (Figure S2C), indicating that PIPKIγi5 is not required for CME.

To examine later sorting steps, the endosomal trafficking of EGFR was investigated. This demonstrated that after EGF stimulation, there was colocalization of EGFR with the early endosome marker early endosomal antigen 1 (EEA1) in both control and PIPKIγi5-knockdown cells (Figures 2A and 2B). This indicated that PIPKIγi5 knockdown did not alter EGFR trafficking to the early endosome. However, 60 min after EGF stimulation, EGFR-EEA1 colocalization in PIPKIγi5-knockdown cells was significantly greater than in control cells (Figures 2A and 2C). This indicated that loss of PIPKIγi5 impeded EGFR sorting from the early endosome.

Under those same conditions, EGFR was also costained with the late endosome/lysosome marker lysosomal-associated membrane protein 1 (LAMP1). The trafficking of EGFR to the lysosome indicated by EGFR-LAMP1 colocalization was diminished in PIPKIγi5-knockdown cells (Figures 2D and 2E). The loss of EGFR trafficking to the late endosome/lysosome is consistent with the decrease in EGFR degradation observed following knockdown of PIPKIγi5.

Internalized EGFR can be recycled back to the plasma membrane from early endosomes or the limiting membrane of MVB

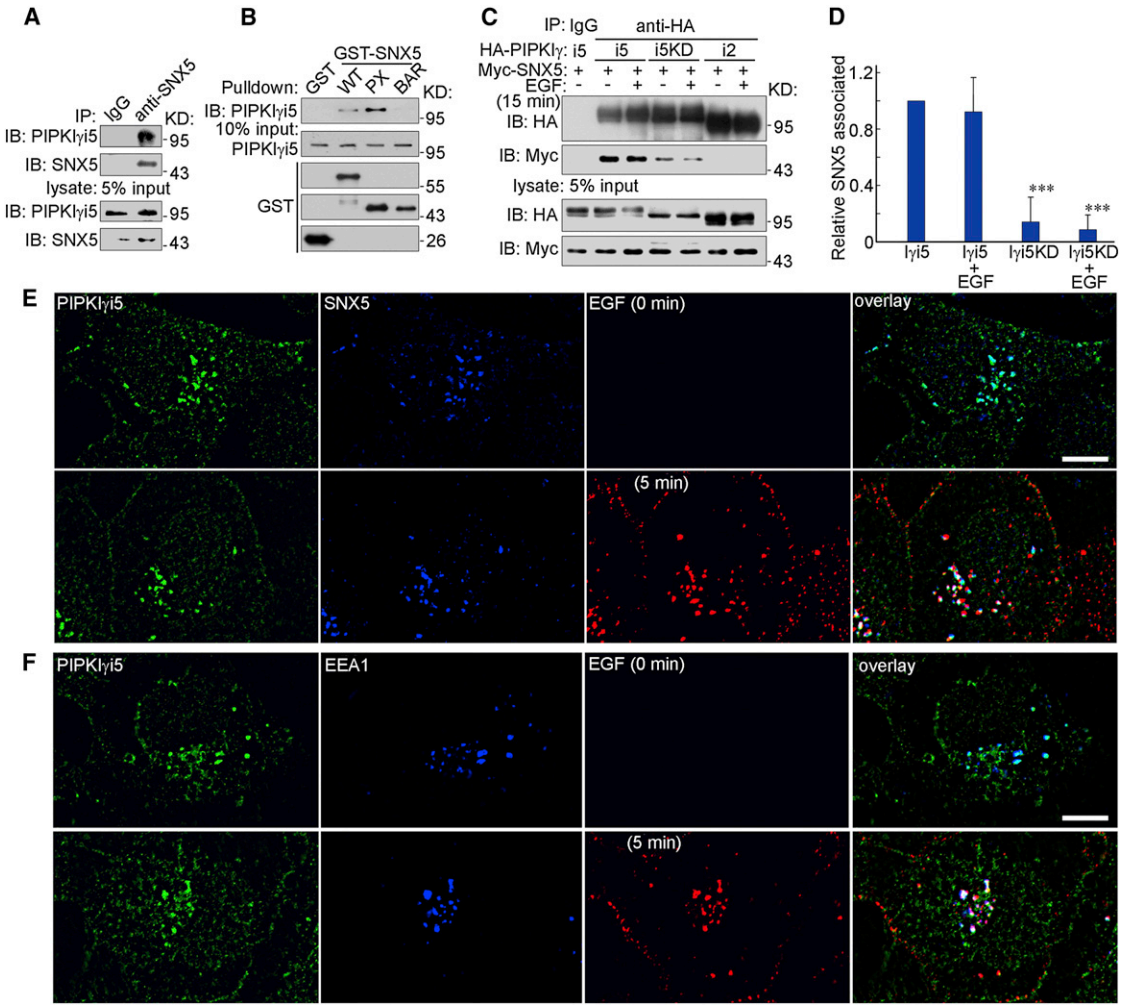

Figure 3. PIPKIγi5 Interacts with SNX5, and Both Localize to the Endosome

(A) MDA-MB-231 cells were subjected to immunoprecipitation with SNX5 antibody and then immunoblotted with antibodies as indicated.

(B) Recombinant GST-SNX5, GST-SNX5-PX, GST-SNX5-BAR, and full-length His_6-PIPKIγi5 were purified from *E. coli* and subjected to GST pull-down assays.

(C) Hemagglutinin (HA)-tag fusion of PIPKIγi2, PIPKIγi5, or PIPKIγi5KD was coexpressed with Myc-SNX5, and HA antibody was used for immunoprecipitation from cell lysates.

(D) Quantification of SNX5 interaction with PIPKIγi5 or PIPKIγi5KD (n = 3). Error bars indicate mean ± SEM. ***$p < 0.001$.

(E) Immunofluorescence staining of HA-PIPKIγi5 (green), Myc-SNX5 (blue), and internalized EGF (Alexa555-EGF, red).

(F) Immunofluorescence staining of HA-PIPKIγi5 (green), EEA1 (blue), and internalized EGF (Alexa555-EGF, red). Scale bar represents 10 μm.

IB, immunoblot; IgG, immunoglobulin G; IP, immunoprecipitation; WT, wild-type. See also Figure S3.

(Sorkin et al., 1991). In PIPKIγi5-knockdown cells, the impeded EGFR trafficking from endosomes to lysosomes may enhance receptor recycling; therefore, EGFR recycling was quantified. As shown in Figures S2D–S2F, there was a significant increase in internalized EGFR recycling back to the plasma membrane in PIPKIγi5-knockdown cells.

PIPKIγi5 Interacts with SNX5

PIPKIγ splice variants usually regulate biological functions by associating with specific binding partners, often PtdIns4,5P_2 effectors, via their distinct C termini (Heck et al., 2007). These PIPKIγ interactions lead to spatial generation of PtdIns4,5P_2 that regulates specific effectors (Ling et al., 2002; Sun et al., 2007; Thapa et al., 2012). To identify PIPKIγi5-binding partners, a yeast two-hybrid screen was performed using the C terminus of PIPKIγi5 as bait. SNX5, a phosphoinositide-binding protein, was identified as an interacting protein. SNX5 is composed of a PX domain and a Bin/Amphiphysin/Rvs (BAR) domain. SNX5 is a component of the mammalian retromer complex and is an endosomal trafficking protein (Wassmer et al., 2009). Additionally, overexpression of SNX5 has been reported to inhibit EGFR degradation (Liu et al., 2006), but the exact role of SNX5 in EGFR endosomal trafficking remains unclear. Endogenous SNX5 was immunoprecipitated from cell lysates and examined by western blot for association of PIPKIγi5. PIPKIγi5 was detected with the SNX5 complex (Figure 3A). Direct binding was confirmed using glutathione S-transferase (GST) pull-down assays with GST-SNX5 and full-length His_6-PIPKIγi5. PIPKIγi5

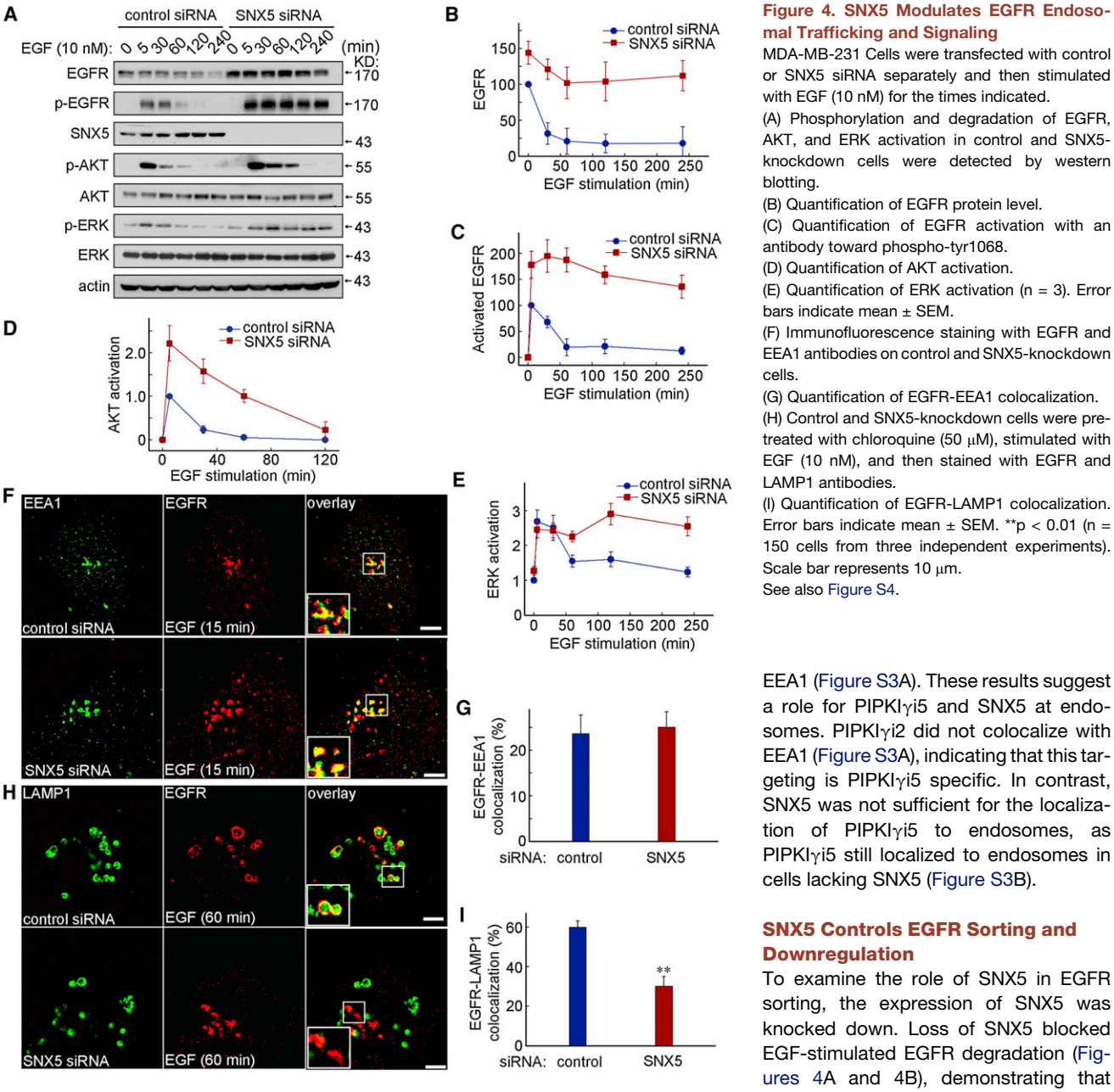

Figure 4. SNX5 Modulates EGFR Endosomal Trafficking and Signaling

MDA-MB-231 Cells were transfected with control or SNX5 siRNA separately and then stimulated with EGF (10 nM) for the times indicated.

(A) Phosphorylation and degradation of EGFR, AKT, and ERK activation in control and SNX5-knockdown cells were detected by western blotting.

(B) Quantification of EGFR protein level.

(C) Quantification of EGFR activation with an antibody toward phospho-tyr1068.

(D) Quantification of AKT activation.

(E) Quantification of ERK activation (n = 3). Error bars indicate mean ± SEM.

(F) Immunofluorescence staining with EGFR and EEA1 antibodies on control and SNX5-knockdown cells.

(G) Quantification of EGFR-EEA1 colocalization.

(H) Control and SNX5-knockdown cells were pretreated with chloroquine (50 μM), stimulated with EGF (10 nM), and then stained with EGFR and LAMP1 antibodies.

(I) Quantification of EGFR-LAMP1 colocalization. Error bars indicate mean ± SEM. **$p < 0.01$ (n = 150 cells from three independent experiments). Scale bar represents 10 μm.

See also Figure S4.

associated directly with the SNX5-PX, but not the SNX5-BAR domain in vitro (Figure 3B).

PIPKIγi5, but not PIPKIγi2 (Figure 3C) or other variants (not shown), was coimmunoprecipitated with SNX5. This result demonstrated that the unique C terminus of PIPKIγi5 is required for its association with SNX5. Although EGF did not regulate the interaction (Figures 3C and 3D), the PIPKIγi5KD interaction with SNX5 was diminished compared to wild-type PIPKIγi5 (Figures 3C and 3D). This indicates that PIPKIγi5 kinase activity regulates the PIPKIγi5-SNX5 interaction. Consistent with their physical association, PIPKIγi5 and SNX5 colocalize in cells (Figure 3E). SNX5 targets to early endosomes (Merino-Trigo et al., 2004) with PIPKIγi5 (Figure 3F), and kinase activity is required for PIPKIγi5 localization, as PIPKIγi5KD did not colocalize with EEA1 (Figure S3A). These results suggest a role for PIPKIγi5 and SNX5 at endosomes. PIPKIγi2 did not colocalize with EEA1 (Figure S3A), indicating that this targeting is PIPKIγi5 specific. In contrast, SNX5 was not sufficient for the localization of PIPKIγi5 to endosomes, as PIPKIγi5 still localized to endosomes in cells lacking SNX5 (Figure S3B).

SNX5 Controls EGFR Sorting and Downregulation

To examine the role of SNX5 in EGFR sorting, the expression of SNX5 was knocked down. Loss of SNX5 blocked EGF-stimulated EGFR degradation (Figures 4A and 4B), demonstrating that SNX5 is required. Knockdown of SNX5 also enhanced and prolonged activation of EGFR, AKT, and ERK (Figures 4A–4E) similar to PIPKIγi5 knockdown. In SNX5-knockdown cells, the endosomal trafficking of EGFR was investigated to determine if loss of SNX5 resulted in a phenotype analogous to the PIPKIγi5 knockdown. Knockdown of SNX5 did not impact EGFR trafficking to early endosomes (Figures 4F and 4G), but did block trafficking to the late endosome/lysosome (Figures 4H and 4I). This phenotype is indistinguishable from that of PIPKIγi5 loss, demonstrating that SNX5 is also required for EGFR lysosomal trafficking.

SNX5 is a component of the retromer complex that regulates retrograde trafficking of cation-independent mannose-6-phosphate receptor (CI-MPR) from the endosome to the *trans*-Golgi network (TGN) (Hara et al., 2008; Wassmer et al., 2007). The

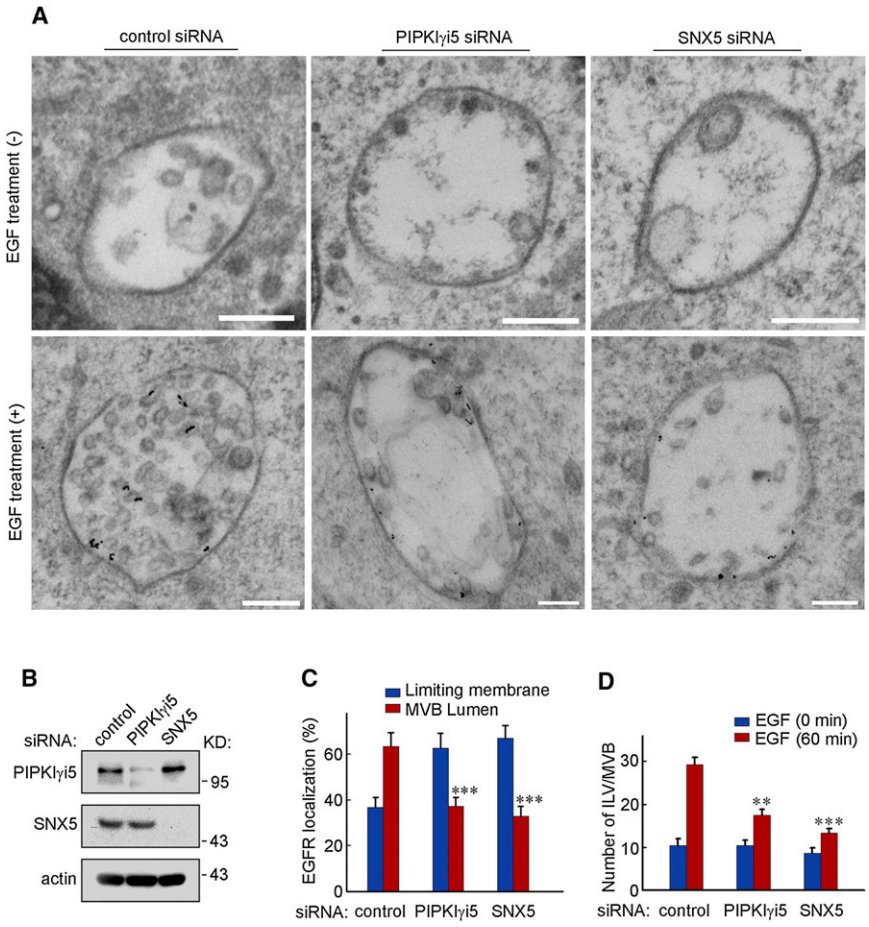

Figure 5. PIPKIγi5 and SNX5 Are Required for EGFR Sorting into ILVs of the MVB

MDA-MB-231 cells were transfected with control, PIPKIγi5 siRNA, or SNX5 siRNA separately, and then the cells were treated with or without EGF (10 nM) for 1 hr and used in the EM study.

(A) MVBs in different siRNA-transfected cells are shown. An MVB containing immunogold-labeled EGFR was seen in EGF-treated cells.

(B) Knockdown efficiency of PIPKIγi5 and SNX5 was confirmed via western blot.

(C) Amount of immunogold-labeled EGFR in the MVB lumen or limiting membrane in EGF-treated cells was quantified.

(D) The number of ILVs in each MVB was quantified. Error bars indicate mean ± SEM. **p < 0.01; ***p < 0.001 (n = 60 MVBs from three independent experiments for each siRNA treatment). Scale bar represents 200 nm.

retromer consists of a Vps26, Vps29, Vps35 heterotrimer and an SNX dimer. To determine if the role of SNX5 in modulating EGFR degradation is dependent on retromer function, two other key retromer components, Vps26 and Vps35, were knocked down and the impact on EGFR degradation was quantified. Loss of Vps26 or Vps35 did not impact EGFR degradation (Figures S4A and S4B), indicating that retromer function is not required for EGFR degradation. The above data suggest that PIPKIγi5 and SNX5 function together to modulate EGFR trafficking, and we explored the role in downregulation of other receptors.

Activation of c-Met by hepatocyte growth factor or PAR1 activation by thrombin also results in receptor degradation in the lysosome (Gullapalli et al., 2006; Hammond et al., 2001). Downregulation of c-Met (Figures S4C and S4D) or PAR1 (Figures S4E and S4F) was unaffected by PIPKIγi5 loss. Similarly, the knockdown of SNX5 blocked the degradation of EGFR, while the degradation of c-Met or PAR1 was not affected (Figures S4G–S4J). This indicates that PIPKIγi5 and SNX5 may modulate the lysosomal sorting of a subset of receptors and that loss of PIPKIγi5 or SNX5 does not disrupt the general function of the endolysosomal system.

PIPKIγi5 and SNX5 Are Required for EGFR Sorting into ILVs of the MVB

PIPKIγi5 and SNX5 are required for EGFR trafficking from endosome to lysosome for degradation (Figures 2 and 4). The sorting of EGFR into ILVs of the MVB is required for its lysosomal sorting and degradation (Eden et al., 2009). To define the role for PIPKIγi5 or SNX5 in EGFR ILV sorting, an electron microscopy (EM) approach was used. Cells were serum starved and then treated with or without EGF (10 nM) for 1 hr. EGF treatment has been shown to stimulate the formation of ILVs and EGFR sorting into ILVs (Eden et al., 2009; White et al., 2006). As shown in Figure 5, EGF-induced ILV formation was decreased in PIPKIγi5- or SNX5-knockdown cells. The ILV sorting of EGFR in EGF-treated cells was tracked via anti-EGFR antibody and 10 nm protein A-gold (see Experimental Procedures). In PIPKIγi5- or SNX5-knockdown cells, the quantity of EGFR was greater at the limiting membrane of the MVB with reduced EGFR in ILVs (Figure 5). This indicates a defect in sorting of EGFR from the limiting membrane to ILVs in PIPKIγi5- or SNX5-knockdown cells.

PIPKIγi5 and Phosphoinositides Modulate Interactions among SNX5, Hrs, and EGFR

Membrane containing EGFR invaginates from the limiting membrane of the MVB to form ILVs, a process dependent on the endosomal sorting complex required for transport (ESCRT) (Katzmann et al., 2002). Hrs is a key component of ESCRT-0 (Henne et al., 2011) that binds to ubiquitinated EGFR and recruits additional ESCRT components to mediate EGFR sorting into ILVs (Eden et al., 2009). Similar to knockdown of PIPKIγi5 or SNX5, Hrs knockdown leads to a defect in EGFR sorting from MVB-limiting membrane to ILVs (Razi and Futter, 2006). To determine if PIPKIγi5 and SNX5 modulate EGFR sorting to ILVs via an Hrs-mediated pathway, the effect of their loss on the Hrs-EGFR interaction was explored. Knockdown of either PIPKIγi5 or SNX5 resulted in a loss of the interaction of EGFR with Hrs (Figures 6A and 6B).

SNX5 associates with Hrs and was coimmunoprecipitated with endogenous Hrs (Figure 6C). Further, the SNX5-Hrs

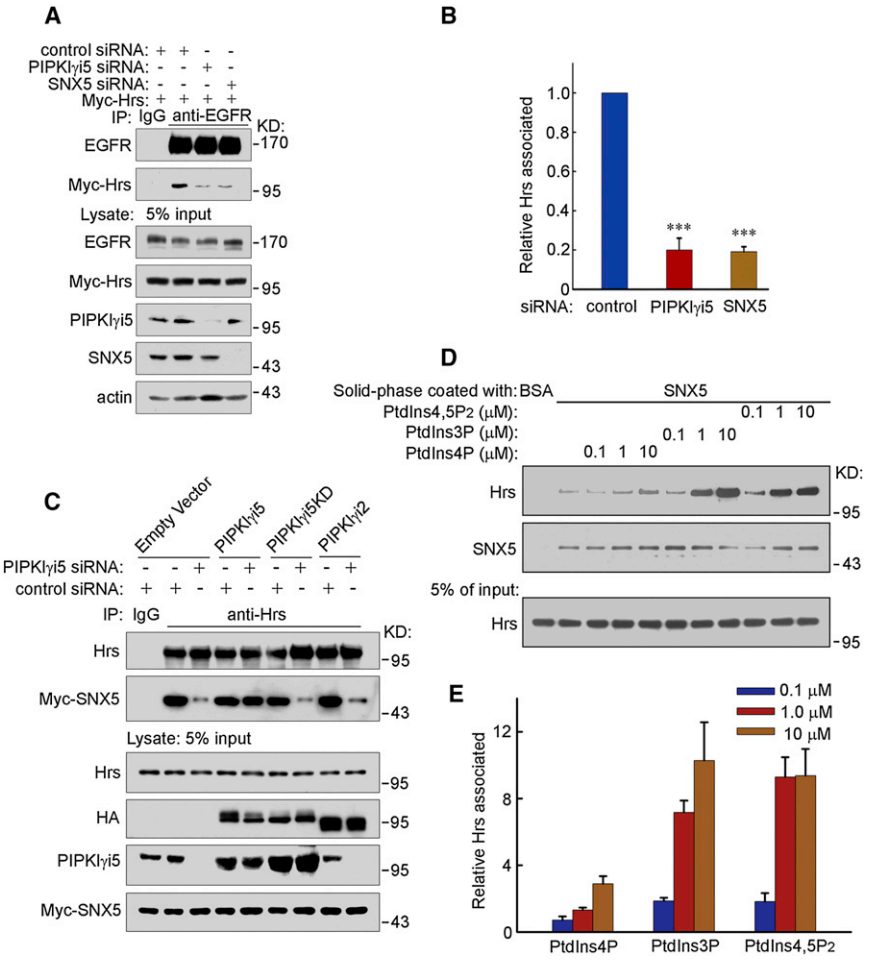

Figure 6. SNX5 and PIPKIγi5 Modulate EGFR-Hrs Interaction

(A) MDA-MB-231 cells were transfected with control, PIPKIγi5 siRNA, or SNX5 siRNA, and the effects on EGFR-Hrs interaction were assessed via coimmunoprecipitation assay.

(B) Quantification of the EGFR-Hrs interaction (n = 3). Error bars indicate mean ± SEM. ***p < 0.001.

(C) MDA-MB-231 cells expressing wild-type PIPKIγi5, PIPKIγi5KD, or PIPKIγi2 were established by lentivirus infection. Cells were transfected with control or PIPKIγi5 siRNA, and the effects on the SNX5-Hrs interaction were evaluated via coimmunoprecipitation assay.

(D) Interaction of purified His_6-SNX5 and GST-Hrs was measured in a solid-phase binding assay with or without PtdIns4P, PtdIns3P, or PtdIns4,5P_2 as indicated.

(E) Quantification of Hrs-SNX5 interaction in the solid-phase binding assay. (n = 3). Error bars indicate mean ± SEM.

See also Figure S5.

interaction was PIPKIγi5 dependent as loss of PIPKIγi5 diminished the SNX5-Hrs interaction (Figure 6C). The SNX5-Hrs interaction was rescued by re-expression of PIPKIγi5 but not PIPKIγi5KD (Figure 6C), indicating that PIPKIγi5 kinase activity is required for the SNX5-Hrs interaction. Expression of PIPKIγi2 could not rescue the SNX5-Hrs interaction (Figure 6C), indicating that this function is PIPKIγi5 specific.

Multiple phosphoinositides, including PtdIns3P and PtdIns4,5P_2, have been shown to bind to SNX5 (Koharudin et al., 2009; Pylypenko et al., 2007; van Weering et al., 2010). To determine if PtdIns4,5P_2 modulates the SNX5-Hrs interaction, a solid-phase-based in vitro binding assay was used with purified recombinant SNX5 and Hrs. As shown in Figures 6D and 6E, addition of PtdIns4,5P_2 or PtdIns3P greatly enhanced the SNX5-Hrs interaction. This result suggests that PtdIns4,5P_2 production by PIPKIγi5 modulates the SNX5-Hrs interaction, which is consistent with the loss of SNX5-Hrs interaction observed after PIPKIγi5 knockdown. PtdIns4P had a minimal effect on the SNX5-Hrs interaction compared with PtdIns4,5P_2 or PtdIns3P, indicating a specificity of phosphoinositides in modulating the SNX5-Hrs interaction (Figures 6D and 6E).

To explore the targeting of SNX5 to endosomes, Hrs or PIPKIγi5 was knocked down. This did not significantly change SNX5 targeting (Figure S5A). These data indicate that PtdIns4,5P_2 generation alone does not control SNX5 endosomal targeting. However, inhibition of PI3K impedes SNX5 endosomal targeting, indicating a role for PtdIns3P generation in this process (Figures S5B and S5C). These combined results indicate that SNX5 may be regulated by multiple phosphoinositides.

These results suggest that both PtdIns3P and PtdIns4,5P_2 play critical roles in modulating SNX5 function at endosomes. To assess if phosphoinositide binding is required for the SNX5 modulation of EGFR sorting, we used a structure-function approach to define SNX5 binding to phosphoinositides. Though PX domains of SNXs primarily bind to PtdIns3P (Carlton et al., 2005), the structure of the SNX5-PX domain was solved by nuclear magnetic resonance and X-ray crystallography, and this method found that SNX5-PX interacted with PtdIns4,5P_2 (Koharudin et al., 2009). R42/K44/K46 are positively charged and form a sequence found in the SNX5 PX domain critical for PtdIns4,5P_2 binding (Koharudin et al., 2009). These positively charged residues were mutated to the similar, but uncharged, glutamine. This mutant was named SNX5_PX3. A PIP strip assay showed that wild-type SNX5 protein could bind to multiple phosphoinositides, including PtdIns3P and PtdIns4,5P_2 (Figure S6C). The PX domain of SNX5_PX3 is defective in PtdIns4,5P_2 binding (data not shown). Unexpectedly, the full-length SNX5_PX3 protein still retained the ability to bind PtdIns4,5P_2 via a PIP strip assay (Figure S6C). This indicates that the BAR domain of SNX5 is also capable of interacting with PtdIns4,5P_2.

It was reported that with SNX9, mutations of specific residues in the BAR domain inhibited its phosphoinositide binding and function (Pylypenko et al., 2007). Using a sequence and structural homology approach with SNX9 (see Figure S6A), residues were mutated (K224E/R235E/K324E/K328E/R330E) in the

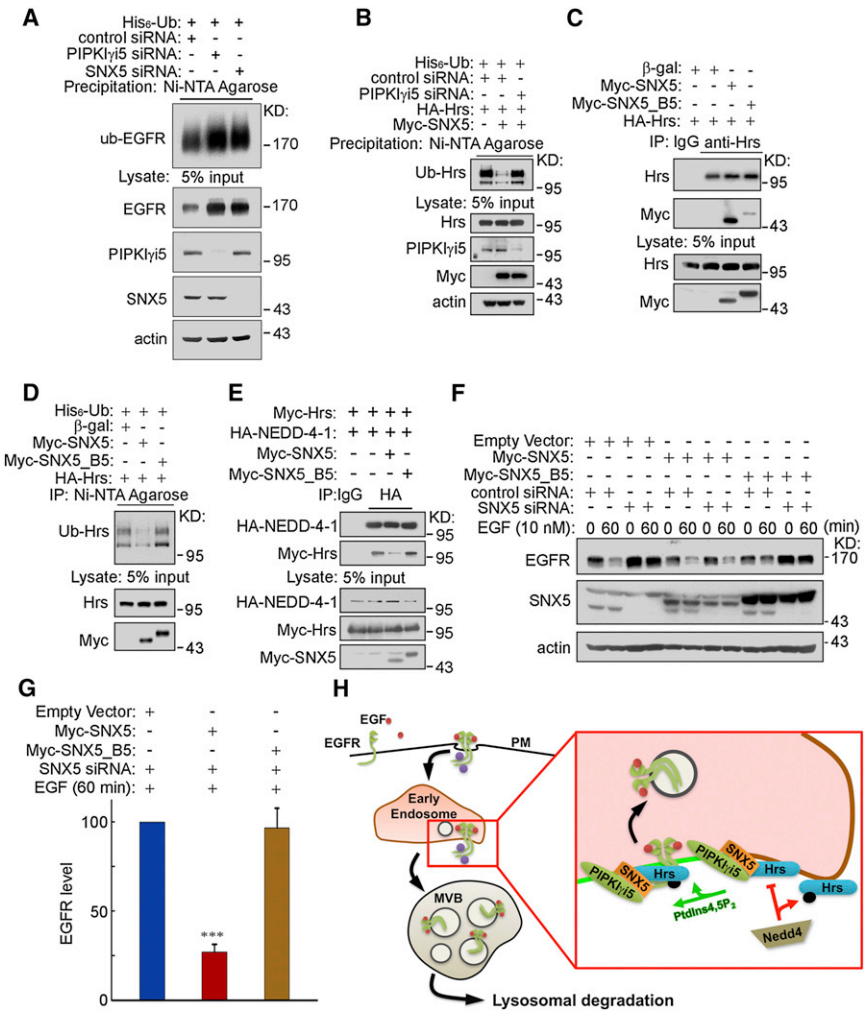

Figure 7. SNX5 and PIPKIγi5 Modulate Hrs Ubiquitination

(A) MDA-MB-231 cells were transfected with control, PIPKIγi5 siRNA, or SNX5 siRNA. Cells were then treated with EGF (10 nM) for 15 min, and the ubiquitination of EGFR was measured.

(B) MDA-MB-231 cells were transfected with or without Myc-SNX5 combined with control or PIPKIγi5 siRNA, and the effects on Hrs ubiquitination were detected.

(C) HA-Hrs was coexpressed with Myc-SNX5 or Myc-SNX5_B5, and the Hrs-SNX5 interaction was detected via coimmunoprecipitation assay.

(D) MDA-MB-231 cells were transfected with β-galactosidase (control), Myc-SNX5, or Myc-SNX5_B5, and the effects on Hrs ubiquitination were detected.

(E) HA-NEDD-4-1 and Myc-Hrs was coexpressed with Myc-SNX5 or Myc-SNX5_B5, and the Hrs interaction with NEDD-4-1 was detected via coimmunoprecipitation assay.

(F) MDA-MB-231 cell lines expressing wild-type SNX5 or SNX5_B5 were established by lentivirus infection. Cells were transfected with control or SNX5 siRNA and then stimulated with EGF (10 nM) for 60 min. The expression of EGFR or SNX5 protein was detected with specific antibodies compared to the actin loading control.

(G) Rescue effect of SNX5 or SNX5_B5 on EGFR downregulation in SNX5 siRNA-transfected cells was quantified. Error bars indicate mean ± SEM. ***$p < 0.001$ ($n = 3$).

(H) Model for PIPKIγi5 and SNX5 regulation of EGFR endosomal trafficking and degradation. PIPKIγi5 directly interacts with SNX5 and generates PtdIns4,5P_2, which modulates SNX5-Hrs interaction. The SNX5-Hrs interaction inhibits NEDD-4 recruitment to Hrs and blocks Hrs ubiquitination and facilitates Hrs interaction with EGFR to initiate EGFR sorting to ILVs for downstream lysosomal degradation.

See also Figure S6.

SNX5-BAR domain (SNX5_B5). This mutant exhibited reduced phosphoinositide binding, including decreased binding to PtdIns4,5P_2 and PtdIns3P (Figure S6C). The abundance of positive charges along the concave face of the BAR domain is conducive to binding negatively charged lipid membrane surfaces (Frost et al., 2009). Consistently, by liposome binding assay, full-length SNX5 could bind to multiple phosphoinositides, including PtdIns4,5P_2, PtdIns3P, and other PtdInsP_n isomers (Figure S6F).

PIPKIγi5, Phosphoinositides, and SNX5 Modulate Hrs Ubiquitination

The interaction between Hrs and EGFR is required for lysosomal sorting, and these interactions are regulated by ubiquitination of Hrs and EGFR (Eden et al., 2009; Komada and Kitamura, 2005; Sorkin and Goh, 2009; Zwang and Yarden, 2009). The ubiquitination of EGFR is required for interaction with Hrs and EGFR sorting to the ILV (Eden et al., 2012). EGFR ubiquitination was not inhibited by loss of PIPKIγi5 or SNX5 (Figure 7A). Ubiquitination of Hrs inhibits its ability to interact with ubiquitinated cargos such as EGFR (Hoeller et al., 2006). SNX5 overexpression blocked Hrs ubiquitination, and this required PIPKIγi5 (Figure 7B). Consistently, loss of PIPKIγi5 dramatically decreased the interaction of SNX5 with Hrs (Figure 6C) and increased Hrs ubiquitination (Figure 7B). These data indicate that PIPKIγi5 and SNX5 together regulate the ubiquitination of Hrs and thus the interaction of Hrs with EGFR (Figures 6A and 6B), an interaction required for sorting of EGFR to the ILV (Eden et al., 2012).

PIPKIγi5 and SNX5 did not regulate c-Met or PAR1 degradation (Figures S4C–S4J), and loss of Hrs also did not impact c-Met or PAR1 degradation (Figures S4G–S4J), but Hrs is required for EGFR degradation (Eden et al., 2012). This suggests that PIPKIγi5, SNX5, and Hrs form a nexus that regulates EGFR degradation. Phosphoinositides regulate the SNX5-Hrs interaction (Figure 6D), and this interaction blocks Hrs ubiquitination (Figure 7B). In vitro, SNX5 and SNX5_B5 indistinguishably interact with Hrs without phosphoinositides (data not shown), while the addition of PtdIns4,5P_2 or PtdIns3P did not enhance Hrs-SNX5_B5 interaction (Figures S6D and S6E). This is consistent with the finding that SNX5_B5 lost phosphoinositides binding (Figure S6C). In vivo, SNX5_B5 interacts poorly with Hrs compared to wild-type (Figure 7C). Expression of SNX5 but not

SNX5_B5 blocked Hrs ubiquitination (Figure 7D). This is consistent with the data showing that SNX5 interaction with Hrs is regulated by phosphoinositide binding.

The E3 ubiquitin ligase NEDD-4-1 ubiquitinates Hrs, and this ubiquitination inhibits Hrs interaction with ubiquitinated EGFR (Hoeller et al., 2006; Katz et al., 2002; Lin et al., 2010). NEDD-4-1 interacts with Hrs, but this interaction is reduced upon expression of SNX5 but not the SNX5_B5 mutant (Figure 7E). As the interaction of NEDD-4-1 is required for Hrs ubiquitination, this defines a mechanism for SNX5 control of Hrs ubiquitination (Hoeller et al., 2006; Katz et al., 2002; Lin et al., 2010).

To determine if SNX5 requires phosphoinositide binding for EGFR sorting and degradation, a knockdown and rescue assay was established. This approach demonstrated that SNX5 rescued the EGFR degradation defect in SNX5-knockdown cells but the SNX5_B5 mutant did not (Figures 7F and 7G). This is consistent with the deficiency of SNX5_B5 to interact with Hrs in vivo and its inability to modulate Hrs ubiquitination. These data are consistent with a model in which PIPKIγi5 directly interacts with SNX5 and subsequent PtdIns4,5P_2 generation enhances the SNX5-Hrs interaction. The SNX5-Hrs interaction inhibits NEDD-4-1 recruitment to Hrs and blocks Hrs ubiquitination. Thus, PIPKIγi5 and SNX5 collaborate to facilitate Hrs interaction with ubiquitinated EGFR, which initiates EGFR sorting to ILVs for subsequent lysosomal degradation (Figure 7H).

DISCUSSION

PtdIns3P plays essential roles in the trafficking of EGFR and other receptors through the endosomal and lysosomal pathway (Clague et al., 2009; de Lartigue et al., 2009; Lindmo and Stenmark, 2006; Sorkin and Goh, 2008). We have shown that PIPKIγi5 and its kinase activity are also required for EGFR sorting to the ILVs of the MVB, supporting a role for PtdIns4,5P_2 in EGFR endosomal trafficking. Hrs, a PtdIns3P binding protein, also binds ubiquitinated EGFR and is required for sorting EGFR to ILVs (Sorkin and Goh, 2008). PIPKIγi5, SNX5, and PtdIns4,5P_2 synthesis regulates the interaction of EGFR with Hrs by regulating the ubiquitination of Hrs, a process known to block the interaction of Hrs with EGFR (Hoeller et al., 2006). As the Hrs interaction with EGFR is essential for EGFR sorting to ILVs, this represents a key regulatory step in this pathway (see Figure 7H).

PtdIns4,5P_2 modulates many biological processes, including adhesion and cytoskeletal dynamics (Ling et al., 2006), vesicular trafficking (Downes et al., 2005), secretion (Martin, 2001), ion channel regulation (Delmas et al., 2005), nuclear signaling, and gene expression (Barlow et al., 2010; Mellman et al., 2008). These activities are regulated by PtdIns4,5P_2 synthesis at diverse subcellular sites (Barlow et al., 2010; Heck et al., 2007). The PH domain of PLCδ fused to GFP (PLCδ-PH-GFP) has been used as a PtdIns4,5P_2-specific probe, and it primarily detects PtdIns4,5P_2 at the plasma membrane (Botelho et al., 2000; Várnai and Balla, 1998). It is clear that PLCδ-PH does not detect all cellular PtdIns4,5P_2, for example at focal adhesions or in the nucleus (Barlow et al., 2010; Ling et al., 2002). Consistently, we have not been able to detect PtdIns4,5P_2 at EGFR-containing endosomes with PLCδ-PH-GFP (data not shown).

The inability to detect PtdIns4,5P_2 at some compartments may be explained by a low abundance of PtdIns4,5P_2 or by the mechanism of PIP kinase signaling at these sites. The specificity of PtdIns4,5P_2 signaling can be regulated by PIP kinase interactions with PtdIns4,5P_2 effectors (Anderson et al., 1999; El Sayegh et al., 2007; Heck et al., 2007; Li et al., 2012; Ling et al., 2002, 2007; Mellman et al., 2008; Schill and Anderson, 2009a; Thapa et al., 2012). For this mechanism, we and others have been unable to show a targeting of the PtdIns4,5P_2-specific PLCδ-PH-GFP to locations where the PIP kinases function, including focal adhesions, vesicles for trafficking, and the nucleus (Li et al., 2012; Ling et al., 2002, 2007; Mellman et al., 2008; Sun et al., 2007; Thapa et al., 2012). Potentially, the abundance of PtdIns4,5P_2 at these sites is low because the PtdIns4,5P_2 is bound to effector proteins. Using biochemical approaches, PtdIns4,5P_2 has previously been shown to be synthesized on late endosomes and lysosomes (Arneson et al., 1999; Watt et al., 2002). Recently, it was found that PtdIns4,5P_2 is present at autolysosomes and regulates autophagic lysosome reformation (Rong et al., 2012). The combined results support PtdIns4,5P_2 generation on endosome/lysosome membranes.

PIPKIγ isoforms use PtdIns4P as substrate to synthesize PtdIns4,5P_2 (Anderson et al., 1999). Type II phosphatidylinositol 4-kinase (type II PI-4K) α and β are enzymes that synthesize PtdIns4P and can be targeted to endosomes (Balla et al., 2002), indicating that the PIPKIγ substrate is present at endosomes. Consistent with this role, the type II PI-4Kα has been reported to modulate EGFR trafficking to the late endosome (Minogue et al., 2006). OCRL, a PtdIns4,5P_2 5-phosphatase, is reported to function at endosomes (Vicinanza et al., 2011). Loss of OCRL leads to a decrease of EGFR degradation (Vicinanza et al., 2011), indicating that both PIPKIγi5 and OCRL, the enzymes producing and destroying PtdIns4,5P_2, respectively, play roles in EGFR degradation.

Multiple phosphoinositide phosphate isomers bind to SNX5, including PtdIns3P, PtdIns3,4P_2, and PtdIns4,5P_2 (Koharudin et al., 2009; Liu et al., 2006; Merino-Trigo et al., 2004). Our results are consistent, indicating that SNX5 binds to multiple phosphoinositides through different sites on both the PX and BAR domains. Our results indicate that PtdIns3P and PtdIns4,5P_2 bind to SNX5 and promote its interaction with Hrs (see Figure 6).

SNX5 is a component of the mammalian retromer (Wassmer et al., 2007, 2009) that controls trafficking between the endosome and the TGN (Bonifacino and Hurley, 2008). The retromer is composed of SNX5 and SNX6 in association with SNX1 and SNX2, and these SNXs form complexes with the cargo recognition trimer composed of Vps26, Vps29, and Vps35 (Bonifacino and Hurley, 2008). Loss of Vps26 or Vps35 did not impact EGFR lysosomal degradation (Figure S4), indicating that retromer function was not involved. Yet, overexpression of SNX5 inhibited EGFR degradation (Liu et al., 2006), possibly by disrupting endogenous interactions with other components. Similarly, Hrs mediates EGFR degradation (Lloyd et al., 2002), but its overexpression also inhibited EGFR degradation (Chin et al., 2001). SNX1 and SNX2 may influence the lysosomal sorting of internalized EGFR, but neither protein is essential for this process (Gullapalli et al., 2004). The loss of SNX5, SNX6, or both in HeLa cells was shown to also diminish SNX1 protein levels (Wassmer et al., 2007). In MDA-MB-231 cells, knockdown of SNX5 does not

result in loss of SNX1, SNX2, or SNX6. However, efficient knockdown of SNX1 or SNX2 resulted in loss of SNX5 (but not SNX6), resulting in a block of EGF-stimulated EGFR degradation (unpublished data). Knockdown of SNX6 also decreased SNX1 and SNX2 and blocked EGFR degradation (unpublished data). These results are consistent with the assembly of SNX1, SNX2, SNX5, and SNX6 into a dynamic complex (Wassmer et al., 2009) that stabilizes the proteins within the complex. These SNXs bind phosphoinositides, target to the endosome, and may function together in EGFR endosomal trafficking.

PIPKIγi5, SNX5, and Hrs regulate the degradation of EGFR but not c-Met or PAR1. This suggests that PIPKIγi5, SNX5, and Hrs work in a common pathway that is receptor selective. Previous findings support receptor-specific mechanisms for the formation of ILVs in the MVB (Babst, 2011; White et al., 2006). For example, the sorting of PAR1 into ILVs of the MVB is independent of Hrs (Dores et al., 2012). This supports a model where multiple pathways control receptor sorting into ILVs. The PIPKIγi5 pathway has significant implications for EGFR signaling, as the EGFR remains active as it travels through the endosomal pathway. Changes in expression or regulation of PIPKIγi5, SNX5, or Hrs are positioned to regulate EGFR degradation and signaling. As EGFR plays key roles in cancer biology, therapeutic modulation of this pathway represents a mechanism to control the magnitude and duration of EGFR signaling. Further, this pathway may control the cellular content of EGFR, a key factor in EGFR control of autophagic cell death (Weihua et al., 2008).

EXPERIMENTAL PROCEDURES

Lentivirus Constructs

Generation of replication-defective infectious viral particles and the transduction of the cells were carried out following the protocol provided by Addgene and Invitrogen. In brief, Myc-tagged SNX5 constructs containing silence mutations in the SNX5 siRNA targeting region were cloned into MluI and SalI sites of PWPT vector (Addgene). Hemagglutinin-tagged PIPKIγi5 constructs containing silence mutations in the PIPKIγi5 siRNA targeting region were cloned into pLenti6.3 vector (Invitrogen) following the company's instructions. Stbl3 competent cells (Invitrogen) were used for transformation and DNA purification to minimize the mutagenesis.

Electron Microscopy

The EGFR trafficking into the MVB was detected via EM as described previously (Bache et al., 2006; Hanafusa et al., 2011). MDA-MB-231 cells treated with control or PIPKIγi5 siRNA were serum starved. The cells were then labeled with LA22 EGFR antibody (Millipore) at 4°C for 20 min and washed thrice, followed by 20 min incubation with 10 nm protein A-gold (Electron Microscopy Sciences). After washing, the cells were treated with EGF (10 nM) for 60 min at 37°C. Cells then were fixed in 0.1 M sodium cacodylate containing 2.0% paraformaldehyde and 2.5% glutaraldehyde. The morphology of the MVB was visualized by a JOEL100CX transmission electron microscope at the UW Medical School EM Facility. Three separate experiments were performed for each treatment, and >2,000 μm^2 of cytoplasm was examined in each case. More than 60 MVBs were examined for statistical analysis for each treatment.

Immunoprecipitation and Immunoblotting

Immunoprecipitation was performed as described previously (Ling et al., 2003). Briefly, 24 hr after transfection, MDA-MB-231 cells were starved with serum-free Dulbecco's modified Eagle's medium (DMEM) overnight and then stimulated with or without 10 nM EGF for 15 min. Then cells were harvested and lysed in 25 mM HEPES (pH 7.2), 150 mM NaCl, 0.5% NP-40, 1 mM $MgCl_2$, and protease inhibitor cocktail and then centrifuged and incubated with protein G Sepharose and 2 μg antibody as indicated at 4°C for 4 hr. The immunocomplexes were separated by SDS-PAGE and analyzed as indicated.

Immunofluorescence

Cells were resuspended and then plated on the coverslips in DMEM with 10% fetal bovine serum, allowed to adhere for 4 hr, and then starved in serum-free DMEM for 2 hr. Then, cells were stimulated with 10 nM EGF for a different time course and fixed by 4% paraformaldehyde. Then, cells were permeablized with 0.5% Triton X-100 and blocked by 3% BSA in PBS at room temperature for 30 min, incubated with the primary antibody overnight at 4°C, washed with 0.1% Triton X-100 in PBS, incubated with fluorescence-labeled secondary antibody at room temperature for 30 min, and then washed with 0.1% Triton X-100 in PBS. Cells were maintained and examined using a 60× Plan oil immersion lens on an inverted microscope (Eclipse TE200-U, Nikon). Images were processed as described previously (Ling et al., 2002) using Photoshop 7.0.

Quantification of Colocalization

The background-subtracted images were segmented using a minimal intensity of EEA1- or LAMP1-labeled vesicles as a low threshold. The integrated voxel intensity of EGFR in the segmented image was considered as EGFR localized in EEA1- or LAMP1-labeled vesicles, respectively. The extent of colocalization was calculated as the ratio of the integrated EGFR fluorescence of the segmented image to the total fluorescence of the same fluorochromes.

Solid-Phase Binding Assay

This assay was performed as described previously (Martel et al., 2001). Microtiter plates (96 wells; MaxiSorp Immuno Plate, Nunc) were coated overnight at 4°C with 1 μg of His_6-SNX5 per well in a final volume of 200 μl in PBS and subsequently blocked with 1% fatty-acid-free BSA in PBS for 1 hr at room temperature. The plates were then incubated with or without PtdIns4,5P_2 or PtdIns3P in a final volume of 200 μl in PBS for 30 min at room temperature. Then plates were incubated with GST-Hrs (1 μg in 200 μl PBS) for 1 hr at room temperature. The wells were then washed three times with PBS containing 1% fatty-acid-free BSA, and bound protein was removed by the addition of 40 μl of Laemmli sample buffer followed by incubation of the microtiter plate at 95°C for 7 min.

In Vivo Ubiquitination Assay

The ubiquitination of Hrs was evaluated as described previously (Pan and Chen, 2003). His_6-ubiquitin-conjugated Hrs in MDA-MB-231 cells was purified by Ni^{2+}-nitrilotriacetic acid (NTA) beads. MDA-MB-231 cell was lysed in IP buffer (25 mM HEPES [pH 7.2], 150 mM NaCl, 0.5% NP-40, 1 mM $MgCl_2$, and protease inhibitor cocktail) and incubated with Ni^{2+}-NTA beads (QIAGEN) for 2 hr at 4°C. The beads were washed with IP buffer, buffer A (8 M urea, 0.1 M Na_2PO_4/NaH_2PO_4, 0.01 M Tris-HCl [pH 8.0], and 10 mM β-mercaptoethanol), and buffer B (8 M urea, 0.1 M Na_2PO_4/NaH_2PO_4, 0.01 M Tris-HCl [pH 6.3], and 10 mM β-mercaptoethanol), and bound proteins were eluted with buffer C (200 mM imidazole, 0.15 M Tris-HCl [pH 6.7], 30% glycerol, 0.72 M β-mercaptoethanol, and 5% SDS). The eluted proteins were analyzed by western blotting for the presence of His_6-ubiquitin-conjugated Hrs via using anti-Hrs antibody.

Statistics

All data analysis was performed using SigmaPlot. Bar graphs represent means ± SEM, as indicated. Statistical significance was assessed using the Student's t test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2013.03.010.

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