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Type I γ Phosphatidylinositol Phosphate 5-Kinase i5 Controls the Ubiquitination and Degradation of the Tumor Suppressor Mitogen-inducible Gene 6*

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Mitogen-inducible gene 6 (Mig6) is a tumor suppressor, and the disruption of Mig6 expression is associated with cancer development. Mig6 directly interacts with epidermal growth factor receptor (EGFR) to suppress the activation and downstream signaling of EGFR. Therefore, loss of Mig6 enhances EGFR-mediated signaling and promotes EGFR-dependent carcinogenesis. The molecular mechanism modulating Mig6 expression in cancer remains unclear. Here we demonstrate that type I γ phosphatidylinositol phosphate 5-kinase i5 (PIPKIγi5), an enzyme producing phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2), stabilizes Mig6 expression. Knockdown of PIPKIγi5 leads to the loss of Mig6 expression, which dramatically enhances and prolongs EGFR-mediated cell signaling. Loss of PIPKIγi5 significantly promotes Mig6 protein degradation via proteasomes, but it does not affect the Mig6 mRNA level. PIPKIγi5 directly interacts with the E3 ubiquitin ligase neuronal precursor cell-expressed developmentally down-regulated 4-1 (NEDD4-1). The C-terminal domain of PIPKIγi5 and the WW1 and WW2 domains of NEDD4-1 are required for their interaction. The C2 domain of NEDD4-1 is required for its interaction with PtdIns(4,5)P_2. By binding with NEDD4-1 and producing PtdIns(4,5)P_2, PIPKIγi5 perturbs NEDD4-1-mediated Mig6 ubiquitination and the subsequent proteasomal degradation. Thus, loss of NEDD4-1 can rescue Mig6 expression in PIPKIγi5 knockdown cells. In this way, PIPKIγi5, NEDD4-1, and Mig6 form a novel molecular nexus that controls EGFR activation and downstream signaling.

EGF receptor (EGFR)[2] is a critical receptor tyrosine kinase that controls cell growth and differentiation during embryogenesis and adult homeostasis (1). After stimulation by its agonists, EGFR mediates multiple downstream signaling cascades that control cell proliferation, apoptosis, migration, survival, angiogenesis, and other biological processes (2). The appropriate regulation of EGFR activation is critical to maintain normal physiology, and loss or overactivation of EGFR can lead to multiple diseases (2, 3). The overexpression or aberrant kinase activity of EGFR could result in unregulated growth stimulation, tumorigenesis, and metastasis in various tumor types (4). Therefore, targeted therapies directed against EGFR have been a primary focus of antitumor drug development over the past 10 years (5). An understanding of the molecular mechanisms controlling EGFR activation is required to design efficient anti-EGFR therapies and to identify biomarkers predicting higher sensitivity to anti-EGFR drugs.

Mig6 (also known as RALT or Gene 33) is a widely expressed adaptor protein that directly binds to the kinase domain of EGFR to block its activation (6–8). Agonist binding stimulates the formation of an asymmetric EGFR dimer by the intracellular kinase domains in which the carboxy-terminal lobe (C lobe) of one kinase domain induces an active conformation in the other (9). Mig6 inhibits EGFR activation by blocking formation of the activating EGFR dimer interface (6). Furthermore, Mig6 controls the endosomal trafficking of EGFR (10). Following agonist stimulation, activated EGFR is internalized, and subsequent trafficking events determine the fate of internalized EGFR, including recycling back to the plasma membrane or trafficking to the lysosomes for degradation (11, 12). Mig6 ensures the sorting of internalized EGFR to late endosomes and the subsequent sorting to lysosomes for degradation (10). Mig6 can be transcriptionally induced by growth factors, cytokines, and cell stress (13–16). Deletion of Mig6 leads to hyperactivated EGFR signaling and causes carcinogenesis in multiple organs (14, 17). The disruption of Mig6 in mice accelerates initiation and progression of mutant EGFR-driven lung adenocarcinoma (18). Thus, Mig6 is a tumor suppressor, and its expression is down-regulated in various cancers (14). The molecular mechanisms controlling Mig6 expression in cancer remain to be clarified.

Type I γ phosphatidylinositol phosphate kinase (PIPKIγ) is an enzyme that generates PtdIns(4,5)P_2 (19). The PIPKIγ gene

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[2] The abbreviations used are: EGFR, EGF receptor; PIPKIγi5, type I γ phosphatidylinositol phosphate 5-kinase i5; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; KD, kinase-dead; CRISPR, clustered regularly interspaced short palindromic repeats; sgRNA, single guide RNA; PAM, protospacer adjacent motif; Ni^{2+}-NTA, nickel-nitrilotriacetic acid; HECT, the homologous to the E6AP carboxyl terminus; PtdIns(3,4,5)P_3, phosphatidylinositol 3,4,5-trisphosphate; PTEN, phosphatase and tensin homolog; ILV, intraluminal vesicle; ESCRT, endosomal sorting complex required for transport; Hrs, hepatocyte growth factor-regulated tyrosine kinase substrate; IP, immunoprecipitation; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PIP, phosphatidylinositol phosphate.

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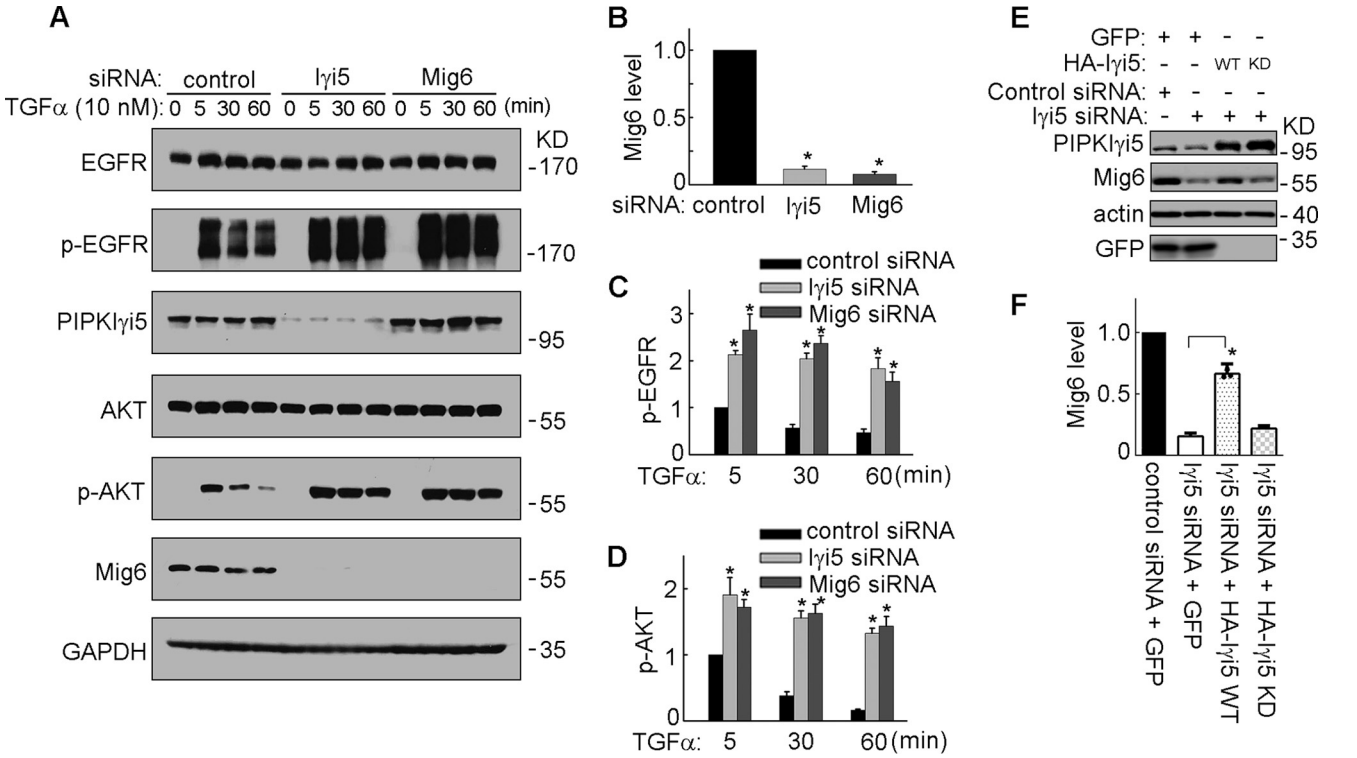

FIGURE 1. **PIPKIγi5 controls Mig6 expression and EGFR signaling.** MDA-MB-231 cells were transfected with control siRNA, PIPKIγi5 (*Iγi5*) siRNA, or Mig6 siRNA separately. 72 h after siRNAs transfection, cells were stimulated with TGFα (10 nM) for the times indicated. *A*, the EGFR protein level, EGFR activation, AKT activation, and Mig6 expression were detected. The following were quantified: Mig6 protein level in TGFα-untreated cells (*B*), EGFR activation detected by Tyr(P)-1068 antibody (*C*), and AKT activation detected by Ser(P)-473 antibody (*D*). Quantification of the Mig6 protein level was normalized with the GAPDH level. Quantification of EGFR or AKT activation was normalized with the total EGFR or AKT level. GFP, HA-tagged wild-type PIPKIγi5, or kinase dead-mutant PIPKIγi5 was constructed into PWPT (Addgene) lentivirus vectors. GFP, HA-PIPKIγi5 WT, or HA-PIPKIγi5 KD was then expressed by lentivirus transfection in PIPKIγi5 knockdown MDA-MB-231 cells, and the effects on Mig6 expression were detected by immunoblotting (*E*) and quantified (*F*). *Error bars* indicate mean ± S.E. from three independent experiments. *, $p < 0.05$.

is alternatively spliced, resulting in at least six protein variants expressed in humans, known as PIPKIγi1-i6 (20). These splicing variants have the same N terminus and kinase domain, and they only differ by different extensions at the C terminus (20, 21). These extensions allow for association with unique binding partners and allow for different PIPKIγ splice variants to produce PtdIns(4,5)P_2 with distinct subcellular distributions necessary to perform specific biological functions. Distinct roles of different PIPKIγ splice variants have been found in mediating or controlling EGFR signaling. For instance, PIPKIγi2 specifically binds to talin at cell adhesions, and the production of PtdIns(4,5)P_2 modulates talin-integrin interaction to regulate inside-out integrin signaling and cell adhesion turnover (22). EGFR phosphorylates PIPKIγi2 to control local PtdIns(4,5)P_2 levels at adhesions. In this way, PIPKIγi2 is required for nascent adhesion formation at the leading edge to facilitate EGFR-induced cell directional migration (22). Unlike PIPKIγi2, PIPKIγi5 is localized at endosomes and is required for EGFR sorting from endosomes to lysosomes for degradation (23). Therefore, the loss of PIPKIγi5 blocks the lysosomal trafficking and degradation of activated EGFR, which dramatically enhances and prolongs EGFR activation and downstream signaling such as AKT and ERK MAPK activation (23). These results indicate that PIPKIγi5 is a critical regulator of EGFR.

Here we show that PIPKIγi5 directly interacts with an E3 ubiquitin ligase, NEDD4-1, to block NEDD4-1-mediated ubiquitination of Mig6. In this way, PIPKIγi5 protects Mig6 from being degraded by the proteasome. Therefore, PIPKIγi5 ensures Mig6 expression and down-regulates EGFR activation. The data uncover a novel molecular nexus composed of PIPKIγi5, Mig6, and NEDD4-1 that controls EGFR signaling.

Results

Loss of PIPKIγi5 Dramatically Decreases Mig6 Expression—PIPKIγi5 is required for down-regulating EGFR activation and signaling (23). However, the molecular mechanism by which PIPKIγi5 controls EGFR is not fully understood. Mig6 is a suppressor of EGFR, and knockdown of PIPKIγi5 by siRNA in MDA-MB-231 cells dramatically decreased Mig6 protein expression levels (Fig. 1, *A* and *B*). Consistently, loss of PIPKIγi5 significantly enhanced and prolonged TGFα-induced autophosphorylation of EGFR on tyrosine 1068 (Fig. 1, *A* and *C*), indicating increased levels of EGFR activation. Downstream AKT activation was also enhanced and prolonged by PIPKIγi5 knockdown (Fig. 1, *A* and *D*). Knockdown of Mig6 by siRNA similarly promoted EGFR activation and AKT activation as the effects induced by PIPKIγi5 knockdown (Fig. 1, *A*, *C*, and *D*). These results indicate that PIPKIγi5 can regulate EGFR signaling by modulating Mig6 expression.

To determine whether PIPKIγi5 lipid kinase activity was required for modulation of Mig6 expression, a knockdown-rescue approach was developed. siRNA was used to knock down endogenous PIPKIγi5, and then wild-type PIPKIγi5 or a kinase-dead mutant (PIPKIγi5KD) construct containing siRNA-

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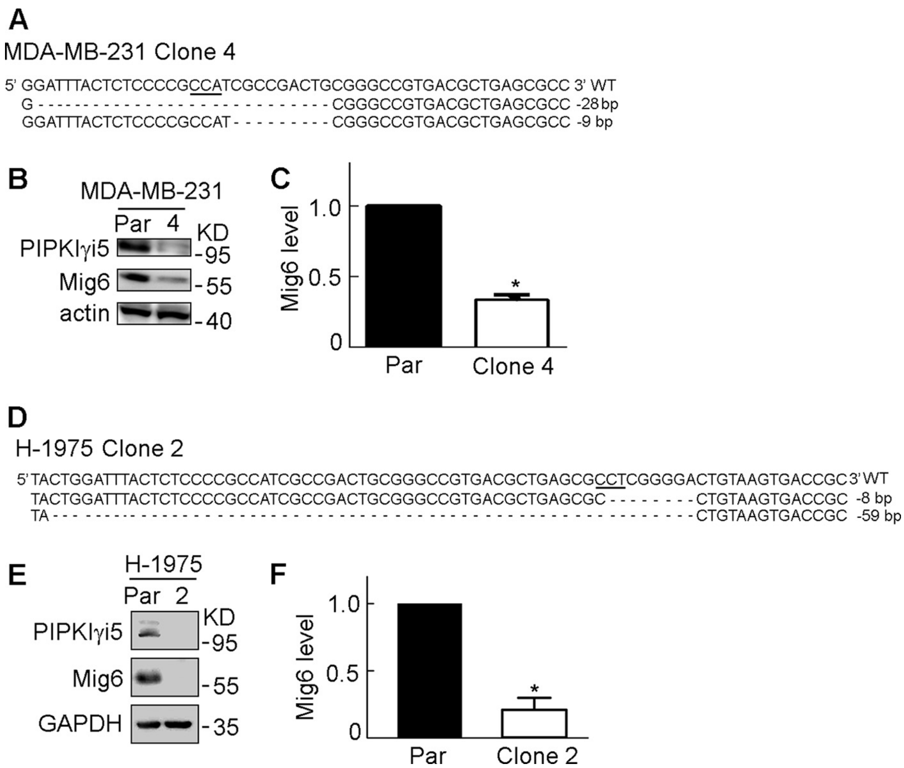

FIGURE 2. **CRISPR-mediated genome disruption of PIPKIγi5 leads to loss of Mig6 expression.** Two different guide RNA sequences were designed to induce genome editing of PIPKIγi5 (*Iγi5*) in MDA-MB-231 or H-1975 cells separately. Single clones were selected and validated by sequencing the targeted PIPKIγi5 loci. The sequences of two mutant alleles in MDA-MB-231 single cell clone 4 are shown in (*A*). PIPKIγI5 and Mig6 were detected from MDA-MB-231 parental (*Par*) or single clone 4 by immunoblotting (*B*), and Mig6 expression levels were quantified (*C*). The sequences of two mutant alleles in H-1975 single cell clone 2 are shown in (*D*). PIPKIγI5 and Mig6 were detected from H-1975 parental or single clone 2 by immunoblotting (*E*), and Mig6 expression levels were quantified (*F*). Protospacer adjacent motif (PAM) sequences are underlined. *Error bars* indicate mean ± S.E. from three independent experiments. *, $p < 0.05$.

resistant silent mutations was re-expressed using lentivirus-mediated infection. Expression of wild-type PIPKIγi5 but not PIPKIγi5KD significantly rescued Mig6 expression in PIPKIγi5 knockdown cells (Fig. 1, *E* and *F*). These results confirm the roles of PIPKIγi5 in Mig6 expression and indicate that kinase activity with resulting production of PtdIns(4,5)P_2 is required for PIPKIγi5 control of Mig6 expression.

To rule out possible PIPKIγi5 siRNA off-target effects, another approach, clustered regularly interspaced short palindromic repeats (CRISPR)-cas9 genome editing (24, 25), was used to disrupt PIPKIγi5 expression. Two different sgRNA sequences were designed to mediate the genome editing of PIPKIγi5 in a breast cancer cell line, MDA-MB-231, and a lung cancer cell line, H-1975. Single clones with CRISPR-cas9-mediated PIPKIγi5 genome editing were selected from MDA-MB-231 and H-1975 cells. The single clone selected from MDA-MB-231 has an in-frame 28-bp deletion at the targeted PIPKIγi5 locus in one allele, leading to full disruption, and an in-frame 9-bp deletion at another allele, leading to a deletion of three amino acids at the C terminus of PIPKIγi5 (Fig. 2*A*). Although it is not a full disruption of PIPKIγi5, PIPKIγi5 protein expression is dramatically decreased and Mig6 expression is significantly decreased in this clone, similar to the siRNA-induced PIPKIγi5 knockdown cells (Fig. 2, *B* and *C*). The H-1975 single clone has an in-frame 8-bp and an in-frame 59-bp deletion at the PIPKIγi5 locus in two different alleles separately (Fig. 2*D*). This is a full disruption of PIPKIγi5, and the Mig6 expression in this clone is significantly decreased (Fig. 2, *E* and *F*). These results further confirm that PIPKIγi5 controls Mig6 expression.

To demonstrate whether PIPKIγi5 regulates steady-state Mig6 mRNA levels, a real-time PCR assay was performed to detect the mRNA levels of Mig6 in control or PIPKIγi5 knockdown cells. As shown in Fig. 3*A*, knockdown of PIPKIγi5 does not significantly change Mig6 mRNA levels. This suggests that PIPKIγi5 may modulate Mig6 protein degradation but not the steady-state Mig6 mRNA levels. The efficiency of PIPKIγi5 knockdown was confirmed by Western blotting (Fig. 3*B*). To define whether PIPKIγi5-regulated Mig6 degradation is dependent on lysosomes or proteasomes, the lysosome inhibitor chloroquine or the proteasome inhibitor MG132 was used to treat the cells. Only inhibition of the proteasome function increased Mig6 expression in PIPKIγi5 knockdown cells (Fig. 3*C*). This shows that PIPKIγi5-regulated Mig6 degradation is dependent on proteasomes but not lysosomes. To confirm the efficiency of chloroquine in inhibiting lysosome function, the LC3A/BII level was detected because its degradation is dependent on lysosomes (26). To test the efficiency of MG132 in blocking proteasome function, the levels of SMAD ubiquitination regulatory factor 1 (Smurf1) and TGFβ receptor I (TGFβRI) were detected because their degradation is dependent on proteasomes (27, 28). Chloroquine treatment increased the LC3A/BII level (Fig. 3*D*), and MG132 treatment enhanced Smurf1 and TGFβRI levels (Fig. 3*E*). This validates the function of these two inhibitors. Exogenously expressed HA-tagged Mig6 was also degraded when endogenous PIPKIγi5 was lost

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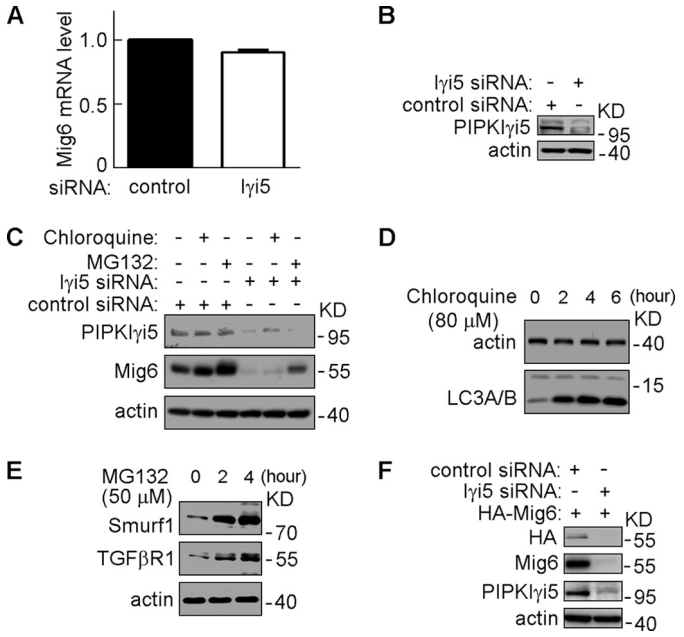

FIGURE 3. **PIPKIγi5 controls the protein degradation of Mig6.** *A*, control siRNA or PIPKIγi5 (*Iγi5*) siRNA was transfected into MDA-MB-231 cells. 72 h after siRNAs transfection, the Mig6 mRNA levels in these cells were quantified by real-time PCR. *B*, PIPKIγi5 expression in control or PIPKIγi5 knockdown cells was detected by immunoblotting. *C*, control or PIPKIγi5 knockdown MDA-MB-231 cells were treated with the lysosome inhibitor chloroquine (80 μM) or the proteasome inhibitor MG132 (50 μM) for 4 h, and then Mig6 expression was detected. *D*, MDA-MB-231 cells were treated with chloroquine (80 μM) for the times indicated, and then the LC3A/BII protein level was detected. *E*, MDA-MB-231 cells were treated with MG132 (50 μM) for the times indicated, and then the Smurf1 or TGFβRI protein levels were detected. *F*, HA-Mig6 was transfected into control or PIPKIγi5 knockdown MDA-MB-231 cells, and then the expression of HA-Mig6 was detected by anti-HA antibody or anti-Mig6 antibody via immunoblotting. *Error bars* indicate mean ± S.E. from three independent experiments.

(Fig. 3*F*). This further supports that PIPKIγi5 controls Mig6 protein degradation but not the mRNA level.

The E3 Ubiquitin Ligase NEDD4-1 Is Responsible for PIPKIγi5-regulated Mig6 degradation—Mig6 possesses two peptide sequences that are rich in proline, glutamic acid, serine, and threonine (PEST sequences). These PEST sequences promote Mig6 ubiquitination and proteasomal degradation (29). However, the E3 ubiquitin ligase responsible for Mig6 ubiquitination has not been defined. PIPKIγi5 is involved in the modulation of E3 ligase NEDD4-1-mediated ubiquitination of the hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) (23). Therefore, the effect of NEDD4-1 on PIPKIγi5-regulated Mig6 expression was detected. As shown in Fig. 4, *A* and *B*, knockdown of NEDD4-1 rescued Mig6 expression in PIPKIγi5 knockdown cells. NEDD4-1 belongs to the NEDD4 family E3 ligases. Knockdown of other NEDD4 family E3 ligases such as NEDD4–2, Smurf1, or SMAD ubiquitination regulatory factor 2 (Smurf2) cannot rescue Mig6 expression in PIPKIγi5 knockdown cells (Fig. 4, *A* and *B*). These results indicate that NEDD4-1 is the E3 ubiquitin ligase responsible for PIPKIγi5-regulated Mig6 expression. To rule out siRNA off-target effects, three different NEDD4-1 siRNAs were used. All of these siRNAs similarly knocked down NEDD4-1 expression and rescued Mig6 expression in PIPKIγi5 knockdown cells (Fig. 4, *C* and *D*). These results suggest that PIPKIγi5 may modulate Mig6 ubiquitination and degradation by regulating NEDD4-1.

To determine whether NEDD4-1 can use Mig6 as a substrate and mediate Mig6 ubiquitination, Mig6-NEDD4-1 interaction was first investigated. Endogenous Mig6 was immunoprecipitated from cell lysates and examined by Western blotting for association of endogenous NEDD4-1. As shown in Fig. 5*A*, NEDD4-1 was detected with the Mig6 complex. Their direct binding was confirmed using *in vitro* pulldown assays with purified GST-NEDD4-1 and His_6-Mig6 (Fig. 5*B*). An *in vivo* ubiquitination assay was then performed to demonstrate whether NEDD4-1 can promote Mig6 ubiquitination. Myc-Mig6 and His_6-ubiquitin were co-transfected into HEK-293 cells, and the ubiquitinated proteins were pulled down by Ni^{2+}-NTA beads. Unubiquitinated proteins may also be pulled down by NTA beads via nonspecific binding or via interaction with ubiquitinated protein. To remove the unubiquitinated proteins, the NTA beads were washed under strong denaturing conditions as described under "Experimental Procedures." The amount of Mig6 in total ubiquitinated proteins was then detected by anti-Myc antibody via Western blotting. NEDD4-1 expression enhanced the Mig6 poly-ubiquitination level (Fig. 5*C*). To further confirm that NEDD4-1 directly ubiquitinates Mig6, an *in vitro* ubiquitination assay was used with purified NEDD4-1 and GST-Mig6 proteins. Addition of Mig6 and NEDD4-1 dramatically enhanced total ubiquitinated protein levels (Fig. 5*D*). Mig6 in the reaction system was immunoprecipitated by anti-Mig6 antibody, and then the levels of ubiquitinated Mig6 were detected by anti-ubiquitin antibody (Fig. 5*E*) via Western blotting. Addition of NEDD4-1 induced Mig6 ubiquitination (Fig. 5*E*). These results indicate that NEDD4-1 ubiquitinates Mig6.

PIPKIγi5 Directly Interacts with NEDD4-1—The *in vitro* binding assay by purified His_6-PIPKIγi5 and GST-NEDD4-1 indicated the direct interaction of PIPKIγi5 with NEDD4-1 (Fig. 6*A*). To determine whether the PIPKIγi5-NEDD4-1 interaction is specific, the interaction of NEDD4-1 with other PIPKIγ splicing variants was examined by co-IP assay. As shown in Fig. 6*B*, PIPKIγi1 and PIPKIγi2 do not have the ability to bind with NEDD4-1. This result demonstrated that the unique C terminus of PIPKIγi5 is required for its association with NEDD4-1. Consistently, knockdown of PIPKIγi2 does not affect Mig6 expression (Fig. 6*C*). This suggests that binding with NEDD4-1 is required for PIPKIγi5 to modulate Mig6 expression. PIPKIγi5KD still interacts with NEDD4-1 (Fig. 6*B*). This demonstrates that the kinase activity producing PtdIns(4,5)P_2 is not required for PIPKIγi5-NEDD4-1 interaction. A series of truncations in the PIPKIγi5-specific C terminus were made (Fig. 7*A*), and their ability to interact with NEDD4-1 was examined. The truncation mutant PIPKIγi5 1–652 (the C terminus after amino acid 652 was deleted) lost the ability to interact with NEDD4-1 (Fig. 7*B*). This further supports that the C terminus of PIPKIγi5 is required for NEDD4-1 interaction. NEDD4-1 is composed of three functional regions: an N-terminal C2 domain for membrane binding, a central region containing four WW domains for protein-protein interactions, and a C-terminal homologous to the E6AP carboxyl terminus (HECT) domain for ubiquitin protein ligation (30). A series of NEDD4-1 truncation mutants were made (Fig. 7*C*), and the deletion of WW1 and WW2 decreased

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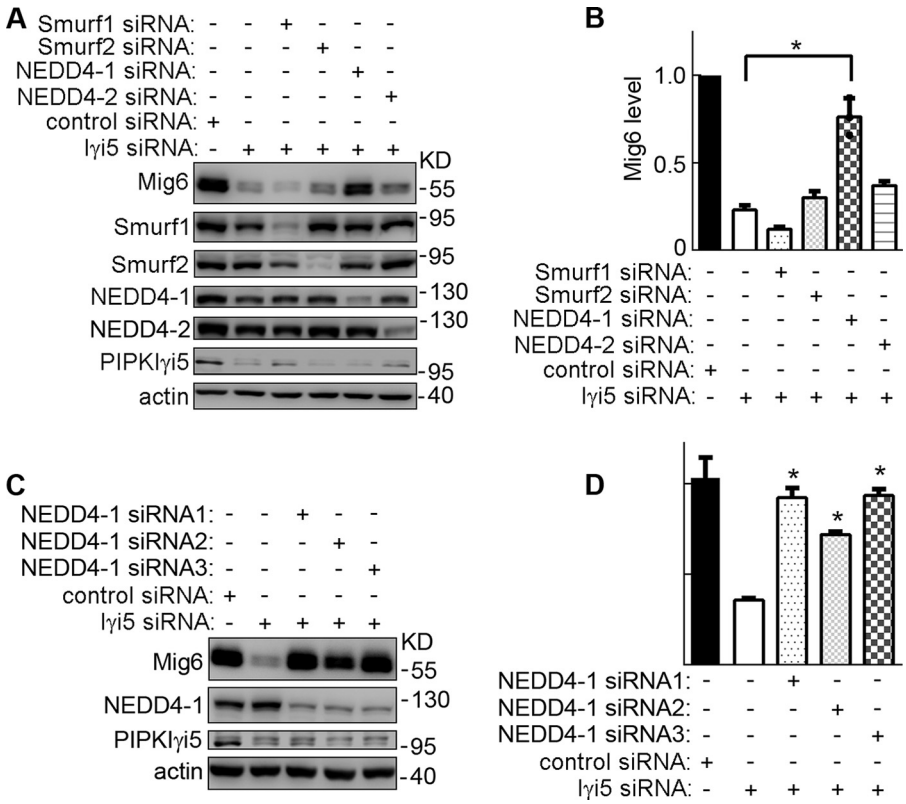

FIGURE 4. **Loss of the E3 ubiquitin ligase NEDD4-1 rescues Mig6 expression in PIPKIγi5 knockdown cells.** Smurf1 siRNA, Smurf2 siRNA, NEDD4-1 siRNA, or NEDD4–2 siRNA were co-transfected with PIPKIγi5 (*Iγi5*) siRNA into MDA-MB-231 cells as indicated. 72 h after siRNA transfection, the Mig6 and different E3 ubiquitin ligase expression levels were detected by immunoblotting (*A*), and the Mig6 expression levels were quantified (*B*). Three different siRNAs targeting NEDD4-1 were separately transfected into PIPKIγi5 knockdown MDA-MB-231 cells. The Mig6 and NEDD4-1 expression levels were detected by immunoblotting (*C*), and the Mig6 expression levels were quantified (*D*). The NEDD4-1 siRNA used in *A* and *B* was NEDD4-1 siRNA1. Quantification of Mig6 protein levels was normalized with actin levels. *Error bars* indicate mean ± S.E. from three independent experiments. *, $p < 0.05$.

NEDD4-1 association with PIPKIγi5 (Fig. 7, *D* and *E*). This indicates that the WW1 and WW2 domains are required for NEDD4-1-PIPKIγi5 interaction.

PIPKIγi5 Blocks NEDD4-1-mediated Mig6 Ubiquitination—The effect of PIPKIγi5 on NEDD4-1-mediated ubiquitination was tested by *in vitro* ubiquitination assays. Addition of purified wild-type His_6-PIPKIγi5 or kinase-dead mutant His_6-PIPKIγi5 proteins dramatically decreased NEDD4-1-mediated ubiquitination *in vitro* (Fig. 8*A*). Both wild-type and kinase-dead mutant PIPKIγi5 interact with NEDD4-1 (Fig. 6*B*). This suggests that PIPKIγi5-NEDD4-1 interaction blocks NEDD4-1-mediated ubiquitination in the *in vitro* system.

Unlike the *in vitro* system, although kinase-dead mutant PIPKIγi5 was expressed at a similar level as wild-type PIPKIγi5, it was less efficient to decrease NEDD4-1-mediated Mig6 polyubiquitination compared with wild-type PIPKIγi5 in the *in vivo* system (Fig. 8*B*). This indicates that kinase activity to produce PtdIns(4,5)P_2 is required for PIPKIγi5 to efficiently modulate NEDD4-1-mediated Mig6 ubiquitination *in vivo*. This is consistent with the result that only wild-type PIPKIγi5 but not the kinase-dead mutant can rescue Mig6 expression in PIPKIγi5 knockdown cells (Fig. 1, *E* and *F*). Expression of wild-type PIPKIγi5 but not the KD mutant decreased endogenous Mig6-NEDD4-1 interaction (Fig. 8, *C* and *D*). This suggests that PtdIns(4,5)P_2 produced by PIPKIγi5 can disturb Mig6-NEDD4-1 interaction *in vivo*.

PtdIns(4,5)P_2 Binds to NEDD4-1 and Decreases NEDD4-1-mediated Mig6 Ubiquitination—The C2 domain of the yeast NEDD4 homolog could bind phosphoinositides (31). To determine whether NEDD4-1 is a PtdIns(4,5)P_2 binding protein, a protein-lipid overlay assay was performed. As shown in Fig. 9*A*, purified GST-NEDD4-1 can bind to multiple species of phospholipids, including PtdIns(4,5)P_2. The C2 domain deletion mutant GST-NEDD4-1 lost the ability to interact with PtdIns(4,5)P_2 (Fig. 9*A*). This indicates that the C2 domain of NEDD4-1 is required for binding with PtdIns(4,5)P_2.

To further confirm the NEDD4-1-PtdIns(4,5)P_2 interaction, a liposome binding assay was performed. Adding PtdIns(4,5)P_2 into the liposomes significantly enhanced liposome binding with GST-NEDD4-1 (Fig. 9, *B* and *C*), although adding PtdIns(4,5)P_2 did not increase liposome binding with C2 domain deletion mutant GST-NEDD4-1 (Fig. 9, *D* and *E*). These results further support that NEDD4-1 binds PtdIns(4,5)P_2 and that the C2 domain is critical for this interaction.

To determine whether PtdIns(4,5)P_2 binding can affect NEDD4-1-mediated ubiquitination, an *in vitro* NEDD4-1 E3 ubiquitin ligase activity assay was performed. As shown in Fig. 9*F*, addition of PtdIns(4,5)P_2-containig liposomes into the system decreased NEDD4-1-mediated ubiquitination. Liposomes not containing PtdIns(4,5)P_2 did not affect NEDD4-1-mediated

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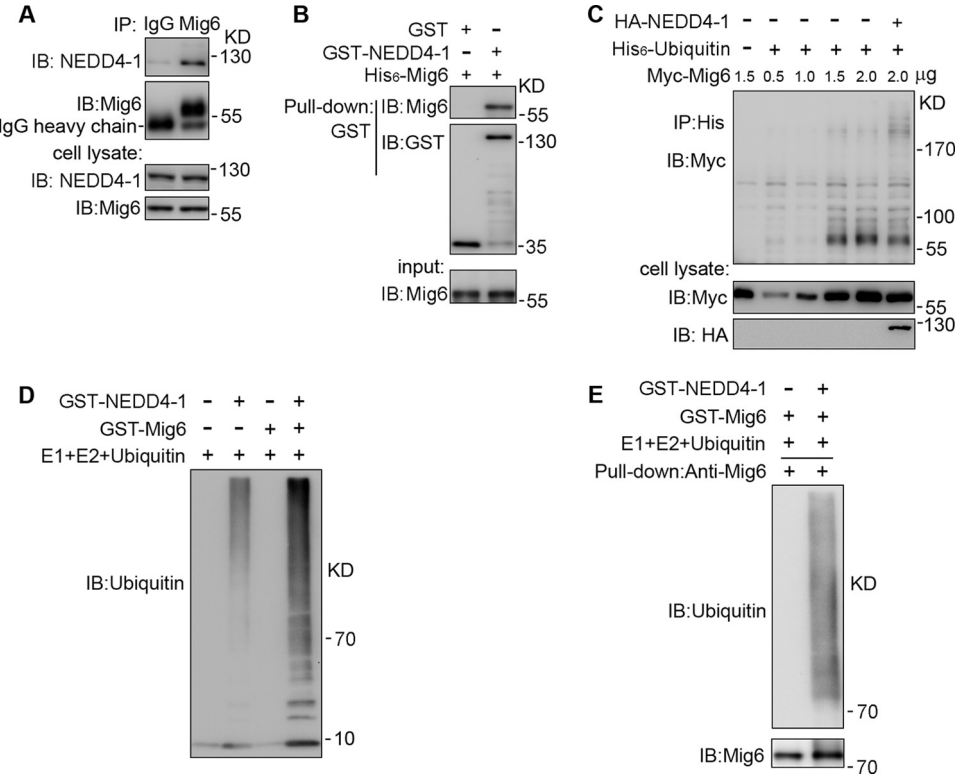

FIGURE 5. **NEDD4-1 directly interacts with Mig6 and mediates the poly-ubiquitination of Mig6.** *A*, MDA-MB-231 cells were subjected to immunoprecipitation with Mig6 antibody and then immunoblotted (*IB*) with NEDD4-1 or Mig6 antibodies. *B*, recombinant GST-NEDD4-1 and His_6-Mig6 were purified from *E. coli*. 2 μg of His_6-Mig6 was mixed with 1 μg of GST-NEDD4-1 or 1 μg of GST and subjected to GST pulldown assay with glutathione-Sepharose 4B (GE Healthcare Life Sciences). *C*, different amounts of Myc-Mig6, combined with or without HA-NEDD4-1 and His_6-ubiquitin, were transfected into HEK-293T cells, and then Mig6 ubiquitination was detected by an *in vivo* ubiquitination assay. *D*, the *in vitro* ubiquitination reaction of Mig6 was carried out in the presence of E1, UbcH5b, ubiquitin, and ATP with or without E3 NEDD4-1, and the whole protein ubiquitination level was tested by immunoblotting. *E*, Mig6 was purified by anti-Mig6 antibody from the *in vitro* ubiquitination reaction system and followed by Western blotting to detect the ubiquitination levels.

ubiquitination (Fig. 9*F*). These results further support a role of PtdIns(4,5)P_2 in regulating NEDD4-1-mediated ubiquitination.

Altogether, our results are consistent with a model where PIPKIγi5 blocks NEDD4-1-mediated Mig6 ubiquitination by direct interaction with NEDD4-1 and producing PtdIns(4,5)P_2. Therefore, PIPKIγi5 protects Mig6 from being degraded by proteasomes. Loss of PIPKIγi5 leads to increased Mig6 degradation and enhances EGFR signaling (Fig. 10).

Discussion

Phosphoinositides are crucial regulators of most cellular processes (32, 33). Among them, the function of phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P_3) and PtdIns(4,5)P_2 are the most widely investigated. PtdIns(3,4,5)P_3 is a major regulator of cell survival, and constitutively active PtdIns(3,4,5)P_3 signaling often promotes cancer (34). Somatic mutations of PI3K and phosphatase and tensin homolog (PTEN), the enzymes controlling PtdIns(3,4,5)P_3 generation and metabolism, occur frequently in cancer (35). Based on these findings, the research on phosphoinositides in cancer has largely focused on PI3K-, PTEN-, and PtdIns(3,4,5)P_3-mediated signaling. Concomitantly, the function of other phosphoinositides and the enzymes producing these phosphoinositides in cancer is largely underexplored. However, more recent studies suggest that PtdIns(4,5)P_2 and PtdIns(4,5)P_2-producing enzymes have critical roles in tumorigenesis. A subset of breast cancers expresses high levels of PIPKIIα and β, two PtdIns(4,5)P_2-producing kinases, and the function of these kinases is essential for p53-negative breast cancer progression (36). Here our research indicates that PIPKIγi5, NEDD4-1, and Mig6 form a novel molecular nexus to control EGFR signaling. The elevated expression and overactivation of EGFR in patients correlate with cancer metastasis, recurrence, and poor prognosis (37). PIPKIγi5 and Mig6 prevent EGFR overactivation. As such, this PIPKIγi5-regulated pathway may have an important role in EGFR-dependent cancer progression.

In the siRNA knockdown-rescue assay, expression of exogenous wild-type PIPKIγi5 can rescue 70% but not full Mig6 expression in PIPKIγi5 knockdown cells (Fig. 1, *E* and *F*). There might be an optimal PIPKIγi5 expression level required to mediate its normal function. Inappropriate expression levels of a particular protein would either not rescue or could cause artificial effects (38). Although we used a lentivirus vector for attenuated PIPKIγi5 expression compared with other types of protein expression vectors, the level of exogenous PIPKIγi5 was still higher than the normal endogenous level. This may lead to some artificial effects that prevent exogenous PIPKIγi5 to fully rescue endogenous PIPKIγi5 function. Another possible reason is that individual cells may take up different amounts of siRNA-*versus* cDNA-expressing constructs. In cells with a high siRNA-*versus* cDNA ratio, the expression of PIPKIγi5 may not be adequate to rescue Mig6 expression. Although it is not a full rescue, expression of exogenous wild-type PIPKIγi5 does significantly

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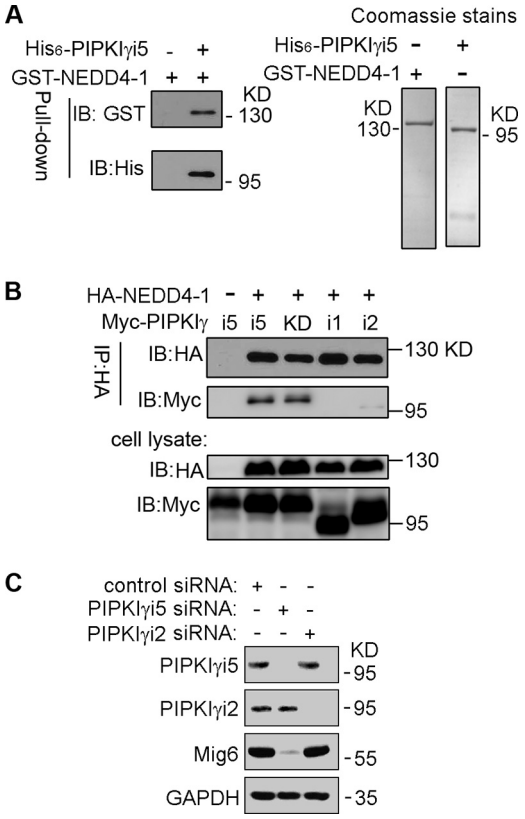

FIGURE 6. **PIPKIγi5 directly interacts with NEDD4-1.** *A*, recombinant GST-NEDD4-1 (1 μg) and His_6-PIPKIγi5 (1 μg) were purified from *E. coli* and subjected to pulldown assays with His_6-affinity Ni^{2+}-NTA beads. The quality of purified proteins was verified by Coomassie staining. *IB*, immunoblot. *B*, HA-NEDD4-1 was co-transfected with Myc-PIPKIγi5, Myc-PIPKIγi5 kinase dead mutant, Myc-PIPKIγi1, or Myc-PIPKIγi2 separately into HEK-293T cells, subjected to immunoprecipitation with anti-HA antibody, and then immunoblotted with antibodies as indicated. *C*, control, PIPKIγi5-, or PIPKIγi2-specific siRNAs were transfected into MDA-MB-231 cells, and the Mig6 expression levels were detected by Western blotting 72 h after siRNA transfection.

increase Mig6 expression in PIPKIγi5 knockdown cells (Fig. 1, *E* and *F*). Furthermore, disturbing PIPKIγi5 expression by another approach, CRISPR-cas9 genome editing, similarly decreases the Mig6 level as using PIPKIγi5 siRNA (Fig. 2). These results clearly indicate that PIPKIγi5 controls Mig6 expression.

Kinase-dead mutant PIPKIγi5 interacts with NEDD4-1 to the same extent as wild-type PIPKIγi5 (Fig. 6*B*), and it can block NEDD4-1-mediated ubiquitination similarly as wild-type PIPKIγi5 in the *in vitro* system (Fig. 8*A*). But in the *in vivo* system, kinase-dead mutant PIPKIγi5 cannot decrease NEDD4-1-mediated Mig6 ubiquitination as efficiently as wild-type PIPKIγi5 (Fig. 8*B*), and only wild-type PIPKIγi5 can significantly rescue Mig6 expression in PIPKIγi5 knockdown cells (Fig. 1*F*). These results suggest that PtdIns(4,5)P_2 plays an important role in modulating Mig6 degradation. PtdIns(4,5)P_2 binds to NEDD4-1 and decreases NEDD4-1-mediated ubiquitination (Fig. 9). Furthermore, PtdIns(4,5)P_2 can modulate NEDD4-1-Mig6 interaction *in vivo*, as only wild-type PIPKIγi5 but not kinase-dead PIPKIγi5 decreased endogenous NEDD4-1-Mig6 interaction (Fig. 8, *C* and *D*). In the *in vivo* system, PtdIns(4,5)P_2 may also modulate NEDD4-1 interaction with other components to affect Mig6 ubiquitination, and this cannot be reflected in the *in vitro* ubiquitination assay system.

Although all PIPKIγ splicing variants produce the same product, PtdIns(4,5)P_2, they play distinct biological functions by interacting with specific effector proteins (39). This work identified NEDD4-1 as a novel specific effector binding with PIPKIγi5 (Fig. 6). Consistently, PIPKIγi5 specifically controls Mig6 expression; other PIPKIγ splicing variants such as PIPKIγi2 do not play the similar role (Fig. 6*C*). PIPKIγi5 and PIPKIγi2 may differentially modulate cancer progression. PIPKIγi5 down-regulates EGFR signaling and can function as a tumor suppressor, whereas PIPKIγi2 promotes anchorage-independent cancer growth, cancer cell migration, and invasion (22, 40–43). NEDD4-1 is an oncoprotein and has been found to be frequently overexpressed in many types of human cancers such as non-small cell lung cancer (44), gastric carcinomas (45), and breast cancer (46). Suppression of NEDD4-1 expression can significantly reduce tumor cell growth (47, 48). Our research indicates that NEDD4-1 mediates degradation of the tumor suppressor Mig6 (Fig. 4 and 5), which provides a novel mechanism for NEDD4-1 promotion of tumor progression. This work also identified PIPKIγi5 as a suppressor of NEDD4-1 function.

PIPKIγi5 is required for EGFR sorting from the limiting membrane of endosomes into intraluminal vesicles (ILVs) of the multivesicular bodies, which is a key step for EGFR sorting to lysosomes for degradation (23). Hrs is a key component of the endosomal sorting complexes required for transport (ESCRT)-0 (49), which binds to ubiquitinated EGFR and recruits additional ESCRT components to mediate EGFR sorting into ILVs (50). PIPKIγi5 facilitates Hrs interaction with Sorting Nexin 5 (SNX5), which blocks NEDD4-1-mediated Hrs ubiquitination and promotes Hrs association with EGFR to mediate EGFR sorting into ILV (23). Here we found that PIPKIγi5 directly interacts with NEDD4-1 to modulate Mig6 ubiquitination and degradation. As such, the ubiquitination events are critical for PIPKIγi5 in modulating EGFR. PIPKIγi2 can be ubiquitinated by E3 ligase HECT domain E3 ubiquitin protein ligase 1 (HECTD1) in the N terminus at lysine 97 and resulted in PIPKIγi2 degradation (51). Blocking PIPKIγi2 ubiquitination by mutating lysine 97 to arginine perturbs focal adhesion assembly and cancer cell migration (51). PIPKIγi5 has exactly the same N terminus and lysine 97 as PIPKIγi2. Therefore, PIPKIγi5 itself has the potential to be modified by ubiquitination, and this can modulate its function.

EGFR mutations have been identified in lung cancer, including the point mutation L858R and an in-frame short deletion mutation, del746–750 (52–55). These mutations render EGFR with constitutive kinase activity, and they are deficient for being sorted to the lysosomes for degradation (56, 57). Mig6 still interacts with these EGFR mutants and plays essential roles in down-regulating signaling mediated by them in cancer. Loss of Mig6 dramatically accelerates the initiation and progression of EGFR L858R-driven lung adenocarcinomas (18). By modulating Mig6 expression, PIPKIγi5 can regulate EGFR mutant-initiated cancer progression.

Mig6 not only interacts with EGFR but also interacts with other members of ErbB family receptors such as ErbB2 (7).

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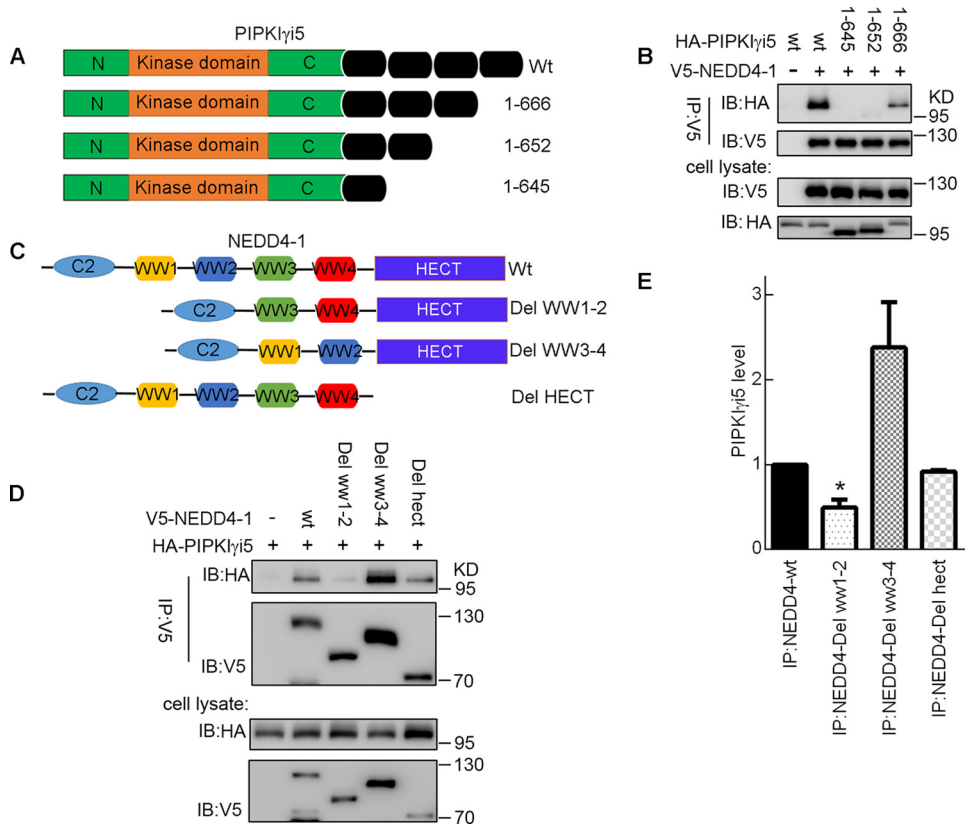

FIGURE 7. **Identification of the regions responsible for PIPKIγi5-NEDD4-1 interaction.** *A*, schematic of a series of PIPKIγi5 C terminus truncation mutants. *B*, V5-NEDD4-1 was co-transfected with HA-tagged wild type PIPKIγi5 (*wt*) or a series of PIPKIγi5 C terminus truncation mutants into HEK-293T cells, subjected to immunoprecipitation with anti-V5 antibody, and then immunoblotted (*IB*) with antibodies as indicated. *C*, schematic of the domains of NEDD4-1 and a series of NEDD4-1 deletion mutants. *D*, HA-PIPKIγi5 was co-transfected with V5-tagged wild-type NEDD4-1 (*wt*) or a series of NEDD4-1 deletion mutants into HEK-293T cells, subjected to immunoprecipitation with anti-V5 antibody, and then immunoblotted with antibodies as indicated. *E*, the amount of PIPKIγi5 co-immunoprecipitated with wild-type NEDD4-1 or NEDD4-1 deletion mutants was quantified. *Error bars* indicate mean ± S.E. from three independent experiments. *, $p < 0.05$.

Thus, Mig6 suppresses the signaling from ErbB2 (29). As an adaptor protein, Mig6 also interacts and modulates the function of many important components of different signaling pathways, including cdc42, IκBα, GRB2, Src, PI3K p85, and 14-3-3ζ (7, 58–60). This suggest that PIPKIγi5 can function in multiple signaling pathways by controlling the expression of Mig6.

Experimental Procedures

Cell Cultures and Transfection—The breast cancer cell line MDA-MB-231 was cultured using DMEM supplemented with 10% FBS. The lung cancer cell line H-1975 was cultured using 1640 medium supplemented with 10% FBS. For plasmid transfection, cells were transfected using Lipofectamine 2000 (Invitrogen) following the instructions of the manufacturer. For siRNA transfection, cells were transfected with Oligofectamine (Invitrogen) for 72 h following the instructions of the manufacturer.

Reagents—TGFα (catalog no. GF022) was purchased from EMD Millipore (Billerica, MA). Antibodies to EGFR (catalog no. sc-03), Mig6 (catalog no. sc-137154), Myc-tag (catalog no. sc-40), NEDD4–2 (catalog no. sc-514954), and GAPDH (catalog no. sc-32233) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to NEDD4-1 (catalog no. 2740), total AKT (catalog no. 4685), phosphorylated AKT (catalog no. 4060), and phosphorylated EGFR (catalog no. 3777) were from Cell Signaling Technology (Danvers, MA). Anti-HA antibody (catalog no. 901502) was from BioLegend (San Diego, CA). Anti-FLAG antibody (catalog no. F3165) was from Sigma-Aldrich (St. Louis, MO). Anti-GFP (catalog no. 11814460001) antibody was from Roche Life Science (Indianapolis, IN). Anti-PIPKIγi2- and -i5-specific antibodies were generated as described previously (21). Secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA).

Constructs—PIPKIγi5 (GenBank accession no. NM_001300849.1) was amplified by PCR, ligated into the pcDNA3.0 vector (Invitrogen) with an HA tag at the N terminus, and subcloned into the PWPT lentivirus vector (Addgene) through MluI/SalI sites. Mig6 (GenBank accession no. NM_018948.3) was subcloned into the pcDNA3.0 vector via BamHI/XhoI sites. PIPKIγi5 C terminus truncation mutants were generated by regular PCR, and NEDD4-1 truncation mutants were generated using PCR primer overlap extension with primers containing the desired mutations. To generate V5-tag-NEDD4-1, NEDD4-1 (GenBank accession no. NM_006154.3) was amplified by PCR and inserted into the pcDNA3.1 vector (Invitrogen) through BamHI/XhoI sites. For expression in *Escherichia coli*, PIPKIγi5 was subcloned into pET28c(+) (Novagen), Mig6 was subcloned

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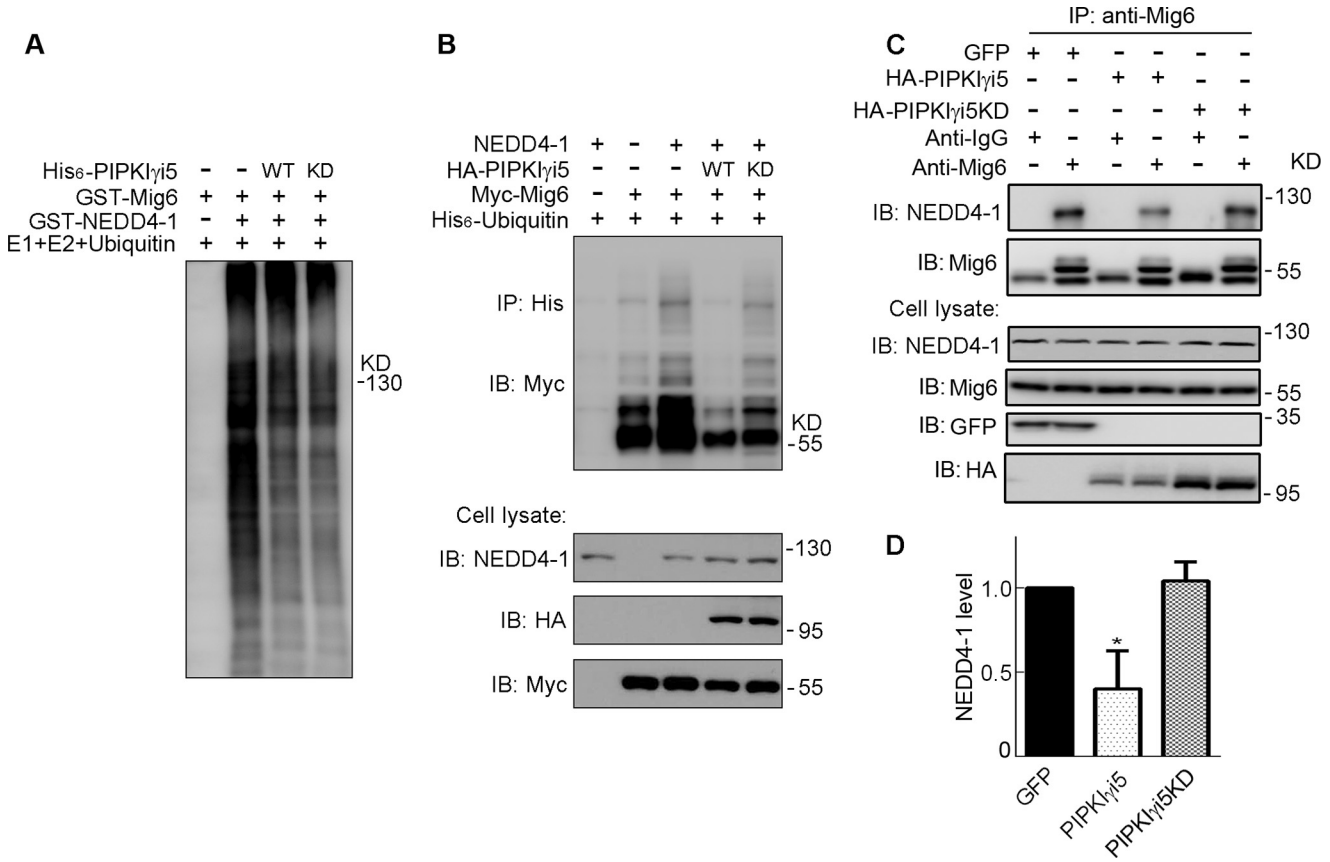

FIGURE 8. **PIPKIγi5 blocks NEDD4-1-mediated Mig6 ubiquitination.** *A*, the ubiquitination reaction of GST-Mig6 was carried out in the presence of E1, UbcH5b, NEDD4-1, ubiquitin, and ATP with or without purified wild-type His_6-PIPKIγ5 or kinase dead mutant His_6-PIPKIγi5 *in vitro*, and the whole ubiquitination level was detected by immunoblotting. *B*, Myc-Mig6 was co-transfected with His_6-ubiquitin, NEDD4-1, wild-type HA-PIPKIγi5, or kinase dead mutant PIPKIγi5 into HEK-293T cells as indicated, and Myc-Mig6 ubiquitination was detected by an *in vivo* ubiquitination assay. *IB*, immunoblot. *C*, GFP, HA-tagged wild-type PIPKIγi5 or kinase-dead mutant PIPKIγi5 was constructed into PWPT lentivirus vectors. GFP, HA-PIPKIγi5 WT, or HA-PIPKIγi5 KD was then expressed via lentivirus transfection in MDA-MB-231 cells, and then cells were subjected to immunoprecipitation with Mig6 antibody. The amount of endogenous NEDD4-1 co-immunoprecipitated with endogenous Mig6 was detected by immunoblotting. *D*, quantification of the amount of endogenous NEDD4-1 co-immunoprecipitated with endogenous Mig6 as in *C*. *Error bars* indicate mean ± S.E. from three independent experiments. *, $p < 0.05$.

into pET52b(+) (Novagen) or pGEX 5x-2 (GE Healthcare), and NEDD4-1 were subcloned into pGEX 5x-2.

Recombinant Protein Purification—E. coli strain BL21 (DE3) was transformed with pET28c-PIPKIγi5 (His_6-tagged), pET52b-Mig6 (His6-tagged), pGEX 5x-2-Mig6 (GST-tagged) or pGEX 5x-2-NEDD4-1 (GST-tagged), respectively. Single clones were picked, and 0.1 mM isopropyl β-D-thiogalactopyranoside was added when A_{600} reached 0.4–0.6. The culture was then further incubated at 30 °C overnight. The cells was harvested and sonicated in buffer (50 mM Tris-HCl, 150 mM NaCl, 1%Nonidet P-40, and 1 mM DTT). The GST-tagged proteins were purified by glutathione Sepharose™ 4B resin (GE Healthcare). The His_6-tagged proteins were purified by Ni^{2+}-NTA-agarose resin (Thermo Fisher Scientific).

siRNA—The sequence of control scrambled siRNA was 5′-GUACCUGUACUUCAUGCAG-3′. The siRNA sequence for human PIPKIγi5 was 5′-GGAUGGGAGGUACUGGAUU-3′. The siRNA sequence specific targeting human PIPKIγi2 was 5′-GAGCGACACAUAAUUUCUA-3′. The siRNA sequence for Smurf1 was 5′-GCAUCGAAGUGUCCAGAGAAG-3′. The siRNA sequence for Smurf2 was 5′-CCUUCUGUGUUGAACAUAA-3′. The NEDD4-2 siRNA sequence was 5′-AACCACAACACAAAGUCACAC-3′. The NEDD4-1 siRNA1 sequence was 5′-UUCCAUGAAUCUAGAAGAACA-3′. The NEDD4-1 siRNA2 sequence was 5′-UAGAGCCUGGCUGGGUUGUUU-3′. The NEDD4-1 siRNA3 sequence was 5′-CAGCAUCGAGCUCAAAUCA-3′. Mig6 siRNA was purchased from Santa Cruz Biotechnology (catalog no. sc-45704).

CRISPR-cas9 Genome Editing—Genomic disruptions were created in MDA-MB-231 and H-1975 cells using the CRISPR)/Cas9 system as described previously (61). The lentiCRISPR v2 plasmid (Addgene, plasmid no. 52961) was used to mediate the expression of the single guide RNA (sgRNA) and cas9 protein by lentivirus infection. The sgRNAs targeting the PIPKIγi5-specific sequence were designed using online software. Two different sgRNA-targeting sequences were chosen: 5′-TCACGGCCCGCAGTCGGCGA-3′ and 5′-GCGGTCACTTACAGTCCCCG-3′. Lentivirus-infected cells were cultured in medium containing 1.0 μg/ml puromycin for 2 days for selection. Surviving cells were then trypsinized, and a single cell was isolated by limited dilution of the selected cells in 96-well plates. Each single clone was passaged and expanded. The total DNA of each colony was extracted and genotyped. The genomic region surrounding the CRISPR/Cas9 target site for

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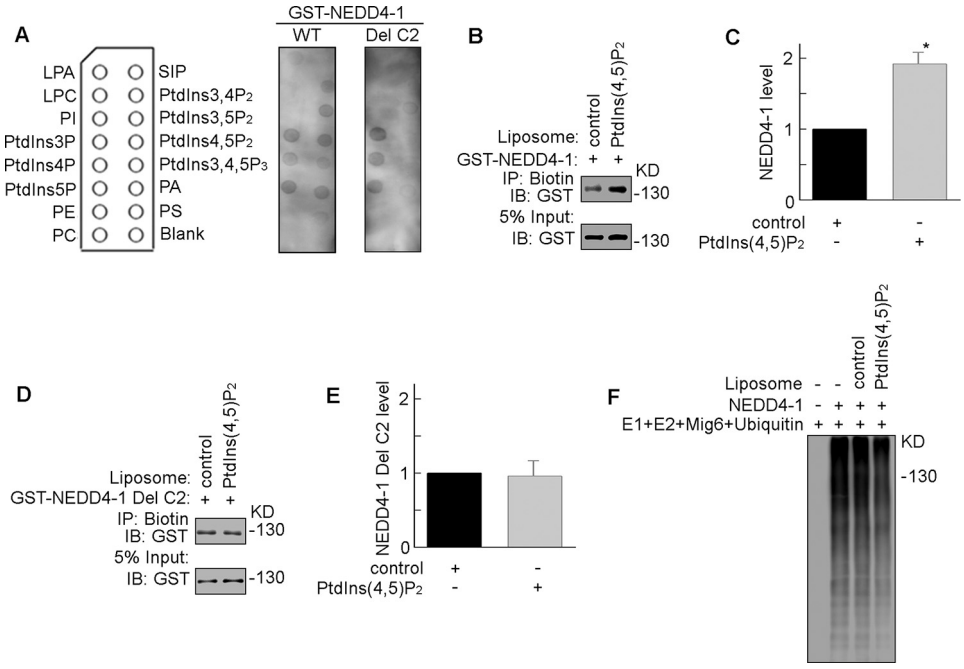

FIGURE 9. **PtdIns(4,5)P_2 binds NEDD4-1 and decreases NEDD4-1-mediated ubiquitination.** Wild-type GST-NEDD4-1 and C2 domain-deletion mutant GST-NEDD4-1 (*Del C2*) were purified from *E. coli*. *A*, binding of GST-NEDD4-1 (*WT*) or GST-NEDD4-1 (*Del C2*) with different lipids was detected by protein-lipid overlay assay with PIP strips. *PI*, phosphatidylinositol. *B* and *C*, the binding of GST-NEDD4-1 (*WT*) with PtdIns(4,5)P_2-containing or non-containing liposomes (*control*) was detected (*B*) and quantified (*C*). *IB*, immunoblot. *D* and *E*, the binding of GST-NEDD4-1 with PtdIns(4,5)P_2-containing or non-containing liposomes was detected (*D*) and quantified (*E*). *F*, the ubiquitination reaction of GST-Mig6 was carried out in the presence of E1, UbcH5b, NEDD4-1, ubiquitin, and ATP with or without PtdIns(4,5)P_2-containing or non-containing liposomes *in vitro*, and the whole ubiquitination level was detected by immunoblotting. *LPA*, lysophosphatidic acid; *LPC*, lysophosphocholine; *S1P*, sphingosine 1-phosphate.

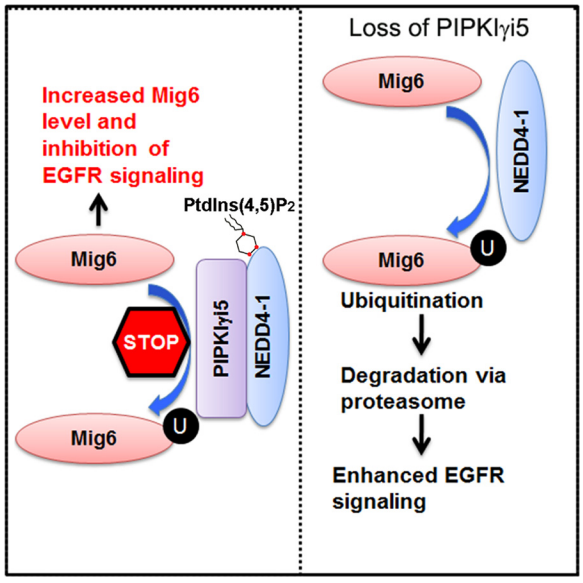

FIGURE 10. **Model for PIPKIγi5 regulation of Mig6 expression and EGFR signaling.** By directly interacting with NEDD4-1 and producing PtdIns(4,5)P_2, PIPKIγi5 blocks NEDD4-1-mediated Mig6 ubiquitination. This protects Mig6 from being degraded by proteasomes. As a result, increased Mig6 protein levels inhibit EGFR activation and signaling. Loss of PIPKIγi5 promotes NEDD4-1-mediated Mig6 ubiquitination and the subsequent Mig6 degradation by proteasomes. This enhances EGFR signaling.

PIPKIγi5 was PCR-amplified. The amplicons were cloned into the pCR™ 2.1 Vector (Invitrogen) and validated by sequencing.

RNA Extraction and Real-time PCR—MDA-MB-231 cells were harvested, and total RNA was extracted using the ISOLATE RNA mini kit (Bioline, catalog no. BIO-52072). 2 μg of total RNA was used for cDNA reverse transcription with the kit (Applied Biosystems, catalog no. 4368814). SYBR Green master mix (Applied Biosystems, catalog no. A25742) was used for real-time PCR, and the results was analyzed by QUANTSTUDIO 3 software. The Mig6 mRNA abundance was normalized to the expression of GAPDH. Primers used for the PCR were as follows: 5′-CTACTGGAGCAGTCGCAGTG-3′ (forward) and 5′-CCTCTTCATGTGGTCCCAAG-3′ (reverse) for Mig6 and 5′-AATCCCATCACCATCTTCCA-3′ (forward) and 5′-TGGACTCCACGACGTACTCA-3′ (reverse) for GAPDH.

Immunoprecipitation and Immunoblotting—Immunoprecipitation was performed as described previously (62). Briefly, cells were harvested and lysed in 25 mM HEPES (pH 7.2), 150 mM NaCl, 0.5% Nonidet P-40, 1 mM $MgCl_2$, and protease inhibitor mixture and then centrifuged and incubated with protein G-Sepharose and 2 μg of antibody as indicated at 4 °C for 4 h. The immunocomplexes were separated by SDS-PAGE and analyzed as indicated.

In Vivo Ubiquitination Assay—The ubiquitination of Mig6 was evaluated as described previously (63). His_6-ubiquitin-conjugated Mig6 in MDA-MB-231 cells was purified by Ni^{2+}-NTA beads (Qiagen). MDA-MB-231 cell was lysed in IP buffer (25 mM HEPES (pH 7.2), 150 mM NaCl, 0.5% Nonidet P-40, 1 mM $MgCl_2$, and protease inhibitor mixture) and incubated with Ni^{2+}-NTA beads for 2 h 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 5.3),

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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 Mig6 by anti-Mig6 antibody.

In Vitro E3 Ubiquitin Ligase Activity Assay—The E3 ubiquitin ligase activity assay was based on a method described in previous studies (64, 65) with minor modifications. The ubiquitination reaction mixture contained 0.2 μM E1, 1 μM UBCH5C (E2), 0.5 μM NEDD4-1, and 10 μM ubiquitin in buffer (50 mM Tris-HCl (pH 7.5), 2 mM ATP, and 5 mM $MgCl_2$). After incubation at 30 °C for 1 h, the reaction was terminated by SDS loading sample buffer. Mig6 poly-ubiquitination chains were prepared as above. To isolate Mig6 from the mixture, SDS was added to the reaction mixture to a final concentration of 1%, and the sample was incubated at 90 °C for 10 min. After a 10-fold dilution of SDS with buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, and 10% glycerol), Mig6 antibody (1.0 μg, Santa Cruz Biotechnology) and protein G-Sepharose beads (5 μl) were added. The mixture was incubated for 4 h at room temperature and spun at 1000 × *g* to collect protein G-Sepharose beads. The beads were washed three times with buffer plus 0.1% SDS, and then proteins were eluted with SDS loading sample buffer and detected by immunoblotting with ubiquitin antibody or Mig6 antibody.

Protein-Lipid Overlay Assay—PIP strips (catalog no. P-6001, Echelon) are 2 × 6 cm hydrophobic membranes that have been spotted with 100 picomoles of all eight phosphoinositides and seven other biological important lipids. PIP strips were first blocked with PBS including 3% BSA for 1 h and then incubated with GST-NEDD4-1 (WT) or GST-NEDD4-1 (Del C2) (1 μg/ml) in PBS with 3% BSA. Subsequently, the PIP strips were washed extensively in PBS containing 0.1% Tween 20, and the bound protein was detected with anti-GST antibody by immunoblotting.

Liposome Binding Assay—Liposome binding assays were carried out essentially as described previously (66, 67). PtdIns(4,5)P_2-containing PolyPIPosomes (catalog no. Y-P045, Echelon) are composed of 65% phosphatidylcholine, 29% phosphatidylethanolamine (PE), 1% biotin-PE, and 5% PtdIns(4,5)P_2. Control PolyPIPosomes (catalog no. Y-P000, Echelon) are composed of 65% phosphatidylcholine, 29% PE, 1% biotin-PE, and 5% phosphatidylinositol. 15 μmol of PolyPIPosomes were diluted in 100 μl of binding buffer (50 mM Tris (pH 7.8), 150 mM NaCl, 0.05% Nonidet P-40 (v/v), and 20 mM iodoacetamide) and incubated for 15 min at room temperature with 10 μg of GST-NEDD4-1 (WT) or GST-NEDD4-1 (Del C2). Liposome-protein complexes were recovered by 10-min centrifugation at room temperature (11,356 × *g*), and pellets were resuspended in 500 μl of binding buffer and further incubated with streptavidin-agarose (Invitrogen) for 1 h at room temperature. Biotinylated liposomes bound to GST proteins were washed with binding buffer and eluted with Laemmli sample buffer, separated by SDS-PAGE, and immunoblotted with anti-GST antibody.

Statistics—All data analyses were performed using SigmaPlot. Bar graphs represent mean ± S.E. as indicated. Statistical significance was assessed using Student's *t* test.

Author Contributions—M. S. conducted most of the experiments and analyzed the results. J. C. conducted the CRISPR-cas9 genome editing and selected single clones. R. A. A. provided assistance with the preparation of the manuscript. Y. S. conceived the idea for the project, designed the experiments, and wrote the paper with M. S.

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