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Phosphoinositide Signaling Regulates the Exocyst Complex and Polarized Integrin Trafficking in Directionally Migrating Cells

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SUMMARY

Polarized delivery of signaling and adhesion molecules to the leading edge is required for directional migration of cells. Here, we describe a role for the PIP_2-synthesizing enzyme, PIPKIγi2, in regulation of exocyst complex control of cell polarity and polarized integrin trafficking during migration. Loss of PIPKIγi2 impaired directional migration, formation of cell polarity, and integrin trafficking to the leading edge. Upon initiation of directional migration, PIPKIγi2 via PIP_2 generation controls the integration of the exocyst complex into an integrin-containing trafficking compartment that requires the talin-binding ability of PIPKIγi2, and talin for integrin recruitment to the leading edge. A PIP_2 requirement is further emphasized by inhibition of PIPKIγi2-regulated directional migration by an Exo70 mutant deficient in PIP_2 binding. These results reveal how phosphoinositide generation orchestrates polarized trafficking of integrin in coordination with talin that links integrins to the actin cytoskeleton, processes that are required for directional migration.

INTRODUCTION

Cell migration is critical for many biological processes, including embryogenesis, inflammation, and the metastasis of cancer cells. At the onset of migration, cells undergo a spatial reorganization of the cytoskeleton and membrane proteins to establish polarity (Insall and Machesky, 2009; Ling et al., 2006; Ridley et al., 2003; Rørth, 2009; Vicente-Manzanares et al., 2009). Coordinated cell migration hinges on the ability of cells to traffic signaling molecules and proteins toward the leading edge (Caswell and Norman, 2008; Fletcher and Rappoport, 2010; Ulrich and Heisenberg, 2009), a process that requires the tight regulation of cytoskeletal and vesicle-trafficking machineries. The trafficking of newly synthesized or recycled integrin molecules to and from the plasma membrane is required for directional cell migration (Caswell and Norman, 2008; Caswell and Norman, 2006; Ulrich and Heisenberg, 2009). A prevailing theory is that migrating cells assemble adhesion sites at the leading edge and disassemble at the trailing edge, resulting in a continual endo- and exocytosis of integrins (Bretscher, 1984, 1989; Ridley et al., 2003). Impairment of the endo-exocytic trafficking of integrins profoundly affects the polarity and directionality of cell migration (Caswell et al., 2009; Kuo et al., 2006; Nishimura and Kaibuchi, 2007).

Phosphatidylinositol-4,5-biphosphate (PIP_2) is a lipid messenger that modulates many diverse biological processes, including regulation of actin cytoskeletal dynamics, cell migration, cell-cell contact formation, endocytosis, and exocytosis (Heck et al., 2007; Ling et al., 2006; van den Bout and Divecha, 2009). PIP_2 is a lipid messenger that is spatially and temporally generated, making it an ideal messenger for polarized signaling (Anderson et al., 1999; Heck et al., 2007; Ling et al., 2006). Type I PIPKs (α, β, and γ isoforms) represent the predominant class of PIP_2-generating enzymes in mammalian cells (Anderson et al., 1999). The spatiotemporal generation of PIP_2 by the coordinated activity and/or recruitment of PIPKIs and phosphatases is a central hypothesis in PIP_2 signaling (Anderson et al., 1999; Heck et al., 2007; Ling et al., 2006). PIPKIγ has roles in vesicle trafficking both at the plasma membrane and in endosomal structures (Bairstow et al., 2006; Ling et al., 2007; Schill and Anderson, 2009a). In addition, PIP_2 generation is required for vesicle exocytosis (Hay et al., 1995; Martin, 1998) and endocytosis (Jost et al., 1998).

PIP_2 is generated at many cellular compartments, although its cellular content does not vary significantly, suggesting that PIP_2 signals differently than other messengers (Anderson et al., 1999). The signaling specificity of PIP_2 is defined by the interaction of the PIP kinases with PIP_2 effectors or compartments containing PIP_2 effectors (Anderson et al., 1999; Heck et al., 2007; Ling et al., 2006; Schill and Anderson, 2009a). Multiple PIPKIγ isoforms exist in mammals that have different C-terminal extensions, and these sequences specifically interact with PIP_2 effectors; in addition, the PIP_2 generated regulates these effectors (Heck et al., 2007; Schill and Anderson, 2009b). Previously, PIPKIγi2 has been demonstrated to interact with adaptor molecules AP2 and AP1B, regulating the endocytosis and basolateral trafficking of E-cadherin molecules in polarized epithelial cells (Bairstow et al., 2006; Ling et al., 2007; Schill and Anderson, 2009a, 2009b; Thieman et al., 2009). PIPKIγi2 is also specifically recruited to focal adhesions by an association with talin (Ling et al., 2002), and this requires

the same sequence in the PIPKIγi2 C terminus that interacts with the AP complexes (Bairstow et al., 2006; Ling et al., 2007; Thieman et al., 2009). PIPKIγi2 is also specifically required for chemotaxis toward growth factors, and the interaction of PIPKIγi2 with talin appears to be required for chemotaxis (Sun et al., 2007).

The exocyst protein complex has a pivotal function in polarized trafficking of membrane proteins during cell migration (He and Guo, 2009). The exocyst complex consists of eight different subunits (Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, and Exo84) that mediate tethering of post-Golgi and endocytic-recycling endosomes to the plasma membrane (He and Guo, 2009; Yeaman et al., 2001) and is associated with all stages of endosomal trafficking (Oztan et al., 2007). The exocyst complex is important for the polarized trafficking of LDL receptor, E-cadherin, integrin, and Glut4-containing vesicles (Grindstaff et al., 1998; Inoue et al., 2003; Spiczka and Yeaman, 2008) and serves as an effector of the small GTPases Rab11 and Arf6 (Oztan et al., 2007). The small GTPases Rab11 and Arf6 also regulate integrin trafficking (Powelka et al., 2004). Two subunits, Sec3 and Exo70, directly interact with PIP_2 via conserved basic residues in their C terminus, suggesting that PIP_2 generation could be an important mechanism in regulating the exocyst complex in vesicle trafficking (Liu et al., 2007).

Here, we report that PIPKIγi2 regulates the exocyst complex trafficking of β1-integrin to the leading edge in directionally migrating cells. In this pathway, PIPKIγi2 interacts with the exocyst complex and β1-integrin upon initiation of directional cell migration and regulates β1-integrin trafficking to focal adhesion complexes at the leading edge membrane. This requires an interaction between PIPKIγi2 and talin.

RESULTS

PIPKIγi2 Is Required for Directional Cell Migration

To define the mechanistic role of PIPKIγi2 in cell migration, we specifically knocked down endogenous PIPKIγi2 expression using a lentiviral vector-mediated delivery system. The expression levels of PIPKIγi2 were reduced >90% using this approach (Figure 1A). PIPKIγi2 knockdown cells were morphologically indistinguishable from control cells and showed no obvious effect on cell proliferation (see Figures S1A and S1B available online). We quantified the impact of PIPKIγi2 knockdown on cell migration using both wound healing and haptotactic migration assays using a modified Boyden chamber. PIPKIγi2 knockdown significantly impaired cell migration in MDA-MB-231 cells (Figures 1B and 1C) and HeLa cells (Figures S1D–S1F). Haptotactic cell migration was performed to assess the role of PIPKIγi2 during integrin-dependent cell migration toward extracellular matrix (ECM) proteins. These data show that PIPKIγi2 knockdown impaired cell migration toward fibronectin (FN) and collagen I (Col.I) (Figure 1D), suggesting that PIPKIγi2 knockdown could regulate integrin dynamics. The re-expression of PIPKIγi2, but not a kinase dead mutant, rescued integrin-dependent cell migration (Figure 1E). However, PIPKIγi2 knockdown did not show any obvious defect on directionality or velocity in nondirectionally migrating cells (Figure S1C; Movies S1 and S2).

Knockdown of PIPKIγi2, Exocyst Complex Components, or Rab11 Impairs Polarized Recruitment of β1-Integrin and Cell Migration

During migration the reorganization of the actin cytoskeleton, microtubules, and the Golgi apparatus induces polarity in the direction of migration, resulting in polarized membrane trafficking toward the leading edge (Caswell and Norman, 2008; Caswell et al., 2009; Ulrich and Heisenberg, 2009). In directionally migrating cells, PIPKIγi2 is recruited to the leading edge (Figure S1G). Knockdown of PIPKIγi2 resulted in impaired actin assembly at the leading edge and impaired microtubule orientation (Figure 1F). These cells also lost Golgi orientation in the direction of migration (Figures 1F and 1G), indicating that PIPKIγi2 is required for cell polarization during migration.

Polarization of cells during migration is regulated by vesicular trafficking, cytoskeletal dynamics, small G proteins, and cell adhesion receptors (Caswell and Norman, 2008; Etienne-Manneville, 2008; Ridley et al., 2003). The endosomal recycling of integrin molecules controlled by Rab11 plays an integral role in polarity (Caswell and Norman, 2008; Powelka et al., 2004). Similarly, the exocyst complex has been implicated in polarized vesicle trafficking and integrin recruitment to focal adhesions (Spiczka and Yeaman, 2008). Because PIPKIγi2 modulates both focal adhesion dynamics and membrane trafficking (Bairstow et al., 2006; Ling et al., 2006, 2007; Sun et al., 2007), we used siRNA-mediated knockdown of PIPKIγi2, Rab11, or Exo70 (Figure 2A) to compare the role of each of these molecules in establishing polarity and cell migration. Individual knockdown of PIPKIγi2, Rab11, or Exo70 similarly impaired cell orientation toward the direction of migration (Figures 2B and 2C) and haptotactic cell migration toward FN, a β1-integrin-dependent process (Figure 2D).

The polarized trafficking of integrins is required for formation of nascent focal adhesion complexes and the stabilization of the leading edge in migrating cells (Caswell and Norman, 2008; Caswell and Norman, 2006; Choma et al., 2004). As shown in Figure 2E, the knockdown of PIPKIγi2, exocyst components, or Rab11 disrupted focal adhesion complex assembly, as evidenced by a loss of FAK at the migrating cell front. Furthermore, the loss of PIPKIγi2, Exo70, or Rab11 all impaired the polarized recruitment/trafficking of β1-integrin to the leading edge (Figures 2F and 2G). The phenotypes resulting from the loss of PIPKIγi2 were specific because knockdown of PIPKIγi5 isoform had no impact on these processes (Figures S2A–S2E). PIPKIγi2 knockdown cells lost accumulation of β1-integrin at membrane ruffles/protrusions and a loss of colocalization with cortactin (Figures S3A and S3B). These data indicate a role for PIPKIγi2 in the targeting of β1-integrin to the leading edge in migrating cells.

The adhesion of cells to ECM protein mimics some events that take place in cell migration such as activation of integrins (Ginsberg et al., 2005). PIPKIγi2 knockdown cells were morphologically indistinguishable from control cells and showed no obvious impairment in adhering or spreading when plated on FN (10 μg/ml) or Col.I (20 μg/ml)-coated plates for 30 min (Figures S3D and S3E). There were subtle changes in cell adhesion at lower FN concentration with shorter incubation time (Figures S3F and S3G). These data are also consistent with a selective role for PIPKIγi2 in polarized β1-integrin trafficking and cell migration.

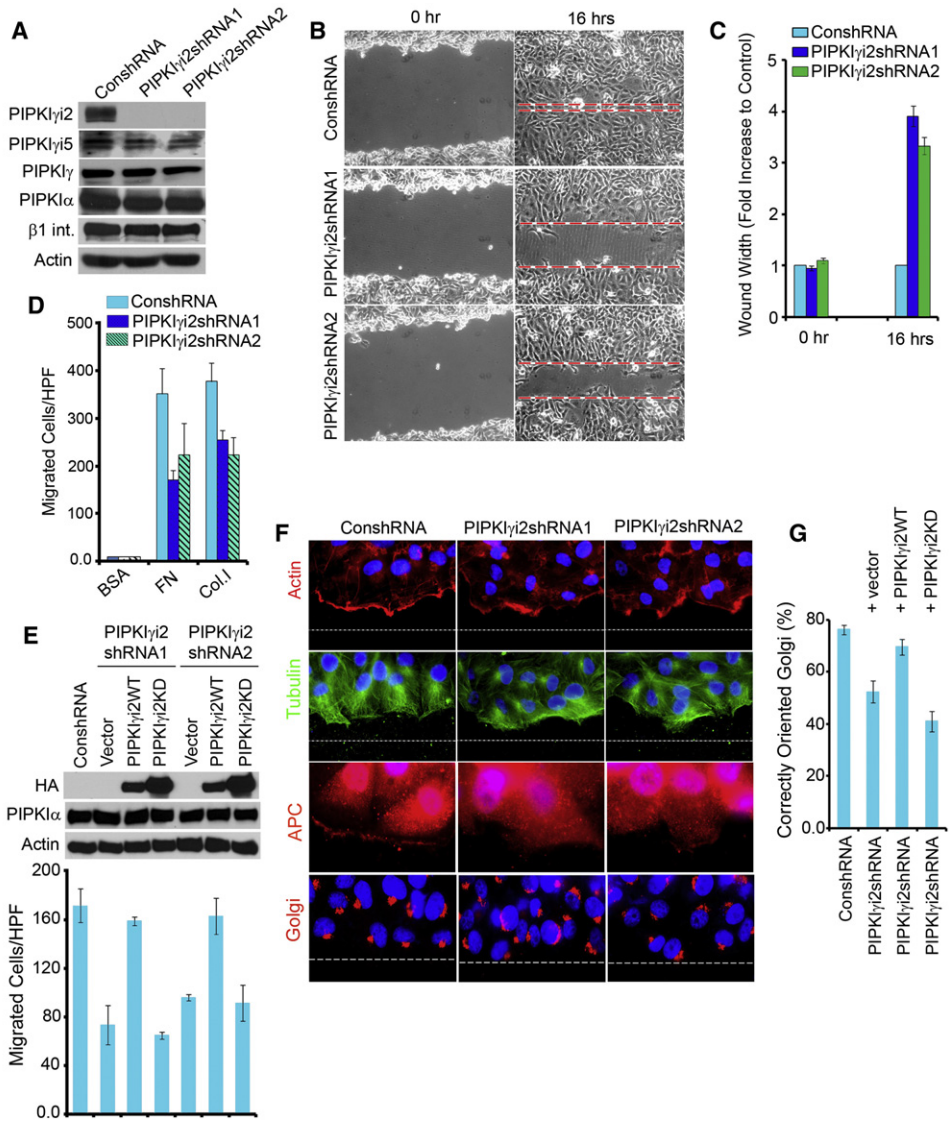

Figure 1. PIPKIγi2 Is Required for Directional Cell Migration

(A) shRNA/lentiviral system was used to knock down endogenous PIPKIγi2. Isolated cell lines (designated as PIPKIγi2shRNA1 and PIPKIγi2shRNA2) were examined by immunoblotting for knockdown of PIPKIγi2. β1 int., β1-integrin.

(B) Control or PIPKIγi2 knockdown cells grown to confluency were wounded, and wound width was measured at 0 and 16 hr postwounding (representative images at 0 and 16 hr postwounding).

(C) The results are expressed as average fold increase in wound width compared with control cells at 0 and 16 hr postwounding (mean ± SD from three independent experiments). Error bars represent SD.

(D) For haptotactic cell migration toward FN or Col.I, the modified Boyden chamber was used. Results expressed as the total number of cells migrated/HPF (mean ± SD from three independent experiments). Error bars represent SD.

(E) Rescue of cell migration defect in PIPKIγi2 knockdown cells. Lentiviral expression system was used to express PIPKIγi2 or its kinase dead mutant into the PIPKIγi2 knockdown cells. Haptotactic cell migration was examined as described above (results are mean ± SD from three independent experiments). Error bars represent SD.

(F) Confluent cell cultures were wounded and processed 6 hr postwounding to examine orientation of actin (red), microtubules (green), APC (red), and Golgi (red) toward the direction of cell migration.

(G) Quantitative data for Golgi orientation (mean ± SD of three independent experiments). Error bars represent SD.

See also Figure S1 and Movies S1 and S2.

Cell Migration Stimulates a PIPKIγi2 Association with β1-Integrin

In nonmigrating confluent MDA-MB-231 or HeLa cells, PIPKIγi2 and β1-integrin are localized at the cell periphery in addition to intracellular compartments (Figure 3A). Upon initiation of directional migration, PIPKIγi2 and β1-integrin relocalized to the leading edge and perinuclear vesicle-like compartments (Figure 3A). Consistent with previous data, PIPKIγi2 colocalizes

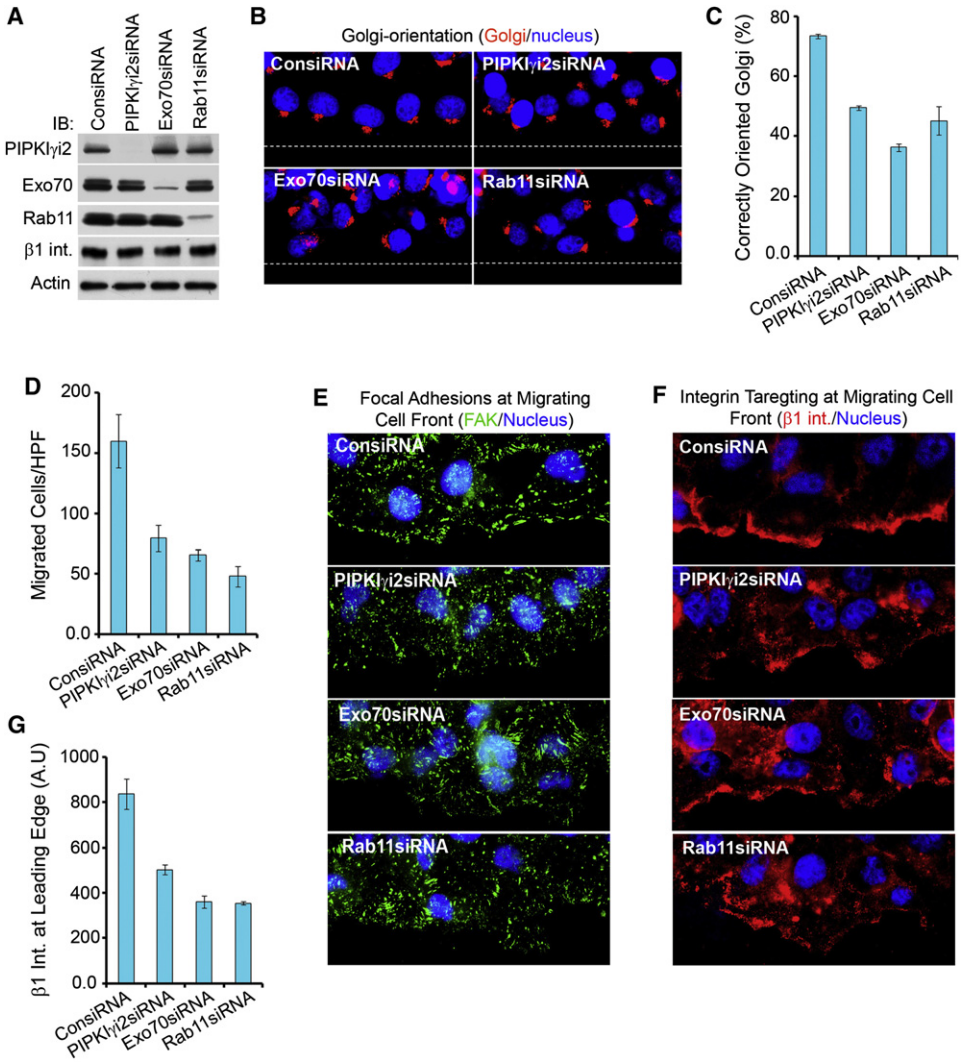

Figure 2. Knockdown of PIPKIγi2, Exocyst Complex Components, or Rab11 Impairs Polarized Recruitment of β1-Integrins and Cell Migration

(A) Knockdown of PIPKIγi2, Exo70, or Rab11 in MDA-MB-231 cells. Cells were transfected with specific siRNAs, and knockdown of indicated proteins was examined by immunoblotting. β1 int., β1-integrin.

(B) Confluent cell culture (48–72 hr posttransfection with siRNA) were wounded and processed 6 hr postwounding to examine Golgi (red) orientation toward the direction of cell migration.

(C) Quantitative data of Golgi orientation (mean ± SD of three independent experiments). Error bars represent SD.

(D) The modified Boyden chamber assay as described above was used to examine the migration of siRNA-treated cells toward FN. Results were expressed as the total number of cells migrated/HPF (mean ± SD from three independent experiments). Error bars represent SD.

(E and F) Confluent cell culture (48–72 hr posttransfection with siRNA) was fixed 5–6 hr postwounding and immunostained for FAK (green) and β1-integrin (red) to examine the focal adhesion complex formation and β1-integrin recruitment at the leading edge.

(G) Quantitative data of β1-integrin recruitment at the migrating cell fronts. The average fluorescence intensity (AU) of β1-integrin at migrating cell front was measured using the MetaMorph (mean ± SD of three independent experiments). Error bars represent SD.

See also Figure S2.

with talin at focal adhesion complexes of migrating cell fronts and also the recycling endosome (Ling et al., 2002, 2007) but did not localize to the Golgi, early endosomes, or lysosomes (data not shown). PIPKIγi2 also showed partial colocalization with Rab4 and Rab11-containing compartments (Figure 3B), GTPases with established roles in endosomal recycling of integrins (Caswell and Norman, 2006; Powelka et al., 2004). At the onset of migration, a large increase in PIPKIγi2 association with β1-integrin and talin was observed in both MDA-MB-231 and HeLa cells (Figure 3C). This is consistent with the colocalization of PIPKIγi2 with these molecules.

PIPKIγi2 directly interacts with talin, and talin directly associates with the cytoplasmic domain of β1-integrin; therefore, a role for talin in mediating complex assembly between PIPKIγi2 and β1-integrin in migrating cells was examined. PIP_2 modulates the talin interaction with β1-integrin (Martel et al., 2001), and

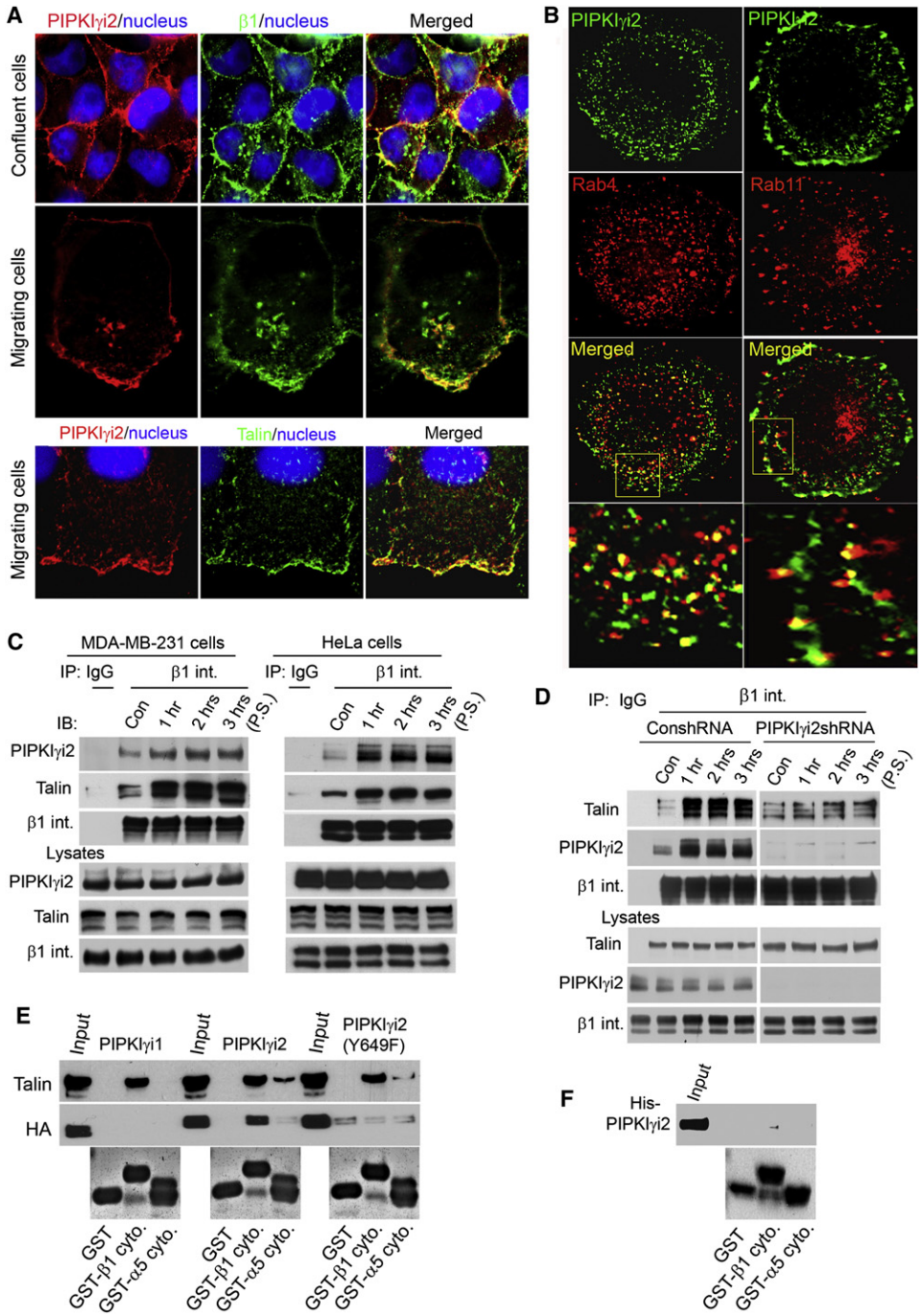

Figure 3. Cell Migration Promotes PIPKIγi2 Reorganization and Association with β1-Integrin Complexes

(A) PIPKIγi2 colocalizes with β1-integrins and talin. MDA-MB-231 cells expressing moderate level of HA-tagged PIPKIγi2 were wounded and processed for immunofluorescence (4–5 hr postscratching). PIPKIγi2 (red) and β1-integrin or talin (green) are recruited to and colocalize at migrating cell fronts and at intracellular compartments.

(B) PIPKIγi2 colocalizes with Rab4 and Rab11. MDA-MB-231 cells expressing PIPKIγi2 were seeded on FN-coated coverslips and cultured for 2–3 hr prior to cell fixation and immunostaining for PIPKIγi2 (green) and Rab4 or Rab11 (red). Boxes are selected regions for magnified view.

(C) Cell migration enhances a PIPKIγi2 association with β1-integrin and talin. Confluent MDA-MB-231 or HeLa cells were wounded extensively so that about 50% of cells were detached from culture plates. Cells were harvested at different time points, and β1-integrin was immunoprecipitated followed by immunoblotting to examine the coimmunoprecipitation of PIPKIγi2 and talin. β1 int., β1-integrin; Con, control; IP, immunoprecipitate; P.S., postscratch.

(D) PIPKIγi2 is required for talin association with β1-integrin in migrating cells. Confluent cultures of control or PIPKIγi2 knockdown cells (HeLa) were wounded as described above before immunoprecipitation of β1-integrin to examine the coimmunoprecipitation of talin and PIPKiγi2 by immunoblotting.

consistent with this, PIPKIγi2 knockdown severely impaired the association of talin with β1-integrin in migrating cells (Figure 3D). PIPKIγi2 and β1-integrin associate with talin's FERM domain (de Pereda et al., 2005; Ling et al., 2003; Wegener et al., 2007), and the interaction of PIPKIγi2 with talin is required for chemotaxis (Sun et al., 2007). Talin forms a homodimer that would interact with both PIPKIγi2 and β1-integrin in vivo, and talin also contains a second β1-integrin binding site in the rod domain (Critchley and Gingras, 2008). GST pull-down approach was used to demonstrate that talin can bind both PIPKIγi2 and β1-integrin. For this, GST fused to the cytoplasmic domain of β1-or α5-integrin was purified and incubated with cell lysates prepared from cells expressing PIPKIγi1 or PIPKIγi2 or PIPKIγi2Y649F mutant defective in talin binding (Ling et al., 2003). The GST-β1 cytoplasmic domain pulled down both talin and PIPKIγi2, but not PIPKIγi1 (lacking the C-terminal talin binding region) or PIPKIγi2Y649F, indicating the requirement of talin in mediating PIPKIγi2 association with β1-integrin (Figure 3E). Similarly, direct binding assays using GST-β1 or α5-integrin with purified His-tagged PIPKIγi2 indicated no binding (Figure 3F). These data demonstrate that PIPKIγi2 forms a complex with talin, and the PIPKIγi2-talin interaction enhanced the binding of β1-integrin to talin. Knockdown of PIPKIγi2 results in loss of β1-integrin targeting to the leading edge (Figures 2F and 2G), indicating a defect in trafficking.

PIPKIγi2 Knockdown Impairs β1-Integrin Exocytosis

To define the role of PIPKIγi2 in integrin trafficking, we examined the recycling of β1-integrin in control and PIPKIγi2 knockdown cells (Powelka et al., 2004). When β1-integrin was surface labeled and then internalized, there was enhanced accumulation of β1-integrin in the perinuclear region of PIPKIγi2 knockdown cells (Figures 4A–4C). The isolation of the β1-integrin-antibody complex following endocytosis at 37°C for 10 min did not show a difference in the endocytosis of β1-integrin in PIPKIγi2 knockdown cells (Figure 4D). This demonstrated that internalization of β1-integrin was not impaired in PIPKIγi2 knockdown cells, suggesting that PIPKIγi2 regulates exocytosis.

To define if exocytosis was impacted by PIPKIγi2 loss, we quantified the trafficking of perinuclear β1-integrin to the plasma membrane upon stimulation of serum-starved cells with 10% FBS. PIPKIγi2 knockdown cells resulted in diminished plasma membrane trafficking of β1-integrins (Figures 4E–4G), indicating a role for PIPKIγi2 in integrin exocytosis. These data were also confirmed biochemically by demonstrating more internal β1-integrin remaining in PIPKIγi2 knockdown cells after FBS stimulation (Figure 4H). In addition, we measured the β1-integrin recycling using a cell surface biotinylation approach. Quantification of β1-integrin recycling indicated that the exocytosis of β1-integrin was diminished in PIPKIγi2 knockdown cells but was rescued by re-expression of PIPKIγi2 (Figures 4I and 4J). Yet, there was no detectable change in the total surface content of β1- or α5-integrin in either confluent or migrating cells upon knockdown of PIPKIγi2 (Figure S3C), supporting a role for PIPKIγi2 in polarized trafficking of integrin. We focused on β1-integrin trafficking because it represents the predominant integrin in epithelial cells and interacts with the most abundant ECM proteins, FN and collagen (Caswell and Norman, 2006; Caswell et al., 2007). The loss of β1-integrin impaired microtubule orientation, nascent focal adhesion, complex formation at migrating cell fronts and haptotactic cell migration toward FN (Figures S2F and S2G).

PIPKIγi2 Directly Associates with the Exocyst Complex

The data indicate a role for PIPKIγi2 in the polarized trafficking of integrins and the involvement of PIP_2-regulated proteins in β1-integrin trafficking during cell migration. The exocyst is a conserved octomeric protein complex involved in polarized vesicle trafficking and is required for directional cell migration (Hertzog and Chavrier, 2011; Zuo et al., 2006). Components of the exocyst complex also serve as effectors of Rab11 and Arf6 GTPases, which regulate integrin trafficking and cell migration (Caswell and Norman, 2006). In addition, the docking of the exocyst complex to membrane is regulated by PIP_2 through interactions with Exo70 and Sec3 (He et al., 2007; Liu et al., 2007). Because PIP kinases often associate with PIP_2 effectors (Anderson et al., 1999; Heck et al., 2007), an interaction of PIPKIγi2 with the exocyst complex was explored. The exocyst components were coimmunoprecipitated with PIPKIγi2 (Figure 5A). Cell migration induced the association between PIPKIγi2, exocyst complex and β1-integrin (Figure 5B). This migration-induced association was also observed between endogenous PIPKIγi2, the exocyst complex and β1-integrin (Figure 5C). PIPKIγi2 expression specifically promoted the complex formation between β1-integrin and the exocyst complex in migrating cells, whereas expression of PIPKIγi1, PIPKIγi2KD (kinase dead mutant), or PIPKIγi2Y649F (mutant deficient in talin binding) poorly enhanced these associations (Figure 5D). Consistent with this, coimmunoprecipitation of the exocyst complex with β1-integrin was also reduced in PIPKIγi2 knockdown cells (Figure S6B). The immunoprecipitation of PIPKIγ further confirmed these associations and showed that PIPKIγi2Y649F lost interactions with both talin and β1-integrin (Figure 5E). This indicates a requirement for PIP_2 generation and talin binding ability of PIPKIγ in regulating the complex formation. Yet, PIPKIγi1 and PIPKIγi2Y649F were equally efficient in their interaction with exocyst complex in migrating cells (Figure 5E). PIPKIγi2KD had a reduced association with exocyst components, but not talin (Figure 5E), supporting a requirement for both talin interaction and PIP_2 production. These data indicate that PIPKIγi2 and PIP_2 generation specifically provide the platform for association of exocyst complex with β1-integrin in migrating cells and this requires PIPKIγi2 interaction with talin.

To investigate direct interactions between PIPKIγi2 and the exocyst complex, components of the exocyst complex were purified as GST fusion proteins. GST pull-down assays

(E) Cytoplasmic domain of β1-integrin pulled down both talin and PIPKIγi2. GST fusion protein of cytoplasmic domain of β1- or α5-integrin was incubated with cell lysates prepared from MDA-MB-231 cells expressing PIPKIγi1, PIPKIγi2, or PIPKIγi2Y649F. Pull down of talin and PIPKIγi2 was examined by immunoblotting. (F) In vitro binding study. GST fusion protein of cytoplasmic domain of β1- or α5-integrin was incubated with His-tagged PIPKIγi2 purified from bacteria, and PIPKIγi2 binding was examined by immunoblotting using anti-His antibody.
See also Figure S3.

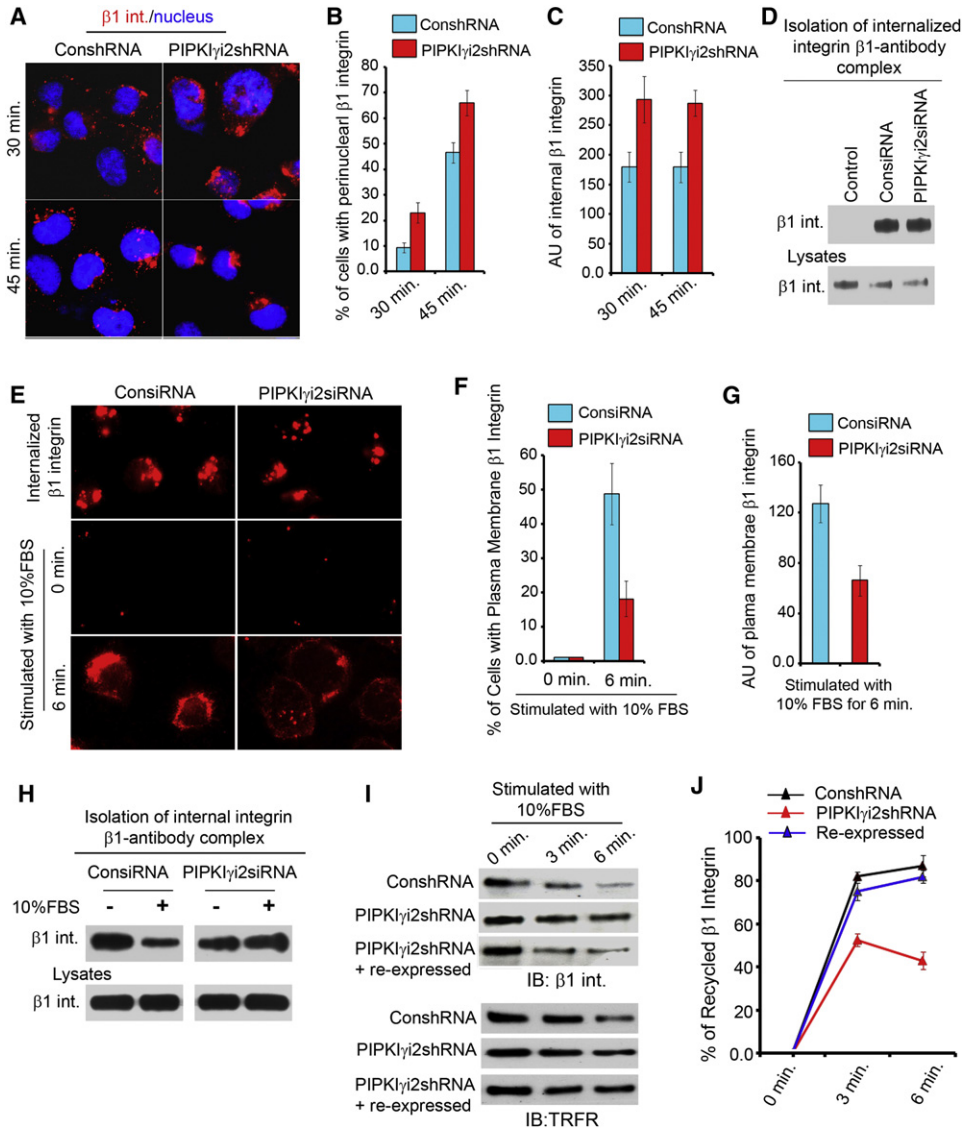

Figure 4. PIPKIγi2 Knockdown Impairs β1-Integrin Exocytosis

(A) For β1-integrin endocytosis, cell surface β1-integrins were labeled with anti-β1 antibody at 4°C. Cells were incubated at 37°C to induce internalization. Shown is the β1-integrin internalized after 30 and 45 min incubation.

(B) Cells with distinct perinuclear accumulation of β1-integrin-antibody complex were counted and expressed as percentage of total cells. A total of 150–200 cells were counted for each condition (results are mean ± SD of three independent experiments). Error bars represent SD.

(C) Average fluorescence intensity (AU) of internalized β1-integrin in knockdown and control cells was measured (around 150 cells included for each condition; results are mean ± SD of three independent experiments). Error bars represent SD.

(D) For biochemical assay of β1-integrin endocytosis, cell surface β1-integrins were labeled with anti-β1 antibody at 4°C followed by incubation of cells at 37°C for 10 min to induce internalization. The content of internalized β1-integrins in control or PIPKIγi2 knockdown cells was examined by immunoblotting.

(E) For examining β1-integrin accumulation at perinuclear regions, cells were permeabilized (top) before immunostaining as described in Experimental Procedures. Cells were processed for immunostaining without cell permeabilization to examine the β1-integrin (red) trafficking to the plasma membrane before (middle panels) or after (bottom panels) cell stimulation with FBS.

(F) The number of cells with distinct plasma membrane localization of β1-integrin in control versus PIPKIγi2 knockdown cells was quantified (around 150 cells counted each time; values are mean ± SD of three independent experiments). Error bars represent SD.

(G) Average fluorescence intensity (AU) of plasma membrane localization of β1-integrin in control versus PIPKIγi2 knockdown cells (around 150 cells counted each time; values are mean ± SD of three independent experiments). Error bars represent SD.

(H) The content of internal β1-integrin after FBS stimulation. Representative image of three independent experiments showing that PIPKIγi2 knockdown slowed β1-integrin trafficking to the plasma membrane.

(I) Integrin recycling was examined by cell surface biotinylation assay as described in Experimental Procedures. Biotinylated cell surface proteins remaining inside the cells were isolated using Streptavidin Affinity Gel followed by examination of β1-integrin and transferine receptor (TRFR) by immunoblotting.

(J) Quantitative data of β1-integrin recycling. The percentage of β1-integrin recycled was calculated as described in Experimental Procedures (values are mean ± SD from three independent experiments). Error bars represent SD.

β1 int., β1-integrin; IB, immunoblotting.

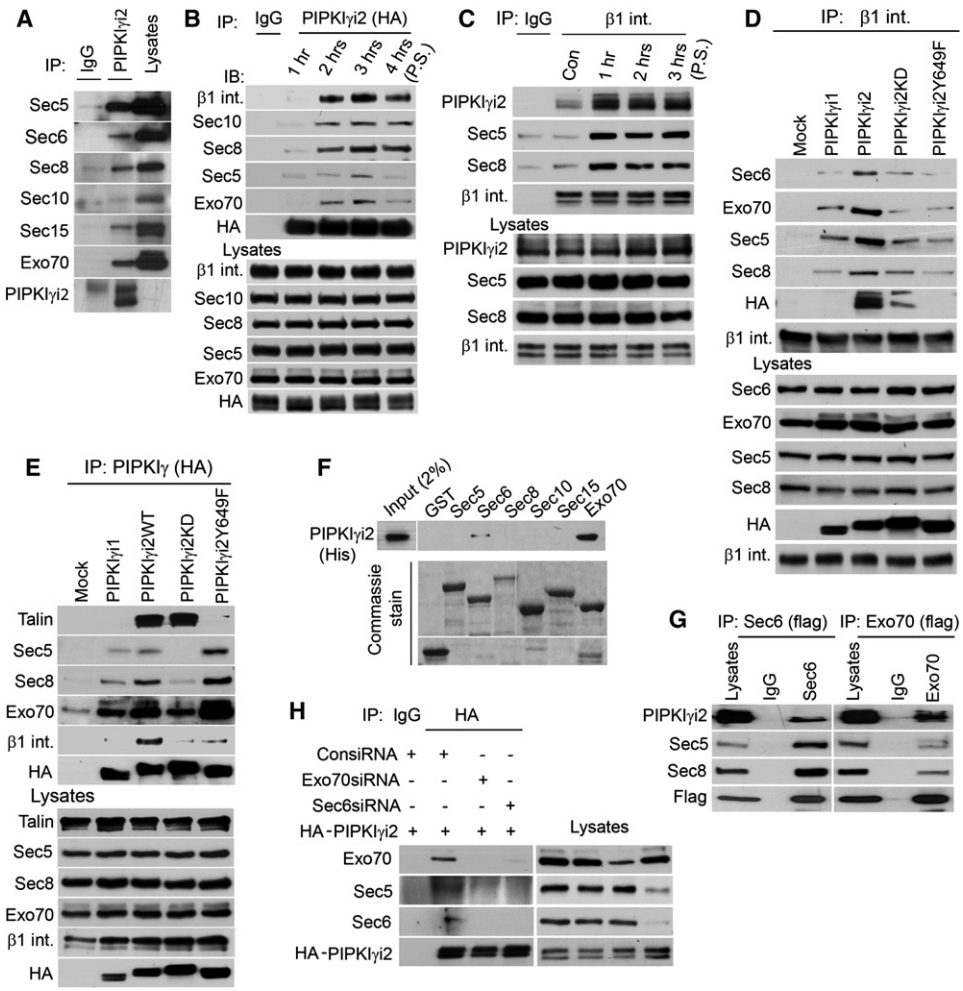

Figure 5. PIPKIγi2 Directly Associates with the Exocyst Complex

(A) Endogenous PIPKIγi2 was immunoprecipitated from MDA-MB-231 cells, and coimmunoprecipitation of exocyst complex was examined by immunoblotting.

(B) MDA-MB-231 cells expressing moderate level of HA-tagged PIPKIγi2 grown to confluence were harvested at different time points after scratch wounding. PIPKIγi2 was immunoprecipitated using anti-HA antibody, and coimmunoprecipitation of β1-integrin and exocyst complex was examined by immunoblotting.

(C) HeLa cells grown to confluence were harvested at different time points after scratch wounding. β1-integrin was immunoprecipitated, and coimmunoprecipitation of PIPKIγi2 and exocyst components was examined by immunoblotting.

(D) MDA-MB-231 cells expressing PIPKIγi1, PIPKIγi2, PIPKIγi2KD, or PIPKIγi2Y649F were harvested 2–3 hr postwounding. β1-integrin was immunoprecipitated, and coimmunoprecipitation of exocyst complex proteins was examined by immunoblotting.

(E) MDA-MB-231 cells expressing PIPKIγi1, PIPKIγi2, PIPKIγi2KD, or PIPKIγi2Y649F were harvested 2–3 hr postwounding. PIPKIγi2 and other mutants were immunoprecipitated using anti-HA antibody, and coimmunoprecipitation of exocyst complex was examined by immunoblotting.

(F) Sec6 and Exo70 directly interact with PIPKIγi2. GST fusion protein of exocyst complex components was incubated with His-PIPKIγi2 purified from bacteria. PIPKIγi2 binding was examined by immunoblotting using anti-His antibody.

(G) Exo70 and Sec6 coimmunoprecipitate endogenous PIPKIγi2. HeLa cells were transiently transfected with Flag-tagged Exo70 or Sec6 and immunoprecipitated using anti-Flag antibody. Coimmunoprecipitation of PIPKIγi2 and other components of exocyst complex was examined by immunoblotting.

(H) Knockdown of Exo70 or Sec6 impairs PIPKIγi2 association with exocyst complex. HeLa cells were transfected with siRNA for Exo70 or Sec6. Twenty-four hours after the siRNA transfection, cells were transfected with HA-tagged PIPKIγi2. Next day, cells were harvested to immunoprecipitate PIPKIγi2, and coimmunoprecipitation of exocyst complex was examined by immunoblotting.

β1 int., β1-integrin; IB, immunoblotting; IP, immunoprecipitate. See also Figure S4.

demonstrated Sec6 and Exo70 as direct binding partners of PIPKIγi2 (Figure 5F), although Exo70 interacted more strongly than Sec6. The interaction between Sec6 or Exo70 with PIPKIγi2 in vitro was not specific for PIPKIγi2 because all splice variants interacted (data not shown). Coexpression and coimmunoprecipitation studies in HEK293 cells indicated that all the isoforms of PIPKIγ interact with Sec6 and Exo70 (Figures S4A and S4B). This indicates that PIPKIγ interacts with Sec6 and Exo70 through regions conserved in all PIPKIγ isoforms (Heck et al., 2007). Expression of Flag-tagged Sec6 or Exo70 in HeLa cells coimmunoprecipitated endogenous PIPKIγi2 along with other exocyst complex components (Figure 5G). In cells, Sec6 and Exo70 mediate the PIPKIγi2 association with the exocyst complex because knockdown of either Sec6 or Exo70

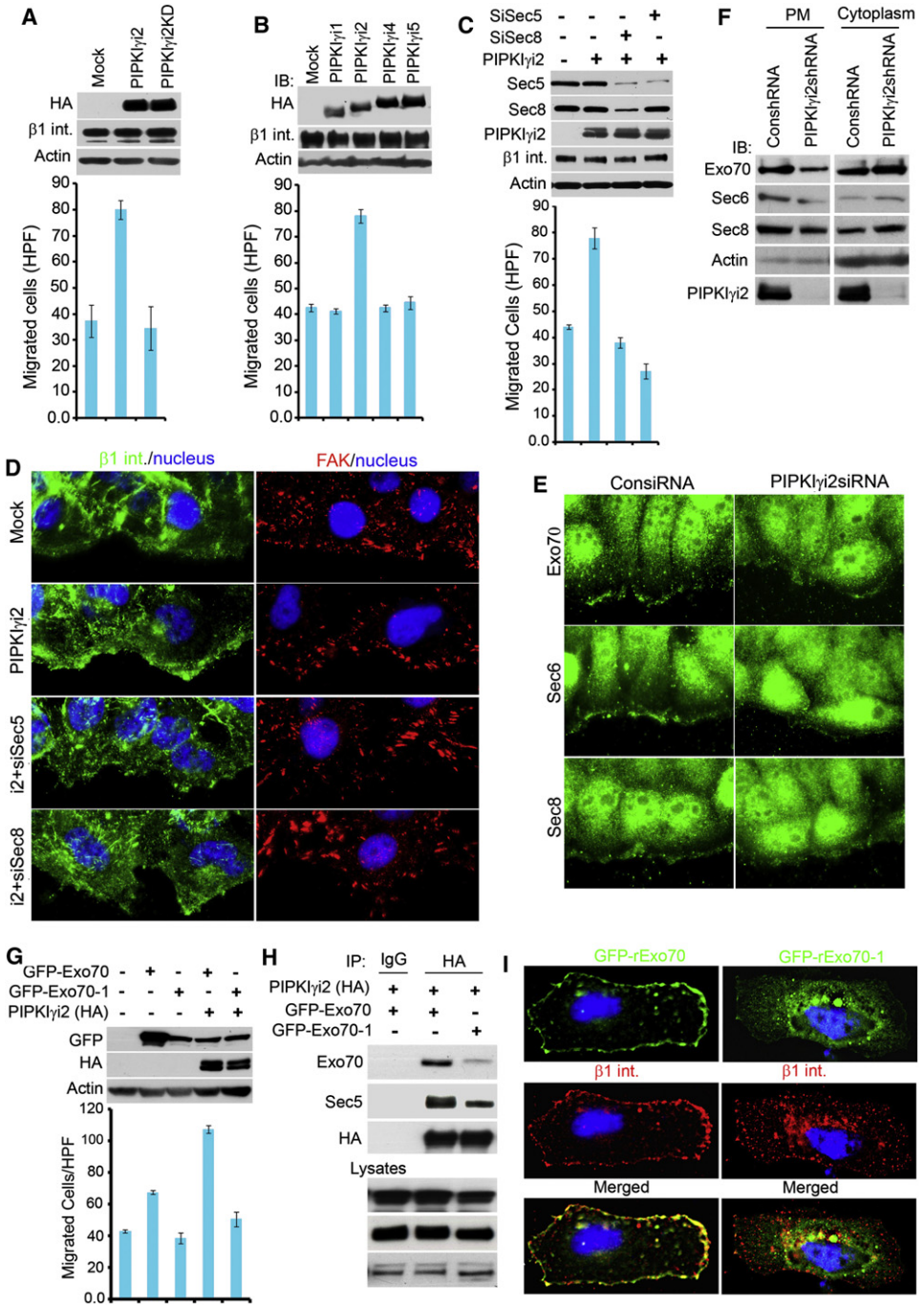

Figure 6. The Exocyst Complex Is Required for PIPKIγi2-Regulated Cell Migration

(A) PIPKIγi2 expression promotes cell migration. HeLa cells transiently transfected with PIPKIγi2 or PIPKIγi2KD were monitored for changes in haptotactic cell migration using a modified Boyden chamber assay. The results are expressed as migrated cells/HPF (mean ± SD of three experiments). Immunoblots were used to examine PIPKIγ expression using anti-HA antibody. Error bars represent SD.

(B) Cell migration assays were performed as above in HeLa cells transiently transfected with PIPKIγ isoforms, and results were expressed as migrated cells/HPF (mean ± SD of three experiments). Immunoblots were used to examine PIPKIγ expression using anti-HA antibody. Error bars represent SD.

(C) Cell migration assays were performed in HeLa cells treated with siRNA to knock down exocyst components (Sec5 or Sec8) followed by PIPKIγi2 overexpression as described above. Results expressed as migrated cells/HPF (mean ± SD of three experiments). Knockdown of Sec5 or Sec8 and expression of PIPKIγi2 were monitored by immunoblotting. Error bars represent SD.

(D) Exocyst complex is required for polarized recruitment of β1-integrin. HeLa cells stably expressing PIPKIγi2 were treated with siRNA to knock down Sec5 or Sec8. At 48–72 hr posttransfection, cells were scratch wounded and immunostained for β1-integrin (green) and FAK (red) to examine the recruitment of β1-integrin and focal adhesion formation at migrating cell fronts.

abrogated coimmunoprecipitation of the exocyst complex with PIPKIγi2 (Figure 5H).

The Exocyst Complex Is Required for PIPKIγi2-Regulated Cell Migration

PIPKIγi2 forms a complex with β1-integrin and exocyst components, but PIPKIγi2KD is poorly incorporated into this complex, indicating that PIP_2 generation is required (Figures 5D and 5E). Consistent with this, the expression of PIPKIγi2, but not PIPKIγi2KD, promoted the haptotactic cell migration of HeLa cells toward FN (Figure 6A). Similar results were obtained using a HeLa Tet-off cell line expressing PIPKIγi2 or PIPKIγi2KD (data not shown). The expression of PIPKIγ isoforms is variable between cell lines and tissues (Schill and Anderson, 2009b). In breast cancers, increased PIPKIγ expression correlates with disease progression (Schramp et al., 2011; Sun et al., 2010), indicating that changes in PIPKIγ content is an in vivo mechanism to modulate cellular function.

To define the specificity of PIPKIγi2 in promoting cell migration, each PIPKIγ isoform was expressed in HeLa cells. Enhanced cell migration toward FN was promoted exclusively in cells expressing PIPKIγi2 (Figure 6B), and only the PIPKIγi2 isoform associated with talin and β1-integrin (Figures S4E and S4F). PIPKIγi2 expression specifically promoted complex formation between β1-integrin and the exocyst complex in migrating cells (Figures 5D and 5E). To define the functional link between the exocyst complex and PIPKIγi2 in cell migration, we used siRNAs to knockdown exocyst components (Sec5 or Sec8) in cells ectopically expressing PIPKIγi2. As shown in Figure 6C, knockdown of exocyst components reduced PIPKIγi2-stimulated cell migration toward FN. Furthermore, knockdown of Sec5 or Sec8 blocked PIPKIγi2-enhanced β1-integrin recruitment to the migrating cell front (Figure 6D). The knockdown of any exocyst complex component impaired the PIPKIγi2-regulated cell migration and polarized β1-integrin targeting (Figures 6C and 6D; data not shown). Overexpression of individual exocyst complex components did not rescue the cell migration defect in PIPKIγi2 knockdown cells (data not shown). These data indicate that PIPKIγi2 and PIP_2 generation regulates the assembly of exocyst complex required for driving polarized recruitment/trafficking of integrin molecules required for directional migration. To explore this, the polarized recruitment of Exo70, Sec6, and Sec8 to the migrating cell fronts was examined. As shown in Figures 6E and 6F, PIPKIγi2 knockdown impaired the polarized recruitment of exocyst complex components to the leading edge membrane.

PIPKIγi2 and PIP_2 generation regulates cell migration, and the exocyst complex is required for driving polarized recruitment/trafficking of β1-integrin. The exocyst complex binds to PIP_2 and is regulated by this interaction (He et al., 2007; Liu et al., 2007). The role of PIP_2 binding was explored using the Exo70 mutant, Exo70-1, deficient in PIP_2 binding (He et al., 2007). Expression of Exo70 modestly enhanced directional migration, but coexpression with PIPKIγi2 synergistically increased migration (Figure 6G). However, expression of Exo70-1 did not enhance migration, and coexpression of Exo70-1 with PIPKIγi2 blocked PIPKIγi2-induced cell migration (Figure 6G). This demonstrates that PIP_2 binding to Exo70 regulates PIPKIγi2-induced directional cell migration.

PIPKIγi2 directly associates with Exo70, is a link to the exocyst complex, and these interactions are enhanced during cell migration (Figures 5B and 5C). The PIP_2 generation by PIPKIγi2 is required for its association with the exocyst complex in migrating cells because PIPKIγi2KD weakly associated with the exocyst complex (Figure 5E). When coexpressed, PIPKIγi2 and Exo70 tightly colocalize in cells, but PIPKIγi2KD did not colocalize with Exo70, which was diffusely localized (Figure S6C). PIP_2 binding is required for Exo70 localization to the membrane, and the Exo70-1 mutant loses this localization (Liu et al., 2007). Similarly, the Exo70-1 mutant poorly interacted with PIPKIγi2 compared to Exo70 (Figure 6H). Yet, Exo70-1 retained the ability to interact with other exocyst components (Figure S4G). These data indicate that the PIPKIγi2 interaction with Exo70 and the exocyst complex is regulated by PIPKIγi2 generation of PIP_2 and PIP_2 binding to Exo70.

Integrated Role of PIPKIγi2, Exocyst Complex, and Talin in Integrin Trafficking

The exocyst regulates polarized membrane trafficking (He and Guo, 2009) and cell migration (Hertzog and Chavrier, 2011; Zuo et al., 2006). Expressed GFP-Exo70 targets to the plasma membrane and colocalized with β1-integrin at the plasma membrane (Figure 6I). GFP-Exo70-1 poorly targeted to plasma membrane and did not colocalize with β1-integrin at plasma membrane (Figure 6I). This is consistent with previous reports demonstrating that Exo-70 binding to PIP_2 is required for the trafficking of membrane proteins (Liu et al., 2007). GFP-Exo70 colocalizes with α5-integrin (a β1-integrin partner) both at the plasma membrane and intracellular compartments (Figure S6A), but in PIPKIγi2 knockdown cells, GFP-Exo70 poorly colocalized with α5-integrin, specifically in the intracellular compartment (Figure S6A).

(E) Confluent culture of cells (48–72 hr posttransfection with siRNA) were processed 2–3 hr postwounding. The polarized recruitment of endogenous exocyst complex (Exo70, Sec6, and Sec8) (green) to migrating cell fronts was examined using their specific antibodies.

(F) Crude plasma membrane was isolated from control or PIPKIγi2 knockdown cells followed by examination of exocyst complex components in plasma membrane (PM) and cytosol.

(G) HeLa cells were transfected with GFP-Exo70 or GFP-Exo70-1 or cotransfected with PIPKIγi2. Haptotactic cell migration toward FN was examined as described above. The results expressed as migrated cells/HPF (mean ± SD of three experiments). Error bars represent SD.

(H) Exo70-1 poorly associates with PIPKIγi2 and impairs the PIPKIγi2 association with the exocyst complex. HeLa cells were cotransfected with PIPKIγi2 and Exo70 or Exo70-1. Cells were harvested 24 hr posttransfection to immunoprecipitate PIPKIγi2, and coimmunoprecipitation of exocyst complex was examined by immunoblotting.

(I) HeLa cells were transfected with either GFP-Exo70 or GFP-Exo70-1. GFP-Exo70 colocalized with β1-integrin (red) at plasma membrane, whereas GFP-Exo70-1 was found either diffusely distributed into the cytoplasma or accumulated around perinuclear regions. β1-integrin was poorly recruited to plasma membrane and accumulated around perinuclear regions in GFP-Exo70-1 expressing cells.

β1 int., β1-integrin; IB, immunoblotting; IP, immunoprecipitate. See also Figure S5.

The role of PIPKIγi2 and the exocyst complex to drive polarized trafficking of integrin molecules to focal adhesion complexes was further supported by in vivo colocalization. PIPKIγi2 and components of the exocyst complex (Exo70, Sec6, and Sec8) colocalized at focal adhesion complexes in HeLa cells adhering and spreading to FN (Figure 7A). In PIPKIγi2-expressing cells, Exo70 and α5-integrin colocalized with PIPKIγi2 at plasma membrane and vesicular intracellular compartments (Figure 7B, top), whereas Exo70 poorly colocalized with α5-integrin in cells not ectopically expressing PIPKIγi2 (Figure 7B, middle). As a control, Exo70 and PIPKIγi2 were highly colocalized, but not with GFP (Figure 7B, bottom). These data indicate that PIPKIγi2 expression promotes Exo70 localization/association with α5-integrin. Inconsistent to this, the knockdown of PIPKIγi2 resulted in diminished exocyst association with β1-integrin (Figure S6B).

PIPKIγi2 directly and specifically interacts with talin (Di Paolo et al., 2002; Ling et al., 2002, 2003) (Figure S4E), and this interaction mediates PIPKIγi2 association with β1-integrin in migrating cells (Figures 3E, 5D, and 5E). This interaction is also required for growth factor-stimulated chemotaxis (Sun et al., 2007). Talin physically links integrins at focal adhesions to the actin cytoskeleton, a process controlled by PIP_2 (Gilmore and Burridge, 1996; Ling et al., 2006; Martel et al., 2001). Talin is required for focal adhesion targeting of PIPKIγi2, and talin knockdown cells were defective in focal adhesion formations (Figure S5A). Also, targeting of the exocyst complex and β1-integrin to the migrating cell front and polarization of cells was impaired in talin knockdown cells (Figures S5B–S5D). Exo70 is targeted to talin containing focal adhesions in PIPKIγi2-expressing cells (Figure 7C, top) compared to nonexpressing cells (Figure 7C, bottom). Consistent with this observation, PIPKIγi2 expression specifically promoted the association of talin with the exocyst complex and β1-integrin (Figure 7D). These linkages are further supported by the migration-dependent integration of PIPKIγi2 into a complex with talin, α5-, and β1-integrin, but not PIPKIγi2KD, indicating that these are PIP_2-regulated processes (Figure 7E).

DISCUSSION

The precisely controlled trafficking of integrin molecules to and from plasma membrane is a fundamental process of migrating cells (Caswell and Norman, 2008; Caswell et al., 2009; Muller et al., 2009). The mechanisms for trafficking of integrins toward the membrane front in directionally migrating cells are emerging with roles for Rab4, Rab11, ARF6, Rab25, and spatial growth factor receptor signaling (Caswell and Norman, 2008; Caswell and Norman, 2006; Caswell et al., 2009). The exocyst complex plays a role in polarized secretion and also cell migration (He and Guo, 2009; Zuo et al., 2006). Upon initiation of migration, components of the exocyst complex are redistributed from cell-cell contact sites to focal adhesions (Spiczka and Yeaman, 2008). Here, we describe a role of PIPKIγi2 in the polarized trafficking of integrin molecules in directionally migrating cells via its association with and regulation of the exocyst complex.

Generation of PIP_2 in a spatiotemporal manner controls vesicle trafficking at the plasma membrane (Di Paolo et al., 2004; Schill and Anderson, 2009a). A role for PIP_2 in trafficking to the plasma membrane or between intracellular compartments is also emerging because PIP_2 is synthesized on intracellular membrane compartments (Vicinanza et al., 2008), and PIP_2 generation modulates E-cadherin sorting to the basolateral membrane from the recycling endosome (Ling et al., 2007). Exocyst complex components also bind PIP_2 and may regulate trafficking (Liu et al., 2007). This suggests that the exocyst complex coordinates with PIP_2-synthesizing enzymes to modulate integrin trafficking during cell migration.

PIPKIγi2 directly interacts with exocyst components Sec6 and Exo70. The association of PIPKIγi2 with both Sec6 and Exo70 may be functionally significant because Sec6 is associated with vesicle-containing cargo, whereas Exo70 may mediate plasma membrane docking via PIP_2 interactions (Yu and Hughson, 2010). The interaction with both Sec6 and Exo70 is consistent with the localization of PIPKIγi2 in cytosolic compartments and at the plasma membrane/focal adhesions. This indicates that PIPKIγi2 regulates the exocyst complex in multiple compartments, positioning PIPKIγi2 to modulate polarized trafficking of molecules required for cell migration.

The exocyst complex, PIPKIγi2, β1-integrin, and talin are all individually required for cell migration (Ling et al., 2006; Sun et al., 2007; Zuo et al., 2006). We show that these components integrate together to orchestrate directional migration. Based on these results, upon migration PIPKIγi2 integrates the exocyst complex with β1-integrin. The ability of PIPKIγi2 to interact with talin through its unique C-terminal domain enables the targeting of the exocyst/β1-integrin complex to the leading edge where integrin delivery/activation is required for nascent focal adhesion complex formation (Figure 7F).

The interaction between PIPKIγi2 and talin targets the exocytosis of β1-integrin to talin-enriched focal adhesion complexes at leading edge plasma membrane (Figure 7F), suggesting that talin serves as a tethering factor to guide β1-integrin trafficking (Yu and Hughson, 2010). In this context, PIPKγi2 acts both as a signaling scaffold that links the exocyst complex and β1-integrin vesicle to talin-based adhesive complexes and generates PIP_2 that regulates vesicle docking with the plasma membrane through PIP_2 regulation of Exo70. This would place β1-integrin, talin, PIPKIγi2, and PIP_2 generation in spatial proximity, where PIP_2 enhances the interaction of talin with β1-integrin (Martel et al., 2001). Talin mediated the PIPKIγi2 interaction with β1-integrin, and PIP_2 generation enhanced the interaction of the exocyst complex with β1-integrin. The intrinsic ability of talin to integrate into focal adhesion complexes in concert with PIPKIγi2 regulation of exocyst function facilitates the polarized delivery of β1-integrin to the leading edge of migrating cells. This would lead to the formation of adhesive complexes at the leading edge an event critical for cell migration.

Vinculin links adhesive complexes to actin, and its incorporation into the talin/integrin complexes required PIPKIγi2 kinase activity (PIP_2 synthesis). The interaction of vinculin with talin has been reported to be both PIP_2 dependent and independent (Chandrasekar et al., 2005; Gilmore and Burridge, 1996). Talin interaction with vinculin is also enhanced by talin stretching (del Rio et al., 2009). Because PIP_2 regulates the interaction of talin with integrin, this interaction would serve as an anchor for talin such that stretching that would expose vinculin binding sites, indicating that PIP_2 regulates multiple talin interactions.

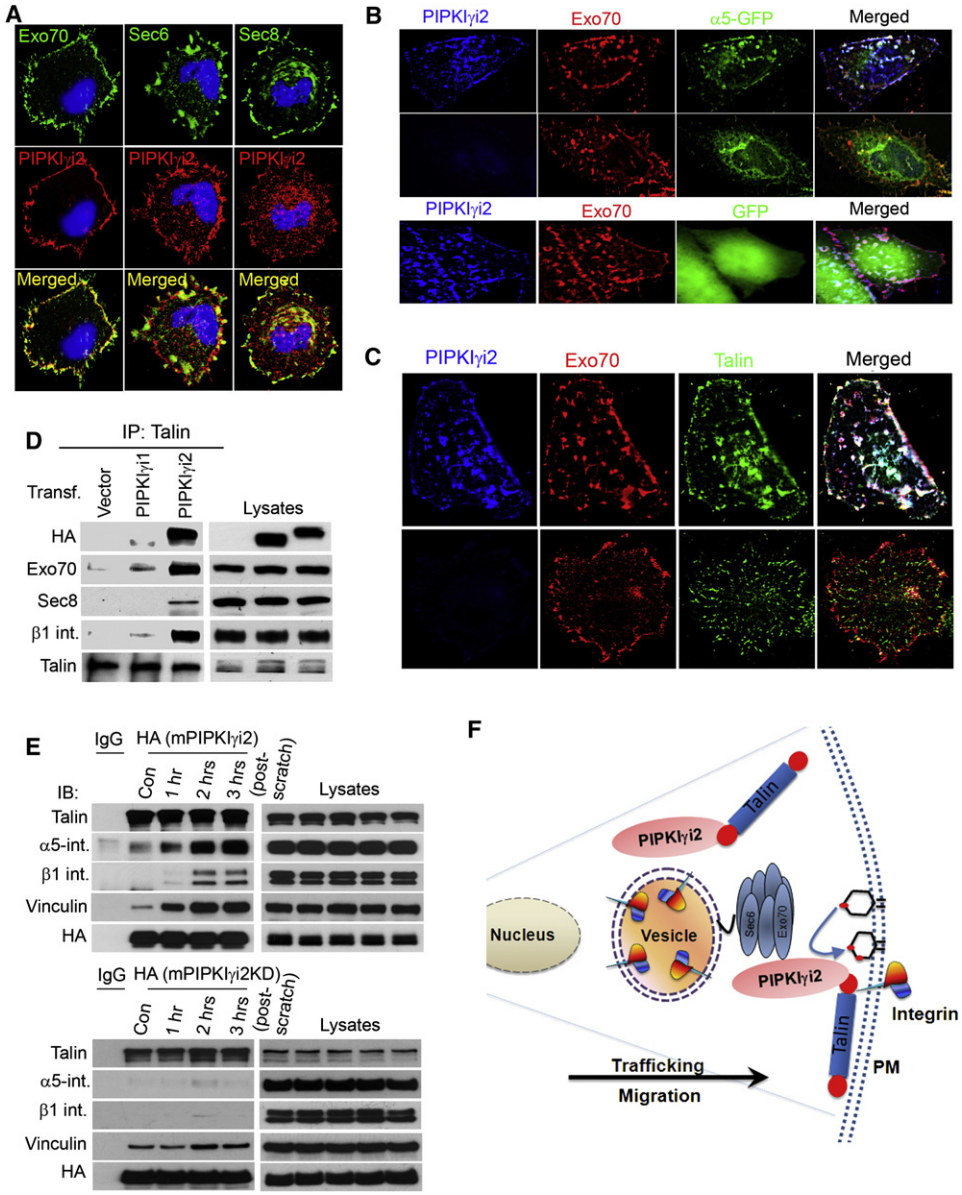

Figure 7. PIPKIγi2 Integrates Exocyst Complex and Talin with Integrin and Is Required for Integrin Trafficking

(A) HeLa cells cotransfected with Flag-tagged exocyst complex components (Sec6 or Sec8 or Exo70) and HA-tagged PIPKIγi2 were allowed to adhere on FN-coated coverslips for 1–2 hr before immunostaining with anti-Flag (green) and anti-HA (red) antibodies.

(B) PIPKIγi2, Exo70, and α5-GFP integrin colocalize at cell membrane and intracellular compartments. HeLa cells (bottom) or HeLa cells stably expressing HA-tagged PIPKIγi2 (top) were cotransfected with Flag-tagged Exo70 and α5-integrin-GFP or GFP. Cells were fixed and immunostained using anti-HA (blue) or anti-Flag (red) antibodies.

(C) Exo70 colocalizes with talin at focal adhesions and intracellular sites. HeLa cells transiently transfected with Flag-tagged Exo70 alone or cotransfected with HA-tagged PIPKIγi2 were allowed to adhere on FN-coated coverslips for 1–2 hr before immunostaining with anti-HA (blue), anti-Flag (red), and anti-talin (green) antibodies.

(D) PIPKIγi2 integrates talin, β1-integrin, and exocyst complex in the same complex. HeLa cells were transfected with PIPKIγi1 or PIPKIγi2. Talin was immunoprecipitated 24 hr posttransfection, followed by immunoblotting for exocyst complex and β1-integrin.

(E) PIPKIγi2 was immunoprecipitated from HeLa cells stably expressing PIPKIγi2 or PIPKIγi2KD at different time points following wounding to induce migration. Immunocomplexes were examined for presence of integrins and/or talin by immunoblotting.

(F) Model depicting the role of PIPKIγi2 in integrin trafficking in directionally migrating cells. Cell migration induces the integration of PIPKIγi2, talin, and β1-integrin into the complex either in plasma membrane (PM) or in intracellular recycling compartments. Furthermore, PIP_2 generation by PIPKIγi2 into the complex facilitates the assembly of the exocyst complex. Thus, coordinated activity of PIPKIγi2 and the exocyst complex in concert with talin promotes the polarized recruitment and trafficking of integrin molecules to migrating cell fronts. Loss of PIPKIγi2 or the exocyst complex or talin compromises the polarized recruitment/trafficking of integrin impairing cell polarization and directional cell migration.

α5 int., α5-integrin; β1 int., β1-integrin; IB, immunoblotting; IP, immunoprecipitate; Transf, transfected. See also Figure S6.

In vivo migration and invasion occur in three-dimensional (3D) matrices. This requires cells to form highly polarized membrane projections to migrate or invade through the matrix. Compared to two-dimensional migration, the role of PIPKIγi2, the exocyst, integrin trafficking, and talin in 3D migration is likely to be accentuated because membrane structures are more polarized, and the polarized trafficking of molecules to the leading edge is essential. The increased expression of PIPKIγi2 enhanced cell migration and formation of the exocyst/integrin complexes. Increased PIPKIγ expression also correlates with disease progression in patients with breast cancer (Schramp et al., 2011; Sun et al., 2010). This implicates a role for PIPKIγi2 in the metastasis of breast cancers, a process requiring cell migration and invasion.

EXPERIMENTAL PROCEDURES

Cell Migration and Wound-Healing Assays

Cell migration assays were performed using modified Boyden chambers. The underside of polycarbonate membrane (8 μm pore size; Neuro Probe) was coated overnight in 10 μg/ml FN or Col.I, air-dried, and placed in the chamber filled with DMEM containing 0.1% BSA, with coated surface facing down. Cells after overnight serum starvation were suspended in DMEM containing 0.1% BSA, and then introduced into the upper compartment and incubated for 8–12 hr at 37°C in the incubator. Membranes were fixed after removing the nonmigrated cells from upperside with cotton swab, stained with crystal violet stain. Migrated cells were counted from at least ten randomly selected areas at 200× microscopic fields (HPF). Each experiment was reproduced at least in triplicate for each cell type and matrix. For wound-healing assay, MDA-MB-231 or HeLa cells grown to confluence on FN-coated culture dishes were wounded using 200 μl pipette tips and incubated for 12–16 hr before taking several randomly selected fields to measure wound width.

Immunoprecipitation and GST Pull-Down Assay

Cells were lysed in lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% NP40, 1 mM EDTA, 10 mM NaF, 5 mM Na_3VO_4, and proteases inhibitors). Clear supernatant was incubated with indicated antibodies for overnight followed by isolation of immunocomplexes using protein G Sepharose 4B beads (Amersham). The beads were washed three times with lysis buffer before eluting immunocomplexes with 2× sample buffer and then subjected to immunoblotting with indicated antibodies.

GST fusion proteins of exocyst complex components were purified from *E. coli*. GST fusion proteins (5 μg each) immobilized on glutathione-agarose beads were incubated with His-tagged PIPKIγi2 (purified from BL21) in binding buffer (20 mM HEPES [pH 7.4], 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, and protease inhibitors) at 4°C for 1 hr. After washing the beads three times with binding buffer, bound PIPKIγi2 was analyzed by immunoblotting using anti-His antibody.

For pull-down assay using cell lysates, GST fusion proteins (5 μg each) of the cytoplasmic domain of β1- or α5-integrin immobilized on glutathione-agarose beads were incubated with cell lysates prepared from MDA-MB-231 cells stably expressing PIPKIγi1, PIPKIγi2, or PIPKIγi2Y649F. After incubation at 4°C for 2–3 hr, beads were washed two to three times with lysis buffer before eluting the bound proteins for immunoblotting.

Immunofluorescence Microscopy

To examine the polarized recruitment of PIPKIγi2, β1-integrin, exocyst complex, or talin, the MDA-MB-231 or HeLa cells were cultured into FN-coated coverslips until confluency. The scratch wounds were created using 200 μl pipette tips, and detached cells were removed. The remaining cells were allowed to migrate toward the denudated area by incubating the cells at 37°C for 4–5 hr before fixing the cells with 3.7% PFA. The cells were permeabilized with 0.1% Triton-X before blocking with 3% BSA, followed by overnight incubation with indicated antibodies at 4°C.

For examining the recruitment of PIPKIγi2 and exocyst complex at focal adhesion sites, cells were seeded onto FN-coated coverslips in serum-free DMEM and incubated for 2–3 hr at 37°C in the incubator before processing the cells for immunofluorescence study as described above. Images were acquired using MetaMorph in fluorescence microscope (Nikon Eclipse TE2000-U).

Integrin-Trafficking Assay

Control or PIPKIγi2 knockdown MDA-MB-231 cells grown on FN-coated coverslips were labeled with anti-β1 (MAB2000; Millipore) or anti-α5-integrin (610633; BD Biosciences) at concentration of 10 μg/ml in serum-free DMEM/0.1%BSA by incubating the cells at 4°C for 1 hr. After removing the unbound antibody with cold DMEM, internalization of antibody-integrin complex was initiated by incubating the cells at 37°C in DMEM/10%FBS. At different time points, cell surface antibodies were removed by acid wash (0.5% glacial acetic acid and 0.5 M NaCl [pH 3.0]), followed by fixation with 3.7% PFA and cell permeabilization with 0.1% Triton-X. Cells were incubated with Alexa 555-labeled goat anti-mouse antibody (Molecular Probes) to visualize internalized antibody-integrin complex. Fluorescence intensity of internalized integrin was measured using MetaMorph. For biochemical assay of endocytosis, cells were lysed after removing the cell surface antibodies by acid wash. The internalized β1-integrin antibody complex was isolated from clear supernatants using protein G Sepharose beads followed by SDS-PAGE and immunoblotting of β1-integrin in the isolated complex.

For integrin exocytosis assay, cell surface β1-integrins were labeled as described above. Integrin-antibody complexes were allowed to internalize and accumulate at perinuclear region by incubating the cells at 37°C for 1–2 hr in DMEM/0.1%BSA medium. The β1-integrin antibody remaining on cell surface was removed by acid wash. Then, internalized integrin β1-antibody complexes were induced to recycle to plasma membrane by stimulating cells with DMEM/10% FBS for 3–6 min. Cells were processed for staining as above without cell permeabilization (except for examining the internalized β1-integrin before cell stimulation). For biochemical assay, recycled β1-integrin antibody on cell surface was removed by acid wash before cell lysis.

For cell surface biotinylation approach to assess integrin recycling, cells were serum starved overnight and incubated with biotin (0.2 mg/ml) (Sulf-NHS-SS-Biotin; Thermo Scientific) to label surface protein at 4°C for 30 min. Then, biotinylated cell surface proteins were allowed to undergo endocytosis by incubating the cells at 37°C for 1–2 hr in DMEM. After incubation, biotin remaining on cell surface was removed by MeSNa wash. The recycling of internalized integrins to cell surface was stimulated by incubating the cells in DMEM containing 10% FBS at 37°C. Biotin from recycled integrins to cell surface was removed by second wash with MeSNa. Biotinylated cell surface proteins remaining inside the cells were isolated using Streptavidin Affinity Gel (Sigma-Aldrich), followed by immunoblotting to examine β1-integrin and transferrin receptor (TRFR). The percentage of β1-integrin recycled was calculated as percentage of the difference in the amount of β1-integrin in zero time and selected time point divided by total internalized integrin.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, Supplemental Experimental Procedures, and two movies and can be found with this article online at doi:10.1016/j.devcel.2011.10.030.

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Supplemental Information

Phosphoinositide Signaling Regulates the Exocyst Complex and Polarized Integrin Trafficking in Directionally Migrating Cells

Narendra Thapa, Yue Sun, Mark Schramp, Suyong Choi, Kun Ling, and Richard A. Anderson

Supplemental Inventory

Figure S1 (related to Figure 1). It describes effect of PIPKIγi2 knockdown on directional cell migration of HeLa cells and on directional persistence/velocity of non-directionally migrating cells, and PIPKIγi2 recruitment to migrating cell fronts.

Figure S2 (related to Figure 2). It describes the effect of PIPKIγi2 and PIPKIγi5 knockdown on Golgi orientation, and effect of β1-integrin knockdown on cell migration and microtubule orientation.

Figure S3 (related to Figure 3). It describes the effect of PIPKIγi2 knockdown on β1-integrin recruitment to membrane protrusions/ruffles, on total surface β1-integrin level and on cell adhesion.

Figure S4 (related to Figure 5). It describes the PIPKIγ association with Sec6 and Exo70, and specific interaction of PIPKIγi2 with talin and β1-integrin.

Figure S5 (related to Figure 6). It describes the talin requirement for PIPKIγi2 recruitment and PIP_2 generation at focal adhesion complex.

Figure S6 (related to Figure 7). It describes the PIPKIγi2 requirement for Exo70 colocalization with a5 integrin, and kinase activity of PIPKIγi2 is required for Exo70 recruitment to vesicle-like intracellular compartments.

Supplemental Experimental Procedures

Cell culture and transfection, Antibodies, siRNA, shRNA system for generation of PIPKIγi2 knockdown cells, lentiviral system for expression of PIPKIγ isoforms and PIPKIγi2 mutants, cloning of exocyst complex components, cell adhesion assay, isolation of crude plasma membrane and live cell imaging.

Supplemental References

References cited exclusively in “Supplemental Information”

Figure S1

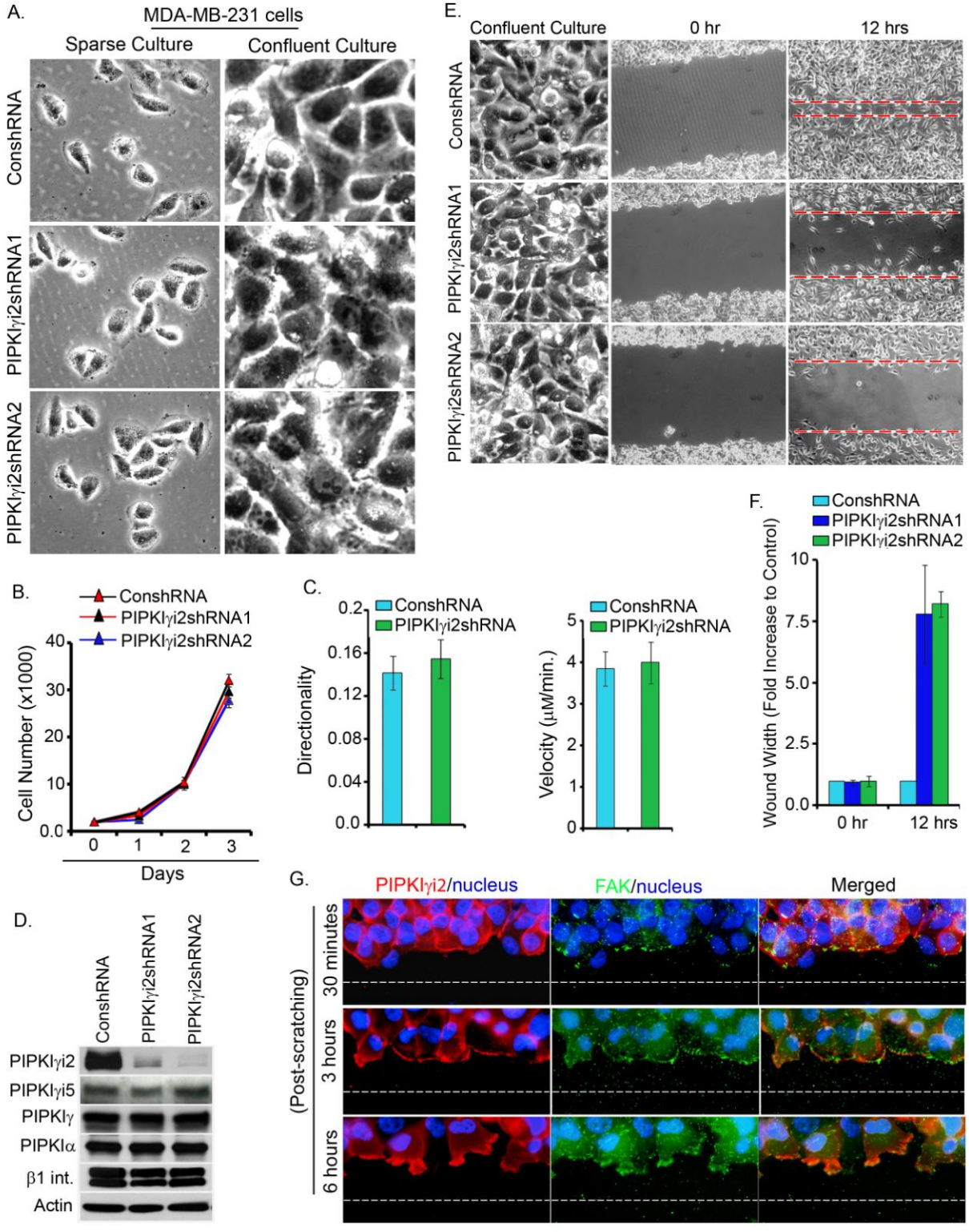
A.
MDA-MB-231 cells
Sparse Culture
Confluent Culture
ConshRNA
PIPKIγi2shRNA1
PIPKIγi2shRNA2
E.
Confluent Culture
0 hr
12 hrs
ConshRNA
PIPKIγi2shRNA1
PIPKIγi2shRNA2
B.
ConshRNA
PIPKIγi2shRNA1
PIPKIγi2shRNA2
Cell Number (x1000)
Days
C.
ConshRNA
PIPKIγi2shRNA
Directionality
Velocity (μM/min.)
F.
ConshRNA
PIPKIγi2shRNA1
PIPKIγi2shRNA2
Wound Width (Fold Increase to Control)
0 hr
12 hrs
D.
ConshRNA
PIPKIγi2shRNA1
PIPKIγi2shRNA2
PIPKIγi2
PIPKIγi5
PIPKIγ
PIPKIα
β1 int.
Actin
G.
PIPKIγi2/nucleus
FAK/nucleus
Merged
(Post-scratching)
30 minutes
3 hours
6 hours

Figure S1 (related to Figure 1). PIPKIγi2 Knockdown Impairs Directional Cell Migration.

(A) Left, PIPKIγi2 knockdown cells are morphologically indistinguishable from control cells. Control or PIPKIγi2 knockdown cells were seeded into the culture plates and incubated overnight before taking the picture at light microscopy (x200). Right, confluent culture of control or PIPKIγi2 knockdown cells before the start of scratch-wounding.

(B) PIPKIγi2 knockdown does not affect cell proliferation. Control or PIPKIγi2 knockdown cells were seeded into 12-well culture plate in DMEM containing 10%FBS (2000 cells/well). Total number of cells were counted manually at 24, 48 and 72 hours post-seeding.

(C) Directional persistence and velocity were not affected in PIPKIγi2 knockdown cells in non-directional migration assay. Control or PIPKIγi2 knockdown cells were sparsely seeded into Col.I-coated culture plates and their motility monitored by live microscopy for 2 hours. At least 30 cells were included for each cell type to obtain the directional persistence and velocity of the cells.

(D) shRNA/lentiviral knockdown of endogenous PIPKIγi2 in HeLa cells. Isolated cells (two different cells designated as PIPKIγi2shRNA1 and PIPKIγi2shRNA2) were examined by immunoblotting for knockdown of PIPKIγi2.

(E) PIPKIγi2 knockdown in HeLa cells impairs cell migration. Control or PIPKIγi2 knockdown cells grown to confluency were wounded and wound width measured at zero and 12 hours post-wounding (representative images at zero and 12 hours post-scratching).

(F) Average fold increase in wound width compared with control cells at zero and 12 hours post-scratching (mean±SD from three independent experiments). Error bars represent the SD.

(G) PIPKIγi2 is recruited to migrating cell fronts. Confluent monolayer of MDA-MB-231 cells stably expressing moderate levels of HA-tagged PIPKIγi2 were wounded and processed at different time points. PIPKIγi2 (red) recruitment at migrating cell front and co-localization with FAK (green) was examined.

Figure S2

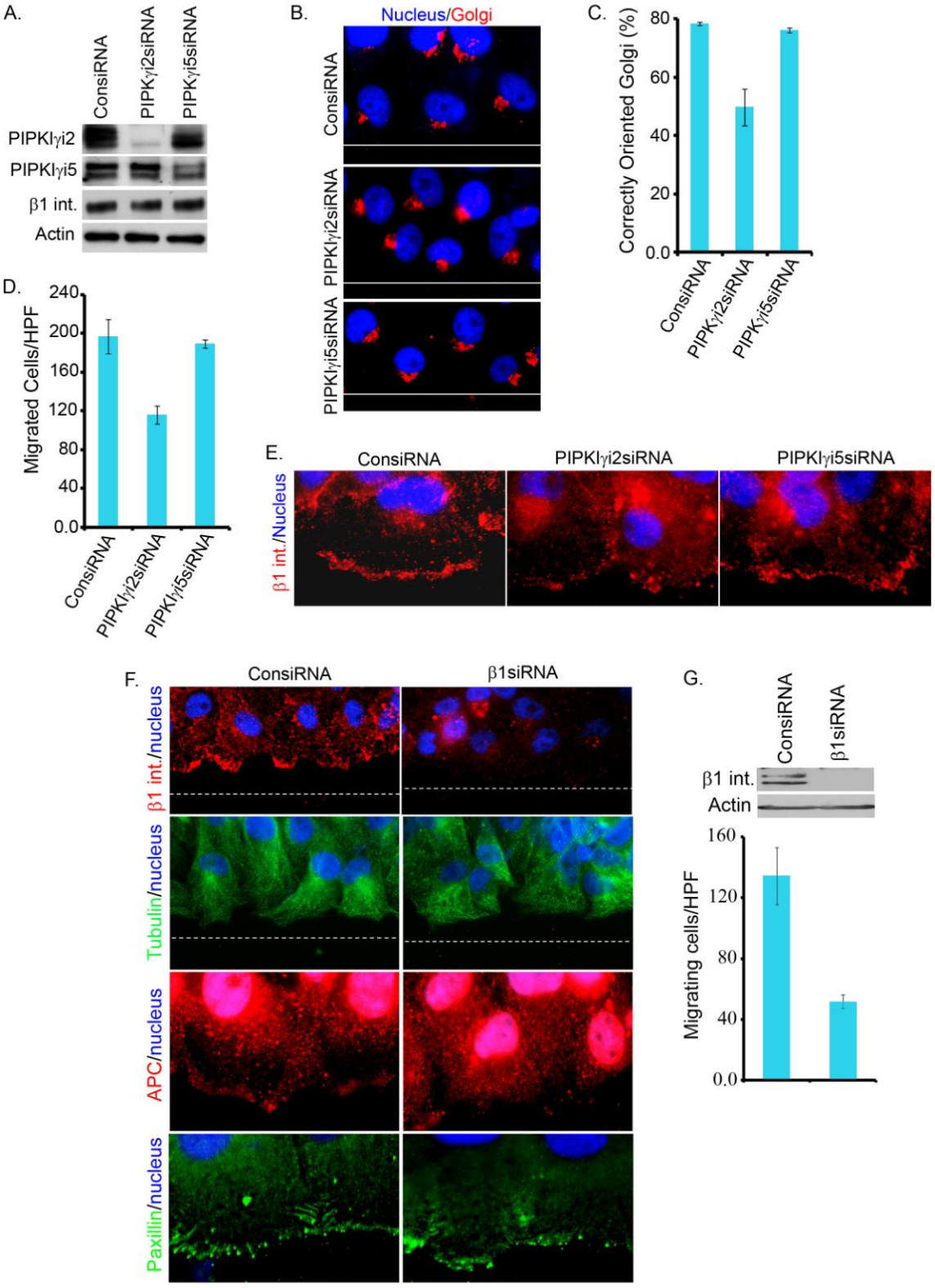
A.
ConsiRNA
PIPKγi2siRNA
PIPKγi5siRNA
PIPKIγi2
PIPKIγi5
β1 int.
Actin
B.
Nucleus/Golgi
ConsiRNA
PIPKIγi2siRNA
PIPKIγi5siRNA
C.
Correctly Oriented Golgi (%)
80
60
40
20
0.0
ConsiRNA
PIPKγi2siRNA
PIPKγi5siRNA
D.
Migrated Cells/HPF
240
200
160
120
80
40
0.0
ConsiRNA
PIPKIγi2siRNA
PIPKIγi5siRNA
E.
ConsiRNA
PIPKIγi2siRNA
PIPKIγi5siRNA
β1 int./Nucleus
F.
ConsiRNA
β1siRNA
β1 int./nucleus
Tubulin/nucleus
APC/nucleus
Paxillin/nucleus
G.
ConsiRNA
β1siRNA
β1 int.
Actin
Migrating cells/HPF
160
120
80
40
0.0

Figure S2 (related to Figure 2). PIPKIγi2 Knockdown Specifically Impairs Haptotactic Cell Migration and β1-integrin Recruitment to Migrating Cell Fronts.

(A) MDA-MB-231 cells were transfected with siRNA for PIPKIγi2 (second set of siRNA) or PIPKIγi5. Knockdown of PIPKIγi2 and PIPKIγi5 was examined 48 hours post-transfection.

(B) PIPKIγi2 knockdown specifically affects the polarity of migrating cells. Confluent cell cultures of MDA-MB-231 cells were wounded and processed 4-6-hours post-wounding to examine the orientation of Golgi (red) towards the direction of migration.

(C) Quantitative data of Golgi orientation in migrating cells after PIPKIγi2 or PIPKIγi5 knockdown. Values are expressed as mean±SD from three independent experiments. Error bars represent the SD.

(D) PIPKIγi2 knockdown specifically impairs haptotactic cell migration. MDA-MB-231 cells were transfected with siRNA for PIPKIγi2 or PIPKIγi5. Haptotactic cell migration was examined 48-72 hours post-transfection with siRNA using modified Boyden chamber assay. The results expressed as migrated cells/HPF (mean±SD from three independent experiments). Error bars represent the SD.

(E) β1-integrin recruitment to migrating cell fronts. 48-72 hours post-transfection with siRNA, cells were scratch-wounded and β1-integrin (red) recruitment to the migrating cell fronts examined.

(F) β1-integrin knockdown affects microtubule orientation and nascent focal adhesion formation of migrating cells. 48-72 hours post-transfection with siRNA, confluent cultures of MDA-MB-231 cells were wounded and processed 4-6-hours post-scratching to examine the recruitment of β1-integrin (red), microtubule (green), APC (red) and paxillin (green) at migrating cell fronts.

(G) β1-integrin knockdown impairs cell migration in MDA-MB-231 cells. 48-72 hours post-transfection with siRNA, haptotactic cell migration examined as described above. Error bars represent the SD.

Figure S3

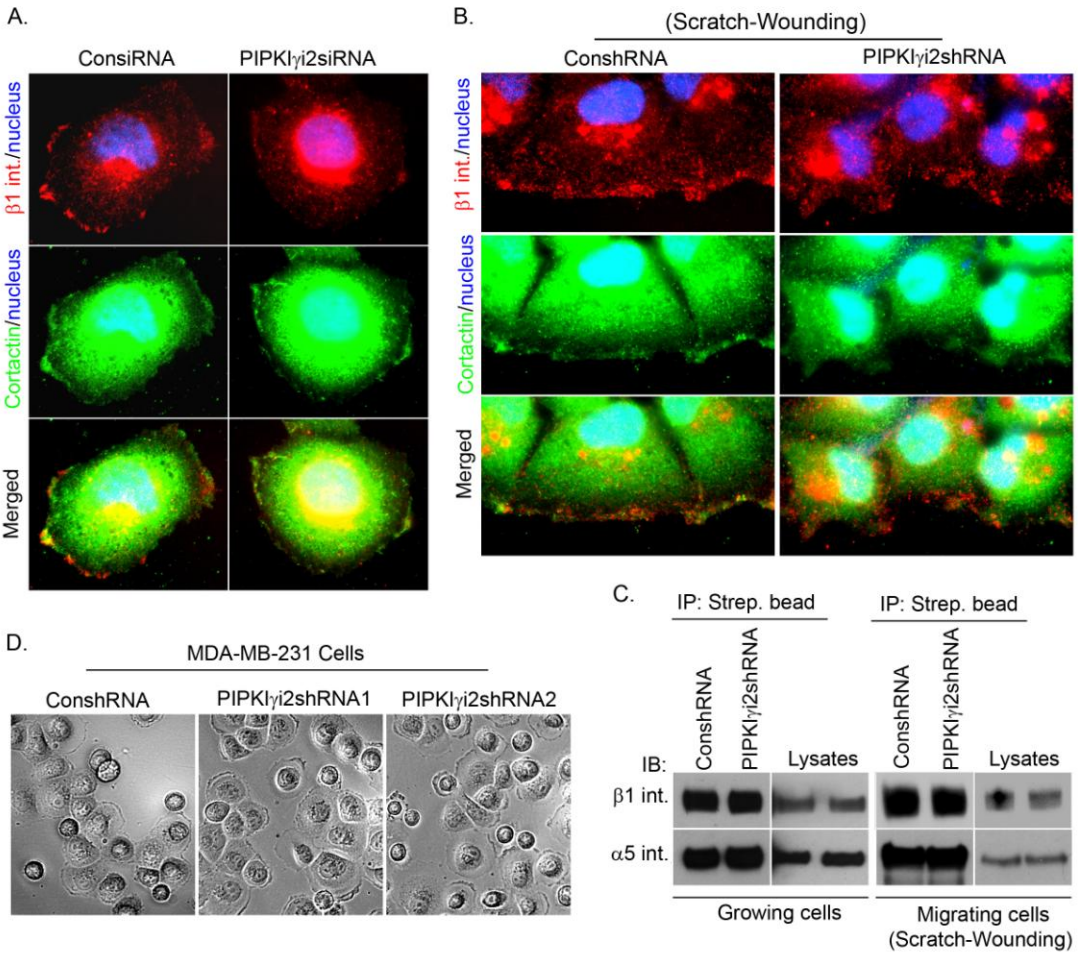
A.
ConsiRNA
PIPKIγi2siRNA
β1 int./nucleus
Cortactin/nucleus
Merged
B.
(Scratch-Wounding)
ConshRNA
PIPKIγi2shRNA
β1 int./nucleus
Cortactin/nucleus
Merged
C.
IP: Strep. bead
IP: Strep. bead
ConshRNA
PIPKIγi2shRNA
Lysates
ConshRNA
PIPKIγi2shRNA
Lysates
IB:
β1 int.
α5 int.
Growing cells
Migrating cells
(Scratch-Wounding)
D.
MDA-MB-231 Cells
ConshRNA
PIPKIγi2shRNA1
PIPKIγi2shRNA2
E.
BSA
FN (10 μg/ml)
Absorbance (595 nm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
ConshRNA
PIPKIγi2shRNA1
PIPKIγi2shRNA2
F.
ConshRNA
PIPKIγi2shRNA1
PIPKIγi2shRNA2
Number of Adherent Cells/Field (20X)
120
100
80
60
40
20
0.0
BSA
1
2.5
5
10
20
FN (μg/ml)
G.
ConshRNA
PIPKIγi2shRNA1
PIPKIγi2shRNA2
Number of Adherent Cells/Field (20X)
100
80
60
40
20
0.0
10
15
20
40
(Min.)
FN (2 μg/ml)

Figure S3 (related to Figure 3). PIPKIγi2 Knockdown Affects β1-integrin Recruitment to the Cell Protrusion/ruffles and Result in Subtle Defect on Cell Adhesion at Lower Concentration of FN and Shorter Incubation Time.

(A) Impaired β1-integrin recruitment at cell protrusions/ruffles in PIPKIγi2 knockdown cells. PIPKIγi2-specific siRNA was used to knock down PIPKIγi2 expression in MDA-MB-231 cells grown into coverslips. 48 hours-post transfection, cells were fixed with 3.7% PFA and immunostained with β1-integrin (red) and cortactin (green).

(B) Impaired recruitment of β1-integrin (red) and cortactin (green) into the migrating cell front of PIPKIγi2 knockdown cells.

(C) Total surface integrin levels remain unaffected in PIPKIγi2 knockdown cells. Confluent culture of MDA-MB-231 cells or migrating (4-5 hours post-scratching) cells were surface-labeled by incubating the cells with biotin (0.5 mg/ml) (Sulf-NHS-LC-Biotin, Thermo Scientific) at 4ºC for 1 hour. Cells were washed 2-3 times with cold PBS before lysis. Biotinylated cell surface proteins were captured by incubating cell lysates with streptavidin affinity gel (Sigma) followed by immunoblotting for integrins.

(D) Adhesion/spreading of PIPKIγi2 knockdown cells at 10 μg/ml FN is indistinguishable from control cells. Cells were seeded into FN-coated culture plates and incubated at 37°C for 30 minutes.

(E) Cell adhesion of PIPKIγi2 knockdown cells is not affected at higher concentration of FN. Control or PIPKIγi2 knockdown cells were seeded into 12-well culture plates at concentration of 1×10^5 cells per well. Cells were incubated at 37°C for 30 minutes before removing the unattached cells. The attached cells to the culture plates were stained with 0.2% crystal violet. The dye retained by cells was measured in spectrophotometer. The results expressed as OD at 595 nm (mean±SD from three independent experiments). Error bars represent the SD.

(F) Subtle defect of PIPKIγi2 knockdown on cell adhesion at lower concentration of FN. Control or PIPKIγi2 knockdown cells were seeded into 12-well culture plates coated with different concentration of

FN and incubated at 37°C for 15 minutes before removing the unattached cells. Attached cells were fixed before counting. At least 10 randomly selected fields for each cell were counted to obtain average cell number per field. Error bars represent the SD.

(G) Subtle defect of PIPKIγi2 knockdown on cell adhesion at shorter incubation time. Control or PIPKIγi2 knockdown cells were seeded into 12-well culture plates coated with 2 μg/ml FN and incubated at 37°C for different time periods before removing the unattached cells. Average cell number attached per field obtained as described above. Error bars represent the SD.

Figure S4

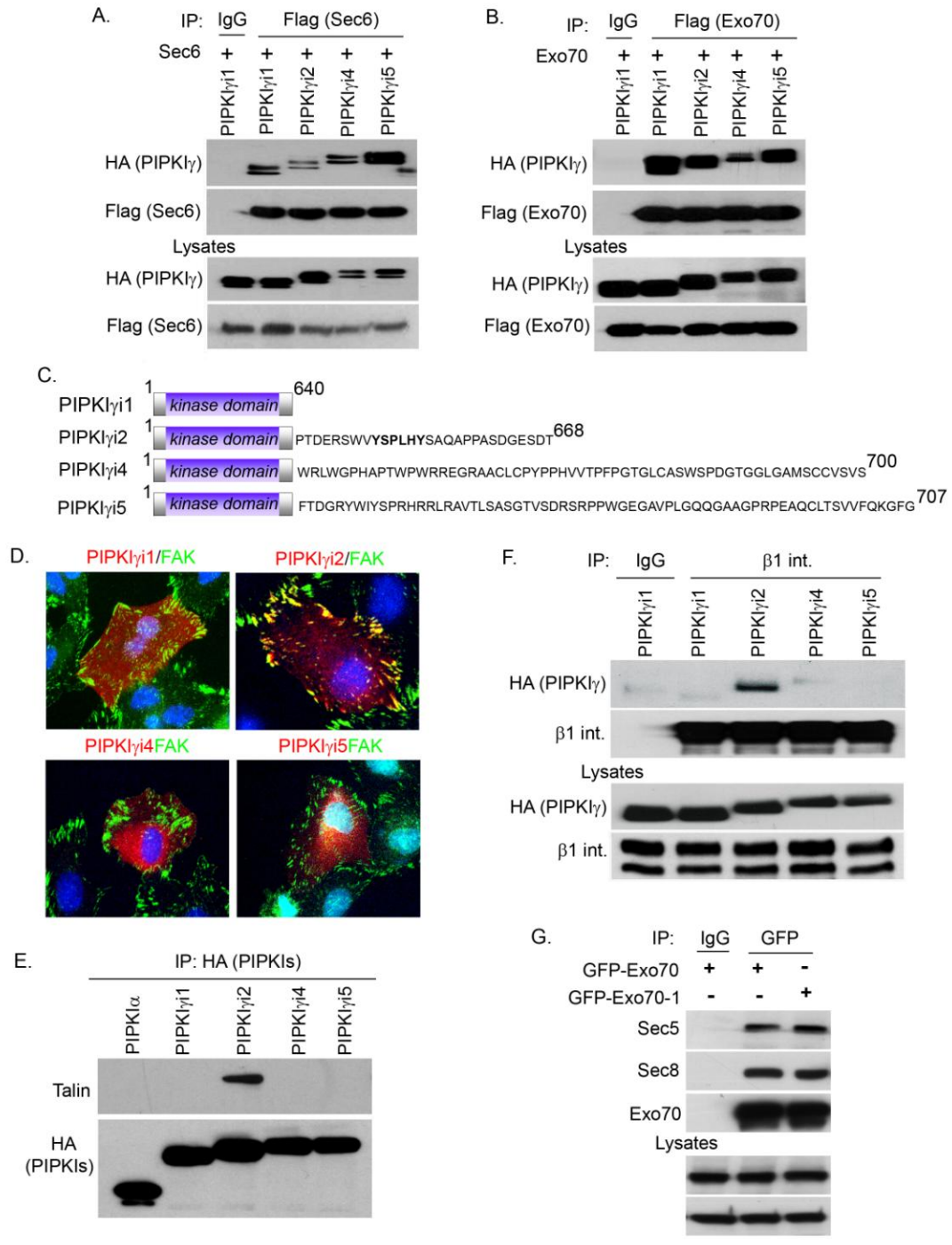
A.
IP: IgG Flag (Sec6)
Sec6 + + + + +
PIPKIγi1
PIPKIγi1
PIPKIγi2
PIPKIγi4
PIPKIγi5
HA (PIPKIγ)
Flag (Sec6)
Lysates
HA (PIPKIγ)
Flag (Sec6)
B.
IP: IgG Flag (Exo70)
Exo70 + + + + +
PIPKIγi1
PIPKIγi1
PIPKIγi2
PIPKIγi4
PIPKIγi5
HA (PIPKIγ)
Flag (Exo70)
Lysates
HA (PIPKIγ)
Flag (Exo70)
C.
PIPKIγi1 1 kinase domain 640
PIPKIγi2 1 kinase domain PTDERSWVYSPLHYSAQAPPASDGESDT 668
PIPKIγi4 1 kinase domain WRLWGPHAPTWPWRREGRAACLCPYPPHVVTPFPGTGLCASWSPDGTGGLGAMSCCVSVS 700
PIPKIγi5 1 kinase domain FTDGRYWIYSPRHRRLRAVTLSASGTVSDRSRPPWGEGAVPLGQQGAAGPRPEAQCLTSVVFQKGFG 707
D.
PIPKIγi1/FAK
PIPKIγi2/FAK
PIPKIγi4FAK
PIPKIγi5FAK
E.
IP: HA (PIPKIs)
PIPKIα
PIPKIγi1
PIPKIγi2
PIPKIγi4
PIPKIγi5
Talin
HA (PIPKIs)
F.
IP: IgG β1 int.
PIPKIγi1
PIPKIγi1
PIPKIγi2
PIPKIγi4
PIPKIγi5
HA (PIPKIγ)
β1 int.
Lysates
HA (PIPKIγ)
β1 int.
G.
IP: IgG GFP
GFP-Exo70 + + -
GFP-Exo70-1 - - +
Sec5
Sec8
Exo70
Lysates

Figure S4 (related to Figure 5). Different Isoforms of PIPKIγ Associate with Sec6 and Exo70; PIPKIγi2 Specifically Interacts with Talin and β1-integrin.

(A) Flag-tagged Sec6 was cotransfected with different isoforms of PIPKIγ into HEK293 cells. Cells were harvested 24 hours post-transfection and Sec6 immunoprecipitated to examine coimmunoprecipitation of PIPKIγ.

(B) Flag-tagged Exo70 was cotransfected with different isoforms of PIPKIγ into HEK293 cells. Cells were harvested 24 hours post-transfection and Exo70 immunoprecipitated to examine coimmunoprecipitation of PIPKIγ.

(C) C-terminal extensions of different PIPKIγ isoforms. The talin-binding motif in PIPKIγi2 is shown in bold letter.

(D) PIPKIγi2 is a focal adhesion-targeting isoform of PIPKIγ. HeLa or NRK cells transfected with HA-PIPKIγ isoforms including PIPKIγi2. Cells were immunostained 8-12 hours post-transfection with anti-HA (red) and FAK (green).

(E) PIPKIγi2 specifically interacts with talin. HEK293 cells were transfected with HA-tagged PIPKIs. Cells were harvested 24 hours post-transfection. PIPKIs were immunoprecipitated using anti-HA antibody and coimmunoprecipitation of talin examined.

(F) PIPKIγi2 specifically associates with β1-integrin. HeLa cells were transiently transfected with HA-PIPKIγ isoforms including PIPKIγi2. The β1-integrin was immunoprecipitated from the transfected cells and coimmunoprecipitation of PIPKIγ examined by immunoblotting using anti-HA antibody.

(G) Deficiency in PIP_2 binding does not affect the association of Exo70-1 with other components of the exocyst complex. HeLa cells were transfected with Exo70 or Exo70-1. Cells were harvested 24 hours post-transfection. Exo70 and Exo70-1 were immunoprecipiated to examine the coimmunoprecipitation of other components of the exocyst complex.

Figure S5

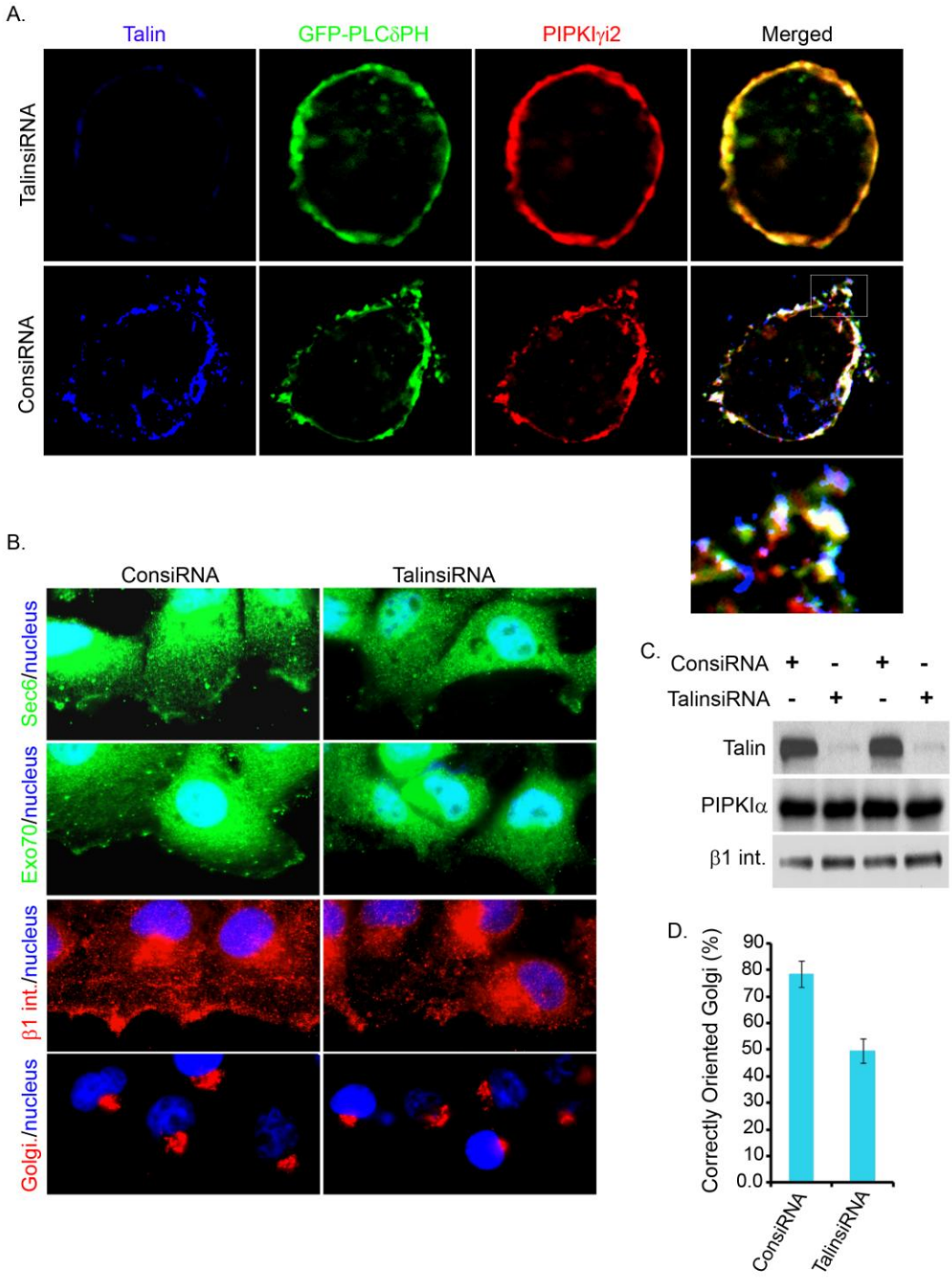
A.
Talin
GFP-PLCδPH
PIPKIγi2
Merged
TalinsiRNA
ConsiRNA
B.
ConsiRNA
TalinsiRNA
Sec6/nucleus
Exo70/nucleus
β1 int./nucleus
Golgi./nucleus
C.
ConsiRNA + - + -
TalinsiRNA - + - +
Talin
PIPKIα
β1 int.
D.
Correctly Oriented Golgi (%)
90
80
70
60
50
40
30
20
10
0.0
ConsiRNA
TalinsiRNA

Figure S5 (related to Figure 6). Talin is Required for PIPKIγi2 Recruitment and PIP_2 Generation at Focal Adhesion Complexes; Talin Knockdown Impairs Polarized Recruitment of Exocyst Complex and β1-integrin.

(A) PIPKIγi2 recruitment and PIP_2 generation at newly formed focal adhesion complexes are impaired in talin knockdown cells. MDA-MB-231 cells were transfected with control siRNA (lower panel) or siRNA for talin (upper panel). 24-hours post-transfection, cells were cotransfected with HA-tagged PIPKIγi2 and GFP-PLC-δ-PH. 24 hours later, cells were trypsinized and seeded onto the coverslips-coated with FN. After incubating the cells at 37°C for 30-40 minutes, cells were fixed and processed for immunostaining using anti-talin (blue) and anti-HA (red) antibodies.

(B) Talin knockdown impairs polarized recruitment of exocyst complex and β1-integrin in MDA-MB-231 cells. 48-72 hours post-transfection, confluent cells were scratched and polarized recruitment of Sec6 (green), Exo70 (green) and β1-integrin (red) to migrating cell fronts were examined using specific antibodies. Orientation of Golgi towards the direction of migration was examined after staining the cells with anti-GM130 (red) antibody.

(C) Cells transfected with siRNA examined for talin knockdown by immunoblotting.

(D) Quantitative data of Golgi (red) orientation; value expressed as mean±SD from three independent experiments. Error bars represent the SD.

Figure S6

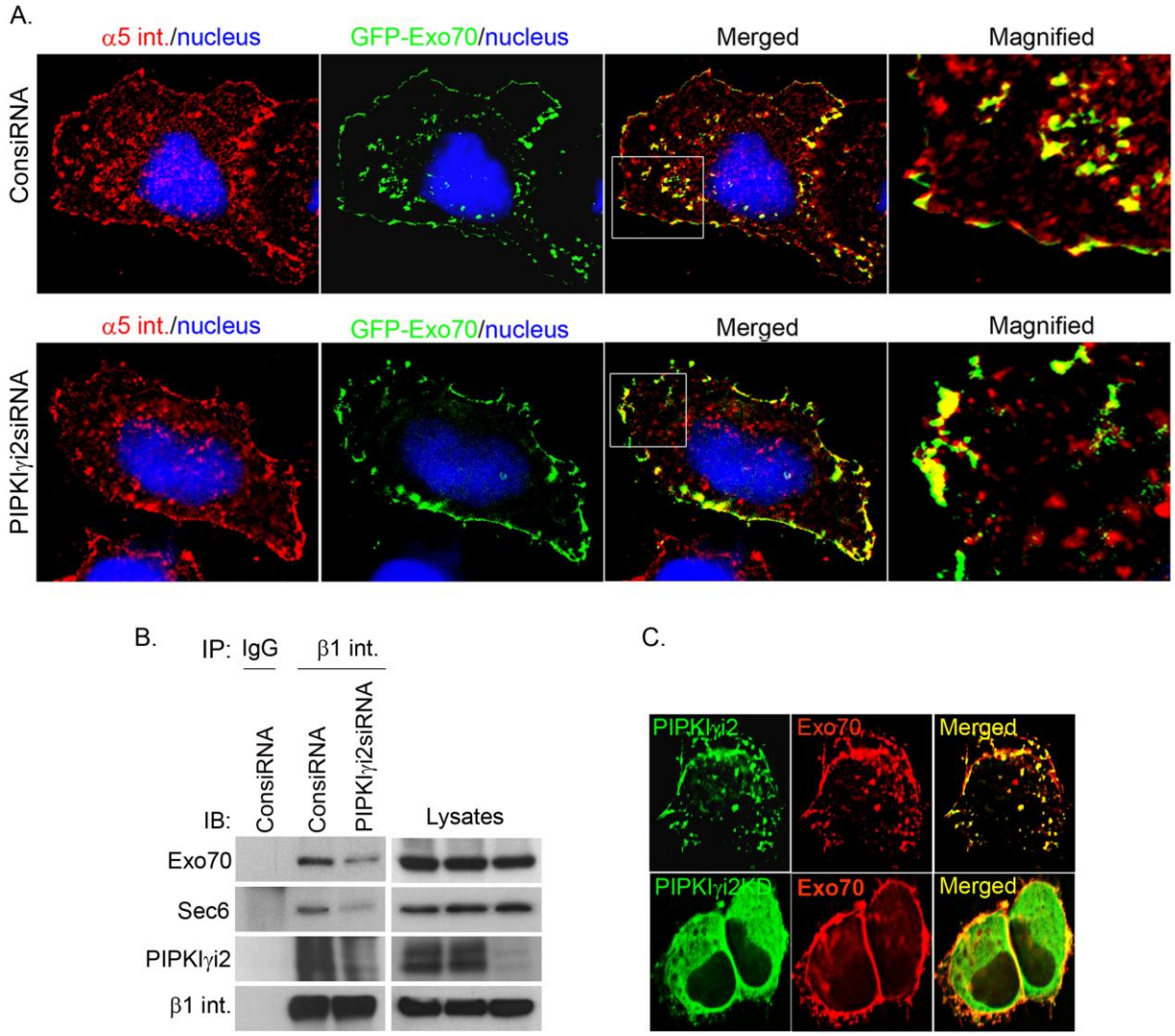

Figure S6 (related to Figure 7). PIPKIγi2 Knockdown Impairs Exo70 Colocalization with α5 Integrin; Kinase Activity of PIPKIγi2 is Required for Recruiting Exo70 into the Vesicle-like Intracellular Compartments.

(A) Exo70 colocalization with endogenous α5 integrin. HeLa cells were transfected with control or PIPKIγi2 specific siRNA. 24-48 hours post-transfection, cells were transfected with GFP-Exo70 followed by immunostaining with anti-α5 integrin antibody (red). Exo70 extensively colocalized with

endogenous α5 integrin both in focal adhesion and vesicle-like intracellular compartments. GFP-Exo70 poorly colocalized with α5 integrin in the intracellular compartments in PIPKIγi2 knockdown cells.

(B) PIPKIγi2 knockdown decreases β1-integrin association with exocyst complex. MDA-MB-231 cells were transfected with specific siRNA for PIPKIγi2 knockdown. Cells were harvested 48-72 hours post-transfection and β1-integrin immunoprecipitated to examine the coimmunoprecipitation of exocyst complex.

(C) Kinase activity of PIPKIγi2 modulates its association with Exo70. HeLa cells were co-transfected with Flag-Exo70 and HA-tagged PIPKIγi2 or its kinase dead mutant; 12 hours post-transfection, cells were fixed and immunostained with anti-HA (green) and anti-Flag (red) antibodies.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cell Culture and Transfection

MDA-MB-231, HeLa and HEK293T cells were cultured in Dubelbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml pencillin G and 100 µg/ml streptomycin at 37ºC with 5% CO_2. Transient transfection of HeLa cells was performed using FuGENE6 or Lipofectamine following manufacturer's instructions.

Antibodies

The antibodies used were: anti-β1 (MAB2000, Millipore), anti-β1 (610467, BD Bioscience), anti-β1 (MAB2247, Millipore), anti-α5 (610633, BD Bioscience), anti-α5 (sc-59761, Santa Cruz Biotech.), anti-HA (MMS-101P, Covance), anti-tubulin (#2148 Cell Signaling), anti-Rab4 (610889, BD Bioscience), anti-Rab11 (610656, BD Bioscience), anti-Sec8 (610658, BD Bioscience), anti-Sec6 (55924, Calbiochem.), antibodies to Exo70, Sec5, Sec10 and Sec15 (kind gift of Dr. Shu-Chan Hsu at Rutgers University), anti-Flag (#2368, Cell Signaling), anti-GM130 (610822, BD Biosciences), anti-TRFR (61214, BD Bioscience), anti-EEA1 (610456, BD Bioscience), anti-LAMP1 (ab25630, AbCAM), anti-talin (05-385, Millipore), anti-FAK (Invitrogen), anti-APC (sc-896, Santa Cruz Biotech.) anti-paxillin (610051, BD Bioscience), anti-vinculin (sc-55465, Santa Cruz Biotech.) and anti-cortactin (C7112, Sigma-Aldrich). The anti-pan PIPKIγ, anti-PIPKIγi2, anti-PIPKIγi5 and anti-PIPKIα were developed in the lab.

siRNA

For siRNA-mediated knockdown of genes, LipofectamineRNAiMAX (Invitrogen) was used following the protocol from manufacturer and cells were used 48-72 hours post-transfection. The siRNA sequences used were:

siPIPKIγi2: GAGCGACACAUAAUUUCUAUU

siSec6: CCUGAUGGUUCAGUGCUUUTT

siSec8: AAUUUGCUUCAACUCCUGCTT

siSec5: AAUGGUCAAGCCUAUGAGGTT

siExo70: CCAUUGUGCGACACGACUUTT

Control siRNA: CCUUGGUGACUCGUAGUUUTT

The siRNA for Rab11, talin, β1-integrin and also that of exocyst complex components were purchased from Santa Cruz Biotechnology.

The Short Hairpin RNA (shRNA) for Knockdown of PIPKIγi2

Oligonucleotide sequences used for generation of short hairpin RNA specific for PIPKIγi2 were: GAGCGACACATAATTTCTA (PIPKIγi2shRNA) and GCCTTCTTCGCTAAACGAA (ConshRNA). Generation of replication-defective infectious viral particles and the transduction of the cells were carried out following the protocol provided by Addgene and as described previously (Lee et al., 2004; Rubinson et al., 2003). In brief, synthesized oligonucleotides were annealed and cloned into HpaI and XhoI sites of pLL3.7 vector (Addgene). Stabl3 competent cells (Invitrogen) were used for transformation and DNA purification to minimize the mutagenesis. The integrity of lentiviral vector-containing cloned shRNA sequences were validated by DNA sequencing. For generation of viral particles, lentiviral vector along with other accessory plasmids (pCMV-VSVG, pRSV-Rev and pMD2.G) required for the production of infectious viral particles were cotransfected into HEK 293T cells using calcium phosphate. Conditioned medium was collected 48 hours post-transfection, cleared of debris by centrifugation at low speed, filtered through 0.45 μM filter and viral particles were concentrated by centrifugation at 24,000 rpm in Beckman SW28 centrifuge for 2 hours at 4℃. For the transduction of the target cells (MDA-MB-231), sub-confluent cells cultured into 6-well culture plates

were infected with viral supernatant in the presence of 0.5 µg/ml polybrene (Sigma). The infected cells were either sorted using cell sorter (expression of GFP is driven by CMV promoter in the vector) or individual clone isolated. The knockdown of PIPKIγi2 expression was examined by immunoblotting using PIPKIγi2 specific antibody.

Lentiviral Vector System for Expression of PIPKIγ Isoforms and PIPKIγi2 Mutants

cDNAs for PIPKIγi1, PIPKIγi2, kinase dead PIPKIγi2 or PIPKIγi2Y649F were subcloned into MluI and Sal I sites in frame with HA-tag in N-terminus of PWPT vector (Addgene). The kinase dead PIPKIγi2 mutant and PIPKIγi2Y649F (deficient in talin binding) were created by substituting aspartic acids at 253/316 with alanine and tyrosin with phenylalanine at 649, respectively using Quickchange Site-Directed Mutagenesis kits (Stratagene). For generation of infectious viral particles, lentiviral vector along with accessory plasmids, psPAX2 and pMD2.G (Addgene) were cotransfected into HEK293T cells using calcium phosphate. The harvesting of viral particles and infection of target cells (MDA-MB-231 or HeLa cells) were performed as described above. The same procedures were followed for re-expression of PIPKIγi2 or kinase dead PIPKIγi2 having silent mutations into the PIPKIγi2shRNA1 or PIPKIγi2shRNA2 cells.

Cloning of Exocyst Complex Components

The open reading frame for human exocyst complex components [GenBank accession numbers: NM_018261 (*Sec3*), NM_018303 (*Sec5*), NM_007277 (*Sec6*), BC067263 (*Sec8*), NM_006544 (*Sec10*), BC028395 (*Sec15*) and NM_015219 (*Exo70*)] were amplified by polymerase chain reaction (PCR) from cDNA prepared from MDA-MB-231 cells and cloned into pCMV-Tag2b vector (Stratagene) in frame with Flag-tag in the N-terminus. The integrity of the DNA sequences was verified by DNA sequencing. For purification of GST-fusion proteins, exocyst complex components were subcloned into pGEX-6P-1 vector (Amersham Bioscience).

Cell Adhesion Assay

For cell adhesion assay, 12-well cell culture plates were coated by incubating with either FN or Col. I (10 µg/ml in PBS) at 4℃ overnight. Then, plates were blocked with 2% BSA in PBS for 1 hour at room temperature before seeding the cells. Cells were lifted from culture plates using 4 mM EDTA and suspended into serum-free DMEM containing 0.1% BSA, and seeded into wells at the density of $1x10^5$ cells per well. After incubating at 37℃ for 25-30 minutes, unattached and feebly attached cells were removed by washing the wells with DMEM. Then, attached cells were fixed with methanol before staining with 0.1% crystal violet for 5 minutes. After washing the well several times with the PBS, crystal violet dye retained by attached cells was extracted using 0.1% Triton-X in PBS and absorbance measured at 595 nm using spectrophotometer. For examining cell adhesion in ligand- and -time dependent manner, at least 10 randomly selected fields were selected for counting the cells using x40 objective lens and average cell number adhered per field obtained. Since less cells were adherent in these assays, crystal violet dye retained non-specifically by side walls/empty spaces of the culture plates interfered the precise measurement of adherent cells as described above.

Live Cell Imaging

For random migration analysis, cells were seeded on Col.I (10 µg/ml)-coated Delta TPG dish (Fisher Scientific) at a density of 10^4 cells/disk in L15 culture medium and placed in a temperature-controlled chamber of a microscope (TE2000-U; Nikon). Time-lapse recording started 4 h after cell plating. Images were collected at 1-min intervals over 120 minutes with a cooled charge-coupled device (CCD) video camera (CollSNAP fx; Roper Scientific) operated by Metamorph image analysis software (Molecular Devices). Motility parameters including migration path, distance and directional persistence were obtained from time-lapse movies. To track the migration path of individual cells, cells were manually traced for each frame and the geographical centers were recorded using ImageJ. The rates of cell

migration (velocity) were calculated as a ratio of the total migration distance and the duration of migration. Migration distances were determined as the net translocation during a 120-min. period. Directional persistence was calculated as a ratio of the direct distance during a 120-min period and the total length of the migration path.

Isolation of Crude Plasma Membrane

Subcellular fractionation protocol published by abcam was used: Cells growing into 100 mm culture dishes were washed with cold PBS and put onto the ice. Cells were collected from culture plates by adding 1 ml of subcellular fractionation buffer (250 mM sucrose, 20 mM Hepes, pH7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and protease/phosphatase inhibitors). Cells were homogenized by using Dounce homogenizer (50 strokes using tight-fitting glass pestle) and put on ice for 20 minutes before centrifuging at 3000 rpm for 5 minutes to remove nuclear fraction. The nuclear-free supernatants were further centrifuged at 8000 rpm for 5 minutes at 4ºC to remove the mitochondrial fraction. The clear supernatant containing cytosolic and membrane fractions were further centrifuged at 100,000G for 1 hour using ultracentrifuge (Beckman Le-80 ultracentrifuge). The membrane fraction obtained was washed with fractionation buffer before solubilizing with cell lysis buffer-containing 0.5% SDS, then, followed by SDS-PAGE and immunoblotting.

Supplemental References

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