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**PIPKIγ Regulates Focal Adhesion Dynamics and Colon Cancer Cell Invasion**

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**Abstract**

Focal adhesion assembly and disassembly are essential for cell migration and cancer invasion, but the detailed molecular mechanisms regulating these processes remain to be elucidated. Phosphatidylinositol phosphate kinase type Iγ (PIPKIγ) binds talin and is required for focal adhesion formation in EGF-stimulated cells, but its role in regulating focal adhesion dynamics and cancer invasion is poorly understood. We show here that overexpression of PIPKIγ promoted focal adhesion formation, whereas cells expressing either PIPKIγ¹K188,200R or PIPKIγ¹D316K, two kinase-dead mutants, had much fewer focal adhesions than those expressing WT PIPKIγ in CHO-K1 cells and HCT116 colon cancer cells. Furthermore, overexpression of PIPKIγ, but not PIPKIγ¹K188,200R, resulted in an increase in both focal adhesion assembly and disassembly rates. Depletion of PIPKIγ by using shRNA strongly inhibited formation of focal adhesions in HCT116 cells. Overexpression of PIPKIγ¹K188,200R or depletion of PIPKIγ reduced the strength of HCT116 cell adhesion to fibronectin and inhibited the invasive capacities of HCT116 cells. PIPKIγ depletion reduced PIP2 levels to ~40% of control and PIP3 to undetectable levels, and inhibited vinculin localizing to focal adhesions. Taken together, PIPKIγ positively regulates focal adhesion dynamics and cancer invasion, most probably through PIP2-mediated vinculin activation.

**Introduction**

Focal adhesions (FAs, also called cell-matrix adhesions) are specific types of large macromolecular assemblies at the ventral surface of cells, functioning as both mechanical machineries and regulatory signaling hubs [1,2]. Temporal and spatial regulation of focal adhesion assembly/disassembly is required for cell migration [3]. During cell migration, nascent focal adhesions (also called focal complexes) are formed to stabilize lamellipodia at the front of cells while focal adhesions are dissolved at the trailing edges of cells [4,5].

Focal adhesion assembly and disassembly are also implicated in cancer invasion, a prerequisite for metastasis. DRR (Down-Regulated in Renal cell carcinoma) associates with actin and microtubules and stimulates glioma invasion by promoting focal adhesion disassembly [6]. The actin cross-linking protein filamin A suppresses focal adhesion disassembly and breast cancer cell invasion [7]. Rho/Rock signaling promotes tumor cell migration and invasion by regulating focal adhesion dynamics through caveolin-1 phosphorylation [8]. FAK also promotes focal adhesion disassembly and cancer invasion [9,10,11]. Focal adhesion dynamics and signaling pathways that regulate this process could therefore be an attractive target for cancer therapy.

Many molecules have been shown to regulate focal adhesion dynamics. FAK regulates focal adhesion turnover [9,12], probably through a dynamin and microtubule-dependent pathway [13]. Paxillin, a focal adhesion adaptor, also modulates focal adhesion dynamics by JNK and PAK-mediated phosphorylation [14,15]. Talin activates integrins and initiates focal adhesion formation [16,17,18], whereas cleavage of talin by calpain mediates focal adhesion disassembly [19]. Calpain also cleaves FAK and paxillin to modulate focal adhesion dynamics [20,21]. We have shown that Smurf1-mediated ubiquitination of the talin head domain, one of the two cleavage products, plays an important role in focal adhesion disassembly and cell migration [22]. ACF7, a microtubule and filamentous actin binding protein, regulates focal adhesion assembly/disassembly through its ATPase activity [23]. However, the molecular mechanisms that control focal adhesion assembly/disassembly are not fully understood.

Phosphatidylinositol phosphate kinase type Iγ (PIPKIγ) is an enzyme that catalyzes ATP-dependent phosphorylation of phosphatidylinositol 4-phosphate (PI(4)P) to generate PI(4,5)P₂, which regulates a variety of biological processes, including focal adhesion formation [24]. PIPKIγ has a well-conserved kinase catalytic domain at the central region [25]. Within the catalytic domain, there is a subdomain called the activation loop, which determines site of phosphorylation on the inositol ring of the substrate. PIPKIγ strongly interacts with talin and competes for talin binding with the β integrin tail [26,27]. It is localized at adherens junctions in epithelial cells [28,29]. It has been reported that PIPKIγ is

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required for focal adhesion formation in migratory cells [30]. However, the precise roles of the lipid kinase activities of PIPKιγ in focal adhesion dynamics are not defined.

In the present study, we investigated the requirement for the lipid kinase activity of PIPKιγ in focal adhesion dynamics and colon cancer invasion. Our results identify an essential role of PIPKιγ in focal adhesion assembly/disassembly and cancer invasion.

**Results**

**PIPKιγ promotes focal adhesion formation**

To examine a possible role for PIPKιγ in focal adhesion formation, CHO-K1 cells were transfected with EGFP-PIPKιγ or EGFP vector, respectively. The cells were re-plated on fibronectin, fixed with paraformaldehyde, incubated with an anti-paxillin monoclonal antibody, and then stained with Dylight 549 conjugated goat anti-mouse IgG. Focal adhesions were lined up around the edges of the EGFP vector-transfected cells, with few focal adhesions in the centers of the cells, whereas PIPKιγ expression dramatically stimulated focal adhesion formation in the centers of the cells (Fig. 1A). Over-expression of PIPKιγ stimulates an increase in focal adhesion numbers (Fig. 1B) and the increase was mainly contributed by small focal adhesions (<3 μm²) (Fig. 1C). The stimulation of focal adhesion formation by PIPKιγ relies on its interaction with talin, because PIPKιγ<sup>316K</sup>, a mutant that is deficient in the interaction with talin, was not able to promote focal adhesion formation (Fig. 1A, B, & C).

**PIPKιγ kinase activity is required for stimulation of focal adhesion formation**

PI(4,5)P₂ binds talin and strengthens the interaction between talin and the β integrin tail, stimulating integrin clustering [31]. PI(4,5)P₂ also binds vinculin and unmasks the actin and talin binding sites on vinculin, promoting focal adhesion formation [32]. Therefore, PIPKιγ-stimulated PI(4,5)P₂ synthesis could be essential for promoting focal adhesion formation. To test this hypothesis, we mutated two lysine residues, K188 and K200, to arginine residues within the ATP-binding site of PIPKιγ. The mutant PIPKιγ<sup>K188,R,K200,R</sup> and the WT were purified from CHO-K1 cells and the kinase activities were examined in vitro using mass spectrometry to quantify production of PI(4,5)P₂ from PI(4)P. As shown in Fig. 2A, mutation at K188 and K200 reduced kinase activity by 95%. EGFP-tagged mutant PIPKιγ<sup>K188,R,K200,R</sup> and WT PIPKιγ were stably expressed in CHO-K1 cells, and their effects on focal adhesion formation were examined after paxillin staining. Cells that stably express WT PIPKιγ formed focal adhesions around the edges and in the centers of cells, whereas cells that express PIPKιγ<sup>K188,R,K200,R</sup>, similar to parental cells, possessed small focal adhesions, most of which were around the edges of the cells, and had a defect in spreading (Fig. 2B). Quantitative analysis indicated that PIPKιγ increased focal adhesions by more than 2 fold, whereas PIPKιγ<sup>K188,R,K200,R</sup> did not significantly promote focal adhesion formation (Fig. 2C&D). These results indicate that PIPKιγ activity is essential for focal adhesion formation in CHO-K1 cells.

To further verify the requirement of PIPKιγ activity for focal adhesion formation, we tested the capacity of another kinase dead mutant, PIPKιγ<sup>316K</sup> [33], to promote focal adhesion formation as described above. As shown in Fig. S1, the average focal adhesion number (per cell) in cells expressing PIPKιγ<sup>316K</sup> was approximately 45, which is approximately the same as that in cells expressing EGFP vector (Fig. 1). The focal adhesions in cells expressing PIPKιγ<sup>316K</sup> accumulated at the edges of the cells, with very few focal adhesions in the center of the cells. This result confirms the essential role of PIPKιγ activity in focal adhesion formation.

**The activity of PIPKιγ is essential for its promoting focal adhesion dynamics**

To examine whether PIPKιγ lipid kinase activity is required for focal adhesion dynamics, CHO-K1 cells that stably express EGFP-PIPKιγ or -PIPKιγ<sup>K188,R,K200,R</sup> were plated with mDsRed-paxillin and then plated on MatTek dishes (with a glass coverslip at the bottom) precoated with fibronectin (5 μg/ml) and grown for 3 hr. TIRF images of mDsRed-paxillin were taken using a Nikon TIRF microscope and the temperature was maintained at 37°C using an INU-TIZ-F1 microscope incubation system (Tokai Hit). Images were recorded at 1-min intervals for a 120 min period. Focal adhesion assembly and disassembly rate constants were calculated as described previously [22]. The FA assembly and disassembly rate constants in cells expressing PIPKιγ<sup>K188,R,K200,R</sup> were 0.083±0.014 and 0.072±0.010 min⁻¹, respectively, which are similar to those in normal CHO-K1 cells that we reported previously [22], whereas FA assembly and disassembly rate constants were 0.190±0.019 and 0.136±0.014 min⁻¹, respectively, in cells expressing WT PIPKιγ (Fig. 3). This result indicates that the inositol lipid kinase activity of PIPKιγ is required for its stimulation of focal adhesion assembly and disassembly.

**An essential role for PIPKιγ in focal adhesion formation in colon cancer cells**

Focal adhesions have been implicated in regulating cancer invasion, while the role of focal adhesions in colon cancer cells has not been well defined. To determine if the activity of PIPKιγ influences focal adhesion formation in colon cancer cells, HCT116 cells that stably express EGFP-PIPKιγ or -PIPKιγ<sup>K188,R,K200,R</sup> were plated on fibronectin and stained for paxillin. As shown in Fig. 4A, most of the focal adhesions were located to the peripheral region, and HCT116 cells expressing PIPKιγ<sup>K188,R,K200,R</sup> had much fewer focal adhesions than those expressing the WT enzyme. The average focal adhesion number in cells expressing WT PIPKιγ was 22.5/cell, whereas that in cells expressing PIPKιγ<sup>K188,R,K200,R</sup> was 11.5/cell, both of which were fewer than those observed in CHO-K1 cells (Fig. 4B). In addition, PIPKιγ<sup>K188,R,K200,R</sup> had more effect on the smaller focal adhesions than the larger ones (Fig. 4C).

To test whether PIPKιγ is essential for focal adhesion formation in HCT116 cells, HCT116 cells were infected with recombinant lentiviruses that express PIPKιγ shRNA or shRNA control and were selected with puromycin. As shown in Fig. 5A, expression of PIPKιγ shRNA resulted in dramatic reduction in the endogenous PIPKιγ level of HCT116 cells. The cells were then plated on fibronectin and stained for paxillin. Surprisingly, PIPKιγ knockdown almost abolished focal adhesion formation in HCT116 cells (Fig. 5B). PIPKιγ depletion reduced focal adhesion number by about 74% (Fig. 5C). Different from PIPKιγ<sup>K188,R,K200,R</sup>, PIPKιγ shRNA had more effect on the larger focal adhesions than the smaller ones (Fig. 5D). These results indicate an essential role of PIPKιγ in focal adhesion formation in HCT116 colon cancer cells. This dramatic effect of PIPKιγ knockdown did not occur in MDA-MB-231 human breast cancer cells, where PIPKιγ knockdown only partially inhibited focal adhesion formation (data not shown).

**PIPKιγ positively modulates adhesion strength of colon cancer cells to fibronectin**

To examine whether the activity of PIPKιγ regulates cell adhesion strength on fibronectin, HCT116 cells that stably express
EGFP-PIPKI<sub>c</sub> or -PIPKI<sub>c</sub><sub>K188,200R</sub> were stained with calcein-AM and seeded on a 96-well plate that was pre-coated with different concentrations of fibronectin. The calcein fluorescence was read, and then the plate was inverted and centrifuged at 1500 g for 5 min. The calcein fluorescence was read again. As shown in Fig. 6A, the cell adhesion strength difference between the cells expressing PIPKI<sub>c</sub><sub>K188,200R</sub> and the WT was not significant at high concentrations of fibronectin. However, at low concentrations of fibronectin, the cells expressing PIPKI<sub>c</sub><sub>K188,200R</sub> had significantly lower adhesion strength as compared to those expressing the WT. PIPKI<sub>c</sub> knockdown also caused a significant decrease in cell adhesion strength in HCT116 cells (Fig. 6B).

These results indicate that PIPKI<sub>c</sub> activity regulates cell adhesion strength in colon cancer cells.

PIPKI<sub>c</sub> is essential for invasion but not the migration of colon cancer cells

It has been reported that PIPKI<sub>c</sub> plays a role in cell migration. To test whether PIPKI<sub>c</sub> regulates the migration of HCT116 colon cancer cells, we employed time-lapsed wound-healing assays to examine the migration of HCT116 cells expressing EGFP-PIPKI<sub>c</sub> and -PIPKI<sub>c</sub><sub>W647F</sub>, respectively, in the presence of HGF. As shown in Fig. S2 A&B, cells expressing PIPKI<sub>c</sub><sub>K188,200R</sub> migrated slightly faster than those expressing the WT enzyme as measured
by time-lapse wound-healing assays. Also, depletion of PIPKIγ had no significant effect on the migration of HCT116 cells in transwell assays (Fig. S2 C&D). These results suggest that PIPKIγ activity is not essential for the migration of HCT116 colon cancer cells.

To test the role of PIPKIγ in cancer invasion, the invasion of HCT116 cells that stably express PIPKIγ shRNA or a shRNA control in the absence and presence of HGF was examined by using Matrigel invasion assays. As shown in Fig. 7 (A&B), PIPKIγ knockdown resulted in significant reduction in the invasion of HCT116 cells either in the absence or in the presence of HGF. The basal and HGF-stimulated invasive capacities of HCT116 cells stably expressing PIPKIγK188,200R are also significantly lower than those of the cells expressing the WT enzyme (Fig. 7C&D). These results indicate that PIPKIγ positively regulates the invasion of HCT116 colon cancer cells.
PIPKI\(\gamma\) regulates focal adhesion formation through activating vinculin by P(4,5)P\(_2\).

To dissect the mechanism by which PIPKI\(\gamma\) regulates focal adhesion formation, we set out to analyze the levels of polyphosphoinositides in HCT116 cells that express PIPKI\(\gamma\) shRNA or a shRNA control by using mass spectrometry. Depletion of PIPKI\(\gamma\) in HCT116 cells had no significant effect on the level of PIP, but caused significant reduction in PIP\(_2\) level (note that these methods cannot distinguish positional enantiomers of these lipids so it is possible that P(3,4)P\(_2\) may contribute significantly to residual levels of PIP\(_2\) in these cells). Interestingly, in support of this idea knockdown of PIPKI\(\gamma\) reduced PIP\(_3\) levels below the detection limit of our assay (Fig. 8). These results indicate that PIPKI\(\gamma\) is a key enzyme responsible for the production of P(4,5)P\(_2\) and P(3,4,5)P\(_3\) in HCT116 cells.

To see whether P(3,4,5)P\(_3\) is essential for focal adhesion formation in colon cancer cells, HCT116 cells were treated with LY294002, a specific inhibitor of phosphatidylinositol 3-kinase, and focal adhesion formation in these cells was examined after paxillin staining. LY294002 (20 \(\mu\)M) had no significant effect on focal adhesion formation (data not shown). Taken together with the above observations, this finding indicates that P(4,5)P\(_2\), but not P(3,4,5)P\(_3\), is required for focal adhesion formation.
PI(4,5)P2 binds and activates vinculin and is implicated in regulating focal adhesion formation [32]. If PIPKIγ-mediated production of PI(4,5)P2 is essential for focal adhesion formation in HCT116 cells, depletion of PIPKIγ would reduce PI(4,5)P2 levels and prevent vinculin from localizing to focal adhesions. To test this hypothesis, endogenous PIPKIγ-depleted cells were infected with retroviruses expressing a codon-modified PIPKIγ (rescue) (Fig. 9A), and the cells were stained for vinculin. Vinculin was rarely localized to focal adhesions in PIPKIγ-depleted HCT116 cells, whereas re-expression of PIPKIγ in PIPKIγ-depleted cells resulted in a dramatic increase in focal adhesion-localized vinculin (Fig. 9B). Quantitative analysis indicated that PIPKIγ rescue restored focal adhesion formation in PIPKIγ-depleted cells (Fig. 9C&D), as compared to the data in Fig. 5. These data suggest that PIPKIγ may regulate focal adhesion formation through PI(4,5)P2-mediated vinculin activation.

Figure 4. PIPKIγK188,200R was incapable of mediating focal adhesion formation in HCT116 cells. (A) TIRF images of CHO-K1 cells expressing PIPKIγ or PIPKIγK188,200R. Cells that stably express EGFP-PIPKIγ WT or -PIPKIγK188,200R were stained for paxillin. Scale bar, 20 μm. (B) PIPKIγK188,200R failed to stimulate an increase in focal adhesion number (n = 10, error bar = mean ± s.e.m; paired t-test, P<0.005). (C) Area distribution of focal adhesions in cells expressing PIPKIγ and PIPKIγK188,200R. doi:10.1371/journal.pone.0024775.g004
Discussion

Besides serving as the precursor of other second messengers, PI(4,5)P2 itself binds many cytoskeletal and focal adhesion proteins and is believed to be a key regulator of focal adhesion dynamics [34]. PI(4,5)P2 binds vinculin to unmask the talin-binding sites on vinculin [32]; it also binds talin thus stabilizing talin-integrin interactions [35]. PIPKIγ is thought to be the enzyme that generates PI(4,5)P2 spatially and temporally for focal adhesion formation during cell migration [27,34]. On the other hand, PI(4,5)P2 has not been detected at focal adhesions and the role of PIPKIγ in regulating focal adhesion dynamics is controversial [36]. PIPKIγ has been shown to be required for focal adhesion formation during EGF-stimulated cell migration [30], whereas it has also been reported that expression of PIPKIγ caused cell rounding and focal adhesion disassembly [26]

We show here that expression of PIPKIγ at low levels in CHO-K1 cells stimulated focal adhesion formation (Fig. 1), whereas kinase-dead mutants, PIPKIγK188,200R and PIPKIγD316K failed to promote focal adhesion formation (Fig. 2, Fig. 4 and Fig. S1). Furthermore, PIPKIγ knockdown almost completely abolished focal adhesion formation in HCT116 colon cancer cells (Fig. 5). In addition, expression of PIPKIγ promoted focal adhesion assembly and also disassembly rates, while PIPKIγK188,200R was unable to do so (Fig. 3). These results identify an essential role of PIPKIγ in regulating focal adhesion dynamics.

Although direct evidence is lacking, PI(4,5)P2 has been well implicated in regulating integrin activation. PI(4,5)P2 binds talin and blocks its self-inhibition (head-tail interaction) thus promoting its interaction with the β integrin tail [35]. It also stimulates integrin clustering [31]. The increase in both talin-integrin interaction and integrin clustering should stimulate integrin activation. We found that HCT116 cells expressing PIPKIγK188,200R have significantly lower adhesion strength as compared to those expressing the WT, and depletion of PIPKIγ by shRNA also caused a significant decrease in cell adhesion strength in HCT116 cells (Fig. 6), suggesting that PIPKIγ may modulate integrin activation in colon cancer cells.

It has been reported that PIPKIγ is required for EGF-stimulated migration of MDA-MB-231 human breast cancer cells and HeLa human ovarian cancer cells [30,37]. However, our results here show that neither expressing PIPKIγK188,200R, a kinase-dead mutant, nor depletion of PIPKIγ inhibit the migration of HCT116 cells (Fig. S2). The MDA-MB-231 and HeLa cells migrate much faster than HCT116 cells, suggesting that PIPKIγ is essential for fast-moving cells, but not for slow-migrating cells like HCT116 cells. This is supported by our unpublished result that PIPKIγK188,200R dramatically inhibits the migration of Clone A cells, a fast-moving colon cancer cell line.

On the other hand, PIPKIγ seems to be required for the invasion of both fast- (such as MDA-MB-231) and slow-invading
PIPKIγ is a major enzyme that controls polyphosphoinositide metabolism in HCT116 cells. PIPKIγ knockdown results in significant reduction in the level of PI(4,5)P2 and decreases PI(3,4,5)P3 levels even more substantially (Fig. 8). PI(3,4,5)P3 plays an important role in tumorigenesis and cancer metastasis. However, PIPKIγ knockdown does not affect the activation of Akt, a major target of PI(3,4,5)P3, in HCT116 cells (data not shown), probably because Akt can be activated by PI(3,4)P2, which is not directly affected by PIPKIγ.

Inhibition of PI 3-kinase using LY294002 does not influence focal adhesion formation in HCT116 cells, suggesting that PI(4,5)P2, instead of PI(3,4,5)P3, is responsible for PIPKIγ-mediated focal adhesion formation. PI(4,5)P2 promotes vinculin binding to talin and actin and has been shown to be essential for focal adhesion formation [32]. Our result shows that PIPKIγ regulates vinculin localization to focal adhesions in HCT116 cells (Fig. 9). Taken together, it is most likely that PIPKIγ regulates focal adhesion formation through PI(4,5)P2-mediated vinculin activation.

Cancer invasion is a complicated process, requiring spatial and temporary regulation of cell-matrix adhesions, cell protrusions and matrix-degradation [38]. PI(4,5)P2 generated by PIPKIγ regulates cancer invasion through modulating cell adhesion dynamics. Although PI(3,4,5)P3 is not essential for focal adhesion formation, it may also play a role in other aspects of cancer invasion, probably through activating Rac [39].

**Materials and Methods**

**Reagents**

Anti-paxillin antibody (clone 5H11) was from Millipore; Anti-PIPKIγ polyclonal antibody was from Cell Signaling Technology; Anti-tubulin antibody and pLKO1 lentivirus PIPKIγ shRNA (sequence: CCG GCC AGT CCT ACA GGT TGA TCA ACT) from Dr. Mark Ginsberg (University of California-San Diego); Pfu Ultra was from Agilent Technologies; DNA primers were synthesized by Integrated DNA Technologies; Pfu Ultra was from Agilent Technologies; DNA primers were synthesized by Integrated DNA Technologies.

**Plasmid construction**

The plasmid pEGFP-PIPKIγ was generated by pfu Ultra-based PCR using pEGFP-PIPKIγ as template and 5'-GAT GAG AGG AGC TTT GTG TAC TCC CGC CTC-3'/5'-GAG CGG GGA GTA GAC AAA GGT CCT CTC ATC-3' as primers. pEGFP-PIPKIγK188,200R and pZZ-PIPKIγK188,200R were created by PCR using pEGFP-PIPKIγ and pZZ-PIPKIγ [40] as templates, respectively, and sequentially 5'-GAC GAG TTC and 5'-GTT CCT GAT GAG as primers. pEGFP-PIPKIγK188,200R and pZZ-PIPKIγK188,200R were generated by PCR using pEGFP-PIPKIγ and pZZ-PIPKIγ [40] as templates, respectively, and sequentially 5'-GAC GAG TTC and 5'-GTT CCT GAT GAG as primers. pEGFP-PIPKIγK188,200R and pZZ-PIPKIγK188,200R were created by PCR using pEGFP-PIPKIγ and pZZ-PIPKIγ [40] as templates, respectively, and sequentially 5'-GAC GAG TTC and 5'-GTT CCT GAT GAG as primers. pEGFP-PIPKIγK188,200R and pZZ-PIPKIγK188,200R were generated by PCR using pEGFP-PIPKIγ and pZZ-PIPKIγ [40] as templates, respectively, and sequentially 5'-GAC GAG TTC and 5'-GTT CCT GAT GAG as primers.

**Cell culture**

The pLKO1-shRNA-A2 was from BD Biosciences; The plasmid pEGFP-PIPKIγ was a gift from Dr. Mark Ginsberg (University of California-San Diego); Pfu Ultra was from Agilent Technologies; DNA primers were synthesized by Integrated DNA Technologies.
PIPKIγ with Age1, blunting with Klenow, and digesting with Sal1. The smaller fragments were sub-cloned into pBabe-neo vector that had been treated with BamH1, Klenow, and Sal1. pBabe-ZZ-PIPKIγ were created by PCR using pBabe-ZZ-PIPKIγ as template and sequentially 5'GAG CTG TAC AGG TTC -3' and 5'GTA CGA TTA TCA TCA CTG TAC GA-3' as primers. All plasmids were sequenced by Eurofins MWG Operon (Huntsville, AL).

Cell culture, transfections and infections

CHO-K1 Chinese hamster ovary cells, HCT116 human colon cancer cells and 293T human embryonic kidney cells were from the American Type Culture Collection and were maintained in DMEM medium (Mediatech, Inc.) containing 10% fetal bovine serum (FBS), penicillin (100 U ml⁻¹) and streptomycin (100 μg ml⁻¹). CHO-K1 cells were transfected with SafeCTine RU50 (Syd Labs) according to the manufacturer’s protocol. HCT116 cells that stably express EGFP-PIPKIγ WT, or –PIPKIγK188,200R, were obtained by transfecting the cells with TurboFect (Fermentas) and sorting EGFP-positive cells after G418 selection in the University of Kentucky Flow Cytometry Facility, or by infecting with pBabe retrovirus and selecting with puromycin. HCT116 cells stably expressing shRNA control or PIPKIγ shRNA were obtained by infecting with pLKO1 lentivirus and selected with puromycin.

Preparation of viruses and cell infection

The 293T cells were transfected with pBabe retrovirus or pLKO1 lentivirus system using SafeCTine RU50 transfection reagent according to the manufacturer’s protocol. The medium of transfectants was collected at 48 and 72 h, filtered through 0.45-

Immunofluorescence staining and TIRF imaging

Cells were plated on glass coverslips that were precoated with 5 μg/ml fibronectin. For immunofluorescence staining, the cells were fixed with 4% paraformaldehyde permeabilized with 0.5% Triton X-100.
Figure 8. PIPKιγ is a major enzyme responsible for PIP2 and PIP3 production in HCT116 cells. Polyphosphoinositides from cells stably expressing shRNA control or PIPKιγ shRNA were extracted and derivatized using trimethylsilyl diazomethane, and then were measured using mass spectrometry. N = 4, error bar = mean ± s.e.m; PIP, P > 0.05; PIP2, P = 0.0034; PIP3, P = 0.001.

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Figure 9. PIPKιγ is required for vinculin localizing to focal adhesions in HCT116 cells. (A) Re-expression of rescue ZZ-tagged PIPKιγ in cells that PIPKιγ has been knocked down (KD) by stably expressing PIPKιγ shRNA. The PIPKιγ-KD cells were infected with retroviral particles carrying rescue ZZ-tagged PIPKιγ selected with neomycin. ZZ-PIPKιγ was detected by Western blotting with an anti-PIPKιγ polyclonal antibody. (B) TIRF images of PIPKιγ-KD cells and the KD cells that express rescue PIPKιγ. Cells were plated on glass-bottomed dishes pre-coated with fibronectin (5 µg/ml), fixed and stained for vinculin. Scale bar, 20 µm. (C) PIPKιγ rescue restored focal adhesion formation in PIPKιγ-KD cells. (n = 11, error bar = mean ± s.e.m; paired t-test, P < 0.0001) (D) Area distribution of focal adhesions in PIPKιγ-KD cells and the KD cells expressing rescue PIPKιγ.

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blocked with 5% BSA in PBS, and then incubated with anti-paxillin mAb. Paxillin was then visualized by incubating with DyLight 549 Conjugated goat Anti-mouse IgG (H+L). Paxillin staining and EGF fluorescence were viewed by using a Nikon Eclipse Ti TIRF microscope equipped with a 60×, 1.45 NA objective, CoolSNAP HQ2 CCD camera (Roper Scientific). Images were acquired and analyzed by using NIS-Elements (Nikon). To quantify the number and area of focal adhesions, paxillin immunofluorescence images were thresholded to include only focal adhesions and the number and area were calculated by using the software.

**Time-lapse live fluorescence imaging**

CHO-K1 cells that stably express EGFP-PIPKIγ WT, or -PIPKIγK188,200R were transfected into CHO-K1 cells. At 24 h post-transfection, the cells were trypsinized and plated on MatTek dishes (with a glass coverslip at the bottom) that had been precoated with fibronectin (5 μg ml⁻¹). The cells were cultured for 3 hours and TIRF images were taken using the Nikon Eclipse Ti TIRF microscope stated earlier and the temperature and humidity were maintained by using an INU-TIZ-F1 microscope incubation system (Tokai Hit). Images were recorded at 1-min intervals for a 120 min period. Focal adhesion assembly and disassembly rate constants were analyzed as described previously [22].

**PIPKIγ activity assays**

pZZ-PIPKIγ and pZZ-PIPKIγK188,200R were transfected into CHO-K1 cells. At 24 h post-transfection, the cells were harvested in a lysis buffer (50 mM Tris-HCl, pH8.1, 140 mM NaCl, 50 mM NaF, 1% Triton X-100, 10 mM 2-mercaptoethanol, 0.5 mM AEBSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 5 μg/ml E-64, 5 μg/ml pepstatin, 5 μg/ml bestatin.). Cell lysates were cleared by centrifugation and pZZ-PIPKIγ and pZZ-PIPKIγK188,200R in supernatants were immuno-precipitated using IgG-Agarose beads. The beads were washed three times with lysis buffer and washed once with a kinase buffer (50 mM Tris-HCl, pH7.5, 5 mM MgC2, 25 mM KCl, 0.5 mM EGTA, and 0.5 mM ATP). The beads were incubated with 100 μl of the kinase buffer containing 100 μM PI(4)P for 30 min at 37°C. PI(4)P formed in these assays was extracted using modified Bligh-Dyer extraction [41]. Phosphoinositides were quantitated using a Shimadzu UFLC equipped with an ABI 4000-Qtrap hybrid linear ion trap triple quadrupole mass spectrometer in multiple reaction monitoring (MRM) mode. 17:0–20:4 PI(4)P, 17:0–20:4 PI(4,5)P2 and 17:0–20:4 PI(3,4,5)P3 were used as internal standards. Detailed experimental procedures are described in Materials S1.

**Quantitation of Polyphosphoinositides in cells**

Polyphosphoinositides were extracted using a modified Bligh-Dyer extraction [41] and were derivatized using trimethylsilyl diazomethane as described [42]. Polyphosphoinositides were measured as their TMS-diazomethane derivatives using a Shimadzu UFLC equipped with a Vydac 214MS C4, 5 μm, 4.6×250 mm column, coupled with an ABI 4000-Qtrap hybrid linear ion trap triple quadrupole mass spectrometer in multiple reaction monitoring (MRM) mode. 17:0–20:4 PI(4)P, 17:0–20:4 PI(4,5)P2 and 17:0–20:4 PI(3,4,5)P3 were used as internal standards. Detailed experimental procedures are described in Materials S1.

**Centrifugation assays**

HCT116 cells growing on 60-mm dishes were incubated in 2 ml of 2 μg/ml calcim-AM in opti-MEM for 20 min at 37°C. The Cells were then trypsinized, washed and re-suspended in normal growth media containing 10%FBS. The cell suspensions (100 μl, 40,000 cells/well) were added to a 96-well plate coated with different concentrations of fibronectin, centrifuged at 180g in a Beckman Coulter Allegra X-13R centrifuge (5X1750A rotor) and allowed to attach for 1h in 37°C CO2 incubator. Each well was then carefully aspirated to remove floating cells and refilled with fresh PBS-dextrose. An initial fluorescence (F0) was read to determine the density of cells before detachment using a Promega Glomax multi+ Detection system (490 nm excitation, 510–570 nm emission). The lid was then removed, and the plate was covered with sealing tape and centrifuged upside down at 150 g for 5 min to detach the cells. The wells were carefully aspirated and refilled with fresh PBS-dextrose. The fluorescence after centrifuging (Fc) was read to determine the density of cells that remain attached.

**Invasion Assays**

One hundred microliters of Matrigel (1:30 dilution in serum-free DMEM medium) was added to each Transwell polycarbonate filter (6 mm diameter, 8 μm pore size, Costar) and incubated with the filters at 37°C for 4 h. HCT116 cells were trypsinized and washed 3 times with DMEM containing 1% FBS. The cells were resuspended in DMEM containing 1% FBS at a density of 1×10⁶ cells/ml. The cell suspensions (100 μl) were seeded into the upper chambers, and 600 μl of DMEM medium containing 1% FBS and 5 μg/ml Fibronectin with or without 50 ng/ml HGF were added to the lower chambers. The cells were allowed to invade for 36 h in a CO2 incubator. The invaded cells were fixed for 15 min with 3.7% formaldehyde and stained using 0.1% crystal violet in 10% ethanol for 30 min. The number of invaded cells per field was counted under a light microscope at ×200.

**Supporting Information**

**Figure S1** PIPKIγD316K, a kinase-dead mutant, was unable to promote focal adhesion formation in CHO-K1 cells. (A) TIRF images of CHO-K1 cells expressing PIPKIγ or PIPKIγD316K. Cells were transiently transfected with pEGFP-PIPKIγ WT or –PIPKIγD316K, and then stained for paxillin. Scale bar, 20 μm. (B) PIPKIγD316K was deficient in promoting an increase in focal adhesion number (n = 10, error bar = mean ± s.e.m; P<0.005). (C) Area distribution of focal adhesions in cells expressing PIPKIγ and PIPKIγD316K. (TIF)

**Figure S2** Neither expression of PIPKIγK188,200R nor depletion of PIPKIγ impaired the migration of HCT116 cells. (A) Expression of PIPKIγK188,200R slightly enhanced the migration of HCT116 cells. The cells that stably express EGFPP-PIPKIγ or –PIPKIγK188,200R were plated on 35 mm Mat'fek glass bottom dishes coated with 5 μg/ml fibronectin, and grown to 90% confluency. The medium was then changed to DMEM containing 1%FBS and 10 ng/ml HGF for 6 h. A wound was made on the confluent monolayer, and time-lapse cell migration was recorded using a Nikon Biostation IMQ. The pictures were extracted from time-lapse movies. (B) Quantification of the migration speed of HCT116 cells that stably express PIPKIγ or PIPKIγK188,200R using NIS-Elements AR 3.2. (n = 4, *P<0.05). (C) Depletion of PIPKIγ by using shRNA had no significant effect on the migration of HCT116 cells. The migration of cells stably expressing shRNA control or PIPKIγ shRNA were examined in the absence and presence of HGF (50 ng/ml) by Transwell migration assays. In brief, transwell polycarbonate filters (6 mm diameter, 8 μm pore size, Costar) were coated with fibronectin (5 μg/ml) over night. HCT116 cells were trypsinized and washed 3 times with DMEM containing 1% FBS. The cells were resuspended in DMEM
containing 1% FBS at a density of 1×10^6 cells/ml. The cell suspensions (100 µl) were seeded into the upper chambers, and 600 µl of DMEM medium containing 1% FBS and 5 µg/ml Fibronectin with or without 50 ng/ml HGF were added to the lower chambers. The cells were allowed to migrate for 24 h in a CO2 incubator. The migrated cells were fixed for 15 min with 3.7% formaldehyde and stained using 0.1% crystal violet in 10% ethanol for 30 min. The number of migrated cells per membrane was counted under a light microscope at ×200. (D) Quantification of the migration of HCT116 cells that stably express shRNA control or PIPKiy shRNA. n = 3, error bar = mean ± s.e.m, P<0.05. (TIF)

Materials S1 Quantitation of phosphoinositides by HPLC ESI tandem Mass Spectrometry. (DOCX)

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Author Contributions

Conceived and designed the experiments: CH. Performed the experiments: ZW XL MS CH. Analyzed the data: HS MS. Wrote the paper: CH AJM.