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IQGAP1 is a novel phosphatidylinositol 4,5 bisphosphate effector in regulation of directional cell migration

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Phosphatidylinositol 4,5 bisphosphate (PIP_2) is a key lipid messenger for regulation of cell migration. PIP_2 modulates many effectors, but the specificity of PIP_2 signalling can be defined by interactions of PIP_2-generating enzymes with PIP_2 effectors. Here, we show that type Iγ phosphatidylinositol 4-phosphate 5-kinase (PIPKIγ) interacts with the cytoskeleton regulator, IQGAP1, and modulates IQGAP1 function in migration. We reveal that PIPKIγ is required for IQGAP1 recruitment to the leading edge membrane in response to integrin or growth factor receptor activation. Moreover, IQGAP1 is a PIP_2 effector that directly binds PIP_2 through a polybasic motif and PIP_2 binding activates IQGAP1, facilitating actin polymerization. IQGAP1 mutants that lack PIPKIγ or PIP_2 binding lose the ability to control directional cell migration. Collectively, these data reveal a synergy between PIPKIγ and IQGAP1 in the control of cell migration.

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Introduction

Cell migration is a highly orchestrated, multistep process requiring the establishment of polarity, the regulation of cytoskeleton dynamics and spatiotemporal signalling (Ridley *et al*, 2003; Parsons *et al*, 2010). Cell migration is initiated in response to extracellular stimuli, such as cytokines and signals from the extracellular matrix (ECM). These extracellular signals activate intracellular signalling cascades that promote changes in the cytoskeleton. A diverse array of proteins are implicated in these processes, but scaffold proteins that integrate signals from multiple structural and signalling molecules play pivotal roles in transmitting cellular information (Rodriguez *et al*, 2003; Good *et al*, 2011). Previous work has focussed on how scaffold proteins coordinate different signals. However, the exact mechanism of how scaffold proteins themselves are targeted and activated remains largely unknown.

IQ motif containing GTPase activating protein 1 (IQGAP1) is a multidomain protein that regulates cytoskeletal dynamics, proliferation, adherens junction integrity and vesicular trafficking, by serving as a scaffold for key signals (Brown and Sacks, 2006; Brandt and Grosse, 2007; Osman, 2010). IQGAP1 targets to the leading edge, where it promotes actin polymerization through Rac1 and Cdc42 and their effectors, such as N-WASP and Dia1 (Ho *et al*, 1999; Li *et al*, 1999; Brown and Sacks, 2006; Brandt *et al*, 2007; Le Clainche *et al*, 2007). IQGAP1 also controls microtubule (MT) behaviour. IQGAP1 interacts with MT plus end regulators, CLIP-170 and adenomatous polyposis coli (APC), and recruits MTs to the leading edge membrane (Fukata *et al*, 2002; Watanabe *et al*, 2004). By targeting MTs to the leading edge, IQGAP1 is believed to facilitate the polarized trafficking of protein to the migrating front (Watanabe *et al*, 2005; Osman, 2010). Yet, how IQGAP1 interacts with the leading edge membrane is largely undefined. A recent study has shown that phosphatidylinositol 4,5 bisphosphate (PIP_2)-dependent microdomains are required for the recruitment of MTs to the plasma membrane (PM), and Cdc42, N-WASP and IQGAP1 are also required in this process (Golub and Caroni, 2005). However, the exact role for PIP_2 in IQGAP1 regulation of the cytoskeleton at the PM is unknown.

At a molecular level, IQGAP1 is kept inactive through an autoinhibitory interaction between the GRD domain and RGCT domain (Brandt and Grosse, 2007). This autoinhibition can be relieved by RhoGTPase binding to the GRD domain or phosphorylation on Ser1443 to activate IQGAP1 (Grohmanova *et al*, 2004; Li *et al*, 2005). In agreement with this model of activation, a mutant IQGAP1, defective in RhoGTPase binding on the GRD domain, induces multiple leading edges (Fukata *et al*, 2002) and a phosphomimetic variant of IQGAP1 on Ser1443 stimulates neurite outgrowth (Li *et al*, 2005).

PIP_2 comprises $\sim 1\%$ of membrane phospholipids and is the most abundant phosphoinositide species at the PM. Besides serving as a precursor for other lipid messengers, PIP_2 exerts direct signalling roles by interacting with protein targets (Anderson *et al*, 1999; Heck *et al*, 2007). Though PIP_2 binding is often achieved by defined modules on proteins, including C2, pleckstrin homology (PH) and band 4.1/ezrin/radixin/moesin (FERM) domains, many PIP_2-interacting proteins lack canonical modules and instead contain clusters of basic amino acids, known as polybasic motifs (PBMs) that bind PIP_2 (McLaughlin *et al*, 2002). The interaction of PBMs

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with phosphoinositides is largely mediated by the positively charged residues in the PBM that interact with the phosphate head group. Therefore, these interactions in some cases can be promiscuous for phosphoinositides (McLaughlin and Murray, 2005). Recent advances in proteomic analyses have identified hundreds of putative PIP_2 binding proteins, but most of them do not contain canonical modules (Catimel *et al*, 2008; Dixon *et al*, 2011), and thus many PBMs or atypical phosphoinositide binding motifs remain to be characterized.

PIP_2 modulates the activity and targeting of cytoskeleton regulatory proteins, controlling cytoskeletal dynamics and, ultimately, migration (Yin and Janmey, 2003; Zhang *et al*, 2012). Although the roles for PIP_2 in cytoskeleton regulation are extensively studied, the roles for PIP_2-generating enzymes in this process are still emerging (Ling *et al*, 2006; Zhang *et al*, 2012). In mammalian cells, PIP_2 is primarily generated by type I PIP kinases (PIPKIs), and three isoforms, α, β and γ, are expressed in humans with multiple isoforms (van den Bout and Divecha, 2009). For example, four different isoforms of PIPKIγ are expressed in humans and each displays unique cellular distribution. PIPKIγi1 is the most abundant isoform in most cell types and largely locates to the PM (Mao and Yin, 2007). PIPKIγi2 is found at focal adhesions and cell–cell contacts (Ling *et al*, 2002, 2007). PIPKIγi4 is found largely in the nucleus, while PIPKIγi5 localizes to cell–cell contacts and intracellular compartments (Schill and Anderson, 2009). Often, protein–protein interactions recruit PIPKI isoforms to specific cellular regions, and many of these targeting proteins are themselves PIP_2 effectors (Anderson *et al*, 1999; Heck *et al*, 2007). For example, talin recruits PIPKIγi2 to focal adhesions, while the site-specific generation of PIP_2 by PIPKIγi2 strengthens talin binding to β1-integrin (Ling *et al*, 2006).

PIPKIγ and IQGAP1 are implicated in cancer progression and metastasis (Johnson *et al*, 2009; Sun *et al*, 2010). Overexpression of PIPKIγ in breast cancer was found to correlate with poor prognosis (Sun *et al*, 2010). Loss of the PIPKIγi2 isoform from metastatic breast cancer cell lines reduces cell motility (Thapa *et al*, 2012), but the role of other PIPKIγ isoforms and molecular mechanisms remain elusive. Similarly, loss of IQGAP1 from malignant breast epithelial cells reduces cell motility (Mataraza *et al*, 2003) and cell growth (Jadeski *et al*, 2008). IQGAP1 overexpression is reported in cancers originating from many different tissues (White *et al*, 2009). IQGAP1 is shown to regulate the function of many oncoproteins. Notably, IQGAP1 is found at the invasive front of aggressive cancers (Johnson *et al*, 2009) without knowing the underlying mechanism.

Here, we report IQGAP1 as a novel PIP_2 effector that is tightly regulated by PIP_2-generating enzyme PIPKIγ. PIPKIγ and IQGAP1 interact and function together in regulation of directional cell migration. Mechanistically, IQGAP1 requires PIPKIγ for targeting to the leading edge membrane of migrating cells. Also, IQGAP1 is activated specifically by PIP_2, disrupting IQGAP1 autoinhibition to induce actin polymerization. Directional cell migration is dramatically attenuated in cells expressing IQGAP1 mutants that lack PIPKIγ or PIP_2 interaction. Given that expression of both proteins is deregulated in cancers, this study identifies the PIPKIγ/IQGAP1 signalling nexus as a putative therapeutic target in the early steps of cancer progression.

Results

IQGAP1 and PIPKIγ interact

Interacting proteins often determine the function and intracellular targeting of PIPKIs (Heck *et al*, 2007). To identify interacting proteins for PIPKIγ, i1 and i5 isoforms were inducibly expressed and immunoprecipitated (IP'ed) from MDCK cell lysates. The isolated complexes were separated by SDS–PAGE and the gels visualized by Coomassie staining. Then, protein bands were analysed by mass spectrometry. IQGAP1 was identified to interact with the PIPKIγi1 and i5 complexes (Figure 1A).

The interaction between PIPKIγ and IQGAP1 was confirmed in human cell lines. Endogenous proteins were IP'ed and association was examined by immunoblotting. IQGAP1 co-IP'ed with PIPKIγ, and vice versa, from HEK 293 and MDA-MB-231 cell lysates (Figure 1B). The cellular location of the proteins was examined via immunostaining. DsRed-PIPKIγi1 colocalized with endogenous IQGAP1 at the periphery of MCF7 cells and to a lesser extent at a perinuclear compartment (Figure 1C). To characterize binding, His-PIPKIγi1 and GST-IQGAP1 were expressed in *E. coli*, purified and *in vitro* binding was assessed. As shown in Figure 1D, the binding was saturable and Scatchard analysis revealed that the dissociation constant (K_d) for the interaction is ~175 nM, demonstrating that *in vitro* PIPKIγ directly interacts with IQGAP1 with a moderate affinity.

PIPKIγ interacts with the IQ domain

IQGAP1 integrates many signalling pathways by forming interactions through its calponin homology (CHD), WW, IQ, GAP-related (GRD) and RasGAP C-terminal (RGCT) domains (Brown and Sacks, 2006). To identify the PIPKIγ binding site on IQGAP1, we coexpressed Myc-IQGAP1 wild type (WT) or deletion mutants of each domain with HA-PIPKIγi1 in HEK 293 cells and performed an IP. Deletion of the IQ domain (ΔIQ) abrogated IQGAP1 co-IP with PIPKIγ (Figure 1E), and *in vitro* the ΔIQ mutant also failed to interact with PIPKIγ (Figure 1F). Furthermore, the IQ domain alone was capable of interacting with IQGAP1 (Figure 1F and Supplementary Figure S2A). These data indicate that the IQ domain is both necessary and sufficient to interact with PIPKIγ.

The IQ domain is composed of four tandem IQ motifs. The CaM^- mutant, which contains point mutations in the IQ motifs and abrogates calmodulin binding (Li and Sacks, 2003), bound PIPKIγ to a lesser extent than WT (Figure 1F). Furthermore, deletion or mutation of individual motifs reduced binding to PIPKIγ, compared to WT, and the combined mutation of multiple IQ motifs further reduced binding (Figure 1F; and S Choi, unpublished observations). These data indicate that the intact IQ domain is required for the interaction with PIPKIγ. Further studies used the ΔIQ mutant to examine the functional importance of the PIPKIγ interaction.

Migration and lamellipodium formation require PIPKIγ

A role for PIPKIγi2 in migration is emerging (Sun *et al*, 2007; Thapa *et al*, 2012). To further define a role of other PIPKIγ isoforms in the regulation of migration, we stably knocked down PIPKIγ in MDA-MB-231 cells using two different shRNAs (Thapa *et al*, 2012). ShRNA 1 and 2 reduced total PIPKIγ (panPIPKIγ) expression by ~75 and 90%, respectively. PIPKIγi2 expression was also slightly

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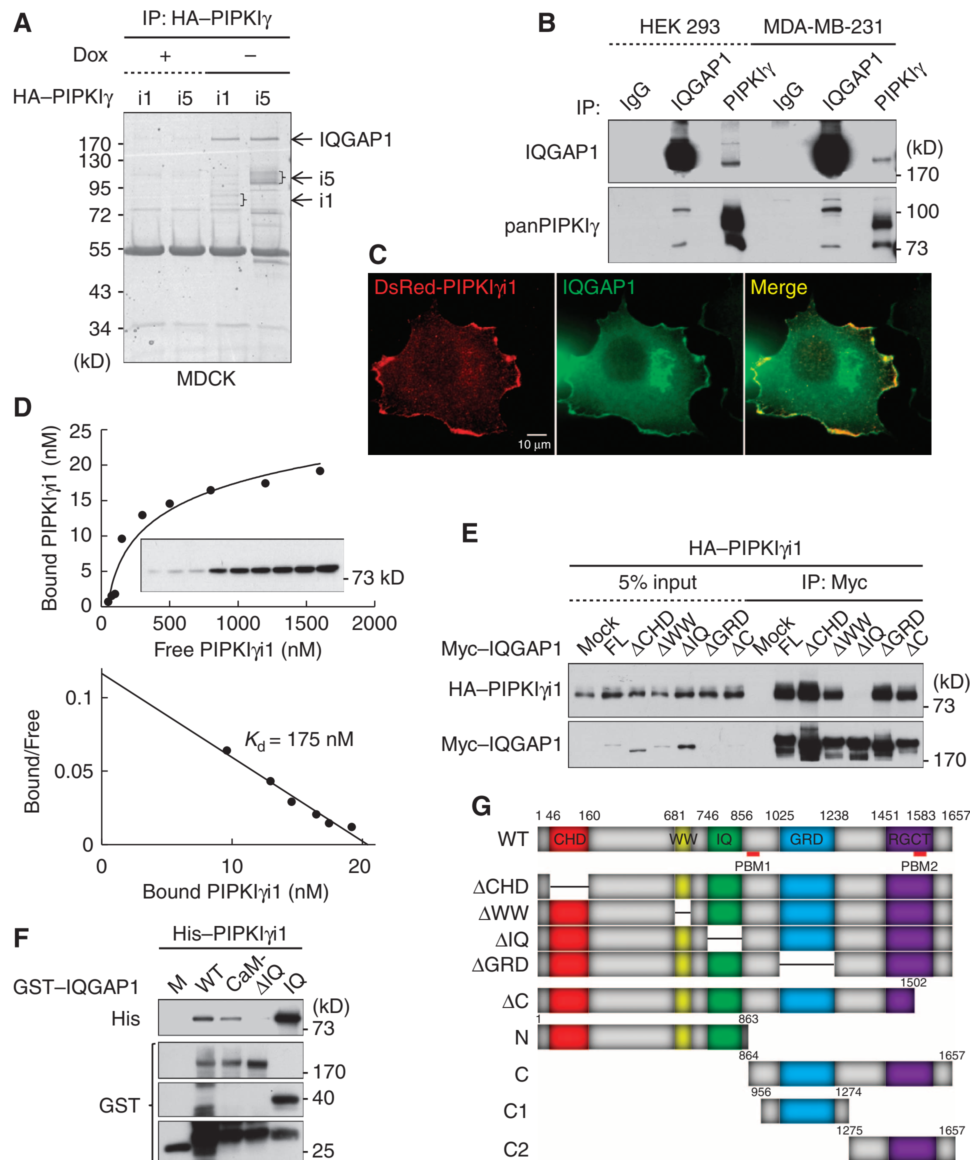

Figure 1 PIPKIγ interacts with the IQ motif of IQGAP1. (**A**) HA–PIPKIγi1 and i5 were expressed in tet-off MDCK cells, and an anti-HA antibody used to IP i1- and i5-containing complexes. Samples were resolved by SDS–PAGE and protein bands visualized by Coomassie staining. Dox, doxycycline. (**B**) PIPKIγ and IQGAP1 were separately IP'ed and association of the other protein examined by immunoblotting. IgG, isotype immunoglobulin control. (**C**) DsRed-PIPKIγi1 was transiently expressed in MCF-7 cells and endogenous IQGAP1 was immunostained. Cells were photographed under $\times$600 magnification. (**D**) GST-IQGAP1 (50 pM) was incubated with 5 to 1600 nM His–PIPKIγi1. Binding was detected by immunoblotting with an anti-His antibody (top). K_d was determined by standard Scatchard analysis (bottom). (**E**) Myc–IQGAP1 proteins were coexpressed with HA–PIPKIγi1 in HEK293 cells and proteins were IP'ed with an anti-Myc antibody. Associated PIPKIγi1 was analysed by immunoblotting with an anti-HA antibody. (**F**) Purified GST–IQGAP1 proteins were incubated with His–PIPKIγi1. The associated protein complex was examined by immunoblotting with the indicated antibodies. Some degraded products of GST–IQGAP1 proteins were detected by immunoblotting with an anti-GST antibody. Data above are representative of at least four independent experiments. (**G**) Schematic representation of IQGAP1 domains and IQGAP1 constructs used for this study. Source data for this figure is available on the online supplementary information page.

reduced (∼24 and 36%, respectively), whereas i4 and i5 expression were not changed (Supplementary Figure S1B), as reported previously (Wang *et al*, 2004). These data indicate that PIPKIγi1 is the predominant isoform in these cells (Mao and Yin, 2007). By bright field microscopy, PIPKIγ knockdown cells were less spread than control cells with fewer protrusions (Supplementary Figure S1A). Serum-induced migration using a Transwell assay was significantly attenuated by PIPKIγ knockdown (Supplementary Figure S1B). These data indicate that PIPKIγ is required for proper spreading and migration.

Knockdown of PIPKIγi2 has a defined migration defect (Sun *et al*, 2007; Thapa *et al*, 2012), but PIPKIγi1 could not be knocked down specifically as it is a splice variant with no unique coding sequence compared to the other isoforms. To explore the role of PIPKIγi1 and i2, we separately re-expressed these two isoforms to determine if they restore migration. The shRNA-resistant DsRed-PIPKIγ was stably re-expressed in PIPKIγ knockdown cells. Cells were then sorted to isolate cells with expression levels similar to endogenous PIPKIγ in control cells. Re-expression of PIPKIγi2 rescued migration (Supplementary Figure S1C), as

reported previously (Thapa *et al*, 2012). Interestingly, PIPKIγi1 WT also rescued the migration whereas i1 kinase dead (KD) did not rescue, indicating that PIPKIγi1 or i2 are sufficient for serum-induced migration, and PIP_2 synthesis is required for this process.

Migrating cells extend lamellipodia at the leading edge and persistent formation of lamellipodia is critical for directional migration (Ridley, 2011). To test how PIPKIγ regulates lamellipodium formation, a lamellipodial marker ARPC2 (Le Clainche *et al*, 2007) was immunostained following initiation of migration by scratch-wounding confluent cells. At 3 h after scratching, ARPC2 localized at the periphery of protrusions in the control cells (Supplementary Figure S1D). In PIPKIγ knockdown cells, formation of protrusions was retarded and ARPC2 no longer localized at the membrane extensions. PIPKIγi1 or i2 re-expression could recover lamellipodium formation, whereas PIPKIγi1 KD had no effect. Early protrusion formation was indistinguishable in different cells but persistent formation was diminished (Supplementary Figure S1E). This demonstrates that PIPKIγ, by generation of PIP_2, regulates persistent lamellipodium formation that is required for migration.

PIPKIγ and IQGAP1 interdependently control cell motility

Upon stimulation, IQGAP1 targets to the leading edge and recruits regulators of the cytoskeleton that control migration (Watanabe *et al*, 2005; Brown and Sacks, 2006). As described above, PIPKIγ also regulates migration (Thapa *et al*, 2012). *Pip5k1c*, a gene coding PIPKIγ in mice, knockout (KO) mice are embryonic lethal with migration defects of cardiovascular cell precursors (Wang *et al*, 2007), and cells from these mice have a defective association between the membrane and the cytoskeleton (Wang *et al*, 2008). To investigate how PIPKIγ and IQGAP1 control cell motility, serum-induced cell motility was measured using a Transwell system. Individual knockdown of PIPKIγ or IQGAP1 significantly reduced both migration and invasion (Figure 2A). Knockdown of both proteins dramatically reduced cell motility, indicating a synergistic role. To better define the relationship of the two proteins, we overexpressed IQGAP1 that is reported to enhance cell motility (Mataraza *et al*, 2003). Overexpression of IQGAP1 in MDA-MB-231 cells increased cell motility over three-fold, whereas knockdown of PIPKIγ in IQGAP1 overexpressing cells reduced cell motility to the basal level. Consistently overexpression of PIPKIγi1 increased cell motility and this increase was inhibited by knockdown of IQGAP1 (Figure 2B). Similar results were obtained in HeLa cells. Here, inducible expression of PIPKIγi1 increased cell motility and depletion of IQGAP1 under these conditions returned motility to the basal level (Figure 2C). Together these data indicate that PIPKIγ and IQGAP1 interdependently control cell motility.

The PIPKIγ–IQGAP1 interaction is required for migration

To investigate how PIPKIγ and IQGAP1 function together, we tested if their association is altered by stimuli that promote migration. Migration is initiated by a variety of extracellular stimuli, including chemokines or ECM (Ridley *et al*, 2003). To define the pathway in which PIPKIγ and IQGAP1 function, cells were stimulated with type I collagen (COL) or serum and changes in the association were examined by IP. In response to either stimulus there was an increase in the panPIPKIγ-IQGAP1 complex, whereas the Rac1 interaction with IQGAP1 remained unchanged (Figure 2D). This demonstrates that the PIPKIγ interaction with IQGAP1 is enhanced by factors that stimulate migration. Furthermore, phosphorylation of Ser1441 and Ser1443 residues of IQGAP1 (Grohmanova *et al*, 2004; Li *et al*, 2005) is required to enhance the interaction (Supplementary Figure S2C). Interestingly, the PIPKIγi2 interaction was unaffected, suggesting that migration enhances IQGAP1 interaction with the predominant isoform, PIPKIγi1 (Mao and Yin, 2007). This is consistent with results indicating that PIPKIγi2 modulates cell migration by a different mechanism (Sun *et al*, 2007; Thapa *et al*, 2012).

The IQGAP1 mutant that lacks interaction with PIPKIγ (ΔIQ) was examined to determine if this interaction is required for migration. For this, *Iqgap1* KO mouse embryonic fibroblasts (MEFs) (Ren *et al*, 2007) were reconstituted with WT or ΔIQ IQGAP1 and migration was examined under various conditions (Keely, 2001). To avoid nonspecific effects from overexpression, we maintained IQGAP1 expression levels similar to the WT MEFs by the cell sorting method as above (Supplementary Figure S1). *Iqgap1* KO MEFs showed $>50\%$ reduction in migration in response to serum, fibronectin or epidermal growth factor (EGF) stimuli. WT IQGAP1 fully rescued migration under all of these conditions, while the ΔIQ mutant showed no recovery of migration induced by fibronectin or EGF (Figure 2E). This indicates that the PIPKIγ–IQGAP1 interaction is necessary for integrin- and EGF receptor-mediated migration. Intriguingly, the ΔIQ mutant still rescued serum-induced migration. Serum contains a collection of factors that induce migration and the contribution of each factor in PIPKIγ-regulated migration varies by cell types (Sun *et al*, 2007). Collectively, the PIPKIγ–IQGAP1 interaction specifically regulates fibronectin- or EGF-induced migration in MEFs (Supplementary Figure S2D), indicating that the PIPKIγ–IQGAP1 nexus is regulated by these pathways.

PIPKIγ controls IQGAP1 translocation to the leading edge membrane

At the onset of migration, many cytoskeleton regulatory proteins translocate to the leading edge membrane to mediate directional migration (Del Pozo *et al*, 2002; Ling *et al*, 2006; Ridley, 2011). To further define how PIPKIγ and IQGAP1 regulate migration, we examined their targeting to the membrane by cell fractionation. Cells were plated on COL, then lysed and fractionated into membrane and cytosolic components (Chao *et al*, 2010). In response to integrin activation, both PIPKIγ and IQGAP1 increased in the membrane fraction (Figure 3A). Rac1 also increased in the membrane fraction, as reported previously (Del Pozo *et al*, 2002). However, membrane proteins, such as calnexin, GM-130 and Na^+K^+ channel, remained unchanged (Figure 3A).

In response to receptor activation, IQGAP1 translocates to the leading edge membrane (Brandt and Grosse, 2007; White *et al*, 2012). Yet, the mechanism for IQGAP1 interaction with the membrane is largely unknown. To examine if PIPKIγ regulates IQGAP1 membrane targeting, PIPKIγ was knocked down using RNAi and cells were fractionated. Knockdown of PIPKIγ significantly reduced IQGAP1 in the membrane fraction upon COL and/or EGF stimulation (Figure 3B). The knockdown of PIPKIγ also reduced the membrane content of

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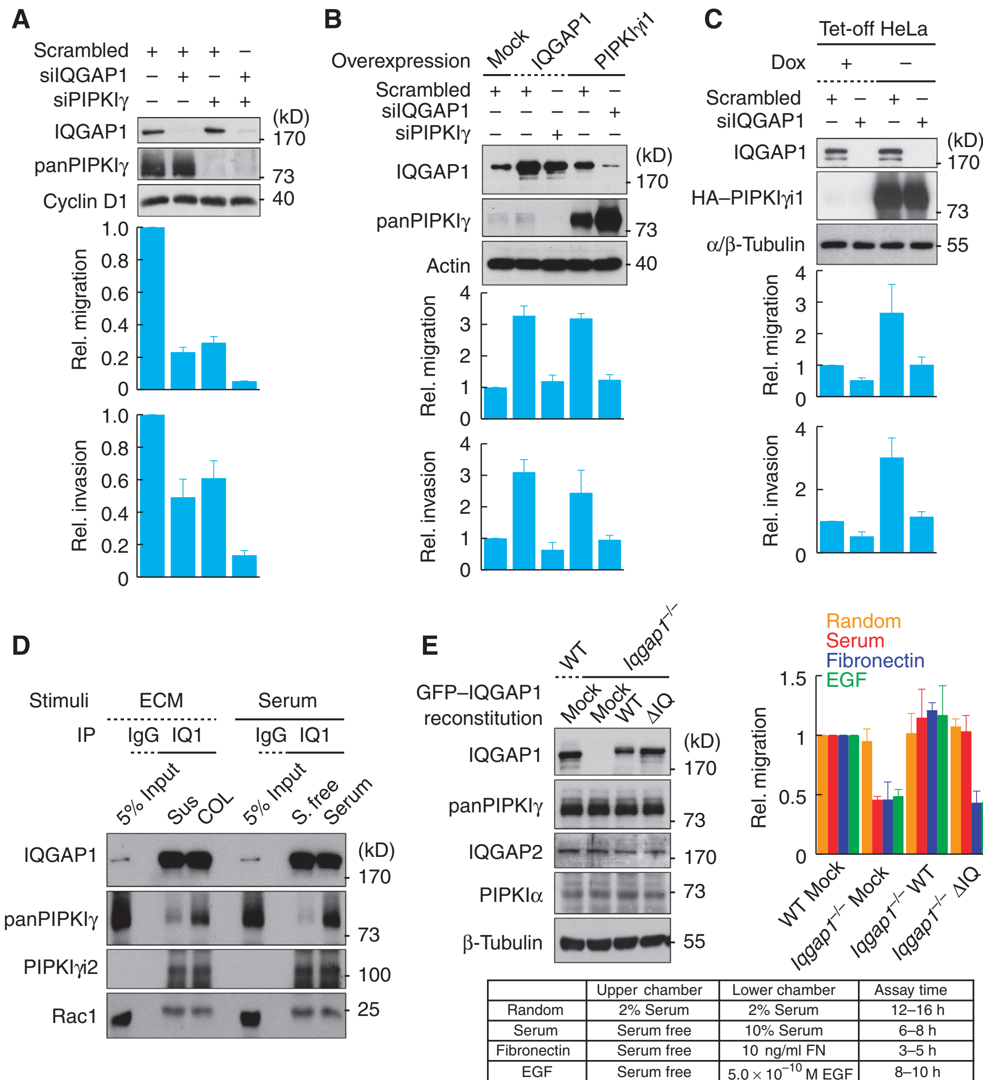

	Upper chamber	Lower chamber	Assay time
Random	2% Serum	2% Serum	12–16 h
Serum	Serum free	10% Serum	6–8 h
Fibronectin	Serum free	10 ng/ml FN	3–5 h
EGF	Serum free	5.0×10^{-10} M EGF	8–10 h

Figure 2 PIPKIγ and IQGAP1 cooperate to regulate migration. (**A**) MDA-MB-231 cells were transfected with the indicated siRNA for 48 h. Knockdown was confirmed by immunoblotting with the indicated antibodies (top). Using Transwell, 10% serum-induced migration (middle) and invasion through 2 mg/ml Matrigel (bottom) were measured. (**B**) Cells were transfected with the indicated DNA and siRNA combinations for 24 h. Expression level was analysed by immunoblotting with the indicated antibodies (top). Migration and invasion were measured as in (**A**) (bottom). (**C**) PIPKIγi1 was expressed in HeLa tet-off cells by removing doxycycline from media for 24 h. Protein expression and cell motility were measured as above. Data are shown as mean ± s.d. for four independent experiments. (**D**) Cells maintained in suspension were either plated on 10 ng/ml collagen I or kept in suspension for 30 min. Serum-starved cells were treated with or without 10% serum for 15 min. Endogenous IQGAP1 was IP'ed and associated PIPKIγ was analysed by immunoblotting. COL, type I collagen; IQ1, IQGAP1; S, serum; Sus, suspension. (**E**) *Iqgap1* KO MEFs were stably reconstituted with the indicated IQGAP1 proteins, and four different modes of migration were measured with a Transwell (top right). Protein expression was analysed by immunoblotting (top left). Conditions used for treating Transwells (bottom). Data are shown as mean ± s.d. of four independent experiments. Data above are representative of at least four independent experiments. Source data for this figure is available on the online supplementary information page.

Rac1, supporting reports that PIPKI and Rac1 interdependently control PM targeting (Chao *et al*, 2010; Halstead *et al*, 2010). To test the contribution of PIPKIγ in IQGAP1 targeting, we utilized a Rac1 binding defective mutant PIPKIγ (E111L) (Halstead *et al*, 2010). The mutant co-immunoprecipitated with IQGAP1 similar to WT PIPKIγ (Supplementary Figure S2E), indicating that Rac1 binding to PIPKIγ is not required for PIPKIγ interaction with IQGAP1. Notably, the E111L mutant enhanced IQGAP1 association with the membrane fraction similar to WT PIPKIγ (Supplementary Figure S2F). These data suggest that the IQGAP1 recruitment to the leading edge is largely regulated by PIPKIγ independent of Rac1. Knockdown of IQGAP1 reduced Rac1 in the membrane fraction, but had no effect on PIPKIγ accumulation in the membrane fraction.

To assess targeting *in vivo*, serum-starved cells were treated with EGF to induce lamellipodia formation (Baumgartner *et al*, 2006) and IQGAP1 localization was observed by immunostaining. As shown in Figure 3C, the number of PIPKIγ knockdown cells with IQGAP1-positive protrusions was reduced by >50% compared to the control cells. To assess PIPKIγ regulation of IQGAP1 localization in migrating cells, endogenous IQGAP1 was immunostained in cells migrating into the scratch wound. IQGAP1 nicely localized at the leading edge in the control cells, but in PIPKIγ knockdown cells the IQGAP1 staining at the cell periphery was

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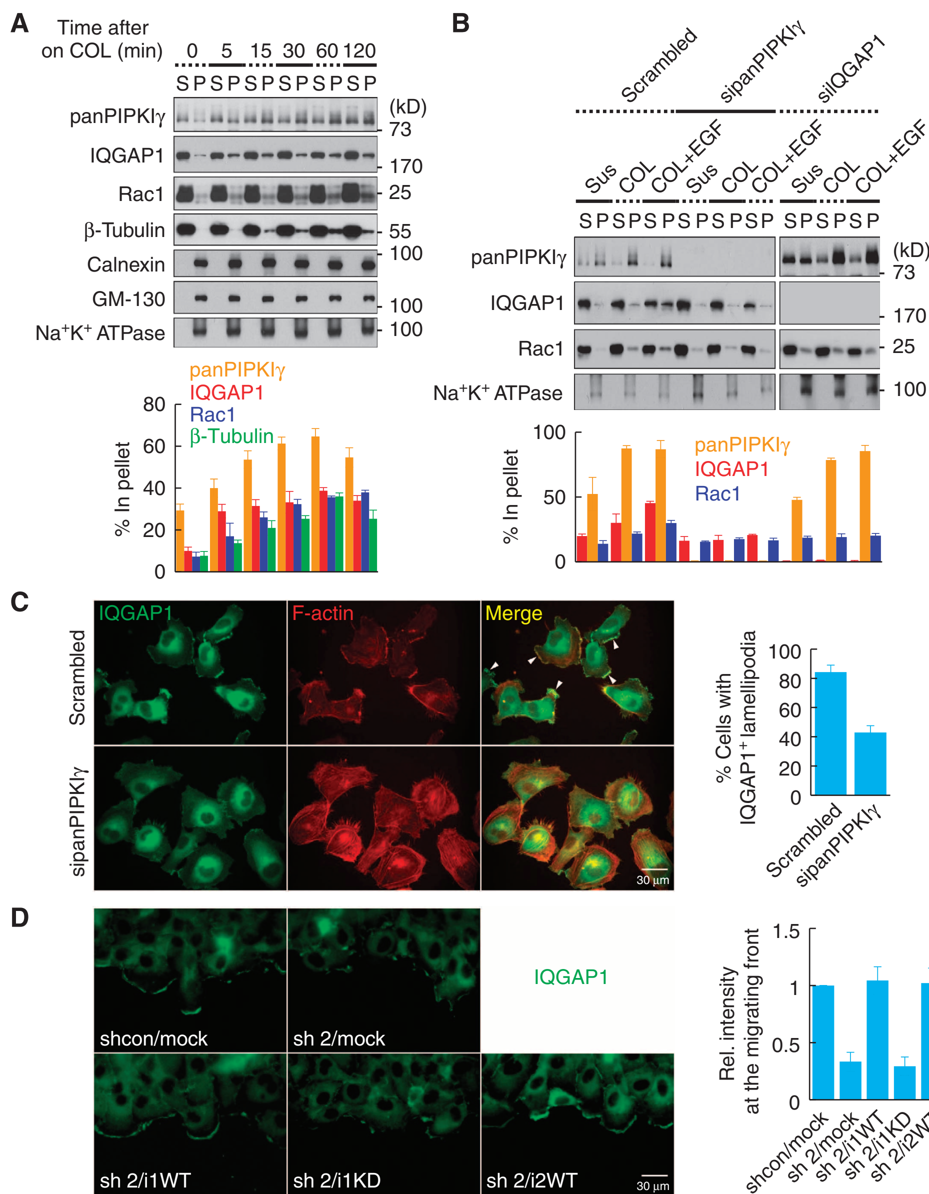

Figure 3 PIPKIγ regulates IQGAP1 targeting to the leading edge membrane. (**A**) MDA-MB-231 cells maintained in suspension were plated on 10 ng/ml COL for the indicated times. Cells were lysed with a hypotonic buffer and the membrane fraction was separated from the cytosolic fraction by centrifugation. Then, 10 μg of each protein was resolved by SDS–PAGE and analysed by immunoblotting with the indicated antibodies (top). The percentage of protein bound in the pellet relative to total (S + P) was calculated by quantifying the immunoblots (bottom). S, supernatant. P, pellet. (**B**) After transient knockdown with the indicated siRNA, cells were treated as in (**A**) in the presence or absence of 50 ng/ml EGF for 30 min. Cells were fractionated and analysed as above. (**C**) Serum-starved control or PIPKIγ knockdown cells were treated with 20 ng/ml EGF for 1 h. Cells were fixed and stained for IQGAP1 and F-actin. Cells were photographed at × 400 magnification. For quantification, at least 300 cells were counted. White arrowheads indicate IQGAP1-positive lamellipodia. Data are shown as mean ± s.d. of three independent experiments. (**D**) Cells grown to confluence were wounded and fixed 3 h later, followed by immunostaining for IQGAP1. Cells were photographed at × 400 magnification. Intensity of fluorescent signal at the migrating front was measured from at least 10 different images of each condition and quantified using ImageJ software. Data are shown as mean ± s.d. of three independent experiments. All the experiments described above were performed independently at least three times. Source data for this figure is available on the online supplementary information page.

significantly reduced (Figure 3D). Reconstitution with either PIPKIγi1 or i2 WT, but not i1 KD, rescued IQGAP1 localization at the leading edge. The difference between WT and KD is not due to an improper interaction with IQGAP1 because the amount of PIPKIγi1 KD that co-IP'ed with IQGAP1 was indistinguishable from that of WT (Supplementary Figure S2B). Taken together, these results demonstrate that PIPKIγ and generation of PIP_2 are required for IQGAP1 targeting to the leading edge membrane in response to migratory signals.

IQGAP1 interacts with PIP_2 through a polybasic motif

Signalling specificity of PIP_2 can be defined by interaction of PIPKIs with PIP_2 effectors (Anderson *et al*, 1999; Heck *et al*, 2007). There is emerging evidence that PIPKIγ controls the cytoskeleton by interacting with cytoskeleton regulatory proteins, which are PIP_2 effectors, such as talin (Ling *et al*, 2002) and trafficking components (Bairstow *et al*, 2006; Thapa *et al*, 2012). Because PIPKIγ associated with IQGAP1 physically (Figure 1) and functionally (Figures 2 and 3), we

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hypothesized that IQGAP1 could be a PIP_2 effector. Consistent with this hypothesis, two independent proteomic analyses suggest that IQGAP1 interacts with PIP_2 (Catimel *et al*, 2008; Dixon *et al*, 2011). To understand how IQGAP1 interacts with PIP_2, their cellular distributions were examined by immunostaining. PH domain from phospholipase Cδ1 (PLCδ1) has been extensively used to probe cellular PIP_2 (Czech, 2000; Raucher *et al*, 2000; Di Paolo and De Camilli, 2006) but excessive expression prevents targeting of PIP_2 binding proteins to the plasma membrane (Raucher *et al*, 2000). Thus, we titrated the GFP-PLCδ1-PH expression and analysed endogenous IQGAP1 localization (Supplementary Figure S4B). In the optimal amount of expression, endogenous IQGAP1 partially colocalized with GFP-PLCδ1-PH (Figure 4A and Supplementary Figure S4B), indicating that both IQGAP1 and PIP_2 are present at regions of the PM containing PIP_2. To define PIP_2 binding, liposomes were synthesized containing membrane lipids (57.5% of phosphatidylcholine, 20% of phosphatidylethanolamine and 20% of phosphatylserine in molar ratio) and 2.5% $PI4,5P_2$. A co-sedimentation assay was used to define the PIP_2 binding site on IQGAP1. IQGAP1-N or -C (Figure 1G) were examined and only IQGAP1-C co-sedimented with PIP_2 liposomes, indicating that PIP_2 binds to the C-terminal half (Figure 4B).

A lysine cluster mediates IQGAP1 interaction with PIP_2

IQGAP1 does not contain known PIP_2 binding modules, but we found at least two potential PBMs within AA 921-970 and 1491-1560, named PBM1 and PBM2, respectively (Figure 1G). Deletion of PBM2 dramatically reduced IQGAP1 interaction with PIP_2 liposomes, whereas deletion of PBM1 had little effect, indicating that IQGAP1 interacts with PIP_2 through PBM2 (Figure 4C). To define a putative PIP_2 binding site on PBM2, human IQGAP1, 2 and 3 sequences were aligned with

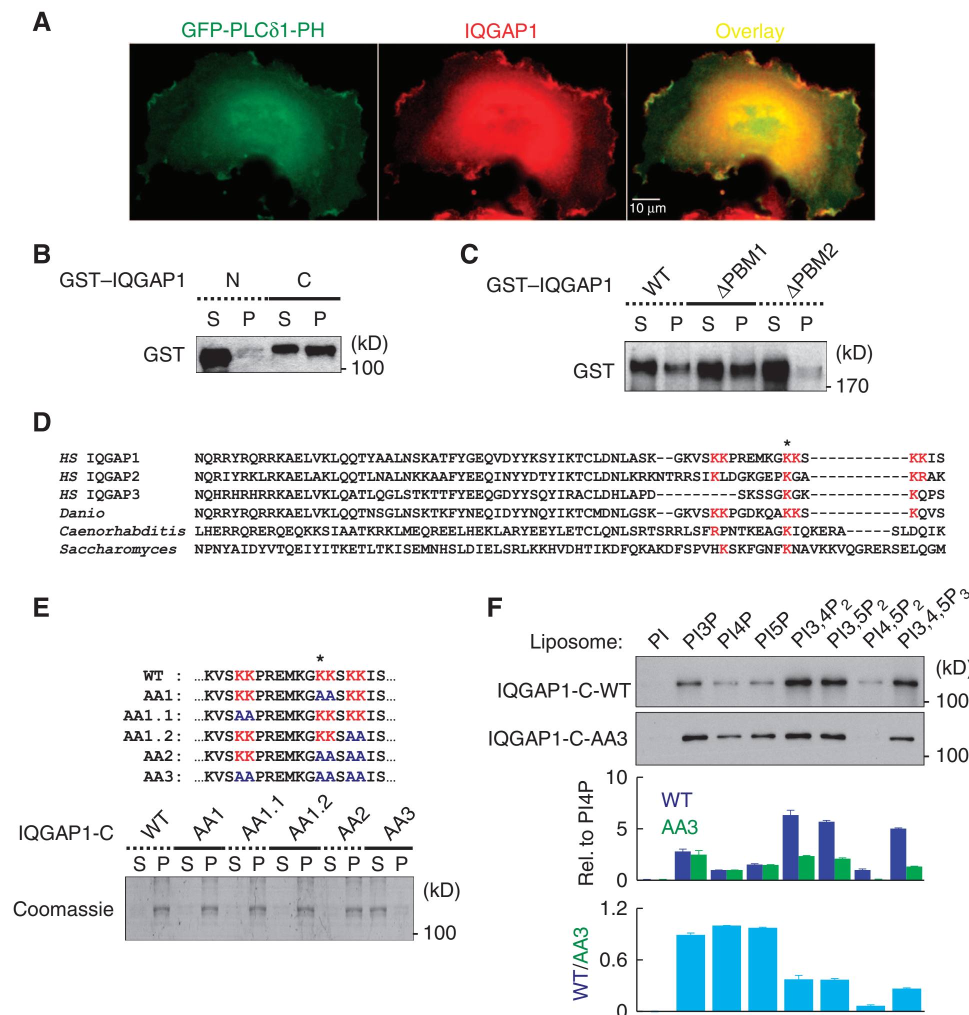

Figure 4 IQGAP1 interacts with the phosphoinositides through a polybasic motif. (**A**) GFP-PLCδ1-PH was transiently expressed in MDA-MB-231 cells and endogenous IQGAP1 was immunostained. Cells were photographed at $\times 600$ magnification. (**B**) PIP_2 liposomes (2.5%) were incubated with 0.5 μM GST-IQGAP1-N or -C for 10 min. Liposome-bound IQGAP1 was pelleted by centrifugation. Equal volume of the supernatant and the pellet were resolved by SDS–PAGE and IQGAP1 in each fraction was analysed by immunoblotting with an anti-GST antibody. (**C**) GST–tagged WT or deletion mutants were used for a sedimentation assay with 2.5% PIP_2 liposomes. (**D**) Amino acid sequence alignment of the PBM2 region among IQGAPs from the indicated species. (**E**) Selected lysine residues were mutated to alanines to generate a series of AA mutants (top). Binding of WT and the AA mutants to 5% PIP_2 liposomes were tested (bottom). (**F**) Binding of GST–tagged WT and the AA3 mutant to 5 μM of 5% phosphoinositide liposomes were tested. Samples were analysed as above and liposome-bound proteins were detected by immunoblotting with anti-GST antibody. Immunoblots were quantified and the graph is shown as mean ± s.d. of three independent experiments. All the experiments described above were performed independently at least four times. Source data for this figure is available on the online supplementary information page.

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IQGAP sequences from multiple species. As shown in Figure 4D, the sequence alignment identified a lysine residue, marked by an asterisk, which is conserved in PBM2. Around this lysine, there are other conserved basic residues, highlighted in red. We mutated these residues to alanines as illustrated in Figure 4E and tested the impact on PIP_2 liposome binding. Mutating two or four lysine residues had little effect, whereas mutating all six residues (termed AA3) eliminated IQGAP1 binding to the PIP_2 liposomes.

To examine phospholipid binding specificity, a lipid overlay assay was performed. IQGAP1 WT and -C bound to multiple phosphoinositides but not other phospholipids (Supplementary Figure S3A). To better define IQGAP1 phosphoinositide binding, the IQGAP1-C fragment was used in liposome sedimentation assays, with liposomes containing 5% phosphoinositide (Papayannopoulos *et al*, 2005). In this assay, PI3,4P_2, PI3,5P_2 and PI3,4,5P_3 bound with a higher affinity than PI3P, PI4P, PI5P and PI4,5P_2 (Figure 4F). Although the apparent affinity for other bis- and tris-phosphate species is up to seven-fold higher than PI4,5P_2, PI4,5P_2 is estimated to be present in the PM at a concentration 20- to 100-fold higher than other phosphoinositide species (Papayannopoulos *et al*, 2005), indicating that PI4,5P_2 is the major *in vivo* ligand for IQGAP1. The AA3 mutation reduced binding to PI3,4P_2, PI3,5P_2 and PI3,4,5P_3 but not monophosphate species (Figure 4F). Strikingly, the AA3 mutant lost binding to PI4,5P_2. The combined data indicate that IQGAP1-C has multiple distinct phosphoinositide binding sites (Dixon *et al*, 2012) and the lysine cluster mutated in AA3 defines a specific PI4,5P_2 binding site.

The IQGAP1 PIP_2 binding mutant exhibited multiple leading edges and loss of migration

To determine how PIP_2 binding modulates IQGAP1 function, the AA3 mutant was expressed in *Iqgap1* KO MEFs and the cell morphology was examined. When plated on a stiff substratum (glass or plastic) coated with COL, fibronectin or gelatin, all types of cells indistinguishably highly spread and formed massive stress fibres (S Choi, unpublished observations). Cytoskeleton organization and cell shape are greatly influenced by substrate stiffness (Solon *et al*, 2007), and therefore cells were plated on pliant gelatin gel and cell morphology was observed by staining F-actin. Three distinct cell morphologies were observed compared to the star-shaped cells (type 1 morphology) that were predominant in WT MEFs (Figure 5A). *Iqgap1* KO resulted in an increase in the number of cells with a single leading edge (type 2). Reconstitution of IQGAP1 WT partially recovered shapes of WT MEFs, whereas the ΔIQ mutant had a limited effect. Interestingly, the number of cells with multiple leading edges (type 3) was increased in the AA3-reconstituted cells (Figure 5A). To closely examine localization of the reconstituted proteins, IQGAP1 was immunostained. WT IQGAP1 localized at the leading edge where active actin polymerization occurs. The ΔIQ mutant was largely cytoplasmic and failed to localize at the leading edge (Figure 5B, arrowhead), supporting the results in Figure 3 indicating that the interaction with PIPKIγ controls IQGAP1 targeting.

The AA3-reconstituted cells formed multiple leading edges and the AA3 mutant localized at these sites (Figure 5B). Consistent with this morphological phenotype, the AA3-reconstituted cells did not rescue haptotactic migration (Figure 5C). The functional defects of AA3 were not due to a change in interaction with PIPKIγ as co-IP of the AA3 mutant with PIPKIγ was indistinguishable from that of WT IQGAP1 (Supplementary Figure S3B and C). Rather, the defects result from the loss of directional persistence (Figure 5D, Supplementary Figure S4 and Supplementary Movies 1–3). This indicates that the IQGAP1 interaction with PIPKIγ is required for IQGAP1 targeting to the leading edge, but PIP_2 binding is required for the role of IQGAP1 in normal membrane protrusions (lamellipodia formation) and migration.

IQGAP1–PIP_2 interaction regulates actin polymerization

Knockdown of PIPKIγ reduced IQGAP1 targeting to the leading edge membrane. Also, in knockdown cells actin polymerization at the leading edge, indicated by strong F-actin staining, was lost and stress fibre formation was increased (Figures 3C and 6A), signifying that PIPKIγ controls actin polymerization at the leading edge by regulating IQGAP1 targeting. However, the AA3 mutant is capable of interacting with PIPKIγ and localizes at the leading edge membrane, but forms multiple leading edges (Figure 5B). These data suggest that PIPKIγ regulates activity of IQGAP1 required for persistent formation of a single leading edge.

IQGAP1 folds into an inactive conformation through an intramolecular interaction between the GRD and the RGCT domains (Brandt and Grosse, 2007). RhoGTPase binding to the GRD or phosphorylation of Ser1443 disrupts autoinhibition and activates IQGAP1 (Grohmanova *et al*, 2004). We identified a PIP_2 binding PBM within the RGCT domain close to Ser1443, suggesting that PIP_2 binding to this PBM may open the inactive conformation (Brandt *et al*, 2007; Le Clainche *et al*, 2007). To test this hypothesis, we examined how phosphoinositides affect binding between the GRD and the RGCT domains. For this analysis, His-C2 was incubated with immobilized GST-C1 (Figure 1G) in the presence or absence of phosphoinositide liposomes. In the absence of liposomes, C1 bound to C2 as reported previously (Grohmanova *et al*, 2004). Intriguingly, the binding was dramatically decreased in the presence of PI4,5P_2 liposomes, while other phosphoinositides or phosphatidylinositol had no significant effect. Introduction of the AA3 mutation in the C2 fragment eliminated the effect of PI4,5P_2 on the C1–C2 binding (Figure 6B and Supplementary Figure S5C). Although the AA3 IQGAP1-C interacts with other phosphoinositide species, it lacks PI4,5P_2 binding (Figure 4F). This indicates that there are multiple phosphoinositide binding sites in IQGAP1-C (Dixon *et al*, 2012), but only PI4,5P_2 binding to the PBM modulates the activation of IQGAP1 as indicated by a loss of the C1–C2 interaction.

The C-terminal fragment of IQGAP1 (AA 746–1657) enhances actin polymerization by activating N-WASP (Le Clainche *et al*, 2007). Using this system, the influence of phosphoinositides in IQGAP1-mediated actin polymerization was assessed. Since the actin polymerization activity of N-WASP is also regulated by PI4,5P_2, a N-WASP-ΔB mutant, which lacks the PI4,5P_2-responsive element (Rohatgi *et al*, 2000), was used for this assay. Addition of PI4,5P_2 liposomes had no effect while addition of IQGAP1-C enhanced actin polymerization as shown previously (Le Clainche *et al*, 2007). Introduction of PI4,5P_2 liposomes in combination with WT IQGAP1-C significantly enhanced actin polymerization

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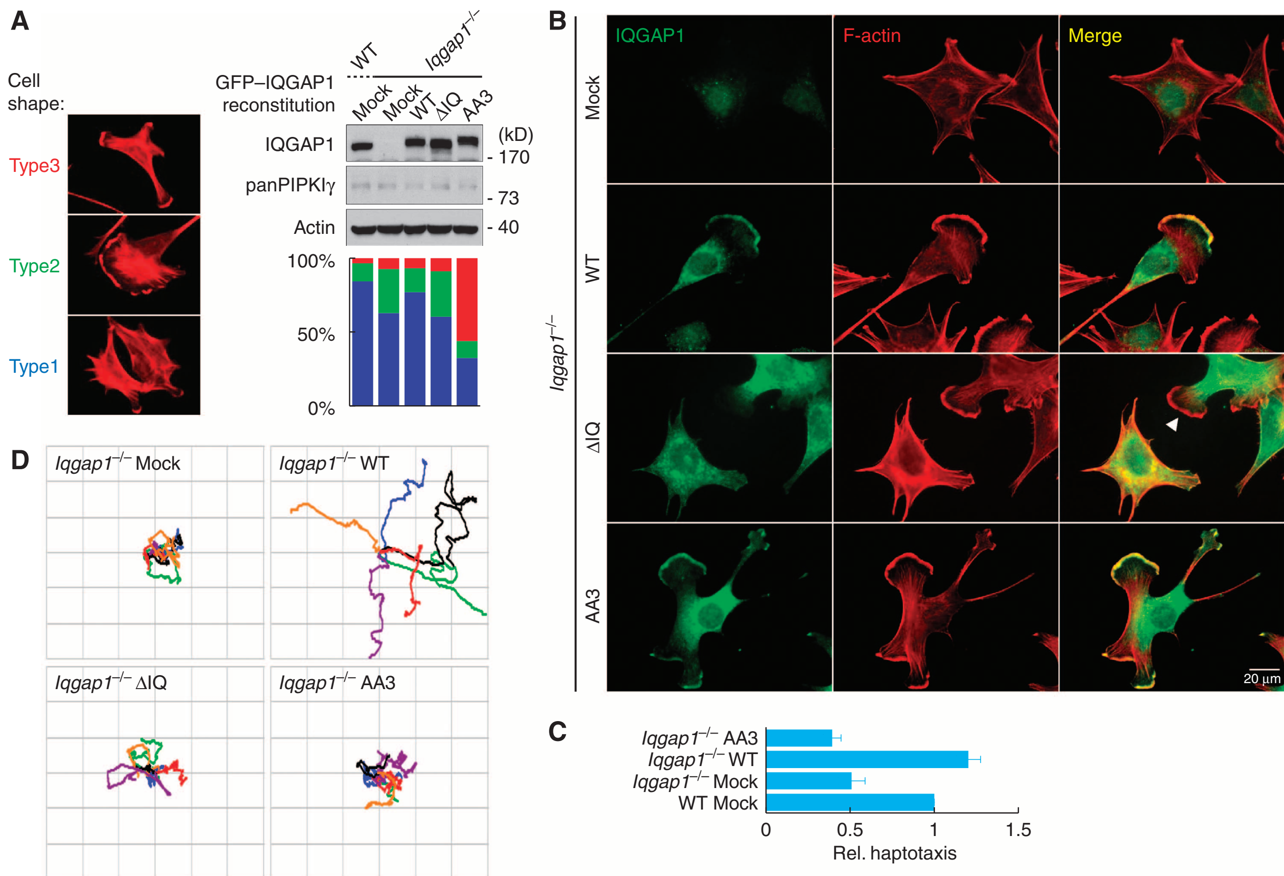

Figure 5 PIP_2 binding of IQGAP1 is important for cell morphology and migration. For both (**A**) and (**B**), *Iqgap1* KO MEFs, reconstituted with the indicated proteins, were plated on 0.2% gelatin gel for 3 h. Fixed cells were stained for IQGAP1 and F-actin. Cells were photographed at ×600 magnification. (**A**) At least 300 cells were counted for each condition and categorized based on cell morphology (left). The graph is shown as mean of three independent experiments (right bottom). Expression levels of the proteins were analysed by immunoblotting with antibodies against the indicated molecules (right top). (**B**) IQGAP1 and F-actin staining. Arrowhead indicates the lamellipodium that is deficient of the ΔIQ mutant. (**C**) With the reconstituted MEFs, fibronectin-induced haptotaxis was measured as described in Figure 2E. (**D**) Reconstituted MEFs were plated on gelatin gel for 3 h before recording using time-lapse microscopy. Images were collected every min for 6 h at ×100 magnification and combined into a time-lapse movie. The migration path of six individual cells was then traced and plotted on a grid, with the origin of each cell placed in the centre of the grid. All the experiments described above were performed independently at least three times. Source data for this figure is available on the online supplementary information page.

activity, whereas PI4,5P_2 had a limited effect on actin polymerization by the AA3 mutant (Figure 6C). Strikingly, stimulation of actin polymerization was highly specific for PI4,5P_2 (Supplementary Figure S5A–D).

Discussion

Here, we define a novel mechanism of how PIPKIγ and IQGAP1 function together as a signalling nexus to regulate migration (Figure 7). In polarized epithelial cells, IQGAP1 is largely localized to cell–cell contacts (Li *et al*, 1999; Fukata *et al*, 2001; Watanabe *et al*, 2004; Noritake *et al*, 2005). In directionally migrating cells, IQGAP1 translocates to the leading edge (Mataraza *et al*, 2003) and facilitates actin polymerization. In response to receptor signalling, PIPKIγ associates with IQGAP1 and recruits IQGAP1 to the leading edge membrane. There, generation of PIP_2 by PIPKIγ activates IQGAP1, as PIP_2 binding to a PBM relieves autoinhibition between the RGD and RGCT domains. This allows the RGCT domain to recruit N-WASP and the Arp2/3 complex to facilitate actin polymerization (Supplementary Figure S3E) (Brandt and Grosse, 2007). Overall, extracellular stimuli control the spatiotemporal activation of the PIPKIγ/IQGAP1 nexus to regulate actin polymerization required for persistent formation of lamellipodia and migration.

All PIPKIγ isoforms have the potential to interact with IQGAP1 (Figure 1A) and this suggests that IQGAP1 may mediate isoform-specific functions at different compartments. For example, IQGAP1 is found in the nucleus and ectopic expression of IQGAP1 enhances transcriptional activity of β-catenin (Briggs *et al*, 2002). Similarly, PIPKIγ also modulates β-catenin-mediated transcriptional co-activation (Schramp *et al*, 2011). IQGAP1 associates with the exocyst complex and regulates cancer cell invasion, a function also regulated by PIPKIγi2 (Sakurai-Yageta *et al*, 2008). Here, we demonstrate that receptor signalling stimulates the recruitment of IQGAP1 to the leading edge through an interaction with PIPKIγ, likely the PIPKIγi1 isoform (Figure 2D). PIPKIγi2 isoform plays an analogous role by interaction with talin, linking the trafficking of integrin-containing vesicles to talin-rich adhesions (Thapa *et al*, 2012).

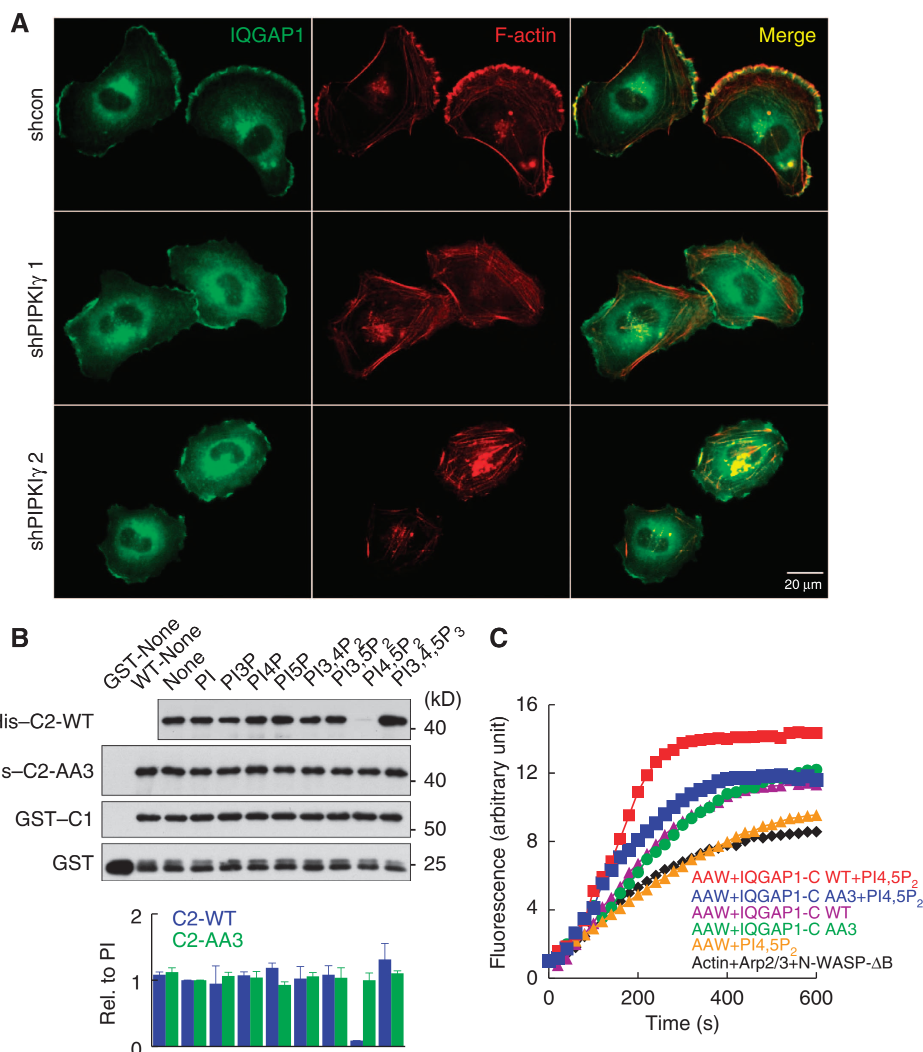

Figure 6 Phosphoinositide binding regulates IQGAP1 function in actin polymerization. (**A**) Control or PIPKIγ knockdown MDA-MB-231 cells were grown on cover glass for 24 h. Cells were fixed and endogenous IQGAP1 and F-actin were stained. Cells were photographed at $\times 600$ magnification. (**B**) A total of 0.05 μM of His–C2 WT or AA3 mutant was incubated with 1 μM of GST–C1 immobilized on glutathione beads in the absence or presence of the indicated phosphoinositide liposomes (2 μM) for 10 min. Liposome-bound proteins were detected by immunoblotting with an anti-His antibody. Immunoblots were quantified and the graph is shown as mean ± s.d. of three independent experiments. (**C**) Actin polymerization in the presence of the indicated combinations of GST–IQGAP1-C (50 nM) or 5% PI4,5P$_2$ liposomes (2 μM). The experiments described above were performed independently at least four times. Source data for this figure is available on the online supplementary information page.

The C-terminal half of IQGAP1 (IQGAP1-C) binds to different phosphoinositide species with a varying binding affinity (Figure 4F). A recent study shows that the distal portion of the C-terminus of IQGAP1 (AA 1559–1657) forms a pseudo C2 domain fold and binds to class I phosphoinositide 3-kinase products, PI3,4P$_2$ and PI3,4,5P$_3$ (Dixon *et al*, 2012). According to the solved structure, Lys1562 and Lys1604 are important for ligand recognition. Here we define a distinct PI4,5P$_2$-binding site at Lys1546, Lys1547, Lys1554, Lys1555, Lys1557 and Lys1558 (Figure 4). These data indicate that there could be multiple phosphoinositide binding sites on IQGAP1-C. Consistent with this possibility, the IQGAP1-C1 interaction with IQGAP1-C2 is specifically inhibited by PI4,5P$_2$, while mutating the six lysine residues blocks the inhibition (Figure 6B). Further work is necessary to define other phosphoinositide binding sites on IQGAP1. These studies will give us mechanistic insight into how IQGAP1 is found at the intracellular compartments where different phosphoinositide species are predominant (Di Paolo and De Camilli, 2006; Osman, 2010).

PIPKIγ regulates IQGAP1 targeting to the leading edge and this event requires PIP$_2$ generation (Figure 3). IQGAP1 is widely believed to target to the PM by association with Rac1 and Cdc42 (Fukata *et al*, 2002; Watanabe *et al*, 2004; Brandt and Grosse, 2007). Rac1 and Cdc42 contain PBMs near the C-termini and these PBMs contribute to membrane targeting (Del Pozo *et al*, 2002; Heo *et al*, 2006). This raises the possibility that PIP$_2$ controls IQGAP1 targeting to the PM by indirectly regulating Rac1 targeting. Consistently, sequestration of cellular PIP$_2$ by either neomycin treatment (Gabev *et al*, 1989) or PLCδ1-PH expression (Raucher *et al*, 2000) blocks both Rac1 and IQGAP1 translocation to membrane in response to integrin activation (Supplementary Figure S3D). To examine the sole contribution of PIP$_2$ binding

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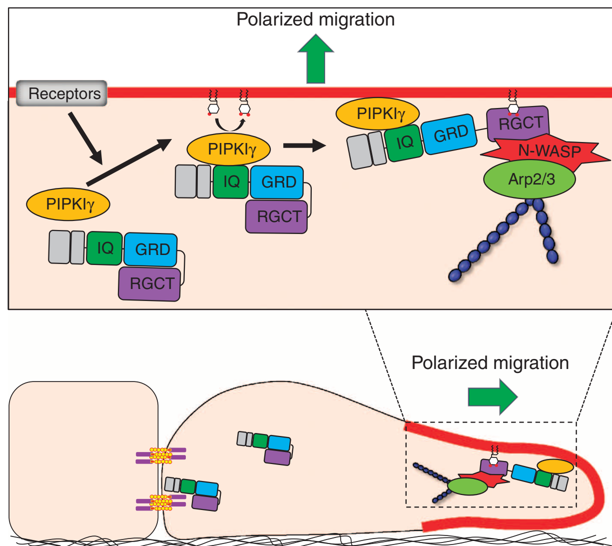

Figure 7 Model of PIP_2-mediated IQGAP1 activation. In response to receptor activation, PIPKIγ recruits IQGAP1 to the leading edge membrane of migrating cells. Then, PIP_2 generated by PIPKIγ interacts with a PBM of IQGAP1 to block the autoinhibitory interaction between the GRD and RGCT domains. The relieved RGCT domain mediates actin polymerization by recruiting N-WASP and the Arp2/3 complex.

for IQGAP1 targeting, we generated and expressed a PIP_2-binding-defective mutant of IQGAP1 in *Iqgap1*$^{-/-}$ MEFs. The PIP_2-binding-defective mutant still localizes to the PM, while the PIPKIγ-binding-defective (ΔIQ) mutant is largely cytosolic (Figure 5B). These data indicate that the physical interaction between the two proteins is more important than PIP_2 binding for targeting IQGAP1 to the PM.

Cells expressing the PIP_2 binding IQGAP1 mutant (AA3) form multiple leading edges, suggesting that PIP_2 regulation of IQGAP1 is important for maintaining polarity and leading edge integrity (Figure 5B). These cells exhibit perpetual formation and retraction of leading edges but display little movement (Figure 5D and Supplementary Movie 3). Consistent with this observation, IQGAP1 is suggested to maintain polarity of migrating cells through local capture of MTs at the leading edge by interaction with MT regulators (Watanabe *et al*, 2005). The interaction sites for these proteins are within the RGCT domain, which also contains the PIP_2 binding site (Brown and Sacks, 2006). We envision that the autoinhibitory interaction between the GRD and RGCT domains may also block MT recruitment, and PIP_2 binding may relieve this (Figure 3A). In this model, the AA3 mutant may remain inactive at the leading edge and fail to recruit MTs, which would result in loss of cell polarity. Alternatively, multiple leading edges could be induced by perturbation of actin dynamics. In support of this possibility, cells display multiple leading edges after manipulation of certain actin regulatory proteins. For example, multiple leading edges also form in Cdc42 KO dendritic cells (Lammermann *et al*, 2009) and in Vero cells after expression of an IQGAP1 mutant that is defective in Rac1/Cdc42 binding (Fukata *et al*, 2002).

Finally, multiple reports suggest roles for both PIPKIγ and IQGAP1 in cancer metastasis (Johnson *et al*, 2009; White *et al*, 2009; Sun *et al*, 2010). The current findings define a molecular mechanism of how these two proteins interact and function together in migration and invasion, and potentially other processes required for cancer progression.

Materials and methods

Cell culture and constructs

MDA-MB-231, HEK 293, MCF-7 and MEF cells were maintained in DMEM supplemented with 10% fetal bovine serum (Gibco). MDCK and HeLa tet-off cells were cultured as previously described (Ling *et al*, 2007) and induction of transgene was achieved by removing of doxycycline from media for 24 h. The constructs used for this work have been described previously (Sokol *et al*, 2001; Li *et al*, 2005; Papayannopoulos *et al*, 2005; Le Clainche *et al*, 2007; Ren *et al*, 2007).

Stable cell line generation

To generate stable MDA-MB-231 cell lines, cells were transfected with vectors expressing DsRed-PIPKIγ isoforms using Lipofectamine 2000 (Invitrogen) and selected with 1.2 mg/ml Geneticin (Gibco) for 15 days, and further selected for DsRed expression using cell sorter. Cells expressing the transgene at a level similar to the endogenous level of PIPKIγ were used for experiments. For generation of stable cell lines in MEFs, cells were infected with retrovirus for 24 h. Then, cells expressing GFP-IQGAP1 were first selected for GFP expression, and then further sorted by expression level.

Antibodies and siRNAs

Monoclonal antibodies against IQGAP1, β-tubulin, Myc-tag, Na^+K^+ ATPase, GST–tag, His–tag (Millipore), α/β-tubulin, cyclin D1 (Cell Signaling Technology), Rac1, calnexin, GM-130 (BD Biosciences), HA–tag (Covence Biotechnology), actin (MP Biomedicals) and polyclonal antibody against IQGAP2 (Santa Cruz Biotechnology) were used for this study. Polyclonal and monoclonal antibodies against total and specific isoforms of PIPKIγ were produced as described previously (Schill and Anderson, 2009). Pooled siRNAs against PIPKIγ were obtained from Dharmacon and IQGAP1 from Santa Cruz Biotechnology.

IP and immunoblotting

Cells were lysed in a buffer containing 1% Brij58, 150 mM NaCl, 20 mM HEPES, pH 7.4, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 1 mM Na_3VO_4, 1 mM Na_2MoO_4 and protease inhibitors. Protein concentration of lysates was measured by the BCA method (Pierce) and equal amounts of protein were used for further analysis. For IP, 0.5 to 1 mg of proteins were incubated with 1 μg of antibodies at 4°C for 8 h and then incubated with a 50% slurry of Protein G Sepharose (GE Life Sciences) for another 2 h. After washing 5 × with lysis buffer, the protein complex was eluted with SDS sample buffer. For immunoblotting, 10 to 20 μg of proteins were loaded. After developing immunoblots, the film was scanned using a transmitted light scanner (resolution = 600 d.p.i.). Protein bands were quantified using ImageJ, and statistical analysis of the data was performed with Microsoft Excel. The statistical analysis was performed using data from at least three independent experiments.

In vitro binding assay

Recombinant proteins were expressed in BL21 *E. coli* strain. GST–tagged proteins were then purified with GST Sepharose 4B (GE Life Sciences) and His–tagged proteins were purified with His-Bind Resin (Novagen). GST–tagged proteins were incubated with glutathione beads before binding assays. The binding assay was performed in the lysis buffer used for IP by adding 10 nM to 5 μM of His–tagged proteins and 20 μl of GST–tagged protein bound to glutathione beads. After incubation for 1 h at 25°C, unbound proteins were washed out and the protein complex was analysed by immunoblotting. For the binding assay with liposomes, analysis was performed for 10 m at 25°C without detergent (150 mM KCl, 50 mM HEPES, pH 7.4, 2 mM $MgCl_2$, 2 mM $CaCl_2$ and protease inhibitors) to maintain the integrity of liposomes.

Transwell motility assay

Motility assays were performed with a Transwell (Corning) as described before (Keely, 2001). Briefly, equal numbers of cells were loaded on the upper chamber and cells that migrated towards attractants were fixed with 4% paraformaldehyde followed by

staining with 0.5% crystal violet. Cells were counted in photographs taken from at least five random fields with a Nikon Eclipse TE2000U at ×200 resolution. Statistical analysis was performed with Microsoft Excel, using data from at least three independent experiments. A Transwell with 3.0 μm pores was used for migration assay and 8.0 μm pores for invasion assay.

Subcellular fractionation assay

Cells were lysed in a hypotonic lysis buffer (Del Pozo *et al*, 2002) for 10 min. Then cell lysates were homogenized with 15 strokes of a Dounce homogenizer. Homogenates were centrifuged at 700 *g* for 3 min to pellet nuclei and intact cells. The supernatants were spun at 100 000 *g* for 30 min at 4°C to sediment particulates. The cytosol-containing supernatant was removed and the crude membrane pellet was gently washed with the lysis buffer. Protein concentration was determined in the membrane and cytosolic fractions. Equal amounts of protein were resolved by SDS–PAGE and further analysed by immunoblotting.

Fluorescence microscopy

Glass coverslips were coated with 10 ng/ml COL, fibronectin, gelatin or 10% serum before seeding cells. For Figure 5, coverslips were coated as described previously (Sakurai-Yageta *et al*, 2008). Cells were grown on coverslips placed inside six-well plates until experimental manipulation. Coverslips were washed twice in 37°C PBS, and then fixed with 4% paraformaldehyde, followed by permeabilization with 0.5% Triton X-100 in PBS. The cells were then blocked for 1 h at 25°C in 3% BSA. Primary antibody incubation was performed at 4°C for 12 h, while incubation with fluorophore-conjugated secondary antibodies occurred at 37°C for 45 min. Fluorescence microscopy was performed using a ×60 plan-fluor objective on a Nikon Eclipse TE2000U equipped with a Photometrics CoolSNAP ES CCD camera. Images were captured using MetaMorph v6.3 (Molecular Devices). Images were exported to Photoshop CS2 (Adobe) for final processing and assembly.

Liposome sedimentation assay

Liposomes were prepared as previously described (Papayannopoulos *et al*, 2005). Dried lipids were resuspended with a buffer containing 150 mM KCl, 50 mM HEPES, pH 7.4, 2 mM $MgCl_2$, 2 mM $CaCl_2$ and 300 mM sucrose. After bath sonication for 20 min, the rehydrated lipids were subjected to at least five cycles of freezing and thawing and extruded through a 0.1 μm filter with a lipid extruder (Avanti). Liposome co-sedimentation assay was performed by mixing 0.5 μM of proteins with 2.5 μM of liposomes in the buffer without sucrose. After 10 min of incubation at 25°C, samples were centrifuged at 100 000 *g* for 30 min at 4°C. Pellets were gently washed and resuspended in SDS sample buffer for a final volume equal to the supernatant. Samples were resolved by SDS–PAGE and proteins were detected by either Coomassie staining or immunoblotting.

Live cell imaging

Delta TPG dish (Fisher Scientific) were coated with a gelatin gel as described previously (Sakurai-Yageta *et al*, 2008). Cells were seeded at a density of 1.0×10^4 cells/dish in L15 culture medium and placed in a temperature-controlled chamber of a Nikon Eclipse TE2000U. Time-lapse recording started 3 h after cell plating. Images were collected every 30 or 60 s for over 5 h with a Photometrics CoolSNAP ES CCD camera (Roper Scientific) operated by Metamorph image analysis software (Molecular Devices). Analyses of collected images including tracking the migration path of individual cells and generation of movies were performed with Metamorph.

Actin polymerization assay

Actin polymerization assay was performed as described before (Le Clainche *et al*, 2007). Pyrene-conjugated G-actin (Cytoskeleton) was prepared according to the manufacturer's instructions. Then, 12.5 nM of Arp2/3 complex and 40 nM of N-WASP-ΔB in the presence of GST-IQGAP1-C (50 nM) and/or 5% phosphoinositide-liposomes (2 μM) were incubated for 5 min before the addition of 1.5 μM of pyrene-conjugated G-actin stock. Fluorescence was read immediately after the addition of actin using a PC1 photon counting spectrofluorometer (ISS) set on kinetic mode to read every 20 s for the duration of the assay. PC1 setting was as follows: excitation, 365 nm; emission, 407 mm. Obtained fluorescence density was converted to arbitrary units.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Author contributions: SC and RAA designed experiments; SC, NT and ZL performed experiments; SC, ACH, DBS and RAA wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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SUPPLEMENTARY INFORMATION

Figure S1. PIPKIγ is required for migration and lamellipodium formation

(A) MDA-MB-231 breast cancer cells were infected with lentivirus expressing short hairpin (sh) RNAs against either human PIPKIγ or scrambled control. Infected cells were selected by a cell sorter (viral vector contains GFP coding sequence). Either parental or virus infected cells maintained in normal culture conditions were photographed under an inverted microscope at 200X magnification. White arrows indicate ruffle-like structures.

(B) Either parental or virus infected cells were placed in the upper chamber of a Transwell and cells were allowed to migrate for 12-16 h towards 10% serum as a chemoattractant in the lower chamber. Cells were fixed and stained with a 0.5% crystal violet (CV) solution. CV-positive cells that had migrated across 3.0 μm pores were counted from photographs taken from at least five random fields (bottom). Expression levels of the endogenous proteins were analyzed by immunoblotting of cell lysates with isoform specific PIPKIγ antibodies (top).

(C) ShRAN-resistant DsRed-tagged PIPKIγ isoforms were stably expressed in shPIPKIγ#2 cells. Cells expressing a similar amount of PIPKIγ compared to the control cells were isolated using a cell sorter. With these reconstituted cells, serum-induced chemotaxis was measured with a Transwell as described above (top). Protein expression was confirmed by immunoblotting against the indicated molecules (bottom). WT, wild type. KD, kinase dead. Data are shown as mean ± SD of four independent experiments.

(D) The reconstituted cells allowed to migrate into a scratch wound were fixed after 3 hours and immunostained with the Arp2/3 complex component ARPC2. Images were taken at 400X magnification and the representative images are shown.

(E) Either control or shPIPKIγ#2 cells were grown to confluence. Lawn of cells was scratched and boundaries between cells and cell-free space were photographed at 5, 30,

60, 120, 180 and 240 min after scratching. At least 200 cells were counted for disk-like protrusions. Data are shown as mean ± SD of four experiments.

The experiments described above were performed independently at least four times.

Figure S2. PIPKIγ interacts with IQGAP1 regardless of kinase activity

(A) Identification of the PIPKIγ binding site on IQGAP1. Equal amounts of [^{35}S]methionine-labeled IQGAP1-N, IQGAP1-(2-764), IQGAP1-(763-864) or IQGAP1-C were incubated with 4 μg GST-PIPKIγi1 or GST alone on glutathione beads. Complexes were washed, resolved by SDS-PAGE and processed by autoradiography. An aliquot of [^{35}S]methionine-labeled TNT product that was not subjected to chromatography was processed in parallel (Input).

(B) Control vector or HA-tagged PIPKIγi1 wild type (WT) or kinase dead (KD) mutant was expressed with in MDA-MB-231 cells and endogenous IQGAP1 proteins were immunoprecipitated with an anti-IQGAP1 antibody. Immunoprecipitates were resolved by SDS-PAGE and the associated PIPKIγi1 was analyzed by immunoblotting with an anti-HA antibody.

(C) HA-PIPKIγi1 was co-transfected with Myc-IQGAP1 WT or S1441S/S1443A mutant in MDA-MB-231 cells for 36 h. Then, cells were serum starved for 12 h before treating with 10% FBS for 30 m. Myc-IQGAP1 WT or mutant was immunoprecipitated with an anti-Myc antibody and the associated PIPKIγi1 was analyzed by immunoblotting with an anti-HA antibody (top). Data are shown as mean ± SD of three independent experiments (bottom).

(D) The reconstituted MEFs were used for 5 μM lysophosphatidic acid induced cell migration using a Transwell. Data are shown as mean ± SD of three independent experiments.

(E) Myc-IQGAP1 was co-transfected with HA-PIPKIγi1 WT or E111L mutant in MDA-MB-

231 cells for 48 h. Cells were harvested and exogenous IQGAP1 was immunoprecipitated with an anti-Myc antibody and the associated PIPKIγi1 was analyzed by immunoblotting with an anti-HA antibody.

(F) MDA-MB-231 cells were transfected with the indicated IQGAP1 proteins or mock control for 48 h. Cells were harvested with a hypotonic buffer and the membrane fraction was separated from the cytosolic fraction by centrifugation. 10 μg of each protein was resolved by SDS-PAGE and analyzed by immunoblotting with the indicated antibodies (top). The percentage of protein bound in the pellet relative to total (S+P) was calculated by quantifying the immunoblots (bottom).

The experiments described above were performed independently at least four times.

Figure S3. IQGAP1 interacts with PIP_2 and sequestration of PIP_2 blocks IQGAP1 targeting to membrane

(A) 0.01 μM of GST-tagged IQGAP1 full length (FL), N- or C-terminal half was incubated with Pip Strips (Eschelon Bioscience) for 1 h at room temperature and bound proteins were detected by immunoblotting with an anti-GST antibody. LPA, lysophosphatidic acid. LPC, lysophosphocholine. PE, phosphatidylethanolamine. PC, phosphatidylcholine. S1P, sphingosine 1-phosphate. PA, phosphatidic acid. PS, phosphatidylserine.

(B) Myc-tagged IQGAP1 wild type or mutants was co-expressed with HA-tagged PIPKIγi1 in HEK293 cells and exogenous IQGAP1 proteins were immunoprecipitated with an anti-Myc antibody. Immunoprecipitates were resolved by SDS-PAGE and the associated PIPKIγi1 was analyzed by immunoblotting with an anti-HA antibody.

(C) IQGAP1 proteins from the reconstituted MEFs were immunoprecipitated with an anti-GFP antibody. Immunoprecipitates were resolved by SDS-PAGE and the associated PIPKIγ was analyzed by immunoblotting with an anti-PIPKIγ antibody.

(D) Before plating, MDA-MB-231 cells were treated with either vehicle or 1 mM neomycin

(Calbiochem) for 10 min, or transfected with either vector control or GFP-PLCδ1-PH for 24 h. Cells were plated on 10 ng/ml collagen I-coated culture dish for 1 h and similar fractionation assay was performed as Fig. 3 A. Equal amount of proteins (10 μg each) were resolved by SDS-PAGE and analyzed by immunoblotting with the indicated antibodies.

(E) MDA-MB-231 cells were transfected with Myc-IQ domain alone or mock control for 48 h. Cells were harvested with a hypotonic buffer and the membrane fraction was separated from the cytosolic fraction by centrifugation. 10 μg of each protein was resolved by SDS-PAGE and analyzed by immunoblotting with the indicated antibodies (top). The percentage of protein bound in the pellet relative to total (S+P) was calculated by quantifying the immunoblots (bottom). Data are shown as mean ± SD of three independent experiments.

(F) Cell lysates from reconstituted MEFs were used for immunoprecipitating endogenous N-WASP. Immunoprecipitates were resolved by SDS-PAGE and the associated IQGAP1 was analyzed by immunoblotting with an anti-IQGAP1 antibody.

All the experiments described above were performed independently at least three times.

Figure S4. PIPKIγ- and PIP_2-binding of IQGAP1 are required for directionally persistent migration

(A) The reconstituted MEFs were plated on gelatin gel for 3 h before recording using time-lapse microscopy at 400X. To locate cells expressing GFP-positive IQGAP1 proteins, cells were first photographed under a fluorescent channel. Immediately after, cells were imaged every 5 min for 3 h to generate the movies shown in videos 1-3.

(B) MDA-MB-231 cells were transfected with mock control or increasing amount of GFP-PLCδ1-PH DNA for 4h. Cells plated on collagen I for 1h were fixed and immunostained with endogenous IQGAP1. Cells were photographed at 400X magnification.

All the experiments described above were performed independently at least three times.

Figure S5. Enhancement of actin polymerization is specific to PI4,5P$_2$

(A and B) Actin polymerization (1.5 μM of pyrene-conjugated G-actin, 12.5 nM of Arp2/3 comple and 40 nM of N-WASP-ΔB) in the presence of the indicated combinations of GST-IQGAP1-C (50 nM) or 5% phosphoinositide-liposomes (2 μM).

(C) 0.1 μM of His-C2 WT or AA3 mutant were incubated with 1 μM of GST-C1 immobilized on glutathione beads in the absence or presence of the indicated phosphoinositide-liposomes for 10 m. Liposome-bound proteins were detected by immunoblotting with an anti-His antibody.

(D) Actin polymerization was performed in the presence of 50 nM GST-IQGAP1-C with the indicated liposomes.

All the experiments described above were performed independently at least three times.

Supplementary Movie 1. Migration of wild type IQGAP1-reconstituted *Iqgap1*$^{-/-}$ MEFs

Iqgap1 KO MEFs were reconstituted with WT IQGAP1. Then, reconstituted MEFs were plated on gelatin gel for 3 h before recording using time-lapse microscopy. Images were collected every 5 min for 3 h at 400X magnification and combined into a time-lapse movie.

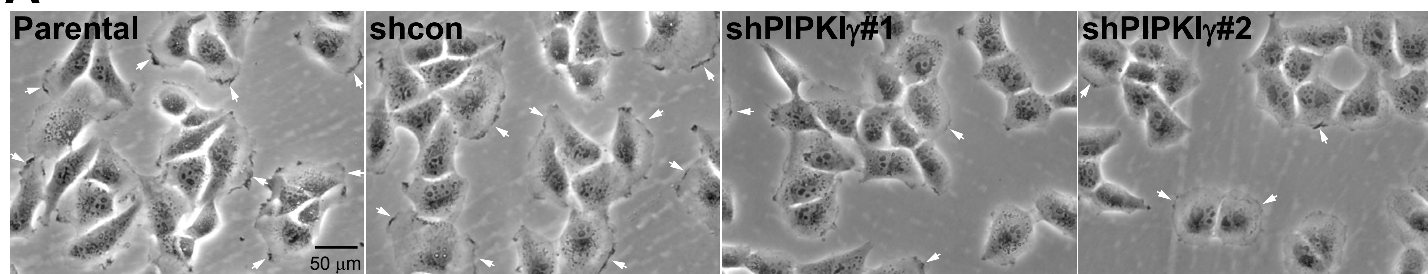
Supplementary Movie 2. Migration of ΔIQ-reconstituted *Iqgap1*$^{-/-}$ MEFs

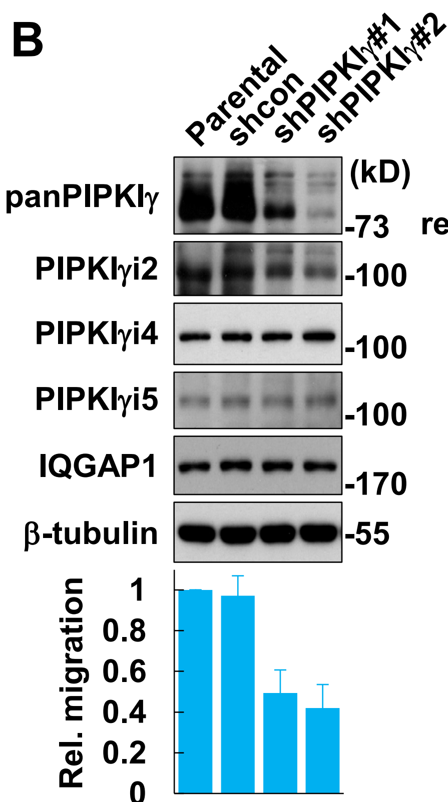
Iqgap1 KO MEFs were reconstituted with the ΔIQ mutant IQGAP1. Then, reconstituted MEFs were plated on gelatin gel for 3 h before recording using time-lapse microscopy. Images were collected every 5 min for 3 h at 400X magnification and combined into a time-lapse movie.

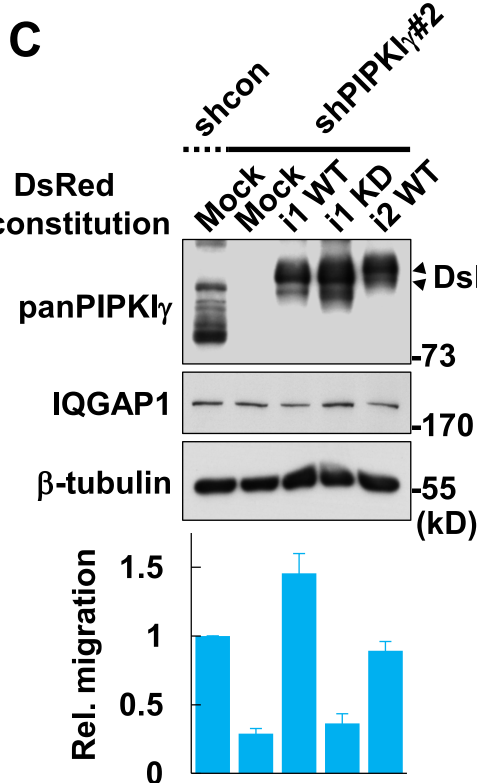
Supplementary Movie 3. Migration of AA3-reconstituted *Iqgap1*$^{-/-}$ MEFs

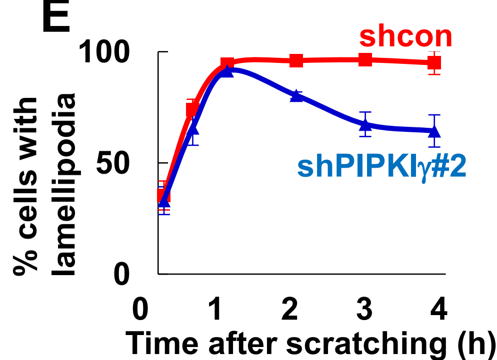
Iqgap1 KO MEFs were reconstituted with the AA3 mutant IQGAP1. Then, reconstituted MEFs were plated on gelatin gel for 3 h before recording using time-lapse microscopy. Images were collected every 5 min for 3 h at 400X magnification and combined into a time-lapse movie.

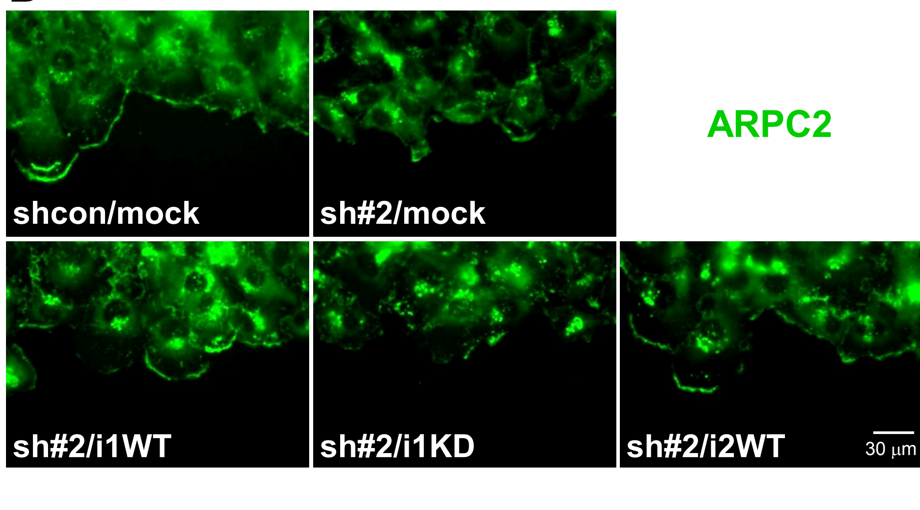
Figure S1

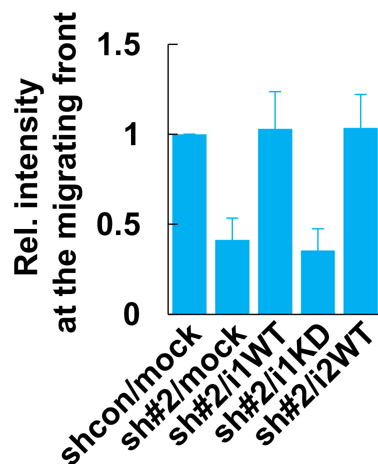












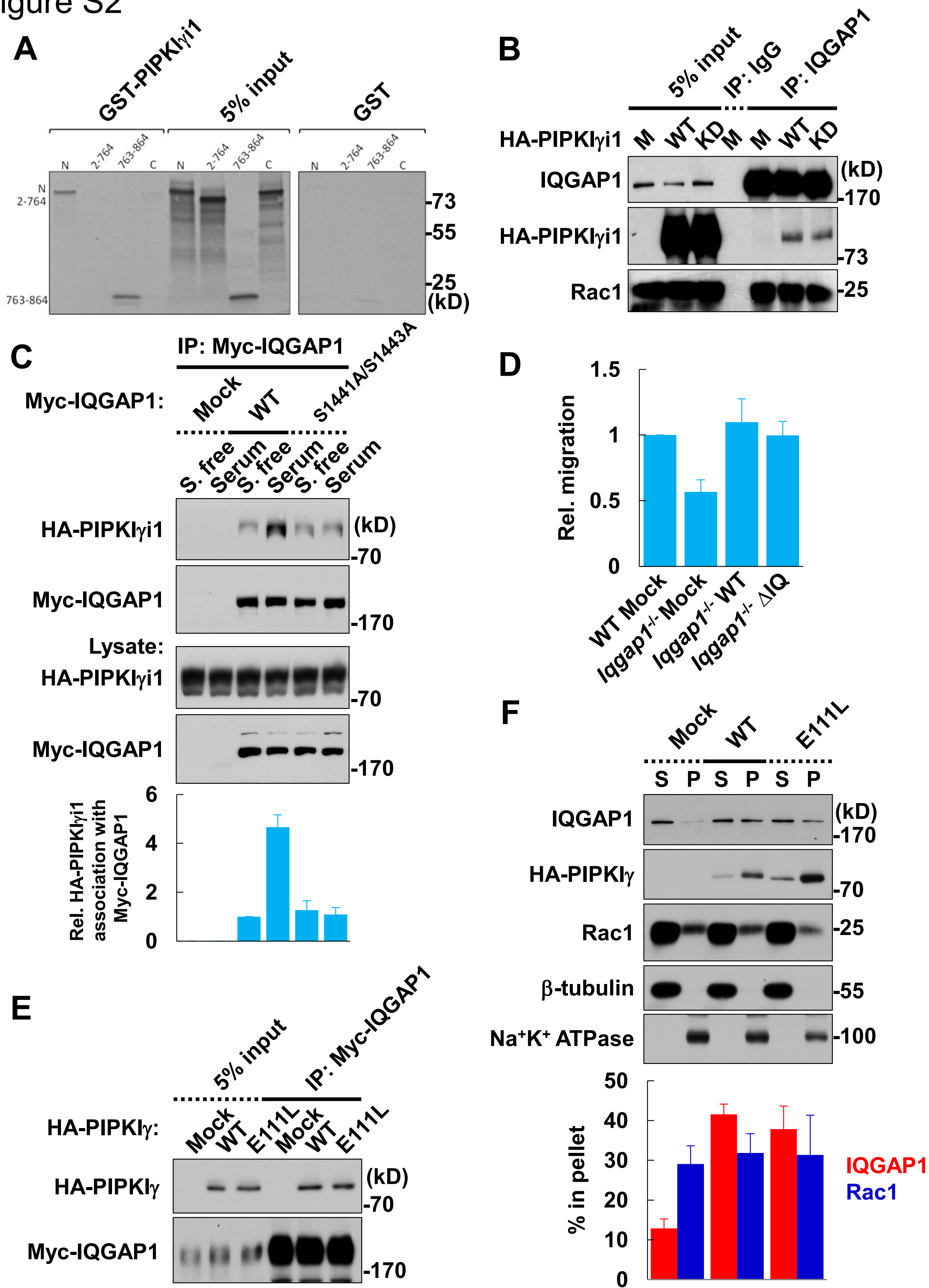
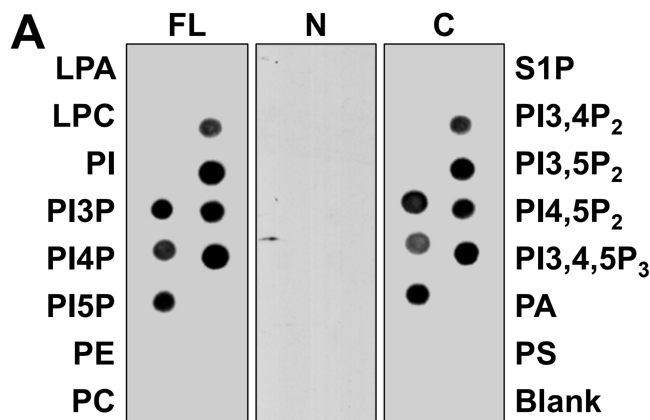
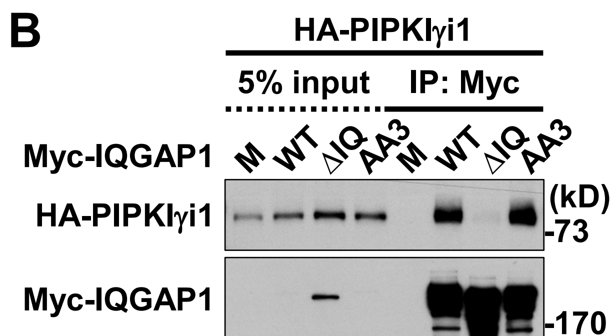
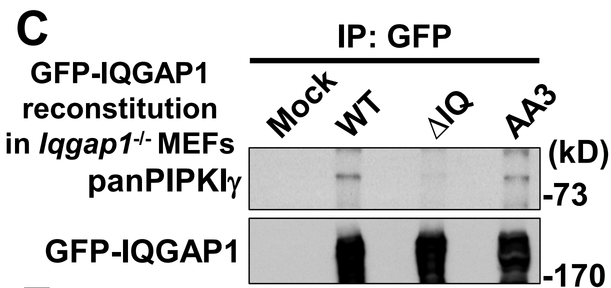


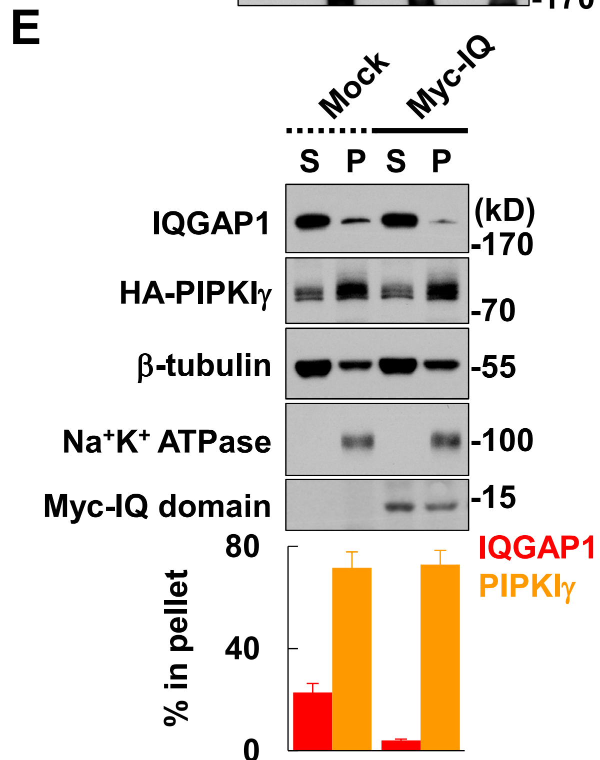
Figure S2
A
GST-PIPKIγi1
5% input
GST
N
2-764
763-864
C
-73
-55
-25
(kD)
B
5% input
IP: IgG
IP: IQGAP1
HA-PIPKIγi1
M
WT
KD
IQGAP1
HA-PIPKIγi1
Rac1
(kD)
-170
-73
-25
C
IP: Myc-IQGAP1
Myc-IQGAP1:
Mock
WT
S1441A/S1443A
S. free
Serum
HA-PIPKIγi1
Myc-IQGAP1
Lysate:
(kD)
-70
-170
Rel. HA-PIPKIγi1 association with Myc-IQGAP1
6
4
2
0
D
Rel. migration
1.5
1
0.5
0
WT Mock
Iqgap1-/- Mock
Iqgap1-/- WT
Iqgap1-/- ΔIQ
E
5% input
IP: Myc-IQGAP1
HA-PIPKIγ:
Mock
WT
E111L
HA-PIPKIγ
Myc-IQGAP1
(kD)
-70
-170
F
Mock
WT
E111L
S
P
IQGAP1
HA-PIPKIγ
Rac1
β-tubulin
Na+K+ ATPase
(kD)
-170
-70
-25
-55
-100
% in pellet
50
40
30
20
10
0
IQGAP1
Rac1

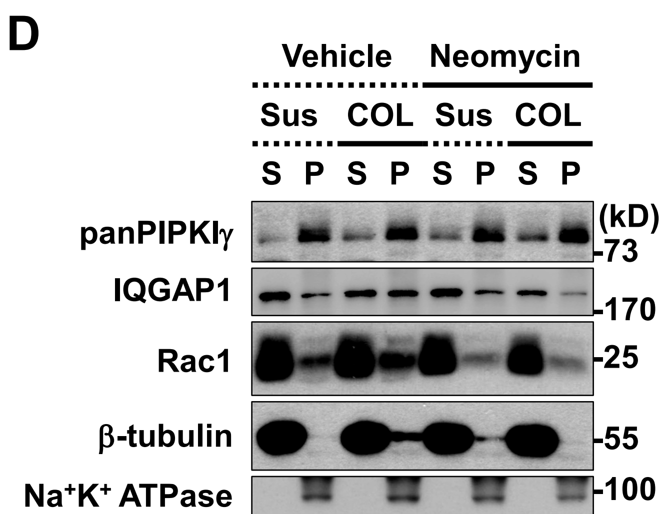
Figure S3

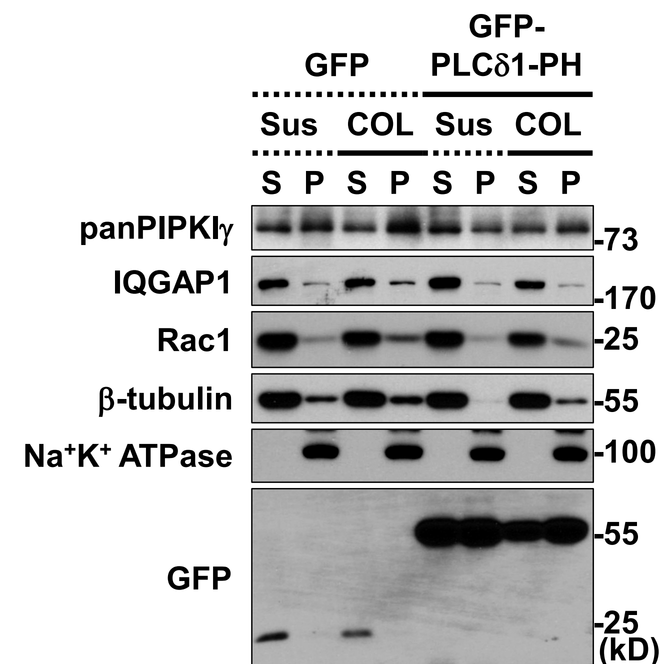












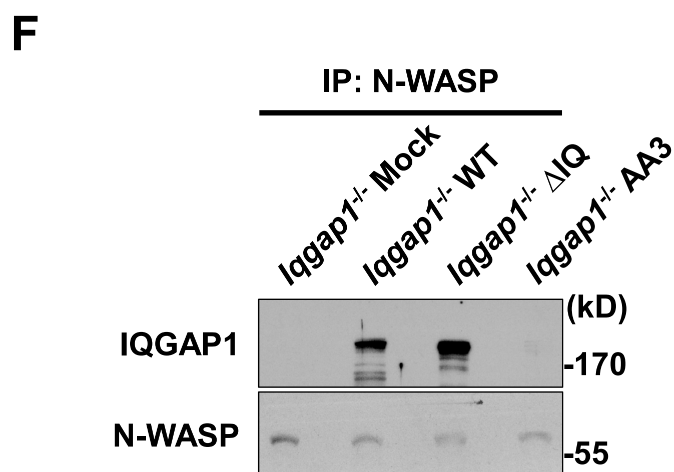
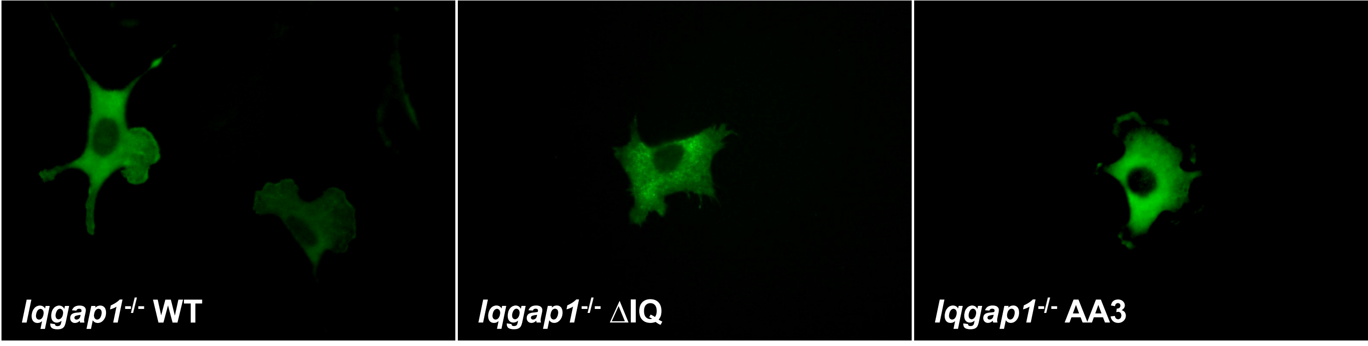


Figure S4

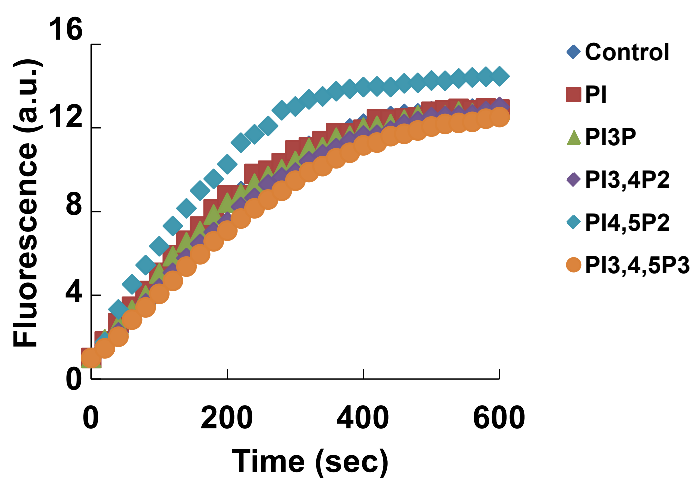
A

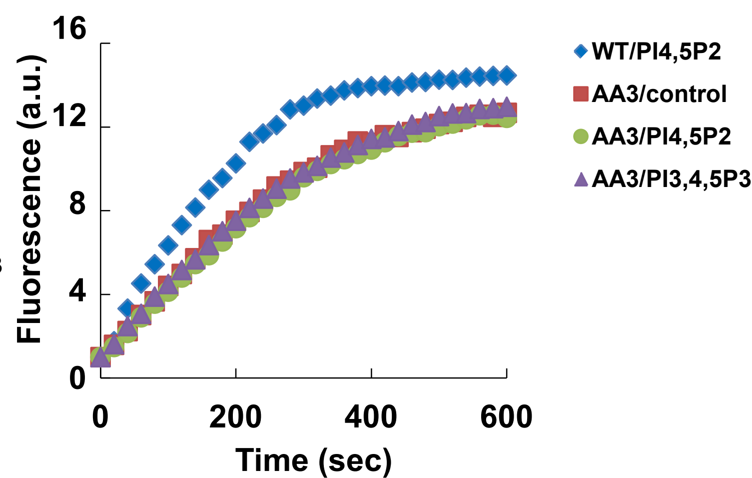


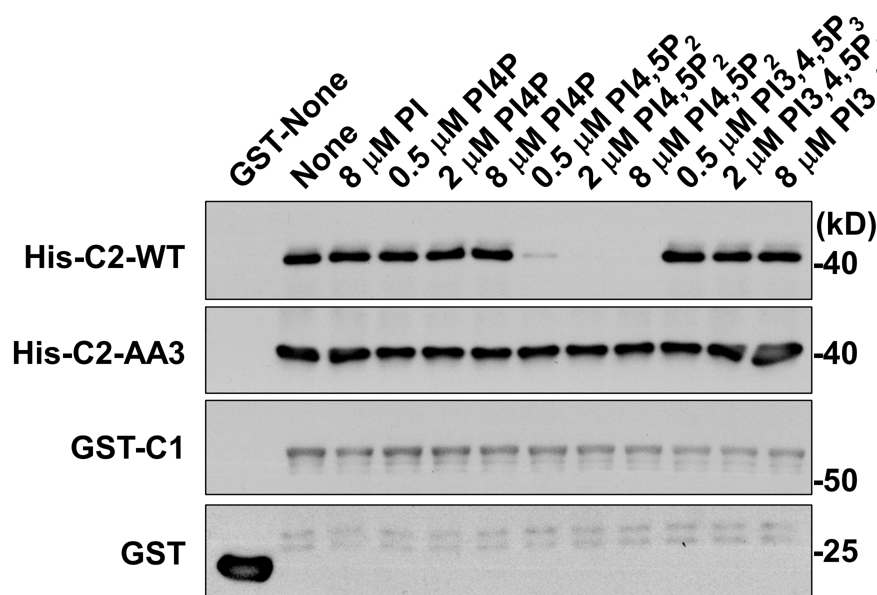
B

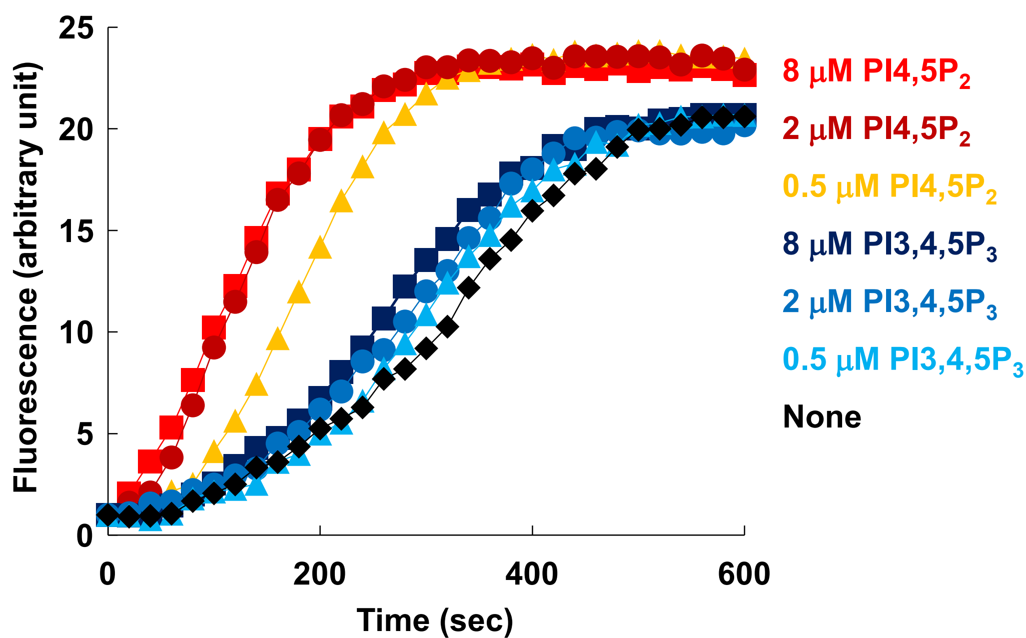
GFP-PLCδ1-PH
IQGAP1
Overlay
-
+
++
+++
30 μm
GFP-PLCδ1-PH

Figure S5









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IQGAP1 is a novel phosphatidylinositol 4,5 bisphosphate effector in regulation of directional cell migration

Suyong Choi, Narendra Thapa, Andrew C. , Zhigang Li, David B. Sacks and Richard A. Anderson

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Editor: Andrea Leibfried

1st Editorial Decision 02 April 2013

Thank you for submitting your manuscript entitled 'IQGAP1 is a novel PIP2 effector in regulation of directional cell migration'. I have now received the three reports on your paper.

As you can see below, all referees value your results but have some technical concerns or would like to have some additional information to substantiate the data. Given the comments provided, I would like to invite you to submit a revised version of the manuscript, addressing the concerns of the referees.

I should also add that it is EMBO Journal policy to allow only a single round of revision and that it is therefore important to address the concerns raised at this stage.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE COMMENTS

Referee #1

This is an interesting, data-rich manuscript reporting a novel interaction between PIPKIg (type I gamma phosphatidylinositol 4-phosphate 5-kinase) and IQGAP1, which is found to be required for persistent cell migration. This IQGAP1/PIPKIg interaction, which involves the IQ domain of IQGAP1, is required for recruitment of IQGAP1 to the cell leading edge, while PI4,5P2 produced by PIPKIg contributes to the opening and activation of IQGAP1 by counteracting an intramolecular interaction of the GRD and RGCT domains in the carboxy-terminal region of IQGAP1. As a consequence, IQGAP1-dependent actin assembly can be restricted spatially ensuring protrusion formation and persistent cell migration. Appropriate controls are provided and data support the main conclusions of the manuscript.

Specific comments

1- Proper quantification of Arp2/3 complex accumulations and the effect of PIPKIg knockdown should be provided (Fig. S1D). Along the same line, experiments showing rescue of IQGAP1 recruitment to the cell edge by expression of PIPKIgi1 and i2 should be properly quantified (Fig. 3D).

2- The finding (Fig. 2E) that optimal serum-induced migration of iqgap-null MEFs can be rescued by IQGAP1 independently of the presence of the IQ domain and thus does not require the interaction of IQGAP1 with PIPKIg seems to contradict data in Fig. 2A-C indicating synergistic functions of the two proteins during serum-induced migration.

3- The authors conclude that PIP2 (produced by PIPKIg) activates IQGAP1-mediated actin assembly. Silencing of PIPKIg interferes with the recruitment of IQGAP1 but also Rac1 recruitment to the leading edge (Fig. 3B), which is likely to affect cell migration and possibly IQGAP1 recruitment of the leading edge.

Referee #2

This manuscript identifies a new interaction between PIPKIgamma and the IQ domain of the cytoskeletal regulator IQGAP1. Evidence is provided that this interaction is potentiated by appropriate ECM/growth factor stimulation and is important for the localisation of IQGAP1 at the pm, where interaction with the product of PIPKIgamma, PI45P2, 'de-represses' IQGAP1, allowing it to stimulate actin polymerisation.

A lot of technically well executed work is presented. The conceptual novelty is limited; PIPKIgamma, IQGAP1 and PIP2 are known to regulate actin polymerisation at emerging lamellipods, PIP2 has been shown to bind IQGAP1 and the concept that PIPKIs recruit effectors of PIP2 is established. However, this study does bring together several strands of research into a potentially satisfying explanation of the role of PIPKIgamma and IQGAP1 in cell migration.

Specific comments:

1. The data presented in Fig1 showing PIPKIgamma and IQGAP1 interact as endogenous proteins, 'in transfecto' and as recombinant proteins is both compelling and significant. The data presented in Fig 2 suggesting this interaction is important for cell migration is less easy to interpret. The relative effects of PIPKIgamma and IQGAP1 overexpression/knock-down on migration do not directly address this point and, whilst the inability of the 'delta-IQ' mutant to rescue the migration defects of the IQGAP1-KO MEFs provides essential corroborative evidence, it only says that the IQ domain is

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needed, not that the domain is needed for interaction with PIPKIgamma (the IQ domain is known to interact with other proteins). In this regard, the demonstration that the interaction between PIPKIgamma and IQGAP1 is stimulated by collagen and serum is important (fig 2D); given that the recombinant proteins interact constitutively, do the authors have any evidence as to how this is regulated?

2. The binding of phosphoinositides to IQGAP is obviously complicated, as illustrated by the data presented in this manuscript and in the recent work from the Dundee groups (Dixon et al 2012). The 'AA3' mutant does however seem to be specifically deficient in its ability to bind PI45P2 (Figs 4F and 6B). These assays are presented at a single concentration of phosphoinositides, which makes relative comparisons difficult; do the authors have data which illustrates the extent of this specificity? I would also be interested in knowing how PI345P3 behaves in the actin polymerisation assay (Fig 6C).

Minor points

1. It would be easier for the non-expert if the various isoforms of PIPKIgamma (i1-5) were explained before the discussion.

2. The legend to Fig 7 needs to explain more clearly what the non- PIPKIgamma-complexed IQGAP is doing.

Referee #3

The paper by Choi et al. describes the discovery of a novel interaction of the cytoskeletal scaffold protein IQGAP1 and the type Igamma phosphatidylinositol 4-phosphate 5-kinase (PIPKIgamma) with important consequences for motile cell behavior. An affinity approach, using PIPKIgamma as a bait protein, identified IQGAP1 as a potential interacting partner. This interaction was verified using co-immunoprecipitation experiments and also by the use of purified proteins and the IQ-motif of IQGAP1 was mapped as a region required for interaction with PIPKIgamma. Furthermore the authors' show that this interaction is dynamically regulated in response to extracellular signals like serum or adhesion to collagen and seems to work as important signal hub for the regulation of lamellipodia formation and subsequent cellular motility. The authors show that PIPKIgamma is critical for IQGAP1 plasma membrane localization where IQGAP1 binds to PIP2, which relieves the autoinhibitory interaction of the IQGAP1 Gap Related Domain (GRD) and the C-terminus (RGCT). Relieve of this intramolecular interaction through PIP2 binding is proposed to facilitate activation of N-WASP for localized actin polymerization via Arp2/3.

This study is overall well conducted and the data provided in this manuscript are definitively very interesting and would be important to publish, but there are significant issues that need to be addressed as outlined below.

The PIP2-mediated activation is an important claim and should be further substantiated by additional controls. The effects on in vitro actin polymerization are rather minor. In this line it would be good to map the interaction interface of PIPKI required to interact with IQGAP1 and test whether this mutant is still able to promote changes in actin dynamics. The authors also propose that PIP2-mediated activation of IQGAP1 promotes interaction with downstream effectors like N-WASP, but data regarding this issue are missing. It would be interesting to see how silencing of PIPKI, mutation of the PIP2 binding motif or the IQ motif affects N-WASP binding in cells.

Another point that puzzles me is the effect of the PIP2-binding deficient mutant on cellular polarity. In principle, the use of IQGAP1 deficient MEFs reconstituted with IQGAP variants is a very elegant approach to address the functional role of specific IQGAP1 regions with regard to cellular motility. I

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wonder, however, how the PIP2-binding deficient mutant is able to promote multiple lamella formation. Since the authors propose that PIP2-binding may contribute to IQGAP1 activation by opening up the intramolecular GRD/RGCT interaction, which facilitates binding to downstream effector proteins like N-WASP to promote local actin polymerization, the multiple lamella phenotype seems contradictory to me. How is this working? These structures are highly dynamic and depend on actin polymerization. The PIP2-binding deficient mutant of IQGAP1 is clearly enriched in these structures. How does IQGAP1 coordinate actin polymerization in these structures if binding to PIP2 is abolished?

If PIPKI is a critical factor for IQGAP1 localization, does the overexpression of the isolated IQ-motif changes the subcellular localization of endogenous IQGAP1?

Data regarding the effect of expression of PH-PLCgamma are contradictory to me. The authors show that endogenous IQGAP1 co-localizes with GFP-PH-PLCgamma at the plasma membrane. In the supplement the authors propose on the basis of fractionation experiments, that expression of PH-PLCgamma strips IQGAP1 off the plasma membrane. These fractionation data are not very convincing and miss quantifications and they should be done using membrane flotation assays. Also, the authors claim that PIPKI-binding is more important for localization of IQGAP1 and not PIP2 binding. I suggest clarifying this issue e.g. by titrating the PH-PLC and analyze endogenous IQGAP1 localization under these conditions.

Minor points:

I had problems reading the manuscript, the figure legends should be more informative. E.g. the direct association of IQGAP1 and PIPKI show in figure 1f contains a control blot for GST at the bottom, but it is not clear and not clarified what is shown here.

The immunoblots shown in this work are largely overexposed and it would be more informative to show lower exposures of the films. The input levels for many of the interaction data are missing and should be included.

Would it be possible to provide more informations on the invasion data? It would be interesting to see, how these cells behave, how the cellular morphology is changed in a 3-D matrix after silencing of IQGAP1 or PIPKI. The assay is not well described and it is not clear to me how this was done. Why is there a synergistic effect after silencing of both, IQGAP1 and PIPKI, if the authors postulate a linear signal transduction cascade, starting with the recruitment of IQGAP1 by PIPKI?

The schematic view in figure 7 is, at least to this reviewer, not helpful and I suggest overworking this cartoon.

1st Revision - authors' response 30 June 2013

We would like to thank the referees for their invaluable comments and suggestions. Below we detail the changes to the revised manuscript that address the *referees' comments* followed by the revisions that we have made.

Referee #1:

This is an interesting, data-rich manuscript reporting a novel interaction between PIPKIg (type I gamma phosphatidylinositol 4-phosphate 5-kinase) and IQGAP1, which is found to be required for persistent cell migration. This IQGAP1/PIPKIg interaction, which involves the IQ domain of IQGAP1, is required for recruitment of IQGAP1 to the cell leading edge, while PI4,5P$_2$ produced by PIPKIγ contributes to the opening and activation of IQGAP1 by counteracting an intramolecular interaction of the GRD and RGCT domains in the carboxy-terminal region of IQGAP1. As a

consequence, IQGAP1-dependent actin assembly can be restricted spatially ensuring protrusion formation and persistent cell migration. Appropriate controls are provided and data support the main conclusions of the manuscript.

Specific comments:
Proper quantification of Arp2/3 complex accumulations and the effect of PIPKIg knockdown should be provided (Fig. S1D). Along the same line, experiments showing rescue of IQGAP1 recruitment to the cell edge by expression of PIPKIgi1 and i2 should be properly quantified (Fig. 3D).
Intensity of fluorescent signals at the migrating front was measured from at least 10 different images of each condition and quantified using ImageJ software (Fig. 3D and Fig. S1D).

The finding (Fig. 2E) that optimal serum-induced migration of Iqgap-null MEFs can be rescued by IQGAP1 independently of the presence of the IQ domain and thus does not require the interaction of IQGAP1 with PIPKIg seems to contradict data in Fig. 2A-C indicating synergistic functions of the two proteins during serum-induced migration.
We are also intrigued by this result. For Fig. 2A-C, serum-induced migration was measured in MDA-MB-231 and HeLa, whereas, MEFs were used for Fig. 2E. These seemingly contradictory observations could be in part explained by cell type specificity. In other words, in MEFs the ΔIQ mutant could mediate serum-induced cell migration independent of PIPKIγ. In support of this notion, in our previous study (Sun et al, 2007), we showed that epidermal growth factor (EGF)-induced cell migration requires PIPKIγ, whereas lysophosphatidic acid (LPA)-induced migration is independent of PIPKIγ. As LPA is one of the most important factors in serum that induces MEF migration (Kim et al, 2008), it is likely that LPA mediates serum-induced MEF migration of the ΔIQ mutant (Fig. S2D).

The authors conclude that PIP_2 (produced by PIPKIg) activates IQGAP1-mediated actin assembly. Silencing of PIPKIg interferes with the recruitment of IQGAP1 but also Rac1 recruitment to the leading edge (Fig. 3B), which is likely to affect cell migration and possibly IQGAP1 recruitment of the leading edge.
We totally agree with the referee's comment. Silencing of PIPKIγ blocks IQGAP1 recruitment to the leading edge (Fig. 3B-D and), which might be indirectly through interference of Rac1 recruitment (Fig. 3B). This is consistent with previous report that the physical interaction of PIPKIs with Rac1 regulates plasma membrane targeting of Rac1 (Chao et al, 2010). To test the sole contribution of PIPKIg for IQGAP1 targeting, we utilized a Rac1 binding defective mutant PIPKIg (E111L) (Halstead et al, 2010). The mutant co-immunoprecipitated with IQGAP1 similar to wild type PIPKIg (Fig. S2E) indicating that Rac1 binding to PIPKIg is not required for the PIPKIg interaction with IQGAP1. Notably, the E111L mutant enhanced IQGAP1 association with the membrane fraction similar to wild type PIPKIg (Fig. S2F). These data suggest that the IQGAP1 recruitment to the leading edge is largely regulated by PIPKIg independent of Rac1.

Referee #2:
This manuscript identifies a new interaction between PIPKIg and the IQ domain of the cytoskeletal regulator IQGAP1. Evidence is provided that this interaction is potentiated by appropriate ECM/growth factor stimulation and is important for the localisation of IQGAP1 at the plasma membrane, where interaction with the product of PIPKIg, $PI4,5P_2$, 'de-represses' IQGAP1, allowing it to stimulate actin polymerisation.

A lot of technically well executed work is presented. The conceptual novelty is limited; PIPKIg, IQGAP1 and PIP_2 are known to regulate actin polymerization at emerging lamellipods, PIP_2 has been shown to bind IQGAP1 and the concept that PIPKIs recruit effectors of PIP_2 is established. However, this study does bring together several strands of research into a potentially satisfying explanation of the role of PIPKIg and IQGAP1 in cell migration.

Specific comments:
The data presented in Fig. 1 showing PIPKIg and IQGAP1 interact as endogenous proteins, 'in transfecto' and as recombinant proteins is both compelling and significant. The data presented in Fig. 2 suggesting this interaction is important for cell migration is less easy to interpret. The relative effects of PIPKIg and IQGAP1 overexpression/knock-down on migration do not directly address this point and, whilst the inability of the 'delta-IQ' mutant to rescue the migration defects of the IQGAP1-KO MEFs provides essential corroborative evidence, it only says that the IQ domain is needed, not that the domain is needed for interaction with PIPKIg (the IQ domain is known to

interact with other proteins). In this regard, the demonstration that the interaction between PIPKIg and IQGAP1 is stimulated by collagen and serum is important (Fig 2D); given that the recombinant proteins interact constitutively, do the authors have any evidence as to how this is regulated?
Many common signaling pathways are activated in response to serum and collagen stimuli. Among them, PKC is reported to relieve the autoinhibitory fold of IQGAP1, between the N and C termini, by phosphorylation of Ser1441 and Ser1443 upon activation of GPCRs, RTKs or integrins (Brandt & Grosse, 2007). Because the PIPKIγ binding site within the IQ domain is likely masked by the autoinhibitory fold, Ser1441 and Ser1443 phosphorylation might be required for the PIPKIγ binding. To test this possibility, a phosphorylation defective mutant (S1441A/S1443A) was expressed and the interaction with IQGAP1 was examined by immunoprecipitation. The IQGAP1 interaction with wild type PIPKIg was increased ~4.5 fold in response to serum activation, whereas binding of the phosphorylation defective mutant was not altered (Fig. S2C). These data indicate that the phosphorylation on Ser1441 and Ser1443 of IQGAP1 is required for the PIPKIg binding in response to membrane receptor activation.

The binding of phosphoinositides to IQGAP is obviously complicated, as illustrated by the data presented in this manuscript and in the recent work from the Dundee groups (Dixon et al 2012). The 'AA3' mutant does however seem to be specifically deficient in its ability to bind $PI4,5P_2$ (Fig. 4F and 6B). These assays are presented at a single concentration of phosphoinositides, which makes relative comparisons difficult; do the authors have data which illustrates the extent of this specificity? I would also be interested in knowing how $PI3,4,5P_3$ behaves in the actin polymerization assay (Fig. 6C).
The binding experiments between GST-C1 and His-C2 (WT or AA3) were performed with varying concentrations of different liposomes. As shown in Fig. S5C, PI, PI4P and $PI3,4,5P_3$ had no apparent effect in 0.5 to 8 mM concentration. In contrast, $PI4,5P_2$ dramatically blocked the C1 and C2 interaction even in the lowest concentration (0.5 mM). Consistent with these binding data, $PI4,5P_2$ enhanced actin polymerization of IQGAP1-C in a dose dependent manner, whereas $PI3,4,5P_3$ was much less effective (Fig. S5D).

Minor points:
It would be easier for the non-expert if the various isoforms of PIPKIg (i1-5) were explained before the discussion.
Information on the various PIPKIγ isoforms is included in the introduction section.

The legend to Fig 7 needs to explain more clearly what the non-PIPKIg-complexed IQGAP is doing.
IQGAP1 that is non-complexed with PIPKIγ may accumulate at cell-cell contacts. We have modified the figure to illustrate this possibility (Fig. 7).

Referee #3:
The paper by Choi et al. describes the discovery of a novel interaction of the cytoskeletal scaffold protein IQGAP1 and the type Ig phosphatidylinositol 4-phosphate 5-kinase (PIPKIg) with important consequences for motile cell behavior. An affinity approach, using PIPKIg as a bait protein, identified IQGAP1 as a potential interacting partner. This interaction was verified using co-immunoprecipitation experiments and also by the use of purified proteins and the IQ-motif of IQGAP1 was mapped as a region required for interaction with PIPKIg. Furthermore the authors show that this interaction is dynamically regulated in response to extracellular signals like serum or adhesion to collagen and seems to work as important signal hub for the regulation of lamellipodia formation and subsequent cellular motility. The authors show that PIPKIg is critical for IQGAP1 plasma membrane localization where IQGAP1 binds to PIP_2, which relieves the autoinhibitory interaction of the IQGAP1 Gap Related Domain (GRD) and the C-terminus (RGCT). Relieve of this intramolecular interaction through PIP_2 binding is proposed to facilitate activation of N-WASP for localized actin polymerization via Arp2/3. This study is overall well conducted and the data provided in this manuscript are definitively very interesting and would be important to publish, but there are significant issues that need to be addressed as outlined below.

Specific comments:
The PIP_2-mediated activation is an important claim and should be further substantiated by additional controls.
Dose dependence experiments were performed using multiple phosphoinositide species to test the specificity of $PI4,5P_2$ in regulation of the C1 and C2 interaction and actin polymerization. Data

presented in Fig. S5C-D indicate that $PI4,5P_2$ specifically blocks the C1 interaction with C2 and, as a result, enhances actin polymerization activity of IQGAP1.

The effects on in vitro actin polymerization are rather minor. In this line it would be good to map the interaction interface of PIPKIg required to interact with IQGAP1 and test whether this mutant is still able to promote changes in actin dynamics.

We have evidence that both PIPKIa and PIPKIγ interact with IQGAP1 through the IQ domain (Choi et al., manuscript in preparation). PIPKIa and PIPKIγ have high sequence similarity in the kinase domain, whereas they have highly variable N- and C-terimini (Heck et al, 2007). Thus, it is likely that the IQGAP1 interaction with PIPKIs is mediated by the kinase domain. However, the isolated PIPKI kinase domain is very unstable and truncations or mutations in the kinase domain result in unstable and kinase defective proteins (Coppolino et al, 2002). Thus, we mapped the PIPKIγ binding site on IQGAP1 instead of the IQGAP1 binding site on PIPKIγ.

The authors also propose that PIP_2-mediated activation of IQGAP1 promotes interaction with downstream effectors like N-WASP, but data regarding this issue are missing. It would be interesting to see how silencing of PIPKIg, mutation of the PIP_2 binding motif or the IQ motif affects N-WASP binding in cells.

We tested how mutation of IQGAP1 affects interaction with N-WASP using immunoprecipitation (Fig. S3F). Wild type IQGAP1 interacts with N-WASP, whereas the PIP_2-binging defective (AA3) mutant interaction is dramatically reduced. This is consistent with our hypothesis as these data suggest that PIP_2-binding regulates the recruitment of actin polymerizing machinery to IQGAP1. Intriguingly, the PIPKIg-binding defective (ΔIQ) mutant is able to interact with N-WASP. As the N-WASP binding site of the ΔIQ mutant is intact (Fig. 1G), it is likely that the ΔIQ mutant interacts with the cytosolic pool of N-WASP (Cai et al, 2012; Taunton et al, 2000).

Another point that puzzles me is the effect of the PIP_2-binding deficient mutant on cellular polarity. In principle, the use of IQGAP1 deficient MEFs reconstituted with IQGAP variants is a very elegant approach to address the functional role of specific IQGAP1 regions with regard to cellular motility. I wonder, however, how the PIP_2-binding deficient mutant is able to promote multiple lamella formation. Since the authors propose that PIP_2-binding may contribute to IQGAP1 activation by opening up the intramolecular GRD/RGCT interaction, which facilitates binding to downstream effector proteins like N-WASP to promote local actin polymerization, the multiple lamella phenotype seems contradictory to me. How is this working? These structures are highly dynamic and depend on actin polymerization. The PIP_2-binding deficient mutant of IQGAP1 is clearly enriched in these structures. How does IQGAP1 coordinate actin polymerization in these structures if binding to PIP_2 is abolished?

We totally agree with the referee's comment. As PIP_2-binding of IQGAP1 is critical for N-WASP-mediated actin polymerization at the leading edge, we also predicted that a PIP_2-binding defective mutant might lose its ability to form lamellipodia instead of inducing multiple leading edges. However, this conceptually contradictory observation is not surprising. Previous studies demonstrate that multiple leading edges are induced by perturbation of factors that are important for leading edge formation. For example, Rac1-null neutrophils (Sun et al, 2004) and Cdc42-null dendritic cells (Lammermann et al, 2009) form multiple leading edges. Also, FAK knockdown in Rat-2 cells induces multiple leading edges, and migration is retarded in these cells (Tilghman et al, 2005). Most noteworthy, a previous study (Fukata et al, 2001) reported that an IQGAP1 mutant defective of interaction with Rac1 or Cdc42 induces multiple leading edges in Vero cells. Based on the literature we reason that the PIP_2-binding defective IQGAP1 mutant, AA3, induces multiple leading edges by loss of its ability to maintain persistent lamellipodium formation. The AA3 mutant targets to the leading edge by interaction with PIPKIγ (Fig. 5B) but remains inactive, and that might increase the instability of the lamellipodium as a result of improper actin polymerization (Tilghman et al, 2005).

If PIPKIg is a critical factor for IQGAP1 localization, does the overexpression of the isolated IQ-domain changes the subcellular localization of endogenous IQGAP1?

The IQ domain was expressed in MDA-MB-231 cells and endogenous IQGAP1 targeting was analyzed by fractionation (Fig. S3E). Indeed, the isolated IQ domain significantly reduces the association of endogenous IQGAP1 with the membrane fraction.

Data regarding the effect of expression of PLCd1-PH are contradictory to me. The authors show that endogenous IQGAP1 co-localizes with GFP-PLCd1-PH at the plasma membrane. In the

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supplement the authors propose on the basis of fractionation experiments, that expression of PLCd1-PH strips IQGAP1 off the plasma membrane. These fractionation data are not very convincing and miss quantifications and they should be done using membrane flotation assays. Also, the authors claim that PIPKIg-binding is more important for localization of IQGAP1 and not PIP_2 binding. I suggest clarifying this issue e.g. by titrating the PH-PLCd1 and analyze endogenous IQGAP1 localization under these conditions.

We totally agree with the referee's comment. The PH domain of phospholipase Cδ1 (PLCδ1) has been extensively used to probe cellular PIP_2 (Czech, 2000; Di Paolo & De Camilli, 2006; Raucher et al, 2000) but excessive expression of PLCd1-PH limits the targeting of PIP_2 binding protein to the plasma membrane (Raucher et al, 2000). Thus, we initially titrated the PLCd1-PH expression by transfecting with varying amounts of DNA to define an experimental condition for probing PIP_2 or limiting IQGAP1 targeting to the plasma membrane (Fig. S4B). In the optimal expression condition, endogenous IQGAP1 colocalizes with GFP-PLCd1-PH (white arrowhead). In the excessive expression condition, ~30% of cells seem retracted (yellow arrowhead) and ~20% of cells form lamellipodia that lack IQGAP1 at the periphery (white arrow). We had performed experiments for Fig. 4A and Fig. S3D in the separate conditions (for either probing PiP_2 or limiting IQGAP1 targeting to the plasma membrane) defined.

The data in Fig S3D and Fig. S4B clearly suggest that the PIP_2-binding also contributes to IQGAP1 targeting to the plasma membrane. However, the data in Fig. S3D and Fig. S4B rely on overexpression of PLCd1-PH that possibly strips off all PIP_2-binding proteins from the plasma membrane. Because several factors targeting IQGAP1 to the plasma membrane are PIP_2-binding proteins (Brandt & Grosse, 2007; Fukata et al, 2002; Watanabe et al, 2004), the data in Fig. S3D and Fig. S4B could be misleading. To better understand the sole contribution of the PIP_2-binding for IQGAP1 targeting, we expressed a PIP_2-binding defective mutant (AA3) in *Iqgap1*$^{-/-}$ MEFs. The PIP_2-binding defective mutant still localizes to the plasma membrane, while the PIPKIg-binding defective (ΔIQ) mutant is largely cytosolic (Fig. 5B). These data indicate that the physical interaction between the two proteins is more important than PIP_2-binding for IQGAP1 plasma membrane targeting.

The currently employed membrane fractionation assay has been used extensively to monitor association of proteins with membrane (Chao et al, 2010; Del Pozo et al, 2002). We tried to repeat some fractionation experiments with membrane flotation assay. However, it was technically challenging and failed to detect IQGAP1 in our preparation. This could be due to technical errors or IQGAP1 might be hard to float in the assay, similar to other actin or microtubule associated proteins (Schollenberger et al, 2012; Watanabe et al, 2005).

Minor points:

I had problems reading the manuscript, the figure legends should be more informative. E.g. the direct association of IQGAP1 and PIPKIγ show in Fig. 1F contains a control blot for GST at the bottom, but it is not clear and not clarified what is shown here.

We changed the figure legends to be more informative. For Fig. 1F, all our GST-tagged recombinant proteins expressed in bacterial have some degraded products that are detected by immunoblotting with an anti-GST antibody. To overcome this, we also expressed recombinant IQGAP1 proteins using the baculoviral system that produces less degradation product (Fig. S2A). By both bacterial and baculoviral expression systems, it is shown that the IQ domain alone is sufficient to interact with PIPKIg.

The immunoblots shown in this work are largely overexposed and it would be more informative to show lower exposures of the films. The input levels for many of the interaction data are missing and should be included.

We changed immunoblots with lower exposure images if available. Also, we included input levels for the interaction data.

Would it be possible to provide more informations on the invasion data? It would be interesting to see, how these cells behave, how the cellular morphology is changed in a 3-D matrix after silencing of IQGAP1 or PIPKI.

Cancer cells extend actin-rich protrusions called invadopodia as they invade into a 3-D matrix and IQGAP1 is required for this process (Sakurai-Yageta et al, 2008). PIPKIγ localizes at invadopodia and PIPKIγ knockdown significantly reduces invadopodia formation (Choi et al. manuscript preparation). We have not examined morphological changes in a 3-D matrix after manipulation of IQGAP1 or PIPKIg but it would be very interesting to study.

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The assay is not well described and it is not clear to me how this was done.
The invasion assay was performed as previously described (Keely, 2001). Matrigel (BD Bioscience) is a liquid form on ice. Elevating temperature by incubating at 37°C will induce gelling. By doing so, we coated the top part of a Transwell insert (Corning) with 2 mg/ml of low serum Matrigel. Serum induced cell invasion through the gel was measured by placing 10% serum in the lower chamber of a Transwell.

Why is there a synergistic effect after silencing of both, IQGAP1 and PIPKIg, if the authors postulate a linear signal transduction cascade, starting with the recruitment of IQGAP1 by PIPKIg?
We totally agree with the referee's comment. Although this study defines how PIPKIg may contribute toward IQGAP1 regulated migration, we do not postulate a linear pathway. As shown in Fig. 2B, overexpression of PIPKIγ or IQGAP1 enhances cell motility, and that is dependent on the expression of the other protein. Additionally, Fig. 2A demonstrates functional synergism of the two proteins in cell motility. The defined mechanism in this study is that PIPKIγ recruits IQGAP1 to the leading edge and activates IQGAP1 by production of PIP_2, and this seems to support a linear pathway. However, we envision that IQGAP1 might regulate PIPKIg function in cell motility. For example, among the diverse proteins that interact with IQGAP1 are many that can activate PIPKI's kinase activity, such as Arf6 (Hu et al., Cancer Res, 2009). Thus, we postulate the association of PIPKIγ with IQGAP1 might enhance PIPKIγ's kinase activity to enhance PIP_2 levels that can both directly regulate IQGAP1 activity, but also stimulate migration, such as by modulating actin regulatory proteins. This is currently under investigation as a part of different project.

The schematic view in Figure 7 is, at least to this reviewer, not helpful and I suggest overworking this cartoon.
We changed the model to make it more informative (Fig. 7).

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2nd Editorial Decision 29 July 2013

I have now received comments from two of the original referees of your manuscript that are both satisfied with the amount of revisions and thus support publication.
I would be grateful at this stage if you were to provide original source data, particularly uncropped/-processed electrophoretic blots for the main figures of your manuscript. This is in accord with our policy to make original results better accessible for the community and thus increase reliability of published data. We would welcome one PDF-file per figure for this information. These will be linked online as supplementary "Source Data" files.
Please allow me to congratulate you to this study at this point. The editorial office will be in touch soon with an official acceptance letter.

REFEREE COMMENTS

Referee #1

The revised submission clearly improved a lot and addressed all points to my satisfaction. I therefore recommend publication of the manuscript in its present state.

Referee #3

All our initial concerns have been addressed by the authors.

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