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

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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

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

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

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.

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).

References:

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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.

