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Production of phosphatidylinositol 5-phosphate via PIKfyve and MTMR3 regulates cell migration

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 21 June 2012

Thank you very much for the submission of your research manuscript to our editorial office. We have now received the full set of reports from the referees that were asked to assess it.

As the detailed reports are pasted below I will only repeat the main points here. You will see that while the reviewers acknowledge the potential interest of the findings, they also agree that in some instances additional work is needed to substantiate the idea that PI5P regulates cell migration. Referee 1 feels that further insight into the mechanism by which PI5P regulates migration should be provided and suggests possible ways of how to achieve this. S/he also states that it would need to be excluded that migration defects in cells with altered PI5P levels are due to reduced survival/increased toxicity. In addition, s/he mentions that some additional controls and further clarifications are needed. Both referees 1 and 3 feel that the link to FGD proteins as effectors of PI5P should be strengthened. Referee 3 also states that s/he did not consider the data indicating that PIKfyve and MTM3 exert their effect via PI5P to be fully convincing yet. The main concern of referee 2 is that stronger evidence for the role of PIKfyve on cell migration should be provided.

Overall, given these evaluations, the reviewers constructive comments and the potential interest of the study, I would like to give you the opportunity to revise your manuscript, with the understanding

that the main concerns of the referees (as outlined above and in their reports) must be addressed. Acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports policy to allow a single round of revision only and that therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

I look forward to seeing a revised form of your manuscript when it is ready.

Yours sincerely

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

In this manuscript, the authors identify a novel role for phosphatidylinositol-5-phosphate (PI5P) in promoting cell migration. Using an siRNA screen targeting genes that encode putative PI3P-binding proteins/effectors (due to the presence in their sequence of FYVE or PX modules) in combination with a cell migration assay, they identify two lipid enzymes controlling levels of PI3P/PI5P/PI(3,5)P2, namely MTMR3 and PIKfyve. Based on data showing that PI5P levels increase in response to migratory stimulation, they speculate that PI5P, rather than his precursors PI3P and PI(3,5)P2, may be the PI species directly mediating this effect. To test this, in vitro and in vivo siRNA models are used to place the production of PI5P in phosphoinositide interconversion pathway involving the enzymatic activities of the lipid kinases Vps34 and PIKFyve and the phosphatase MTMR3. In part using elegant synthetic lipid "add back" experiments, the authors convincingly show that PI5P is a key regulator of cell migration.

PI5P is the most enigmatic species of the phosphoinositide family; therefore, the author's findings provide an exciting investigation into determining one of PI5P's potential roles. The authors do a comprehensive job showing PI5P is a positive regulator of cellular migration and identify a potential pathway for PI5P production in this context. Overall, this investigation is well done, although a key question is the nature of PI5P effectors involved in cell migration. Additionally, the mechanism by which PI5P is regulating cell migration should be further explored.

Main issues:
1) The authors convincingly show that PI5P is important for cell motility, but they provide little information on why this is happening. The authors should at the very least examine the integrity and potentially dynamics of focal adhesions in fibroblasts containing lower PI5P levels using markers, such as FAK, talin and vinculin. Additionally, does PI5P control the trafficking of integrins? This is particularly important because all the enzymes directly or indirectly involved in PI5P production also control the traffic/degradation of integrins presumably via PI3P-dependent processes. For instance, are there differences in total and cell surface levels of the relevant integrins in response to PI5P changes?

2) A decrease in PI5P during migratory stimulation is observed upon knock-down of MTMR3 and PIKfyve when compared to the control in Figure 4B; however, it is important to know the basal levels of PI5P for all the conditions not just the control. Whether there is still a burst in PI5P upon migration in the knock down cells cannot be determined from the data, which is key in ruling out alternative sources here.

3) The proposed source of PI5P synthesis during migration consists of enzymes known to be crucial for maintaining normal endo-/lysosomal functions. Can the authors rule out simple effects of cell toxicity on the migration phenotypes? In other words, wouldn't dying or sick cells be expected to migrate less efficiently? The authors should show that the poor health of the manipulated cells is not a confounding factor in the interpretation of the phenotype by assessing the extent of cell death using standard techniques.

4. While the identification of PI5P effectors mediating cell migratory processes goes beyond the scope of this manuscript, the authors identified FGD1, a PI3P and PI5P effector, as another hit. At the very least, they should test whether overexpressing this factor can rescue the cell migration phenotype observed in the Mtmr3/Pikfyve KD cells and whether this rescue depends on its lipid-binding module.

Minor comments:
5) It is mentioned that HRS was identified as a strong hit in the siRNA cell migration screen; however, it is not listed in Supplemental Figure 1A. Is HRS on the x-axis mislabeled HGS?
6) Previous work from the De Camilli and Anderson labs has connected PI metabolism to focal adhesion dynamics and cell migration. These studies should perhaps be cited to make a stronger case about the involvement of PIs in cell migration.

Referee #2:

Oppelt et al report here a role of PtdIns5P in cell migration. Via a migration screen for proteins containing PtdIns3P-binding motifs, the authors identified PI-5 kinase PIKfyve and PI-3 phosphatase MTMR3. The authors indicate that the two proteins constitute a phosphoinositide loop producing PtdIns5P via PtdIns3,5P2 and modulate cell migration. The results are potentially very interesting but there are significant issues that need to be clarified:

Major comments:

1. The mechanism of Ptdlns5P-regulation of cell migration is poorly defined. Vps34 and PIKfyve were found to localize at endosomes. What is the spatio-temporal distribution of Ptdlns(5)P in migrating cells? Could PIKfyve and MTMR3 modulate PtdIns5P level at the leading edge? These need to be, at least discussed in the manuscript.

2. In this study, FGF1 was used to induce BJ cell migration. Is PtdIns5P required for random migration without agonist treatment? The author indicated that PIKfyve and MTMR3 constituted a phosphoinositide loop producing PtdIns5P via PtdIns3,5P2. Does PIKfyve and MTMR3 colocalize to the same compartments?

3. In the migration screening (supplementary Figure 1), were different ON-TARGET plus Control siRNA (ON) or the same control siRNA used in the screen? Why the ratios (migrated cells per total cells) for each control siRNA are quite different? If different control siRNA were used, did it indicate that different control siRNA could lead to quite different results? If the same control siRNA used, did it indicate there were variations between each different experiment?

4. The authors observe only minute increases in cell migration for the rescue after exogenous addition of Ptdlns(5)P to the cells. How can this be explained?

5. In Figure S1, the western and mRNA expression show that the knockdown effect of PIKfyve siRNA 1 and 2 are similar or even better than siRNA 3 and 4. But PIKfyve siRNA 1 and 2 have no effect on cell migration. The results are also inconsistent with the author claimed that PIKfyve siRNA 3 and 4 yielded strongest knockdown. Knockdown and rescue assay should be used for detecting the role of PIKfyve and MTMR3 in cell migration.

Other points:

1. In Figure 4, it is confusing that basal level of PtdIns5P does not change in PIKfyve and MTMR3 knockdown cells.

2. The knockdown result should be shown first and then its impact on cell migration (change the position and labeling of figure 1 A and B).

Referee #3:

The manuscript reports that silencing VPS34 or selected FYVE-domain-containing proteins, including FGD1, FGD2, PIKfyve and MTMR3, inhibits cell migration. As VPS34, PIKfyve and MTM3 have the potential to control PtdIns5P levels and as the FYVE domain of FGD1 has been shown to be able to bind PtdIns5P, the authors reach the conclusion that PtdIns5P controls cell migration possibly via FGD1/FGD2.
The set of data concerning the involvement of PIKfyve and MTM3 (both in cells and in the Drosophila oogenesis model) is convincing. However, the demonstration that this role is exerted via PtdIns5P is less convincing, and the hypothesis that FGD1 and 2 might be the PtdIns5P effectors is even weaker.
The body of evidence for a role of PtdIns5P is mainly correlative: FGF1, which stimulates migration, also increases PtdIns5P and PIKfyve and MTM3 silencing, which inhibit migration, also decrease PtdIns5P levels. The more direct proof the authors provide is the administration of exogenous PtdIns5P or expression of the IpgD 4-phosphatase which apparently increase the migratory performance of the MTM3 KD cells. However, this increase is really tiny and an important control is lacking, that is the effect of PtdIns5P or lpgD on control cells.
As regards the involvement of FGD1 and 2 (two of the best hits from the initial screening), the authors hypothesize that they might mediate the PtdIns5P effects in cell migration. However, these are complex proteins with multiple phosphoinositide binding motifs, including two PH domains in addition to the FYVE domain. The FYVE domain is non-conventional in FGD1, but it is conventional in FGD2, as it possesses the W..D motif (the authors should amend the claim that it is non-conventional or specify what feature they consider to define it as non-conventional). The lipid binding profile of the FYVE domain of FGD1 has been characterized and it has been shown to bind PtdIns5P. However, only the lipid binding profile of the full-length FGD2 protein (thus including also 2 PH domains) has been studied (unless the authors have their own data on the FGD2 FYVE domain that should then be shown). The full-length protein also binds PtdIns45P2, PtdIns345P3 and weakly PtdIns35P2, thus the specificity for PtdIns5P binding is questionable.
In order to establish a link between the set of effects of MTM3/PIKfyve on migration and on PtdIns5P and the FGDs the authors should assess whether the FGDs are affected in their localization by the silencing of MTM3 or PIKfyve or by the PIKfyve inhibitor and whether FGD mutants unable to bind PtdIns5P lose the ability to rescue the migratory defects in FGD-depleted cells, as compared to wt FGDs.

1st Revision - authors' response 21 September 2012

Replies to points raised by referees

Refereee #1

In this manuscript, the authors identify a novel role for phosphatidylinositol-5-phosphate (PI5P) in promoting cell migration. Using an siRNA screen targeting genes that encode putative PI3P-binding proteins/effectors (due to the presence in their sequence of FYVE or PX modules) in combination with a cell migration assay, they identify two lipid enzymes controlling levels of PI3P/PI5P/PI(3,5)P2, namely MTMR3 and PIKfyve. Based on data showing that PI5P levels increase in response to migratory stimulation, they speculate that PI5P, rather than his precursors PI3P and PI(3,5)P2, may be the PI species directly mediating this effect. To test this, in vitro and in vivo siRNA models are used to place the production of PI5P in phosphoinositide interconversion pathway involving the enzymatic activities of the lipid kinases Vps34 and PIKFyve and the phosphatase MTMR3. In part using elegant synthetic lipid "add back" experiments, the authors convincingly show that PI5P is a key regulat or of cell migration.

PI5P is the most enigmatic species of the phosphoinositide family; therefore, the author's findings provide an exciting investigation into determining one of PI5P's potential roles. The authors do a

comprehensive job showing PI5P is a positive regulator of cellular migration and identify a potential pathway for PI5P production in this context. Overall, this investigation is well done, although a key question is the nature of PI5P effectors involved in cell migration. Additionally, the mechanism by which PI5P is regulating cell migration should be further explored.

Main issues:
1) The authors convincingly show that PI5P is important for cell motility, but they provide little information on why this is happening. The authors should at the very least examine the integrity and potentially dynamics of focal adhesions in fibroblasts containing lower PI5P levels using markers, such as FAK, talin and vinculin. Additionally, does PI5P control the trafficking of integrins? This is particularly important because all the enzymes directly or indirectly involved in PI5P production also control the traffic/degradation of integrins presumably via PI3P-dependent processes. For instance, are there differences in total and cell surface levels of the relevant integrins in response to PI5P changes?

Reply: As suggested by the referee, we have now investigated further how PtdIns5P regulates cell migration. First we tested if α5β1 integrin recycling was changed upon knockdown of MTMR3 and PIKfyve inhibition (YM201636). We find no differences in integrin trafficking under knockdown conditions compared to control, clearly indicating that PtdIns5P does not act on recycling of integrins (new supplementary Fig S5A). Furthermore, we could not detect any changes in total and cell-surface levels of α5β1 or αvβ5 integrins (BJ fibroblasts do not express αvβ3) in response to PtdIns5P changes (upon PIKfyve and MTMR3 knockdown; new supplementary Fig S5B). Additionally, α5 integrin localization remained unchanged as observed by confocal microscopy and immuno-EM (new supplementary Fig S5C). These experiments also provide important controls, indicating that the effects we observe on cell migration are not due to changes in PtdIns3P-dependent endosomal processes as suggested by the referee.
We have also closely investigated the role of focal adhesions. In immunofluorescence microscopy studies of migrating BJ cells, we cannot detect any differences in distribution of focal adhesions using markers like FAK, talin and vinculin between control and knockdown cells. The size and number of focal adhesions seems to be the same independent of PtdIns5P levels (new supplementary Fig S5D, E, F). Furthermore, to investigate their dynamics we tested the ability of siRNA-depleted cells to spread on a fibronectin-coated surface, a process mediated by focal complexes. As shown in the new Supplementary Fig S5G, the knockdown cells spread similarly to control cells (no difference in cell surface area). Interestingly, however, here we observe a clear difference in the organization of the actin skeleton (See new Fig 2D). We observe actin "knots" in a very high proportion compared to control cells. This was also further investigated by structured illumination super-resolution microcopy (SIM). We then see that the "knots" look like nucleation centers from where actin fibers sprout out. We do not know at present precisely what these structures are, but this observation clearly supports that PtdIns5P is implicated in the remodeling of the actin cytoskeleton, which is obviously important for cell migration. This is also supported by the data in Fig 2D showing that the actin fibers in knockdown cells are more parallel to the wound than in control cells which are more perpendicular.

2) A decrease in PI5P during migratory stimulation is observed upon knock-down of MTMR3 and PIKfyve when compared to the control in Figure 4B; however, it is important to know the basal levels of PI5P for all the conditions not just the control. Whether there is still a burst in PI5P upon migration in the knock down cells cannot be determined from the data, which is key in ruling out alternative sources here.

Reply: We thank the referee for pointing out this important issue, and we measured PtdIns5P levels in cells that had not been stimulated with FGF1 (new supplementary Fig S2C). We find in this case a small reduction of PtdIns5P when PIKfyve and MTMR3 are knocked down although this was not statistically significant. This fits with our new data showing that knocking down these proteins in unstimulated cells also decreases migration velocity (new supplementary Fig S4). Thus, it seems that PtdIns5P is also important in basal migration without direct stimulation. FGF1 merely increases the level of PtdIns5P and thereby also cell speed.

3) The proposed source of PI5P synthesis during migration consists of enzymes known to be crucial for maintaining normal endo-/lysosomal functions. Can the authors rule out simple effects of cell toxicity on the migration phenotypes? In other words, wouldn't dying or sick cells be expected to migrate less efficiently? The authors should show that the poor health of the manipulated cells is not a confounding factor in the interpretation of the phenotype by assessing the extent of cell death using standard techniques.

Reply: This is an important point and we have now tested these toxicity issues by two different assays. First, we used the classical MTT assay and then we measured the level of protein synthesis in the cells under the different conditions. As seen in new supplementary Fig S3 we couldn't detect any significant differences between control and treatment conditions. In addition, we observed in the live-cell imaging experiments that the siRNA-depleted cells still underwent cell division showing that both knockdown of MTMR3 and PIKfyve and the imaging conditions were not toxic (see example in supplementary Movie 1).

4. While the identification of PI5P effectors mediating cell migratory processes goes beyond the scope of this manuscript, the authors identified FGD1, a PI3P and PI5P effector, as another hit. At the very least, they should test whether overexpressing this factor can rescue the cell migration phenotype observed in the Mtmr3/Pikfyve KD cells and whether this rescue depends on its lipid-binding module.

Reply:
We tested whether FGD1 overexpression could rescue the cell migration phenotype observed in MTMR3 knockdown cells. We were not able to rescue the cell migration velocity defect with FGD1 in MTMR3 knockdown cells, suggesting that FGD1 does not perform its effect on cell migration through this PtdIns5P pathway. Our speculations in the original manuscript that FGD1 could potentiality be an effector as it was previously found to bind PtdIns5P (in addition to other phosphoinositides) is now removed. However, FGD1 is still a validated hit in the screen and we have kept it in the manuscript.
In conclusion, even though identifying PtdIns5P effectors goes beyond the scope of this manuscript as the referee points out, this will obviously be an important focus of our future research.

Minor comments:
5) It is mentioned that HRS was identified as a strong hit in the siRNA cell migration screen; however, it is not listed in Supplemental Figure 1A. Is HRS on the x-axis mislabeled HGS?

Reply: It was not mislabeled since the human gene name of HRS is actually HGS. We agree that this easily can cause confusion and we have now changed it in the figure.

6) Previous work from the De Camilli and Anderson labs has connected PI metabolism to focal adhesion dynamics and cell migration. These studies should perhaps be cited to make a stronger case about the involvement of PIs in cell migration.

Reply: We are now citing these two important papers. We thank the reviewer for pointing them out to us!

Referee #2:

Oppelt et al report here a role of PtdIns5P in cell migration. Via a migration screen for proteins containing PtdIns3P-binding motifs, the authors identified PI-5 kinase PIKfyve and PI-3 phosphatase MTMR3. The authors indicate that the two proteins constitute a phosphoinositide loop producing PtdIns5P via PtdIns3,5P2 and modulate cell migration. The results are potentially very interesting but there are significant issues that need to be clarified:

Major comments:

1. The mechanism of Ptdlns5P-regulation of cell migration is poorly defined. Vps34 and PIKfyve were found to localize at endosomes. What is the spatio-temporal distribution of Ptdlns(5)P in migrating cells? Could PIKfyve and MTMR3 modulate PtdIns5P level at the leading edge? These need to be, at least discussed in the manuscript.

Reply: We have now investigated further the mechanisms of PtdIns5P-regulation of cell migration (See point 1, referee #1.). We have attempted to visualize the spatio-temporal distribution of PtdIns5P by using the 2xPH-GRAM domain probe (Lorenzo O, Urbé S, Clague MJ. (2005)). Unfortunately, this probe is not effective in detecting normal levels of PtdIns5P and we were not able to determine the localization of PtdIns5P by microscopy. A co-author of the paper, Dr Lucia Rameh, has previously found PtdIns5P predominantly at the plasma membrane (Sarkes D, Rameh LE (2010)). PtdIns5P could very well act there and in the leading edge to control cell migration through the actin cytoskeleton as indicated by our findings. These are very interesting questions and we now discuss this in the paper.

2. In this study, FGF1 was used to induce BJ cell migration. Is PtdIns5P required for random migration without agonist treatment? The author indicated that PIKfyve and MTMR3 constituted a phosphoinositide loop producing PtdIns5P via PtdIns3,5P2. Does PIKfyve and MTMR3 colocalize to the same compartments?

Reply: We have now studied random migration without agonist treatment and find that knockdown of PIKfyve and MTMR3 inhibits migration, indicating that PtdIns5P is also important for random migration (new supplementary Fig S4). This also fits with decreasing PtdIns5P levels upon knockdown in non-stimulated cells (see new supplementary Fig S2C).

It is puzzling that PIKfyve is found in endosomal compartments, while MTMR3 is not (Lorenzo O, Urbé S, Clague MJ. (2005) J. Cell Sci.). As available MTMR3 antibodies are unfortunately not good enough for immunofluorescence microscopy of endogenous MTMR3, we must overexpress it to be able to visualize it. Under these conditions we see it localizing to many membranes, for instance ER and Golgi, as reported before. As this is overexpression, it is difficult to conclude anything, as we see it more or less everywhere. We actually think it is possible that a fraction of MTMR3 and PIKfyve produce PtdIns5P at the plasma membrane, but this has to be investigated further when better antibodies become available.

3. In the migration screening (supplementary Figure 1), were different ON-TARGET plus Control siRNA (ON) or the same control siRNA used in the screen? Why the ratios (migrated cells per total cells) for each control siRNA are quite different? If different control siRNA were used, did it indicate that different control siRNA could lead to quite different results? If the same control siRNA used, did it indicate there were variations between each different experiment?

Reply: We used several different control siRNAs (labeled: ON, SG and SG#2). However, it is correct that we included several values where we used the same ON control. We did this to show the variation of the data and as the referee correctly pointed out, there is some variation in the different experiments. The screen results are composed of three independent replications and it is not surprising that there is some variation between experiments. Importantly, however, the hits investigated further were statistically different from all the control siRNAs. The values for the hits were lower than the controls in all three experiments.

4. The authors observe only minute increases in cell migration for the rescue after exogenous addition of Ptdlns(5)P to the cells. How can this be explained?

Reply: The referee raises a valid point, and we think it is likely to be due to the topology of PtdIns5P after exogenous administration. The PtdIns5P added from outside will probably be in many membranes and therefore has a somewhat different spatio-temporal behavior than endogenous

PtdIns5P produced in the cell. However, even if it is not a complete rescue, it is a clear and specific effect. It is also important that other relevant phosphoinositides do not rescue.

5. In Figure S1, the western and mRNA expression show that the knockdown effect of PIKfyve siRNA 1 and 2 are similar or even better than siRNA 3 and 4. But PIKfyve siRNA 1 and 2 have no effect on cell migration. The results are also inconsistent with the author claimed that PIKfyve siRNA 3 and 4 yielded strongest knockdown. Knockdown and rescue assay should be used for detecting the role of PIKfyve and MTMR3 in cell migration.

Reply: It is true that in the case of PIKfyve, we do not see much effect of any of the four single siRNAs when administered alone and we therefore combined two siRNAs, which then gave nice results, as reported before (Rutherford et al. (2006), J. Cell Sci.). We chose siRNA 3 and 4 because there seemed to be more variation in knockdown for siRNA 1 and 2.
We agree that rescue assays are important to ensure that the effects observed by the knockdown are specific. We have performed experiments showing that MTMR3 knockdown is rescued by an siRNA-resistant EGFP-MTMR3 construct (see Fig 1H). We have also tried to rescue PIKfyve by a similar approach. Unfortunately, we were not able to transfect BJ cells by the PIKfyve plasmid construct probably due to its large size (2098 amino acids). We then also attempted to perform rescue using a Lentiviral approach. We tried to generate lentivirally transduced BJ cells expressing either wildtype EGFP-PIKfyve or a siRNA-resistant EGFP-PIKfyve version. Unfortunately, the generation of EGFP-PIKfyve expressing cell lines was not successful, probably due to size restrictions of the gene payload space within HIV-1 based lentiviral vectors, as the payload size should be maximally 7.5 kb to allow proper virus particle assembly (Tolmachov et al. 2011). In our case, the payload size was 10.8 kb, despite using a small promoter (PGK promoter) and a small selection marker (Blasticidine resistance).

Finally, we were able to rescue the PIKfyve knockdown effect by adding exogenous PtdIns5P or PtdIns3,5P_2 (new Fig 4D). These results are really important as they, in addition to showing that the effect is specific and rescuable, also directly implicate PIKfyve in the production of PtdIns5P necessary for proper cell migration. Importantly, the effect was not rescued by PtdIns3P, as expected.

Other points:

1. In Figure 4, it is confusing that basal level of PtdIns5P does not change in PIKfyve and MTMR3 knockdown cells.

Reply: We have now measured PtdIns5P basal levels in PIKfyve and MTMR3 knockdown cells (See point 2, Referee #1 and new supplementary FigS2C).

2. The knockdown result should be shown first and then its impact on cell migration (change the position and labeling of figure 1 A and B).

Reply: We totally agree and have changed the order.

Referee #3:

The manuscript reports that silencing VPS34 or selected FYVE-domain-containing proteins, including FGD1, FGD2, PIKfyve and MTMR3, inhibits cell migration. As VPS34, PIKfyve and MTM3 have the potential to control PtdIns5P levels and as the FYVE domain of FGD1 has been shown to be able to bind PtdIns5P, the authors reach the conclusion that PtdIns5P controls cell migration possibly via FGD1/FGD2.
The set of data concerning the involvement of PIKfyve and MTM3 (both in cells and in the Drosophila oogenesis model) is convincing. However, the demonstration that this role is exerted via PtdIns5P is less convincing, and the hypothesis that FGD1 and 2 might be the PtdIns5P effectors is even weaker.

The body of evidence for a role of PtdIns5P is mainly correlative: FGF1, which stimulates migration, also increases PtdIns5P and PIKfyve and MTM3 silencing, which inhibit migration, also decrease PtdIns5P levels. The more direct proof the authors provide is the administration of exogenous PtdIns5P or expression of the IpgD 4-phosphatase which apparently increase the migratory performance of the MTM3 KD cells. However, this increase is really tiny and an important control is lacking, that is the effect of PtdIns5P or lpgD on control cells.

Reply: We agree that this is an important control and we have now performed the control experiments. We find that exogenous PtdIns5P or IpgD do not cause any significant differences in migration of control cells (new Fig 4C and E).

As regards the involvement of FGD1 and 2 (two of the best hits from the initial screening), the authors hypothesize that they might mediate the PtdIns5P effects in cell migration. However, these are complex proteins with multiple phosphoinositide binding motifs, including two PH domains in addition to the FYVE domain. The FYVE domain is non-conventional in FGD1, but it is conventional in FGD2, as it possesses the W..D motif (the authors should amend the claim that it is non-conventional or specify what feature they consider to define it as non-conventional). The lipid binding profile of the FYVE domain of FGD1 has been characterized and it has been shown to bind PtdIns5P. However, only the lipid binding profile of the full-length FGD2 protein (thus including also 2 PH domains) has been studied (unless the authors have their own data on the FGD2 FYVE domain that should then be shown). The full-length protein also binds PtdIns45P2, PtdIns345P3 and weakly PtdIns35P2, thus the specificity for PtdIns5P binding is questionable.
In order to establish a link between the set of effects of MTM3/PIKfyve on migration and on PtdIns5P and the FGDs the authors should assess whether the FGDs are affected in their localization by the silencing of MTM3 or PIKfyve or by the PIKfyve inhibitor and whether FGD mutants unable to bind PtdIns5P lose the ability to rescue the migratory defects in FGD-depleted cells, as compared to wt FGDs.

Reply: We agree that the hypothesis that FGD1 is an effector of PtdIns5P is speculative and with our new data probably not correct (See Referee #1, point 4). We have therefore not included in the revised manuscript that FGD1 is a possible effector. The role of FGD1 and FGD2 and their phosphoinositide binding specificities in cell migration need to be better clarified and a thorough study of all its domains would deserve a new project along the lines suggested by the referee.

We apologize for categorizing the FYVE domain of FGD2 as non-conventional. The referee correctly points out that it is conventional, while FGD1 is non-conventional. It is also true that only full-length FGD2 has been used in lipid binding profiles. Again we oversimplified and this has now been corrected in the manuscript.

References

Lorenzo O, Urbé S, Clague MJ.(2005) Analysis of phosphoinositide binding domain properties within the myotubularin-related protein MTMR3. *J Cell Sci*., **118**(9):2005-12

Rutherford AC, Traer C, Wassmer T, Pattni K, Bujny MV, Carlton JG, et al. (2006) The mammalian phosphatidylinositol 3-phosphate 5-kinase (PIKfyve) regulates endosome-to-TGN retrograde transport. *J Cell Sci* **119:** 3944-57

Sarkes D, Rameh LE (2010) A novel HPLC-based approach makes possible the spatial characterization of cellular PtdIns5P and other phosphoinositides. *Biochem J* **428:** 375-84

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2nd Editorial Decision 19 October 2012

Thank you for the submission of your revised manuscript to our offices. We have now received the enclosed reports from the referees that were asked to assess it. Referee 2 still has two minor suggestions (one concerning showing the efficiency of the MTMR3 knock-down and one with regard to an additional clarification) that I would like you to incorporate before we proceed with the official acceptance of your manuscript.

Once you have made these minor changes, please upload your manuscript to our website again. If it is easier for you, you may also send all files as email attachments.

I look forward to seeing a new revised version of your manuscript as soon as it is ready.

Yours sincerely,

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

The authors have addressed all my concerns. I now recommend publication in Embo Reports.

Referee #2:

In this manuscript, Oppelt et al., have demonstrated the role of PtdIns5P in cell migration. Authors have shown that MTMR3 and PIKfyve constitute a phosphoinositide loop producing PtdIns5P via PtdIns3,5P2 and modulate cell migration. The revised manuscript has been significantly improved, and our concerns have been addressed in the revised manuscript except two minor issues:

1. Figure 1H, the protein level of MTMR3 in knockdown and rescue cells needs to be shown by Western Blot.

2. Are there any literatures that show the relationship between actin "knots" and cell migration?

Referee #3:

The authors have ruled out that FGD1 might be one of the PI5P effectors involved in cell migration and have also excluded that integrin trafficking or regulation of focal adhesions might play a role in PI5P-mediated migration. Thus the message of the manuscript remains limited to the original and very intriguing finding that PI5P plays a key role in migration, however both the PI5P targets and the mechanisms by which PI5P promotes cell migration remain undefined.

2nd Revision - authors' response 24 October 2012

Replies to points raised by Referee 2.

Referee #2:

In this manuscript, Oppelt et al., have demonstrated the role of PtdIns5P in cell migration. Authors have shown that MTMR3 and PIKfyve constitute a phosphoinositide loop producing PtdIns5P via PtdIns3,5P2 and modulate cell migration. The revised manuscript has been significantly improved, and our concerns have been addressed in the revised manuscript except two minor issues:

1. Figure 1H, the protein level of MTMR3 in knockdown and rescue cells needs to be shown by Western Blot.

Reply: We agree with the referee that it is important to show the protein levels before and after siRNA knockdown. We have now included a representative Western Blot showing the knockdown levels of MTMR3 and PIKfyve in Fig 1F. For the rescue, we used transient transfection of a GFP-MTMR3 cDNA fusion-construct. Because of the low transfection efficiency of BJ fibroblasts, it is not possible to show rescue by Western Blot. Transfected cells were therefore selected based on their expression of GFP and we only tracked cells that expressed GFP-MTMR3 for the rescue. We were careful not to overexpress the GFP-MTMR3 construct to a too high level. The fact that we obtained full rescue of the phenotype clearly indicates that adequate levels of the fusion protein were expressed in the cells.

2. Are there any literatures that show the relationship between actin "knots" and cell migration?

Reply: We have not been able to find any relevant literature showing a relationship between actin “knots” and cell migration. Their role will be an important focus of our future research.

3rd Editorial Decision 26 October 2012

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