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## Plasticity of PI4KIII interactions at the plasma membrane

Jeeyun Chung, Fubito Nakatsu, Jeremy M. Baskin and Pietro De Camilli

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### Review timeline:

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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.)

Editor: Barbara Pauly

1st Editorial Decision

04 July 2014

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Thank you very much for the submission of your research manuscript to our editorial office and for your patience while we were waiting to hear back from the referees. We have now received the full set of reviews on your manuscript.

As the detailed reports are pasted below I will only repeat the main points here. You will see that all reviewers appreciate the interest of your findings and are, in principle, supportive of publication of your study in our journal. However, they are also in agreement that several aspects of your data need to be improved before the paper can be published. For example, referee 1 feels that the rate of PI4P and the contribution of PI4KIIa in its synthesis need to be measured in a different way and suggests possible approaches on how to achieve this. Reviewer 2 recommends analyzing the composition of the PI4KIIa complex in the absence of TMEM150A and referee 3 also points out some aspects that would need to be clarified and extended.

Overall, and given the reviewers' constructive comments, I would like to give you the opportunity to revise your manuscript, with the understanding that the main concerns of the reviewers should 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. Should you in the meantime have any questions, please do not hesitate to contact me.

#### REFEREE REPORTS:

##### Referee #1:

This study identifies a novel Pi4K interactor TMEM150 which the authors assert regulates the functional activity of PI4K to impact on PI45P2 synthesis at the plasma membrane. The study is interesting but the data do not adequately back up the the authors conclusions.

##### Points

Figure 3B and C. The authors need to show where PI4K is localised alone, and in the presence of 1. efr3B, 2 mem150A 3, TTC7B and in various combinations.

figure 3D the blot does not show the expression of flag-TTC7 when it is expressed alone. does expression of EFR3B effect the expression of TTC7B? Figure 3D implicates the role of TMEM150A in the interaction of EFR3B and TTC7B but as it stands does not implicate the role of any of these proteins in the interaciotn of TMEM150A with PI4KIIIa. other studies need to be carried out in order to conclude that "these data suggest that the interaction of TMEM150A with PI4KIII $\alpha$  is mutually exclusive with the presence of TTC7B"

Contrary to what the authors suggest the rate of PI4P synthesis cannot be determined by measuring PI45P2 synthesis. it can only be inferred and certainly in this situation there is room for doubt. The authors assert that the resynthesis of PI45P2 is dependent on PI4P after atropine mediated inhibition of M1 stimulation presumably as this there suggestion is that TMEM150 is involved it assumes that this would occur at the plasma membrane and the requirement of plasm membrane PI4P in this process is quoted as being based on the hammond et al paper (PI4P and PI(4,5)P2 are essential but independent lipid determinants of membrane identity. ) however in this paper the authors show that plasma membrane PI4P is not required (see quote from paper below)

"We therefore used transient over-expression of M1 receptors in COS-7 cells to investigate re-synthesis of PI(4,5)P2 ([HYPERLINK "/pmc/articles/PMC3646512/figure/F3/"](#)Fig. 3A). Stimulation of M1-expressing cells led to reduced PI(4,5)P2 and PI4P staining, which returned to pre-stimulation levels after addition of the M1 receptor antagonist atropine ([HYPERLINK "/pmc/articles/PMC3646512/figure/F3/"](#)Fig. 3B). PM-recruited PJ-Sac had no effect on this recovery of PI(4,5)P2 staining, despite sustained depletion of PM PI4P ([HYPERLINK "/pmc/articles/PMC3646512/figure/F3/"](#)Fig. 3B). Likewise, PI(4,5)P2 biosensors showed translocation from the PM upon PLC activation, but their return to the PM after atropine addition was unaffected by PJ-Sac recruitment ([HYPERLINK "/pmc/articles/PMC3646512/figure/F3/"](#)Figs 3C, [HYPERLINK "\i "SD1"S6"](#))

Therefore the authors need to detail the requirement of PI4KIIIa and its function in this biological response. The requirement based on PAO is not good enough to implicate PI4KIIIa mediated activation. The authors also need to measure the levels of PI4P directly at the plasma membrane to determine how TMEM150 depletion /overexpression effect PI4P levels. this could be done using the microscopic methodology show in the Hammond manuscript.

##### Referee #2:

The paper by Chung et.al examines the control of PI4KIIIa localization at the plasma membrane. PI4KIIIa (and its yeast counterpart Stt4) are likely the main biochemical activity that underpins the synthesis of PI4P at the plasma membrane. Despite the evident importance of PI4P in multiple cell membrane functions, the biochemistry underlying its regulation is poorly understood. This paper

examines the role of TMEM150A, the mammalian ortholog of Sfk1 (from yeast) in the control of PI4KIIIa localization and function in mammalian cells. The role of Sfk1 in regulating Stt4 localization and activity in yeast has been previously studied.

The authors identify the mammalian ortholog of PI4KIIIa by bioinformatics and show that one isoform, TMEM150A when expressed as a GFP tagged protein localizes to the plasma membrane; further they show that a C-terminal fragment of TMEM150A is sufficient to send another family member TMEM150B, which is not normally localized to the plasma membrane to this location. The consequence of deleting the C-terminal tail of TEME150A on its localization has not been shown in this study. The authors show that when both proteins are overexpressed in mammalian cells, PI4KIIIa and TMEM150A physically interact most likely via the C-terminal tail.

The authors then examine the interaction between TMEM150A and two other proteins previously shown to be members of the PI4KIIIa complex, namely EFR3 and TTC7. They show that in experiments using IPs with PI4KIIIa antibodies the overexpression of TMEM150A in mammalian cells results in the apparent absence of TTC7 from the PI4KIIIa complex. This observation is addressed further in IPs using TTC7 and EFR as the primary IP molecule. It is concluded on the basis of these experiments that there is a dynamic interplay between TTC7 and TMEM150A regarding their presence in the PI4KIIIa complex.

Finally the using TMEM150A KD and overexpression, the authors show that this molecule regulates the recovery of PIP2 levels following receptor active PLC mediated PIP2 hydrolysis in mammalian cells.

While this set of experiments are instructive, the authors should examine the composition of the PI4KIIIa complex in cells where TMEM150A has been down-regulated using the siRNA reagent for this molecule described in Fig 4. This would strengthen the conclusion that endogenous TMEM150A has a role in regulating the composition of the PI4KIIIa complex in mammalian cells.

Minor point: Some of the referencing to Fig 3 panels in the text may be misplaced and should be made clearer.

Referee #3:

This is an interesting manuscript reporting the identification of TMEM150A as the functional homolog of Sfk1, identified in yeast as a regulator of the PI4K Stt4. The authors report that TMEM150A is a component of a multimolecular complex that includes EFR3B responsible for the plasma membrane targeting of PI4KIIIalpha. This complex is distinct from the one the authors have previously identified that included EFR3B, PI4KIIIalpha and TTC7B; in fact the presence of TMEM150A and TTC7B are mutually exclusive in the complex. Functionally, TMEM50A participates in the homeostatic response involving PI4KIIIalpha and generating PI4P & PI45P2 in response to agonist stimulation.

The data are clear and convincing, however some aspects deserve further clarification

1. The authors identify a domain in the C-terminus of TMEM150A that is relevant for entering the complex with PI4KIIIalpha and EFR3B. Is the C-terminus the only domain of TMEM150A that is relevant for its functional role in controlling PI4KIIIalpha activity? Do the other TMEM150A domains take part in this regulation? This question could be addressed by testing the chimerae engineered by the authors (Fig.2) in comparison with the wt TMEM150A. Are the chimerae as active as the wt form in increasing PI45P2 re-synthesis in response to agonist; can they rescue the decreased response in cells knocked-down for TMEM150A?
2. In the model presented in Fig. 4, the authors envisage that TMEM150A contacts both PI4KIIIalpha and EFR3B. Have they tested this possibility? Can TMEM50a co-IP with PI4KIIIa in the absence of EFR3B?
3. The relationships existing between the two different complexes of PI4KIIIa/EFR3B with TMEM150A or with TTC7 should be clarified:
  - are the two complexes localized in similar or different regions of the plasma membrane?

- do they have a different functional impact on PI4KIII $\alpha$ -dependent PI4P & PI45P2 pools? What is the impact of TTC7B on PI4P-PI45P2 under basal or stimulated conditions? The authors report that TMEM150A overexpression displaces TTC7B from the complex, yet it increases PI45P2 re-synthesis (thus presumably PI4KIII $\alpha$  activity) after agonist stimulation. Does this mean that TTC7B might be a negative regulator of the response? How do the authors interpret their observation?

1st Revision - authors' response

02 October 2014

### Point-by-point response to the reviewers' comments

Reviewers' comments are in gray italics and our response in regular black font.

#### Referee #1:

*1. Figure 3B and C. The authors need to show where PI4K is localised alone, and in the presence of 1. efr3B, 2 tmem150A 3, TTC7B and in various combinations.*

As suggested, we analyzed the localization pattern of PI4KIII $\alpha$  when overexpressed in various combinations with the other components of the complex: EFR3B, TMEM150A, and TTC7B. Consistent with our previous report (Nakatsu *et al.*, JCB, 2012), both EFR3B and TTC7B are required for PI4KIII $\alpha$  targeting to the plasma membrane (Supplementary Fig. S2). We also examined whether TMEM150A affects the localization of PI4KIII $\alpha$ . PI4KIII $\alpha$  had a cytosolic distribution when co-expressed with TMEM150A alone or with both TMEM150A and TTC7B but without EFR3 (Fig. 3A).

*2. Figure 3D the blot does not show the expression of flag-TTC7 when it is expressed alone. does expression of EFR3B effect the expression of TTC7B?*

First, we realized that in the original Fig 3D (western blots) some bands were somewhat distorted, making it difficult to compare the intensity of each band. Thus, we replaced this figure with a new figure (now Fig. 4D) that shows exactly same results of former Fig. 3D, but with better bands. The new Fig 4D clearly shows that that EFR3B expression does not affect the expression level of TTC7B.

*3. Figure 3D implicates the role of TMEM150A in the interaction of EFR3B and TTC7B but as it stands does not implicate the role of any of these proteins in the interaciotn of TMEM150A with PI4KIII $\alpha$ . other studies need to be carried out in order to conclude that "these data suggest that the interaction of TMEM150A with PI4KIII $\alpha$  is mutually exclusive with the presence of TTC7B"*

We have further assessed the composition of the PI4KIII $\alpha$  complex upon TMEM150A overexpression. We overexpressed TTC7B, PI4KIII $\alpha$  and EFR3B with/without TMEM150A and performed TTC7B co-immunoprecipitations. These experiments demonstrated that TTC7B specifically interacted with both of EFR3B and PI4KIII $\alpha$  in the absence of TMEM150 overexpression, as reported (Nakatsu *et al.*, JCB, 2012) (Fig. 4E, see lane 5). However, TMEM150A co-overexpression abolished the recovery in the anti-TTC7 immunoprecipitates of ERF3, and strongly decreased the recovery of PI4KIII $\alpha$ , (Fig. 4E, see lane 6) revealing a plasticity of the PI4KIII $\alpha$  complex.

*4. Contrary to what the authors suggest the rate of PI4P synthesis cannot be determined by measuring PI45P2 synthesis. it can only be inferred and certainly in this situation there is room for doubt. The authors assert that the resynthesis of PI45P2 is dependent on PI4P after atropine mediated inhibition of M1 stimulation presumably as this there suggestion is that TMEM150 is*

*involved it assumes that this would occur at the plasma membrane and the requirement of plasma membrane PI4P in this process is quoted as being based on the hammond et al paper (PI4P and PI(4,5)P<sub>2</sub> are essential but independent lipid determinants of membrane identity). however in this paper the authors show that plasma membrane PI4P is not required (see quote from paper below)*

*"We therefore used transient over-expression of M1 receptors in COS-7 cells to investigate re-synthesis of PI(4,5)P<sub>2</sub> (HYPERLINK "/pmc/articles/PMC3646512/figure/F3/"Fig. 3A). Stimulation of M1-expressing cells led to reduced PI(4,5)P<sub>2</sub> and PI4P staining, which returned to pre-stimulation levels after addition of the M1 receptor antagonist atropine (HYPERLINK "/pmc/articles/PMC3646512/figure/F3/"Fig. 3B). PM-recruited P-J-Sac had no effect on this recovery of PI(4,5)P<sub>2</sub> staining, despite sustained depletion of PM PI4P (HYPERLINK "/pmc/articles/PMC3646512/figure/F3/"Fig. 3B). Likewise, PI(4,5)P<sub>2</sub> biosensors showed translocation from the PM upon PLC activation, but their return to the PM after atropine addition was unaffected by P-J-Sac recruitment (HYPERLINK "/pmc/articles/PMC3646512/figure/F3/"Figs 3C, HYPERLINK \l "SD1"SD1)"*

*Therefore the authors need to detail the requirement of PI4KIII $\alpha$  and its function in this biological response. The requirement based on PAO is not good enough to implicate PI4KIII $\alpha$  mediated activation.*

We thank the reviewer for this comment that prompted us to clarify this important issue. As shown in previous studies by other groups, PI(4,5)P<sub>2</sub> maintenance at the plasma membrane depends on the precursor pool of plasma membrane PI4P generated by PI4KIII $\alpha$  (Bojjireddy *et al.*, JBC, 2014 and Dickson *et al.*, PNAS, 2014). Also, in our own previous paper (Nakatsu *et al.*, JCB, 2014), we showed that loss of PI4KIII $\alpha$  resulted in a dramatic reduction of plasma membrane PI(4,5)P<sub>2</sub>, indicating the importance of PI4KIII $\alpha$ -mediated PI4P production in PI(4,5)P<sub>2</sub> synthesis at the plasma membrane. These references are quoted in the manuscript. However, we do recognize the importance of showing more directly this point in our present manuscript. Thus, to further confirm that PI4KIII $\alpha$  is responsible for maintenance of the plasma membrane PI(4,5)P<sub>2</sub> pool, we performed an experiment in which we inhibited PI4KIII $\alpha$  activity using compound A1, a recently described highly specific and potent PI4KIII $\alpha$  inhibitor (Bojjireddy *et al.*, JBC, 2014) and monitored recovery rate of PI(4,5)P<sub>2</sub>. As new Fig. 5A shows, the Oxo-M stimulation-dependent depletion is followed by a rapid resynthesis of PI(4,5)P<sub>2</sub> upon atropine treatment in control cells, a process known to be dependent on PI4P consumption and resynthesis (Fig. 5A). In contrast, the addition of the compound A1 completely blocked PI(4,5)P<sub>2</sub> resynthesis (Fig. 5A). This result supports the conclusion that PI4KIII $\alpha$ -dependent PI4P synthesis is responsible for PI(4,5)P<sub>2</sub> recovery in the acute PI(4,5)P<sub>2</sub> perturbation assay.

*5. The authors also need to measure the levels of PI4P directly at the plasma membrane to determine how TMEM150 depletion /overexpression effect PI4P levels. this could be done using the microscopic methodology show in the Hammond manuscript.*

We performed the PI4P immunofluorescence staining according to Hammond *et al* (Science, 2013) as the reviewer suggested. Knockdown of TMEM150A decreased plasma membrane PI4P levels by 19 $\pm$ 6.4%, compared to controls (Supplementary Fig S5). These results confirms that TMEM150A affects plasma membrane PI4P production.

#### **Referee #2:**

*The paper by Chung et. al examines the control of PI4KIII $\alpha$  localization at the plasma membrane. PI4KIII $\alpha$  (and its yeast counterpart Stt4) are likely the main biochemical activity that underpins the synthesis of PI4P at the plasma membrane. Despite the evident importance of PI4P in multiple cell membrane functions, the biochemistry underlying its regulation is poorly understood. This paper examines the role of TMEM150A, the mammalian ortholog of Sfk1 (from yeast) in the control of*

*PI4KIIIa localization and function in mammalian cells. The role of Sfk1 in regulating Stt4 localization and activity in yeast has been previously studied.*

*The authors identify the mammalian ortholog of PI4KIIIa by bioinformatics and show that one isoform, TMEM150A when expressed as a GFP tagged protein localizes to the plasma membrane; further they show that a C-terminal fragment of TMEM150A is sufficient to send another family member TMEM150B, which is not normally localized to the plasma membrane to this location.*

The reviewer seems to have misinterpreted our results concerning the chimera. Our study shows that both TMEM150A-GFP and TMEM150B-GFP localize to the plasma membrane (Fig 1B) and that only TMEM150A interacts with the PI4KIII $\alpha$  complex. To determine whether the C-terminal fragment of TMEM150A is responsible for the interaction with PI4KIII $\alpha$ , we generated a chimeric construct in which the C-terminal tail of TMEM150A was appended at the C-terminal region of TMEM150B. This experiment showed that the C-tail of TMEM150A is sufficient to interact with the PI4KIII $\alpha$  complex (Fig 2D). These results were reported in the original paper.

*The consequence of deleting the C-terminal tail of TEME150A on its localization has not been shown in this study. The authors show that when both proteins are overexpressed in mammalian cells, PI4KIIIa and TMEM150A physically interact most likely via the C-terminal tail.*

As suggested, we tried to examine the effect of the deletion of the C-terminal tail of TMEM150A. To this aim, we generated several C-terminal deletion mutants of TMEM150A. Unfortunately, all of these constructs were not properly targeted to the plasma membrane

*The authors then examine the interaction between TMEM150A and two other proteins previously shown to be members of the PI4KIIIa complex, namely EFR3 and TTC7. They show that in experiments using IPs with PI4KIIIa antibodies the overexpression of TMEM150A in mammalian cells results in the apparent absence of TTC7 from the PI4KIIIa complex. This observation is addressed further in IPs using TTC7 and EFR as the primary IP molecule. It is concluded on the basis of these experiments that there is a dynamic interplay between TTC7 and TMEM150A regarding their presence in the PI4KIIIa complex.*

*Finally the using TMEM150A KD and overexpression, the authors show that this molecule regulates the recovery of PIP2 levels following receptor active PLC mediated PIP2 hydrolysis in mammalian cells.*

*2. While this set of experiments are instructive, the authors should examine the composition of the PI4KIIIa complex in cells where TMEM150A has been down-regulated using the siRNA reagent for this molecule described in Fig 4. This would strengthen the conclusion that endogenous TMEM150A has a role in regulating the composition of the PI4KIIIa complex in mammalian cells.*

We performed TMEM150A siRNA experiments, followed by coprecipitations to address this issue. However, due to the poor reactivity of antibodies we could only performed these experiments in cells where components of the complex are overexpressed. Under these conditions we found that knockdown of TMEM150A did not affect the composition or localization of PI4KIII $\alpha$  complex components (Supplementary Fig. S3).

*3. Minor point: Some of the referencing to Fig 3 panels in the text may be misplaced and should be made clearer.*

Thank you. We corrected this error.

**Referee #3:**

*This is an interesting manuscript reporting the identification of TMEM150A as the functional homolog of Sfk1, identified in yeast as a regulator of the PI4K Stt4. The authors report that TMEM150A is a component of a multimolecular complex that includes EFR3B responsible for the plasma membrane targeting of PI4KIIIalpha. This complex is distinct from the one the authors have previously identified that included EFR3B, PI4KIIIalpha and TTC7B; in fact the presence of TMEM150A and TTC7B are mutually exclusive in the complex. Functionally, TMEM50A participates in the homeostatic response involving PI4KIIIalpha and generating PI4P & PI45P2 in response to agonist stimulation.*

*The data are clear and convincing, however some aspects deserve further clarification*

*1. The authors identify a domain in the C-terminus of TMEM150A that is relevant for entering the complex with PI4KIIIalpha and EFR3B. Is the C-terminus the only domain of TMEM150A that is relevant for its functional role in controlling PI4KIIIalpha activity? Do the other TMEM150A domains take part in this regulation? This question could be addressed by testing the chimerae engineered by the authors (Fig.2) in comparison with the wt TMEM150A. Are the chimerae as active as the wt form in increasing PI45P2 re-synthesis in response to agonist; can they rescue the decreased response in cells knocked-down for TMEM150A?*

To determine whether the C-tail of TMEM150A is important for its function in the control of PI4KIII $\alpha$  activity, we examined the effect of the TMEM150A/TMEM150B chimera on PI(4,5)P<sub>2</sub> resynthesis in response to Oxo-M-stimulation and atropine treatment. This chimera stimulated the PI(4,5)P<sub>2</sub> recovery rate to same levels as wild type TMEM150 (Fig. 5B). In addition, the chimeric construct rescued the effect of TMEM150A knockdown (Fig. 5D). Taken together, these results suggest that presence of TMEM150A at the plasma membrane affects the homeostatic response to PI(4,5)P<sub>2</sub> depletion, likely by positively regulating PI4KIII $\alpha$ -dependent PI4P synthesis.

*2. In the model presented in Fig. 4, the authors envisage that TMEM150A contacts both PI4KIIIalpha and EFR3B. Have they tested this possibility? Can TMEM50a co-IP with PI4KIIIa in the absence of EFR3B?*

We examined the effect of siRNA-mediated knockdown of EFR3 on the interaction between TMEM150A and PI4KIII $\alpha$ . We knocked down both mammalian EFR3 isoforms (EFR3A and EFR3B) in HeLa cells, co-transfected them with 3XFLAG-PI4KIII $\alpha$  and TMEM150A-GFP, and then generated anti-GFP immunoprecipitates. PI4KIII $\alpha$  was recovered in the immunoprecipitate even in the absence of EFR3A and B (Fig. 3B). In a complementary experiment, siRNA-mediated knockdown of PI4KIII $\alpha$  did not affect the interaction between TMEM150A-GFP and EFR3B, once again as assessed by anti GFP immunoprecipitation (Fig. 3D). These results are consistent with an independent interaction of TMEM150 with both PI4KIII $\alpha$  and EFR3B, although it remains to be proven that the interaction is direct. We also simplified the cartoon. (Now Fig. 5F)

*3. The relationships existing between the two different complexes of PI4KIIIa/EFR3B with TMEM150A or with TTC7 should be clarified:*

*- are the two complexes localized in similar or different regions of the plasma membrane?*

We have no evidence either in favor of, or against, this possibility

*- do they have a different functional impact on PI4KIIIalpha-dependent PI4P & PI45P2 pools? What is the impact of TTC7B on PI4P-PI45P2 under basal or stimulated conditions? The authors report that TMEM150A overexpression displaces TTC7B from the complex, yet it increases PI45P2 re-synthesis (thus presumably PI4KIIIalpha activity) after agonist stimulation. Does this mean that TTC7B might be a negative regulator of the response? How do the authors interpret their observation?*

As we previously reported (Nakatsu *et al.*, JCB, 2014) and, as we demonstrate in our current manuscript (Fig. 3A and Supplementary Fig. 2D), TTC7B is required for proper plasma membrane recruitment of PI4KIII $\alpha$ . Once PI4KIII $\alpha$  has been targeted to the plasma membrane by EFR3 and TTC7, the kinase forms a complex with TMEM150A that mutually excludes TTC7 (See Fig. 5F). How TTC7 performs this function (a molecular chaperone?) remains unclear and will be the object of future studies.

## REFERENCES

1. Bojjireddy N, Botyanszki J, Hammond G, Creech D, Peterson R, Kemp DC, Snead M, Brown R, Morrison A, Wilson S, et al. (2014) Pharmacological and Genetic Targeting of the PI4KA Enzyme Reveals Its Important Role in Maintaining Plasma Membrane Phosphatidylinositol 4-Phosphate and Phosphatidylinositol 4,5-Bisphosphate Levels. *J Biol Chem* **289**: 6120–6132.
2. Nakatsu F, Baskin JM, Chung J, Tanner LB, Shui G, Lee SY, Pirruccello M, Hao M, Ingolia NT, Wenk MR, et al. (2012) PtdIns4P synthesis by PI4KIII $\alpha$  at the plasma membrane and its impact on plasma membrane identity. *The Journal of Cell Biology* **199**: 1003–1016.
3. Dickson EJ, Jensen JB, Hille B (2014) Golgi and plasma membrane pools of PI(4)P contribute to plasma membrane PI(4,5)P<sub>2</sub> and maintenance of KCNQ2/3 ion channel current. *Proc Natl Acad Sci USA* **111**: E2281–E2290.

2nd Editorial Decision

05 November 2014

Many thanks for your patience while we were waiting for the referee comments on the revised version of your manuscript. While referees 1 and 3 now support publication of your study, referee 2 is still not convinced about the relevance of the proposed TMEM150-mediated regulation *in vivo*, since the depletion of the endogenous protein does not have an effect on the composition of the PI4KIII $\alpha$  complex. We see your point that this might be due to the necessity of performing this experiment in the background of overexpressed proteins and after additional discussions with one of the other reviewers (referee 1), I have the following suggestion: could you test the effects of an endogenous agonist in TMEM150 control and knockdown cells instead of overexpressing the acetylcholine receptor? Referee 1 feels that this would strengthen the idea that TMEM150 regulates the PI4KIII $\alpha$  complex *in vivo*.

In addition, referee 1 also suggests discussing in more detail how you think that TMEM150 and TTC7 collaborate to regulate PI45P<sub>2</sub> synthesis. Please do not worry about the character count too much. I think it is important to discuss this issue.

Please do not hesitate to get in touch with me if you have any questions at this point.

## REFeree REPORTS:

Referee #1:

The authors have adequately revised the manuscript and have attempted to answer a number of criticisms and I would recommend publication of the manuscript. However, It is still not clear how expression of TMEM150, which leads to TTC7 delocalisation into the cytoplasm enhances PI4P



synthesis and PI45P2 production, especially as TT7C is required for plasma membrane localisation of PI4KIIIa. While the authors have provided a cartoon that summarises their data, it does not suggest how they think the plasticity of the PI4K complex regulates the production of PI45P2. I suggest that they include a final discussion of how they think TMEM150 and TTC7 collaborate to regulate PI45P2 synthesis.

Referee #2:

This paper describes a potentially interesting new protein TMEM150A that might regulate the activity of PI4KIIIa, an enzyme that has been identified as regulating a plasma membrane pool of PI4P.

In this paper the physical interaction between TMEM150 and the known members of the PI4KIIIa complex are described. The findings do demonstrate potentially interesting protein-protein interactions. However these interactions are done with over-expressed proteins working in a cellular background in which the endogenous protein complex of PI4KIIIa is present. Limited experiments have been done with the endogenous complex itself. While this does not impact the description of a potential protein-protein interaction these experiments are less instructive with regard to the existence of such an endogenous complex and its functional significance in vivo.

In the revised version the authors report the outcome of a key experiment, namely the impact of siRNA depletion of TMEM150A on the composition of the PI4KIIIa complex. They find that knockdown of endogenous TMEM150A does not impact the composition of the PI4KIIIa complex. This finding questions the importance of TMEM150A at endogenous levels in regulating PI4KIIIa complex in vivo.

Referee #3:

The Authors have satisfactorily addressed the concerns I raised in my previous review.

Correspondence - author

11 November 2014

Thank you for your message concerning our manuscript. We have thoroughly considered and discussed the reviews and your editorial comments. Thank you for your very constructive comments and suggestions and also for consulting referee #1 toward a possible solution to the remaining points raised by the review. Please see below our response.

Reviewer #1: we will modify the cartoon and expand the discussion on potential interpretations of our results.

Reviewer #2: the reviewer is puzzled by the apparent lack of impact of the absence of TMEM150A on the "composition of the PI4HIIIalpha complex", which is by the fact that in the absence of TMEM150, all three components of the complex (EFR3, TTC7 and PI4KIIIalpha) are still recruited to the plasma membrane as in naïve cells. However, we know that EFR3 is the main anchor of the complex at the plasma membrane and we would not have expected a change. An impact on the recovery of PI(4,5)P2 after its depletion could be explained by an indirect effect of TMEM150A on the enzymatic activity of PI4KIIIalpha. We note that TMEM150A is a protein with 6 transmembrane regions, unlikely to simply act as an anchor for the complex. We could make this point more clear.

Concerning the idea that you have discussed with reviewer #1, i.e. to show that absence of TMEM150A has an impact on the recovery from the PI(4,5)P2 depletion induced by a physiological agonist, this would be a relatively simple experiment to do (and we thank you for this suggestion). However, we are not aware of a good agonist for endogeneous receptors coupled to PLC in HeLa

cells. Overexpression of the muscarinic receptor is a well established method to assess basic mechanisms in the recovery from a stimulus-induced PI(4,5)P<sub>2</sub> depletion, see for example PMID:20519312(Hille group) or PMID 22722250 (Balla group). Importantly, since your message appropriately stresses the importance of providing "in vivo" evidence, this is indeed an experiment in living cells.

If you agree, we will go carefully through the manuscript to make sure we present and discuss our data keeping these comments in mind and we will resubmit a manuscript where these changes are outlined for your review and consideration.

I thank you in advance for your help.

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Correspondence - editor

12 November 2014

Many thanks for your message and sorry for my delayed response. I discussed the problem with the endogenous agonists with the referee who suggested the experiment and s/he suggests to use histamine or endothelin might work.

I am attaching a paper that this reviewer sent to me; maybe this is helpful. I do think that adding this experiment would strengthen the paper if it is possible to perform.

Do let me know what you think.

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2nd Revision - authors' response

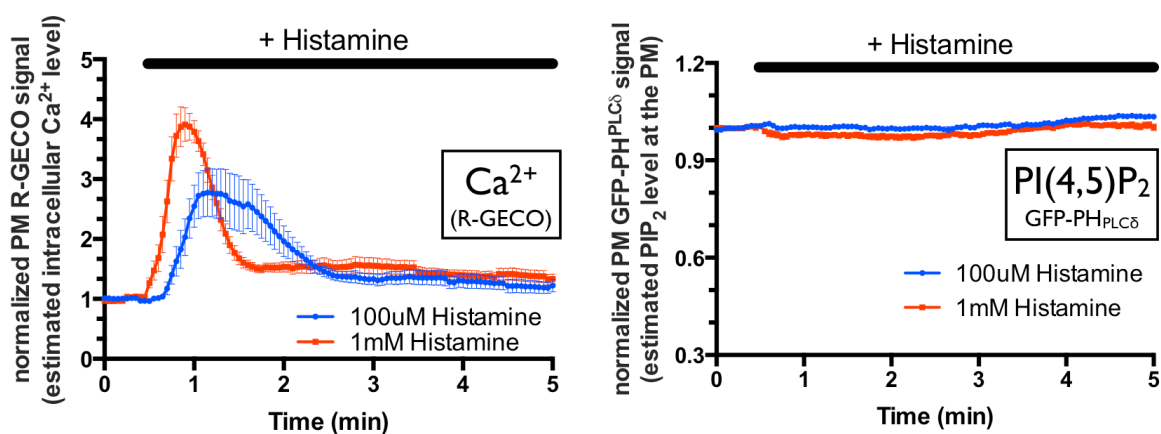
26 November 2014

Thank you again for your constructive suggestions concerning our manuscript (Chung *et al.* # EMBOR-2014-39151V2) and our response to the reviewers' comments. A remaining issue was our experiment concerning the defect in resynthesis of PI(4,5)P<sub>2</sub> following an acute depletion by activation of a PLC-coupled receptor. This experiment was performed in HeLa cells overexpressing a PLC-coupled receptor, the muscarinic acetylcholine receptor. You had consulted with reviewer #1 who had proposed to perform the same experiment by stimulation of an endogenous PLC-coupled receptor, as such experiment would represent a more physiological stimulus. You had also sent us, based on the referee's suggestions, two references reporting the occurrence of such receptors (histamine receptors) in HeLa cells. We have now performed such experiments. We did confirm the occurrence of histamine-dependent PLC activation, as revealed by an increase in R-GECO fluorescence (which reports elevation of cytosolic Ca<sup>2+</sup> in response to PI(4,5)P<sub>2</sub> cleavage and IP<sub>3</sub> generation). However, the loss of PI(4,5)P<sub>2</sub> from the plasma membrane, as reported by (GFP-PH<sub>PLCδ</sub>), was below detectability using TIRF microscopy, the best and most sensitive method to monitor such changes. More specifically, we were unable to detect reliable dissociation of GFP-PH<sub>PLCδ</sub> from the plasma membrane ( $n = 8$ ; please see the attached figure) using 100 μM histamine, which is the concentration typically used in the literature (including in the two papers the reviewer suggested) and which is already in excess relative to the endogenous range of histamine in rodent and human subjects (0.01 ~ 1 μM). In any case, we found that even by increasing the concentration of histamine to 1 mM, no obvious loss of PI(4,5)P<sub>2</sub> from the plasma membrane was detected in our system ( $n = 10$ ; please see the attached figure). A figure summarizing these results is appended below.

These findings are not in disagreement with the two studies you sent us, as in those studies changes in intracellular  $\text{Ca}^{2+}$  levels, but not in  $\text{PI}(4,5)\text{P}_2$  levels as assessed by GFP- $\text{PH}_{\text{PLC}\delta}$  dissociation, were analyzed. To our knowledge, the only paper that used histamine to monitor a change in the level of the GFP- $\text{PH}_{\text{PLC}\delta}$  marker at the plasma membrane (a study of HEK cells) involved over-expression of the H1 receptor (Liu *et al.*, 2008, PMID:18448631). Thus, we think that detectable dissociation of the  $\text{PI}(4,5)\text{P}_2$  reporter GFP- $\text{PH}_{\text{PLC}\delta}$  occurs only upon robust loss of plasma membrane  $\text{PI}(4,5)\text{P}_2$ , as it occurs when receptors are overexpressed. While these conditions may not closely reflect a physiological state, they represent an optimal model system to monitor  $\text{PI}(4,5)\text{P}_2$  resynthesis. We note that such overexpression systems have been used extensively to investigate the biology of  $\text{PI}(4,5)\text{P}_2$ , its metabolites and their effectors.

In view of these considerations, we hope that the use of the muscarinic receptor overexpression system in our study will not preclude its publication in EMBO Reports. We have addressed the other final comments of the re-review (referee #1) with additions to the concluding remarks and to the legend of Fig. 5F.

We hope that you will now consider our manuscript suitable for publication in your Journal and we look forward to hearing from you.



3rd Editorial Decision

09 December 2014

Many thanks for submitting the revised version of your manuscript to our office and for your patience while we were assessing it. I did discuss the outcome of the histamine experiment with reviewer 1, as it had been his/her suggestion. While it would, of course, have been nice had it worked, we both appreciate your willingness to try it and will be happy to accept the paper with just the results of the muscarinic acetylcholine receptor.

Nevertheless, reviewer 1 still feels that the mechanism on how TMEM150 regulates  $\text{PI}4\text{K}$  (i.e. by changing the TTC7 localization) remains somewhat unclear, also because of the lack of sufficiently strong data that this is how it happens in a fully endogenous situation.

Referee 1 therefore suggests to modify your concluding remarks accordingly and, for example, point

out that while TTC7 is required for the localization/recruitment of PI4K at the membrane, its presence is not needed for the maintenance of it there. Once it is there, TMEM150 could interact and activate PI4K. If you are happy with those changes, please modify the text accordingly and simply send the final version as an email attachment. I will then replace the old version and officially accept the manuscript for publication. Reviewer 1 also pointed out that s/he didn't understand what the term 'plasticity' in the title and abstract means. I guess, what you meant to say is that the complex composition and localization is dynamic, eg TMEM150 can interfere with TTC7's localization and interaction with ERF3 etc. Is this correct?

I hope you can live with this compromise. I do think your study is very interesting and believe it got strengthened during the review process. Thanks again for your contribution to EMBO reports.

3rd Revision - authors' response

15 December 2014

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Thank you again for handling this manuscript and for your most constructive help. We have modified the concluding remarks as suggested. I am attaching the final revised text of the manuscript including main text, references, and figure legends.

About the word "plasticity", you are correct: we mean "that the complex composition and localization is dynamic". We cannot find a better word and we prefer to leave it.

Please let me know if everything is OK.

4th Editorial Decision

17 December 2014

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I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.