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Phosphatidylinositol 4-kinase II function at endosomes is regulated by the ubiquitin ligase Itch

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1st Editorial Decision 05 January 2012

Thank you very much for the submission of your research manuscript to our editorial office. I am sorry for the slight delay in the review process of your manuscript, which was due to the holiday season. 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. While the referees agree on the potential interest of the findings, they are concerned that the study relies too much on in vitro assays and overexpression experiments and that additional evidence is needed to show that Itch-mediated ubiquitination of PI4KIIa occurs in cells and that this modification affects the enzymes' function in the cellular context. The referees also point out that stronger proof for the suggested functional interaction of Itch and PI4KIIa in the regulation of endocytosis and endosomal sorting of Fz receptors is needed. In addition, several technical concerns (missing controls, statistical analysis etc) will need to be addressed.

From the analysis of these comments it becomes clear that significant revision is required before the manuscript may become suitable for publication in EMBO reports. However, given the potential interest of your study, I would like to give you the opportunity to revise your manuscript, with the understanding that the main concerns of the referees must be addressed and their suggestions (as detailed above) taken on board. 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.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. If you feel that this period is insufficient for a successful submission of your revised manuscript I can potentially extend this period slightly. Also, the length of the revised manuscript should not exceed roughly 28,000 characters (including spaces).

I noticed that the current manuscript does not contain a materials and methods section. I would therefore like to kindly ask you to incorporate the main experimental details that are required for the repetition of the key experiments into the main body of the manuscript, rather than displaying them as supplementary information only.

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

Yours sincerely

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

The authors identify a novel and direct interaction between the E3 ubiquitin ligase Itch and lipid kinase PI4KII-alpha. They demonstrate both multiubiquitination of PI4KII-alpha and binding-induced negative regulation of PI4KII-alpha kinase activity by Itch. Interestingly, this direct interaction between PPxY motif of PI4KII-alpha and the WW domains of Itch reciprocally controls both enzymes' catalytic function. Lastly, the authors propose that the predominantly endosomal PI4KII-alpha-Itch complex offers a link between phosphoinositide-regulated trafficking and ubiquitin-mediated protein sorting, which they implicate in Wnt-activated endosomal trafficking and downstream signal transduction.

The authors do a comprehensive job showing that PI4KII-alpha is a direct substrate for Itch and identifying the interaction's modulatory consequences. Further work is necessary, however, to clarify the regulatory role of the PI4KII-alpha-Itch complex and its precise step in endosomal trafficking, particularly in the context of Wnt-signaling.
Main issues:

1. Although the authors demonstrate in vitro ubiquitination of PI4KII-alpha by Itch, surprisingly this finding has not been confirmed in intact cells. Is there a decrease in PI4KII-alpha ubiquitination upon knock-down of Itch?

2. Much of the data concerns the identification of this new post-translation modification for PI4KII-alpha, yet a functional role for PI4KII-alpha ubiquitination has not been elucidated. They conclude that binding of the Itch WW-domain alone decreases PI4K activity, but does the presence of the full length Itch protein and its ubiquitination of PI4KII-alpha cause a further decrease in activity? Does the modulation of kinase activity by Itch translate into alterations of PI(4)P levels in intact cells?

3. The authors demonstrate a reciprocal regulatory relationship between PI4KII-alpha and Itch and that this complex predominantly localizes to endosomes; however, whether either proteins localization changes upon knock-down of the other is not addressed. For instance, is there hierarchical recruitment of either protein to the endosome? In fact, the localization of Itch does appear to be more perinuclear in the PI4KII-alpha siRNA-treated compared to the control in Figure 3E. Recruitment of Itch to its site of activity by an activator has been demonstrated in other contexts (i.e. the recruitment of Itch by spartan to lipid droplets and binding induced activation). Does PI4KII-alpha perhaps recruit Itch to the endosome in this scenario?

4. In Figure 4B-C the authors attribute the PI4KIISF mutant's ability to partially rescue the trafficking of the Fz4 receptor in the PI4KII-alpha knockdown background to its decreased binding to Itch. Since this mutant disrupts Itch binding and they show that Itch negatively regulates PI4KII-alpha, an overactivation of PI4KII-alpha and increased rate of Fz4 endocytosis would be expected. In fact it seems likely that there may be other characteristics of this mutant that effect Fz endocytosis, such as decreased PI4K activity or another disrupted conformation required for efficient endocytosis of Fz4. From this rescue experiment it cannot be concluded that the PI4KII-alpha-Itch complex is regulating the Wnt pathway at the level of endocytosis. To confirm a role for Itch in endocytosis, Itch siRNA experiments should also be performed in parallel. In fact, all of the co-localization immunofluorescence evidence points to a more downstream role for the complex in endosomal sorting.

5. In Figure 4A-C, it is unclear whether the authors intend to study the complex's role in endocytosis, endosomal sorting or both. The authors suggest the complex has a role in both throughout the text; however, their experiments are more appropriate for studying endocytosis and do not conclusively show a role for the complex in endosomal sorting. In fact, this complex seems to have a more obvious role in endocytic sorting. Although the concluding remarks claim that the PI4KII-alpha-Itch complex regulates endocytic sorting of activated Fz4, key experiments needed to draw this conclusion are missing. For instance, in order to determine the complex's role in endosomal sorting and the degradative pathway, experiments such as pulse-chase antibody uptake experiments to Fz4 in the presence and absence of lysosomal inhibitors and/or co-localization studies with lysosomal markers should be performed upon Itch or PI4KII-alpha siRNA knock-down.

Other comments:

6. A hypothetical diagram describing the role of the PI4KII-Itch complex in the Wnt activated pathway would be helpful as a concluding figure, perhaps in supplementary information.

7. What is the rationale for substituting the first proline of the PPxY motif with a serine in the SPxF mutant (instead of alanine)?

8. Page 4, last paragraph: the reference for the GST-UBA construct is missing.

9. Figure 2G: The typical Rab5Q phenotype of enlarged endosomes is not apparent. The authors should improve the quality of the images and provide a higher magnification inset of the endosomal co-localization.

10. Figure 4B-C: Expression levels of the protein constructs should be included.

11. Several bar graphs show control conditions in the normalized form (100%). However, no error bars are shown. How was the statistics done? In the absence of variability in the control conditions, the one sample t-test is more appropriate.

12. Molecular weight markers are missing in key Western blots, including Figures 1A,B and E.

13. In one panel (Fig. 3D), depletion of itch with RNAi causes a reduction in the levels of PI4KII-alpha; in another panel (Fig. 4F), the decrease is not obvious, perhaps because the knockdown of itch is not as efficient. The LRP6 blots should be repeated on samples showing a better knockdown.

14. Page 7: For the experiments involving the internalization of Fz4-eGFP into endosomes, a control image showing the localization of Fz4 in the absence of Wnt5a should be included.

15. Figure 3G,H: the inhibition of kinase activity of PI4KII-alpha by the WW domain of itch is an important result; however, an important control is missing: a WW domain deficient in binding to the ppxy motif should be included (for instance, the WW4 domain can be used as it is not capable of binding PI4KII-alpha according to Figure 2D).

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Referee #2:

PI4KIIalpha has been suggested to be involved in ubiquitin-mediated protein sorting in the endosomal system. However, the mechanism(s), by which PI4KII may control ubiquitin-mediated protein sorting, remain obscure. In this manuscript, Mossinger et al. report that ectopically expressed PI4KII is subjected to ubiquitylation and that this ubiquitylation is mediated by the HECT ubiquitin ligase Itch/AIP4. Furthermore, the authors provide evidence to indicate that Itch and PI4KII affect each other's function and that both are involved in controlling Fz4 trafficking and Wnt signaling. The notion that PI4KII function is regulated by Itch-mediated ubiquitylation is indeed of general interest. However, the data with respect to Itch-induced ubiquitylation of PI4KII are rather preliminary and its role in regulation of PI4KII function remains obscure (as my expertise with respect to the mechanisms involved in endosomal sorting is limited, I refrain from commenting on this issue).

Comments:

1. All the binding experiments were performed with ectopically expressed proteins in HEK293 cells or with recombinant proteins in vitro. Evidence (other than colocalization studies) to indicate that endogenous Itch physically interacts with endogenous PIK4II would significantly increase the quality of the manuscript.

2. Does Itch affect the ubiquitylation pattern of PI4KII within cells? This may be difficult to determine with endogenous proteins but should be rather straightforward in cotransfection experiments.

3. The authors indicate that several HECT ubiquitin ligases containing WW domains exist. Thus, it seems mandatory to determine if these (e.g. NEDD4-1 or NEDD4-2) have the potential to interact with PI4KII and to ubiquitylate PI4KII in vitro and within cells (to provide evidence for the specificity of the proposed Itch-PI4KII interaction).

4. Assuming that Itch-mediated ubiquitylation is physiologically relevant, the function of it remains entirely obscure.

5. Figs. 3I and 3J: The quality of the ubiquitylation experiment is not convincing (i.e. if there is any ubiquitylation, the efficiency is so low that quantitation appears to be impossible).

Referee #3:

In this paper Mossiger et al. identify the E3 ligase Itch as an interactor and regulator of PI4KIIα, a TGN/endosomal protein responsible for PI(4)P synthesis. Results show that PI4KIIα is a direct binding partner of Itch (through the canonical PPxY-WW domain interaction) and is a substrate of its ligase activity. By IF analysis the authors suggest that Itch might be an endosomal protein involved in the regulation of Wnt-activated frizzled 4 (Fz4) receptor trafficking but these assumptions lack convincing experimental evidence.
While the subject is interesting, this is a rather preliminary manuscript that fails to convey a clear message on the functional consequences of this interaction. Several relevant issues need to be addressed before publication.

Cleaner and more conclusive experiments are needed in order to conclude that PI4KIIα is an Itch substrate. The In vitro ubiquitination assays (Fig. 3 A,B,I) are far from convincing. None of the experiments include an anti-Ub blot and no time 0 of the reaction is reported. The authors should repeat this entire set of experiments following the standard in vitro ubiquitination procedure (see for example Mund&Pelham, 2009, cited by the authors themselves). This assay should be performed with Ub wt and K0 to confirm that is indeed multi-monoubiquitination. ITCH wt and C830A overexpression in vivo may help in validating PI4KIIα as an Itch substrate.

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No convincing data on the reciprocal regulation of PI4KIIα and Itch enzymatic activity are provided (Fig. 3G and I). Time course and dose response experiments are required. Regulation seems to be provided by interaction surfaces. Are other WW domains or PPXY motifs able to do the same? How is specificity achieved?

From the data presented is not clear if the involvement of Itch protein on Fz4 internalization is related to its E3 ligase activity on PI4KIIα. From the initial set of data, it appears that KD of Itch causes a reduction in the level of PI4KIIα. Thus, one might assume a similar phenotype promoted by the two KDs on Fz4 internalization, while results show the opposite. Testing the overexpression of Itch wt and C830A mutant in experiments shown in Fig.4 B, F and G would clinch the issue, and is required to, at least, propose a model related to the role of Itch in this context.

Minor points:

1. Please systematically indicate in all figures the molecular weight markers.
2. Authors, please comment on the appearance of the slow migrating band in the IP samples of Fig 1A, B and in the input of Fig. 1E and Fig. 2C. Is this monoubiquitination? It seems not to be affected by SF mutation, but then the (same?) band disappears in the presence of Itch C830A (Fig. 3B). Are other post-translational modifications involved?
3. Endosomal localization of Itch is limited. Authors, please tone-down related sentences.

1st Revision - authors' response 05 June 2012

Response to reviewers:

We wish to thank all three referees for their critical reading of our Ms and for their thoughtful suggestions that clearly have helped to improve the paper. Our detailed response to these comments is given below.

Reviewer 1 (comments in italics):

The authors do a comprehensive job showing that PI4KII-alpha is a direct substrate for Itch and identifying the interaction's modulatory consequences. Further work is necessary, however, to clarify the regulatory role of the PI4KII-alpha-Itch complex and its precise step in endosomal trafficking, particularly in the context of Wnt-signaling.

Response: We thank the referee for his/ her positive remarks. We have carried out a number of additional experiments to clarify the role of PI4KIIα and Itch in endocytosis and endosomal trafficking. We have re-analyzed the effect of Itch and PI4KIIα loss-of-function in Fz internalization and in subsequent Fz degradation. In agreement with the known role of PI4KIIα in the PI(4,5)P2-dependent initiation of Wnt/ Fz signaling we show that PI4KIIα-KD cells show impaired endocytosis of Wnt-activated Fz, whereas KD of Itch is without effect. Importantly, we further demonstrate that depletion of either PI4KIIα or Itch delays Fz degradation (see revised Fig. 4). Together with the observed co-localization of Itch and PI4KIIα on Fz-containing endosomes these data argue for a role of Itch, presumably together with PI4KIIα in degradative sorting of Fz at the level of endosomes (now schematically depicted in fig. S4A). These observations complement and are in agreement with recent data showing that depletion of the de-ubiquitinating enzyme UBPy/ USP8 attenuates Wnt signaling (Mukai et al., EMBO J, 2010). We further show - consistent with the above data - that overexpression of PI4KIIα facilitates recruitment of Itch to TGN/ endosomal membranes (revised fig. S4B).

1. Although the authors demonstrate in vitro ubiquitination of PI4KII-alpha by Itch, surprisingly this finding has not been confirmed in intact cells. Is there a decrease in PI4KII-alpha ubiquitination upon knock-down of Itch?

Response: We have performed the suggested KD experiments using siRNA against Itch but have observed little effect on PI4KIIα ubiquitination. This likely is explained by the functional redundancy of HECT domain E3 ligases. Indeed, in the revised Ms we show that several of these enzymes including NEDD4 can associate with PI4KIIα in vitro and might functionally compensate for loss of Itch when it comes to the total level of PI4KIIα ubiquitination. This is not uncommon in the ubiquitin field. To nonetheless address the point we have created a stable HEK293 cell line that allows for the inducible expression of Itch together with PI4KIIα. In the new fig. 3C we show that doxycyclin-induced expression of Itch leads to elevated levels of ubiquitinated PI4KIIα in cells, supporting our in vitro data.

2. Much of the data concerns the identification of this new post-translation modification for PI4KII-alpha, yet a functional role for PI4KII-alpha ubiquitination has not been elucidated.

Response: As said above we have carried out a number of additional experiments to clarify the role of PI4KIIα and Itch in endosomal trafficking. We have re-analyzed the effect of Itch and PI4KIIα loss-of-function in Fz internalization and in subsequent Fz degradation. In agreement with the known role of PI4KIIα in the PI(4,5)P2-dependent initiation of Wnt/ Fz signaling we show that PI4KIIα-KD cells show impaired endocytosis of Wnt-activated Fz, whereas KD of Itch is without effect. Importantly, we further demonstrate that depletion of either PI4KIIα or Itch delays Fz degradation (see revised fig. 4). Together with the observed co-localization of Itch and PI4KIIα on Fz-containing endosomes these data argue for a role of Itch, presumably together with PI4KIIα in degradative sorting of Fz at the level of endosomes (now schematically depicted in fig. S4A).

They conclude that binding of the Itch WW-domain alone decreases PI4K activity, but does the presence of the full length Itch protein and its ubiquitination of PI4KII-alpha cause a further decrease in activity?

Response: We have investigated this issue and show in the new fig. S3D that full-length Itch similar to the isolated WW domains also suppresses PI4KIIa-mediated PI(4)P formation. However, Itch-mediated ubiquitination did not further reduce the PI(4)P-synthesizing activity of PI4KIIα beyond the inhibition induced by catalytically inactive Itch (Fig. S3D). We, thus, favor a model whereby ubiquitination of PI4KIIα does not primarily serve to regulate its enzymatic activity but may rather facilitate its targeting to the Ub-based sorting machinery at endosomes, although other functions (i.e. regulation of its half-life) cannot be ruled out.

Does the modulation of kinase activity by Itch translate into alterations of PI(4)P levels in intact cells?

Response: Previous work, i.e. by the Balla and Yin labs (i.e. Wang et al, Cell 2003) have shown that the global cellular levels of PI(4)P are largely dependent on PI 4-kinase type IIIβ at the Golgi. There are additional type III enzymes that are considered to be the main source of PI(4)P at the ER and the plasma membrane. Consistent with these results we and others have not found evidence that knockdown of PI4KIIα cause a global loss of PI(4)P.

3. The authors demonstrate a reciprocal regulatory relationship between PI4KII-alpha and Itch and that this complex predominantly localizes to endosomes; however, whether either proteins localization changes upon knock-down of the other is not addressed. For instance, is there hierarchical recruitment of either protein to the endosome? In fact, the localization of Itch does appear to be more perinuclear in the PI4KII-alpha siRNA-treated compared to the control in Figure 3E.

Response: As the majority of Itch appears to be cytoplasmic and we observe an overall

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reduction of Itch expression in PI4KIIα-KD cells and conversely of PI4KIIα upon depletion of Itch, we feel that these issues are difficult to address by siRNA experiments. We have therefore opted for inducible overexpression PI4KIIα in cells. In the new fig. S4B we show that overexpression of PI4KIIα indeed facilitates recruitment of Itch to the TGN/ endosomal boundary. An action of PI4KIIα upstream of Itch is also consistent with our data regarding Fz endocytosis and degradative sorting (shown in revised fig. 4). To better convey these results to the readers of *EMBO Rep*. we have added a hypothetical model in fig. S4A.

4. In Figure 4B-C the authors attribute the PI4KIISF mutant's ability to partially rescue the trafficking of the Fz4 receptor in the PI4KII-alpha knockdown background to its decreased binding to Itch.

Response: We show in the new fig. S3C that the enzymatic activities of WT and SF mutant PI4KIIa are very similar. As our new functional data clearly place PI4KIIα upstream of Itch in Fz internalization and endosomal sorting (see fig. 4) we feel that this result is entirely compatible with a hypothetical model whereby PI4KIIα via its PPxY motif recruits Itch to endosomes to facilitate degradative sorting of Fz.

From this rescue experiment it cannot be concluded that the PI4KII-alpha-Itch complex is regulating the Wnt pathway at the level of endocytosis.
To confirm a role for Itch in endocytosis, Itch siRNA experiments should also be performed in parallel. In fact, all of the co-localization immunofluorescence evidence points to a more downstream role for the complex in endosomal sorting.

Response. We agree with the reviewer and have therefore conducted a number of additional experiments to clarify the role of PI4KIIα and Itch in endosomal trafficking. We have re-analyzed the effect of Itch and PI4KIIα loss-of-function in Fz internalization and in subsequent Fz degradation. In agreement with the known role of PI4KIIα in the PI(4,5)P2-dependent initiation of Wnt/ Fz signaling we show that PI4KIIα-KD cells show impaired endocytosis of Wnt-activated Fz, whereas KD of Itch is without any effects. Importantly, we further demonstrate that depletion of either PI4KIIα or Itch delays Fz degradation (see revised fig. 4). Together with the observed co-localization of Itch and PI4KIIα on Fz-containing endosomes these data argue for a role of Itch, presumably together with PI4KIIα in degradative sorting of Fz at the level of endosomes (now schematically depicted in fig. S4A). These observations complement and are in agreement with recent data showing that depletion of the de-ubiquitinating enzyme UBPy/USP8 attenuates Wnt signaling (Mukai et al., EMBO J, 2010). We further show - consistent with the above results - that overexpression of PI4KIIα facilitates recruitment of Itch to TGN/ endosomal membranes (revised fig. S4B).

5. In Figure 4A-C, it is unclear whether the authors intend to study the complex's role in endocytosis, endosomal sorting or both. The authors suggest the complex has a role in both throughout the text; however, their experiments are more appropriate for studying endocytosis and do not conclusively show a role for the complex in endosomal sorting. In fact, this complex seems to have a more obvious role in endocytic sorting. Although the concluding remarks claim that the PI4KII-alpha-Itch complex regulates endocytic sorting of activated Fz4, key experiments needed to draw this conclusion are missing.

Response: We have substantially improved the Ms in this respect by the addition of new data (see below).

For instance, in order to determine the complex's role in endosomal sorting and the degradative pathway, experiments such as pulse-chase antibody uptake experiments to Fz4 in the presence and absence of lysosomal inhibitors and/or co-localization studies with lysosomal markers should be performed upon Itch or PI4KII-alpha siRNA knock-down.

Response: Please see our comments to the above points that pertain to the same issue. Overexpression of PI4KIIα is shown to facilitate recruitment of Itch to TGN/ endosomal membranes. Furthermore, we demonstrate for the first time a specific role for PI4KIIα but not Itch in Fz internalization. Itch together with PI4KIIα is required for degradative sorting of Fz4, in agreement with elevated Wnt signaling in Itch-depleted cells. We feel that the new data indeed provide a coherent mechanistic framework for the role of PI4KIIα and Itch in Fz sorting at endosomes.

6. A hypothetical diagram describing the role of the PI4KII-Itch complex in the Wnt activated pathway would be helpful as a concluding figure, perhaps in supplementary information.

Response: We thank the referee for this suggestion. A tentative model is now provided in fig. S4A and discussed in the text.

7. What is the rationale for substituting the first proline of the PPxY motif with a serine in the SPxF mutant (instead of alanine)?

Response: The SPxF mutation was designed based on interaction studies previously performed by Macias *et al.* (Structure of the WW domain of a kinase-associated protein complexed with a proline-rich peptide; Maria J. Macias, Marko Hyvönen, Elena Baraldi, Johan Schultz, Marius Sudol, Matti Saraste & Hartmut Oschkinat). The first proline of the motif is changed to a serine in order to disrupt the poly-proline type 2 helix and to further introduce a polar side chain to completely abolish the binding of this particular poly-proline helix to the xP groove of the binding site within the Itch WW domains. Tyrosine within PPxY is exchanged for phenylalanine to keep an aromatic residue at this position in order to maintain the overall secondary structure, but to disrupt binding of the motif to the specificity patch of the WW domain by removal of the hydroxyl group (i.e. exchange of tyrosine for phenylalanine).

8. Page 4, last paragraph: the reference for the GST-UBA construct is missing.

Response: The missing reference has been added to the text.

9. Figure 2G: The typical Rab5Q phenotype of enlarged endosomes is not apparent. The authors should improve the quality of the images and provide a higher magnification inset of the endosomal co-localization.

Response: We have arranged the figure including an outline of the transfected cell (now fig. 2G) and hope that the referee will agree that the phenotype is quite overt.

10. Figure 4B-C: Expression levels of the protein constructs should be included.

Response: Included now in revised fig. S4D.

11. Several bar graphs show control conditions in the normalized form (100%). However, no error bars are shown. How was the statistics done? In the absence of variability in the control conditions, the one sample t-test is more appropriate.

Response: The lacking error bars in the controls of several quantifications are due to the fact that the corresponding values were set to 100% as large variations in the primary parameters occur (i.e. different absolute grey values in western blots). Although we believe that the statistical evaluation originally used was legitimate we have re-quantified the primary data without normalization; these are included now in revised fig. 1C, D. We have

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also re-evaluated the data shown in fig. 3 using the one-sample t-test. The corresponding statistical analyses confirm all of our conclusions from the original manuscript.

12. Molecular weight markers are missing in key Western blots, including Figures 1A,B and E.

Response: We have added the missing molecular weight markers in all figures.

13. In one panel (Fig. 3D), depletion of itch with RNAi causes a reduction in the levels of PI4KII-alpha; in another panel (Fig. 4F), the decrease is not obvious, perhaps because the knockdown of itch is not as efficient. The LRP6 blots should be repeated on samples showing a better knockdown.

Response: Indeed, knockdown efficiencies are generally lower in HEK293 cells used in for the experiments shown in fig. 4 compared to cell types and, hence, this effect is not as overt. We have re-analyzed the corresponding samples from HEK 293 cells shown in fig. 4 and now depict a slightly shorter exposure of the PI4KIIα blot, which is consistent with the conclusion drawn from fig. 3 (although the effect indeed remains less prominent in HEK293).

14. Page 7: For the experiments involving the internalization of Fz4-eGFP into endosomes, a control image showing the localization of Fz4 in the absence of Wnt5a should be included.

Response. We kindly refer the referee 1 to the left panel of fig. 4D, which contains the control asked for.

15. Figure 3G,H: the inhibition of kinase activity of PI4KII-alpha by the WW domain of itch is an important result; however, an important control is missing: a WW domain deficient in binding to the ppxy motif should be included (for instance, the WW4 domain can be used as it is not capable of binding PI4KII-alpha according to Figure 2D).

Response. We have done the requested control experiment using WW4 that has now been incorporated into the new fig. 3G, H. Indeed, WW4 does not supress PI4K activity.

<u>*Reviewer 2 (comments in italics):*</u>

PI4KIIalpha has been suggested to be involved in ubiquitin-mediated protein sorting in the endosomal system. However, the mechanism(s), by which PI4KII may control ubiquitin-mediated protein sorting, remain obscure. In this manuscript, Mossinger et al. report that ectopically expressed PI4KII is subjected to ubiquitylation and that this ubiquitylation is mediated by the HECT ubiquitin ligase Itch/AIP4. Furthermore, the authors provide evidence to indicate that Itch and PI4KII affect each other's function and that both are involved in controlling Fz4 trafficking and Wnt signaling. The notion that PI4KII function is regulated by Itch-mediated ubiquitylation is indeed of general interest. However, the data with respect to Itch-induced ubiquitylation of PI4KII are rather preliminary and its role in regulation of PI4KII function remains obscure (as my expertise with respect to the mechanisms involved in endosomal sorting is limited, I refrain from commenting on this issue).

Response: We thank the referee for his/ her kind remarks and the helpful comments. Regarding endosomal sorting we have added a large body of data that demonstrate that depletion of either PI4KIIα or Itch delays Fz degradation (see revised fig. 4). Together with the observed co-localization of Itch and PI4KIIα on Fz-containing endosomes these data argue for a role of Itch, presumably together with PI4KIIα in degradative sorting of Fz at the

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level of endosomes (now schematically depicted in fig. S4A). These observations complement and are in agreement with recent data showing that depletion of the de-ubiquitinating enzyme UBPy/USP8 attenuates Wnt signaling (Mukai et al., EMBO J, 2010). We further show- consistent with the above data- that overexpression of PI4KIIα facilitates recruitment of Itch to TGN/ endosomal membranes (revised fig. S4B).

1. All the binding experiments were performed with ectopically expressed proteins in HEK293 cells or with recombinant proteins in vitro. Evidence (other than colocalization studies) to indicate that endogenous Itch physically interacts with endogenous PIK4II would significantly increase the quality of the manuscript.

Response. We now show in the new panel B of fig. 2 that endogenous PI4KIIα and Itch form a complex in native tissue lysates.

2. Does Itch affect the ubiquitylation pattern of PI4KII within cells? This may be difficult to determine with endogenous proteins but should be rather straightforward in cotransfection experiments.

Response. We thank the reviewer for this suggestion. We have created a stable HEK293 cell line that allows for the inducible expression of Itch together with PI4KIIα. In the new fig. 3C we show that doxycyclin-induced expression of Itch leads to elevated levels of ubiquitinated PI4KIIα in cells, supporting our in vitro data.

3. The authors indicate that several HECT ubiquitin ligases containing WW domains exist. Thus, it seems mandatory to determine if these (e.g. NEDD4-1 or NEDD4-2) have the potential to interact with PI4KII and to ubiquitylate PI4KII in vitro and within cells (to provide evidence for the specificity of the proposed Itch-PI4KII interaction).

Response: In revised fig. S1 we provide evidence from both MS/MS-based proteomics and from in vitro binding studies that other E3 ligases such as NEDD4.1 and NEDD4.2 are capable of forming a complex with PI4KIIα. It is thus possible that some of these enzymes may functionally overlap with Itch, at least in part. However, we hope that the referee will agree that following all of these in detail is beyond the scope of the present paper.

4. Assuming that Itch-mediated ubiquitylation is physiologically relevant, the function of it remains entirely obscure.

Response: To tackle this point we have first investigated whether ubiquitination of PI4KIIα further regulates its enzymatic activity beyond the level already seen upon binding of the WW domains of Itch. In the new fig. S3D we show that Itch-mediated ubiquitination did not further reduce the PI(4)P-synthesizing activity of PI4KIIα beyond the inhibition induced by catalytically inactive Itch (Fig. S3D). We thus favor a model whereby ubiquitination of PI4KIIα does not primarily serve to regulate its enzymatic activity but may rather facilitate its targeting to the Ub-based sorting machinery at endosomes, although additional functions (i.e. regulation of its half-life) cannot be ruled out.

In agreement with this proposal we find a role for Itch or PI4KIIα in the regulation of Fz degradation (see revised fig. 4). Together with the observed co-localization of Itch and PI4KIIα on Fz-containing endosomes these data argue for a role of Itch, presumably together with PI4KIIα in degradative sorting of Fz at the level of endosomes (now schematically depicted in fig. S4A). These observations complement and are in agreement with recent data showing that depletion of the de-ubiquitinating enzyme UBPy/USP8 attenuates Wnt signaling (Mukai et al., EMBO J, 2010).

5. Figs. 3I and 3J: The quality of the ubiquitylation experiment is not convincing (i.e. if there is any ubiquitylation, the efficiency is so low that quantitation appears to be

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impossible).

Response: We have repeated these experiments and the new data are presented in the revised figs. 3A, B, I, J. All data are derived from multiple independent experiments with statistically significant results. We hope that the referee will find the new data set more convincing.

Reviewer 3 (comments in italics):

In this paper Mossiger et al. identify the E3 ligase Itch as an interactor and regulator of PI4KIIα, a TGN/endosomal protein responsible for PI(4)P synthesis. Results show that PI4KIIα is a direct binding partner of Itch (through the canonical PPxY-WW domain interaction) and is a substrate of its ligase activity. By IF analysis the authors suggest that Itch might be an endosomal protein involved in the regulation of Wnt-activated frizzled 4 (Fz4) receptor trafficking but these assumptions lack convincing experimental evidence. While the subject is interesting, this is a rather preliminary manuscript that fails to convey a clear message on the functional consequences of this interaction. Several relevant issues need to be addressed before publication.

Response: We hope that the referee will appreciate the large body of new data that has gone into this manuscript including both in vitro experiments as well as cellular assays. We also provide evidence for complex formation between native endogenous proteins and have done all specific experiments requested by this referee and all other reviewers. We hope that the referee will agree that the revised version of this paper can hardly be considered preliminary any more.

Cleaner and more conclusive experiments are needed in order to conclude that PI4KIIα is an Itch substrate. The In vitro ubiquitination assays (Fig. 3 A,B,I) are far from convincing. None of the experiments include an anti-Ub blot and no time 0 of the reaction is reported.

Response: We have repeated these assays and added the requested information: revised fig. 3A contains a 4°C control (time point 0), in which no ubiquitination is observed and anti-Ub blots are included now in revised fig. 3I.

The authors should repeat this entire set of experiments following the standard in vitro ubiquitination procedure (see for example Mund&Pelham, 2009, cited by the authors themselves). This assay should be performed with Ub wt and K0 to confirm that is indeed multi-monoubiquitination.

Response: The new experiments shown in fig. 3A, B have indeed been conducted under the conditions described by Pelham and colleagues and the requested K0 control (termed 7R here) confirms that PI4KIIα is indeed subject to multi-monoubiquitination.

ITCH wt and C830A overexpression in vivo may help in validating PI4KIIα as an Itch substrate.

Response: To address the point we have created a stable HEK293 cell line that allows for the inducible overexpression of Itch. In the new fig. 3C we show that doxycyclin-induced expression of Itch leads to elevated levels of ubiquitinated HA- PI4KIIα in cells, supporting our in vitro data.

No convincing data on the reciprocal regulation of PI4KIIα and Itch enzymatic activity are provided (Fig. 3G and I). Time course and dose response experiments are required.

Response: We apologize for not having included such controls in the earlier version of the paper as these are routinely done in our laboratory. In the revised fig. S3A, B we provide

time- and dose-response curves for PI4KIIα-mediated PI(4)P synthesis, which confirm that all subsequent experiments using the WW domains of Itch have been conducted in the linear range of the assay. We have also added additional experiments showing that full-length Itch similar to its WW domains negatively regulates PI4KIIα activity (fig. S3D) and that WW domain-mediated inhibition of PI4KIIα activity is dose-dependent and specific (i.e. not observed with WW4, which does not bind to PI4KIIa).

Regulation seems to be provided by interaction surfaces. Are other WW domains or PPXY motifs able to do the same? How is specificity achieved?

Response. We believe that our NMR analysis provides molecular insights into how specificity is achieved. We also show in revised fig. S1 based on MS/MS-analyses and in vitro binding studies that other WW domain containing E3 ligases such as NEDD4.1 and NEDD4.2 are capable of forming a complex with PI4KIIα. It is thus possible that some of these enzymes may functionally overlap with Itch. However, we hope that the referee will agree that following all of these in detail is beyond the scope of the present paper.

From the data presented is not clear if the involvement of Itch protein on Fz4 internalization is related to its E3 ligase activity on PI4KIIα. From the initial set of data, it appears that KD of Itch causes a reduction in the level of PI4KIIα. Thus, one might assume a similar phenotype promoted by the two KDs on Fz4 internalization, while results show the opposite. Testing the overexpression of Itch wt and C830A mutant in experiments shown in Fig.4 B, F and G would clinch the issue, and is required to, at least, propose a model related to the role of Itch in this context.

Response. We agree with the reviewer and have therefore conducted a number of additional experiments to clarify the role of PI4KIIα and Itch in endosomal trafficking. We have re-analyzed the effect of Itch and PI4KIIα loss-of-function in Fz internalization and in subsequent Fz degradation. In agreement with the known role of PI4KIIα in the PI(4,5)P2-dependent initiation of Wnt/ Fz signaling we show that PI4KIIα-KD cells show impaired endocytosis of Wnt-activated Fz, whereas KD of Itch is without any effects. Importantly, we further demonstrate that depletion of either PI4KIIα or Itch delays Fz degradation (see revised fig. 4). Together with the observed co-localization of Itch and PI4KIIα on Fz-containing endosomes these data argue for a role of Itch, presumably together with PI4KIIα in degradative sorting of Fz at the level of endosomes (now schematically depicted in fig. S4A). These observations complement and are in agreement with recent data showing that depletion of the de-ubiquitinating enzyme UBPy/USP8 attenuates Wnt signaling (Mukai et al., EMBO J, 2010). We further show - consistent with the above data - that overexpression of PI4KIIα facilitates recruitment of Itch to TGN/ endosomal membranes (revised fig. S4B).

Minor points:

1. *Please systematically indicate in all figures the molecular weight markers.*

Response: Done in all revised figures.

2. *Authors, please comment on the appearance of the slow migrating band in the IP samples of Fig 1A, B and in the input of Fig. 1E and Fig. 2C. Is this monoubiquitination? It seems not to be affected by SF mutation, but then the (same?) band disappears in the presence of Itch C830A (Fig. 3B). Are other post-translational modifications involved?*

Response: We do indeed see a band of similar size in ubiquitination assays. However, the referee is right in that the band does not appear to be affected by the SF mutation. It, hence, remains possible that PI4KIIα is subject to other post-translational modifications (in fact PI4KIIα is known to be palmitoylated and this required for targeting and for function).

One possibility would be modification by another ubiquitin-related protein. Future studies will be needed to address this issue in detail.

3. Endosomal localization of Itch is limited. Authors, please tone-down related sentences.

Response: We have toned down the corresponding statements and rather argue that Itch and PI4KIIα partially colocalize at the TGN/ endosomal boundary, a conclusion backed up by Pearson correlation analysis with various subcellular markers (see fig. S2).

2nd Editorial Decision 27 June 2012

Many thanks for submitting the revised version of your manuscript to EMBO reports.

It was sent back to two of the original referees and we have now received their comments on it. Both reviewers support publication in EMBO reports, but referee 2 still feels that the functional consequence of Itch-mediated ubiquitination of PI4KII remains insufficiently elucidated. Upon further consultation with referee 1, s/he agreed that the two relatively minor experiments suggested by referee 2 should be performed.

Therefore I would like to kindly ask you to conduct these experiments in a second round of revision. Formally, your manuscript would need to be accepted by July 5th (as this would be 6 months after the initial decision), but if no similar study comes out in the meantime and since the requested experiments seem rather straightforward, I do not envisage problems.

I also noticed that in some instances, you performed statistical analysis on experiments that have only been performed twice (eg Fig 1C/D, 3J, 4G,I). Please note that in order to calculate meaningful standard deviations, an experiment should be repeated at least three independent times (please also refer to David Vaux paper in JCB, Vol. 177, No. 1, April 9, 2007 'Error bars in experimental biology'). May I kindly ask you to either repeat these experiments at least one more time or omit the error bars?

Please do not hesitate to contact me if you want to discuss this further.

Yours sincerely,

Editor
EMBO reports

REFEREE REPORTS:

Referee #1:

The authors have significantly improved their manuscript and have adequately addressed all my concerns.

Referee #2:

This is a significantly improved manuscript (i.e. many of the concerns have been adequately addressed). Nonetheless, a few issues remain that the authors may want to consider;

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1. As indicated in my original comments, the actual consequence of Itch-mediated ubiquitylation of PI4KII remains obscure. Rescue experiments as shown for PI4KII in Fig. 4E would be ideally suited to provide evidence that the catalytic activity of Itch is indeed required for the (cellular) effects observed (i.e. as it stands, the data show that Itch can induce ubiquitylation of PI4KII but if this is functionally relevant for PI4KII function, at least for the ones measured, remains unclear).

2. In response to the reviewers' comments, the authors provide evidence to indicate that two additional members of the Nedd4-like family of E3s are found in a common complex with PI4KII. I agree with the authors that it is the beyond the scope of this manuscript to follow up these potential interactions in detail. However, an siRNA experiment (e.g., half-life of Fz4) and/or a ubiquitylation assay (in vitro or within cells) would be valid controls to prove the physiological significance of the Itch-PI4KII interaction.

2nd Revision - authors' response 10 September 2012

Editorial comment (in italics):

I also noticed that in some instances, you performed statistical analysis on experiments that have only been performed twice (eg Fig 1C/D, 3J, 4G,I). Please note that in order to calculate meaningful standard deviations, an experiment should be repeated at least three independent times (please also refer to David Vaux paper in JCB, Vol. 177, No. 1, April 9, 2007 'Error bars in experimental biology'). May I kindly ask you to either repeat these experiments at least one more time or omit the error bars?

Response: We agree and have thus modified the corresponding figures as follows: We have deleted figures 1C/D and 3J from the manuscript and instead now cite the mean values obtained from two independent experiments in the text. Moreover, we have omitted the error bars from revised figures 4G and 4I as suggested.

Response to reviewers:

1. As indicated in my original comments, the actual consequence of Itch-mediated ubiquitylation of PI4KII remains obscure. Rescue experiments as shown for PI4KII in Fig. 4E would be ideally suited to provide evidence that the catalytic activity of Itch is indeed required for the (cellular) effects observed (i.e. as it stands, the data show that Itch can induce ubiquitylation of PI4KII but if this is functionally relevant for PI4KII function, at least for the ones measured, remains unclear).

Response: We thank the referee for his/ her suggestion. As the experiment shown in fig. 4E has been obtained with stably transfected HEK293 cells overexpressing Fz4 meaningful rescue experiments would require the generation of new cell lines that stably overexpress not only Fz4 but also siRNA-resistant versions of Itch. This would have been very difficult to achieve within the time frame allowed for revision. Moreover, as Itch likely has other substrates apart from PI4KIIα the results from such experiments might be difficult to interpret with respect to the role of PI4IIα ubiquitination.

To nonetheless address the referee's concern we have carried out an extensive quantitative proteomic analysis of the interaction partners of PI4IIα ubiquitinated by Itch in comparison with the non-modified form. As shown in the new supplementary table 1 we find that ubiquitinated PI4KIIα preferentially associates with endosomal proteins such as myoferlin and Tom1, a VHS-GAT domain protein implicated in degradative sorting via binding to ubiquitin and clathrin. Conversely, ubiquitination of PI4KIIα inhibited its association with endocytic proteins including SNX9, SNX18, endophilin, and SGIP1. We thus hypothesize that Itch-mediated ubiquitination of PI4KIIα may act as a switch that redirects the enzyme from the plasma membrane to a degradative endosomal pathway, in

agreement with our functional data.

2. In response to the reviewers' comments, the authors provide evidence to indicate that two additional members of the Nedd4-like family of E3s are found in a common complex with PI4KII. I agree with the authors that it is the beyond the scope of this manuscript to follow up these potential interactions in detail. However, an siRNA experiment (e.g., half-life of Fz4) and/or a ubiquitylation assay (in vitro or within cells) would be valid controls to prove the physiological significance of the Itch-PI4KII interaction.

Response: We thank the referee for this suggestion that we have gladly followed. We have compared side-by-side the ability of NEDD4.1 and Itch to ubiquitinate PI4KIIα. As shown in the new fig. S1G we observe that recombinant Nedd4.1 fails to ubiquitinate PI4KIIα although it undergoes efficient auto-ubiquitination. These data shown in the new fig. S1G indicate that NEDD4.1 in spite of its ability to bind to PI4KIIα is unable to catalyze its ubiquitination, at least *in vitro*. These results further support the physiological significance of the Itch-PI4KIIα interaction.

3rd Editorial Decision 28 September 2012

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

Yours sincerely,

Editor
EMBO Reports

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