EMBO reports

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An essential Role for Grk2 in Hedgehog signalling downstream of Smoothened

Zhonghua Zhao, Raymond Teck Ho Lee, Ganesh Pusapati, Audrey Iyu, Rajat Rohatgi and Philip Ingham

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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 11 November 2015

Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports as well as cross-comments, which are copied below.

As you will see, the referees acknowledge that the study is well done and the findings are of interest. However, they also point out that the phosphorylation target of Grk2 remains unclear. I therefore think that it should be tested whether GPR161 is a target of Grk2, at least in vitro, as referee 2 suggests. It would also clearly strengthen the study if other potential targets are tested, as referee 2 mentions in her/his cross-comments below. Referee 1 further agrees with referee 2 that zebrafish-specific phosphorylation sites of Smo should be identified. The quality of figure 6 should further be improved and all information regarding statistics provided.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Please address all referee comments in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

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REFEREE REPORTS

Referee #1:

This is important work that shows the strength of targeted mutagenesis in a powerful vertebrate system. Zhao et al. impressively take advantage of the ZFN system in zebrafish vivo complemented with the CRISPR system in mouse 3T3 cells in vitro. The authors address the function of Grk2 in the Hh pathway. The authors demonstrate that Grk2 mediated phosphorylation is essential for the activation of the Hh pathway and operates downstream of the receptor but upstream of the transcription factors Gli. However, in contrast to the present hypothesis based on knock-down experiments, c-terminal phosphorylation of the co-receptor smoothened is not required for activation. This raises the question which phosphorylation target is essential for signal transduction? The authors suggest a GPCR but, unfortunately, the substrate of Grk2 remains unknown and requires more detailed investigation in the future. Although the ZFN technology is not widely used in ZF anymore due to its complexity and technical demands, this manuscript will serve as a very interesting example in the zebrafish. This work contains a set of elegant and nicely executed experiments that do support the overall contentions.

Major points:
The authors have raised the major question in the discussion: How does Grk2 interact with the Hh pathway? The authors suggest that a GPCR may serve as a phosphorylation target for the kinase activity of Grk2. Is there any further evidence the authors can provide to strengthen this hypothesis?

Furthermore, the Shh pathway has been discussed to act and be regulated in a feedback loop. However, in the CNS it seems that the expression of the ligands are unchanged (i.e. Scholpp et al., 2007). Therefore it would be interesting to see if the expression of Shh as well as of Twhh are altered in the MZGrk2.

Minor points:
Check citations in "Materials and Methods": i.e. page 5 first paragraph : (ref:17)
Check references. i.e. In 32 the authors are missing.

Referee #2:

Zhao and colleagues analyze the phenotype of GRK2 maternal and zygotic null zebrafish and show that HH response is completely abolished. This was accomplished using a ZFN-mediated stop allele, and then germ cell transplantation to generate a maternal and zygotic null. Epistatic experiments show lack of gain of function response to SHH and SMOA1 alleles and a response from loss of PKA, showing that GRK2 using its kinase domain, acts upstream of PKA and that the downstream pathway remains intact in GRK2 mutants. The surprising finding is that previous studies linked GRK2 to SMO tail phosphorylation and endocytic recycling, but mutations of GRK2 substrate sites of SMO previously shown to revert the SMOA1 dominant, show no phenotype, leading the authors to propose the intriguing possibility that GRK2 acts on an alternative GPCR rather than Smo.

Overall, the work is well written and the data is clean with adequate controls. However, the work is essentially a zebrafish genetics analysis using previously identified GRK2 variants from mouse, human and zebrafish to make conclusions. While the pathway logic remains similar, it is clear from the included experiments that not everything operates the same way in zebrafish. Consequently, independent biochemical confirmation of the key conclusions of the paper should be performed. For example, the argument that SMOSA lacks all the key phosphorylation sites would be true if zebrafish Smo functioned exactly like mouse Smo. Western or Mass Spec data showing that all GRK2 dependent phosphorylation sites have been mutated is needed prior to turning attention to a non -SMO target for GRK2. What is an alternative residue is used in zebrafish? Also the authors point out the possibility that GRK2 phosphorylates GPR161 to turn it off, a tantalizing hypothesis. However, using the in vitro system in Fig 4, the authors can easily test whether GPR161 is a target of GRK2. This should be included in the paper.

Other Comments:

1. SmoA1SA and SMO SA fails to restore SMO- function but still has constitutive activity (althought slightly less) in zebrafish embryos. Are the protein levels in the fish similar?

2. The finding that the SmoSD and SmoKRA mutants lack constitutive activity in MEFs, together with the ability of Smo to localise to the PC of Grk2-/- cells is consistent with this view. However, the basis of the disparity in the activity of the SmoSA mutant between zebrafish embryos and MEFs is currently unclear. A further exploration of the discrepancy is needed.

Minor Considerations:

1. Abstract should be 175 words.
2. pg 15 "smo reportedly fails to localize to the PC of cultured mammalian cells" needs a reference.

Referee #3:

This manuscript describes the production of zygotic and maternal-zygotic zebrafish mutants for the Grk2 gene. Analysis confirms a role for Grk2 in HH signalling, as previously shown in cells, morpholino knockdown zebrafish, Drosophila and mice. This study suggests that this function is kinase dependent, but that phosphorylation is not sufficient or necessary for Smo function (this, along with the production of zebrafish grk2 mutants, is the main novel aspect of this work). The authors speculate that Grk2 may regulate Hh signalling by downregulating an unknown GPCR, but this is not specifically shown. While this study has generated some novel insight into Grk2 function over what was previously known, I have some specific issues with the way some of the data have been presented. I believe these issues would need to be addressed before this manuscript would be suitable for publication.

Specific Comments:
The authors produced both zygotic and maternal-zygotic mutants for the Grk2 gene, the function of which was previously investigated in zebrafish using morpholino knockdown. This is a rigorous approach and while the difference between MOs and mutants is discussed briefly in the discussion, it would help if the results section included a description of the MO phenotype in comparison to both zygotic grk2 and MZgrk2 mutants (maybe include this in a Table?)

Throughout the manuscript the authors tend to use phrases like "response to hedgehog signalling is completely abolished" (abstract, page 2), "failed to rescue", "fully rescued"
Firstly, I am not sure it is correct to say signalling is completely abolished, rather that it is reduced to undetectable levels. As a more general point, throughout this manuscript there is little if any quantification of data to support such sweeping statements, and N numbers are not always given to indicate either the number of embryos analysed (eg in in situ experiments), or in the case of the biochemical assays, the number of replicates and independent experiments that contributed to the statistical analyses. The authors should address these points.

On a similar point: Page 11. "..transcripts...., were ABSENT from both the neural tube and brain". In situ hybridisation is not a particularly sensitive technique and I would advise against concluding that transcripts are absent as opposed to undetectable (or severely reduced) by this method.
Similarly, in the next sentence "Almost all of the Prox1a+ve superficial slow-twitch.....were absent from the myotome...". "almost all" is a very qualitative description, and absent is a very strong word given the limitations of the detection methods used.

Figure 6. The data in this figure are very poor quality. Cilia staining with acetylated-alpha-tubulin is very indistinct in the wild-type fish, and there is generally very little staining for Smo (GFP) - strangely in the merged images the orange overlap appears more than would be suggested from the individual images. In the SHH/Smo panel a large percentage of the Smo staining does not correlate with acetylated-alpha-tubulin staining even though it looks to be cilia staining. Strangely the staining for both cilia and GFP is clearer in the MZgrk2 mutant it is unclear why the mutant would have more cilia. The authors should closely review the data in this figure. Preferably more convincing data should be presented and the co-localisation quantified.

Page 10, "most mutant larvae failed to form a normal swim bladder". Please be more specific in the

% of larvae that show this phenotype. Also, it would be helpful to point to this defect in Figure 1d.

Figure 5d. The error bars are extremely large - please comment. Also, please state in the legend what they represent i.e SEM or SD. Do these data represent multiple experiments? What are the replicates? Please give more experimental details.

Similarly, the error bars for the RT-PCR data shown in supp figure 1 are enormous - as for all experiments please provide experimental details of the replicates used. Please also provide statistical analysis - it is not clear that increased Ptch2 expression by SmoA1SA is significant (say compared to Smo or SmoSD). Only the statistical analysis will tell this.

Page 17: "The dramatic loss of Hh signalling observed in the zebrafish MZgrk2 mutants stands in contrast to the rather mild phenotype of the mouse Grk2 mutation, this mild phenotype might be explained by partial redundancy between Grk2 and the paralogous Grk3 gene. Notably, our Grk2 /- cells, which show a strong loss of Shh responsiveness, do not express Grk3 (unpublished data)." I am confused by this statement. If the authors are proposing that Grk3 is compensating for the loss of Grk2, surely you should see enhanced expression (or at least some expression) in Grk2-/- cells.

Minor points:
In the materials and methods please state what cell line was used to make the SHH-N conditioned medium.

On page 10 please use correct standardised annotation to describe the mutation in the grk2 mutant. ie. c.111delC (and also include the corresponding annotation at the protein level to denote a frameshift)

Page 10 "...Figure 1c, the truncated region includes almost all the...". This statement is confusing. I would recommend changing this to "...Figure 1c, the truncated protein would lack almost all the...".

Page 12: "MZgrk2 embryos injected with shh or SmoA1 mRNA, by contrast, showed little response; only a few additional slow-twitch fibres were detected scattered throughout the length of the trunk, in contrast to those restricted to the anterior somites of uninjected controls (Fig. 3a)." There appears to be no uninjected controls shown for this experiment - please add these images.
Pages 12,13: The authors should state the rationale for looking at Gli2 processing in zebrafish and Gli1 and Gli3 in mammalian cells
The manuscript, including the materials and methods, should be thoroughly proof-read for grammatical errors

Further comments from referee 1:

I would suggest that either the authors provide some evidence for their hypothesis that GPCR (eg GPR161) serves as a substate for Grk2 or they characterize specific phosphorylation sites in zebrafish Smo (compared with mouse). Both findings would increase equally the novelty of the paper and this would make a strong argument for publication in EMBOR.

Referee 2:

I think there is novelty in the strong GRK2 phenotype that places greater emphasis on GRK2 within the pathway. However the work suffers from a lot of "negative" data saying that findings from previous reports are not seen in the authors' system. A positive finding demonstrating a GRK2 target such as GPR161 (or Nrp, BOC, or KIF7 etc...) would significantly strengthen the work.

Referee 3

I agree that finding a GRK2 target would greatly enhance the novelty of this work. However, as the authors acknowledge in the final sentence of the manuscript, this would require significantly extra work in generating mutations in the two zebrafish GPR161 orthologues. If the authors were asked to

do this and GPR161 was not a target, I don't think this should necessarily preclude publication of the manuscript as they would have excluded an excellent candidate.

1st Revision - authors' response 15 February 2016

Point-by-point response

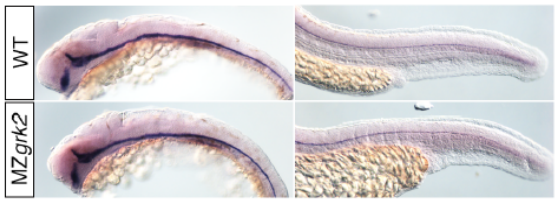
Referee #1:

This is important work that shows the strength of targeted mutagenesis in a powerful vertebrate system. Zhao et al. impressively take advantage of the ZFN system in zebrafish vivo complemented with the CRISPR system in mouse 3T3 cells in vitro. The authors address the function of Grk2 in the Hh pathway. The authors demonstrate that Grk2 mediated phosphorylation is essential for the activation of the Hh pathway and operates downstream of the receptor but upstream of the transcription factors Gli. However, in contrast to the present hypothesis based on knock-down experiments, c-terminal phosphorylation of the co-receptor smoothened is not required for activation. This raises the question which phosphorylation target is essential for signal transduction? The authors suggest a GPCR but, unfortunately, the substrate of Grk2 remains unknown and requires more detailed investigation in the future. Although the ZFN technology is not widely used in ZF anymore due to its complexity and technical demands, this manuscript will serve as a very interesting example in the zebrafish. This work contains a set of elegant and nicely executed experiments that do support the overall contentions.

Major points:
The authors have raised the major question in the discussion: How does Grk2 interact with the Hh pathway? The authors suggest that a GPCR may serve as a phosphorylation target for the kinase activity of Grk2. Is there any further evidence the authors can provide to strengthen this hypothesis?

• Furthermore, the Shh pathway has been discussed to act and be regulated in a feedback loop. However, in the CNS it seems that the expression of the ligands are unchanged (i.e. Scholpp et al., 2007). Therefore it would be interesting to see if the expression of Shh as well as of Twhh are altered in the MZGrk2.

The negative feedback loop that regulates Shh actvity is based on the upregulation of the receptor Ptch and, in vertebrates, the Hh binding protein Hhip in response to pathway activation. As we have shown, Ptch2 expression is reduced in MZgrk2 embryos – however, this does not result in pathway upregulation as Grk2 is itself required for pathway activity. In response the Reviewer's specific question about Shh expression, we have now analysed this by WISH in MZgrk2 embryos and see no difference from wild type. Because of space constraints, we have no included this in the Supplementary data, but supply a representative image for the Reviewers (Appendix 1).



Appendix 1:
ISH of Shh in AB (wild type) and MZ*grk2* 26hpf embryos.
(n=10)

• Minor points:
Check citations in "Materials and Methods": i.e. page 5 first paragraph : (ref:17)
Check references. i.e. In 32 the authors are missing.

We have checked and corrected the references as requested.

Referee #2:

• independent biochemical confirmation of the key conclusions of the paper should be performed. For example, the argument that SMOSA lacks all the key phosphorylation sites would be true if zebrafish Smo functioned exactly like mouse Smo. Western or Mass Spec data showing that all GRK2 dependent phosphorylation sites have been mutated is needed prior to turning attention to a non -SMO target for GRK2. What is an alternative residue is used in zebrafish?

The mouse GRK2 and zebrafish GRK2 has 84.6% identity suggesting that phosphorylation sites should be highly conserved. Moreover, as perhaps we failed to make clear in our original manuscript, we actually assayed the activity of the mouse Smo in zebrafish embryos, the mutations being based on the sites identified and mutated by Chen et al. (2011).We agree that it would be desirable to use Mass Spec analysis to identify the phosphorylation sites in Smo and this is in fact something that we have previously attempted; unfortunately, however, we have found it impossible to purify sufficient amounts of tagged Smo protein from injected embryos to perform such analyses.

Mindful of the possibility that there may be other Grk2 sites not previously identified, we re-analysed the Smo CTT sequence and identified two further putative Grk2 phosphorylation sites (on the basis of a study by Asai et al 2014 which identify peptide substrates of Grk2) in the Smo CTT. Mutation of all 14 sites in Smo CTT still shows activity in rescuing Smo mutant phenotype. These new data are presented in revised Figure 5.

• Other Comments:

1. SmoA1SA and SMO SA fails to restore SMO- function but still has constitutive activity (althought slightly less) in zebrafish embryos. Are the protein levels in the fish similar?

We have checked that the mutant forms of Smo are expressed in MEFs and have performed western blot analyses in zebrafish embryos; the latter shows that the mutant forms are expressed at similar levels, but are much reduced compared to the wild type (GFP-tagged) form of Smo. This now presented in Supplementary Fig. 2.

2. The finding that the SmoSD and SmoKRA mutants lack constitutive activity in MEFs, together with the ability of Smo to localise to the PC of Grk2-/- cells is consistent with this view. However, the basis of the disparity in the activity of the SmoSA mutant between zebrafish embryos and MEFs is currently unclear. A further exploration of the discrepancy is needed.

We have repeated this assay in both systems multiple times and are confident that the observed differences are real. Unfortunately, we still cannot exactly explain the basis for this disparity, but believe it is an important finding to report.

• Minor Considerations:

1. Abstract should be 175 words.
We have shortened the abstract to within this limit.

2. pg 15 "smo reportedly fails to localize to the PC of cultured mammalian cells" needs a reference.
We have added the reference.

Referee #3:

• it would help if the results section included a description of the MO phenotype in comparison to both zygotic grk2 and MZgrk2 mutants (maybe include this in a Table?)

We have repeated the morpholino knock down of grk2 as previously reported by Philipp et al (2008) and find a reduction in the number of Prox1+ve cells very similar to that observed by these authors. These data, along with those for the MZgrk2 mutants and rescued mutants are presented in Suppl. Figure 1.

• Throughout the manuscript the authors tend to use phrases like "response to hedgehog signalling is completely abolished" (abstract, page 2), "failed to rescue", "fully rescued"
Firstly, I am not sure it is correct to say signalling is completely abolished, rather that it is reduced to undetectable levels. As a more general point, throughout this manuscript there is little if any quantification of data to support such sweeping statements, and N numbers are not always given to indicate either the number of embryos analysed (eg in in situ experiments), or in the case of the biochemical assays, the number of replicates and independent experiments that contributed to the statistical analyses. The authors should address these points.

We acknowledge the previous lack of precision in the presentation of our data. In addition to the analysis referred to above, we have repeated the Smo overexpression experiments and now present quantitative data (numbers of Prox1+ve cells) in each case. We have indicated samples sizes (N) for each experiment and indicated replicates in the Material & Methods section where relevant; we have also revised our description of phenotypes in accord with the Reviewer's comments.

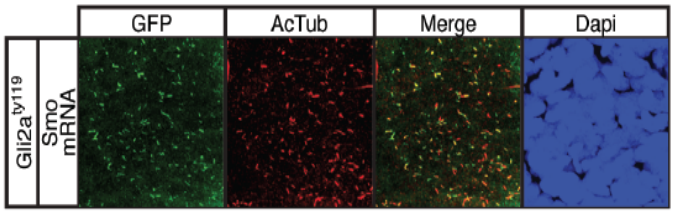
• On a similar point: Page 11. "..transcripts...., were ABSENT from both the neural tube and brain". In situ hybridisation is not a particularly sensitive technique and I would advise against concluding that transcripts are absent as opposed to undetectable (or severely reduced) by this method. Similarly, in the next sentence "Almost all of the Prox1a+ve superficial slow-twitch.....were absent from the myotome...". "almost all" is a very qualitative description, and absent is a very strong word given the limitations of the detection methods used.

We have revised our descriptions accordingly

• Figure 6. The data in this figure are very poor quality. Cilia staining with acetylated-alpha-tubulin is very indistinct in the wild-type fish, and there is generally very little staining for Smo (GFP) - strangely in the merged images the orange overlap appears more than would be suggested from the individual images. In the SHH/Smo panel a large percentage of the Smo staining does not correlate with acetylated-alpha-tubulin staining even though it looks to be cilia staining. Strangely the staining for both cilia and GFP is clearer in the MZgrk2 mutant it is unclear why the mutant would have more cilia. The authors should closely review the data in this figure. Preferably more convincing data should be presented and the co-localisation quantified.

We agree that the original images had become pixelated after compression and were not of sufficient quality to allow the reader to evaluate our claims. To circumvent this difficulty, we have repeated the analysis of the various forms of Smo in wild type embryos, focusing on their localisation to the PC of notochord cells, which can be imaged more readily than the myotome. We hope that the Revieiwer will agree that these preparations are clearer. Our conclusion that phosphorylation of Smo does not affect cilia localization is consistent with our observations in NIH 3T3 cells. We have been unable to repeat the analysis of Smo localisation in MZgrk2 mutants due to their unavailability.

However, we now provide DAPI images of the original preparations, which help explain the apparent difference in cilia number. This reflects the increased cell density in the mutant specimens, which in turn is due to a delay in differentiation of muscle fibres in the MZgrk2 mutants. Moreover, in the absence of Ptch2 mediated negative feedback on Shh distribution, the ligand presumably acts over longer distances, which could explain the apparent increase in PC localization of Smo. Consistent with this hypothesis, we have found a similar increase in cilia localization of Smo in embryos in which the negative ffdback loop is blocked by the dominant negative Gli2 mutant, you-too. Because of space constraints, we have no included this in the Supplementary data, but supply representative images for the Reviewers (Appendix 2).



Appendix 2:
PC localization of Smo in myotome of *gli2a*^{ty119} mutants. AcTub is used to label PC. These mutants also show increased PC localization of Smo. (n=4)

• Page 10, "most mutant larvae failed to form a normal swim bladder". Please be more specific in the % of larvae that show this phenotype. Also, it would be helpful to point to this defect in Figure 1d.

We have now genotyped individual larvae from a grk2 incross and identified 16 grk2 homozygotes, all of which lacked a swim bladder. The text has been revised accordingly.

• Figure 5d. The error bars are extremely large - please comment. Also, please state in the legend what they represent i.e SEM or SD. Do these data represent multiple experiments? What are the replicates? Please give more experimental details.

The error bars represent the standard deviation (SD) and we have added the description in legends. The data are derived from three biological repeats.

• Similarly, the error bars for the RT-PCR data shown in supp figure 1 are enormous - as for all experiments please provide experimental details of the replicates used. Please also provide statistical analysis - it is not clear that increased Ptch2 expression by SmoA1SA is significant (say compared to Smo or SmoSD). Only the statistical analysis will tell this.

We have performed more biological repeats; the statistical analysis is based on the unpaired Student's T-test, as now described in the Figure Legend.

• Page 17: "The dramatic loss of Hh signalling observed in the zebrafish MZgrk2 mutants stands in contrast to the rather mild phenotype of the mouse Grk2 mutation, this mild phenotype might be explained by partial redundancy between Grk2 and the paralogous Grk3 gene. Notably, our Grk2 /- cells, which show a strong loss of Shh responsiveness, do not express Grk3 (unpublished data)." I am confused by this statement. If the authors are proposing that Grk3 is compensating for the loss of Grk2, surely you should see enhanced expression (or at least some expression) in Grk2-/- cells.

No, the point we intended to make here is that GRK3 is not expressed in the 3T3 cells, therefore there is nor compensation for the loss of Grk2 as there is in the whole animal (where GRK3 is expressed). We also have added RT-PCR data in supplementary data.

• Minor points:
In the materials and methods please state what cell line was used to make the SHH-N conditioned medium.

The cell line used was HEK293 cells, as now indicated in the Materials and Methods.

On page 10 please use correct standardised annotation to describe the mutation in the grk2 mutant. ie. c.111delC (and also include the corresponding annotation at the protein level to denote a frameshift)

We have revised the text accordingly.

Page 10 "...Figure 1c, the truncated region includes almost all the...". This statement is confusing. I would recommend changing this to "...Figure 1c, the truncated protein would lack almost all the...".

We have revised the text accordingly.

Page 12: "MZgrk2 embryos injected with shh or SmoA1 mRNA, by contrast, showed little response; only a few additional slow-twitch fibres were detected scattered throughout the length of the trunk, in contrast to those restricted to the anterior somites of uninjected controls (Fig. 3a)." There appears to be no uninjected controls shown for this experiment - please add these images.

We have now included images of the uninjected controls in Fig 3.

Pages 12,13: The authors should state the rationale for looking at Gli2 processing in zebrafish and Gli1 and Gli3 in mammalian cells.

Pragmatically, Gli2a was analysed since this is the only Gli protein for which antibody is available. However, previous studies by our lab (eg. Ben et al 2011; Maurya et al 2013) have established that the processing of Gli2a in response to Hh pathway activity in zebrafish resembles that of Gli3 in mammals. We have now included a brief reference to these earlier findings in the text to make the rationale for the Gli2a analysis clearer.

We now turn to the issue of how Grk2 functions in the pathway and its possible interaction with GPR161. Understandably, this was a point specifically raised by two of the Referees:

Referee 1 states that "The authors have raised the major question in the discussion: How does Grk2 interact with the Hh pathway? The authors suggest that a GPCR may serve as a phosphorylation target for the kinase activity of Grk2. Is there any further evidence the authors can provide to strengthen this hypothesis?" but also observed that this issue will require "more detailed investigation in the future".

Subsequently, however, this Referee suggests "that either the authors provide some evidence for their hypothesis that GPCR (eg GPR161) serves as a substate for Grk2 or they characterize specific phosphorylation sites in zebrafish Smo (compared with mouse)."

Referee 2 suggests that "using the in vitro system in Fig 4, the authors can easily test whether GPR161 is a target of GRK2. This should be included in the paper" and sunsequently comments that "a positive finding demonstrating a GRK2 target such as GPR161 (or Nrp, BOC, or KIF7 etc...) would significantly strengthen the work."

We were, of course, mindful of the limitations to our study when we submitted our paper, which is partly why we felt it more appropriate to present it as a "Research Report". Nevertheless, we were keen to explore the proposed link between Grk2 and GPR161 further in response to the Referees' comment: to this end we set out to generate more fish chimeric for grk2 homozygous germ cells (as our original chimeras had in the meantime died) with the intention of investigating the distribution of tagged GPR161 in the absence of Grk2 activity. Unfortunately, despite significant efforts (my postdoctoral colleagues Zhao and Lee performed over 300 transplants and identified 20 chimeric larvae) we were unsuccessful in generating a single additional chimeric adult female. At the same time, our collaborators in the US were, however, able to investigate the distribution of GPR161 in their grk2 knock our cells as suggested by Referee 2; as predicted, the internalization of the protein in response to Shh is blocked in these cells; however, the significance of this finding is somewhat undermined by their additional finding that knock-out of GPR161 in the same 3T3 cells has no effect on their response to Shh. Moreover we have now succeeded in generating and isolating mutant alleles of both gpr161 paralogues in zebrafish; however our analysis of these to date has also failed to reveal any effect of either mutation on Hh signaling. Further analysis, including the generation of double mutant germ line chimeras, will be required but this is a significant undertaking that we feel is well beyond the scope of the current analysis. Given this lack of functional data, we feel reluctant to make strong claims about the significance of the apparent dependence of GPR161 internalisation on Grk2 in 3T3 cells and would prefer not to include these data; we feel significantly more work needs to be done to clarify the contribution of GPR161 to the Hh response.

As for interactions with other Hh pathway components: we have also attempted to investigate the epistatic relationship between Kif7 and Grk2 by making double mutant embryos; however, this also entails making kif7; grk2 double mutant germ line chimeras, a non trivial feat that has so far eluded us.

We hope that you will agree that the further investigation of the putative Grk2-GPR161 interaction constitutes a major undertaking beyond the scope of this analysis, as recognized by Referee 3.

2nd Editorial Decision 29 February 2016

Thank you for the submission of your revised manuscript to our journal. We have now received the reports from the referees that were asked to assess it, and both support its publication now.

Referee 2 suggests that you improve the discussion, and I would like you to address this point before we can proceed with the official acceptance of your manuscript.

Please also add scale bars to all microscopy images, and specify the bars, error bars, *, and tests used to calculate p-values in the legends for figures 4B,C, 5B,E, 7C, EV1 and EV2A. This information must be provided in the figure legends.

I look forward to seeing a final revised version of your manuscript as soon as possible. Please let me know if you have any questions.

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REFEREE REPORTS

Referee #1:

The manuscript has been substantially revised, and may now be accepted for publication in EMBO reports.

-I am glad to see that the expression of the principal signal of the ZLI , Shh, is unaltered in MZGrk2.

Referee #2:

The revised manuscript adds several supplementary figures to address reviewers comments about protein stability and expression of hh ligands. A major concern remains whether mouse and zebrafish hedgehog pathway use Smo differently, so that mouse mutations do not have the same functional meaning in zebrafish. The authors respond that they have tried mouse SMO in zebrafish, but that doesn't demonstrate that the pathways use Smo the same way. Overall the data presented is of high quality and provides an interesting analysis of GRK2 function in zebrafish. Because of the discrepancy between mouse and zebrafish Smo mutations, the authors should at least include a discussion of the possibilty that the two systems diverge (as they have with cilia genes) slightly and further studies are needed identifying how GRK2 interacts with Smo function.

2nd Revision - authors' response 07 March 2016

Author Response to Editor and Referees:

In response to Referee 2 and following your request, we have expanded the part of the discussion dealing with the disparity between the activity of the SmoSA mutant in fish embryos and mammalian cells. Based on our analysis, we cannot provide a definitive explanation of this difference: however, we now refer to another well documented divergence in Hh signaling between zebrafish and mouse

and suggest additional analyses that should shed further light on this interesting issue. We hope that this is what you had in mind.

*As requested, we have added scale bars to all microscopy images, and specified in the Figure Legends the bars, error bars, *, and tests used to calculate p-values in the legends for figures 4B,C, 5B,E, 7C, EV1 and EV2A. We apologise for omitting these previously*

3rd Editorial Decision 07 March 2016

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

Corresponding Author Name: Philip W Ingham
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2015-41532

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	The sample sizes were based on past precedent of similar analyses. No power calculations were performed.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	All sample sizes were given. The information could be found in following pages: pg 5, 6,7,11,21, figure legend 1, 2, 3, 5, 6, 7,EV1, EV2,EV3, EV4
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	N/A
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	All experiments were performed using embryos generated by outbred wild type lines or mutant lines maintained as heterozygotes. Any genetic variation within these lines was randomised between samples.
For animal studies, include a statement about randomization even if no randomization was used.	see above
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No, except in the case of mutants that were identified by gentoyping after phenotypic analysis
4.b. For animal studies, include a statement about blinding even if no blinding was done	see above
5. For every figure, are statistical tests justified as appropriate?	yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes: the samples are independent
Is there an estimate of variation within each group of data?	Yes
Is the variance similar between the groups that are being statistically compared?	Yes

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-reporting-the-arrive-

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumour-marker-prc

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Western blot antibodies for zebrafish experiments: (Page 19) anti-zebrafish Gli2a (Ben et al 2011), anti-γ-tubulin (Sigma), mouse anti-GFP (632569, Clontech), anti-myc (9E10, Santa Cruz) All the above antibodies have been previously validated in other publications listed. anti-GRK3 (sc-563, Santa Cruz): the specificity of this was validated using the Grk2 null allele descriebd in this study Western blot antibodies for cell line analysis (Page 19) Anti-Grk2 (Sc-13143, Santa Cruz), Anti-Gli1 (2643, Cell Signaling), Anti-Gli3 (AF3690, R&D) Anti-α-tubulin (T6199, Sigma), Anti-Ptch1 (Rohatgi et al 2007) All the above antibodies have been previously validated in other publications as descriebd in the text Antibodies for immunofluorescence in zebrafish (Page 18) Anti-Engrailed, 4D9 antibody (DSHB), Anti-Prox1 (Millipore) Anti-acetylated α-tubulin (Sigma), Chicken Anti-GFP (Abcam) The specificity of the above antibodies for the relevant zebrafish proteins has been previously validated in multiple publications by the Ingham lab and by other investigators. Antibodies for immunofluorescence in cell lines (Page 18) Anti-mouse Smo (Rohatgi et al 2007) All the above antibodies have been previously validated in other publications
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Flp-In-3T3 were obtained from Life technologies and has been test free of mycoplasma contamination.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	All the animals used in the study are of the Danio rerio. Embryos were obtained form matings between males and females 3months to 1 year old. Adult fish were maintained on a 14-hour light/10-hour dark cycle at 28°C in the AVA (Singapore) certificated IMCB Zebrafish Facility. Zebrafish strains used were grk2i283/+; smohi1640/+; yotty119/+;Tg(Eng2a:eGFP)i233. All information is shown on page 15, first section of "Material and Methods"
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	The experiments performed in this study have been reviewed by the A*STAR BMRC Institutional Animal Care and Use Committee review committee (IACUC Project #140912).
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Note that most of our experiments were performed on embryos or larvae prior to hatching and therfore fall outside the NIH PHS restrictions on animal experimentation

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	NA
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA