EMBO reports

Map7/7D1 and Dvl form a feedback loop that facilitates microtubule remodeling and Wnt5a signaling.

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1st Editorial Decision 22 December 2017

Thank you for the submission of your manuscript to EMBO reports. So far, we have received two referee reports that are copied below. Given that both referees are in fair agreement that you should be given a chance to revise the manuscript, I would like to ask you to begin revising your study along the lines suggested by the referees. Please note that this is a preliminary decision made in the interest of time, and that it is subject to change should the third referee offer very strong and convincing reasons for this. If we receive the final report on your manuscript within the next couple of weeks, we will forward it to you as well.

As you will see, the referees acknowledge that the findings are potentially interesting. However, referee 1 also points out several technical concerns and has a number of suggestions for how the study should be strengthened, and I think that all of them should be addressed.

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 concerns 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.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further.

Supplementary/additional data: The Expanded View format, which will be displayed in the main

HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

Regarding data quantification, can you please specify the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends? This information is currently incomplete and must be provided in the figure legends. Please also include scale bars in all microscopy images.

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. You have already used this opportunity, which is very much appreciated.

When submitting your revised manuscript, we will require:

- a complete author checklist, which you can download from our author guidelines (http://embor.embopress.org/authorguide#revision). Please insert page numbers in the checklist to indicate where the requested information can be found.
- a letter detailing your responses to the referee comments in Word format (.doc)
- a Microsoft Word file (.doc) of the revised manuscript text
- editable TIFF or EPS-formatted figure files in high resolution
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- a separate PDF file of any Supplementary information (in its final format)
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I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #2:

βCatenin independent, non-canonical Wnt signaling is key to the formation or oriented structures or directional migration of cells in many cases. In particular, 'wound healing' assays in culture have served as model for Wnt5 mediated singling during directional cell migration.
Kikuchi et al performed screen of selected MT binding proteins that are relevant for cell spreading and 'wound healing' in a scratch assay in HeLa cells. They identified MAP7 and its paralog

MAP7D1 (from now on 7/D1) as required for directed cell migration downstream of the typically non-canonical Wnt5a ligand. They also show that 7/D1 are required for lamellipodia induction and MT organization in the leading edge of migrating cells, without affecting MT plus end growth speed. Furthermore, 7/D1 interact with Dvl 2, a critical adapter of Wnt signaling and are required, with their binding partner Kif5b, for Dvl's recruitment to the cell cortex/leading edge. In turn, Dvl stabilizes 7/D1 and Wnt5a signaling (i.e. 'wounding') promotes delivery of 7/D1 to the leading edge. The authors then show that the function of 7/D1 is likely conserved in flies during the establishment of PCP in the wing, showing that mutants in the Drosophila homolog ensconsin (ens) show aberrant localization of PCP components (incl. Dsh-GFP) in pupal wings. The authors propose a feedback loop between Wnt5a signaling, Dvl, and 7/D1 resulting in asymmetric protein localization and directed cell migration (although it is not well explained, where the feedback idea is coming from and a more clear interpretation of the significance of each of the experiments would be helpful for the reader; see note about epistasis and discussion).

Overall, the data presented is very interesting and of clear value to a large field of scientists. Significantly, the data is of high quality and well controlled, except in cases mentioned below. The use of endogenously tagged proteins is very clean and Kikuchi et al. generally have done a good job at showing specificity of their reagents.

Major points:

• The authors mention βCatenin independent signaling frequently, but fail to address βCatenin in the paper. They either should do that or talk about Wnt5 induced signaling.

• Fig 2 (e.g. D): since 7/D1 are required for lamellipodia (LP) formation, the reduced polarity could be an indirect effect (to some part this concern is addressed in Fig. 4/5). Furthermore, the quantification showed that MTs appear still polarized and are not random, just rotated in line with the cell cortex rather than perpendicular (the Wnt5 KD looks more random). In addition, indicate the leading edge/migration direction in the panels that show cells and be more explicit when using scratch assays, as this information has to be looked for or inferred.

• 7/D1 in Dvl leading edge localization (Figs. 4/5) part 1: Unclear when Wnt5 was added or when a 'healing' assay was performed. 4C lacks a control without Wnt5 (unclear if Dvl-KI accumulates at cortex upon signaling activation or is or is only absent from cortex in 7/D1 knock-down. Why are ratios of Dvl/Actin measured and plotted in Fig 4 F/G? How/why are the three regions chosen in Fig. 4F?

• 7/D1 in Dvl leading edge localization (Figs. 4/5) part 2(perhaps the most serious issue): the authors bypass the requirements for LP formation of 7/D1 by induction of LPs with activated Rac1 and address hierarchy in the pathway (e.g. for 7/D1 and APC). However these arguments are severely flawed in the opinion of this reviewer. As Rac 1 is downstream of Wnt5 too, Dvl2 recruitment to the cortex should be independent of a Wnt5 knock-down (as Rac 1 is sufficient to promote cortical Dvl localization). Similar arguments can be made for the requirement of 7/D1 for Dvl cortical recruitment.

• Fig 7/p10/11: The authors make the argument that Wnt5 promotes + end localization/movement of MAP7 based on FRAP assays in which a faster recovery was found for leading edge signal. However, since all curves shown plateau towards the end of the period shown, it is possible that there simply is less recovery overall and there is no recovery speed difference.

• Conservation in flies: Kikuchi et al show protein asymmetry of Ensconsin (Ens) during fly wing development and state proximal localization of Ens. This should be shown in mosaics, which they can easily do as they have a knock-in (Fig 8E). Furthermore, in the PCP field, people started to quantify protein asymmetry, which would beneficial (alternatively, show mutant mosaics in all cases with clearly outlined wild-type versus mutant areas; even in the clone shown in Fig. 8D, Dsh asymmetry is barely visible and close to impossible to compare to WT areas). Why are no adult wings shown (see next point)? Do they show a PCP defect?

• Fig. S9: There are general issues with respect to ens fly genetics. Where does the Delta C allele come from? What is Df(3L)ensDelta3277? I assume that they are from Sung 2008. If so, both of

these are likely is a null mutants as they showed similar phenotypes in the original paper (e.g. reduced viability) and Delta 3277 removes all of the ens ORF. Furthermore, based on the (badly) described complementation analysis, the Crispr alleles generated for this paper have an off-target, second site lethal on the chromosome (or the same lethal on the original FRT82 chromosome they were made on). This is a simple explanation of why the new alleles are sub-viable over the 3277 and lethal over themselves and as transhets. I thus suggest using the Sung alleles for analysis.

Minor items:

• The authors state that 7/D1 have redundant roles: they likely have only partially redundant roles and show additive effects.
• p6: siRNA specificity: Be more clear that specificity of D1 siRNA was tested too (Fig S3B shows a Western that likely shows that).
• Fig. 3: Interpretation of interaction domain mapping not clear. Dvl DIX and PDZ domains are required in CoIP assays, but in the direct assay, the DEP domain is sufficient. How is that possible? Fig. 3C: MBP construct not explained, but likely a sufficiency construct used in panel E. Does panel E show a Coomassie stained gel?
• Fig. S5A: Test states that MAP7D2 does not interact with Dvl2. The figure shows it does so, just weakly. Adjust text.
• p8 Discussion about DEP requirement for non-canonical signaling: although known for a while, but never really acknowledged, the DEP domain is also critical for canonical signaling and the argument thus futile (see most recent papers e.g. Gammons, Mol. Cell 2016, Paclikova, PNAS 2017, Kaur, Sci Rep 2017).
• p9: ... Map7/7D1-Dvl interaction is also required for Map7/7D1's stability... The experiments do not show that the interaction is required for stability, just that Dvls are.
• Fig. 8A: polarization of 7D1 in ovarian cells not clear. Also, the authors should show a merge to allow judgment of overlap with the counterstain (Fz6 or Celsr1). What is the significance of this finding? Better integrate into paper.
• Fig. 9A: wing panel not really helpful and required WT control missing (Fig 9B too).
• Fig. 9D: adjust orientation of panels to have proximal to the left, distal to the right, as in other panels (and as is convention).
• Discussion: ....suggesting that Map7/7D1 is dispensable for transducing the Wnt5a signal to Dvl.... How is this reconciled with the idea that Wnt5 signaling leads to membrane recruitment of Dvl, for which they show that 7/D1 are required?
• Table S1: Antibody table would be more helpful if actual dilutions would be incorporated.

Referee #3:

The work of K. Kikuchi and colleagues describes an interesting and novel role for mikrotubule-associated proteins MAP7 and MAP7D1. They report that MAP7/7D1 bind to Dishevelled, the key regulator of Wnt pathways. Especially in the non-canonical Wnt pathway that is driven by Wnt-5a and that controls cell polarization and migration, the molecular details are poorly understood - despite clear biological and also clinical importance. The report by K. Kikuchi sheds an interesting light how DVL and other proteins coordinate with the MT-dynamics and how assymmetric trafficking of pathway components in the polarizing cell is controlled. The solid cell biology and life imaging part is also complemented by the in vivo evidence that the effects observed in cell culture are relevant in vivo. I found the experiments well-designed and sufficiently controlled. It is a timely work and I have enjoyed reading this manuscript.

I found few inaccurate statements that should be corrected:
Fig. 3E - The author label the part of Dvl1 281-484 as a DEP domain - both in the figure and the accompanying text. This is innacurate because this part of Dvl1 contains also part of PDZ domain and a highly conserved linker between the PDZ and DEP domain.
Page 8, last paragraph - The authors state: „DEP domain is critical for b-catenin-independent Wnt signaling" - this is true, but it has been convincingly demonstrated recently that in mammalian cells DEP domain is critical also for b-catenin-dependent pathway (see Gammons et al. https://www.ncbi.nlm.nih.gov/pubmed/27744318, and Paclikova et al. https://www.ncbi.nlm.nih.gov/pubmed/28674183). This needs to be corrected.

Our responses to the referees' comments for EMBOR-2017-45471-T

Referee #2

βCatenin independent, non-canonical Wnt signaling is key to the formation or oriented structures or directional migration of cells in many cases. In particular, 'wound healing' assays in culture have served as model for Wnt5 mediated singling during directional cell migration.
Kikuchi et al performed screen of selected MT binding proteins that are relevant for cell spreading and 'wound healing' in a scratch assay in HeLa cells. They identified MAP7 and its paralog MAP7D1 (from now on 7/D1) as required for directed cell migration downstream of the typically non-canonical Wnt5a ligand. They also show that 7/D1 are required for lamellipodia induction and MT organization in the leading edge of migrating cells, without affecting MT plus end growth speed. Furthermore, 7/D1 interact with Dvl 2, a critical adapter of Wnt signaling and are required, with their binding partner Kif5b, for Dvl's recruitment to the cell cortex/leading edge. In turn, Dvl stabilizes 7/D1 and Wnt5a signaling (i.e. 'wounding') promotes delivery of 7/D1 to the leading edge. The authors then show that the function of 7/D1 is likely conserved in flies during the establishment of PCP in the wing, showing that mutants in the Drosophila homolog ensconsin (ens) show aberrant localization of PCP components (incl. Dsh-GFP) in pupal wings. The authors propose a feedback loop between Wnt5a signaling, Dvl, and 7/D1 resulting in asymmetric protein localization and directed cell migration (although it is not well explained, where the feedback idea is coming from and a more clear interpretation of the significance of each of the experiments would be helpful for the reader; see note about epistasis and discussion).

Overall, the data presented is very interesting and of clear value to a large field of scientists. Significantly, the data is of high quality and well controlled, except in cases mentioned below. The use of endogenously tagged proteins is very clean and Kikuchi et al. generally have done a good job at showing specificity of their reagents.

Major points:

1) *The authors mention βCatenin independent signaling frequently, but fail to address βCatenin in the paper. They either should do that or talk about Wnt5 induced signaling.*

Response: In response to the referee's comment "*fail to address β-Catenin in the paper*", we examined whether Map7/7D1 were involved in the β-catenin dependent pathway. As described in Sato et al., *EMBO J.*, 2010, in HeLa cells, the induction of *AXIN2* expression by Wnt3a administration into culture medium is β-catenin dependent. We therefore analyzed the effect of the Wnt3a administration on *AXIN2* expression in control and Map7/7D1-depleted cells, and found that *AXIN2* mRNA was increased in both control and Map7/7D1-depleted cells. These results clearly demonstrate that Map7/7D1 are dispensable for the β-catenin dependent pathway in response to Wnt3a. The results are shown in new Appendix Figure S5E and described in the text (page 8, line 10 through 15).

2) *Fig 2 (e.g. D): since 7/D1 are required for lamellipodia (LP) formation, the reduced polarity could be an indirect effect (to some part this concern is addressed in Fig. 4/5). Furthermore, the quantification showed that MTs appear still polarized and are not random, just rotated in line with the cell cortex rather than perpendicular (the Wnt5 KD looks more random). In addition, indicate the leading edge/migration direction in the panels that show cells and be more explicit when using scratch assays, as this information has to be looked for or inferred.*

Response: We apologize for our confusing figures, which has been revised in new Figure 2D. Rose diagrams in original Figure 2D were made according to a procedure described in Harumoto et al., *Dev. Cell*, 2010. By simply applying this procedure, vector information of EB1-GFP comets was partly lost, because several bins overlapped. Therefore, according to a procedure described in Shi et al., *Mech. Dev.*, 2016, we revised rose diagrams in new Figure 2D together with scheme of

measurement of the vector angle. The revised rose diagrams in new Figure 2D clearly support our interpretation that "the proportion of EB1-GFP comets moving toward the leading edge was severely decreased in Map7/7D1-depleted cells", described in the text (page 7, line 15 through 16). We also re-arranged the direction of all panels in new Figure 2 to show that cells are migrating in an upward direction.

3) *7/D1 in Dvl leading edge localization (Figs. 4/5) part 1: Unclear when Wnt5 was added or when a 'healing' assay was performed. 4C lacks a control without Wnt5 (unclear if Dvl-KI accumulates at cortex upon signaling activation or is or is only absent from cortex in 7/D1 knock-down. Why are ratios of Dvl/Actin measured and plotted in Fig 4 F/G? How/why are the three regions chosen in Fig. 4F?*

Response: We apologize that our description of figure legend for original Figure 4A and C might have been confusing. We inserted the tag "Cell migration induced by wounding" into new Figure 4A, and added the text explaining "wound healing" or "Wnt5a addition" in figure legend for new Figure 4A and E (page 30, line 2 or 19, respectively).
In response to the referee's comment "*original Figure 4C lacks a control without Wnt5a*", we have also added new data on a cortical accumulation of Dvl2-EGFP^{KI} without Wnt5a administration into culture medium (new Figure 4E). Because Wnt5a is endogenously expressed at a detectable level in HeLa cells (Matsumoto et al., *EMBO J.*, 2010; Nishita et al., *J. Cell Biol.*, 2006; Nomachi et al., *J. Biol. Chem.*, 2008), the Dvl2-EGFP^{KI} accumulation was observed in 10.5% of control cells even in the absence of the Wnt5a administration. In contrast, the Dvl2-EGFP^{KI} accumulation was significantly reduced in Map7/7D1-depleted cells. These data clearly indicate that the Dvl2-EGFP^{KI} accumulation requires Map7/7D1 upon both endogenous and ectopic signaling activation.
Regarding the referee's comment "*Why are ratios of Dvl/Actin measured and plotted in original Figure 4F and G* (new Figure EV1B and Figure 5B, respectively)", we analyzed the accumulation of Dvl2-EGFP^{KI} at the lamellipodia, by quantifying the intensity of cortical Dvl2-EGFP^{KI}. We normalized the signal intensities against those of cortical F-actin at the corresponding lamellipodia. As described in figure legend, we carefully distinguished the lamellipodium/ruffling structures from other peripheral actin structures by confocal z-sectioning. Furthermore, to make data statistically reliable, we quantified Dvl2-EGFP^{KI} and F-actin levels at the three different lamellipodia in each cell and calculated average.

4) *7/D1 in Dvl leading edge localization (Figs. 4/5) part 2(perhaps the most serious issue): the authors bypass the requirements for LP formation of 7/D1 by induction of LPs with activated Rac1 and address hierarchy in the pathway (e.g. for 7/D1 and APC). However these arguments are severely flawed in the opinion of this reviewer. As Rac 1 is downstream of Wnt5 too, Dvl2 recruitment to the cortex should be independent of a Wnt5 knock-down (as Rac 1 is sufficient to promote cortical Dvl localization). Similar arguments can be made for the requirement of 7/D1 for Dvl cortical recruitment.*

Response: Although the reviewer states "*Rac 1 is sufficient to promote cortical Dvl localization,*" this is not the case as shown in original Figure 4E (new Figure 5A). Constitutively active form of Rac1 (Rac1^{CA}) failed to rescue cortical targeting of Dvl2 in Wnt5a-depleted cells. Thus, most likely additional factors in Wnt5a signaling are involved in this process. We think that the data in original Figures 4D, 4E, 5A and 5B (new Figure 5A-D) allow us to conclude that components upstream of Dvl such as Wnt5a and Map7/7D1 are required for cortical Dvl localization. Both Wnt5a and Dvl are known to act upstream of Rac1 for lamellipodia formation in the Wnt5a signaling pathway (e.g. Nishida et al., *Mol. Cell. Biol.*, 2010). Consistent with this, both lamellipodia formation and targeting of MTs to the lamellipodia in Wnt5a- or Dvls-depleted cells were rescued when Rac1^{CA} was ectopically expressed (in original Figure 5A and 5B; new Figure 5C and 5D). However, the defects in cortical Dvl localization in Wnt5a-depleted cells was not rescued even when Rac1^{CA} was ectopically expressed (in original Figure 4E and D; new Figure 5A and B). Thus, lamellipodia formation and MT targeting are insufficient for cortical Dvl localization, and dynamic movement of Map7/7D1 on MT toward cell cortex is important for this process. We state the interpretation of our results in page 10, line 13 through 15.
In addition, to deepen our understanding on the role of Map7/7D1 in the Wnt5a signaling pathway, we dissected the functional differences between Map7/7D1 and APC, which is another MT-binding protein that binds to Dvl in Wnt5a signaling and acts downstream of Rac1 (Matsumoto et al., *EMBO J.*, 2010; Watanabe et al., *Dev. Cell*, 2004). Differently from Wnt5a or Dvls depletion, the Rac1^{CA}-

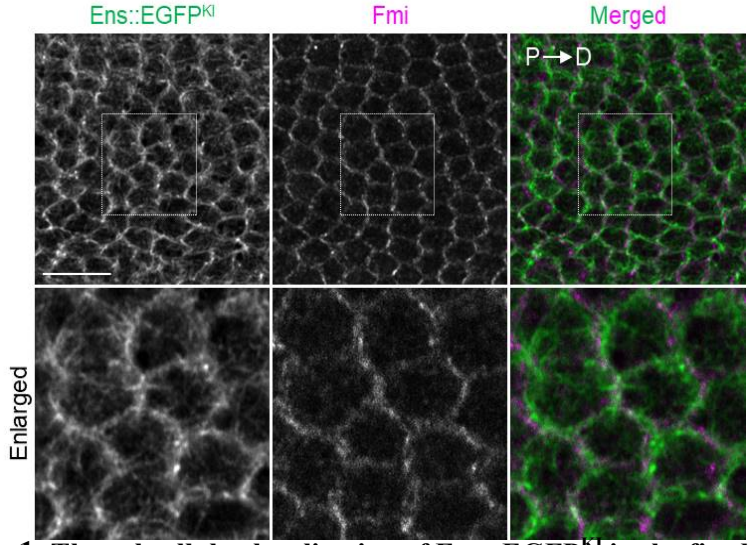
induced MT targeting was compromised by APC depletion, though the lamellipodia formation appeared to be normal even in APC-depleted cells, as shown in original Figure 5A and B (new Figure 5C and D). Since the Map7/7D1-depleted cells responded to $Rac1^{CA}$ in a similar fashion as Wnt5a- or Dvls-depleted cells in in original Figures 4D, 4E, 5A and 5B (new Figure 5A-D), Map7/7D1 are likely to act upstream of Rac1 in the Wnt5a signaling pathway to regulate lamellipodia formation and the targeting of MTs. We state the interpretation of our results in page 10, line 24 through 27.

5) *Fig 7/p10/11: The authors make the argument that Wnt5 promotes + end localization/movement of MAP7 based on FRAP assays in which a faster recovery was found for leading edge signal. However, since all curves shown plateau towards the end of the period shown, it is possible that there simply is less recovery overall and there is no recovery speed difference.*

Response: In response to the referee's comments, we rephrased the corresponding text to "the recovery rate of Map7-$EGFP^{KI}$ fluorescence at the leading edge was higher in migrating cells than in non-migrating cells" (page 11, line 7 through 9). In addition, fitted curves from each dataset were integrated into each graph in original Figure 7, according to the procedure in Kemmer and Keller, *Nat. Prot.*, 2010. The results are shown in new Figure 7 and new Appendix Figure S8A.

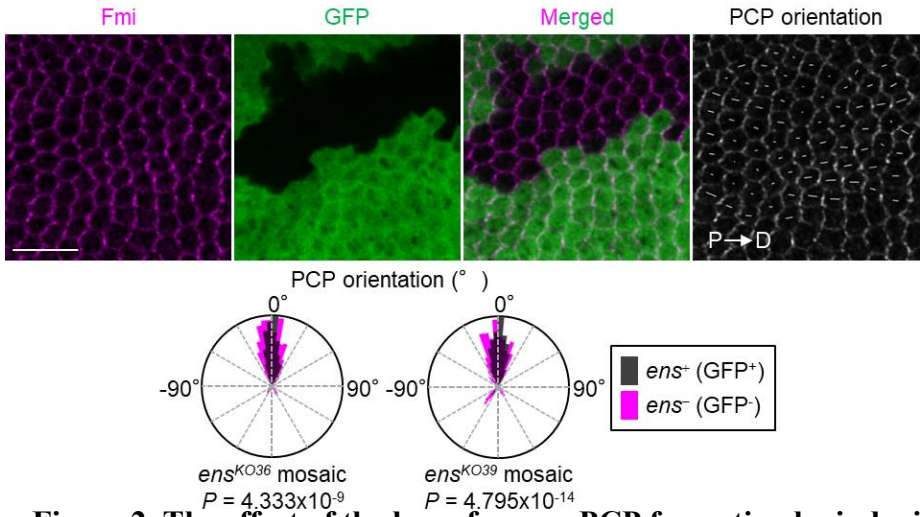
6) *Conservation in flies: Kikuchi et al show protein asymmetry of Ensconsin (Ens) during fly wing development and state proximal localization of Ens. This should be shown in mosaics, which they can easily do as they have a knock-in (Fig 8E). Furthermore, in the PCP field, people started to quantify protein asymmetry, which would beneficial (alternatively, show mutant mosaics in all cases with clearly outlined wild-type versus mutant areas; even in the clone shown in Fig. 8D, Dsh asymmetry is barely visible and close to impossible to compare to WT areas). Why are no adult wings shown (see next point)? Do they show a PCP defect?*

Response: As shown in original Figure 8C (new Figure 8C), Ens::$EGFP^{KI}$ is enriched close to the proximal side of the cytoplasm. This localization pattern is different from that of core PCP components, which reside just in close proximity to the proximal cortex. The unique Map7/7D1 localization likely reflects dynamic nature of Map7/7D1 movement, as described in our HeLa cell assay (new Figure 6 and 7).
Also, although the mosaic analysis of protein asymmetry is normally performed using fixed tissues (*e.g.* Strutt, *Mol. Cell*, 2001; Strutt et al, *Dev.Cell*, 2011), we found that cell fixation disrupted the proper distribution of Ens::$EGFP^{KI}$ in pupal wings (**Referee Figure 1**). Thus, we conducted live cell imaging of the Ens::$EGFP^{KI}$ localization. Although the Ens::$EGFP^{KI}$ localization in mosaic live cells could be conducted by three-color imaging, it would be unfeasible from a technical viewpoint. Instead of the mosaic analysis, the planar-polarization of Ens::$EGFP^{KI}$ was quantified using live imaging data by the method as previously described (Arata et al., *Dev. Cell*, 2017). The quantification clearly indicates that Ens::$EGFP^{KI}$ enriched to the proximal side of each pupal wing cell. We have included these results at the bottom panel in new Figure 8C, and added the description



Referee Figure 1. The subcellular localization of Ens::$EGFP^{KI}$ in the fixed pupal wing.
30 h APF pupal wings were stained with anti-Fmi and anti-GFP antibodies.

for the quantification of Ens::EGFP^{KI} localization in the text (page 19, line 13, and page 33, line 9 through 14).
Regarding the quantification of PCP formation in mosaic pupal wings, we analyzed the effect of the loss of *ens* on PCP formation by inducing *ens*$^{KO/KO}$ (hereafter, *ens*$^{-}$) mosaic clones in the pupal wings. The axis of Fmi polarization (hereafter, PCP orientation) in the *ens*$^{-}$ cells was significantly misoriented (**Referee Figure 2**). However, the defective PCP orientation in *ens*$^{-}$ mutants was somewhat weaker than that seen in mutants for core PCP machinery. This is probably because residual *ens* activity remains in *ens*$^{-}$ mosaic clones and attenuates the defects. Therefore, we analyzed wing hair orientation in *ens*^{KO36}*/Df(3L)BSC735* hemizygous or *ens*^{KO36}*/ens*^{KO39} transheterozygous pupae as shown in new Figure 9A.
As for the referee's concern "*even in the clone shown in original Figure 9D, Dsh asymmetry is barely visible and close to impossible to compare to WT areas*", this is likely to be due to low expression of the Dsh::GFP transgene in mosaics. Therefore, we tried to analyze Dsh::GFP localization in wild-type or *ens*^{KO36}*/ens*^{KO39} pupae in the *P{Dsh::GFP}* homozygous background. The *ens*^{KO36}*/ens*^{KO39} pupae were identified by the loss of mCherry fluorescence from the balancer chromosome. Expectedly, Dsh asymmetry became much clearer in pupal wings of wild-type homologously expressing Dsh::GFP. Even under these conditions, Dsh::GFP asymmetry was compromised in pupal wing cells of *ens*^{KO36}*/ens*^{KO39} mutants. These results clearly indicate that Ens is required for proper Dsh localization in epithelial cell. We have included these results in new Figure 9C and described in the text (page 13, line 11 through 15).
In regard with PCP phenotypes of adult mutant flies, we were unable to analyze them because of their pupal lethality as described in next part.



Referee Figure 2. The effect of the loss of *ens* on PCP formation by inducing *ens*$^{KO/KO}$.
PCP orientation defects in *ens*$^{-}$ mosaic clones (top panels). Images were taken of 30-h APF pupal wings stained for Fmi and GFP. The degrees of PCP orientation were analysed using in two different *ens* null mutants (bottom graphs). *P* values were calculated with the Mardia-Watson-Wheeler test.

7) *Fig. S9: There are general issues with respect to ens fly genetics. Where does the Delta C allele come from? What is Df(3L)ensDelta3277? I assume that they are from Sung 2008. If so, both of these are likely is a null mutants as they showed similar phenotypes in the original paper (e.g. reduced viability) and Delta 3277 removes all of the ens ORF. Furthermore, based on the (badly) described complementation analysis, the Crispr alleles generated for this paper have an off-target, second site lethal on the chromosome (or the same lethal on the original FRT82 chromosome they were made on). This is a simple explanation of why the new alleles are sub-viable over the 3277 and lethal over themselves and as transhets. I thus suggest using the Sung alleles for analysis.*

Response: We apologize our descriptions on fly genetics are misleading, partly due to space restriction of the original manuscript. We used *Df(3L)ens*$^{\Delta 3277}$ as a deficiency line that uncovers the

ens locus (Barlan et al., *Curr. Biol.*, 2013). Note that the *Df(3L)ens*$^{\Delta 3277}$ allele is not a sole *ens*-null allele, because this allele lacks whole *ens* gene and the 5′ end of an adjacent gene encoding a calponin-like protein, Chd64 (Sung et al., *Dev. Cell*, 2008). As shown in original Appendix Figure S9D (new Figure S10A), a few *ens*^{KO} *neoFRT80B/Df(3L)ens*$^{\Delta 3277}$ escapers were eclosed, but they died immediately after eclosion, before wing expansion occurs. *ens*^{KO} *neoFRT80B* homozygotes or transheterozygtes between independent alleles died before eclosion. In contrast, consistent with Barlan et al., *Curr. Biol.*, 2013, *ens*$^{\Delta C}$ *neoFRT80B/Df(3L)ens*$^{\Delta 3277}$ adults survived up to two weeks. These genetic data indicate that *ens*$^{\Delta C}$ is a hypomorph. Therefore, we used *ens*^{KO} alleles as amorphic alleles for *ens*. The results are shown in new Appendix Figure S10A and described in the text (page 12, line 28 through 29).
Concerning an off-target effect of CRISPR/Cas9-mediated mutagenesis, recent papers reported that no off-target mutations have been so far detected as a result of CRISPR/Cas9-mediated mutagenesis in *Drosophila* (Bassett et al., *Cell Rep.*, 2013; Kondo and Ueda, *Genetics*, 2013; Gratz et al., *Genetics*, 2014). It appears that in *Drosophila*, sequence complementarity between target and sgRNA is extremely strict for CRISPR/Cas9-mediated target cleavage, and three mismatches in sequence result in the failure in inducing mutations at the target site (Bassett et al., *Cell Rep.*, 2013). Therefore, we think it unlikely that our *ens*^{KO} alleles have off target mutations. In addition, as shown in new Figure 9A, we analyzed wing hair orientation in *ens*^{KO36}*/Df(3L)BSC735* hemizygotes as well as *ens*^{KO36}*/ens*^{KO39} transheterozygtes. Both allelic combinations clearly showed wing hair misorientation, indicating that Ens is involved, directly or indirectly, in PCP formation.

Minor items:

8) The authors state that 7/D1 have redundant roles: they likely have only partially redundant roles and show additive effects.

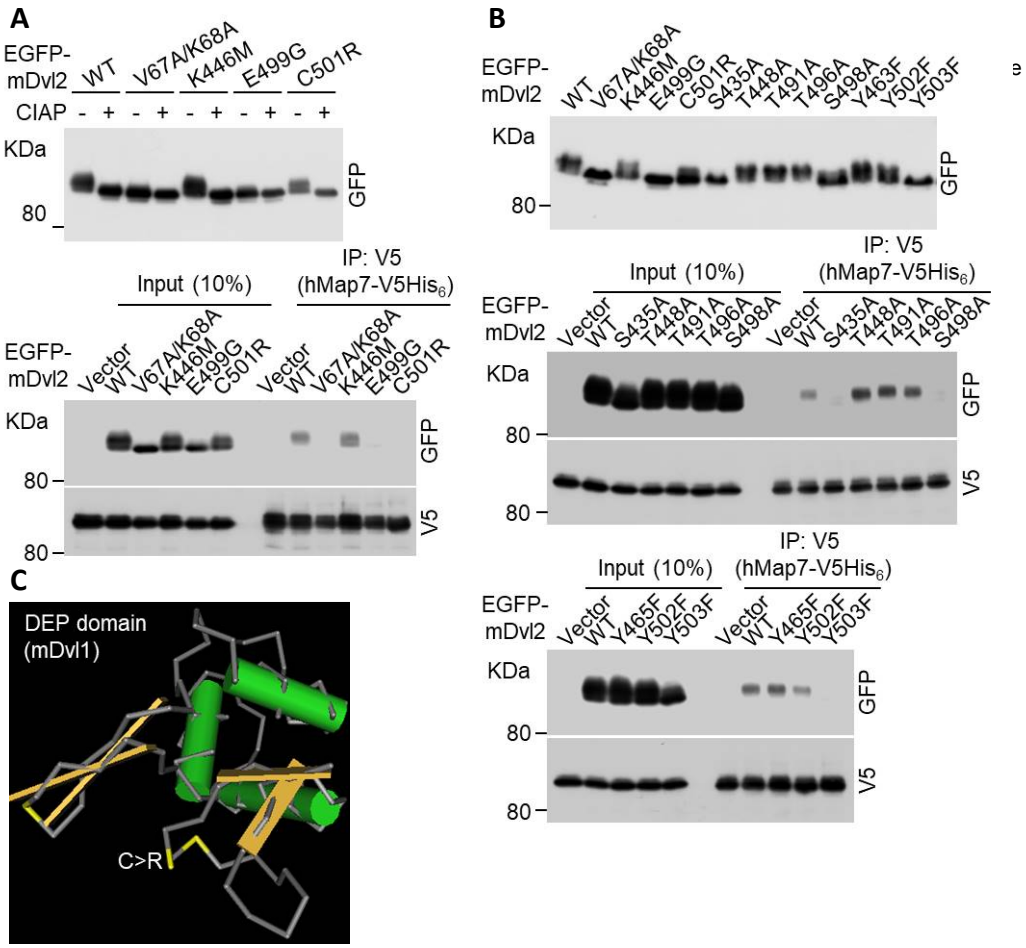
Response: In response to the referee's comments, we rephrased to "overlapping functions" (page 6, line 27 through 28, and page 14, line 22).

9) p6: siRNA specificity: Be more clear that specificity of D1 siRNA was tested too (Fig S3B shows a Western that likely shows that).

Response: According to the referee's comment "*siRNA specificity*", we tested the specificity of *MAP7D1* siRNAs together with *MAP7* and *MAP7D3* siRNAs by RT-qPCR. All of siRNAs used in this study worked specifically against each gene (in the original manuscript, we already stated that si*MAP7D1*#3 was not used in this study because of the less effective depletion). Also, double depletion of Map7/7D1 did not affect the expression of *MAP7D3* mRNA. We have included these results in new Appendix Figure S3C.

10) Fig. 3: Interpretation of interaction domain mapping not clear. Dvl DIX and PDZ domains are required in CoIP assays, but in the direct assay, the DEP domain is sufficient. How is that possible? Fig. 3C: MBP construct not explained, but likely a sufficiency construct used in panel E. Does panel E show a Coomassie stained gel?

Response: In regard with the referee's concern "*Dvl DIX and DEP domains are required in CoIP assays, but in the direct assay, the DEP domain is sufficient. How is that possible?"*, we think that the conformational change of Dvl2 arising from the DIX deletion affects the Map7-Dvl2 interaction. It is well-known that overexpression of Dvl2 in HeLa cells becomes highly phosphorylated. This phosphorylation was abolished in truncated Dvl2 that lacks the DIX domain. In contrast, another truncated Dvl2 with DEP deletion was phosphorylated, as shown in new Appendix Figure S6C. Also, our above interpretation is supported by the fact that the Map7-Dvl2 interaction was compromised by the point mutations in the DIX domain that abolished Dvl2 phosphorylation (**Referee Figure 3**). In addition, we extensively analyzed the effect of point mutations in the DEP domain on the Map7-Dvl2 interaction. Point mutations in the DEP domain that abolished Dvl2 phosphorylation, also spoiled the Map7-Dvl2 interaction. Even though C501R mutation did not affect Dvl2 phosphorylation, C501R mutation compromised the Map7-Dvl2 interaction, suggesting that the region including C501 in the DEP domain, which makes the loop structure, may responsible for the Map7-Dvl2 interaction. Our interpretation about DIX deletion is described in the text (page 8, line 28 through page 9, line 2). However, the experiments using point mutations shown below are



Referee Figure 3. The effect of point mutations in the DIX or DEP domain on the Map7-Dvl2 interaction.

A. Top panel, The phosphorylation state of the indicated Dvl2 mutants. Lysates from HeLa cells expressing point mutants of mDvl2-EGFP were immunoprecipitated with an anti-GFP antibody, and the immunoprecipitates were treated with (+) or without (-) alkaline phosphatase (CIAP). V67A/K68A, point mutations in the DIX domain. E499G or C501R, a point mutant in the DEP domain. Bottom panel, Lysates from HeLa cells co-expressing point mutants of EGFP-mDvl2 with hMap7-V5His$_6$ were immunoprecipitated with an anti-V5 antibody, and the immunoprecipitates were probed with anti-GFP and anti-V5 antibodies.

B. Top panel, The phosphorylation state of point mutants in the DEP domain. Lysates from HeLa cells expressing point mutants in the DEP domain were subjected to SDS-PAGE, and the phosphorylation state was detected by the mobility shift. Middle and bottom panels, Lysates from HeLa cells co-expressing point mutants in the DEP domain with hMap7-V5His$_6$ were immunoprecipitated with an anti-V5 antibody, and the immunoprecipitates were probed with anti-GFP and anti-V5 antibodies.

C. The 3D structure of mouse Dvl1 DEP domain. C>R indicates a point mutation corresponding to C501R mutation of Dvl2.

not essential to establish our conclusion, and we feel that these data are a bit far from the scope of our manuscript.

Regarding the explanation of MBP construct in original Figure 3C, we moved into new Figure 3E, to incorporate the referee's suggestion. Panels in original Figure 3E (new Figure 3E) were analyzed by immunoblotting with anti-GST and anti-MBP antibodies as described in figure legend.

11) *Fig. S5A: Test states that MAP7D2 does not interact with Dvl2. The figure shows it does so, just weakly. Adjust text.*

Response: In response to the referee's comments, we rephrased the corresponding text to "a trace amount of overexpressed Map7D2 was co-immunoprecipitated with Dvl2" (page 8, line 19).

12) *p8 Discussion about DEP requirement for non-canonical signaling: although known for a while, but never really acknowledged, the DEP domain is also critical for canonical signaling and the argument thus futile (see most recent papers e.g. Gammons, Mol. Cell 2016, Paclikova, PNAS 2017, Kaur, Sci Rep 2017).*

Response: According to the referees' suggestion, we cited two papers, "Gammons et al, 2016; Paclikova et al, 2017". Also, we rephrased the corresponding text to "The DEP domain in Dvls is critical for both β-catenin-dependent and -independent Wnt signaling [25-28]. Because Map7/7D1 are not involved in β-catenin-dependent Wnt3a signaling (Appendix Fig. S5E), these results further support the idea that Map7/7D1 play a role in the Wnt5a signaling pathway through interaction with Dvl." (page 9, line 5 through 9).

13) *p9: ... Map7/7D1-Dvl interaction is also required for Map7/7D1's stability... The experiments do not show that the interaction is required for stability, just that Dvls are.*

Response: We changed to "Dvl is" (page 9, line 12).

14) *Fig. 8A: polarization of 7D1 in ovarian cells not clear. Also, the authors should show a merge to allow judgment of overlap with the counterstain (Fz6 or Celsr1). What is the significance of this finding? Better integrate into paper.*

Response: According to the referee's comments for original Figure 8A, we integrated merged images into new figure 8A. In merged image of Map7D1 and Celser1, Map7D1 signals (green) appeared in close proximity to the inside of Celsr1 signals (magenta), indicating that Map7D1 localizes to the ovary side along the planer axis of multiciliated cells in the mouse oviduct.

15) *Fig. 9A: wing panel not really helpful and required WT control missing (Fig 9B too).*

Response: According to the referee's suggestion, original Figure 9A was removed. And the result of wild-type hair polarity was added in new Figure 9A.

16) *Fig. 9D: adjust orientation of panels to have proximal to the left, distal to the right, as in other panels (and as is convention).*

Response: According to the referee's suggestion, we adjusted orientation of all panels related to the analysis of epithelial tissues, to have ovary/proximal to the left, uterus/distal to the right.

17) *Discussion: ....suggesting that Map7/7D1 is dispensable for transducing the Wnt5a signal to Dvl.... How is this reconciled with the idea that Wnt5 signaling leads to membrane recruitment of Dvl, for which they show that 7/D1 are required?*

Response: It is an open question where Wnt5a-induced phosphorylation of Dvl occurs, though it has been reported that several kinases such as PKC and CK1δ/ϵ are involved in the phosphorylation of Dvl (Chen et al., *Science*, 2003; Bryja et al., *J. Cell Sci.*, 2007). In this study, we found that Wnt5a-induced phosphorylation of Dvl2 was observed in Map7/7D1-depleted cells (original Appendix Figure S7A; new Figure 4C), indicating that Wnt5a signaling is transduced to Dvls' phosphorylation even in the absence of Map7/7D1. Therefore, we propose that regulation of Map7 dynamics in response to Wnt5a is operated by the interaction between Map7 and Dvl. Unfortunately, we are currently unable to examine whether Dvl instructs the Map7 dynamics in response to Wnt5a, because Map7/7D1 are destabilized in Dvls-depleted cells independently of Wnt5a (original Figure 3F and G; new Figure 3F and G).

18) *Table S1: Antibody table would be more helpful if actual dilutions would be incorporated.*

Response: We added the information of the actual dilutions in new Appendix Table S1.

Referee #3

The work of K. Kikuchi and colleagues describes an interesting and novel role for mikrotubule-associated proteins MAP7 and MAP7D1. They report that MAP7/7D1 bind to Dishevelled, the key regulator of Wnt pathways. Especially in the non-canonical Wnt pathway that is driven by Wnt-5a and that controls cell polarization and migration, the molecular details are poorly understood - despite clear biological and also clinical importance. The report by K. Kikuchi sheds an interesting light how DVL and other proteins coordinate with the MT-dynamics and how assymmetric trafficking of pathway components in the polarizing cell is controlled. The solid cell biology and life imaging part is also complemented by the in vivo evidence that the effects observed in cell culture are relevant in vivo. I found the experiments well-designed and sufficiently controlled. It is a timely work and I have enjoyed reading this manuscript.

1) I found few inaccurate statements that should be corrected:
Fig. 3E - The author label the part of Dvl1 281-484 as a DEP domain - both in the figure and the accompanying text. This is innacurate because this part of Dvl1 contains also part of PDZ domain and a highly conserved linker between the PDZ and DEP domain.

Response: We understand that the interaction assays we conducted were complicated, but we believe that our results from both co-immunoprecipitation and *in vitro* binding assays support our conclusion that DEP domain of Dvl is sufficient the Map7-Dvl interaction. As the referee pointed, GST-hDvl1^{DEP} used in *in vitro* binding assays contains part of PDZ domain and a highly conserved linker between the PDZ and DEP domain (original Figure 3E; new Figure 3E). However, by co-immunoprecipitation assays in original Figure 3C (new Figure 3C), we also found that Dvl2 lacking the DEP domain failed to associate with Map7 (concerning our interpretation about DIX deletion, please see page 9 in this letter). Thus, we conclude that the DEP domain of Dvl is sufficient the Map7-Dvl interaction.

2) Page 8, last paragraph - The authors state: „DEP domain is critical for b-catenin-independent Wnt signaling" - this is true, but it has been convincingly demonstrated recently that in mammalian cells DEP domain is critical also for b-catenin-dependent pathway (see Gammons et al. https://www.ncbi.nlm.nih.gov/pubmed/27744318, and Paclikova et al. https://www.ncbi.nlm.nih.gov/pubmed/28674183). This needs to be corrected.

Response: According to the referees' suggestion, we cited two papers, "Gammons et al, 2016; Paclikova et al, 2017". Also, we rephrased the corresponding text to "The DEP domain in Dvls is critical for both β-catenin-dependent and -independent Wnt signaling [25-28]. Because Map7/7D1 are not involved in β-catenin-dependent Wnt3a signaling (Appendix Fig. S5E), these results further support the idea that Map7/7D1 play a role in the Wnt5a signaling pathway through interaction with Dvl." (page 9, line 5 through 9).

2nd Editorial Decision 13 April 2018

Thank you for the submission of your revised manuscript to our journal. It was sent back to referee 2 and we have meanwhile receive this referee's report, which I include below for your information.

As you will see, this referee is overall positive and supports publication of your study in EMBO reports after the clarification of a few remaining issues. Please address these remaining issues in the manuscript and please also provide a point-by-point response upon resubmission.

From the editorial side, there are also a few things that we need before we can proceed with the final acceptance of your study.

REFEREE REPORTS

Referee #2:

βCatenin independent, non-canonical Wnt signaling is key to the formation or oriented structures or directional migration of cells in many cases. In particular, 'wound healing' assays in culture have served as model for Wnt5 mediated singling during directional cell migration.
Kikuchi et al performed screen of selected MT binding proteins that are relevant for cell spreading and 'wound healing' in a scratch assay in HeLa cells. They identified MAP7 and its paralog MAP7D1 (from now on 7/D1) as required for directed cell migration downstream of the typically non-canonical Wnt5a ligand. They also show that 7/D1 are required for lamellipodia induction and MT organization in the leading edge of migrating cells, without affecting MT plus end growth speed. Furthermore, 7/D1 interact with Dvl 2, a critical adapter of Wnt signaling and are required, with their binding partner Kif5b, for Dvl's recruitment to the cell cortex/leading edge. In turn, Dvl stabilizes 7/D1, and Wnt5a signaling (i.e. 'wounding') promotes delivery of 7/D1 to the leading edge. The authors then show that the function of 7/D1 is likely conserved in flies during the establishment of PCP in the wing, showing that mutants in the Drosophila homolog ensconsin (ens) show aberrant localization of PCP components (incl. Dsh-GFP) in pupal wings. The authors propose a feedback loop between Wnt5a signaling, Dvl, and 7/D1 resulting in asymmetric protein localization and directed cell migration

Overall, the data presented in this revised paper is of high quality and still very interesting and of clear value to a large field of scientists. As mentioned in the initial round of review, the use of endogenously tagged proteins is very clean and Kikuchi et al. generally have done a good job at showing specificity of their reagents. In the revision, many issues have been addressed, although some remain. In particular, the fly ens frame-shift (KO) mutants have an off-target of the Crisprs on the same chromosome, in spite of what the authors wrote in their rebuttal (see issue below). Importantly, the authors now address that 7/D1 do not affect Wnt3a mediated canonical signaling. What is still confusing/lacking is a better link of the adhesion/wounding assays with Wnt5 and non-canonical Wnt signaling. As such, it is not clear from the methods whether Wnt5 is added to the screening assays or whether those assays are simply known to depend on endogenous Wnt5a. This should be clarified by improving the text. I apologize for my initial issue 4 (RacCA overexpression). I now understand that the recruitment of Dvl seen in the baseline is likely due to endogenous Wnt5a (and thus making the interpretation of what is now Fig 5 correct).

Major remaining points:
• Fig 7/p10/11: FRAP experiments: Most addressed, but there is one remaining issue that at least should be discussed. One interpretation of the faster recovery in presence of Wnt5a is due to faster MT dynamics as suggested. However, wouldn't a slower 7/D1 off-rate in the absence of Wnt5a (and thus a more stable MT-7/D1 complex) also show the same slower recovery? FRAP can also reflect slow off-rates of the bleached product. The latter may also be consistent with the more stable focal adhesions shown in Fig. 2G.
• Ens localization in flies: Localization is now quantified and wing hair defects in pupal discs are nicely shown. As Ens localizes differently form core PCP genes, the authors are correct and mosaic analyses are less important. What, however, should be more explicitly discussed in the model, is the discrepancy between MT + end transport of Dvl in HeLa cells and opposing localization in flies (Ens proximal, Dsh distal). This needs a bit more explanation than given in discussion on p15.
• Ens mutations: There still is an issue with fly genetics and second site lethals on the ensKO alleles. I understand that the ens Df removes more than ens, but it is a null for ens by definition (it lacks the gene). I agree with the authors that DeltaC is clearly hypomorphic, as homozygous flies that hatch survive longer than the KO alleles /Df. However, homozygous and transhet KO combinations fail to produce hatching embryos which they do over a Df (even though those flies die very shortly after birth). Those phenotypes are therefore stronger than over Df. There are two explanations: either the KO alleles are neomorphic or the Crispr alleles have an additional, shared second site pupal lethal (i.e. off-target). As the KO alleles have frame-shifts right after the start codon, the first explanation is highly unlikely. Whatever is published about Crisprs not having off-targets in flies must be wrong from experience I know of. Using Crisprs, three LOF alleles were obtained for a gene that were all lethal in trans, but viable over a Df. In addition, the second site lethal could be recombined away,

proving that it was a second site lethal due to Crispr off-target (as all three alleles had the same second site lethal). I am willing to share more of that data with the editor should the authors not agree with my arguments. Nevertheless, even the authors' own data show the presence of a second site lethal.
Therefore, I suggest to remove the transhet data and to simply use the ens/Df that is already in the paper and shows the author's conclusion. The authors may leave the mutant mosaic data in Fig S10, as there is no way around using the KO alleles for this. However, they should state that, formally, the second site mutation could have an effect as well (although highly unlikely, as KO/Df in whole wings shows similar phenotypes).

Minor items:
• Old major item 2: Cells in Fig 2 now oriented with migration upwards. Please state this in legend or text, and not only in the rebuttal (rest resolved).
• p9: ....aa 159-246 of Map7 are sufficient.... This is not shown by the data presented. Those amino acids seem to be required for full binding, but there is no sufficiency construct. Adjust text.
• Fig. S8B: indicate leading edge direction to judge recovery direction.
• Drosophila ovary data of Fig S9B in not mentioned in the text, thus remove panel or state relevance (Ens localizes to MT minus ends?).

2nd Revision - authors' response 17 April 2018

Referee #2

βCatenin independent, non-canonical Wnt signaling is key to the formation or oriented structures or directional migration of cells in many cases. In particular, 'wound healing' assays in culture have served as model for Wnt5 mediated singling during directional cell migration.

Kikuchi et al performed screen of selected MT binding proteins that are relevant for cell spreading and 'wound healing' in a scratch assay in HeLa cells. They identified MAP7 and its paralog MAP7D1 (from now on 7/D1) as required for directed cell migration downstream of the typically non-canonical Wnt5a ligand. They also show that 7/D1 are required for lamellipodia induction and MT organization in the leading edge of migrating cells, without affecting MT plus end growth speed. Furthermore, 7/D1 interact with Dvl 2, a critical adapter of Wnt signaling and are required, with their binding partner Kif5b, for Dvl's recruitment to the cell cortex/leading edge. In turn, Dvl stabilizes 7/D1, and Wnt5a signaling (i.e. 'wounding') promotes delivery of 7/D1 to the leading edge. The authors then show that the function of 7/D1 is likely conserved in flies during the establishment of PCP in the wing, showing that mutants in the Drosophila homolog ensconsin (ens) show aberrant localization of PCP components (incl. Dsh-GFP) in pupal wings. The authors propose a feedback loop between Wnt5a signaling, Dvl, and 7/D1 resulting in asymmetric protein localization and directed cell migration

Overall, the data presented in this revised paper is of high quality and still very interesting and of clear value to a large field of scientists. As mentioned in the initial round of review, the use of endogenously tagged proteins is very clean and Kikuchi et al. generally have done a good job at showing specificity of their reagents. In the revision, many issues have been addressed, although some remain. In particular, the fly ens frame-shift (KO) mutants have an off-target of the Crisprs on the same chromosome, in spite of what the authors wrote in their rebuttal (see issue below). Importantly, the authors now address that 7/D1 do not affect Wnt3a mediated canonical signaling. What is still confusing/lacking is a better link of the adhesion/wounding assays with Wnt5 and non-canonical Wnt signaling. As such, it is not clear from the methods whether Wnt5 is added to the screening assays or whether those assays are simply known to depend on endogenous Wnt5a. This should be clarified by improving the text. I apologize for my initial issue 4 (RacCA overexpression). I now understand that the recruitment of Dvl seen in the baseline is likely due to endogenous Wnt5a (and thus making the interpretation of what is now Fig 5 correct).

Response: In regard with the referee's concern "it is not clear from the methods whether Wnt5a is added to the screening assays or whether those assays are simply known to depend on endogenous Wnt5a.", we apologize that our description of the adhesion/wound healing assays in this study might have been confusing. We did most experiments without Wnt5a administration, except for original Figure 4C and E, because HeLa cells express Wnt5a, and this autocrine signaling regulates cell-substrate adhesion and directional cell migration. We thus added our statement "In HeLa cells, cell-substrate adhesion and directional cell migration (hereafter, cell adhesion and migration, respectively) is regulated by endogenously expressing Wnt5a." in the first paragraph of the Results section of the new manuscript (page 6, line 6 through 8). Furthermore, we rephrased the text in the Materials and Methods section of the new manuscript (page 16, line 3 through 5) to "Wnt5a used in Fig. 4C and E was purified from the conditioned medium of L cells stably expressing Wnt5a, as described [47].", to clarify differences of experimental condition.

Major remaining points:

1) Fig 7/p10/11: FRAP experiments: Most addressed, but there is one remaining issue

that at least should be discussed. One interpretation of the faster recovery in presence of Wnt5a is due to faster MT dynamics as suggested. However, wouldn't a slower 7/D1 off-rate in the absence of Wnt5a (and thus a more stable MT-7/D1 complex) also show the same slower recovery? FRAP can also reflect slow off-rates of the bleached product. The latter may also be consistent with the more stable focal adhesions shown in Fig. 2G.

Response: We agree with the referee's comment "FRAP can also reflect slow off-rates of the bleached product.". Therefore, we rephrased the corresponding text to "the association/dissociation cycle of Map7/7D1 with MTs" (page 11, line 21).

2) Ens localization in flies: Localization is now quantified and wing hair defects in pupal discs are nicely shown. As Ens localizes differently form core PCP genes, the authors are correct and mosaic analyses are less important. What, however, should be more explicitly discussed in the model, is the discrepancy between MT + end transport of Dvl in HeLa cells and opposing localization in flies (Ens proximal, Dsh distal). This needs a bit more explanation than given in discussion on p15.

Response: In response to the referee's comment "What, however, should be more explicitly discussed in the model, is the discrepancy between MT + end transport of Dvl in HeLa cells and opposing localization in flies (Ens proximal, Dsh distal)", we modified the third paragraph in the Discussion section of the original manuscript. According to our results obtained from HeLa cells, we propose that Map7/7D1 promote the loading of Kinesin-1 family protein onto MTs for the Dvl localization. As shown in original Figure 8C, in pupal wing cells, Ens localized to the MT minus-end enriched proximal side, whereas Dsh enriches in the distal cortex where the MT plus-ends are known to accumulate. Despite their non-overlapping distributions, we found that, similarly to HeLa cells, Ens is required for Dsh localization to the distal cortex, as shown in original Figure 9C. Intriguingly, Dsh is known to distribute to the entire cell cortex before the onset of PCP formation, and becomes redistributed asymmetrically at the distal cortex during PCP formation, the process in which Ens is involved. Therefore, we also propose that Ens in the proximal side promotes the MT loading of Kinesin-1, which carries Dsh-containing cargo to the distal cortex. Our interpretation about Ens localization in pupal wing cells is described in the fourth paragraph of the Discussion section of the new manuscript (page 15, line 5 through 12).

3) Ens mutations: There still is an issue with fly genetics and second site lethals on the ensKO alleles. I understand that the ens Df removes more than ens, but it is a null for ens by definition (it lacks the gene). I agree with the authors that DeltaC is clearly hypomorphic, as homozygous flies that hatch survive longer than the KO alleles /Df. However, homozygous and transhet KO combinations fail to produce hatching embryos which they do over a Df (even though those flies die very shortly after birth). Those phenotypes are therefore stronger than over Df. There are two explanations: either the KO alleles are neomorphic or the Crispr alleles have an additional, shared second site pupal lethal (i.e. off-target). As the KO alleles have frame-shifts right after the start codon, the first explanation is highly unlikely. Whatever is published about Crisprs not having off-targets in flies must be wrong from experience I know of. Using Crisprs, three LOF alleles were obtained for a gene that were all lethal in trans, but viable over a Df. In addition, the second site lethal could be recombined away, proving that it was a second site lethal due to Crispr off-target (as all three alleles had the same second site lethal). I am willing to share more of that data with the editor should the authors not agree with my arguments. Nevertheless, even the authors' own data show the presence of a second site lethal.

Therefore, I suggest to remove the transhet data and to simply use the ens/Df that is already in the paper and shows the author's conclusion. The authors may leave the mutant mosaic data in Fig S10, as there is no way around using the KO alleles for this. However, they should state that, formally, the second site mutation could have an effect as well (although highly unlikely, as KO/Df in whole wings shows similar phenotypes).

Response: According to the referee's suggestion "remove the transhet data and to simply use the ens/Df that is already in the paper and shows the author's conclusion.", we removed data of wing hair orientation in ensKO36/ensKO39 mutants from original Figure 9A. Please note that defects in wing hair orientation in ensKO36/ensKO39 mutants were virtually identical to those in wing hair orientation in ens hemizygotes. Therefore, off target mutations in ensKO36 or ensKO39 chromosomes, if any, should not affect planar cell polarity on wing epithelium. Since we used ensKO36/ensKO39 pupae expressing Dsh::GFP to investigate the effect of the loss of ens on the Dsh localization in wing cells, we would like to remain data on wing hair orientation in ensKO36/ensKO39 mutants as a new Appendix Figure S10C.

Minor items:

4) Old major item 2: Cells in Fig 2 now oriented with migration upwards. Please state this in legend or text, and not only in the rebuttal (rest resolved).

Response: In response to the referee's comment "Please state this in legend or text", we added the text "Panels in B-D, F, and G are arranged to show that cells are migrating in an upward direction." in figure legend (page 30, line 1 through 2).

5) p9: ....aa 159-246 of Map7 are sufficient.... This is not shown by the data presented. Those amino acids seem to be required for full binding, but there is no sufficiency construct. Adjust text.

Response: Regarding to the referee's concern "Those amino acids seem to be required for full binding, but there is no sufficiency construct.", we rephrased the corresponding text to "the DEP domain of Dvl is sufficient for, and the aa 159-246 region of Map7 is required for their interaction." (page 9, line 5 through 7).

6) Fig. S8B: indicate leading edge direction to judge recovery direction.

Response: According to the referee's suggestion, we added identical panels shown in original Figure 7B to new Appendix Figure S8B.

7) Drosophila ovary data of Fig S9B in not mentioned in the text, thus remove panel or state relevance (Ens localizes to MT minus ends?).

Response: In response to the referee's comment "Drosophila ovary data of Fig S9B in not mentioned in the text, thus remove panel or state relevance", we added the text "Endogenous Ens accumulates in the region where MT minus-ends are known to enrich, such as the anterior side of oocytes and the apical side of epithelial follicle cells [36]. The localization of Ens::EGFPKI was indistinguishable from that of endogenous Ens (Appendix Fig. S9B), indicating that the added EGFP moiety did not affect Ens's localization and functions." in the Results section (page 12, line 16 through 20). As the referee said, Ens localizes to MT minus-ends in oocytes, similar to the Ens localization in wing cells.

3rd Editorial Decision 28 April 2018

Thank you for your patience while your manuscript has undergone a final round of review. As you will see from the report below, former referee 2 is now all positive about its publication in EMBO reports. I am therefore writing with an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once two minor issues/corrections have been addressed, as follows.

- Please indicate the off-target effects of the ens alleles either in the figure legend or alternatively in the methods section.

- Please review the synopsis image. Note that the final size will be 550 x 400 pixels, which is rather small. I have copied the image from the .pptx file into a .tif file of this size. I think the text is just about legible but it could be bigger. Moreover, I kindly ask you to provide a file with higher resolution.

If all remaining corrections have been attended to, you will then receive an official decision letter from the journal accepting your manuscript for publication in the next available issue of EMBO reports. This letter will also include details of the further steps you need to take for the prompt inclusion of your manuscript in our next available issue.

Thank you for your contribution to EMBO reports.

REFEREE REPORT

Referee #2:

The authors have sufficiently addressed most of the issues.

Regarding the off-target issue, I suggest that the authors state in legend of Figure what they wrote in the rebuttal: [N]ote that defects in wing hair orientation in ensKO36/ensKO39 mutants were virtually identical to those in wing hair orientation in ens hemizygotes [and] therefore, off target mutations in ensKO36 or ensKO39 chromosomes should not affect planar cell polarity on wing epithelium.
Note that the statement should NOT contain the 'if any'.

In addition, they should state that there is an offtarget on the KO chromosome in the supplementary figure legend S10A.

As I stated previously, this does not alter their relevant conclusion, but should not be ignored.

1) - Please indicate the off-target effects of the ens alleles either in the figure legend or alternatively in the methods section.

Response: We added the text "Note that defects in wing hair orientation in ens^{KO36}/ens^{KO39} pupae were virtually identical to those in wing hair orientation in $ens^{KO36}/Df(3L)BSC735$ pupae. Therefore, off-target mutations in ens^{KO36} or ens^{KO39} chromosomes should not affect planar cell polarity on wing epithelium." in the figure legend for original Appendix Figure S10C.

2) - Please review the synopsis image. Note that the final size will be 550 x 400 pixels, which is rather small. I have copied the image from the .pptx file into a .tif file of this size. I think the text is just about legible but it could be bigger. Moreover, I kindly ask you to provide a file with higher resolution.

Response: We placed new synopsis image converted to 550 x 401 pixels, together with original synopsis image, in the PowerPoint file.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Koji Kikuchi, Hiroyuki Nakanishi
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2017-45471-T

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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 χ2 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.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

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?	Sample size was chosen based on similar experiments in the previous reports.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	The number of female mice used in each experiment was reduced to the minimum necessary to obtain physiologically relevant results.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
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.	Female mice were purchased from the SLC in Japan. The mice were allocated according to their age (older than 8 weeks), because PCP formation in the mouse oviduct occurs during postnatal development.
For animal studies, include a statement about randomization even if no randomization was used.	Except for age, we used an unbiased approch in the animal experiments for our study.
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.	Whenever possible, each experiment was performed in a blinded manner.
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Statistical tests were chosen based on the distribution of the datasets. The information for statistical test of each experiment is described in a section of Materials and Methods, "Statistics", and figure legends.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	The datasets were assessed for normal distribution using Shapiro-Wilk test.
Is there an estimate of variation within each group of data?	An estimate of variation within a dataset was done by standard deviation .
Is the variance similar between the groups that are being statistically compared?	Similar variance between the groups was tested by F-test.

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

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

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-tun

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).	We provide the catalog numbers and references in Appendix Table S1. As described in Materials and Methods section, rabbit polyclonal antibodies against Map7D1 were raised using recombinant GST fused with a region of Map7D1 (aa 529-803) as an antigen.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	HeLa cells and HeLa cells stably expressing EB1-GFP were provided by M. Nakao in IMEG, Kumamoto Univ. and Y. Mimori-Kiyosue in CLST, RIKEN, respectively. Cell lines are routinely tested for 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.	Female mice (Slc:ICR; Japan SLC, Japan) were used for analysis. The animals were kept in a light and temperature controlled room with a 12-hour light/dark cycle at 22 ± 1 °C.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Animal care and experiments were conducted in accordance with the Guidelines of Animal Experiment of the National Institutes of Natural Sciences. The experiments using animals were approved by The Institutional Animal Care and Use Committee of National Institutes of Natural Sciences.
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.	We are confident that our experiments are in coordance with ARRIVE guidelines.

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 a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for '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).	Datasets for main figures are provided as the Source Data file.
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. 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

22. 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