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Balanced ubiquitylation and deubiquitylation of Frizzled regulate cellular responsiveness to Wg/Wnt

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1st Editorial Decision 04 November 2009

Thank you very much for submitting your research manuscript for consideration to The EMBO Journal editorial office.

As you will see from the comments enclosed below, all expert referees appreciate the insight provided by your study reporting on how ubiquitylation regulates cellular localization of Fz and thus modulates Wnt signals. Irrespective of this overall assessment, all three scientists request important further experimentation that will corroborate your proposal and therefore increase potential impact of your study. As the requirements are very explicitly presented in the referee comments, there is no need for me to repeat them here in much detail. Conditioned on these clearly essential amendments we would be happy to assess a revised version of your paper in the future. Given that some of the experiments seem rather time-consuming, I would also like to mention that we are able to extend the usual deadline for necessary revisions upon request by the authors. I also have to remind you that it is EMBO_J policy to allow a single round of major revisions only, and that the final decision on acceptance or rejection entirely depends on the content within the final version of your manuscript!

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

This is a very nicely laid out story making a significant advance in understanding of how ubiquitylation regulates the cellular location of Frizzled receptors and thus Wnt signaling. The manuscript makes use of a combination of fly genetics and mammalian cell culture to validate the importance of the UBPY/USP8 deubiquitylating enzyme and to probe the mechanism by which it regulates Frizzled intracellular trafficking. While I like the manuscript, I do have some significant concerns about specific experiments. The authors would have to fully address these concerns before I would recommend acceptance for publication.

Major concerns/criticisms

1) The rescue of the UBPY/USP8 RNAi phenotype shown in Fig. 1C is not a valid way to perform this experiment. A proper rescue of RNAi phenotype involves the use of a transgene that is not targeted by the dsRNA. This is not the case in this experiment. The way it is done, overexpressing the UBPY/USP8 mRNA could simply titrate out the dsRNA-RNAi machinery. I absolutely urge that this faulty piece of data be removed from the manuscript. I am satisfied with the authors reporting that two distinct dsRNAs cause the same phenotype, and with the hypomorphic allele used in Fig. 1F.

2) I am still confused by the result in Fig. 4B, where the dominant negative UBPY causes a decrease in Fx4 degradation. The text on page 11 states that this will be discussed further in the discussion, but I am not sure where this occurred.

3) In Fig. 4C-F, some cells express Flag-Fz4 but do not express UBPY. This provides a nice internal control for their measurement of cell surface Fz4. But how do the authors achieve this differential expression? This is not clear. It was my impression that when two constructs are co-transfected, most cells express both transgenic proteins.

4) In Fig. 2A, expression of the constitutively active form of UBPY(S680A) causes an order of magnitude increase in Wnt3a signaling. But in Fig. 4H, expression of Fz4 K0 causes less than two-fold increase. Both experiments use the same readout (TOPFLASH). Doesn't this STRONGLY suggest that UBPY/USP8 affect on Wnt signaling cannot be explained solely by modification of Fz. This is a major point of contention for me: I think the data in the manuscript clearly shows the importance of UBPY/USP8 in Wnt signaling. They also show that this enzyme controls the ubiquitylation and stability of Fz. But the experiment in Fig. 4H is the key to connecting these two bodies of data. I think the modest increase is unimpressive and suggests that more is going on.

5) Fig. 6 would be greatly improved by showing that Wg targets are reduced in the clones removing UBPY/USP8.

Minor comments:

The significance of FK2 is not clearly explained.

On page 11, line 14, I believe "UBPY" should be "Fz4"

Referee #2 (Remarks to the Author):

Mukai et al analyze the role of the UBPY/USAP8 deubiquitylating enzyme in regulating Frizzled protein levels in the Drosophila wing primordium as well as in mammalian cells. Their data indicate

that the membrane levels of the Frizzled receptor are regulated by ubiquitylation to modulate the activity of the Wnt/wg signalling pathway. In general, I believe they present consistent data to demonstrate their model and the topic is of interest to the EMBO Journal. However, I wonder how specific to Fz is the role of UBPY/USAP8. In other terms, the data to demonstrate the specificity are not very convincing. This is fine with me (in case other receptors are affected as well), but the authors should be clear in this regard.
This and other issues should be addressed in a satisfactory manner:

Major points

(1) Monitoring Wg activity in dUBPY KO:

1.1. Figure 1G, H: Other Wg target genes should be analyzed (eg. Senseless, Dll, Vg, Armadillo) to demonstrate the requirement of dUBPY in Wg signalling. What is the phenotype in other imaginal discs (leg or eyes)? Does the dUBPY kO have any phenotype in the embryo?

1.2 Authors should monitor the activity of the Wg pathway in clones of cells mutant for dUBPY (as they have done for other purposes in Fig. 6).

(2) Specificity of dUBPY to Fz receptors: Authors postulate that dUBPY specifically regulates cell surface Fz levels but not the levels of other receptors.
1.1 Authors overexpressed the protein and analyze the turnover of Fz2-FLAg cell surface levels at time 0 or 3 hours after induction of Fz2-FLAg expression (Fig 5). Other membrane receptors (eg. Notch or Smo) should be tested to demonstrate the specificity if any.
1.2. They overexpressed dUBPY and analyze the range of activation of Wg signalling by monitoring Dll expression. Another Gal4 drivers should be used (eg. ci, en, ptc) to have an internal control of cells expressing the transgene and cells in the adjacent compartment not expressing it.
1.3 Other pathways should be monitored after overexpression of dUBPY (eg. Hh, Notch, Dpp)?
1.3. In Fig 6 the authors analyze the levels of other receptors (Notch, Smo and Arrow) in dUBPY mutant clones and they conclude that these are not affected. A Z section of these clones should be included to demonstrate this is the case.
1.4. They state in the ms (pg. 14) that dUBPY knockdown caused enlargement of the Rab7 endosomal compartment (Fig. 7), but the levels of Notch and Smo are not affected. This seems contradictory, as these receptors are regulated by endocytosis.

(3) Some in vivo epistatic analysis should be provided to demonstrate the causal relationship between elevated levels of Fz at the cell surface and increased Wg activity. For example, is it possible to rescue the dUBPY knockdown phenotype by providing extra Fz, or the other way around....

Minor points:
(1) Several mistakes are found throughout the ms (eg. pg 9".....it remains unknown whether Fz is ubiquityated, to say nothing of regulation by UBPY..." is not understandable.)
(2) pg 13. ..." but also enhanced cell proliferation in the wing pouch..." This is just an interpretation and as such should be either deleted or demonstrated (eg. PH3, BrdU, etc).
(3) In the acknowledgements section, DGRC, BDSC and DSHB deserve full names.

Referee #3 (Remarks to the Author):

This paper describes the identification of the deubiquitylating enzyme, UBPY, as a regulator of trafficking of the Wnt receptor, to the lysosomes, thereby promoting Wg/Wnt signaling. Taking advantage of the Drosophila RNAi library, the authors performed a screen in the wing pouch by driving the dsRNA construct with the scalloped-Gal4 driver. As a readout for Wg signaling, the authors scored for the formation of sensory bristles at the wing margin. Wg signaling induces differentiation of the sensory organ precursors to mechanosenory and chemosensory bristles. Of the nine genes identified by the authors for which downregulation leads to defects in bristle formation, the authors focused on the deubiquitylating enzyme, UBPY/USP8. The authors nicely demonstrated specificity. They show that a second set of shRNAs also cause sensory bristle defects and that co-expression of wild-type UBPY rescues the shRNA phenotype. The authors also generated a UBPY

knockout and demonstrated loss of sensory bristles. To provide further evidence that UBPY is involved in the transmission of a Wg/Wnt signal, the authors demonstrated that UBPY facilitates Wg /Wnt signaling in Drosophila wing discs and mammalian cells.

Next, the authors tackled the mechanism of action of UBPY in the Wnt pathway. Taking a page from previous studies demonstrating the role of UBPY in endosomal trafficking of RTKs to lysosomes, the authors asked whether UBPY may similarly be involved in trafficking the Wg receptor, Frizzled. The authors show that Fz is ubiquitylated and degraded over time and that this is reversed by UBPY. They show that this ubiquitin modification results in less Fz at the membrane and more Fz in late endosomes, whereas a non-ubiquitylatable Fz does not turn over and is present in higher amounts on the cell surface. They show that noverexpression of a constitutively active UBPY results in more Fz at the membrane and suggest a new model in which Fz membrane levels are regulated by ubiquitylation and de-ubiquitylation. Overall this is a solid paper although a few things need to be addressed.

Major concerns:

1. The nature of the Ub modification on Fz is unclear. What kind of Ub is being added, and is the type of Ub consistent with what is known about lysosomal sorting? This may simply be addressed by using K0-Ub and other mutant forms of Ub (K48R, K63R, etc) to determine if it is a mono-Ub, multi-mono Ub, or chain and the type of linkage. This is an important point that is not mentioned in the paper.

2. It will be important to distinguish between proteasomal-mediated degradation versus lysosomal-mediated degradation in order to clarify the mechanism. That can be accomplished by using inhibitors that distinguish between the two. According to the authors' model, proteasomal inhibition should have not an effect on Fz turnover, whereas lysosomal inhibition should inhibit Fz turnover.

3. Quantification of the in vivo studies is lacking. For example, Fig 6. is not very convincing and needs to be quantified.

Other concerns...

1. Fig. 1E. Rescue of dsRNA was performed with dUBPY-how can you rescue with the Drosophila gene that is also the target of the dsRNA?

2. Fig 2 shows that catalytically dead UBPY has effects on Topflash, Dvl2 phosphorylation and beta-catenin levels. What about UBPY RNA? It would be nice to demonstrate that knock down of the DUB also has the same effects. The authors demonstrate this in Fig. 3C, so we know that this is possible.

3. Fig. 2B/C. Quantification of the bands is needed. This is especially true for Fig. 2C where the results are not obvious and where there is a lot more Flag-UBPY expressed in the S-A mutant where the biggest change in beta-catenin is detected. Also there is much less Flag-UBPY expressed in the C-A mutant where beta-catenin levels are also presumed to decrease. Also, the authors don't explain why Flag-UBPY is only found in the membrane fraction-is it already known that UBPY is membrane localized?

4. Fig 3E. Does the reciprocal CO-IP work? (i.e. can the authors IP UBPY and blot for Fz4) ?

5. Fig 3. An in vitro de-ubiquitylation assay would directly test the model that UBPY deubiquitylates Fz. One possibility would be to IP Fz4 from cells, add purified UBPY, and show decreased Fz4 ubiquitylation.

6. Fig. 4C/D. Quantification is needed. How many cells showed this phenotype? Along these lines, can the authors provide biochemical evidence for increased levels of Fz at the membrane? For example, could one isolate membranes from the S-A and C-A mutants and blot for Fz?

7. Fig. 4H-can you show that the UBPY C-A and S-A mutants have no effect on the Flag-Fz4K0 mutant but do on the WT Fz4 mediated Topflash activation (i.e. show that UBPY no longer affects

the Topflash if Fz can't be ubiquitinated) ?

8. Fig 5. Again, numbers! How many wing discs were analyzed and what percentage showed these phenotypes? What about quantification of the staining here? How much more Fz is present in the presence of overexpressed UBPY?

9. Fig. 6. What is the intensity of Fz staining in the UBPY-/- vs -/+ cells? There does not appear to be much of a difference in the figure shown. Again, no numbers as to how many cells were analyzed. Quantification is required in order to be convincing. I don't see any difference between panels A, C, E and G where they claim a large effect on Fz and no effect on the other receptors. Without quantification, the authors have not made a convincing case.

10. Fig 7. It would strengthen the study if the authors could overexpress the UBPY S-A mutant. Their model predicts that there would be less Fz in the late endosome, more Fz in the recycling endosomes, and more Fz on the surface.

1st Revision - Authors' Response 26 March 2010

Response to referee 1

Major #1: The rescue of the UBPY/USP8 RNAi phenotype shown in Fig. 1C is not a valid way to perform this experiment. A proper rescue of RNAi phenotype involves the use of a transgene that is not targeted by the dsRNA. This is not the case in this experiment. The way it is done, overexpressing the UBPY/USP8 mRNA could simply titrate out the dsRNA-RNAi machinery. I absolutely urge that this faulty piece of data be removed from the manuscript. I am satisfied with the authors reporting that two distinct dsRNAs cause the same phenotype, and with the hypomorphic allele used in Fig. 1F.

RE: According to the suggestion, we deleted previous Fig. 1E.

Major #2: I am still confused by the result in Fig. 4B, where the dominant negative UBPY causes a decrease in Fx4 degradation. The text on page 11 states that this will be discussed further in the discussion, but I am not sure where this occurred.

RE: We apologize that the discussion was not clearly written in the previous manuscript. We rewrote the part regarding why Fz4 degradation was inhibited by dominant-negative UBPY in the DISCUSSION (page 20, line 22 - page 21, line 14). It has been shown that expression of dominant-negative UBPY causes morphological aberration (enlargement) of endosomes in mammalian cells, accompanying compromised lysosomal trafficking and degradation of ubiquitylated proteins (Mizuno et al, Traffic 7: 1017-, 2006; Row et al, JBC 281: 12618-, 2006). We also observed similar morphological changes to late endosomes after UBPY knockdown in Drosophila wing disc cells (Fig. 7C' and E'). We therefore speculate that dominant-negative UBPY inhibited Fz4 degradation as a secondary effect of compromised lysosomal trafficking. The enlargement of late endosomes is likely due to an accelerated trafficking from early to late endosomes, as previously demonstrated in other contexts (Seto and Bellen, JCB 173: 95-, 2005), which is consistent with our proposed model that UBPY suppresses early-to-late endosomal trafficking of ubiquitylated cargo proteins.

Major #3: In Fig. 4C-F, some cells express Flag-Fz4 but do not express UBPY. This provides a nice internal control for their measurement of cell surface Fz4. But how do the authors achieve this differential expression? This is not clear. It was my impression that when two constructs are co-transfected, most cells express both transgenic proteins.

RE: Under the conditions used in these experiments, the efficiency of co-transfection was indeed quite high. However, we could still find cells transfected only with one construct (such as the cells indicated by arrowheads in Fig. 4C and 4D). We have thus compared cells expressing Flag-Fz4 alone and those expressing both Flag-Fz4 and UBPY, quantified the signal intensities of cell surface Fz4 in more than 10 cells for each sample, and detected statistically significant differences between these two samples (Fig. 4E and 4F).

Major #4: In Fig. 2A, expression of the constitutively active form of UBPY(S680A) causes an order of magnitude increase in Wnt3a signaling. But in Fig. 4H, expression of Fz4 K0 causes less than two-fold increase. Both experiments use the same readout (TOPFLASH). Doesn't this STRONGLY suggest that UBPY/USP8 affect on Wnt signaling cannot be explained solely by modification of Fz. This is a major point of contention for me: I think the data in the manuscript clearly shows the importance of UBPY/USP8 in Wnt signaling. They also show that this enzyme controls the ubiquitylation and stability of Fz. But the experiment in Fig. 4H is the key to connecting these two bodies of data. I think the modest increase is unimpressive and suggests that more is going on.

RE: We agree with the referee that this is a very important point. Since Fz4-K0 lacks all 12 cytoplasmic Lys residues, we thought that mutation of these residues might compromise the signaling function of Fz4. Indeed, K499 of Fz4 has been reported to play an essential role in transmitting Wnt signaling (Umbhauer et. al., EMBO J. 19: 4944-, 2000). We therefore constructed a mutant, Fz4-R499K, in which Arg replaces the 11 cytoplasmic Lys residues except for K499. This Fz4-R499K mutant was not detectably ubiquitylated. Importantly, expression of the Fz4-R499K mutant markedly enhanced Wnt signaling: it increased TOP-Flash 8-fold higher than that of wild-type Fz4, comparable to the level observed when constitutively active UBPY was expressed (Fig. 4H). This result strongly supports the notion that UBPY activates Wnt signaling through deubiquitylation of Fz. This point is newly stated in the text (page 13, line 18 - page 14, line 3).

Major #5: Fig. 6 would be greatly improved by showing that Wg targets are reduced in the clones removing UBPY/USP8.

RE: We examined the expression of two Wg targets, Senseless and Armadillo, in the wing disc. We show that their expression levels are decreased in the dUBPY knockout clones. These results are now shown in the Fig. 1J-J" and K-K" and mentioned in the text (page 7, lines 15-20).

Minor: The significance of FK2 is not clearly explained. On page 11, line 14, I believe "UBPY" should be "Fz4"

RE: We explained the significance of FK2 in the text (page 17, lines 16-17). We corrected "UBPY" to "Fz4" on page 11, as pointed out.

Response to referee 2

Main comment: In general, I believe they present consistent data to demonstrate their model and the topic is of interest to the EMBO Journal. However, I wonder how specific to Fz is the role of UBPY/USAP8. In other terms, the data to demonstrate the specificity are not very convincing. This is fine with me (in case other receptors are affected as well), but the authors should be clear in this regard.

RE: To test the specificity of dUBPY, we additionally analyzed cell surface localization of different types of membrane proteins such as DE-cadherin, Flamingo, and beta-integrin in the dUBPY knockout clones. The cell surface levels of these proteins in the knockout clones were indistinguishable from those in the neighboring wild-type cells (Fig. S6). These data suggest that dUBPY selectively regulates the cell surface level of Fz among the membrane proteins examined in this study. We further examined the specificity of dUBPY by overexpression experiments, as described in our response to major point (2).

(1) Monitoring Wg activity in dUBPY KO:

Major (1):

1.1. Figure 1G, H: Other Wg target genes should be analyzed (eg. Senseless, Dll, Vg, Armadillo) to demonstrate the requirement of dUBPY in Wg signalling. What is the phenotype in other imaginal discs (leg or eyes)? Does the dUBPY kO have any phenotype in the embryo?

RE: We performed immunostaining of other Wg target proteins, Senseless and Armadillo, in the wing discs. We found that their levels were reduced in the dUBPY knockout clones (Fig. 1J-J", K-K") and the level of Armadillo protein was elevated in the dUBPY-overexpressing cells (Fig. 5O-O"). These results support our conclusion that dUBPY is required for Wg signaling, and are now also mentioned in the text (page 7, lines 15-20 and page 15, lines 10-11).

Regarding the effects of dUBPY RNAi in other tissues, we examined dUBPY knockout clones in the leg discs, but found that the levels of Dachshund (Dac), a Wg target in this tissue, was not obviously affected. We speculate that the sensitivity of Wg signaling to the loss of dUBPY might differ in different tissues possibly due to differential expression levels of Wg pathway components or dUBPY itself, or due to differential threshold levels of Wg signaling required for target expression. We also speculate that some compensating signals (e.g., Dpp) might compensate for Dac expression.

Embryos homozygous for dUBPY knockout develop normally until late embryonic stages (~st.16/17) probably because the maternal contribution of dUBPY compensates the requirement at the early stage when Wg is required for the segment polarity formation. To examine the phenotype caused by the loss of dUBPY at the early stage, we tried to generate the germ line clones of dUBPY but failed, most likely due to the requirement of dUBPY for oogenesis. Therefore, we could not analyze the effect of dUBPY on Wg-dependent embryonic segmentation.

1.2. Authors should monitor the activity of the Wg pathway in clones of cells mutant for dUBPY (as they have done for other purposes in Fig. 6).

RE: As described above, we monitored the activity of Wg signaling by detecting Armadillo protein and found that its level was decreased in the dUBPY mutant cells (Fig.1K-K") and increased in the dUBPY-overexpressing cells (Fig.5O-O"). These results provide direct evidence for the role of dUBPY in regulating Wg signaling in vivo.

Major (2) Specificity of dUBPY to Fz receptors: Authors postulate that dUBPY specifically regulates cell surface Fz levels but not the levels of other receptors.

2.1. Authors overexpressed the protein and analyze the turnover of Fz2-FLAg cell surface levels at time 0 or 3 hours after induction of Fz2-FLAg expression (Fig 5). Other membrane receptors (eg. Notch or Smo) should be tested to demonstrate the specificity if any.

RE: We overexpressed dUBPY in the wing disc, and examined the cell-surface levels of Notch by both conventional immunostaining and immunostaining before membrane permeabilization to specifically detect cell surface proteins. In both cases, the surface levels of Notch were unchanged by dUBPY overexpression (Fig. S7A-E). We also found that the surface levels of Arrow were also unchanged by dUBPY overexpression (Fig. S7F-G'). We mention this in the text (page16, lines 8-11). Taken together with the results regarding DE-cadherin, Flamingo and beta-integrin mentioned above, these results support the notion that the cell surface level of Fz is selectively regulated by dUBPY.

2.2. They overexpressed dUBPY and analyze the range of activation of Wg signalling by monitoring Dll expression. Another Gal4 drivers should be used (eg. ci, en, ptc) to have an internal control of cells expressing the transgene and cells in the adjacent compartment not expressing it.

RE: According to the referee's suggestion, we overexpressed dUBPY by the use of a dpp-Gal4 driver and monitored the expression of Dll and Arm, targets of Wg signaling. The levels of Dll and Arm were clearly elevated in cells overexpressing dUBPY compared to the adjacent compartment not expressing it. We include these results in Fig. 5M-M", O-O" and in the text (page 15, lines 8-11).

2.3. Other pathways should be monitored after overexpression of dUBPY (eg. Hh, Notch, Dpp)?

RE: As suggested by the referee, we examined the expression levels of Dpp and Sal to monitor the Hh and Dpp pathways, respectively, in the wing disc. We found that their levels were unchanged by overexpression of dUBPY. We include these results in Fig. 5P-S and in the text (page 15, lines 11-13).

2.4. In Fig 6 the authors analyze the levels of other receptors (Notch, Smo and Arrow) in dUBPY mutant clones and they conclude that these are not affected. A Z section of these clones should be included to demonstrate this is the case.

RE: We included the Z section images of Notch, Smo and Arrow in the Fig. S5. The results confirmed that the surface levels of these proteins are not affected by the knockout of dUBPY. We mention this in the text (page 16, lines 4-6).

2.5. They state in the ms (pg. 14) that dUBPY knockdown caused enlargement of the Rab7 endosomal compartment (Fig. 7), but the levels of Notch and Smo are not affected. This seems contradictory, as these receptors are regulated by endocytosis.

RE: As the referee correctly pointed out, the cell surface levels of Notch and Smo have been reported to be regulated at the early step of endocytosis from the plasma membrane to the early endosome, but not at later steps from the early endosome to the lysosome (Vaccari et. al., JCB 180: 755-, 2008; Chen et al., Science 306: 2257-, 2004). The enlargement of Rab7-positive late endosomes in dUBPY knockdown cells suggests impairment in the late endosomal step. Therefore, our observation of normal cell surface levels of these proteins is not contradictory to the enlargement of the late endosome by dUBPY knockdown.

Major (3): Some in vivo epistatic analysis should be provided to demonstrate the causal relationship between elevated levels of Fz at the cell surface and increased Wg activity. For example, is it possible to rescue the dUBPY knockdown phenotype by providing extra Fz, or the other way around....

RE: According to the referee's suggestion, we have performed a rescue experiment in adult wings by co-expression of DFz2 with dsRNA for dUBPY using the sd-Gal4 driver. We found that expression of extra DFz2 effectively rescued the loss of sensory bristles caused by dUBPY depletion, which strongly suggests that dUBPY functions through the regulation of Fz in vivo and demonstrates a causal relationship between elevated levels of Fz at the cell surface and increased Wg activity regulated by dUBPY. We include this result in Fig. 6I-M and in the text (page 16, lines 12-16).

Minor (1): Several mistakes are found throughout the ms (eg. pg 9".....it remains unknown whether Fz is ubiquityated, to say nothing of regulation by UBPY..." is not understandable.)

RE: We apologize for the mistakes. We asked a native English speaker to correct grammatical errors and improve the manuscript.

Minor (2): pg 13. ..." but also enhanced cell proliferation in the wing pouch..." This is just an interpretation and as such should be either deleted or demonstrated (eg. PH3, BrdU, etc).

RE: We deleted this statement and the corresponding results (previous Fig. 5L, M) accordingly.

Minor (3): In the acknowledgements section, DGRC, BDSC and DSHB deserve full names.

RE: We wrote the full names for DGRC, BDSC, and DSHB in the ACKNOWLEDGEMENTS.

Response to referee 3

Major #1: The nature of the Ub modification on Fz is unclear. What kind of Ub is being added, and is the type of Ub consistent with what is known about lysosomal sorting? This may simply be addressed by using K0-Ub and other mutant forms of Ub (K48R, K63R, etc) to determine if it is a mono-Ub, multi-mono Ub, or chain and the type of linkage. This is an important point that is not mentioned in the paper.

RE: We agree with the referee that this is an important point. We thus compared the patterns of Fz4 ubiquitylation in cells expressing wild-type Ub and several mutant forms of Ub. Ub-K0 is an Ub mutant in which all seven Lys residues are replaced by Arg, and can be used only for mono-ubiquitylation, not for poly-ubiquitylation, due to the lack of Lys residues. We found that when either Flag-tagged wild-type Ub or Ub-K0 mutant was expressed, the patterns of Fz4 ubiquitylation (high-molecular-weight band sifts) detected by the Flag tag were very similar. This strongly indicates that Fz4 harbors multi-mono-Ub. This notion is further supported by the result that the ubiquitylation patterns were very similar when Lys residues necessary for poly-ubiquitylation (K48, K63) are specifically mutated. Ub-K48R and Ub-K63R are Ub mutants in which only K48 and K63, respectively, are replaced by Arg, and Ub-R48K and Ub-R63K are Ub mutants in which all Lys residues but K48 and K63, respectively, are replaced by Arg. All these Ub mutants exhibited similar ubiquitylation patterns on Fz4 when overexpressed. Together, we conclude that Fz4 mainly undergoes monoubiquitylation at multiple sites. This is consistent with the role of monoubiquitylation as a lysosomal sorting signal (Haglund and Dikic, EMBO J. 24: 3353-, 2005). This new result is shown in Fig. 3C and stated in the text (page 9, line 21 - page 10, line 11).

Major #2: It will be important to distinguish between proteasomal-mediated degradation versus lysosomal-mediated degradation in order to clarify the mechanism. That can be accomplished by using inhibitors that distinguish between the two. According to the authors' model, proteasomal inhibition should have not an effect on Fz turnover, whereas lysosomal inhibition should inhibit Fz turnover.

RE: We examined the effects of proteasome and lysosome inhibitors, MG132 and bafilomycin A1, respectively, on Fz4 degradation. The addition of bafilomycin A1 inhibited degradation of cell surface Fz4. In contrast, MG132 treatment did not affect the rate of Fz4 degradation, whereas it clearly inhibited that of beta-catenin. These results suggest that Fz is degraded by a lysosome-dependent mechanism but not a proteasome-dependent mechanism. This result is shown in Fig. S3 and stated in the text (page 12, lines 3-7).

Major #3: Quantification of the in vivo studies is lacking. For example, Fig 6. is not very convincing and needs to be quantified.

RE: We quantified immunofluorescence intensities in Fig. 6 and S6. The intensities of the staining were determined and compared between the knockout clones and neighboring wild-type cells. The immunofluorescence intensity of Dfz2, but not that of Smo, Notch, Arr, Fmi, beta-integrin or DE-cadherin, was significantly decreased in dUBPY knockout clones (Fig. 6H and S6G).

Other #1: Fig. 1E. Rescue of dsRNA was performed with dUBPY-how can you rescue with the Drosophila gene that is also the target of the dsRNA?

RE: Since we cannot exclude the possibility that the rescue with wild-type cDNA is due to titration of the dsRNA-RNAi machinery, we deleted this rescue experiment in the revised manuscript. However, we think that the phenotype is not caused by an off-target effect, given that the same phenotype was obtained with two independent dsRNAs for dUBPY (Fig. 1C, D) and also observed in (partially rescued) dUBPY knockout wings (Fig. 1E).

Other #2: Fig 2 shows that catalytically dead UBPY has effects on Topflash, Dvl2 phosphorylation and beta-catenin levels. What about UBPY RNA? It would be nice to demonstrate that knock down of the DUB also has the same effects. The authors demonstrate this in Fig. 3C, so we know that this is possible.

RE: As suggested by the referee, we examined the effects of UBPY knockdown on Wnt signaling in mammalian cells. We found that UBPY knockdown reduced the level of beta-catenin protein in Wnt-stimulated cells (Fig. 2D). This further demonstrates the necessity of UBPY in Wnt signaling, not just in Drosophila cells, but also in mammalian cells. This result is stated in the text (page 9, lines 6-8). We could not detect the activity of Topflash or Dvl2 phosphorylation in HeLa cells, in which we could effectively knock down UBPY. Unfortunately, we could not knock down UBPY in HEK293T and NIH3T3 cells, in which we could detect the activity of Topflash and Dvl2 phosphorylation.

Other #3: Fig. 2B/C. Quantification of the bands is needed. This is especially true for Fig. 2C where the results are not obvious and where there is a lot more Flag-UBPY expressed in the S-A mutant where the biggest change in beta-catenin is detected. Also there is much less Flag-UBPY expressed in the C-A mutant where beta-catenin levels are also presumed to decrease. Also, the authors don't explain why Flag-UBPY is only found in the membrane fraction-is it already known that UBPY is membrane localized?

RE: We quantified the intensity of the bands in Fig. 2B-D, which is now indicated below each panel. As regards the localization of UBPY, we have previously reported that UBPY is only found in the membrane fraction in subcellular fractionation experiments by using cultured human cells (Mizuno et al, MBC 16: 5163-, 2005). This is stated in the legend for Fig. 2.

Other #4: Fig 3E. Does the reciprocal CO-IP work? (i.e. can the authors IP UBPY and blot for Fz4) ?

RE: We have tried the reciprocal co-IP, but failed, since we could not detect Flag-Fz4 with immunoblotting due to its electrophoretical overlap at ~55kDa with the IgG heavy chain used for IP. We have tried different types/species of IgG to immunoprecipitate UBPY, but could not avoid this problem.

Other #5: Fig 3. An in vitro de-ubiquitylation assay would directly test the model that UBPY deubiquitylates Fz. One possibility would be to IP Fz4 from cells, add purified UBPY, and show decreased Fz4 ubiquitylation.

RE: According to the referee's suggestion, we performed the in vitro deubiquitylation assay. We immunopurified Flag-tagged UBPY proteins (by immunoprecipitation and subsequent elution with Flag peptides) and incubated them with immunoprecipitated Fz4. We found that wild-type UBPY, but not its catalytically inactive mutant C748A, deubiquitylated Fz4. In addition, the constitutively active mutant S680A exhibited higher activity than wild-type UBPY. These results have clarified that Fz4 is a direct substrate of UBPY (Fig. 3G and page 10, line 21 - page 11, line 4).

Other #6: Fig. 4C/D. Quantification is needed. How many cells showed this phenotype? Along these lines, can the authors provide biochemical evidence for increased levels of Fz at the membrane? For example, could one isolate membranes from the S-A and C-A mutants and blot for Fz?

RE: Quantification of immunofluorescence in Fig. 4C and D is shown in Fig. 4E and F. In these experiments, 10~25 pairs of UBPY-expressing and non-expressing cells were examined as we mention in the legend for Fig. 4.

Regarding biochemical quantification, it is difficult to isolate plasma membranes with no contamination of intracellular endosomal membranes. We therefore specifically biotinylated cell surface Fz4 and compared the level of biotinylated Fz4 in control cells and in cells expressing

UBPY-C748A or UBPY-S680A. We found that expression of UBPY-C748A reduced the surface level of Fz4, whereas UBPY-S680A had an opposite effect. This provides biochemical evidence for UBPY regulation of Fz levels at the cell surface. These results are shown in Fig. S4 and mentioned in the text (page 13, lines 6-12).

Other #7: Fig. 4H-can you show that the UBPY C-A and S-A mutants have no effect on the Flag-Fz4K0 mutant but do on the WT Fz4 mediated Topflash activation (i.e. show that UBPY no longer affects the Topflash if Fz can't be ubiquitinated)?

RE: As suggested by the referee, we compared the effects of UBPY mutants on wild-type Fz and a non-ubiquitylatable Fz mutant (Fz-R449K, an Fz mutant in which all Lys residues but K449 are replaced by Arg; please see our response to the referee 1's major point #4). The effects of UBPY C748A and S680A mutants on Wnt signaling were much weaker when the non-ubiquitylatable Fz mutant was expressed, in comparison to when wild-type Fz was expressed. This supports the notion that UBPY affects Wnt signaling via deubiquitylation of Fz. This result is shown in Fig. 4I and mentioned in the text (page 14, lines 3-10).

Other #8: Fig 5. Again, numbers! How many wing discs were analyzed and what percentage showed these phenotypes? What about quantification of the staining here? How much more Fz is present in the presence of overexpressed UBPY?

RE: We analyzed approximately 30 wing discs for each sample, and all dUBPY-overexpressing discs showed DFz2 accumulation. We also quantified the fluorescence intensity in the wing pouches and found that dUBPY overexpression increased the surface level of DFz2 by 4.5 ± 0.46-fold ($p < 0.02$). We included this information in the legend for Fig. 5 and in the text (page 15, lines 1-3).

Other #9: Fig. 6. What is the intensity of Fz staining in the UBPY-/- vs -/+ cells? There does not appear to be much of a difference in the figure shown. Again, no numbers as to how many cells were analyzed. Quantification is required in order to be convincing. I don't see any difference between panels A, C, E and G where they claim a large effect on Fz and no effect on the other receptors. Without quantification, the authors have not made a convincing case.

RE: We quantified the fluorescence intensity, and the results are depicted in Fig. 6H. The level of surface Fz in the dUBPY knockout region was 54.5 ± 8.59% ($p < 0.01$) of that in the control region. No such difference was observed for the other receptors. We measured the intensities of 4 independent areas in 3 independent discs (n=12 in total) for each sample. This is now specified in the legend for Fig. 6.

Other #10: Fig 7. It would strengthen the study if the authors could overexpress the UBPY S-A mutant. Their model predicts that there would be less Fz in the late endosome, more Fz in the recycling endosomes, and more Fz on the surface.

RE: Since dUBPY does not contain a Ser residue comprising a 14-3-3-binding site like S680 in mammalian UBPY, and the overexpression of the wild-type dUBPY causes strong effects on the localization of Fz (Fig. 5), we examined the effect of dUBPY overexpression on the intracellular localization of DFz2. We found that dUBPY overexpression markedly reduces the level of DFz2 localization to Rab7-positive late endosomes in the wing disc cells. The percentage of DFz2/Rab7 double-positive puncta among total DFz2-positive puncta was 90.32 ± 3.5% in control cells vs. 23.73 ± 20.63% in dUBPY-overexpressing cells (Fig. 7G).

Together with our finding that dUBPY overexpression increased the surface level of DFz2 (Fig. 5G, H), these results further support our model. We mention this in the text (page16, line 22 - page17, line 8).

2nd Editorial Decision 06 April 2010

Your revised manuscript has now been re-assessed by one of the original referees whose comments you will find enclosed. This scientist suggests to include a lower magnification figure to orient readers on the position of the clone related to the DV-boundary. I kindly ask you to take this point into consideration and provide us with the ultimate version of the work to enable final acceptance.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #2 (Remarks to the Author):

The authors have addressed all my concerns. I strongly endorse its publication in EMBO Journal. A minor point, however, should be addressed. A lower magnification of Figs 1J-J' and 1K-K' should be included and the actual location of the clone shown in Figs 1K-K' should be clarified. In other terms, is the clone located closed to the DV boundary, in the wing pouch or in the notum? Since the phenotype shown in Fig 1B-E is specific to the DV boundary, it might make sense to show a clone abutting the DV boundary.

