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Wnt5a regulates distinct signaling pathways by binding to Frizzled2

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1st Editorial Decision 01 July 2009

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are enclosed. As you will see, all three referees recognise the interest in your work, but vary somewhat in their overall assessment of the current version. While referee 1 finds that your study is essentially suitable for publication without significant revision, both reviewers 2 and 3 raise a number of concerns that would need to be addressed in a revised version of your manuscript, before we can consider publication.

In the light of the referees' positive recommendations, I would therefore like to invite you to submit a revised version of the manuscript, addressing all the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision. Acceptance of your manuscript will thus depend on the completeness of your responses included in the next, final version of the manuscript.

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

Yours sincerely,
Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

This is another fine ms of the Kikuchi lab, where they follow up on their analysis of endocytic routes involved in Wnt signaling pathways. Specifically they addressed the relationship between antagonistic Wnt3 and Wnt5a signaling in canonical and Wnt/PCP signaling. They provide convincing evidence for a clathrin mediated Wnt5a/PCP route and show that the Wnt3a and Wnt5a antagonism plays at the level of Wnt ligands competing for receptors. This suggests a novel model for Wnt pathway selection and determinants of the specificity.

The ms can essentially be published as is.

Minor comments:

1. Fig. 1D: Please calculate the Kd of Wnt3 binding to LRP6DN
2. p. 10 1st para: The reference for Dvl-dependent Thr1479 phosphorylation is Bilic et al 2007

Referee #2 (Remarks to the Author):

A. Sato et al.
Wnt5a regulates distinct signaling pathways by binding to Frizzled2

This manuscript describes a dual effect of Wnt5a on Rac activation and β-catenin pathway inhibition mediated by the receptor Frizzled 2 (Fz2). The authors show that Wnt5a stimulates Rac through Fz2 and Ror1/2, by a mechanism that involves Fz2 internalization mediated by clathrin, dishevelled 2 (Dvl2) and β-arrestin2. In the second part of the paper, the authors show that Wnt5a inhibits Wnt3a-induced activation of the β-catenin pathway in vitro and in vivo, and that this is due to competition for binding to Fz2, which does not require Wnt5 induced internalization. The results presented are of potential interest but do not justify the conclusions drawn. Major points that need to be addressed experimentally are indicated below.

1. It has been shown recently that Wnt3a activates Rac GTPase, which is required for nuclear translocation of stabilized β-catenin (Wu et al., Cell 2008). Thus, the authors need to determine whether or not Wnt3a activates Rac in their cell system to determine how specific is this biochemical read-out for Wnt5a as opposed to Wnt canonical signaling.

2. In the discussion, the authors suggest that Wnt3a and Wnt5a induce internalization of Fz through the caveolin or clathrin dependent pathways, respectively. This is an important issue that should be experimentally addressed by investigating the effects of Wnt3a treatment on Fz2 subcellular localization and the role of caveolin and clathrin in these events.

3. The authors suggest that overexpression of Dvl2 or β-arrestin2 induces activation of Rac through internalization of Fz2. These effects are not necessarily connected. In order to support their hypothesis, they need to test the effects of Dvl2 and β-arrestin2 overexpression in the presence or absence of Fz2 siRNA.

4. The siRNAs used to knockdown gene expression need to be tested for specificity. For example, does Ror1 siRNA have any off-target effects on Ror2 expression and vice versa? Also, are Ror1/2 receptors required for Fz2 internalization?

5. The lack of effect of clathrin inhibition on Wnt5a-induced inhibition of LRP6 phosphorylation does not justify a full figure (Fig.7). Also, the experimental design chosen by the authors, i.e. co-administration of Wnt3a and Wnt5a is not sufficient to answer this question. For example, it is conceivable that endogenous Wnt5a present as suggested by the experiments performed with wt and Wnt5a-/-MEFs could induce Fz2 internalization, resulting in a decreased Fz2 availability for Wnt3a signaling. Therefore, a better experimental design would be to pre-treat with Wnt5a, in the presence or the absence of clathrin inhibition, prior to addition of Wnt3a and Wnt5a.

6. Previous studies, including by the authors (Kurayoshi et al., 2007), have indicated that Wnt5a inhibits Wnt3a signaling at the β-catenin /transcription factor level. Thus, it would be important to determine whether Fz2 internalization and Rac activation are involved in this alternative inhibitory mechanism.

7. The fact that Fz2 knockdown is sufficient to block Wnt-induced phosphorylation of LRP6 in cells expressing Fz5 as well is not consistent with the reported role of this receptor in mediating LRP6 phosphorylation. Also, the authors have previously reported that Wnt5a induced Fz5 internalization, while in the present manuscript Fz5 expression does not seem to be required for Wnt5a-induced activation of Rac. Is it possible that Fz5 mediates a different Wnt5a pathway? Do Wnt3a and Wnt5a have much lower affinities for Fz5 compared to Fz2?

8. The authors need to perform a time-course experiment to test whether the lack of effect of Wnt5a on JNK and Rho activation may be due to use of only a single time point.

9. The authors should determine expression level of Wnt5a in wild-type MEFs to support their conclusion as to the basis for increased levels of axin2 expression induced by Wnt3a in Wnt5a-/- cells.

Referee #3 (Remarks to the Author):

The authors establish FZD2 as a WNT-5A receptor and identify agonist-induced receptor internalization as an important means of signal transduction to e g RAC1. The experiments were performed very carefully with suitable controls. Results are novel and fill important gaps in our knowledge of WNT/Frizzled signaling. Conclusions drawn are supported by the presented data. The authors also address the functional interaction of WNT-3A and WNT-5A with regard to β-catenin signaling suggesting a model of WNT-3A/-5A competition at the level of FZD. This issue has been addressed by several groups and underlying mechanisms are - as pointed out in the manuscript - a matter of debate.
In the following list I will address my concerns and additions.

1. For the investigation of the clathrin-mediated endocytosis of FZD, the authors employ MDC and CZ. It is known that hyperosmolaric sucrose and potassium depletion, two inhibitors of clathrin-dependent endocytosis downregulate DVL expression in several cell lines (Bryja et al 2007, Acta Phsyiologica). Therefore, I suggest to monitor the DVL levels in the Fig 2D, 3A,B, 7A,B by Immunoblotting. In case MDC and CZ reduce DVL levels, conclusions on their mechanisms have to be changed (page 6 and 10).
2. The authors aim to clarify the mechanisms underlying the WNT-5A-mediated inhibition of WNT-3A signaling via LRP and β-catenin . This is a matter of debate in the field and the authors provide interesting and novel aspects in this issue. However, the authors should put their findings into context with a recent publication (Bryja et al 2009, Mol Biol Cell.;20(3):924-36), showing the functional interaction of WNT-5A with LRP regulating WNT/P-LRP/β-catenin signaling in vitro and in vivo. These changes should be implemented throughout the manuscript e g p 7 (last paragraph), p8/9, p 9 third paragraph, p 10 (third paragraph), p11/top of page, p12, last paragraph, p14 etc.
Sato et al mainly discuss the possibility of intracellular crosstalk and the WNT/FZD competition model. However, WNT-5A binding to LRP also serves as an important modulator of WNT-3A signaling in this context and could even be responsible for functional selectivity of WNT/FZD interaction. The data should be taken into consideration in both the results and the discussion section.
3. The data shown in Fig. 3 F are intriguing and implicate that DVL and β-arrestin can induce agonist-independent FZD internalization. It is important to exclude the requirement of endogenously expressed WNTs to support this hypothesis. This could e g be done by co-expression or treatment of cells with sFRPs in sufficient amounts.

Minor points:
1. The IUPHAR (www.iuphar.org) recommends a general nomenclature for Frizzleds: capital letters FZD with number as subscript.
2. provide 95% confidence interval for Kd values of WNT binding to the FZD CRD (page 6, first

paragraph).
3. page 10, 2nd paragraph should read: ...WNT-3A and WNT-5A bound to FZD2-CRD.... in order to clarify that binding studies were performed with the CRD only and not the whole receptor.
4. Fig. 3 D: the immunoblot does not agree with the bar graphs of RAC1 activation, since there is still an increase in active RAC1 visible.
5. Fig. 5E: This is the only bar graph summarizing immunoblot data, which lacks error bars? Was this experiement only performed once or did the error bars disappear during image assembly?
6. For the sake of clarity it should be stated - even though it is rather obvious - that the precipitates of the binding assays are analyzed by SDS-PAGE and then probed with WNT-3A/-5A antibodies.

1st Revision - authors' response 10 August 2009

Our responses to the referees' comments for EMBO J-2009-71556

Referee #1

(1) The referee requested to calculate the Kd of Wnt3a binding to LRP6 in Fig. 1D.

It is important to calculate the Kd of Wnt3a to LRP6. However, as shown in Figure 1D, the binding of Wnt3a to LRP6 was not saturated even though 800 ng/ml (approximately 20 nM) Wnt3a was used. It seems that the affinity of Wnt3a to LRP6 is low, and the Kd must be higher than 20 nM. This value is quite high as compared to the concentration used for Wnt3a-dependent LRP6 phosphorylation or β-catenin accumulation (0.5~2.5 nM). The maximal concentration of our purified Wnt3a that can be used in this assay is 1 μg/ml. Therefore, it was difficult to calculate the Kd value of Wnt3a to LRP6 practically. Since we used the extracellular region of LRP6 in the present study, this may lower the Wnt3a binding activity. What we would like to emphasize in Figure 1D is that Wnt5a bound to LRP6 little under the same conditions. These results are consistent with our present observations that Wnt3a activated the β-catenin pathway but Wnt5a did not in ten different cell lines. These statements were described in the text (page 6, lines 9 and 10, page 16, lines 8 through 13).

(2) The referee requested to cite the reference correctly.

According to the referee's suggestion, we cited Bilic's paper (Science, 2007).

Referee #2

(1) The referee asked whether Wnt3a activates Rac, which leads to nuclear localization of β-catenin , in our cells system.

In the paper (Cell, 133, 340-353, 2008) pointed out by the referee, Wnt3a conditioned medium activated Rac in ST2 cells. We used purified Wnt3a and HeLaS3 and L cells. In these cells it was difficult to detect Wnt3a-dependent activation of Rac. The results were shown in Supplementary Figure S2D and described in the text (page 5, lines 4 and 5 from bottom). It was also reported that overexpression of Wnt3a activates Rac in HEK293 cells (Genes Dev. 15, 295-309, 2003). Therefore, whether Wnt3a activates Rac may be dependent on cell types. Alternatively, other factor(s) in addition to Wnt3a might be necessary for the activation of Rac. These statements were described in the text (page 16, lines 7 through 10 from the bottom).

(2) The referee requested to investigate the effects of Wnt3a treatment on Fz2 subcellular localization.

According to the referee's comment, FLAG-Fz2 was expressed in HeLaS3 cells and the cells were stimulated with Wnt3a. When cells expressing FLAG-Fz2 alone were stimulated with Wnt3a, FLAG-Fz2 was colocalized mainly with clathrin and hardly, if at all, with caveolin. However, when FLAG-Fz2 and LRP6-GFP were coexpressed, the receptor complex was internalized with caveolin

at high efficiency. Therefore, Fz2 may be internalized through a clathrin-mediated pathway probably with endogenous Ror1 or Ror2, and when both Fz2 and LRP6 are activated by Wnt3a, the complex may be internalized through the caveolin-mediated pathway. These results support our data that Wnt3a activates both the β-catenin and Rho (PCP) pathways (Mol. Cell. Biol. 24, 4487-4501, 2004) and are consistent with our previous observations using FLAG-Fz5 (Dev. Cell, 11, 213-223, 2006). The results were shown in Supplementary Figure S10 and described in the text (page 14, the bottom through page 15, line 8).

(3) The referee requested to test effects of Dvl2 and β-arrestin2 overexpression on Rac activation in the presence or absence of Fz2 siRNA.

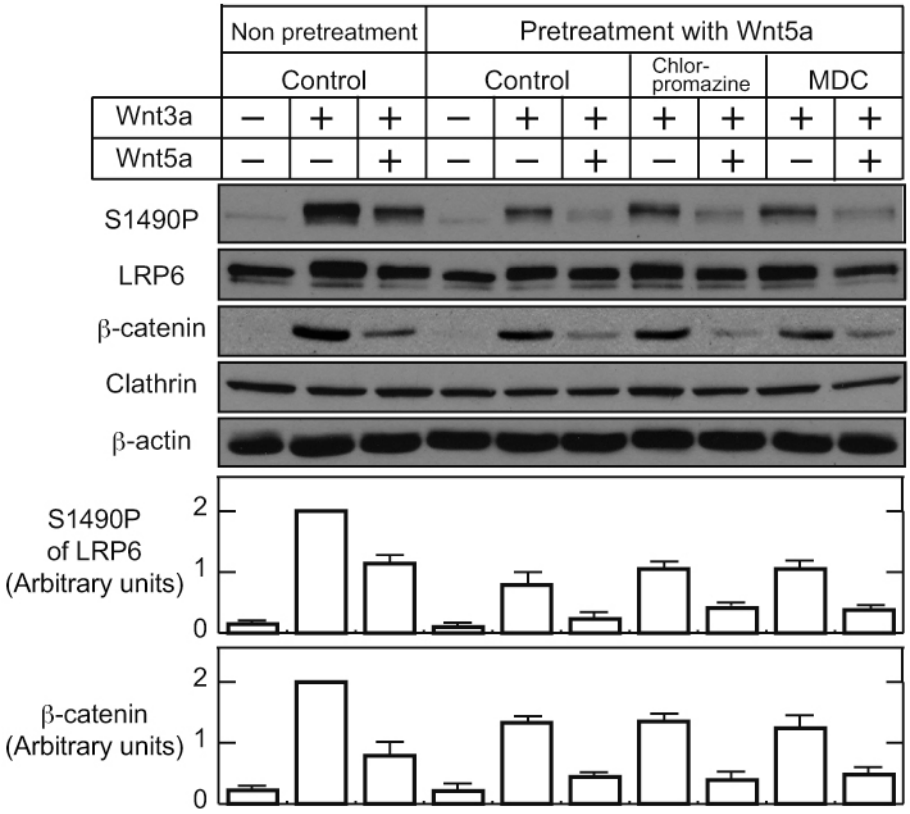
According to the referee's suggestion, we examined whether overexpression of Dvl2 or β-arrestin2 activates Rac in HeLaS3 cells treated with Fz2 siRNA. Dvl2-induced Rac activation was observed in Fz2 knockdown cells, while β-arrestin2-induced Rac activation was lost by knockdown of Fz2. Taken together with the observations that Dvl2 and β-arrestin2 induce the internalization of Fz2, these results suggest that the clathrin-dependent internalization of Fz2 plays a role in Wnt5a-dependent activation of Rac, and it is likely that overexpressed Dvl2 itself has another way to activate Rac. The results were shown in Supplementary Figure S7B and described in the text (page 8, lines 4 through 9).

(4) The referee pointed out to test specificity of siRNA.

We realized that it is important to test specificity of siRNA, because one siRNA for Fz5 reduced the expression levels of LRP6 (data not shown). We confirmed that our siRNA for Ror1 does not affect Ror2 expression and Ror2 siRNA does not have off-target effect on Ror1 expression. Fz2 siRNA did not affect the expression of Fz6. The results were shown in Supplementary Figure S1B and described in the text (page 5, lines 9 and 10 from the bottom).
The referee also asked whether Ror1 and Ror2 are required for Fz2 internalization. We found that siRNA for Ror1 and/or Ror2 decreases Wnt5a-dpendent internalization of Fz2. The results were shown in Figure 2C and Supplementary Figure S3A and described in the text (page 6, lines 5 through 7 from the bottom).

(5) The referee suggested a better experimental design in Fig. 7.

According to the referee's suggestion, we pretreated HeLaS3 with 100 ng/ml Wnt5a for 30 min in the presence or absence of chlorpromazine or MDC and then stimulated the cells with 100 ng/ml Wnt3a in the presence or absence of 300 ng/ml Wnt5a. Pretreatment with Wnt5a decreased the levels of phosphorylation of LRP6 induced by Wnt3a, compared to conditions without pretreatment. This result seems to reflect the results in Figure 5E using MEFs. In addition, irrespective of any pretreatment conditions with or without chlorpromazine or MDC, Wnt5a further inhibited Wnt3a-dependent phosphorylation of LRP6, and chlorpromazine or MDC did not affect the inhibitory action of Wnt5a (see an attached figure).
As pointed out by the referee, it can not be excluded the possibility that endogenous Wnt5a binds to and internalizes Fz2 via the clathrin-dependent route, resulting in the decrease in the protein levels of Fz2 on cell surface. However, it can also be speculated that Wnt5a binds to and occupies Fz2 in the lipid raft, but rarely induces the internalization of it by the caveolin-dependent route as shown in Figure 2B. The protein levels of Fz2 in the lipid raft on cell surface was unaffected by Wnt5a as shown in Supplementary Figure S9. Therefore, it is difficult to conclude the points raised by the referee at present. Because to show the results with pretreatment conditions may make the readers confuse, we would like to use original results in Figure 7. Instead, we show the actual results as follows.



Effects of Wnt5a on phosphorylation of LRP6 and accumulation of β-catenin induced by Wnt3a after pretreatment with Wnt5a in the presence of inhibitors of clathrin-mediated endocytosis.

(Figure legend) HeLaS3 cells were pretreated with 10 μg/ml chlorpromazine or 50 M MDC for 30 min, and then treated with 100 ng/ml Wnt5a for 30 min in the presence or absence of chlorpromazine or MDC. After pretreatment steps, the cells were washed with PBS three times, and then stimulated with 100 ng/ml Wnt3a with or without 300 ng/ml Wnt5a for 1 h in the presence or absence of chlorpromazine or MDC. The lysates were probed with the indicated antibodies. The signals of S1490P and β-catenin were quantified using NIH Image. The results are expressed as arbitrary units compared with the signal intensities in control cells stimulated by Wnt3a in condition without pretreatments, and indicate means ± SE from three independent experiments.

(6) The referee said that it would be important to determine whether Fz2 internalization or Rac activation are involved in Wnt5a-dependent inhibition of Tcf transcriptional activity downstream of β-catenin .

As pointed out by the referee, we showed previously that Wnt5a inhibits β-catenin -dependent Tcf transcriptional activity in HEK293 cells downstream of β-catenin (Biochem. J., 402, 515-523, 2007). Knockdown of clathrin in HEK293 cells expressing stably expressing constitutively active β-catenin (SA- β-catenin) suppressed the Wnt5a-dependent inhibition on TOP-fos-Luc reporter activity induced by SA- β-catenin . Thus, some intracellular signal transduction pathways induced by Wnt5a may inhibit the transcription activated by β-catenin in the nucleus in HEK293 cells, and this inhibition may require the clathrin-dependent internalization. Therefore, the inhibitory mechanism by Wnt5a may depend on the cell type. At present, it is conceivable that Wnt5a can interfere with different steps of the β-catenin pathway. The results were shown in Supplementary Figure S8 and described in the text (page 14, lines 10 through 18).

(7) The referee asked the role of Fz5 in the Wnt signaling pathway.

Basically we believe that Fz5 is an important receptor for Wnt5a signaling. The reason why we did not mention Fz5 in this manuscript was that our siRNAs for Fz5 did not reduce its mRNA levels efficiently due to technical problem. As shown in Figure 1B, Wnt3a still induced the phosphorylation of LRP6 in Fz2 knockdown cells. These results suggest that other Fzs including Fz5 may also play a role in Wnt3a signaling with LRP6.
As pointed out by the referee, we showed that Wnt5a induces the internalization of Fz5 in HEK293 cells (Biochem. J., 402, 515-523, 2007). In the present manuscript, knockdown of Fz2 suppressed Wnt5a-dependent Rac activation almost completely in HeLaS3 cells. Therefore, it is unlikely that Fz5 mediates Wnt5a-dependent Rac activation in this cell line. Fz5 may mediate a different Wnt5a pathway in this cell line. However, to clarify the specificity between various Fzs and the Wnt5a pathway is beyond the aim of this manuscript. I believe that these are important issues in Wnt signaling field. We would like to clarify how Wnt5a activates different signaling pathway selectively by interacting with distinct Fzs. These statements were described in the text (page 15, lines 10 through 15 from the bottom).
The referee also asked whether Wnt3a and Wnt5a have lower affinities for Fz5 compared to Fz2. In vitro binding studies showed that Wnt3a and Wnt5a have similar affinities for Fz5 and Fz2. The results were shown in Supplementary Figure S11 and described in the text (page 15, lines 11 and 12).

(8) The referee said that we need to perform a time-course experiment of Wnt5a-dependent activation of JNK and Rho.

According to the referee's comment, Wnt5a-dependent activation of JNK and Rho in L cells was examined in various time points. However, it was hard to detect the activation of JNK and Rho by Wnt5a compared with Wnt5a-dependent activation of Rac. The results were shown in Supplementary Figure S2, B and C, and described in the text (page 5, lines 4 and 5 from bottom).

(9) The referee said that we should determine expression levels of Wnt5a in wild-type MEFs.

According to the referee's comment, we measured protein levels of Wnt5a in wild-type and Wnt5a knockout MEFs. The result was shown in Figure 5D and described in the text (page 9, lines 7 and 8 from the bottom).

Referee #3

(1)The referee said that we should monitor the Dvl levels in the cells treated with MDC or chlorpromazine.

It has been reported that the protein levels of Dvl reduced in the cells treated with hyperosmotic sucrose and potassium depletion which inhibits the clathrin-dependent internalization. According to the referee's comment, we examined the Dvl levels in the cells treated with MDC or chlorpromazine. These reagents that suppress clathrin-mediated endocytosis did not affect the Dvl levels in all experiments in Figures 3B and 7B and Supplementary Figure S3B. Clathrin knockdown in Figures 3A and 7A did not reduce the levels of Dvl, either. The results were shown in Supplementary Figure S5 and described in the text (page 6, line 2 from the bottom through page 7, line 2).

(2) The referee required that we should consider the possibility of the functional interaction of Wnt5a with LRP6, which was reported by Bryja et al (Mol Biol. Cell 2009, 20, 924-936).

The paper by Bryja et al. reported that extracellular domain of LRP6 inhibits the β-catenin - independent pathway by binding to Wnt5a. Their in vitro studies were performed using cells expressing with HA-Wnt5a and LRP6N-Fc. Wnt5a was indeed coprecipitated with LRP6N-Fc in this condition, but the assay was not quantitative, and comparison with the binding activity of Wnt3a to LRP6N-Fc was never done. Furthermore, a large amount of Wnt5a (1000 ng/ml) was necessary for the inhibition of Wnt3a (20 ng/ml)-dependent phosphorylation of LRP6 in their paper. Our in vitro studies showed that the binding activity of Wnt5a to LRP6N is much weaker than that of Wnt3a using purified Wnt proteins and LRP6N-IgG. In our assay Wnt5a (100 ng/ml) could

decrease Wnt3a (50 ng/ml)-dependent LRP6 phosphorylation to around 50 %. Bryja et al. also showed that the phenotypes by deletion of LRP6 in mice and Xenopus embryos were rescued by additional deletion of Wnt5a. These are interesting findings, but further experiments are necessary to conclude that these phenotypes are due to LRP6-dependent suppression of the β-catenin -independent pathway.
Since the possibility that LRP6 extracellular domain inhibits the β-catenin -independent pathway can not be excluded, we cited the paper and discussed about this possibility in the text (page 16, lines 2 through 7 from the bottom).

(3) The referee asked whether Dvl2- or β-arrestin2-induced Fz2 internalization requires endogenously expressed Wnt5a in Fig. 3F.

According to the referee's suggestion, we examined whether Dvl2 or β-arrestin2 can still induce the internalization of Fz2 in the presence of sFRP2 conditioned medium. sFRP2 conditioned medium could suppress Wnt5a-dependent migration (Cancer Res., 66, 10439-10448, 2006). Although after HeLaS3 cells were treated with sFRP2 for 1 h, Dvl2 or β-arrestin2 was able to induce the internalization of Fz2. These results suggest that Dvl2 or β-arrestin2 can induce the Wnt-independent internalization of Fz2. The results were shown in Supplementary Figure S7A and described in the text (page 8, lines 2 through 4).

(4) The referee suggested to use a general nomenclature for Frizzled recommended by the IUPHAR.

As far as we have searched recent publications (from January, 2006 to July, 2009) using "Frizzled and Fz" or "Frizzled and FZD" as keywords in NCBI home page, 63 publications still have used "Fz" for a nomenclature for Frizzled, while 51 have used FZD. Since we also used "Fz" in our previous reports, we would like to describe the same nomenclature in the present manuscript.

(5) The referee required to provide 95% confidence interval for Kd values of Wnt binding to the Fz CRD.

According to the referee's comment, we calculated means±SE. The data was described in the text (page 6, lines 7 through 9).

(6) The referee requested to state clearly that the CRD only was used for the Wnt binding assay in vitro.

We agree to the referee's comment. We rewrote the description (page 10, line 3 from the bottom).

(7) The referee did not accept the bar graphs of Rac activation in Fig. 3D.

We re-calculated Wnt5a-dependent Rac activation in CHO cells and CHO cells expressing ENTH domain in four independent experiments. The results were shown in Figure 3D.

(8) The referee commented that Fig. 5E lacks error bars.

As described in the legend, we performed this experiments using MEFs from four different pairs of Wnt5a-/- and the littermate Wnt5a+/+ mice. All experiments showed that Wnt3a-dependent LRP6 phosphorylation is enhanced in Wnt5a-/- MEFs. According to the referee's comment, we added the error bars in Figure 5E.

(9) The referee said that we should describe the binding assay more correctly.

According to the referee's suggestion, the statement was changed to "the precipitates of the binding assay are analyzed by SDSPAGE and then probed with anti-Wnt3a, anti-Wnt5a, and anti-FLAG antibodies" in Materials and methods (page 17, lines 2 and 3 from the bottom, page 18, lines 7 and 8).

2nd Editorial Decision 04 September 2009

Many thanks for submitting the revised version of your manuscript EMBOJ-2009-71556R. It has now been seen again by referees 2 and 3, whose comments are appended below. As you will see, referee 3 is now happy with the paper, save for a couple of minor corrections to be made to the text. Referee 2, on the other hand, still has a number of major concerns. I have looked into this in detail, and have discussed the study with my colleagues. Generally, we do not allow a second round of major revision, but in this case we feel that the referee raises an important point: the fact that Wnt3a stimulates both canonical and non-canonical pathways complicates the picture and throws some doubt upon your conclusions. However, you should be able to address these concerns relatively easily, by analysing colocalisation of Fz2 with caveolin vs. clathrin under conditions where cells expressing LRP6 are treated with Wnt5a. If your model is correct, LRP6, while able to shift Fz2 to the caveolin pool upon Wnt3a treatment, should not be able to induce relocalisation of Fz2 upon Wnt5a treatment.

The referee also highlights questions as to the relative effects of Wnt3a on canonical vs. non-canonical signalling - contingent upon LRP6 expression. I recognise that this may entail a significant amount of further work, and given that you have previously shown that Wnt3a is able to activate both pathways, would not insist on additional experiments along these lines. However, if you do have such data, I would encourage you to incorporate them, and would in any case ask you to respond to the referee's comments on these points.

I would therefore ask you to revise your manuscript as outlined above, and to prepare a point-by-point response to the reviewers' comments. Please do not hesitate to get in touch should you have any questions or wish to discuss this further.

I look forward to receiving your revision.

Best wishes,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #2 (Remarks to the Author):

This revised manuscript addressed many of the questions raised concerning the initial submission. However, the authors' response to point 2 does not sufficiently address the points raised, and some major concerns remain. In particular, the authors argue that different endocytic pathways in response to specific Wnts determine the activation of β-catenin dependent or independent signaling (page 12, diagram in Fig.8). However, new Fig.S10 shows that Wnt3a induced the internalization of Flag-Fz2 mostly through clathrin, which is exactly what happens with Wnt5a treatment (Fig.2B). These results clearly contradict the authors' conclusion. Even though they report that Wnt3a triggered caveolin-mediated internalization of Flag-Fz2 when LRP6-GFP was co-expressed, they should investigate the effects of Wnt5a under these same conditions. To support different mechanisms for internalization of Fz2 by Wnt3a and Wnt5a, additional experiments, including a comparison of the effects of these two Wnts under the same conditions, need to be performed.
The authors also claim that in the absence of exogenous LRP6, Wnt3a induces clathrin-mediated internalization of Flag-Fz2 and activation of a β-catenin independent pathway (page 15 and Fig.8). This is an important point that needs to be supported by stronger data. For example, the authors need to show that in cells expressing Flag-Fz2, both ligands stimulate Rac and/or Rho, but not the β-catenin pathway, whereas when Flag-Fz2 and LRP6 are co-expressed, Wnt3a and Wnt5a activate distinct signaling pathways.

Minor point (point 5 in the first review).
The authors admit that it is difficult to exclude the possibility that endogenous Wnt5a induces the

internalization of Fz2, resulting in decreased levels of the receptor at the cell surface. Thus, the negative results with the clathrin inhibitors shown in Fig.7 do not justify a full figure and should be more appropriately incorporated in Fig.6 and/or as a supplementary figure.

Referee #3 (Remarks to the Author):

The revision has improved the paper substantially. The authors responded carefully to the referees comments.
minor comments:
1. p 14 last row: ... cells expressing FLAG-Fz2 alone were....
2. error bars in Fig 5A or state that bar graphs shows resilts from one exp representative of ... experiments.

2nd Revision - authors' response 30 September 2009

Our responses to the referees' comments for EMBO J-2009-71556R1

Referee #2

(1) The referee said that the experiments to compare the effects of Wnt3a and Wnt5a on the internalization of Fz2 under the same conditions need to be performed.

Since it was hard to detect endogenous Fzs or LRP6 in immunocytochemical studies, we have to overexpress them. Summary of our findings in this study is as follows.
[1] Wnt5a induces the internalization of Fz2 with clathrin when it is expressed alone. (Figure 2B)
[2] Wnt3a induces the internalization of Fz2 with clathrin when it is expressed alone. (Figure S11A)
[3] Wnt3a induces the internalization of Fz2 and LRP6 with caveolin when both proteins are expressed. (Figure S11B)
These results suggest that Wnt3a and Wnt5a induce the internalization of Fz2 via a clathrin-mediated pathway when it was expressed alone. Referee #2 pointed out that these results are contradict to our conclusion. We believe that Wnt5a triggers the internalization of the Fz2/Ror2 complex with clathrin, while Wnt3a may induce the internalization of the unknown single-pass transmembrane receptor with Fz2 via a clathrin-mediated route. Therefore, there may be different binding proteins to the Wnt5a/receptor and Wnt3a/receptor complexes even though they are in clathrin-coated vesicles. These differences may activate the distinct pathways. Consistent with this speculation, Wnt3a activated Rho, while Wnt5a activated Rac. Thus, it is possible that Wnt3a and Wnt5a activate distinct signaling pathways even though both Wnts induce the internalization of Fz2 with clathin.
According to the referee's comment, FLAG-Fz2 was expressed with LRP6 in HeLaS3 cells and the cells were stimulated with Wnt5a. FLAG-Fz2 was internalized and colocalized with clathrin mainly, while LRP6-GFP remained on the cell surface membrane. In addition, it is notable that the numbers of Fz2 puncta internalized by Wnt5a decreased when Fz2 and LRP6 were coexpressed as compared with that when Fz2 was expressed alone. This suggests that the coexpression of LRP6 causes unknown effects to lead to the retention of Fz2 on the cell surface membrane when the cells were stimulated with Wnt5a that had low affinity for LRP6. Although to detect the localization of endogenous receptors will be necessary in the future, we assume that Wnt5a can not induce the internalization of LRP6 with Fz2 through a caveolin-mediated route at least in HeLaS3 cells. The results are shown in Supplementary Figure S12 and described in the text (page 15, line 9 through line 6 from the bottom).
The referee also asked to examine whether Wnt3a and Wnt5a activate the β-catenin -dependent and/or independent pathways under overexpression of Fz2 alone and of both Fz2 and LRP6. So far we have already reported that Wnt3a activates both the β-catenin and Rho (PCP) pathways, and that Wnt5a activates the β-catenin independent pathway but not β-catenin pathway (Kishida et al, 2004, and Kurayoshi et al, 2007). In this study we focused on Wnt5a signaling, because it has not yet been examined systematically whether Wnt5a regulates multiple pathways through the receptor-mediated endocytosis. To compare effects of Wnt3a and Wnt5a on the receptor internalization and signaling pathways is beyond the aim of this study. Therefore, it seems that we do not need to

perform the experiments to respond to this point at present. We would like to clearly show how distinct Wnt ligands regulate distinct signaling pathways through different endocytic routes in the future.

(2) The referee suggested that Fig. 7 should be incorporated in Fig. 6.

According to the referee's suggestion, we combined Fig. 6 and Fig. 7, A and C in one figure and moved Figure 7B to Supplementary Figure S8 (page 11, lines 8 and 9).

Referee #3

(1) The referee could kindly our careless mistake in page 14.

We corrected it.

(2) The referee said to show error bars in Fig. 6A.

According to the referee's comment, we added the error bars from three independent experiments in Figure 6A.

2nd editorial decision 1 October 2009

Many thanks for submitting the revised version of your manuscript - having now looked at the latest version, I appreciate that you have included the specific experiment requested, and that it gives the expected result. I am therefore pleased to tell you that we can now accept the manuscript without further delay.

You will receive the official acceptance message shortly.

