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Interaction with both ZNRF3 and LGR4 is required for the signaling activity of R-spondin

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

Editor: Barbara Pauly

1st Editorial Decision 10 July 2013

Thank you for the submission of your research manuscript to our editorial offices and your patience while we were conducting the peer review. We have now received the three enclosed reports on it.

While referee 2 is in general more positive about the study, both referees 1 and 3 feel that -despite its merits- the paper would be better suited for publication in a more specialized journal and they communicate this evaluation in the manuscript assessment form that all referees submit to our office directly. Nevertheless, it also becomes clear from their reports that the study could potentially become suitable for publication here if the novel aspects of it were to be developed further. Currently, the referees do not consider them to be sufficiently developed yet. Examples include the data on the SOMAmer inhibitor and the need to show that the proposed mechanism is also supported by combined treatment of cells with Wnt and R-spondin, with the latter point having been raised also by referee 2. All referees also point out instances in which additional controls and clarifications are needed. Referees 1 and 3 also state that the mechanism by which LGR4/5 that lacks the transmembrane and cytoplasmic domain regulates R-spondin signaling should be elucidated further.

From the analysis of these comments it is clear that publication of your manuscript in our journal cannot be considered at this stage and that further work would be required before the study could be published here. On the other hand, given the potential interest of your study, I would like to give you the opportunity to address the reviewers concerns and submit a revised manuscript with the understanding that the main referee concerns must be fully addressed and that acceptance of the manuscript would entail a second round of review. With regard to the mode by which membrane tethered LGR4/5 affect R-spondin signaling we would not require you to provide a full analysis, but it would significantly strengthen the study if you could devise experiments that would give first clues as to which of the proposed mechanisms account for this interesting observation.

I should also remind you that it is EMBO reports policy to allow a single round of revision only and that therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. I do realize that you are seeking rapid publication of your work and I would thus also understand if you decided to submit your manuscript to another journal.

Should you decide to embark on such a revision, revised manuscripts should be submitted within roughly three months; they will otherwise be treated as new submissions, except under exceptional circumstances in which a short extension is obtained from the editor. Also, the length of the revised manuscript should not exceed roughly 29,000 characters (including spaces). Shortening can, for example, be achieved by combining the results and discussion section as this will avoid redundancies. I would also kindly ask you to move one of the currently 6 figures to the supplementary information, as we can only have five main figures.

I should also point out that we recently changed our reference style to a number-based one. I am sorry for having to ask you to do this, but could you please change the style before submitting your revised manuscript? This will also free up space to incorporate a more detailed M&M section. Details and the relevant end-note file can be found here:

http://www.nature.com/embor/about/authors.html#refformat

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We also welcome the submission of cover suggestions or motifs that might be used by our Graphics Illustrator in designing a cover.

I look forward to seeing a revised form of your manuscript when it is ready. Should you choose to submit your paper elsewhere, I would welcome a message to this effect.

REFEREE REPORTS:

Referee #1:

This paper reports on the mechanism of action of R-spondin binding LGR4 and ZNFR3 via different motifs and reports the requirement of both LGR4/5 and ZNFR3/RNF4 for R-spondin function. Moreover, they present a dominant negative function of the extracellular domain of LGR5 and aim to characterize a RSPO1 neutralizing SOMAmer.

The paper is a follow-up of the recently published paper "ZNRF3 promotes Wnt receptor turnover in an R-spondin-sensitive manner" Nature, 2012 from the same group. The general outline of the paper supports the model that R-spondins form a complex with LGR4/5 and RNF43/ZNRF3, where LGR4/5 are the engagement receptors and RNF43/ZNRF3 are the effector receptors. These in principle interesting findings extend the data of the group's previous paper and partially complement the recently published structural analyses of R-spondins and LGR5/RNF43 receptors. However, the reader may feel a lack of coherence within the manuscript and the need of a clear thread linking the individual experiments presented in this paper. Parts of the manuscript look more like the supplementary materials of the group's Nature paper rather than an independent story, while other parts could be developed into individual manuscripts, but are yet unfinished. As it stands, the figures appear to be quite preliminary and the manuscript is not suitable for publication.

Specific comments:

1. The authors present a binding model, where the FU1 domain of RSPO1 binds to ZNRF3 and the FU2 domain to LGR4. Arg66 and Gln77 of RSPO1 have been shown to form hydrogen bonds with RNF43 in the paper by Chen et al., hence the mutation data shown by the authors match their model. Regarding the binding of LGR4, the importance of Phe110 and Phe106 is also in concordance with the structural analyses by Wang et al., however, R87A mutation did not affect binding of LGR4 in this study, while it was shown to significantly decrease binding affinity in the Wang et al. paper. This led Wang et al to the conclusion, that both FU domains are important for LGR4 binding, while the Xie et al only relate the FU2 domain to LGR4. The authors should discuss these divergent results.

2. The authors show a very interesting result by expressing the membrane-tethered LGR5, which had a dominant negative function. This would suggest a more prominent role of LGR4/5, rather than only acting as an engagement receptor for R-spondin and presenting it to ZNRF3. However, the mechanism of LGR4 for R-spondin function remains elusive in this paper.

3. Figure 1-2. It would be good to show IP and FACS experiments for mutants as in Figure 5 of the Nature paper.

4. Figure 1A; 4C. Stainings of LGR4, ZNFR and FZD4 are missing (at least it is possible to show that the cells are still there in bright field). For this experiment, condition media were used, so the expression of different mutants in the medium has to be shown. It would be better to purify RSPO1-GFP proteins, to make these experiments with different amounts of protein and to quantify the results showing at least 3 independent experiments.

5. Figure 1C. It is not clear if two different mutated constructs of R-Spondin have been administered at the same time, if double mutants have been used, or if the pictures shown are representative for each two conditions. This should be explained in the figure legend. It would be better, if the experiment would have been performed and presented with one condition per picture as in Fig. 1B.

6. Figure 2. What is the role of LGR4? The panel of mutants tested for figure 1 could be tested with this individual approach. (Q71A mutated constructs are not shown)

7. Figure 3. In table S1 there are other mutants within conserved sites that bind LGR4 and ZNRF3, but could be non-functional or have dominant negative role in Wnt signaling, which effect would they have in the reporter assay?

8. Figure 3. Upon addition of canonical Wnts (Wnt3a or Wnt1), would the mutants interfere with RSPO1 enhancement of the signal or could some of them even block the signal?

9. Figure 4 presents the interesting finding of an inhibitingaAgent (neutralizing SOMAmer - S1) for RSPO1. However, this should be studied in more detail, especially since it raises several questions. If SOMAmer acts by binding FU1 domain of RSPO1, why does it not bind and inhibit other R-Spondins, which share substantial homology and also have FU1 domains for the interaction with ZNRF3? Thus, it appears that the mechanism of SMOAmer inhibition of R-spondin 1 cannot be conclusively explained. Also the reduced binding to LGR4 argues against the hypothesis of the authors. The authors could include RSPO2 and 3 in pulldown assays with SOMAmer to demonstrate

that there is no binding. Moreover, the mutated RSPO constructs should be tested in the reporter assay with SOMAmer to confirm that the decreased binding also translates to decreased function. Furthermore, a rescue with Wnt3a after inhibition of RSPO with S1 could be done. Moreover, it would be interesting to test the effect of S1 on the proliferation of cells that are dependent on R-spondin1, cell survival of stem cells, or to show the effect in vivo for zebrafish/xenopus development. The function of SOMAmer should also be explained briefly in the text. There is now only one sentence which is however not helpful for readers that have no experience with SOMAmers.

Minor comments:

10. Figure 5. Authors used for all previous experiments LGR4 and here they are using LGR5. LGR4 appears to be missing in this experiment.

11. Figure 6. Expression levels of ZNRF3 have to be shown (qPCR/Western). Were ZNRF3 and ZNRF3H102A/P103A mutated at the siRNA binding site?

12. In the figure legends there is no information about the graphs, e.g. whether one representative experiment is shown (as it looks like) or if it is the average of several independent experiments with s.e.m/s.d (Figure 4A is without any error bars).

13. The reference „de et al." should be „de Lau et al."

14. Figure S1 should be better explained: what are the asterixes, the dots and the different types of triangles?

15. Figure 4C: the labeling is misleading. It should be clarified that both SOMAmer and RSP1-Fc are added to the cells.

16. Table S1: Q71Q should be Q71A?

Referee #2:

In this study, Xie et al. take a mutational approach to gain understanding on how R-spondin (RSPO) functionally interacts with LGR4/5 and ZNRF3/RNF43. The ZNRF3/RNF43 membrane E3 ubiquitin ligases were shown previously to inhibit Wnt signaling by inducing Wnt receptor endocytosis and turnover. The protein-protein interactions that underlie the mechanism by which RSPO signaling prevents the activity of ZNRF3/RNF43 are the subject of this study. The authors identify two regions in the RSPO protein FU2 and FU1 domains which mediate binding to LGR4/5 and ZNRF3/RNF43, respectively. A functional comparison of various binding mutants of RSPO, LGR4/5 and ZNRF3 supports the conclusion that LGR4/5 primarily serves as an engagement receptor which promotes the RSPO-ZNRF3 interaction, while ZNRF3/RNF43 functions as an effector receptor. Complex formation of RSPO, ZNRF3/RNF43 and LGR4/5 prevents the inhibitory activity of ZNRF3/RNF43 and potentiates Wnt pathway activation.
This is an important and timely study which complements the recently published structure of RSPO, LGR5 and RNF43 (Chen et al., Genes and Development 2013) by performing a functional analysis of the identified binding interfaces.

A few issues however still need to be addressed:

1)Strikingly, the measured affinity between ZNFR3 ECD and RSPO1 (0.8 μM) differs one order of magnitude from the affinity of RNF43 and RSPO1 (7-10 μM) presented in Chen et al. Genes & Dev 2013. This discrepancy needs to be discussed. Purity of the used recombinant proteins should be mentioned.
2)Wnt reporter assays are performed using the stably transfected STF cell line which carries the super TOPflash plasmid. Did the authors include a control for non-specific effects on luciferase activity in their experiments, e.g. the FOPflash reporter which carries mutated TCF binding sites?
3)Page 8, line 3: The conclusion that "R-spondin needs to interact with both LGR4 and ZNRF3 to be

functional" is not fully justified at this point. The preceding results would allow to conclude that both binding motifs for LGR4 and ZNRF3 on RSPO1 are required for signaling. The presented STF assays however do not rule out participation of other partners that may bind these motifs.
4)As mentioned at various places throughout the Ms., RSPO proteins are established as potent enhancers of Wnt signaling. In Figs 4, 5 and 6, however, RSPO treatment is done in the absence of Wnt, which generally leads to very weak reporter activity. It will be important to test the prediction that the synergistic effect of RSPO on Wnt treatment depends on its interaction with LGR4/5 and ZNRF3/RNF43. Thus, in Fig. 4 and 5, somatomer treatment or LGR5 ECD-TM expression are both expected to bring down RSPO/Wnt-mediated reporter activation to the levels of Wnt alone.
5)The legend and description of Fig 6 is insufficiently detailed to fully understand the experimental conditions. In lane 3 and 4: why is RSPO treatment not as effective as RSPO treatment of ZNRF3 knockdown cells? What would be limiting? In addition, is the H102A/P103A ZNRF3 mutant expressed at the plasma membrane to allow binding with RSPO? Mutation-induced misfolding may lead to ER retention of the protein and thus provide an alternative explanation for the observed RSPO-insensitive phenotype.

Minor errors that need correction:
1)Page 2, line 19: the 'engagement of receptor' should be the 'engagement receptor'
2)Page 9, line 6: 'chrorionic' should be 'chorionic'
3)Page 9, line 9: 'We tested whether that'
4)Page 11, line 10: ZNRF3 'P102A/P103A' should be ZNRF3 'H102A/P103A'
5)Page 16, line 15: 'soup' should be 'supernatant'
6)References of the Clevers lab are lacking the first author name (de Lau et al.), this needs to be corrected
7)Legend for Fig S3 is missing

Referee #3:

This group has recently described a mechanism whereby R-Spondin proteins activate wnt signalling through inhibition of E3 ligase ZNRF4/RNF43. It was also shown that R-Spondin binds to both LGR4 and ZNFR3. Through mutational analysis they show here that R-Spondin uses different furin domains to bind to either LGR4 and ZNFR3 and that R-Spondin mutants of either interaction abolishes R-Spondin signalling activity in TCF/b-catenin dependent reporter assays. Because binding of R-Spondin to ZNFR3 appears to occur with much lower affinity than its binding to LGR4 they propose a model whereby LGR4 acts as an engagement receptor to catch R-Spondin with high affinity thereby promoting its medium-affinity interaction with ZNFR3 which mediates furher downstream effects. Data are in line with a recent publication in Genes and Development by Chen et al. reporting the crystal structure of R-spondin in complex with its receptors because critical residues identified by mutation in the current paper are involved in direct interactions in the structure. In addition, affinity differences between R-Spondin-ZNFR3 and R-Spondin-LGR4 reported here are also shown in the Genes Dev. paper.

General evaluation

As the Genes Dev paper does not report a functional analysis of critical residues, the present manuscript, in particular Figs. 1-3, nicely supplements and confirms the structural study.

Other results are less conclusive or are only loosely connected to the main findings. In Fig. 4 an aptamer-like DNA molecule called SOMAmer is shown to block binding of R-Spondin to ZNRF3. R-Spondin mutants that do not interact with ZNFR3 also do not interact with the SOMAmer, suggesting that these amino acids are important for inhibition by SOMAmer. However, the SOMAmer also blocks interaction R-Spondin with LGR4 but its interaction with R-Spondin is not affected by the specific LGR4 binding mutations of R-Spondin. Thus this experiment is not very conclusive and the purpose of showing it here was not getting clear.

Likewise, in Fig. 5 it is shown that tethering LGR4 to the plasma membrane in the absence of its membrane-spanning and cytoplasmic domains blocks R-Spondin action. The mechanism is not clear

and the main conclusion is that cytoplasmic parts of LGR4 might be required for its function.

Results in Fig. 5 are more interesting because they show that a ZNFR3 mutant incapable of R-Spondin binding blocks Wnt signalling as good as the wild-type protein but cannot be blocked anymore by R-Spondin, supporting that ZNFR3 is a functionally relevant receptor for R-Spondin.

Altogether the merits of the paper lie in the conformation of critical residues from the published crystal structure in functional and binding studies, and in the functional demonstration that both receptors are required for R-Spondin signalling. This is quite clear-cut and there is little place for improvement. In fact the Chen et al. paper answers several critical question that could have been raised here such as whether a triple complex of ZNRF3-R-Spondin-LGR4 can develop, and whether LGR receptors helps in the binding of R-Spondin to ZNRF3.

Specific suggestions
Although space is limited, the authors should give a bit more details about the R-Spondin-ZNFR3-LGR-Frizzled-LRP connection in the introduction from the previous Hao et al 2012 publication.

There are several mistakes in the ms:

Table S1 Q71Q should read Q71A
p. 11: ZNRF3H102A vs. ZNFR3P102A

1st Revision - authors' response 12 September 2013

We are submitting the revised version of our manuscript entitled “Interaction with both ZNRF3 and LGR4 is required for the signaling activity of R-spondin” for consideration for publication in *EMBO Reports*.

We are pleased to see that all reviewers are generally positive on the manuscript. Reviewer #2 indicated that “This is an important and timely study which complements the recently published structure of RSPO, LGR5 and RNF43”. Review #3 indicated that our conclusion that both LGR4 and ZNRF3 are required for R-spondin signaling “is quite clear-cut and there is little place for improvement”. Reviewers also gave us many constructive suggestions to improve the manuscript. Major complains are around LGR5 ECD-TM data and SOMAmer data, as such results are only loosely connected to the rest of the manuscript, and can be distracting. We greatly appreciate these insightful comments and suggestions.

In this revised manuscript, we have made all necessary adjustments to address reviewers’ comments/suggestions. We agree with reviewers that LGR5 ECD-TM data and SOMAmer data are not necessary for the main message of the manuscript. We have removed LGR5 ECD-TM data (see detailed response later). We have revised the SOMAmer section so the purpose of SOMAmer experiment is clearer to readers. We have performed several experiments to further support the main conclusion, so the manuscript is stronger and more focused. Such experiments include demonstrating ZNRF3 and LGR4 binding motifs are both required for RSPO1-induced association of ZNRF3 and LGR4, ZNRF3 and LGR4 binding motifs are both required for RSPO1-induced membrane clearance of ZNRF3, and RSPO1 mutant deficient of ZNRF3 binding has dominant negative activity. We have also performed many control experiments suggested by reviewers, including performing RSPO1 STF reporter assay in the presence of exogenous Wnt3a.

The main message of our paper is that R-spondin needs to interact with both ZNRF3 and LGR4 to be functional AND that ZNRF3 is the major target of R-spondin. This is a very important message and it has never been formally demonstrated before. Beyond LGR4/5 and ZNRF3/RNF43, R-spondin has been reported to bind to several other proteins. The relative contribution of these R-spondin binding proteins to the signaling activity of R-spondin is not clear. Although the crystal structure of RSPO1-RNF43-LGR5 complex has been solved, it does not mean this complex is actually functionally important. It is conceivable that RSPO1, once bound to LGR4/5, can have low affinity interactions with many other proteins, including LRP6, and such interactions are more

important than R-spondin-ZNRF3 interaction. Although we have shown that RSPO1 R66A/Q71A mutant fails to bind to ZNRF3 and has defective signaling activity, it does not prove ZNRF3 interaction is required for R-spondin signaling. Considering that FU1 domain is small, it is quite possible that these mutations disrupt another critical interaction to be identified. To counter this difficult, but important, argument, we have focused on ZNRF3 itself. We have shown that ZNRF3 H102A/P103A mutant, which does not bind to R-spondin, has the same membrane expression as the wild-type protein and inhibits Wnt signaling in ZNRF3 null background just like the wild-type protein. However, cells expressing ZNRF3 H102A/P103A are resistant to the Wnt-potentiating activity of R-spondin (old Fig. 6, now Fig. 5), strongly suggesting that ZNRF3 is the major target of R-spondin. We have done this experiment multiple times, and results are highly consistent. In our mind, this is a defining experiment and represents the most important experiment of our manuscript. It has answered a critical question that neither published structure studies nor our previous work could answer. There are many competing models on the signaling mechanism of R-spondin. In recent reviews of R-spondin, these models are still listed side-by-side, which is confusing to readers. We feel that our current study represents the most rigorous functional study to demonstrate ZNRF3 as the major target of R-spondin, and this conclusion is important for the Wnt field.

We have also shortened the manuscript as suggested by the editor. We hope that our revised manuscript is now suitable for publication in *EMBO Reports*. Should any questions arise, please do not hesitate to contact me.

Detailed responses to reviewers' comments.

Reviewer #1

1. The authors present a binding model, where the FU1 domain of RSPO1 binds to ZNRF3 and the FU2 domain to LGR4. Arg66 and Gln77 of RSPO1 have been shown to form hydrogen bonds with RNF43 in the paper by Chen et al., hence the mutation data shown by the authors match their model. Regarding the binding of LGR4, the importance of Phe110 and Phe106 is also in concordance with the structural analyses by Wang et al., however, R87A mutation did not affect binding of LGR4 in this study, while it was shown to significantly decrease binding affinity in the Wang et al. paper. This led Wang et al to the conclusion, that both FU domains are important for LGR4 binding, while the Xie et al only relate the FU2 domain to LGR4. The authors should discuss these divergent results.

We have also noted the difference. We feel that FU2 is the major LGR4 binding site of RSPO1 although FU1 might play a minor role. We did not observe a contribution of FU1 domain in LGR4 binding. This is likely due to different assay formats and assay sensitivities. We performed the binding experiments using cell-based binding assay. It is possible that the expression of endogenous ZNRF3, which binds to FU1, or proteoglycans, which bind to the TSP domain, contribute to the difference. We have discussed this in the discussion section.

2. The authors show a very interesting result by expressing the membrane-tethered LGR5, which had a dominant negative function. This would suggest a more prominent role of LGR4/5, rather than only acting as an engagement receptor for R-spondin and presenting it to ZNRF3. However, the mechanism of LGR4 for R-spondin function remains elusive in this paper.

Indeed, this is an interesting observation, which suggests that LGR4 has an active signaling role. We have actually spent a huge amount time to understand the 'active' signaling role of LGR4, as elucidation of this mechanism should lead to a major publication. Multiple hypotheses have been examined and eventually all of them have been de-validated. Unfortunately, we don't have any clue at this time, and we do not think we will make any progress on this in the near future. We initially put this data into the manuscript, feeling that this is an important result that the field should know. On the other hand, this figure represents a loose end and would distract readers from the main message, as the reviewer correctly pointed out. We have now decided to remove this figure so the manuscript is more concise and focused. If the reviewer thinks that we should still include this result, we can certainly do so.

3. Figure 1-2. It would be good to show IP and FACS experiments for mutants as in Figure 5 of the Nature paper.
We would thank the reviewer for this excellent suggestion. We have now shown that ZNRF3 and LGR4 binding motifs are required for RSPO1-induced association of ZNRF3 and LGR4 (Fig. 3D). Also note that wild-type RSPO1 and F106A/F110A mutant, but not R66A/Q71A mutant, were co-immunoprecipitated with ZNRF3 (Fig. 3D), consistent with results from cell-based binding assay. Following reviewer's suggestion, we have further shown that unlike wild-type RSPO1, neither R66A/Q71A nor F106A/F110A mutant reduced the membrane level of ZNRF3 in a FACS assay (Fig. 3E). Together, these results suggest that both LGR4 and ZNRF3 binding motifs are required for RSPO1-induced LGR4/ZNRF3 interaction and downregulation of ZNRF3 on the cell surface.

4. Figure 1A; 4C. Stainings of LGR4, ZNFR and FZD4 are missing (at least it is possible to show that the cells are still there in bright field). For this experiment, condition media were used, so the expression of different mutants in the medium has to be shown. It would be better to purify RSPO1-GFP proteins, to make these experiments with different amounts of protein and to quantify the results showing at least 3 independent experiments.

1. We did fluorescence-based quantification to make sure the same amount of RSPO1-GFP mutants were used in binding assays and reporter assays. Following reviewer's suggestion, we have performed western blot assay to show that the same amount of RSPO1-GFP was used (Fig. 1C). We have performed the cell-based binding assay using these mutants multiple times, and results are highly reproducible. However, we would also stress that results from the cell-based binding assay are more qualitative than quantitative, subtle differences will be missed. For this reason, we have only focused on key mutants that gave us clean-cut phenotypes. Please also note that our cell-based binding data is also supported by SPR assay (Fig. 2) and co-immunoprecipitation assay (Fig. 3D).

2. Cells are certainly there in images without strong RSPO1-GFP staining. Outlines of cells caused by non-specific bindings (or background bindings) can be clearly seen in many panels, for example WT/ FZD4, R66A/ZNRF3 ECD-TM of Fig. 1B. RSPO1-GFP has fairly strong background binding to empty vector transfected cells, which we don't see with DKK1-GFP. We speculate that this binding is mediated by the TSP domain of RSPO1, which binds to proteoglycans. It is very easy to distinguish specific binding from non-specific binding as they have different patterns. The signal intensity of non-specific binding is low and is even across all cells. On the hand, signal from specific binding is not even because LGR4 or ZNRF3 was transiently transfected into cells and their expression is not even. We have included a few large pictures for reviewer's eye (Fig. 1R).

5. Figure 1C. It is not clear if two different mutated constructs of R-Spondin have been administered at the same time, if double mutants have been used, or if the pictures shown are representative for each two conditions. This should be explained in the figure legend. It would be better, if the experiment would have been performed and presented with one condition per picture as in Fig. 1B.
These are double mutants. We have made it clear in the figure legends.

6. Figure 2. What is the role of LGR4? The panel of mutants tested for figure 1 could be tested with this individual approach. (Q71A mutated constructs are not shown)
Unfortunately, we could not produce recombinant LGR4 ECD proteins with good quality. In our hand, LGR4 ECD proteins are very sticky and precipitate easily. For this reason, we could not test the direct binding between RSPO1 mutants and LGR4. It should be noted that the binding between R-spondin and LGR4 and the critical role of LGR4 in R-spondin signaling are well accepted in the field. The focus of this manuscript is to demonstrate ZNRF3 as the major target of R-spondin. The intention of Fig. 2 is to demonstrate a specific binding between RSPO1 and ZNRF3 in a direct binding assay, as single amino acid mutation in either R-spondin or ZNRF3 significantly decreased this binding. We did this experiment in collaboration with a biophysical lab, which has many different requests. Testing more RSPO1 mutants in this experiment would take us a long time but would not add much to the current story.

7. Figure 3. In table S1 there are other mutants within conserved sites that bind LGR4 and ZNRF3, but could be non-functional or have dominant negative role in Wnt signaling, which effect would they have in the reporter assay?
Indeed, these mutants could be non-functional or have dominant negative role in Wnt signaling. However, interpretation of such data would be extremely difficult and it would not contribute to the

main message of the manuscript. R-spondin likely induces a conformation change of LGR4 and ZNRF3 once it binds to its receptors. A mutant that binds to both LGR4 and ZNRF3 might not be functional because it would not cause a functional conformation change of its receptors. It is difficult to predict such effects. For this reason, we have only focused on a set of key mutants to make our points.

8. Figure 3. Upon addition of canonical Wnts (Wnt3a or Wnt1), would the mutants interfere with RSPO1 enhancement of the signal or could some of them even block the signal?
We performed the experiment suggested by the reviewer. R-spondin strongly increases Wnt signaling in HEK293 in the absence of Wnt conditioned medium. This activity can be completely blocked by addition of porcupine inhibitor (see Fig. 2 for reviewer). HEK293 cells actually express a significant amount of Wnt proteins. However, there is no obvious basal STF activity in HEK293 cells because endogenous ZNRF3 strongly suppresses Wnt signaling through downregulating Wnt receptors in these cells. For this reason, the function of R-spondin can be tested without exogenous Wnt in HEK293 cells. Following reviewer's suggestion, we tested the function of R-spondin mutants in the absence or the presence of low dose of Wnt3a CM and results are consistent (Fig. 3A, 3B, 3C). We tested potential dominant negative activity of R-spondin mutant in the absence of Wnt3a CM. We treated HEK293-STF cells with wild-type RSPO1 in the absence or the presence of 10-fold excess of RSPO1 R66A/Q71A, F106A/F110A, or R66A/Q71A/F106A/F110A mutant (Fi. 3F). We observed that RSPO1 R66A/Q71A mutant, which binds to LGR4 but not ZNRF3, reproducibly decreased Wnt-promoting activity of wild-type RSPO1 (Fig. 3F). R66A/Q71A mutant most likely exerts its dominant negative activity through affecting the interaction between wild-type RSPO1 and LGR4. Consistent with this hypothesis, R66A/Q71A/F106A/F110A mutant, which would not bind to LRG4, lost this dominant negative activity (Fig. 3F). Interestingly, RSPO1 F106A/F110A mutant, which binds to ZNRF3 but not LGR4, showed minimal dominant activity in this assay condition. This is consistent with the observation that RSPO1-ZNRF3 affinity is much low than RSPO1-LGR4/5 affinity. Presumably, LGR4-bound RSPO1 would have easier access to ZNRF3, rendering it resistant to the dominant activity of RSPO1 F106A/F110A. Together, these results provide further support to our main conclusion. We thank the reviewer for this excellent suggestion.

9. Figure 4 presents the interesting finding of an inhibitingaAgent (neutralizing SOMAmer - S1) for RSPO1. However, this should be studied in more detail, especially since it raises several questions. If SOMAmer acts by binding FU1 domain of RSPO1, why does it not bind and inhibit other R-Spondins, which share substantial homology and also have FU1 domains for the interaction with ZNRF3? Thus, it appears that the mechanism of SMOAmer inhibition of R-spondin 1 cannot be conclusively explained. Also the reduced binding to LGR4 argues against the hypothesis of the authors. The authors could include RSPO2 and 3 in pulldown assays with SOMAmer to demonstrate that there is no binding. Moreover, the mutated RSPO constructs should be tested in the reporter assay with SOMAmer to confirm that the decreased binding also translates to decreased function. Furthermore, a rescue with Wnt3a after inhibition of RSPO with S1 could be done. Moreover, it would be interesting to test the effect of S1 on the proliferation of cells that are dependent on R-spondin1, cell survival of stem cells, or to show the effect in vivo for zebrafish/xenopus development. The function of SOMAmer should also be explained briefly in the text. There is now only one sentence which is however not helpful for readers that have no experience with SOMAmers.

1. FU1 domain of RSPO1 and FU1 domain of RSPO3 only share 59% identity, which is not very high. SOMAmers were generated through multiple rounds of affinity maturation, which can achieve an extremely level of specificity. We only consider cross activity when proteins have over 70% identity. In practice, we have generated highly specific SOMAmers that can distinguish proteins with 98% identity without cross-panning. Inability of RSPO1 SOMAmer to inhibit RSPO3-induced STF reporter is consistent with the finding that RSPO1 SOMAmer has no specific binding to RSPO3 (now shown in Fig. S3). The finding that SOMAmer S1 blocks RSPO1-LGR4 binding is actually not inconsistent with our hypothesis. The size of SOMAmer is 18KD while the size of FU1-FU2 domain combined is only 11KD. It is possible that SOMAmer blocks RSPO1-LGR4 interaction through creating steric hindrance or induce a conformation change.

2. We could not test RSPO1 mutants together with RSPO1 SOMAmer. Since RSPO1 mutants have drastically decreased Wnt stimulatory activity, there is nothing to be inhibited.

3. RSPO1 and RSPO3 data showing in Fig. 4A was actually done in the presence of 5% Wnt3a CM. A lower dose of RSPO1 or RSPO3 is needed when exogenous Wnt is present, so only a low dose of SOMAmer is needed to block the signaling activity of RSPO. We apologize this was

not clear in the early version. We have made this clear in the figure legends. Also note that R-spondin SOMAmer did not suppressed STF activity induced by 50% Wnt3a, suggesting that the activity is specific. In the previous version, we showed normalized data. In the revised manuscript, we have redrawn Fig. 4A and shown the non-normalized data.

4. We agree with the reviewer that testing RSPO1 SOMAmer in stem cell assays or xenopus assays would be interesting. Unfortunately, SOMAmers carry significant negative charges, and long term treatment of cells can be toxic. For this reason, SOMAmers have mostly been used in in vitro biomarker studies, and not used for in vitro or in vivo functional studies like antibodies. Note that Fig. 4A was done using overnight treatment, so the toxicity is minimal. Nevertheless, to satisfy the reviewer, we tested RSPO1 SOMAmer and control SOMAmer in RSPO1-dependent intestinal organoid assay. Unfortunately, SOMAmers have significant non-specific toxicity in this assay as we predicted and this prevents us from drawing any conclusion. Although demonstrating the function of RSPO1 SOMAmer in a more physiological assay is nice, it does not contribute to the main message of our manuscript.

5. We have added more introduction of SOMAmer as suggested by the reviewer.

Minor comments:

10. Figure 5. Authors used for all previous experiments LGR4 and here they are using LGR5. LGR4 appears to be missing in this experiment.
We did not use LGR4 ECD-TM because it does not translocate to the plasma membrane. Note that this figure has been removed.

11. Figure 6. Expression levels of ZNRF3 have to be shown (qPCR/Western). Were ZNRF3 and ZNRF3H102A/P103A mutated at the siRNA binding site?
We have now shown that wild-type ZNRF3 and ZNRF3 H102A/P103A are expressed at the same level on the cell surface and in the total cell lysates by Western blot (shown as Fig. 5B). Indeed, these constructs carry silent mutations at the siRNA binding site and their expression is not affected by ZNRF3 siRNA. We have made this clear in the figure legends.

12. In the figure legends there is no information about the graphs, e.g. whether one representative experiment is shown (as it looks like) or if it is the average of several independent experiments with s.e.m/s.d (Figure 4A is without any error bars).
This has been corrected.

13. The reference "de et al." should be "de Lau et al."
This has been corrected.

14. Figure S1 should be better explained: what are the asterixes, the dots and the different types of triangles?
We have now explained this in the figure legends. The asterisks indicate identical amino acid residues, the dots indicate homologous amino acid residues, the filled triangles indicate two residues critical for ZNRF3 binding in the FU1 domain, and the non-filled triangles indicate two residues critical for LGR4 binding in the FU2 domain.

15. Figure 4C: the labeling is misleading. It should be clarified that both SOMAmer and RSP1-Fc are added to the cells.
This has been fixed.

16. Table S1: Q71Q should be Q71A?
This has been fixed.

Reviewer #2
1)Strikingly, the measured affinity between ZNFR3 ECD and RSPO1 (0.8 µM) differs one order of magnitude from the affinity of RNF43 and RSPO1 (7-10 µM) presented in Chen et al. Genes & Dev 2013. This discrepancy needs to be discussed. Purity of the used recombinant proteins should be mentioned.

We also noticed this difference. The discrepancy is likely due to different proteins used (ZNRF3 vs RNF43), different methods of measurement (SPR vs ITC), or both. This is now discussed in page 14. The purity of our proteins is over 95% (mentioned in page 6)

2)Wnt reporter assays are performed using the stably transfected STF cell line which carries the super TOPflash plasmid. Did the authors include a control for non-specific effects on luciferase activity in their experiments, e.g. the FOPflash reporter which carries mutated TCF binding sites?
We did not use super Super FOPflash as a control. STF reporter has been extensively examined by us and many other labs, and it is very specific. In general, we prefer not to use Super FOPflash as control. Even something nonspecifically decreases STF reporter would not necessarily decrease Super FOPflash, as the activity of Super FOPflash is exceedingly low. We prefer to use alternative stimuli to demonstrate the specificity of the assay. For example, we have shown that RSPO1 SOMAmer decreased RSPO1-induced STF, but not RSPO3 or 50% Wnt3a-induced STF.

3)Page 8, line 3: The conclusion that "R-spondin needs to interact with both LGR4 and ZNRF3 to be functional" is not fully justified at this point. The preceding results would allow to conclude that both binding motifs for LGR4 and ZNRF3 on RSPO1 are required for signaling. The presented STF assays however do not rule out participation of other partners that may bind these motifs.
Agree. We have changed the conclusion to "Taken together, these results suggest that both LGR4 and ZNRF3 binding motifs are required for the ability of RSPO1 of inhibiting ZNRF3 and promoting Wnt signaling."

4)As mentioned at various places throughout the Ms., RSPO proteins are established as potent enhancers of Wnt signaling. In Figs 4, 5 and 6, however, RSPO treatment is done in the absence of Wnt, which generally leads to very weak reporter activity. It will be important to test the prediction that the synergistic effect of RSPO on Wnt treatment depends on its interaction with LGR4/5 and ZNRF3/RNF43. Thus, in Fig. 4 and 5, somatomer treatment or LGR5 ECD-TM expression are both expected to bring down RSPO/Wnt-mediated reporter activation to the levels of Wnt alone.
5% Wnt3a CM was actually used together with RSPO1 or RSPO3 in Fig. 4A. 5% Wnt3a was added in all wells in Fig. 6A. We apologize that we did not make this clear in the previous version. This has been now clarified in figure legends. Indeed, RSPO1 SOMAmer can only inhibit STF to the level of 5% Wnt3a alone. We have replotted Fig. 4A to make it clear. Fig. 5 has now been removed.

5)The legend and description of Fig 6 is insufficiently detailed to fully understand the experimental conditions. In lane 3 and 4: why is RSPO treatment not as effective as RSPO treatment of ZNRF3 knockdown cells? What would be limiting? In addition, is the H102A/P103A ZNRF3 mutant expressed at the plasma membrane to allow binding with RSPO? Mutation-induced misfolding may lead to ER retention of the protein and thus provide an alternative explanation for the observed RSPO-insensitive phenotype.

1. We have added detailed description in the legends of Fig. 6: "HEK293 cells were first transfected with control pGL2 siRNA or ZNRF3 siRNA to eliminate endogenous ZNRF3. Twenty four hours after siRNA transfection, cells were transfected with empty vector (EV), Myc-ZNRF3 WT, or Myc-ZNRF3 H102A/ P103A expression plasmid. Twenty four hours after plasmid transfection, all wells were treated with 5% Wnt3a and selected wells were treated with 200ng/ml of RSPO2 protein. STF luciferase activity was measured 24 hours later. ZNRF3 WT and ZNRF3 H102A/P103A carry silent mutations at the siRNA binding site and their expression is not affected by ZNRF3 siRNA."

2. We think that R-spondin cannot completely inhibit endogenous ZNRF3 and RNF43 in the condition that we used. This could be due to the dose of R-spondin used or the mechanism involved (for example, R-spondin cannot completely abolish membrane expression of ZNRF3). The exact limiting factor is still not clear. In addition, ZNRF3 siRNA would not completely eliminate the expression of endogenous ZNRF3, and there could be a minor role of RNF43 in HEK293 cells. This would explain why R-spondin can further increase STF reporter in cells treated with ZNRF3 siRNA.

3. We have now shown that ZNRF3 H102A/P103A is expressed at the same level as the wild-type protein on the plasma membrane (determined by membrane biotinylation assay) or in the total cell lysates (shown as Fig. 5B). Therefore, different activities of wild-type ZNRF3 and ZNRF3 H102A/P103A in response to R-spondin cannot be explained by altered localization of ZNRF3 mutant.

Minor errors that need correction:
1)Page 2, line 19: the 'engagement of receptor' should be the 'engagement receptor'
This has been fixed.

2)Page 9, line 6: 'chrorionic' should be 'chorionic'
The LGR5 ECD-TM section has been eliminated.

3)Page 9, line 9: 'We tested whether that'
The LGR5 ECD-TM section has been eliminated.

4)Page 11, line 10: ZNRF3 'P102A/P103A' should be ZNRF3 'H102A/P103A'
This has been fixed.

5)Page 16, line 15: 'soup' should be 'supernatant'
This has been fixed.

6)References of the Clevers lab are lacking the first author name (de Lau et al.), this needs to be corrected
This has been fixed.

7)Legend for Fig S3 is missing
This has been fixed.

Reviewer #3

Reviewer appreciates the importance of our work, but complained about LGR5 ECD-TM data and SOMAmer data as they are not central to our conclusion.

We agree with the reviewer that LGR5 ECD-TM data as it is not related to our main message. We have removed this figure and added additional experiments related to our main conclusion, so our revised manuscript is more focused and stronger.

We think that SOMAmer data is relevant, although not central, to our main conclusion. It is interesting that SOMAmer S1 has reduced interaction to R66A/Q71A mutant, suggesting that the SOMAmer might directly bind to ZNRF3binding motif of RSPO1. In agreement with this, SOMAmer S1 strongly blocked RSPO1-ZNRF3 interaction. The fact that SOMAmer S1 also blocked RSPO1-LGR4 interaction is not surprising, considering that SOMAmer is 18 KD, while FU1-FU2 is only 11KD. SOMAmer S1potentially blocks RSPO1-LGR4 interaction through creating steric hindrance or inducing a conformation change of RSPO1. We have revised the SOMAmer section, so the purpose of this section is clearer to readers. We prefer to keep SOMAer data, but we can remove it if reviewer insists.

Regarding the significance of the manuscript, we would like to stress that showing the critical structure of RSPO1-RNF43-LGR5 does not mean this complex is functionally important. Although R66A/Q71A mutant has reduced ZNRF3 interaction and reduced Wnt-promoting activity, it does not mean ZNRF3 is functionally important. It is possible that these mutations affected another critical interaction. R-spondin has many proposed interactors, but relative contribution of these interactors to R-spondin signaling is not clear. One can argue that R-spondin has low affinity interactions with multiple proteins, and ZNRF3 only plays a minor role or is not relevant at all. Our H102A/P103A data clearly demonstrates that ZNRF3 is the major target of R-spondin. This experiment answered a question that published structure studies or our previous study could not answer.

Specific suggestions
Although space is limited, the authors should give a bit more details about the R-Spondin-ZNFR3-LGR-Frizzled-LRP connection in the introduction from the previous Hao et al 2012 publication.

We have added induction on this connection as suggested.

There are several mistakes in the ms:

Table S1 Q71Q should read Q71A
This has been corrected.

p. 11: ZNRF3H102A vs. ZNFR3P102A
This has been corrected.

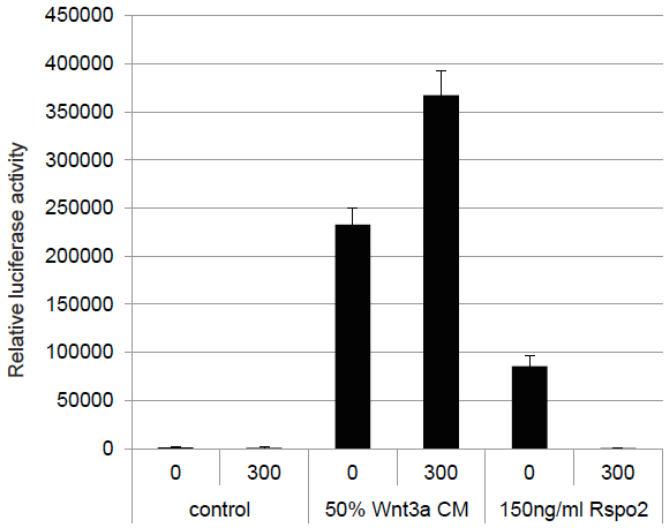

Figure R1 The activity of R-spondin on STF reporter in HEK293 cells is dependent on endogenous Wnt.
HEK293-STF cells were treated with DMSO or 300 nM LGK974 (porcupine inhibitor), together with control conditioned medium, 50% Wnt3a CM, or 150ng/ml RSPO2. STF luciferase activity was measured 24 hours after treatment.

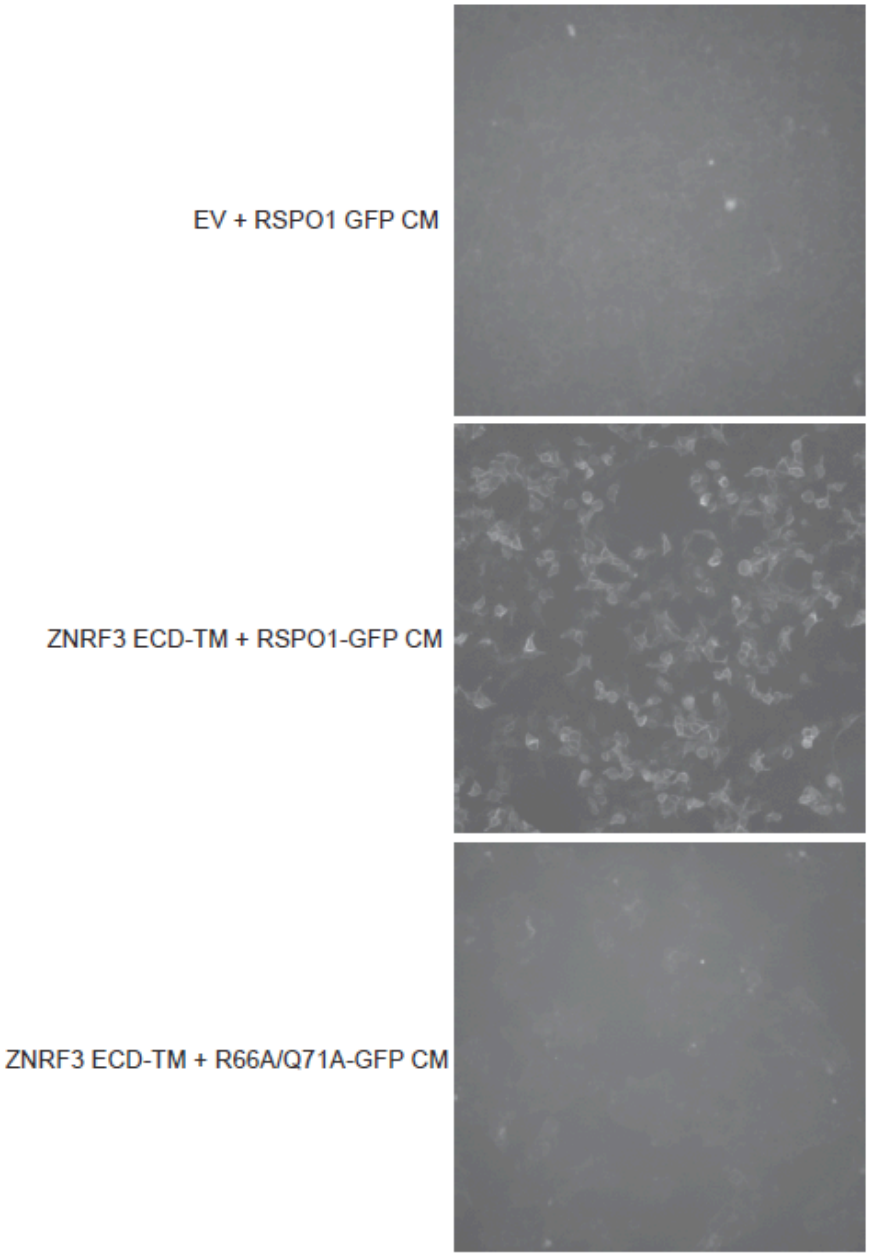

Figure R2 Background staining of RSPO1-GFP.
HEK293 cells were treated with Empty vector (EV) or ZNRF3 ECD-TM, incubated with RSPO1-GFP or RSPO1 R66A/Q71A-GFP CM, and imaged. Dead round-up cells have increased autofluorescence.

2nd Editorial Decision 30 September 2013

Thank you for your patience while we have reviewed your revised manuscript. As you will see from the reports below, the two referees who were asked to review it are now 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 a few minor issues/corrections have been addressed, as follows:

Please specify the statistical tests used in the study and indicate throughout the manuscript how many independent times each experiment has been performed. Please also note that error bars should only be shown if an experiment has been repeated at least three times independently (biological, not technical, replicates).

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 REPORTS:

Referee #2 (Report):

The authors have addressed my questions satisfactorily. The manuscript is improved and in my opinion worthy of publication.

Referee #3 (Report):

The manuscript has clearly profited from the revision. It is much more focused now and includes additional data in fig. 3 that further substantiate the model drawn by the authors and provide functional and biochemical support for the crystal structure published by others.

2nd Revision - authors' response 01 October 2013

We are very happy that both reviewers are positive about our revised manuscript.

I have modified the manuscript as you requested. In relevant sections of figure legends, we have indicated that data are representative of three independent experiments. We have used Student's t-test for statistical analysis. A difference is only considered significant if P is smaller than 0.01. The smallest difference we described in this manuscript is in Fig 3F (EV vs R66A/Q71A). I have added an asterisk symbol in this figure with description in figure legends ($P<0.01$). Differences shown in other figures are much bigger and they all easily pass t-test. Figures will look very ugly if we add asterisks to highlight all relevant comparisons in all figures. For this reason, I chose to add "statistical analysis" section in supplementary materials, indicating that Student's t-tests are used to determine statistical significance and it is only considered significant when P is smaller than 0.01. Please let me know whether this is fine with you.

I have attached revised manuscript, supplementary materials and Fig. 3. Many thanks for your help. Please let me know if you have any question.

3rd Editorial Decision 01 October 2013

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

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. As you are aware, this File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

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

