nature portfolio

Peer Review File

Structural insights into the LGR4-RSPO2-ZNRF3 complexes regulating WNT/β-catenin signaling

Corresponding Author: Professor Yong Geng

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this manuscript, the authors characterized the structure of multiple protein complexes with stoichiometry variability involving LGR4, RSPO2, ZNRF3. To do so the authors accomplished a significant work of protein design, protein purification optimization that deserve to be highlighted. We appreciate the originality of the work that is significantly different from GPCRs-G protein structures routinely published nowadays. The work is of great interest since it highlights new structural features and establishes a rational to understand different complexes stoichiometry in a physiological context explaining LGR4 Wnt signaling regulation. The complex of LGR4, RSPO2, ZNRF3 provides indeed novel structural insight into the functional consequences of RSPO action on WNT signaling by complexation of the ZNRF3 E3 ligases.

We have minor comments that need to be addressed to improve the manuscript.
On the technical aspect (Cryo-EM): Density maps are consistent for the overall resolutions reported. The authors are careful in terms of interpretation and don't overinterpret the data, which is very much appreciated.

1. Final subsets of particles are quite large while the different reconstruction features some apparent dynamic that was not sorted properly. The authors need to retake subsets from Heterotrimer (1,064,324), Pentamer A (407,148), Pentamer B (838,832) and sort it further to get rid of heterogeneity. There are multiple tools that can be appropriate, if you want to extract information on dynamics in the process, 3D variability in cluster mode represents a good strategy, Heterogeneous refinement or Ab-initio with multiple reconstructions (initial res 15, final res 6, and large minibatchs) can also be used. Obtaining a better map will also help to improve the atomic models. Regarding the Di-heterotrimer the C2 symmetry was ignored during refinement while it can significantly improve alignment during refinement and be used to duplicate particles through symmetry expansion for subsequent classification with local sampling or no alignment. Overall, we think that the processing needs to be completed with the suggested strategies.

2. For the Extended Data Fig. 4 Pentamer B, the local resolution reported in the supplementary figures is not consistent with the quality of the map and overall resolution, it looks to be ranging from 8 to 14 in the figure while it is much better based on final map inspection. Can you please correct this discrepancy?

3. The overall quality of the model could be improved to remove or reduce Ramachandran and rotamer outliers while maintaining a descent clash score. This is only a suggestion because the model quality is still acceptable, and since these changes will not alter the conclusions of the work.

On the scientific aspect:
The manuscript is very well written, well-structured and the topic is timely and novel. The methodology is sound and the conclusions are supported by the presented data. We have only very minor more cosmetic criticism:

1. Avoid the term "FZD receptors", they are WNT receptors and should be called "Frizzleds". In this context, it should be taken into consideration that not all WNT receptors are FZDs and that not all WNT receptors (for example LRP5/6, ROR1/", RYK, PTK7) are regulated by ZNRF43/RNF43. Please use G protein, not G-protein.

2. "Understanding the structural principles governing these regulatory modules is crucial for tightly controlling Wnt signaling

output." This sentence needs to be reworded: Understanding does not regulate signaling.

3. For pharmacological clarity, it should be mentioned that LGR4 is classified as a class A orphan GPCR (see https://gpcrdb.org/protein/lgr4_human/). This aspect is especially important when comparing the LGR4 conformation to other GPCRs in Ext Data Fig. 6.

4. "providing the structural platform for drug development of Wnt-driven cancers" …maybe better: "providing a structural platform…."

5. "A pair of horseshoe shaped LGR4 protomers are located at
the periphery of the complex" should be "is located"

6. Page 10, lane 20 "LGR4 induces ZNRF3 into an inactive state" – maybe simpler: "LGR4 deactivates ZNRF32" – or similar.

7. With the statement "Upon analyzing the structures of LGR4 in all complexes, it becomes apparent that LGR4 consistently adopts an inactive conformation" on page 12 lane 16/17 (also discussion page 15, last paragraph), the authors touch upon an interesting point. What does LGR4 activation mean and does this receptor follow the same activation mode as "conventional" class A GPCRs? The authors might want to elaborate a bit more on this pointing out that the active conformation of a GPCR with the TM6 swing out refers to the G protein-coupled (or at least transducer-bound) state.

8. Discussion: The paragraph starts with "The Wnt signal transduction cascade….". Here the authors should underline that RSPOs are mostly connected with amplification of the WNT/b-catenin signaling route. Given the underlying regulatory mechanism of the regulation of FZD surface expression, also b-catenin-independent but FZD-mediated signaling routes should be affected by RSPO. The simple way out here would be to limit the discussion to the WNT/b-catenin pathway. Strategically, this should be done throughout the manuscript replacing WNT signaling with WNT/b-catenin signaling.

9. Page 16, lane 7/8: "In conclusion, our study uncovers the assembly mechanism of LGR4, RSPO2, and ZNRF3 in mediating Wnt signaling." This is an overstatement (since there are not actual experiments putting the novekl srcutural insights into the framework of WNT signaling) and I suggest rewording: In conclusion, our study uncovers the assembly mechanism of LGR4, RSPO2, and ZNRF3 and provides deep mechanistic insights into the RSPO-mediated regulation of Wnt signaling……or similar. Or somehow connect to the sentence afterwards.

10. The mutational analysis of W751A, F804A and Q742K (ionic lock) are a bit lost in the manuscript. The authors state that these mutants were chosen based on their importance for protein-protein interaction or intramolecular interactions. Please show these interactions in detail. The reader is referred to Fig. 4 and Ext data Fig. 5, but essential insight is not provided. The R643-Q742 interaction is actually shown in Ext Data Fig 6. It would make it easier for the reader if the Ballesteros Weinstein nomenclature for GPCRs would be adopted throughout the manuscript. While the functional consequences of the mutations on RSPO-induced TOPFlash are obvious, it remains unclear whether indeed the impairment of protein-protein interactions or other receptor-intrinsic effects are responsible for this shift in RSPO potency.

As a technicality, we could not find any information about WNT addition. Do the authors add WNT to see the RSPO effect on TOPFLASH or do they rely on basal, endogenous WNT secretion in HEK293 cells?

11. Fig. 3c: In the TOPflash data, wt is described as "black" but that is not understandable from the bar graph. Also, the bar graph should be changed to a scatter plot. Statistics? Number of independent experiments, technical replicates? Y axis: % of maxmume should be % of maximum.

12. Fig. 4: The CRC with RSPO – it should be emphasized in the legend that the wt LGR4 datasets in all three panels are identical.

13. The authors propose a regulatory mechanism where a Di-heterotrimer triggers complex internalization and ZNRF3 inactivation. They qualify it as 'a stable complex' that triggers internalization. In this case, I would expect the other conditions to yield subpopulations of Di-heterotrimer. Nonetheless, based on the biochemistry and cryo-EM data, there is no evidence of Di-heterotrimer formation without tethering. Are there any pieces of evidence for the presence of Di-heterotrimer in other datasets, even if they do not yield high-resolution maps? If yes, highlight this point in your manuscript. Could you elaborate more on the presence of Di-heterotrimer in physiological conditions based on ref 35 to strengthen your point? If there are any other publications showing this evidence, it will also strengthen your point and provide translational validation to confirm what was found based on the bivalent agent's strategy. Can you provide a rationale to justify why there is no formation of Di-heterotrimer without tethering if it is the case?

14. (P11 L9) Please represent W751A, F804A, and Q742K mutants on the structures in the figures to provide a better view of their involvement in the interfaces and/or refer to Extended Data Fig. 6e where two of the three residues are already depicted.

15. P35 L10 change "and a blot force of 2 for 3 s" replace blot force by blot time

16. P38 "manufacture " change to "manufacturer"

17. “Moreover, LGR4 strengthens its association with ZNRF3 in the assembly with 2:2:2 stoichiometric ratio (Fig. 4a-d), facilitating their joint endocytosis and promoting Wnt signaling.” – While cointernalization has been pointed out at an important mechanism, the authors do not include experiments assessing the role of stoichiometry for endocytosis efficicency of the individual components. We suggest therefore to be a bit more speculative on this end.

Reviewer #2

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #3

(Remarks to the Author)
Geng et al. present several cryoEM structures of LGR4/RPSO2/ZNRF3 complexes from the Wnt signaling pathway demonstrating a variety of stoichiometries of complexes. From this they attempt to build a picture of how these proteins work together to modulate Wnt signaling. These are potentially very interesting results for a tricky system. However, the paper has substantial issues in the experimental data, its presentation, and its interpretation that would preclude it from publication without additional studies and reworking of the manuscript.

The largest issue arises from the fact that half of the data is described as deriving from a RING-truncated construct with GFP/a GFP binder to cement the complex while the other half of the data is described as deriving from a full length construct cemented with LgBiT HiBit, with poor annotation of which figure panels come from which data set. This results in both significant confusion for the reader and fundamental conclusions that seem like they could plausibly derive from the truncation of the constructs rather than actual molecular physiology. Particularly as RING-dimerization is a key part of ZNRF3 activation and the 1:2:2 complex without RING shows also poor interaction, it seems crucial to demonstrate that the 2:2:2 state observed is indeed a true state and not an artifact of the absence of the dimerizing RING domains. Ideally, additional data should be collected with the full length constructs in order to obtain a sufficient resolution reconstruction of the near-wild-type 2:2:2 complex (Extended Data 3) to at least demonstrate agreement with the truncated 2:2:2 (if not a high resolution reconstruction outright). This would allow for the key conformational changes of the study to be definitively attributed to reality and not construct design. The truncated data, if presented, should be presented in a clearer, more straightforward manner where constructs are better delineated. However, there are still several additional issues detailed further that would also need to be addressed in order for the manuscript to be publishable.

A point by point critique is provided below:

There are several different megabody designs, including several based around HopQ, please state more clearly which one was used (even though none of the rest of the megabody beyond the nanobody was resolved). I think it would be also beneficial to the reader to comment on whether the megabody was really needed (e.g. whether datasets with just Nb52, if they were done, had bad preferred orientation) or if it was just what was tried initially.

Page 6 line 13-14 the punctuation of this sentence is confusing, I think a comma needs to be removed or a semicolon added depending on what the authors were trying to say.

I find the assertion that MB52 stabilizes the complex on page 6 line 19-20 to be potentially problematic, as it seems to bind primarily to the outer extracellular portion of the ECD without contacting ZNRF3 or RSPO2. Additionally there is no direct pharmacological characterization of NB/MB52 to suggest it enhances complex formation and the nanobody was suggested to be raised against LGR4 alone. Indeed, I think if NB/MB52 potentially stabilizes various complexes preferentially that would actually be problematic for the assertions of the study. I would highly suggest the authors provide some level of characterization of if and how MB52/NB52 influences LGR4 complex stoichiometry.

For the section “Structure of LGR4-RSPO2-ZNRF3 complex”, there seems to be currently no justification provided for why the initial work was done with a GFP/anti-GFP and RING truncated version and the following work is done with the RING domain included and LgBiT HiBiT. Some justification/explanation for the swap and why both structures are presented in the introduction of the paragraph is warranted to prevent the reader from being confused. Again, repeating the structure with the full length construct is necessary for the validity of the study.

The sentence on page 7, line 18-19 is confusing; a predominant complex compared to separate components, or of a certain stoichiometry? I assume it is referring to the 1:1:1 but this should be more explicitly stated.

For the section “structures of … complexes with 1:2:2 stoichiometry” I again feel there is insufficient description of how/why this varies from the 1:1:1 work; is this the same constructs, different titer? Same construct but with no specific titer? This should be laid out more clearly.

When pentamer A is discussed, as this comes from ‘the same sample where a 2:2:2’ structure was obtained, if this is the version of the structure the authors intend to use it should more explicitly describe this as from the delta-RING construct, especially in the figures.

In Figure 3, panel d is presented next to panel e (pentamer B, second data set with RING domains) analogous to a and b, even though a, b, and e are actually (derived from) the same map. There seems to be the assertion that there was some fitting the map of an and b into d to generate e, although it is underexplained what this entails and how this was done. Also it should be commented on why the authors seem to assume that the connection between the TMD and RING should be rigid and such docking is fair, even though the RING is not so well resolved. There is this key suggested conclusion that the observed 2:2:2 conformation forces separations of the RING domains, and this prevents catalysis, but it is hard to state definitely with a truncated construct. By eye the 3D and 2D classes in extended data 3 almost look like the full length 2:2:2 complex also has the RING domains interacting, although the resolution is poor. The authors themselves note in the conclusion the 2:2:2 complex might reduce activity through sequestration and/or internalization. Again, even a modest resolution 2:2:2 reconstruction with the full length would help clear this up. Constructs where maps were derived from also needs substantially better labeling.

This also brings me to my second point about Figure 3, the degree of low pass filtering should be provided in the captions as well as the low pass filtered maps. The thickness of the maps for single helices at this degree of low pass filtering/contour threshold casts heavy doubt on the reliability of this portion/interpretation of the map. Personal inspection of the unsharpened map does suggest there may be some map features for the RING. Figure 3 should thus be significantly reworked for both improved clarity and to interpret the map with a more realistic contour/low pass filtering protocol.

The title of ED6 suggests that the panels demonstrate that the 2:2:2 complex is incompatible with LGR4 (TM6?) activation, but there is no overlay that really demonstrates this to be the case as none of the panels show an overlay with the 2:2:2 complex, they all only show a single LGR4 with no ZNRF3 or other subunits.

The modeling on all of the structures needs work. In regions where there is little/poor density period, the protein including the backbone should be truncated. For regions where there is little/poor density for side chains but the backbone can be modeled, sidechains should be stubbed. The modeling is currently liberal for what are 3.2-3.6A resolution maps.

Reviewer #4

(Remarks to the Author)
The LGR4/5-R-spondin module is an essential modulator of adult stem homeostasis. Critically, their target E3 ligases ZNRF3/RNF43 have emerged as potential therapeutical targets, including in cancer, as well as engineering tools for receptor degradation through e.g., PROTABs. Although several structures of these factors are available, including from the ternary complex, little is known about the contribution of the anchoring transmembrane domains (TMDs) to the complex formation and/or stoichiometry.

In this ms, Wang and colleagues present various cryo-EM structures of LGR4-RSPO2-ZNRF3 complexes that include the TMDs of both LGR4-ZNRF3. The authors characterize the properties of the complexes and hypothesize about their role in LGR-RSPO-dependent inactivation of ZNRF3. Furthermore, the authors perform mutagenesis analyses of specific LGR4 and ZNRF3 residues at their TMDs involved in the interaction demonstrating their relevance for WNT signaling activation without affecting their deployment to the PM.

Although I cannot comment on the structural analyses, I found the overall model and validation experiments quite convincing, as well as with relevance for the broad readership of Nature Communications.

I only have a couple of comments for the authors:

1- The authors identify critical residues likely driving TMD-TMD and ligand-receptor interactions for LGR4-RSPO2-ZNRF3. Can the authors discuss whether those interactions are also present/predicted in the other members associated with this module (LGR4/5, RSPO1-4 and RNF43/ZNRF3)? Ligands and TM proteins of this module can be largely exchanged with their paralogous (e.g., RNF43 behaves as ZNRF3 for interaction purposes but it is usually more relevant for cancer research). A broader bioinformatic/predictive analysis of whether the other ternary complexes are potentially governed by the same interactions (conservation of key residues/surfaces) should strengthen the authors functional claims, especially for i) the ionic lock in LGR5, ii) the expected interaction surfaces between LGR4-RNF43 and LGR5-ZNRF3 and iii) equivalent V229-P-S230 mutation in RNF43. These prediction analyses could be directly complemented with WNT reporter assays. Taken together, these analyses should provide important insights on whether the described molecular lock and switches govern the functional interactions of the LGR4/5 - RSPO1-4 - ZNRF3/RNF43 module or they are specific for the LGR4-ZNRF3 pair.

2- LGR4 F804 and Q742K display impaired response to RSPO1 at lower ligand concentrations, but behave similar to wt LGR4 at nM range of RPSO1 (which is also around the Kd). Can the authors comment on the possible implications of these results? It seems that the TMD-TMD interactions could stabilise the complex under limiting [RSPO], but might be fully compensated by the interactions of the extracellular domain under what are likely standard [RSPO1] (nM range)

Minor comments:

3- I believe that the authors only use the furin domain of RSPO2 for all their structural analyses. I think it should be properly indicated in the main figures to avoid confusion. A Figure visually displaying (and properly marking) all constructs used in

this study will be extremely helpful for the reader (Please see Chen et al., Genes & Dev 2013, Figure 1A)

4- Please show the mutated residues in the TMD cartoons of Figure 4.

5- Can the authors further elaborate on why the pentamer A structure could lead to di-heterotrimer and not the other way around?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Overall, the authors thoroughly addressed the comments from the reviewers. Data interpretation and the overall clarity of the manuscript have been improved. A careful check for consistency in abbreviation, wording, and spelling would be necessary, preferentially by a native English speaker. Furthermore, there are still critical issues with the model refinement that should be clarified.

Regarding data processing and model building

1. While the processing was significantly improved, masked local refinements towards transmembrane sections (combined with signal subtraction) might improve the area that is usually of lesser quality in the maps. Nonetheless, this will not change the conclusion of the manuscript, consequently this is only a suggestion.

2. Regarding the model. There are serious geometry issues in the new models. I assume this is because the authors attempted to reach a clash score of zero at all costs, at the expense of other critical metrics. One illustration of that, that is quite telling is the geometry of the cholesterol in the 8Y69 that is completely distorted. Also, geometry analysis that focuses on bond lengths and angles in coot highlights the problem strikingly. You can also refer to metrics from phenix validation like (rmsd angle and length among others). A model with the worst clash score (between 0 and 10) and a more realistic geometry is a better representation of the protein. This needs to be improved before publication.

Points that require the authors' attention before acceptance:

1. Regarding LGR4-RSPO2-ZNRF3(RING)-MB52 complex: FSC is not dropping to 0, this is usually induced by duplicated particles or a too tight mask during FSC calculation. In both cases, it could lead to resolution overestimation. Please try to remove duplicates before your final refinement and if this is not the problem, recompute FSC with a broader mask.

2. Please clarify the end of the following sentence "These structures, RSPO2 domain (PDB code: 4UFR), and 712 ZNRF3 PAD (PDB code: 4UFS) were fit into the composite cryo-EM map of the LGR4-RSPO2- ZNRF3 complexes as starting model in ChimeraX, and then iteratively refined with manual adjustment in Coot , Real-space, and performed reciprocal-space refinements in Phenix ."

3. The sentence "Understanding the structural principles governing these regulatory modules is crucial for tightly controlling Wnt signaling output" was corrected as "It is essential to understand the structural principles governing these regulatory modules to precisely control Wnt signaling output". Unfortunately, this still does not make sense since understanding principles cannot control Wnt signaling. The syntax is incorrect.

4. "The pair of TMHs are drawn close together on the extracellular side but diverge on the cytoplasmic side, creating an inverted V-shape configuration, clipping two distinct elongated densities that are attributed to cholesterol hemisuccinate (CHS) (Fig. 1b, c, e).2" correct for cholesteryl hemisuccinate.

5. In the figure labels, cholesterol hemisuccinate is annotated as CLR while it should be CHS. Please correct that, it is confusing.

6. The authors use two abbreviations TM and TMH that refer to transmembrane helices. We strongly suggest to abbreviate transmembrane with TM and do not abbreviate helix.

7. "TMH,TMHs" is explained multiple times P3 L52 , P8 L140 , P10 L179 and 185, P12 L241. Remove and refine.

8. "Extended Data Fig. 6c (3rd fit)" While the map strongly suggests that two ring domains are clearly separated, the model fit is not convincing. Please adjust the loops orientation or if you can't provide a model that fits better replace the ring models (only for this structure) with a schematic representation.

9. P4. "A higher proportion of LGR4 induces and stabilizes ZNRF3 inactive state, forming a stable assembly, facilitating internalization and consequently promoting WNT/β-catenin signaling" Since the facilitated internalization is speculative rephrase as a hypothesis.

10. Title “Overall architecture of LGR4-RSPO2-ZNRF3complex with a 2:2:2 stoichiometry” lacks a space before complex.

11. Page 17: The authors argue that the lack of a TM6 swing out in LGR4 complexed with RSPO and ZNRF3 would argue against class A-like mechanisms of activation and LGR4-G protein coupling. This statement is indeed an overstatement and should be toned down/removed. Also in class A GPCRs agonist binding does not elicit a TM6 swing out – the opening of the receptor is only observed in the presence of a heterotrimeric G protein stabilizing the TM6 swing out. As a consequence the subsequent argumentation of LGR4 activation mechanisms is pure speculation (for example, an unidentified transducer could stabilize an open and active-like receptor conformation).

12. The legend of Fig. 5 is insufficient. Please elaborate on necessary details.

13. The legend of Extended Data Fig. 7 is insufficient. Please elaborate on necessary details.

Reviewer #2

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #3

(Remarks to the Author)
The authors have reasonably addressed the comments and critiques that I provided and the work would seem suitable for publication.

Reviewer #4

(Remarks to the Author)
The authors have addressed my comments. However, I think that both the referee figure 17 and the associated comments merit space in the manuscript. I suggest to bring the data from referee figure 17 to a extended figure in the paper, and summarise the authors' response to my point #1 (and the last part of point #2) into a paragraph at the end of the results section or the discussion. Although I understand that part of the response is based on modelling and not structural data, i believe that i) it is well-reasoned and structured and ii) discussing its implications for RNF43 and LGR5 will be important for the community, and for future structural studies tackling the formation of these ternary complexes.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have adequately addressed our comments and significantly improved the manuscript, making the work suitable for publication.

Gunnar Schulte and Julien Bous

Reviewer #2

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #1 (Remarks to the Author):

In this manuscript, the authors characterized the structure of multiple protein complexes with stoichiometry variability involving LGR4, RSPO2, ZNRF3. To do so the authors accomplished a significant work of protein design, protein purification optimization that deserve to be highlighted. We appreciate the originality of the work that is significantly different from GPCRs-G protein structures routinely published nowadays. The work is of great interest since it highlights new structural features and establishes a rational to understand different complexes stoichiometry in a physiological context explaining LGR4 Wnt signaling regulation. The complex of LGR4, RSPO2, ZNRF3 provides indeed novel structural insight into the functional consequences of RSPO action on WNT signaling by complexation of the ZNRF3 E3 ligases.

Thank you for your thoughtful and positive feedback on our manuscript. We greatly appreciate your recognition of the significant work involved in protein design and purification optimization. Your comments on the originality of our work and its importance in understanding different complexes' stoichiometry in a physiological context are highly encouraging.

Additionally, we will address any specific comments and suggestions you may have to further improve the clarity and impact of our work.

Thank you once again for your valuable feedback.

We have minor comments that need to be addressed to improve the manuscript. On the technical aspect (Cryo-EM): Density maps are consistent for the overall resolutions reported. The authors are careful in terms of interpretation and don't overinterpret the data, which is very much appreciated.

Thank you for your valuable suggestions and positive feedback. We appreciate your acknowledgment of our careful interpretation of the Cryo-EM density maps and the consistency of our reported resolutions.

We have made the necessary adjustments to our revised manuscript to further clarify and accurately present our data. We have also ensured that our interpretations remain cautious and aligned with the data, as you recommended.

Thank you once again for your insightful feedback, which has helped us improve the quality and clarity of our manuscript.

1. Final subsets of particles are quite large while the different reconstruction features some apparent dynamic that was not sorted properly. The authors need to retake subsets from Heterotrimer (1,064,324), Pentamer A (407,148), Pentamer B (838,832) and sort it further to get rid of heterogeneity. There are multiple tools that can be appropriate, if you want to extract information on dynamics in the process, 3D variability in cluster mode represents a good strategy, Heterogeneous refinement or Ab-initio with multiple reconstructions (initial res 15, final res 6, and large minibatchs) can also be used. Obtaining a better map will also help to improve the atomic models. Regarding the Di-heterotrimer the C2 symmetry was ignored during refinement while it can significantly improve alignment during refinement and be used to duplicate particles through symmetry expansion for subsequent classification with local sampling or no alignment. Overall, we think that the processing needs to be completed with the suggested strategies.

Thank you for your valuable suggestions. We have reprocessed the data following your recommendations and made significant improvements. Here are the updates and the steps taken:

In addition to the conventional workflow, we implemented multiple rounds of **Heterogeneous Refinement** and **Ab-initio reconstruction** (initial resolution 15 Å, final resolution 6 Å) to eliminate heterogeneity in the particle subsets. We also used **CryoSieve software** for further refinement of particle counts. These efforts led to a significant enhancement in the resolution of several density maps, which aided in **model building** and **density map identification**, thus improving the overall quality of the models. Regarding the **Di-heterotrimer**, we utilized **C2 symmetry** during refinement, which greatly improved the alignment and allowed for particle duplication through symmetry expansion for further classification. This approach notably enhanced the refinement of this complex.

Summary of Improvements:

Table 1 - Particle and Resolution Improvements:

Dataset	Number of Particles (Old)	Number of Particles (New)	Resolution (Old)	Resolution (New)
Heterotrimer	1,064,324	106,020	3.24 Å	2.50 Å
Pentamer A	407,148	143,915	3.40 Å	3.21 Å
Pentamer B	838,832	61,066	3.26 Å	3.20 Å
Di-heterotrimer	277,875	47,438	3.59 Å	3.38 Å

Table 2 - Model Quality Improvements:

Dataset	Clash score (Old)	Clash score (New)	Ramachandran outliers (Old)	Ramachandran outliers (New)	Sidechain outliers (Old)	Sidechain outliers (New)
Heterotrimer	7	0	0.3%	0.0%	1.0%	0.0%
Pentamer A	11	2	0.3%	0.4%	2.4%	1.1%
Pentamer B	11	0	0.5%	0.1%	2.8%	0.7%
Di-heterotrimer	13	0	0.2%	0.0%	2.3%	0.3%

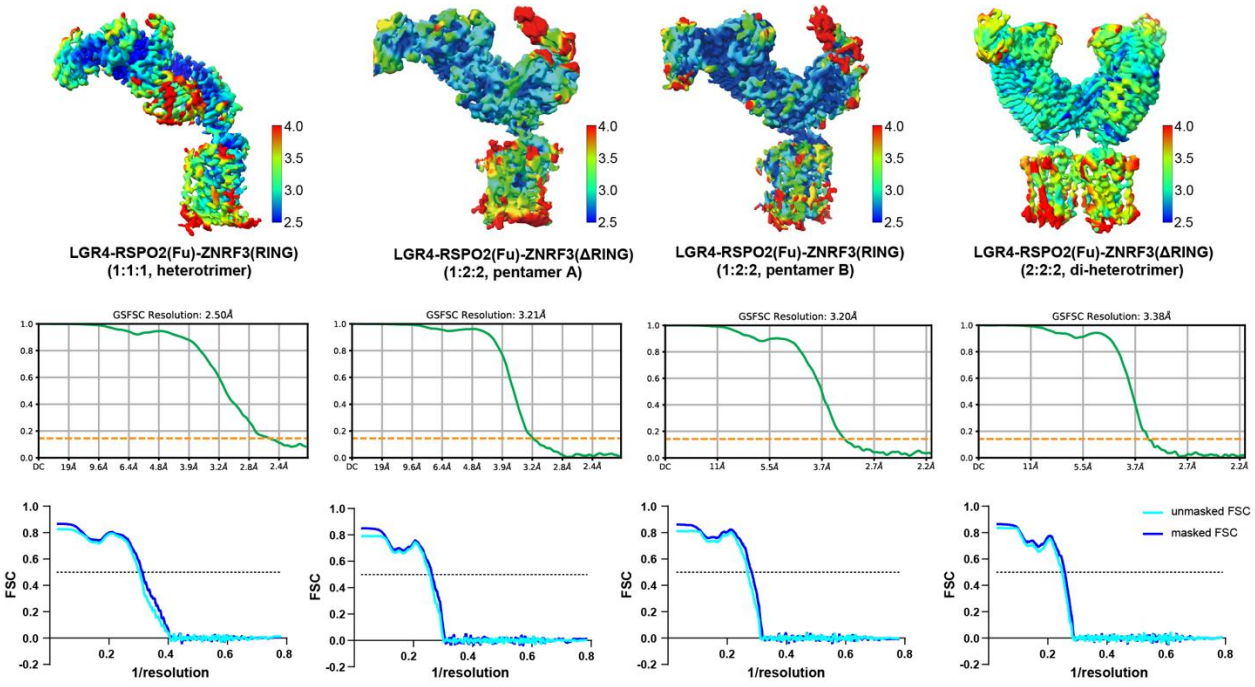
Revised Models:

We have rebuilt the models based on the updated density maps and re-evaluated their quality. The changes resulted in the elimination of **Ramachandran outliers**, a significant reduction in **rotamer outliers**, and substantial improvements in **clash scores**. (Table 1 and 2) These adjustments have bolstered the integrity of the models, ensuring they are more reliable and accurate.

We believe these revisions have significantly strengthened the manuscript, aligning it with the highest standards. We deeply appreciate your constructive feedback, which has greatly contributed to the improvement of this work. Thank you once again for your invaluable suggestions.

2. For the Extended Data Fig. 4 Pentamer B, the local resolution reported in the supplementary figures is not consistent with the quality of the map and overall resolution, it looks to be ranging from 8 to 14 in the figure while it is much better based on final map inspection. Can you please correct this discrepancy?

Thank you for bringing this issue to our attention. We have reprocessed our data based on your above suggestions. This has led to an improved map quality, and we have corrected the local resolution values reported in the supplementary figures accordingly. (Response Fig. 1) We appreciate your careful review and helpful suggestions, which have enabled us to enhance the accuracy of our presentation.



Response Fig. 1 The quality of the map for following complexes LGR4-RSPO2-ZNRF3(RING) (1:1:1, heterotrimer), LGR4-RSPO2-ZNRF3(ΔRING) (1:2:2, pentamer A), LGR4-RSPO2-ZNRF3(RING) (1:2:2, pentamer B), and LGR4-RSPO2-ZNRF3(2:2:2, di-heterodimer). The first row illustrates the local resolution maps of these four complexes, the second row presents the FSC curves for each complex, and the third row displays the model-to-map correlation for all four complexes.

3. The overall quality of the model could be improved to remove or reduce Ramachandran and rotamer outliers while maintaining a descent clash score. This is only a suggestion because the model quality is still acceptable, and since these changes will not alter the conclusions of the work.

Thank you for your valuable suggestion. Based on your feedback, we have reprocessed our data, which has led to an improved map quality. Consequently, we have rebuilt and refined our model. As a result, the overall quality of the model has significantly improved, with reduced Ramachandran and rotamer outliers and a maintained decent clash score (Table 2). We appreciate your careful review and constructive input, which have enabled us to enhance the robustness of our model.

Table 2 - Model Quality Improvements:

Dataset	Clash score (Old)	Clash score (New)	Ramachandran outliers (Old)	Ramachandran outliers (New)	Sidechain outliers (Old)	Sidechain outliers (New)
Heterotrimer	7	0	0.3%	0.0%	1.0%	0.0%
Pentamer A	11	2	0.3%	0.4%	2.4%	1.1%
Pentamer B	11	0	0.5%	0.1%	2.8%	0.7%
Di-heterotrimer	13	0	0.2%	0.0%	2.3%	0.3%

On the scientific aspect:

The manuscript is very well written, well-structured and the topic is timely and novel. The methodology is sound and the conclusions are supported by the presented data. We have only very minor more cosmetic criticism:

Thank you for your positive feedback on our manuscript.

1. Avoid the term “FZD receptors”, they are WNT receptors and should be called “Frizzleds”. In this context, it should be taken into consideration that not all WNT receptors are FZDs and that not all WNT receptors (for example LRP5/6, ROR1/”, RYK, PTK7) are regulated by ZNRF43/RNF43. Please use G protein, not G-protein.

Thank you for pointing out this mistake. Your explanation has clarified these concepts for us. We have corrected the terminology in the manuscript accordingly. We appreciate your thorough review and valuable feedback.

2. “Understanding the structural principles governing these regulatory modules is crucial for tightly controlling Wnt signaling output.” This sentence needs to be reworded: Understanding does not regulate signaling.

Thank you for pointing out this mistake. We have reworded the sentence for clarity. The revised sentence is: “It is essential to understand the structural principles governing these regulatory modules to precisely control Wnt signaling output”

3. For pharmacological clarity, it should be mentioned that LGR4 is classified as a class A orphan GPCR (see https://gpcrdb.org/protein/lgr4_human/). This aspect is especially important when comparing the LGR4 conformation to other GPCRs in Ext Data Fig. 6.

Thank you for your valuable suggestion. We have revised the manuscript to emphasize LGR4's classification as a class A orphan GPCR and clarified its relevance in comparison to other class A GPCRs. This added pharmacological clarity will help readers better understand the structural features and behavior of LGR4 within the context of its complex.

The following description has been incorporated:

LGR4 Classification: “LGR4 is classified as a **class A orphan GPCR**, characterized by a large extracellular domain (ECD) with 17 leucine-rich repeats (LRRs), followed by a seven-helix transmembrane domain (7TMD) typical of the rhodopsin family GPCRs."

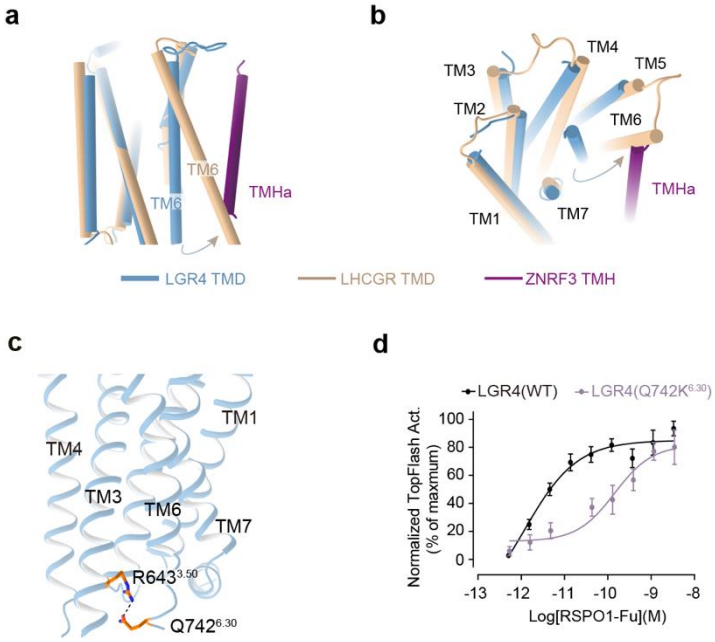
Conformational Comparison: “When comparing LGR4's 7TMD to other class A orphan GPCRs, such as the active LHCGR structure, we noted a significant difference. The outward movement and rotation of **TM6**—a critical step in G protein coupling activation observed in other GPCRs—are restricted in LGR4 due to steric clashes with ZNRF3's single transmembrane domain (TMD). (Response Fig. 2a, b) This restriction prevents LGR4 from adopting an active conformation."

Functional Role of the Ionic Lock: "We also explored the role of the ionic lock, a stabilizing mechanism in rhodopsin-family GPCRs. Our mutation of the ionic lock (Q742K$^{6.30}$) in LGR4’s

transmembrane domain showed reduced TOPFlash activity, suggesting a potential increase in TM6 movement. (Response Fig. 2c, d) However, in the LGR4-RSPO2-ZNRF3 complex (2:2:2), LGR4 appears to remain in an inactive conformation. Unlike other GPCRs that are activated by signal-induced conformational changes, LGR4's function seems to rely more on direct, context-dependent regulation of the assembly."

Conclusion: "These findings indicate that LGR4, unlike other class A GPCRs, may play a more direct role in assembly and signaling regulation, without undergoing the conformational changes typically associated with activation."

This revised explanation enhances clarity around the unique structural and functional properties of LGR4 in the context of its complex and highlights its differences from other GPCRs. We believe this additional context strengthens the manuscript, making it more accessible and informative to the audience. Thank you again for your thoughtful feedback.



Response Fig. 2 The conventional inactive state of LGR4 in di-heterotrimer. **a**, **b**, Conformational comparison of the TMD of LGR4 (light blue) in the di-heterotrimer complex with that of active LHCGR (wheat, PDB:7FII, RMSD= 1.524, 182 to 182 atoms) from the front view (**a**), and bottom view (**b**). The potential steric clash between TM6 of the active LGR4 and the single TMH (violet) of ZNRF3 in the di-heterotrimer complex is shown. **c**, The ionic lock between $R643^{3.50}$ of TM3 and $Q742^{6.30}$ of TM6 in LGR4. **d**, TOPFlash plot illustrating the effect of breaking ionic lock ($Q742K^{6.30}$ mutant) (purple) in the transmembrane domain of LGR4 on the activity of RSPO1,

compared to that of WT (black). Each value represents the mean ± SEM from three independent experiments.

4. “providing the structural platform for drug development of Wnt-driven cancers” …maybe better: “providing a structural platform….”

Thank you for pointing out the error. We have revised the sentence to 'providing a structural platform for drug development of Wnt-driven cancers.' We appreciate your attention to detail and your suggestion for improvement.

5. “A pair of horseshoe shaped LGR4 protomers are located at the periphery of the complex” should be “is located”

Thank you for highlighting the grammatical error. We have corrected it to 'A pair of horseshoe-shaped LGR4 protomers is located at the periphery of the complex.' We appreciate your attention to detail and your valuable suggestion for improvement.

6. Page 10, lane 20 “LGR4 induces ZNRF3 into an inactive state” – maybe simpler: “LGR4 deactivates ZNRF32” – or similar.

Thank you for your excellent suggestion. We agree that it simplifies the statement effectively. We have made the change in the context, revising it to 'LGR4 deactivates ZNRF3.' We appreciate your insightful feedback.

7. With the statement “Upon analyzing the structures of LGR4 in all complexes, it becomes apparent that LGR4 consistently adopts an inactive conformation” on page 12 lane 16/17 (also discussion page 15, last paragraph), the authors touch upon an interesting point. What does LGR4 activation mean and does this receptor follow the same activation mode as “conventional” class A GPCRs? The authors might want to elaborate a bit more on this pointing out that the active conformation of a GPCR with the TM6 swing out refers to the G protein-coupled (or at least transducer-bound) state.

Thank you for highlighting this important aspect. We appreciate your suggestion to elaborate on the mechanism of LGR4 regulating Wnt signaling.

We have expanded the discussion to provide a more detailed explanation of what LGR4 regulating Wnt signaling entails.

The following text has been added on page 13 of the manuscript:

"Upon analyzing the structures of LGR4 in all complexes, it becomes apparent that LGR4 consistently adopts an conventional inactive conformation. In the context of conventional class A GPCRs, activation typically involves the outward swing of TM6, which facilitates G protein coupling or binding to other transducers. Our analysis indicates that LGR4 does not exhibit this outward movement of TM6, even in the presence of RSPO2 and ZNRF3."

Additionally, we have added the following text to page 17 of the manuscript:

"In conventional class A GPCRs, activation typically involves an outward swing of TM6, which facilitates G protein coupling or binding to other transducers. Our analysis indicates that LGR4 does not exhibit this outward movement of TM6, even in the presence of RSPO2 and ZNRF3 (Response Fig. 2a,b). This suggests that LGR4 remains in an conventional inactive state and does not follow the conventional activation mode seen in other class A GPCRs."

Thank you for your valuable feedback.

8. Discussion: The paragraph starts with "The Wnt signal transduction cascade….". Here the authors should underline that RSPOs are mostly connected with amplification of the WNT/b-catenin signaling route. Given the underlying regulatory mechanism of the regulation of FZD surface expression, also b-catenin-independent but FZD-mediated signaling routes should be affected by RSPO. The simple way out here would be to limit the discussion to the WNT/b-catenin pathway. Strategically, this should be done throughout the manuscript replacing WNT signaling with WNT/b-catenin signaling.

Thank you for your insightful suggestion. We agree that emphasizing the connection between

RSPOs and the amplification of the WNT/β-catenin signaling route would enhance the clarity of our discussion. To address this, we have revised the discussion to specifically highlight the role of RSPOs in amplifying the WNT/β-catenin signaling pathway. We have also ensured that references to WNT signaling throughout the manuscript are replaced with WNT/β-catenin signaling where appropriate.

By making these adjustments, we aim to provide a more precise and focused discussion that aligns with the underlying regulatory mechanisms. We appreciate your feedback and believe these changes will strengthen the manuscript.

Thank you once again for your valuable input.

9. Page 16, lane 7/8: “In conclusion, our study uncovers the assembly mechanism of LGR4, RSPO2, and ZNRF3 in mediating Wnt signaling.” This is an overstatement (since there are not actual experiments putting the novekl srcutural insights into the framework of WNT signaling) and I suggest rewording: In conclusion, our study uncovers the assembly mechanism of LGR4, RSPO2, and ZNRF3 and provides deep mechanistic insights into the RSPO-mediated regulation of Wnt signaling……or similar. Or somehow connect to the sentence afterwards.

Thank you for pointing out this overstatement. We appreciate your suggestion for rewording the conclusion to better reflect the scope of our findings.

We have revised the conclusion as follows:

"In conclusion, our study uncovers the assembly mechanism of LGR4, RSPO2, and ZNRF3 and provides deep mechanistic insights into the RSPO-mediated regulation of Wnt/β-catenin signaling. These structural insights lay a foundation for future regenerative therapeutics and cancer treatments."

Thank you once again for your valuable feedback.

10. The mutational analysis of W751A, F804A and Q742K (ionic lock) are a bit lost in the manuscript. The authors state that these mutants were chosen based on their importance for protein-protein interaction or intramolecular interactions. Please show these interactions in detail. The reader is referred to Fig. 4 and Ext data Fig. 5, but essential insight is not provided. The R643-Q742 interaction is actually shown in Ext Data Fig 6. It would make it easier for the reader if the

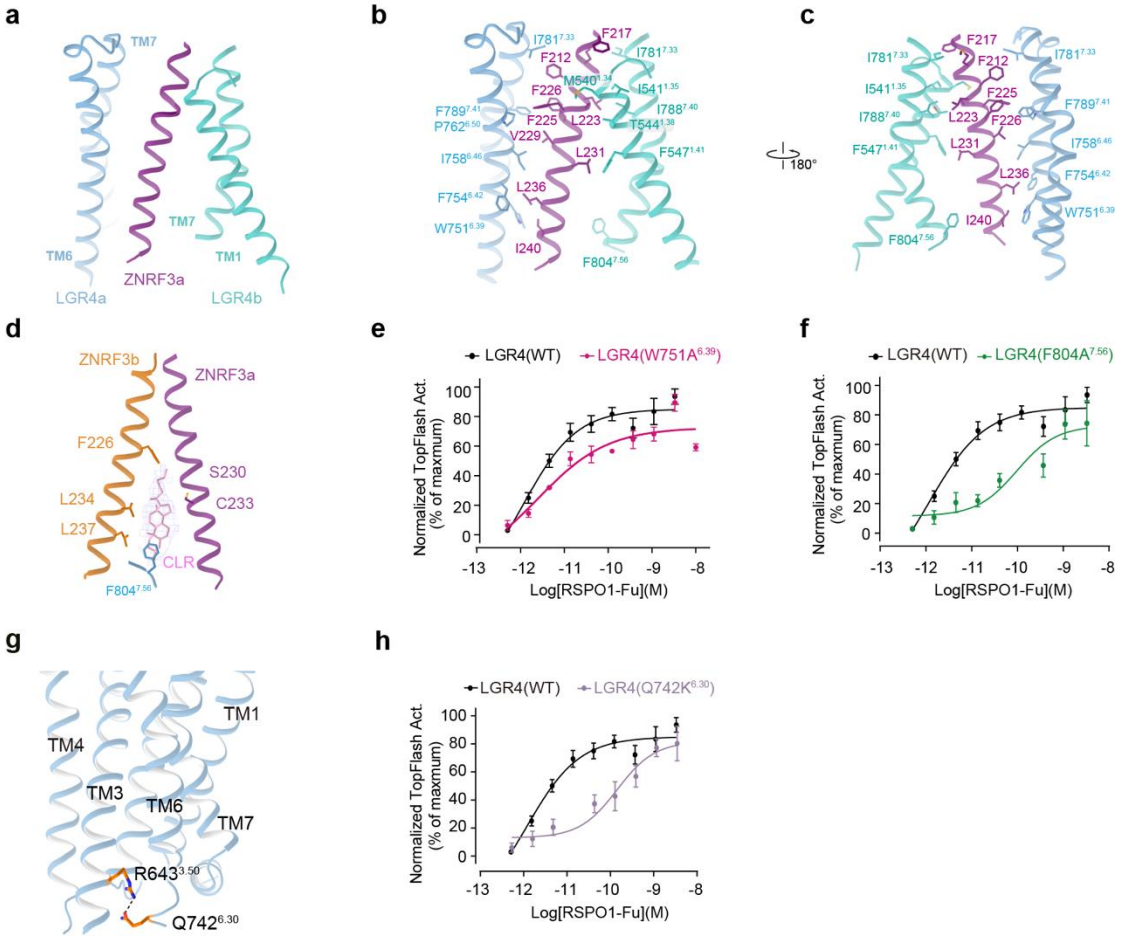
Ballesteros Weinstein nomenclature for GPCRs would be adopted throughout the manuscript. While the functional consequences of the mutations on RSPO-induced TOPFlash are obvious, it remains unclear whether indeed the impairment of protein-protein interactions or other receptor-intrinsic effects are responsible for this shift in RSPO potency.

As a technicality, we could not find any information about WNT addition. Do the authors add WNT to see the RSPO effect on TOPFLASH or do they rely on basal, endogenous WNT secretion in HEK293 cells?

Thank you for your insightful comments. We have addressed your points as follows:

1. Detailed Interaction Descriptions:

We have revised the manuscript to provide detailed descriptions of the interactions involving the W751A$^{6.39}$, F804A$^{7.56}$, and Q742K$^{6.30}$ (ionic lock) mutations. These interactions are now clearly illustrated in Fig. 4d-i and Extended data Fig. 8e.



Response Fig. 3 **a,** The interface between the transmembrane domains of LGR4 and ZNRF3. **b,c**, The side chain interactions between LGR4 and ZNRF3 within the transmembrane region of the LGR4-RSPO2-ZNRF3 complex (2:2:2) are shown in detail.**d**, The specific interactions between the transmembrane domain of LGR4, ZNRF3 and cholesterol within the LGR4-RSPO2-ZNRF3 complex (2:2:2) are highlighted.**e**, **f**, Dose-dependent TOPFlash activity induced by WT (black) or W751A$^{6.39}$ mutant (red, **e**) and F804A$^{7.56}$ mutant (green, **f**) of LGR4 after stimulation with RSPO1. **g**, The ionic lock between R643$^{3.50}$ of TM3 and Q742$^{6.30}$ of TM6 in LGR4. **h**, TOPFlash plot illustrating the effect of breaking ionic lock via Q742K$^{6.30}$ mutant (purple) in the transmembrane domain of LGR4 on the activity of RSPO1, compared to that of WT (black). Each value represents the mean ± SEM from three independent experiments.

2. Adoption of Ballesteros-Weinstein Nomenclature:

To ensure consistency and facilitate understanding, we have adopted the Ballesteros-Weinstein nomenclature for LGR4 mutation sites (Q742K$^{6.30}$, W751A$^{6.39}$, F804A$^{7.56}$) throughout the manuscript.

3. Clarification of Transmembrane Domain Interactions:

We apologize for not clearly illustrating the transmembrane domain interactions between LGR4 and ZNRF3 previously. We have now provided detailed descriptions and illustrations of these interactions. (Response Fig. 3a-f). Specifically, we have presented the hydrophobic interactions between the W751 sidechain from LGR4 and both sidechains of I240 and L236 from the single membrane helix of ZNRF3. The F804 residue on LGR4a's TM6 interacts with a cholesterol molecule positioned between the two ZNRF3 TMHs, which in turn interacts with the other ZNRF3 TMH (TMHb). (Response Fig. 3d). This suggests that the cholesterol molecule regulates both the interaction between the two ZNRF3 TMHs and the transmembrane interaction between LGR4 and ZNRF3, thereby modulating ZNRF3's catalytic activity. Mutations (W751A$^{6.39}$ or F804A$^{7.56}$) affecting the LGR4-ZNRF3 interface impair RSPO1-dependent TOPFlash activity, highlighting the importance of this interaction in the WNT/β-catenin signaling pathway. (Response Fig. 3e, f).

4. Ionic Lock Mechanism:

The "ionic lock" serves as a molecular switch within rhodopsin-family G protein-coupled receptors (GPCRs), connecting the cytoplasmic ends of transmembrane domains 3 and 6 in the inactive state. This stabilizing interaction is essential for maintaining GPCRs in their resting state. Mutating key amino acid residues involved in this stabilization generates constitutively active mutants. The amino acids involved in forming the ionic lock, specifically the Asp/Glu$^{3.49}$-Arg$^{3.50}$ pair at the cytoplasmic end of transmembrane domain 3 (TM3) and Glu$^{6.30}$ at the cytoplasmic end of TM6, are highly conserved throughout the Rhodopsin family. The interaction involves the positively charged guanidinium group of Arg with the negatively charged carboxylic acid group of aspartic or glutamic acid, constituting the ionic lock interaction. This mechanism of receptor activation is referred to as "ionic lock disruption."

In LGR4, the ionic lock between the Glu$^{3.49}$-Arg$^{3.50}$ (E642-R643) pair at TM3 and Gln$^{6.30}$ (Q742) at TM6 is proposed to maintain the inactive state. Mutating Q742, which is involved in stabilizing the receptor in the inactive state, generates constitutively active mutants. (Response Fig. 3g, h).

5. WNT Preparation and Addition in TOPFlash assay:

In our TOPFlash experiments, we utilize the Wnt3a-conditioned medium (Wnt3a-CM) to ensure consistent activation of the Wnt/β-catenin signaling pathway. The preparation of Wnt3a-CM involves culturing L cells stably transfected with Wnt3a (L-Wnt3a cells), which express and secrete the Wnt3a protein.

For the Wnt/β-catenin signaling TOPFlash reporter assay, HEK293 cells are transiently transfected with plasmids encoding either wild-type or mutant forms of LGR4 or ZNRF3, along with the super 8×TOPFlash firefly luciferase and pRL-SV40-renilla luciferase reporter (Beyotime) at a mass ratio of 1:1:0.1. Following a 12-hour transfection period, the cells are serum-starved overnight to synchronize their signaling response.

To assess the RSPO1 effect, the Fu domain of recombinant RSPO1 is serially diluted (5-fold) in either DMEM or a 1:1 mixture of Wnt3a-CM and DMEM. These mixtures are then added to a half-well white 96-well plate containing 5000 cells/well and incubated at 37°C for 8 hours.

The TOPFlash signal is subsequently measured using the Dual-Glo luciferase assay kit, following the manufacturer's protocol. Each experiment is performed in triplicate to ensure reliability and reproducibility of the results.

By adding Wnt3a-CM, we can reliably assess the RSPO effect on the TOPFlash assay under conditions of consistent Wnt pathway activation.

We appreciate your valuable feedback, which has significantly improved our work. Thank you.

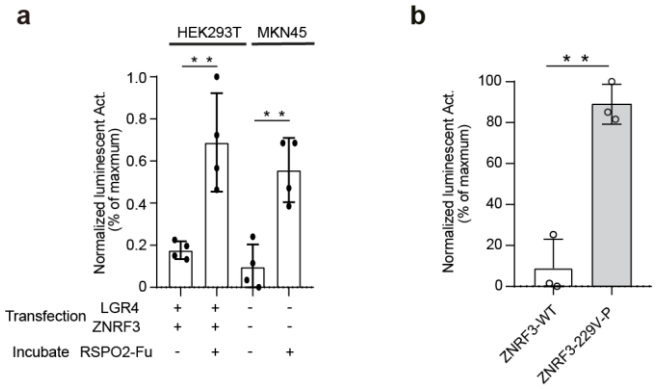
11. Fig. 3c: In the TOPflash data, wt is described as "black" but that is not understandable from the bar graph. Also, the bar graph should be changed to a scatter plot. Statistics? Number of independent experiments, technical replicates? Y axis: % of maxmume should be % of maximum.

Thank you for your detailed feedback. We apologize for not clearly illustrating the bar graph in Fig. 3c. We have addressed your comments as follows:

We have modified our figure to clearly describe the data in the TOPflash assay and have changed the bar graph to a scatter plot for better data visualization.

We have performed three independent experiments with three technical replicates each. The presented figure represents one of these experiments, including error bars to indicate variability. We have corrected the Y-axis label from "% of maxmume" to "% of maximum." (Response Fig. 4).

These changes have been incorporated into the revised manuscript to enhance clarity and accuracy. Thank you for your valuable suggestions.



Response Fig. 4 **a**, The results demonstrate a strong luminescent signal when RSPO2, NB52-LgBiT and NB52-SmBiT, along with furimazine, are added to 293T cells or MKN45 cancer cells expressing LGR4 and ZNRF3. **b**, Wnt3a-stimulated-TOPFlash activity regulated by WT or V229-P-S230 mutant of ZNRF3. Each value represents the mean ± SEM from three independent experiments.

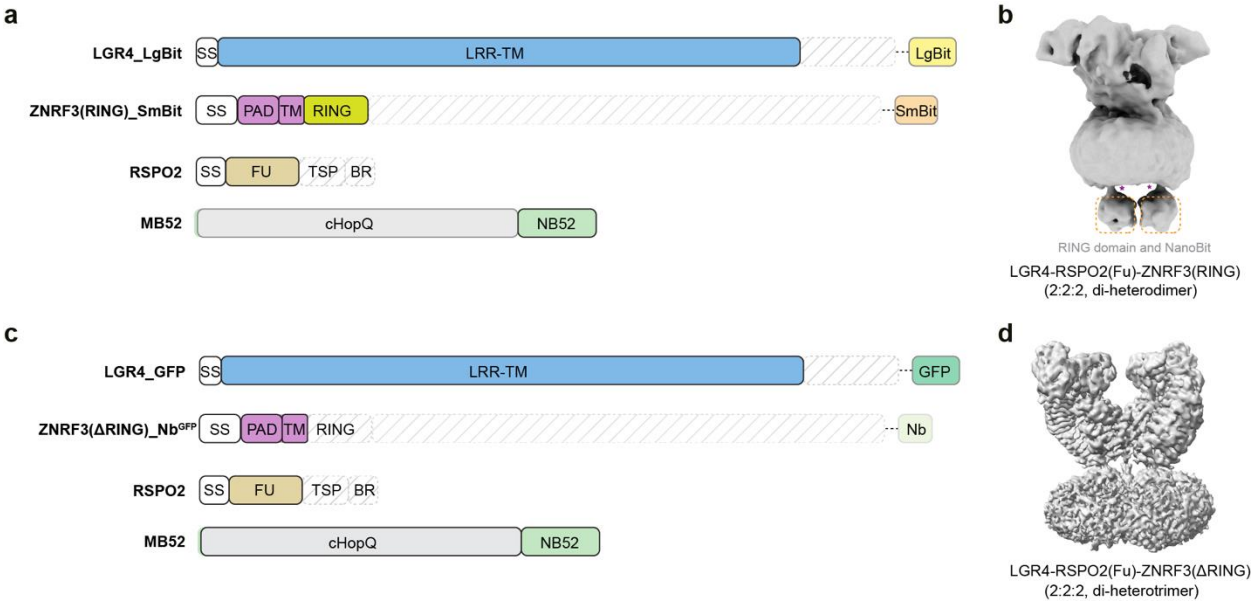
12. Fig. 4: The CRC with RSPO – it should be emphasized in the legend that the wt LGR4 datasets in all three panels are identical.

Thank you for your insightful suggestion. We have updated the figure legend to clearly state that the wt LGR4 datasets in all three panels are identical.

13. The authors propose a regulatory mechanism where a Di-heterotrimer triggers complex

internalization and ZNRF3 inactivation. They qualify it as 'a stable complex' that triggers internalization. In this case, I would expect the other conditions to yield subpopulations of Di-heterotrimer. Nonetheless, based on the biochemistry and cryo-EM data, there is no evidence of Di-heterotrimer formation without tethering. Are there any pieces of evidence for the presence of Di-heterotrimer in other datasets, even if they do not yield high-resolution maps? If yes, highlight this point in your manuscript. Could you elaborate more on the presence of Di-heterotrimer in physiological conditions based on ref 35 to strengthen your point? If there are any other publications showing this evidence, it will also strengthen your point and provide translational validation to confirm what was found based on the bivalent agent's strategy. Can you provide a rationale to justify why there is no formation of Di-heterotrimer without tethering if it is the case?
Thank you for your insightful comments and suggestions.

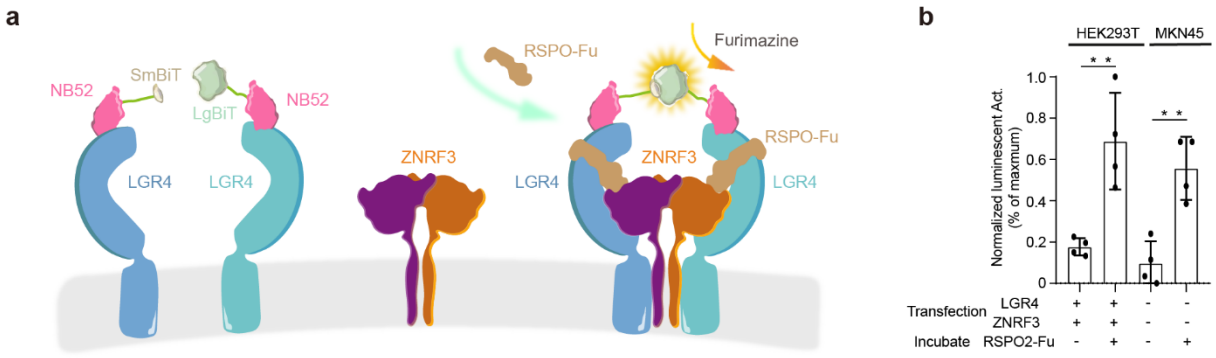
We indeed obtained the structure of the Di-heterotrimer using the same construct as pentamer B (Human LGR4 and human ZNRF3 (including the RING domain), fused with C-terminal LgBiT and HiBiT). However, the particle population of the Di-heterotrimer was low, and the resolution of the reconstruction was very low. When we utilized the construct of ZNRF3 without the RING domain, we obtained enough particles to reconstruct the Di-heterotrimer with high resolution. (Response Fig. 5)



Response Fig. 5 The domain organization of human LGR4, ZNRF3, and RSPO2, along with a schematic representation of their constructs used for structure determination. **a**, The construct used

for the LGR4-RSPO2-ZNRF3(RING) complex in both its heterotrimeric form (1:1:1) and pentameric form B (1:2:2), and (2:2:2, di-heterodimer), where ZNRF3 contains the RING domain, **b,** The map of the LGR4-RSPO2(Fu)-ZNRF3 complex in its di-heterodimer form (2:2:2), with ZNRF3 containing the RING domain. **c**, The construct used for the LGR4-RSPO2-ZNRF3(RING) complex in (1:2:2, pentamer A) and the LGR4-RSPO2-ZNRF3(di-heterodimer, 2:2:2), in which the ZNRF3 RING domain is truncated (**c**). **d**, The map of the LGR4-RSPO2-ZNRF3 complex in its di-heterodimer form (2:2:2), without RING domain of ZNRF3.

Additionally, we attempted to assemble the complex of LGR4 and ZNRF3 without RSPO2, but this effort failed, indicating that LGR4 and ZNRF3 cannot form a stable complex on their own. The presence of RSPO2 increased the association between LGR4 and ZNRF3, facilitating the formation of a stable complex. Our NanoBiT experiments showed that the addition of RSPO2 led to two LGR4 molecules becoming closer, while two LGR4 protomers had no interaction (Response Fig. 6). This is because ZNRF3 brings two LGR4 together through RSPO2, indicating that RSPO2 bridging increases the possibility of Di-heterotrimer assembly.



Response Fig. 6. **a**, Schematic of the NanoBiT cell-based assay. The structure shows the proximity of two NB52 (pink). LgBiT (large subunit, light green) and SmBiT (small subunit, light brown) fragments are fused to the C-terminus of two NB52, respectively. The assembly of LGR4-RSPO2-ZNRF3(2:2:2) complex in the schematic facilitates the luminescence complementation of NB52-LgBiT and NB52-SmBiT. **b**, The results demonstrate a strong luminescent signal when RSPO2,

NB52-LgBiT and NB52-SmBiT, along with furimazine, are added to 293T cells or MKN45 cancer cells expressing LGR4 and ZNRF3. Each value represents the mean ± SEM from three independent experiments.

Furthermore, Q.Y. Liu et al. found that the monovalent RSPO2 furin domain had much lower affinity for binding to LGR4 or RNF43/ZNRF3 than the bivalent form, suggesting that LGR4 and RNF43/ZNRF3 form a 2:2 dimer that accommodates the bivalent binding of RSPOs. However, the LGR4-ZNRF3 heterodimer-dimer on the cell surface is possibly too loose to assemble a stable complex. We have elaborated on this point in the revised manuscript.

In summary, our findings and supporting literature indicate that RSPO2 is crucial for the stable assembly of the complex. We have highlighted these points and the physiological relevance of the Di-heterotrimer in the revised manuscript to strengthen our argument.

Thank you once again for your valuable feedback. We believe these revisions and additional discussions significantly strengthen our manuscript.

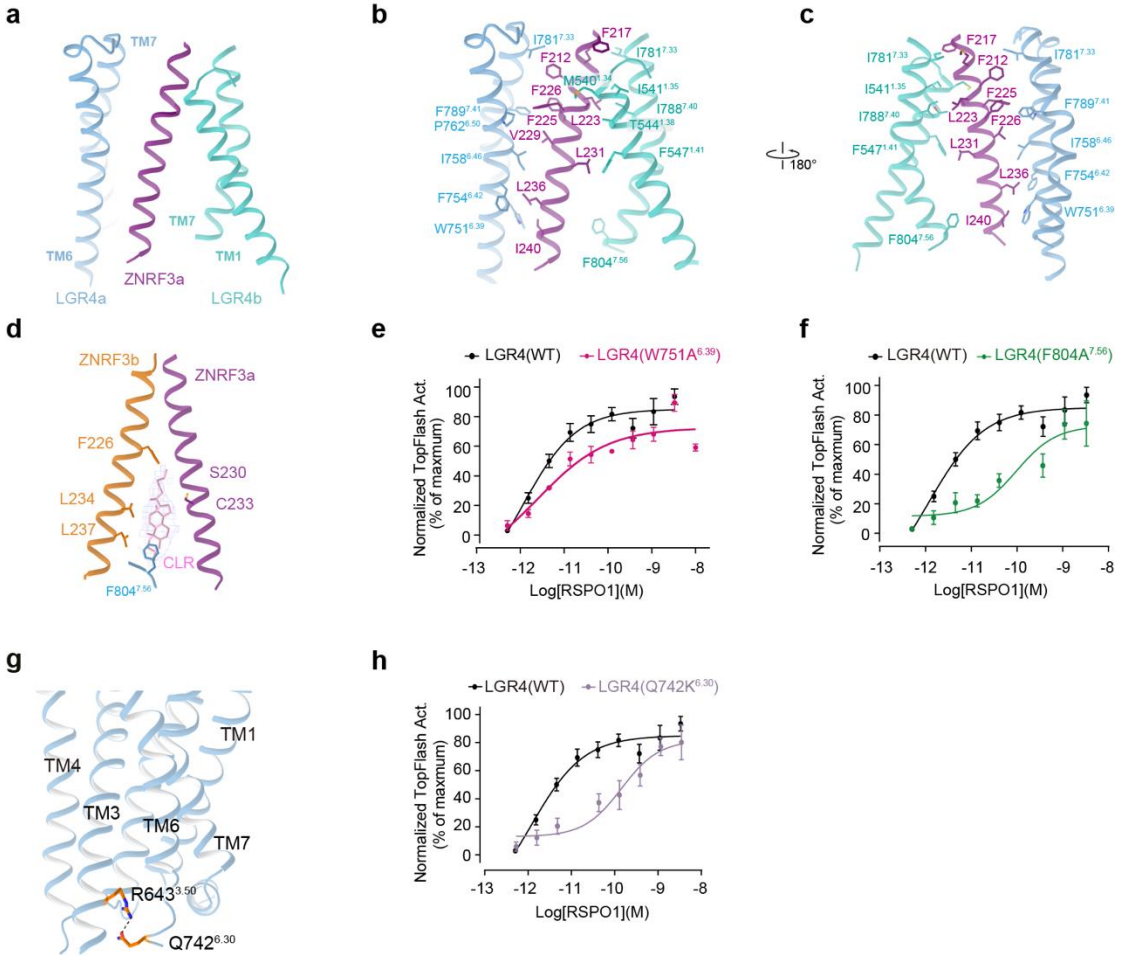
14. (P11 L9) Please represent W751A, F804A, and Q742K mutants on the structures in the figures to provide a better view of their involvement in the interfaces and/or refer to Extended Data Fig. 6e where two of the three residues are already depicted.

Thank you for your insightful feedback. We apologize for not clearly representing W751A$^{6.39}$, F804A$^{7.56}$, and Q742$^{6.30}$ on the structures, as well as their interacting residues in the interface in the figure. We have addressed your comments as follows:

We have now provided detailed descriptions and illustrations of these interactions. Specifically, we have presented the hydrophobic interactions between the W751A$^{6.39}$ sidechain from LGR4 and both sidechains of I240 and L236 from the single membrane helix of ZNRF3. The F804A$^{7.56}$

residue on LGR4a's TM6 interacts with a cholesterol molecule positioned between the two ZNRF3 TMHs, which in turn interacts with the other ZNRF3 TMH (TMHb). This suggests that the cholesterol molecule regulates both the interaction between the two ZNRF3 TMHs and the transmembrane interaction between LGR4 and ZNRF3, thereby modulating ZNRF3's catalytic activity. Mutations ($W751A^{6.39}$ or $F804A^{7.56}$) affecting the LGR4-ZNRF3 interface impair RSPO1-dependent TOPFlash activity, highlighting the importance of this interaction in the WNT/β-catenin signaling pathway. (Response Fig. 7)

We appreciate your attention to detail and valuable suggestions, which have significantly improved the clarity and accuracy of our manuscript.



Response Fig.7 **a,** The interface between the transmembrane domains of LGR4 and ZNRF3. **b, c,** The side chain interactions between LGR4 and ZNRF3 within the transmembrane region of the LGR4-RSPO2-ZNRF3 complex (2:2:2) are shown in detail. **d,** The specific interactions between the transmembrane domain of LGR4, ZNRF3 and cholesterol within the LGR4-RSPO2-ZNRF3

complex (2:2:2) are highlighted. e, f, Dose-dependent TOPFlash activity induced by WT (black) or W751A$^{6.39}$ mutant (red, **e**) and F804A$^{7.56}$ mutant (green, **f**) of LGR4 after stimulation with RSPO1. **g,** The ionic lock between R643$^{3.50}$ of TM3 and Q742$^{6.30}$ of TM6 in LGR4. h, TOPFlash plot illustrating the effect of breaking ionic lock via Q742K$^{6.30}$ mutant (purple) in the transmembrane domain of LGR4 on the activity of RSPO1, compared to that of WT (black). Each value represents the mean ± SEM from three independent experiments. LGR4 (WT) datasets in all three panels are identical.

15. P35 L10 change “and a blot force of 2 for 3 s” replace blot force by blot time

Thank you for your careful review and suggestion. We have made the necessary correction. We appreciate your attention to detail and valuable feedback.

16. P38 “manufacture “change to “manufacturer”

Thank you for pointing out this typographical error. We have corrected "manufacture" to "manufacturer" in the manuscript. We appreciate your careful review and attention to detail. Thank you.

17. “Moreover, LGR4 strengthens its association with ZNRF3 in the assembly with 2:2:2 stoichiometric ratio (Fig. 4a-d), facilitating their joint endocytosis and promoting Wnt signaling.” – While cointernalization has been pointed out at an important mechanism, the authors do not include experiments assessing the role of stoichiometry for endocytosis efficicency of the individual components. We suggest therefore to be a bit more speculative on this end.

Thank you for your insightful suggestions. We have addressed your points as follows:

While we recognize the importance of stoichiometry in the co-internalization and its potential impact on Wnt signaling, we did not perform specific experiments to directly assess the role of

stoichiometry in the endocytosis efficiency of individual components. Therefore, we have revised the manuscript to be more speculative on this point.

Revised sentence:
"Moreover, LGR4 strengthens its association with ZNRF3 in the assembly with a proposed 2:2:2 stoichiometric ratio (Fig. 4a-f), which we hypothesize may facilitate their joint endocytosis and promote Wnt signaling. While co-internalization is highlighted as an important mechanism, further experiments are required to assess the precise role of stoichiometry in the endocytosis efficiency of individual components."

This revision acknowledges the hypothesis regarding stoichiometry's role while clearly stating the need for further experimental validation. Thank you again for your valuable feedback.

Reviewer #2 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Thank you for your valuable suggestions and positive feedback.

Reviewer #3 (Remarks to the Author):

Geng et al. present several cryoEM structures of LGR4/RPSO2/ZNRF3 complexes from the Wnt signaling pathway demonstrating a variety of stoichiometries of complexes. From this they attempt to build a picture of how these proteins work together to modulate Wnt signaling. These are potentially very interesting results for a tricky system. However, the paper has substantial issues in the experimental data, its presentation, and its interpretation that would preclude it from publication without additional studies and reworking of the manuscript.

Thank you for your detailed review and insightful comments regarding our manuscript. We appreciate your recognition of the potential interest in our cryoEM structures of the LGR4/RSPO2/ZNRF3 complexes and their role in modulating Wnt signaling. We understand that there are significant issues that need to be addressed in the experimental data, its presentation, and its interpretation.

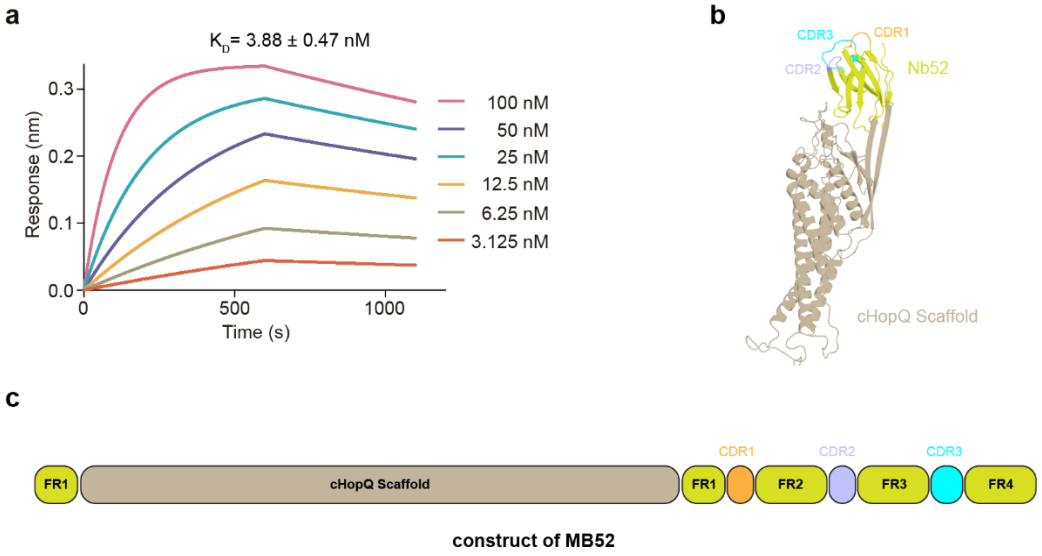
The largest issue arises from the fact that half of the data is described as deriving from a RING-truncated construct with GFP/a GFP binder to cement the complex while the other half of the data is described as deriving from a full length construct cemented with LgBiT HiBit, with poor annotation of which figure panels come from which data set. This results in both significant confusion for the reader and fundamental conclusions that seem like they could plausibly derive from the truncation of the constructs rather than actual molecular physiology. Particularly as RING-dimerization is a key part of ZNRF3 activation and the 1:2:2 complex without RING shows also poor interaction, it seems crucial to demonstrate that the 2:2:2 state observed is indeed a true state and not an artifact of the absence of the dimerizing RING domains. Ideally, additional data should be collected with the full length constructs in order to obtain a sufficient resolution reconstruction of the near-wild-type 2:2:2 complex (Extended Data 3) to at least demonstrate agreement with the truncated 2:2:2 (if not a high resolution reconstruction outright). This would allow for the key conformational changes of the study to be definitively attributed to reality and not construct design. The truncated data, if presented, should be presented in a clearer, more straightforward manner where constructs are better delineated. However, there are still several

additional issues detailed further that would also need to be addressed in order for the manuscript to be publishable.

We sincerely appreciate the reviewer's thorough and insightful feedback on our manuscript. To address these concerns, we have provided a detailed explanation and clarification of our experimental design, data collection, and interpretation. In particular, we have added a new paragraph in the results section of the revised manuscript titled "Structure Determination of LGR4-RSPO2-ZNRF3 Complexes" to elucidate the protein design, purification optimization, and data processing used to obtain structures of the LGR4-RSPO2-ZNRF3 complexes with different stoichiometric ratios.

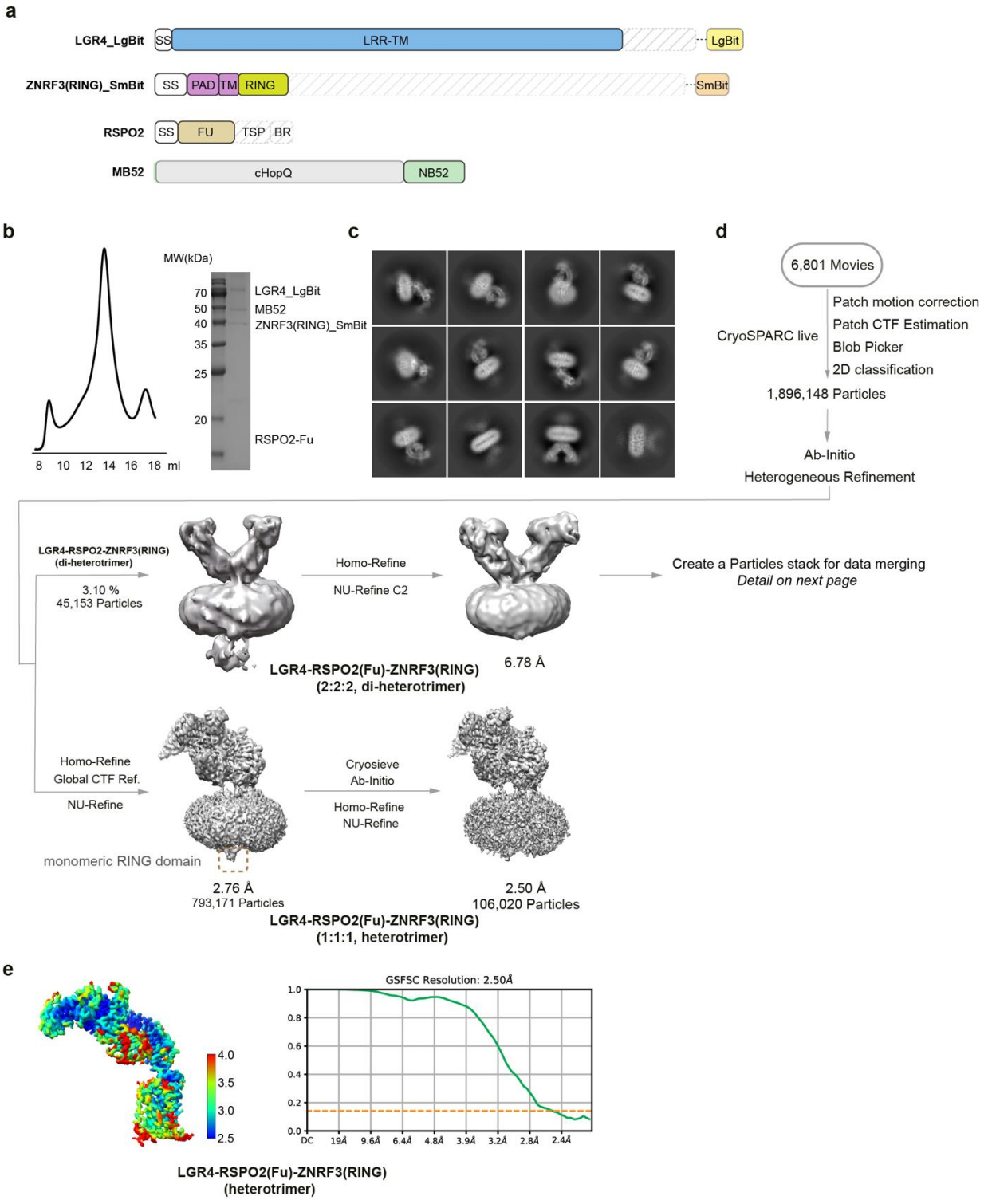
Structure Determination of LGR4-RSPO2-ZNRF3 Complexes

To facilitate structural determination, we developed a high-affinity nanobody (NB52) targeting the ectodomain of LGR4, which we expanded into a larger "megabody" (MB52) to optimize particle orientation. (Response Fig. 8)



Response Fig. 8 **Generation and characterization of camelid nanobody. a,** Measurement of binding affinity between NB52 and LGR4 using biolayer interferometry (BLI) and fitting with a 1:1 binding model. **b**, the model of MB52 predicted by swiss-model. **c**, Domain compositions of MB52 is indicated schematically.

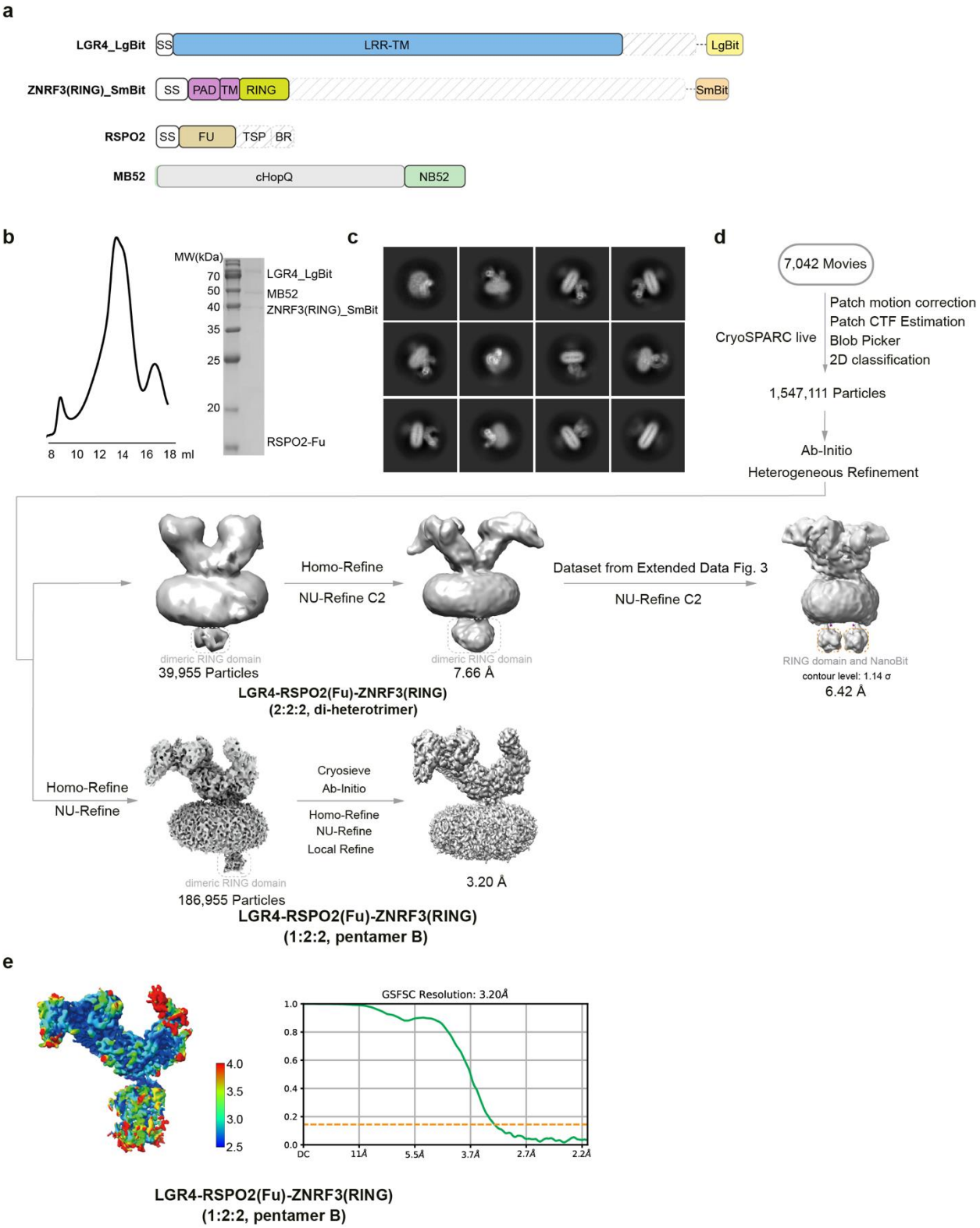
We employed NanoBiT technology to stabilize the interaction between ZNRF3 and LGR4. Specifically, we genetically fused fragments of LgBiT and HiBiT to the C-terminus of LGR4 and the RING domain of ZNRF3, respectively (Extended Data Fig. 2a). LGR4 and ZNRF3 were expressed separately, mixed with RSPO2 during extraction, and subsequently purified to form the LGR4-RSPO2-ZNRF3 complex. We observed different assemblies in 1:1:1 and 2:2:2 stoichiometric ratios. The predominant 1:1:1 particle formation was used for 3D reconstruction, homogeneous refinement, global CTF refinement, and non-uniform refinement, resulting in a reconstruction map with 2.50 Å resolution. The 1:1:1 LGR4-RSPO2-ZNRF3 complex was classified as a heterotrimer. In contrast, the 2:2:2 particle accounted for 3.1% of the good particles, resulting in a final map resolution of approximately 6.78 Å after 3D reconstruction, which was insufficient for high-resolution modeling. (Response Fig. 9) This 2:2:2 complex is referred to as the di-heterotrimer.



Response Fig. 9 **Cryo-EM data analysis of the LGR4-RSPO2(Fu)-ZNRF3(RING)-MB52 complex (1:1:1, heterotrimer). a**, Domain compositions of LGR4, ZNRF3, RSPO2, and MB52 are indicated schematically, along with a schematic representation of their constructs used for

structure determination. **b**, SEC profiles (left) and SDS-PAGE (right) of the LGR4-RSPO2(Fu)-ZNRF3(RING)-MB52 complex (heterotrimer, with a 1:1:1 stoichiometry). Genetic fusions of LgBiT and HiBiT were introduced at the C-terminus of LGR4 and the ZNRF3 RING domain (including the RING domain), the band corresponding to LGR4 appears smeared due to high glycation. **c,** 2D classification of the particles for the LGR4-RSPO2-ZNRF3(RING)-MB52 complex. The particles encompass complexes in ratios of 1:1:1, and 2:2:2. **d, e**, Flowchart illustrating the Cryo-EM image acquisition and data processing steps used to obtain the structure of the LGR4-RSPO2(Fu)-ZNRF3(RING)-MB52 complex (1:1:1, heterotrimer) (**d**), along with their corresponding local resolution and Fourier shell correlation (FSC) curves (**e**).

To enhance the resolution of the 2:2:2 LGR4-RSPO2-ZNRF3 complexes, we optimized the expression system, and purification protocol. These improvements led to the predominant formation of the LGR4-RSPO2-ZNRF3 complex, enabling cryo-EM data collection and processing. Co-expression and purification of LGR4 and ZNRF3 in the presence of RSPO2 primarily yielded pentamer B, with a final map resolution of approximately 3.20 Å. Notably, the RING domain was observed extending into the cytoplasm in low-pass-filtered maps. Cryo-EM analysis also identified a few particles of the di-heterotrimer, achieving a map resolution of approximately 7.66 Å (Response Fig. 10). Despite merging two datasets of the di-heterotrimer, the map resolution did not improve. However, 3D variability analysis in cluster mode revealed that the 2:2:2 LGR4-RSPO2-ZNRF3 complexes exhibit significant dynamics.

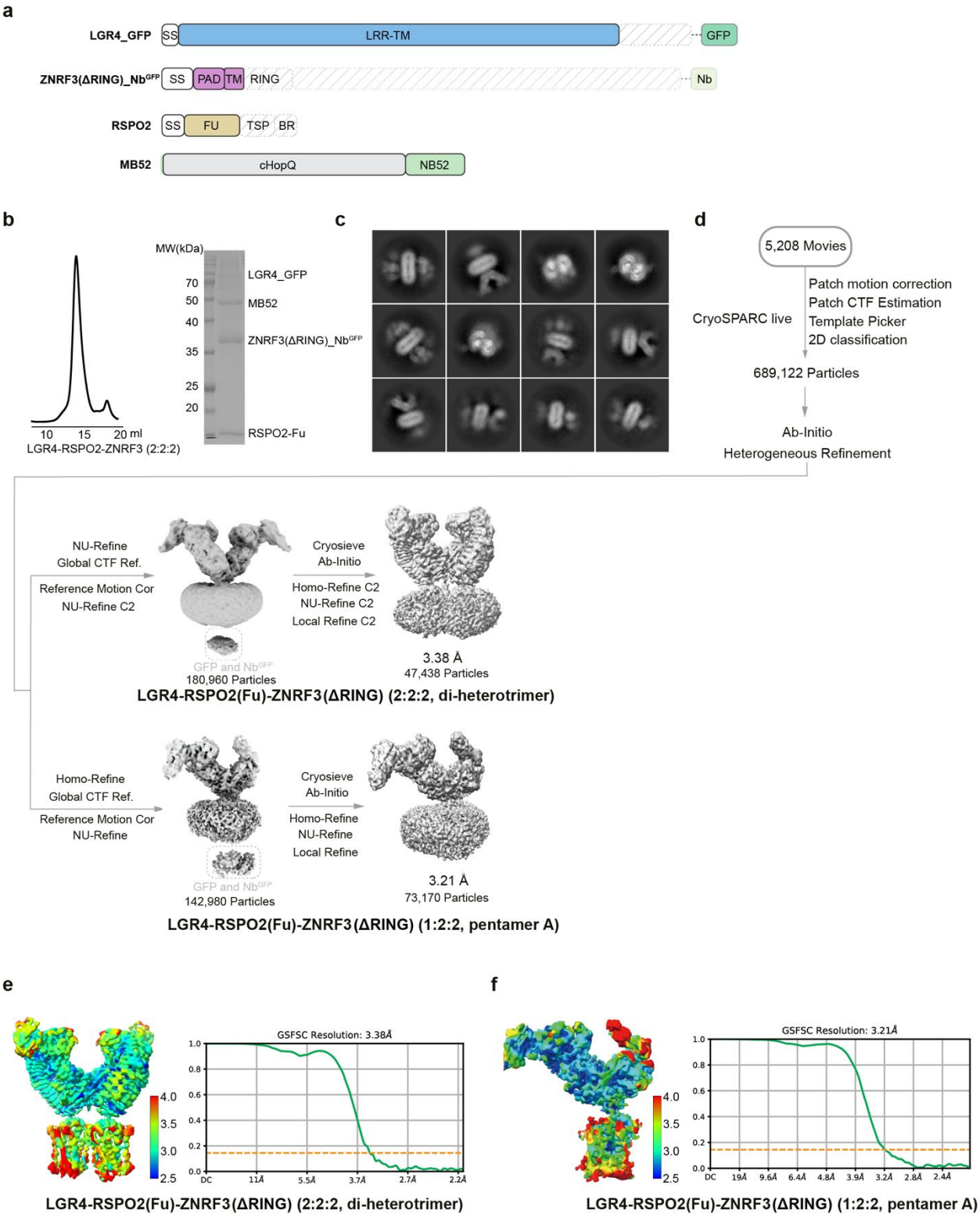


Response Fig. 10 **Cryo-EM data analysis of the LGR4-RSPO2-ZNRF3(RING)-MB52 complex (1:2:2, pentamer B). a**, Domain compositions of LGR4, ZNRF3, RSPO2, and MB52

are indicated schematically, along with a schematic representation of their constructs used for structure determination. **b**, SEC profiles (left) and SDS-PAGE (right) of the LGR4-RSPO2(Fu)-ZNRF3(RING)-MB52 complex (1:2:2, pentamer B), Genetic fusions of LgBiT and HiBiT were attached to the C-terminus of LGR4 and the ZNFR3 RING domain (including the RING domain). The band corresponding to LGR4 appears smeared due to high glycation. **c,** 2D classification of the particles for the LGR4-RSPO2-ZNRF3(RING)-MB52 complex with a 1:2:2 ratio. **d**, **e,** Flowchart illustrating the Cryo-EM image acquisition and data processing steps used to obtain the structure of the LGR4-RSPO2ZNRF3(RING)-MB52 complex (1:2:2, pentamer B) (**d**), along with their corresponding local resolution and Fourier shell correlation (FSC) curves (**e**).

Finaly, we optimized the construct design and purification protocol. Obtaining sufficient particles of the LGR4-RSPO2-ZNRF3 (2:2:2) complex using the ZNRF3 construct with the RING domain proved challenging. To address this, we genetically fused GFP and anti-GFP nanobody to the C-terminus of LGR4 and ZNRF3ΔRING (RING domain truncated), respectively, using appropriately designed linkers. We co-expressed and purified LGR4 and ZNRF3ΔRING in the presence of RSPO2. During 2D analysis, two distinct assemblies of LGR4, RSPO2, and ZNRF3 subunits were observed. Rigorous 3D classification successfully resolved the di-heterotrimer structure (2:2:2) with an overall resolution of 3.38 Å and another assembly map with a 1:2:2 stoichiometry, designated as pentamer A, with a resolution of approximately 3.21 Å (Response Fig. 11). These structures collectively highlight the heterogeneity in the assembly of RSPO2, ZNRF3, and LGR4.

Furthermore, ZNRF3 consists of an extracellular domain, a single transmembrane domain, and a RING domain followed by a long flexible C-terminal tail. Due to the low expression levels of the wild-type ZNRF3 construct, we truncated the long flexible C-terminal tail for structural determination.



Response Fig. 11 **Cryo-EM data analysis of the LGR4-RSPO2(Fu)-ZNRF3(ΔRING)-MB52 complex (2:2:2, di-heterotrimerand 1:2:2, pentamer A). a**, Domain compositions of LGR4,

ZNRF3, RSPO2, and MB52 are indicated schematically, along with a schematic representation of their constructs used for structure determination. **b**, SEC profiles (left) and SDS-PAG (right) of the LGR4-RSPO2-ZNRF3(ΔRING)-MB52 complex (2:2:2, di-heterotrimer, and 1:2:2, pentamer A), In these complexes, GFP and anti-GFP nanobody were genetically fused to the C-terminus of LGR4 and ZNRF3ΔRING (RING domain truncated). The band corresponding to LGR4 appears smeared due to high glycation. **c,** 2D classification of the particles for the LGR4-RSPO2-ZNRF3-MB52 complex (di-heterotrimer, with a 2:2:2 stoichiometry, and pentamer A, with a 1:2:2 stoichiometry). **d**, Flowchart illustrating the Cryo-EM image acquisition and data processing steps used to obtain the structure of the LGR4-RSPO2-ZNRF3(ΔRING)-MB52 complex (2:2:2,di-heterotrimer) and the LGR4-RSPO2-ZNRF3(ΔRING)-MB52 complex (1:2:2, pentamer A), along with their corresponding local resolution and Fourier shell correlation (FSC) curves (**e**, **f**).

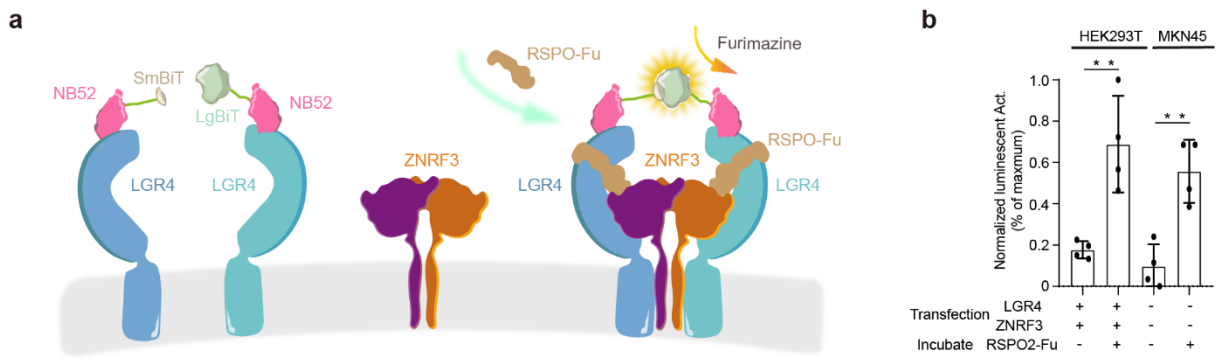
The use of LgBiT/HiBiT fusion has recently become a valuable approach for stabilizing protein complexes in structural studies, as evidenced by recent literature: Duan, J., et al. Nat Commun 11, 4121 (2020); Chisae Nagiri et al. Molecular Cell, Volume 81, Issue 15, 2021, Pages 3205-3215; Ji, SY., et al. Cell Discov 8, 116 (2022); Fumiya K., et al. eLife 12; Xia, R., et al. Nat Commun 12, 2086 (2021); Chen, G., et al. Nat Commun 13, 2375 (2022); Ji, Y., et al. Nat Commun 14, 1268 (2023).

Additionally, we used GFP/anti-GFP nanobody fusion to the C-terminus of LGR4 and ZNRF3 to further stabilize the LGR4-RSPO2-ZNRF3 complex for structural determination (Response Fig. 11a). Importantly, both LgBiT/HiBiT and GFP/anti-GFP nanobody were fused to the C-terminus of LGR4 and ZNRF3 through long flexible linkers (3xGGGGS), which stabilized the interaction between LGR4 and ZNRF3 without creating artificial interactions.

We also obtained the map of the di-heterotrimer with a 2:2:2 stoichiometry using the ZNRF3 construct containing the RING domain. Although this structure had a lower resolution, it demonstrated consistency with the di-heterotrimer structure obtained using the ZNRF3 construct with a truncated RING domain.

The various protein designs and purification optimizations introduced some confusion for readers, but our intent was to acquire sufficient particles with diverse orientations for high-resolution structural determination, necessitating different strategies. We have revised the manuscript to clearly delineate our methods and results.

To detect the presence of the LGR4-RSPO2-ZNRF3 complex (2:2:2) on the cell surface, we designed a NanoBiT sensor based on the binding site of NB52. Structures reveal that the proximity of two NB52 molecules facilitates the complementarity of LgBiT and SmBiT fragments. When NB52-LgBiT and NB52-SmBiT, along with a substrate, were introduced to HEK293T cells (or MKN45 cancer cells) expressing LGR4 and ZNRF3, a robust luminescent signal was generated upon the addition of RSPO2 (Response Fig. 12). This outcome signifies the presence of the LGR4-RSPO2-ZNRF3 complex (2:2:2) on the cell surface.



Response Fig. 12 **a**, Schematic of the NanoBiT cell-based assay. The structure shows the proximity of two NB52 (pink). LgBiT (large subunit, light green) and SmBiT (small subunit, light brown) fragments are fused to the C-terminus of two NB52, respectively. The assembly of LGR4-RSPO2-ZNRF3(2:2:2) complex in the schematic facilitates the luminescence complementation of NB52-LgBiT and NB52-SmBiT. **b**, The results demonstrate a strong luminescent signal when RSPO2, NB52-LgBiT and NB52-SmBiT, along with furimazine, are added to 293T cells or MKN45 cancer cells expressing LGR4 and ZNRF3. Each value represents the mean ± SEM from three independent experiments.

Our findings are in agreement with a recent report indicating that LGR4 and RNF43/ZNRF3 form a 2:2 dimer, allowing for the bivalent binding of RSPO2 on the cellular membrane (Scientific Report 13, 10796, 2023).

Collectively, all evidence demonstrates that the 2:2:2 state observed is indeed a true physiological state and not an artifact of the absence of dimerizing RING domains.

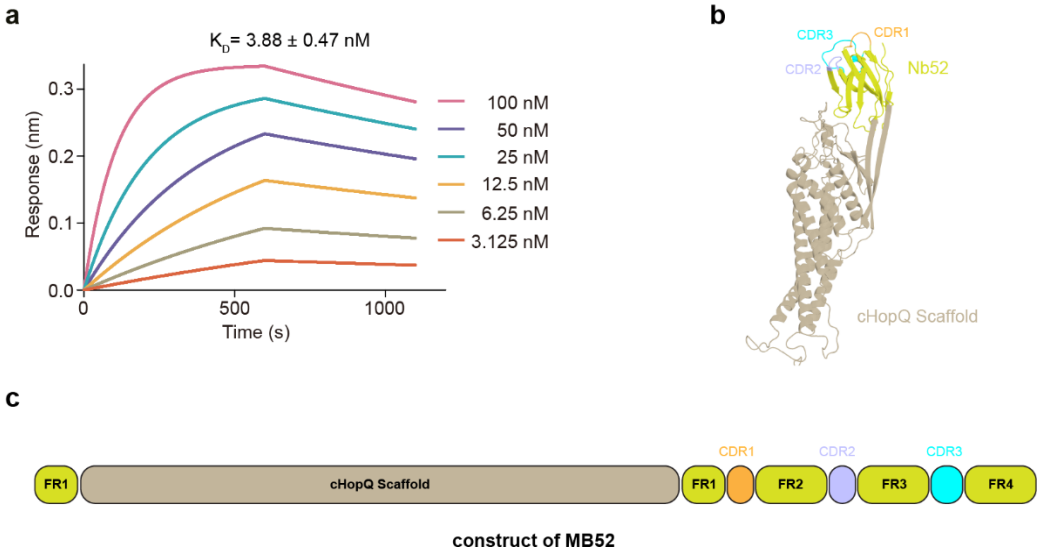
A point by point critique is provided below:

There are several different megabody designs, including several based around HopQ, please state more clearly which one was used (even though none of the rest of the megabody beyond the nanobody was resolved). I think it would be also beneficial to the reader to comment on whether the megabody was really needed (e.g. whether datasets with just Nb52, if they were done, had bad preferred orientation) or if it was just what was tried initially.

Thank you for your insightful comments.

We employed the megabody design developed by Tomasz Uchański et al., which involves grafting nanobodies onto selected protein scaffolds to increase their molecular weight. Specifically, we utilized the adhesin domain of Helicobacter pylori (HopQ, 45 kDa, PDB ID 5LP2).

The VHH gene of NB52 was expanded by fusion to the circular permutated extracellular adhesin domain of Helicobacter pylori (HopQ, 45 kDa) to generate a megabody, referred to as MB52. (Response Fig. 13)



Response Fig. 13 **Generation and characterization of camelid nanobody. a,** Measurement of binding affinity between NB52 and LGR4 using biolayer interferometry (BLI) and fitting with a

1:1 binding model. **b**, the model of MB52 predicted by swiss-model. **c**, Domain compositions of MB52 is indicated schematically.

Initially, we attempted to determine the structure of the LGR4-ZNRF3-RSPO2 complex using just NB52. However, these efforts were unsuccessful due to issues with preferred particle orientation. The use of MB52 proved essential in this context. MB52 helped randomize the orientation of particles, thereby facilitating better 3D cryo-EM reconstructions of the LGR4-ZNRF3-RSPO2 complex. Moreover, the megabody served as a fiducial marker, further aiding in achieving high-quality reconstructions.

We will ensure to clearly state in the revised manuscript that the megabody used was MB52, derived from the HopQ scaffold, and emphasize its necessity in overcoming the challenges posed by preferred orientation in the datasets.

Thank you once again for your valuable feedback. We believe these revisions will significantly enhance the clarity and robustness of our manuscript.

Page 6 line 13-14 the punctuation of this sentence is confusing, I think a comma needs to be removed or a semicolon added depending on what the authors were trying to say.

Thank you for your suggestion. We apologize for the confusion caused by our punctuation. We have revised the sentence for clarity as follows: LGR4 and ZNRF3 were expressed separately, then mixed with RSPO2 during extraction, and subsequently purified to form the LGR4-RSPO2-ZNRF3 complex.

I find the assertion that MB52 stabilizes the complex on page 6 line 19-20 to be potentially problematic, as it seems to bind primarily to the outer extracellular portion of the ECD without contacting ZNRF3 or RSPO2. Additionally there is no direct pharmacological characterization of NB/MB52 to suggest it enhances complex formation and the nanobody was suggested to be raised against LGR4 alone. Indeed, I think if NB/MB52 potentially stabilizes various complexes preferentially that would actually be problematic for the assertions of the study. I would highly

suggest the authors provide some level of characterization of if and how MB52/NB52 influences LGR4 complex stoichiometry.

Thank you for pointing out this issue. We completely agree with the reviewer's assessment. MB52 binds exclusively to the extracellular domain of LGR4 without interacting with RSPO2 or ZNRF3. Furthermore, it does not alter the conformation of LGR4, influence the stoichiometry of the LGR4 complex, or perform any pharmacological functions. Instead, MB52 aids in the randomization of particle orientation, which facilitates better 3D cryo-EM reconstructions of the LGR4-ZNRF3-RSPO2 complex. Additionally, the megabody serves as a fiducial marker, further assisting in achieving high-quality reconstructions.

For the section “Structure of LGR4-RSPO2-ZNRF3 complex”, there seems to be currently no justification provided for why the initial work was done with a GFP/anti-GFP and RING truncated version and the following work is done with the RING domain included and LgBiT HiBiT. Some justification/explanation for the swap and why both structures are presented in the introduction of the paragraph is warranted to prevent the reader from being confused. Again, repeating the structure with the full length construct is necessary for the validity of the study.

Thank you for your valuable feedback. We understand the need for clarity regarding the different constructs used in our study. Here is a detailed explanation:

We employed NanoBiT technology to stabilize the interaction between ZNRF3 and LGR4. Specifically, we genetically fused fragments of LgBiT and HiBiT to the C-terminus of LGR4 and the RING domain of ZNRF3, respectively (Extended Data Fig. 2a). LGR4 and ZNRF3 were expressed separately, mixed with RSPO2 during extraction, and subsequently purified to form the LGR4-RSPO2-ZNRF3 complex. We observed different assemblies in 1:1:1 and 2:2:2 stoichiometric ratios. The predominant 1:1:1 particle formation was used for 3D reconstruction, homogeneous refinement, global CTF refinement, and non-uniform refinement, resulting in a reconstruction map with 2.50 Å resolution. The 1:1:1 LGR4-RSPO2-ZNRF3 complex was classified as a heterotrimer. In contrast, the 2:2:2 particle accounted for 3.1% of the good particles,

resulting in a final map resolution of approximately 6.78 Å after 3D reconstruction, which was insufficient for high-resolution modeling. (Response Fig. 9)

To enhance the resolution of the 2:2:2 LGR4-RSPO2-ZNRF3 complexes, we optimized expression system, and purification protocol. These improvements led to the predominant formation of the LGR4-RSPO2-ZNRF3 complex, enabling cryo-EM data collection and processing. Co-expression and purification of LGR4 and ZNRF3 in the presence of RSPO2 primarily yielded pentamer B, with a final map resolution of approximately 3.20 Å. Cryo-EM analysis also identified a few particles of the di-heterotrimer, achieving a map resolution of approximately 7.66 Å (Response Fig. 10). Despite merging two datasets of the di-heterotrimer, the map resolution did not improve. However, 3D variability analysis in cluster mode revealed that the 2:2:2 LGR4-RSPO2-ZNRF3 complexes exhibit significant dynamics.

Obtaining sufficient particles of the LGR4-RSPO2-ZNRF3 (2:2:2) complex using the ZNRF3 construct with the RING domain proved challenging. To address this, we genetically fused GFP and anti-GFP nanobody to the C-terminus of LGR4 and ZNRF3ΔRING (RING domain truncated), respectively, using appropriately designed linkers. We co-expressed and purified LGR4 and ZNRF3ΔRING in the presence of RSPO2. During 2D analysis, two distinct assemblies of LGR4, RSPO2, and ZNRF3 subunits were observed. Rigorous 3D classification successfully resolved the di-heterotrimer structure (2:2:2) with an overall resolution of 3.38 Å and another assembly map with a 1:2:2 stoichiometry, designated as pentamer A, with a resolution of approximately 3.21 Å (Response Fig. 11).

We have revised the introduction of the relevant paragraph to include this justification and explanation for the use of different constructs. This ensures that readers understand the rationale behind presenting both structures and the importance of each approach in validating our study.

We appreciate your suggestion and have made the necessary revisions to clarify these points in the manuscript.

The sentence on page 7, line 18-19 is confusing; a predominant complex compared to separate

components, or of a certain stoichiometry? I assume it is referring to the 1:1:1 but this should be more explicitly stated.

Thank you for your valuable feedback. We apologize for any confusion caused by the sentence on page 7, lines 18-19. You are correct in assuming that it refers to the 1:1:1 stoichiometry. We have revised the sentence for clarity as follows:

"The cryo-EM analysis revealed that the predominant complex observed was the LGR4-RSPO2-ZNRF3 complex with a 1:1:1 stoichiometry, in comparison to other assemblies."

We hope this revision makes our intention clear. Thank you again for your careful review and helpful suggestion.

For the section "structures of … complexes with 1:2:2 stoichiometry" I again feel there is insufficient description of how/why this varies from the 1:1:1 work; is this the same constructs, different titer? Same construct but with no specific titer? This should be laid out more clearly.

Thank you for your insightful comments. We acknowledge the need for a clearer explanation regarding the differences in constructs, expression, and purification methods used for the 1:1:1 and 1:2:2 stoichiometric ratios of the LGR4-RSPO2-ZNRF3 complexes. Below is a detailed clarification:

For the **LGR4-RSPO2-ZNRF3 complex with a 1:1:1 stoichiometry**, LGR4 and ZNRF3 were expressed separately in HEK293 cells, and RSPO2 was added during the extraction process. The components were then purified together, resulting in the formation of the LGR4-RSPO2-ZNRF3 complex. Cryo-EM analysis revealed that the predominant assembly formed in this condition was the LGR4-RSPO2-ZNRF3 complex with a 1:1:1 stoichiometry. The 1:1:1 complex was thus more prevalent compared to other assemblies, including the 2:2:2 complex, which appeared in a minor population (Response Fig. 9).

In contrast, for the **LGR4-RSPO2-ZNRF3 complex with a 1:2:2 stoichiometry**, we used a different approach. LGR4 and ZNRF3 (with the RING domain) were **co-expressed** in HEK293 cells, rather than being expressed separately. RSPO2 was added during the purification process.

Under these conditions, the LGR4-RSPO2-ZNRF3 complex with a 1:2:2 stoichiometry predominated, achieving a final map resolution of approximately 3.20 Å. A small number of particles representing the LGR4-RSPO2-ZNRF3 complex with a 2:2:2 stoichiometry was also detected, though the resolution for this assembly was lower at around 7.66 Å (Response Fig. 10).

In summary, while the constructs used for both the 1:1:1 and 1:2:2 complexes were identical, the **difference in expression and purification methods** led to the formation of different stoichiometric ratios. The **1:1:1 complex** was formed by mixing separately expressed LGR4 and ZNRF3 with RSPO2 during extraction, whereas the **1:2:2 complex** resulted from co-expressing LGR4 and ZNRF3 and then adding RSPO2 during purification. Importantly, both approaches contained a minor population of the LGR4-RSPO2-ZNRF3 complex with a 2:2:2 stoichiometry, but in neither case were the particles sufficient for high-resolution structural reconstruction.
Furthermore, 3D variability analysis using cluster mode revealed that the LGR4-RSPO2-ZNRF3 complexes with 2:2:2 stoichiometry exhibited **significant conformational dynamics**, which may explain the lower resolution observed for this complex. To improve resolution, we undertook several **construct optimizations** aimed at stabilizing the complex and increasing the particle population with the 2:2:2 stoichiometry.
We hope this revised explanation provides a clearer understanding of the distinctions between the two experimental setups and the resulting structural data. Thank you again for your valuable feedback.

When pentamer A is discussed, as this comes from ‘the same sample where a 2:2:2’ structure was obtained, if this is the version of the structure the authors intend to use it should more explicitly describe this as from the delta-RING construct, especially in the figures.

Thank you for your insightful feedback. We have revised our manuscript to clarify this point.

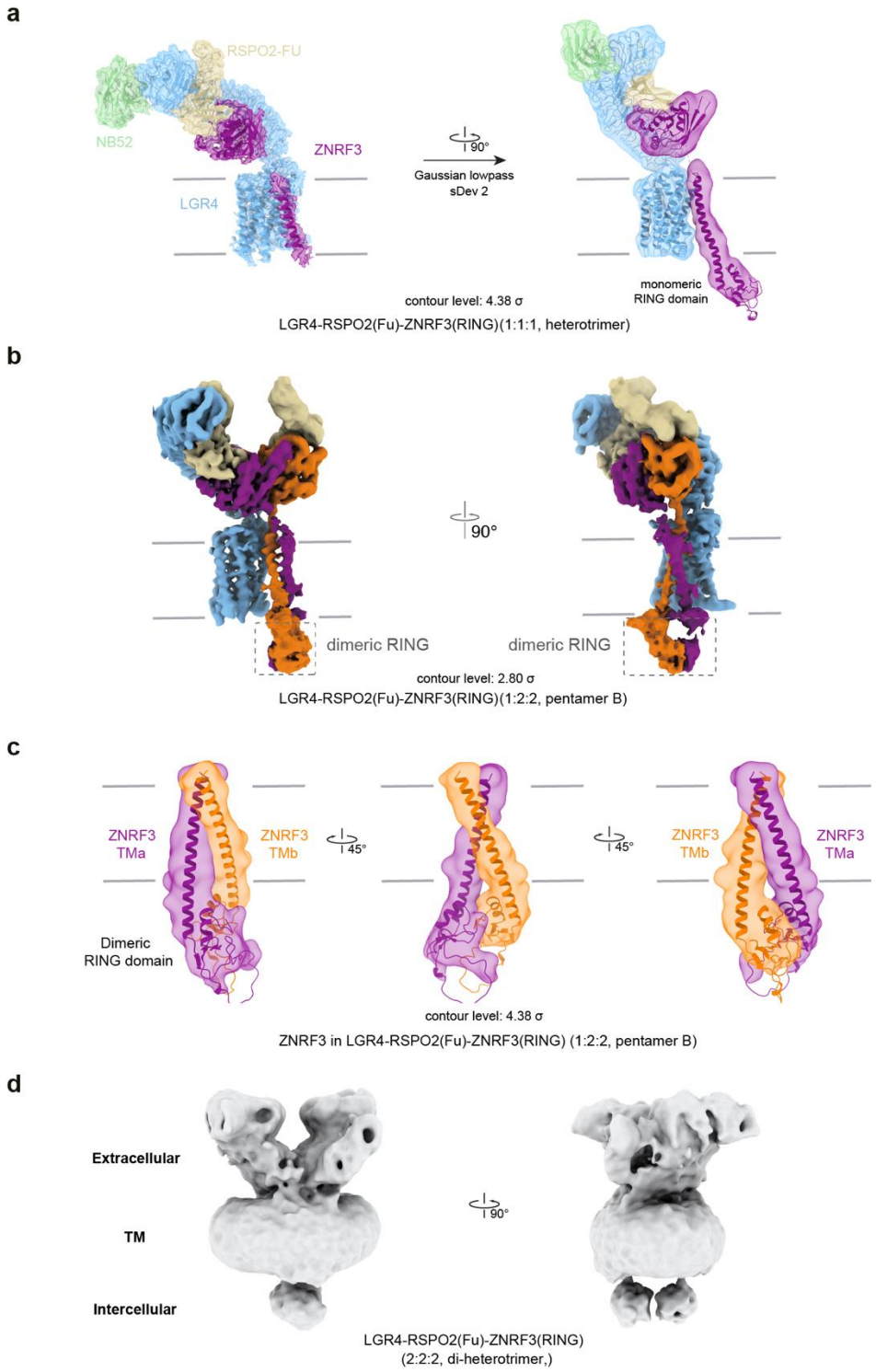
When discussing Pentamer A, we have now explicitly stated that it comes from the same sample where the 2:2:2 structure was obtained, specifically using the delta-RING construct. This clarification has also been added to the relevant figures to ensure consistency and transparency.

We hope this revision addresses the reviewer's concerns and provides a clearer understanding of the experimental context. Thank you again for your valuable feedback.

In Figure 3, panel d is presented next to panel e (pentamer B, second data set with RING domains) analogous to a and b, even though a, b, and e are actually (derived from) the same map. There seems to be the assertion that there was some fitting the map of an and b into d to generate e, although it is underexplained what this entails and how this was done. Also it should be commented on why the authors seem to assume that the connection between the TMD and RING should be rigid and such docking is fair, even though the RING is not so well resolved. There is this key suggested conclusion that the observed 2:2:2 conformation forces separations of the RING domains, and this prevents catalysis, but it is hard to state definitely with a truncated construct. By eye the 3D and 2D classes in extended data 3 almost look like the full length 2:2:2 complex also has the RING domains interacting, although the resolution is poor. The authors themselves note in the conclusion the 2:2:2 complex might reduce activity through sequestration and/or internalization. Again, even a modest resolution 2:2:2 reconstruction with the full length would help clear this up. Constructs where maps were derived from also needs substantially better labeling.

Thank you for your thorough review and insightful comments. We understand the need for clarity regarding the presentation and interpretation of the data in Figure 3, and we have revised the figure accordingly.

While achieving high resolution for the ubiquitylation domain of ZNRF3 has been challenging, we used AlphaFold predictions to gain structural insights. Notably, there is a stable cytoplasmic helix connecting the transmembrane helix (TMH) to the RING domain, which supports our assumption of a relatively rigid connection. Using these AlphaFold results, we fit the predicted structure of ZNRF3(RING) into the low-pass-filtered map of both the 1:1:1 (heterotrimer) and the 1:2:2 (pentamer B) complexes. The dimerization of the PA domain (PAD), TMH, and RING domains in the ZNRF3 dimer is clearly observed, and despite the lower resolution of the RING domain, the fit is reasonable within the available map.

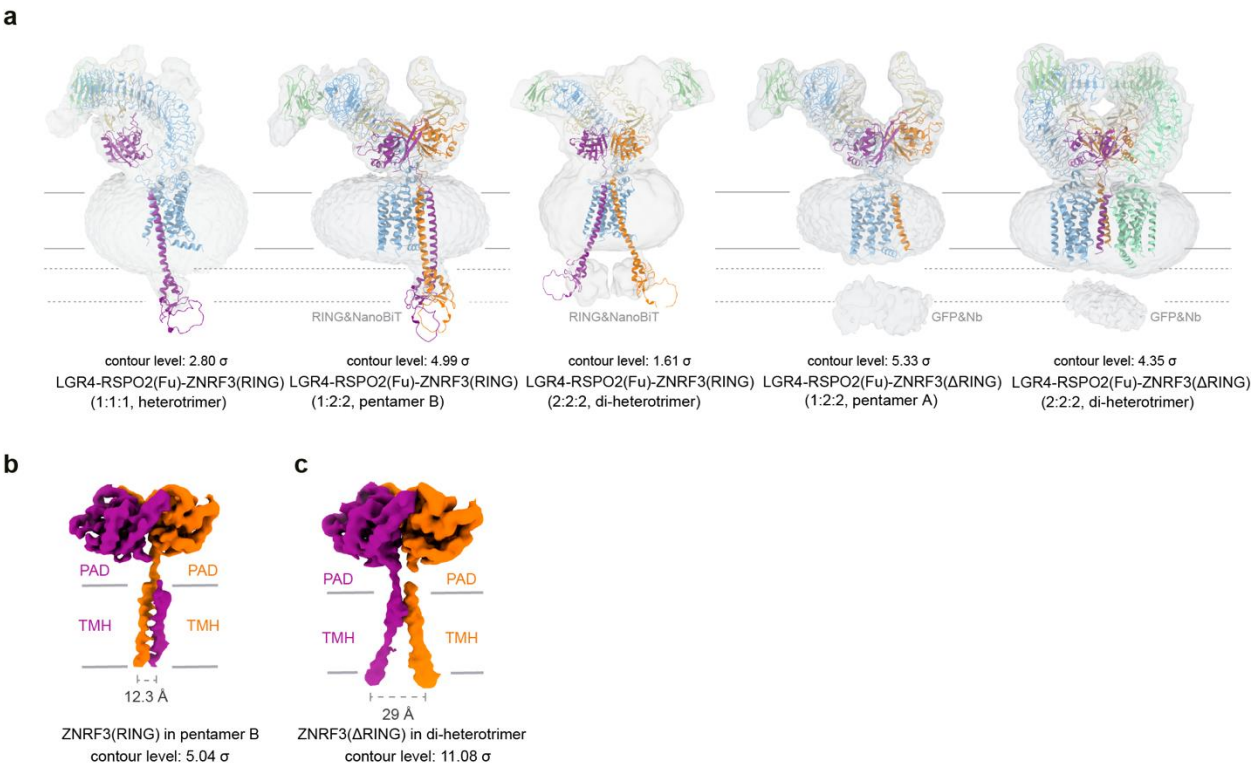


Response Fig. 14 The rearrangement RING domain in different complexes. **a**, Superposition of the map and model of the LGR4-RSPO2-ZNRF3 heterodimer (contour level: 2.8σ). **b**, Map of the LGR4-RSPO2-ZNRF3 complex in pentamer B (contour level: 2.8σ). **c**, Superposition of the map and model of the transmembrane helix (TMH) and RING domain in pentamer B, shown in the

low-pass map (contour level: 4.38σ) from different perspectives. **d**, Superposition of the map and model of the LGR4-RSPO2-ZNRF3 complex in its di-heterodimer form (2:2:2), with ZNRF3 containing the RING domain.

Regarding the LGR4-RSPO2-ZNRF3 complex with a 2:2:2 stoichiometry (including the RING domain), we extracted particles and obtained a structure at very low resolution (Response Fig. 14d). As you correctly noted, the map shows some features below the transmembrane domain, which we interpreted as the separated RING domains and its fused NanoBiT. Our analysis indicates that the map is divided into two distinct parts, corresponding to the separation of the two RING domains and its fused NanoBiT, and it is clear that the cytoplasmic portions of the ZNRF3 TMHs are also separated. In contrast, the 1:2:2 complex (pentamer B) shows a more compact map, with the cytoplasmic portions closely associated. Additionally, the NanoBiT fusion tags may have contributed to the observed map of the 1:2:2 complex.

Interestingly, we observed a similar low-pass-filtered map feature at the bottom of the LGR4-RSPO2-ZNRF3 complex with a 2:2:2 stoichiometry, even in the construct where ZNRF3 lacks the RING domain. We hypothesize that the fused GFP and anti-GFP nanobodies contributed to this map. However, there is a noticeable empty space between the bottom map and the detergent micelle/transmembrane domain in the 2:2:2 complex lacking the RING domain. In contrast, in the 1:1:1, 1:2:2 (pentamer B), and 2:2:2 complexes (with RING domain), the map of the cytoplasmic helix is continuous, connecting the bottom map with the detergent micelle.



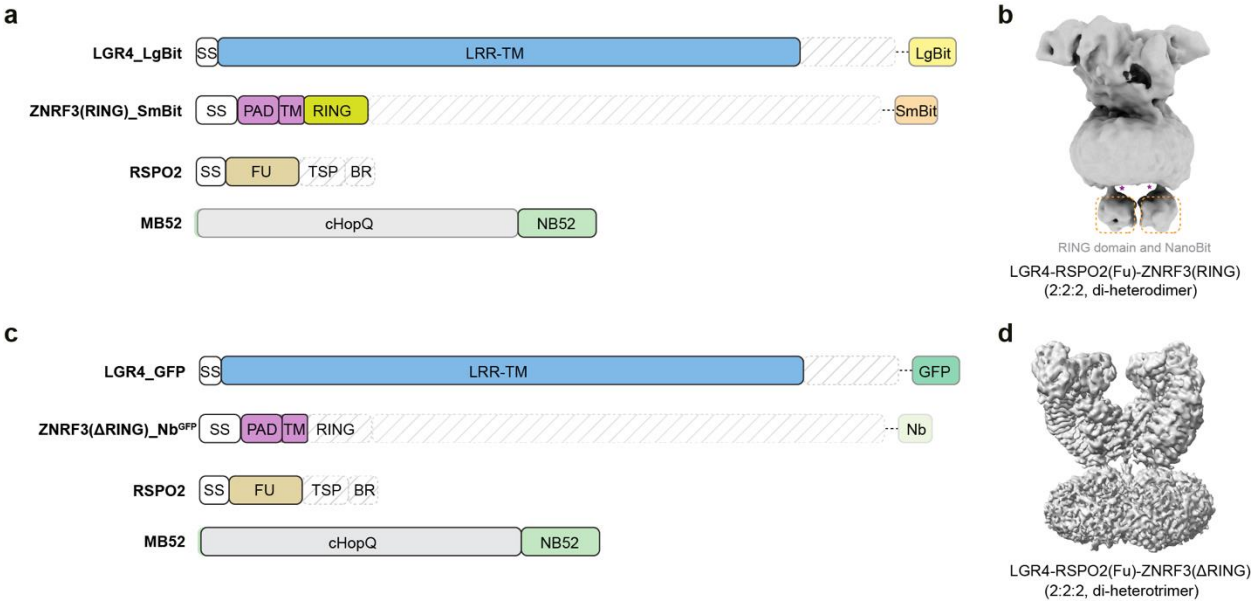
Response Fig. 15 **a**. Superposition of the map and model of the LGR4-RSPO2-ZNRF3 complexes. **b**, Map of ZNRF3 in the 1:2:2 complex (pentamer B, with ZNRF3 containing the RING domain). **c**, Map of ZNRF3 in the 2:2:2 complex (di-heterotrimer, with the ZNRF3 RING domain truncated).

In the low-pass-filtered map of the 1:2:2 complex (pentamer B), we observed the two RING domains crossing and interacting with each other, while the C-terminal ends of the two helices linking the RING domains are separated by approximately 12.3 Å. In contrast, in the 2:2:2 complex (without the RING domain), the distance between the corresponding helices increases to around 29 Å, resulting in the separation of the two RING domains and a significant change in their interaction mode (Response Fig. 15b, c). This separation provides strong evidence that, in the inverted V-shape TMH configuration of the 2:2:2 complex, the RING domains cannot dimerize, which likely affects ZNRF3 activity.

We also appreciate your suggestion regarding the mechanism by which the 2:2:2 complex regulates WNT/β-catenin signaling. You are correct that this configuration likely prevents catalysis, and we will incorporate this more precise terminology into our manuscript. The term

"preventing catalysis" more effectively captures the functional state of the 2:2:2 complex, which we had previously described as an "inactive state."

Lastly, we acknowledge the need for clearer labeling of the constructs from which the maps were derived. We will update the figure legends and text to provide explicit details about each construct and its corresponding map (Response Fig. 16). This will ensure that readers can easily follow the data presented and understand the experimental design.



Response Fig. 16 The domain organization of human LGR4, ZNRF3, and RSPO2, along with a schematic representation of their constructs used for structure determination. **a,** The constructs are used for the LGR4-RSPO2-ZNRF3(RING) complex in its heterotrimeric form (1:1:1) and pentameric form B (1:2:2), and di-heterotrimer (2:2:2), where ZNRF3 contains the RING domain, **b**, The map of the LGR4-RSPO2-ZNRF3 complex in its di-heterodimer form (2:2:2), with ZNRF3 containing the RING domain. **c**, The constructs are used for the LGR4-RSPO2-ZNRF3 (1:2:2, pentamer A) and the LGR4-RSPO2-ZNRF3(2:2:2, di-heterodimer), in which the ZNRF3 RING domain is truncated. **d,** The map of the LGR4-RSPO2-ZNRF3(ΔRING) complex in its di-heterodimer form (2:2:2), without RING domain of ZNRF3.

Thank you again for your valuable feedback. Your comments have significantly improved the clarity and quality of our work.

This also brings me to my second point about Figure 3, the degree of low pass filtering should be provided in the captions as well as the low pass filtered maps. The thickness of the maps for single helices at this degree of low pass filtering/contour threshold casts heavy doubt on the reliability of this portion/interpretation of the map. Personal inspection of the unsharpened map does suggest there may be some map features for the RING. Figure 3 should thus be significantly reworked for both improved clarity and to interpret the map with a more realistic contour/low pass filtering protocol.

Thank you for your thoughtful review and insightful comments regarding Figure 3. We appreciate your attention to the need for more precise data presentation and interpretation. We have carefully revised Figure 3 and the corresponding captions to address your concerns.

Regarding the LGR4-RSPO2-ZNRF3 complex with a 2:2:2 stoichiometry (including the RING domain), we processed the particles and obtained a structure at very low resolution. As you correctly noted, the map shows some features below the transmembrane domain that we interpret as the separated RING domains and its fused NanoBiT (Response Figs. 14d and 15a). Our analysis indicates that the map can be divided into two distinct regions, corresponding to the separation of the two RING domains and its fused NanoBiT. This observation aligns with the fact that the cytoplasmic portions of the ZNRF3 transmembrane helices (TMHs) are also clearly separated in this configuration.

In contrast, the 1:2:2 complex (pentamer B) presents a more compact map, with closely associated cytoplasmic regions.

Interestingly, a similar low-pass-filtered map feature was observed at the bottom of the LGR4-RSPO2-ZNRF3 complex with a 2:2:2 stoichiometry, even in the construct lacking the RING domain. We hypothesize that this feature could be attributed to the fused GFP and anti-GFP nanobodies, which likely contributed to the density observed in this region. However, there is a clear empty space between the map at the bottom and the detergent micelle/transmembrane domain in the 2:2:2 complex without the RING domain. This gap contrasts with the 1:1:1, 1:2:2 (pentamer B), and 2:2:2 complexes (including the RING domain), where the map of the cytoplasmic helix is continuous, seamlessly connecting the bottom of the map with the detergent micelle (Response Fig. 15a).

To address your concerns about the reliability of the low-pass-filtered maps, we have reworked Figure 3 and adjusted the contour levels and low-pass filtering protocols to ensure a more realistic and accurate representation of the data. The degree of low-pass filtering has now been provided in the figure captions for better transparency.
We hope that these revisions enhance the clarity and quality of Figure 3, meeting your expectations for a more accurate and realistic presentation of our data. Once again, thank you for your valuable feedback, which has been instrumental in improving our work.

The title of ED6 suggests that the panels demonstrate that the 2:2:2 complex is incompatible with LGR4 (TM6?) activation, but there is no overlay that really demonstrates this to be the case as none of the panels show an overlay with the 2:2:2 complex, they all only show a single LGR4 with no ZNRF3 or other subunits.

Thank you for your insightful feedback. We understand the importance of accurately representing the data in our figures. We have revised the title of Extended Data Figure 8 (ED8) to more accurately reflect the content of the panels. The original phrase "and the LGR4-RSPO2-ZNRF3 (2:2:2) complex is incompatible with the active conformation of LGR4" has been removed to avoid any misinterpretation.

The revised title now reads: "RSPO2 cannot activate LGR4."

We appreciate your feedback and believe these revisions will enhance the clarity and accuracy of our manuscript.

The modeling on all of the structures needs work. In regions where there is little/poor density period, the protein including the backbone should be truncated. For regions where there is little/poor density for side chains but the backbone can be modeled, sidechains should be stubbed. The modeling is currently liberal for what are 3.2-3.6A resolution maps.

Thank you for your insightful suggestions regarding the modeling of the structures in our manuscript. We appreciate the time and effort you have taken to review our work. We have

carefully re-evaluated regions with little or poor density and truncated the protein backbone accordingly. For regions where there is little or poor density for side chains but sufficient density for the backbone, we have stubbed the side chains to better reflect the limited resolution of the map. We have conducted additional rounds of refinement to ensure that our model is consistent with the resolution maps. We have validated the revised model and the results show an improvement in the overall quality and reliability of the model. Additional supporting data and figures have been included in the revised manuscript to provide clearer evidence of the model's accuracy. We believe that these modifications have significantly improved the quality of the structural model and have addressed the concerns you raised. We are grateful for your constructive feedback.

Table 1 Particle and Resolution Improvements:

Dataset	Number of Particles (Old)	Number of Particles (New)	Resolution (Old)	Resolution (New)
Heterotrimer	1,064,324	106,020	3.24 Å	2.50 Å
Pentamer A	407,148	143,915	3.40 Å	3.21 Å
Pentamer B	838,832	61,066	3.26 Å	3.20 Å
Di-heterotrimer	277,875	47,438	3.59 Å	3.38 Å

Table 2 Model Quality Improvements:

Dataset	Clash score (Old)	Clash score (New)	Ramachandran outliers (Old)	Ramachandran outliers (New)	Sidechain outliers (Old)	Sidechain outliers (New)
Heterotrimer	7	0	0.3%	0.0%	1.0%	0.0%
Pentamer A	11	2	0.3%	0.4%	2.4%	1.1%
Pentamer B	11	0	0.5%	0.1%	2.8%	0.7%
Di-heterotrimer	13	0	0.2%	0. 0%	2.3%	0.3%

Reviewer #4 (Remarks to the Author):

The LGR4/5-R-spondin module is an essential modulator of adult stem homeostasis. Critically, their target E3 ligases ZNRF3/RNF43 have emerged as potential therapeutical targets, including in cancer, as well as engineering tools for receptor degradation through e.g., PROTABs. Although several structures of these factors are available, including from the ternary complex, little is known about the contribution of the anchoring transmembrane domains (TMDs) to the complex formation and/or stoichiometry.

In this ms, Wang and colleagues present various cryo-EM structures of LGR4-RSPO2-ZNRF3 complexes that include the TMDs of both LGR4-ZNRF3. The authors characterize the properties of the complexes and hypothesize about their role in LGR-RSPO-dependent inactivation of ZNRF3. Furthermore, the authors perform mutagenesis analyses of specific LGR4 and ZNRF3 residues at their TMDs involved in the interaction demonstrating their relevance for WNT signaling activation without affecting their deployment to the PM.

Although I cannot comment on the structural analyses, I found the overall model and validation experiments quite convincing, as well as with relevance for the broad readership of Nature Communications.

Thank you very much for your thoughtful and encouraging feedback. We are delighted that you found our structural models and validation experiments convincing, as well as relevant to a broad audience.

Regarding your comment on the role of the transmembrane domains (TMDs) in complex formation and stoichiometry, we fully agree that this is an important aspect that remains underexplored. One of our primary aims was to provide novel insights into the contributions of the TMDs of LGR4 and ZNRF3 in stabilizing the complex, as well as their potential role in the regulation of WNT signaling pathways. Our cryo-EM structures were designed to address these gaps, and we are glad that our findings on TMD involvement in LGR-RSPO-dependent ZNRF3 inactivation were of interest.

We appreciate your acknowledgment of the relevance of our work to potential therapeutic applications, particularly in the context of E3 ligases such as ZNRF3 and RNF43. These proteins indeed hold significant promise as therapeutic targets, and we hope that our study can contribute to ongoing efforts in this field, including receptor degradation tools like PROTABs.

Thank you again for your kind remarks. We believe that the findings presented in this manuscript will provide a solid foundation for future studies on LGR4/5-R-spondin signaling and its therapeutic implications. Your valuable input has further reinforced our confidence in the relevance and robustness of our work.

I only have a couple of comments for the authors:

1- The authors identify critical residues likely driving TMD-TMD and ligand-receptor interactions for LGR4-RSPO2-ZNRF3. Can the authors discuss whether those interactions are also present/predicted in the other members associated with this module (LGR4/5, RSPO1-4 and RNF43/ZNRF3)? Ligands and TM proteins of this module can be largely exchanged with their paralogous (e.g., RNF43 behaves as ZNRF3 for interaction purposes but it is usually more relevant for cancer research). A broader bioinformatic/predictive analysis of whether the other ternary complexes are potentially governed by the same interactions (conservation of key residues/surfaces) should strengthen the authors functional claims, especially for i) the ionic lock in LGR5, ii) the expected interaction surfaces between LGR4-RNF43 and LGR5-ZNRF3 and iii) equivalent V229-P-S230 mutation in RNF43. These prediction analyses could be directly complemented with WNT reporter assays. Taken together, these analyses should provide important insights on whether the described molecular lock and switches govern the functional interactions of the LGR4/5 - RSPO1-4 - ZNRF3/RNF43 module or they are specific for the LGR4-ZNRF3 pair.

Thank you for your insightful comment. We appreciate your suggestion to expand the analysis of the transmembrane domain (TMD) interactions and conservation across other members of the LGR4/5-RSPO-ZNRF3/RNF43 module. We have undertaken the following steps to address this.

Sequence Conservation and Predicted Interactions

Our sequence alignment of LGR4/LGR5 and ZNRF3/RNF43 reveals that key residues at the TMD-TMD interface are indeed conserved (Response Fig. 17a). Notably, in LGR4, residues W751 and F804 play critical roles in mediating the interaction with ZNRF3. We demonstrated that mutations at these sites impair RSPO1-dependent TOPFlash activity, indicating the importance of this interface in modulating WNT/β-catenin signaling. In LGR5, the corresponding residues, L770 and F822, are similarly conserved, suggesting that the molecular interaction mechanisms are shared across LGR4 and LGR5(Response Fig. 17a).

However, previous reports have indicated that RSPO1 is significantly more potent in cells expressing LGR4 than in those expressing LGR5, pointing to subtle functional differences between these receptors despite the high conservation of key residues.

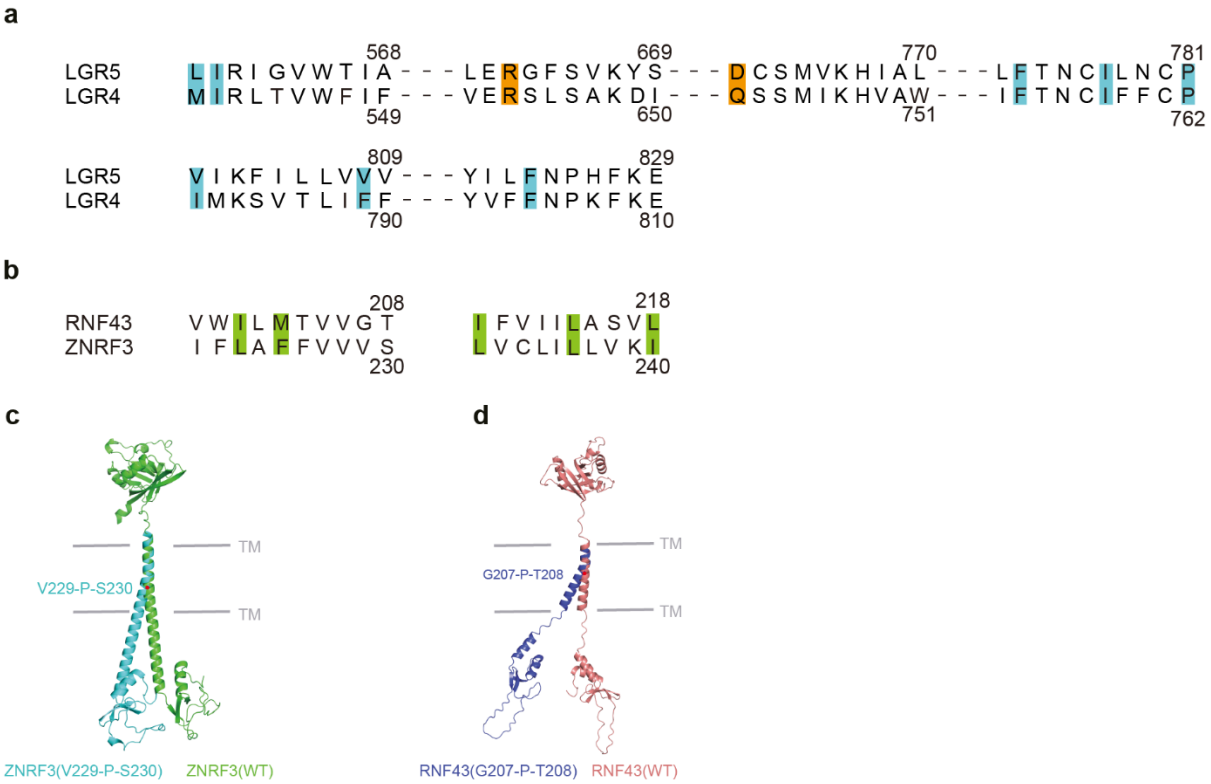
Ionic Lock and Its Role

We explored the "ionic lock," a well-known stabilizing interaction within the rhodopsin-family GPCRs, involving transmembrane domains 3 and 6. In LGR4, this lock is formed by $R^{3.50}$ and $Q^{6.30}$, while in LGR5, the equivalent residues are $R^{3.50}$ and $D^{6.30}$. Given the conservation of these residues, the "ionic lock" likely functions similarly in LGR5, maintaining the receptor in its inactive state and contributing to the overall conformational dynamics of LGR4 and LGR5 (Response Fig. 17a).

Structural Disruption via Mutations

Our insertion of a proline residue between V229 and S230 in ZNRF3 (mutant V229-P-S230) significantly enhanced Wnt3a-stimulated TOPFlash activity by disrupting the helical structure. This indicates that the structural integrity of ZNRF3's transmembrane coiled-coil helices is essential for regulating its ubiquitination of Frizzled, thereby modulating WNT signaling. In RNF43, the corresponding residues G207 and T208 are less conserved, though structural modeling

via AlphaFold suggests that these residues also lie at the middle of the transmembrane helix. A similar proline insertion here is expected to disrupt RNF43's coiled-coil helix structure, although RNF43 may have a slightly different functional mechanism due to the flexible loop between the transmembrane helix and RING domain forms a cytoplasmic helix (Response Fig. 17b).



Response Fig. 17 **a**, Sequence alignment between LGR4 and LGR5. **b**, Sequence alignment between ZNRF3 and RNF43. Specific interaction residues within the transmembrane regions of LGR4 and ZNRF3 are highlighted: LGR4 residues are shown in light blue, and ZNRF3 residues in green. The ionic lock is indicated in orange. **c, d**, A comparison of the AlphaFold-predicted structures between the wild-type and proline-inserted mutant is shown. **c**. The structural comparison between ZNRF3 (green) and the ZNRF3 V229-PS230 mutant (cyan) **d**. The comparison between RNF43 (orange) and the RNF43 G207-P-T208 mutant (dark blue).

Broader Structural Considerations

ZNRF3 and RNF43 share many structural similarities, though important differences exist. For instance, ZNRF3's linker region between the transmembrane helix and RING domain forms a cytoplasmic helix, while RNF43’s linker region is more flexible (Response Fig. 17c, d). This could

result in different rearrangements in the ternary complex, potentially affecting their catalytic activity. Additionally, while ZNRF3's extracellular domain can dimerize, RNF43's ability to form dimers is more context-dependent. This may explain why the mechanisms governing WNT signaling differ slightly between ZNRF3 and RNF43, even though both are capable of interacting with LGR4/5.

Conclusion

In summary, while the key residues mediating TMD-TMD and receptor-ligand interactions are conserved across LGR4, LGR5, ZNRF3, and RNF43, functional differences likely arise due to variations in structural dynamics, such as the flexible linker in RNF43 and the distinct potencies of RSPO1 in LGR4 versus LGR5. These distinctions point to slightly divergent mechanisms in the regulation of WNT/β-catenin signaling by these receptor-ligand systems.

Thank you for your suggestion, which has allowed us to deepen our analysis and provide a broader context for the molecular interactions governing the LGR4/5-RSPO-ZNRF3/RNF43 module.

2- LGR4 F804 and Q742K display impaired response to RSPO1 at lower ligand concentrations, but behave similar to wt LGR4 at nM range of RPSO1 (which is also around the Kd). Can the authors comment on the possible implications of these results? It seems that the TMD-TMD interactions could stabilise the complex under limiting [RSPO], but might be fully compensated by the interactions of the extracellular domain under what are likely standard [RSPO1] (nM range)

Thank you for your insightful and thought-provoking question. We completely agree with the reviewer's reasoning and analysis. The LGR4-RSPOs-ZNRF3 complex is stabilized by the TMD-TMD interactions between LGR4 and ZNRF3 under limiting [RSPOs]. This stabilization may be fully compensated by the association of the extracellular domain under standard [RSPO1] conditions (nM range).

We believe that even without RSPOs, LGR4 and ZNRF3 may directly interact through their TMDs, although this interaction might be too weak to form a stable complex. Nevertheless, LGR4 can still internalize some ZNRF3 from the cell surface, reducing its abundance on the membrane.

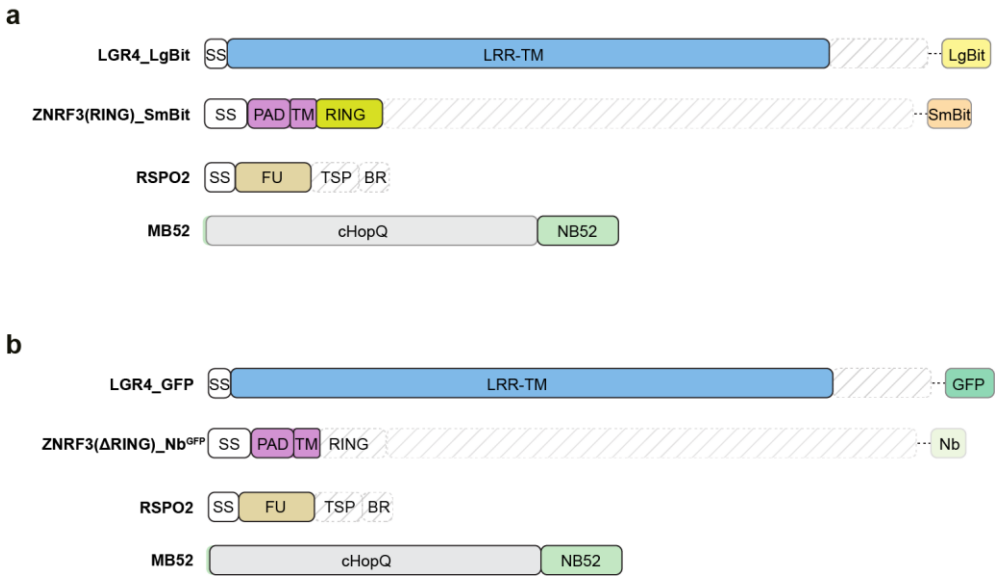
RSPOs bridge the extracellular domains of LGR4 and ZNRF3, strengthening their TMD-TMD interactions. These interactions, in turn, facilitate the association of their extracellular domains. Both the bridging by RSPOs and the TMD-TMD interactions contribute to the assembly of the LGR4-RSPOs-ZNRF3 complex.

Therefore, LGR4 F804A$^{7.56}$ and Q742K$^{6.30}$ display an impaired response to RSPO1 at lower ligand concentrations, which diminishes the potency of RSPOs. However, increased RSPO molecules can effectively bridge the extracellular domains of LGR4 and ZNRF3, compensating for the impaired TMD-TMD interactions. This results in a maximal response of RSPOs for the endocytosis of ZNRF3 with LGR4 mutants similar to that of wild-type LGR4.

Minor comments:

3- I believe that the authors only use the furin domain of RSPO2 for all their structural analyses. I think it should be properly indicated in the main figures to avoid confusion. A Figure visually displaying (and properly marking) all constructs used in this study will be extremely helpful for the reader (Please see Chen et al., Genes & Dev 2013, Figure 1A)

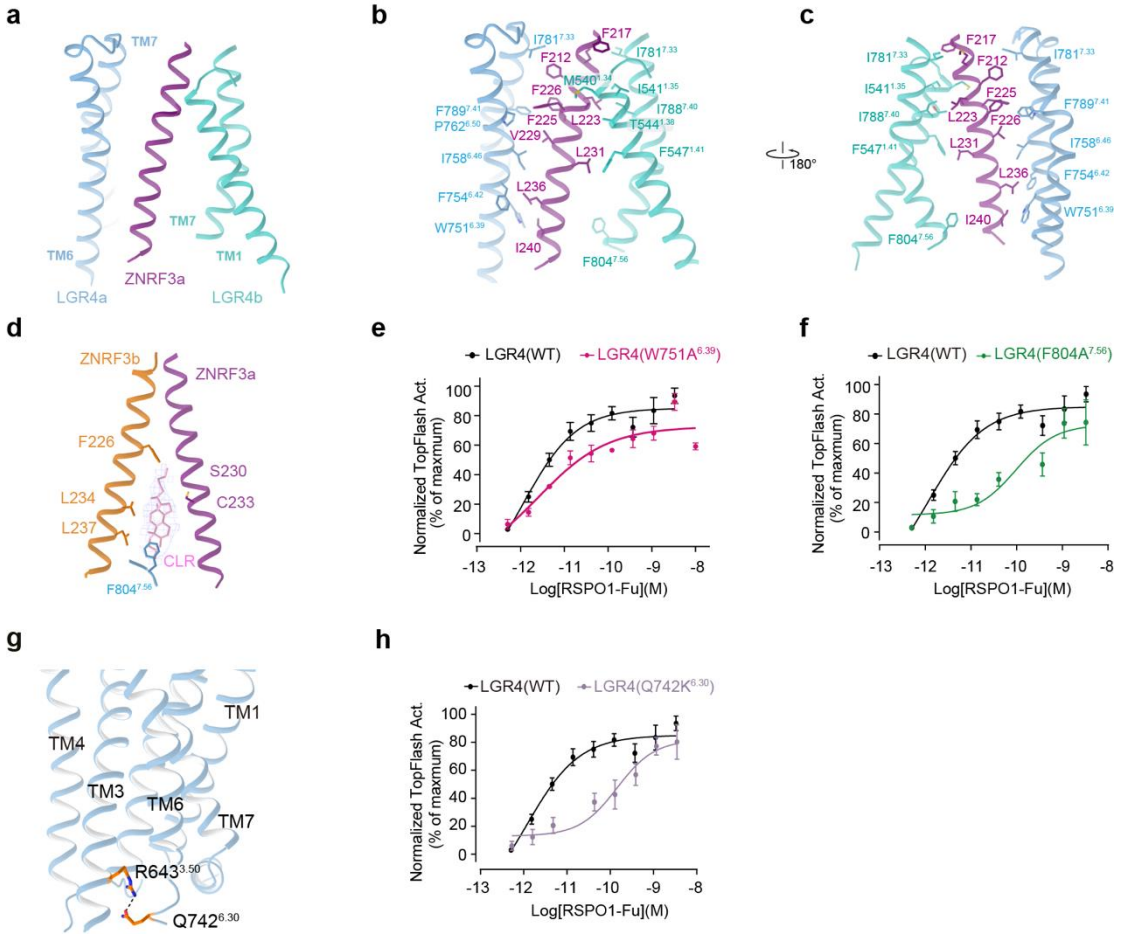
Thank you for your great suggestions. We have indicated all constructure in Extended Figure 2-4.



Response Fig. 18 Domain compositions of LGR4, ZNRF3, RSPO2, and MB52 are indicated schematically, along with a schematic representation of their constructs used for structure determination. The LGR4-RSPO2-ZNRF3(RING) complex in both its heterotrimeric form (1:1:1) and pentameric form B (1:2:2), where ZNRF3 contains the RING domain (**a**), the LGR4-RSPO2-ZNRF3 (1:2:2, pentamer A) and the LGR4-RSPO2-ZNRF3(2:2:2, di-heterodimer,), in which the ZNRF3 RING domain is truncated (**b**).

4- Please show the mutated residues in the TMD cartoons of Figure 4.

"Thank you for your valuable suggestion. We have now labeled the mutated residues in the TMD cartoons of Figure 4 to clearly illustrate their positions and impacts (Response Fig. 19).



Response Fig. 19 **a,** The interface between the transmembrane domains of LGR4 and ZNRF3. **b, c,** The side chain interactions between LGR4 and ZNRF3 within the transmembrane region of the LGR4-RSPO2-ZNRF3 complex (2:2:2) are shown in detail. **d,** The specific interactions between the transmembrane domain of LGR4, ZNRF3 and cholesterol within the LGR4-RSPO2-ZNRF3 complex (2:2:2) are highlighted. **e, f**, Dose-dependent TOPFlash activity induced by WT (black) or W751A$^{6.39}$ mutant (red, **e**) and F804A$^{7.56}$ mutant (green, **f**) of LGR4 after stimulation with RSPO1. **g,** The ionic lock between R643$^{3.50}$ of TM3 and Q742$^{6.30}$ of TM6 in LGR4. **h**, TOPFlash plot illustrating the effect of breaking ionic lock via Q742K$^{6.30}$ mutant (purple) in the transmembrane domain of LGR4 on the activity of RSPO1, compared to that of WT (black). Each value represents the mean ± SEM from three independent experiments. LGR4 (WT) datasets in all three panels are identical.

5- Can the authors further elaborate on why the pentamer A structure could lead to di-heterotrimer and not the other way around?

Thank you for your insightful question. Our analysis indicates that one LGR4 protomer approaches a pentamer A to form a di-heterotrimer. However, as you suggested, it is indeed possible that one LGR4 protomer detaches from the di-heterotrimer, leading to the formation of the pentamer A structure.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Overall, the authors thoroughly addressed the comments from the reviewers. Data interpretation and the overall clarity of the manuscript have been improved. A careful check for consistency in abbreviation, wording, and spelling would be necessary, preferentially by a native English speaker. Furthermore, there are still critical issues with the model refinement that should be clarified.

Thank you for your thoughtful review and valuable feedback on our manuscript. We appreciate your acknowledgment of our efforts to address the reviewers' comments and improve the data interpretation and clarity.

In response to your suggestion regarding consistency in abbreviations, wording, and spelling, we will ensure a thorough review of the manuscript. We plan to have it checked by a native English speaker to enhance its overall readability.

Regarding the critical issues with the model refinement that you mentioned, we recognize the importance of clarifying these aspects. We will provide additional details and explanations in the revised manuscript to address any remaining concerns.

Thank you once again for your constructive feedback. We are committed to improving our manuscript and addressing all critical points raised.

Regarding data processing and model building

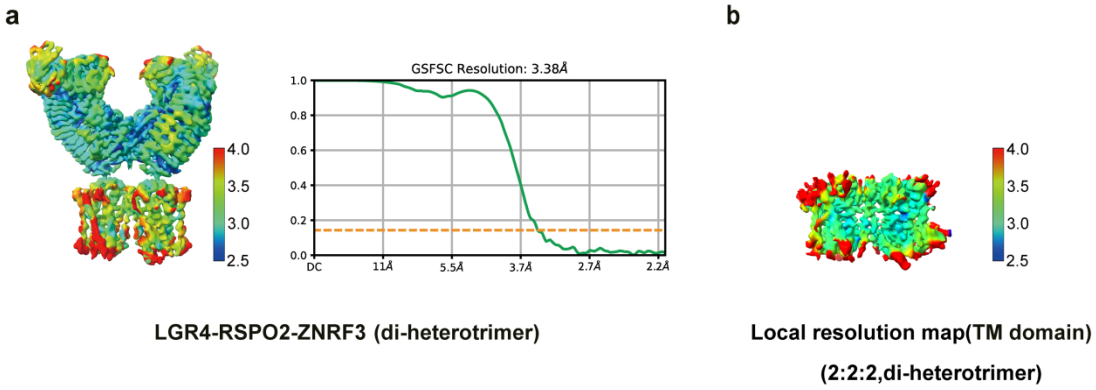
1. While the processing was significantly improved, masked local refinements towards transmembrane sections (combined with signal subtraction) might improve the area that is usually of lesser quality in the maps. Nonetheless, this will not change the conclusion of the manuscript, consequently this is only a suggestion.

Thank you for your insightful comments regarding our manuscript.

We generated a mask for the low-resolution transmembrane region and performed local refinements. This approach resulted in a significantly improved density map compared to our

previous version (Response Fig. 1). Additionally, we optimized the map using EMReady to further enhance the quality and interpretability of the cryo-EM images.

We appreciate your constructive feedback. Thank you once again for your guidance.



Response Fig. 1 The structure of the LGR4-RSPO2-ZNRF3(ΔRING)-MB52 complex (2:2:2**,** di-heterotrimer) with their corresponding local resolution and Fourier shell correlation (FSC) curves (**a**) and local refinements towards transmembrane sections (**b**).

2. Regarding the model. There are serious geometry issues in the new models. I assume this is because the authors attempted to reach a clash score of zero at all costs, at the expense of other critical metrics. One illustration of that, that is quite telling is the geometry of the cholesterol in the 8Y69 that is completely distorted. Also, geometry analysis that focuses on bond lengths and angles in coot highlights the problem strikingly. You can also refer to metrics from phenix validation like (rmsd angle and length among others). A model with the worst clash score (between 0 and 10) and a more realistic geometry is a better representation of the protein. This needs to be improved before publication.

Thank you for your detailed feedback regarding the geometry issues in our revised models. We sincerely appreciate your insights, which are crucial for enhancing the quality of our manuscript.

In response to your comments, we will revisit our model refinement process to address the geometry concerns. We plan to conduct a comprehensive geometry analysis using both Coot and Phenix validation metrics to ensure that bond lengths and angles are accurately represented while maintaining a reasonable clash score.

The following table summarizes the clash scores and geometry metrics for all complexes:

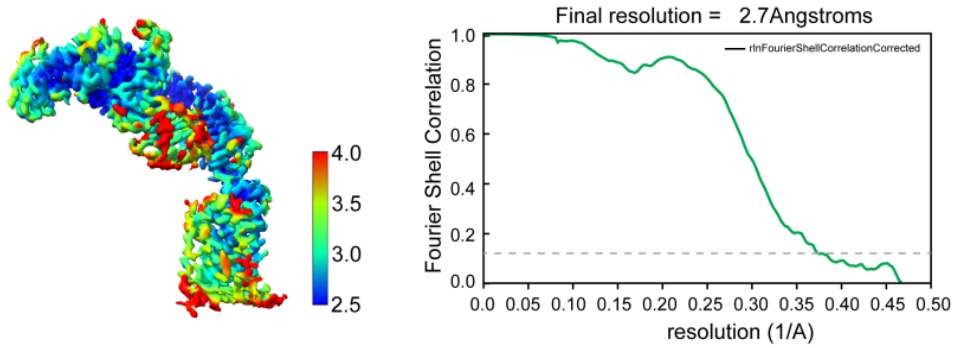
Dataset	Clash score (New)	Ramachandran outliers (New)	Sidechain outliers (New)	Bonds length (Å) (New)	Bonds Angle (°) (New)
Heterotrimer	7	0%	1.2%	0.003	0.526
Pentamer A	7	0.1%	1.7%	0.002	0.491
Pentamer B	6	0.1%	3.0%	0.002	0.515
Di-heterotrimer	8	0.1%	2.6%	0.003	0.661

Thank you again for your constructive critique.

Points that require the authors' attention before acceptance:

1. Regarding LGR4-RSPO2-ZNRF3(RING)-MB52 complex: FSC is not dropping to 0, this is usually induced by duplicated particles or a too tight mask during FSC calculation. In both cases, it could lead to resolution overestimation. Please try to remove duplicates before your final refinement and if this is not the problem, recompute FSC with a broader mask.

Thank you for your valuable feedback on the LGR4-RSPO2-ZNRF3(RING)-MB52 complex. We appreciate your insights regarding the FSC calculations. We reprocessed the data for the LGR4-RSPO2-ZNRF3(RING)-MB52 complex (1:1:1) as before and then imported it into RELION for post-processing (Response Fig. 2). We applied a broader mask during the FSC calculation, which has resolved the issue, and the FSC drops to 0.



Response Fig. 2 The structure of the LGR4-RSPO2-ZNRF3-MB52 complex (heterotrimer) with their corresponding local resolution and Fourier shell correlation (FSC) curves.

2. Please clarify the end of the following sentence "These structures, RSPO2 domain (PDB code: 4UFR), and 712 ZNRF3 PAD (PDB code: 4UFS) were fit into the composite cryo-EM map of the LGR4-RSPO2- ZNRF3 complexes as starting model in ChimeraX, and then iteratively refined with manual adjustment in Coot , Real-space, and performed reciprocal-space refinements in Phenix ."

Thank you for your attention to detail and for highlighting the need for clarification in our manuscript. We appreciate your valuable feedback.

We will revise the sentence for clarity. The revised version will read: "The RSPO2 domain (PDB code: 4UFR) and the ZNRF3 PAD (PDB code: 4UFS) structures were fit into the composite cryo-EM map of the LGR4-RSPO2-ZNRF3 complexes as starting models in ChimeraX. These models were then iteratively refined with manual adjustments in Coot, followed by real-space refinements in Phenix."

Thank you once again for your constructive suggestion.

3. The sentence "Understanding the structural principles governing these regulatory modules is crucial for tightly controlling Wnt signaling output" was corrected as "It is essential to understand the structural principles governing these regulatory modules to precisely control Wnt signaling output". Unfortunately, this still does not make sense since understanding principles cannot control Wnt signaling. The syntax is incorrect.

Thank you for your constructive feedback. We appreciate your observation regarding the syntax of the sentence. We have revised it to clarify the intended meaning. The updated sentence now reads: “Understanding the structural principles governing these regulatory modules is essential for developing strategies to precisely control Wnt signaling output.” Thank you for your careful review.

4. “The pair of TMHs are drawn close together on the extracellular side but diverge on the cytoplasmic side, creating an inverted V-shape configuration, clipping two distinct elongated densities that are attributed to cholesterol hemisuccinate (CHS) (Fig. 1b, c, e).2” correct for cholesteryl hemisuccinate.

Thank you for your feedback. We have corrected the text to use “cholesteryl hemisuccinate” instead of “cholesterol hemisuccinate” to ensure accurate terminology. We appreciate your careful attention to detail.

5. In the figure labels, cholesterol hemisuccinate is annotated as CLR while it should be CHS. Please correct that, it is confusing.

Thank you for bringing this to our attention. We have corrected the figure labels to replace "CLR" with "CHS" for cholesteryl hemisuccinate.

6. The authors use two abbreviations TM and TMH that refer to transmembrane helices. We strongly suggest to abbreviate transmembrane with TM and do not abbreviate helix.

Thank you for this helpful suggestion. We have revised the manuscript to use “TM” consistently to abbreviate “transmembrane” and have removed the abbreviation “TMH” to avoid confusion.

7. “TMH,TMHs” is explained multiple times P3 L52 , P8 L140 , P10 L179 and 185, P12 L241. Remove and refine.

Thank you for pointing this out. We have removed the redundant explanations of “TMH” and “TMHs” and retained a single, clear definition at its first mention.

8. “Extended Data Fig. 6c (3rd fit)“ While the map strongly suggests that two ring domains are

clearly separated, the model fit is not convincing. Please adjust the loops orientation or if you can't provide a model that fits better replace the ring models (only for this structure) with a schematic representation.

Thank you for your insightful feedback. We agree that the fit of the RING domains in Extended Data Fig. 6c could be improved. After further analysis, we adjust the loop orientations to enhance the model fit.

9. P4. "A higher proportion of LGR4 induces and stabilizes ZNRF3 inactive state, forming a stable assembly, facilitating internalization and consequently promoting WNT/β-catenin signaling" Since the facilitated internalization is speculative rephrase as a hypothesis.

Thank you for this suggestion. We have revised the statement to present it as a hypothesis: "A higher proportion of LGR4 may induce and stabilize the inactive state of ZNRF3, potentially forming a stable assembly that facilitates internalization and, consequently, promotes WNT/β-catenin signaling."

10. Title "Overall architecture of LGR4-RSPO2-ZNRF3complex with a 2:2:2 stoichiometry" lacks a space before complex.

Thank you for pointing out this formatting issue. We have corrected the title to include a space before 'complex' and now it reads: 'Overall architecture of LGR4-RSPO2-ZNRF3 complex with a 2:2:2 stoichiometry.

11. Page 17: The authors argue that the lack of a TM6 swing out in LGR4 complexed with RSPO and ZNRF3 would argue against class A-like mechanisms of activation and LGR4-G protein coupling. This statement is indeed an overstatement and should be toned down/removed. Also in class A GPCRs agonist binding does not elicit a TM6 swing out – the opening of the receptor is only observed in the presence of a heterotrimeric G protein stabilizing the TM6 swing out. As a consequence the subsequent argumentation of LGR4 activation mechanisms is pure speculation (for example, an unidentified transducer could stabilize an open and active-like receptor conformation).

Thank you for highlighting this point. We agree that our initial statement may have been overstated, and we have accordingly removed it.

12. The legend of Fig. 5 is insufficient. Please elaborate on necessary details.

Fig. 5 | Different conformational states of ZNRF3 in various assemblies of the LGR4-RSPO2(Fu)-ZNRF3 complexes. **a**, ZNRF3 in the heterotrimer: the linker between the extracellular domain of ZNRF3 and its transmembrane (TM) helix is absent. **b**, ZNRF3 in pentamer A: one of the transmembrane helices (TM helix) in the ZNRF3 dimer is completely missing, and the linker between the extracellular domain and the TM helix is also absent. **c**, ZNRF3 in pentamer B: the two TM helices exhibit a "finger-crossed" arrangement. **d**, ZNRF3 in the di-heterotrimer: the two TM helices adopt an inverted V-shaped configuration. ZNRF3 is depicted in purple or orange.

13. The legend of Extended Data Fig. 7 is insufficient. Please elaborate on necessary details.

Extended Data Fig. 7 | Flow cytometric analysis of cell surface expression levels shows that LGR4 mutants exhibit approximately 100% expression relative to the wild-type (WT) LGR4. HEK293T cells were transiently transfected with Flag-tagged WT LGR4 or the indicated mutants for 12 hours and subsequently treated with RSPO1. Cells were stained with an anti-Flag monoclonal antibody, followed by washing and incubation with Alexa Fluor® 488 Goat Anti-Mouse IgG. After further washing, samples were analyzed using a BD Fortessa™ flow cytometer, and data were processed with FlowJo Software (FlowJo, LLC). In the flow cytometry histograms, unstained cells are represented by dashed lines, while WT LGR4 is displayed in black. Mutants are color-coded as follows: W751A in cyan (a), F804A in green (b), and Q742K in pink (c). The near-complete overlap of each mutant's histogram with WT indicates that the cell surface expression levels of the mutants are unaffected, demonstrating comparable expression to WT LGR4.

Reviewer #2 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #3 (Remarks to the Author):

The authors have reasonably addressed the comments and critiques that I provided and the work would seem suitable for publication.

Reviewer #4 (Remarks to the Author):

The authors have addressed my comments. However, I think that both the referee figure 17 and the associated comments merit space in the manuscript. I suggest to bring the data from referee figure 17 to a extended figure in the paper, and summarise the authors' response to my point #1 (and the last part of point #2) into a paragraph at the end of the results section or the discussion. Although I understand that part of the response is based on modelling and not structural data, i believe that i) it is well-reasoned and structured and ii) discussing its implications for RNF43 and LGR5 will be important for the community, and for future structural studies tackling the formation of these ternary complexes.

Thank you for your valuable suggestions.

In the 'LGR4 Deactivates ZNRF3' section, we have expanded the discussion to clarify how TMD-TMD interactions may stabilize the complex under limiting RSPO concentrations, while higher concentrations (in the nM range) allow extracellular domain interactions to fully support complex formation. We added the following paragraph:

“Even without RSPOs, LGR4 and ZNRF3 may directly interact through their TMDs, although this interaction might be too weak to form a stable complex. Nevertheless, LGR4 can still internalize some ZNRF3 from the cell surface, reducing its abundance on the membrane. RSPOs bridge the extracellular domains of LGR4 and ZNRF3, strengthening their TMD-TMD interactions. These interactions, in turn, facilitate the association of their extracellular domains. Both the bridging by RSPOs and the TMD-TMD interactions contribute to the assembly of the LGR4-RSPOs-ZNRF3 complex. Therefore, LGR4 F804A$^{7.56}$ and W751A$^{6.39}$ display an impaired response to RSPO1 at lower ligand concentrations, which diminishes the potency of RSPOs. However, increased RSPO molecules can effectively bridge the extracellular domains of LGR4 and ZNRF3, compensating for the impaired TMD-TMD interactions. This results in a maximal response of RSPOs for the endocytosis of ZNRF3 with LGR4 mutants similar to that of wild-type LGR4.”

In the 'Discussion' section, we added a paragraph to address the conserved interactions within the LGR4/5-RSPO1-4-ZNRF3/RNF43 module:

“Our sequence alignments of LGR4 and LGR5, as well as ZNRF3 and RNF43, reveal a significant conservation of key residues at the TMD-TMD interface. In LGR4, residues W751 and F804 are

essential for interactions with ZNRF3, while the corresponding residues in LGR5, L770 and F822, suggest a shared interaction mechanism across both receptors. The transmembrane coiled-coil helices of ZNRF3 are crucial for regulating Frizzled ubiquitination, and the AlphaFold-predicted structure of RNF43 displays a similarly conserved helix, supporting functional parallels. However, RNF43 may operate through a slightly different mechanism, as the flexible loop between its transmembrane helix and RING domain forms a cytoplasmic helix, potentially impacting catalytic activity."