nature portfolio

Peer Review File

Conformational Coupling Between Extracellular and Transmembrane Domains Modulates Holo-adhesion GPCR Function

Corresponding Author: Professor Demet Arac

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)
Manuscript NCOMMS-24-05828

Reviewer´s comments
The manuscript entitled “Structural analysis and conformational dynamics of a holo-adhesion GPCR reveal interplay between extracellular and transmembrane domains” by Kordon at al vows to address one of the most contentious matters in the adhesion GPCR field: is ECR dissociation required for receptor activation to take place? Here, they provide the structural basis of a liganded adhesion GPCR to settle this conundrum using Cryo-EM and single-molecule FRET. This timely study inserts itself in the context of recent structural characterizations of various adhesion GPCRs which have been truncated at the hallmark cleavable GPS site just upstream of the tethered agonist (TA), also known as the Stachel peptide. While these studies were based on the a priori model of the TA exposure arising from ECR dissociation, the majority of these studies exemplified mutant receptors devoid of their ECR thus unable to display an associative state and leaving all but one default state to be adopted by these model truncated receptors. The present study would be the first to present Cryo-EM densities for both the transmembrane domains and the ECR comprising the signature GAIN domain, albeit of low resolution. The authors determine that the GAIN domain of adhesion GPCR ADGRL3 adopts limited membrane-proximal configurations in its liganded state, that these states can be described by the GAIN domain proximity to the receptor’s 7TM and that these states can be dynamically modulated by ligands or inactivating mutations. An important limitation of this manuscript stems from the fact that the receptor construct used for Cryo-EM determination lacks a considerable portion of the ECR domains and consequently the volume of the ECR and its positioning towards the membrane could be a result of its reduced size. Another limitation denoted by the authors is the inability to resolve the 7TM structure at high resolution due to high flexibility thus impeding a determination of contact points between the ECR and 7TM. An additional limitation is that the resolved structure does not correspond to a holo-activated state thus not providing the optimal conditions for testing the TA exposure hypothesis. However, the authors overcome these limitations by conducting smFRET experiments in the context of the full-length receptor which provides further support for their structural static model and allows them to test their hypothesis with structural dynamic data. On the same line of idea, other observations extracted from the structural determination assays require additional testing in the context of the full-length receptor. Also, the synthetic binders need to be better characterized in terms of their agonistic/neutral activity. What follows is a description of issues which need to be addressed:

Major Revision

A. Because the construct described in reference 24 (Nazarko et al, IScience, 2018) corresponds to “Lphn3 GAIN+TM domains” and no mention of the inclusion of the HormR domain is made, it is not clear if the construct HormR/GAIN ADGRL3 has been previously tested for activity towards G protein as the authors claim on Line 126. We suggest that the authors characterize both the SRE pathway and cAMP pathway using this construct in mammalian cells compared to full-length ADGRL3.

B. In view of the smFRET assays which provide a FRET signature for agonist-like binders vs neutral agonist-like binders, a better characterization of the binders activity is needed. Authors should provide results from assaying the effect of LK3 and LK1 on other known pathways activated by ADGRL3: cAMP inhibition, cAMP production, G protein coupling, etc.

C. Structural characterization uncovered N-glycan moieties that are critically positioned at the interface between the GAIN and the lipidic membrane. The authors should test their hypothesis by mutating these consensus N-glycosylation sites and conducting signaling assays and smFRET experiments. The hypothesis being that if an association can be made between GAIN dynamics and 7TM-dependent signaling thus N-glycans at the interface could be modulating GAIN positioning and receptor signaling.

D. Given that these findings challenge the dissociative model, the use of a non-dissociable ADGRL3 mutant in which the GPS site is made non-cleavable should be included into the full-length ADGRL3 in order to compare the distribution of the FRET states with the ones generated by the cleavable receptor.

E. The differential activity of the synthetic binders LK1 and LK3 should be tested in the context of the GAIN cancer mutants with smFRET assays.

Minor Revision

1. Please indicate which ADGRL3 splicing isoform was used for the full-length constructs.
2. Line 545, please indicate the incubator's settings (temperature, %CO2)

Reviewer #2

(Remarks to the Author)
In this work, the authors study the important problem of how aGPCR ECRs couple with TM domains allowing signaling to regulate downstream activity. In particular, the authors aim to distinguish between mechanisms focused on TA(GAIN)-7TM or larger-scale reversible ECR-7TM interactions, as well as to probe structure in the holoreceptor including the full-length protein. To address this problem, the authors here use a combination of cryo-EM, smFRET and biochemical perturbation in the aGPCR Latrophilin3/ADGRL3. The authors' data favor the ECR-7TM interaction mechanism, while providing evidence for multiple conformational states (relatively discrete within the resolution) whose distribution is linked to downstream activity in the system, and that these can be perturbed by antibodies and disease mutants. The authors include an interesting discussion of the biological implications of their results in the context of the forces experienced by the protein and coupled function in an in vivo setting, as well as possible allosteric effects in the ECR-7TM coupling. The combination of new insights into this important biological problem using a strong combination of methods will make this of substantial interest to the field. However, I note some issues below that the authors should address.

1) The authors have discussed several interesting aspects of the multistate distributions from the smFRET analysis. In the results, authors should also discuss dwell time analysis as linked to transitions between states in more detail.

2) The state count analysis provides some information along these lines (point 1), however less directly. In addition, other factors such as length of trace, photobleaching (stochastic), which states were occupied, could also influence state counts. Authors should analyze/clarify further.

3) For completion, authors, please note whether acceptor direct excitation was corrected for during smFRET analysis (this is not expected to change conclusions).

4) Authors may want to state in their cryo-EM results section that LK3 used there was a neutral binder in terms of activity, i.e., the structure information acquired is not expected to be perturbed (tested later by smFRET).

Reviewer #3

(Remarks to the Author)
This is a nice body of scientific work that uses cryoEM, smFRET, synthetic antibodies (neutral & activating) and mutational analysis to examine conformational states of Latrophilin3 (ADGRL3) receptor ECR relative to the TMH core.

While I enjoyed the manuscript, I did have some issues, that should be addressed. (1) The lack of TMH evidence presented for the cryoEM data and (2) the overstatement of three defined states with respect to the smFRET studies.

Figure S2b and S3 lack images showing the receptor TMH which should be visible at the reported ~nanometer resolution for this region. Thus, it is unclear to this reviewer in the absence of the maps if the rotation of the ECR is relative to the TMHs or an empty micelle based on the pictoral information provided. Even the 3D variability analysis is only relative to the micelle. This relative orientation (to the TMH) is critical to the conclusions of the manuscript and should be addressed and made more clear to the reader.

Moreover, the 3D variability analysis used requires much more details in the methods and perhaps its own section. If possible, the change relative to the TMHs is critical for this analysis to be meaningful.

Additionally, the LK1 cryoEM looks promising, however it is only shown in Fig5j. No specifics of this were obvious to me in the methods and standard cryoEM table. While a different FAB orientation is observed it is unclear from the overlay how the

ECR is affected, except as stated on line 261.

As for the smFRET studies being able to be fit to three defined states I find this data compelling (given the differences between FABs and mutants) but it is of my opinion that it is overstated in the manuscript (especially line 219). As such I would recommend using language that would state can be modeled to 3 states. Additionally, it would improve the rigor to also have chi squared surface plots in the supplemental demonstrating that 3 states is indeed the optimal fit over the other number of states (i.e. 1,2,4 and 5) tested.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have adequately addressed all of my comments. As for the results regarding the GPS-uncleavable ADGRL3 mutant (T855G), the authors have uncovered a very interesting finding giving that this issue is still very contentious in the Adhesion GPCR field. This shift in smFRET populations warrants further characterization and, as noted by the authors, is beyond the scope of this study. I agree that these results not be included as part of the manuscript to allow authors to further analyze this in the future.

Reviewer #2

(Remarks to the Author)
The authors have done a good job of making revisions in response to my comments. The work will be of substantial interest to the field.

Reviewer #3

(Remarks to the Author)
I have now reviewed the EM map files. Overall, the authors provide some evidence that suggests multiple ECD orientations can be observed in the cryoEM ensemble relative to a mobile and/or absent 7TM core. The smFRET studies provide support for multiple orientational ECD distributions relative to a probes located on the receptor. The data from these two complementary techniques (cryoEM and smFRET) provide a suggestive explanation for a plausible scenario describing how this receptor may exist. However, other scenarios or weaknesses are not discussed.

My major concern is the TM helices are absent and the general location of the TMH core is vague at best. Also of concern are statements such as: “With current model, we can unambiguously define the position of the 167 GAIN domain in relation to the 7TM (Fig. 1d, e).” This bold over-statement makes me question the author’s scientific rigor and brings the entire body of work and conclusions into question. Parts of Figure 1 are very misleading. As it stands now, the paper should not be accepted for publication in its current form. Since there is not clear receptor density in the maps and the conclusions of ECD domain movements that are relative to this unresolved receptor density are again misleading.

If the authors plan to move forward with publication; it is my opinion that this lack of cryo-EM receptor density caveat needs to be *explicitly stated in the text*. Additionally, other possible interpretations should be suggested in the discussion.

Importantly, “hypothetical location” should be added to all figures showing any receptor placement so as not to mislead readers on the quality of the cryo-EM data.

I have listed some comments related to these concerns:
Line 34 – cannot say 7TM as they are not visible in the cryo-EM – can say hypothetical position of the 7TM
Line 35 – hypothetical 7TM (as above)
Line 112 movement is with respect to residues XXX from smFRET studies (can list specific 7TM helix or loop)
Line 118 with respect to residues XXX (as above)
Line 165 – there is no receptor density present in the maps - – adjust the text to appropriately describe the map quality in this region
Line 166 – Major Concern! “With current model, we can unambiguously define the position of the 167 GAIN domain in relation to the 7TM (Fig. 1d, e).” I find this statement grossly overstated given the maps provided.
Line 172 – The low resolution density for the TMDs – it is absent – tone down the rhetoric and describe the data
174 - hypothetically place (what density?)
185 – 3D Variability is relative to the detergent micelle – cannot make claims about receptor 7TM density based on the maps provided – adjust language to describe the data
190 close to the hypothetical 7TM placement

234 could be helpful here to list what TMHs or loops you placed the fluorophores on (also can label in figure)

289 – hypothetical 7TM orientation

342 hypothetical 7TMs from cryoEM and follow up smFRET studies help define the relative position

346 can be modeled up to 3 states with respect to residues XXX on TM helix XXX

Version 2:

Reviewer comments:

Reviewer #3

(Remarks to the Author)
The authors have adequately addressed all of my comments. I look forward to their publication.

Response to Reviewer Comments

Structural analysis and conformational dynamics of a holo-adhesion GPCR reveal interplay between the extracellular and transmembrane domains

NCOMMS-24-05828

Dear Dr. MacRae,

Thank you very much for the prompt handling of our manuscript. We are delighted that the Reviewers appreciate the importance of our work for a variety of fields and thank them for their constructive comments. We have revised the manuscript accordingly. Below we provide our detailed responses:

Reviewer #1 (Remarks to the Author):

Manuscript NCOMMS-24-05828

Reviewer´s comments
The manuscript entitled “Structural analysis and conformational dynamics of a holo-adhesion GPCR reveal interplay between extracellular and transmembrane domains” by Kordon at al vows to address one of the most contentious matters in the adhesion GPCR field: is ECR dissociation required for receptor activation to take place? Here, they provide the structural basis of a liganded adhesion GPCR to settle this conundrum using Cryo-EM and single-molecule FRET.

We want to send our special thanks to the reviewer for seeing through this manuscript, and understanding how essential it is to answer the question he/she mentions. Indeed, we have spent more than 10 intense years completing the work we present here. We are glad we did because we believe this will be a seminal paper for the adhesion GPCR field and shape future research.

This timely study inserts itself in the context of recent structural characterizations of various adhesion GPCRs which have been truncated at the hallmark cleavable GPS site just upstream of the tethered agonist (TA), also known as the Stachel peptide. While these studies were based on the a priori model of the TA exposure arising from ECR dissociation, the majority of these studies exemplified mutant receptors devoid of their ECR thus unable to display an associative state and leaving all but one default state to be adopted by these model truncated receptors. The present study would be the first to present Cryo-EM densities for both the transmembrane domains and the ECR comprising the signature GAIN domain, albeit of low resolution. The authors determine that the GAIN domain of adhesion GPCR ADGRL3 adopts limited membrane-proximal configurations in its liganded state, that these states can be described by the GAIN domain proximity to the receptor’s 7TM and that these states can be dynamically modulated by ligands or inactivating mutations. An important limitation of this manuscript stems from the fact that the receptor construct used for Cryo-EM determination lacks a considerable portion of the ECR domains and consequently the volume of the ECR and its positioning towards the membrane could be a result of its reduced size. Another limitation denoted by the authors is the inability to resolve the 7TM structure at high resolution due to high flexibility thus impeding a determination of contact points between the ECR and 7TM. An additional limitation is that the resolved structure does not correspond to a holo-activated state thus not providing the optimal conditions for testing the TA exposure hypothesis. However, the authors overcome these limitations by conducting smFRET experiments in the context of the full-length receptor which provides further support for their structural static model and allows them to test their hypothesis with structural dynamic data. On the same line of idea, other observations extracted from the structural determination assays require additional testing in the context of the full-length receptor. Also, the synthetic binders need to be better characterized in terms of their agonistic/neutral activity.

What follows is a description of issues which need to be addressed:

We thank the reviewer for their appreciation of our work and enthusiasm; and for providing valuable comments. Please see below for specific answers and a general comment about our overall understanding of aGPCR activation.

Major Revision

A. Because the construct described in reference 24 (Nazarko et al, IScience, 2018) corresponds to “Lphn3 GAIN+TM domains” and no mention of the inclusion of the HormR domain is made, it is not clear if the construct HormR/GAIN ADGRL3 has been previously tested for activity towards G protein as the authors claim on Line 126.

We would like to clarify that the construct described in Nazarko et al. *iScience* paper includes both HormR and GAIN domains in the extracellular region. It is indeed the identical construct that was used for the cryo-EM reconstruction in this manuscript.

We suggest that the authors characterize both the SRE pathway and cAMP pathway using this construct in mammalian cells compared to full-length ADGRL3.

To further analyze this construct activity as the reviewer suggested, we performed the signaling experiments with the HormR+GAIN/7TM construct (this is a mammalian expression construct suitable for signaling assays) using our SRE and cAMP assays; and found that the HormR+GAIN/7TM construct is active in both assays. The activity of the HormR+GAIN/7TM ADGRL3 is similar to the WT receptor in the SRE-luciferase assay. In the cAMP assay, the HormR+GAIN/7TM construct has higher activity than the WT receptor, as seen by the inhibition of cAMP production (updated Supplementary Fig. 1e, f). Please see section C below for our interpretation of this data.

B. In view of the smFRET assays which provide a FRET signature for agonist-like binders vs neutral agonist-like binders, a better characterization of the binders activity is needed. Authors should provide results from assaying the effect of LK3 and LK1 on other known pathways activated by ADGRL3: cAMP inhibition, cAMP production, G protein coupling, etc.

Thank you for this suggestion. In addition to the SRE assay already presented in the manuscript, we have now performed cAMP assay to test the effect of LK3 and LK1 on the inhibition/stimulation of cAMP production. In the cAMP assay, LK1 or LK3 did not lead to significant changes in cAMP levels downstream of ADGRL3 when compared to the untreated control sample (updated Supplementary Fig. 2e, f). Please see section C below for our interpretation of this data.

C. Structural characterization uncovered N-glycan moieties that are critically positioned at the interface between the GAIN and the lipidic membrane. The authors should test their hypothesis by mutating these consensus N-glycosylation sites and conducting signaling assays and smFRET experiments. The hypothesis being that if an association can be made between GAIN dynamics and 7TM-dependent signaling thus N-glycans at the interface could be modulating GAIN positioning and receptor signaling.

This is an interesting point and we thank the reviewer for this question. We mutated the N-linked glycosylation sites on ADGRL3. We then tested the mutant ADGRL3 constructs using our signaling assays and using smFRET sensors at positions 2 and 8. Compared to the wildtype receptor, the glycosylation mutant does not significantly change the SRE signaling but shows higher receptor activity in the cAMP assay as seen by the inhibition of cAMP production. Interestingly, the FRET histogram distribution and occupancy of the three identified conformational states were not affected by the N843A mutation, similar to what was observed with the SRE assay. This suggests that the conformational changes seen by smFRET correspond more closely to the activation of the SRE pathway (new Supplementary Fig. 15).

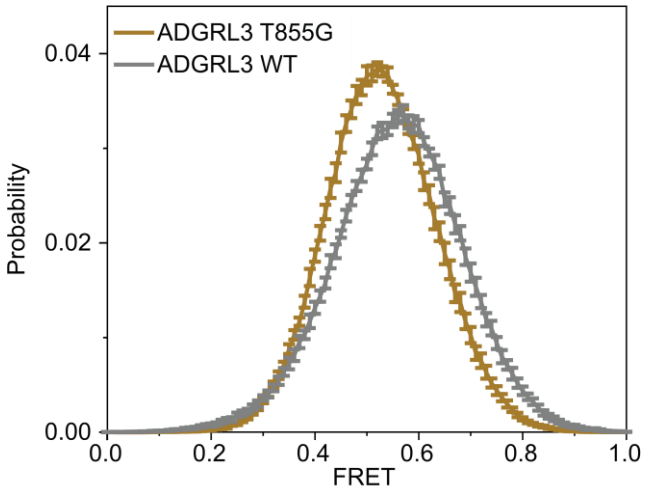
General Comment:
We interpret the results from parts A, B and C as below. We have included the below section to the manuscript:

"…Our data suggest that the highest FRET state corresponds to the lower receptor signaling and the lowest FRET state corresponds to the higher basal receptor signaling. Thus, our results suggest that ECR conformation and dynamics correlate with downstream receptor signaling as measured by the SRE assay. Because the SRE assay is commonly used as a likely readout for $G\alpha_{12/13}$ signaling, we also tested the effect of ECR perturbations using an additional assay, the cAMP assay that is used as a readout for $G\alpha_{s/i}$ signaling. Our observations hinted at signaling bias that is caused by ECR conformation. We observed that the ADGRL3 construct containing HormR+GAIN and 7TM had higher basal activity than the full-length ADGRL3 in the cAMP assay, unlike in the SRE assay (Supplementary Fig. 1e, f); and that both the LK3 and LK1 sABs acted as neutral binders in the cAMP assay although LK1 was an agonist in the SRE assay (Supplementary Fig. 2e, f). These observations suggested that while ECR conformation and dynamics affect downstream signaling, the effect is not identical in different signaling readouts. This idea was strengthened when we tested the N-linked glycosylation site mutations in the SRE and cAMP assays and observed no effect on SRE assay and smFRET conformations, but observed an effect on the cAMP signaling (Supplementary Fig. 15). We hypothesize that observed conformations of the holo-receptor can activate a certain signaling pathway while the same conformations are inactive for the other signaling pathways and vice versa. This potential signaling bias opens new directions for further studies on aGPCRs. "

D. Given that these findings challenge the dissociative model, the use of a non-dissociable ADGRL3 mutant in which the GPS site is made non-cleavable should be included into the full-length ADGRL3 in order to compare the distribution of the FRET states with the ones generated by the cleavable receptor.

We and other labs have reported signaling of non-cleavable construct previously (Perry-Hauser et al. 2022 - PMID: 36244455, Kordon et al. 2023 - PMID: 36746957, Ojeda-Muñiz et al. 2023 - PMID: 37464463).

To address the reviewer's question, we performed smFRET experiments on the non-cleavable ADGRL3 mutant (T855G). We found that this mutant shows a slight shift in probability histograms to the lower FRET values with a slight increase in the occupancy of the FRET state 0.44 and a decrease in the occupancy of the FRET state at 0.65 compared to the wild type (Reviewer Fig. 1). This is in fact consistent with the idea that autoproteolysis introduces bias to the receptor signaling that was introduced by the Boucard Lab in Ojeda-Muñiz et al. 2023 - PMID: 37464463. In other words, a cleaved receptor and an uncleaved receptor might activate different G proteins. Our current data is in agreement with this idea, however fully characterizing the effect of autoproteolysis requires further careful in-vivo and in-vitro experiments and thus is beyond the scope of the current study.



***Reviewer Fig. 1**: Single-molecule FRET population histograms of ADGRL3 WT and T855G autoproteolysis mutant.*

With the reviewer's approval, we want to exclude this data from this manuscript because we are planning a detailed analysis of the effect of autoproteolysis on receptor structure, function and dynamics.

E. The differential activity of the synthetic binders LK1 and LK3 should be tested in the context of the GAIN cancer mutants with smFRET assays.

We have now tested the GAIN domain cancer mutant constructs with smFRET assays in the presence of LK1 or LK3 binders. The result of the this experiment is now included in the new Supplementary Fig. 13 and Fig. 6. Addition of LK3 antibody to the mutant receptor with S810L+E811Q mutations did not significantly change smFRET distribution as expected for the neutral binder LK3.

Minor Revision

1. Please indicate which ADGRL3 splicing isoform was used for the full-length constructs.

The detailed information was included in the method section (ADGRL3 isoform: Q9HAR2-4).

2. Line 545, please indicate the incubator's settings (temperature, %CO2)

We have added the required information in the methods section - cells were grown at 37C, 5% CO

Reviewer #2 (Remarks to the Author):

In this work, the authors study the important problem of how aGPCR ECRs couple with TM domains allowing signaling to regulate downstream activity. In particular, the authors aim to distinguish between mechanisms focused on TA(GAIN)-7TM or larger-scale reversible ECR-7TM interactions, as well as to probe structure in the holoreceptor including the full-length protein. To address this problem, the authors here use a combination of cryo-EM, smFRET and biochemical perturbation in the aGPCR Latrophilin3/ADGRL3. The authors' data favor the ECR-7TM interaction mechanism, while providing evidence for multiple conformational states (relatively discrete within the resolution) whose distribution is linked to downstream activity in the system, and that these can be perturbed by antibodies and disease mutants. The authors include an interesting discussion of the biological implications of their results in the context of the forces experienced by the protein and coupled function in an in vivo setting, as well as possible allosteric effects in the ECR-7TM coupling. The combination of new insights into this important biological problem using a strong combination of methods will make this of substantial interest to the field. However, I note some issues below that the authors should address.

We thank the reviewer for their appreciation of our work and address their comments below.

1) The authors have discussed several interesting aspects of the multistate distributions from the smFRET analysis. In the results, authors should also discuss dwell time analysis as linked to transitions between states in more detail.

We thank the reviewer for this question regarding additional analysis of smFRET data. We performed dwell time analysis for the WT and the cancer mutant as suggested and the results are now included in Supplementary Fig. 11 and the discussion is added to the main text as following:

“We found that addition of LK1 increases the dwell time of FRET state 0.44 by 77% while reducing the dwell time of the FRET state at 0.65 by 58%. Importantly we did not observe significant change in the dwell time of different states in the presence of LK3, as expected from a neutral binder.”

2) The state count analysis provides some information along these lines (point 1), however less directly. In addition, other factors such as length of trace, photobleaching (stochastic), which states were occupied, could also influence state counts. Authors should analyze/clarify further.

We thank the reviewer for bring up this point about technical factors that could affect the analysis. We have used the same imaging parameters such as the laser intensity, data acquisition resolution for all the conditions and samples and repeated the experiments at least 3 independent times and treated the data in the same way. To avoid bias from very short traces we excluded traces that were shorter than 4 seconds (less than 5% of good traces). We have now added this information to the Methods section of our manuscript to clarify.

3) For completion, authors, please note whether acceptor direct excitation was corrected for during smFRET analysis (this is not expected to change conclusions).

We did not correct for this but as the reviewer noted this does not change the conclusions of this study. We have now added this information in the Methods section.

4) Authors may want to state in their cryo-EM results section that LK3 used there was a neutral binder in terms of activity, i.e., the structure information acquired is not expected to be perturbed (tested later by smFRET).

We included a short clarification in the main text of the manuscript, with appropriate figure callout.

Reviewer #3 (Remarks to the Author):

This is a nice body of scientific work that uses cryoEM, smFRET, synthetic antibodies (neutral & activating) and mutational analysis to examine conformational states of Latrophilin3 (ADGRL3) receptor ECR relative to the TMH core.

We thank the reviewer for their appreciation of our work.

While I enjoyed the manuscript, I did have some issues, that should be addressed. (1) The lack of TMH evidence presented for the cryoEM data and (2) the overstatement of three defined states with respect to the smFRET studies.

Thank you for your comments. These points are indeed extremely important to clarify. Please see our answers below.

Figure S2b and S3 lack images showing the receptor TMH which should be visible at the reported ~nanometer resolution for this region. Thus, it is unclear to this reviewer in the absence of the maps if the rotation of the ECR is relative to the TMHs or an empty micelle based on the pictoral information provided. Even the 3D variability analysis is only relative to the micelle. This relative orientation (to the TMH) is critical to the conclusions of the manuscript and should be addressed and made more clear to the reader.

We are sorry that reviewer 3 seems to not have received the maps and other files that we had provided during the review process. We are now providing these files in the revised manuscript as Supplementary Movies 1 and 2. In case there is a problem with access to the files, the reviewer can also access these files in this dropbox link (please be aware that the authors are not sure whether your identity will be compromised if you click on this dropbox link that belongs to Demet Araç):

https://www.dropbox.com/scl/fo/nx1b1yh10n1j4kl283w69/AHDmimFlGno7rcaLo41c8Ac?rlkey=y0di4gf3v3d9zllb1ptcunm29&dl=0

The reviewer is correct about the lack of a very distinct density related to 7TM helices in our reconstruction. However, at the current resolution and with the quality of our model we can observe a lower-resolution density in the middle of the micelle that corresponds to the 7TM region. Thus, we docked the available structure of apo-state ADGRL3 (PDB 8jmt) by rigid-body fitting into the available density and made sure that the first α-helix is placed in close vicinity to linker connecting it to the GAIN domain. In other words, we used the covalent connection between the C-terminus of the GAIN domain and the N-terminus of the TM1 as an anchor point to unambiguously place the 7TM structure into the low resolution 7TM density within the micelle. Our visual inspection shows that this fit is indeed the best possible fit when the overall shape of the 7TM is considered. We now provide a better description of the fitting process in the manuscript and provide Supplementary Files. Please see the new Supplementary Movie 1, that we hope better represents the 3D model of ADGRL3 in complex with the sAB LK3. Additionally, please refer to the submission maps and ChimeraX session files that we provide:

1. The sharpened map of ADGRL3 HormR/GAIN/7TM in detergent micelle in complex with LK3 from the Non-Uniform Refinement job (as in Fig. 1).
2. The same map as in (1), gaussian filtered to 6A - this map allows for better visualization of the low-resolution 7TM density within the micelle.
3. The 3.9A map of HormR/GAIN domains from local refinement job, along with the pdb file with the atomic model (as in Fig. 3).

Additionally, we include files:

4. & 5. ChimeraX session files with the models of HormR/GAIN and 7TM (pdb: 8jmt), rigid-body fitted into the maps described in points (1) and (2). Models are colored as in the manuscript, the start of TM1 in pdb 8jmt is colored purple to show the proximity to the TA.

As stated in the manuscript, we believe that the reason we were unable to visualize the separate helices in the 7TM domain is the absence of G-proteins to lock the 7TM region in an active state and the general flexibility of the GAIN domain in relation to 7TM. As a result, GAIN with sAB bound, being a more distinctive feature, dominated the alignment, causing the density for the 7TM to be washed out during averaging. To resolve TMs, we would have to include another antibody (binding to 7TM) or G-protein to stabilize the helices and facilitate the alignment process. However, we would like to point out that this approach was carried out by others (Barros-Álvarez et al. 2022 - PMID: 35418682) and resulted in averaged-out density for the ECR, which was the main focus of our study. We would like to clarify that while the overall resolution is ~5.5 Å, in the 7TM region, the resolution is only ~10 Å, and helices resolve at around ~8 Å.

Finally, we want to remind the reviewer that the cryo-EM analysis and smFRET analysis are in complete agreement about the position of the GAIN domain relative to the 7TM. Although we did not include the data in the manuscript, we have designed at least 4 other FRET pairs between ECR and 7TM as controls and tested them for any mismatch with the cryo-EM data and the 3 FRET pairs in the manuscript and found that all FRET pairs matched with the cryo-EM data and with each other.

Moreover, the 3D variability analysis used requires much more details in the methods and perhaps its own section. If possible, the change relative to the TMHs is critical for this analysis to be meaningful.

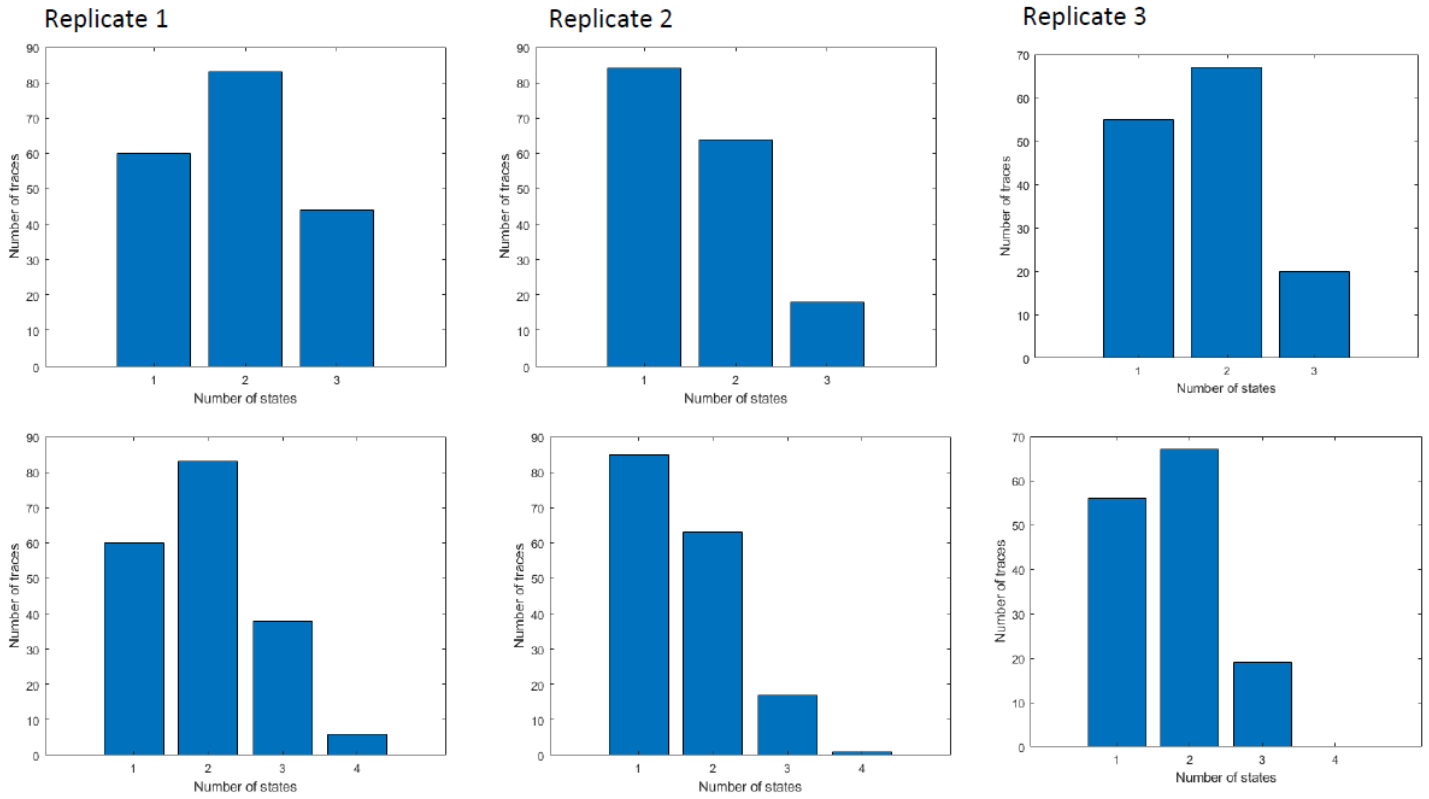
We now included a separate section for the 3D Variability Analysis in the Methods, that describes the data analysis process in detail. As in the point above, though we cannot resolve TMs to high resolution, we still observe a low-resolution density in the middle of the micelle in our 3D Variability Analysis that can accommodate 7TM region (blue density within the micelle in Fig. 2a-c). We also provide a new Supplementary Movie 2, that further presents the extent of the movement of the ECR in relation to the micelle and the 7TM region.

Additionally, the LK1 cryoEM looks promising, however it is only shown in Fig5j. No specifics of this were obvious to me in the methods and standard cryoEM table. While a different FAB orientation is observed it is unclear from the overlay how the ECR is affected, except as stated on line 261.

We have included an additional description in the method section for the ADGRL3 in complex with LK1, provided a clearer depiction of the model in the new Supplementary Fig. 12, and included the data collection statistics in Supplementary Table 1.

As for the smFRET studies being able to be fit to three defined states I find this data compelling (given the differences between FABs and mutants) but it is of my opinion that it is overstated in the manuscript (especially line 219). As such I would recommend using language that would state can be modeled to 3 states. Additionally, it would improve the rigor to also have chi squared surface plots in the supplemental demonstrating that 3 states is indeed the optimal fit over the other number of states (i.e. 1,2,4 and 5) tested.

We thank reviewer for the suggestion, and we have adjusted the wording in the text in line 219 as suggested. Regarding the choice of the number of states for fitting, we chose three states because: 1) Manual inspection of all the traces by two independent people consistently showed three states. 2) Qualitatively idealized traces showed three states consistently between replicates and conditions. 3) Permitting fitting up to 4 states for the FRET data found only very few traces that had 4 states as shown in the figure below for three replicates.



We thank all reviewers for their constructive comments and questions. We believe the manuscript is now in a much better shape after addressing these comments. We are very excited about this manuscript and we hope to first convince the reviewers and then the field about these exciting results!

Response to Reviewer Comments

Structural analysis and conformational dynamics of a holo-adhesion GPCR reveal interplay between the extracellular and transmembrane domains

NCOMMS-24-05828A

Reviewer #1 (Remarks to the Author):

The authors have adequately addressed all of my comments. As for the results regarding the GPS-uncleavable ADGRL3 mutant (T855G), the authors have uncovered a very interesting finding giving that this issue is still very contentious in the Adhesion GPCR field. This shift in smFRET populations warrants further characterization and, as noted by the authors, is beyond the scope of this study. I agree that these results not be included as part of the manuscript to allow authors to further analyze this in the future.

We thank the reviewer for their helpful comments and suggestions and allowing for further studies of the autoproteolysis mutant of ADGRL3.

Reviewer #2 (Remarks to the Author):

The authors have done a good job of making revisions in response to my comments. The work will be of substantial interest to the field.

We thank the reviewer for their appreciation of our work.

Reviewer #3 (Remarks to the Author):

I have now reviewed the EM map files. Overall, the authors provide some evidence that suggests multiple ECD orientations can be observed in the cryoEM ensemble relative to a mobile and/or absent 7TM core. The smFRET studies provide support for multiple orientational ECD distributions relative to a probes located on the receptor. The data from these two complementary techniques (cryoEM and smFRET) provide a suggestive explanation for a plausible scenario describing how this receptor may exist. However, other scenarios or weaknesses are not discussed.

We would like to thank the reviewer for the detailed review and highlighting the complementary insights provided by our cryo-EM and smFRET data.

My major concern is the TM helices are absent and the general location of the TMH core is vague at best. Also of concern are statements such as: “With current model, we can unambiguously define the position of the 167 GAIN domain in relation to the 7TM (Fig. 1d, e).” This bold over-statement makes me question the author’s scientific rigor and brings the entire body of work and conclusions into question. Parts of Figure 1 are very misleading. As it stands now, the paper should not be accepted for publication in its current form. Since there is not clear receptor density in the maps and the conclusions of ECD

domain movements that are relative to this unresolved receptor density are again misleading.

Thank you for the critical discussion of the weaknesses in our data.

The reviewer is correct in noting the lack of distinct density for the transmembrane region in our cryo-EM reconstruction. The proposed location of the transmembrane region is based on rigid-body fitting of the available structure of ADGRL3 7TM into a weak density within the micelle, as observed in the 6 Å low-pass filtered map of the ADGRL3.

However, we would like to clarify that we do not make any detailed claims regarding the movement of the ECR relative to specific parts of the transmembrane core of the receptor. The description of the GAIN domain's position above the transmembrane region/micelle (line 166, as mentioned by the reviewer) was intended to describe the flat orientation, with the main surface of the GAIN domain facing the membrane, as stated in the following sentence (lines 167-169). We recognize that this statement could be misinterpreted as an overstatement, and we have revised the text and Figures 1 and 2 accordingly, to avoid misleading the readers. Additionally, we have provided a more detailed description of the data and the use of the low-pass filtered map for the proposed (or hypothetical) placement of the 7TM structure.

In Figure 1d, we now present the 3D map of ADGRL3 in the micelle, low-pass filtered to 6Å (with the threshold level set to 0.0375). There, we color-coded the regions of the map according to their respective domains, and replaced the “7TM” label with a more general “TM region” description. This figure aims to demonstrate that all parts of the receptor are present in our sample, with the vertical density in the middle of the micelle corresponding to the dynamic 7TM core.

Figure 1e shows a composite of the atomic model of the ECR positioned above the 6 Å micelle region.

In the new Figure 1f, we present a distinctly labeled composite model of ADGRL3 fitted into the 6 Å cryo-EM density, with the hypothetical location of the 7TM (PDB: 8jmt) greyed out.

In Figure 2, we have removed the fitted 7TM structure from the micelle densities and replaced it with featureless shape representing the transmembrane region. All interpretations of 3D Variability Analysis are now specifically related only to the micelle.

If the authors plan to move forward with publication; it is my opinion that this lack of cryo-EM receptor density caveat needs to be *explicitly stated in the text*. Additionally, other possible interpretations should be suggested in the discussion.

We adjusted the result section accordingly and included the possibility of other receptor positions within the micelle in the discussion section.

Importantly, “hypothetical location” should be added to all figures showing any receptor placement so as not to mislead readers on the quality of the cryo-EM data.

As mentioned above, we now labeled the 7TM placement accordingly in Figure 1.

I have listed some comments related to these concerns:

Line 34 – cannot say 7TM as they are not visible in the cryo-EM – can say hypothetical position of the 7TM
Line 35 – hypothetical 7TM (as above)

As smFRET uses sensors placed within ECR and transmembrane domain, we suggest using the general term “transmembrane region” in the abstract instead of “hypothetical 7TM”.

Line 112 movement is with respect to residues XXX from smFRET studies (can list specific 7TM helix or loop)
Line 118 with respect to residues XXX (as above)

Based on available structures and AlphaFold prediction, the smFRET sensors used in this study - R933UAA, E1099UAA and A871UAA – are indeed located within the 7TM domain. While we believe that this level of detail in the introductory paragraph may be excessive, we have now named the specific sensors from the GAIN and 7TM in the text as suggested.

Line 165 – there is no receptor density present in the maps - – adjust the text to appropriately describe the map quality in this region

Line 166 – Major Concern! “With current model, we can unambiguously define the position of the 167 GAIN domain in relation to the 7TM (Fig. 1d, e).” I find this statement grossly overstated given the maps provided.

Adjusted the text accordingly as stated in the response above.

Line 172 – The low resolution density for the TMDs – it is absent – tone down the rhetoric and describe the data
174 - hypothetically place (what density?)

We adjusted the text accordingly, using 6 Å low-pass filtered map of ADGRL3 as a guide for the proposed placement of the available 7TM structure.

185 – 3D Variability is relative to the detergent micelle – cannot make claims about receptor 7TM density based on the maps provided – adjust language to describe the data
190 close to the hypothetical 7TM placement

We made appropriate changes in the text.

234 could be helpful here to list what TMHs or loops you placed the fluorophores on (also can label in figure)

We have included the placements of the fluorophores in the text and detailed their locations in Figure 4a.

289 – hypothetical 7TM orientation

Revised accordingly.

342 hypothetical 7TMs from cryoEM and follow up smFRET studies help define the relative position
346 can be modeled up to 3 states with respect to residues XXX on TM helix XXX

Adjusted the text accordingly.