Manuscript Title: Autoantibody mimicry of hormone action at the thyrotropin receptor

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referees' comments:

Referee #1 (Remarks to the Author):

In this manuscript, Faust and colleagues describe atomic models of the active and inactive conformations of the TSHR using cryo-EM. To my knowledge, this is the most complete description of these different structures reported to date. Most importantly, the differences in the interactions among ligand, TSHR ECDs and 7-TMHs, and the lipid bilayer allow for plausible working hypotheses of the mechanisms of TSHR activation by TSH and an activatory autoantibody M22 and the inhibition by a neutral antagonist antibody K1-70 and inverse agonist antibody CS-17.
The data are clearly presented and appear valid. The findings are original and the conclusions drawn are appropriate.
Specific comments:
Line 42 – sensing of thyroid hormones occurs at the hypothalamus and at the pituitary thyrotroph
Line 53 – it is important to include that TSIs, which are not regulated by physiologic processes, persistently activate TSHRs.
Line 356 – The model in Figure 5 is not as clear as it could be. At the least, I suggest the cartoon contain the abbreviations (TSH, M22, CS-17).

Referee #2 (Remarks to the Author):

The TSHR regulates the release of thyroid hormones that play critical roles in growth, metabolism etc., and dysregulation of TSHR activity (e.g. through activation by autoantibodies) leads to disease. As such the mode of activation by TSH and disease-causing antibodies is of clinical relevance. In the current study, Faust et al, have applied cryo-EM to determine structures of TSHR bound to TSH or the agonist autoantibody M22, in complex with Gs protein. To understand activation mechanisms, they also solve the structure of an inactive state TSHR in complex with an allosteric inhibitor, together with an inverse agonist Fab (CS-17). The authors propose a mechanism for activation based on these structures.
The structures are of moderate resolution (low for the TMD of the inactive), and while they are consistent with the proposed "steric clash" mechanism, direct evidence is limited. Overall, the work is interesting, but the mechanistic advance is currently overly speculative relative to the available data, while many of the global structural features are similar to previously published structures of the related glycoprotein hormone receptors (LHCGR, FSHR), including inactive and active LHCGR. The current work provides additional confidence in the likely generality of the ECD movements associated with glycoprotein hormone receptor activation. There is also only limited (and largely

speculative) insight into how the ECD movements propagate to activation of the TMD.

Specific comments:

Figure 1 (legend) – sulfotyrosine residue Y335 (sY335) is labelled "pY335" in the figure

Lines 131-132 – from the map and pdb supplied, the modelling of the "seatbelt" loop is problematic, particularly in the vicinity of D93.

Lines 134-138 – given the map resolution and problems with modelling, it is difficult to draw conclusions on TSH engagement in this region. The low resolution suggests that this region may also be conformationally dynamic creating additional issues in interpretation. It would be useful for the authors to try the 3D variance analysis (3DVA) in Cryosparc to see if they can gain further insight into the complex.

Lines 153-155 - How much of this might be to do with the way the data were reconstructed, conditions of vitrification etc. What does the 3DVA look like?

Line 171 – what is being defined as "high resolution features", in the map provided there were not a lot of what would generally be considered "high resolution" features (e.g. unambiguous sidechain rotamer placement)

Lines 172-173 – "enabled an accurate model…" is an overstatement. The resolution (in the map provided) is such that the rotamer is ambiguous for most residues. Also many residues have been modelled that are not well supported by density.

Lines 188-189 – How well does the ECD location modelled here fit with the low-resolution map from the structure with the allosteric inhibitor but without CS-17?

Lines 206-209 – In my opinion, there is insufficient evidence to support this claim on mechanism. The resolutions are not good enough to have confidence in the extent of order/disorder between the 2 states…

Figure 3F – both the A644K and A647K mutants have substantially lower cell surface expression as assessed by FACS. As such, it is difficult to interpret the impact on these mutations on receptor activation by ligands in the types of functional assays that have been performed.

Lines 257-260, While this may be true, better evidence is required that this is a true difference versus an artefact of processing and sample preparation/vitrification that has impacted on the resolution.

Lines 269-271, what do the mutations do to activity of the NAM?

Lines 272-278, as per comment above, this is over-interpreted. There is no data on the extent to which the cell surface expression is driving the pharmacology, and no ability to link

mutants/expression and lipid binding. They need to (minimally) do mass spectrometry experiments to compare relative lipid binding. Also, what do less dramatic mutations do to TSHR function?

Lines 309-311, it would be useful to report the glycosylation status of the TSH used in the currently study.

Lines 322-324, while the speculation is not unreasonable, this has not been sufficiently established in the current study.

Lines 326-339, While the increase in agonism seen with insertion of a glycosylation site into K1-70 is consistent with the authors hypotheses of steric hindrance from the membrane driving changes to the ECD orientation required for activation, the observation that K1-70 is an agonist is in contrast to the hypothesis being tested by the insertion of a glycosylation site (their modelling is not consistent with WT K1-70 being an agonist by steric hindrance). It is also inconsistent with the prevailing literature on the pharmacology of K1-70. As such, this requires further experimentation to enable interpretation of the data.

Lines 338-339, as per the argument above, this conclusion is at odds with their observation of K1-70 agonism. Moreover, this is somewhat simplistic as it does not robustly address how this is propagated to the changes in the TMD that are required for activation.

Lines 351-353, While I agree that steric hindrance of interactions with the lipid bilayer appear to be important, I don't think the authors present anywhere near enough evidence to support this being the primary mechanism for activation.

Lines 363-364, can the authors please illustrate how their data "demonstrate" the hypothesis around the hinge regions. This appears to be inferred rather than demonstrated.

Lines 365-366, again this is inferred rather than demonstrated. Do they have data on the actual glycosylation status of N52 for the TSH used in the current study?

Lines 367-368, more robust data is required. The authors infer the orientation of the mutated residue and membrane (based on isolated ECD xtal structure), they really need to demonstrate that glycosylation at other sites does not change K1-70 activity, or solve a complex of the TSHR with the modified Fab.

Lines 369-370, again this is inferential. The data show distinct orientations but do not specifically speak to activation.

Lines 374-375, the data is currently purely observational and not sufficient to conform likely mechanism w.r.t. phospholipids in the TMD.

Supp. Fig. 7. Title is incorrect (this is not TSH-bound TSHR-Gs complex).

Line 820, what specifically does "samples" refer to here?

Line 851, "cAMP accumulation" – the description does not mention PDE inhibitors. Do they really mean accumulation or "production"? What is the basal cAMP? Does this vary for mutants?

A substantial weakness of the study is that there is no clear insight into how the changes in ECD drive receptor activation (i.e. conformational change in the TMD).

Suppl. Figs. 2, 5, 7 all display local resolution maps that suggest a large proportion of the maps are close to 2 angstrom resolution. The maps are completely at odds with this (most appear to be ~3 A at best – which is also what is reported in the structure statistics Table, suppl. Table 1). None of the maps have features consistent with near atomic resolution. As such, this is wrong.

Does CS-17 block activation of TSHR by small molecule agonists such as ML-109? Does this affect understanding of the mode of receptor activation?

Does the allosteric inhibitor Org 274179-0 inhibit activation by the M22 antibody? Is the inhibition equivalent or different from the inhibition of TSH activation?

TSHR is reported to dimerise, with negative cooperativity on binding by TSH to multiple protomers. Can the authors speculate on how their models for activation work in the context of dimers?

There are a lot of sidechains (across all structures) that have been modelled where there is no robust density to support the modelling. In the seatbelt loop region, there are also issues with the backbone modelling relative to the map density. The authors need to provide strong justification for why these are included in the deposited PDB file (I understand why they need to be present for model fitting). If they are to be included, the authors need to provide some sort of record that explicitly states where the density supports the modelling and also where it does not.

Referee #3 (Remarks to the Author):

Faust et al. provide novel insights into the mechanism of activation for the thyrotropin receptor (TSHR) and beyond a molecular understanding of how autoantibodies activate the TSHR and pathologically increase thyroid hormones in Graves' disease. For this, the authors determined cryogenic-electron microscopy structures of the active TSHR in complex with thyrotropin (TSH) and M22 - an autoantibody isolated in patients with Graves' disease as well as the inactive TSHR bound to CS-17. One of the most important structural finding is the role of steric clashes between protein agonists/inverse agonists and the membrane that drive either active- or inactive-like conformations of the extracellular domain of the TSHR. Overall, the obtained structural insights are highly relevant in particular as the reported mechanism may have implications for other GPCRs with large extracellular domains.

Comments:

1) Authors write that in the inactive state, the down conformation of the TSHR ECD would be unable to bind TSH due to clashes between the glycosylated Asn52 residue in the common GPHα chain. At times ECD domains can also anchor the membrane with hydrophobic residues. Is it possible that in addition to steric clashes with the N-linked glycans, another important function of this highly polar chains could be to impede membrane interaction/penetration of the ECD? Could authors comment on this scenario?

2) The authors resolved densities for a phospholipid buried within the 7TM domain of the receptor in a region that overlaps with the likely binding side for Org 274179-0 in TSHR and Org43553 in LH/CGR. Additional mutational (A644K, A647K) experiments provide evidence for a functional role of a lipid in this transmembrane pocket. This is an extremely interesting finding and has important implication for the TSHR function in different cell membrane environments. Can the authors conclude about lipid specificity? Would it be possible to measure agonist-mediated cAMP accumulation in the presence of different types of lipids considering different tail lengths and saturation degrees?

3) Authors present models of how the membrane bilayer may interact with TSH, M22, or CS-17 when bound to the TSHR ECD in either the active up or inactive down conformations. Please provide more information about how the complexes (e.g. M22 bound to inactive TSHR, etc.) were modeled. I would also propose to provide the modeled complexes as supplemental PDB files.

4) Authors measure rotations, e.g. "The TSHR ECD is rotated 38° towards the 7TM domain and the membrane bilayer in the inactive state when compared to the active state bound to TSH". Can authors please specify how are the rotation angles were obtained?

5) Authors measure distances, e.g. "TM6 of active TSHR is displaced outward by 14 Å to accommodate the α5 helix of miniGαs and TM7 moves ~4 Å inward relative to the transmembrane core of the receptor". For reproducibility, which exact points were used to measure these distances?

Other:
6) Caption 4: "…Modeling of TSH binding to inactive TSHR with the ECD in the down conformation with either TSH (b) or M22 Fab (c)…."
Remove TSH from "TSH binding"

Author Rebuttals to Initial Comments:

Referee #1:

Remarks to the Author:
In this manuscript, Faust and colleagues describe atomic models of the active and inactive conformations of the TSHR using cryo-EM. To my knowledge, this is the most complete description of these different structures reported to date. Most importantly, the differences in the interactions among ligand, TSHR ECDs and 7-TMHs, and the lipid bilayer allow for plausible working hypotheses of the mechanisms of TSHR activation by TSH and an activatory autoantibody M22 and the inhibition by a neutral antagonist antibody K1-70 and inverse agonist antibody CS-17.
The data are clearly presented and appear valid. The findings are original and the conclusions drawn are appropriate.

We thank the reviewer for careful reading of our manuscript and evaluation of the mechanisms proposed. Importantly, we agree that the manuscript provides a working hypothesis that is itself an important advance for the field. We have now added significant new evidence to this working model based on a new, higher resolution structure of a TSH analog (TR1402) bound to TSHR and additional mutagenesis studies to examine how ECD rotation leads to activation of the 7TM domain. Additionally, we have performed new molecular dynamics simulations to provide further evidence for our hypothesis that the membrane bilayer is a critical component of receptor activation.

Specific comments:
Line 42 – sensing of thyroid hormones occurs at the hypothalamus and at the pituitary thyrotroph

This is helpful and we have updated the manuscript accordingly.

Line 53 – it is important to include that TSIs, which are not regulated by physiologic processes, persistently activate TSHRs.

This is helpful and we have updated the manuscript accordingly.

Line 356 – The model in Figure 5 is not as clear as it could be. At the least, I suggest the cartoon contain the abbreviations (TSH, M22, CS-17).

We have now revised this figure to make the depictions clearer.

Referee #2:

Remarks to the Author:
The TSHR regulates the release of thyroid hormones that play critical roles in growth, metabolism etc., and dysregulation of TSHR activity (e.g. through activation by autoantibodies) leads to disease. As such the mode of activation by TSH and disease-causing antibodies is of clinical relevance. In the current study, Faust et al, have applied cryo-EM to determine structures of TSHR bound to TSH or the agonist autoantibody M22, in complex with Gs protein. To understand activation mechanisms, they also solve the structure of an inactive state TSHR in complex with an allosteric inhibitor, together with an inverse agonist Fab (CS-17). The authors propose a mechanism for activation based on these structures.

We thank the reviewer for summarizing the main scope of our manuscript and its clinical relevance. To recap our goal was to describe how TSH activates the TSHR and how autoantibodies can mimic this activity, a molecular medicine puzzle that has remained unclear for decades.

The structures are of moderate resolution (low for the TMD of the inactive), and while they are consistent with the proposed "steric clash" mechanism, direct evidence is limited. Overall, the work is interesting, but the mechanistic advance is currently overly speculative relative to the available data, while many of the global structural features are similar to previously published structures of the related glycoprotein hormone receptors (LHCGR, FSHR), including inactive and active LHCGR. The current work provides additional confidence in the likely generality of the ECD movements associated with glycoprotein hormone receptor activation.

Based on the summary statements provided here by the reviewer, and specific comments outlined below, we have now significantly revised the manuscript to support the key mechanistic advances proposed in the manuscript. Key additions include:

1) *A new, higher resolution structure of a TSH analog that improves our interpretation of key hormone-receptor contacts. With this structure, we now appreciate additional structural motifs in the receptor hinge region previously undescribed by prior publications.*
2) *New mutagenesis experiments that directly assess how ECD conformational changes impinge on receptor activation and how ECD rotation propagates a signal to the 7TM domain. Based on comments from the reviewer, these revised experiments were done with carefully matched expression levels of TSHR.*
3) *Molecular dynamics simulations that demonstrate the effect of the M22 and CS-17 Fab fragments on controlling the flexibility of the TSHR ECD. Simulations on apo, M22, and CS-17-bound TSHR directly interrogate how these ligands influence ECD dynamics and provide further evidence for the proposed steric clash mechanism.*

A critical differentiating feature of our work compared to the recent cryo-EM structures of LH/CGR is how glycosylation drives signaling for the eponymous glycoprotein hormones.

Furthermore, we provide fundamental insights into how antibodies can control TSHR signaling, which is distinct from prior work on the glycoprotein hormone receptors.

There is also only limited (and largely speculative) insight into how the ECD movements propagate to activation of the TMD.

We agree that our prior manuscript provided little experimental evidence for signal propagation from the ECD to the TMD. Due to the limited resolution of our inactive-state structure, we previously refrained from making speculative claims on how ECD movements propagate to activation of the TMD. Based on a higher resolution structure of TR1402 (a high affinity TSH analog) bound to TSHR, we now have greater confidence in modeling of multiple hormone receptor contacts (specifically in the hinge region and the hormone-selectivity "seatbelt loop") and in critical ECD-7TM interfaces (specifically in the ECL1-hinge helix and p10-TM7/ECL3 regions). With the improved reconstruction in the ECD-7TM interface, we have designed specific structure-guided mutagenesis to interrogate how conformational changes in the TSHR ECD propagate to the 7TM domain. Our signaling experiments suggest that ECD rotation to the "up" state is sufficient to activate the receptor, and that multiple points of contact in the p10 and ECL1 interfaces contribute to hormone-mediated signal transduction into the 7TM domain. These experiments connect ECD rotation to 7TM activation and are outlined in detail below.

Comments from separate email:
The steric clash component of the activation mechanism for TSH and activating antibodies is similar to that previously proposed for other glycoprotein hormone receptors. With the limited resolution of some of the structures, in the absence of molecular detail and mechanism, this did not appear sufficient for publication in Nature.

The aim of our manuscript is to describe how the TSH hormone and autoantibodies control TSHR activity, which is distinct from prior work on the LH/CGR. Our work explains a broadly known medical puzzle that the vast majority in the medical community have encountered, we therefore believe this will be of significant interest to a broad readership not limited to only the GPCR structural biology community. We have now significantly added new experiments and revised the text to provide more support for our proposed mechanism, which we hope the reviewer will agree yield a significantly strengthened manuscript.

It is clear that the receptors can be activated without an ECD-steric clash mechanism - as evidenced by small molecule agonists.

We agree with the reviewer that the action of small molecule ligands at TSHR and other GPCRs is important. However, the confluence of GPCR structural biology over the past decade has taught the field that small molecules can engage GPCRs in completely novel ways as compared to endogenous hormones (outlined nicely by Sexton and Christopoulos in https://pubmed.ncbi.nlm.nih.gov/29973731/). The present manuscript is not an attempt to provide a comprehensive set of mechanisms for TSHR or glycoprotein hormone activation, but instead to understand the fundamental question of how autoantibodies mimic the action of TSH

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and to provide a refined model for how glycosylation drives glycoprotein hormone function. As noted below, we believe that a deeper inquiry of how small molecule agonists activate TSHR, and whether this occurs independent of ECD-steric clash, deserves sufficient space for a future body of work/manuscript.

What was not clear to me was (i) the mechanistic link between steric repositioning of the ECD and how this propagates to the conformational changes required for activation and transducer engagement and,

We agree that this was previously not well articulated. We have now performed new structure-guided mutagenesis experiments to better understand the mechanistic link between rotation of the ECD and activation of the 7TM domain. These studies provide a working model for signal propagation.

(ii) whether sterically driven shifts in the ECD alone versus other conformational effects that might be associated with the binding of ECD activators might also be required/contribute. The presented data did not address this.

If we understand correctly, the reviewer is concerned that ECD activators may directly change the conformation of the ECD, leading to receptor activation independent of the very dramatic rotation observed with activators. We don't believe this is the case for several reasons. First, the conformation of isolated TSHR ECD in the active and inactive states is highly similar with a root mean squared deviation (RMSD) of 0.64 Å. This suggests that the entire ECD moves as a rigid body between the active and inactive states with relatively minimal conformational changes within the ECD itself. We have now clarified this further in lines 246-249.

To address this concern more directly, we have now designed a strategy to "lock" the ECD in the upright conformation in the absence of a TSHR activator. To do so, we designed a disulfide bridge between positions 262 and 483 that is only compatible with the active TSHR state. Consistent with ECD rotation being sufficient for activation, we find that the double cysteine mutant is constitutively active. Individual cysteine mutations at these positions have similar constitutive activity as wild-type TSHR. This experiment is now included in revised Fig 2i, 2j (double cysteine mutant modeling + signaling), and Supplementary Figure 1 (individual cysteine mutations), and provides clear evidence that domain rotation is sufficient to activate the receptor in the absence of further conformational changes induced by TSH or the M22 antibody.

I would also like the authors to explain activation that occurs in the absence of ECD binding (small molecule allosteric agonists) and what happens with respect to the ECD in this process. Obviously, this mode of activation does not require a steric clash mechanism

We agree that this is an interesting line of inquiry in understanding control of TSHR signaling by small molecule ligands, which has important implications for the development of therapeutics targeting TSHR. However, our current study does not map where any of the presently known small molecule allosteric modulators bind TSHR. As such, we are reluctant to speculate on how

these molecules act at TSHR. Furthermore, we believe these studies are outside the scope of our present manuscript, which aims to explain how hormones and antibodies targeting the ECD control TSHR activity. Even if we were successful in obtaining such data, we believe this line of inquiry would require an entire manuscript with sufficient space to carefully explain the complex allosteric relationship between ECD conformational change and allosteric small molecule binding.

Specific comments:

Figure 1 (legend) – sulfotyrosine residue Y335 (sY335) is labelled "pY335" in the figure

We thank the reviewer for picking up on this - our new higher resolution reconstruction of TR1402-bound to TSHR has subsequently resulted in a large revision of Figure 1. This panel has been removed. Importantly, we now specifically resolve residue Y385 in TSHR and are more confident that this residue likely is not sulfated when interacting with the hormone.

Lines 131-132 – from the map and pdb supplied, the modelling of the "seatbelt" loop is problematic, particularly in the vicinity of D93.

Lines 134-138 – given the map resolution and problems with modelling, it is difficult to draw conclusions on TSH engagement in this region. The low resolution suggests that this region may also be conformationally dynamic creating additional issues in interpretation. It would be useful for the authors to try the 3D variance analysis (3DVA) in Cryosparc to see if they can gain further insight into the complex.

We agree with the reviewer that our prior map of TSHR bound to native TSH purified from human pituitary glands was of low overall resolution, and not particularly good in the seatbelt region of TSH. To gain higher resolution insights into how the hormone interacts with the TSHR ECD, we have now obtained a new cryo-EM structure of TSHR bound to a recombinantly produced TSH analog (TR1402; Mueller S et al JBC 2009; 284(24); https://pubmed.ncbi.nlm.nih.gov/19386596/). TR1402 has four substitutions in the common glycoprotein alpha (GPHα) chain which increase its potency at the TSHR by ~50-fold, but otherwise signals similarly to TSH. The resulting structure of TR1402-bound TSHR has a nominal local resolution of 2.4 Å in the 7TM/G protein region and 2.7 Å in TSHR ECD-TR1402 region. This new structure provides a much clearer view of interactions between the receptor and the hormone. In particular, the seatbelt loop is more clearly visualized in this map, as are interactions between the TSHR hinge region and the hormone. The text and figures are heavily revised taking this new structure into account.

Lines 153-155 - How much of this might be to do with the way the data were reconstructed, conditions of vitrification etc. What does the 3DVA look like?

This section of the manuscript highlighted interactions between the hinge region of TSHR and the TSH hormone. Our prior structure of native TSH bound to TSHR was not sufficiently high resolution to fully understand this interaction. This is likely because of potential flexibility of TSH bound to the TSHR ECD. Unfortunately 3DVA of the native TSH-TSHR complex was not particularly illuminating while trying to understand this complex. However, our new structure of TR1402-bound TSHR provides a higher resolution view of the interaction between the TSHR hinge region and the hormone. This higher resolution view also provides a much clearer perspective for how the native TSH hormone binds TSHR. We have now revised the text and figures to highlight the higher resolution features observed in our TR1402-TSHR structure. Furthermore, we have conducted 3DVA analysis of the TR1402-TSHR complex, which reveals some evidence of flexibility between the TSHR hinge region and the hormone. We anticipate that this flexibility is significantly higher for TSHR bound to native TSH, which precluded a high resolution reconstruction with the native hormone. One of the major components identified in 3DVA shows relative flexibility of the TSHR ECD and 7TM domains, which we have included as Supplementary Movie 1.

Line 171 – what is being defined as "high resolution features", in the map provided there were not a lot of what would generally be considered "high resolution" features (e.g. unambiguous sidechain rotamer placement)

We thank the reviewer for bringing up this subjective interpretation of the EM density map quality. We have removed the term "high resolution" from the text describing the improved reconstruction quality of the CS-17 Fab:TSHR ECD in comparison to the transmembrane domain.

Lines 172-173 – "enabled an accurate model…" is an overstatement. The resolution (in the map provided) is such that the rotamer is ambiguous for most residues. Also many residues have been modelled that are not well supported by density.

We apologize for an oversight in the provided maps. The map we previously provided to the reviewer was an unsharpened map of the entire TSHR-CS-17 complex. Our description of "high resolution features for the TSHR ECD and CS-17 Fab" was based on a focused refinement of the TSHR ECD bound to the CS-17 Fab. This sharpened map enabled more accurate modeling of sidechain rotamers, and we've provided some example figures from this region in Revision Fig. 1. We have also provided this map in an updated Box folder and will also upload this map to the EMDB. We opt to model side chains even in poorly resolved regions to make the model more informative. Please see the final section of this response for our approach to highlighting regions that are well or poorly resolved.
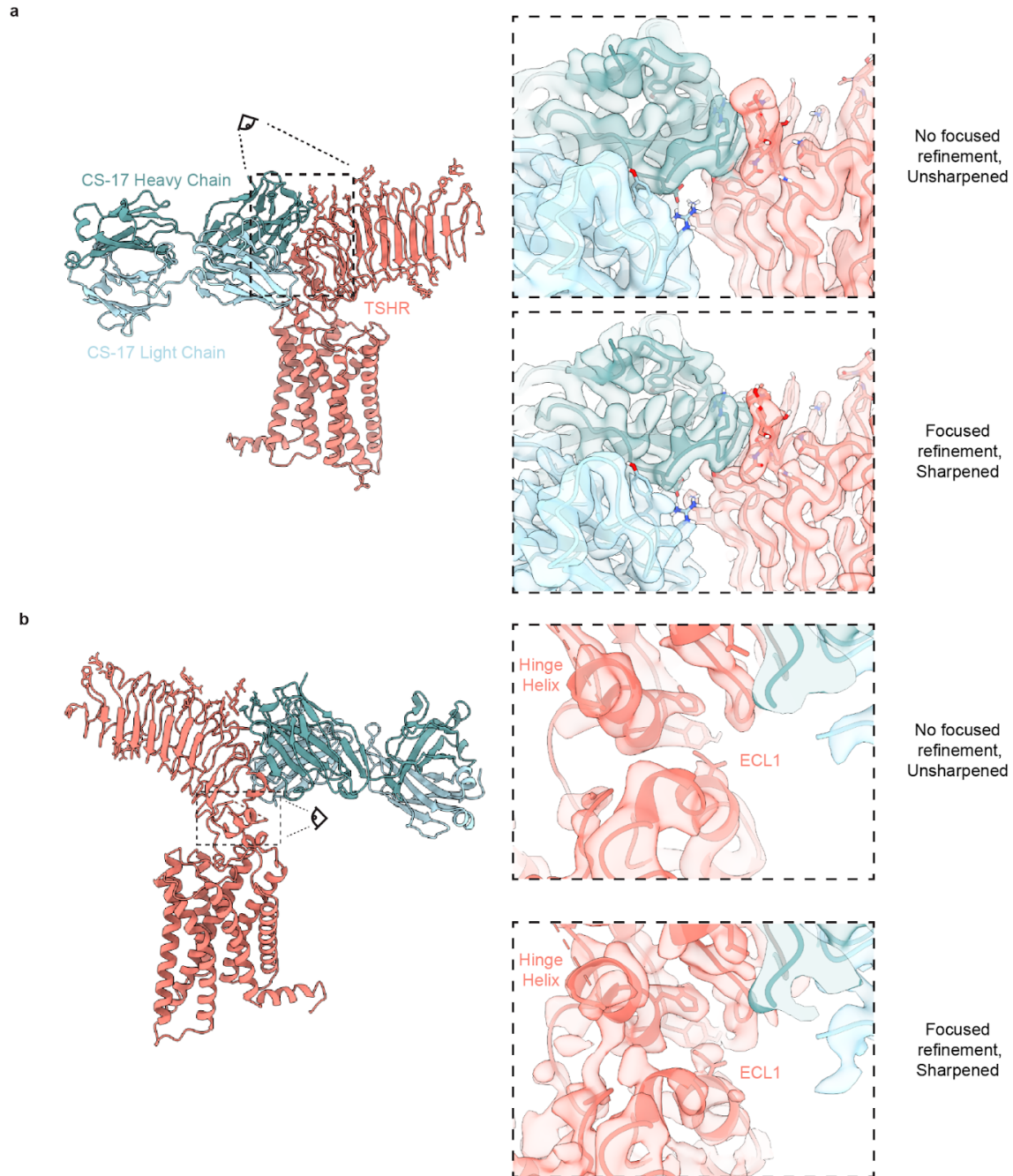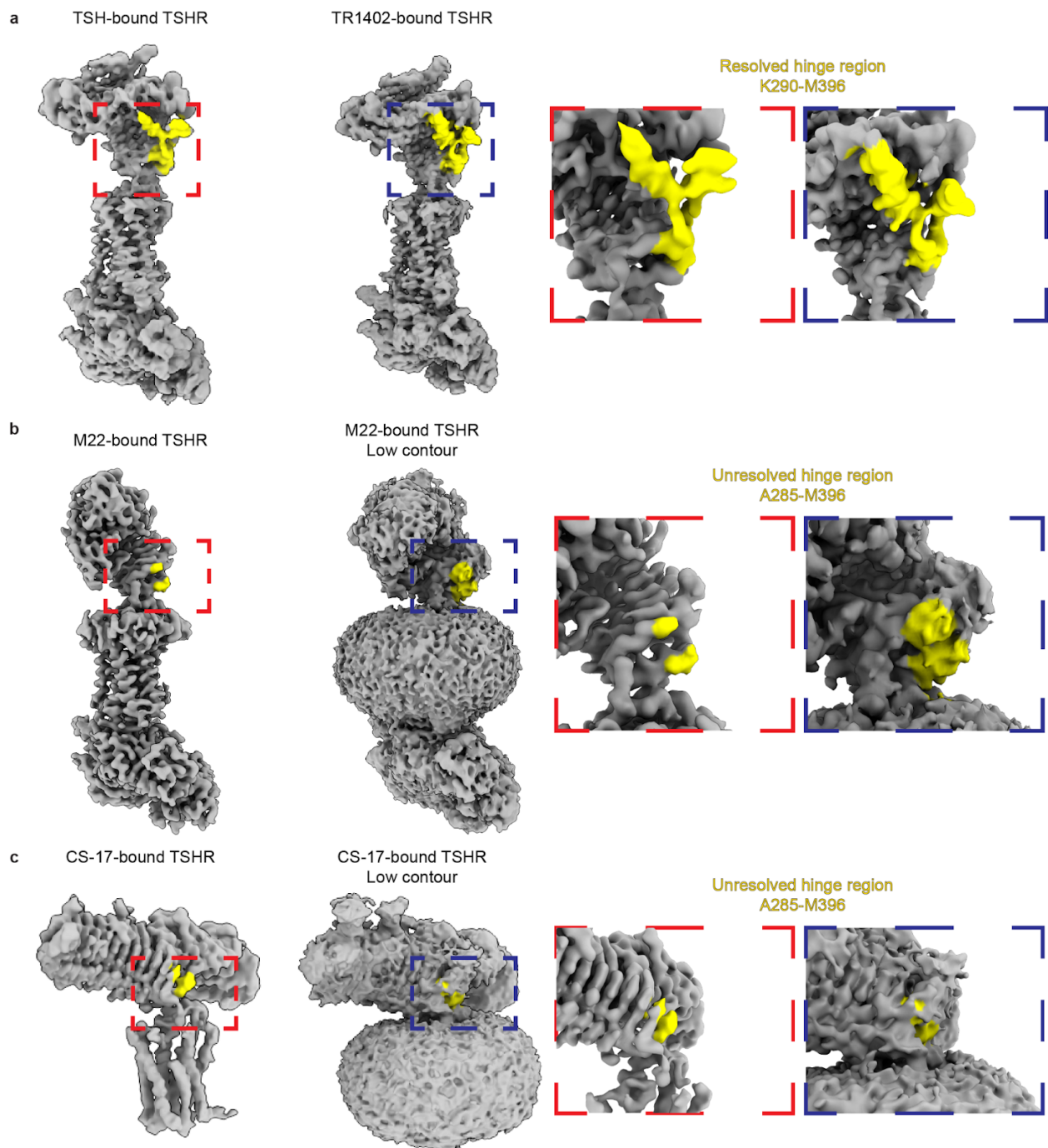
Revision Fig. 1. Comparison of CS-17-bound TSHR map pre-/post- focused refinement and sharpening. *Comparison of the initially deposited CS-17 map without focused refinement reconstruction or sharpening to highlight regions that highlight improved in reconstruction quality upon focused refinement and sharpening.* **a)** *view of the CS-17:TSHR-ECD interface and* **b)** *view of the Hinge Helix:ECL1 interface*

Lines 188-189 – How well does the ECD location modelled here fit with the low-resolution map from the structure with the allosteric inhibitor but without CS-17?

We thank the reviewer for this suggestion. We have now added a comparison of the low-resolution map of TSHR without CS-17 to the higher resolution reconstruction with CS-17 in Supplementary Fig. 9. We see a reasonably good alignment of the ECD density relative to the micelle density in both maps, suggesting that the "down" state of the ECD is reconstructed in the absence of CS-17. We have revised the text to highlight this comparison (lines 249-251, Supplementary Figure 9 in the revised manuscript).

Lines 206-209 – In my opinion, there is insufficient evidence to support this claim on mechanism. The resolutions are not good enough to have confidence in the extent of order/disorder between the 2 states…

We proposed a disorder-to-order transition of the TSHR hinge region in this section of the manuscript. We agree that our prior structure of TSHR bound to native TSH made it challenging to have high confidence in whether a disorder-to-order transition occurs upon hormone binding as the density was not particularly strong in this region. In our new structure of TSHR bound to TR1402, we see two clearly defined alpha helices in the hinge region. With this higher resolution insight, it is now easier to appreciate that a similar interaction likely also occurs for native TSH, but with some inherent flexibility that likely limits a high resolution reconstruction. Comparing the cryo-EM reconstructions of active TSH and TR1402-bound TSHR to inactive CS-17-bound TSHR shows a clear difference in density for the hinge region as seen in Revision Fig. 2. At low contours, there is no discernable structured density observed for the hinge region for inactive TSHR bound to CS-17. By contrast, there is clear density for this region in both the TSH and TR1402-bound structures. We believe this data provides direct evidence for a transition between a disordered state in the inactive state and an ordered state while bound to TSH or TR1402.

Revision Fig. 2. TSHR hinge region reconstruction comparisons a) *EM density map of TSH (L) and TR1402 (R) with hinge region residues (K290-M396) highlighted provided for comparison with the **b)** M22-bound TSHR map and **c)** CS-17-bound TSHR map at contour levels comparable to modeling threshold (L) and at lower volume contour thresholds (R) with hinge region-bound residues (A285-M396) highlighted. At low contour levels, no hinge region structural features (i.e. alpha helices) comparable to those observed in hormone-bound maps are interpretable.*

Lines 257-260, While this may be true, better evidence is required that this is a true difference versus an artefact of processing and sample preparation/vitrification that has impacted on the resolution.

In this section, we previously indicated that the hinge region is unresolved for the M22 structure but resolved in the TSH structure. With a higher resolution structure of TR1402-bound TSHR, this is even more clear - we can now very clearly define a significant portion of the hinge region that interacts with the GPHα chain in TR1402. By contrast, even at low contour, there is no density for this region in the M22-bound TSHR reconstruction (see Revision Figure 2 above).

We do not believe that the absence of resolved hinge region contacts with M22 could be explained by an artifact of processing and sample preparation/vitrification for several reasons. First, TSHR was prepared in an identical manner for TSH, TR1402, and M22-bound complexes with identical final buffer components and detergent concentrations. Vitrification of all of these samples was performed on the same Vitrobot with identical freezing parameters (blot force/time and chamber temperature/humidity). The data collection parameters and computational processing workflows are highly similar between these samples, as highlighted in Supplementary Figs. 2,3, and 11. Importantly, we do not observe any hint of density for the hinge region for the M22-bound TSHR reconstruction. Finally, given the comparable overall resolutions of the TR1402-bound and M22-bound TSHR reconstructions, we believe it is highly unlikely that the dramatic difference in hinge region density can be ascribed to sample preparation or vitrification differences.

Lines 269-271, what do the mutations do to activity of the NAM?

We anticipate that these mutations would alter the ability of the NAM to bind, but cannot be certain since we have not mapped the NAM binding site in our inactive-state structure. While this may be an interesting line of inquiry, we believe this is out of scope to our central reason for making these mutations, i.e. to identify a potential functional role for a putative lipid that binds within the TSHR 7TM domain.

Lipid observed in TM domain and its relevance to function:
Figure 3F – both the A644K and A647K mutants have substantially lower cell surface expression as assessed by FACS. As such, it is difficult to interpret the impact on these mutations on receptor activation by ligands in the types of functional assays that have been performed.

Lines 272-278, as per comment above, this is over-interpreted. There is no data on the extent to which the cell surface expression is driving the pharmacology, and no ability to link mutants/expression and lipid binding. They need to (minimally) do mass spectrometry experiments to compare relative lipid binding. Also, what do less dramatic mutations do to TSHR function?

Lines 374-375, the data is currently purely observational and not sufficient to conform likely mechanism w.r.t. phospholipids in the TMD.

Regarding receptor expression levels: We appreciate this observation and the potential caveat of receptor expression influencing signaling results. To confirm that our conclusions from all Glosensor experiments performed in this study are not confounded by differences in receptor expression levels, we have now carefully controlled for cell-surface expression in all signaling experiments. More specifically, we have titrated transfection DNA concentrations for every TSHR construct and measured receptor cell-surface expression levels by flow cytometry using anti-FLAG antibody (Supplementary Fig. 1). In expression matched cells, we still observe that the A644K and A647K mutations impair the ability of TSH and M22 to activate TSHR. This new data is included in Figure 1i and Supplementary Fig. 1.

Regarding mass spectrometry to compare relative binding for wild-type and mutant: we note that this request is outside the norm for understanding how endogenous lipids may influence GPCR activity. For example, two recent publications identified an endogenous lipid that co-purifies with the inactive GABA$_B$ receptor (Park et al., Nature (2020), Papasergi-Scott, et al. Nature (2020)). In these studies, lipid-displacing mutations were used to understand the relative importance of these lipids in receptor function. We chose dramatic mutations to have the highest probability of displacing the lipid in the cavity - more conservative mutations would confound our interpretation as they would decrease our confidence in whether the mutations displace binding of the lipid.

Lines 374-375 of the original manuscript stated two main conclusions: 1) that the lipid observed within TSHR is in an overlapping region as compared to the LH/CGR structure and 2) that our observation that this lipid site important for TSHR activation may also extend to the other glycoprotein hormone receptors. We respectfully disagree that the data is solely observation of a lipid in our structures. Our revised experiments with A644K and A647K, now accounting for potential caveats arising from differential expression levels, show that this lipid is important for TSHR activation. This evidence is commensurate with the technical capabilities of the field, and reflects similar studies that have identified lipids that act as key cofactors for other GPCRs.

Glycosylation status of TSH:
Lines 309-311, it would be useful to report the glycosylation status of the TSH used in the currently study.

Lines 365-366, again this is inferred rather than demonstrated. Do they have data on the actual glycosylation status of N52 for the TSH used in the current study?

We have several lines of direct evidence that the TSH used in this study is glycosylated.
1) Our structure of TSHR bound to TSH used native human TSH purified from pituitary, which has been extensively characterized in the literature previously. Numerous studies have demonstrated that the common glycoprotein hormone α chain (GPHα) is glycosylated at

Gain of function experiment with K1-70:

Lines 326-339, While the increase in agonism seen with insertion of a glycosylation site into K1-70 is consistent with the authors hypotheses of steric hindrance from the membrane driving changes to the ECD orientation required for activation, the observation that K1-70 is an agonist is in contrast to the hypothesis being tested by the insertion of a glycosylation site (their modelling is not consistent with WT K1-70 being an agonist by steric hindrance). It is also inconsistent with the prevailing literature on the pharmacology of K1-70. As such, this requires further experimentation to enable interpretation of the data. Lines 338-339, as per the argument above, this conclusion is at odds with their observation of K1-70 agonism.

Lines 367-368, more robust data is required. The authors infer the orientation of the mutated residue and membrane (based on isolated ECD xtal structure), they really need to demonstrate that glycosylation at other sites does not change K1-70 activity, or solve a complex of the TSHR with the modified Fab.

We thank the reviewer for helping us think critically about this pivotal experiment. It is common in structure-function work to obtain loss-of-function phenotypes, which are used to support models. By contrast, it is much more rare to devise a gain-of-function based on a precise hypothesis built on a presumptive model from static structures. To recap, the critical experimental result here supporting our proposal that the membrane bilayer is key to TSHR activation is that addition of a glycosylation at a specific position dramatically _increases_ the weak partial agonism of K1-70 into the full agonist activity of K1-70^{glyco}. We appreciate the reviewer's efforts to "kick the tires" to make sure this result is robust.

We interpret three concerns posed by the reviewer for this experiment, which we restate to make sure we address fully: 1) Our modeling of K1-70 based on the prior X-ray structure of the K1-70 and ECD complex using a bilayer boundary defined by OPM is not fully consistent with its activity as a partial agonist as one would expect some clashes with the bilayer in our membrane steric clash model. 2) Our modeling is not sufficiently constrained to enable the precise positioning of a glycan that we desire for this gain-of-function experiment and 3) Glycosylation itself, independent of the specific position it is introduced on K1-70, may directly activate TSHR.

To address #1, we first revisited the difference in activity for K1-70 in our hands compared to prior literature on K1-70. We first reasoned that our TSHR cell lines expressed the receptor at high levels, leading to high receptor reserve that would amplify very weak partial agonism. Titrating receptor expression to lower levels decreased the Emax from ~35% to ~20% (using TSH to define system Emax). Even with these lower expression levels, however, K1-70 is clearly a weak partial agonist in our hands. We are unable to explain the difference in signaling activity in our hands vs prior work. It is possible that the distinction may arise from differential signal amplification in human HEK293 cells (used in our work) and CHO cells (used in all of the prior work we were able to identify). We conclude that K1-70 can indeed have partial agonist efficacy at TSHR. The revised signaling data is included in Figure 4.

As suggested by the reviewer, we next re-evaluated whether the OPM modeling approach is compatible with our proposed mode of action for K1-70. We agree with the reviewer that the partial agonism of K1-70 is at odds with a simple model for no clash between K1-70 and the membrane bilayer for inactive TSHR. This discrepancy is likely because our modeling is a simple static snapshot that does not reflect the full range of orientations the TSHR ECD can adopt. Our new molecular dynamics simulations of unliganded TSHR show that the TSHR ECD can extend further "down" or "up" from our experimental structures of active and inactive TSHR. We therefore now provide a clear caveat to our OPM modeling approach in the text (lines 397-399, lines 407-410), and highlight that these models may not account for conformational flexibility between the receptor and membrane bilayer. We also highlight that there may be additional mechanisms for the weak partial agonist activity of K1-70 not described by a membrane steric clash model (lines 460-463).

Regarding #2: We do not completely understand this concern. If our modeling is completely inaccurate, it is unlikely to give a spurious gain of function (barring a caveat like raised in point #3). We have tried to come up with other caveats that could explain the improved potency and increase in Emax for K1-70^{glyco} compared to K1-70 and have not been successful.

To address concern #3: As suggested by the reviewer, we generated a new K1-70 glycosylation construct (K1-70^{glycoB}) that positions a glycan more distally to the membrane bilayer (Supplementary Fig. 14). Unlike the full agonist K1-70^{glyco}, the K1-70^{glycoB} Fab has a similar efficacy and potency to the unglycosylated K1-70 Fab. Glycosylation itself, is therefore not a sufficient explanation for the gain in potency for K1-70^{glyco}, and we conclude that precise positioning of the glycosylation is required for the improved activity of K1-70^{glyco}. This new experimental result has been included in Supplementary Fig. 14.

With these clarifications and additional experiments, we believe that this result provides evidence that a clash between an appropriately positioned glycan and the membrane bilayer can itself be an important driver of TSHR activation.

Steric hindrance with the bilayer as a mechanism of efficacy
Lines 322-324, while the speculation is not unreasonable, this has not been sufficiently established in the current study.

Lines 351-353, While I agree that steric hindrance of interactions with the lipid bilayer appear to be important, I don't think the authors present anywhere near enough evidence to support this being the primary mechanism for activation.

Lines 369-370, again this is inferential. The data show distinct orientations but do not specifically speak to activation.

A central conclusion our manuscript reaches is that TSH and antibodies exert their efficacy at TSHR by sterically clashing with the membrane bilayer, which leads to conformational selection of either the "up" or the "down" state of the ECD, with the "up" state associated with receptor activation and the "down" state associated with inactivation. In other parts of the manuscript, we have now strengthened the connection for how changes in ECD orientation lead to activation of the 7TM domain of TSHR.

These comments by the reviewer, as well as comments provided in the summary statements above, assert that there is not sufficient information to reach this key conclusion. Unfortunately, the reviewer does not provide a clear alternative model that is consistent with the data provided or fundamental reasons why this model is invalid. In the absence of a clear alternative mechanism consistent with the data, it is unclear to us what would be considered sufficient evidence to provide a molecular mechanism. A full understanding of any structural mechanism will undoubtedly require continuous study for years to come - in the context of the present manuscript, however, we believe we have provided a complementary set of experiments to reach this conclusion. We recap these here:

1) *Our structures directly show conformational differences in the ECD orientation in the presence of either agonistic (TSH, TR1402, and M22) or inverse agonist (CS-17) modulators. Many recent GPCR structural biology studies use similar structural biology results to connect conformation to the efficacy of GPCR modulators. Key examples of similar studies recently published include the agonist, antagonist, and apo structures of Class D fungal GPCRs (Velazhahan et al., Nature (March 2022)), the structural characterization of agonist-bound mGlu2 and mGlu4 heterodimers (Lin et al., Nature (June 2021)) and the structural, functional, and MD-mediated elucidation on the activity of various peptide agonists at the GLP1 receptor (Cary et. al, Nature Chemical Biology (December 2021)). In each of these studies, structural methods reveal a change in conformation associated with binding of a modulator. A combination of structural analysis and functional studies provide further evidence that these conformational changes are important to receptor activation. Our study falls in line with such analyses.*

2) *We previously provided a modeling approach to most parsimoniously explain prior data for the role of the Asn52 residue in glycoprotein hormone action, the action of the M22 antibody, and the action of the CS-17 antibody. Each of these separate observations strengthens the central conclusion that steric clash with the membrane is an important factor in TSHR activation. We have now added text to provide context for the utility of this modeling approach.*

3) *We devised a gain-of-function experiment using the K1-70 antibody to assess whether an appropriately positioned glycan can influence TSHR agonism. We thank the reviewer for bringing up potential caveats for this experiment, which have been addressed above.*

This experiment provides an independent line of evidence for the membrane steric-clash model.

4) *We highlight that the conformational changes within the ECD itself are minimal, with r.m.s.d. changes of <1 Å. This observation, combined with no contacts between M22/CS-17 and the 7TM domain in our structures leave us with the proposed mechanism as the most logical conclusion.*

To further support this claim, our revised manuscript now adds the following lines of evidence:

1) *We have conducted molecular dynamics simulations of TSHR in a membrane bilayer to assess whether antibody binding to TSHR ECD restricts the conformational orientation of the TSHR ECD. Simulations of apo TSHR started from either the ECD "up" or "down" state in the absence of either M22 or CS-17 show that the ECD samples multiple orientations between our solved active and inactive structures in comparison to the 7TM domain. Simulations of TSHR with M22 stabilize the ECD in the active, "up" conformation while simulations with CS-17 stabilize the ECD in the inactive, "down" conformation. These simulations therefore directly highlight how agonistic and inverse agonistic antibodies conformationally select the ECD conformation to exert their efficacy. Data from these simulations are now included in Fig. 4 and Supplementary Fig. 12.*

2) *We have now performed experiments to directly assess how ECD conformation influences signaling. To do so, we engineered a TSHR construct with a disulfide bridge between positions 262 and 483 - our structures indicate that these two residues are only sufficiently close to form a disulfide bond in the active conformation of TSHR (Cα distance 4.6 Å), but not in the inactive state (Cα distance 10.4 Å). Introduction of this disulfide bridge leads to a dramatic increase in TSHR constitutive activity, supporting the contention that trapping the ECD "up" state is sufficient to activate the receptor. This result directly indicates that ECD rotation, and not some other effect imparted by TSH and M22 is the driving force for TSHR activation.*

Transmission of conformational changes from the ECD to the TMD
Moreover, this is somewhat simplistic as it does not robustly address how this is propagated to the changes in the TMD that are required for activation.

A substantial weakness of the study is that there is no clear insight into how the changes in ECD drive receptor activation (i.e. conformational change in the TMD).

We agree with the reviewer that our prior explanation of how changes in the ECD result in activation of the 7TM domain was lacking. Enabled by a higher resolution structure of TSHR bound to TR1402, we have now identified that the extracellular tip of TM7 is moved ~4 Å inward toward the transmembrane core in the active state when compared to the inactive state.

Furthermore, we identify a key ionic interaction between the tip of TM7 and the highly conserved receptor p10 peptide at the interface of the ECD and 7TM domain. In brief, we find that changes in ECD conformation lead to a reorientation of the p10 peptide that, in the active state, mediates a direct p10-TM7 contact. In the inactive state, this ionic interaction is broken. Mutagenesis at

this site also confirms it as an important conduit in mediating ligand binding signals at the ECD into the 7TM domain.

Additionally, we generated a constitutively active TSHR construct via mutation in the ECL1-hinge helix interface that is expected to preclude down, or inactive, states of the TSHR ECD.

We believe the discussion on the re-orientation of the p10 peptide mediated by changes in ECD orientation coupled with new signaling data implicating p10-TM7 and ECL1-hinge helix interactions as important components in ligand-directed receptor activation improves our description of why ECD re-orientation matters for receptor activation. The resolution of our inactive state structure precludes further speculation on TMD specific features of activation.

Lines 363-364, can the authors please illustrate how their data "demonstrate" the hypothesis around the hinge regions. This appears to be inferred rather than demonstrated.

We agree with the reviewer that this statement was poorly worded and not clearly demonstrated by data provided in the manuscript. Our intent is to highlight that our structures provide context into the key role that the GPHα N52 glycan plays in glycoprotein hormone efficacy. We have now removed this line and reworded this text and cited the prior literature to highlight this point more clearly.

Supp. Fig. 7. Title is incorrect (this is not TSH-bound TSHR-Gs complex).

We thank the reviewer for catching this - this has now been fixed in the revised manuscript.

Line 820, what specifically does "samples" refer to here?

We thank the reviewer for catching this typo. It should have read "Samples of" not "Samples or". The text has now been fixed.

Line 851, "cAMP accumulation" – the description does not mention PDE inhibitors. Do they really mean accumulation or "production"? What is the basal cAMP? Does this vary for mutants?

This is a helpful clarification. We did not use PDE inhibitors in our cAMP assays and have therefore changed the text to indicate "cAMP production". We have now measured basal cAMP levels in our expression matched mutants.

Suppl. Figs. 2, 5, 7 all display local resolution maps that suggest a large proportion of the maps are close to 2 angstrom resolution. The maps are completely at odds with this (most appear to be ~3 A at best – which is also what is reported in the structure statistics Table, suppl. Table 1). None of the maps have features consistent with near atomic resolution. As such, this is wrong.

We thank the reviewer for this feedback. The prior color scaling was too shallow in the range of the reported GS-FSC resolution. We have replotted the outputs from each local resolution estimation output to provide a better visualization of their location resolution.

Does CS-17 block activation of TSHR by small molecule agonists such as ML-109? Does this affect understanding of the mode of receptor activation? Does the allosteric inhibitor Org 274179-0 inhibit activation by the M22 antibody? Is the inhibition equivalent or different from the inhibition of TSH activation?

The reviewer brings up an intriguing line of investigation. However, we believe this is outside of the scope of our current manuscript, which aims to understand how the native hormone and autoantibodies act at TSHR. A prior study has demonstrated Org 274179-0 inhibits the activity of both TSH and M22 antibody with similar potency and allosteric parameters K_A and K_B (van Zeijl, et al. 2012, JCEM; https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3413865/). These results suggest that TSH and M22 share a common mechanism of activation at the site that Org 274179-0 acts upon. To our knowledge, CS-17 has not been tested against allosteric agonists like ML-109. Importantly, although it is likely that the binding site for Org 274179-0 and ML-109 overlap with the lipid-binding site within the 7TM domain, neither of these have been mapped experimentally by our work. As a result, we are reluctant to speculate on activation mechanisms given that we don't know precisely where these allosteric modulators bind.

TSHR is reported to dimerise, with negative cooperativity on binding by TSH to multiple protomers. Can the authors speculate on how their models for activation work in the context of dimers?

The data on TSHR oligomerization is not all concordant and therefore it is hard to speculate on a specific model for how dimerization or oligomerization influences TSH activity. As is common for many family A GPCRs there are varying reports of oligomerization driving cooperativity. Unfortunately, most cryo-EM or X-ray structures don't provide clear understanding of this behavior. We are reluctant to speculate because we do not have a specific dimerization or oligomerization model to work with at this point for TSHR.

There are a lot of sidechains (across all structures) that have been modelled where there is no robust density to support the modelling. In the seatbelt loop region, there are also issues with the backbone modelling relative to the map density. The authors need to provide strong justification for why these are included in the deposited PDB file (I understand why they need to be present for model fitting). If they are to be included, the authors need to provide some sort of record that explicitly states where the density supports the modelling and also where it does not.

With the advent of relatively accurate side chain predictions by AlphaFold2 in GPCR 7TM domains (often with RMSDs to experimental structures below 1 Å), we prefer to keep side chains in models with moderate to poor resolution. Importantly, including side chains in poorly resolved regions of models constrains the overall backbone structure in various refinement packages, leading to better quality models. While we tried to communicate regions of poor

density directly in the main text previously, we agree that a more explicit record documenting regions supported by good density and highlighting poor density would be valuable. We have therefore now provided a graph of residue-level information on map-vs-model correlation coefficients (Supplementary Fig. 15). For example, these graphs directly show the difference in quality for the ECD vs 7TM domains in our reconstruction of inactive TSH as well as the variable regions of the M22 and CS-17 Fabs compared to the constant regions.

Referee #3:

Faust et al. provide novel insights into the mechanism of activation for the thyrotropin receptor (TSHR) and beyond a molecular understanding of how autoantibodies activate the TSHR and pathologically increase thyroid hormones in Graves' disease. For this, the authors determined cryogenic-electron microscopy structures of the active TSHR in complex with thyrotropin (TSH) and M22 - an autoantibody isolated in patients with Graves' disease as well as the inactive TSHR bound to CS-17. One of the most important structural finding is the role of steric clashes between protein agonists/inverse agonists and the membrane that drive either active- or inactive-like conformations of the extracellular domain of the TSHR. Overall, the obtained structural insights are highly relevant in particular as the reported mechanism may have implications for other GPCRs with large extracellular domains.

We thank the reviewer for carefully reading our manuscript and for highlighting the novelty of the mechanism proposed and the evidence used to support it.

Comments:
1) Authors write that in the inactive state, the down conformation of the TSHR ECD would be unable to bind TSH due to clashes between the glycosylated Asn52 residue in the common GPHα chain. At times ECD domains can also anchor the membrane with hydrophobic residues. Is it possible that in addition to steric clashes with the N-linked glycans, another important function of this highly polar chains could be to impede membrane interaction/penetration of the ECD? Could authors comment on this scenario?

This is an interesting proposition. We don't believe this is likely for the TSHR as there do not appear to be any strongly hydrophobic residues (e.g. Trp, Phe) that are on the "lip" of the ECD closest to the membrane. To answer some of the concerns brought up by reviewer 2, we have now performed molecular dynamics simulations of apo TSHR. Here, too, we do not see evidence of a persistent interaction between the TSHR ECD and the membrane bilayer.

2) The authors resolved densities for a phospholipid buried within the 7TM domain of the receptor in a region that overlaps with the likely binding side for Org 274179-0 in TSHR and Org43553 in LH/CGR. Additional mutational (A644K, A647K) experiments provide evidence for a functional role of a lipid in this transmembrane pocket. This is an extremely interesting finding and has important implication for the TSHR function in different cell membrane environments. Can the authors conclude about lipid specificity? Would it be possible to measure agonist-mediated cAMP accumulation in the presence of different types of lipids considering different tail lengths and saturation degrees?

Our mass spectrometry studies suggest that this lipid is DPPC, which is highly abundant in eukaryotic plasma membranes. This may, however, simply reflect that we purified the TSHR from HEK293 cells. We agree that it is intriguing that a lipid binding site within TSHR may be relevant for integrating lipid biology with thyroid homeostasis, and prior physiology supports this notion (e.g. Lu S et al, Lipids Health Dis, 2012). We have now added this reference to the

manuscript to highlight this possibility. However, due to space constraints, we have limited this speculation and are reluctant to add any further speculation to the manuscript based on comments from Reviewer 2. Regarding agonist-mediated cAMP accumulation studies in the presence of different lipids - we are not aware of a technically tractable way to specifically control lipid species in HEK293 cells sufficient to enable a direct connection between different types of lipids and signaling via TSHR. Important caveats would be that changing the lipid composition could have an effect on other aspects of Gs signaling, and we would not be able to directly attribute changes to the proposed lipid binding site on TSHR.

3) Authors present models of how the membrane bilayer may interact with TSH, M22, or CS-17 when bound to the TSHR ECD in either the active up or inactive down conformations. Please provide more information about how the complexes (e.g. M22 bound to inactive TSHR, etc.) were modeled. I would also propose to provide the modeled complexes as supplemental PDB files.

This is a very helpful suggestion. We have now added further description in the methods section on modeling of the TSHR ECD in the active/inactive states bound to either M22, TSHR, or CS-17. We have also added these PDB files as supplemental material. Importantly, based on some of the suggestions from reviewer 2, we have now more carefully worded our interpretation of the modeling as it does not fully explain the weak partial agonist activity of the K1-70 antibody.

4) Authors measure rotations, e.g. "The TSHR ECD is rotated 38° towards the 7TM domain and the membrane bilayer in the inactive state when compared to the active state bound to TSH". Can authors please specify how are the rotation angles were obtained?

We thank the reviewer for this suggestion. We had initially used a plane within the membrane bilayer to define a rotation axis. But based on potential caveats where one draws the membrane, we have now instead used the DynDom program to calculate domain rotation. This does not require a membrane reference, and we believe is a better approach to quantifying the rotation. The resulting angular change across this newly defined axis is 55°. We have now added a rotation axis in Fig. 2c to clarify this point for the reader and revised the text.

5) Authors measure distances, e.g. "TM6 of active TSHR is displaced outward by 14 Å to accommodate the α5 helix of miniGαs and TM7 moves ~4 Å inward relative to the transmembrane core of the receptor". For reproducibility, which exact points were used to measure these distances?

We have added these descriptors now in the text to enable readers to calculate this precisely.

Other:
6) Caption 4: "…Modeling of TSH binding to inactive TSHR with the ECD in the down conformation with either TSH (b) or M22 Fab (c)…."

Remove TSH from "TSH binding"

These are now fixed in the revised text.

Reviewer Reports on the First Revision:

Referees' comments:

Referee #2 (Remarks to the Author):

The authors have appropriately addressed my major concerns. I believe the manuscript in its current form provides an important advance for the field that will be of broad interest.

Referee #3 (Remarks to the Author):

The authors have carefully addressed all my concerns and comments.