

## Peer Review Information

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**Journal:** Nature Structural & Molecular Biology

**Manuscript Title:** Structural insights into the activation and inhibition of CXC chemokine receptor 3

**Corresponding author name(s):** Hongli Hu, Ruobing Ren, Ying-Chih Chiang

### Reviewer Comments & Decisions:

#### Decision Letter, initial version:

**Message:** 27th May 2023

Dear Dr. Hu,

Thank you again for submitting your manuscript "Structural insights into the activation and inhibition of CXC chemokine receptor 3". I apologize for the delay in responding, which resulted from the difficulty in obtaining suitable referee reports. Nevertheless, we now have comments (below) from the 2 reviewers who evaluated your paper. In light of those reports, we remain interested in your study and would like to see your response to the comments of the referees, in the form of a revised manuscript.

You will see that while the reviewers find the results interesting, they raise several concerns which will need to be addressed in a revision. Specifically, we agree with reviewer #1 that further mechanistic investigation would strengthen the study, particularly exploring receptor aspects of receptor activation and downstream signalling, if feasible. Furthermore, we would request biochemical and functional characterisation of the chimeric protein construct, in line with reviewer's #1 comments, and comparison of expression levels between WT and mutant proteins as pointed out by reviewer #2. To further strengthen conclusions pertaining to the allosteric regulation, we would encourage further validation of these experiments.

Please be sure to address/respond to all concerns of the referees in full in a point-by-point response and highlight all changes in the revised manuscript text file. If you have comments that are intended for editors only, please include those in a separate cover letter.

We are committed to providing a fair and constructive peer-review process. Do not hesitate to contact us if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.

We expect to see your revised manuscript within 10 weeks. If you cannot send it within

this time, please contact us to discuss an extension; we would still consider your revision, provided that no similar work has been accepted for publication at NSMB or published elsewhere.

As you already know, we put great emphasis on ensuring that the methods and statistics reported in our papers are correct and accurate. As such, if there are any changes that should be reported, please submit an updated version of the Reporting Summary along with your revision.

Please follow the links below to download these files:

Reporting Summary:

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Please note that the form is a dynamic 'smart pdf' and must therefore be downloaded and completed in Adobe Reader.

When submitting the revised version of your manuscript, please pay close attention to our [Digital Image Integrity Guidelines](https://www.nature.com/nature-portfolio/editorial-policies/image-integrity) and to the following points below:

- that unprocessed scans are clearly labelled and match the gels and western blots presented in figures.
- that control panels for gels and western blots are appropriately described as loading on sample processing controls
- all images in the paper are checked for duplication of panels and for splicing of gel lanes.

Finally, please ensure that you retain unprocessed data and metadata files after publication, ideally archiving data in perpetuity, as these may be requested during the peer review and production process or after publication if any issues arise.

If there are additional or modified structures presented in the final revision, please submit the corresponding PDB validation reports.

Please note that all key data shown in the main figures as cropped gels or blots should be presented in uncropped form, with molecular weight markers. These data can be aggregated into a single supplementary figure item. While these data can be displayed in a relatively informal style, they must refer back to the relevant figures. These data should be submitted with the final revision, as source data, prior to acceptance, but you may want to start putting it together at this point.

**SOURCE DATA:** we request authors to provide, in tabular form, the data underlying the graphical representations used in figures. This is to further increase transparency in data reporting, as detailed in this editorial (<http://www.nature.com/nsmb/journal/v22/n10/full/nsmb.3110.html>). Spreadsheets can be submitted in excel format. Only one (1) file per figure is permitted; thus, for multi-paneled figures, the source data for each panel should be clearly labeled in the Excel file; alternately the data can be provided as multiple, clearly labeled sheets in an Excel file.

When submitting files, the title field should indicate which figure the source data pertains to. We encourage our authors to provide source data at the revision stage, so that they are part of the peer-review process.

Data availability: this journal strongly supports public availability of data. All data used in accepted papers should be available via a public data repository, or alternatively, as Supplementary Information. If data can only be shared on request, please explain why in your Data Availability Statement, and also in the correspondence with your editor. Please note that for some data types, deposition in a public repository is mandatory - more information on our data deposition policies and available repositories can be found below: <https://www.nature.com/nature-research/editorial-policies/reporting-standards#availability-of-data>

We require deposition of coordinates (and, in the case of crystal structures, structure factors) into the Protein Data Bank with the designation of immediate release upon publication (HPUB). Electron microscopy-derived density maps and coordinate data must be deposited in EMDB and released upon publication. Deposition and immediate release of NMR chemical shift assignments are highly encouraged. Deposition of deep sequencing and microarray data is mandatory, and the datasets must be released prior to or upon publication. To avoid delays in publication, dataset accession numbers must be supplied with the final accepted manuscript and appropriate release dates must be indicated at the galley proof stage.

While we encourage the use of color in preparing figures, please note that this will incur a charge to partially defray the cost of printing. Information about color charges can be found at <http://www.nature.com/nsmb/authors/submit/index.html#costs>

Nature Structural & Molecular Biology is committed to improving transparency in authorship. As part of our efforts in this direction, we are now requesting that all authors identified as 'corresponding author' on published papers create and link their Open Researcher and Contributor Identifier (ORCID) with their account on the Manuscript Tracking System (MTS), prior to acceptance. This applies to primary research papers only. ORCID helps the scientific community achieve unambiguous attribution of all scholarly contributions. You can create and link your ORCID from the home page of the MTS by clicking on 'Modify my Springer Nature account'. For more information please visit [www.springernature.com/orcid](http://www.springernature.com/orcid).

Please use the link below to submit your revised manuscript and related files:

[redacted]

**Note:** This URL links to your confidential home page and associated information about manuscripts you may have submitted, or that you are reviewing for us. If you wish to forward this email to co-authors, please delete the link to your homepage.

We look forward to seeing the revised manuscript and thank you for the opportunity to review your work.

Sincerely,

Katarzyna Ciazynska  
(she/her)  
Associate Editor  
Nature Structural & Molecular Biology  
<https://orcid.org/0000-0002-9899-2428>

Referee expertise:

Referee #1: structural biology, GPCRs

Referee #2: biochemistry, GPCRs

Reviewers' Comments:

Reviewer #1:

Remarks to the Author:

This study provides a comprehensive analysis of four CXCR3 structures bound to various ligands, including an endogenous chemokine, two small molecule agonists, and an antagonist. The authors confirmed the binding interfaces of each agonist on CXCR3 using mutagenesis and discovered an allosteric binding site in the antagonist-bound structure that had not been previously identified in class A GPCRs. While these findings offer valuable insights into receptor-ligand interaction, a more in-depth exploration of the mechanisms beyond the binding interface and the newly discovered allosteric binding sites would further enhance the study's impact. To achieve this, it is recommended that the manuscript discusses the impact of these different binding modes on receptor activation and signaling, as well as their potential applications in future drug development efforts.

Major points:

1) Page 2, line 22 "Structural analysis reveals that PS372424 shares a similar orthosteric binding pocket with the N terminus of CXCL11, while VUF11222 buries deeper and activates the receptor in a different way."

The authors concluded that CXCL11 and PS372424 have a similar binding pocket, while VUF11222 binds deeply, resulting in a distinct activation mechanism for the receptor. While the structures do show conformational changes in the extracellular domain to accommodate different ligands, the intracellular sites appear to be highly similar across all three structures. I would appreciate further clarification on the statement that VUF11222 activates the receptor in a distinct manner. I am also interested in learning more about the downstream signaling regulation properties of the three agonists discussed in the paper. I am curious about whether they are neutral or biased agonists, whether they have similar affinity to the receptor and whether the structures provide a possible explanation for their unique properties.

2) In order to obtain the antagonist-bound structure, the authors generated a chimeric protein that involved swapping the ICL3 of CXCR3 with the ICL3 from the kappa-opioid receptor. However, the paper lacks any biochemical data that functionally characterizes

this chimeric protein. Furthermore, there is no mutagenesis data to verify the observed allosteric binding site for the antagonist in the wild-type protein, as was done for the agonists in cell-based assays. These omissions should be addressed experimentally.

3) Page 4, line 99 "The interactions between the proximal N-terminus (chemokine recognition site 1, CRS1) of the receptor and the core domain (chemokine site 1, CS1) of chemokine were reported to be weak and reversible."

Further clarification is needed on what is meant by "reversible" in this context. Please provide a citation to the original paper that supports this claim, rather than relying on a review paper.

4) Page 5, line 115 "Mutation of Trp1092.60, Phe1313.32, Phe1353.36, Tyr2716.51, Ser3047.39, and Tyr3087.43 results in reduced CXCL11-induced signaling response (Fig. 2c, Extended Data Fig. 4, Extended Data Table 2), confirming the essential roles of these residues in CXCL11 binding and receptor activation."

The data presented in Figure 2c suggests that the mutants exhibit the same maximum signal as the wild-type protein, indicating that there is no change in CXCL11-induced signaling responses, but potentially reduced binding affinity. The mutations made on the receptor are helpful in validating the binding interface between the receptor and CXCL11. However, to gain a more complete understanding of the observed interaction, it would be useful to investigate the role of the residues on the chemokine. Therefore, it would be worth summarizing existing literature on how mutations in CXCL11 affect CXCR3 signaling and compare them to the structural observations.

5) The numbering of residues in CXCL11 and receptor in the structure of SCH546738 presented in the provided PDB files do not match the numbering described in the manuscript. For instance, residue E40 in the manuscript is labeled as E61 in the provided PDB file. This discrepancy can be quite confusing and needs to be addressed by correcting the numbering in the PDB file or vice versa.

6) P6, line 145 "In a previous study, three-dimensional alignments of PS372424 to pentapeptides of CXCL10 suggest that PS372424 may mimic the residues 35-39 or 19-23 of CXCL1036." And the conclusion from this paragraph: "we suggest the binding pattern of PS372424 is similar to CXCL11 rather than CXCL10."

The paper cited in the manuscript clearly states that "We emphasize that there is no direct evidence that any small molecule agonist binds to CXCR3 in the same manner as any portion of IP-10. Rather we present this example to show how three-dimensional alignment can be used in a nonstandard manner to generate reasonable testable hypotheses" The paper primarily focuses on the geometry of PS372424 and suggests that its 3D conformation might be similar to either 35-39 or 19-23 of CXCL10. In fact, the structure of PS372424 in your model aligns well with one of their predictions. Therefore, it may not be appropriate to conclude that "the binding pattern of PS372424 is similar to CXCL11 rather than CXCL10" based solely on the cited paper.

7) Page 7, line 184 "Compared with the VUF11222 coupled receptor, a kink in TM1 could be observed in the CXCL11 coupled receptor, making the N-terminus of TM1 bend toward TM7 (Fig.5c). "

In the structure of VUF11222, there is disorder in part of TM1 and the N-terminus when compared to the CXCL11 and PS372424 structures. It is unclear what may have caused this disorder, particularly since the N-terminus is tethered to TM7 by a disulfide bond in the other two structures.

8) Page 9, line 254 "As no structure of chemokine receptors activated by small molecule agonists is available at present, the structures of CXCR3 coupled with small molecule agonists PS372424 and VUF11222 provide an opportunity for comparing the binding pattern of small molecule agonists with chemokines."

This statement is incorrect, and it would be appropriate to refer to a study that demonstrates the structure of an atypical chemokine receptor (ACKR3) bound to a small molecule agonist (PMID: 35857509). The author should cite this paper and make essential revisions to their comment. It may also be advantageous for the author to compare their study's results with those of the ACKR3 study to highlight the current study's significance.

9) Page 9, line 257 "The binding mode of PS372424 is similar to the intrinsic agonist CXCL11, while VUF11222 inserts deeper and triggers a distinct conformational change in Trp2686.48. CXCL11 and PS372424 were reported to be agonists biased toward receptor internalization<sup>31</sup>. The similar conformation of Trp2686.48 in CXCR3 activated by CXCL11 and PS372424 may provide a structural explanation."

The conformation of side chain Trp268 seems to be different in the VUF11222-bound structure to accommodate the biaryl moiety. However, it is not clear how this observation provides an explanation for the biased agonist activity of CXCL11 and PS372424.

Minor points:

1) Page 4, line 85 "To facilitate the determination of the inhibited CXCR3 structure through cryo-EM ..." It is suggested to use antagonist bound CXCR3 instead of inhibited CXCR3.

2) Page 5, line 123-124 "suggesting that the salt bridge interactions between the KRGR motif and the receptor are crucial for receptor activation." should be revised to "suggesting that the salt bridge interactions between the KRGR motif and the receptor are crucial for CXCL11-induced receptor activation" to make it clear that it pertains specifically to CXCL11.

3) Page 5, line 129 "diminished CXCL11 activity on CXCR3," It might be more appropriate to use the term "CXCL11 affinity" instead of "activity" in this context.

4) I recommend including a WT curve in figure 2F, as in fig 2C, to allow for a better comparison.

5) Page 5, line 134 "PS372424 is a peptidomimetic agonist of CXCR3, consisting of a cyclohexanemethylamine group, a natural amino acid Arg, an unnatural amino acid Tic, and a deamino-Asp(Ph) group (Fig. 3a)." Need to add a citation for this compound.

6) Page 6, line 166 "In contrast to the deeply buried biaryl group, the bicycloaliphatic group only forms weak hydrophobic interactions with the sidechains of Trp1092.60 and Tyr601.39 (Fig. 4c)." It would be helpful to include the EC50 values for the W109A and Y60A mutants, described in Table S2, in the main text to support this statement.

Additionally, it would be useful to add the corresponding curves of these mutants to the figure 4d where this interaction is depicted.

7) Page 7, line 175 "The binding patterns of CXCL11 and PS372424 are also very similar, with all TMs of CXCR3 could be aligned well, and only a subtle conformational difference in the ECL2 loop could be observed (Fig. 5b). The TMs of VUF11222-activated CXCR3 go through a more distinct conformational change than CXCL11 or PS372424-activated CXCR3." Please provide RMSD values to support this statement as it would help to better understand the extent of conformational differences observed between CXCL11/PS372424-activated CXCR3 and VUF11222-activated CXCR3.

8) Page 9, line 254 "As no structure of chemokine receptors activated by small molecule agonists is available at present, the structures of CXCR3 coupled with small molecule agonists PS372424 and VUF11222 provide an opportunity for comparing the binding pattern of small molecule agonists with chemokines."

Typo "binding pattern" should be binding pattern.

9) Extended data Table 1, the % favored and outliers in Ramachandran plot appears to be listed in the opposite manner.

10) Extended data Table 2, please include the unit for pEC50. Please also make the use of significant figures realistic and consistent in all columns of the table (vary from 2-4 in pEC50 column and 1-3 in Fold column). For example, 2 for the pEC50 value and 1 for SEM (which probably should be reported as SD instead) is probably more accurate way of listing how reproducible the data is.

11) Include in the table a column that lists the specific number of measurements used for each data point. Also, the table legend says 2-4, but 2 measurements is probably not sufficient for attaining accurate values. Hopefully this only pertains to a few of the data points.

Reviewer #2:

Remarks to the Author:

This paper contains important information regarding the structure of chemokine receptor CXCR3/G protein in complex with chemokine CXCL11, small molecule agonists PS72424 and VUF1122; and a CXCR3 chimeric receptor in complex with antagonist SCH546738. These complexes reveal some unique features that are of interest to the field and are clearly discussed in the manuscript. However, I have concerns about the study in its present form, which I address below. However, even if my concerns are addressed, I still wonder about the suitability of this manuscript for NSMB.

1. Y60A and W109A receptor variants express to the same degree as WT CXCR3 on the cell surface, however, all other variants, when considering the large SD have fewer than 40% or less receptors on the cell surface when compared to WT (extended data Fig. 4). For example, Y271A is barely expressed on the cell surface (extended data Fig. 4) and yet it is able to robustly inhibit cAMP production, albeit with reduced potency and efficacy, relative to WT (Fig. 4D). This makes me wonder about the validation of the key interaction sites in terms of whether the altered signaling is due to binding defects. Why not directly

test binding using a pharmacological approach?

2. A chimeric CXCR3-KOR was generated by replacing the ICL3 of CXCR3 with ICL3 from KOR to facilitate structural determination because nanobody Nb6, which recognizes the ICL3 of  $\kappa$ OR, could be used to stabilize a complex with antagonist SCH546738 (Extended Data Fig. 1j-l). This was important to discover the extra density between TM5 and TM6 that could accommodate antagonist SCH546738. Although this strategy is clever, one wonders about the realistic nature of this presumed allosteric site, given the extensive engineering required to solve this structure.

3. The rationale for suggesting an allosteric site for SCH546738 is interesting, although not directly observed. Although potentially exciting, this lack of direct evidence diminishes enthusiasm for this possibility. Also, key validation/orthogonal experiments have not been pursued.

4. An important feature of the N-terminus of CXCR3 is that the proximal 16 amino acid residues are critical for recognition by CXCL11. A major limitation of the structure with CXCL11 is that the densities of the N-terminus (residues 1-39) of CXCR3 were not observed, thereby missing key structural insight that can further explain receptor-ligand interactions.

5. I'm curious about the other CXCR3 chemokines CXCL9 and CXCL10. Do they show a similar interaction pose as CXCL11? Can this be tested experimentally and/or computationally? How is their binding/signaling impacted by the various mutations that have been introduced here in CXCR3? It seems as though there is something to learn from such experiments/discussion.

**Author Rebuttal to Initial comments**



1 **Reviewer #1:**

2 **Remarks to the Author:**

3 **This study provides a comprehensive analysis of four CXCR3 structures bound to various**  
4 **ligands, including an endogenous chemokine, two small molecule agonists, and an**  
5 **antagonist. The authors confirmed the binding interfaces of each agonist on CXCR3**  
6 **using mutagenesis and discovered an allosteric binding site in the antagonist-bound**  
7 **structure that had not been previously identified in class A GPCRs. While these findings**  
8 **offer valuable insights into receptor-ligand interaction, a more in-depth exploration of**  
9 **the mechanisms beyond the binding interface and the newly discovered allosteric binding**  
10 **sites would further enhance the study's impact. To achieve this, it is recommended that**  
11 **the manuscript discusses the impact of these different binding modes on receptor**  
12 **activation and signaling, as well as their potential applications in future drug**  
13 **development efforts.**

14

15 **Response to the reviewer:**

16 We appreciate very much for your constructive comments and suggestions on our manuscript  
17 “Structural insights into the activation and inhibition of CXC chemokine receptor 3” (ID:  
18 NSMB-A47307-T ). During the revision, the resolution of the CXCR3<sup>KOR</sup>-SCH546738-Nb6  
19 complex has been improved to 3.6 Å by combining newly collected CryoEM data. The density  
20 map and the coordinate have been updated in the PDB data bank. The updated map and  
21 coordinate will not influence our conclusions and could be provided if requested by the  
22 reviewer. Point-to-point responses are provided below for your further consideration. We have  
23 tried our best to revise our manuscript according to the comments, and all changes made to the  
24 paper are highlighted in yellow color in the revised manuscript.

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27 **Major points:**

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29 **1) Page 2, line 22 “Structural analysis reveals that PS372424 shares a similar orthosteric**  
30 **binding pocket with the N terminus of CXCL11, while VUF11222 buries deeper and**  
31 **activates the receptor in a different way.”**

32 **The authors concluded that CXCL11 and PS372424 have a similar binding pocket, while**  
33 **VUF11222 binds deeply, resulting in a distinct activation mechanism for the receptor.**  
34 **While the structures do show conformational changes in the extracellular domain to**

35 accommodate different ligands, the intracellular sites appear to be highly similar across  
36 all three structures. I would appreciate further clarification on the statement that  
37 VUF11222 activates the receptor in a distinct manner. I am also interested in learning  
38 more about the downstream signaling regulation properties of the three agonists  
39 discussed in the paper. I am curious about whether they are neutral or biased agonists,  
40 whether they have similar affinity to the receptor and whether the structures provide a  
41 possible explanation for their unique properties.

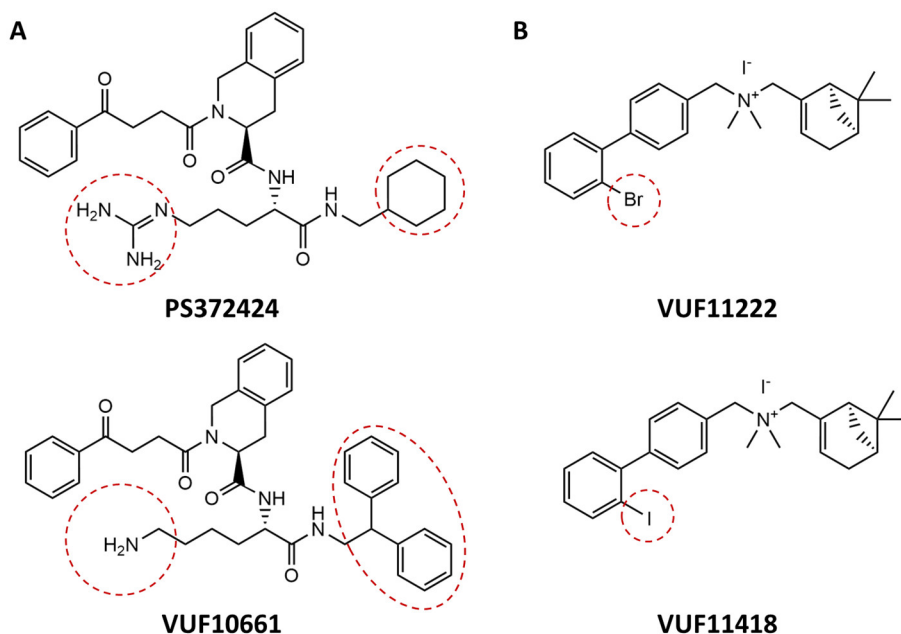
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43 **Response to the reviewer:**

44 Thank you very much for the constructive comments. The corresponding description  
45 “VUF11222 buries deeper and activates the receptor in a different way” (page 2, line 22) has  
46 been corrected to “VUF11222 buries deeper and activates the receptor in a distinct manner” in  
47 the revised manuscript (page 2, line 23).

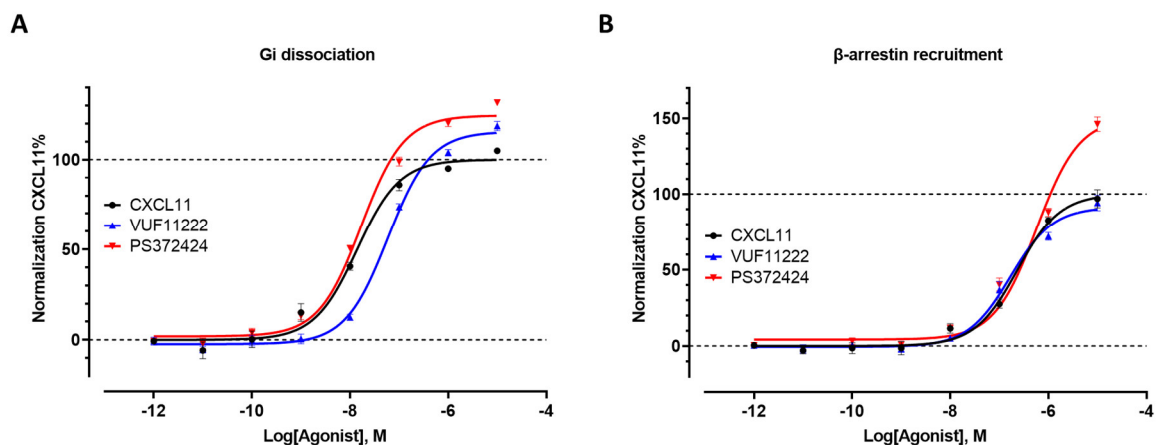
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49 According to previous studies, CXCL11 is more efficacious at activating  $G_i$  signaling and  $\beta$ -  
50 arrestin signaling than CXCL10 and CXCL9, and is slightly biased toward  $\beta$ -arrestin  
51 signaling<sup>1,2</sup>. PS372424 is efficacious at inducing receptor internalization, while its analog  
52 VUF10661 (Response Figure 1.1A) was reported to recruit  $\beta$ -arrestin with greater efficacy than  
53 CXCL11<sup>3,4</sup>. The biased signaling of VUF11222 has not been studied, but its analog VUF11418  
54 (Response Figure 1.1B) is reported to be a G-protein biased agonist<sup>5,6</sup>. We also validated the  
55  $G_i$  dissociation and  $\beta$ -arrestin recruitment kinetics of CXCR3 in the presence of CXCL11,  
56 PS372424, and VUF11222 (Response Figure 1.2A, B). As the bias signaling of CXCL11 has  
57 been validated, the data of CXCL11 is used as a contrast. In both assays, the potency of  
58 PS372424 is similar to that of CXCL11, while the efficacy of PS372424 increases (with a  
59 greater increase in the efficacy for  $\beta$ -arrestin recruitment) (Response Figure 1.2A, B).  
60 Therefore, similar to CXCL11, PS372424 appears to be biased towards  $\beta$ -arrestin signaling.  
61 For VUF11222, increases in both  $E_{max}^{G_i}$  and  $EC_{50}^{G_i}$  are observed (Response Figure 1.2A, B),  
62 thus it is difficult to estimate the signaling bias of VUF11222. Systematic works in the future  
63 are required to illustrate the bias signaling of these three ligands. A proposed structural  
64 explanation for the bias signaling was presented in the response to major point 9.



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**Response Figure 1.1 Chemical structures of PS372424, VUF10661, VUF11222, and VUF11418. (A) Chemical structures of PS372424 and VUF10661. (B) Chemical structures of VUF11222 and VUF11418. The differences in the structures are indicated by red circles.**



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**Response Figure 1.2 Bias signaling of CXCL11, PS372424, and VUF11222.**

74 The affinity of CXCL11 for CXCR3 measured by radiolabeled CXCL11 displacement binding  
75 assay is  $0.3 \text{ nM}^7$ . The affinity of PS372424 measured by radiolabeled CXCL10 displacement  
76 assay is  $42 \pm 21 \text{ nM}^8$ . The  $\text{pK}_i$  value of VUF11222 measured by radiolabeled CXCL10  
77 displacement binding assay is  $7.2 \pm 0.1$  (about  $63 \text{ nM}$  in  $\text{K}_i$  value)<sup>9</sup>. Analysis by PISA shows  
78 that the buried surface areas for CXCL11, PS372424, and VUF11222 are  $1473.7 \text{ \AA}^2$ ,  $521.0 \text{ \AA}^2$ ,  
79 and  $437.2 \text{ \AA}^2$ , respectively. A larger buried surface area generally indicates higher binding

80 affinity. Therefore, the reported binding affinities of CXCL11, PS372424, and VUF11222  
81 match well with our structure analysis. A paragraph has been added in the revised manuscript  
82 to illustrate the binding affinity of CXCL11, PS372424, and VUF11222 (page 7, line 189-192)

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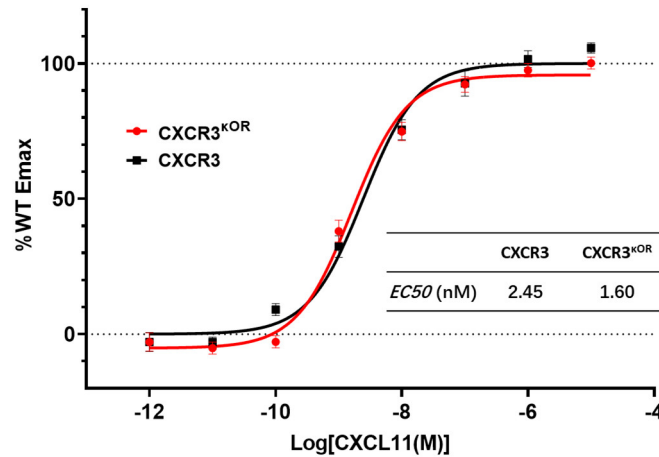
85 **2) In order to obtain the antagonist-bound structure, the authors generated a chimeric**  
86 **protein that involved swapping the ICL3 of CXCR3 with the ICL3 from the kappa-opioid**  
87 **receptor. However, the paper lacks any biochemical data that functionally characterizes**  
88 **this chimeric protein. Furthermore, there is no mutagenesis data to verify the observe**  
89 **allosteric binding site for the antagonist in the wild-type protein, as was done for the**  
90 **agonists in cell-based assays. These omissions should be addressed experimentally.**

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92 **Response to the Reviewer:**

93 Thank you for the constructive comments. The function of chimeric CXCR3<sup>KOR</sup> has been  
94 evaluated by cAMP assay. As shown in Response Figure 1.3, the EC<sub>50</sub> value is 1.60 nM for  
95 CXCR3<sup>KOR</sup> and 2.45 nM for wild-type CXCR3. Therefore, the activity of chimeric CXCR3<sup>KOR</sup>  
96 is comparable to that of the wild-type CXCR3. A sentence has been added in the revised  
97 manuscript (page 4, line 89-91) and a panel has been added in Extended Figure 4 to illustrate  
98 the activity of the chimeric protein.

99 The allosteric binding site has been evaluated by mutagenesis in the revised manuscript.  
100 Residues Val261 and Ala265 were mutated to phenylalanine, which contains a larger  
101 hydrophobic side chain. The mutations were expected to reduce the space of the allosteric  
102 binding site and interfere with the binding of SCH546738. The CXCR3<sup>V261F</sup> and CXCR3<sup>A265F</sup>  
103 mutants retain receptor activity as shown in Response Figure 1.4A-D. The EC<sub>50</sub> for  
104 CXCR3<sup>V261F</sup> is 3.33 nM, a value comparable with that of CXCR3<sup>WT</sup> (Response Figure 1.4D).  
105 The EC<sub>50</sub> for CXCR3<sup>A265F</sup> increases to 34.2 nM (Response Figure 1.4D). For CXCR3<sup>WT</sup>, an  
106 increase in the SCH546738 concentration resulted in a decrease in the potency of CXCL11  
107 (Response Figure 1.4A). For CXCR3<sup>V261F</sup> and CXCR3<sup>A265F</sup>, in contrast, the antagonism of  
108 SCH546738 is less evident (Response Figure 1.4B, C). The assay indicates that mutation of  
109 Val261 and Ala265 to phenylalanine may interfere with the binding of SCH546738.  
110 Corresponding results have been added in the revised manuscript (page 9, line 244-252), and  
111 three panels have been added in Figure 6e-g.



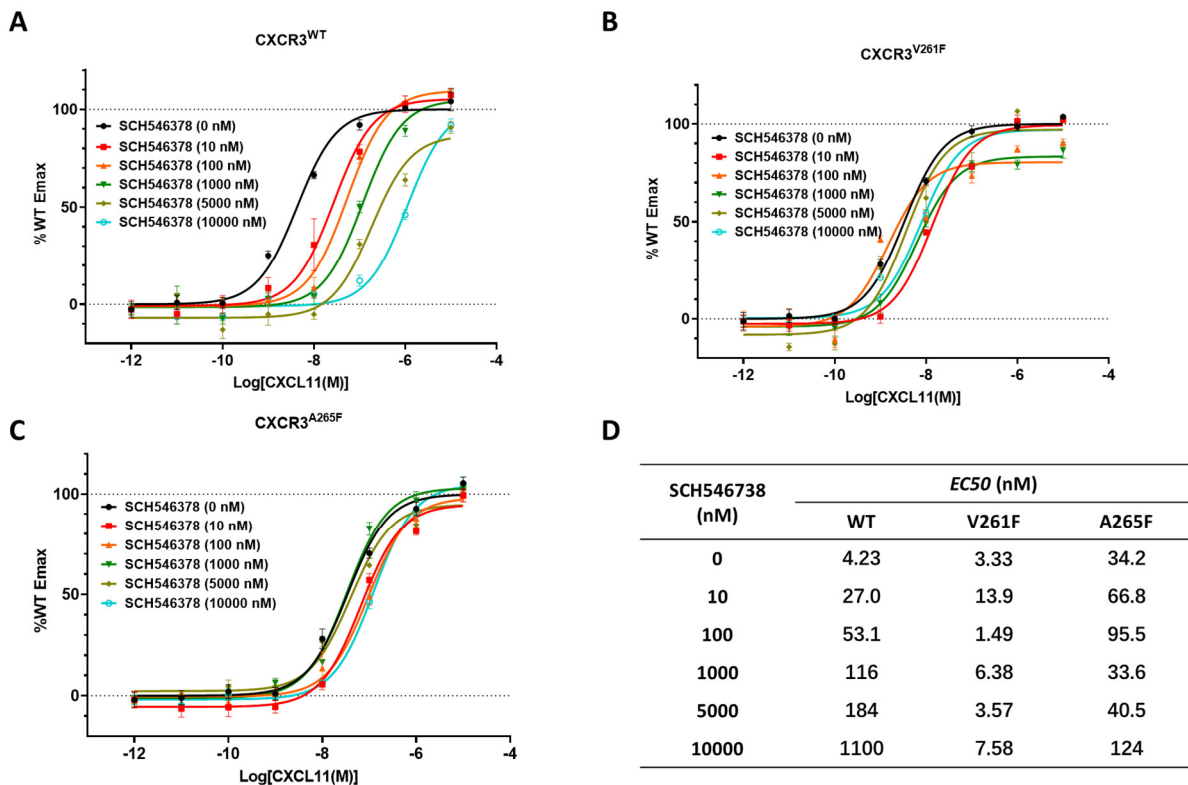
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**Response Figure 1.3 Validation of the activity of CXCR3<sup>KOR</sup>.** cAMP responses of CXCR3 and CXCR3<sup>KOR</sup> to CXCL11. cAMP responses are normalized to the percent agonist activity of wild-type CXCR3.



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**Response Figure 1.4 Validation of the allosteric binding site.** (A) cAMP responses of CXCR3 to CXCL11 in the presence of SCH546738 at different concentrations. (B) cAMP responses of CXCR3<sup>V261F</sup> to CXCL11 in the presence of SCH546738 at different concentrations. (C) cAMP responses of CXCR3<sup>A265F</sup> to CXCL11 in the presence of SCH546738 at different concentrations. (D) EC<sub>50</sub> values summarized for CXCR3, CXCR3<sup>V261F</sup>, and CXCR3<sup>A265F</sup>.

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124 **3) Page 4, line 99 “The interactions between the proximal N-terminus (chemokine**  
125 **recognition site 1, CRS1) of the receptor and the core domain (chemokine site 1, CS1) of**  
126 **chemokine were reported to be weak and reversible.”**

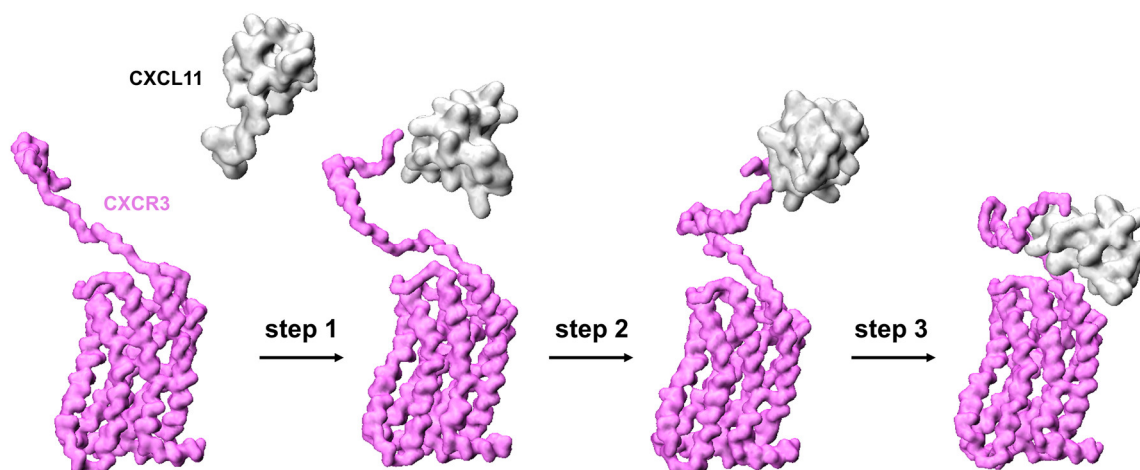
127 **Further clarification is needed on what is meant by “reversible” in this context. Please**  
128 **provide a citation to the original paper that supports this claim, rather than relying on a**  
129 **review paper.**

130

131 **Response to the reviewer:**

132 Thank you for the constructive comments. The interactions between the proximal N-terminus  
133 of the chemokine receptor (known as chemokine recognize site 1.0, CRS1.0) and the core  
134 domain of the chemokine (known as chemokine site 1.0, CS1.0) have been widely studied in  
135 the chemokine receptor family. In the case of CXCR2, although the interaction between  
136 CRS1.0 and CS1.0 has been demonstrated, the N-terminus of CXCR2 was not traced in the  
137 CryoEM structure of CXCR2 complexed with CXCL8<sup>10</sup>. To investigate the interaction  
138 between the proximal N-terminus of CXCR3 and CXCL11, coarse-grained (CG) molecular  
139 dynamics simulations were performed. CXCL11 recruitment could be observed in half of 20  
140 independent CG simulations, and one of them is presented in Extended Data Fig 5 (also  
141 presented in Response Figure 1.5). Therefore, the N-terminus of CXCR3 may play a key role  
142 in the initial recruitment of CXCL11. The corresponding description has been included in the  
143 revised manuscript (page 4, line 101-109).

144 A paragraph was included in the Method section (page 32, line 731-742) to illustrate the  
145 simulation process as follow “The missing residues 1-39 of CXCR3 were modeled using  
146 AlphaFold structure, while the CXCL11 was placed at least 30 Å away from CXCR3 structure.  
147 The system was mapped into the EIneDyn22 CG model using the CHARMM-GUI Martini  
148 Maker<sup>11</sup>. The system was embedded in a 150 Å x 150 Å membrane, which is composed of 90%  
149 1,2-dioleoyl-sn-glycero-3-phosphocholine and 10% cholesterol lipids. The system was then  
150 further solvated in salt water of 150 mM salt concentration. Prior to the production run, a 5000-  
151 step energy minimization and a restrained 5-ns NPT equilibration were performed. Then, 1 μs  
152 production run was performed using the velocity rescaling thermostat and the Parrinello-  
153 Rahman barostat to maintain the temperature at 300 K and 1 bar. The integration time step is  
154 chosen to be 20 fs. To avoid the N-terminus aggregation at the entrance of the binding pocket,  
155 the backbone of residue 35-44 was restrained during the simulations<sup>10</sup>. 20 independent CG  
156 simulations were conducted.”



157

158 **Response Figure 1.5 The dynamic process of CXCL11 recruitment revealed by**  
 159 **coarse-grained molecular dynamic simulation. The backbone of CXCR3 is shown**  
 160 **as surface and colored violet, CXCL11 is shown as surface and colored gray.**

161

162

163 **4) Page 5, line 115 “Mutation of Trp1092.60, Phe1313.32, Phe1353.36, Tyr2716.51,**  
 164 **Ser3047.39, and Tyr3087.43 results in reduced CXCL11-induced signaling response (Fig.**  
 165 **2c, Extended Data Fig. 4, Extended Data Table 2), confirming the essential roles of these**  
 166 **residues in CXCL11 binding and receptor activation.”**

167 **The data presented in Figure 2c suggests that the mutants exhibit the same maximum**  
 168 **signal as the wild-type protein, indicating that there is no change in CXCL11-induced**  
 169 **signaling responses, but potentially reduced binding affinity. The mutations made on the**  
 170 **receptor are helpful in validating the binding interface between the receptor and**  
 171 **CXCL11. However, to gain a more complete understanding of the observed interaction,**  
 172 **it would be useful to investigate the role of the residues on the chemokine. Therefore, it**  
 173 **would be worth summarizing existing literature on how mutations in CXCL11 affect**  
 174 **CXCR3 signaling and compare them to the structural observations.**

175

176 **Response to the Reviewer:**

177 Thank you for the insightful suggestions. The importance of the N-terminus of CXCL11 has  
 178 been extensively studied through mutation. Truncation of dipeptide in the N-terminus of  
 179 CXCL11 by CD26 resulted in reduced CXCR3-binding property and chemotactic potency<sup>12,13</sup>.  
 180 A potent antagonist for CXCR3 could be obtained by truncation of three or four amino acids  
 181 in the N-terminus of CXCL11, which lose the agonistic activity but retain marked binding

182 affinity<sup>14,15</sup>. Mutation of <sup>5</sup>KRGR<sup>8</sup> to <sup>5</sup>AAGA<sup>8</sup>, <sup>46</sup>KENKGQR<sup>52</sup> to <sup>46</sup>AENAGQA<sup>52</sup>,  
183 <sup>57</sup>KSKQAR<sup>62</sup> to <sup>57</sup>ASAQAA<sup>62</sup>, and <sup>66</sup>KKVERK<sup>71</sup> to <sup>66</sup>AAVEAA<sup>71</sup> lead to 225, 30, 3, and 6-  
184 fold loss in binding affinity compared to wild-type CXCL11<sup>16</sup>. These studies suggest that both  
185 the <sup>1</sup>FPMF<sup>4</sup> motif and the <sup>5</sup>KRGR<sup>8</sup> motif in the N-terminus of CXCL11 are essential for  
186 receptor binding and activation. Several sentences have been added in the revised manuscript  
187 (page 5, line 123-126, and line132-134).

188  
189

190 **5) The numbering of residues in CXCL11 and receptor in the structure of SCH546738**  
191 **presented in the provided PDB files do not match the numbering described in the**  
192 **manuscript. For instance, residue E40 in the manuscript is labeled as E61 in the provided**  
193 **PDB file. This discrepancy can be quite confusing and needs to be addressed by correcting**  
194 **the numbering in the PDB file or vice versa.**

195

196 **Response to the Reviewer:**

197 Thank you for pointing this out. In the previous PDB file, the residues of CXCL11 were  
198 numbered starting from the signal peptide. In the revised PDB file of CXCR3-CXCL11-DNGi-  
199 scFv16, the residues of CXCL11 have been renumbered to be consistent with the figures.

200 For the numbering of CXCR3 in the structure of CXCR3<sup>κOR</sup>-SCH546738-Nb6, the mismatch  
201 is caused by the replacement of ICL3. Compared to the ICL3 of CXCR3, the ICL3 of κOR  
202 contains 8 extra residues, so the residue number after ICL3 shifts by 8. The only way to  
203 renumber the residues is to apply a different chain ID for the fragment from κOR, but this may  
204 cause some confusion when depositing the PDB file. Therefore, the numbering of CXCR3 has  
205 not been changed in the PDB file of CXCR3<sup>κOR</sup>-SCH546738-Nb6.

206  
207

208 **6) P6, line 145 “In a previous study, three-dimensional alignments of PS372424 to**  
209 **pentapeptides of CXCL10 suggest that PS372424 may mimic the residues 35-39 or 19-23**  
210 **of CXCL1036.” And the conclusion from this paragraph: “we suggest the binding pattern**  
211 **of PS372424 is similar to CXCL11 rather than CXCL10.”**

212 **The paper cited in the manuscript clearly states that “We emphasize that there is no**  
213 **direct evidence that any small molecule agonist binds to CXCR3 in the same manner as**  
214 **any portion of IP-10. Rather we present this example to show how three-dimensional**  
215 **alignment can be used in a nonstandard manner to generate reasonable testable**



216 **hypotheses” The paper primarily focuses on the geometry of PS372424 and suggests that**  
217 **its 3D conformation might be similar to either 35-39 or 19-23 of CXCL10. In fact, the**  
218 **structure of PS372424 in your model aligns well with one of their predictions. Therefore,**  
219 **it may not be appropriate to conclude that "the binding pattern of PS372424 is similar to**  
220 **CXCL11 rather than CXCL10" based solely on the cited paper.**

221

222 **Response to the Reviewer:**

223 Thank you for pointing it out. We are sorry for our misinterpretation. The corresponding  
224 description “we suggest the binding pattern of PS372424 is similar to CXCL11 rather than  
225 CXCL10” (page 6, line 151-152) has been corrected to “In conclusion, we suggest that the  
226 binding pattern of PS372424 and the N-terminal pentapeptides of CXCL11 share similarity”  
227 (page 6, line 164-166).

228

229

230 **7) Page 7, line 184 “Compared with the VUF11222 coupled receptor, a kink in TM1 could**  
231 **be observed in the CXCL11 coupled receptor, making the N-terminus of TM1 bend**  
232 **toward TM7 (Fig.5c). “**

233 **In the structure of VUF11222, there is disorder in part of TM1 and the N-terminus when**  
234 **compared to the CXCL11 and PS372424 structures. It is unclear what may have caused**  
235 **this disorder, particularly since the N-terminus is tethered to TM7 by a disulfide bond in**  
236 **the other two structures.**

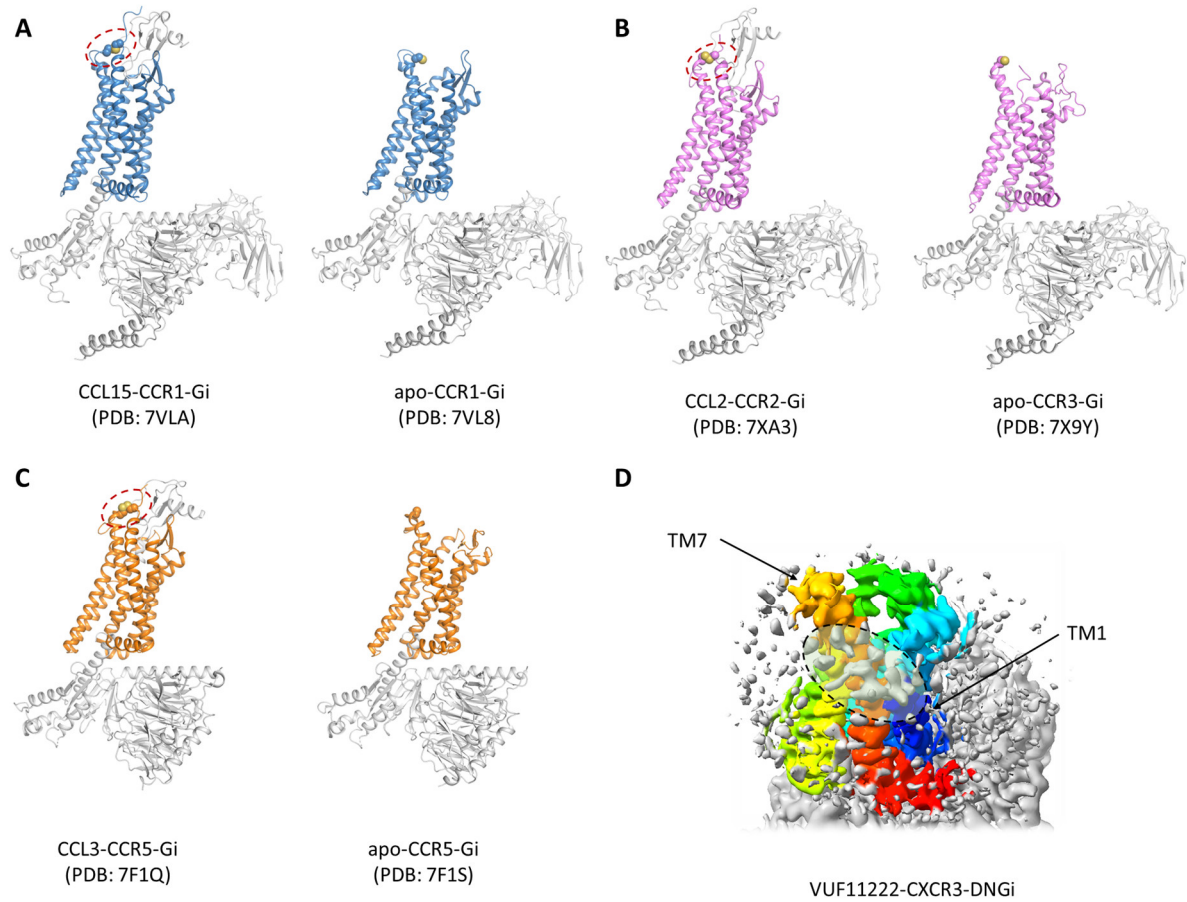
237

238 **Response to the Reviewer:**

239 Thanks for the insightful comments. Looking through the structures of chemokine receptors,  
240 we found that a similar phenomenon could be observed in other chemokine receptors. In the  
241 structure of chemokine-CCR1/CCR2/CCR5-G<sub>i</sub> complex, the disulfide bond between the N-  
242 terminus of TM1 and TM7 could be traced (Response Figure 1.6A-C). However, in the apo-  
243 CCR1/CCR3/CCR5-G<sub>i</sub> structure, the disulfide bond could not be modeled (Response Figure  
244 1.6A-C). This suggests that the disulfide bond alone may not be sufficient for stabilizing the  
245 N-terminus of TM1. The interactions with the agonist may play an essential role.

246 In the unsharpened map of VUF11222-CXCR3-DNG<sub>i</sub> (Response Figure 1.6D), weak and  
247 discontinuous densities could be observed between TM1 and TM7, suggesting the flexibility  
248 of the N-terminus of TM1. The presence of the disulfide bond is not sufficient to stabilize the

249 N-terminus of TM1. Additional interactions between the N-terminus of TM1 and  
 250 CXCL11/PS372424 would help to further stabilize the conformation.



251  
 252 **Response Figure 1.6 The disulfide bond between the N-terminus of TM1 and**  
 253 **TM7. (A)** The disulfide bond is observed in the CCL15-CCR1-G<sub>i</sub> complex but not  
 254 the apo-CCR1-G<sub>i</sub> complex. **(B)** The disulfide bond is observed in the CCL2-CCR2-  
 255 G<sub>i</sub> complex but not the apo-CCR3-G<sub>i</sub> complex. **(C)** The disulfide bond is observed  
 256 in the CCL3-CCR5-G<sub>i</sub> complex but not the apo-CCR5-G<sub>i</sub> complex. In B-D, the  
 257 disulfide bonds are shown as spheres and indicated by red circles. **(D)** Weak densities  
 258 between TM1 and TM7 indicate the bending of TM1 toward TM7.

259  
 260

261 **8) Page 9, line 254 “As no structure of chemokine receptors activated by small molecule**  
 262 **agonists is available at present, the structures of CXCR3 coupled with small molecule**  
 263 **agonists PS372424 and VUF11222 provide an opportunity for comparing the binding**  
 264 **pattern of small molecule agonists with chemokines.”**

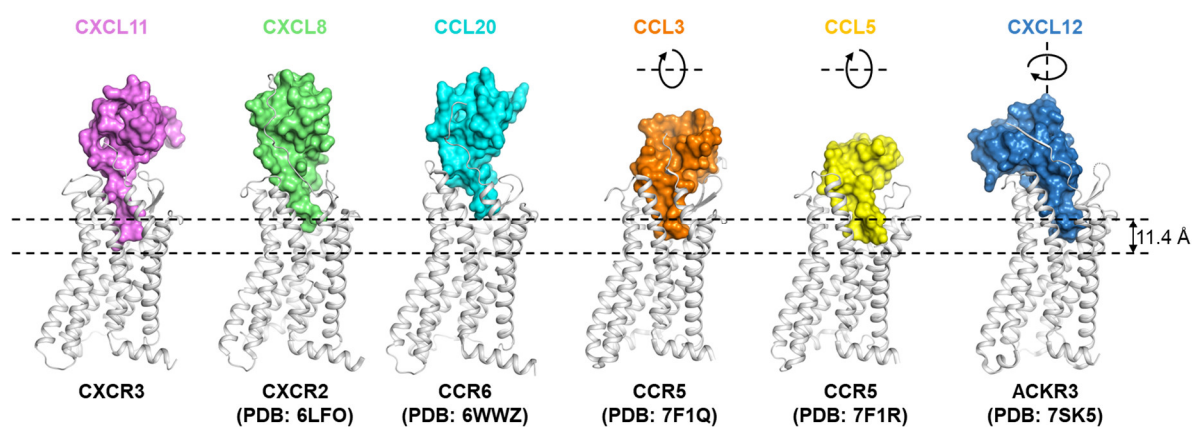
265 This statement is incorrect, and it would be appropriate to refer to a study that  
266 demonstrates the structure of an atypical chemokine receptor (ACKR3) bound to a small  
267 molecule agonist (PMID: 35857509). The author should cite this paper and make essential  
268 revisions to their comment. It may also be advantageous for the author to compare their  
269 study's results with those of the ACKR3 study to highlight the current study's significance.  
270

271 **Response to the Reviewer:**

272 Thank you for pointing it out. The structure of ACKR3 complexed with a small molecule  
273 CCX662 has been cited in the revised manuscript and the corresponding description has been  
274 corrected to “At present, only one structure of chemokine receptor activated by small molecular  
275 agonist was available, which is the structure of atypical chemokine receptor ACKR3  
276 complexed with a small molecule agonist CCX662<sup>49</sup>. The structures of CXCR3 coupled with  
277 small molecule agonists PS372424 and VUF11222 would provide more information for  
278 comparing the binding pattern of small molecule agonists with chemokines targeting the typical  
279 chemokine receptors.” (page 10, line 289-291).

280 In addition, the structure of CXCL12 bound ACKR3 was included in Extended Data Fig. 6a  
281 (Response Figure 1.7). A corresponding description has been added in the revised manuscript  
282 as follow: “In addition, CXCL11 shares a binding pose similar to CXCL8 and CCL20 with  
283 minor deviations. Compared to CXCL11, CCL5 and CCL3 undergo a rotation along a pseudo  
284 axis parallel to the membrane surface, while CXCL12 undergoes a rotation along a pseudo axis  
285 perpendicular to the membrane surface. The difference in the insertion depth and binding pose  
286 suggests that the binding patterns of chemokines differ widely” (page 10, line 271-275).

287



289 **Response Figure 1.7 Comparison of the insertion depth of CXCL11 (violet),**  
290 **CXCL8 (green), CCL20 (cyan), CCL3 (orange), CCL5 (yellow), and CXCL12**

291 (blue). The receptors are shown as cartoon and colored gray, while the chemokines  
292 are shown in surface model. The pseudo rotation axis and orientation for CCL3,  
293 CCL5, and CXCL12 compared to CXCL11 are shown.

294

295

296 **9) Page 9, line 257 “The binding mode of PS372424 is similar to the intrinsic agonist**  
297 **CXCL11, while VUF11222 insets deeper and triggers a distinct conformational change in**  
298 **Trp2686.48. CXCL11 and PS372424 were reported to be agonists biased toward receptor**  
299 **internalization<sup>31</sup>. The similar conformation of Trp2686.48 in CXCR3 activated by**  
300 **CXCL11 and PS372424 may provide a structural explanation.”**

301 **The conformation of side chain Trp268 seems to be different in the VUF11222-bound**  
302 **structure to accommodate the biaryl moiety. However, it is not clear how this observation**  
303 **provides an explanation for the biased agonist activity of CXCL11 and PS372424.**

304

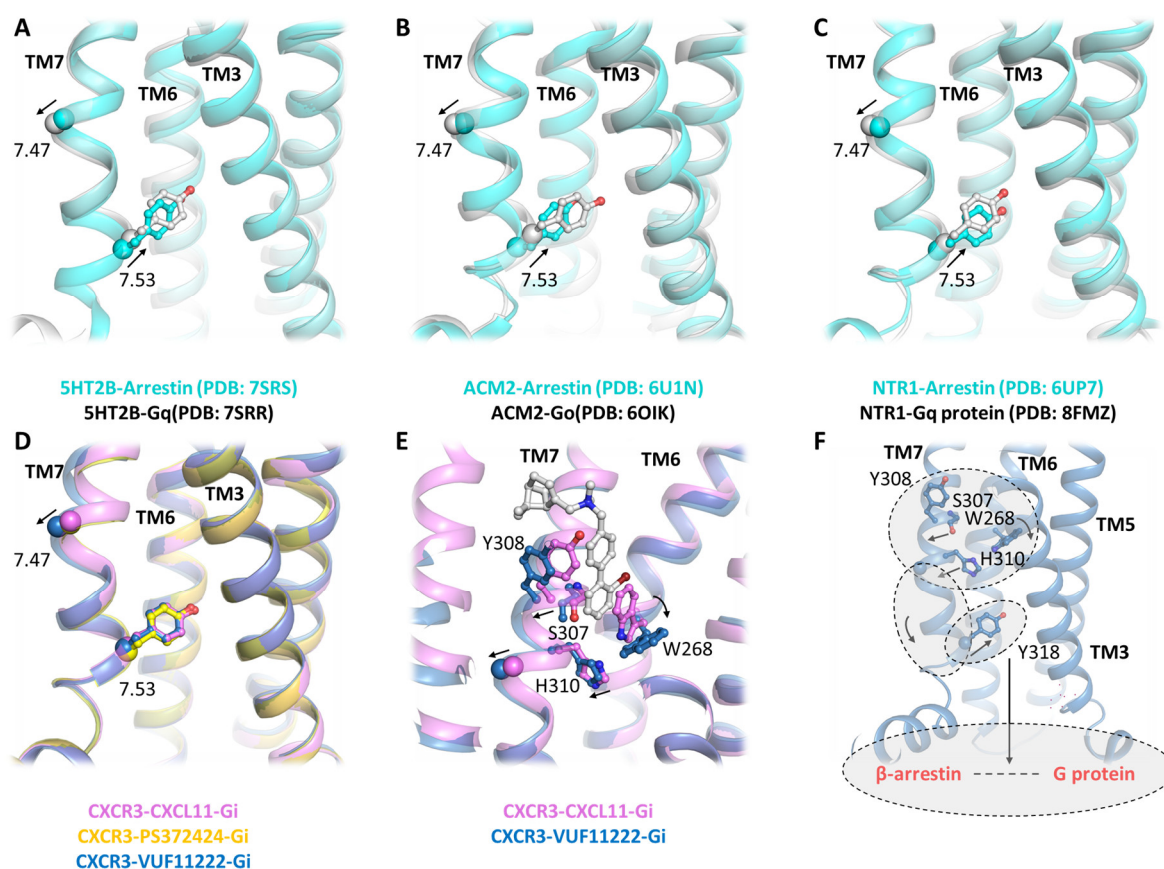
#### 305 **Response to the Reviewer:**

306 Thank you very much for the constructive comments. In the absence of a structure of  $\beta$ -arrestin  
307 coupled CXCR3, it is difficult to interpret the molecular basis of bias signaling of CXCR3.  
308 Structural analysis of available structures of  $\beta$ -arrestin coupled GPCRs may give some clues.  
309 When superposed with the structure of  $\beta$ -arrestin coupled 5HT2B, a micro-tilt in the lower half  
310 of TM7 (named TM<sup>7.47-7.53</sup>) is observed in the structure of G<sub>q</sub>-coupled 5HT2B (Response  
311 Figure 1.8A)<sup>17</sup>. Superposition of  $\beta$ -arrestin-coupled with G<sub>o</sub>-coupled ACM2 and superposition  
312 of  $\beta$ -arrestin-coupled with G<sub>q</sub>-coupled NTR1 show similar micro-tilt in TM<sup>7.47-7.53</sup> (Response  
313 Figure 1.8B, C)<sup>18-21</sup>. Therefore, it appears that TM<sup>7.47-7.53</sup> tilt to a greater degree in the G  
314 protein-coupled receptor than in the  $\beta$ -arrestin-coupled receptor. The micro-tilt in TM<sup>7.47-7.53</sup>  
315 extends the side chain of 7.53 (a conserved Tyr in the NPxxY motif) into a different spatial  
316 position in the intracellular binding cavity. Since residue 7.53 is sandwiched between TM3 and  
317 TM6, the spatial position of 7.53 may affect the overall property of the signaling protein  
318 binding cavity and ultimately influence the signaling bias.

319 In our structures, no obvious difference could be observed in the spatial position of 7.53. This  
320 is mainly due to the coupling of G protein in all three structures (Response Figure 1.8D).  
321 However, a micro-tilt at 7.47 is observed in the VUF11222-coupled CXCR3 when superposed  
322 with the CXCL11- and PS372424-coupled CXCR3 (Response Figure 1.8D). As shown in  
323 Response Figure 1.8E, the insertion of VUF11222 causes a microswitch in Trp268 that repels  
324 His310 away. Together with the repulsion of Ser307 and Tyr308 directly caused by VUF11222,

325 the N-terminus of TM7<sup>7.47-7.53</sup> is tilted (Response Figure 1.8E). A possible mechanism for the  
 326 biased signaling of CXCR3 is shown in Response Figure 1.8F. The insertion of VUF11222  
 327 initiates the tilt in the N-terminus of TM7<sup>7.47-7.53</sup>, which is transferred to the C-terminus of  
 328 TM7<sup>7.47-7.53</sup>, moves Tyr318<sup>7.53</sup> towards the central of the signaling protein binding cavity, and  
 329 ultimately influence the binding of signaling proteins.

330 A paragraph has been included in the Discussion section to discuss the mechanism for the  
 331 biased signaling of CXCR3 (page 11, line 297-327). We are struggling to determine the  
 332 structure of CXCR3 coupled with  $\beta$ -arrestin and expect to validate the hypothesis in the future.  
 333 In addition, discovering of biased agonists will also be helpful for understanding the bias  
 334 agonism of CXCR3.



335 **Response Figure 1.8 A proposed mechanism for the bias signaling of CXCR3.**  
 336 (A) Superposition of the  $\beta$ -arrestin-coupled and G protein-coupled 5HT2B. (B)  
 337 Superposition of the  $\beta$ -arrestin-coupled and G protein-coupled ACM2. (C)  
 338 Superposition of the  $\beta$ -arrestin-coupled and G protein-coupled NTR1. In A-C, the  
 339 receptors are shown as cartoon, and colored cyan for  $\beta$ -arrestin-coupled receptor and  
 340 gray for G protein-coupled receptor. The CA atoms of 7.47 and 7.53 are shown as  
 341 spheres, and the side chains of 7.53 are shown as sticks. The tilt in TM7<sup>7.47-7.53</sup> is  
 342

343 indicated by black arrows. **(D)** Superposition of G<sub>i</sub>-coupled CXCR3 activated by  
344 CXCL11 (violet), PS372424 (yellow), and VUF11222 (blue). **(E)** The tilt of TM7<sup>7.47-</sup>  
345 <sup>7.53</sup> initiated by the insertion of VUF11222. **(F)** A proposed model for the mechanism  
346 for the biased signaling of CXCR3.

347

348

349 **Minor points:**

350

351 **1) Page 4, line 85 “To facilitate the determination of the inhibited CXCR3 structure**  
352 **through cryo-EM ...” It is suggested to use antagonist bound CXCR3 instead of inhibited**  
353 **CXCR3.**

354

355 **Response to the Reviewer:**

356 Thank you very much for the kind suggestion. The description “To facilitate the determination  
357 of the inhibited CXCR3 structure through cryo-EM single particle analysis” (page 4, line 85)  
358 in the previous manuscript has been revised to “To facilitate the determination of the antagonist  
359 bound CXCR3 structure through cryo-EM single particle analysis” (page 4, line 85).

360

361

362 **2) Page 5, line123-124 “suggesting that the salt bridge interactions between the KRGR**  
363 **motif and the receptor are crucial for receptor activation.” should be revised to**  
364 **“suggesting that the salt bridge interactions between the KRGR motif and the receptor**  
365 **are crucial for CXCL11-induced receptor activation” to make it clear that it pertains**  
366 **specifically to CXCL11.**

367

368 **Response to the Reviewer:**

369 Thank you very much for the kind suggestion. The description “suggesting that the salt bridge  
370 interactions between the KRGR motif and the receptor are crucial for receptor activation” (page  
371 5, line 123-124) in the previous manuscript has been revised to “suggesting that the salt bridge  
372 interactions between the KRGR motif and the receptor are crucial for CXCL11-induced  
373 receptor activation” (page 5, line 136-137).

374

375

376 3) Page 5, line 129 “diminished CXCL11 activity on CXCR3,” It might be more  
377 appropriate to use the term “CXCL11 affinity” instead of “activity” in this context.

378

379 **Response to the Reviewer:**

380 Thank you very much for the kind suggestion. The description “Mutation of Arg197<sup>ECL2</sup> and  
381 Arg212<sup>5.35</sup> diminished CXCL11 activity on CXCR3” (page5, line129) in the previous  
382 manuscript has been revised to “Mutation of Arg197<sup>ECL2</sup> and Arg212<sup>5.35</sup> results in reduced  
383 CXCL11 binding affinity” (page 6, line 142).

384

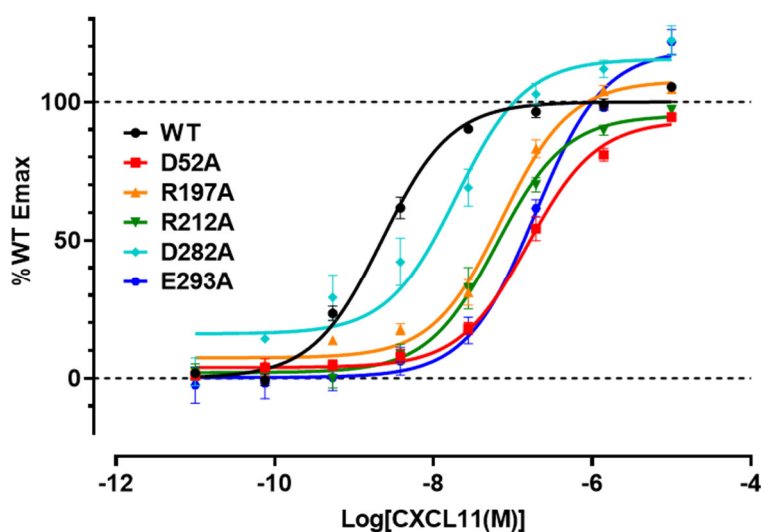
385

386 4) I recommend including a WT curve in figure 2F, as in fig 2C, to allow for a better  
387 comparison.

388

389 **Response to the Reviewer:**

390 Thank you very much for the kind suggestion. A WT curve has been included in Figure 2F  
391 (Also presented in Response Figure 1.9).



392

393 **Response Figure 1.9 cAMP responses of CXCR3 mutants to CXCL11.** cAMP  
394 responses are normalized to the percent agonist activity of wild-type CXCR3.

395

396 5) Page 5, line 134 “PS372424 is a peptidomimetic agonist of CXCR3, consisting of a  
397 cyclohexanemethylamine group, a natural amino acid Arg, an unnatural amino acid Tic,  
398 and a deamino-Asp(Ph) group (Fig. 3a).” Need to add a citation for this compound.

399

400 **Response to the Reviewer:**

401 Thank you very much for the kind suggestion. The paper which identified the agonist  
402 PS372424 (“Stroke, I. L. *et al.* Identification of CXCR3 receptor agonists in combinatorial  
403 small-molecule libraries. *Biochem Biophys Res Commun* **349**, 221-228,  
404 doi:10.1016/j.bbrc.2006.08.019 (2006).”) has been cited (page 6, line 148).

405

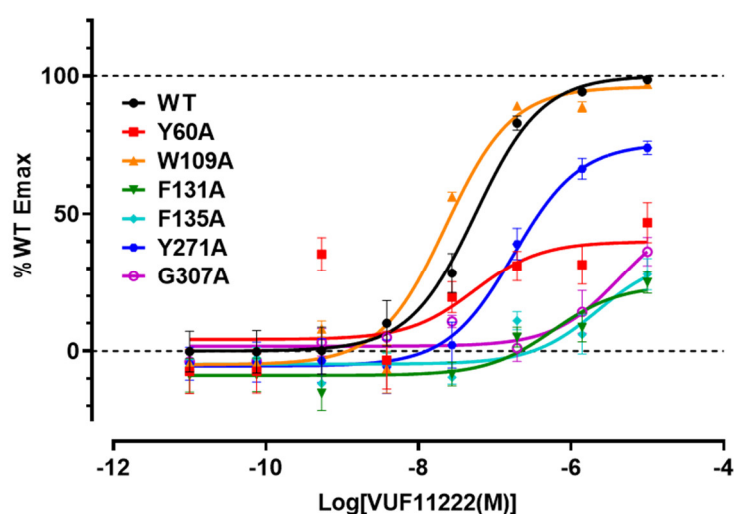
406

407 **6) Page 6, line 166 “In contrast to the deeply buried biaryl group, the bicycloaliphatic**  
408 **group only forms weak hydrophobic interactions with the sidechains of Trp1092.60 and**  
409 **Tyr601.39 (Fig. 4c).” It would be helpful to include the EC<sub>50</sub> values for the W109A and**  
410 **Y60A mutants, described in Table S2, in the main text to support this statement.**  
411 **Additionally, it would be useful to add the corresponding curves of these mutants to the**  
412 **figure 4d where this interaction is depicted.**

413

414 **Response to the Reviewer:**

415 Thank you very much for the kind suggestion. A sentence “As comparison, mutation of  
416 Trp109<sup>2.60</sup> and Tyr60<sup>1.39</sup> results in no obvious changes in the EC<sub>50</sub> values (Fig. 4d, Extended  
417 Data Fig. 4, Extended Data Table 2).” has been added in the revised manuscript (page 7, line  
418 184-186). The corresponding curves of Trp109<sup>2.60</sup> and Tyr60<sup>1.39</sup> mutant have been included in  
419 Figure 4d as well (Also presented in Response Figure 1.10).



420

421 **Response Figure 1.10 cAMP responses of CXCR3 mutants to VUF11222. cAMP**  
422 **responses are normalized to the percent agonist activity of wild-type CXCR3.**



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**7) Page 7, line 175 “The binding patterns of CXCL11 and PS372424 are also very similar, with all TMs of CXCR3 could be aligned well, and only a subtle conformational difference in the ECL2 loop could be observed (Fig. 5b). The TMs of VUF11222-activated CXCR3 go through a more distinct conformational change than CXCL11 or PS372424-activated CXCR3.” Please provide RMSD values to support this statement as it would help to better understand the extent of conformational differences observed between CXCL11/PS372424-activated CXCR3 and VUF11222-activated CXCR3.**

**Response to the Reviewer:**

Thank you very much for the kind suggestion. The RMSD between PS372424- and CXCL11-activated CXCR3 is 0.398 Å, while the RMSD between VUF11222- and CXCL11-activated CXCR3 is 0.938 Å, and the RMSD between VUF11222- and PS372424-activated CXCR3 is 0.984 Å. The RMSD values further confirm that VUF11222-activated CXCR3 adopts a distinct conformation compared to CXCL11- and PS372424-activated CXCR3. A sentence “The RMSD (Root-Means-Square Deviation) between PS372424- and CXCL11-activated CXCR3 is 0.398 Å, while the RMSD between VUF11222- and CXCL11/PS372424-activated CXCR3 is 0.938/0.984 Å.” has been added in the revised manuscript (page 7, line 197-200).

**8) Page 9, line 254 “As no structure of chemokine receptors activated by small molecule agonists is available at present, the structures of CXCR3 coupled with small molecule agonists PS372424 and VUF11222 provide an opportunity for comparing the binging pattern of small molecule agonists with chemokines.”**

**Typo “binging pattern” should be binding pattern.**

**Response to the Reviewer:**

Thank you for pointing it out. We are very sorry for our incorrect writing. The typo “binging pattern” (page 9, line256) has been corrected as “binding pattern” (page 10, line 292).

**9) Extended data Table 1, the % favored and outliers in Ramachandran plot appears to be listed in the opposite manner.**

457 **Response to the Reviewer:**

458 Thank you for pointing it out. We are very sorry for our incorrect writing. The error in the %  
459 favored and outliers in the Ramachandran plot in Extended Data Table 1 has been corrected.

460

461

462 **10) Extended data Table 2, please include the unit for pEC50. Please also make the use of**  
463 **significant figures realistic and consistent in all columns of the table (vary from 2-4 in**  
464 **pEC50 column and 1-3 in Fold column). For example, 2 for the pEC50 value and 1 for**  
465 **SEM (which probably should be reported as SD instead) is probably more accurate way**  
466 **of listing how reproducible the data is.**

467

468 **Response to the Reviewer:**

469 Thank you for your correction. The Extended Data Table has been updated, and the  $\log EC_{50} \pm$   
470 Std.error and  $EC_{50}$  (M) values calculated in Graphpad Prism are listed. 3 significant figures  
471 were used for the  $\log EC_{50}$  and  $EC_{50}$  values. 2 significant figures were used for the Std.error of  
472  $\log EC_{50}$  and the fold values. As explained by GraphPad Prism  
473 ([https://www.graphpad.com/support/faq/why-does-prism-report-the-standard-error-for-  
474 logec50-but-not-for-the-ec50-itself-when-it-fits-dose-response-curves/](https://www.graphpad.com/support/faq/why-does-prism-report-the-standard-error-for-logec50-but-not-for-the-ec50-itself-when-it-fits-dose-response-curves/)), the Std.error of  $EC_{50}$   
475 could not be calculated and thus not listed in the revised Table.

476

477 **11) Include in the table a column that lists the specific number of measurements used for**  
478 **each data point. Also, the table legend says 2-4, but 2 measurements is probably not**  
479 **sufficient for attaining accurate values. Hopefully this only pertains to a few of the data**  
480 **points.**

481

482 **Response to the Reviewer:**

483 Thank you very for the kind suggestion. The values of measurements have been included in  
484 Extended Data Table 2 in the revised manuscript. It should be noted that the measurements for  
485 CXCL11 have been replenished to three measurements. For the revised  $EC_{50}$  values for  
486 CXCL11, small variations are observed but have little impact on our conclusion.

487

488

489 **Reviewer #2:**

490 **Remarks to the Author:**

491 **This paper contains important information regarding the structure of chemokine**  
492 **receptor CXCR3/G protein in complex with chemokine CXCL11, small molecule agonists**  
493 **PS72424 and VUF1122; and a CXCR3 chimeric receptor in complex with antagonist**  
494 **SCH546738. These complexes reveal some unique features that are of interest to the field**  
495 **and are clearly discussed in the manuscript. However, I have concerns about the study in**  
496 **its present form, which I address below. However, even if my concerns are addressed, I**  
497 **still wonder about the suitability of this manuscript for NSMB.**

498

499 **Response to the reviewer:**

500 We appreciate very much for your constructive comments and suggestions on our manuscript  
501 “Structural insights into the activation and inhibition of CXC chemokine receptor 3” (ID:  
502 NSMB-A47307-T ). During the revision, the resolution of the CXCR3<sup>KOR</sup>-SCH546738-Nb6  
503 complex has been improved to 3.6 Å by combining newly collected CryoEM data. The density  
504 map and the coordinate have been updated in the PDB data bank. The updated map and  
505 coordinate will not influence our conclusions and could be provided if requested by the  
506 reviewer. Point-to-point responses are provided below for your further consideration. We have  
507 tried our best to revise our manuscript according to the comments, and all changes made to the  
508 paper are highlighted in yellow color in the revised manuscript.

509

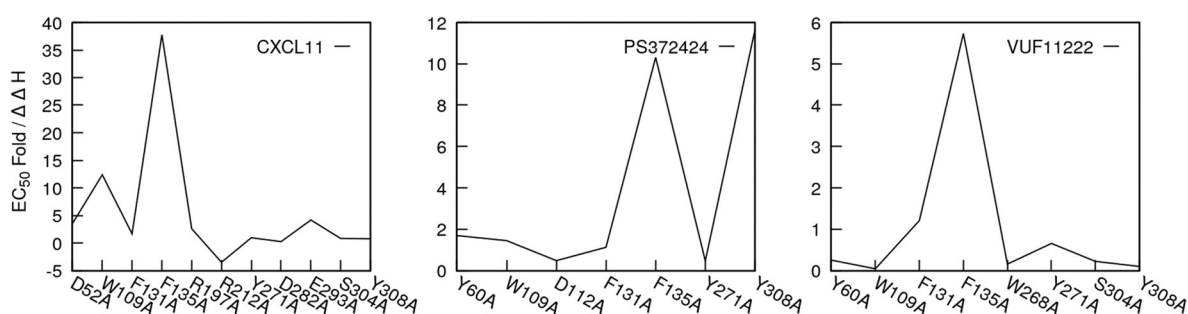
510 **1. Y60A and W109A receptor variants express to the same degree as WT CXCR3 on the**  
511 **cell surface, however, all other variants, when considering the large SD have fewer than**  
512 **40% or less receptors on the cell surface when compared to WT (extended data Fig. 4).**  
513 **For example, Y271A is barely expressed on the cell surface (extended data Fig. 4) and yet**  
514 **it is able to robustly inhibit cAMP production, albeit with reduced potency and efficacy,**  
515 **relative to WT (Fig. 4D). This makes me wonder about the validation of the key**  
516 **interaction sites in terms of whether the altered signaling is due to binding defects. Why**  
517 **not directly test binding using a pharmacological approach?**

518

519 **Response to the Reviewer:**

520 Thanks a lot for the constructive comments. The binding assay is very useful to illustrate the  
521 activity of CXCR3 mutants, however, the assay was not performed in our research for two

522 reasons. Firstly, the CXCR3 apo-protein is unstable and couldn't be purified without the  
 523 addition of antagonists or agonists. Therefore, it is less likely to conduct a binding assay in  
 524 vitro using purified CXCR3 protein. And secondary, radiolabeled CXCL11, PS372424, and  
 525 VUF11222 are not commercialized and thus are very difficult to obtain.  
 526 Alternatively, we use a computational method to estimate the binding energy change upon  
 527 different mutations. Here we use "molecular mechanics-Poisson Boltzmann surface area"  
 528 calculations. In short, if the mutation only affects the ligand binding but does not contribute to  
 529 conformational dynamics, the trend of EC50 increased fold over delta (delta H) should be linear.  
 530 As the Response Figure 2.1 suggested, except for F135A, other residues likely affect ligand  
 531 binding most. F135A locates in the middle of TM3 and bottom of the orthosteric pocket,  
 532 closely associates with W268, which is an essential residue for receptor activation, and may  
 533 contribute to both the ligand binding and conformational change of the receptor.



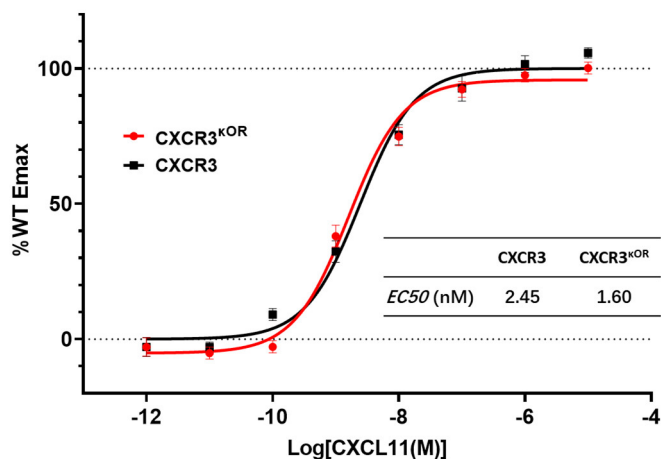
534  
 535 **Response Figure 2.1 Molecular mechanics-Poisson Boltzmann surface area**  
 536 **calculations of CXCR3 mutants bound to three agonists.** The trend of EC50  
 537 increased fold over delta (delta H) is linear except for F135A in three cases.

538  
 539 **2. A chimeric CXCR3-KOR was generated by replacing the ICL3 of CXCR3 with ICL3**  
 540 **from KOR to facilitate structural determination because nanobody Nb6, which**  
 541 **recognizes the ICL3 of κOR, could be used to stabilize a complex with antagonist**  
 542 **SCH546738 (Extended Data Fig. 1j-l). This was important to discover the extra density**  
 543 **between TM5 and TM6 that could accommodate antagonist SCH546738. Although this**  
 544 **strategy is clever, one wonders about the realistic nature of this presumed allosteric site,**  
 545 **given the extensive engineering required to solve this structure.**

546  
 547 **Response to the Reviewer:**

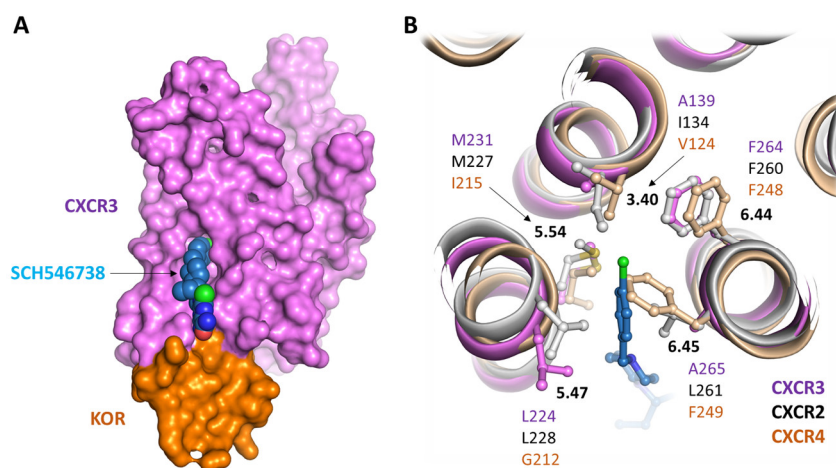
548 Thank you for the constructive comments. The function of chimeric CXCR3<sup>KOR</sup> has been  
 549 evaluated by cAMP assay. As shown in Response Figure 2.2, the EC50 value is 1.60 nM for

550 CXCR3<sup>KOR</sup> and 2.45 nM for wild-type CXCR3. Therefore, the activity of chimeric CXCR3<sup>KOR</sup>  
551 is comparable to that of the wild-type CXCR3. A sentence has been added in the revised  
552 manuscript (page 4, line 89-91) and a panel has been added in Extended Figure 4 to illustrate  
553 the activity of the chimeric protein.



554  
555 **Response Figure 2.2 Validation of the activity of CXCR3<sup>KOR</sup>.** cAMP responses of  
556 CXCR3 and CXCR3<sup>KOR</sup> to CXCL11. cAMP responses are normalized to the percent  
557 agonist activity of wild-type CXCR3. Data are shown as means  $\pm$  *S.E.M.*

558  
559 As shown in Response Figure 2.3A, the chimeric fragment has little contribution to the  
560 formation of the allosteric binding site. By comparing the amino acid residues around the  
561 allosteric binding site in CXCR2, CXCR3, and CXCR4, the side chains of residue Ala265<sup>6,45</sup>  
562 in CXCR3 are smaller than Leu261<sup>6,45</sup> and Phe249<sup>6,45</sup> in CXCR2 and CXCR4 (Response  
563 Figure 2.3B). In addition, a microswitch is observed in the side chain of Leu224<sup>5,47</sup> in CXCR3  
564 compared to Leu228<sup>5,47</sup> in CXCR2, resulting in a larger opening between the TM5 and TM6 in  
565 CXCR3. Within the pocket, CXCR3 also contains a smaller residue Ala139<sup>3,40</sup> (Ile134<sup>3,40</sup> in  
566 CXCR2 and Val124<sup>3,40</sup> in CXCR4 for comparison), making the space large enough to  
567 accommodate SCH546738. In conclusion, the formation of the allosteric binding site is mainly  
568 attributed to the constitutional and conformational uniqueness of the residues surrounding the  
569 allosteric binding site in CXCR3.



570

571 **Response Figure 2.3 The allosteric binding site in CXCR3.** (A) The allosteric  
 572 binding pocket is mainly contributed by CXCR3. CXCR3<sup>κOR</sup> is shown as surface,  
 573 the fragment from CXCR3 and the fragment from κOR are color violet and orange,  
 574 respectively. SCH546738 is shown as spheres and colored blue. (B) The formation  
 575 of the allosteric binding site in CXCR3 is mainly attributed to the small size of side  
 576 chains around the pocket. The structure of CXCR3, CXCR2, and CXCR4 are shown  
 577 as cartoon and colored violet, gray, and orange, respectively. The side chains around  
 578 the allosteric binding pocket are shown as sticks.

579

580

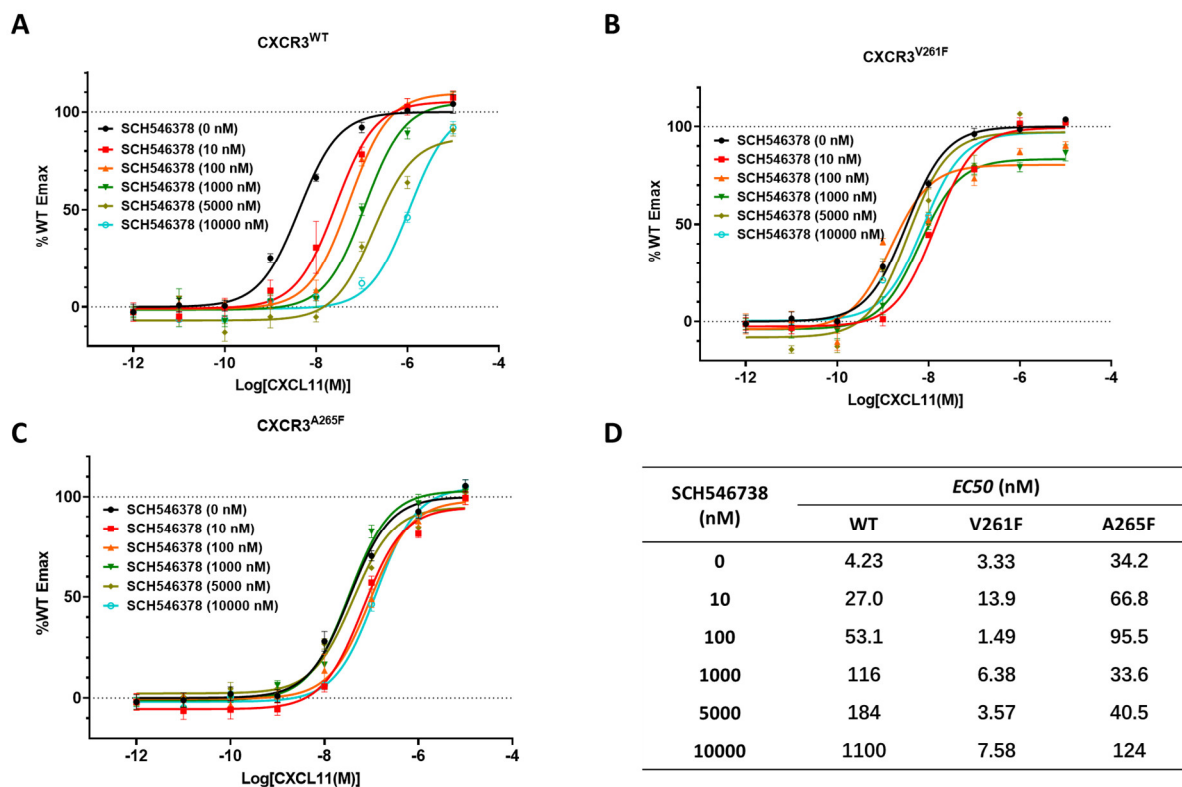
581 **3. The rationale for suggesting an allosteric site for SCH546738 is interesting, although**  
 582 **not directly observed. Although potentially exciting, this lack of direct evidence**  
 583 **diminishes enthusiasm for this possibility. Also, key validation/orthogonal experiments**  
 584 **have not been pursued.**

585

586 **Response to the Reviewer:**

587 The allosteric binding site has been evaluated by mutagenesis in the revised manuscript.  
 588 Residues Val261 and Ala265 were mutated to phenylalanine, which contains a larger  
 589 hydrophobic side chain. The mutations were expected to reduce the space of the allosteric  
 590 binding site and interfere with the binding of SCH546738. The CXCR3<sup>V261F</sup> and CXCR3<sup>A265F</sup>  
 591 mutants retain receptor activity as shown in Response Figure 2.4A-D. The EC<sub>50</sub> for  
 592 CXCR3<sup>V261F</sup> is 3.33 nM, a value comparable with that of CXCR3<sup>WT</sup> (Response Figure 2.4D).  
 593 The EC<sub>50</sub> for CXCR3<sup>A265F</sup> increases to 34.2 nM (Response Figure 2.4D). For CXCR3<sup>WT</sup>, an  
 594 increase in the SCH546738 concentration resulted in a decrease in the potency of CXCL11

595 (Response Figure 2.4A). For CXCR3<sup>V261F</sup> and CXCR3<sup>A265F</sup>, however, the antagonism of  
 596 SCH546738 is less evident (Response Figure 2.4B, C). The assay indicates that mutation of  
 597 Val261 and Ala265 to phenylalanine may interfere with the binding of SCH546738.  
 598 Corresponding results have been added in the revised manuscript (page 9, line 244-252), and  
 599 three panels have been added in Figure 6e-g.



600  
 601 **Response Figure 2.4 Validation of the allosteric binding site. (A)** cAMP responses  
 602 of CXCR3 to CXCL11 in the presence of SCH546738 at different concentrations.  
 603 **(B)** cAMP responses of CXCR3<sup>V261F</sup> to CXCL11 in the presence of SCH546738 at  
 604 different concentrations. **(C)** cAMP responses of CXCR3<sup>A265F</sup> to CXCL11 in the  
 605 presence of SCH546738 at different concentrations. **(D)** EC<sub>50</sub> values summarized for  
 606 CXCR3, CXCR3<sup>V261F</sup>, and CXCR3<sup>A265F</sup>. Data are shown as means ± *S.E.M.*.

607  
 608  
 609 **4. An important feature of the N-terminus of CXCR3 is that the proximal 16 amino acid**  
 610 **residues are critical for recognition by CXCL11. A major limitation of the structure with**  
 611 **CXCL11 is that the densities of the N-terminus (residues 1-39) of CXCR3 were not**  
 612 **observed, thereby missing key structural insight that can further explain receptor-ligand**  
 613 **interactions.**

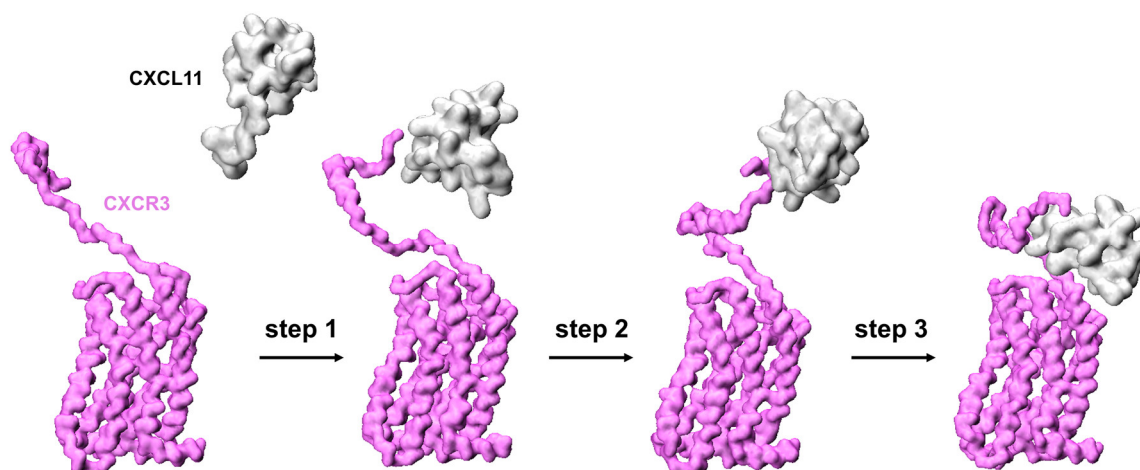
614

615 **Response to the Reviewer:**

616 Thank you for the constructive comments. The interactions between the proximal N-terminus  
617 of the chemokine receptor (known as chemokine recognize site 1.0, CRS1.0) and the core  
618 domain of the chemokine (known as chemokine site 1.0, CS1.0) have been widely studied in  
619 the chemokine receptor family. In the case of CXCR2, although the interaction between  
620 CRS1.0 and CS1.0 has been demonstrated, the N-terminus of CXCR2 was not traced in the  
621 CryoEM structure of CXCR2 complexed with CXCL8<sup>10</sup>. To investigate the interaction  
622 between the proximal N-terminus of CXCR3 and CXCL11, coarse-grained (CG) molecular  
623 dynamics simulations were performed. CXCL11 recruitment could be observed in half of 20  
624 independent CG simulations, and one of them is presented in Extended Data Fig 5 (also  
625 presented in Response Figure 2.5). Therefore, the N-terminus of CXCR3 may play a key role  
626 in the initial recruitment of CXCL11. The corresponding description has been included in the  
627 revised manuscript (page 4, line 101-109).

628 A paragraph was included in the Method section (page 32, line 731-742) to illustrate the  
629 simulation process as follow “The missing residues 1-39 of CXCR3 were modeled using  
630 AlphaFold structure, while the CXCL11 was placed at least 30 Å away from CXCR3 structure.  
631 The system was mapped into the EIneDyn22 CG model using the CHARMM-GUI Martini  
632 Maker<sup>11</sup>. The system was embedded in a 150 Å x 150 Å membrane, which is composed of 90%  
633 1,2-dioleoyl-sn-glycero-3-phosphocholine and 10% cholesterol lipids. The system was then  
634 further solvated in salt water of 150 mM salt concentration. Prior to the production run, a 5000-  
635 step energy minimization and a restrained 5-ns NPT equilibration were performed. Then, 1 μs  
636 production run was performed using the velocity rescaling thermostat and the Parrinello-  
637 Rahman barostat to maintain the temperature at 300 K and 1 bar. The integration time step is  
638 chosen to be 20 fs. To avoid the N-terminus aggregation at the entrance of the binding pocket,  
639 the backbone of residue 35-44 was restrained during the simulations<sup>10</sup>. 20 independent CG  
640 simulations were conducted.”





641

642 **Response Figure 2.5 The dynamic process of CXCL11 recruitment revealed by**  
 643 **coarse-grained molecular dynamic simulation.** The backbone of CXCR3 is shown  
 644 as surface and colored violet, CXCL11 is shown as surface and colored gray.

645

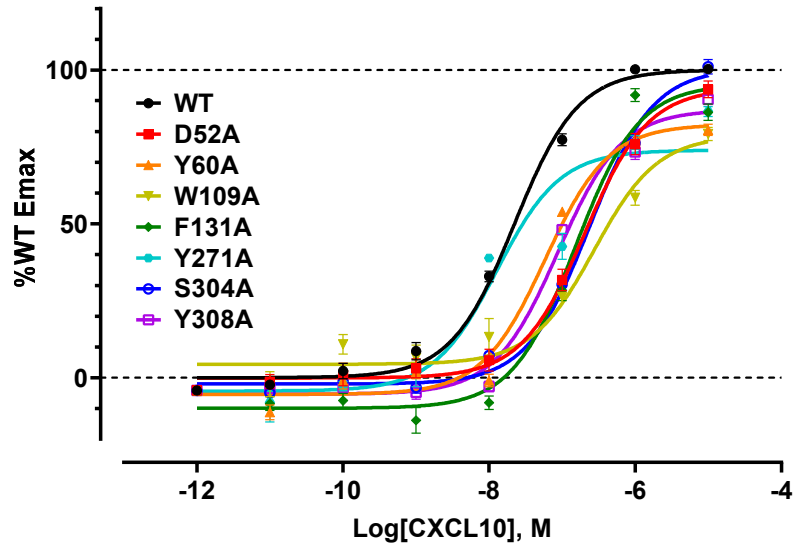
646

647 **5. I'm curious about the other CXCR3 chemokines CXCL9 and CXCL10. Do they show**  
 648 **a similar interaction pose as CXCL11? Can this be tested experimentally and/or**  
 649 **computationally? How is their binding/signaling impacted by the various mutations that**  
 650 **have been introduced here in CXCR3? It seems as though there is something to learn**  
 651 **from such experiments/discussion.**

652

653 **Response to the Reviewer:**

654 Thank you for the constructive comments. During the revision, several CXCR3 mutants have  
 655 been tested for CXCL10. Among the mutants tested, mutations at D52, W109, F131, S304, and  
 656 Y308 result in reduced potency of CXCL10 (Response Figure 2.6 and Response Table 2.1).  
 657 However, the EC<sub>50</sub> fold increase for CXCL10 is generally lower than that for CXCL11. In  
 658 addition, Y271A mutant has little effect on the potency of CXCL10, but reduces the potency  
 659 of CXCL11. Therefore, we suggest that CXCL10 may bind to CXCR3 in a binding pocket that  
 660 overlaps with that of CXCL11. However, the precise interaction network between CXCR3 and  
 661 CXCL10 may be different from that between CXCR3 and CXCL11.



662

663

**Response Figure 2.6** cAMP responses of CXCR3 mutants to CXCL10. cAMP responses are normalized to the percent agonist activity of wild-type CXCR3. Data are shown as means  $\pm$  *S.E.M.*.

666

667

**Response Table 2.1** EC<sub>50</sub> values for CXCL11 and CXCL10.

EC <sub>50</sub> (nM) for CXCL11				EC <sub>50</sub> (nM) for CXCL10			
Mutants	EC <sub>50</sub> (nM)	N	Fold	Mutants	EC <sub>50</sub> (nM)	N	Fold
WT	2.25	4	1.0	WT	22.3	6	1.0
D52A	155	3	69	D52A	205	6	9.2
W109A	186	3	83	W109A	276	6	12
F131A	24.2	3	11	F131A	153	6	6.9
271A	11.9	3	5.3	Y271A	12.8	6	0.6
S304L	34.8	3	15	S304L	239	6	11
Y308A	9.46	3	4.2	Y308A	87.9	6	3.9

668

669

670 **Response References**

671

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733

734

**Decision Letter, first revision:**

**Message:** Our ref: NSMB-A47307A

12th Sep 2023

Dear Dr. Hu,

Thank you for submitting your revised manuscript "Structural insights into the activation and inhibition of CXC chemokine receptor 3" (NSMB-A47307A). It has now been seen by the original referees and their comments are below. The reviewers find that the paper has improved in revision, and therefore we'll be happy in principle to publish it in Nature Structural & Molecular Biology, pending minor revisions to satisfy the referees' final requests and to comply with our editorial and formatting guidelines.

We are now performing detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements in about a week. Please do not upload the final materials and make any revisions until you receive this additional information from us.

To facilitate our work at this stage, it is important that we have a copy of the main text as a word file. If you could please send along a word version of this file as soon as possible, we would greatly appreciate it; please make sure to copy the NSMB account (cc'ed above).

Thank you again for your interest in Nature Structural & Molecular Biology Please do not hesitate to contact me if you have any questions.

Sincerely,

Katarzyna Ciazynska  
(she/her)  
Associate Editor  
Nature Structural & Molecular Biology  
<https://orcid.org/0000-0002-9899-2428>

Reviewer #1 (Remarks to the Author):

The majority of the comments I provided have been appropriately addressed by the author in the revised manuscript. I am satisfied with the modifications. There are a few minor suggestions that I would like to point out:

1) In fig. 4d, although Y60A exhibits a comparable EC50 to the WT, its efficacy (Emax) is significantly reduced. This trend is similarly observed in fig. 3d, where there's a reduction in affinity as well. Since the surface expression level of Y60A is comparable to that of WT, the reduced efficacy of Y60A might be worth discussing within the manuscript.

2) It would be beneficial to include the full name of PISA and provide a citation or the link

to its website. This would enable readers to easily access it.

3) Within the figure legend of Extended fig. 8, I recommend using "Ca atoms" instead of CA atoms.

Reviewer #2 (Remarks to the Author):

I am happy with the rebuttal and revisions. I have no additional comments.

**Author Rebuttal, first revision:**

## **Reviewer #1 (Remarks to the Author):**

**The majority of the comments I provided have been appropriately addressed by the author in the revised manuscript. I am satisfied with the modifications. There are a few minor suggestions that I would like to point out:**

Response to the reviewer: We appreciate very much for your constructive comments and suggestions on our manuscript "Structural insights into the activation and inhibition of CXC chemokine receptor 3" (ID: NSMB-A47307A). Point-to-point responses are provided below for your further consideration, and changes made to the paper are highlighted in yellow color in the revised manuscript.

**1) In fig. 4d, although Y60A exhibits a comparable EC50 to the WT, its efficacy (Emax) is significantly reduced. This trend is similarly observed in fig. 3d, where there's a reduction in affinity as well. Since the surface expression level of Y60A is comparable to that of WT, the reduced efficacy of Y60A might be worth discussing within the manuscript.**

Response: Thank you very much for the constructive comments. A sentence “Notably, the mutation of Tyr60<sup>1.39</sup> significantly reduced the  $E_{\max}$  of VUF11222, suggesting that the mutation may change the conformational landscape of the receptor or the binding kinetics of the ligand, perhaps by affecting the dissociation rate constant  $K_{\text{off}}$ .” has been added in the manuscript to discuss the reduced efficacy of Y60A. (page 7, line185-187)

**2) It would be beneficial to include the full name of PISA and provide a citation or the link to its website. This would enable readers to easily access it.**

Response: Thank you for pointing it out. The full name of PISA, which is “Proteins, Interfaces, Structures and Assemblies” as well as the citation “Krissinel, E. & Henrick, K. Inference of macromolecular assemblies from crystalline state. *J Mol Biol* 372, 774-797, doi:10.1016/j.jmb.2007.05.022 (2007).” has been added in the revised manuscript. (page 7, line 191)

**3) Within the figure legend of Extended fig. 8, I recommend using “C $\alpha$  atoms” instead of CA atoms.**

Response: The “CA atoms” has been corrected to “C $\alpha$  atoms” in the legend of Extended Fig. 8 in the revised manuscript.

## **Reviewer #2 (Remarks to the Author):**

**I am happy with the rebuttal and revisions. I have no additional comments.**

Response to the reviewer: We appreciate very much for your constructive comments and suggestions on our manuscript "Structural insights into the activation and inhibition of CXC chemokine receptor 3" (ID: NSMB-A47307A).

**Final Decision Letter:**

**Message** 3rd Nov 2023

:

Dear Dr. Hu,

We are now happy to accept your revised paper "Structural insights into the activation and inhibition of CXC chemokine receptor 3" for publication as an Article in Nature Structural & Molecular Biology.

Acceptance is conditional on the manuscript's not being published elsewhere and on there being no announcement of this work to the newspapers, magazines, radio or television until the publication date in Nature Structural & Molecular Biology.

Over the next few weeks, your paper will be copyedited to ensure that it conforms to Nature Structural & Molecular Biology style. Once your paper is typeset, you will receive an email with a link to choose the appropriate publishing options for your paper and our Author Services team will be in touch regarding any additional information that may be required.

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<http://www.nature.com/authors/policies/availability.html>.

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About one week before your paper is published online, we shall be distributing a press release to news organizations worldwide, which may very well include details of your work. We are happy for your institution or funding agency to prepare its own press release, but it must mention the embargo date and Nature Structural & Molecular Biology. If you or your Press Office have any enquiries in the meantime, please contact [press@nature.com](mailto:press@nature.com).

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(she/her)  
Associate Editor  
Nature Structural & Molecular Biology  
<https://orcid.org/0000-0002-9899-2428>

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