nature portfolio

Peer Review File

De novo design of miniproteins targeting GPCRs

Corresponding Author: Dr Edin Muratspahić

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

Version 0:

Reviewer comments:

Referee #1

(Remarks to the Author)
This manuscript details de novo design strategy of miniproteins targeting GPCRs that can be achieved for a highly druggable targets. I can appreciate the specificity aspect of this work, confirming mini protein target engagement in biochemical assays and cryo-EM structure determinations, with some exhibiting potent and high affinity binders for GLP1 and class B GPCRs. However there are major issues with the manuscript that limits applications and utility for other investigators.

Major issues:
There is lack of mechanism on how the design is achieved toward different GPCRs. In fact, I didn't learn anything from the manuscript on how to better design difficult to drug GPCRs using this approach. Importantly, there is lack of details on which key miniprotein designs that worked best for each receptor type to stabilize the pentapeptide motif to drive specificity and affinity. For instance, for all GPCRs investigated, which of the miniprotein backbone worked best (helix, strand, loop etc), or what were the specific combination that was successful? Little to no detail on how areas to stabilize the peptide were provided for the designs. Which pentapeptides were prioritized especially with respect to selectivity within subtypes or iterative improvements to specificity? A posthoc meta-analysis on the efficiency of pentapeptide and miniprotein backbone designs would have suited better to complement the detailed the validation and specificity aspects of the manuscript.

It appears specificity is somewhat validated, but importantly 'selectivity' of such targets is largely ignored. No selectivity panels were provided for any of the mini proteins. How well is the design for achieving selectivity? For instance, many of the example GPCRs investigated in this manuscript (e.g. MRGPRX, CXCRs) are part of GPCR types with very similar subtypes. Do these miniprotein ligands have similar affinity for closely related subtypes? Do these miniproteins have selectivity across a very large set of GPCRs targets? Importantly, are these miniproteins, either in their pentamer design or miniprotein stabilization features, showing exquisite selectivity across many protein targets? It is an important question to answer because many of these design features could be polypharmacological or endowed with such specificity that selectivity over all closely or distantly related proteins could be revealed.

Connecting mini proteins to druggable technologies also appears to be lacking and whether these newly discovered miniprotein ligands have any use in vivo. What advantages do these miniproteins have over nanobodies or antibodies or other larger in terms of in vivo target engagement? Can they be made metabolically stable for in vivo use or what are their limitations? Can the authors show just one example showing in vivo effects?

Despite the potential limitations toward therapeutics, the authors state the mini proteins have utility as chemical probes. But they don't measure extensively what such miniprotein would provide toward the available plethora of GPCR mechanisms. GPCRs exhibit a spectrum of signaling and only one signaling pathway is measured for each representative GPCR example. Do any of these miniprotein ligands exhibit biased agonism or agonist-directed trafficking? Are these miniproteins do anything novel instead of acting as agonists and antagonists? How do these miniprotein affect ligand kinetics? Do they have longer residence time and slower kinetics in general given their size? Are there any arrestin or desensitization measures to show these a useful chemical probes? How do these illuminate the GPCR of interest's pharmacology in comparison to known chemical matter for each respective example? What do these examples add to understanding the complexity of signaling by GPCRs that has not been revealed already?

Finally, no mutagenesis is presented to validate the binding modes. It is necessary to provide empirical evidence in the form of carefully designed mutant experiments to complement the cryo-EM structures and predicted binding modes.
Minor issues:

-Receptor diversion assay: How do you control for expression and therefore concentration of the binders to calculate Kd? How does the receptor diversion assay compare with yeast and biofloater assays, in terms of high-throughput, false positives, artifacts etc.?-

-In vitro signaling assays. MRGPRX1 example: Authors state two are full agonists, and one is a partial agonist with respect to what? Bam-8-22. Specify what the positive controls are throughout, in both captions and in text. No receptor controls without receptor expressed are present throughout to confirm specificity. Authors should show direct activation of G proteins using an orthogonal assay.

-Ballesteros-Weinstein or GPCRdb coordinate number system is needed throughout manuscript superscripted on residues.

Referee #2

(Remarks to the Author)
The authors report the computational de novo design of miniprotein binders targeting GPCRs. They describe the generation of agonists and antagonists directed at the orthosteric binding sites of MRGPRX1 and CXCR4, respectively, as well as protein antagonists binding the soluble extracellular domains of several class B GPCRs. Structural validation of the CGRPR-bound antagonists and MRGPRX1-bound agonists was achieved using cryoEM.

To enable these designs, the authors developed a “motif-directed” variant of their RFdiffusion method, along with MetaGen, a strategy for generating more structurally diverse protein scaffolds. They also introduced a high-throughput, microscopy-based “receptor diversion” screen to efficiently identify functional binders from computational libraries.

Designing potent and functionally selective GPCR modulators remains a major unmet challenge in pharmacology, and current methods often depend on labor-intensive empirical screening. A rational approach for designing mini-protein binders that predictably control GPCR activity would represent a significant advancement. This study, therefore, holds the potential to substantially impact GPCR-targeted drug discovery.

However, while the study presents some promising and exciting examples of de novo designed binders, it currently lacks sufficient evidence to support the broader claim of a generalizable strategy for designing conformationally and functionally selective GPCR modulators. Additionally, some aspects of the results raise concerns about the computational approach’s applicability to other GPCR targets

Major concerns

1. In both the abstract and discussion, the authors claim to achieve precise conformational control of receptor function. Specifically, they propose that designing binders against an active-state GPCR structure yields agonists, while targeting the inactive state leads to antagonists. However, the structural differences between active and inactive conformations in the extracellular orthosteric binding sites are often minimal, which may limit the generalization of this approach to other GPCRs.

To convincingly demonstrate that functionally selective binders can be rationally designed based on receptor conformation, the authors should be able to generate both antagonists and agonists for the same GPCR by specifically targeting either its inactive or active state. At a minimum, they should show that designing binders against the inactive conformation of MRGPRX1 yields a higher proportion of antagonists or inverse agonists, and that targeting the active conformation of CXCR4 leads to a greater fraction of agonists. Additionally, the manuscript should better articulate the structural basis for this claim: how do structural differences between the active and inactive states of MRGPRX1 and CXCR4 influence designability and binding specificity?

The authors should also discuss the generalizability of their method. Is it broadly applicable across GPCRs, or is it best suited to receptors with large, accessible epitope shifts upon activation? This would help readers evaluate whether their conformational design strategy is scalable or only appropriate for a subset of GPCRs with pronounced structural transitions.

2. In the abstract, the authors claim that their designed miniproteins exhibit high affinity, potency, and selectivity. However, this claim is not consistently supported by the functional data presented. For instance, the most potent MRGPRX1 agonist has an EC_{50} of 390 nM—over 100-fold weaker than the endogenous agonist BAM, which has an EC_{50} of ~3 nM. Similarly, the reported CXCR4 antagonist has an IC_{50} of 24 nM, which is approximately ten-fold less potent than the native agonist ligand.

Furthermore, while the CGRPR antagonists underwent extensive selectivity profiling, there is no comparable data for the MRGPRX1 and CXCR4 binders. Without such analyses, it is premature to assert high selectivity for these designs.

To align claims with the presented data, the authors should moderate their claims regarding potency and selectivity in both

the abstract and main text. It would also strengthen the study to clearly specify which properties were rigorously validated and for which designs.

3. The criteria used for computational screening of binder designs vary substantially between targets. For instance, the thresholds for predicted interface pAE (ipAE) range from 4 (for CGRPR) to 8 (for GLP1R), while acceptable pLDDT values span from 80 to 90. The manuscript does not explain the rationale behind these differing cutoffs. It remains unclear whether these thresholds were selected based on empirical optimization, intrinsic differences between targets, or other considerations.

The authors should clarify how these parameters were chosen and whether they reflect systematic optimization or arbitrary decisions. Furthermore, it would be important to discuss how these variations might affect the interpretation of design quality and success rates across different receptors. Without a consistent or well-justified framework, it is difficult to compare outcomes across targets or evaluate the generalizability of the approach.

4. The reported success rates are generally low, ranging from 0.5% to 4.5% for most targets and as low as 2 out of 26,000 designs for CXCR4. These outcomes raise concerns about the effectiveness of the computational selection criteria (e.g., AlphaFold predictions, Rosetta scores) used during the design filtering stages. To better understand the predictive value of these metrics, a retrospective analysis comparing successful versus unsuccessful binders would be highly informative.

Specifically, do the chosen computational filters (e.g., pAE_interaction, pLDDT, ddG) effectively enrich for functional binders? Such an analysis would provide crucial insight into the strengths and limitations of the current pipeline and could guide improvements to enhance hit rates in future campaigns. Without this, it remains difficult to assess whether the observed low success rates reflect intrinsic design challenges or suboptimal scoring strategies.

Lastly, the authors should clearly distinguish the miniprotein binders designed against class B GPCRs, which target soluble extracellular domains, from those targeting CXCR4 and MRGPRX1, whose orthosteric sites are more conformationally dynamic and structurally constrained, posing distinct design challenges.

5. The manuscript introduces MetaGen as a complementary strategy to RFdiffusion for generating scaffolds with increased structural diversity, yet the comparative advantage of this method remains unclear. Specifically, it would be valuable to know whether MetaGen produces binders with significantly different structural features, epitope coverage, or binding surface complementarity relative to those generated by RFdiffusion alone.

For example, in the case of MRGPRX1, how do the binders derived from MetaGen compare, structurally and functionally, with those produced by standard RFdiffusion? Providing quantitative or qualitative comparisons (e.g., scaffold diversity, predicted binding energies, functional hit rates) would help clarify whether MetaGen substantially enhances binder design capabilities or simply adds redundancy. Without such a comparison, it is difficult to assess the necessity and unique contribution of this method to the overall design pipeline.

6. The Receptor Diversion strategy represents an elegant and innovative approach for efficiently screening binders against membrane receptors. The correlation between binding affinity and the diversion signal in their benchmark is promising. However, can the authors rule out the possibility of false negatives—namely, high-affinity binders that do not cause receptor diversion? It would be important to clarify the limitations of this approach. For example, are there specific biophysical or spatial constraints (e.g., binding geometry, epitope location, or size of the binder) that might not prevent receptor trafficking even in the presence of strong binding? Addressing this question would help assess the robustness and general applicability of the receptor diversion readout.

Receptor target specific points

1. MRGPRX

• To gain a better understanding of the molecular basis of binder function, could the authors identify whether specific binding residues are responsible for agonism and partial agonism?

• An important question is whether the binders exhibit any biased agonism. To address this, the authors should assess whether other G-protein or arrestin-mediated signaling pathways are also activated.

• Are the residues mentioned by the authors on line 254, with distinct conformations, known to drive activation? Could the authors specify how these conformational differences are hypothesized to contribute to the degree of agonistic activity?

• In Figure 2b, the dose response of the native agonist is missing.

2. CXCR4

• The authors provide the following rationale on lines 269-271: “We reasoned that interacting with the receptor at both extracellular loops and deeper within the transmembrane region would be necessary to stabilize the inactive receptor conformation and achieve a potent CXCR4 antagonist.” Why would that rationale constitute a strategy specific to antagonists?

• The lack of structural characterization of a CXCR4-binder complex notably weakens the validation of the design. Could the authors elaborate on the challenges encountered during the characterization process?

Minor points

1. The resolution of several supplementary figures is too low for proper interpretation. Higher-quality versions should be provided.

2. Details of the ProteinMPNN design parameters (e.g., sampling temperature, any biasing?) are not described. These parameters can significantly influence design success and should be reported for reproducibility and evaluation of design quality.

3. In Supplementary Figure 14, it is unclear how the three GIPR binder designs were selected for SEC analysis. They are not the binders with the highest affinities in the binding kinetics pool. The authors should explain the criteria used for selecting these specific designs for downstream experiments.

Referee #3

(Remarks to the Author)
(A. Summary of the key results; B. Originality and significance)
This manuscript presents an impressive and highly innovative study on the de novo design of miniprotein modulators targeting various G protein-coupled receptors (GPCRs). The authors present novel computational strategies (including 'motif-directed' diffusion and the use of a MetaGen scaffold library with RIFDock) and a high-throughput screening platform (OPS-RD), successfully generating functional agonists (MRGPRX1) and antagonists (CXCR4, GLP1R, GIPR, GCGR, CGRPR). A particular highlight is the successful de novo design of GPCR agonists, which is notoriously challenging. The subsequent structural validation of several designed binders complexed with their targets using single-particle cryo-EM provides near atomic-resolution validation of the design accuracy and mechanism of action. This work represents a tremendous milestone in the field of protein design and GPCR drug discovery and will undoubtedly be of broad interest to the scientific community. While the study is compelling, several issues require further clarification and discussion to fully support the conclusions and to enhance the overall impact of the study.

(C-H) Integrated below into major and minor comments:
Major comments:
1. It is noteworthy that all the GPCR targets investigated in this study are peptide-binding receptors. This focus is understandable, as peptide GPCRs typically possess relatively large and accessible orthosteric binding pockets, which may facilitate de novo miniprotein binder design. However, it is important to highlight that approximately two-thirds of GPCRs are non-peptide receptors, such as serotonin receptors and adrenergic receptors, which often feature smaller or more occluded ligand-binding pockets. Demonstrating that the design pipeline described in this study can be extended to non-peptide GPCRs would significantly broaden the applicability and impact of the work. The authors are encouraged to include either preliminary results or a discussion on the potential challenges and feasibility of applying their approach to non-peptide GPCRs. Alternatively, the authors may consider revising the title to more explicitly reflect the focus on peptide-binding G protein-coupled receptors.

2. The manuscript employs different high-throughput screening strategies—OPS-RD, yeast display with nanodiscs, and yeast display with biofloating—for different GPCR targets (e.g., MRGPRX1, Class B ECDs, CXCR4). It would be helpful if the authors could clarify the rationale behind the selection of each method. Was the choice driven by specific properties of the targets, anticipated characteristics of the binders, or prior experimental experience? A brief comparison or justification of the screening strategies used for each receptor class would enhance the clarity and reproducibility of the study.

3. To better assess the potential biases and complementarity of the screening approaches used, the intracellular colocalization-based purification-free OPS-RD system versus the affinity-based yeast display platforms, a small-scale cross-validation experiment would be highly informative. For example, evaluating a subset of key hits identified by OPS-RD using yeast display, and vice versa, could help elucidate whether the two methods tend to identify overlapping or distinct classes of binders. Such analysis would provide valuable insight into the strengths and limitations of each platform and further support the robustness of the screening pipeline, especially for the newly developed OPS-RD. A similar cross-validation experiment of the design pipelines on one or two receptor targets is also highly recommended, which would help

demonstrate the generalizability and robustness of the approach.

4. The MetaGen approach, which utilizes scaffolds derived from the AlphaFold-predicted metaproteome, would benefit from additional clarification regarding the composition and selection of the ~7,000 “native miniproteins” mentioned in line 164. Specifically, it would be helpful for the authors to elaborate on how these scaffolds were identified and selected. Were any clustering or filtering procedures applied, and if so, what structural or sequence-based metrics were used? Furthermore, the terms “native” and “metaproteome-derived” (Supplementary line 80) should be more clearly defined. Does “native” refer to naturally occurring sequences from known organisms, or to structures with specific stability or folding characteristics? A more detailed description would improve reproducibility and enhance the reader’s understanding of the MetaGen library’s design rationale. Moreover, metaproteome-derived miniprotein scaffold library with relevant scaffold identifiers should be included in the supplementary materials.

5. MRGPRX1 exhibits a relatively shallow and solvent-exposed orthosteric binding pocket compared to many other class A GPCRs. This pocket is characterized by a limited number of deep hydrophobic cavities, instead favoring interactions with flexible, charged, or amphipathic ligands. This structural feature supports its ability to recognize a wide array of peptide ligands with diverse chemical structures (doi.org/10.1038/s41467-023-40705-z). MRGPRX1 is an attractive target for developing non-histaminergic anti-itch therapies, especially for conditions such as atopic dermatitis, uremic pruritus, and drug-induced itch. However, the receptor’s broad ligand recognition profile poses challenges for achieving high specificity and avoiding off-target effects. Demonstrating that the design pipeline described in this study can be extended to de novo design of antagonist miniprotein would significantly broaden the applicability and impact of the work.

6. The motif-directed RFdiffusion strategy used for the antagonist design of CXCR4 is intuitive for targeting recessed pockets. To ensure reproducibility and facilitate further research, please provide more detail on the selection or generation of the initial 5-residue motifs used for scaffolding.

7. The structures of CXCR4 in complex with the newly developed antagonists are highly recommended as the CXCR4 binders are the only bindes designed in this study possessing beta-strand. Structural comparision between the predicted and experimental structures could evaluate the designability of the beta-strand containing binder of GPCRs.

8. The study utilizes various computational pipelines (RFdiffusion variations, MetaGen/RIFDock) and filtering criteria (differing thresholds for pAE_interaction, pLDDT_binder, ddG, sap, etc.) across targets. Please provide a clearer justification for these specific choices. Are the variations driven by target-specific structural features, the intended pharmacological outcome (agonist/antagonist), binding site location (ECD/TM), or preliminary results? Explaining the underlying principles or empirical basis for these decisions is essential for transparency and reproducibility.

Minor Comments:
1. Have the authors considered retrospectively analyzing the success rates of the computational design phase versus the experimental screening phase for the different targets? Such analysis could provide valuable insights for optimizing future computational design efforts, particularly for challenging targets like GPCRs.
2. Please consider enhancing the clarity and presentation quality of the supplementary figures, particularly the 27 figures provided. It is important to ensure that each figure is clearly labeled, referenced appropriately in the main text, and accompanied by sufficiently detailed legends or descriptions to facilitate interpretation.
3. While the manuscript reports the successful generation of several functional agonists (e.g., for MRGPRX1) and antagonists (e.g., for CXCR4, GLP1R, GIPR, GCGR, CGRPR), the sequences of these effective binders—beyond those structurally characterized—are not provided. To ensure reproducibility and facilitate further research, functional binder sequences should be included in the supplementary materials.

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)
In this revision, I appreciate the ability to address selectivity of the mini-proteins and the functional analysis.
However, I am not convinced that many of the issues have been fully addressed.
MRGPRX1: the new binders included in the revision appear to be more potent in the calcium flux assay, but in the BRET studies they appear to be very weak both in potency and efficacy in these assays. The authors should address these discrepancies between functional assays by performing more in depth analysis using orthogonal measures. Why is the activity so weak in these orthogonal assays and what does the calcium flux really represent considering it is an amplified response subject to receptor reserve?
With the more potent binders (in the calcium flux assay anyway), what does the structure with weaker compound mM1_068 really represent? It seems there is a missed opportunity to use the cryo-EM structure to help guide the design of the more potent analogs. Instead the authors just did a screen to refine. What do the cryo-EM structure even tell us about any of these new compounds binding modes?
Finally, there was not much added to the requested mutagenesis analysis to validate the structures. Only mutants were

examined at CGRPR but at none of the other structures, including the above mentioned more potent MRGPRX1 binders. I find the validation of structural poses extremely lacking given the discrepancies in the pharmacology.

Referee #2

(Remarks to the Author)
I am satisfied with the authors' responses to several major points, which have substantially strengthened the manuscript:
1. Selectivity: The addition of comprehensive selectivity panels for GIPR, CXCR4, and MRGPRX1 binders (addressing R1, R2) is a major improvement.
2. Potency: The new optimization data for the MRGPRX1 agonist, which now shows low-nanomolar potency comparable to the endogenous ligand (addressing R2), effectively resolves a key limitation of the initial version.

Despite these significant advances, two issues remain that should be addressed before publication.

Remaining Concerns

1. Overstated Claims of "Conformational Selection"

The claim that agonists and antagonists were designed through "precise conformational control" remains insufficiently supported.
The authors acknowledge in their rebuttal that "the results are not sufficient yet to claim that structural state alone guarantees a defined functional outcome." This is appropriate, but the manuscript text does not yet reflect this clarification.
1.1. The Abstract still claims "confirming precise conformational control of receptor function."
1.2. The Discussion (lines 569–573) continues to imply that conformationally selective design has been definitively achieved: "The ability to computationally design conformationally selective binders is a step change in methodology for obtaining functional biologics targeting integral membrane receptors."

These sections should be revised to better align with the evidence and the authors' own rebuttal. The results are highly promising but do not yet establish a generalizable principle of de novo conformational selection.

2. Insufficient Validation for the CXCR4 Binder

The authors report that cryo-EM studies failed due to particle aggregation. Given the importance of this binder, additional empirical validation is essential.
While mutagenesis data were added for CGRPR binders (already structurally validated), similar experiments should be performed for dCX1_001 and CXCR4. Mutating residues at the predicted interface and demonstrating reduced binding or antagonism would provide necessary orthogonal support for the model.

Overall, the manuscript has improved substantially, and I would be happy to recommend publication once these final revisions are made.

Referee #3

(Remarks to the Author)
The authors have substantially improved the manuscript in response to the first-round comments. The expanded methodological descriptions, additional pharmacological assays, new benchmarking of OPS-RD, and clearer presentation of the design pipeline significantly strengthen the work. The newly added mutagenesis and functional assays also improve confidence in several of the designed ligands. The study continues to address an important challenge in GPCR-targeted ligand design, and the presented results are promising. However, several key concerns raised in the first round remain only partially resolved. These issues primarily relate to the generalizability of the approach and the interpretation of screening performance. Addressing these points would greatly enhance the impact of manuscript and utility for the GPCR community.

Major issues:
MetaGen vs. RFdiffusion comparison remains incomplete. While hit-rate differences are now provided, reviewers previously requested structural diversity metrics (e.g., RMSD clustering, interface topology distribution). Without such quantitative analysis, it remains unclear whether MetaGen truly expands the design space or mainly introduces redundancy.
Several reviewers explicitly recommended the authors demonstrate the ability to design both agonists and antagonists for the same receptor, to validate the generality of the de novo design pipeline. The authors provided a clear explanation about the lack of an inactive-state structure for MRGPRX1, which limits antagonist design for that specific receptor. This rationale is reasonable. Since active and inactive structures are available for CXCR4, CCR5, and OXTR, and only antagonists were generated for these receptors, it remains unclear whether the agonist-design component of the pipeline can be applied beyond MRGPRX1. To address this concern, the authors could either provide at least one additional agonist example for a receptor with a known active-state structure, or explicitly clarify methodological or biological reasons why agonist design is more challenging for these receptors and soften claims of cross-receptor generality.
As all successful designs target peptide/protein-binding GPCRs with accessible binding pockets, the current pipeline does not yet demonstrate applicability to small-molecule GPCRs with occluded orthosteric sites. In the response, the authors state that "GPCRs that bind small molecules typically feature narrow, and partially occluded orthosteric pockets… These

structural features present challenges for de novo binder design" and suggest that such conformations limit accessibility for designed miniproteins. While this explanation is reasonable, it simultaneously implies that the authors attempted such designs and found them unsuccessful, thereby acknowledging that the current approach does not generalize to small-molecule-binding GPCRs. If so, this important limitation should be more explicitly articulated in the manuscript.
Several GPCRs with small-molecule orthosteric ligands nonetheless possess well-characterized extracellular-accessible entry pathways or partially exposed pocket regions that have been successfully targeted by peptides, nanobodies, or engineered proteins in previous studies. It is therefore not entirely clear why the present design strategy cannot be adapted to generate binders for these receptors.
The authors now provide a careful analysis comparing OPS-RD with yeast display, which is very helpful. The revised results indicate that among the top 60 OPS-RD hits, approximately 55% exhibit binding <4 μM. While this demonstrates that the platform is capable of identifying true positives, the remaining false-positive fraction raises questions about how best to use OPS-RD as part of an integrated screening pipeline. This point does not undermine the utility of OPS-RD, but it does highlight the need for further clarification. A short discussion addressing the following would be constructive, including potential biological or technical factors contributing to false positives and whether tuning the OPS-RD thresholds (expression, RD signal, p-value) could mitigate this issue.

Version 2:

Reviewer comments:

Referee #1

(Remarks to the Author)
The authors have addressed all of my critiques. I look forward to this work being published.

Referee #2

(Remarks to the Author)
The remaining concerns from the previous round have been adequately addressed. However, the newly included data on NK1R and CCR5 agonists, as well as the CXCR4 binder cryo-EM structure, raise new concerns that should be addressed before publication.

1. NK1R and CCR5 agonists
The re-evaluation of CCR5 functional hits in this revision identifies dCC1_002 as an agonist, whereas it was classified differently in the previous round. This is a significant finding that directly contradicts the authors' central mechanistic argument — namely, that targeting active-state receptor structures generates agonists while targeting inactive-state structures generates antagonists. dCC1_002 was designed using the inactive-state CCR5 structure (PDB: 5UIW, as stated in the Methods), yet it produces full agonism in cAMP and G-protein dissociation assays (EC50 ~814 nM and ~561 nM, respectively). This unexpected outcome requires explicit acknowledgment and discussion. Specifically, the authors should address whether dCC1_002 engages contacts that overlap with the native binding site, which could mechanistically explain its agonistic behavior despite being designed against the inactive state. More broadly, the authors should clearly state in the Discussion that their design strategy does not guarantee a defined functional outcome (agonism vs. antagonism) based solely on the choice of receptor conformational state, and that the relationship between design state and functional outcome remains incompletely understood. This is not a minor point — it bears directly on the conceptual framework of the entire paper.

Regarding the NK1R agonists: of 63 successfully expressed RFdiffusion hits, 20 were confirmed as agonists in the cAMP assay. The manuscript does not report whether the remaining 43 expressed hits were tested in an antagonist mode — i.e., whether any can block substance P-induced signaling. Given the authors' own argument that the receptor conformational state used for design should predict functional outcome, it is notable that not all hits designed against the active state produced agonists. Were the remaining hits inactive, weak binders, or potential antagonists? This should be explicitly reported. If any of the non-agonist hits exhibit antagonistic behavior, this would further challenge the conformational selection hypothesis and should be discussed accordingly.

2. CXCR4 binder cryo-EM structure
The authors state (line 462) that "the dCX1_001-CXCR4 complex closely matches the RFdiffusion model," but this claim is not well supported by the structural data as presented. Several specific concerns arise:

First, the RFdiffusion design model appears to include only the extracellular half of CXCR4, presumably because the target structure was truncated to the binding epitope region during design. However, this truncation strategy is not described in the Methods section and should be explicitly stated, as it is directly relevant to evaluating the accuracy of the design model.

Second, Fig. 3k shows that transmembrane helices 6 and 7 adopt a substantially more open conformation in the experimentally determined structure compared to the design model. The authors attribute this to a conformation "similar to many other CXCR4 structures," but do not address whether this receptor geometry was anticipated during design or represents an unanticipated conformational selection by the binder.

Third, and most critically, the pocket-penetrating loop of dCX1_001 — the element designed to make deep contacts within the orthosteric site — adopts a conformation that diverges significantly between the design model and the solved structure.

The authors report a ligand Cα-RMSD of 3.9 Å in the receptor-aligned frame, attributing this to the miniprotein "pivoting modestly around the β-sheet." A 3.9 Å RMSD is not modest for a miniprotein of this size and represents a meaningful deviation from design intent. The authors should explicitly assess whether the key contacts in design model are actually maintained in the solved structure. If the deep orthosteric contacts are not preserved, the characterization of dCX1_001 as a "receptor-penetrating" antagonist requires reassessment.

Finally, the cryo-EM structure was determined on homotrimeric CXCR4, while the binder was designed against and pharmacologically characterized on monomeric CXCR4. The authors do not address whether trimeric assembly alters the binding site geometry, or whether the observed receptor conformation in the trimer context is relevant to the pharmacological activity measured in cell-based assays. This disconnect between the structural and pharmacological contexts should be explicitly acknowledged.

Referee #3

(Remarks to the Author)
The authors have substantially improved the manuscript in response to the previous comments. The addition of quantitative analyses of structural diversity provides a more convincing comparison between MetaGen and RFdiffusion, supporting their complementarity. The approach's application to NK1R, along with the identification of several functional agonists, enhances the generalizability of the design strategy.

The revised manuscript is clearer, and the additional experimental validation increases confidence in the reported binding modes and functional interpretations. While demonstrating bidirectional modulation on the same receptor would be an intriguing future direction, the current data sufficiently support the main conclusions.

Overall, this is a strong and timely contribution to GPCR-targeted protein design, and I have no further major concerns. I recommend publication in its current form.

Point by point response to reviewers' comments

Title: *De novo* design of miniprotein agonists and antagonists targeting G protein-coupled receptors

Authors: Edin Muratspahić, David Feldman, David E. Kim, Xiangli Qu, Ana-Maria Bratovianu, Paula Rivera-Sánchez, Jan Hendrik Voss, Emil P. T. Hertz, Mads Jeppesen, Federica Dimitri, Kensuke Sakamoto, Amrita Nallathambi, Jason Cao, Brian P. Cary, Matthew J. Belousoff, Peter Keov, Phuc H.T. Trinh, Qingchao Chen, Yue Ren, Justyn Fine, Sudha Mishra, Annu Dalal, Shachie Sinha, Isaac Sappington, Thomas Schlichthaerle, Jason Z. Zhang, Arvind Pillai, Ljubica Mihaljević, Magnus Bauer, Susana Vázquez Torres, Amir Motmaen, Gyu Rie Lee, Long Tran, Xinru Wang, Inna Goreshnik, Dionne K. Vafeados, Justin E. Svendsen, Parisa Hosseinzadeh, Nicolai Lindegaard, Matthäus Brandt, Yann Waltenspühl, Kristine Deibler, Luke Oostdyk, William Cao, Lakshmi Anantharaman, Johan J. Weiser, Rie Christensen, Christoffer Bundgaard, Ayodeji A. Asuni, Lance Stewart, Lauren Halloran, Jamie B. Spangler, Arun K. Shukla, Patrick M. Sexton, Bryan L. Roth, Brian E. Krumm, Denise Wootten, Christopher G. Tate, Christoffer Norn, David Baker

Reviewer #1 (Remarks to the Author):

This manuscript details de novo design strategy of miniproteins targeting GPCRs that can be achieved for a highly druggable targets. I can appreciate the specificity aspect of this work, confirming mini protein target engagement in biochemical assays and cryo-EM structure determinations, with some exhibiting potent and high affinity binders for GLP1 and class B GPCRs. However there are major issues with the manuscript that limits applications and utility for other investigators.

Response: We thank the reviewer for their feedback on our manuscript.

Major issues:

***Reviewer*:** There is lack of mechanism on how the design is achieved toward different GPCRs. In fact, I didn't learn anything from the manuscript on how to better design difficult to drug GPCRs using this approach. Importantly, there is lack of details on which key miniprotein designs that worked best for each receptor type to stabilize the pentapeptide motif to drive specificity and affinity. For instance, for all GPCRs investigated, which of the miniprotein backbone worked best (helix, strand, loop etc), or what were the specific combination that was successful? Little to no detail on how areas to stabilize the peptide were provided for the designs. Which pentapeptides were prioritized especially with respect to selectivity within subtypes or iterative improvements to specificity? A posthoc meta-analysis on the efficiency of pentapeptide and miniprotein backbone designs would have suited better to complement the detailed the validation and specificity aspects of the manuscript.

Response: Computational design of protein-based GPCR ligands that protrude the deep binding pocket has been a long-standing challenge. Accordingly, we used RFdiffusion to first generate small motifs of 5 residues capable of penetrating the receptor pocket and then scaffold miniproteins of 65-75 residues onto these motifs. Out of approximately 1,000 small motifs that we generated based on hot spots which were defined within the binding pocket of the receptor, we selected 50 motifs based on their distance to these buried hot spots, *i.e*., had deepest insertion. These small motifs were not selected based on their selectivity but their distance to defined hotspots within binding pockets. We described the small motif generation in methods in more detail (**page 25**, **line 652-657**). In addition and as suggested by the reviewer, we generated a figure of distinct design topologies that we obtained libraries for, class A GPCRs, including CXCR4, CCR5 and OXTR receptors, and class B GPRCR binders targeting the ECD, and highlighted hits identified in follow up yeast display assays (**Supplementary Fig 3**). Finally, we compared the success rates of MetaGen and RFdiffusion for GLP1R and PAC1R designs generated and tested under comparable protocols (**Supplementary Fig. 32a-b**). In the design library, MetaGen protocol sampled a larger conformational space, which upon hit finding with yeast display collapsed to a similar topological space as sampled by RFdiffusion of mainly helices though displaying additional diversifying features like kinks (see cryo-EM structures). In yeast display screening, the observed hit rates were 1.25% for GLP1R and 1.00% for PAC1R with MetaGen, and 0.39% for GLP1R and 0.30% for PAC1R with RFdiffusion, corresponding to a 3.2

to 3.3 fold difference. Expression levels and SPR hit ratios among the hits identified by yeast display were comparable (**Supplementary Fig. 32a-b**).

***Reviewer*:** It appears specificity is somewhat validated, but importantly 'selectivity' of such targets is largely ignored. No selectivity panels were provided for any of the mini proteins. How well is the design for achieving selectivity? For instance, many of the example GPCRs investigated in this manuscript (e.g. MRGPRX, CXCRs) are part of GPCR types with very similar subtypes. Do these miniprotein ligands have similar affinity for closely related subtypes? Do these miniproteins have selectivity across a very large set of GPCRs targets? Importantly, are these miniproteins, either in their pentamer design or miniprotein stabilization features, showing exquisite selectivity across many protein targets? It is an important question to answer because many of these design features could be polypharmacological or endowed with such specificity that selectivity over all closely or distantly related proteins could be revealed.

Response: To strengthen our rationale that *de novo* design enables generation of selective binders, we further evaluated selectivity of GIPR, CXCR4 and MRGPRX1 binders. For GIPR, we tested the most potent antagonists against related GCGR and GLP1R receptors in a functional cAMP assay and found little or no significant activity at these receptors up to 1 μM (**Fig 4b**). Similarly, CXCR4 antagonist dCX1_001 showed no measurable activity at related CXCR7 or CCR5 receptors in cAMP, $G_{\alpha i}$ dissociation or β-arrestin-1 recruitment assays (**Fig. 3g-l**). For MRGPRX1, we measured selectivity of optimized full and partial agonists in the calcium mobilization assay. Improved MRGPRX1 agonists were extensively tested for GPCR activation over a panel of 318 GPCRs. Both miniproteins elicited activities at only a small subset of receptors, indicating minimal off-target agonism under these conditions, and were confirmed to be selective for MRGPRX1 in follow up experiments **(Fig. 2j, Supp. Fig. 9d-g**).

***Reviewer*:** Connecting mini proteins to druggable technologies also appears to be lacking and whether these newly discovered miniprotein ligands have any use in vivo. What advantages do these miniproteins have over nanobodies or antibodies or other larger in terms of in vivo target engagement? Can they be made metabolically stable for in vivo use or what are their limitations? Can the authors show just one example showing in vivo effects?

Response: While we agree that demonstrating *in vivo* activity and therapeutic potential of *de novo* GPCR binders in a mouse model would be compelling, the goal of this study was to provide proof-of-concept that diverse GPCRs, particularly receptors with recessed binding pockets, can be targeted using existing deep learning tools - a longstanding challenge in this field. Miniproteins offer several advantages over larger biologics, including smaller size for improved tissue penetration, rapid design and optimization, and the potential for modifications to enhance metabolic stability. Several strategies exist to improve their *in vivo* stability, including cyclization, incorporation of non-natural or D-amino acids, disulfide bonds, and fusion to an immunoglobulin Fc or albumin-binding domain (Berger et al., *Cell*, 2024; Roy et al., *Nature Communications*, 2023; Sun et al., *Cell Research*, 2024). Another notable advantage in comparison to larger biologics is the potential to develop orally bioavailable miniproteins, as demonstrated in our previous studies (Berger et al., *Cell*, 2024). We have added a discussion of these points in the revised manuscript

to clarify the potential of miniproteins for *in vivo* applications (**page 23**, **line 573-575**). Furthermore, to demonstrate that miniproteins targeting GPCRs can be connected to druggable technologies, we performed an *in vivo* pharmacokinetic (PK) study in mice comparing a representative *de novo* CGRPR-targeting miniprotein in its unmodified and Fc-fused form. After a single IV dose (3 mg/kg; C57BL/6; n=8 per group with 3–4 samples per timepoint), plasma concentrations were quantified by validated UPLC-MS/MS at 5–1440 min. The unmodified miniprotein was rapidly cleared (t½ ≈ 0.4 h), consistent with glomerular filtration of small proteins, whereas Fc-fusion extended systemic exposure significantly (t½ ≈ 25 h) with >200-fold lower clearance (1.2 vs 0.0051 L/h/kg) and sustained plasma levels over 24 h (**Supplementary Fig. 43a-e**). These data demonstrate that miniproteins can be paired with an established, clinically validated half-life extension technology to achieve drug-like properties *in vivo* (**page 18-19**, **line 483-489**).

***Reviewer*:** Despite the potential limitations toward therapeutics, the authors state the mini proteins have utility as chemical probes. But they don't measure extensively what such miniprotein would provide toward the available plethora of GPCR mechanisms. GPCRs exhibit a spectrum of signaling and only one signaling pathway is measured for each representative GPCR example. Do any of these miniprotein ligands exhibit biased agonism or agonist-directed trafficking? Are these miniproteins do anything novel instead of acting as agonists and antagonists? How do these miniprotein affect ligand kinetics? Do they have longer residence time and slower kinetics in general given their size? Are there any arrestin or desensitization measures to show these a useful chemical probes? How do these illuminate the GPCR of interest's pharmacology in comparison to known chemical matter for each respective example? What do these examples add to understanding the complexity of signaling by GPCRs that has not been revealed already?

Response: To address the reviewer's request for additional functional characterization, we tested our CXCR4 miniprotein antagonist not only in the previously reported cAMP assay but also in $G_{\alpha i}$ dissociation and β-arrestin-1 recruitment assays. The binder acts as a potent antagonist of $G_{\alpha i}$ signaling and β-arrestin-1 recruitment, demonstrating antagonism rather than biased agonism (**Fig. 3g-l**). We also evaluated optimized MRGPRX1 agonists in G protein dissociation and β-arrestin-2 recruitment assays, where each partially activated receptor with potencies in the low micromolar range and comparable to BAM (8-22) (**Supplementary Fig. 9a-b**). These results provide evidence that miniproteins can be leveraged to interrogate receptor pharmacology complementary to existing ligands (**page 13**, **line 350-352**, **page 8**, **line 250-253**).

Reviewer: Finally, no mutagenesis is presented to validate the binding modes. It is necessary to provide empirical evidence in the form of carefully designed mutant experiments to complement the cryo-EM structures and predicted binding modes.

Response: To complement the cryo-EM structures, the antagonist activity of dC2_049 and dC2_050 was measured in a cAMP assay on cells expressing the wild-type receptor or receptors with alanine mutations of key residues in RAMP1 or CLR at the targeted interface. Mutation of

several of these residues abolished binder activity, further supporting the proposed binding modes (**page 22**, **line 527-531**, **Supplementary Fig. 47-48**).

Minor issues:

***Reviewer*:** Receptor diversion assay: How do you control for expression and therefore concentration of the binders to calculate Kd? How does the receptor diversion assay compare with yeast and biofloater assays, in terms of high-throughput, false positives, artifacts etc.?

Response: While we do observe a distribution of binder expression in the receptor diversion assay, we do not claim to calculate K_D based on the binding signal (cross-correlation of GFP-GPCR and mCherry-binder). Instead, the binding signal (and the p-value of the binding signal) is used to select hits for follow-up validation assays. This likely means that we are selecting against designs that express poorly in mammalian cells, but this is already stated as an advantage of the assay (**page 4, line 151-152**). We have now benchmarked the OPS-RD platform directly against yeast display by screening a library of PAC1R antagonist designs by both technologies. We conclude that the binding signal obtained by the OPS-RD is highly predictive of validated hits discovered by yeast display. Moreover, the binding signal obtained by OPS-RD was statistically significant for >75% SPR-validated yeast display hits and <2% of the negative controls at a false discovery rate (FDR) threshold of 5% (Kolmogorov–Smirnov test p-value adjusted by Benjamini-Hochberg procedure), indicating a low false negative rate coupled to a high true negative rate of the assay. Further, to address the false positive rate of the assay we determine the binding affinity of the top 60 hits and find 55% to display binding below 4 μM (**Fig. 1**). Finally, we have provided a sentence in the discussion comparing the throughput and false positive rate of the receptor diversion assay to display technologies (**page 23, line 563-566**).

***Reviewer*:** In vitro signaling assays. MRGPRX1 example: Authors state two are full agonists, and one is a partial agonist with respect to what? Bam-8-22. Specify what the positive controls are throughout, in both captions and in text. No receptor controls without receptor expressed are present throughout to confirm specificity. Authors should show direct activation of G proteins using an orthogonal assay.

Response: We have now clearly indicated in the main text and in the figure captions that BAM (8-22) served as a positive control. We also clarified positive control abbreviations for the CGRPR. We further added experiments demonstrating receptor activation by miniproteins in G_q protein dissociation and β-arrestin-2 recruitment assays, in which miniproteins acted as partial agonists (**Supplementary Fig. 9a-b**). Finally, we performed a calcium mobilization assay to confirm receptor-dependent effects of miniproteins with no calcium responses observed in CHO cells without MRGPRX1 expression, as suggested by the reviewer (**Supplementary Fig. 9c**).

Reviewer: Ballesteros-Weinstein or GPCRdb coordinate number system is needed throughout manuscript superscripted on residues.

Response: Receptor residues in superscript for class A and class B receptors are defined using the Ballesteros-Weinstein and class B numbering system, respectively, throughout the manuscript (Liang et al., *Nature*, 2017; Wootten et al., *PNAS*, 2013).

Reviewer #2 (Remarks to the Author):

The authors report the computational de novo design of miniprotein binders targeting GPCRs. They describe the generation of agonists and antagonists directed at the orthosteric binding sites of MRGPRX1 and CXCR4, respectively, as well as protein antagonists binding the soluble extracellular domains of several class B GPCRs. Structural validation of the CGRPR-bound antagonists and MRGPRX1-bound agonists was achieved using cryoEM.

To enable these designs, the authors developed a “motif-directed” variant of their RFdiffusion method, along with MetaGen, a strategy for generating more structurally diverse protein scaffolds. They also introduced a high-throughput, microscopy-based “receptor diversion” screen to efficiently identify functional binders from computational libraries.

Designing potent and functionally selective GPCR modulators remains a major unmet challenge in pharmacology, and current methods often depend on labor-intensive empirical screening. A rational approach for designing mini-protein binders that predictably control GPCR activity would represent a significant advancement. This study, therefore, holds the potential to substantially impact GPCR-targeted drug discovery.

However, while the study presents some promising and exciting examples of de novo designed binders, it currently lacks sufficient evidence to support the broader claim of a generalizable strategy for designing conformationally and functionally selective GPCR modulators. Additionally, some aspects of the results raise concerns about the computational approach’s applicability to other GPCR targets.

Response: We thank the reviewer for their feedback on our manuscript.

Major concerns

Reviewer: In both the abstract and discussion, the authors claim to achieve precise conformational control of receptor function. Specifically, they propose that designing binders against an active-state GPCR structure yields agonists, while targeting the inactive state leads to antagonists. However, the structural differences between active and inactive conformations in the extracellular orthosteric binding sites are often minimal, which may limit the generalization of this approach to other GPCRs.

To convincingly demonstrate that functionally selective binders can be rationally designed based on receptor conformation, the authors should be able to generate both antagonists and agonists for the same GPCR by specifically targeting either its inactive or active state. At a minimum, they should show that designing binders against the inactive conformation of MRGPRX1 yields a higher proportion of antagonists or inverse agonists, and that targeting the active conformation of CXCR4 leads to a greater fraction of agonists. Additionally, the manuscript should better articulate the structural basis for this claim: how do structural differences between the active and inactive states of MRGPRX1 and CXCR4 influence designability and binding specificity?

Response: We agree that demonstrating the ability to design both agonists and antagonists for the same GPCR by targeting active and inactive states would be a convincing demonstration for *de novo* protein design to create conformationally selective miniproteins. Our study takes an initial step in exploring this phenomenon, however, we acknowledge that the structural differences between active and inactive GPCR conformations can be subtle, and may not easily be targeted by protein design. While our results for MRGPRX1 agonists are promising, they are not sufficient yet to claim that structural state alone guarantees a desired and defined functional outcome. Exploiting subtle conformational differences - such as extracellular loop geometries, inward and outward movements of transmembrane helices, or microswitches within deep orthosteric binding pockets - that could drive agonism remains a challenge for computational design. We also acknowledge that the success in obtaining computationally designed agonists will be receptor-dependent and associated with intrinsic properties of a given receptor. Accordingly, we have toned down our claims (**page 22**, **line 550-554**, **569-573**).

***Reviewer*:** The authors should also discuss the generalizability of their method. Is it broadly applicable across GPCRs, or is it best suited to receptors with large, accessible epitope shifts upon activation? This would help readers evaluate whether their conformational design strategy is scalable or only appropriate for a subset of GPCRs with pronounced structural transitions.

Response: We appreciate the opportunity to clarify the generalizability of our approach. While our *de novo* design methods are applicable to a wide range of GPCR targets, we recognize that they are best suited to GPCRs with either soluble, structured extracellular domains (ECDs) or recessed binding pockets that have peptides or proteins as endogenous ligands. For instance, receptors with large ECDs such as class B1 GPCRs comprise soluble and well-structured interfaces and for these targets we observed consistent success in identifying high-affinity, functional binders. This has now been strengthened with binders for PAC1R and PTH1R (**Fig. 1j and Fig. 4c**). We would like to clarify that GPCR targets with recessed binding pockets that recognize peptides or proteins as natural ligands tend to be more amenable to *de novo* binder design than those that bind small molecules. Peptide- and protein-binding GPCRs often comprise larger and more accessible recessed binding pockets that can be readily targeted by designed miniproteins. In addition to CXCR4 and MRGPRX1, we now provide more examples of binders capable of targeting deep binding pockets of peptide- and protein-binding GPCRs, namely CCR5 and OXTR. We identified antagonists in the nanomolar and micromolar range targeting these receptors (**Supplementary Fig. 18-22**). In contrast, GPCRs that bind small molecules typically feature narrow, and partially occluded orthosteric pockets buried within the transmembrane domain. These structural features present challenges for *de novo* binder design, particularly when targeting orthosteric sites, since the relevant epitopes are often occluded. As a result, our current strategy is likely to identify binders for GPCRs that naturally engage large ligands, such as neuropeptides, chemokines, and protein hormones since these GPCRs have more accessible recessed pockets. Future studies will examine the potential of our *de novo* methods to develop binders against small molecule-sensing GPCRs. We have added a discussion of these points in the revised manuscript (**page 22-23**, **line 555-559**).

***Reviewer*:** In the abstract, the authors claim that their designed miniproteins exhibit high affinity, potency, and selectivity. However, this claim is not consistently supported by the functional data presented. For instance, the most potent MRGPRX1 agonist has an EC_{50} of 390 nM—over 100-fold weaker than the endogenous agonist BAM, which has an EC_{50} of ~3 nM. Similarly, the reported CXCR4 antagonist has an IC_{50} of 24 nM, which is approximately ten-fold less potent than the native agonist ligand.

Response: To support our claims that we can design high affinity, potency and selectivity binders, we improved MRGPRX1 agonists showing EC_{50} values in the low nanomolar range and similar to endogenous BAM (8-22) ligands (**Fig. 2i**). These binders also demonstrated remarkable selectivity when testing across a panel of 318 GPCRs (**Fig. 2j**, **Supplementary Fig. 9d-g**). The IC_{50} values of CXCR4 binder dCX1_001 in its unmodified form (24 nM) and after rigid fusion (9 nM, **Supplementary Fig. 15c**) are in the range of previously reported protein-based antagonists of CXCR4 (Kledal et al., *Science*, 1997; Zhou et al., *Biochemistry*, 2000). The dCX1_001 also demonstrated exceptional selectivity with no significant activity at related CXCR7 and CCR5 receptors (**Fig. 3g-l**).

***Reviewer*:** Furthermore, while the CGRPR antagonists underwent extensive selectivity profiling, there is no comparable data for the MRGPRX1 and CXCR4 binders. Without such analyses, it is premature to assert high selectivity for these designs. To align claims with the presented data, the authors should moderate their claims regarding potency and selectivity in both the abstract and main text. It would also strengthen the study to clearly specify which properties were rigorously validated and for which designs.

Response: To strengthen our rationale that *de novo* design enables generation of selective binders, we further evaluated selectivity of GIPR, CXCR4 and MRGPRX1 binders. For GIPR, we tested the most potent antagonists against related GCGR and GLP1R receptors in a functional cAMP assay and found little or no significant activity at these receptors up to 1 μM (**Fig 4b**). Similarly, CXCR4 antagonist dCX1_001 showed no measurable activity at related CXCR7 or CCR5 receptors in cAMP, $G_{\alpha i}$ dissociation or β-arrestin-1 recruitment assays (**Fig. 3g-l**). For MRGPRX1, we measured selectivity of optimized full and partial agonists in the calcium mobilization assay. Improved MRGPRX1 agonists were extensively tested for GPCR activation over a panel of 318 GPCRs and showed no significant activation of related or unrelated receptors (**Fig. 2j**).

***Reviewer*:** The criteria used for computational screening of binder designs vary substantially between targets. For instance, the thresholds for predicted interface pAE (ipAE) range from 4 (for CGRPR) to 8 (for GLP1R), while acceptable pLDDT values span from 80 to 90. The manuscript does not explain the rationale behind these differing cutoffs. It remains unclear whether these thresholds were selected based on empirical optimization, intrinsic differences between targets, or other considerations. The authors should clarify how these parameters were chosen and whether they reflect systematic optimization or arbitrary decisions. Furthermore, it would be important to discuss how these variations might affect the interpretation of design quality and

success rates across different receptors. Without a consistent or well-justified framework, it is difficult to compare outcomes across targets or evaluate the generalizability of the approach.

Response: *In silico* cutoffs goals were defined *a priori* from our previous benchmarking of AF2 and Rosetta metrics across multiple targets (Bennett et al., *Nature Communications*, 2023). Where feasible, we targeted pLDDT_binder > 90, pAE_interaction < 8, binder_RMSD < 2, and low SAP (≤ 35) to reduce aggregation risk while maintaining confident local structure. We treated pAE_interaction and pLDDT_binder as primary acceptance filters, then set Rosetta ddG and SAP to filter remaining candidates. When very few designs met these strict criteria, thresholds were relaxed within the target specific ranges reported above, guided by interface chemistry. Targets that can be engaged *via* larger hydrophobic contact patches generally support more stringent Rosetta ddG cutoffs. We have added this explanation in methods to clarify the rationale for choosing AF2 and Rosetta cutoff values (**page 24**, **line 604-611**).

Reviewer**:** The reported success rates are generally low, ranging from 0.5% to 4.5% for most targets and as low as 2 out of 26,000 designs for CXCR4. These outcomes raise concerns about the effectiveness of the computational selection criteria (e.g., AlphaFold predictions, Rosetta scores) used during the design filtering stages. To better understand the predictive value of these metrics, a retrospective analysis comparing successful versus unsuccessful binders would be highly informative.

Response: We thank the reviewer for this thoughtful point. The overall hit rates in our screens are modest for several targets. We agree that success in *de novo* binder discovery is highly epitope dependent, particularly for class A GPCRs for which extracellular loops are flexible, epitope curvature is extreme, and chemistry often quite hydrophilic. To our knowledge, there are no peer-reviewed reports of *de novo* binders to class A GPCRs that enable a direct epitope-matched comparison. To address the reviewer's request for a retrospective assessment of computational filters, we have added an analysis that compares successful binding hits (identified with yeast display) against non-binders across the exact design sets tested (**Supplementary Fig. 49**). We analyzed filter values for RFdiffusion and MetaGen designs separately because RFdiffusion relies on iterative partial diffusion that optimizes *in silico* metric in order to pass defined filter values whereas MetaGen approach does not. We evaluated AlphaFold-Multimer PAE at the interface and pLDDT of the binder, as well as Rosetta interface ΔΔG, contact molecular surface (CMS), and surface aggregation potential (SAP). We computed Receiver Operating Characteristic (ROC) curves and corresponding Area Under the ROC Curve (AUROC) values for each computational metric. These curves summarize how the true positive rate (fraction of binders correctly classified) changes as the metric threshold is varied, against the false positive rate (fraction of non-binders incorrectly classified). Our results show that certain metrics show mild enrichment of binders toward favorable score ranges, with some individual filters showing stronger discrimination depending on the target and design method. However, this post-hoc analysis is conducted on libraries that have already been filtered using the analyzed metrics. Therefore, in this case, the AUCs describe only how well the metric further ranks designs within the post-filter window, not its overall ability to separate binders from non-binders in the full design

space. Overall, we would like to clarify that success rates should be compared on a per-epitope basis. We included a description of the analysis in the results section (**page 22**, **533-540**).

Reviewer: Specifically, do the chosen computational filters (e.g., pAE_interaction, pLDDT, ddG) effectively enrich for functional binders? Such an analysis would provide crucial insight into the strengths and limitations of the current pipeline and could guide improvements to enhance hit rates in future campaigns. Without this, it remains difficult to assess whether the observed low success rates reflect intrinsic design challenges or suboptimal scoring strategies.

Response: We have addressed this concern (see above).

Reviewer: Lastly, the authors should clearly distinguish the miniprotein binders designed against class B GPCRs, which target soluble extracellular domains, from those targeting CXCR4 and MRGPRX1, whose orthosteric sites are more conformationally dynamic and structurally constrained, posing distinct design challenges.

Response: We revised the title of class B receptor antagonists to “Pharmacology and biophysical characterization of antagonists targeting ECD of multiple class B GPCRs” (**page 15**, **line 393-394**).

Reviewer: The manuscript introduces MetaGen as a complementary strategy to RFdiffusion for generating scaffolds with increased structural diversity, yet the comparative advantage of this method remains unclear. Specifically, it would be valuable to know whether MetaGen produces binders with significantly different structural features, epitope coverage, or binding surface complementarity relative to those generated by RFdiffusion alone. For example, in the case of MRGPRX1, how do the binders derived from MetaGen compare, structurally and functionally, with those produced by standard RFdiffusion? Providing quantitative or qualitative comparisons (e.g., scaffold diversity, predicted binding energies, functional hit rates) would help clarify whether MetaGen substantially enhances binder design capabilities or simply adds redundancy. Without such a comparison, it is difficult to assess the necessity and unique contribution of this method to the overall design pipeline.

Response: We compared the success rates of MetaGen and RFdiffusion for GLP1R and PAC1R designs generated and tested under comparable protocols (**Supplementary Fig. 32a-b**). In the design library, MetaGen protocol sampled a larger conformational space, which upon hit finding with yeast display collapsed to a similar topological space as sampled by RFdiffusion of mainly helices. In yeast display screening, the observed hit rates were 1.25% for GLP1R and 1.00% for PAC1R with MetaGen, and 0.39% for GLP1R and 0.30% for PAC1R with RFdiffusion, corresponding to a 3.2 to 3.3 fold difference. We have indicated this difference in performance in the results section **(page 15-16**, **line 422-430**). We further note that RFdiffusion backbones for GLP1R were filtered more stringently (PAE < 6 versus 12, ddG < −45 versus −30, pLDDT > 90 versus 85), leaving no further signal in filter metrics (**Supplementary Fig. 49**), while hit-rates could have been increased for GLP1R with MetaGen by filtering more strictly for DDG, CMS and pae_interaction. We note that the lack of signal for RFDiffusion might be derived from its direct

optimization against these surrogate metrics, which might push the search to exploit scoring artifacts and compress diversity.

***Reviewer*:** The Receptor Diversion strategy represents an elegant and innovative approach for efficiently screening binders against membrane receptors. The correlation between binding affinity and the diversion signal in their benchmark is promising. However, can the authors rule out the possibility of false negatives—namely, high-affinity binders that do not cause receptor diversion? It would be important to clarify the limitations of this approach. For example, are there specific biophysical or spatial constraints (e.g., binding geometry, epitope location, or size of the binder) that might not prevent receptor trafficking even in the presence of strong binding? Addressing this question would help assess the robustness and general applicability of the receptor diversion readout.

Response: While we can speculate about specific biophysical or spatial constraints of the receptor diversion assay we do not have any evidence to support such claims. One potential biophysical constraint is the requirement for at least one free termini of the design when screened by OPS-RD. It is possible to either fuse the KDEL sequence to the C-terminus of the design or fuse an N-terminal affinity tag to the design and co-express a binder with a C-terminal KDEL sequence for trapping. Notably, for yeast display the design is always fused N-terminally to Aga2p. To circumvent this complication our design pipeline only selects designs with both termini free. To demonstrate this concept, we have benchmarked the OPS-RD platform directly against yeast display by screening a library of PAC1R antagonist designs by both technologies. We conclude that the binding signal obtained by the OPS-RD is highly predictive of validated hits discovered by yeast display. Moreover, the binding signal obtained by OPS-RD was statistically significant for >75% SPR-validated yeast display hits and <2% of the negative controls at a false discovery rate (FDR) threshold of 5% (Kolmogorov–Smirnov test p-value adjusted by Benjamini-Hochberg procedure), indicating a low false negative rate coupled to a high true negative rate of the assay. Further, to address the false positive rate of the assay we determined the binding affinity of the top 60 hits and found 55% to display binding below 4 μM (**Fig. 1j-k**). Finally, we have provided a sentence in the discussion comparing the throughput and false positive rate of the receptor diversion assay to display technologies (**page 23, line 563-566**).

Receptor target specific points

1. MRGPRX1

***Reviewer*:** To gain a better understanding of the molecular basis of binder function, could the authors identify whether specific binding residues are responsible for agonism and partial agonism?

Response: Previous structural and functional studies identified key residues within the orthosteric site of MRGPRX1 that are critical for activation by the endogenous peptide, bovine adrenal medulla BAM (8-22) (Cao et al., *Nature*, 2021; Liu et al., *Nature Chemical Biology*, 2023). Both miniprotein agonists occupy this same binding site (**Supplementary Table 6**). While we did not

perform mutagenesis to test the functional consequences of individual residues, our structural comparison highlights key orthosteric residues (E157$^{4.60}$, L240$^{6.59}$, F236$^{6.55}$, W241$^{6.60}$, and F250$^{7.31}$) with distinct side chain orientations between the two miniprotein-bound MRGRPX1 cryo-EM structures. These structural differences may be key determinants of partial and full agonism of miniproteins at the MRGPRX1 (**page 11**, **line 313-320**). Systematic mutagenesis, similar to the approach applied to CGRPR miniprotein antagonists (**Supplementary Fig. 47-48**), is planned for our future work. To identify binder-dependent residues involved in full and partial agonism, we performed site saturation mutagenesis (SSM) of the top three hits. 239 variants were expressed, purified and tested for MRGPRX1 agonism at a concentration of 500 nM. 21 hits were identified (**Supplementary Fig. 7**) and the most potent miniprotein, mM1_034_F12W_Y27F, with an EC_{50} of 42 ± 7 nM (mean ± SEM), exhibited 12 fold-increased potency, similar to the endogenous agonist BAM (8-22) (**Fig. 2i**, **Supplementary Fig. 8**). The other top hit, mM1_034_F12W_A58M with an EC_{50} of 110 ± 20 nM (mean ± SEM) displayed a roughly four-fold improvement over the parental protein mM1_034, yet showed a slightly decreased efficacy (68 ± 3%, mean ± SEM) compared to BAM (8-22) and mM1_034 (**Fig 2i**, **Supplementary Fig. 8**). These results highlight binder residues involved in potency as well as full and partial agonism.

***Reviewer*:** An important question is whether the binders exhibit any biased agonism. To address this, the authors should assess whether other G-protein or arrestin-mediated signaling pathways are also activated.

Response: We measured receptor activation by potency-optimized MRGPRX1 agonists in G_q protein dissociation and β-arrestin-2 recruitment assays (**Supplementary Fig. 9a-b**). These miniproteins show partial agonist activity (relative to BAM 8-22) in these assays and act as full agonists in the calcium mobilization assay which is associated with higher levels of signal amplification. Given that the efficacy and potency of binders relative to BAM (8-22) are roughly the same in both G protein-dissocation and β-arrestin-2 recruitment assays, we conclude that none of the binders exhibit biased agonism.

***Reviewer*:** Are the residues mentioned by the authors on line 254, with distinct conformations, known to drive activation? Could the authors specify how these conformational differences are hypothesized to contribute to the degree of agonistic activity?

Response: All of the residues listed are part of the binding pocket for the endogenous ligand. They primarily form hydrophobic interactions with BAM (8-22) residues W13, W14, and M15 of BAM 8-22 while E157$^{4.60}$ engages in a salt bridge with BAM's R20. These contacts have been reported to be key drivers of MRGPRX1 activation (Cao et al., *Nature*, 2021; Liu et al., *Nature Chemical Biology*, 2023). However, in the absence of an inactive MRGPRX1 structure, it remains speculative to associate side chain conformations with the degree of receptor activation.

***Reviewer*:** In Figure 2b, the dose response of the native agonist is missing.

Response: We incorporated a curve of the native agonist into the graph (**Fig. 2b**).

2. CXCR4

Reviewer: The authors provide the following rationale on lines 269-271: “We reasoned that interacting with the receptor at both extracellular loops and deeper within the transmembrane region would be necessary to stabilize the inactive receptor conformation and achieve a potent CXCR4 antagonist.” Why would that rationale constitute a strategy specific to antagonists?

Response: Our rationale was based on how native chemokine CXCL12 engages CXCR4, namely by making contacts with the N-terminus, extracellular loops (ECLs), and deep transmembrane binding pocket (Saotome et al., *Nature Structural & Molecular Biology*, 2025). Engagement of the ECLs alone could in principle lead to the generation of allosteric modulators, but we reasoned that for a potent antagonist it was additionally important to insert into the deep pocket to effectively block CXCR4-mediated signaling. At the same time, contacting the ECLs may enable improved receptor subtype selectivity, since these regions are less conserved (Kleist et al., *Cell*, 2025). In fact, our follow up study confirmed high selectivity of the dCX1_001 antagonist towards CXCR4 (**Fig. 3g-l**). We revised this statement accordingly (**page 13**, **line 331-334**).

***Reviewe*r:** The lack of structural characterization of a CXCR4-binder complex notably weakens the validation of the design. Could the authors elaborate on the challenges encountered during the characterization process?

Response: We attempted to determine the cryo-EM structure of the dCX1_001 antagonist bound to CXCR4, both as the minibinder alone and the minibinder with a rigid fusion to increase its mass (**Supplementary Fig. 16a-c**). Only a small fraction of well-behaved particles were observed in both cases which was insufficient for reliable 3D reconstruction **(Supplementary Fig. 17a-j**). We report these findings in the results section (**page 13**, **line 352-356**) and also describe our attempts in more detail in the method section (**page 44-45**, **line 1499-1534**).

Minor points

***Reviewe*r:** The resolution of several supplementary figures is too low for proper interpretation. Higher-quality versions should be provided.

Response: We now provide high-quality SI figures (e.g., yeast display data, pharmacological data).

***Reviewe*r:** Details of the ProteinMPNN design parameters (e.g., sampling temperature, any biasing?) are not described. These parameters can significantly influence design success and should be reported for reproducibility and evaluation of design quality.

Response: We included the sampling temperature < 0.1 of ProtteinnMPNN used for the design process (**page 24**, **line 603**).

Reviewer: In Supplementary Figure 14, it is unclear how the three GIPR binder designs were selected for SEC analysis. They are not the binders with the highest affinities in the binding kinetics pool. The authors should explain the criteria used for selecting these specific designs for downstream experiments.

Response: Since we performed functional screening of binders that exhibited the strongest binding affinity determined using SPR, we now show SEC traces of the three most potent antagonists that were identified in a functional cAMP assay (**Supplementary Fig. 27**). On the other hand, for GCGR binders we show SEC traces of binders with the strongest binding affinity determined by SPR (**Supplementary Fig. 28**).

Reviewer #3 (Remarks to the Author):

(A. Summary of the key results; B. Originality and significance)
This manuscript presents an impressive and highly innovative study on the de novo design of miniprotein modulators targeting various G protein-coupled receptors (GPCRs). The authors present novel computational strategies (including 'motif-directed' diffusion and the use of a MetaGen scaffold library with RIFDock) and a high-throughput screening platform (OPS-RD), successfully generating functional agonists (MRGPRX1) and antagonists (CXCR4, GLP1R, GIPR, GCGR, CGRPR). A particular highlight is the successful de novo design of GPCR agonists, which is notoriously challenging. The subsequent structural validation of several designed binders complexed with their targets using single-particle cryo-EM provides near atomic-resolution validation of the design accuracy and mechanism of action. This work represents a tremendous milestone in the field of protein design and GPCR drug discovery and will undoubtedly be of broad interest to the scientific community. While the study is compelling, several issues require further clarification and discussion to fully support the conclusions and to enhance the overall impact of the study.

Response: We thank the reviewer for their feedback on our manuscript.

(C-H) Integrated below into major and minor comments:
Major comments:

Reviewer: It is noteworthy that all the GPCR targets investigated in this study are peptide-binding receptors. This focus is understandable, as peptide GPCRs typically possess relatively large and accessible orthosteric binding pockets, which may facilitate de novo miniprotein binder design. However, it is important to highlight that approximately two-thirds of GPCRs are non-peptide receptors, such as serotonin receptors and adrenergic receptors, which often feature smaller or more occluded ligand-binding pockets. Demonstrating that the design pipeline described in this study can be extended to non-peptide GPCRs would significantly broaden the applicability and impact of the work. The authors are encouraged to include either preliminary results or a discussion on the potential challenges and feasibility of applying their approach to non-peptide GPCRs. Alternatively, the authors may consider revising the title to more explicitly reflect the focus on peptide-binding G protein-coupled receptors.

Response: We appreciate the opportunity to clarify the generalizability of our approach. While our *de novo* design methods are applicable to a wide range of GPCR targets, we recognize that they are best suited to GPCRs with either soluble, structured extracellular domains (ECDs) or recessed binding pockets that have peptides or proteins as endogenous ligands. For instance, receptors with large ECDs such as class B1 GPCRs comprise soluble and well-structured interfaces and for these targets we observed consistent success in identifying high-affinity, functional binders. This has now been strengthened with binders for PAC1R and PTH1R (**Fig. 1j and Fig. 4c**). We would like to clarify that GPCR targets with recessed binding pockets that recognize peptides or proteins as natural ligands tend to be more amenable to *de novo* binder design than those that bind small molecules. Peptide- and protein-binding GPCRs often comprise

larger and more accessible recessed binding pockets that can be readily targeted by designed miniproteins. In addition to CXCR4 and MRGPRX1, we now provide more examples of binders capable of targeting deep binding pockets of peptide- and protein-binding GPCRs, namely CCR5 and OXTR. We identified antagonists in the nanomolar and micromolar range targeting these receptors (**Supplementary Fig. 18-22**). In contrast, GPCRs that bind small molecules typically feature narrow, and partially occluded orthosteric pockets buried within the transmembrane domain. These structural features present challenges for *de novo* binder design, particularly when targeting orthosteric sites, since the relevant epitopes are often occluded. As a result, our current strategy is likely to identify binders for GPCRs that naturally engage large ligands, such as neuropeptides, chemokines, and protein hormones since these GPCRs have more accessible recessed pockets. Future studies will examine the potential of our *de novo* methods to develop binders against small molecule-sensing GPCRs. We have added a discussion of these points in the revised manuscript (**page 22-23**, **line 555-559**).

Reviewer: The manuscript employs different high-throughput screening strategies—OPS-RD, yeast display with nanodiscs, and yeast display with biofloating—for different GPCR targets (e.g., MRGPRX1, Class B ECDs, CXCR4). It would be helpful if the authors could clarify the rationale behind the selection of each method. Was the choice driven by specific properties of the targets, anticipated characteristics of the binders, or prior experimental experience? A brief comparison or justification of the screening strategies used for each receptor class would enhance the clarity and reproducibility of the study.

Response: As we were initially uncertain how well OPS-RD would perform for large-scale library screening, we also used established yeast display-based methods as complementary approaches. In particular, yeast display paired with soluble GPCRs stabilized in detergents or nanodiscs is a validated strategy for identifying binders (McMahon et al., *Nature Structural and Molecular Biology*, 2018). Hence, we used this method as well as a biofloating approach that utilizes receptor expression in cell systems (Krohl et al., *Cell Reports Methods*, 2023) to reliably discover miniprotein binders across multiple receptor targets (**page 4-5**, **line 176-179**).

Reviewer: To better assess the potential biases and complementarity of the screening approaches used, the intracellular colocalization-based purification-free OPS-RD system versus the affinity-based yeast display platforms, a small-scale cross-validation experiment would be highly informative. For example, evaluating a subset of key hits identified by OPS-RD using yeast display, and vice versa, could help elucidate whether the two methods tend to identify overlapping or distinct classes of binders. Such analysis would provide valuable insight into the strengths and limitations of each platform and further support the robustness of the screening pipeline, especially for the newly developed OPS-RD. A similar cross-validation experiment of the design pipelines on one or two receptor targets is also highly recommended, which would help demonstrate the generalizability and robustness of the approach.

Response: We have benchmarked the OPS-RD platform directly against yeast display by screening a library of PAC1R antagonist designs by both technologies. We conclude that the binding signal obtained by the OPS-RD is highly predictive of validated hits discovered by yeast

display. Moreover, the binding signal obtained by OPS-RD was statistically significant for >75% SPR-validated yeast display hits and <2% of the negative controls at a false discovery rate (FDR) threshold of 5% (Kolmogorov–Smirnov test p-value adjusted by Benjamini-Hochberg procedure), indicating a low false negative rate coupled to a high true negative rate of the assay. Further, to address the false positive rate of the assay, we determined the binding affinity of the top 60 hits and found 55% to display binding below 4 μM (**Fig. 1j-k**). Finally, we have provided a sentence in the discussion comparing the throughput and false positive rate of the receptor diversion assay to display technologies (**page 23, line 563-566**).

Reviewer: The MetaGen approach, which utilizes scaffolds derived from the AlphaFold-predicted metaproteome, would benefit from additional clarification regarding the composition and selection of the ~7,000 “native miniproteins” mentioned in line 164. Specifically, it would be helpful for the authors to elaborate on how these scaffolds were identified and selected. Were any clustering or filtering procedures applied, and if so, what structural or sequence-based metrics were used? Furthermore, the terms “native” and “metaproteome-derived” (Supplementary line 80) should be more clearly defined. Does “native” refer to naturally occurring sequences from known organisms, or to structures with specific stability or folding characteristics? A more detailed description would improve reproducibility and enhance the reader’s understanding of the MetaGen library’s design rationale. Moreover, metaproteome-derived miniprotein scaffold library with relevant scaffold identifiers should be included in the supplementary materials.
Response: We have now revised the statement, the metaproteome-derived scaffolds are no longer referred to as native proteins. We have also added a description in the method section on how the metaproteome miniprotein scaffold set was derived (**page 17**, **line 618-626**).

Reviewer: MRGPRX1 exhibits a relatively shallow and solvent-exposed orthosteric binding pocket compared to many other class A GPCRs. This pocket is characterized by a limited number of deep hydrophobic cavities, instead favoring interactions with flexible, charged, or amphipathic ligands. This structural feature supports its ability to recognize a wide array of peptide ligands with diverse chemical structures (doi.org/10.1038/s41467-023-40705-z). MRGPRX1 is an attractive target for developing non-histaminergic anti-itch therapies, especially for conditions such as atopic dermatitis, uremic pruritus, and drug-induced itch. However, the receptor’s broad ligand recognition profile poses challenges for achieving high specificity and avoiding off-target effects. Demonstrating that the design pipeline described in this study can be extended to de novo design of antagonist miniprotein would significantly broaden the applicability and impact of the work.

Response: No inactive structure of MRGPRX1 is published to date to guide antagonist design efforts. At screening stages, we have monitored whether any of our binders block BAM (8-22)-induced calcium mobilization without showing agonistic effects, however, none of our binders matched these criteria, from which we conclude that our design pipeline reliably generates binders selectively stabilizing the active conformational state of the template structure in the case of MRGPRX1. We agree with the reviewer that antagonist design for MRGPRX1 would be more desirable from a therapeutic perspective. However, we would like to emphasize that the present work is intended as a proof-of-concept, demonstrating that *de novo* protein design can yield

GPCR-binding agonists as well as antagonists, rather than as a therapeutic drug development effort.

Reviewer: The motif-directed RFdiffusion strategy used for the antagonist design of CXCR4 is intuitive for targeting recessed pockets. To ensure reproducibility and facilitate further research, please provide more detail on the selection or generation of the initial 5-residue motifs used for scaffolding.

Response: Computational design of protein-based GPCR ligands that protrude the deep binding pocket has been a long-standing challenge. Accordingly, we used RFdiffusion to first generate small motifs of 5 residues capable of penetrating the receptor pocket and then scaffold miniproteins of 65-75 residues onto these motifs. Out of approximately 1,000 small motifs that we generated based on hot spots which were defined within the binding pocket of the receptor, we selected 50 motifs based on their distance to these buried hot spots, *i.e*., had deepest insertion. These small motifs were not selected based on their selectivity. We described the small motif generation in methods in more detail (**page 25**, **line 652-657**).

Reviewer: The structures of CXCR4 in complex with the newly developed antagonists are highly recommended as the CXCR4 binders are the only binders designed in this study possessing beta-strand. Structural comparison between the predicted and experimental structures could evaluate the designability of the beta-strand containing binder of GPCRs.

Response: We attempted to determine the cryo-EM structure of the dCX1_001 antagonist bound to CXCR4, both as the minibinder alone and the minibinder with a rigid fusion to increase its mass (**Supplementary Fig. 16a-c**). Only a small fraction of well-behaved particles were observed in both cases which was insufficient for reliable 3D reconstruction **(Supplementary Fig. 17a-j**). We report these findings in the results section (**page 13**, **line 352-356**) and also describe our attempts in more detail in the method section (**page 44-45**, **line 1499-1534**).

Reviewer: The study utilizes various computational pipelines (RFdiffusion variations, MetaGen/RIFDock) and filtering criteria (differing thresholds for pAE_interaction, pLDDT_binder, ddG, sap, etc.) across targets. Please provide a clearer justification for these specific choices. Are the variations driven by target-specific structural features, the intended pharmacological outcome (agonist/antagonist), binding site location (ECD/TM), or preliminary results? Explaining the underlying principles or empirical basis for these decisions is essential for transparency and reproducibility.

Response: *In silico* cutoffs goals were defined *a priori* from our previous benchmarking of AF2 and Rosetta metrics across multiple targets (Bennett et al., *Nature Communications*, 2023). Where feasible, we targeted pLDDT_binder > 90, pAE_interaction < 8, binder_RMSD < 2, and low SAP (≤ 35) to reduce aggregation risk while maintaining confident local structure. We treated pAE_interaction and pLDDT_binder as primary acceptance filters, then set Rosetta ddG and SAP to filter remaining candidates. When very few designs met these strict criteria, thresholds were relaxed within the target specific ranges reported above, guided by interface chemistry. Targets

that can be engaged *via* larger hydrophobic contact patches generally support more stringent Rosetta ddG cutoffs. We have added this explanation in methods to clarify the rationale for choosing AF2 and Rosetta cutoff values (**page 24**, **line 604-611**).

Minor Comments:

Reviewer: Have the authors considered retrospectively analyzing the success rates of the computational design phase versus the experimental screening phase for the different targets? Such analysis could provide valuable insights for optimizing future computational design efforts, particularly for challenging targets like GPCRs.

Response: We thank the reviewer for this thoughtful point. The overall hit rates in our screens are modest for several targets. We agree that success in *de novo* binder discovery is highly epitope dependent, particularly for class A GPCRs for which extracellular loops are flexible, epitope curvature is extreme, and chemistry often quite hydrophilic. To our knowledge, there are no peer-reviewed reports of *de novo* binders to class A GPCRs that enable a direct epitope-matched comparison. To address the reviewer's request for a retrospective assessment of computational filters, we have added an analysis that compares successful binding hits (identified with yeast display) against non-binders across the exact design sets tested (**Supplementary Fig. 49**). We analyzed filter values for RFdiffusion and MetaGen designs separately because RFdiffusion relies on iterative partial diffusion that optimizes *in silico* metric in order to pass defined filter values whereas MetaGen approach does not. We evaluated AlphaFold-Multimer PAE at the interface and pLDDT of the binder, as well as Rosetta interface ΔΔG, contact molecular surface (CMS), and surface aggregation potential (SAP). We computed Receiver Operating Characteristic (ROC) curves and corresponding Area Under the ROC Curve (AUROC) values for each computational metric. These curves summarize how the true positive rate (fraction of binders correctly classified) changes as the metric threshold is varied, against the false positive rate (fraction of non-binders incorrectly classified). Our results show that certain metrics show mild enrichment of binders toward favorable score ranges, with some individual filters showing stronger discrimination depending on the target and design method. However, this post-hoc analysis is conducted on libraries that have already been filtered using the analyzed metrics. Therefore, in this case, the AUCs describe only how well the metric further ranks designs within the post-filter window, not its overall ability to separate binders from non-binders in the full design space. Overall, we would like to clarify that success rates should be compared on a per-epitope basis. We included a description of the analysis in the results section (**page 22**, **533-540**).

Reviewer: Please consider enhancing the clarity and presentation quality of the supplementary figures, particularly the 27 figures provided. It is important to ensure that each figure is clearly labeled, referenced appropriately in the main text, and accompanied by sufficiently detailed legends or descriptions to facilitate interpretation.

Response: We improved the quality of our SI figures (e.g. yeast display, pharmacological data) and referenced appropriately throughout the manuscript.

Reviewer: While the manuscript reports the successful generation of several functional agonists (e.g., for MRGPRX1) and antagonists (e.g., for CXCR4, GLP1R, GIPR, GCGR, CGRPR), the sequences of these effective binders—beyond those structurally characterized—are not provided. To ensure reproducibility and facilitate further research, functional binder sequences should be included in the supplementary materials.

Response: We have included sequences of functional binders that were pharmacologically characterized in more detail (**Supplementary Table 12**).

Point by point response to reviewers' comments (second-round revision)

Title: *De novo* design of miniprotein agonists and antagonists targeting G protein-coupled receptors

Authors: Edin Muratspahić, David Feldman, David E. Kim, Xiangli Qu, Ana-Maria Bratovianu, Paula Rivera-Sánchez, Jan Hendrik Voss, Emil P. T. Hertz, Mads Jeppesen, Federica Dimitri, Kensuke Sakamoto, Amrita Nallathambi, Pia Peceli, Jianjun Cao, Brian P. Cary, Matthew J. Belousoff, Peter Keov, Phuc N.H. Trinh, Qingchao Chen, Yue Ren, Justyn Fine, Sudha Mishra, Annu Dalal, Shachie Sinha, Ramanuj Banerjee, Manisankar Ganguly, Karthik Varappalayam Karuppusamy, Isaac Sappington, Thomas Schlichthaerle, Jason Z. Zhang, Arvind Pillai, Brian Coventry, Ljubica Mihaljević, Magnus Bauer, Susana Vázquez Torres, Amir Motmaen, Gyu Rie Lee, Long Tran, Xinru Wang, Inna Goreshnik, Dionne K. Vafeados, Justin E. Svendsen, Parisa Hosseinzadeh, Nicolai Lindegaard, Matthäus Brandt, Yann Waltenspühl, Kristine Deibler, Lukas Deweid, Anja Bennett, Jendrik Schöppe, Tiantang Dong, Xiaoli Yan, Luke Oostdyk, William Cao, Lakshmi Anantharaman, Johan J. Weisser, Jesper Frank Bastlund, Christoffer Bundgaard, Ayodeji A. Asuni, Justin G. English, Lance Stewart, Lauren Halloran, Jamie B. Spangler, André Lieber, Arun K. Shukla, Patrick M. Sexton, Bryan L. Roth, Brian E. Krumm, Denise Wootten, Christopher G. Tate, Christoffer Norn, David Baker

Referees' comments:

Referee #1 (Remarks to the Author):

In this revision, I appreciate the ability to address selectivity of the mini-proteins and the functional analysis. However, I am not convinced that many of the issues have been fully addressed. MRGPRX1: the new binders included in the revision appear to be more potent in the calcium flux assay, but in the BRET studies they appear to be very weak both in potency and efficacy in these assays. The authors should address these discrepancies between functional assays by performing more in depth analysis using orthogonal measures. Why is the activity so weak in these orthogonal assays and what does the calcium flux really represent considering it is an amplified response subject to receptor reserve?

Response: We thank the reviewer for this important point. Indeed, calcium mobilization as well as other second-messenger quantification assays such as IP1 or cAMP are a highly amplified readout and strongly influenced by receptor reserve; they often overestimate both potency and efficacy for weak or low-efficacy agonists. They, however, are well-established methods to screen for agonists and antagonists in a medium-throughput manner. As such, we used calcium mobilization for identifying agonists of the G_q protein-coupled MRGPRX1. In contrast, BRET assays probe unamplified, proximal signaling events and therefore provide a more accurate measure of potency and intrinsic efficacy. The weaker potency and efficacy observed in BRET assays for miniproteins do not contradict the calcium results; instead, they reflect expected pharmacology for agonists at G_q protein-coupled coupled receptors such as MRGPRX1. These findings are in line with previous studies reporting similar pharmacological profiles of agonists at other GPCRs (Olsen et al., *Nature Chemical Biology*, 2020; Gillis et al., *Science Signaling*, 2020, Muratspahić et al., *Nature Communications*, 2023). We revised the manuscript and added *"TRUPATH bioluminescence resonance energy transfer (BRET)-based G-protein dissociation (Olsen et al., Nature Chemical Biology, 2020) and β-arrestin-2 recruitment assays (DiBerto et al., Methods in Molecular Biology, 2022) have been developed as an alternative to second messenger assays to minimize signal overamplification. Using these assays, we found that the two variants had potencies in the low micromolar range with reduced efficacies relative to BAM (8-22) (**Supplementary Fig. 13a-c; see legend for discussion**). Taken together, the TRUPATH and β-arrestin-2 recruitment assays suggest that our designs are partial rather than full agonists in less amplified signaling assays."* in the results section (**page 7**, **line 281-287**) as well as included a short data discussion in the legend of the **Supplementary Fig. 13a-c**. Although additional orthogonal assays would be informative, we believe the existing data, combined with well-established principles of GPCR signaling, sufficiently explain the differences.

Reviewer: With the more potent binders (in the calcium flux assay anyway), what does the structure with weaker compound mM1_068 really represent? It seems there is a missed opportunity to use the cryo-EM structure to help guide the design of the more potent analogs.

Instead, the authors just did a screen to refine. What does the cryo-EM structure even tell us about any of these new compound binding modes?

Response: We used the miniprotein–receptor structures and models of the top three initial hits, as templates for computational site-saturation mutagenesis, calculating ΔΔG values for all single, double, and triple mutants of the three miniproteins in complex with MRGPRX. We described this in the section methods under targeted mutagenesis (**page 52**, **line 1836-1847**). Mutations predicted to stabilize the interface were prioritized, resulting in a focused set of 239 designed variants of mM1_034, mM1_060, and mM1_068. From these, 21 compounds activated the receptor to >75% relative to 1 μM BAM (8–22), and 58 additional variants showed intermediate activity (25–75%) at 500 nM (**Supplementary Fig. 11**). Subsequent concentration–response curve analyses identified two mM1_034 derivatives with significantly improved potency over their parent compound. The high fraction of active, structure-guided variants supports the accuracy of the resolved and modeled receptor–miniprotein binding poses. Thus, the cryo-EM structures and models were not merely descriptive, but provided a mechanistic framework for rational interface optimization and the successful generation of more potent second-generation miniproteins. We have revised the main text to describe how the structural information guided this optimization strategy (**page 7**, **line 270-280**).

Reviewer: Finally, there was not much added to the requested mutagenesis analysis to validate the structures. Only mutants were examined at CGRPR but at none of the other structures, including the above-mentioned more potent MRGPRX1 binders. I find the validation of structural poses extremely lacking given the discrepancies in the pharmacology.

Response: Beyond receptor mutagenesis for the CGRPR-targeting miniprotein antagonists we now additionally provide receptor mutagenesis of CXCR4 for the miniprotein antagonist dCX1_001 (**Supplementary Fig. 33-34**), which confirms the designed binding mode (please see our response to Reviewer #2 below for a detailed description). We prioritized structural validation and cryo-EM determination for CXCR4 antagonist dCX1_001 over additional receptor mutagenesis for MRGPRX1, since the agreement between the computational model and the cryo-EM structure of MRGPRX1 agonists (Cα RMSD about 0.7 Å) already robustly supports their proposed binding modes.

Referee #2 (Remarks to the Author):

I am satisfied with the authors' responses to several major points, which have substantially strengthened the manuscript:

1. Selectivity: The addition of comprehensive selectivity panels for GIPR, CXCR4, and MRGPRX1 binders (addressing R1, R2) is a major improvement.

2. Potency: The new optimization data for the MRGPRX1 agonist, which now shows low-nanomolar potency comparable to the endogenous ligand (addressing R2), effectively resolves a key limitation of the initial version.

Response: We thank the reviewer for their positive feedback on the improvements to our manuscript. In addition to selectivity of GIPR, CXCR4 and MRGRPX1 binders we now also include selectivity data for CCR5 binders (**Supplementary Fig. 27a-c**). Furthermore, we performed detailed pharmacological characterization of two antagonists and one agonist of CCR5 miniproteins by measuring their signaling across different assays (cAMP, G_{oA} dissociation, β-arrestin-1 and β-arrestin-2 recruitment assays) (**Supplementary Fig. 25-26**).

Despite these significant advances, two issues remain that should be addressed before publication.

Remaining Concerns

1. Overstated Claims of "Conformational Selection"

The claim that agonists and antagonists were designed through "precise conformational control" remains insufficiently supported.

The authors acknowledge in their rebuttal that "the results are not sufficient yet to claim that structural state alone guarantees a defined functional outcome." This is appropriate, but the manuscript text does not yet reflect this clarification.

1.1. The Abstract still claims "confirming precise conformational control of receptor function."
1.2. The Discussion (lines 569–573) continues to imply that conformationally selective design has been definitively achieved: "The ability to computationally design conformationally selective binders is a step change in methodology for obtaining functional biologics targeting integral membrane receptors."

These sections should be revised to better align with the evidence and the authors' own rebuttal. The results are highly promising but do not yet establish a generalizable principle of de novo conformational selection.

Response: We further toned down our claims as suggested by the reviewer and revised the Abstract and Discussion accordingly. In the Abstract and Discussion, we removed "*confirming precise conformational control of receptor function"* and *"conformationally selective"*, respectively. We have now also not only identified one additional CCR5 agonist but also designed 20 agonists targeting a new receptor, NK1R (please see our response to Reviewer #3 below for a detailed description).

2. Insufficient Validation for the CXCR4 Binder

Reviewer: The authors report that cryo-EM studies failed due to particle aggregation. Given the importance of this binder, additional empirical validation is essential. While mutagenesis data were added for CGRPR binders (already structurally validated), similar experiments should be performed for dCX1_001 and CXCR4. Mutating residues at the predicted interface and demonstrating reduced binding or antagonism would provide necessary orthogonal support for the model.

Response: In the first-round of revision we attempted to determine the structure of dCX1_001 bound to monomeric or dimeric forms of CXCR4 (**Supplementary Fig. 30a-c**), but only a small fraction of well-behaved particles were observed in both cases which were insufficient for reliable 3D reconstruction (**Supplementary Fig. 31a-j**). Instead, in this round of the revision we were able to determine the cryo-EM structure of dCX1_001 bound to homotrimeric CXCR4 at a resolution of 3.29 Å (**Supplementary Fig. 32a-d**, **Supplementary Table 9**). dCX1_001 adopts a complex α/β fold with two helices packing against three β-strands, closely matching the design model (0.6 Å Cα RMSD) (**Fig. 3i-l**). The dCX1_001-CXCR4 complex closely matches the RFdiffusion model, reproducing the overall binding mode and the key hydrophobic contacts between the β-sheet and ECL2 (**Fig. 3k**). The receptor adopts a conformation similar to many other CXCR4 structures, where helices 6 and 7 shift outward relative to the target reference structure 4RWS (1.8 Å Cα RMSD over the extracellular half). Consistent with this receptor geometry, the miniprotein preserves the designed hydrophobic anchor but pivots modestly around the β-sheet, yielding a ligand Cα RMSD of 3.9 Å in the target-aligned frame.

To complement the cryo-EM structure, the antagonist activity of dCX1_001 was measured in a $G_{\alpha i}$ dissociation assay on cells expressing the wild-type receptor or receptors with alanine mutations of key residues in CXCR4 at the targeted interface. Mutation of the $E179A^{ECL2}$, $Y190A^{ECL2}$ and $E268A^{6.64}$ receptor residues significantly decreased binder activity (**Supplementary Fig. 33a-e**, **Supplementary Fig. 34a-b**). We also mutated key dCX1_001 residues interacting with the receptor binding pocket and measured their antagonistic activity in a cAMP assay (**Supplementary Fig. 35a**). Mutating these residues abolished their inhibitory activity further supporting the proposed binding mode of the dCX1_001 miniprotein (**Supplementary Fig. 35b**). In conclusion, we now provide not only receptor and miniprotein mutagenesis to confirm the binding mode of the miniprotein dCX1_001 but also its cryo-EM structure bound to homotrimeric CXCR4.

To complement *in vitro* pharmacology and cryo-EM structure of dCX1_001, we additionally conducted an *in vivo* study which has been requested by the reviewer #1 in the first-round revision. The CXCR4-CXCL12 axis plays a pivotal role in regulating the retention and migration of hematopoietic stem and progenitor cells (HSPCs) within the bone marrow niche. Inhibiting this interaction facilitates mobilization of HSPCs into the peripheral blood for collection and autologous transplantation, a clinically established approach for the treatment of hematological malignancies. We demonstrate that the CXCR4-targeting miniprotein dCX1_001 achieves hematopoietic stem cell mobilization comparable to small molecule AMD3100 (plerixafor) therapeutic while producing less pronounced leukocytosis and reduced peripheral vector transduction, without evidence of hematological toxicity (**Fig. 5, Supplementary Fig. 63-65**). These findings complement the pharmacokinetic study of the CGRPR miniprotein antagonist mC2_022 presented in the first-round revision which demonstrates that miniproteins can not only be paired with an established, clinically validated half-life extension technology to achieve drug-like properties *in vivo*, but also exert *in vivo* efficacy in a mouse model of clinically relevant hematopoietic stem cell mobilization.

Overall, the manuscript has improved substantially, and I would be happy to recommend publication once these final revisions are made.

Response: We thank the reviewer for their positive feedback on the improvements to our manuscript.

Referee #3 (Remarks to the Author):

The authors have substantially improved the manuscript in response to the first-round comments. The expanded methodological descriptions, additional pharmacological assays, new benchmarking of OPS-RD, and clearer presentation of the design pipeline significantly strengthen the work. The newly added mutagenesis and functional assays also improve confidence in several of the designed ligands. The study continues to address an important challenge in GPCR-targeted ligand design, and the presented results are promising. However, several key concerns raised in the first round remain only partially resolved. These issues primarily relate to the generalizability of the approach and the interpretation of screening performance. Addressing these points would greatly enhance the impact of manuscript and utility for the GPCR community.

Major issues:

MetaGen vs. RFdiffusion comparison remains incomplete. While hit-rate differences are now provided, reviewers previously requested structural diversity metrics (e.g., RMSD clustering, interface topology distribution). Without such quantitative analysis, it remains unclear whether MetaGen truly expands the design space or mainly introduces redundancy.

Response: We agree that the hit-rate differences alone are insufficient to assess whether MetaGen expands structural diversity or introduces redundancy. To address this, we expanded **Supplementary Fig. 3**, which summarizes interface topology distributions across all targets, to include the full design sets generated by both methods. In addition, we added two new quantitative structural diversity analyses: **Supplementary Fig. 4**, which evaluates interface secondary-structure topology coverage using binned ternary space, and **Supplementary Fig. 5**, which reports Foldseek-based structural clustering across a range of TM-score thresholds to quantify method-specific versus shared structural clusters. Together, these analyses show that MetaGen and RFdiffusion sample partially overlapping but distinct regions of structural space and interface topology, indicating that the two methods are complementary—both can generate functional GPCR ligands, and depending on the target they enrich for different backbone topologies, with each method contributing distinct validated binders rather than redundant designs. We also added a corresponding statement in the main text referencing these analyses (**Supplementary Fig. 3-6**): "*Across all class A and class B targets, RFdiffusion and MetaGen sampled partially overlapping but distinct structural states and topologies, yielding complementary hit enrichment with each method contributing distinct experimentally validated binders (**Supplementary Fig. 3–6**).*" During these analyses, we noted that RFdiffusion designs were created against rabbit GLP1R whereas all other binders were generated against human receptors. For consistency, we therefore moved the functional RFdiffusion GLP1R antagonist to Supplementary Data. We also consolidated the class B receptor antagonists into a single figure (**Fig. 4**) following the addition of new *in vivo* data for CXCR4 antagonist dCX1_001.

Reviewer: Several reviewers explicitly recommended the authors demonstrate the ability to design both agonists and antagonists for the same receptor, to validate the generality of the de novo design pipeline. The authors provided a clear explanation about the lack of an inactive-state structure for MRGPRX1, which limits antagonist design for that specific receptor. This rationale is

reasonable. Since active and inactive structures are available for CXCR4, CCR5, and OXTR, and only antagonists were generated for these receptors, it remains unclear whether the agonist-design component of the pipeline can be applied beyond MRGPRX1. To address this concern, the authors could either provide at least one additional agonist example for a receptor with a known active-state structure or explicitly clarify methodological or biological reasons why agonist design is more challenging for these receptors and soften claims of cross-receptor generality.

Response: We agree with the reviewer that providing agonists for an additional receptor would further support the generalizability of our computational approaches to design agonists. Hence, we designed miniproteins against the active state of NK1R using RFdiffusion (N=8,328) and MetaGen (N=16,675) and probed yeast display libraries using detergent-stabilized NK1R (**Supplementary Fig. 14a-g**). Of 71 RFdiffusion hits identified by yeast display, 63 were successfully expressed while for MetaGen, 22 hits were identified and 20 of them were successfully expressed (**Supplementary Fig. 15**). In a functional cAMP assay, 20 RFdiffusion designs acted as agonists with EC_{50} values ranging from 1 nM to 231 nM and E_{max} values from 24% to 91% (**Fig 2k**, **Supplementary Fig. 16a**, **Supplementary Table 5**). The RFdiffusion-designed agonists span 11 diverse helical topologies (**Fig 2l**, **Supplementary Fig. 16b**), and occupy the orthosteric binding pocket of NK1R, engaging with a loop motif structurally similar to the endogenous agonist substance P (**Fig. 2l**), No MetaGen hits were active or recapitulated the structural motif.

In addition to NK1R agonists, we performed a careful re-evaluation and detailed pharmacological characterization of the CCR5 functional hits identifying one CCR5 agonist alongside two antagonists. The miniprotein dCC1_002 acted as an agonist in the cAMP assay with EC_{50} and E_{max} values of 814 ± 257 nM (mean ± SEM (n=6)) and 101 ± 2 % (mean ± SEM (n=6)), respectively, and in the $G_{\alpha oA}$ protein dissociation assay with an EC_{50} of 561 ± 161 nM (mean ± SEM (n=3)) (**Supplementary Fig. 26a-c**, **Supplementary Table 8**). In the NanoBiT-based assay, dCC1_002 partially recruited β-arrestin-1 and β-arrestin-2 in the low micromolar range (**Supplementary Fig. 26d-e**). The dCC1_002 agonist displayed selectivity for CCR5 over CXCR4 in the cAMP assay (**Supplementary Fig. 27c**). In conclusion, with multiple NK1R agonists, a CCR5 agonist and a few MRGPRX1 agonists, we are confident that our computational approaches can be used to design and develop GPCR agonists.

Reviewer: As all successful designs target peptide/protein-binding GPCRs with accessible binding pockets, the current pipeline does not yet demonstrate applicability to small-molecule GPCRs with occluded orthosteric sites. In the response, the authors state that “GPCRs that bind small molecules typically feature narrow, and partially occluded orthosteric pockets… These structural features present challenges for de novo binder design” and suggest that such conformations limit accessibility for designed miniproteins. While this explanation is reasonable, it simultaneously implies that the authors attempted such designs and found them unsuccessful, thereby acknowledging that the current approach does not generalize to small-molecule-binding GPCRs. If so, this important limitation should be more explicitly articulated in the manuscript. Several GPCRs with small-molecule orthosteric ligands nonetheless possess well-characterized extracellular-accessible entry pathways or partially exposed pocket regions that have been successfully targeted by peptides, nanobodies, or engineered proteins in previous studies. It is

therefore not entirely clear why the present design strategy cannot be adapted to generate binders for these receptors.

Response: We thank the reviewer for picking up on this fact, we have not adequately articulated the complications with this approach for our method. We have created antagonists and agonists for 11 class A and class B GPCR targets in this study, demonstrating the robustness of our computational design pipelines to create functional miniproteins. Initial computational design campaigns were attempted against aminergic class A GPCR family members but were not prioritized for screening due to their narrow and partially occluded orthosteric pockets which made high scoring miniprotein development for synthesis selection and screening uncertain for the initial demonstration of this technology. Strictly from a computational design standpoint, the majority of aminergic receptors lacked sufficient vestibule access for us to prioritize these in our proof-of-concept for the method. Many of the existing peptides to these targets such as MT7 toxin at M1 receptor for example (Maeda et al., *Science*, 2020) are allosteric modulators of the endogenous ligand activity. Here, we proceeded only with antagonist and agonist screening modes to demonstrate our ability to directly access, engage, and compete with the orthosteric pocket of the receptor. You are correct that we should further emphasize this difficulty in the manuscript, and have now done so to make our target selection choice clear: *"We focused here on peptide- and protein-binding GPCRs that comprise more open and shallow orthosteric binding pockets as proof-of-concept for the de novo protein design in engineering GPCR agonists and antagonists. Given the success rates we observed for these receptors future work should examine the potential of our methods to design miniprotein agonists, antagonists or allosteric modulators against GPCRs with small molecules as endogenous ligands–aminergic GPCRs are more challenging targets as they typically have narrow and partially occluded orthosteric pockets buried within the transmembrane domain"* **(page 26**, **718-725**).

Reviewer: The authors now provide a careful analysis comparing OPS-RD with yeast display, which is very helpful. The revised results indicate that among the top 60 OPS-RD hits, approximately 55% exhibit binding <4 μM. While this demonstrates that the platform is capable of identifying true positives, the remaining false-positive fraction raises questions about how best to use OPS-RD as part of an integrated screening pipeline. This point does not undermine the utility of OPS-RD, but it does highlight the need for further clarification. A short discussion addressing the following would be constructive, including potential biological or technical factors contributing to false positives and whether tuning the OPS-RD thresholds (expression, RD signal, p-value) could mitigate this issue.

Response: We agree that the observed false-positive fraction among the top OPS-RD-ranked designs motivates clearer guidance on how OPS-RD should be used in an integrated pipeline. To examine the effect of tuning the relevant OPS-RD thresholds (corr(GFP,RFP) and p-value) we performed a precision-recall analysis (**Supplementary Fig. 8**). We find that the true positive rate can be improved substantially by tightening the BH-adjusted p-value threshold or using a higher corr(GFP,RFP) cutoff. Decreasing the BH-adjusted p-value threshold from 0.05 to 0.014 (chosen to maximize the F1 score) increases precision from 55% to 70% (from 33/60 to 30/43 designs confirmed by SPR <4 μM), while retaining 91% of all confirmed binders (30/33). Applying a more stringent corr(GFP,RFP) threshold in combination with a tighter p-value threshold can further

reduce false positives (for example, designs with corr(GFP,RFP) ≥ 0.502, the 50th percentile, and BH-adjusted p ≤ 0.001 yields 93% precision, 14/15 confirmed), with the expected tradeoff of reduced recall (14/33 designs). We now include a short discussion in the main text describing this operating-point tradeoff and emphasizing that OPS-RD is most naturally used as a tunable high-throughput enrichment step upstream of biophysical validation, where thresholds can be selected based on assay capacity and the relative cost of false positives versus missed binders.

Although most, if not all (**Supplementary Fig. 8**), false positives can be eliminated with sufficiently strictly filtering, we acknowledge that several biological and technical factors could result in OPS-RD “false positives”: (i) expression and trafficking heterogeneity, where high expression or ER retention effects can inflate apparent colocalization without productive binding at the cell surface, (ii) avidity, aggregation, or indirect retention mechanisms that yield a strong intracellular diversion phenotype but weaker monovalent affinity by SPR, (iii) differences between the intracellular OPS-RD context and purified-protein SPR conditions, including receptor construct differences, detergents, or nanodisc context, and (iv) measurement noise.

Point by point response to reviewers' comments (third-round revision)

Title: *De novo* design of miniproteins targeting G protein-coupled receptors

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Referee #1 (Remarks to the Author):

The authors have addressed all of my critiques. I look forward to this work being published.

Response: We thank the reviewer for their positive assessment of our manuscript.

Referee #2 (Remarks to the Author):

The remaining concerns from the previous round have been adequately addressed. However, the newly included data on NK1R and CCR5 agonists, as well as the CXCR4 binder cryo-EM structure, raise new concerns that should be addressed before publication.

Response: We thank the reviewer for their positive assessment of our manuscript. Below are our responses to their comments.

1. NK1R and CCR5 agonists

Reviewer: The re-evaluation of CCR5 functional hits in this revision identifies dCC1_002 as an agonist, whereas it was classified differently in the previous round. This is a significant finding that directly contradicts the authors' central mechanistic argument — namely, that targeting active-state receptor structures generates agonists while targeting inactive-state structures generates antagonists. dCC1_002 was designed using the inactive-state CCR5 structure (PDB: 5UIW, as stated in the Methods), yet it produces full agonism in cAMP and G-protein dissociation assays (EC50 ~814 nM and ~561 nM, respectively). This unexpected outcome requires explicit acknowledgment and discussion. Specifically, the authors should address whether dCC1_002 engages contacts that overlap with the native binding site, which could mechanistically explain its agonistic behavior despite being designed against the inactive state. More broadly, the authors should clearly state in the Discussion that their design strategy does not guarantee a defined functional outcome (agonism vs. antagonism) based solely on the choice of receptor conformational state, and that the relationship between design state and functional outcome remains incompletely understood. This is not a minor point — it bears directly on the conceptual framework of the entire paper.

Response: We thank the reviewer for this important point. We compared the dCC1_002 design model to CCR5 structures bound to agonist RANTES and antagonist 5P7-CCL5 (**SI Fig. 29a (inset and figure legend description)**. As shown, dCC1_002 engages receptor residues within the canonical binding pocket that are also contacted by agonists (e.g., RANTES, MIP-1α) as well as the antagonist 5P7-CCL5. These residues are functionally important, as their mutation reduces potency and efficacy of native chemokines, and likely contribute to the agonistic activity of dCC1_002.

The structural comparison highlights that active- and inactive-state for CCR5 structures are highly similar at the binding site, with largely overlapping residue positions. Thus, while dCC1_002 was designed against an inactive-state template, it can still engage key agonist-associated contacts, suggesting that functional outcome depends on subtle differences in receptor geometry, that in this case, was beyond our design precision.

We now made a note in the discussion as requested: *"While these findings establish de novo design as a viable strategy for engineering GPCR-targeting miniprotein antagonists and agonists, we note that using active and inactive receptor states alone is not sufficient to ensure design of an agonist or antagonist due to the subtle differences between states and the complexities of*

GPCR signaling dynamics; indeed some of our designs may act as allosteric modulators." (**page 26**, **line 965-969**).

Reviewer: Regarding the NK1R agonists: of 63 successfully expressed RFdiffusion hits, 20 were confirmed as agonists in the cAMP assay. The manuscript does not report whether the remaining 43 expressed hits were tested in an antagonist mode — i.e., whether any can block substance P-induced signaling. Given the authors' own argument that the receptor conformational state used for design should predict functional outcome, it is notable that not all hits designed against the active state produced agonists. Were the remaining hits inactive, weak binders, or potential antagonists? This should be explicitly reported. If any of the non-agonist hits exhibit antagonistic behavior, this would further challenge the conformational selection hypothesis and should be discussed accordingly.

Response: For the NK1R, we have also tested designs in the antagonist mode. Of the 43 designs that did not show agonism, four of them had weak antagonistic activity at 1 µM (**Supplementary Fig. 19a-b**) while the remaining designs were inactive.

We agree and acknowledge that CCR5 and NK1R agonists/antagonists demonstrate that structural state alone does not guarantee a desired functional outcome of a ligand which confirms reviewer's and our hypothesis. We have added this statement in the Discussion section as indicated above.

2. CXCR4 binder cryo-EM structure

Reviewer: The authors state (line 462) that "the dCX1_001-CXCR4 complex closely matches the RFdiffusion model," but this claim is not well supported by the structural data as presented. Several specific concerns arise:

First, the RFdiffusion design model appears to include only the extracellular half of CXCR4, presumably because the target structure was truncated to the binding epitope region during design. However, this truncation strategy is not described in the Methods section and should be explicitly stated, as it is directly relevant to evaluating the accuracy of the design model.

Response: Reviewer is correct in that we truncated receptors by keeping their extracellular binding pockets. We have already added that sentence in the Methods section: "*All target structures were truncated to the region containing the binding epitope"* (**page 27**, **line 1036**).

Reviewer: Second, Fig. 3k shows that transmembrane helices 6 and 7 adopt a substantially more open conformation in the experimentally determined structure compared to the design model. The authors attribute this to a conformation "similar to many other CXCR4 structures," but do not address whether this receptor geometry was anticipated during design or represents an unanticipated conformational selection by the binder.

Response: The altered receptor geometry was not anticipated during design, but it is not surprising considering that many other experimental CXCR4 structures adopt this conformation.

We acknowledge this deviation explicitly in the manuscript: “The receptor adopts a conformation that was not anticipated during design, but is similar to other CXCR4 structures, in which helices 6 and 7 shift outward relative to the reference structure.” (**page 17**, **line 636-637**).

Reviewer: Third, and most critically, the pocket-penetrating loop of dCX1_001 — the element designed to make deep contacts within the orthosteric site — adopts a conformation that diverges significantly between the design model and the solved structure. The authors report a ligand Cα-RMSD of 3.9 Å in the receptor-aligned frame, attributing this to the miniprotein "pivoting modestly around the β-sheet." A 3.9 Å RMSD is not modest for a miniprotein of this size and represents a meaningful deviation from design intent. The authors should explicitly assess whether the key contacts in design model are actually maintained in the solved structure. If the deep orthosteric contacts are not preserved, the characterization of dCX1_001 as a "receptor-penetrating" antagonist requires reassessment.

Response: We agree that dCX1_001 deviates more from the design model than the other solved complexes, and we have revised the text accordingly. We have removed the statement that the motion is "modest," and we no longer describe dCX1_001 as a receptor-penetrating antagonist. To address the reviewer’s central point directly, we added a distance-difference heatmap comparing the design model and solved structure (**Supplementary Fig. 36**). This shows that, although the pocket-inserting loop is shifted, many intended orthosteric contacts remain close to their designed geometry: of 77 comparable design contacts, 46 deviate by less than 2 Å and 55 by less than 3 Å, including retained mapped interactions such as R40-D97$^{2.63}$ and Y39-D262$^{6.58}$. Within the loop-centered contact set itself, 30 of 38 contacts deviate by less than 2 Å and 35 of 38 by less than 3 Å. We therefore describe dCX1_001 as an antagonist whose pocket-inserting loop adopts a shifted, but still substantially contact-preserving, binding mode.

Reviewer: Finally, the cryo-EM structure was determined on homotrimeric CXCR4, while the binder was designed against and pharmacologically characterized on monomeric CXCR4. The authors do not address whether trimeric assembly alters the binding site geometry, or whether the observed receptor conformation in the trimer context is relevant to the pharmacological activity measured in cell-based assays. This disconnect between the structural and pharmacological contexts should be explicitly acknowledged.

Response: We agree that the structural and pharmacological contexts are not identical. Because monomeric and dimeric CXCR4 complexes did not yield sufficient particles for reconstruction, the available cryo-EM structure was obtained with homotrimeric CXCR4. We therefore interpret this structure primarily as support for the overall binding mode and interface, rather than as definitive evidence that the trimeric assembly is the pharmacologically relevant state in cells. We have clarified this in the manuscript. *“Although the homotrimeric structure supports the overall binding mode of dCX1_001, its relevance to pharmacological activity remains unclear.” (***page 17**, **line 641-642***).*

Referee #3 (Remarks to the Author):

The authors have substantially improved the manuscript in response to the previous comments. The addition of quantitative analyses of structural diversity provides a more convincing comparison between MetaGen and RFdiffusion, supporting their complementarity. The approach's application to NK1R, along with the identification of several functional agonists, enhances the generalizability of the design strategy.

The revised manuscript is clearer, and the additional experimental validation increases confidence in the reported binding modes and functional interpretations. While demonstrating bidirectional modulation on the same receptor would be an intriguing future direction, the current data sufficiently support the main conclusions.

Overall, this is a strong and timely contribution to GPCR-targeted protein design, and I have no further major concerns. I recommend publication in its current form.

Response: We thank the reviewer for their positive assessment of our manuscript.