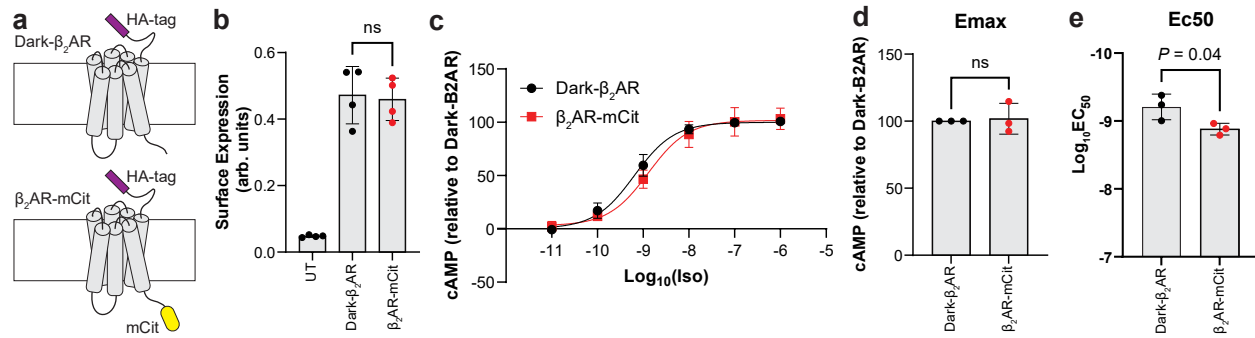
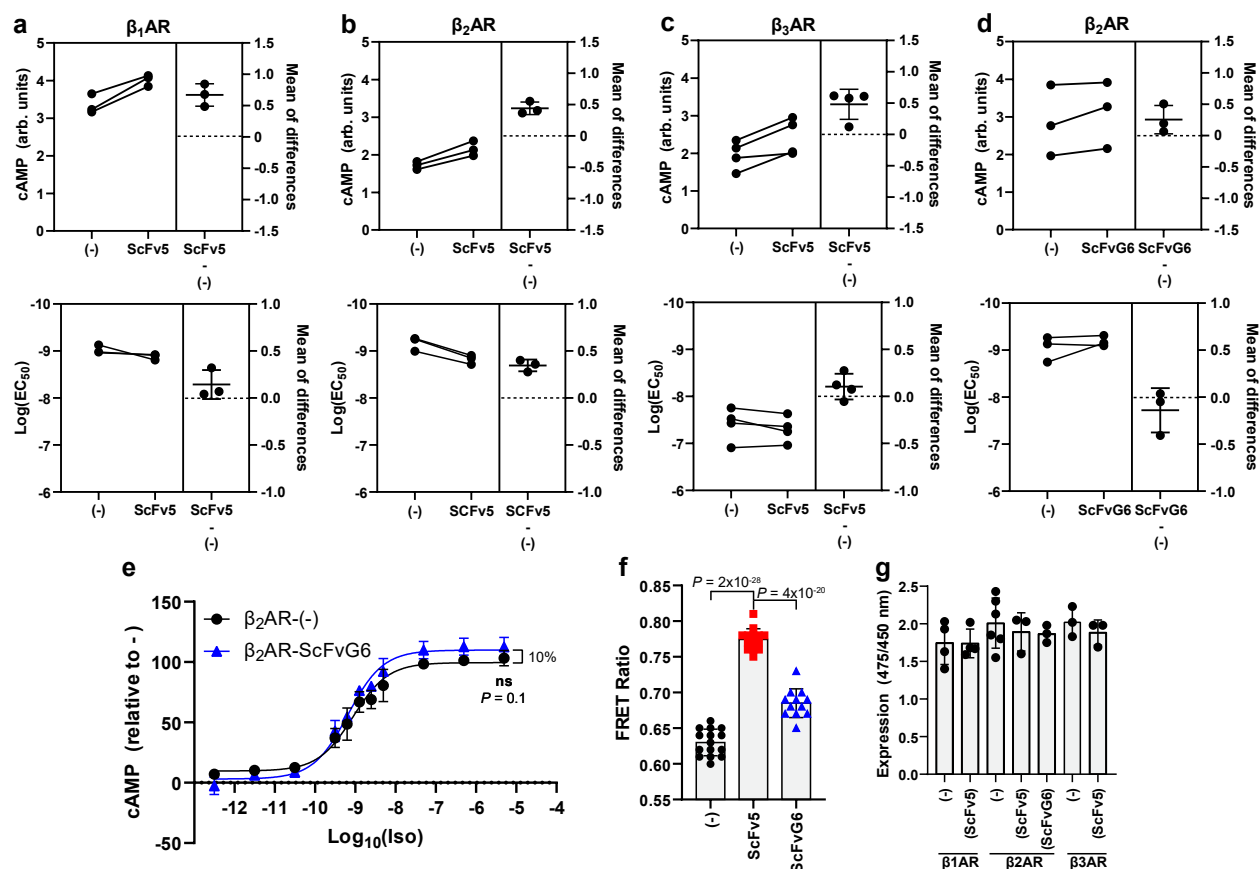
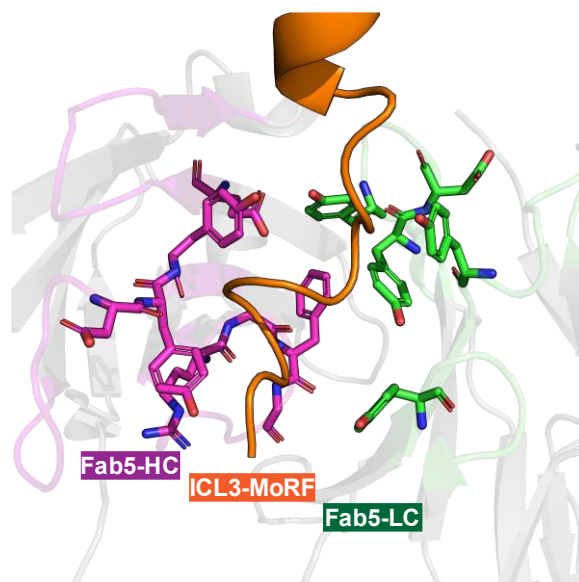




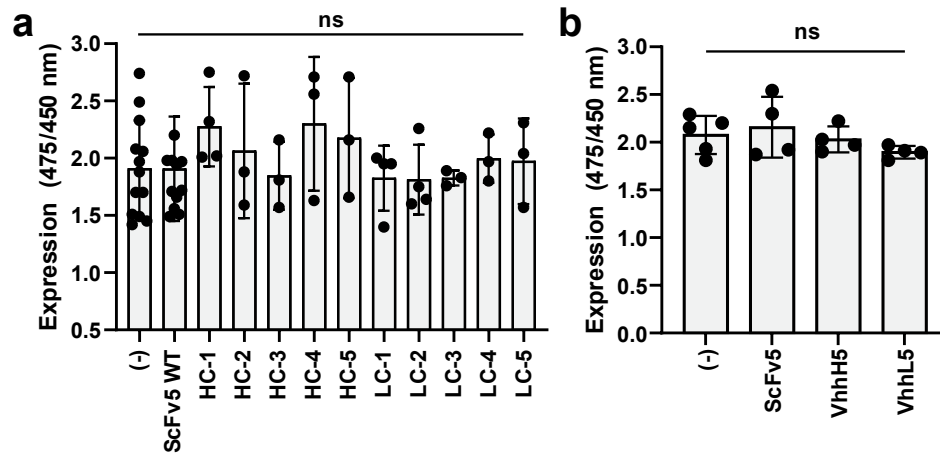
Supplementary Figure 1. Validation of receptor pharmacology upon C-tail fusion. **a** Schematic of the untethered (Dark- β_2 AR) or C-terminally tagged with mCitrine (β_2 AR-mCit). **b** Surface expression of both construct against non-transfected cells (UT) after 24 hours transfection (n = 4). **c** Isoproterenol dose response for cAMP accumulation on both constructs (n = 3). Pairwise t-test was performed for the **d** Emax and **e** EC₅₀ from dose response in **c**.



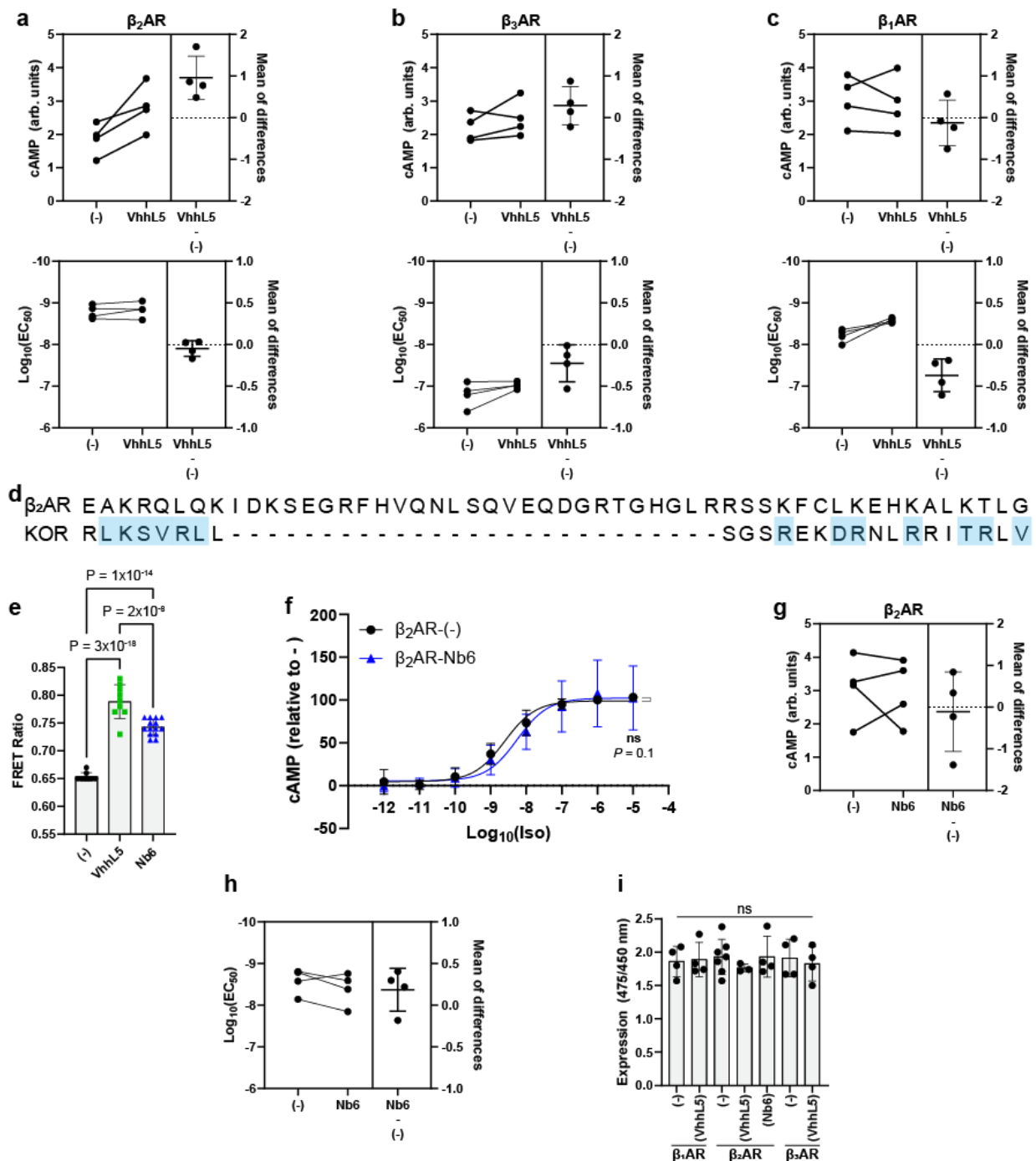
Supplementary Figure 2. **a-d**, Comparative Emax and EC50 data from $\beta_x\text{AR}$ -ScFv dose response curves (Fig. 1c-e, SFig. 2e). **e**, isoproterenol induced cAMP downstream of $\beta_2\text{AR}$ -SPASM sensors fused to 4xGSG control (-) or VEGF binding ScFvG6 (n = 3). **f**, Ratiometric FRET measurement of the interaction strength for the fusion of 4xGSG control and ScFvG6 to $\beta_2\text{AR}$. **g**, Expression quantification for dose response curves (Fig. 1c-e, SFig. 2e).



Supplementary Figure 3. Fab5 interacts through a molecular recognition feature (MoRF) in the N-terminus of β_2 AR's ICL3 (PDB: 2R4S). The end of TM5 and the MoRF in ICL3 (middle) are sandwiched between the heavy chain (HC, left) and the light chain (LC, right) of the Fab. Amino acid residues in HC and LC that form contacts with the MoRF are depicted as sticks.

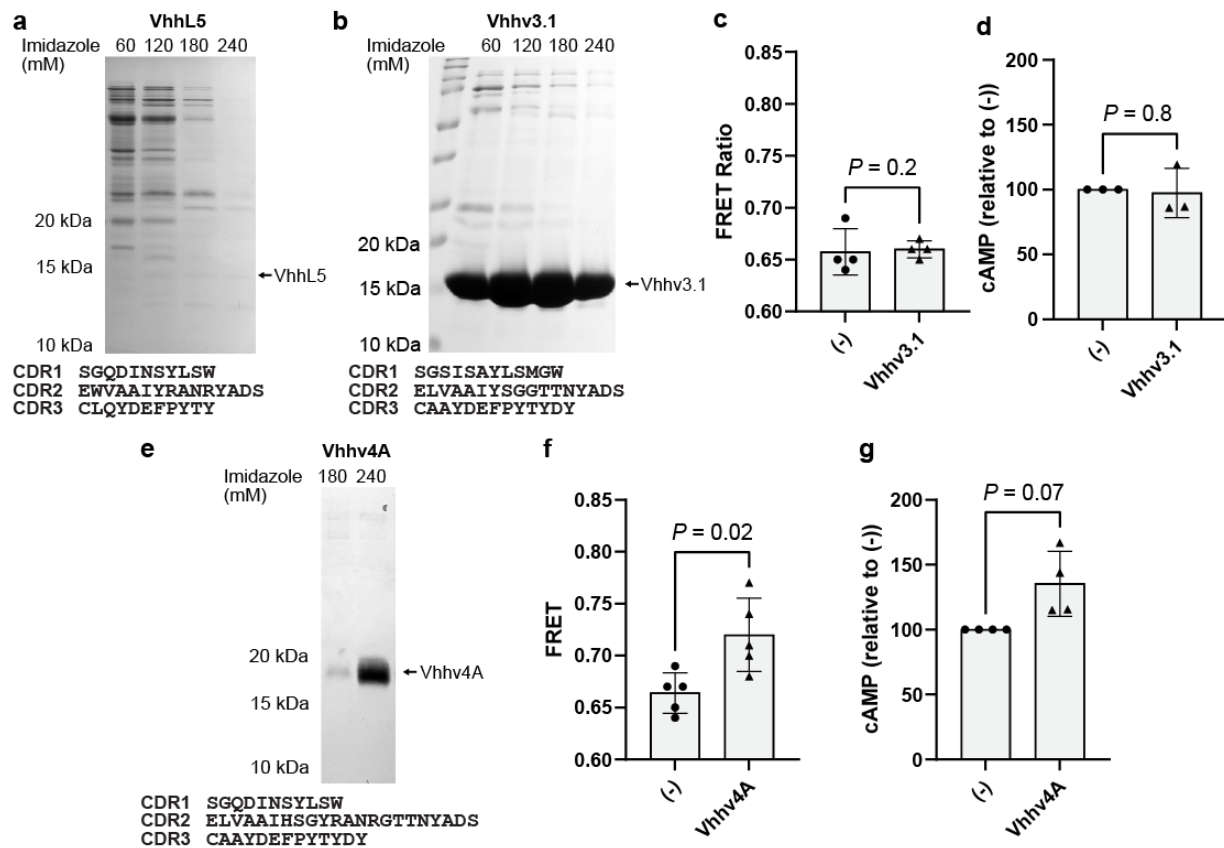


Supplementary Figure 4. a, Expression quantification of β_2 AR SPASM sensors for ScFv5 mutagenesis (Fig. 2c-d) 4xGSG control (n = 13), ScFv5 (n = 12), and ScFv5 alanine mutants(LC-1 n = 4, LC-2 n = 4, LC-3 n = 3, LC-4 n = 3, LC-5 n = 3, HC-1 n = 4, HC-2 n = 3, HC-3 n = 3, HC-4 n = 3, HC-5 n = 3). **b**, Expression quantification of β_2 AR SPASM sensors for Vhh constructs (Fig. 2f-g) 4xGSG control (n = 5), ScFv5 (n = 4), a heavy chain Vhh (VhhH5) (n = 4), or a light chain Vhh (VhhL5) (n = 4).

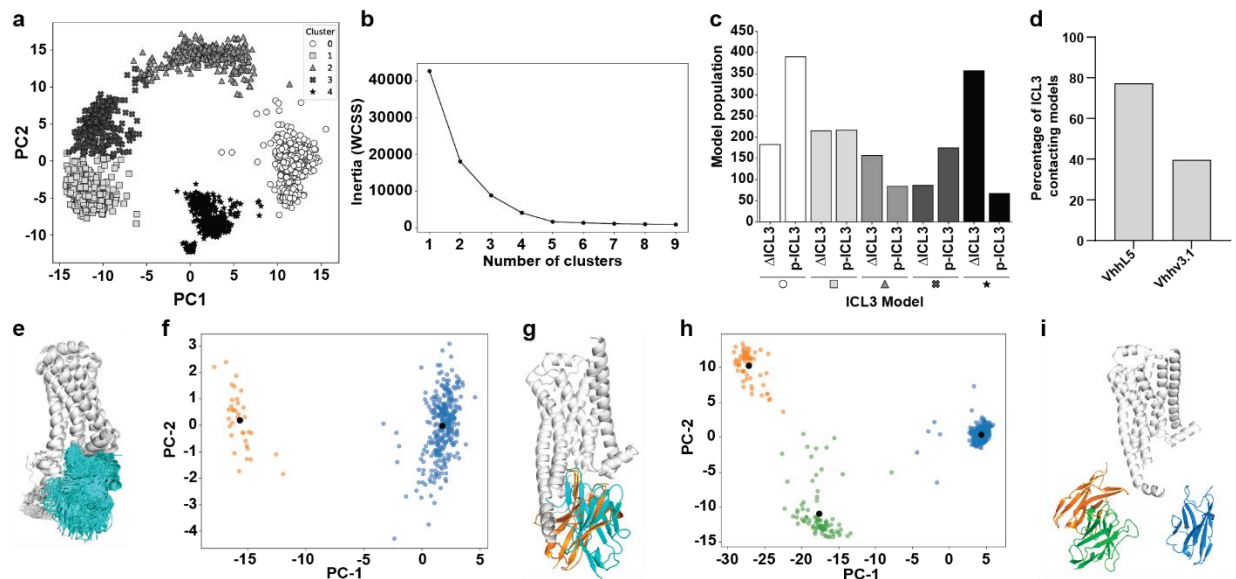


Supplementary Figure 5. **a-c**, Comparative Emax and EC50 data from $\beta_x\text{AR}$ -VhhL5 dose response curves (Fig. 3a; S.Fig 3a). **d**, Sequence alignment of a TM5-ICL3-TM6 segment of $\beta_2\text{AR}$ and Kappa opioid receptor (KOR). Blue shading in the sequence of KOR corresponds to the KOR-Nb6 interface residues within a 4 Å radius (PDB: 6VI4). **e**, Ratiometric FRET measurement of the interaction strength for the fusion of 4xGSG control, VhhL5, and Nb6 to $\beta_2\text{AR}$. **f**, Isoproterenol dose response induced cAMP accumulation of $\beta_2\text{AR}$ -SPASM sensors fused to 4xGSG control (-) or KOR binding Nb6

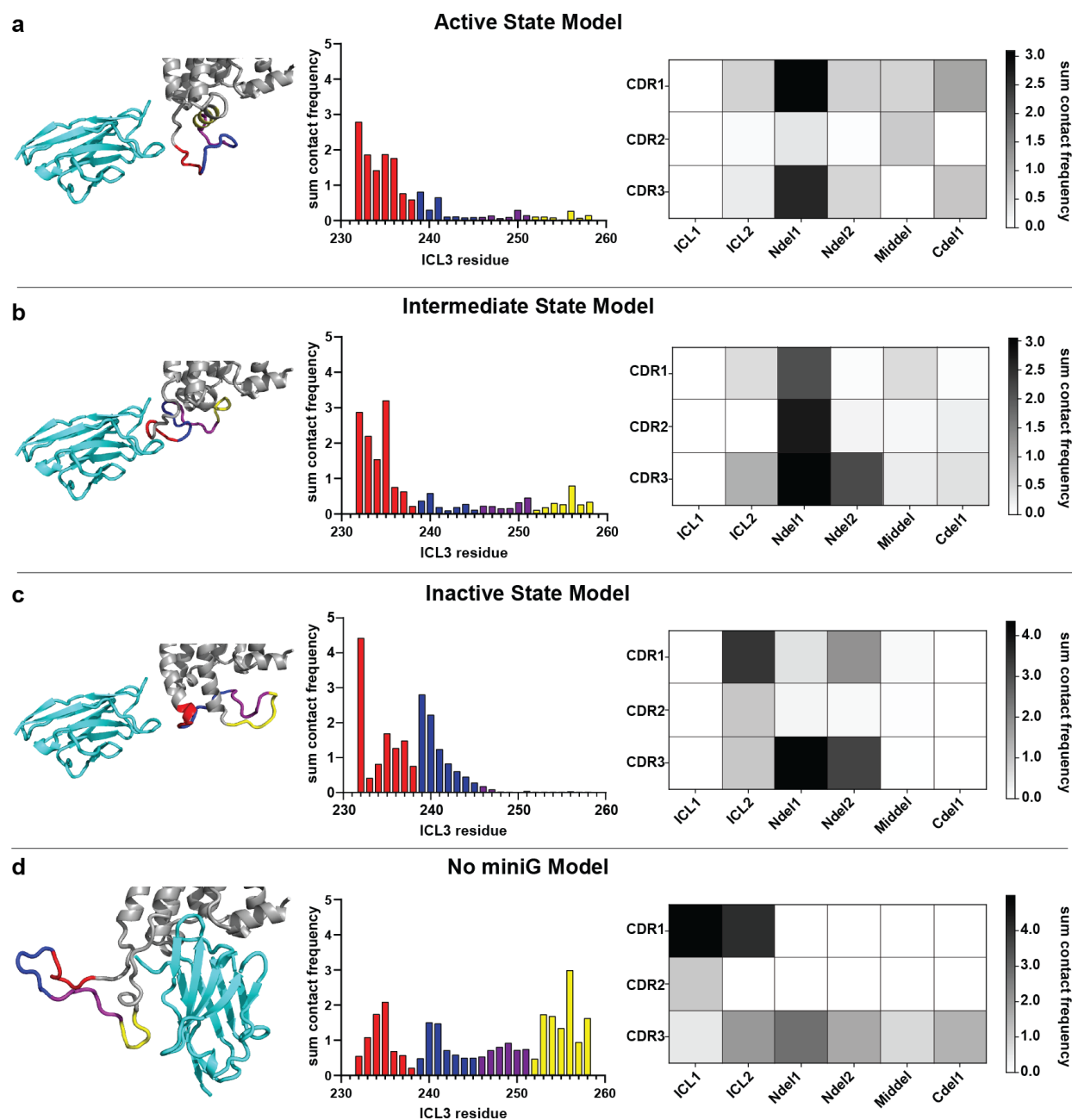
(n = 4). **g-h**, Comparative Emax and EC50 data from β_2 AR-Nb6 dose response curve (SFig. 5f). **i**, Expression quantification for dose response curves (Fig. 3a; SFig. 5f).



Supplementary Figure 6. a-b. Top, denaturing SDS-PAGE of elution fractions of VhhL5 (~14 kDa) (**a**) and Vhhv3.1 (~14 kDa) (**b**) from bacterial expression and purification. Concentration of imidazole in each fraction indicated on top of gel. 1% of total fraction loaded (5 μ L/500 μ L total). Bottom, CDR sequences of each Vhh CDR1 – Kabat residues H25-H36, CDR2- Kabat residues H46-H61, CDR3 – Kabat residues H92-H102. **c**, Ratiometric FRET measurement of the interaction strength for the fusion of 4xGSG control and Vhhv3.1 to β_2 AR (n = 4). **d**, saturating concentrations of isoproterenol induced cAMP accumulation of β_2 AR-SPASM sensors fused to 4xGSG control (-) and Vhhv3.1 (n = 3). **e**, Top, denaturing SDS-PAGE of elution fractions of Vhhv4A (~15 kDa) from bacterial expression and purification. Concentration of imidazole in each fraction indicated on top of gel. 1% of total fraction loaded (5 μ L/500 μ L total). Bottom, CDR sequences of each Vhh CDR1 – Kabat residues H25-H36, CDR2- Kabat residues H46-H61, CDR3 – Kabat residues H92-H102. **f**, Ratiometric FRET measurement of the interaction strength for the fusion of 4xGSG control and Vhhv4A to β_2 AR (n = 5). **g**, saturating concentrations of isoproterenol induced cAMP accumulation of β_2 AR-SPASM sensors fused to 4xGSG control (-) and Vhhv4A (n = 4). Statistical comparisons shown for (**c,d,f,g**) are paired t-tests.

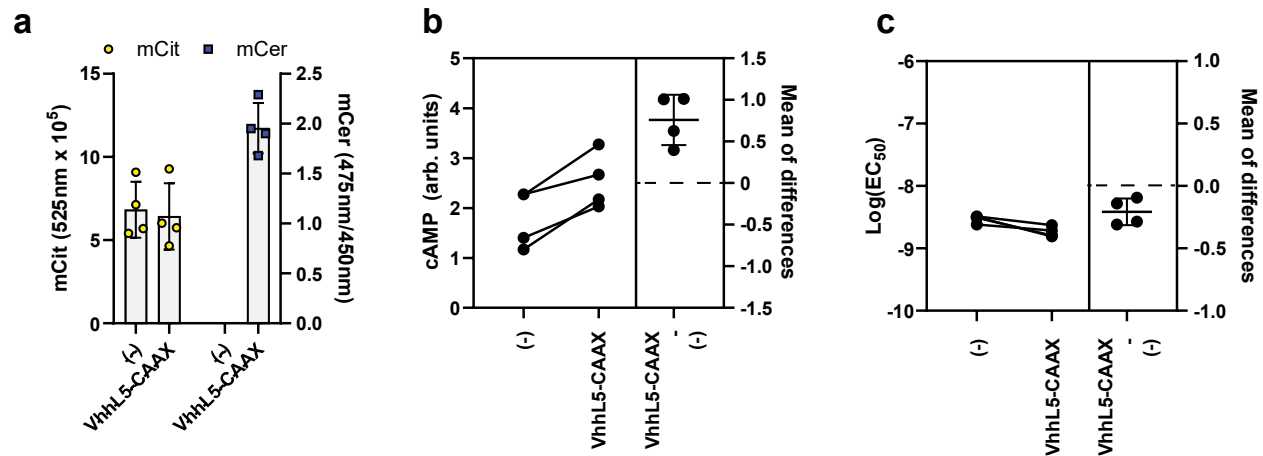


Supplementary Figure 7. **a**, PCA Clustering of structural models from AlphaFold3. The AF3 models were generated using β_2 AR sequence with no ICL3, and with two different partial ICL3 sequences (see S.Table 2 and Methods section for details). **b**, Elbow plot used for K-means clustering in PC space shown in **(a)**. The within-cluster sum of squares (WCSS) is shown on the y-axis. WCSS represents the sum of the square of the Euclidean distance between data points and their assigned cluster centroids, quantifying the variance within the cluster. WCSS is plotted as a function of the number of clusters ($k = 1-10$) applied to the PC-1 and PC-2 space. The elbow $k = 5$ suggests an optimal balance between cluster compactness and model complexity and this was used. **c**, Number of AF3 structural models (Model Population) that show residue contacts between residues in ICL3, intracellular end of TM5 and the antibody fragments VhhL5 and Vhh3.1 (see Supplementary Table 2) for various clusters obtained from PC analysis. **d**, Percentage of structural models for VhhL5 and Vhh3.1 corresponding to clusters 0 and 3 in **(a)**. **e**, Three-dimensional structural models generated using AF3 with input amino acid sequence of β_2 AR+p2ICL3+VhhL5 (shown in cyan) without the mini- G_s protein. **f**, Principal component analysis of the 369 AF3 structural models of β_2 AR+p2ICL3+VhhL5 complex (done without the mini- G_s sequence) showing two clusters of conformations. **g**, Representative structures extracted from each of the two conformational clusters shown in the PCA landscape. **h**, Principal component analysis of the 849 AF3 structural models of β_2 AR+p2ICL3+VhhL5 complex (done with the mini- G_s sequence) showing three clusters of conformations. **i**, Representative structures extracted from each of the three conformational clusters shown in the PCA landscape.

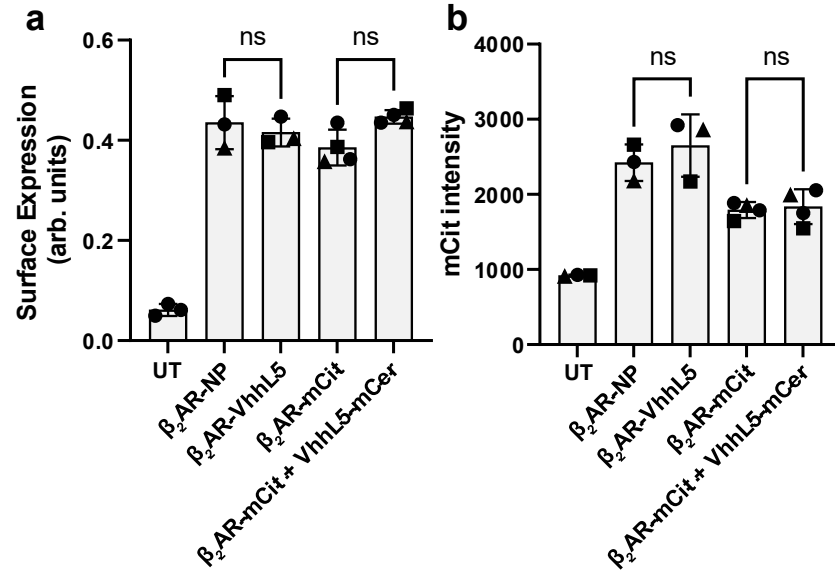


Supplementary Figure 8: a-d, The four structural models of $\beta_2\text{AR}+\text{Full ICL3}+\text{VhhL5}$ used for starting the coarse grain MD simulations. Panels **(a–c)** starting structures derived from VhhL5 orientations derived from mini- G_s dependent AF3 models and panel **(d)** shows the orientation of VhhL5 predicted in the absence of mini- G_s sequence. (middle) Histogram of the sum of the frequencies of the residue contacts made by residues in ICL3 are shown next to the structures. Colors used in the structural models and histograms indicate highlighted regions correlated to the block deletion constructs of ICL3 tested using SPASM sensor FRET experiments (Fig. 4a).

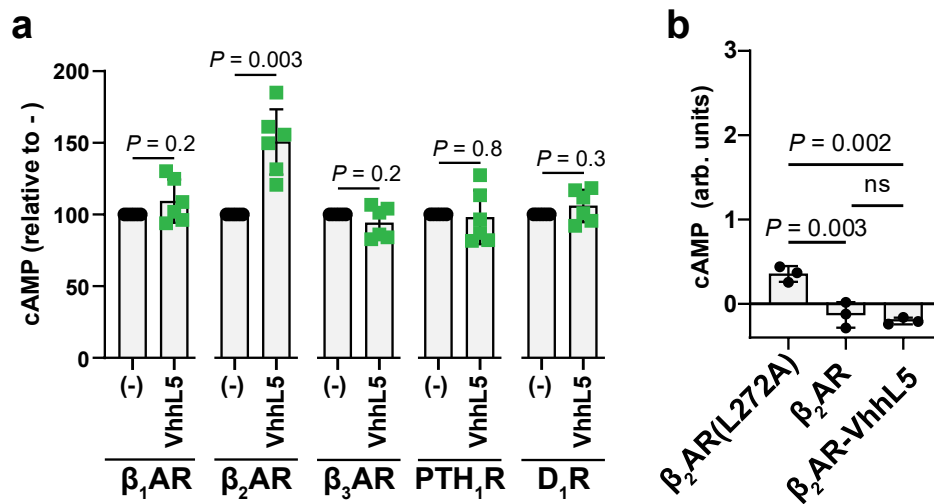
(left) The contact heat map between residues in the CDR loops of VhhL5 and residues in the three intracellular loops (ICL) of β_2 AR+full ICL3+VhhL5 calculated from CGMD simulation trajectories.



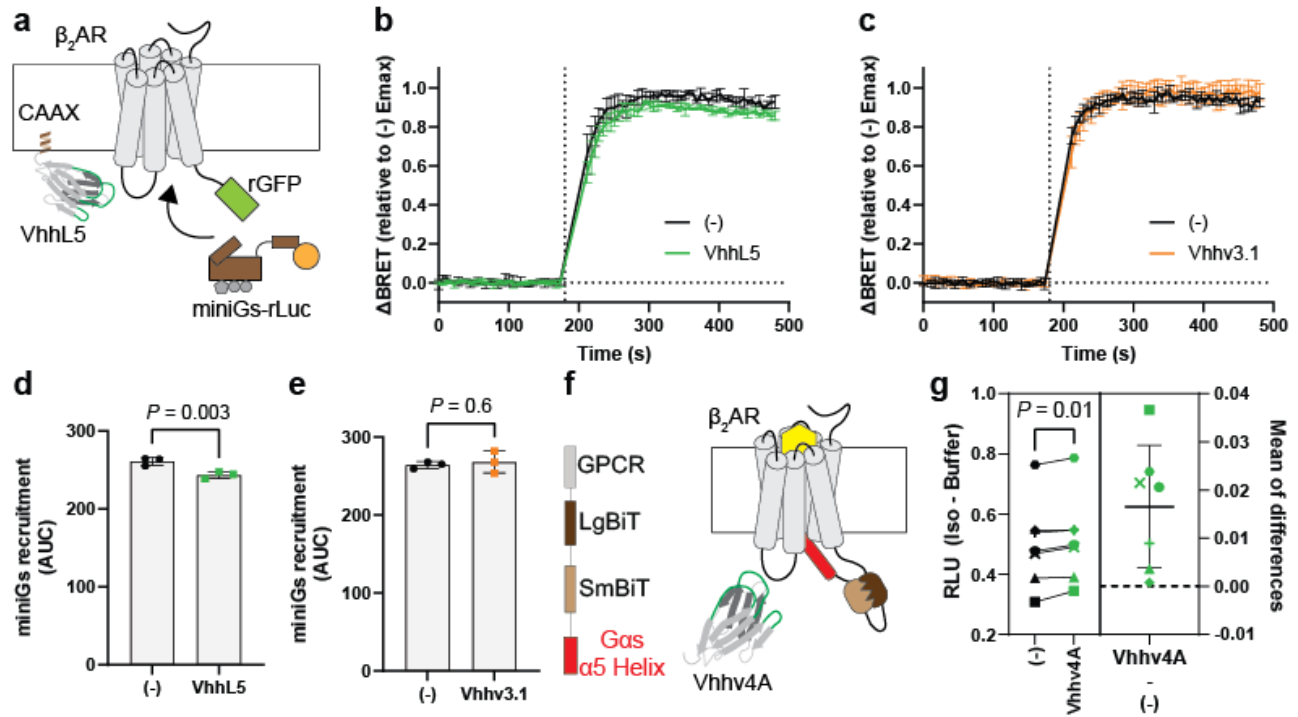
Supplementary Figure 9. **a**, Expression of VhhL5-mCerulean-CAAX (475 nm / 450 nm) and max intensity at 525 nm β_2 AR-mCit for HEK293 WT dose responses (Fig. 4b). **b-c**, Comparative Emax and EC50 data from β_2 AR-mCit $\pm$ VhhL5-mCer-CAAX dose response curves for HEK293 WT cell line (Fig. 4b).



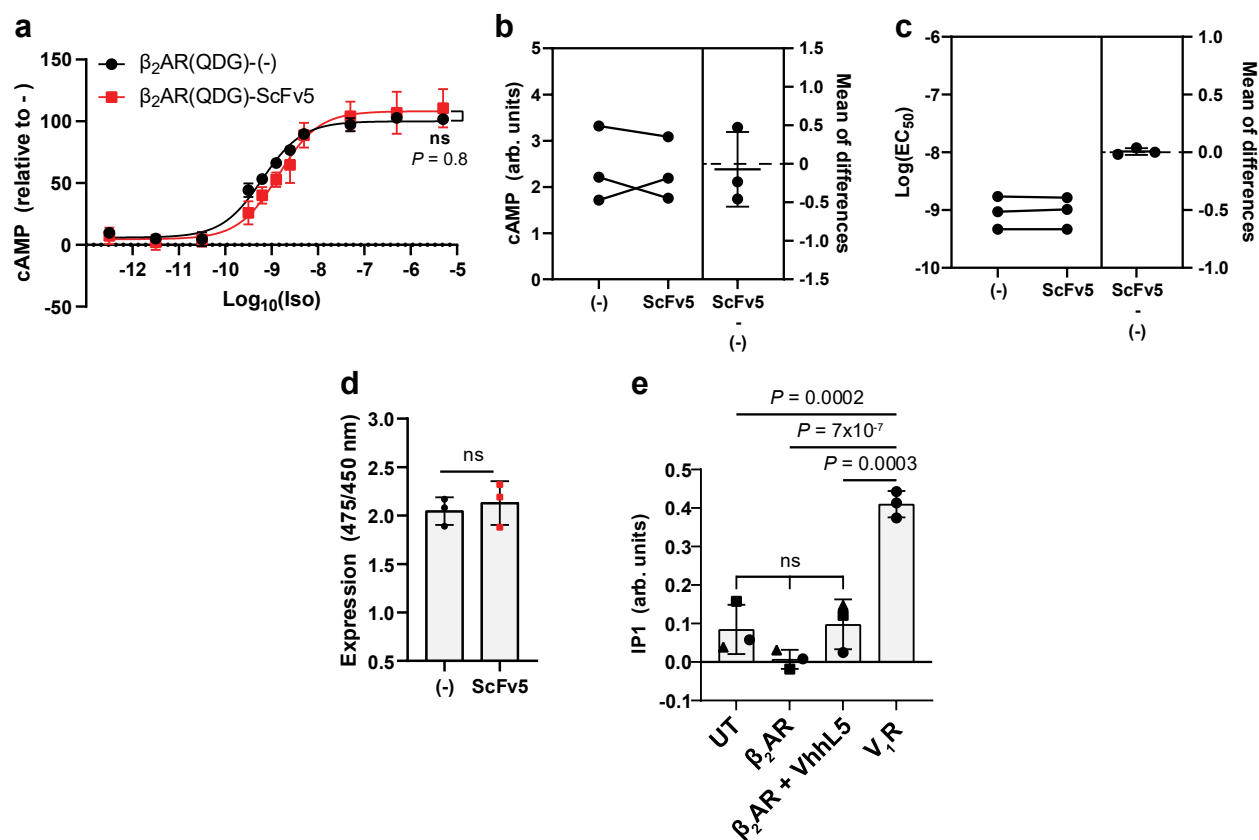
Supplementary Figure 10. Surface expression upon fusion or co-transfection of VhhL5. **a** Surface expression (anti-HA-HRP) and **b** mCitrine intensity (receptor fused) of the expression of non-transfected cells (UT), SPASM sensor fusions (β_2 AR-NP and β_2 AR-VhhL5) ($n = 3$), and co-transfected β_2 AR-mCit with VhhL5-mCer ($n = 4$). Statistical analysis was One-way ANOVA for both panel figures.



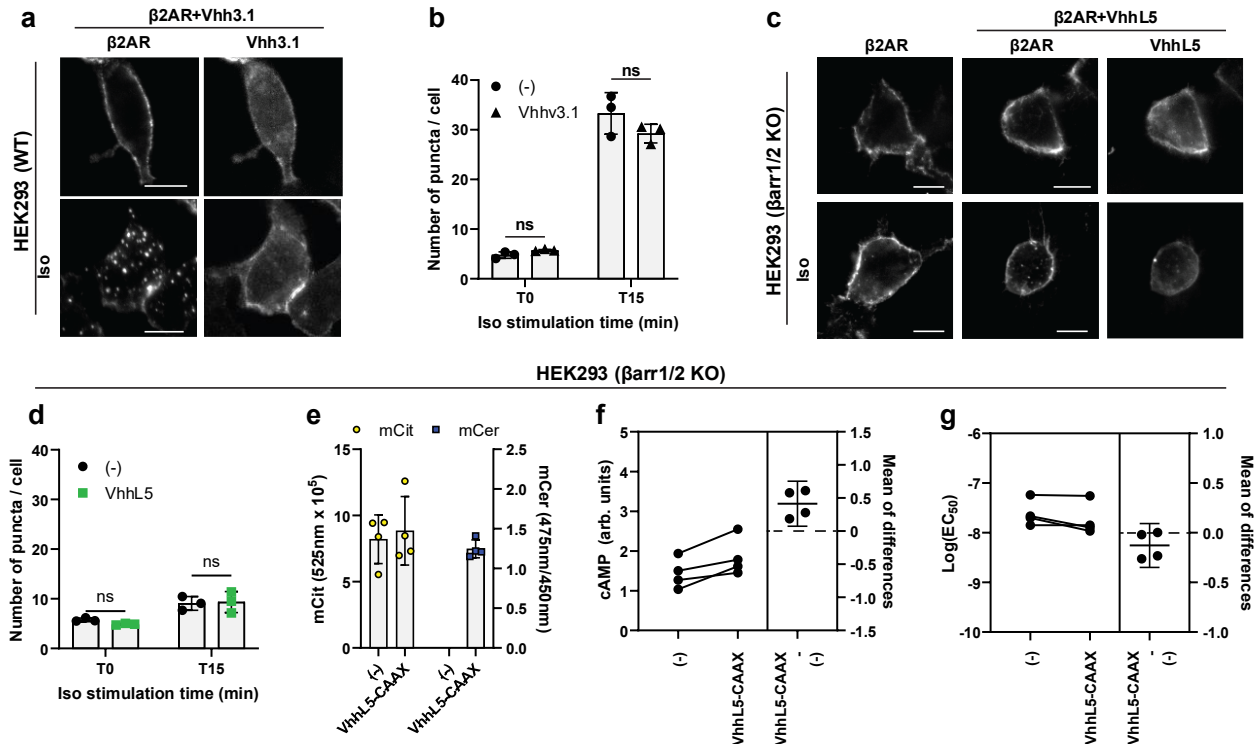
Supplementary Figure 11. a cAMP accumulation normalized to the absence of VhhL5 (-) for multiple receptors (n = 6). Statistical comparisons shown are paired t-tests for each receptor (n = 6). **b**, cAMP accumulation of β_2AR -NP L272A mutant which exhibits constitutive activity in the absence of isoproterenol compared to undetectable levels of basal activity of wild type β_2AR -NP and receptor fusion to VhhL5 (n = 3). Statistical comparison shown is a one-way ANOVA.



Supplementary Figure 12. **a**, Schematic of co-transfection for HEK393 cells to express β_2 AR-rGFP and miniGs-rLuc as the BRET pair for a miniG protein recruitment BRET assay. **b-c**, HEK293 cells expressing miniGs-rLuc and β_2 AR alone (black) or co-expressed with Vhh-CAAX ((**b**) VhhL5, green or (**c**) Vhhv3.1, orange), measuring Iso-induced miniGs recruitment normalized to the maximal BRET response of β_2 AR alone. Data is represented as mean $\pm$ SD of $n = 3$. **d-e**, Statistical analysis of area under the curve (AUC) for miniGs recruitment of receptor alone (-) or co-transfection with Vhh-CAAX ((**d**) VhhL5, green or (**e**) Vhhv3.1, orange) ($n = 3$). **f**, Schematic of luciferase complementation-based reporter of β_2 AR-G protein coupling. Luciferase complementation components (LgBiT, dark brown and SmBiT, light brown) are linked by an ER/K α helix, with the $\alpha 5$ helix of Gas fused C-terminally to SmBiT. The sensor is stimulated with saturating concentrations of the agonist Iso (100 μ M) and treated with Vhhv4A (1 μ M), a derivative of VhhL5 optimized for recombinant expression. **g**, Luminescence signal of sensor shown in (**f**), paired symbols for biological replicates ($n = 7$). Both panels, **d**, **e**, and **g**, were analyzed with paired t-test.



Supplementary Figure 13. **a**, Dose response curve for β_2 AR QDG(AAA) mutant fused to 4xGSG control (black) or ScFv5 (red) ($n = 3$). **c-d**, Comparative Emax and EC50 data for the dose response curve in **(a)**. Statistical comparison shown is a paired t-test. **d**, Expression quantification for dose response curves in **(a)**. **e**, IPx accumulation assay. Receptors were stimulated by isoproterenol (β_2 AR constructs) or AVP (V_1 R construct). Each symbol represents a biological replicate, and bars represent the mean $\pm$ SD for $n = 3$. Statistical comparison shown is from a one-way ANOVA.



Supplementary Figure 14. **a**, Representative images of β_2 AR internalization in HEK293 cells co-expressing β_2 AR and Vhhv3.1-tRFP-CAAX, stimulated with isoproterenol (Iso, 10 μ M). The first column illustrates receptor's fluorescence while the far-right column represents tRFP fluorescence from VhhL5. **b**, Number of puncta per cell for β_2 AR internalization in HEK293 cells expressing β_2 AR alone or co-expressed with Vhhv3.1-tRFP-CAAX under basal or Iso conditions. Data is represented as mean $\pm$ SD of $n = 3$ ($n > 10$ cells analyzed for each replicate), two-way ANOVA used to compare the effect of iso stimulation and co-expression of VhhL5. **c**, Representative images of β_2 AR internalization in HEK293 (β arr1/2 KO) cells expressing β_2 AR alone or co-expressed with VhhL5-tRFP-CAAX, stimulated with isoproterenol (Iso, 10 μ M). The first two columns illustrate receptor's fluorescence while the far-right column represents tRFP fluorescence from VhhL5. **d**, Number of puncta per cell for β_2 AR internalization in HEK293 (β arr1/2 KO) cells expressing β_2 AR alone or co-expressed with VhhL5-tRFP-CAAX under basal or Iso conditions. Data is represented as mean $\pm$ SD of $n = 3$ ($n > 10$ cells analyzed for each replicate), two-way ANOVA used to compare the effect of iso stimulation and co-expression of VhhL5. **e**, Expression of VhhL5-mCerulean-CAAX (475 nm / 450 nm) and max intensity at 525 nm β_2 AR-mCit for HEK293 (β arr1/2 KO) dose response (Fig. 6f). **f**, **g**, Comparative E_{max} and EC_{50} data from β_2 AR-mCit $\pm$ VhhL5-mCer-CAAX dose response curve for HEK293 (β arr1/2 KO) cell line (Fig. 6f).

Supplementary Table 1. Protein sequences of the ScFv and Vhh used in the experiments of this study.

Construct	Sequence
ScFv5	EVQLQQSGAELARPGASVKLSCKASGYIFTDYYINWVRQRTGQGFIEWIG EIYPGSGNIDYNERFKDKATLTADKSSSTAYMQLSSLTSEDSAVYFCVRG FGYWGGQTTLTVSSGGGGSGGGGSGGGGSGGGGSGGGGSDIKMTQS PSSMYASLGERVTITCKASQDINSYLSWFQQKPGKSPKTLIYRANRLVDG VPSRFITGSGGDYSLTISSLDYADMGIYYCLQYDEFPYTFGGGTKLEIK
ScFvG6	MEVQLVESGGGLVQPGGSLRLSCAASGFTISDYWIHWVRQAPGKGLEW VAGITPAGGYTTYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCA RFVFFLPYAMDYWGQGTLLTVSSSSASGGGGSGGGGSGGGGSDIQMTQ SPSSLASVGDRTITCRASQDVSTAVAWYQQKPGKAPKLLIYSASFLYS GVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYTTPPTFGQGKVEI KRLE
VhhL5	QVQLQESGGGLVQAGGSLRLSCAASGQDINSYLSWYRQAPGKEREWVA AIYRANRYADSVKGRFTISRDNANKNTVYLQMNSLKPEDTAVYYCLQYDEF PYTYWGQGTQVTVSS
VhhH5	QVQLQESGGGLVQAGGSLRLSCAASGYIFTDYYINWYRQAPGKEREWV AAIYPGSGNIDYADSVKGRFTISRDNANKNTVYLQMNSLKPEDTAVYYCVR GFGYWGGGTQVTVSS
Nb6	QVQLQESGGGLVQAGESLRLSCAASGTIFRLYDMGWYRRVSGNQRELV ASITSGGSTKYGDSVKGRFTISRDNANKNTVYLQMSSLKPEDTAVYYCNAE YRTGIWEELLDGWGQGTQVTVSS
Vhhv3.1	QVQLQESGGGLVQAGGSLRLSCAASGSISAYLSMGWYRQAPGKQRELV AAIYSGGTTNYADSVKGRFTISRDNAANTVYLQMNSLKPEDTAVYYCAAY DEFPYTYDYWGQGTQVTVSS
Vhhv4A	QVQLQESGGGLVQAGGSLRLSCAASGSISAYLSMGWYRQAPGKQRELV AAIHSGYRANRGTTNYADSVKGRFTISRDNAANTVYLQMNSLKPEDTAVY YCAAYDEFPYTYDYWGQGTQVTVSS

Supplementary Table 2. Protein sequences of the β_2 AR, miniGs and antibody fragments used to generate AF3 models in this study.

Construct	Sequence
β_2 AR-ICL3	TQERDEVWVVGMGIVMSLIVLAIVFGNVLVITAIKFERLQTVTNFYFITS LACADLVMGLAVVPFGAAHILMKMWTFGNFWCEFWTSIDVLCVTASI ETLCVIAVDTRYFAITSPFKYQSLLTKNKARVILMVWIVSGLTSFLPIQM HWYRATHQEAINCYANETCCDFFTQAYAIASSIVSFYVPLVIMVFVY SRVFQEAQRQLQKIDKSEGRFHVQNLSQVEQDGRGTGHGLRRSSSKFC LKEHKALKTLGIIMGTFTLCWLPFFIVNIVHVIQDNLRKEVYILLNWIGY VNSGFNPLIYCRSPDFRIAFQELLCL
ICL3	KRQLQKIDKSEGRFHVQNLSQVEQDGRGTGHGLRRSSKFCLKEHKAL
β_2 AR-p ₁ ICL3	KRQLQKIDKSEGRFHVQNLSQVEQDGRGTGHGLRRSSKFCLKEHKAL
β_2 AR-p ₂ ICL3	KRQLQKIDKSEGRFHVQNLSQVEQDGRGTGHGLRRSSKFCLKEHKAL
β_2 AR- Δ ICL3	KRQLQKIDKSEGRFHVQNLSQVEQDGRGTGHGLRRSSKFCLKEHKAL
Mini-G _s	GIEKQLQKDKQVYRATHRLLLLGADNSGKSTIVKQMRIYHGGSGGSG GTSGIFETKFQVDKVNFMFDVGGQRDERRKWIQCFNDVTAIIFVVD SSDYNRLQEALNDFKSIWNNRWLRTISVILFLNKQDLLAEKVLGKSK IEDYFPEFARYTTPEDATPEPGEDPRVTRAKYFIRDEFRLISTASGDG RHYCYPHFTCAVDTENARRIFNDCRDIIQRMHLRQYELL
VhhL5	MQVQLQESGGGLVQAGGSLRLSCAASGQDINSYLSWYRQAPGKER EWVAAIYRANRYADSVKGRFTISRDNKNTVYLMNSLKPEDTAVYY CLQYDEFPYTYWGQGTQVTVSS

("...") in the table indicates gap between sequence in absence of full-ICL3.

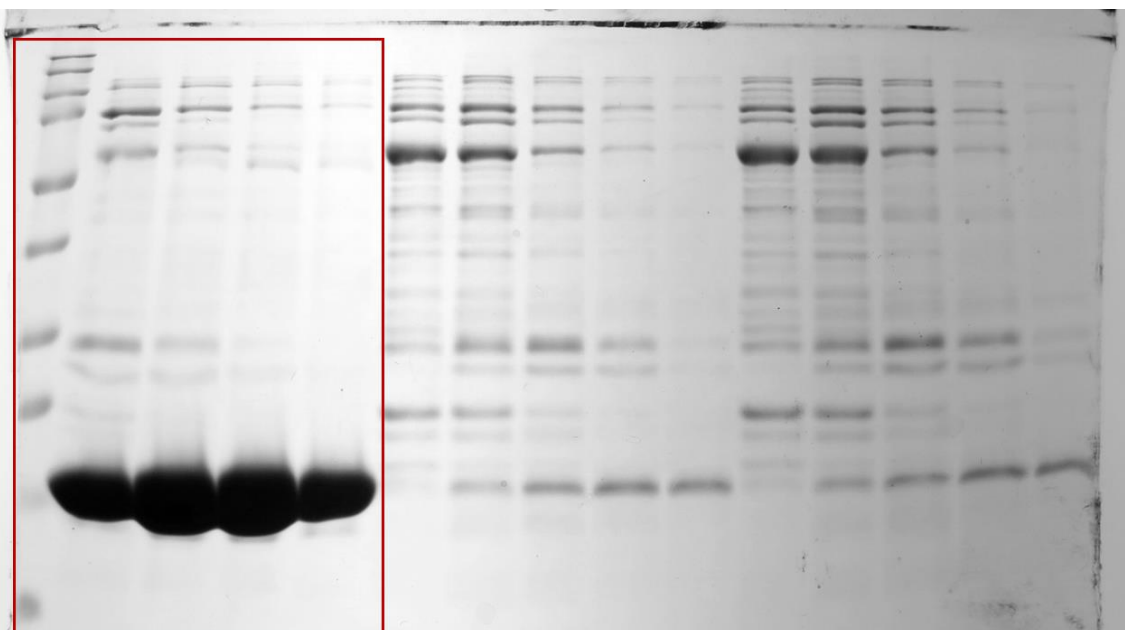
The olive-green highlighted sequence is the full ICL3 sequence

The red, blue, purple, yellow highlighted regions are the block deletion constructs of ICL3 tested using SPASM sensor FRET experiments.

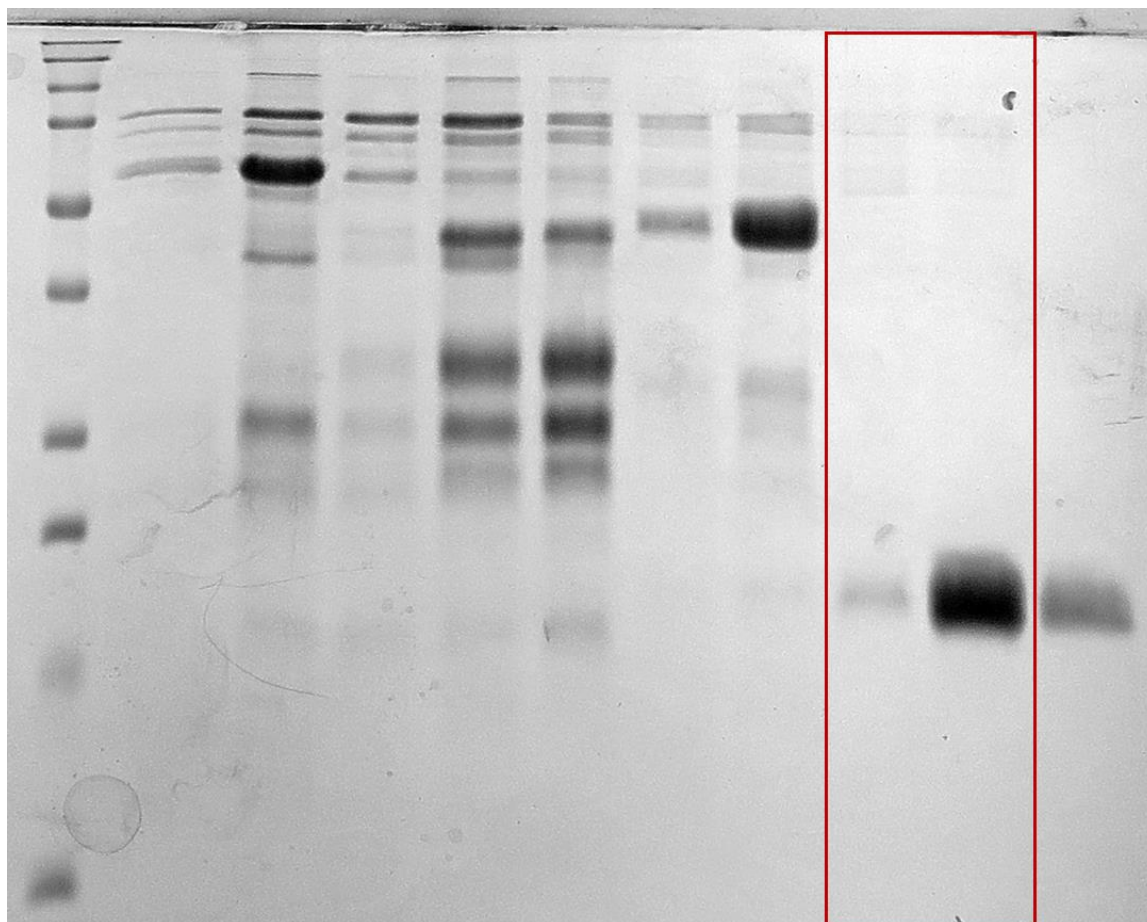
The gray highlighted regions are the residues in ICL1, ICL2 and ICL3 in β_2 AR and the three CDR loops in VhhL5 and Vhhv3.1 used to calculate the residue distances between CDRs and ICLs.



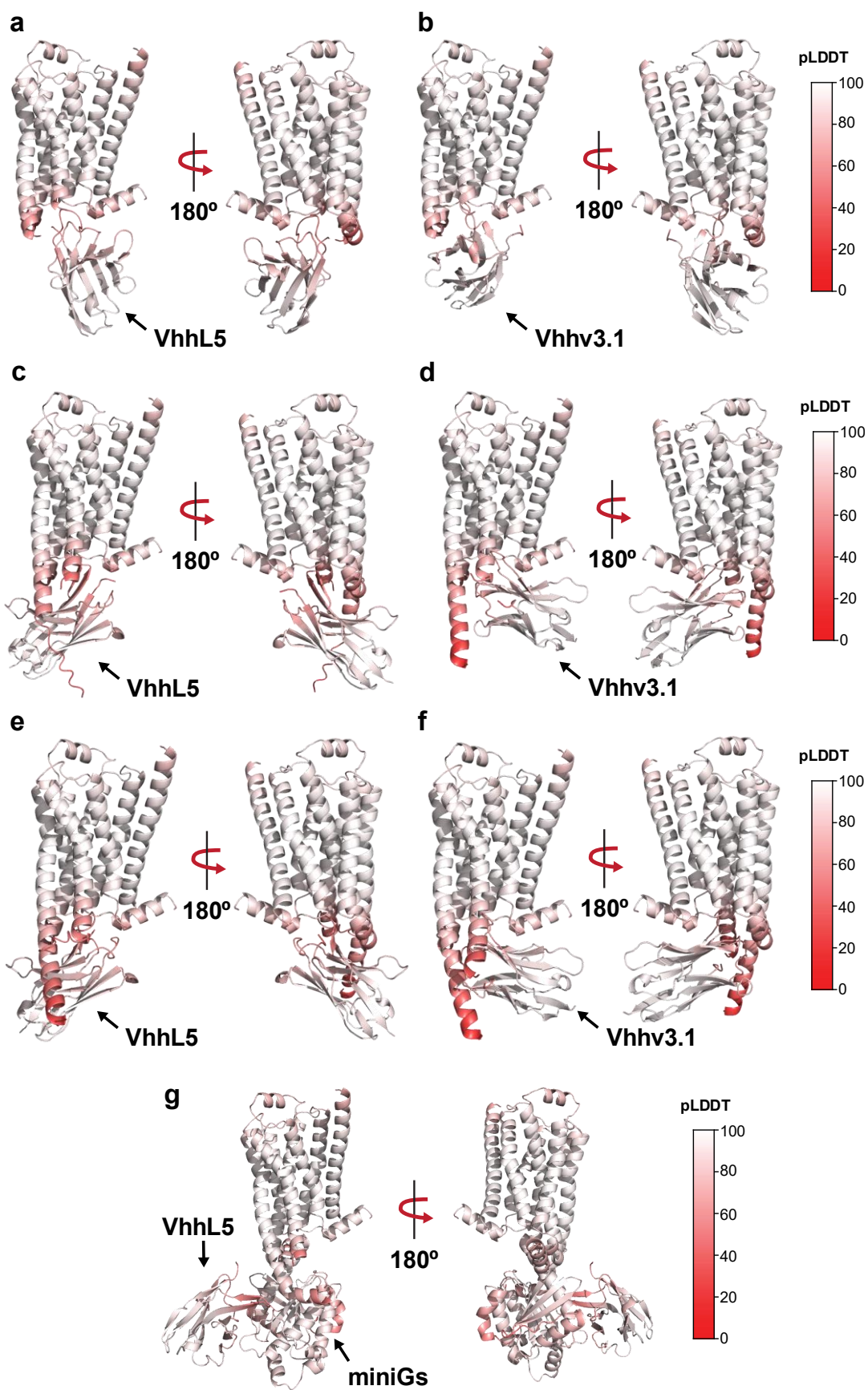
Supplementary Figure 15. Uncropped SDS-PAGE gel from **SFig. 6a**. Red rectangle delineates the cropped portion shown in **SFig. 6a**.



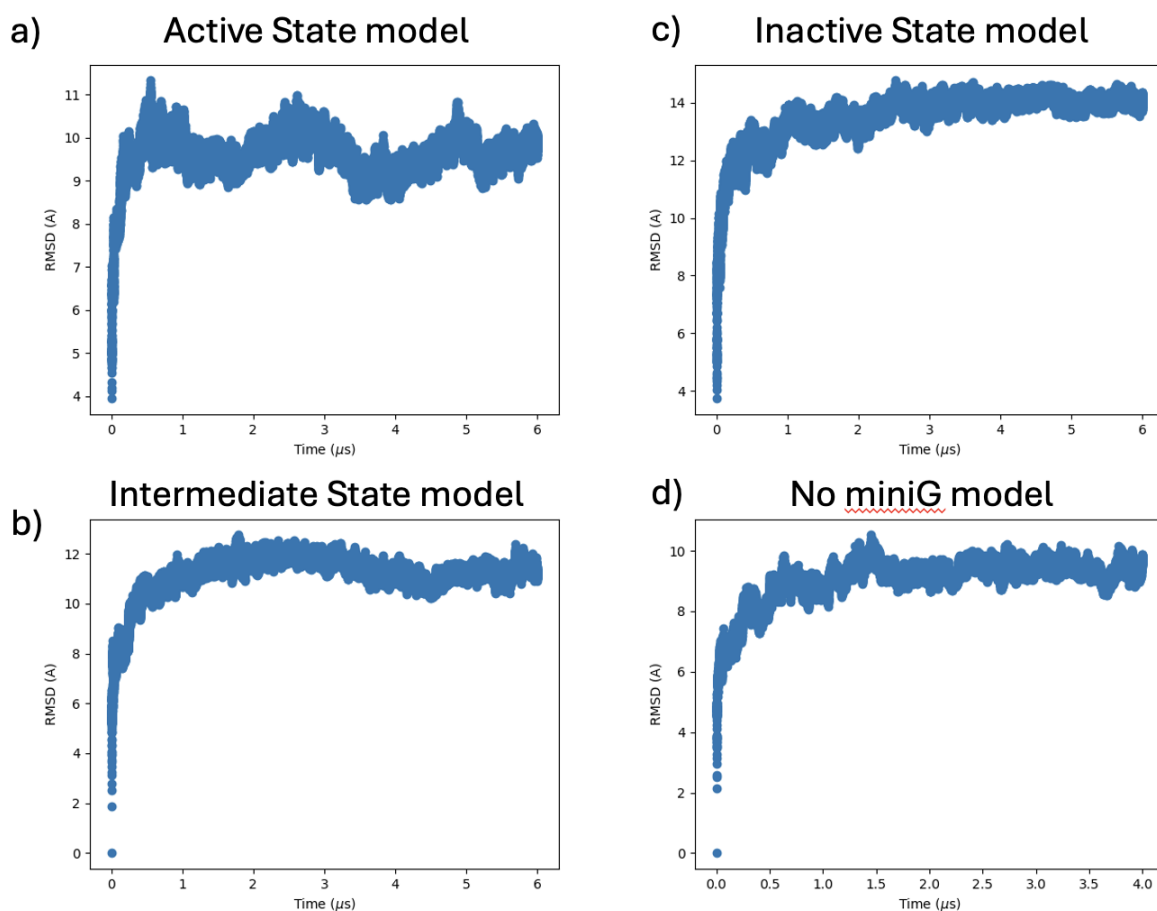
Supplementary Figure 16. Uncropped SDS-PAGE gel from **SFig. 6b**. Red rectangle delineates the cropped portion shown in **SFig. 6b**.



Supplementary Figure 17. Uncropped SDS-PAGE gel from **SFig. 6e**. Red rectangle delineates the cropped portion shown in **SFig. 6e**.



Supplemental Figure 18. pLDDT scores overlaid on representative AlphaFold models used in this study. Each panel represent the following models: **a**, β_2 AR- Δ ICL3 with VhhL5, **b**, β_2 AR- Δ ICL3 with Vhhv3.1, **c**, β_2 AR-p1ICL3 with VhhL5, **d**, β_2 AR-p1ICL3 with Vhhv3.1, **e**, β_2 AR-p2ICL3 with VhhL5, **f**, β_2 AR-p2ICL3 with Vhhv3.1, and **g**, β_2 AR-p1ICL3 with VhhL5 and miniGs.



Supplemental Figure 19: The root mean squared deviation (RMSD) versus simulation time during the coarse grain MD simulations calculated with respect to the starting four structural models of β_2 AR+Full length ICL3+VhhL5. Panels **(a–c)** starting structures from VhhL5 orientations derived using mini-G_s and β_2 AR sequence AF3 models **(d)** RMSD starting from model where the VhhL5 orientation with respect to β_2 AR was derived with AF3 models in the absence of mini-G_s sequence. Each panel shows the RMSD of the coarse grain beads of β_2 AR –VhhL5 complex as a function of time, confirming that the system reaches equilibrium. Coarse grain MD simulations were used to simulate the close association of VhhL5 to β_2 AR-ICL3. The RMSD shift of ~10 angstrom is due to the shift in the position of VhhL5 closer to the receptor.

Supplementary Table 3. Reliability and reproducibility checklist for molecular dynamics simulations

	Yes	No	Response
1. Convergence of simulations and analysis			
1a. Is an evaluation presented in the text to show that the property being measured has equilibrated in the simulations (e.g. time-course analysis)?	☒	☐	Figure 19 in Supporting Information
1b. Then, is it described in the text how simulations are split into equilibration and production runs and how much data were analyzed from production runs?	☒	☐	Methods section: In step 5 of the section titled “2 Computational method for generating structural models of VhhL5 bound to β_2 AR with full ICL3”
1c. Are there at least 3 simulations per simulation condition with statistical analysis?	☒	☐	Methods section: In step 5 of the section titled “2) Computational method for generating structural models of VhhL5 bound to β_2 AR with full ICL3”
1d. Is evidence provided in the text that the simulation results presented are independent of initial configuration?	☒	☐	Methods section: In step 5 of the section titled “2) Computational method for generating structural models of VhhL5 bound to β_2 AR with full ICL3”
2. Connection to experiments			
2a. Are calculations provided that can connect to experiments (e.g. loss or gain in function from mutagenesis, binding assays, NMR chemical shifts, J-couplings, SAXS curves, interaction distances or FRET distances, structure factors, diffusion coefficients, bulk modulus and other mechanical properties, etc.)?	☒	☐	Figure 4. VhhL5 targets the most N terminal part of the β_2 AR’s ICL3 Our contact frequency of VhhL5 to intracellular

			domain of β_2 AR connects with FRET measurements in Fig. 4a
3. Method choice			
3a. Do simulations contain membranes, membrane proteins, intrinsically disordered proteins, glycans, nucleic acids, polymers, or cryptic ligand binding?	☒	☐	Methods section: In step 5 of “2) Computational method for generating structural models of VhhL5 bound to β_2 AR with full ICL3”
3b. Is it described in the text whether the accuracy of the chosen model(s) is sufficient to address the question(s) under investigation (e.g. all-atom vs. coarse-grained models, fixed charge vs. polarizable force fields, implicit vs. explicit solvent or membrane, force field and water model, etc.)?	☒	☐	Methods section Computational methods for generating structural models of β_2 AR-ICL3-VhhL5
3c. Is the timescale of the event(s) under investigation beyond the brute-force MD simulation timescale in this study that enhanced sampling methods are needed?	☒	☐	We used Coarse Grain MD simulation to enhance sampling methods
If YES , are the parameters and convergence criteria for the enhanced sampling method clearly stated?	☒	☐	SI Figure 19
If NO , is the evidence provided in the text?	☐	☐	
4. Code and reproducibility			
4a. Is a table provided describing the system setup that includes simulation box dimensions, total number of atoms, total number of water molecules, salt concentration, lipid composition (number of molecules and type)?	☒	☐	Methods section: In step 5 of “2) Computational method for generating structural models of VhhL5 bound to β_2 AR with full ICL3”
4b. Is it described in the text what simulation and analysis software and which versions are used?	☒	☐	Methods section: In step 5 of section titled “2) Computational method for generating structural models of VhhL5 bound to β_2 AR with full ICL3”

4c. Are other parameters for the system setup described in the text, such as protonation state, type of structural restraints if applied, nonbonded cutoff, thermostat and barostat, etc.?	☒	☐	Methods section: In step 5 of section titled “2) Computational method for generating structural models of VhhL5 bound to β_2 AR with full ICL3”
4d. Are initial coordinate and simulation input files and a coordinate file of the final output provided as supplementary files or in a public repository?	☒	☐	supplementary Figure 8
4e. Is there custom code or custom force field parameters?	☐	☒	Response not needed if No
☐ If YES , are they provided as supplementary files or in a public repository?	☐	☐	