

Supplementary Materials for

Distinct C-tail dynamics between β -arrestin isoforms in forming GPCR tail- and core-engaged complexes

Ruibo Zhai, Jiening Wang, Xianhui Hou, Xiaogang Niu, Changwen Jin, Shan Wu*, Yunfei Hu*

Corresponding author: S.W., wushan91@hubu.edu.cn; Y.H., huyunfei@wipm.ac.cn

The PDF file includes:

Figs. S1 to S15

Table S1

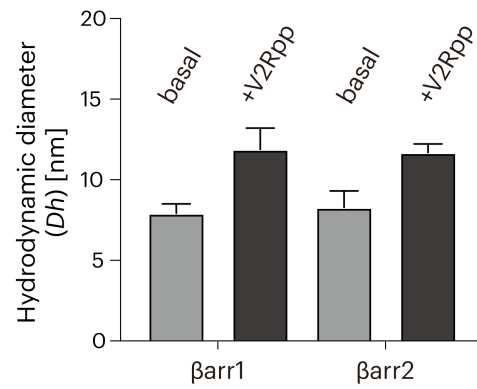


Fig. S3. Protein size assessment by dynamic light scattering (DLS). DLS measurements of the hydrodynamic diameter (Dh) of β arrs (0.5 mg/mL) before and after adding V2Rpp. Data are presented as mean values $\pm$ SD of $n = 7$ technical replicates.

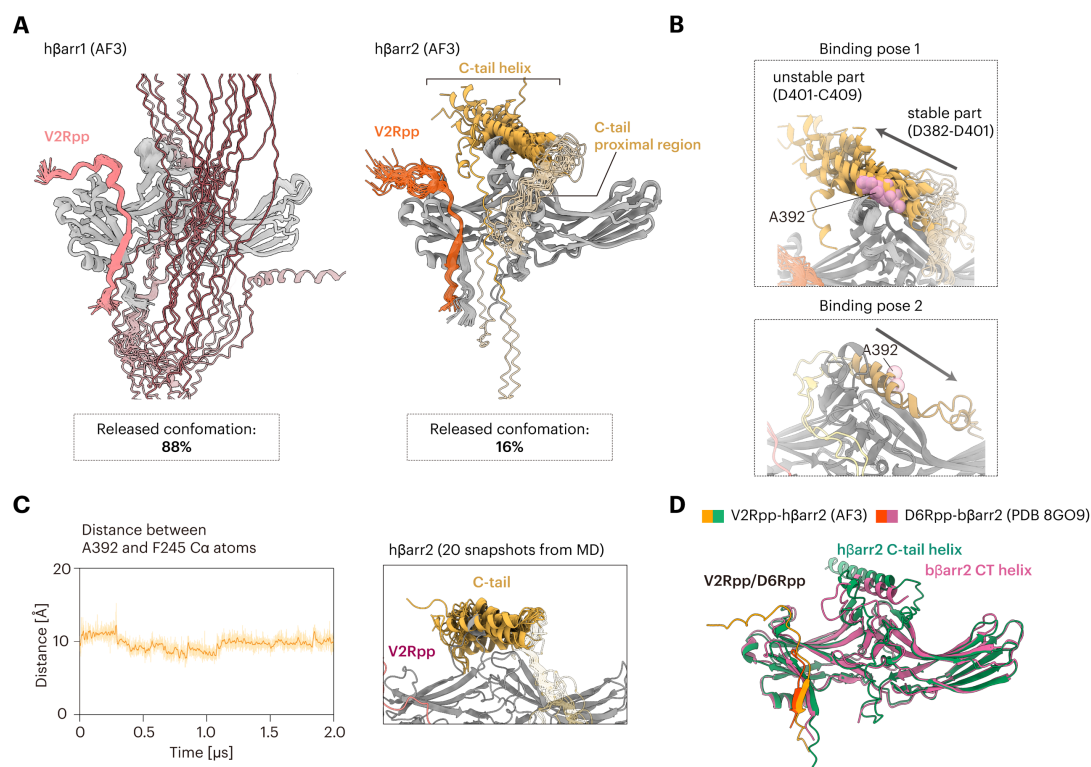


Fig. S4. AlphaFold3 predictions of V2Rpp-activated barr structures. (A) Twenty randomly selected models of V2Rpp-complexed barr1/2 structures from AF3 predictions ($n_{\text{model}} = 92$). The population of the conformations with a free released C-tail is calculated for both barrs based on all 92 predictions. (B) Two different binding poses of the barr2 C-tail helix observed in the AF3 predictions. (C) A time trace showing the fluctuations of the distance between the A392 (C-tail) and F245 (central crest C loop) C α atoms in a 2 μ s MD simulation of V2Rpp–barr2 complex starting from an AF3 model (left), and an overlay of 20 conformational snapshots along the time trace at 100 ns intervals. (D) Overlay of the AF3-predicted V2Rpp–human barr2 structure (major binding pose) with the experimentally determined D6Rpp–bovine barr2 complex structure (PDB 8GO9). The Fab30 molecule in the D6Rpp–bovine barr2 structure is omitted.

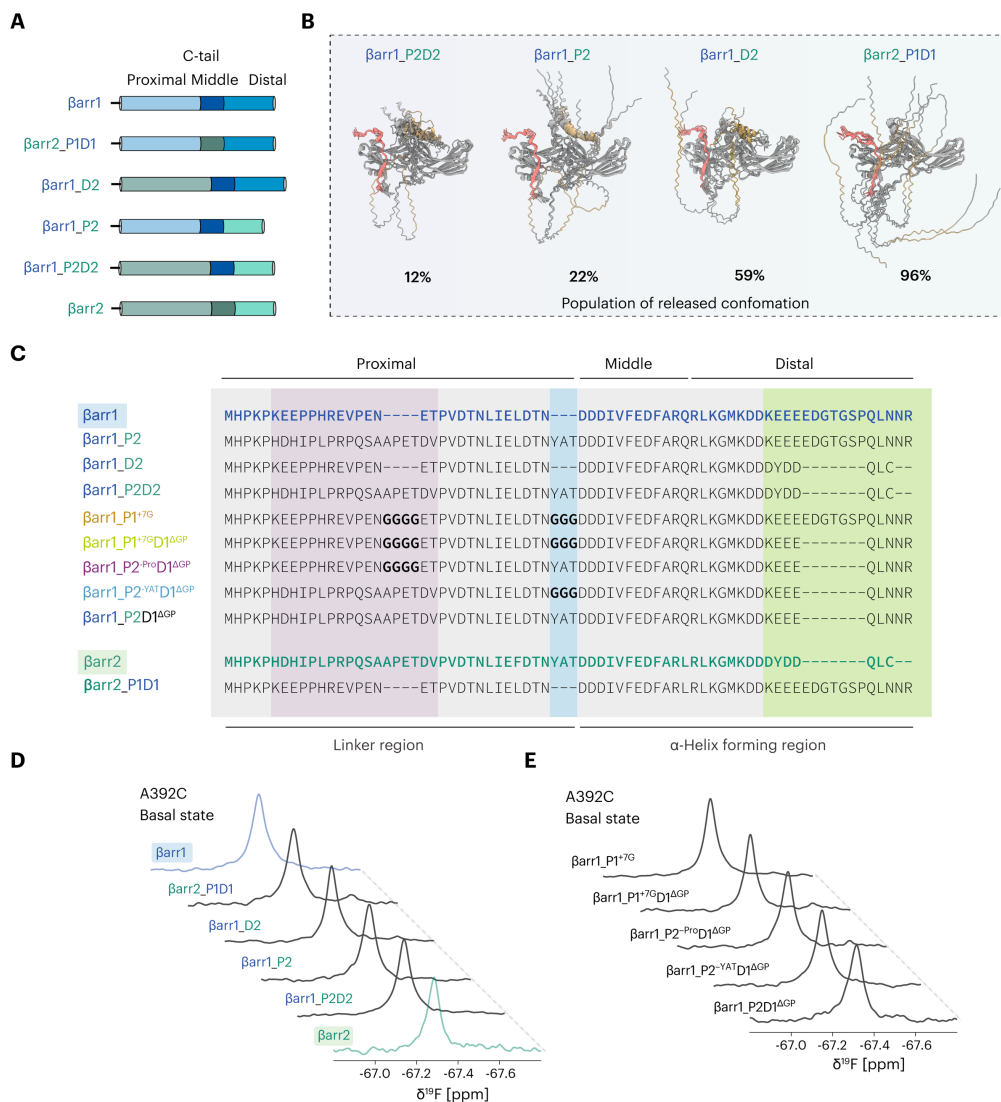


Fig. S5. Preparations of different barr chimeras. (A) A schematic illustration of the construct design of barr C-tail chimeras. (B) Five randomly selected models of barr chimera in the V2Rpp-complexed state from AF3 predictions ($n_{\text{model}} = 92$), with the C-tails colored in orange. The population of the conformations with a released C-tail is calculated based on all 92 predictions. (C) C-tail sequences of the barrs mutants. Conserved regions are shown in gray, the proline-rich region in the proximal segment is shown in purple, the YAT motif is shown in blue, and the distal segment is shown in green. (D-E) The basal-state ^{19}F NMR spectra of the A392C site in barr C-tail chimeras and additional barr1 mutants.

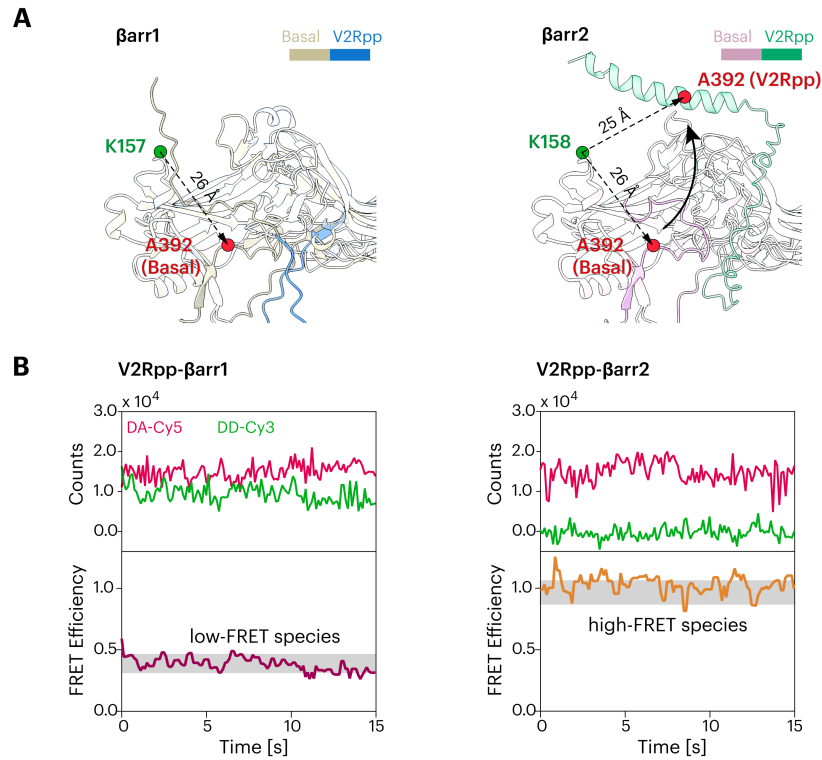


Fig. S6. smFRET reveals distinct C-tail conformations in β arr1 and β arr2. (A) Distances between the FRET sites in the basal state of β arr1 (left), and in both the basal and V2Rpp-activated states of β arr2 (right). (B) Representative single-molecule traces of the fluorescence (donor in green, acceptor in red) and FRET (bottom) results for both β arrs in the presence of 300 μ M V2Rpp. The data were recorded using an objective-based TIRF microscope with 100 ms imaging.

A		
	proximal segment (31 residues)	middle+distal segments (36 residues)
ARRB1_MOUSE	MHPKPKEE PPH REV P ESETPVDTNLIELDTNDDIVFEDFARQLKGMKDDKDEEDD GTG SPHLNNR	
ARRB1_RAT	MHPKPKEE PPH REV P ESETPVDTNLIELDTNDDIVFEDFARQLKGMKDDKDEEDD GTG SPHLNNR	
ARRB1_HUMAN	MHPKPKEE PPH REV P ENETPVDTNLIELDTNDDIVFEDFARQLKGMKDDKEEEDD GTG SPQLNNR	
ARRB1_MACFA	MHPKPKEE PLH REV P ENQTPVDTNLIELDTNDDIVFEDFARQLKGMKDDKEEEN GTG SPQLNNR	
ARRB1_RABIT	MHPKPKEE PPH REV P ENETPVDTNLIELDTNDDIVFEDFARQLKGMKDDKEEEDDV TG SPRLNDR	
ARRB1_PANTR	MHPKPKEE PPH REV P ENETPVDTNLIELDTNDDIVFEDFARQLKGMKDDKEEEDD GTG SPQLNNR	
ARRB1_PANTR	MHPKPKEE PPH REV P EHETPVDTNLIELDTNDDIVFEDFARQLKGMKDDKEEEDD GTDS PRLNDR	
ARRB1_DANIO	MHPKPLEESIYRDA P ENDAPIDNLIEFDNDDIIFEDFARQLIGAKDDKDEED E GADSPKLNDR	
ARRB1_CHICK	MHPKPREE P AHRDV P ENEAPIDTNLIELDTNDDIVFEDFARQLKGMKDDKEDEERTNS P QLNDR	
ARRB1_BOVIN	MHPKPKEE PPH REV P EHETPVDTNLIELDTNDDIVFEDFARQLKGMKDDKEEEDD GTG SPRLNDR	
B		
	proximal segment (≥ 38 residues)	middle+distal segments (27 residues)
ARRB2_MOUSE	MHPKPHDHITL LRP QSA P R-----ETDVPVDTNLIIEFDN YAT DDDIVFEDFARLRLKGMKDDDCDDQFC	
ARRB2_RAT	MHPKPHDHITL LRP QSA P R-----EIDIPVDTNLIIEFDN YAT DDDIVFEDFARLRLKGMKDDDCDDQFC	
ARRB2_HUMAN	MHPKPHDHI PLRP QSA P -----ETDVPVDTNLIIEFDN YAT DDDIVFEDFARLRLKGMKDDDYDDQLC	
ARRB2_PONAB	MHPKPHDHI PLRP QSA P -----ETDVPVDTNLIIEFDN YAT DDDIVFEDFARLRLKGMKDDDYDDQLC	
ARRB2_MACHFA	MHPKPRDHT PLRP QSA P -----ETDVPVDTNLIIEFDN YAT DDDIVFEDFARLRLKGMKDDDYDDQLC	
ARRB2_PANTR	MHPKPHDHI PLRP QSA P -----ETDVPVDTNLIIEFDN YAT DDDIVFEDFARLRLKGMKDDDYDDQLC	
ARRB2_CAPHI	MHPKPHDIAL LRP QSV THPS ALL PSA P ETDAPVDTNLIIEFDN YAT DDDIVFEDFARLRLKGLKDEDYDDQFC	
ARRB2_BOVIN	MHPKPHDIAL LRP QSAATH PPTL PSAV P ETDAPVDTNLIIEFETN YAT DDDIVFEDFARLRLKGLKDEDYDDQFC	

Fig. S7. C-tail sequence alignments of both β arr isoforms across different species.

(A) Sequences of β arr1 were obtained from the UniProt database: Q8BWG8 (ARRB1_MOUSE), P29066 (ARRB1_RAT), P49407 (ARRB1_HUMAN), Q4R562 (ARRB1_MACFA), Q95223 (ARRB1_RABBIT), C3PTE8 (ARRB1_DANIO), A0A8V0XCX3 (ARRB1_CHICK), H2R1Z2 (ARRB1_PANTR), A0A452EHP7 (ARRB1_CAPHI), and P17870 (ARRB1_BOVIN). (B) Sequences of β arr2 were obtained from the UniProt database: Q91YI4 (ARRB2_MOUSE), P29067 (ARRB2_RAT), P32121 (ARRB2_HUMAN), A0A7N9D4J4 (ARRB2_MACFA), H2QBY1 (ARRB2_PANTR), A0A452ESR2 (ARRB2_CAPHI), and P32120 (ARRB2_BOVIN). The Pro-rich sequence in the proximal segment of β arr1/2, the Gly/Pro-rich sequence in the distal segment of β arr1, and the YAT motif in β arr2 are highlighted.

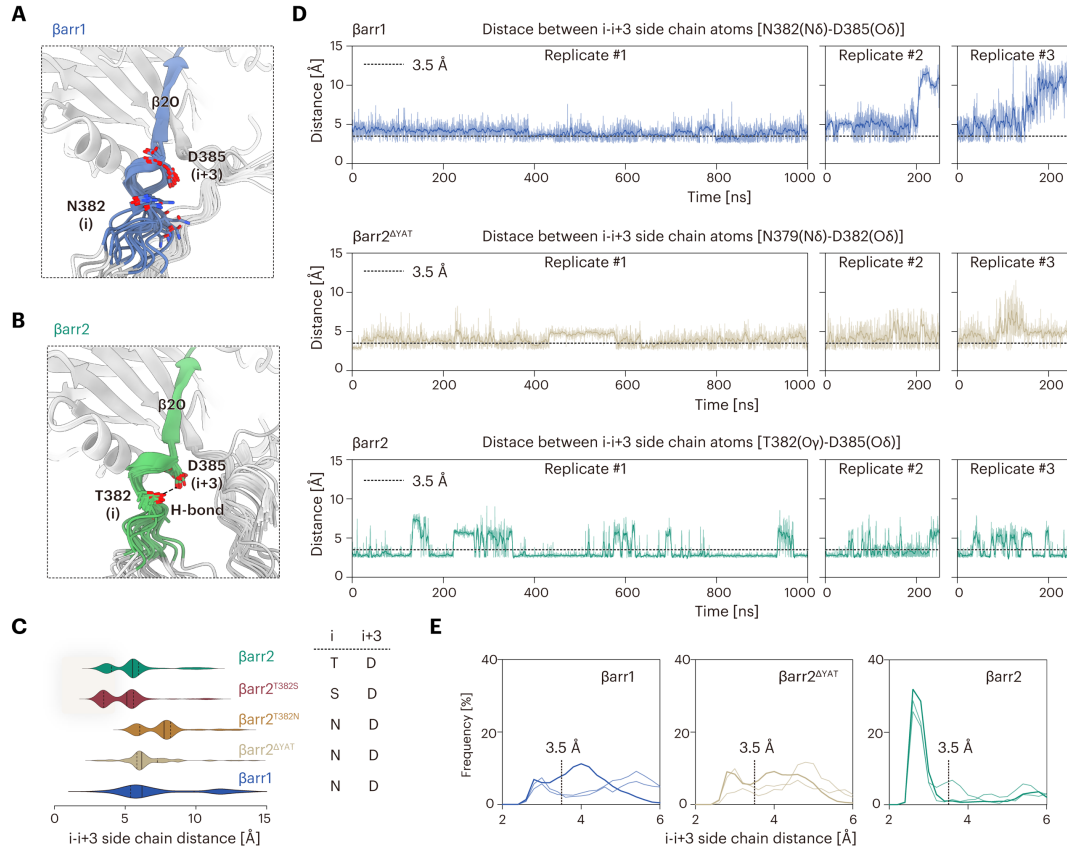


Fig. S8. Conformational analysis of the YAT motif by AF3 predictions and MD simulations. (A–B) AF3 models of full-length β arr1 (A) and β arr2 (B) in the basal state (20 models each), showing the local conformation of the N382/T382–D385 segment upstream of β 20. The side chains of N382/T382 and D385 are shown in sticks. (C) Violin plots showing the distributions of the i→i+3 side chain distances (the i+3 position corresponds to D385 in both β arrs) in β arr1, β arr2 and β arr2 mutants computed from AF3 models ($n_{\text{model}} = 80$), with solid and dashed lines indicating the median and interquartile range. The distances were calculated between the O δ atom of D385 (i+3) and the O γ , O β or N δ atoms of the Thr, Ser or Asn residues at the i position. (D) Time traces of basal-state β arr1, β arr2 and β arr2^{ΔYAT} showing the i→i+3 side chain distances (i+3 corresponds to D385) in three MD trajectories (traj 1: 1 μ s, traj 2&3: 250 ns). All trajectories start from an initial state with a helical-like conformation at the 382–385 region. Dashed lines indicate the threshold of 3.5 Å, a distance below which is favorable for hydrogen bond formation. (E) Frequency distributions of the i→i+3 distance computed from the time traces in (D). Thick and thin lines correspond to results from traj 1 and traj 2–3, respectively.

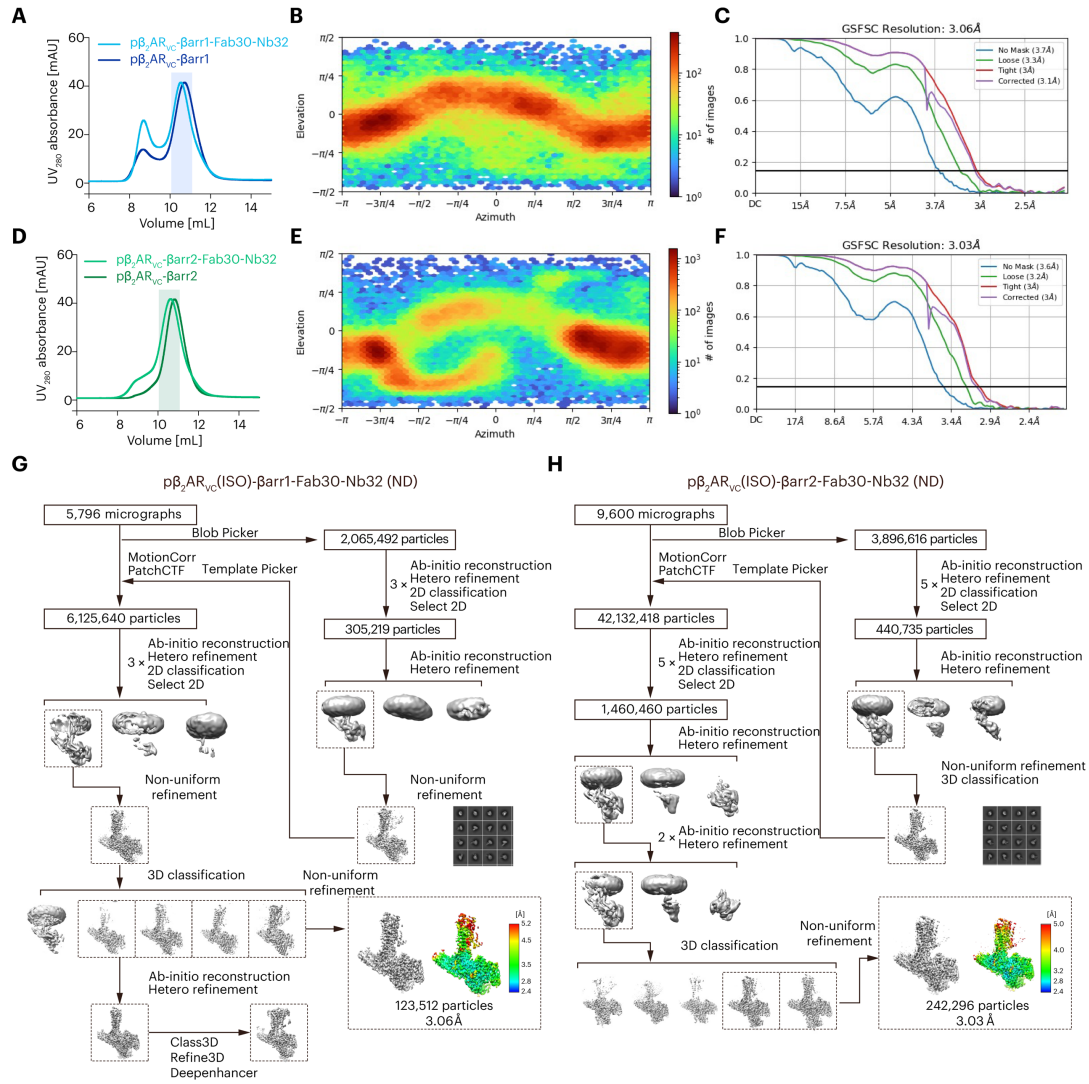


Fig. S9. Protein preparation, cryo-EM data-processing workflow, and resolution assessment for $p\beta_2AR_{vc}\text{-}\beta arr$ complexes. (A–C) $p\beta_2AR_{vc}\text{-}\beta arr1$: size-exclusion chromatography (SEC) profile, angular plot of the particles used for 3D reconstruction, and gold-standard Fourier shell correlation (FSC) curves for the maps. (D–F) $p\beta_2AR_{vc}\text{-}\beta arr2$: SEC profile, angular plot of the particles used for 3D reconstruction, and gold-standard FSC curves for the maps. (G) Cryo-EM data-processing workflow for the $p\beta_2AR_{vc}\text{-}\beta arr1$ complex. (H) Cryo-EM data-processing workflow for the $p\beta_2AR_{vc}\text{-}\beta arr2$ complex.

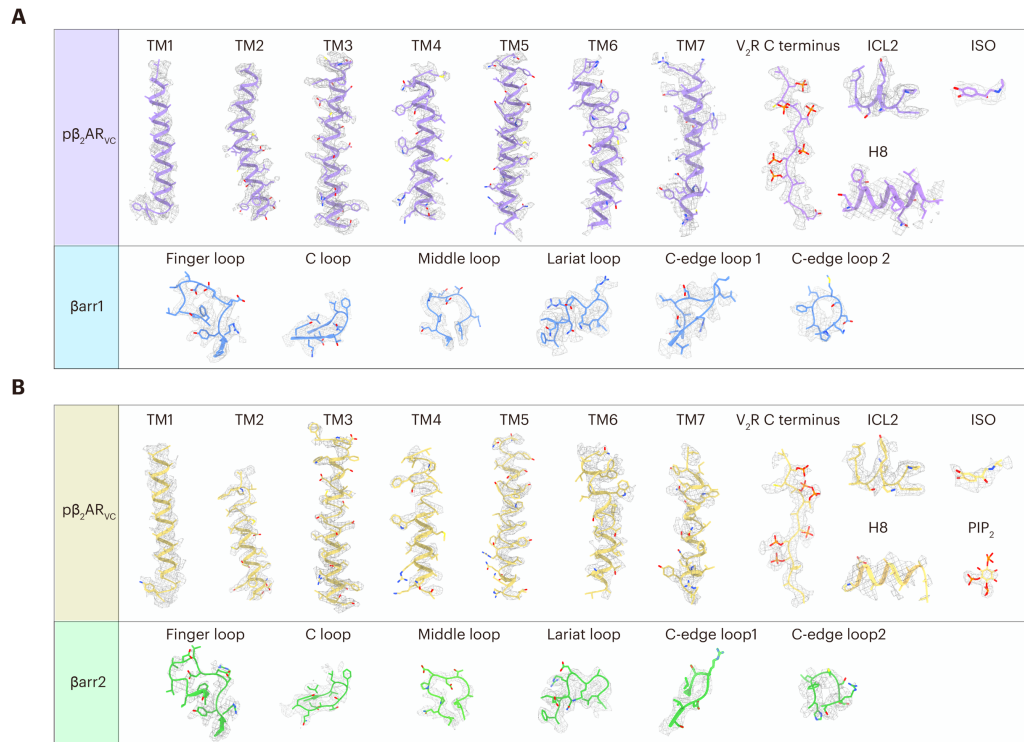


Fig. S10. Cryo-EM density maps with fitted models of the $p\beta_2AR_{VC}$ - βarr complexes. (A) $p\beta_2AR_{VC}$ - $\beta arr1$: cryo-EM density and fitted model are shown for β_2AR TM1–TM7, ICL2, H8, the V_2R C terminus, ISO (top) and for $\beta arr1$ central crest loops and C-edge loops (bottom). Density maps are shown in gray. (B) $p\beta_2AR_{VC}$ - $\beta arr2$: cryo-EM density and fitted model are shown for β_2AR TM1–TM7, ICL2, H8, the V_2R C terminus, ISO and PIP_2 (top), and for $\beta arr2$ central crest loops and C-edge loops (bottom). Density maps are colored gray.

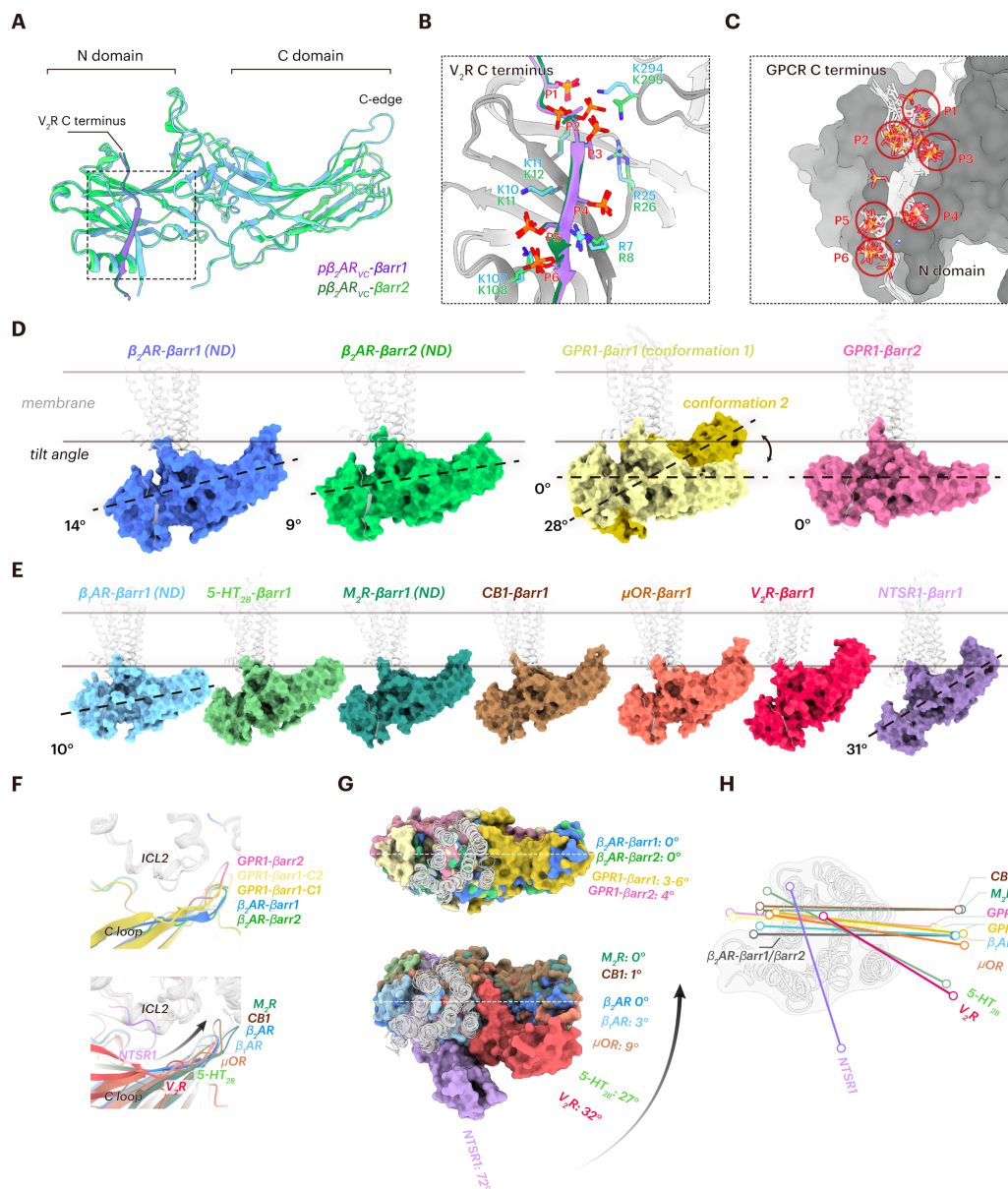


Fig. S11. Comparison of GPCR-βarr complex structures. (A) Overlay of the βarr structures in the $p\beta_2AR_{VC}$ -βarr1 (blue) and $p\beta_2AR_{VC}$ -βarr2 (green) complexes. (B-C) Local structures showing the resolved phosphate groups and their contacts with charged residues in βarr N domain observed in the $p\beta_2AR_{VC}$ -βarr1/2 structures (B), compared with other reported GPCR-arrestin complexes (PDB: 6U1N, 8JRV, 6TKO, 7R0C, 7SRS, 6UP7, 8WU1, 9WSV, 8TIO, 9UYH, 9UYL, 9UYM). (D-E) Tilt angle differences observed in the $p\beta_2AR_{VC}$ -βarr1/2 structures compared with the GPR1-βarr1/2 complexes (PDB: 9UYH, 9UYI, 9UYM) (D), and those observed in other GPCR-βarr1 complexes, including β_1AR -βarr1 (PDB: 6TKO), 5-HT_{2B}-βarr1 (PDB: 7SRS), M₂R-βarr1 (PDB: 6U1N), CB1-βarr1 (PDB: 8WU1), μ OR-βarr1 (PDB: 9WSV), V₂R-βarr1 (PDB: 7R0C), NTSR1-βarr1 (PDB: 6UP7) (E). The dashed line corresponds to the Cα-Cα vector between F149/F150 and L327/L328 of βarr1/2. Structures determined in lipid nanodiscs (ND) are indicated. (F) Comparison of βarr C loop conformations in different GPCR-βarr complexes. The structures are aligned by

the receptor chains. (G) Structural comparison with available GPCR- β arr complexes showing the differences of β arr docking orientation relative to the 7TM core viewed from the extracellular side. For clarity, comparison with the GPR1- β arr1/2 complexes are shown at the top, and comparisons with other GPCR- β arr complexes are shown at the bottom. The structures are aligned by the receptor chains (gray), and the β arrs are shown as surface representations with different colors. The white dashed lines depict the C α -C α vector between β arr1 F149 and L327 (or β arr2 F150 and L328) residues in the p β ₂AR_{VC}- β arr1/2 complex structures, which was used as the reference (0°) to define the in-plane rotation angle of β arrs relative to the 7TM core. (H) A simplified illustration showing both the differences in orientation angle and translational displacement of β arrs relative to the 7TM core in the GPCR- β arr complexes. The lines depict C α -C α vectors between F149/F150 and L327/L328 residues in β arr1/2.

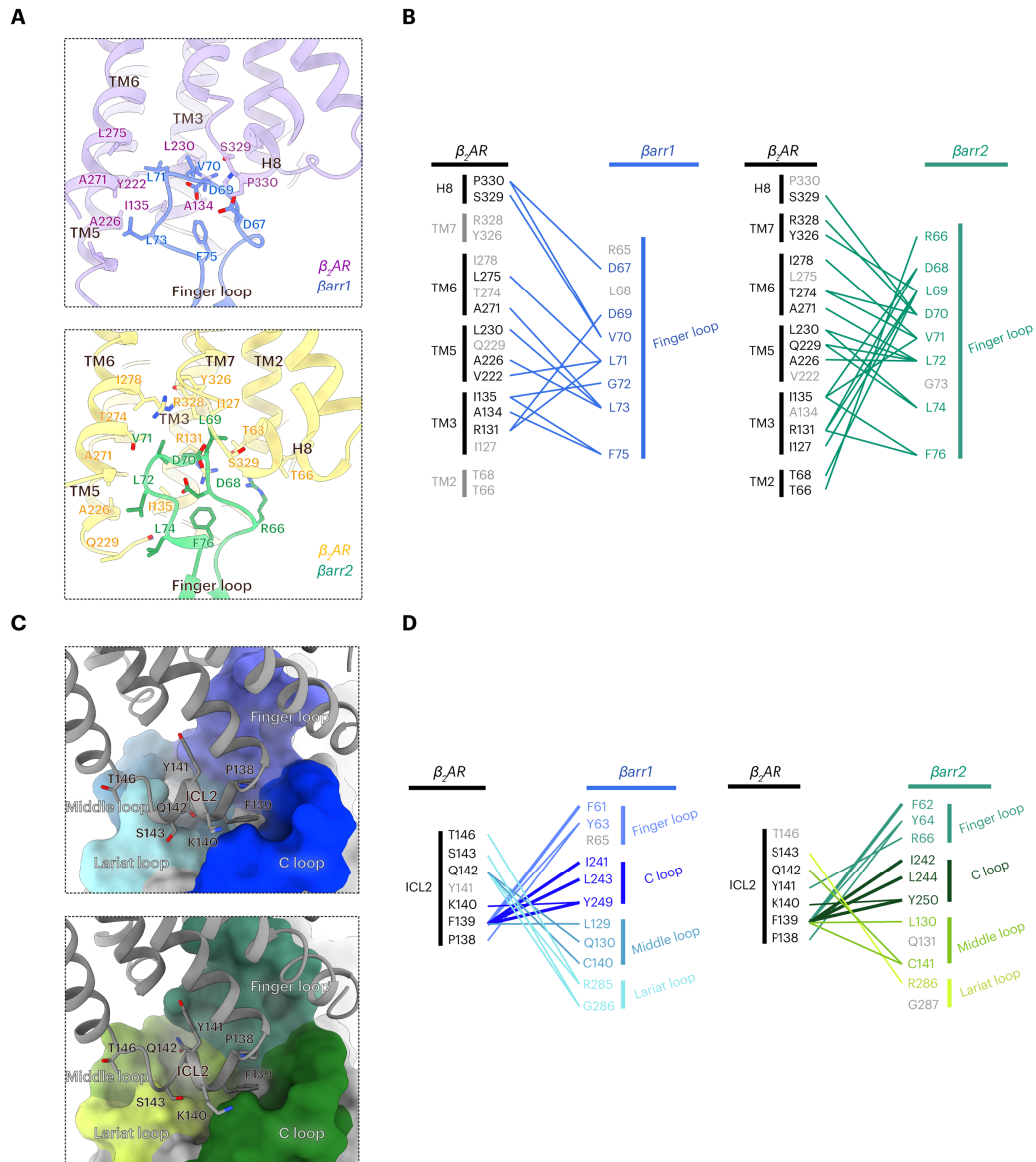


Fig. S12. Comparison of β arr1/2 central crest interactions with the β_2 AR TM core. (A) Local structures showing the interactions of the β arr1 (top) and β arr2 (bottom) finger loops with the β_2 AR TM core. Residues involved in the interactions are shown as sticks. (B) Contact maps between β_2 AR TM core and β arr1/2 finger loops, defined by inter-residue heavy-atom distance cutoff of 4 Å. (C) Detailed interactions between β_2 AR ICL2 and the β arr central crest (top, β arr1; bottom, β arr2). Central crest loops are shown as surface representations, and ICL2 residues are shown as sticks. (D) Contact maps between β_2 AR ICL2 and β arr central crest loops (top, β arr1; bottom, β arr2), using the same color code as in (C).

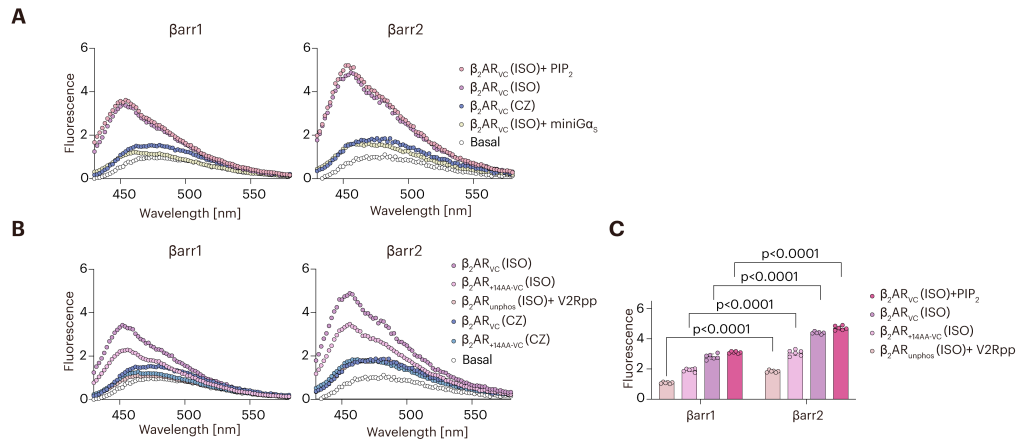


Fig. S13. Finger loop fluorescence of barrs reporting interactions with the receptor TM core under different conditions. (A) Representative mBBR fluorescence measurements of β arr1 V70C and β arr2 V71C in the basal state and in the presence of β_2 AR_{VC} under the following conditions: ISO, CZ, ISO + miniG α_s or ISO + PIP₂. (B) Representative mBBR fluorescence measurements of β arr1 V70C and β arr2 V71C with β_2 AR_{VC} or β_2 AR_{14AA-VC} under CZ- or ISO-bound conditions, or with ISO-bound unphosphorylated β_2 AR supplemented with V2Rpp. (C) Quantification of the mBBR fluorescence measurements shown in panels A and B. Fluorescence was quantified as the area under the curve from 430 to 540 nm. For each β arr construct, fluorescence values were normalized to the mean value of the corresponding basal condition. Dots represent independent experiments and bars indicate mean values $\pm$ SD ($n = 6$ independent experiments). P values were determined by two-way ANOVA followed by Šídák's multiple-comparisons test.

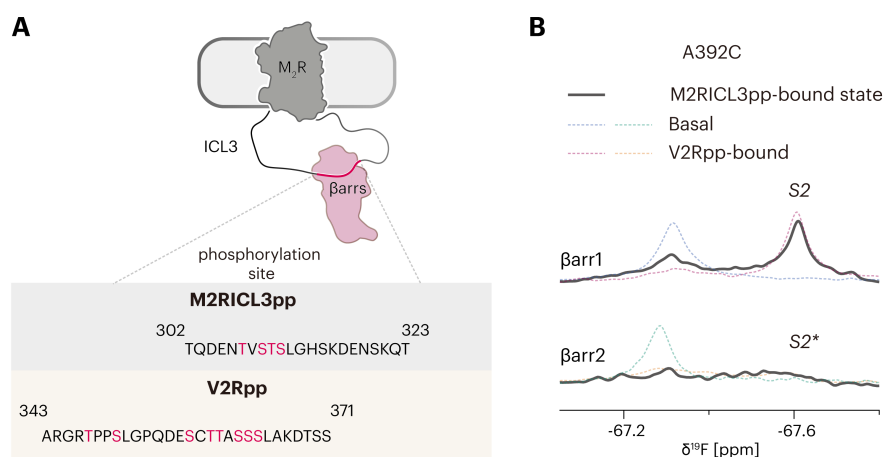


Fig. S14. C-tail conformations of β arr1 and β arr2 upon binding to a phosphopeptide derived from the M₂R ICL3 region. (A) A schematic illustration of β arrs binding to the ICL3 of M₂R in the hanging mode. The sequence of the synthesized peptide M2RICL3pp is shown at the bottom, together with that of V2Rpp. The phosphorylated sites in the peptides are colored in pink. (B) ¹⁹F NMR spectra of the A392C site in both β arrs in the M2RICL3pp-bound state compared with the basal and V2Rpp-bound states.

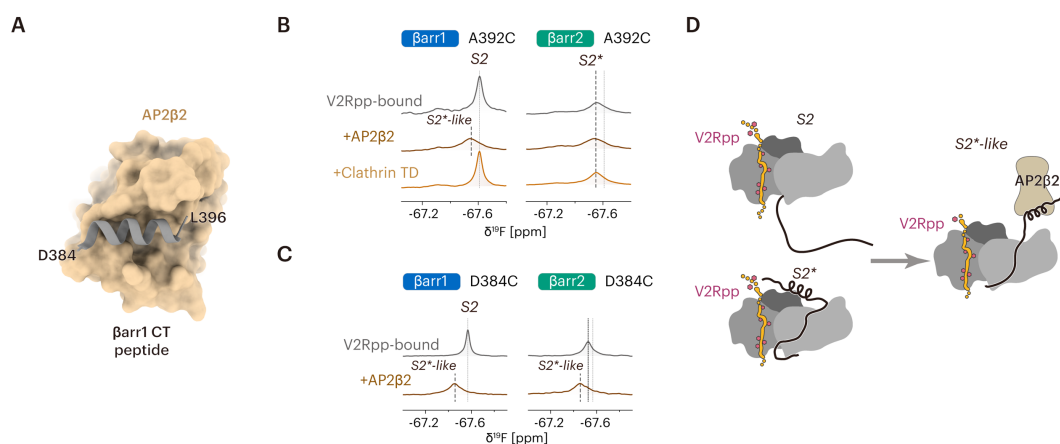


Fig. S15. Monitoring β arr interactions with AP2 β 2 or clathrin-TD by ^{19}F NMR. (A) Structure of a β arr1 C-tail peptide bound to AP2 β 2 (PDB 2IV8). (B) A392C ^{19}F NMR spectra of β arr1 and β arr2 in the V2Rpp-bound state and following the addition of the AP2 β 2 or Clathrin-TD. (C) D384C ^{19}F NMR spectra of β arr1 and β arr2 in the V2Rpp-bound state and following the addition of the AP2 β 2. The experiment was conducted to verify the interaction between β arr2 and AP2 β 2. The D384C resonance in V2Rpp-bound β arr2 is also broadened and appears at an averaged chemical shift between the flexible S2 and the helical S2* state. (D) A schematic model illustrating AP2 β 2 binding to the β arr C-tail following activation.

Table S1. Cryo-EM data collection, refinement and validation statistics

	β_2AR_{VC}-$\beta arr1$ (PDB: 25MU) (EMDB: EMD-80213)	β_2AR_{VC}-$\beta arr2$ (PDB: 25MV) (EMDB: EMD-80214)
Data collection and processing		
Magnification	81,000	81,000
Voltage (kV)	300	300
Electron exposure ($e^-/\text{\AA}^2$)	60	60
Defocus range (μm)	-1.0 ~ -1.5	-1.0 ~ -1.5
Pixel size ($\text{\AA}$)	1.07	1.07
Symmetry imposed	C1	C1
Initial particle projections (no.)	6,125,640	42,132,418
Final particle projections (no.)	123,512	242,296
Map resolution ($\text{\AA}$)	3.06	3.03
FSC threshold	0.143	0.143
Map resolution range ($\text{\AA}$)	2.68 ~ 42.01	2.59 ~ 42.66
Refinement		
Initial model used	AlphaFold 3	β_2AR_{VC} - $\beta arr1$
Model resolution ($\text{\AA}$)	3.21	3.16
FSC threshold	0.5	0.5
Map sharpening B factor ($\text{\AA}^2$)	-84.5	-89.6
Model composition		
Non-hydrogen atoms	8,409	8,634
Protein residues	1,100	1,127
Ligands	1	2
B-factors ($\text{\AA}^2$)		
Protein	12.12/155.16/72.33	20.48/148.45/67.73
Ligand	135.88/135.88/135.88	131.10/136.08/132.84
R.m.s. deviations		
Bond lengths ($\text{\AA}$)	0.003	0.006
Bond angles ($^\circ$)	0.606	0.749
Validation		
MolProbity score	1.86	2.23
Clashscore	10.20	14.79
Rotamer outliers (%)	0.00	0.22
Ramachandran plot		
Favored (%)	95.20	89.89
Allowed (%)	4.61	9.65
Disallowed (%)	0.19	0.46