

Supplementary Figures:

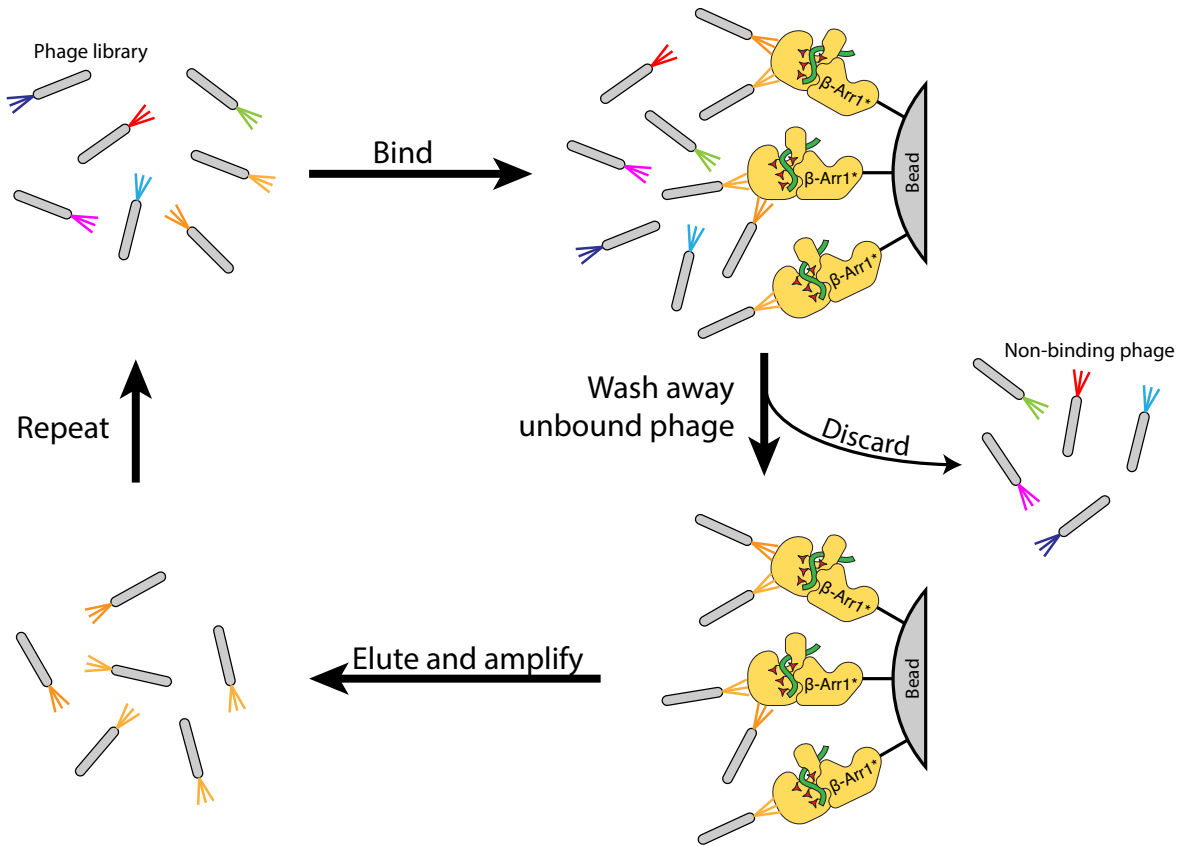


Figure S1. Phage display based selection of Fabs against β -arrestin1:V₂Rpp. Purified β -arrestin1 (full length) was biotinylated, incubated with V₂Rpp and immobilized on magnetic streptavidin beads. Immobilized β -arrestin1 was incubated with the phage library followed by washing to remove the non-specific phages. Specifically bound phages were eluted and amplified in *E. coli* for next round of selection.

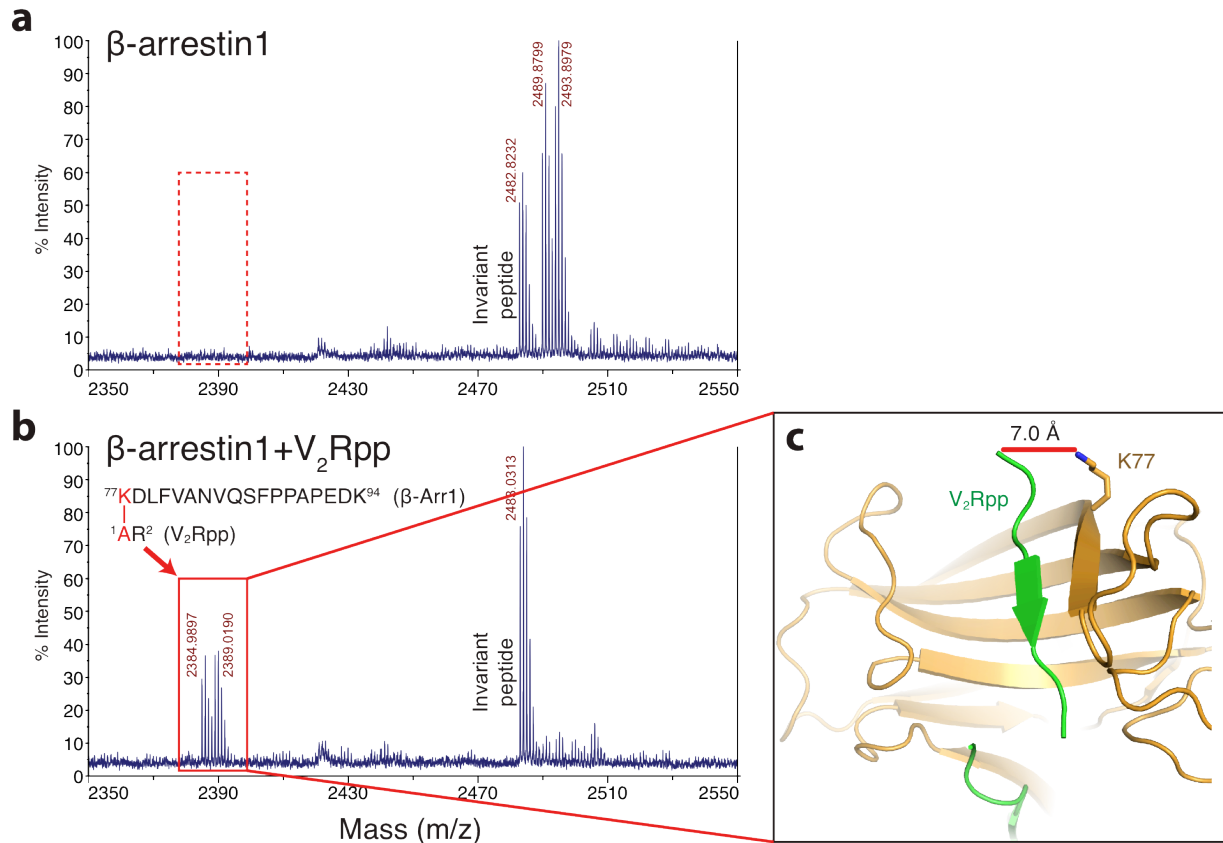


Figure S2. Crosslinking analysis of V₂Rpp binding is consistent with the crystal structure. **a**, Mass spectrometry peaks of β -arrestin1 in the absence of V₂Rpp are shown between m/z of 2350 and 2550. **b**, In the presence V₂Rpp, the homobifunctional amine-reactive crosslinker bisulfosuccinimidyl suberate (BS3) crosslinks K77 of β -arrestin1 with the amino terminus of V₂Rpp. Efficient crosslinking gives rise to a doublet peak separated by an m/z ratio of 4.0193, which is consistent with the 1:1 stoichiometry of BS3-d₀:BS3-d₄ used for this experiment. The peak highlighted here was assigned by MALDI-TOF/TOF MS/MS fragmentation spectra. **c**, Crosslinking data are consistent with the active state crystal structure presented here, where K77 is in close proximity to the resolved amino terminus of V₂Rpp.

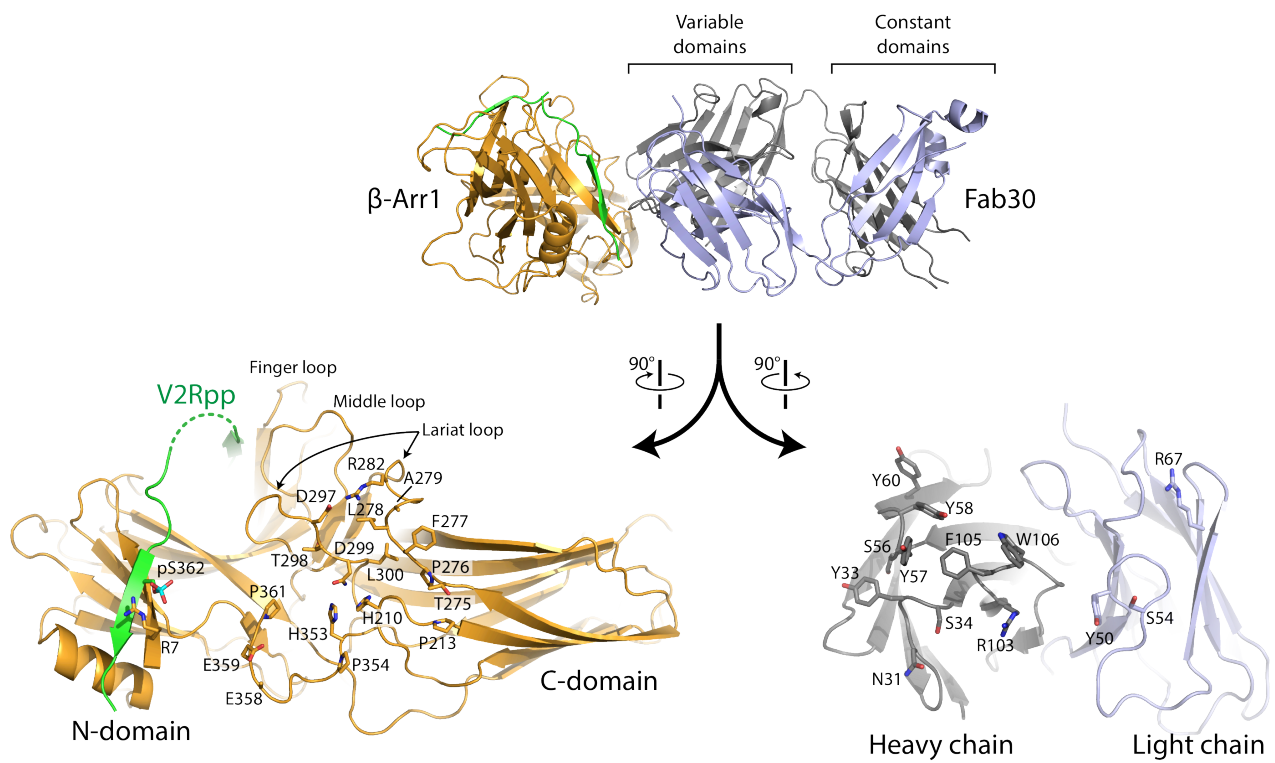


Figure S3. An overview of the β -arrestin1:Fab30 interaction interface. Residues within 4 Å of the interaction surface are shown as sticks on β -arrestin1 and on the heavy and light chains of Fab30.



Lattice contacts (red) Fab contacts (blue)

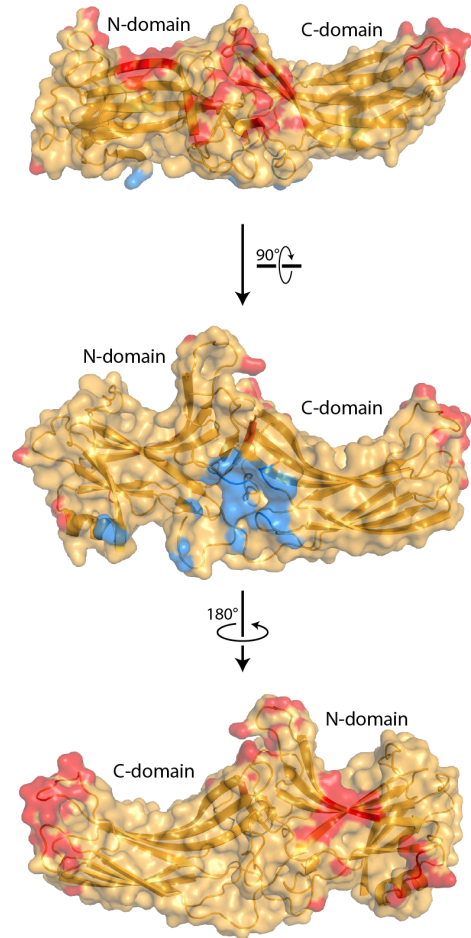


Figure S4. Crystals and lattice contacts of β -arrestin1:V₂Rpp:Fab30. Large three dimensional crystals of the β -arrestin1:V₂Rpp:Fab30 complex in hanging drop crystallization screens. Within the high symmetry $I2_12_12_1$ crystallographic lattice, crystal contacts for the β -arrestin1 molecule are highlighted in red. Contacts between β -arrestin1 and Fab30 are highlighted in blue.

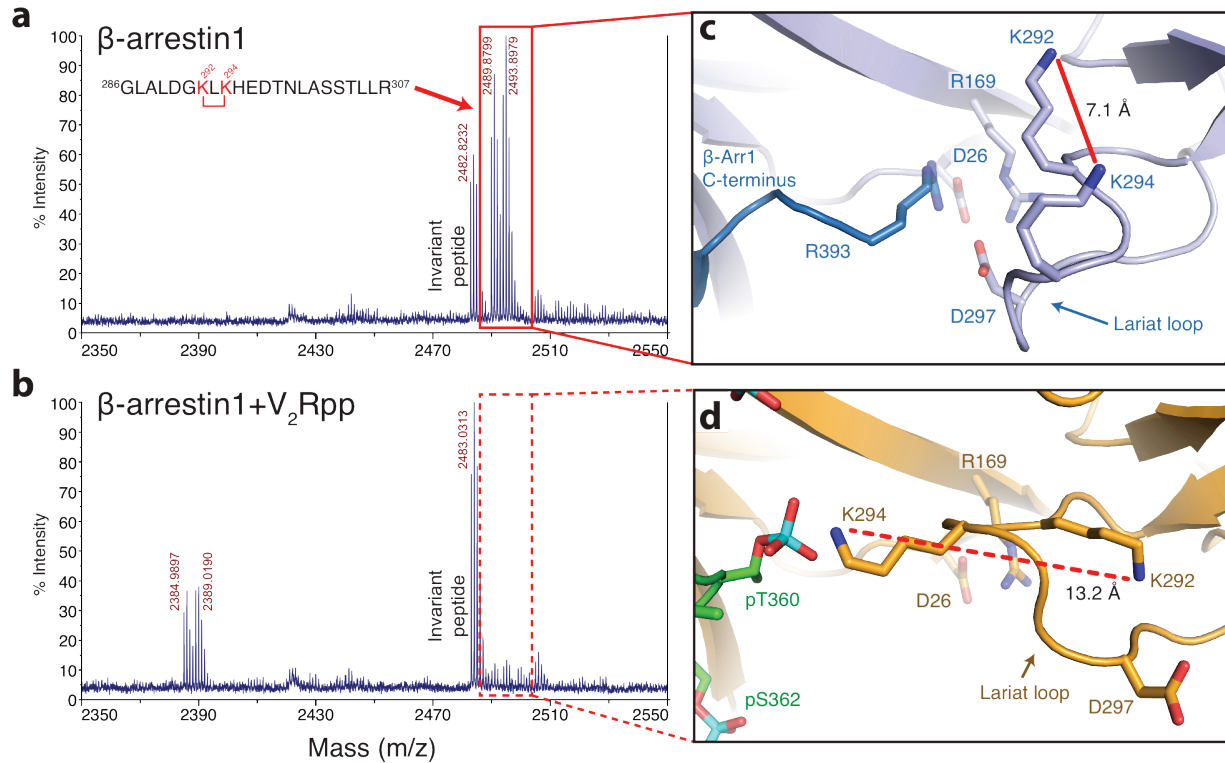


Figure S5. Crosslinking analysis shows changes in lariat loop conformation. **a**, In the absence of V₂Rpp, β -arrestin1 residues K292 and K294 become crosslinked in the presence of the homobifunctional amine-reactive crosslinker bissulfosuccinimidyl suberate (BS3). Efficient crosslinking gives rise to a doublet peak separated by an m/z ratio of 4.0193, which is consistent with the 1:1 stoichiometry of BS3-d₀:BS3-d₄ used for this experiment. **b**, In the presence of V₂Rpp, conformational changes in β -arrestin1 prevent efficient crosslinking. These results are consistent with the distances between K292 and K294 observed in inactive (**c**) and active (**d**) structures of β -arrestin1. The peak highlighted here was assigned by MALDI-TOF/TOF MS/MS fragmentation spectra. Because experiments were performed in the absence of Fab30, they further suggest that the conformation of β -arrestin1 observed crystallographically represents a V₂Rpp induced active state.

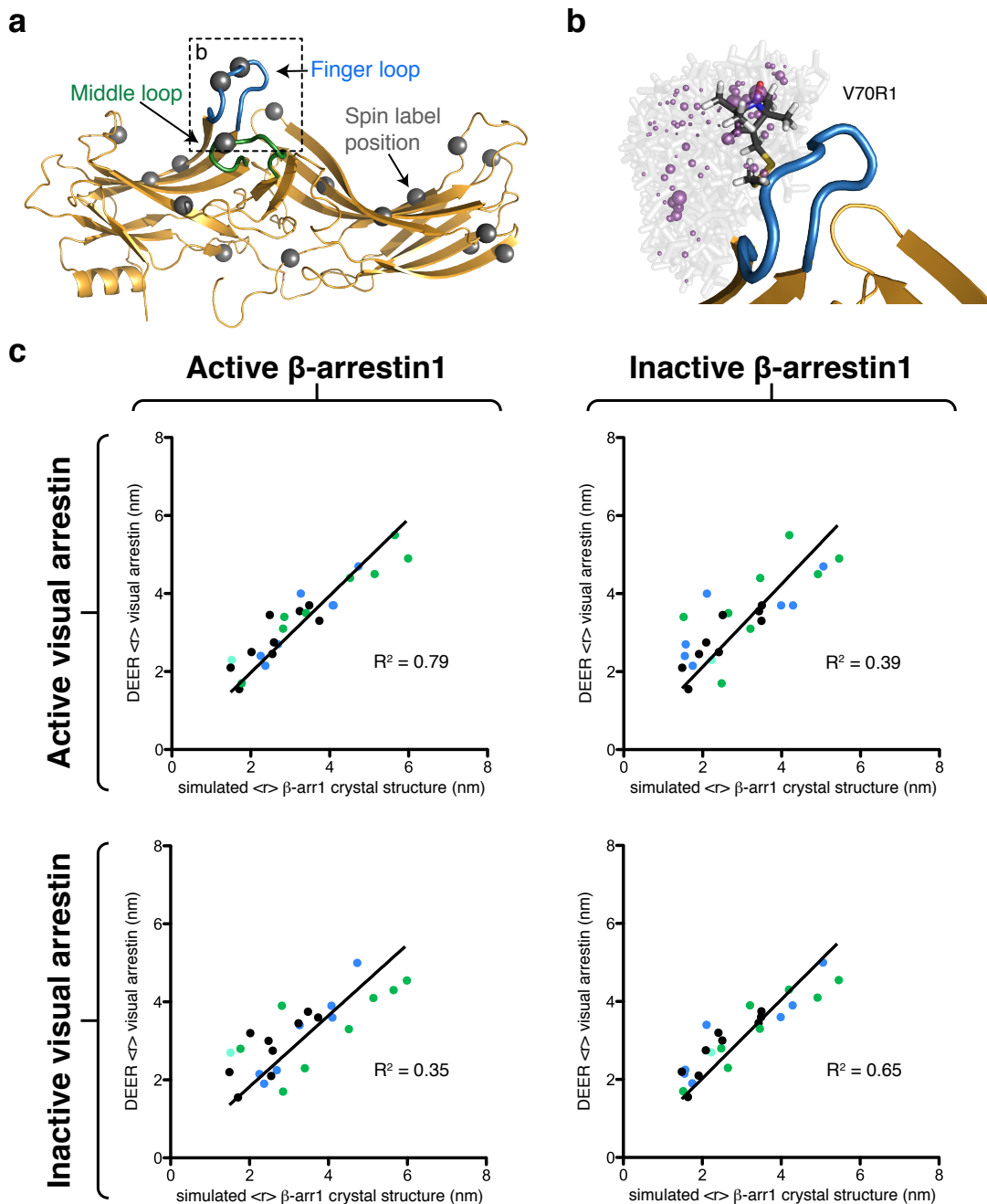


Figure S6. β -arrestin1 crystal structure is consistent with DEER distance measurements on active visual arrestin¹. In order to compare the activated β -arrestin1 crystal structure with mean interspin distances derived from active visual arrestin, we modeled spin labels onto the active β -arrestin1 structure. This analysis utilizes a rotamer library approach within the MMM software package². **a**, Sites that were spin labeled in visual arrestin are shown mapped onto the structure of β -arrestin1 grey spheres at their C α -atoms. Each site shown here was modeled with a rotamer library of spin label conformations, and the predicted interspin distance was then assessed. **b**, Predicted conformational distribution of the methanethiosulfonate spin

label side chain (R1) at position 70 of β -arrestin1. Purple spheres visualize the location of the midpoint of the N-O bonds. Relative sphere volume encodes the relative population of spin label rotamers. The most probable MTSSL rotamer is shown as an opaque stick model. **c**, Comparison of simulated mean interspin distances of β -arrestin1 crystal structures with DEER distances measured for active and inactive visual arrestin. Mean interspin distances between pairs including positions in the finger loop or the middle loop are highlighted in blue or green, respectively. Distances between sites in both the finger and middle loop are colored in cyan, and distances between other positions in the N- and C-domain of β -arrestin1 are shown in black.

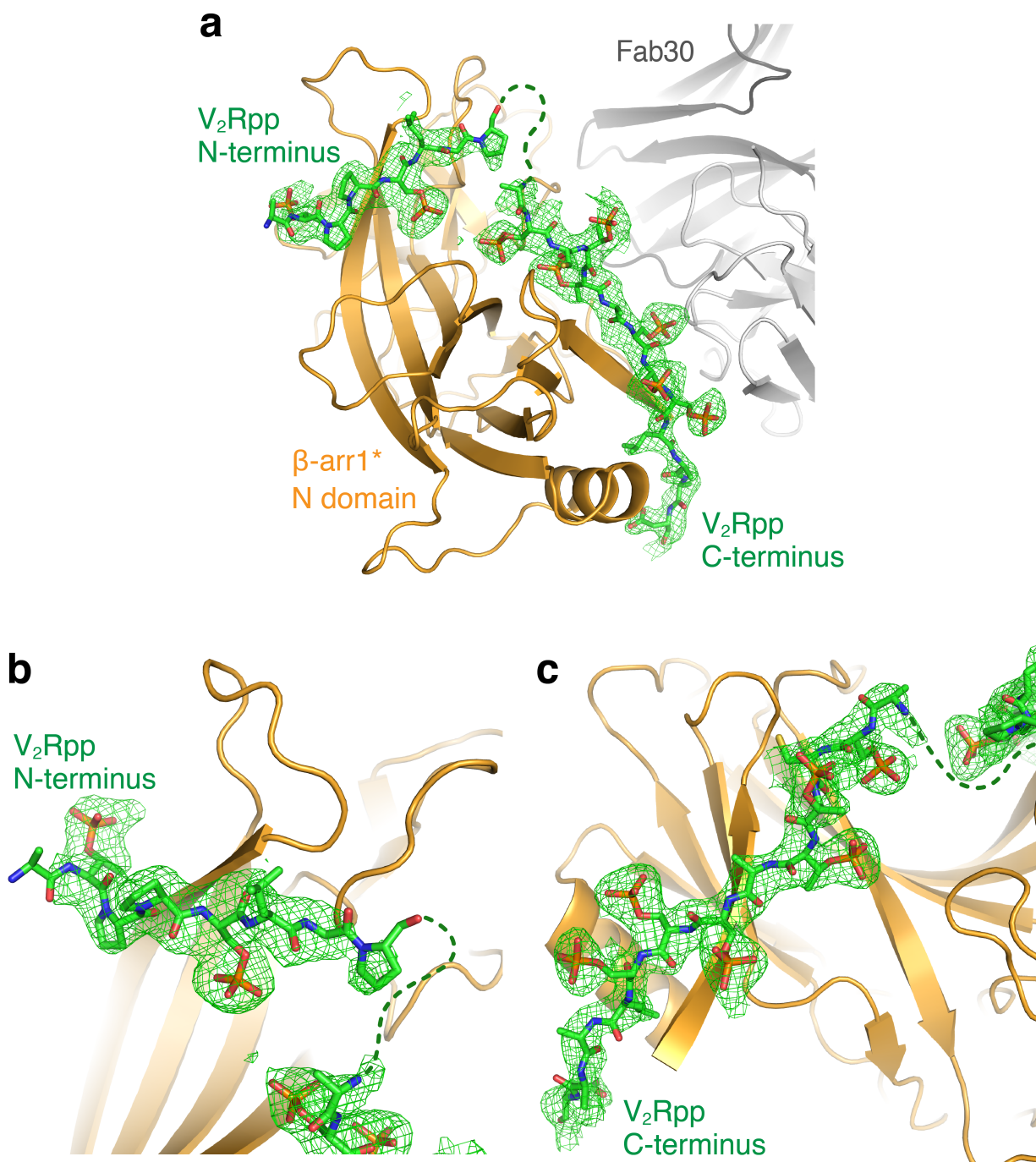


Figure S7. Electron density for V₂Rpp. An F_o-F_c omit map of V₂Rpp contoured at 2 σ and with a carve radius of 2.5 Å is displayed here as green mesh. V₂Rpp residues are represented as sticks. The electron density allowed confident placement of phosphoserine and phosphothreonine residues, and allowed subsequent determination of the V₂Rpp amino acid register. Because the electron density for V₂Rpp is discontinuous, dashed lines here represent intervening residues that could not be resolved.

Supplementary methods:

Selection and characterization of Fabs: Purified β -arrestin1 was biotinylated using EZ-Link Sulfo-NHS-S-S-Biotin (Pierce). Typically, 50 μ L of a 5 mg/mL solution of biotin in 10 mM MOPS pH 6.5 was added to 1 mL of 1 mg/mL solution of purified β -arrestin1 and incubated on ice for 30 min. The reaction was stopped with 0.1 M Tris pH 8.0, and excess biotin was removed either by PD-10 desalting column or overnight dialysis. Biotinylation level was assessed by using Streptavidin MagneSphere magnetic beads (Promega). 1-2 biotins were bound to each molecule of β -arrestin1. Biotinylated protein was flash frozen with 20% glycerol in small aliquots.

Selection of Fabs against β -arrestin1:V₂Rpp complex from a synthetic phage display library was performed essentially as described previously³. Briefly, biotinylated β -arrestin1 was incubated with 1:3 mol:mol V₂Rpp on ice for 30 min. Subsequently, 0.1 nmol V₂Rpp: β -arrestin1 complex was immobilized on streptavidin MagneSphere magnetic beads followed by blocking with biotin. 1 mL phage library solution (approximately 1×10^{12} cfu) was added to the immobilized protein and incubated for 15 min at room temperature. Beads were washed 3 times with 1 mL buffer (20 mM HEPES pH 7.5, 150 mM NaCl, 0.01% dodecyl maltoside, 100 nM V₂Rpp) at room temperature, and phage captured on the beads were amplified. The next day, phage were precipitated and used for second round of panning. The concentration of V₂Rpp: β -arrestin1 complex was reduced to 50 nM for the second round. Beads were washed 5 times and bound phage were eluted by incubating with 100 mM DTT at room temperature for 10 min, followed by phage amplification. In the third round of panning, the concentration of V₂Rpp: β -arrestin1 complex was reduced to 10 nM, and a negative selection was performed to enrich phage selective for V₂Rpp-bound β -arrestin1 conformation. For negative selection, phage bound to V₂Rpp: β -arrestin1 complex on beads were incubated with 1 μ M purified β -arrestin1 for 15 min at RT. Subsequently, the phage were eluted with 100 mM DTT at room temperature for 10 min and amplified.

Amplified phage from third round of selection were incubated with either empty magnetic beads or V₂Rpp: β -arrestin1 complex immobilized on magnetic beads to determine an enrichment ratio (defined as number of phage from the V₂Rpp: β -arrestin1 complex vs. number of phage from the empty beads selection). An enrichment ratio of 50-100 was observed. Phage from third round of panning were used for single point ELISA to test their selectivity towards V₂Rpp-bound β -arrestin1. *E. coli* colonies containing individual phagemids were inoculated into 1 mL of 2xTY broth supplemented with 100 μ g/mL ampicillin and M13-KO7 helper phage (NEB), and cultures were grown overnight at 37 °C. Culture supernatants were buffer adjusted by adding concentrated buffer (20 μ L of 100 mM HEPES pH 7.4, 500 mM NaCl, 0.05% dodecylmaltoside) to 80 μ L of phage. Phage were incubated with either empty neutravidin coated wells, immobilized biotinylated β -arrestin1, or V₂Rpp: β -arrestin1 complex. The plates were washed and incubated for 30 min with horseradish peroxidase coupled anti-M13 antibody. The plates were washed and bound phage were visualized using TMB ELISA substrate (Thermo Scientific). The reactions were stopped with 2M phosphoric acid and the absorbance at 450 nm was measured. ELISA-positive clones selective for V₂Rpp bound β -arrestin1 conformation were sequenced and 5 unique clones were chosen for further characterization.

Purification of Fab: The phagemid DNA from the selected Fab clones was used as the template for Kunkel mutagenesis to add a stop codon between the end of Fab heavy chain coding region and the start of phage coat protein pIII coding region. These expression clones were then transformed into *E. coli* M55244 cells, which were grown in CRAP medium (27 mM ammonium sulfate, 14 mM potassium chloride, 2.4 mM sodium citrate, 5.4 g/L yeast extract, 5.4 g/L HyCase SF Casein, 0.11 M MOPS buffer pH 7.3, 0.55% (w/v) glucose, 7 mM magnesium sulfate) supplemented with 100 µg/mL ampicillin. Cells were grown at 30°C for 20 – 24 h, then harvested by centrifugation. Periplasmic extracts were prepared by incubating the cells first in TES buffer (50 mM Tris pH 8.0, 12.5 mM EDTA and 0.125 M sucrose) and then in TES/4 buffer (TES buffer diluted fourfold in water). Samples were centrifuged, and supernatant was loaded on Protein A agarose. Fabs were eluted with 10 mM sodium acetate pH 3.0 with 40 mM NaCl and neutralized with 0.1 M sodium acetate, pH 5.0. Fabs were subsequently purified on a pre-packed Resource S column followed by dialysis in 20 mM HEPES, pH 7.4, 100 mM NaCl and flash frozen with 10% glycerol.

Crosslinking of β -arrestin1 and V₂Rpp: Homobifunctional amine-reactive water-soluble crosslinkers bissulfosuccinimidyl suberate-d0 (BS3-d0) and bissulfosuccinimidyl 2,2,7,7-suberate-d4 (BS3-d4) were purchased from Thermo Scientific. Light and deuterated BS3 stock solutions were prepared in 25 mM HEPES pH 8.0, 150 mM NaCl. In one reaction, V₂Rpp was added to 5 µg of β -arrestin1 at a 5:1 molar ratio and the reaction was incubated at room temperature for 30 min. In a parallel reaction, the same volume of buffer was added to 5 µg of β -arrestin1. After incubation, a 100-fold molar excess of BS3-d0/d4 equimolar mixture was added to both reactions and incubated at room temperature for another 30 minutes. Unreacted crosslinker was quenched by incubation with 100 mM ammonium bicarbonate for 20 min.

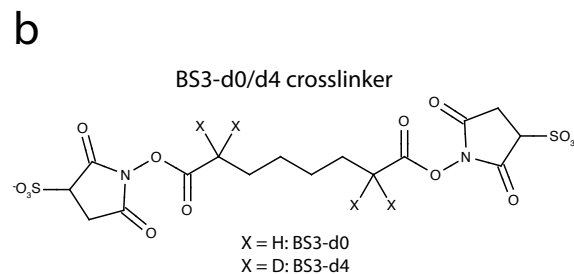
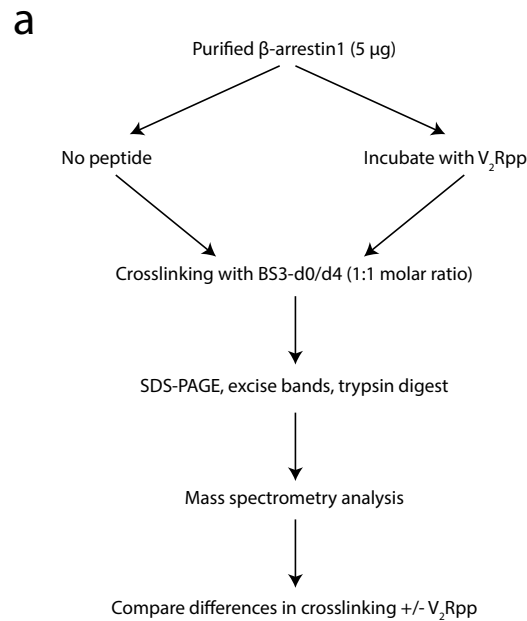
Crosslinked proteins were separated by 4-20% SDS-PAGE gel. Protein bands were excised and subjected to in-gel trypsin digestion. The tryptic peptides were extracted, lyophilized and resuspended in 5% formic acid and desalted on C18 resin, using handmade StageTips. The desalted peptide samples were reconstituted in 50% ACN/0.1% TFA, spotted onto MALDI target plate, followed by addition of equal volume of saturated solution of α -cyano-4-hydroxycinnamic acid in 20 mM ammonium phosphate, 50% ACN and 0.1% TFA. The mixture was allowed to co-crystallize at room temperature before mass spectrometry analysis.

Mass spectrometry analysis of crosslinked peptides: Mass-spectrometry (MS) analysis of crosslinked peptides was performed on an ABI 4700 MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Framingham, MA) equipped with a nitrogen laser operating at 337 nm, a video system, and 4000 Series Explorer software for spectrum acquisition and instrument control. All mass spectra were obtained in the reflection positive-ion mode, at an acceleration voltage of 20 kV and a detector voltage of 2 kV. MS data were automatically acquired over the mass-to-charge range of 800 - 4000 Da, and laser intensity was adjusted to obtain optimized resolution.

Data analyses were performed with Data Explorer software (version 4.3.0.0, Applied Biosystems, Framingham, MA). Peptide peaks from known trypsin auto-proteolytic products were used as internal standards for calibration. After calibration,

data lists of mono-isotopic peptide peak mass and intensity were extracted from baseline corrected spectra. To identify crosslinked peptides, MS spectra were screened manually or with the assistant of DXset program for the presence of ion signal “doublets” in which the mono-isotopic peaks are 4 mass units apart. Doublet peak lists were used as inclusion lists for automatic acquisition of the corresponding MS/MS spectra when the mass spectrometer was in a MALDI-TOF/TOF mode.

For assignment of crosslinked peptides by mass, the MS-Bridge (<http://prospector.ucsf.edu>, Protein Prospector, Mass Spectrometry Facility, University of California San Francisco) was used to predict possible crosslinked peptides. The MS-Bridge analysis was performed by allowing for maximum of two missed cleavage and a mass tolerance of ± 50 ppm. Oxidation of methionines was used as variable modification and carbamidomethylation of cysteines as constant modification. The MALDI-TOF/TOF MS/MS fragmentation spectra were then used to confirm the assigned crosslinked peptides with the help of MS-PRODUCT program in Protein Prospector and ICC-CLASS programs which predict all possible combinations of crosslinked peptide masses.



Supplementary table 1: Data collection and refinement statistics.

Data collection^a	
Number of crystals	1
Space group	I2 ₁ 2 ₁ 2 ₁
Cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	116.4, 125.1, 144.2
α , β , γ (°)	90, 90, 90
Resolution (Å)	40.0 – 2.6 (2.7 – 2.6)
R _{sym} (%)	8.7 (72.0)
$\langle I \rangle / \langle \sigma \rangle$	17.3 (2.1)
Completeness (%)	98.1 (97.5)
Redundancy	5.8 (5.0)
Refinement	
Resolution (Å)	39.3 – 2.6
No. unique reflections	32184 (1628 in test set)
R _{work} /R _{free} (%)	20.3 / 24.9
Number of atoms	5860 (142 in solvent)
Average <i>B</i> -factors (Å ²)	
β-arrestin1	71
Fab30	99
Fab30 Variable	82
Fab30 Constant	124
V ₂ Rpp	92
Solvent	69
R.m.s. deviation from ideality	
Bond length (Å)	0.01
Bond angles (°)	0.78
Ramachandran statistics^b	
Favored regions (%)	95.6
Allowed regions (%)	4.4
Outliers (%)	0

^aHighest shell statistics are in parentheses. ^bAs defined by MolProbity⁴.

Bibliography

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4. Chen, V. B. *et al.* MolProbity: all-atom structure validation for macromolecular crystallography. *Acta. Crystallogr. D. Biol. Crystallogr.* **66**, 12-21 (2010).