Structural basis of ligand binding modes at the neuropeptide Y Y$_1$ receptor

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Neuropeptide Y (NPY) receptors belong to the G-protein-coupled receptor superfamily and have important roles in food intake, anxiety and cancer biology$^{1,2}$. The NPY–Y receptor system has emerged as one of the most complex networks with three peptide ligands (NPY, peptide YY and pancreatic polypeptide) binding to four receptors in most mammals, namely the Y$_1$, Y$_2$, Y$_4$ and Y$_5$ receptors, with different affinity and selectivity$^3$. NPY is the most powerful stimulant of food intake and this effect is primarily mediated by the Y$_1$ receptor (Y$_1$R)$^4$. A number of peptides and small-molecule compounds have been characterized as Y$_1$R antagonists and have shown clinical potential in the treatment of obesity$^6$, tumour$^4$ and bone loss$^3$. However, their clinical usage has been hampered by low potency and selectivity, poor brain penetration ability or lack of oral bioavailability$^4$. Here we report crystal structures of the human Y$_1$R bound to the two selective antagonists UR-MK299 and BMS-193885 at 2.7 and 3.0 Å resolution, respectively. The structures combined with mutagenesis studies reveal the binding modes of Y$_1$R to several structurally diverse antagonists and the determinants of ligand selectivity. The Y$_1$R structure and molecular docking of the endogenous agonist NPY, together with nuclear magnetic resonance, photo-crosslinking and functional studies, provide insights into the binding behaviour of the agonist and for the first time, to our knowledge, determine the interaction of its N terminus with the receptor. These insights into Y$_1$R can enable structure-based drug discovery that targets NPY receptors.

NPY is a highly abundant neuropeptide in the central nervous system$^7$. The first characterized NPY receptor Y$_1$R is widely expressed in a variety of tissues and is involved in the regulation of many physiological functions, some of which are known to be related to obesity$^8$ and cancer$^9$. To better understand the ligand-binding behaviour of NPY receptors and provide a basis for drug discovery, we solved crystal structures of Y$_1$R in complex with two structurally diverse antagonists, UR-MK299, an argininamide with high Y$_1$R selectivity$^{10}$, and BMS-193885, which displays anorectic activity in animal models$^6$ (Fig. 1 and Extended Data Table 1). To facilitate the determination of structure, an engineered Y$_1$R construct was designed (see ‘Cloning and protein expression’ in Methods).

Within the β-branch of class A G-protein-coupled receptors (GPCRs), to which NPY receptors belong, the structures of four receptors, namely the neurotensin receptor NTS1$^{11}$, the OX$_1$ and OX$_2$ orexin receptors$^{12,13}$ and the endothelin ET$_B$ receptor$^{14}$, have been determined so far. These structures reveal differences of ligand-binding modes between different receptors, suggesting that more structural information is needed to develop any consensus about the ligand recognition mechanisms for this GPCR subfamily. The Y$_1$R structure shares a canonical seven-transmembrane helical bundle (helices I–VII) with the other known GPCR structures (Fig. 1 and Extended Data Fig. 1a, b). The Y$_1$R–UR-MK299 and Y$_1$R–BMS-193885 complexes are structurally similar with a C$_{\text{root}}$ mean-square deviation (r.m.s.d.) of 0.75 Å within the helical bundle. Both structures exhibit inactive conformations with helix VI adopting an inward conformation that is similar to other inactive GPCR structures. UR-MK299 binds to Y$_1$R in a cavity within the helical bundle bordered by helices IV, III, IV, V and VII (Fig. 2a, b). The diphenylmethyl moiety of the antagonist interacts with a hydrophobic cluster formed by F282$^{65}$, F286$^{68}$ and F302$^{75}$ (superscripts on residues throughout the text indicate Ballesteros–Weinstein nomenclature$^{15}$) on helices VI and VII of Y$_1$R. The critical role of this hydrophobic patch in recognizing the argininamide-type

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Y₁R antagonist was confirmed by the NPY-induced inositol phosphate accumulation of Y₁R when inhibited by UR-MK299 and several related Y₁R antagonists—BIBP3226, BIBO3304, UR-HU404 and UR-MK289 (Extended Data Fig. 1e–i). The F302Y mutation abolishes the antagonistic activity for all these antagonists, and a two- to fivefold decrease in the antagonistic effect of all tested antagonists was observed for the F286Y mutation (Fig. 3a–c, Extended Data Fig. 2 and Extended Data Table 2). The hydroxyphenyl group of UR-MK299 sits in a groove formed by helices III and VI of the receptor, enabling hydrophobic contacts with residues Q120, C121, I124, W276 and L279. In Y₁R and Y₂R, Q120 is suggested to be the interaction partner for the C terminus of NPY and crucial for receptor activation. In the Y₁R–UR-MK299 structure, this residue forms a hydrophobic contact with the phenyl ring of the hydroxyphenyl group in UR-MK299, potentially blocking the binding of Y₁R to NPY. Mutagenesis data show that the Q120N mutation does not influence the inhibitory effect of Y₁R antagonists on NPY signalling, but a mutation to histidine increases the antagonistic activity of these ligands (Fig. 3d, e and Extended Data Table 2), suggesting that an additional stacking interaction with the antagonist is beneficial at this position. The highly conserved residue W648 represents the ‘toggle switch’ and was suggested to trigger receptor activation through a conformational change in various GPCRs. In the Y₁R–UR-MK299 structure, the residue W276 is in a conformation that is similar to those observed in other inactive class A GPCR structures and is distinct from their active-state conformations. The hydroxyphenyl group of UR-MK299 forms a hydrophobic contact with W276, potentially preventing its activation-related motion thus stabilizing the receptor in an inactive conformation. Compared to the wild-type receptor, the Y₁R mutant W276Δ48 displays an over 2,000-fold decrease in its binding affinity to [³H]UR-MK299 (Extended Data Table 3) and reduced the antagonistic activity of the arginine-type Y₁R antagonists by four- to sevenfold (Fig. 3f and Extended Data Table 2), supporting its important role in antagonist recognition. Residues N283 and D287 were suggested as the most important amino acids for Y₁R ligand recognition. In the Y₁R–UR-MK299 structure, N283 is engaged in two hydrogen bonds with the – nitrogen and the carboxylic oxygen next to the hydroxybenzylamine moiety of UR-MK299. D287 builds a salt bridge with the protonated guanidinyl moiety and a hydrogen bond with the carbamoyl group, in agreement with a decrease in antagonist affinity when the carbamoyl group was replaced by an alkoxycarbonyl, acyl or alky group. The mutants N283D and D287N displayed a notable loss of NPY-induced receptor function, a complete abolishment of antagonistic activity for the small-molecule antagonists (Fig. 3g, h and Extended Data Table 2), and an over 2,000-fold decrease in the binding affinity of Y₁R to [³H]UR-MK299. The latter was reflected by a 30-fold decrease in the binding affinity of Y₁R to UR-MK299 mutants Q219 and Q219EEV (Extended Data Table 3). Extra empty space at the bottom of the sub-pocket is observed in the Y�R–UR-MK299 structure, suggesting that a larger substituent may be allowed (Extended Data Fig. 1c). This is supported by studies showing that other carbamoylated arginine-type Y₁R antagonists containing longer carbamoyl chains, such as UR-MK136 (Extended Data Fig. 1j), bind to the receptor with a relatively high affinity.

UR-MK299 was reported to exhibit high Y₁R selectivity (Y₁R, inhibition constant (Ki) > 3,000 nM; Y₂R and Y₄R, Ki > 10,000 nM) and specificity compared to two related neuropeptide FF (NPFF) receptors (NPFR, Ki > 1,000 nM; NPFFR, Ki > 3,000 nM). Sequence alignment reveals that most of the key residues involved in UR-MK299 binding are conserved between Y₁R, the other NPY receptors and the NPFFRs, except for F4.66, Q5.46, N6.55 and F6.58 (Extended Data Fig. 3), indicating the importance of these four residues in terms of the selectivity and specificity of UR-MK299. Y₁R is the only NPY receptor with F4.66 instead of Q4.66, preventing key polar contacts. In Y₁R, E6.58 disturbs the F6.54, F6.58, F7.35 hydrophobic patch and probably mediates selectivity, supported by the F6.58E mutation in Y₁R reducing binding affinity for BIBP3226, which contains the same diphenylmethyl group as UR-MK299. Similarly, hydrophilic residues at key positions impede high-affinity binding at Y₁R (T6.58 and NPFFR, (S6.58), while the hydrophobic pocket is preserved in NPFFR, (L5.54, I6.58, F7.35), although with less bulk, leading to a moderate affinity of BIBP3226 (Kᵢ = 18 nM).

The ligands BMS-193885 and UR-MK299 occupy a similar binding pocket within the helical bundle of Y₁R (Fig. 2c, d and Extended Data Fig. 1k). The dihydropyridine group of BMS-193885 fits in a sub-pocket formed by helices III, V and VI, which aligns with previous structure–activity relationship studies showing that larger substituents at position three of the dihydropyridine ring reduced Y₁R binding affinity. Residue T280 forms a hydrogen bond with the nitrogen of the dihydropyridine ring as confirmed by our mutagenesis studies, which showed that the T280F mutation decreased the binding affinity of BMS-193885 by about 330-fold (Extended Data Table 3), in agreement with a reported lower affinity N-methylated derivative.

Additionally, the dihydropyridine ring makes a hydrophobic contact with residue I124, which is consistent with a 400-fold decrease in affinity.
the affinity of the mutant I124F3.36A (Extended Data Table 3). It was also reported that methylation of either nitrogen of the urea group of BMS-193885 decreased the binding ability of the methylated derivatives to Y1R24, suggesting that these hydrogen bond donors are critical for Y1R recognition. Indeed, in the BMS-193885-bound Y1R structure, the urea group forms hydrogen bond interactions with D2876.59. Similar to the diphenylmethyl group of UR-MK299, the piperidine and methoxyphenyl rings of BMS-193885 form extensive hydrophobic contacts with the diphenylmethyl group of UR-MK299, the piperidine and methoxyphenyl group forms hydrogen bond interactions with D287 6.59. Similar to the recognition. Indeed, in the BMS-193885-bound Y1R structure, the urea 193885 decreased the binding ability of the methylated derivatives to

Understanding the binding mode of the endogenous agonist NPY at a molecular level will facilitate the rational development of Y1R-selective ligands. The C-terminal pentapeptide of NPY was found to be essential for binding to the NPY receptors25. Because the hydroxyphenyl and the argininamide group of UR-MK299 mimic R35 and Y36 in the C terminus of NPY (Extended Data Fig. II), the crystal structure of Y1–UR-MK299 serves as a good template for molecular docking of the agonist. To aid docking, complementary mutagenesis studies were performed to determine corresponding interaction partners between Y1R and NPY (Extended Data Table 4a). Furthermore, solid-state nuclear magnetic resonance (NMR) chemical shift measurements revealed residue-specific alterations of the secondary structure of NPY upon binding to Y1R (Extended Data Fig. 4). Several key Y1R–NPY contacts identified by the mutagenesis studies were used to guide NPY docking in Rosetta26 with the final models being filtered against the NMR data to generate a final ensemble that best represents the combined data. The NPY-bound model reveals a relatively flat NPY–Y1R binding pose with the C-terminal tetrapeptide R33–Y36, identified as either a random coil or a β-strand structure in NMR, penetrating into the binding pocket (Fig. 4a). The unstructured N terminus (Y1–P13) is in close proximity to the second extracellular loop (ECL2), while the α-helix in the middle region of NPY (A14–T32) lies along ECL1 and ECL3 and points away from ECL2.

Inspection of the binding pocket of NPY reveals that the binding pose of residue R35 of NPY is similar to that of the argininamide of UR-MK299 (Fig. 4b). R35 forms a salt bridge with the D2876.59 residue of Y1R and approaches N2836.55 (Fig. 4c). The NPY mutant R35A displays a decrease in activity of over 6,000-fold, which represents the highest influence on agonist potency of all tested NPY analogues (Extended Data Table 4a), supporting the importance of the positively charged residue in NPY recognition. Aspartate or glutamate residues are not found at position 6.59 in any peptide GPCRs except for the receptors that bind to Arg–Phe-amide peptides, including NPFF, prolactin-releasing peptide and pyroglobularated Arg–Phe-amide peptide, which share a common C-terminal Arg–Phe–NH2 motif, supporting the hypothesis that the arginine residue may function in a manner that
is similar to that of the R35 of NPY by interacting with the conserved D/E98.99 of the respective receptors.27 In contrast to the similarity between the binding modes of the R35 of NPY and the guandine group of UR-MK299, the C-terminal tyrosinamide of NPY and the hydroxyphenyl group of UR-MK299 show different orientations. The hydroxyphenyl ring is oriented towards helix V (Q219–46) in the UR-MK299-bound Y1R structure, whereas Y36 of NPY points towards the Q120–32 residue on helix III in the NPY-docked model (Fig. 4c). This may arise from the opposite configuration of the stereocentre in the R35 of NPY and UR-MK299, as well as by only partial mimicking of the Y36–NH2 of NPY by a 4-hydroxybenzyl group in UR-MK299 (Extended Data Fig. 1g, l). In the Y1R–UR-MK299 structure, residue Q120–32 forms a hydrophobic contact with the hydroxyphenyl group of the antagonist. By contrast, the NPY-bound model shows that the side chain of Q120–32 points almost in the opposite direction and engages in a hydrogen bond with the hydroxy group of Y36–NH2 (Extended Data Fig. 1d), in a similar manner to the previously suggested interaction between the Y6–R residue Q132 and NPY.28 In Y2R, it was also reported that Q132 may interact with the C-terminal amide of NPY16. Inositol phosphate accumulation studies show that the Y1R mutation Q120–32H leads to a 26-fold decrease in the potency of NPY, and NPY–tyramide lacking the C-terminal amide displays a 45-fold loss of activity. Complementary mutagenesis analysis revealed an additional reduction of NPY–tyramide potency at the Q120–32H mutant, and thus rules out a direct contact between the C-terminal amide of NPY and Q120–32 in Y1R (Extended Data Table 4a). Additionally, Y36 of NPY forms hydrophobic contacts with Y100–64 and W106ECL1 in Y1R (Fig. 4c). Although Y100–64 is not involved in antagonist binding, mutagenesis data suggest a critical role in agonist recognition as the Y1R mutant Y100–64A displays a 284-fold decrease in potency for NPY (Fig. 3i and Extended Data Table 2). Furthermore, the model reveals close contacts between L30 of NPY and I293 in ECL3 of Y1R and between R33 of NPY and the Y1R residue N299–32 (Fig. 4c), which align with complementary mutagenesis data showing no further loss of function for combining mutant I293N with [N30]NPY and N299–32A with [A33]NPY (Fig. 3j–m and Extended Data Table 4a).

Previous studies have shown that different NPY receptors behave differently when binding to the N terminus of NPY. Y1R and Y3R can bind to N-terminally truncated NPY, whereas Y5R and Y1R require the complete N terminus of NPY for full agonist potency.25,27 However, these data did not allow conclusions about the interaction of the N terminus of NPY with the receptor. To further explore the involvement of the NPY N terminus in recognition between the receptor and ligand, we performed mutagenesis studies, showing that truncation of the first two residues of NPY (NPY(3–36)) reduces peptide potency by more than 50-fold (Extended Data Table 4b). This decrease in potency, however, is not seen when these residues are mutated to alanine ([(A1A2)NPY, fivefold shift in the half-maximal effective concentration (EC50)], suggesting important contributions of the peptide backbone in binding to the receptor. Our NPY-bound model suggests that the N-terminal region of NPY makes close contacts with the T180–F199 fragment in ECL2 of Y1R and is also in proximity to the receptor N terminus (Fig. 4a, d). To experimentally verify interacting sites in the receptor, we performed photo-crosslinking studies between NPY analogues carrying the highly reactive p-benzoyl-phenylalanine [Bpa], K5[(Ahx)2-biotin]}NPY (in which Ahx denotes aminoheaxanoate) and Y1R. Crosslinking sites were assigned to two regions in Y1R, the N terminus (K21–D32) and the ECL2 (A191–D205) (Extended Data Fig. 5 and Extended Data Table 5). Previous studies demonstrated that deletion of the Y1R N terminus does not interfere with receptor signalling, but reduces NPY binding by about 95% compared to the full-length receptor.23 This creates the possibility that the N terminus of Y1R has a role in recognizing and positioning the peptide ligand, which is in agreement with the photo-crosslinking data. Consistent with the crosslinking hits in receptor ECL2, our mutagenesis data show that the F184A/N and V197N mutations in this region greatly reduce NPY potency (Extended Data Table 4c). Together, these data suggest that the N terminus and ECL2 of Y1R have important roles in the recognition of the N terminus of NPY and receptor activation. This contrasts with NPY binding at Y3R, in which ECL2 may interact with the central α-helix of NPY and the peptide N terminus is flexible and not anchored by the receptor.16 Although this study provides insights into the interactions between Y1R and NPY, further structural details, such as the structures of Y receptors bound to NPY, are required to fully understand the endogenous agonist-binding modes of the NPY receptor family.

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Author contributions Z.Y. and S.H. optimized the construct, developed the purification procedure, purified the Y$_1$R protein for crystallization, performed crystallization trials, solved the structures and wrote the manuscript. M.K., D.W., G.B., N.P. and T.L. synthesized the compounds, designed, performed and analysed the ligand-binding assay. A.K., K.B. and L.M.K. performed peptide synthesis, inositol phosphate accumulation assays, the photo-crosslinking assay and mass spectrometry after crosslinking. B.J.B. helped to refine the Y$_1$R–UR-MK299 structure and modelled the Y$_1$R–NPY complex. M.B. and P.S. performed NMR analysis and analysed NMR data. C.Y. expressed the Y$_1$R proteins. B.L. helped with construct and crystal optimization. S.Y., R.Z., B.X., D.L., R.C.S., D.H., J.M., A.G.B.-S. and A.B. helped with structure analysis, interpretation and edited the manuscript. R.C.S. helped to initiate the project. D.H. overviewed NMR studies. J.M. overviewed molecular docking. Q.Z. collected X-ray diffraction data and solved the structures. A.G.B.-S. oversaw peptide synthesis, inositol phosphate accumulation and photo-crosslinking assays. B.W. and Q.Z. initiated the project, planned and analysed experiments, supervised the research and wrote the manuscript with input from all co-authors.

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METHODS

No statistical methods were used to predetermine sample size. The experiments were not randomized and the investigators were not blinded to allocation during experiments and outcome assessment.

Cloning and protein expression. The DNA sequence of wild-type human Y1R was optimized and synthesized by Genewiz and then cloned into a modified pFastBac vector (Invitrogen), which contains an expression cassette with a haemagglutinin signal sequence followed by a Flag tag before the receptor at the N terminus and a PreScission protease site followed by a 10 × His-tag at the C terminus. An engineered construct was generated by inserting a modified T4 lysozyme (T4L) at the third intracellular loop (ICL3) between residues R241 and D250 and introducing a mutation (F129H, W237C). Twenty-five amino acids (V339–I384) were truncated at the C terminus to further improve protein yield and stability. Bac-to-Bac Baculovirus Expression System (Invitrogen) was used to generate high-titre (>10^8 viral particles per ml) recombinant baculovirus. Spodoptera frugiperda (SB) cells (Invitrogen) at a density of 2 × 10^6 cells per ml were infected by viral stock at an MOI (multiplicity of infection) of 5. As well as the virus, a ligand (UR-MK299 or BMS-193885) was added to the cell culture to a final concentration of 1 μM. Transfected cells were cultured at 27°C for 48 h and then collected by centrifugation and stored at −80°C until use.

Purification of Y1R–UR-MK299 and Y1R–BMS-193885 complexes. Frozen insect cells expressing the Y1R–UR-MK299 complex were disrupted with thawing and repeated dounce homogenization in a hypotonic buffer containing 10 mM HEPES, pH 7.5, 10 mM MgCl2, 20 mM KCl and protease inhibitor cocktail (Roche). After centrifugation at 160,000g for 30 min, cell debris was re-suspended in a high-osmotic buffer containing 100 mM HEPES, pH 7.5, 1 M NaCl, 1% (w/v) dodecyl-β-d-maltopyranoside (DDM, Anatech), 0.2% (w/v) cholesterol hemisuccinate (CHS, Sigma), and then homogenized extensively. Soluble and membrane-associated proteins were removed from the suspension by centrifugation. This procedure was repeated two to three more times and then the hypotonic buffer was used to remove the high concentration of NaCl. Purified membranes were re-suspended in the hypotonic buffer with additional 30% (v/v) glycerol and stored at −80°C until use.

Purified membranes were thawed on ice in the presence of 100 μM UR-MK299, 2 mg ml−1 iodoacetamide (Sigma) and EDTA-free protease inhibitor cocktail (Roche) and incubated at 4°C for 1 h. Equal volume of solubilization buffer containing 100 mM HEPES, pH 7.5, 1 M NaCl, 1% (w/v) dodecyl-β-d-maltopyranoside (DDM, Anatech), 0.2% (w/v) cholesterol hemisuccinate (CHS, Sigma), and then added and incubation was continued for an additional 3 h. The supernatant was isolated by centrifugation at 160,000g for 30 min and incubated with TALON resin (Clontech) supplemented with 10 mM imidazole, pH 7.5 at 4°C overnight. The resin was first washed with ten volumes of 25 mM HEPES, pH 7.5, 500 mM NaCl, 0.05% (w/v) DDM, 0.01% (w/v) CHS, 10% (v/v) glycerol, 30 mM imidazole and 50 μM UR-MK299, then with ten volumes of 50 mM HEPES, pH 7.5, 500 mM NaCl, 0.05% (w/v) DDM, 0.01% (w/v) CHS, 10% (v/v) glycerol, 100 mM NaCl and 50 μM UR-MK299, and then packed into 1 M NaCl, 100 mM HEPES, pH 7.5, 500 mM NaCl and 10% (v/v) glycerol and 30 mM imidazole and 25 μM BMS-193885 complexes were harvested directly from LCP using 150 μm and 50 μm micro mounts (M2-L19-50/100, MiTeGen), respectively, and flash frozen in liquid nitrogen.

Data collection and structure determination. X-ray diffraction data were collected at the Spring-8 beam line 41XU, Hyogo, Japan, on a Pilatus3 6 M detector (X-ray wavelength 1.0000 Å). Crystals were exposed with a 10 μm × 8 μm mini-beam for 0.2 s and 0.2° oscillation per frame. Data from the best-diffracting crystals of the Y1R–UR-MK299 complex and 33 crystals of the Y1R–BMS-193885 complex were processed by XDS35. The structure of the Y1R–UR-MK299 complex was solved by molecular replacement implemented in Phaser34 using the receptor portion of NTSI (Protein Data Bank accession number 4GRV), converted to polyalanines, and T4L structure (PDB accession number IC6P) as search models. The correct molecular replacement solution contained one Y1R–T4L molecule in the asymmetric unit. Initial refinement was performed with REFMAC535 and BUSTER36, and then manual examination and rebuilding of the refined coordinates were carried out in COOT37 using both [Fo − Fc] and |Fo − Fc| maps. The structure has been carefully refined and the Ramachandran plot analysis indicates that 100% of the residues are in favourable (95.5%) or allowed (4.5%) regions (no outliers). The final model includes 306 residues (F18–R241 and S256–F337) of the 384 residues of Y1R and residues N2–Y16 of T4L. The Y1R–BMS-193885 complex structure was solved using Y1R in the Y1R–UR-MK299 complex and T4L as search models and refined using the same procedure. The Ramachandran plot analysis indicates that 100% of the residues are in favourable (95.4%) or allowed (4.6%) regions (no outliers). The final model of the Y1R–BMS-193885 complex contains 301 residues (D31–R241 and D250–D339) of Y1R and the 160 residues of T4L. All hydrogen bonds were used. The structure was rotated towards helix VI by about 90° compared to the BMS-193885-bound structure, this is probably caused by crystal packing (Extended Data Fig. 1).

Immunoblotting. The total solubilized protein of the S9 membrane preparations (see above) used in the radio ligand binding assay was determined using the Bradford method according to the manufacturers’ protocol (BioRad Protein Assay; BioRad). Aliquots of homogenized membrane preparations, corresponding to 100 μg of protein, were centrifuged at 50,000g at 4°C for 15 min, and the pellets were re-suspended in 30 mM Tris, pH 7.4, supplemented with 1 mM EDTA and protease inhibitors (SIGMAFAST Protease Inhibitor cocktail tablets, Sigma) at a protein concentration of 1,600 μg ml−1. Membrane homogenates (15 μl) were processed and subjected to immunoblotting as described previously38 with the following modifications: blotting onto the nitrocellulose membrane was performed at 60 mA for 60 min. Primary antibody anti-Flag M1 from mouse (Sigma, F3040, lot SLBLK1592V) was diluted 1:50. The secondary antibody, an anti-mouse IgG horseradish peroxidase (HRP)–conjugated antibody from goat (Sigma, A0168, lot 08084839) was diluted 1:80,000. The washing steps after incubation with the primary and the secondary antibody were 3 × 10 min each. Control experiments in the absence of the primary antibody were not performed.

Radioligand-binding assay. All binding experiments with [3H]UR-MK299 (synthesis described elsewhere19) were performed on S9 membrane preparations in PP 96-well microplates (Greiner Bio One) at 23 ± 1°C using a sodium-containing, isoo-osmotic HEPES buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM KCl, 2.5 mM CaCl2, 1.2 mM KH2PO4, 1.2 mM Mg2SO4 and 25 mM NaHCO3 supplemented with 1% BSA) for competition-binding studies with antagonists, and a sodium-free, hypo-osmotic HEPES buffer (25 mM HEPES, pH 7.4, 2.5 mM CaCl2, and 1 mM MgCl2 supplemented with 1% BSA) for competition binding studies with agonists. The plate was incubated for 45 min at 37°C. Before competition binding experiments, dissociation constant (Kd) values of [3H]UR-MK299 were determined by saturation binding using the respective binding buffer. In the case of saturation-binding experiments, [3H]UR-MK299 was 1:1
diluted with ‘cold’ UR-MK299 (hereafter, the mixture is referred to as ‘radioligand’). On the day of the experiment, S9 membranes were thawed, re-suspended using a 1-ml syringe equipped with a needle (20 G) and then centrifuged at 16,000 g at 4 °C for 10 min. The supernatant was discarded and the pellets were re-suspended in binding buffer using a 1-ml syringe equipped with a needle (27G/34). The membrane homogenates were stored on ice until use. The total amount of protein per well was between 0.25 and 1 μg, depending on the receptor expression level.

Saturation binding experiments. For the determination of total binding, wells were pre-filled with binding buffer (160 μl), and then 20 μl of binding buffer, containing the radioligand at a concentration tenfold higher than the final concentration, was added. For the determination of unspecific binding (in the presence of UR-MK299 at a 100-fold excess), wells were pre-loaded with binding buffer (140 μl), binding buffer (20 μl) containing UR-MK299 (tenfold concentrated) and binding buffer (20 μl) containing the radioligand (tenfold concentrated). To all wells, 20 μl of the membrane suspension were added, and the plates were shaken at 23 °C for 90 min. The membranes were collected on GF/C filter mats (0.26 mm; Whatman) (pre-treated with 0.3% polyethyleneimine for 30 min) and washed with cold Tris buffer (91 g l⁻¹ Tris base, 25.5 g l⁻¹ MgCl₂·6H₂O and 3.75 g l⁻¹ EDTA) using a Brandel Harvester (Brandel). Filter pieces were punched out and transferred into 1450–401 96-well plates (PerkinElmer). Rotiscint eco plus (Carl Roth) (200 μl) was added, and the plates were sealed with transparent tape (permanent seal for microplates, PerkinElmer), vigorously shaken for at least 3 h and kept in the dark for at least 1 h before the measurement of radioactivity (d.p.m.) with a MicroBeta2 plate counter (PerkinElmer). Specific binding data (d.p.m.) were plotted against the free radioligand nanomolar concentration (obtained by subtracting the amount of bound radioligand (nM) (calculated from the specifically bound radioligand in d.p.m., the specific activity, and the volume per well) from the total radioligand concentration (nM)) and analysed by a two-parameter equation describing hyperbolic binding (SigmaPlot 11.0, Systat Software Inc.) to obtain Kᵦ and receptor density (Bmax) values. For Kᵦ values < 1 nM, the Bmax was kept below 1,200 d.p.m. by choosing an appropriate protein concentration. For Kᵦ values > 1 nM, the Bmax was kept below 3,300 d.p.m.

Competition-binding experiments. Competition-binding experiments were performed according to the procedure for saturation binding with the following modifications: [³²P]UR-MK299 was used undiluted and in the case of Y₁R mutants, for which [³²P]UR-MK299 exhibited a Kᵦ value > 3 nM, the total volume per well was 100 μl, that is, in the case of total binding, wells were pre-filled with binding buffer (80 μl), and 10 μl of binding buffer containing [³²P]UR-MK299 (tenfold concentrated), and the membrane homogenate (10 μl) were added. The following concentrations of [³²P]UR-MK299 were used for competition binding with antagonists: 0.2 nM (wild-type Y₁R, TR208A, T212A, 0.3 nM (F173Y, W19)), 1.1 nM (L279Y, A5), 3 nM (Q219F, A5), 7 nM (L215D, G), 10 nM (I124L, F173Y, A5). [³²P]UR-MK299 was used for competition binding with NPY. The incubation time was adjusted accordingly. Unspecific binding (determined by the addition of binding buffer, 3% (v/v) hydrazine in DMF for 10 min) was determined by two different analytical RP-HPLC systems using 0.1% (v/v) TFA in H₂O (eluant A) and 0.08% (v/v) TFA in acetonitrile (ACN) (eluant B). After acetonitrile (ACN) (eluant B). After acetonitrile (ACN) (eluant B). After acetonitrile (ACN) (eluant B). After acetonitrile (ACN) (eluant B). After acetonitrile (ACN) (eluant B).

Peptide synthesis. Porcine NPY ([γ-3P]DPDPEPDAPADLARYYSALRH YLNILTRQRY–NH₂) and NPY analogues were synthesized by automated solid-phase peptide synthesis on an automated multiple peptide synthesis robot system (Syro, MultiSynTech), using a 9-fluorenylmethoxycarbonyl-tart-butyl (Fmoc/Bu) strategy in 15 μM scale as previously described 46. NPY–tyramide was synthesized as previously described 46. Isotopically labelled NPY variants were prepared as described, 46 and ¹²⁵I⁻N-labelled amino acids were coupled manually with 2 equivalents (equiv.) hydroxybenzotriazole/N,N-diisopropylcarbodiimide (DIC) in DMF overnight. The porcine variant of NPY, which contains a single mutation (M17L) was used. This variant has binding affinity and signalling properties that are identical to human NPY and will therefore be referred to as wild-type NPY 47. It also has increased solubility to assist in handling.

Modified NPY analogues [Bpa₁, Kᵦ(¹⁴Abx₂), biotin] NPY and [Kᵦ(¹⁴Abx₂)] biotin] NPY were synthesized by automated solid-phase peptide synthesis and Bpa, Abx and biotin were coupled manually using orthogonal 1-(4,4-dimethyl-2,6-dioxycyclohexylidene)ethyl (Dde) protection groups, cleaved by freshly prepared 3% (v/v) hydrazine in DMF for 10 min. Manual coupling reactions were performed with a 5 equiv. of the respective amino acid, 3 equiv. HOBt and 5 equiv. DIC in DMF for 2 h.

For biotin labelling, 3 equiv. biotin was dissolved in DMF for 10 min at 60°C. Next, 3 equiv. HOBt and 3 equiv. DIC were added to the mixture. Coupling was performed overnight at room temperature under constant shaking. Bpa containing peptides were cleaved from the resin and completely deprotected with a mixture of trifluoroacetic acid (TFA)/thioanisole (TA)/water (90:5:5 v/v/v). All peptides were purified by preparative reversed-phase high-performance liquid chromatography (RP-HPLC) on a Jupiter 4 µm Proteo RP-C18 column (90 Å, 4 µm, Phenomenex), Kinetex 5 µm XB-C18 column (100 Å, 5 µm, Phenomenex), Kinetex 5 µm Biphenyl (100 Å, 5 µm, Phenomenex), Aeria 3.6 µm WIDEPORTE XB-C18 (200 Å, 3.6 µm, Phenomenex) or Varitide RPC (200 Å, 6 µm, Agilent Technologies). All peptides were characterized by matrix-assisted laser desorption/ionization time of flight (MALDI–TOF) mass spectrometry (Ultrafleck III MALDI–TOF/TOF, Bruker Daltonics) and ESI-HCT (high-capacity ion trap electrospray-ionization mass spectrometry, Bruker Daltonics). Peptide purities were determined in two different analytical RP-HPLC systems using 0.1% (v/v) TFA in H₂O (eluant A) and 0.08% (v/v) TFA in acetonitrile (ACN) (eluant B).

Purity of all peptides was > 95%.

NMR measurements of Y₁R-bound NPY. Fourteen differently isotopically labelled [¹⁴C¹⁵N]porcine NPY peptides were prepared by standard fluoro-2-methylhexylcarboxyl (Fmoc) solid-phase synthesis as described previously 46. On the basis of the structure of the NMR labels, the positions of the NMR labels were chosen to avoid signal overlap in a single quantum double quantum correlation experiments and to allow straightforward signal assignment. Expression of the human Y₁R in Escherichia coli as inclusion bodies, inclusion body preparation, solubilization of the receptor in SDS and receptor purification and expression of Y₁R for 1H- and ¹³C-¹⁵N NMR analysis were as previously described 46. The NPY solution was dried again in the presence of expression medium. To assemble the Y₁R into a functional state, a three-step folding protocol was applied. In step 1, the purified Y₁R receptor was dialysed against a degassed buffer containing 2 mM SDS, 50 mM NaP at pH 8.5, 1 mM EDTA, 1 mM reduced glutathione, and

Using GraphPad Prism 5.0 (GraphPad Software) the determined concentration response curves were analysed. The curves were normalized to the top (100%) and bottom (0%) values of the associated NPY curve. All independent experiments were summarized to one single concentration response curve by the row means total function. Using nonlinear regression (curve fit) the EC₅₀ and pEC₅₀ (s.e.m.) were examined. The shift between the NPY and NPY/antagonist curves is defined as the EC₅₀ ratio and calculated by dividing EC₅₀[NPY/antagonist]/EC₅₀[NPY]. For ECo₅₀ the Hill slope was set to 1. All experiments were performed at least two times independently in technical duplicate.

Live-cell fluorescence microscopy. The membrane localization of Y₁R and receptor mutants was verified by fluorescence microscopy. COS-7 cells were seeded in 8-well-slides (IBIDI treat) and transiently transfected with Lipofectamine 2000 transfection reagent (Invivogen, Toulouse, France). Twenty-four hours after transfection, nuclei were stained with Hoechst 33342 (Sigma-Aldrich) and images were recorded using an ApoTome Imaging System, AxioVert Observer Z1 (YPF: Filter Set 46, DAPI: Filter Set 49, ApoTome, 63 x /1.40 oil objective, ZEISS) in a quasi-confocal setting. The data demonstrate that all the mutants are expressed at similar, and wild-type-like, level in COS-7 cells (Extended Data Fig. 2).

Quantification of receptor surface expression in COS-7 cells. COS-7 cells were seeded in 96-well plates (Greiner), and transiently transfected with a plasmid encoding a receptor–eYFP fusion protein using MetabolicProteo. Twenty-four hours after transfection, cells were washed once with HBSS, and fluorescence was quantified using a plate reader (Tecan Infinite M200, Tecan, Tännesfeld, Switzerland) at excitation 485/5 nm and emission 530/5 nm. Data were normalized to mock transfected (0%) and wild-type Y₁R-eYFP (100%). Results represent mean ± s.d. from independent experiments performed in quadruplicate.
0.5 mM oxidized glutathione at room temperature for 48 h. Subsequently, 25% (w/v) poly(ethylene glycol) (molecular mass 20 kDa) was added to the buffer to concentrate the receptor before reconstitution. In step 2, bicelles consisting of 1,2-dimyristoyl-sn-glycerol-3-phosphocholine (DMPC) and 1,2-dihexanoyl-sn-glycerol-3-phosphocholine (DHPC-c7) (Avanti PolarLipids) with a q value of 0.25 in 50 mM NaP, pH 8.0 were incubated with Y1R, with three subsequent cycles of fast temperature cycles from 42°C to 0°C. Aggregated protein was removed instantly by centrifugation. In step 3, the Y1R samples were concentrated in large bicelles (q > 20), which were used instead of liposomes because of the high achievable receptor packing. In large bicelles, all receptor binding sites are easily accessible. Subsequently, 50 mg ml−1 BioBeadsSM2 was added at least twice to the solution. After removal of the beads with a sieve, samples were washed four times through cycles of pelleting by centrifugation and resolubilization in 50 mM NaP, pH 7.0. Concentration determination of the membrane embedded receptor was performed by solubilization of the bicelles in ten times the volume of 15 mM SDS and 50 mM NaP, pH 7.0 and subsequent measurement of the Y1R intrinsic absorption at 280 nm using UV-visible spectroscopy. Labelled NPY variants in the plasmid were removed with the Y1R after detergent removal but before concentrating.

Assessment of the binding affinity of the Y1R was carried out using homogenous fluorescence assays as described in the literature. The reconstituted receptor was incubated in increasing concentrations with 50 nM fluorescently labelled NPY (NPY-Atto520) overnight at room temperature in 50 mM NaP, pH 7.0 and 0.1% BSA. The fluorescence spectra were recorded on a FluoroMax-2 (JOBIN YVON) in a 10 mm quartz cuvette at 20°C. The maximum signals of each spectrum were determined and normalized and plotted against the receptor concentration. The inflection point for Y1R binding was determined (OriginPro 8 G / DoseResp) at EC50 = 52 nM, demonstrating high functionality of the system. As a control, we used empty bicelles in concentrations that matched the bicelle concentration of the receptor samples, resulting in a lower binding ability to the ligand in comparison to the Y1R.

NMR spectra were recorded on a Bruker Avance III 600 NMR spectrometer. The 13C cross-polarization magic angle spinning (MAS) NMR experiments (0.7 ms contact time) were carried out using a double-resonance MAS probe with a 4 mm spinning module. Typical 90° pulse lengths were 4 μs for 1H and 13C and heteronuclear decoupling SPINAL(64) at a field strength of ~65 kHz. The 13C chemical shifts were referenced relative to tetramethylsilane. The experiments were conducted at ~30°C and an MAS frequency of 7 kHz. The 13C double quantum 13C single quantum correlation spectra were acquired using the SPC-5 recoupling sequence for double quantum excitation and reconversion (set to 0.571 ms each). The relaxation delay was 2.4 s.

**Molecular docking of NPY into Y1R.** Peptide docking of full-length porcine NPY was completed using Rosetta's FlexPepDock application. In brief, low energy backbone conformations were generated from the starting conformations of UR-MK299-bound Y1R. Initially, the trimer of C-terminal NPY was docked into these conformations using full flexible docking guided by mutagenesis data. For each round of docking, 5,000 models were generated. The models were sorted by total energy and binding energy. Top models from a given docking round were used to seed the next round of docking in which the peptide was extended. Fragment docking was performed using the fragment picker application with Rosetta. Secondary structure during fragment docking was guided by the NMR chemical shift data. Additionally, experimentally derived restraints were used to guide docking (R35-D297, R35-N283, Y36-I100, R33-N290, L30-L293). After docking peptides of length 6, 12, 18 and 36, the binding pocket was resampled to allow the ligand binding pocket to adapt to the shape of the peptide. This was accomplished with RosettaDockCM40. The Y1R crystal structure was used as a template along with the docked model to ensure the models did not drift too far from the starting structure though the N terminus was removed until the last docking step to provide steric bulk. Following full-length NPY docking, the N terminus of NPY was localized using loose distance constraints with the peptides identified in crosslinking experiments. Model selection from RosettaCM was accomplished to provide steric bulk. Following full-length NPY docking, the N terminus of the starting structure though the N terminus was removed until the last docking step to provide steric bulk. Following full-length NPY docking, the N terminus of the starting structure was resampled to allow the ligand binding pocket to adapt to the shape of the peptide.

For photo-crosslinking Y1R in binding buffer was incubated with [Bpa1, K(Ahx)-biotin]NPY in a molar ratio of 4:1 (5 mM, 125 mM) for 30 min at room temperature. In addition, the same reaction was performed with an eightfold-excess of NPY (Y1R:NPY, 1:4:1). Subsequently, the opened reaction vessels were placed on ice and irradiated with UV light (UV lamp, Atkas Fluorescent, A = 366 nm, 180 W) for 90 min. 50 μl of photo-crosslinked Y1R sample (~200 μg) was digested with Glu-C and rLys-C (Promega) according to the manufacturer's protocol. Crosslinked fragments were then isolated by affinity purification using Monomeric Avidin Agarose beads (Thermo Fisher Scientific) according to the manufacturer's protocol. Possible fragments of digested Y1R were calculated analogously by adding Q to a. To account for incomplete digestion the tool allowed for a maximum of five missed cleavages. For the analysis the combined option 'Glu C (phosphate) + Lys C' was chosen. The same procedure was used for the calculation of possible NPY fragments. Theoretical masses of fragments after enzymatic cleavage of photo-crosslinked Y1R-[Bpa1, K(Ahx)-biotin]NPY were calculated by adding possible Y1R fragment masses to NPY fragment masses containing the N terminus. The mass of Bpa, two times Aha and biotin reduced by water were added manually to account for the formation of a peptide bond. Peptide fragments of photo-crosslinked Y1R, reassembled against MALDI−TOF−TOF mass spectrometry using an Ultraflex III MALDI−TOF−TOF mass spectrometer (Bruker Daltonics).

Functionality of cell-free expressed Y1R samples was verified by a homogenous binding assay based on fluorescence polarization. We used [Dpr22-Atto 520]NPY (hereafter: NPY-Atto 520) as a fluorescence tracer (inositol phosphate accumulation in transiently transfected COS-7: EC50 = 24 nM, PEPCO = −7.61 ± 0.20). 50 nM of NPY-Atto 520 was incubated with increasing concentrations of Y1R in Brij-58 micelles in buffer (0.1 mM Tris/HCl, pH 7.4, 2.5% glycerol, 0.1% (w/v) Brij-58 and 0.1% bovine serum albumin) for 90 min under gentle agitation in opaque 96-well plates. A fluorescence presence was then measured in a Tecan Spark plate reader (Tecan) using linear polarized light (excitation 510/5 mn, emission 550/10 mn, 90° detection angle). Experiments were conducted at least twice independently in duplicate.

**Reporting summary.** Further information on experimental design is available in the Nature Research Reporting Summary linked to this paper.

**Data availability.** Atomic coordinates and structure factor files for the Y1R−UR-MK299 and Y1R−BMS-193885 complex structures have been deposited in the Protein Data Bank (PDB) with accession codes ZSBQ and ZSBH, respectively.

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Extended Data Fig. 1 | Crystal packing and structural features of Y₁R and chemical structures of Y₁R ligands. a, b. Crystal packing of Y₁R–UR-MK299 (a) and Y₁R–BMS-193885 (b) complexes. Y₁R is shown in cartoon representation and coloured brown and green in the Y₁R–UR-MK299 and Y₁R–BMS-193885 complexes, respectively. The T4L fusion is shown in grey cartoon representation. UR-MK299 and BMS-193885 are displayed as yellow and pink spheres, respectively. c. Cutaway view of the UR-MK299-binding pocket in Y₁R. The receptor is shown in brown cartoon and surface representations. The ligand is shown as yellow sticks. d. Comparison of Y₁R in the Y₁R–UR-MK299 crystal structure (brown) and the Y₁R–NPY model (green). Side chains of Q₁₂₀ and W₂₇₆ are shown as sticks. R₃⁵–Y₃₆ of NPY is displayed as cyan sticks. The hydrogen bond between Q₁₂₀ and Y₃₆ of NPY is shown as a green dashed line. e–j. Chemical structures of the argininamide Y₁R antagonists BIBP3226 (e), UR-HU404 (f), UR-MK299 (g), BIBO3304 (h), UR-MK289 (i) and UR-MK136 (j). k. Chemical structure of BMS-193885. l. Scaffold of NPY C-terminal residues R₃⁵ and Y₃₆. Key differences between R₃⁵–Y₃₆ of NPY and UR-MK299 are chirality of the arginine derivative and alteration of bond connectivity leading to the hydroxyphenyl group.
Extended Data Fig. 2 | Expression of wild-type and mutant Y$_1$ receptors in transiently transfected COS-7 cells. 

**a**, Live-cell fluorescence microscopy verifies all Y$_1$R variants to be properly folded and exported to the cell membrane like the wild-type receptor. Nuclei stained with Hoechst33342. Scale bars, 10 μm. Pictures are representative of two independent experiments with similar results. 

**b**, The total expression level was determined by fluorescence reading and expression was confirmed to be similar to the wild type. Transfection of only 50% or 25% of the DNA amount (with total DNA amount held constant by empty vector), led to a proportional decrease of fluorescence, and thus, expression level. Data represent mean ± s.e.m. of three to five independent experiments performed in technical triplicate (see Source Data for sample size of each mutant). 

**c**, Estimation of the receptor reserve in functional inositol phosphate accumulation assays. Transfection of half of the vector encoding the receptor (with a constant total DNA amount including chimeric G protein, see a) still produces maximum signal, while further reduction results in signal loss at comparable potency. Thus, there is only a small receptor reserve in the functional readout, allowing potency alteration to be directly related to compromised ligand binding. Data represent mean ± s.e.m. of three independent experiments performed in technical duplicate. cNPY, concentration of NPY.
Extended Data Fig. 3 | Sequence alignment of the human NPY receptors and the human NPFF receptors. Colours represent the similarity of residues: red background, identical; red text, strongly similar. Key residues in the UR-MK299-binding pocket, which are conserved or variable among receptors, are indicated by red or black arrows, respectively. The alignment was generated using UniProt (http://www.uniprot.org/align/) and the graphic was prepared on the ESPript 3.0 server (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi).
Extended Data Fig. 4 | Pharmacological characterization of refolded Y1R and NMR studies of Y1R-bound NPY. a, Binding of Atto 520-labelled NPY (50 nM) to increasing amounts of bicelles containing Y1R or empty bicelles. Data reflect fluorescence enhancement upon binding. An inflection point at EC₅₀ = 52 nM was determined. Two independent experiments were performed in technical duplicate with similar results. Data shown are from a representative experiment. a.u., arbitrary units. c(Y1R), concentration of Y1R. b, Typical ¹³C MAS single-quantum (SQ)/double-quantum (DQ) correlation spectrum of NPY in the presence of Y1R reconstituted into large bicelles at −30 °C. NMR spectra were acquired from one to three independent preparations for each labelled amino acid with similar results (see d). Data shown are from a representative experiment. c, Table showing ¹³C-NMR chemical shifts of assigned amino acids of NPY bound to Y1R (referred to tetramethylsilane) as acquired in solid-state NMR experiments. d, ¹³C-chemical-shift index of NPY bound to Y1R in large DMPC/DHPC-c7 bicelles (q > 20) compared with docked models. Plotted in black is the measured chemical shift difference (Cα − Cβ) for each individual residue of NPY minus the chemical shift difference of the same amino acid type in random-coil conformation. Individual data points from one to three independent experiments for each labelled amino acid are shown. Typical experimental error when determining chemical shifts under these conditions are ±1 p.p.m. Chemical shifts were back-calculated for the top docking solutions and filtered against the experimental data to generate a final ensemble of docked poses. Their average chemical-shift index and associated s.d. from the top ten docked poses are shown in red.
Extended Data Fig. 5 | Photo-crosslinking experiments between NPY and Y1R. a, Mass spectra of photo-crosslinked Y1R with [Bpa1, K4[(Ahx)2-biotin]]NPY. Exemplary MALDI–TOF mass spectra of photo-crosslinked samples enzymatically digested by rLys-C and Glu-C. Potential Y1R fragments are labelled. Two independent experiments were performed with similar results. N, N terminus of Y1R (blue); E, ECL2 (red).
b, Respective regions of NPY N terminus at Y1R. Amino acid sequence of Y1R with a C-terminal His-tag. The two detected regions within Y1R (N terminus (blue), ECL2 (red)) after crosslinking with [Bpa1,K4[(Ahx)2-biotin]]NPY are emphasized in boxes. The different sizes of the boxes represent different detected fragments (Extended Data Table 5). Experiments were repeated twice independently with similar results, and only fragments that were observed in both experiments are listed here and in Extended Data Table 5. c, Binding of Atto 520-labelled NPY (50 nM) to increasing amounts of cell-free produced Y1R in Brij-58. Data reflect fluorescence enhancement upon binding. An EC50 value of 69 nM was determined. Data shown are mean ± s.e.m. from six independent experiments performed in technical triplicate. c(Y1R), concentration of Y1R.
**Extended Data Table 1 | Data collection and refinement statistics**

|                      | Y,R–UR-MK299 | Y,R–BMS-193885 |
|----------------------|--------------|----------------|
| **Data Collection**  |              |                |
| Space group          | *P2₁*        | *C₂₂₂₁*        |
| Cell dimensions      |              |                |
| a, b, c (Å)          | 37.8, 100.7, 83.2 | 76.9, 126.8, 170.3 |
| α, β, γ (°)          | 90.0, 98.8, 90.0   | 90.0, 90.0, 90.0   |
| Resolution (Å)       | 50.0-2.70 (2.83-2.70)† | 50.0-3.0 (3.1-3.0)† |
| R_{merge} (%)        | 17.0 (86.1)    | 16.6 (93.7)    |
| I / σI              | 4.78 (0.97)    | 5.30 (1.00)    |
| Completeness (%)     | 97.3 (96.9)    | 92.4 (79.2)    |
| Redundancy           | 4.0 (3.6)      | 3.5 (2.6)      |
| **Refinement**       |              |                |
| Resolution (Å)       | 50.0-2.7      | 50.0-3.0       |
| No. reflections      | 16,520 (790)  | 15,600 (797)   |
| R_{work} / R_{ref} (%) | 22.5 / 24.7  | 22.4 / 24.9    |
| Number of atoms      |              |                |
| Protein              | 3,715         | 3,654          |
| Ligand               | 45            | 43             |
| Overall B values (Å²) |              |                |
| Protein              | 88.2          | 108.0          |
| Ligand               | 65.2          | 81.0           |
| R.m.s. deviations    |              |                |
| Bond lengths (Å)     | 0.010         | 0.009          |
| Bond angles (°)      | 1.04          | 1.00           |

*Diffraction data from 47 Y,R–UR-MK299 crystals and 33 Y,R–BMS-193885 crystals were used to solve the structures.
†Numbers in parentheses refer to the highest-resolution shell.
## Extended Data Table 2 | Inositol phosphate accumulation assays of wild-type and mutant Y1 receptors for NPY and antagonists

| Mutants | NPY | NPY/BIP3226 (10⁻6 M)* | NPY/BIBO3304 (10⁻6 M)* | NPY/UR-HU404 (10⁻6 M) | NPY/UR-MK289 (10⁻6 M) | NPY/UR-MK299 (10⁻7 M) |
|---------|-----|------------------------|-------------------------|------------------------|------------------------|------------------------|
|          | EC₅₀ (pM) ± SEM | n     | EC₅₀ (pM) ± SEM | Kᵢ (nM) ± SEM | Ratio | Kᵢ (nM) ± SEM | n | EC₅₀ (pM) ± SEM | Kᵢ (nM) ± SEM | Ratio | Kᵢ (nM) ± SEM | n | EC₅₀ (pM) ± SEM | Kᵢ (nM) ± SEM | Ratio | Kᵢ (nM) ± SEM | n |
| Wild type | 1.7 | (8.78 ± 0.03) | 20 | 683 (6.43 ± 0.04) | 272 | 36.9 | 14 | 175 (6.76 ± 0.04) | 103 | 9.8 | 16 | 1.09/0.12/0.04 (5.96 ± 0.04) (6.90 ± 0.07) | 646/74/16 | 0.16/0.14/0.14 | 14 | 228 (6.64 ± 0.04) | 134 | 75.2 | 15 | 250 (6.62 ± 0.06) | 147 | 0.69 | 10 |
| Y100F/A | 562 | 404 | 2 | nd nd nd nd nd nd nd nd | nd nd nd | 2 | >50,000 | >50,000 | >3,500 | <0.03 | 2 | >50,000 | >50,000 | >3,500 | <2.86 | 2 | >50,000 | >50,000 | >3,500 | <0.03 | 2 |
| Q120D/E | 12 | 15 | 2 | nd nd nd nd nd nd nd nd | nd nd nd | 2 | >50,000 | >50,000 | >3,500 | <2.86 | 2 | >50,000 | >50,000 | >3,500 | <0.03 | 2 |
| Q120D/E * | 3.8 | (8.42 ± 0.07) | 4 | 836 (6.08 ± 0.06) | 220 | 45.7 | 3 | 414 (6.38 ± 0.09) | 109 | 9.3 | 3 | 3.744 (5.43 ± 0.10) | 985 | 0.10 | 3 | 325 (6.49 ± 0.08) | 86 | 118 | 3 | 743 (6.13 ± 0.06) | 196 | 0.51 | 3 |
| I124E/A | 6.9 | (8.22 ± 0.08) | 3 | 1,877 (5.73 ± 0.06) | 313 | 32.1 | 4 | 306 (6.62 ± 0.06) | 51 | 20.0 | 4 | 1.588 (5.80 ± 0.06) | 265 | 0.38 | 4 | 637 (6.20 ± 0.08) | 106 | 95.2 | 4 | 208 (6.68 ± 0.09) | 35 | 2.94 | 4 |
| Q219A/E | 23 | (7.63 ± 0.05) | 7 | 2,732 (5.56 ± 0.08) | 119 | 84.8 | 4 | 1,156 (5.94 ± 0.07) | 50 | 20.4 | 3 | >10,000 | >1,711/2,197 | >435/96/11 | >2.3/1 | 3 | 7,018 (5.26 ± 0.12) | 240 | 41.8 | 3 | 1,1467 | 3 | 193 | 30 | 2.05 | 2 |
| W276E/A | 3.8 | (8.42 ± 0.06) | 5 | 246 (6.61 ± 0.12) | 65 | 156 | 3 | 84/60 | 22/16 | 47/68 | 2 | 490/254 | 129/87 | 0.6 | 2 | 1,445/1505 | 380/399 | 26/25 | 2 | 140/87 | 37/23 | 2.8 | 2 |
| T280A/F | 2.6 | (8.58 ± 0.08) | 4 | 141 (6.61 ± 0.08) | 54 | 189 | 4 | 124 (6.91 ± 0.05) | 48 | 21.3 | 4 | 871 (6.06 ± 0.06) | 335 | 0.30 | 4 | 278 (6.56 ± 0.07) | 107 | 94.3 | 3 | 360 (6.51 ± 0.06) | 118 | 0.86 | 3 |
| N283D/A | 800 | (6.05 ± 0.06) | 7 | 1,148 (5.94 ± 0.02) | 1 | nd | 3 | 1,036 | 5.98 ± 0.14 | 1 | nd | 3 | 7.622 | 5.12 ± 0.14 | 8 | 14.3 | 3 | 1,193 | 5.92 ± 0.14 | 1 | nd | 3 | 495/571 | 1 | nd | 2 |
| F288W/A | 4.5 | (8.35 ± 0.04) | 7 | 451 (6.31 ± 0.05) | 109 | 92.6 | 3 | 118 (6.93 ± 0.09) | 28 | 40.0 | 3 | 1,553 | (5.81 ± 0.05) | 345 | 0.29 | 3 | 126 | (6.90 ± 0.07) | 28 | 370 | 3 | 180 | (6.74 ± 0.16) | 40 | 2.56 | 3 |
| D287Y/A | 260 | (6.58 ± 0.05) | 7 | 900 | 748 | 3 | 341/142 | 1 | nd | 2 | >20,000/1,571/747 | >77/67 | >1/2 | 3 | 729/504 | 28 | 6.84 | 1 | 38/5.543 | 9.95 | 10.665 | 2 | 13,157 | 2 | 100 | 3 |
| F302Y/A | 4.2 | (8.38 ± 0.09) | 3 | 16/29 | 4 | 3 | 3,570 | 1,685 | 2 | 44/10 | 1 | 24 | nd | 712 | 2 | 33/42 | 8 | 14.6 | 3 | 3.8/5.2 | 1 | nd | 2 | 9.4 | (8.03 ± 0.11) | 2 | 100 | 3 |

*Antagonist concentrations were chosen based on their antagonistic activity on Y1R.
†EC₅₀ values were determined after 1 h stimulation by increasing the concentration of NPY or NPY together with different antagonists. Data are shown as mean values from at least three independent experiments or the results of two individual experiments each performed in technical duplicate.
‡Sample size: the number of independent experiments performed in technical duplicate.
§The EC₅₀ ratio represents the shift between the NPY and NPY + antagonist curve (EC₅₀(NPY + antagonist)/EC₅₀(NPY)) and characterizes the antagonistic effect on the wild-type receptor or receptor mutants. By comparison of EC₅₀ ratios between wild-type and mutant receptor, influences of all tested residues on antagonist activity were determined. A higher ratio indicates higher antagonist activity. A reduced EC₅₀ ratio of mutant compared to the wild-type receptor was interpreted as important for the respective antagonist.
#These data were obtained at a reduced concentration of UR-HU404 (10⁻⁸ M) as concentration response curves did not reach saturation (EC₅₀ > 10,000 nM) when a higher concentration was used (10⁻⁷ M).
### Extended Data Table 3 | Binding of Y₁R antagonists and agonists to membrane preparations from Sf9 cells expressing wild-type and mutant Y₁ receptors

#### a. Binding of antagonists to wild-type and mutant Y₁Rs

| Y₁R mutants               | $K_a$ (nM)$^*$ | BMS-193885 n | BIBP3226 n | BIBO3304 n | UR-MK136 n | UR-MK289 n |
|---------------------------|----------------|-------------|------------|------------|------------|------------|
| Wild type                 | 0.17 ± 0.03    | 3           | 22 ± 6     | 2.4; 3.1   | 1.6 ± 0.3  | 2.8; 4.0   | 25; 28     | 2          |
| Crystallization construct | 0.33 ± 0.06    | 3           | 38 ± 2     | /          | /          | /          | /          | /          |
| C121H22A                  | 1.4; 2.4       | 2           | /          | /          | /          | /          | /          | /          |
| I124H21A                  | 7.0; 8.0       | 2           | 9,500 ± 1,700 | 3 | 15 ± 5     | 11 ± 2     | 12 ± 2     | 80 ± 16   | 3          |
| I124H21F                  | 1.3; 1.9       | 2           | /          | /          | /          | /          | /          | /          |
| F173H46A                  | 9.1; 2.2       | 4           | 590 ± 220  | 68; 84     | 120 ± 17   | 88; 110    | 660 ± 110f | 5          |
| F173H46W                  | 0.31; 0.32     | 2           | 110; 130   | 15; 26     | 13 ± 4     | 3.6; 4.3   | 6.8; 9.2   | 2          |
| T212H46A                  | 0.12; 0.18     | 2           | 150; 150   | 13; 12     | 2.5 ± 0.3  | 5.7; 7.5   | 18; 21     | 2          |
| L215H46G                  | 4.2; 5.6       | 2           | 9.6 ± 1.9  | 43; 23     | 47 ± 4     | 11; 8.4    | 29; 30     | 2          |
| Q219H44A                  | 4.1; 5.1       | 2           | 0.50 ± 0.07| 35; 53     | 6.2 ± 0.7  | 16 ± 4     | 13 ± 4     | 3          |
| Q219H44R                  | 5.1 ± 1.2      | 3           | /          | /          | /          | /          | /          | /          |
| W276H44A                  | >500           | 3           | /          | /          | /          | /          | /          | /          |
| L279H44A                  | 1.0; 1.1       | 2           | 160; 220   | 110; 110   | 13 ± 2     | 120; 150   | 320 ± 40   | 3          |
| T280H44A                  | 0.16 ± 0.04    | 3           | 7,300 ± 1,300| 3 | 32 ± 7     | 2.6 ± 0.4  | 4.2 ± 1.1  | 8.2 ± 4.2 | 3          |
| N283H44A                  | >500; >500     | 2           | /          | /          | /          | /          | /          | /          |
| D287H44A                  | >500; >500     | 2           | /          | /          | /          | /          | /          | /          |
| F302H44A                  | >500; >500     | 2           | /          | /          | /          | /          | /          | /          |

$^*$Dissociation constant determined by saturation binding at Sf9 membranes (receptor expression was confirmed by western blot analysis) using a sodium-containing buffer (a) or a sodium-free buffer (b) (the sodium-free buffer was used for the determination of agonist binding affinity because porcine NPY exhibited approximately tenfold higher affinity in the sodium-free buffer compared to the sodium-containing buffer (data not shown)).

†Dissociation constant determined by competition binding with [3H]UR-MK299 at Sf9 membranes using a sodium-containing buffer (a) or a sodium-free buffer (b).

‡Sample size; the number of independent experiments performed in technical triplicate. If $n > 2$, data are shown as mean ± s.e.m. If $n = 2$, results of two individual experiments are shown.

§The lower curve plateau of the four-parameter logistic fit, amounting to 17 ± 3% of specifically bound [3H]UR-MK299 (mean ± s.e.m. from five independent experiments), was significantly different from zero ($P < 0.005$, one-sample one-tailed $t$-test), which is indicative of a non-competitive mechanism.

#### b. Binding of NPYs to wild-type Y₁R

| Y₁R    | $K_a$ (nM)$^*$ | Human NPY n | Porcine NPY n |
|--------|----------------|-------------|---------------|
| Wild type | 0.89; 1.1       | 4.1; 4.2    | 2.8 ± 0.4     |

$^*$Dissociation constant determined by competition binding with [3H]UR-MK299 at Sf9 membranes using a sodium-containing buffer (a) or a sodium-free buffer (b).
### Extended Data Table 4 | Inositol phosphate accumulation assays of wild-type and mutant Y<sub>1</sub> receptors for NPY and NPY analogues

#### a. IP accumulation assays of complementary mutagenesis between NPY/NPY analogues and WT and mutant Y<sub>1</sub>Rs

| Peptides<sup>*</sup> | WT | Q120<sup>158H</sup> | I293<sup>GLN</sup> | N299<sup>138A</sup> | N283<sup>158A</sup> | D287<sup>158A</sup> |
|-------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                  | EC<sub>50</sub> (nM) ± SEM | X-fold over WT | EC<sub>50</sub> (nM) ± SEM | X-fold over WT | EC<sub>50</sub> (nM) ± SEM | X-fold over WT | EC<sub>50</sub> (nM) ± SEM | X-fold over WT | EC<sub>50</sub> (nM) ± SEM | X-fold over WT | EC<sub>50</sub> (nM) ± SEM | X-fold over WT |
| NPY              | 1.5 (8.83 ± 0.02) | 51              | 26              | 81              | 1,053           | 61              | 6               | 1,384           | 461             | 3               |
| [N30]NPY         | 289 (6.54 ± 0.06) | 9               | /               | /               | /               | /               | /               | /               | /               | /               |
| [A33]NPY         | 5,395 (5.27 ± 0.07) | 1               | 3               | /               | /               | /               | /               | 5,306 (5.28 ± 0.07) | 1               | 3               | /               | /               |
| [A35]NPY         | >10,000          | 1               | 3               | /               | /               | /               | /               | /               | /               | >4,900          | >5              | 3               |
| [A36]NPY         | 1,378 (5.86 ± 0.06) | 1               | 6               | /               | /               | /               | /               | /               | /               | /               | /               |
| NPY-tyramide     | 68 (7.17 ± 0.11) | 1               | 9               | nd              | nd              | 3               | /               | /               | /               | /               | /               |

#### b. IP accumulation assays of WT Y<sub>1</sub>R for NPY/NPY analogues

| Peptides | EC<sub>50</sub> (nM) ± SEM | X-fold over NPY | n |
|----------|-----------------|-----------------|---|
| NPY      | 1.5 (8.83 ± 0.02) | 1               | 51 |
| Ac-NPY   | 0.5; 3.0        | 1.2             | 4 2 |
| [A1]NPY  | 6.5; 2.4        | 5               | 3 4 |
| [A2]NPY  | 7.3 (8.14 ± 0.11) | 5               | 3 4 |
| [A1,A2]NPY | 8.0 (8.10 ± 0.06) | 5               | 3 4 |
| NPY(3-36) | 83 (7.08 ± 0.09) | 5               | 3 4 |
| NPY(13-36) | 477; 744       | 5               | 3 4 |

#### c. NPY-induced IP accumulation assays of WT and mutant Y<sub>1</sub>Rs

| Peptides | EC<sub>50</sub> (nM) ± SEM | X-fold over WT | n |
|----------|-----------------|-----------------|---|
| WT       | 1.5 (8.83 ± 0.02) | 1               | 51 |
| F184<sup>C31A</sup> | 18.7 (7.73 ± 0.06) | 13              | 5 7 |
| F184<sup>CGLN</sup> | 23 (7.64 ± 0.10) | 15              | 3 3 |
| V187<sup>CGLN</sup> | 1.9 (8.72 ± 0.06) | 1               | 5 7 |
| L189<sup>C31S</sup> | 1.9 (8.73 ± 0.16) | 1               | 5 7 |
| Y192<sup>CGLN</sup> | 3.8 (8.42 ± 0.11) | 1               | 5 7 |
| V197<sup>C31A</sup> | 1.9 (8.71 ± 0.13) | 1               | 5 7 |
| V197<sup>CGLN</sup> | 188 (6.73 ± 0.11) | 1               | 5 7 |
| F199<sup>CGLN</sup> | 3.4 (8.47 ± 0.11) | 1               | 5 7 |
| F202<sup>CGLN</sup> | 1.2 (8.93 ± 0.15) | 1               | 5 7 |

**nd,** not determined up to 10<sup>−4</sup> M agonist concentration; /, not tested.

*Peptides were synthesized as described in the ‘Peptide synthesis’ section of the Methods.

†EC<sub>50</sub> values were determined using GraphPad Prism 5.0. All curves were normalized to the top and bottom values of the Y<sub>1</sub>R–NPY curve. Nonlinear regression (curve fit) was performed for normalized response in all assays. All data are shown as mean values from at least three independent experiments or results of two individual experiments each performed in technical duplicate.

‡The EC<sub>50</sub> shifts were determined by EC<sub>50(mutant)/EC<sub>50(wild type)</sub></sub>. The Hill slope was set to one. For the wild-type receptor x-fold (fold change) is set to one. A lower EC<sub>50</sub> shift of the NPY analogue/mutant compared to NPY/mutant was interpreted as no further loss of function and a direct interaction between both positions.

§Sample size; the number of independent experiments performed in technical duplicate.

||Previously published data<sup>27</sup>.

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Extended Data Table 5 | Mass spectromeric signals and calculated mass of photo-crosslinked Y, R with [Bpa\textsuperscript{1}, K\textsuperscript{4}[(Ahx)\textsubscript{2}-biotin]]NPY

| MALDI-ToF MS (m/z)* | Number in Y,R | Position [Bpa\textsuperscript{1},K\textsuperscript{4}[(Ahx)\textsubscript{2}-biotin]]NPY | M\textsubscript{calc} (Da)* | [M\textsubscript{calc} + H]\textsuperscript{+} (Da) | [M\textsubscript{calc} + Na]\textsuperscript{+} (Da) | [M\textsubscript{calc} + K]\textsuperscript{+} (Da) |
|-------------------|----------------|----------------------------------|-----------------|-------------------|-------------------|-------------------|
| 1824.2            | 11 - 32        | 1 - 6                            | 3760.7          | 3761.8            | 3783.7            | 3799.7            |
| 1867.3            | not identified | not identified                   | not identified  | not identified    | not identified    | not identified    |
| 1892.6            | 1 - 11 + 5-6   | 1986.9                           | 1987.9          | 2009.9            | 2022.9            | 2038.9            |
| 2001.1            | 1 - 10 + 5-7   | 2000.0                           | 2001.0          | 2009.9            | 2022.9            | 2038.9            |
| 2059.2            | -               | 2057.9                           | 2058.9          | 2080.9            | 2080.9            | 2086.9            |
| 2073.2            | 1 - 10 + 8-11  | 2057.9                           | 2058.9          | 2080.9            | 2080.9            | 2086.9            |
| 2081.3            | -               | 2057.9                           | 2058.9          | 2080.9            | 2080.9            | 2086.9            |
| 2121.3            | not identified | not identified                   | not identified  | not identified    | not identified    | not identified    |
| 2162.2            | 191 - 194      | 2139.0                           | 2140.0          | 2162.0            | 2178.0            | 2178.0            |
| 2311.4            | not identified | not identified                   | not identified  | not identified    | not identified    | not identified    |
| 2317.4            | not identified | not identified                   | not identified  | not identified    | not identified    | not identified    |
| 2381.4            | 191 - 195      | 2380.1                           | 2381.1          | 2403.1            | 2419.1            | 2419.1            |
| 2381.8            | 195 - 205      | 2381.1                           | 2382.1          | 2404.1            | 2420.1            | 2420.1            |
| 2397.3            | 32 - 32        | 2374.0                           | 2375.0          | 2397.0            | 2413.0            | 2413.0            |
| 2410.3            | 194 - 194      | 2374.0                           | 2375.0          | 2397.0            | 2413.0            | 2413.0            |
| 2414.3            | 194 - 194      | 2374.0                           | 2375.0          | 2397.0            | 2413.0            | 2413.0            |
| 2450.4            | -               | 2427.1                           | 2428.1          | 2450.1            | 2466.1            | 2466.1            |
| 2514.5            | not identified | not identified                   | not identified  | not identified    | not identified    | not identified    |
| 2553.5            | 194 - 200      | 2530.1                           | 2531.1          | 2553.1            | 2569.1            | 2569.1            |
| 2589.6            | not identified | not identified                   | not identified  | not identified    | not identified    | not identified    |
| 2775.7            | not identified | not identified                   | not identified  | not identified    | not identified    | not identified    |
| 2807.7            | not identified | not identified                   | not identified  | not identified    | not identified    | not identified    |
| 3031.7            | 22 - 29        | 3030.4                           | 3031.4          | 3053.4            | 3069.4            | 3069.4            |
| 3377.7            | 191 - 200      | 3376.5                           | 3377.5          | 3399.5            | 3415.5            | 3415.5            |
| 3784.1            | 11 - 32        | 3760.7                           | 3761.8          | 3783.7            | 3799.7            | 3799.7            |
| 3966.3            | 1 - 7 + 17-36  | 3943.1                           | 3944.1          | 3966.1            | 3982.1            | 3982.1            |
| 4524.9            | not identified | not identified                   | not identified  | not identified    | not identified    | not identified    |

* Determined signals by MALDI-TOF mass spectrometry.
† Selected calculated masses in Dalton of possible photo-crosslinked fragments of Y, R with [Bpa\textsuperscript{1}, K\textsuperscript{4}[(Ahx)\textsubscript{2}-biotin]]NPY or [Bpa\textsuperscript{1},K\textsuperscript{4}[(Ahx)\textsubscript{2}-biotin]]NPY with itself. The fragments are selected based on the correlation with the detected signals. For clarity, further calculated masses of possible photo-crosslinked fragments are not shown.
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 Experimental design

1. Sample size
   Describe how sample size was determined.

   Due to radiation damage, X-ray diffraction data collection of the protein crystals was limited to 5-10 degree per crystal. To collect a complete data set for structure determination, diffraction data from multiple crystals were integrated and scaled using XDS. By calculating completeness of the data set, diffraction data from 47 Y1R–UR-MK299 crystals and 33 Y1R–BMS-193885 crystals were used to ensure the completeness was close to 100%. For the ligand binding, IP accumulation assays and NMR measurements, 2-5 independent experiments were performed in technical duplicate/triplicate to ensure each data point was repeatable.

2. Data exclusions
   Describe any data exclusions.

   No data were excluded from the analyses.

3. Replication
   Describe the measures taken to verify the reproducibility of the experimental findings.

   All attempts at replication were successful.

4. Randomization
   Describe how samples/organisms/participants were allocated into experimental groups.

   Randomization is not relevant to this study, as protein and crystal samples are not required to be allocated into experimental groups in protein structural studies, and no animals or human research participants are involved in this study.

5. Blinding
   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

   Blinding is not relevant to this study, as protein and crystal samples are not required to be allocated into experimental groups in protein structural studies, and no animals or human research participants are involved in this study.

Note: all in vivo studies must report how sample size was determined and whether blinding and randomization were used.
6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

- [ ] The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
- [ ] A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- [ ] A statement indicating how many times each experiment was replicated
- [ ] The statistical test(s) used and whether they are one- or two-sided. *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
- [ ] A description of any assumptions or corrections, such as an adjustment for multiple comparisons
- [ ] Test values indicating whether an effect is present. *Provide confidence intervals or give results of significance tests (e.g. P values) as exact values whenever appropriate and with effect sizes noted.*
- [ ] A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
- [x] Clearly defined error bars in all relevant figure captions (with explicit mention of central tendency and variation)

See the web collection on statistics for biologists for further resources and guidance.

- [ ] n/a

- [ ] Confirmed

7. Software

Describe the software used to analyze the data in this study.

- Phaser: version 2.5.6 (CCP4Interface 6.5.000)
- REFMACS: version 5.8.0103
- BUSTER: version 2.10.3
- COOT: version 0.7.2
- SigmaPlot 11.0: Systat Software Inc., Chicago, IL
- GraphPad Prism Software 5.0: GraphPad Software, San Diego, CA

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a third party.

No unique materials were used.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

- ANTI-FLAG M1 from mouse: Sigma, order no. F3040, lot SLBK1592V, diluted 1:500.
- anti-mouse IgG HRP-conjugated antibody from goat: Sigma, order no. A0168, lot 080M4839, diluted 1:80,000.

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

COS-7 cells were obtained from American Type Culture Collection (ATCC). SF9 cells were obtained from Invitrogen.

b. Describe the method of cell line authentication used.

The COS-7 cell line was authenticated using a PCR based multiplex assay based on the use of short tandem repeats (STR) (Authentication of Human Cell Lines: Standardization of STR Profiling, ANSI/ATCC ASN-0002-2011). The SF9 cell line was authenticated through morphology check by microscope and growth curve analysis.

c. Report whether the cell lines were tested for mycoplasma contamination.

The cell lines were negative for mycoplasma contamination.

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

No commonly misidentified cell lines were used.
**Animals and human research participants**

Policy information about *studies involving animals*; when reporting animal research, follow the **ARRIVE guidelines**

11. Description of research animals
   - Provide all relevant details on animals and/or animal-derived materials used in the study.
   - **No animals were used.**

Policy information about *studies involving human research participants*

12. Description of human research participants
   - Describe the covariate-relevant population characteristics of the human research participants.
   - **The study did not involve human research participants.**