Supporting Information

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SI Methods

Protein Production. The cDNA of the human Gαi1 gene (GenBank accession no. BC026326) was inserted into an in-house–modified pQE30 vector (Qiagen) using PCR methods, resulting in a DNA construct encoding (N terminal-to-C terminal) a His_6-tag, GB1, a tobacco etch virus (TEV) protease cleavage site, and Gαi1. For NMR optimization, another construct, Gαi1Δ31, was made in which the first 31 amino acids were deleted. Protein expression was achieved in the *E. coli* Tuner (DE3) pLacI strain (Novagen). Cells were shaken at 37 °C until an OD_{600} of 0.6 was reached, at which point the temperature was lowered to 20 °C before the addition of 150 μM IPTG for the induction of protein production. Cells were shaken for additional 20 h and harvested by centrifugation at 5,000 × *g*. Cells were resuspended in 50 mM NaPi (pH 8.0), 200 mM NaCl, 10 mM β-mercaptoethanol, 1% Triton X-100, and 10% glycerol (buffer A) plus protease inhibitor (Roche). Cells were lysed by lysozyme treatment and sonication and were spun down at 50,000 × *g* for 45 min. Cell extract then was applied to an Ni-NTA column, was washed first with buffer A with 0.5% Triton X-100 and then with buffer A containing 10 mM imidazole and no Triton X-100, and was eluted with buffer B [50 mM NaPi (pH 8.0), 200 mM NaCl, 10 mM β-mercaptoethanol, 10% glycerol, 300 mM imidazole, 20 μM GDP]. Protein was buffer exchanged into 20 mM NaPi (pH 7.5), 50 mM NaCl, 3 mM DTT, 0.5 mM EDTA, 10% glycerol and was digested with TEV protease (1 A_{280} for 100 A_{280} protein). After TEV digestion, the buffer was replaced with 20 mM NaPi (pH 7.8), 200 mM NaCl, 5 mM β-mercaptoethanol, 5% glycerol and was reapplied to an Ni-NTA column. The collected flow-through and wash fractions were pooled, concentrated using 30 kDa MWCO centrifugal devices (Millipore), and applied to a S75 Hi Load 16/600 size-exclusion column (GE Healthcare) equilibrated in 20 mM NaPi (pH 7.0), 50 mM NaCl, 5 mM $MgCl_2$, 5 mM DTT. The protein typically elutes from the column at ∼70 mL (124 mL column volume).

The neurotensin-bound, activated neurotensin receptor 1 variant HTGH4 L167R (28) was produced and purified as described (27, 29). Nanodisc incorporation was achieved by adding decyl maltoside solubilized receptor (concentration = 20 μM) to the nanodisc assembly mixture consisting of 200 μM MSP1D1 (38) and 5 mM POPC lipids solubilized with 20 mM sodium cholate in 20 mM Tris·HCl (pH 7.5), 100 mM NaCl, 0.5 mM EDTA, and 2 mM DTT. Nanodisc assembly then was initiated by dialysis using a 20-kDa MWCO cassette. GPCR-containing nanodiscs were isolated with SP Sepharose and S200 size-exclusion chromatography. Neurotensin peptide (1 μM) was added to the size-exclusion chromatography buffer [20 mM sodium phosphate (pH 7.0), 50 mM NaCl, 5 mM DTT] to keep the receptor in an activated state.

CD Spectroscopy. CD spectra and thermal melting curves were recorded on a Jasco J-715 spectrometer using a cuvette with a 1-mm path length. The protein concentration was 10 μM in 10 mM NaPi (pH 7.0), 20 mM NaCl, 1 mM $MgCl_2$, and 1 mM DTT. The ligand concentration was 50 μM. CD spectra were recorded from 190 to 260 nm using five scans for each sample. The response time was 1 s, and the bandwidth was 1 nm. Thermal scans were run with a heating rate of 60 °C/h. Curve fitting was done with an equation described in ref. 39.

Fluorescence Experiments. Fluorescence experiments were conducted with a Horiba-Jobin Fluoromax-4 instrument. To determine the apparent K_d between GDP and GMP-PNP to Gαi1, we performed a FRET-based binding assay in which 1 μM of MANT-labeled nucleotides (Invitrogen) in buffer [20 mM Hepes (pH 7.0), 100 mM NaCl, 2 mM DTT] were titrated with increasing amounts of Gαi1. During the course of the titration, protein aromatic residues were excited by irradiation at 280 nm (2-nm bandwidth), and the FRET emission signal intensity of the MANT label was detected at 425 nm (5-nm bandwidth). Fluorescence emission and FRET spectra were recorded between 350 and 600 nm and between 320 and 550 nm, respectively.

SAXS Experiments. Measurements in SAXS experiments were performed with Gαi1Δ31 samples on a Rigaku BIOSAXS-1000 instrument using a Pilatus 100K detector (at a distance from the sample of 480 mm) with a HF007 microfocus generator equipped with a Cu target at 40 kV and 30 mA. Transmissions were measured with a photodiode beam stop, and q-calibration was made by a silver-behenate measurement. Samples of 10, 5, and 2 mg/mL protein containing 1 mM of GTPγS, GDP, or no nucleotide were measured. All samples were filtered using a 0.1-μM filter (Whatman) immediately before data collection. Thirty microliters of each sample were loaded in a capillary (1-mm diameter) and exposed to an X-ray beam for 30 min at 20 °C. Circular averaging was done with the Rigaku SAXSLab software v 3.0.1r1. Background subtraction was done within the Origin program (Microcal). Measurements of different concentrations were normalized to exclude concentration-dependent effects. The 10-mg/mL samples then were used for the rigid body modeling with CORAL, ATSAS package v 2.5.0-2 (36). We used the crystal structure of GTP-bound Gα (PDB ID: 1cip) for our modeling calculations. To allow the unrestricted domain movement required to optimize the correlation between experimental and back-calculated SAXS scattering curves, two flexible linkers (five and six amino acid residues in length, respectively) were defined between the Ras and α-H domains. Five independent structural models were calculated, and the model with the lowest χ^2 value was used for further RDC refinement.

NMR Assignment. Sequence-specific assignment of backbone ^{1}HN, ^{15}N, ^{13}Cα, ^{13}Cβ, and ^{13}C′ resonances was first carried out with a 200 μM Gαi1Δ31 + 3 mM GMP-PNP sample labeled with ^{2}H, ^{13}C, and ^{15}N. Triple-resonance experiments used for this purpose include TROSY-based HNCA, HN(CO)CA, HNCACB, HN(CA)CO, and HNCO (20). Additionally, the assignments were validated with a 3D [^{1}H,^{1}H]-NOESY-^{15}N–edited TROSY spectrum recorded with an NOE mixing time of 200 ms. These experiments were recorded in an NUS manner using Poisson-gap sampling (21) at 30 °C on a 600-MHz Bruker spectrometer equipped with a cryogenic TXI probe. Processing of NUS spectra was done with NMRPipe (40) and IST reconstruction (22). Resonance assignments were obtained for ∼85% of all backbone and ^{13}Cβ resonances of the GMP-PNP complex. The same set of experiments was applied to assign resonances in the apo and GDP-bound forms and in the GTPγS complex. Data analysis was done with Sparky 3 software (T. D. Goddard and D. G. Kneller, University of California, San Francisco). Chemical shift perturbations for both ^{15}N and ^{13}C correlation experiments were calculated using the empirical formula $\Delta_{av} = [(\Delta dH^2 + (\Delta N/5)^2)/2]^{0.5}$.

RDCs and Calculation of Structural Models. Weak alignment of ^{2}H,^{15}N-labeled Gαi1Δ31 was induced by the addition of 8 mg/mL Pf1 phage medium (Asla Biotech). RDCs were measured with

TROSY and semi-TROSY experiments in the isotropic and aligned state. RDC values were extracted with the software Sparky.

For RDC refinement, we used the best (lowest χ value) structural model obtained with SAXS in the apo and the GDP-bound form. First, we used the obtained SAXS structural models to back-calculate NOE restraints between heteroatoms in the protein to fix the secondary and tertiary structures of the individual domains during subsequent structure-calculation steps. No restraints between the two domains (Ras and α-H) were included at this point. We then performed structure calculation runs using XPlor-NIH (41) and standard protocols for refinement. RDC back-calculation and fitting of the alignment tensor was done with the program Pales (42). The structures showing the best RDC correlations, i.e., R-factor, were further ranked by their agreement with the initial SAXS scattering curve using Chimera (University of California, San Francisco) (43). The overlay between the final structures and the envelope determined with SAXS using the program DAMMIN [ATSAS package, European Molecular Biology Laboratory Hamburg (EMBL Hamburg) (36)] was done with the program SUBCOMB [ATSAS package, EMBL Hamburg (36)].

NMR Relaxation Dispersion Measurements. Backbone ^{15}N single-quantum CPMG relaxation dispersion experiments were performed on 600-MHz and 700-MHz Agilent DD2 spectrometers equipped with a third-generation cryogenic probe. The CPMG dispersion experiment measures the modulation of the transverse relaxation rate [R_2 ($1/\tau_{cp}$)] as a function of the delay (τ_{cp}) between 180° rf pulses. As shown in the data analysis, the R_2 versus τ_{cp} data can be fitted to determine equilibrium populations (p_a, p_b), kinetics ($k_{ex} = k_1 + k_{-1}$), and chemical-shift differences ($\Delta\omega$) between the two interconverting conformations. The CPMG experiments use a 20-ms constant-time ^{15}N relaxation delay with τ_{cp} spacing corresponding to CPMG-based rf field strengths, ν(CPMG), ranging from 100–900 Hz (44). All spectra were recorded at 30 °C with 0.25 mM [^{2}H,^{15}N]-labeled Gαi1Δ31 in the apo form or in complex with 3 mM GDP or GTPγS, respectively. One hundred twenty-eight complex data points were recorded in the indirect dimension with 96 scans per increment.

For probing side-chain dynamics, a ^{13}C single-quantum CPMG constant-time (20 ms) relaxation dispersion experiment featuring HMQC-based indirect chemical shift evolution optimized for large proteins (35) was recorded on selectively Ileδ1, Leuδ2, Valγ2, Alaβ [^{1}H,^{13}C]-methyl- and otherwise [^{2}H,^{12}C,^{15}N]-labeled Gαi1Δ31 in the apo and GDP-bound forms on Bruker Avance III or HD spectrometers with cryogenic probes operating at proton frequencies of 600 and 900 MHz. CPMG-based RF field strengths ranged from 75 to 975 Hz. For methyl spectra, 96 complex data points were recorded in the indirect dimension with 64 scans. The spectra with different τ_{cp} values were recorded in an interleaved fashion, and duplicate datasets were recorded at two τ_{cp} values for error analysis.

CPMG data were analyzed using the program Relax (45). Initial starting values were determined by grid search, and data subsequently were fitted to motional models [no exchange, fast exchange (46), and a general two-site exchange model (47) for all time scales, and to a two-site numerical solution (44)]. Each spin system was assigned to the best-fitting model based on χ^2 calculations. Finally, the statistical significance of each model was estimated with Monte-Carlo simulations.

MD Simulations. The MD simulation setup with Gαi1Δ31 in its apo form was done with VMD (48). An MD simulation of 1-μs duration was carried out at 310 K with the program NAMD (49) using standard parameters settings. Analysis of the trajectory was performed with VMD or Gromacs (37). Figures were prepared with VMD or Chimera (University of California, San Francisco) (43).

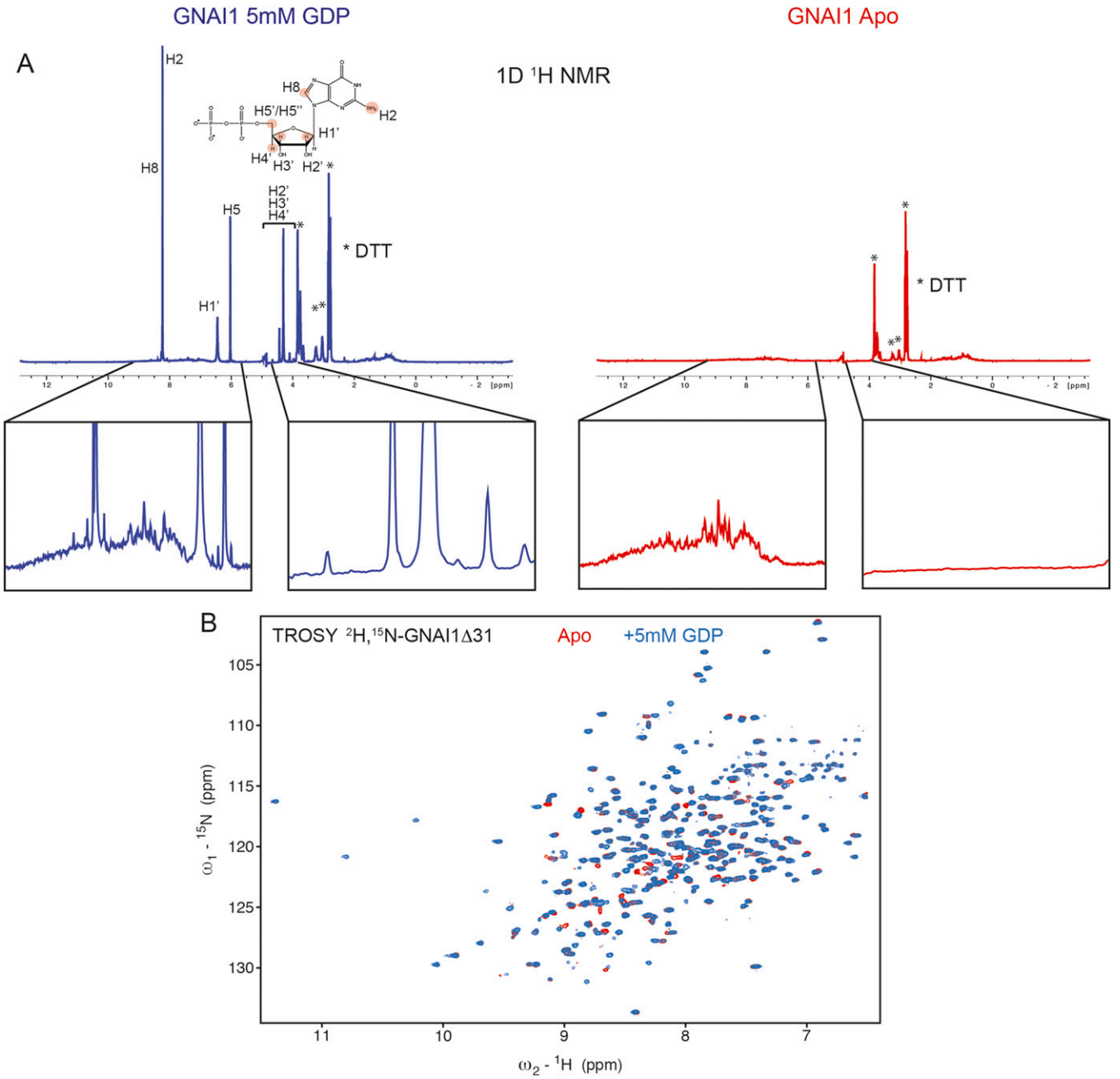

Fig. S1. NMR spectroscopy of Gαi1Δ31 preparations. Gα extracted from *E. coli* culture was purified with Ni-NTA and size-exclusion chromatography. This procedure yielded pure Gαi1 that was free of any bound nucleotides, as can be seen by the spectral differences in 1D [^{1}H]-NMR and 2D [^{15}N,^{1}H]-TROSY in the apo (red spectra) and the GDP-bound (blue spectra) protein. (*A*) Signals arising from GDP are completely absent in the 1D spectrum of the apo sample. Signals labeled with an asterisk originate from the reducing agent DTT in the sample buffer [20 mM sodium phosphate (pH 7.0), 50 mM NaCl, 5 mM $MgCl_2$, 5 mM DTT]. (*B*) A 2D [^{15}N,^{1}H]-TROSY spectrum shows that GDP induced chemical shift perturbations in Gα and indicates that the apo form is able to bind to nucleotides.

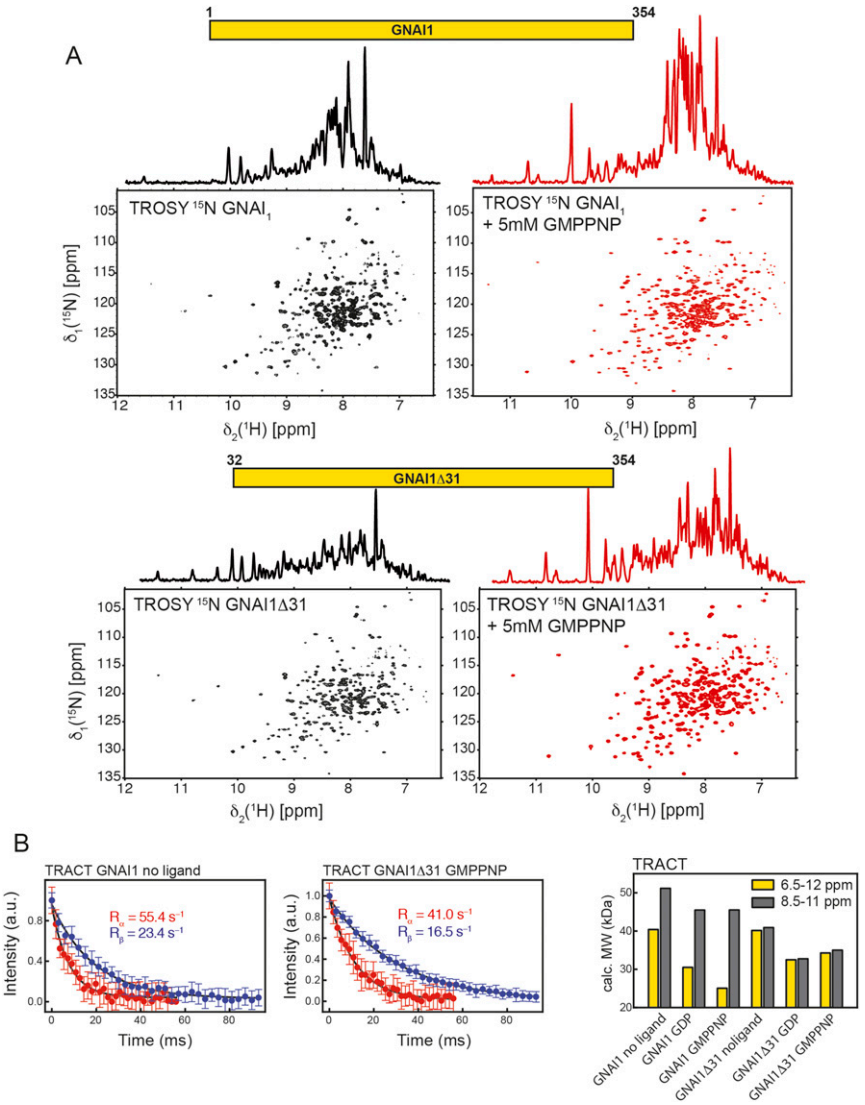

Fig. S2. Initial NMR analysis of Gα constructs. (*A*) 2D TROSY spectra of full-length Gαi1 and the optimized truncated variant Gαi1Δ31. The signal intensity of resonances around 8 ppm is reduced significantly for the Δ31 variant, indicating a lower content of amino acids in an unfolded conformation. The spectral quality in the GMP-PNP–bound form is improved compared with the apo state. (*B*) ^{15}N-TRACT experiments to determine the apparent correlation time and molecular weight of Gαi1 and Gαi1Δ31 in various nucleotide-bound states. Gαi1Δ31 showed improved relaxation properties and thus was used for subsequent NMR experiments.

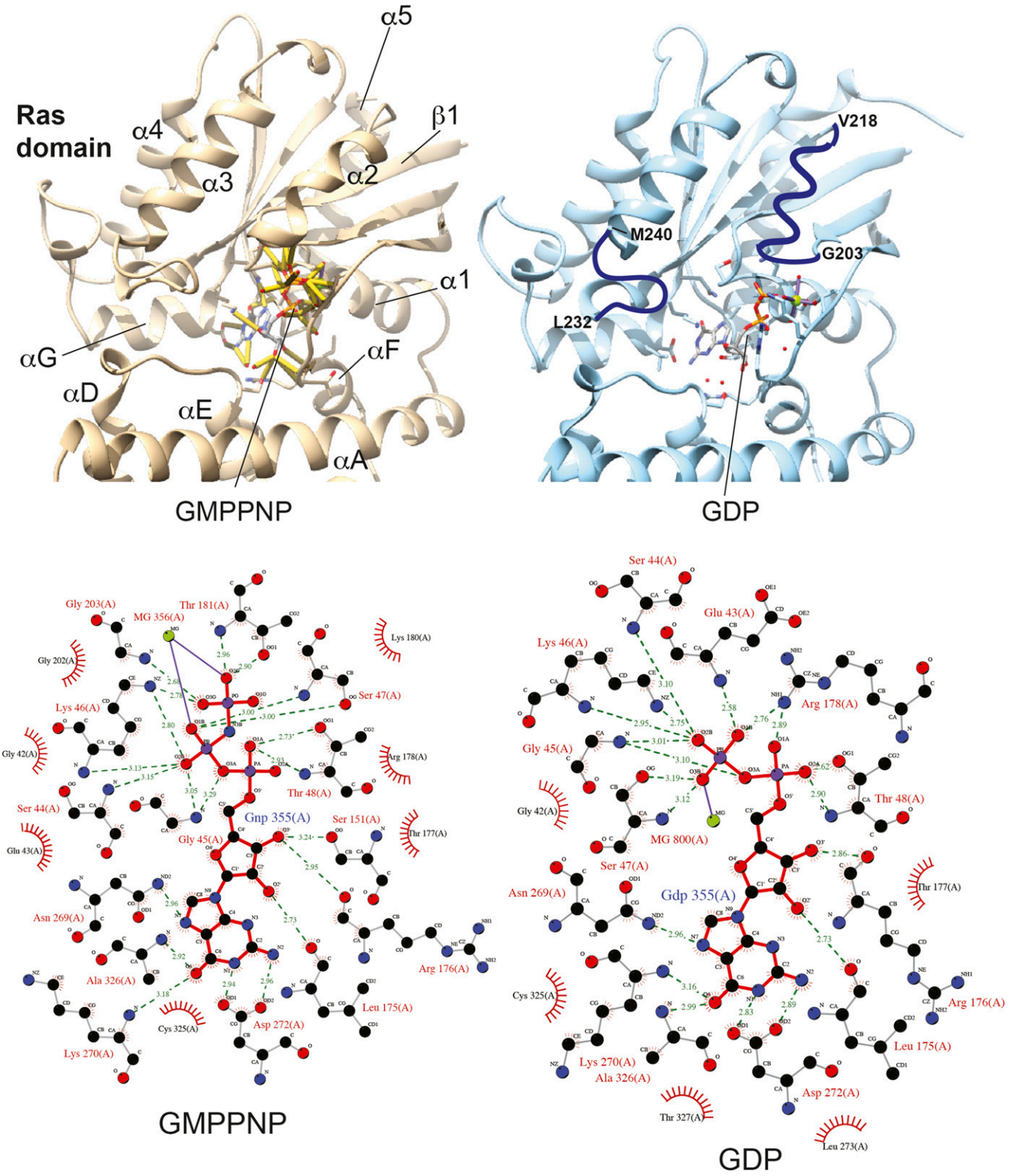

Fig. S3. (*Upper*) Comparison of crystal structures of Gαi1 bound to GMP-PNP (*Left*) and to GDP (*Right*). Regions in the protein not resolved in the GDP-bound structure are marked by lines, and corresponding residues are labeled. (*Lower*) LigPlot+ (50) diagram of the interaction between Gαi1 and GMP-PNP and between Gαi1 and GDP.

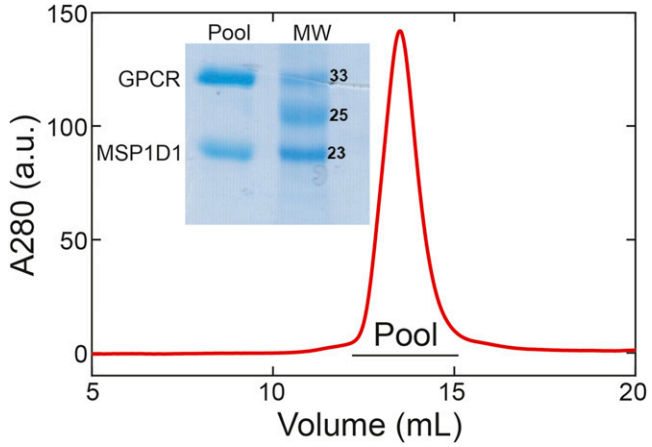

Fig. S4. Assembly of phospholipid nanodiscs containing NTR1. For nanodisc assembly, we used a 20-fold molar excess of MSP1D1 over rat NTR1 (HTGH4 L167R variant) (28) and a POPC-to-MSP ratio of 25:1. After assembly, empty nanodiscs were removed by cation exchange on an SP Sepharose HiTrap column (GE Healthcare). GPCR-containing nanodiscs eluted from the column at a sodium chloride concentration of ~150 mM; empty discs did not bind to the column. Eluted protein was applied to a Superdex S200 10/300 GL size-exclusion column (24 mL bed volume) (GE Healthcare). a.u., arbitrary units.

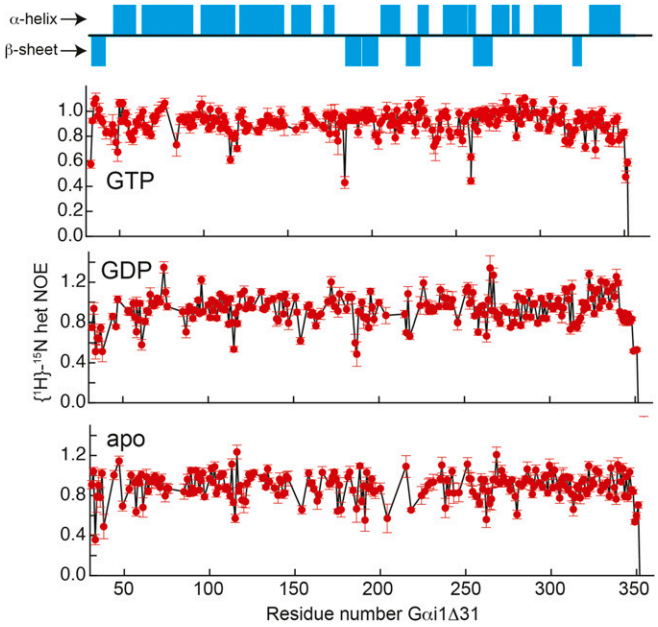

Fig. S5. $\{^1H\}^{15}N$-heteronuclear NOE experiments of Gαi1Δ31 at a field strength of 600 MHz in the apo form and in complex with GDP or GMP-PNP, as indicated, measured at 30 °C. ^1H irradiation time was 2 s. The number of transients was 96 and 128 complex points in the indirect ^{15}N dimension. The error bars underestimate the true uncertainty of the presented data.

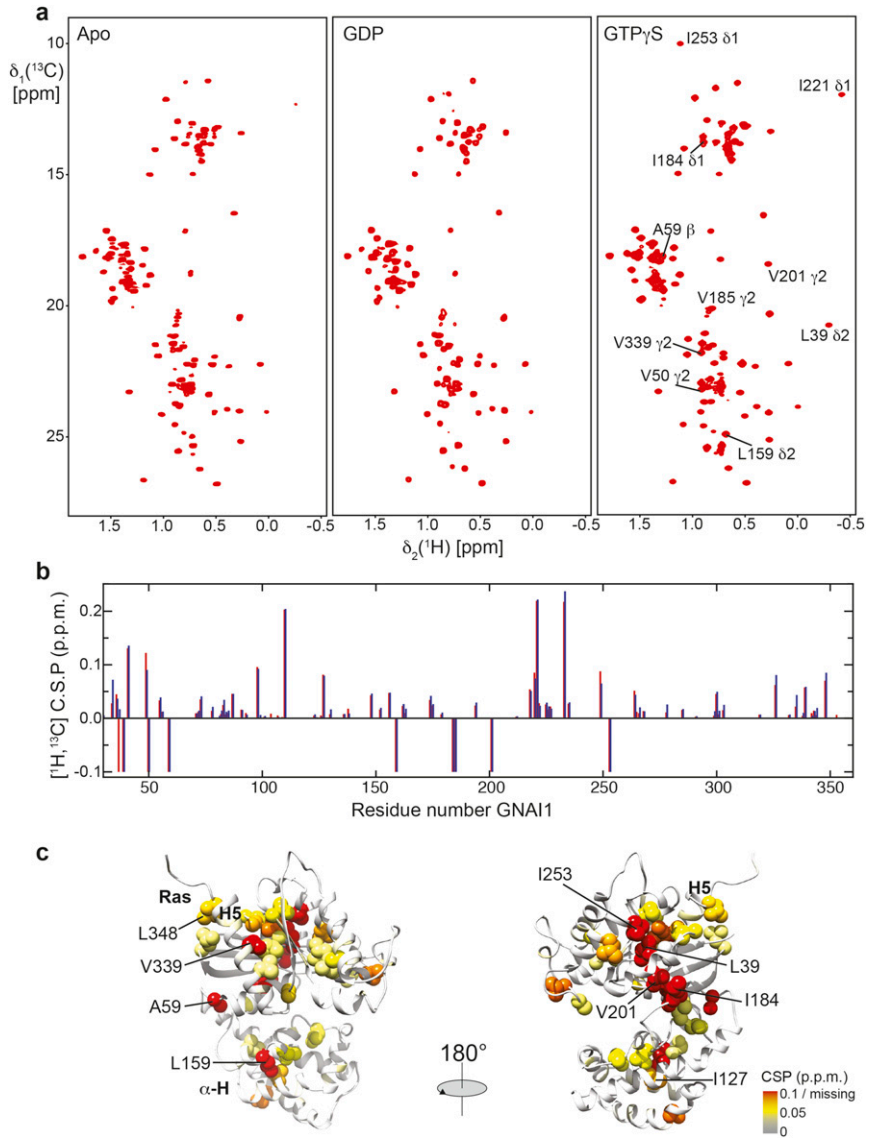

Fig. S6. (*A*) 2D [^{1}H,^{13}C] HMQC experiments of selective Ileδ1, Leuδ2, Valγ2, and Alaβ ^{1}H,^{13}C-methyl– and otherwise [^{2}H,^{12}C,^{15}N]-labeled Gαi1Δ31 in the apo form and in complex with GDP or GTPγS, as indicated, measured at 30 °C. Signals that are severely broadened in the apo and GDP samples are labeled in the GTPγS spectrum. (*B*) Averaged ^{1}H,^{13}C chemical shift perturbation of GNAI1 in the apo (red) or GDP-bound (blue) form versus the GTPγS-bound form. Negative bars indicate missing signals in the apo and GDP-bound samples. (*C*) Gαi1 structure color-coded to show ^{1}H,^{13}C chemical shift perturbations between the apo and the GTPγS-bound forms. The most-affected residues are located within the Ras domain and in the inner core of the α-H domain. Residues in helix 5 are also affected by GTP binding.