Supplementary Materials and Methods

cDNAs

AC2-HA *(8)* and AKAP79-HA *(87)* were described previously. pEF1α-AcGFP-C1 vector was from Clontech, and human M_3R and 3HA-M_3R were from the Bloomsburg University cDNA Resource Centre (www.cdna.org). Human β_2AR *(88)* was a gift from R Summers, and FLAG-β_2AR *(89)* was a gift from R Lefkowitz. M_3R-DREADD (Y149C, A239G) *(39)* was a gift from J Wess. Dominant negative PDE4D3 D484A *(90)* and PDE4D5 D556A *(91)* were gifts from M Houslay. HA-AKAP250 *(92)* was a gift from C Malbon, and pSilencer and AKAP79 shRNA *(56)* were gifts from J Scott.

pmEpac2 *(93)* was a gift from D Cooper. nucEKAR EGFP-mRFP (Addgene plasmid 18682) and cytoEKAR EGFP-mRFP (Addgene plasmid 18680) were gifts from K Svoboda *(94)*. CytoCKAR (Addgene plasmid 14870) and pmCKAR (MyrPalm-CKAR, Addgene plasmid 14862) were gifts from A Newton *(95, 96)*. Raichu-Cdc42 (Raichu-Cdc42/Cdc42CT) was a gift from M Matsuda *(48)*, and was contained within the pCAGGS vector *(97)*, which was a gift from J Miyazaki.

$G\alpha_s$-YFP (Addgene plasmid 55781) and $G\alpha_q$-YFP (Addgene plasmid 55782) were gifts from C Berlot *(98)*. AKAP79-YFP (AKAP79 in a pEYFP-N1 vector) was a gift from M Dell'Acqua *(99)*. YFP-β-arrestin 1 and YFP-β-arrestin 2 were gifts from M Caron *(100)*. PKA catalytic subunit-YFP (PKA-YFP) was a gift from M Zaccolo *(101)*. YFP-PKC-βII-YFP (YFP-PKC; Addgene plasmid 14866) was a gift from A Newton *(96)*.

FLAG-β_2AR D3.32A (D113A) and 3HA-M_3R D3.32A (D148A) were generated using the Quikchange II kit (Agilent Technologies). The D3.32A annotation uses the Ballesteros-Weinstein numbering system *(102)*. FLAG-β_2AR-CFP and 3HA-M_3R-CFP were generated by sub-cloning FLAG-β_2AR and 3HA-M_3R into pECFP-N1. GST-tagged fragments of the β_2AR and M_3R

intracellular regions were generated by amplifying the required region from the full-length cDNA using PCR, and cloning into pGEX-4T1. Shorter regions (β_2AR-ICL1, β_2AR-ICL2, M_3R-ICL1 and M_3R-CT1) were generated by annealing complementary primers, and cloning into pGEX-4T1. The following GST-tagged fragments of the β_2AR were generated: ICL1 (residues 59-71), ICL2 (134-150), ICL3 (221-274), CT (330-413), CT1 (330-357), CT2 (358-386), and CT3 (387-413). The following GST-tagged fragments of the M_3R were generated: ICL1 (91-104), ICL2 (165-185), ICL3 (256-489), ICL3-1 (256-304), ICL3-2 (305-457), ICL3-3 (458-489), CT (546-590), CT1 (546-560), and CT2 (561-590).

RNA sequencing

RNA was extracted from two passages of HEK293 cells (P0 and P37) using the RNeasy Mini Kit (Qiagen), and transcriptome sequencing was performed by the Beijing Genomics Institute.

Fluorescent ligand binding

HEK293 cells were seeded into black, optically clear 96-well plates and grown to 80% confluency. Cells were washed in PBS, then incubated with a nuclear stain (Hoescht 33342, Pierce) and a saturating concentration of antagonist (1 μM ICI-118,551 for β_2AR binding or 100 μM N-methyl scopolamine for M_3R binding) or vehicle control for 1 hour at RT. The fluorescent ligands (1 μM BODIPY-propranolol for β_2AR binding or 100 nM BODIPY-pirenzipine for M_3R binding, both from CellAura) were added for 10 min at RT. Buffer was removed from the cells and replaced with PBS prior to fluorescence imaging using a high-content PerkinElmer Operetta with an Olympus LUCPlanFLN 20x (NA 0.45) objective. Nuclei were visualized using the Hoescht 33342 filter set (excitation 360-400, emission 410-480) and BODIPY fluorescence was visualized using the Cy5 filter set (excitation 620-640, emission 640-680). Four fields of view were captured per well and data were automatically analysed by determining the mean BODIPY fluorescence per well using

Harmony High Content Imaging and Analysis software (v3.5.2). BODIPY fluorescence was expressed relative to the vehicle-treated control in triplicate from *n* biological repeats, as stated.

ELISA

HEK293 cells in 10 cm dishes were transfected with 3 μg pcDNA3.1, FLAG-β_2AR, FLAG-β_2AR D3.32A, 3HA-M_3R or 3HA-M_3R D3.32A, then seeded into 48-well plates 24 hours post-transfection. 48 hours post-transfection, cells were washed with TBS (50 mM Tris pH 7.5, 150 mM NaCl), and fixed (4% paraformaldehyde in TBS, 30 min). Cells were washed in TBS, blocked (1% w/v skim milk, 0.1 M $NaHCO_3$; 4 h, RT with shaking), then incubated with primary antibodies overnight at 4°C (mouse anti-HA or anti-FLAG, both 1:2,000 in 0.1% w/v BSA in TBS). Cells were washed three times with TBS, then incubated with secondary goat anti-mouse-HRP antibody solution (1:2,000, 0.1% w/v BSA in TBS, 2 h, RT). SIGMAFAST^{TM} OPD substrate solution (Sigma-Aldrich) was added and the reaction was terminated with 3M HCl. The samples were transferred to a 96-well plate and optical density at 492 nm was measured using an EnVision Multilabel Reader (PerkinElmer). Data are expressed as the fold change in receptor expression compared to pcDNA3.1 transfected cells from *n* biological repeats as stated.

Confirmation of protein knockdown and dominant negative over-expression

HEK293 cells were seeded into 6-well plates and grown to 70% confluency. Cells were transfected with 25 nM scrambled or targeted siRNA or 1.5 μg pcDNA/pSilencer, targeted shRNA or dominant negative cDNA for 72 hours using PEI. Following transfection, cells were lysed in 100 μL modified RIPA lysis buffer (50 mM Tris pH 7.4, 375 mM NaCl, 1 mM EDTA, 1% v/v Triton X-100, 0.5% w/v sodium deoxycholate, 0.1% w/v SDS) for 30 min on ice. Lysates were centrifuged (10,000*g*, 15 min, 4°C), and protein concentration in the supernatant determined using the Bradford Ultra reagent (Expedeon). Laemmli sample buffer was added to the supernatants, and samples incubated at 37°C for 30 min prior to immunoblotting.

Antibodies for immunoblotting

Immunoblotting was performed using primary antibodies against AKAP79 (Millipore ABS102; rabbit; 1:1,000), β-actin (Abcam ab36956; rabbit; 1:1,000), β-arrestin 1/2 (Cell Signaling 46745; rabbit; 1:1,000), β-arrestin 1 (Abcam ab31868; rabbit; 1:1,000), β-arrestin 2 (Millipore AB6022; rabbit; 1:1,000), β-tubulin (Santa Cruz Biotechnology sc-9104; rabbit; 1:5,000), $G\alpha_{i3}$ (Santa Cruz Biotechnology sc-262; rabbit; 1:1,000), $G\alpha_{q/11}$ (Santa Cruz Biotechnology sc-392; rabbit; 1:1,000), $G\alpha_{s}$ (Millipore 06-237; rabbit; 1:1,000), gravin (AKAP250; Sigma-Aldrich G3795; mouse; 1:1,000), GST (Sigma-Aldrich G1660; mouse; 1:25,000), HA (Abcam ab9110; rabbit; 1:5,000), PDE4D (Abcam ab14613 for GST pulldowns or Santa Cruz Biotechnology sc-25814 for confirmation of protein overexpression; rabbit; 1:1,000), PKA (Santa Cruz Biotechnology sc-903; rabbit; 1:1,000), or PKC (Millipore 05-983; mouse; 1:1,000). Immunoblotting was detected using fluorescent or HRP-conjugated secondary antibodies as follows: goat anti-mouse 680 (LI-COR 926-68070; 1:10,000), goat anti-mouse-HRP (Abcam ab97023; 1:2,000), goat anti-rabbit 800 (LI-COR 926-32211; 1:10,000), goat anti-rabbit-HRP (Cell Signaling 70745; 1:2,000 to detect β-arrestin 2, 1:5,000 to detect AKAP79, β-arrestin 1/2 and PDE4D).

Supplementary Figures

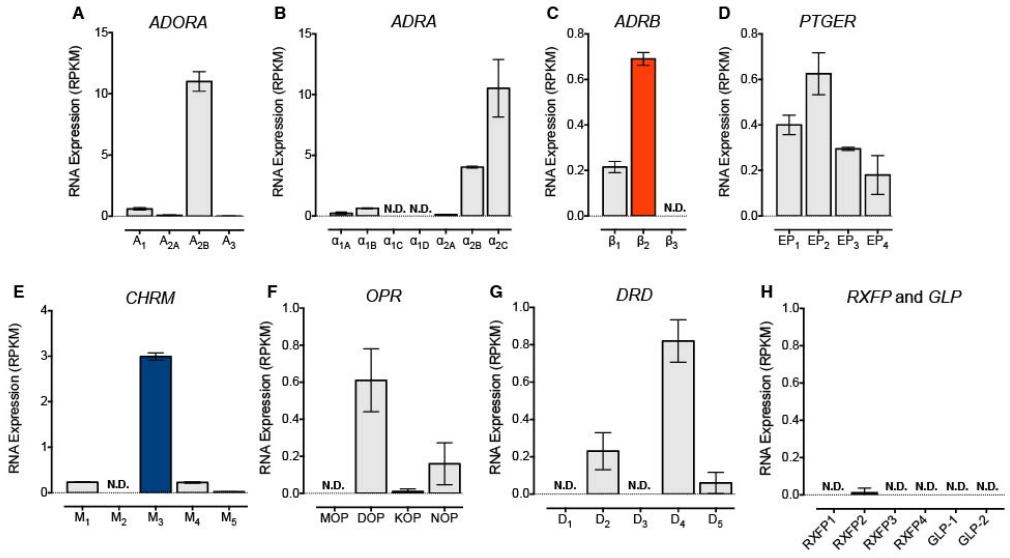

Figure S1. Endogenous expression of GPCRs in HEK293 cells. RNA was isolated from unstimulated native HEK293 cells and the expression of receptor subtypes was determined by RNA sequencing (n=2). (A) adenosine receptors, (B) α-adrenoceptors, (C) β-adrenoceptors, (D) prostanoid receptors, (E) muscarinic receptors, (F) opioid receptors, (G) dopamine receptors and (H) relaxin and glucagon-like peptide receptors. Data are expressed as reads per kilobase of transcript per million mapped reads (RPKM), and shown as mean ± S.D. of two independent experiments. The β_2AR and M_3R are highlighted in red and blue, respectively. N.D. denotes sequence not detected.

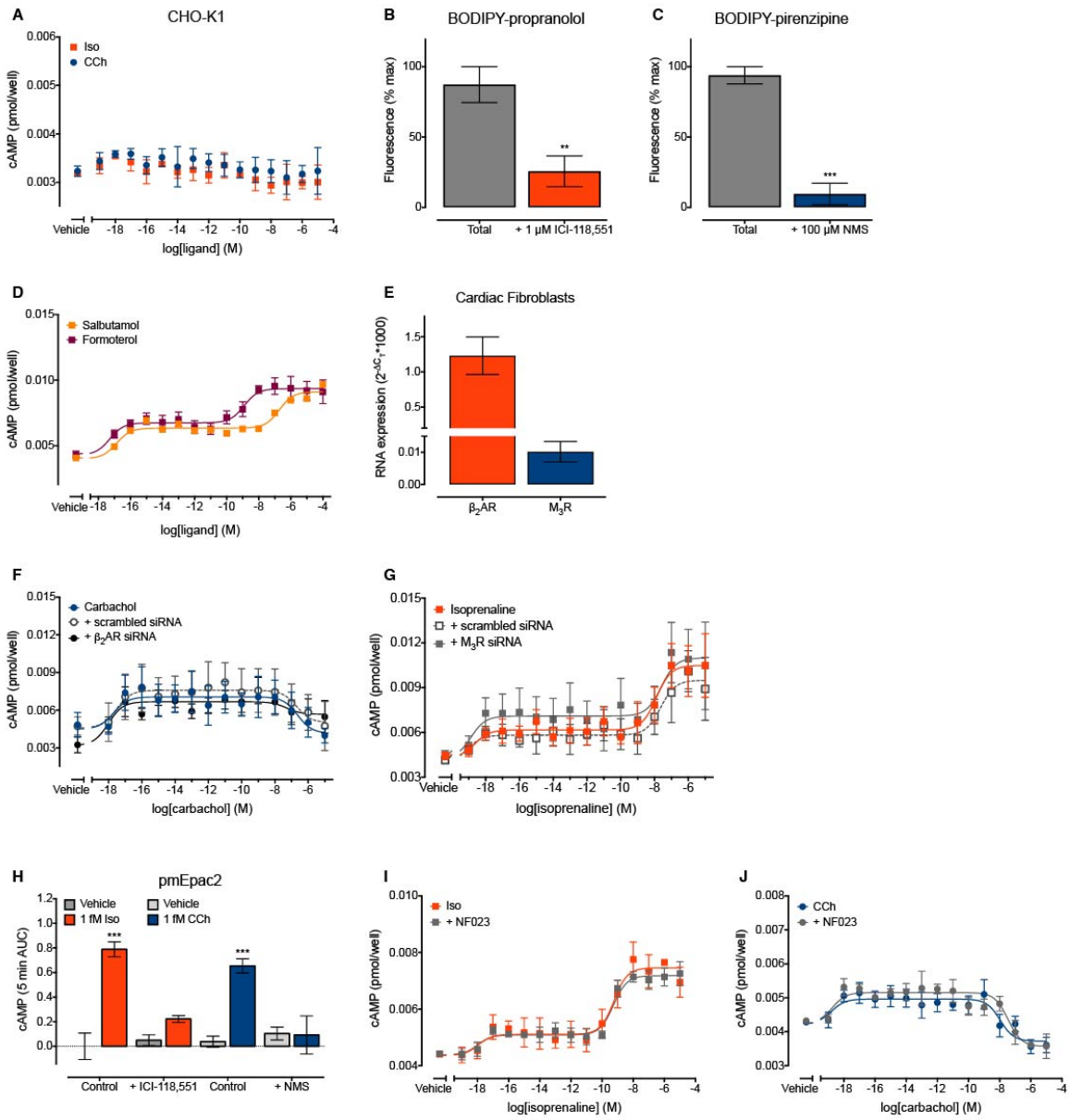

Figure S2. Biphasic changes in cAMP are due to activation of endogenously expressed β_2AR and M_3R. (A) cAMP assay in native CHO-K1 cells stimulated for 30 min in the presence of IBMX by increasing concentrations of Iso or CCh (n=6). (B) Binding of the fluorescent βAR antagonist, BODIPY-propranolol, to the plasma membrane of native HEK293 cells was prevented following pre-incubation with the β_2AR-selective antagonist, ICI-118,551 (used at 100x Ki) (n=3). (C) Binding of the fluorescent MR antagonist, BODIPY-pirenzipine, to the plasma membrane of native HEK293 cells was prevented following pre-incubation with the general cholinergic antagonist, N-methyl scopolamine (NMS) (used at 100x Ki) (n=3). (D) cAMP assay in native HEK293 cells stimulated for 30 min in the absence of IBMX with increasing concentrations of the β_2AR-selective agonists salbutamol or formoterol (n=6). (E) RNA was isolated from unstimulated primary human cardiac fibroblasts and the expression of the β_2AR (red) and M_3R (blue) was determined by qRT-PCR. Data are expressed as $2^{-\Delta CT}$ (difference in Ct value of the gene of interest relative to the housekeeping gene, *ACTB*), and shown as the mean ± S.E.M of three independent experiments. (F-G) cAMP assay in native HEK293 cells in the absence of IBMX following (F) transient transfection with 25 nM scrambled or β_2AR siRNA then stimulation with increasing concentrations of CCh (n=6) or (G) transient transfection with 25 nM scrambled or M_3R siRNA then stimulation with increasing concentrations of Iso (n=6). (H) The area under the curve (AUC) was calculated from Figures 2C and 2F. The effect of antagonist pre-incubation (100 nM ICI-118,551, 100x K_i for

β_2AR; 10 nM N-methyl scopolamine, NMS, 100x Ki for M_3R; 10 min pre-incubation) on cAMP was detected at the plasma membrane in single native HEK293 cells following stimulation with vehicle (0.0001% v/v ascorbic acid for isoprenaline or 0.001% v/v milliQ water for carbachol), 1 fM Iso or 1 fM CCh for 5 min (n=47-79 cells). (I-J) cAMP assay in native HEK293 cells in the absence of IBMX following pre-incubation with the $G\alpha_{i/o}$ inhibitor NF023 (10 μM) then stimulation with increasing concentrations of (I) Iso or (J) CCh (n=6). All data are expressed as the mean ± S.E.M. of n cells or experiments. B, C ** $p<0.01$ and *** $p<0.001$ versus control, unpaired t-test; H *** $p<0.001$ versus vehicle control, two-way ANOVA with Dunnett's multiple comparison test.

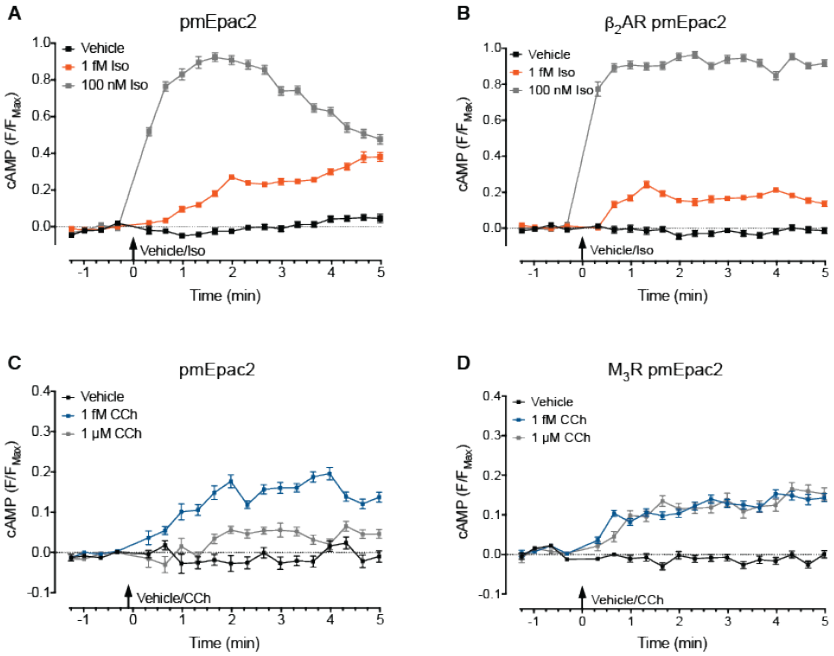

Figure S3. FRET biosensors can detect responses to femtomolar concentrations of ligand with endogenous or exogenous receptor expression. (A-B) cAMP was detected at the plasma membrane in (A) single native HEK293 cells or (B) single HEK293 cells transiently expressing the β_2AR following stimulation with vehicle (0.0001% v/v ascorbic acid), 1 fM or 100 nM Iso for 5 min (n=122-254 cells). The cAMP response to 100 nM Iso in HEK293 cells transiently expressing the β_2AR appears to saturate the pmEpac2 biosensor. (C-D) cAMP was detected at the plasma membrane in (C) single native HEK293 cells or (D) single HEK293 cells transiently expressing the M_3R following stimulation with vehicle (0.001% v/v milliQ water), 1 fM or 1 µM CCh for 5 min (n=164-305 cells). A small increase in cAMP in response to a high concentration of CCh is evident in native HEK293 cells. This returns to baseline by 20 min (see Fig. 5K) and is therefore not detected in cAMP assays in cell populations, which were performed after 30 min stimulation in the absence of IBMX (Fig. 1B). Exogenous expression of the M_3R appears to enhance cAMP responses to high concentrations of CCh (1 µM) suggesting increased coupling of the GPCR to the cAMP pathway. All cells were stimulated at 0 min, and a maximal cAMP response was induced after 5 min (10 µM forskolin with 100 µM IBMX and 100 nM PGE_1). Individual cells were analysed from experiments performed on three separate occasions. Data are expressed as the mean ± S.E.M. of n cells, normalised to the maximal cAMP response induced after 5 min (F/F_{Max}).

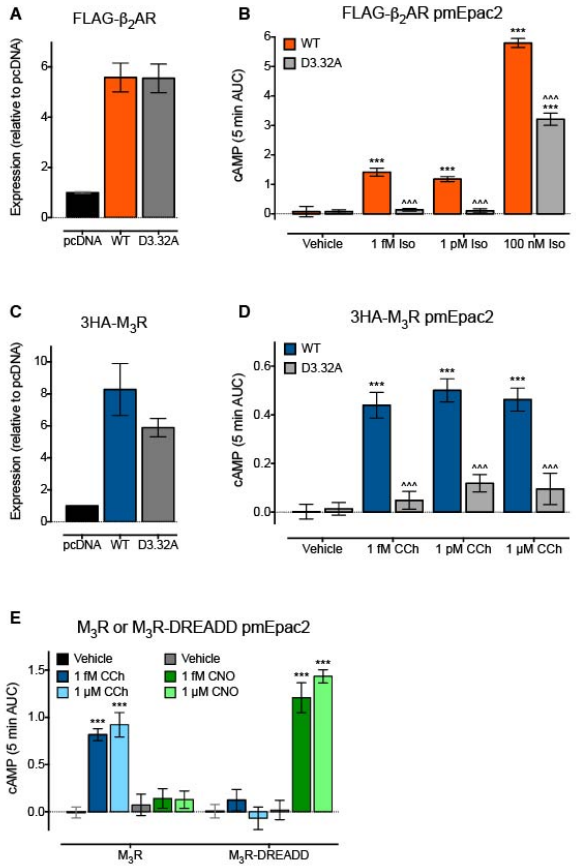

Figure S4. Plasma membrane localization and activity of mutant β2AR and M3R.
(A) The expression of FLAG-β2AR at the cell surface of HEK293 cells transiently expressing pcDNA, wild-type (WT) or mutant D3.32A FLAG-β2AR was determined by ELISA (n=3). Data are expressed relative to pcDNA. (B) The AUC was calculated from Figure 2G. cAMP was detected at the plasma membrane in single HEK293 cells transiently expressing WT or D3.32A mutant FLAG-β2AR following stimulation with vehicle (0.0001% v/v ascorbic acid), 1 fM, 1 pM or 100 nM Iso (n=43-151 cells). (C) The expression of 3HA-M3R at the cell surface of HEK293 cells transiently expressing pcDNA, WT or D3.32A mutant 3HA-M3R was determined by ELISA (n=3). Data are expressed relative to pcDNA control. (D) The AUC was calculated from Fig. 2H. cAMP was detected at the plasma membrane in single HEK293 cells transiently expressing WT or mutant D3.32A 3HA-M3R following stimulation with vehicle (0.001% v/v milliQ water), 1 fM, 1 pM or 1 μM CCh for 5 min (n=119-186 cells). (E) For M3R-DREADD, the AUC was calculated from Fig. 2I. cAMP was detected at the plasma membrane in single HEK293 cells transiently expressing WT M3R or mutant M3R-DREADD following stimulation with vehicle (0.001% v/v milliQ water for CCh, or 0.01% v/v DMSO for CNO), 1 fM or 1 μM CCh, or 1 fM or 1 μM CNO for 5 min (n=57-94 cells). CNO can only activate the M3R-DREADD, whereas CCh cannot. All data are expressed as the mean ± S.E.M. of n cells or experiments. For single cell cAMP measurements, individual cells were analysed from experiments performed on three independent occasions. *** $p<0.001$ versus vehicle control, two-way ANOVA with Dunnett's (B, D) or Tukey's (E) multiple comparison test.

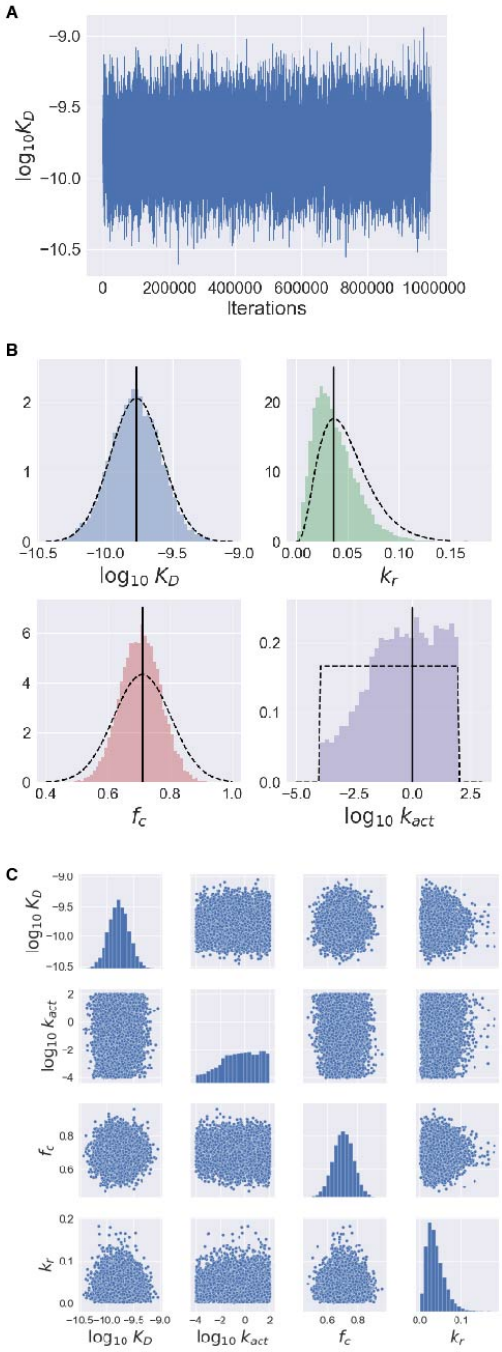

Figure S5. Modeling responses to femtomolar concentrations of Iso. (A) The MCMC sampling trace for $\log_{10} K_D$. The first 10,000 points were discarded as the burn-in period (sampled points prior to reaching stationarity). A total of 990,000 sampled points are shown here. (B) Posterior distributions for the four free parameters (from upper left: $\log_{10} K_D$, k_r, f_c, and $\log_{10} k_{act}$). The black vertical line indicates the parameter value corresponding to the MAP estimate, and the dashed curve indicates the prior distribution. (C) Pairwise relationships between the four free parameters, where the number of points is downsampled for clarity. The diagonal contains downsampled posterior distributions similar to those shown in (B).

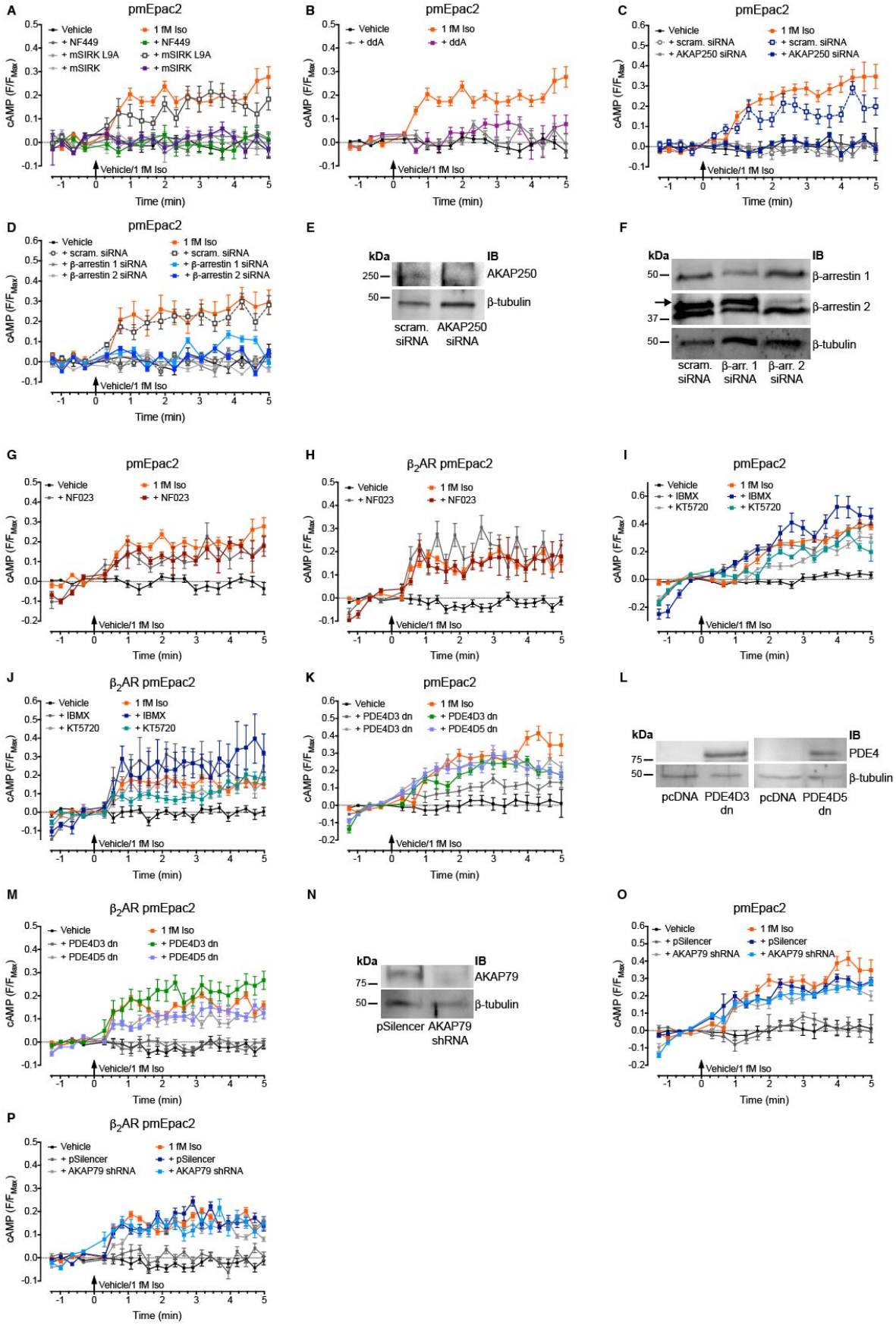

Figure S6. Identification of proteins involved in stimulation and regulation of responses to 1 fM Iso. (A-D) Time courses of cAMP production from which the area under the curve (AUC) presented in Fig. 3A was calculated. cAMP was detected at the plasma membrane in single native HEK293 cells following stimulation with vehicle (0.0001% v/v ascorbic acid) or 1 fM Iso for 5

min. Cells were pre-treated with (A) the $G\alpha_s$ antagonist NF449 (10 μM), the Gβγ inhibitor mSIRK (5 μM) or negative control peptide mSIRK L9A (5 μM) (n=40-85 cells), (B) the AC inhibitor 2',5'-dideoxyadenosine (ddA; 100 μM) (n=28-40 cells), (C) transiently transfected with 25 nM scrambled (scram.) or AKAP250 siRNA (n=47-167 cells), or (D) transiently transfected with 25 nM scram. or β-arrestin 1 or β-arrestin 2 siRNA (n=20-126 cells). (E-F) Immunoblotting shows decreased expression of (E) AKAP250 and (F) β-arrestin 1 and β-arrestin 2 following transfection of HEK293 cells with targeted siRNAs. β-tubulin was used as a loading control.
(G-K, M, O-P) Time courses of cAMP production from which the area under the curve (AUC) presented in Fig. 3B and 3C were calculated. cAMP was detected at the plasma membrane in single HEK293 cells following stimulation with vehicle (0.0001% v/v ascorbic acid) or 1 fM Iso for 5 min. (G) Native cells (n=49-85 cells) or (H) cells transiently transfected with the β_2AR (n=34-84 cells) were pre-treated with the $G\alpha_{i/o}$ antagonist NF023 (10 μM). (I) Native cells (n=22-97 cells) or (J) cells transiently transfected with the β_2AR (n=22-84 cells) were pre-treated with the PDE inhibitor IBMX (100 μM) or the PKA inhibitor KT5720 (1 μM). (K) Native cells (n=47-93) were transiently transfected with PDE4D3 dominant negative (dn) or PDE4D5 dn. (L) Immunoblotting of HEK293 cells shows increased expression of PDE4 following transfection of PDE4D3 dn or PDE4D5 dn. β-tubulin was used as a loading control. (M) Cells transiently transfected with the β_2AR (n=59-124 cells) were transiently transfected with PDE4D3 dn or PDE4D5 dn. (N) Immunoblotting of HEK293 cells shows decreased expression of AKAP79 following transfection of AKAP79 shRNA. β-tubulin was used as a loading control. (O) Native cells (n=47-109 cells) or (P) cells transiently transfected with the β_2AR were transiently transfected with pSilencer control or AKAP79 shRNA. All cells were stimulated at 0 min, and a maximal cAMP response was induced after 5 min (10 μM forskolin with 100 μM IBMX and 100 nM PGE_1). Individual cells were analysed from experiments performed on three independent occasions. Data are expressed as the mean ± S.E.M. of n cells, normalised to the maximal cAMP response induced after 5 min (F/F_{Max}).

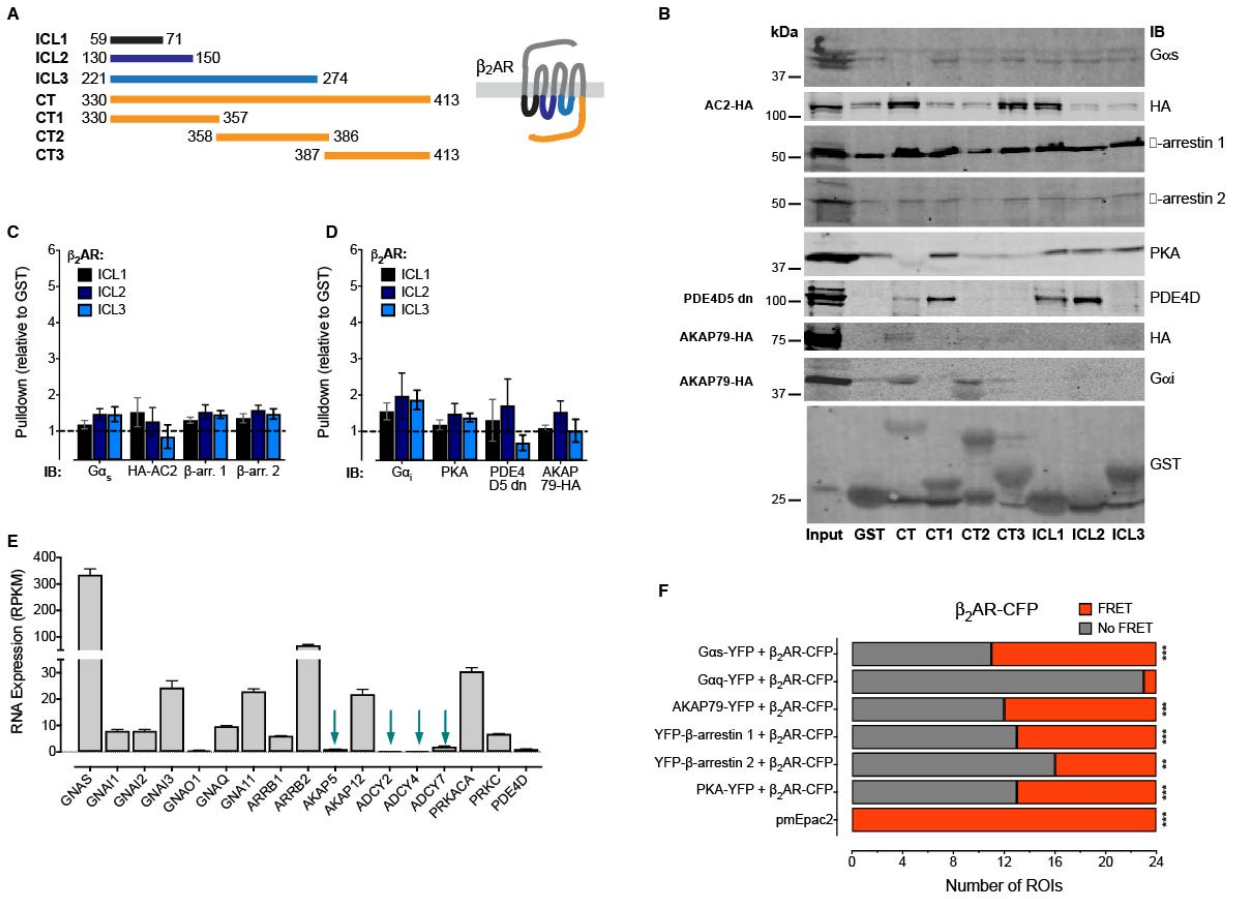

Figure S7. The β_2AR forms a pre-assembled signalling complex. (A) Cartoon showing the regions of the β_2AR ICLs and CT that were tagged with GST and used for GST pull-downs. (B) Representative immunoblots (IB) of GST pulldowns from unstimulated HEK293 cell lysates using GST alone or intracellular fragments of the β_2AR. The immunoblots are the complete version of the immunoblots presented in Fig. 3G, showing GST-tagged fragments of the intracellular loops (ICL) as well as the C-terminus (CT). Immunoblots were probed with antibodies for proteins identified as essential for activation or regulation of responses to 1 fM Iso (from Fig. 3, A to C). Endogenous $G\alpha_s$, β-arrestin 1, β-arrestin 2 and PKA were pulled down from native HEK293 cell lysates. Proteins with low levels of endogenous expression (AC2, PDE4D and AKAP79, see E) could not be pulled down from native HEK293 cell lysates, but could be pulled down from lysates of HEK293 cells transiently expressing the protein of interest (transfection of HEK293 cells indicated by bold font on the left of the immunoblots). While $G\alpha_i$ could not be pulled down from native HEK293 cell lysates, the G protein was pulled down from HEK293 cell lysates transiently expressing AC2-HA, PDE4D5 dn or AKAP79-HA (blot from lysates expressing AKAP79-HA is shown, indicated by bold font on the left of the immunoblot). (C-D) Quantification of GST pulldowns from unstimulated HEK293 cell lysates using ICL fragments for the proteins required for (C) activation of cAMP in response to 1 fM Iso (identified in Fig. 3A), or (D) regulation of the constitutive activity of the pre-assembled β_2AR complex (identified in Fig. 3, B and C). Quantification of GST pulldowns using CT fragments is shown in Fig. 3, E and F. Band densities were normalised for equivalent expression of GST, and expressed relative to GST alone. Data are expressed as the mean ± S.E.M. of five to six independent experiments. (E) RNA was isolated from unstimulated native HEK293 cell populations and the expression of proteins involved in activation and regulation of responses to 1 fM Iso and CCh was determined by RNA sequencing. RNA sequencing revealed expression of multiple PKC isoforms (PKCα, PKCβ_1, PKCγ, PKCδ, PKCε, PKCη, PKCθ, PKCζ and PKCι; *(77)*); the RPKM values were averaged. Green arrows indicate proteins with low levels of endogenous expression. Data are expressed as mean ± S.D. of two

independent experiments. (F) FRET efficiency data for the β_2AR-CFP and YFP-tagged components of the protein complex from Fig. 3J converted to binary values (1 = FRET, 0 = no FRET) (n=24 regions of interest, ROI). pmEpac2 was used as a positive control. ** $p<0.01$ and *** $p<0.001$ versus β_2AR-CFP/Gαq-YFP FRET (negative control), Chi-square test.

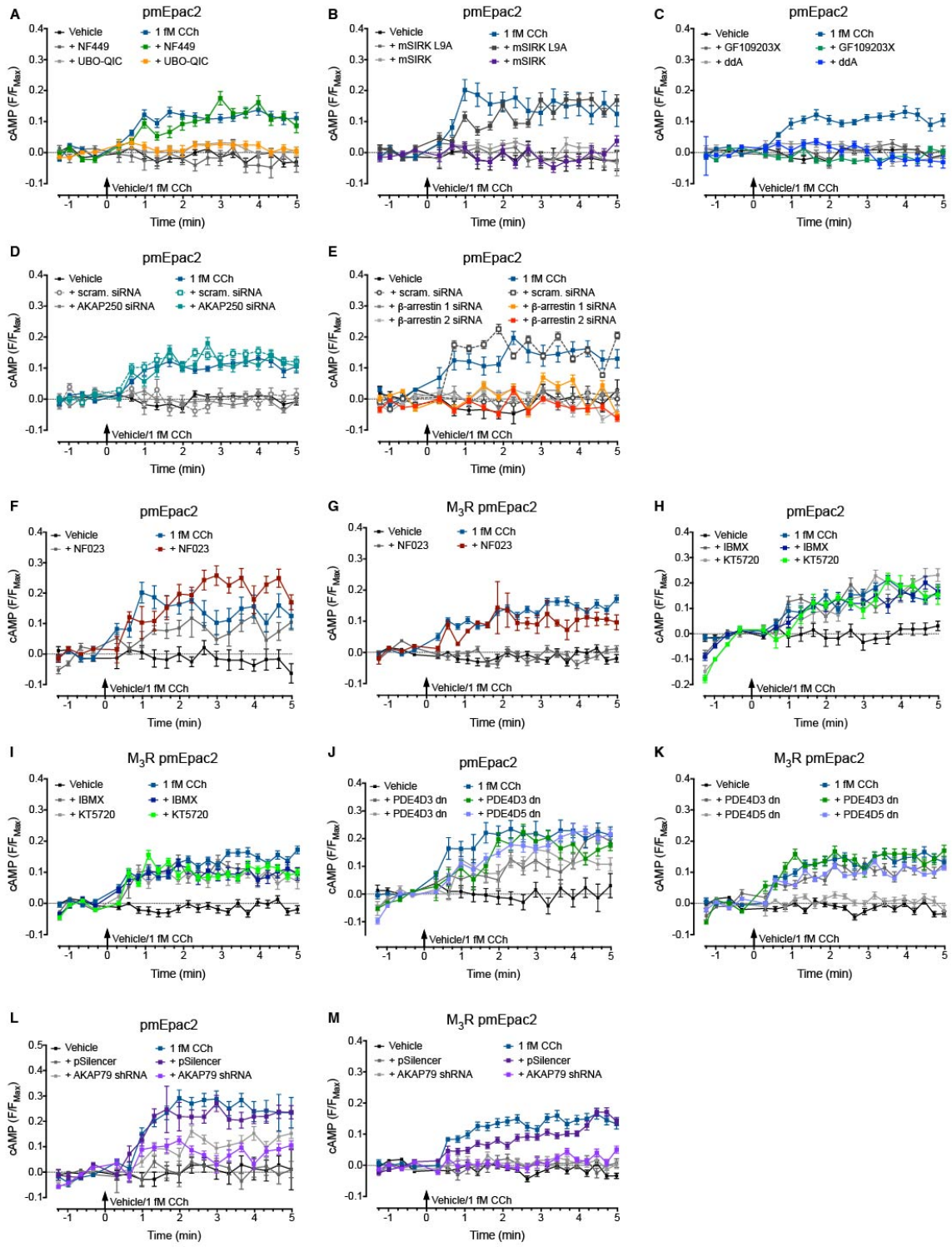

Figure S8. Identification of proteins involved in stimulation and regulation of responses to 1 fM CCh. (A-E) Time courses of cAMP production from which the area under the curve (AUC) presented in Fig. 4A was calculated. cAMP was detected at the plasma membrane in single native HEK293 cells following stimulation with vehicle (0.001% v/v milliQ water) or 1 fM CCh for 5 min. Cells were pre-treated with (A) the $G\alpha_s$ antagonist NF449 (10 μM) or the $G\alpha_{q/11}$ inhibitor UBO-QIC (100 nM) (n=29-105 cells), (B) the Gβγ inhibitor mSIRK (5 μM) or negative control peptide mSIRK L9A (5 μM) (n=39-53 cells), (C) the AC inhibitor 2',5'-dideoxyadenosine (ddA; 100 μM) or the PKC inhibitor GF109203X (1 μM) (n=41-91 cells), (D) transiently transfected with 25 nM scrambled (scram.) or AKAP250 siRNA (n=113-222 cells), or (E) transiently transfected with 25 nM scram. or β-arrestin 1 or β-arrestin 2 siRNA (n=31-120 cells). (F-M) Time courses of cAMP production from which the AUC presented in Fig. 4B and 4C were calculated. cAMP was

detected at the plasma membrane in single native HEK293 cells following stimulation with vehicle (0.001% v/v milliQ) or 1 fM CCh for 5 min. (F) Native cells (n=29-53 cells) or (G) cells transiently transfected with the M_3R (n=72-152 cells) were pre-treated with the $G\alpha_{i/o}$ antagonist NF023 (10 μM). (H) Native cells (n=31-135 cells) or (I) cells transiently transfected with the M_3R (n=65-155 cells) were pre-treated with the PDE inhibitor IBMX (100 μM) or the PKA inhibitor KT5720 (1 μM). (J) Native cells (n=36-81 cells) or (K) cells transiently transfected with the M_3R (n=86-134 cells) were transiently transfected with PDE4D3 dominant negative (dn) or PDE4D5 dn. (L) Native cells (n=34-79 cells) or (M) cells transiently transfected with the M_3R (n=71-134 cells) were transiently transfected with pSilencer control or AKAP79 shRNA. All cells were stimulated at 0 min, and a maximal cAMP response was induced after 5 min (10 μM forskolin with 100 μM IBMX and 100 nM PGE_1). Individual cells were analysed from experiments performed on three independent occasions. Data are expressed as the mean ± S.E.M. of n cells, normalised to the maximal cAMP response induced after 5 min (F/F_{Max}).

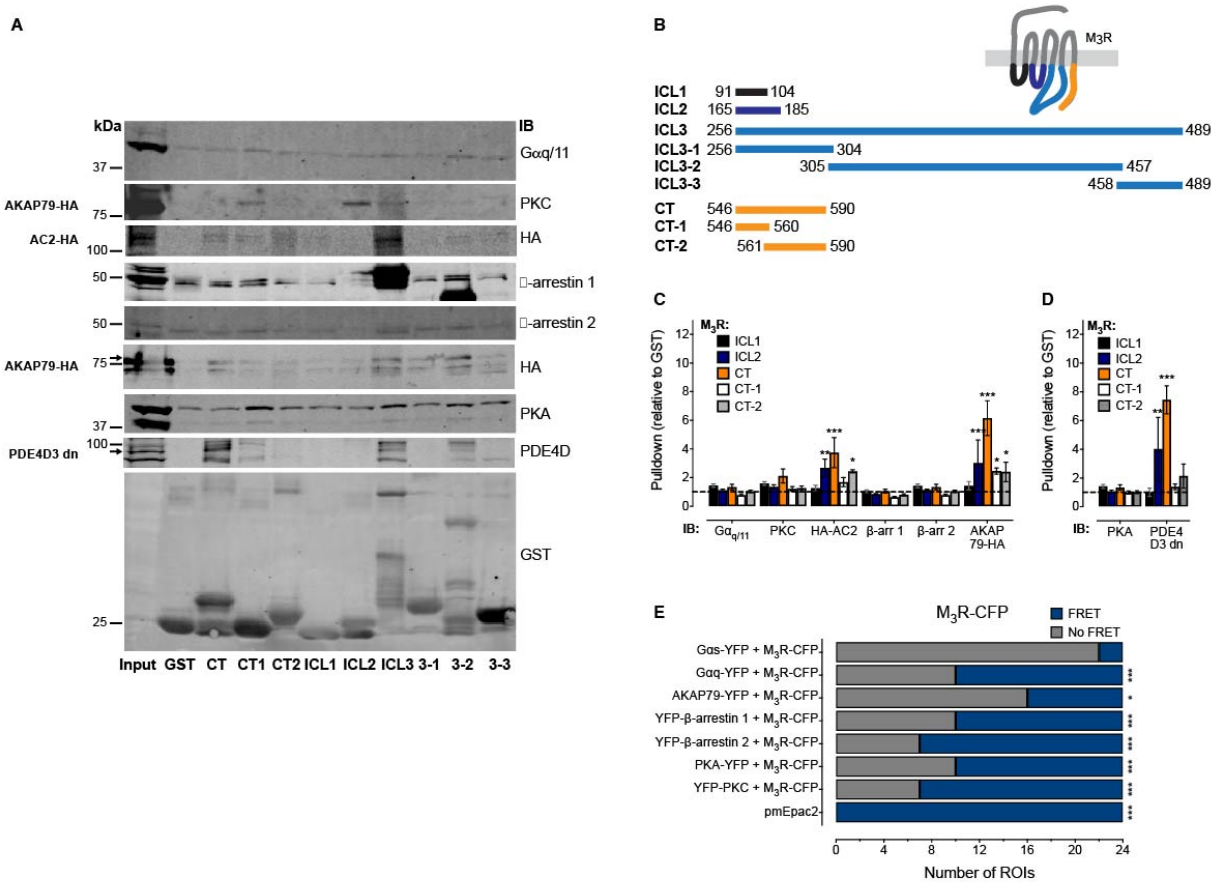

Figure S9. The M_3R forms a pre-assembled signalling complex. (A) Representative immunoblots of GST pulldowns from unstimulated HEK293 cell lysates using GST alone or intracellular fragments of the M_3R. The immunoblots are the complete version of the immunoblots presented in Fig. 4G, showing GST-tagged fragments of the CT, ICL1 and ICL3, in addition to ICL2. Immunoblots were probed with antibodies for proteins identified as essential for activation or regulation of responses to 1 fM CCh (from Fig. 4, A to C). Endogenous $G\alpha_{q/11}$, β-arrestin 1, β-arrestin 2 and PKA were pulled down from native HEK293 cell lysates. Proteins with low levels of endogenous expression (AC2, AKAP79 and PDE4D, see fig. S7E) could not be pulled down from native HEK293 cell lysates, but could be pulled down from lysates of HEK293 cells transiently expressing the protein of interest (transfection of HEK293 cells indicated by bold font on the left of the immunoblots). While PKC could not be pulled down from native HEK293 cell lysates, the kinase was pulled down from HEK293 cell lysates transiently expressing AC2-HA, PDE4D3 dn or AKAP79-HA (blot from lysates expressing AKAP79-HA is shown, indicated by bold font on the left of the immunoblot). (B) Cartoon showing the regions of the M_3R ICLs and CT that were tagged with GST and used for GST pull-downs. (C-D) Quantification of GST pulldowns from unstimulated HEK293 cell lysates using CT, ICL1 and ICL2 fragments for the proteins required for (C) activation of cAMP in response to 1 fM CCh (identified in Fig. 4A), or (D) regulation of the constitutive activity of the pre-assembled M_3R complex (identified in Fig. 4, B and C). Quantification of GST pulldowns using ICL3 fragments is shown in Fig. 4, D and E. In addition to interacting with ICL3, HA-AC2, AKAP79-HA and PDE4D3 dn can also interact with ICL1, ICL2 and the CT. Band densities were normalised for equivalent expression of GST, and expressed relative to GST alone. Data are expressed as the mean ± S.E.M. of five to six independent experiments. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ versus GST alone, two-way ANOVA with Dunnett's multiple comparison test. (E) FRET efficiency data for the M_3R-CFP and YFP-tagged components of the protein complex from Fig. 4I converted to binary values (1 = FRET, 0 = no FRET) (n=24 regions of interest, ROI). pmEpac2 was used as a positive control. * $p<0.05$ and *** $p<0.001$ versus M_3R-CFP/Gαs-YFP FRET (negative control), Chi-square test.

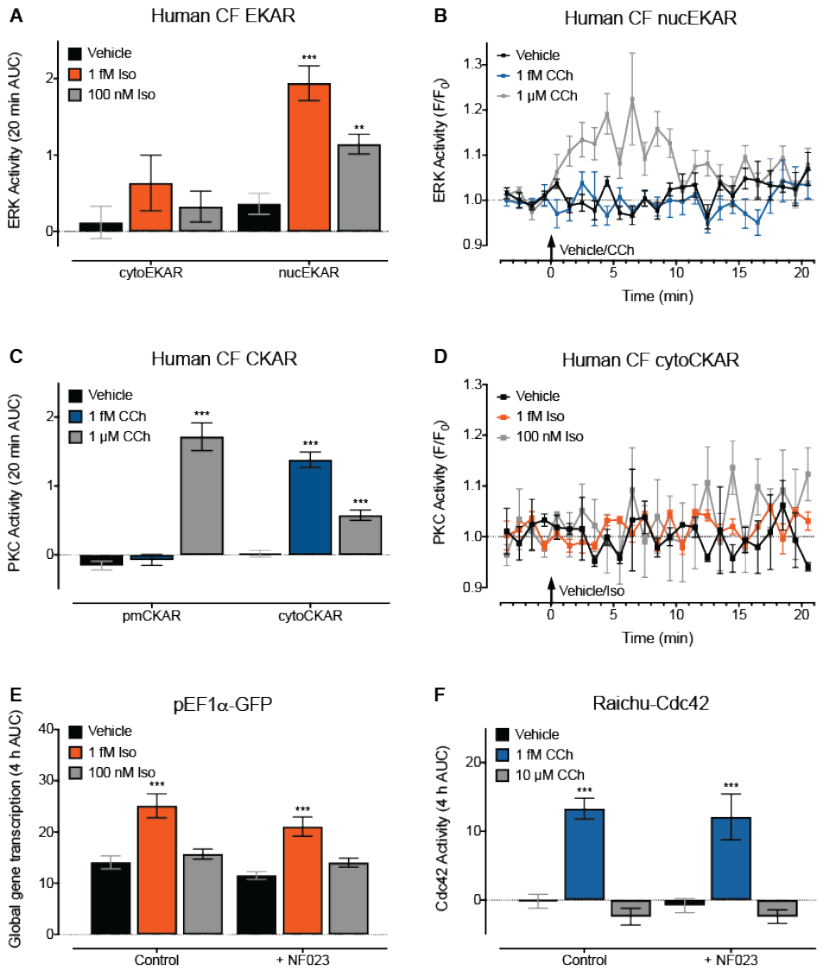

Figure S10. Femtomolar ligand concentrations activate compartmentalised signalling and unique cell responses. (A) The area under the curve (AUC) was calculated from Fig. 5D and ERK activity detected in the cytosol of human cardiac fibroblasts (CF) in response to vehicle (0.0001% v/v ascorbic acid), 1 fM or 100 nM Iso for 20 min (n=12-34 cells). (B) ERK activity was detected in the nucleus of human cardiac fibroblasts (CF) in response to vehicle (0.001% v/v milliQ water), 1 fM or 1 µM CCh for 20 min (n=13-20 cells). Data are expressed relative to baseline fluorescence (F/F_0). (C) The area under the curve (AUC) was calculated from Fig. 5J and PKC activity detected at the plasma membrane of human cardiac fibroblasts (CF) in response to vehicle (0.001% v/v milliQ water), 1 fM or 1 µM CCh for 20 min (n=62-155 cells). (D) PKC activity was detected in the cytosol of human cardiac fibroblasts (CF) in response to vehicle (0.0001% v/v ascorbic acid), 1 fM or 100 nM Iso for 20 min (n=16-18 cells). Data are expressed relative to baseline fluorescence (F/F_0). (E) Effect of pre-incubation with the $G\alpha_{i/o}$ inhibitor, NF023 (10 µM), on the GFP fluorescence in single native HEK293 cells expressing the constitutive promoter pEF1α-GFP reporter gene following stimulation with vehicle (0.0001% v/v ascorbic acid), 1 fM or 100 nM Iso (n=64-107 cells) for 4 hours (n=272-383 cells). Data are expressed as the area under the curve (AUC). (F) Effect of pre-incubation with the $G\alpha_{i/o}$ inhibitor, NF023 (10 µM), on the activation of Cdc42 in single native HEK293 cells following stimulation with vehicle (0.001% v/v milliQ), 1 fM or 10 µM CCh (n=121-335 cells) for 4 hours. Data are expressed as the area under the curve (AUC). For all experiments, individual cells were analyzed from experiments performed on three independent occasions. ** $p<0.01$ and *** $p<0.001$ versus vehicle control, two-way ANOVA with Dunnett's multiple comparisons test.