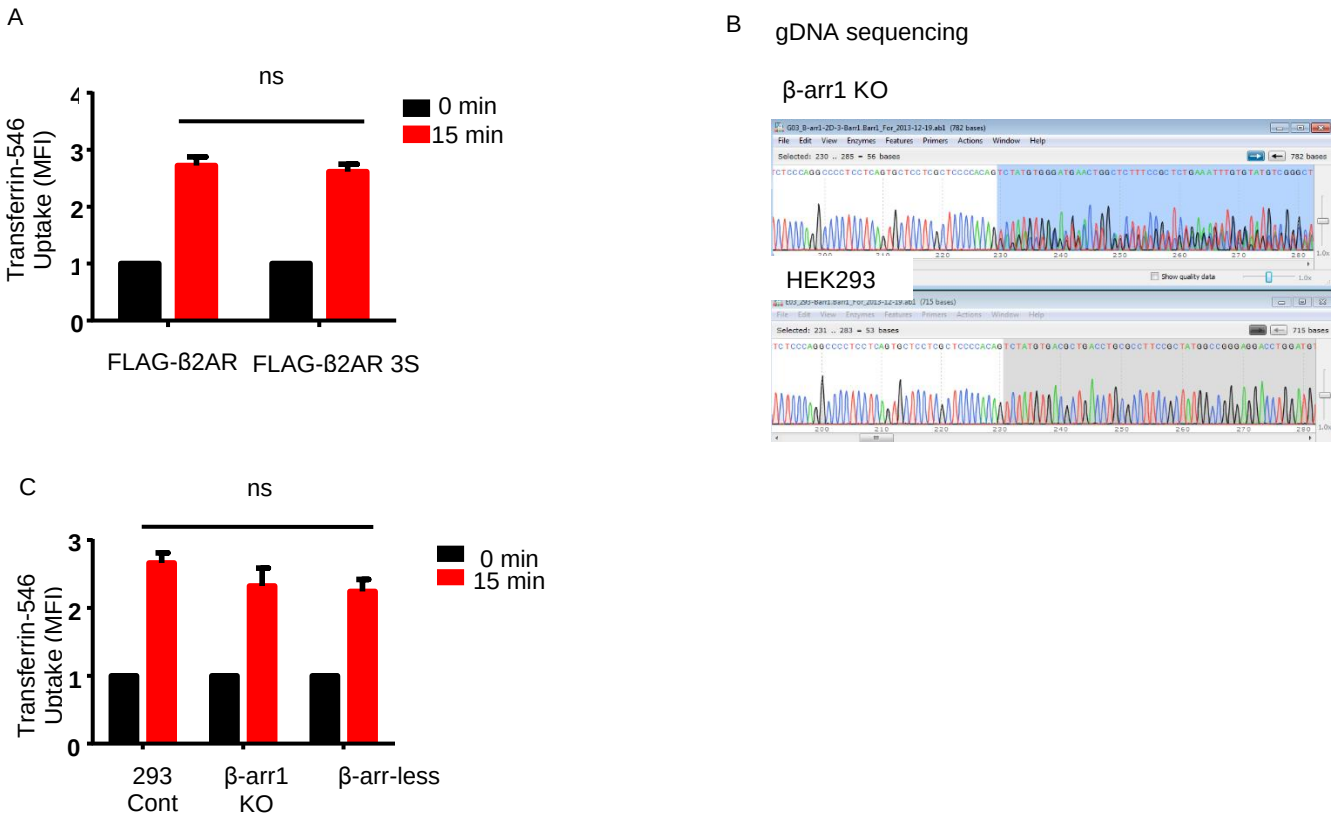

Fig. S1 . Transferrin uptake experiments and genomic DNA sequencing of the β-arr1 KO cell line. A) Quantification of median fluorescence intensity (MFI) of 15 min transferrin-546 uptake by flow cytometry in 293 FLAG-β_2AR and 293 FLAG-β_2AR 3S cells); mean of three independent experiments ± SD. B) Sequence chromatogram of genomic DNA (gDNA) surrounding the β-arr1 TALEN cut site in the β-arr1 KO clone (top) and HEK293 WT control (bottom). C) Flow cytometry quantification of MFI of 15 min transferrin-546 uptake in 293 FLAG-β_2AR, β-arr1 KO, and β-arr1-less cells); mean of three independent experiments ± SD. Statistical significance was determined based on an ANOVA test; ns indicates not significant.

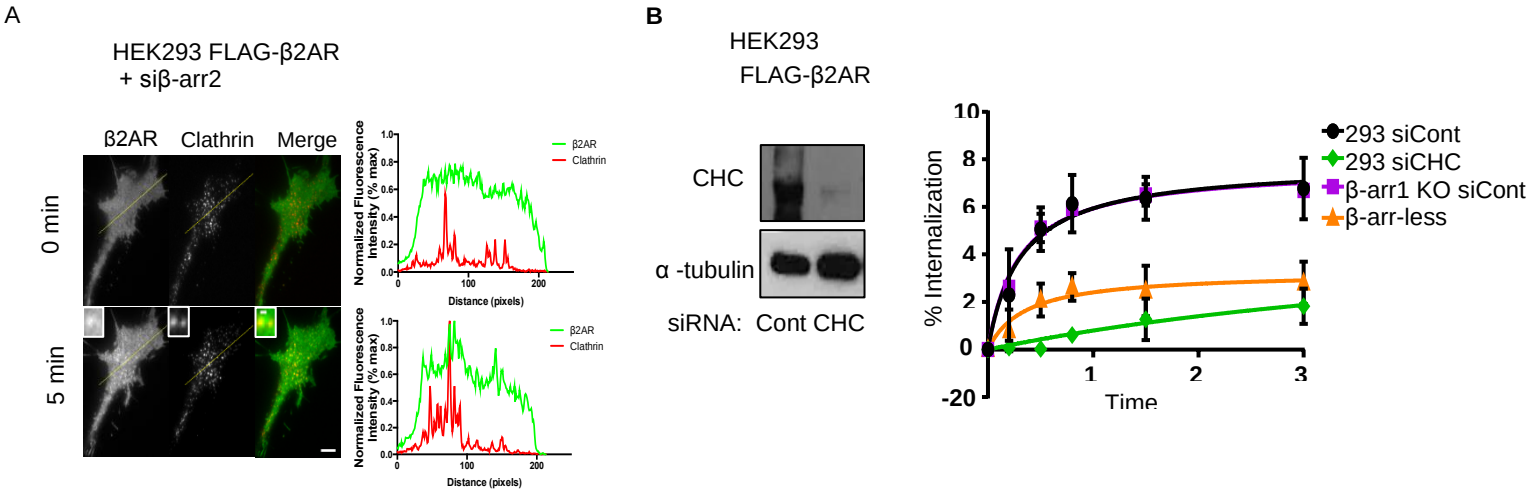

Fig. S2. Clathrin is important for isoproterenol-mediated β_2AR internalization. A) TIRF microscopy image of FLAG-β_2AR (green) and clathrin coated pits (red) in 293 FLAG-β_2AR cells transfected with siRNA against β-arr2, before and after 5 min Iso stimulation. Graphs on the right hand side depict the overlap in fluorescence intensity across the cell, as shown by the designated lines, between FLAG-β_2AR (green) and clathrin coated pits (red) as an indication of colocalization. Representative of three independent experiments. Scale bar, 5 µm, inset scale bar 500 nm. B) Western blot showing knockdown of clathrin heavy chain (siCHC) and α-tubulin loading control (left, representative of three independent experiments) and a flow-cytometry based internalization assay comparing FLAG-β_2AR internalization upon knockdown of CHC with β-arr1-less, β-arr1 KO and 293 siCont cells (right); mean of three independent experiments ± SD.

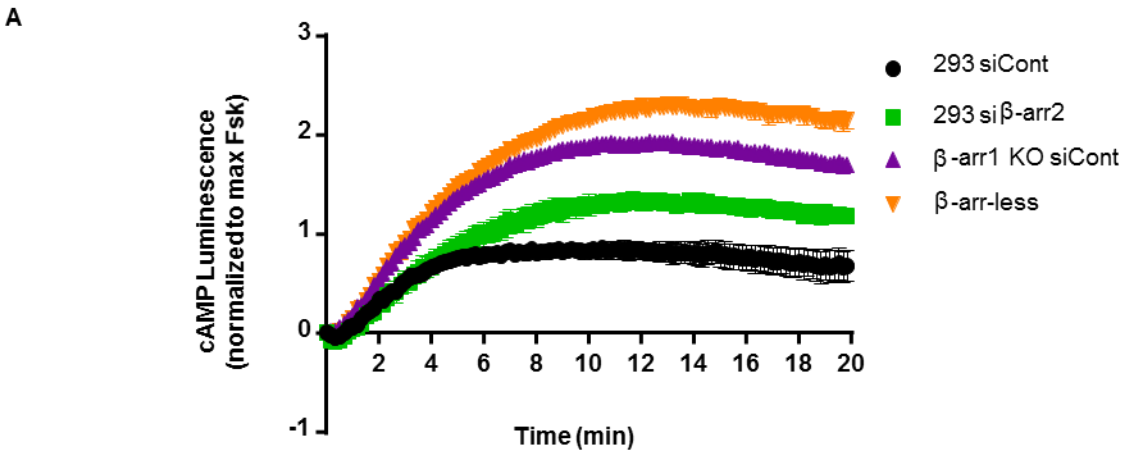

Fig. S3. Depletion of both β-arr1 and β-arr2 increases β_2AR-mediated cAMP production. Measurement of cAMP luminescence over time after Iso stimulation (10 µM) in 293 and β-arr1 KO FLAG-β_2AR cells transfected with siRNA control (siCont) or siβ-arr2. β-arr–less cells are β-arr1 KO cells transfected with siRNA to β-arr2. Representative of three independent experiments.

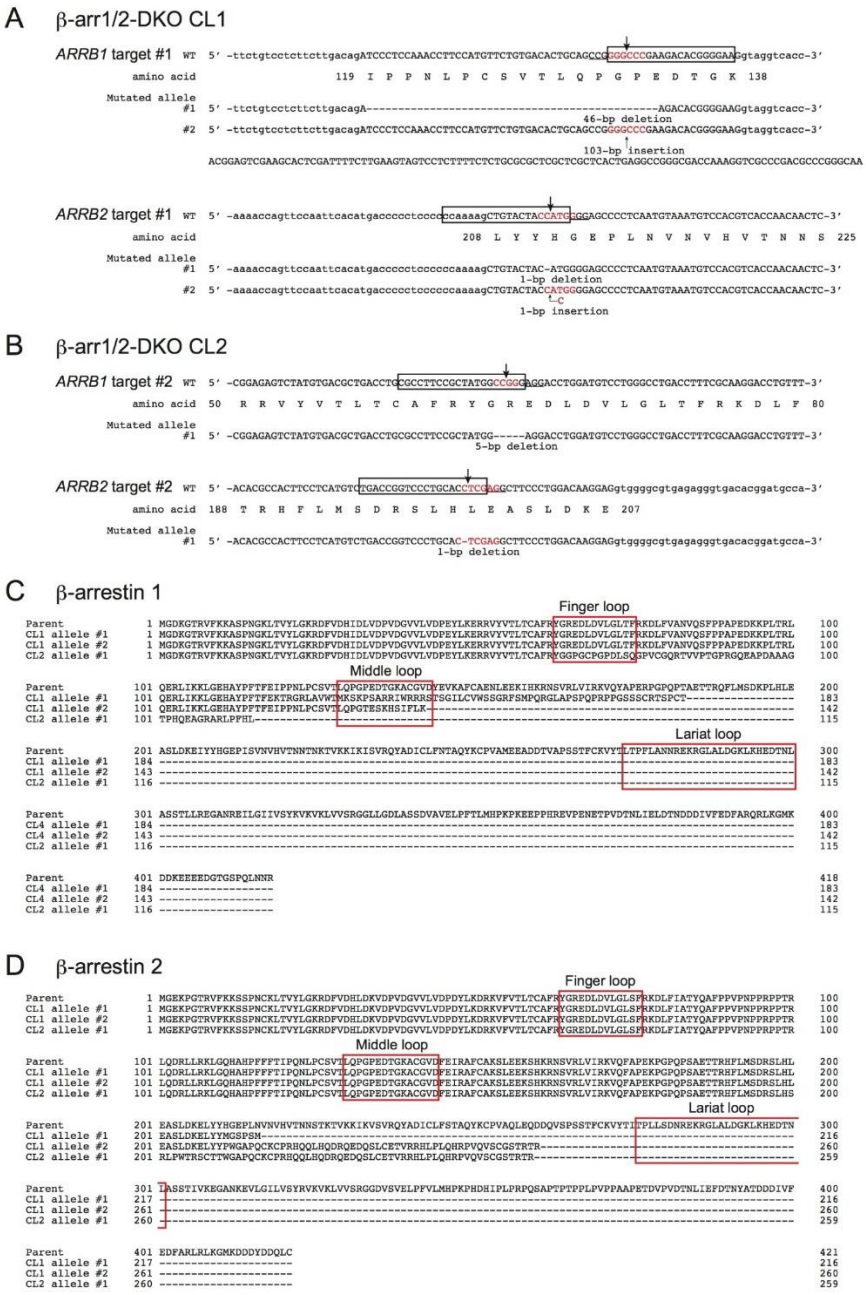

Fig. S4. Sequence of β-arr1/2-double-mutant cells. (A, B) Genomic sequences of the two mutant clones (A, CL1; B, CL2) near sgRNA-targeted sites. sgRNA target sequences are boxed and PAM sequences (NGG) are underlined. Arrows indicate a putative double-stranded break site. Restriction enzyme sites (Apa I (ARRB1 target #1), Nco I (ARRB2 target #2), Hap II (ARRB1 target #2) and Xho I (ARRB2 target #2)) are highlighted in red. Sequences in capital and lower letters indicate exons and introns, respectively. Note that all mutant alleles in CL1 and CL2 carried frame-shift mutations. (C, D) Alignment of deduced amino acids of the β-arrestin1/2-double mutant cells. Three critical loops of β-arrestin are boxed in red. Note that all truncated proteins encoded by the mutant alleles lack the Lariat loop.

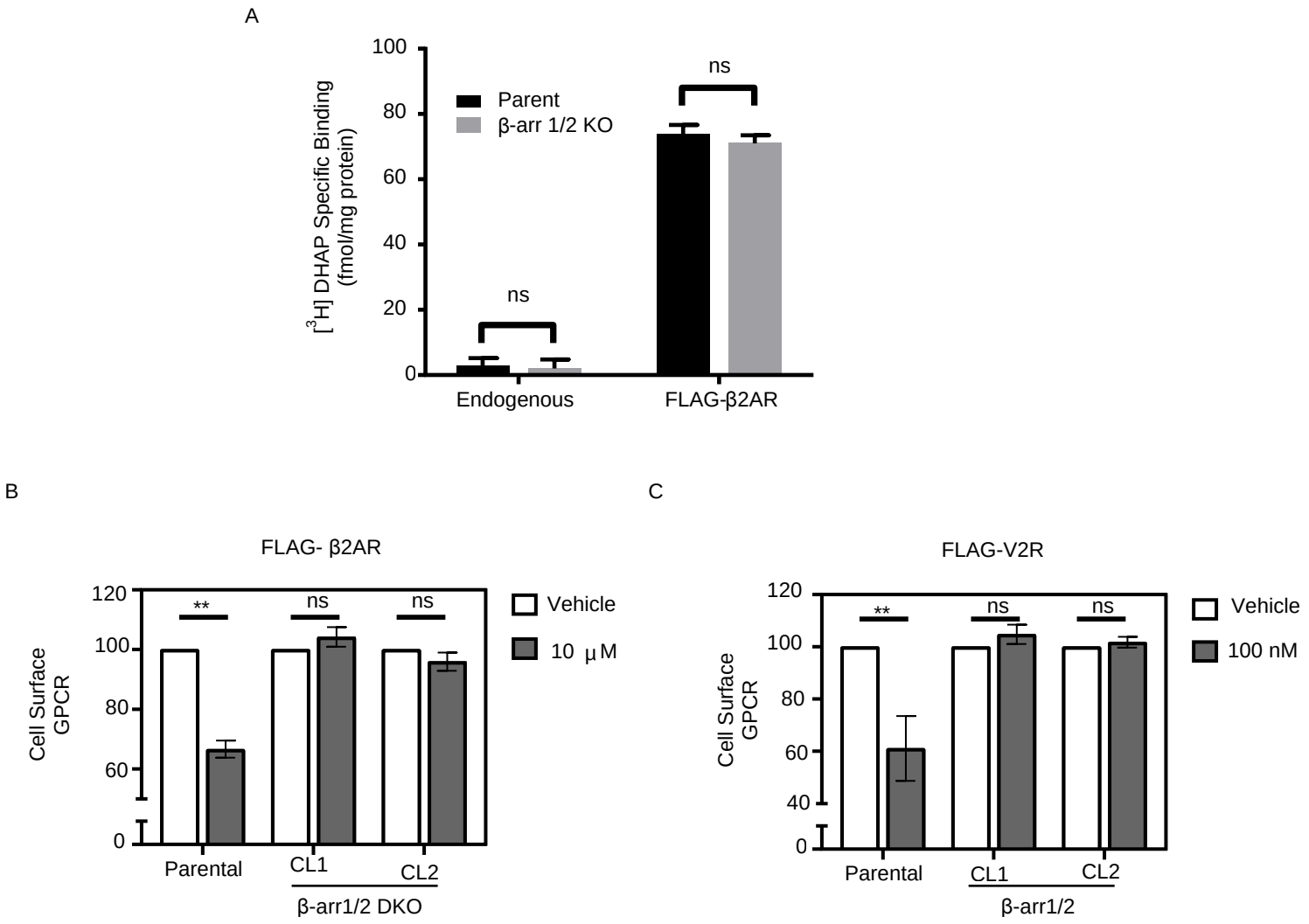

Fig. S5. Endogenous β_2AR in parental HEK293 and β-arr 1/2 KO and β_2AR and V2R internalization in these cells. A) Expression of β_2AR, endogenous and transfected, in parental 293 and β-arr 1/2 KO cell lines. Specific binding of [^{3}H] dihydroalprenolol ([^{3}H] DHAP) at equilibrium in mock transfected parental 293 and β-arr 1/2 KO cells compared with 1 μg FLAG-β_2AR transfected cells. Results shown are an average of three independent triplicate experiments. Mean and SEM values are plotted. (B and C) Parental HEK293 cells or β-arr1/2-DKO cells (CL1 or CL2) were transfected with a plasmid encoding N-terminally FLAG epitope-tagged β_2AR (B) or N-terminally FLAG epitope-tagged V2R (C). The cells were treated with or without ligand (Iso, isoproterenol; AVP, arginine vasopressin) for 1 h and GPCR on cell surface was labeled with a primary antibody against the FLAG epitope tag, followed by a secondary antibody conjugated with Alexa 488. Approximately 20,000 cells were analyzed by a flow cytometer. Mean fluorescent intensity (MFI) was used to represent cell surface GPCRs and normalized to that in vehicle-treated condition. Data are mean ± SEM of four to five independent experiments, each performed in biological triplicates. ***, $p < 0.001$ based on t-test. ns, not significant.

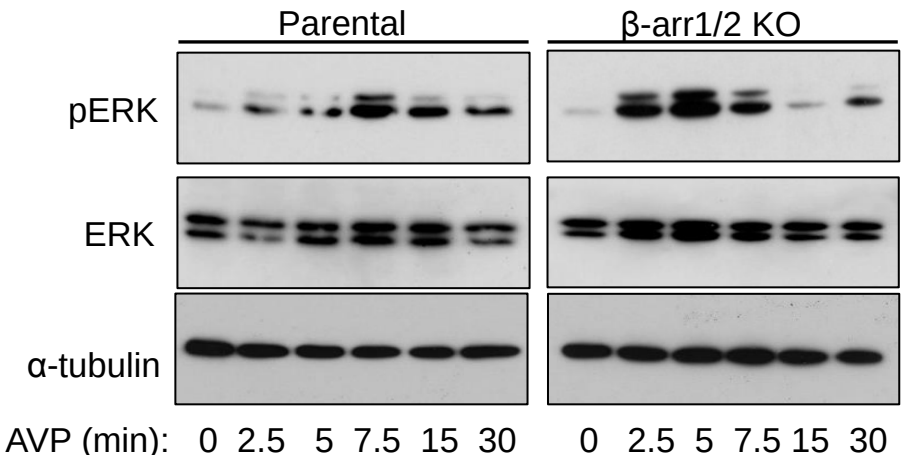

Fig. S6. β-arrestin is not required for ERK phosphorylation in arginine vasopressin receptor 2 (V2R)–expressing cells. Time course of vasopressin (100 nM) stimulation in parental 293 and β-arr 1/2 KO cell lines transfected with 4 μg HA-V2R. Western blot for ERK phosphorylation (pERK), total ERK, and α-tubulin. Results represent three independent experiments.

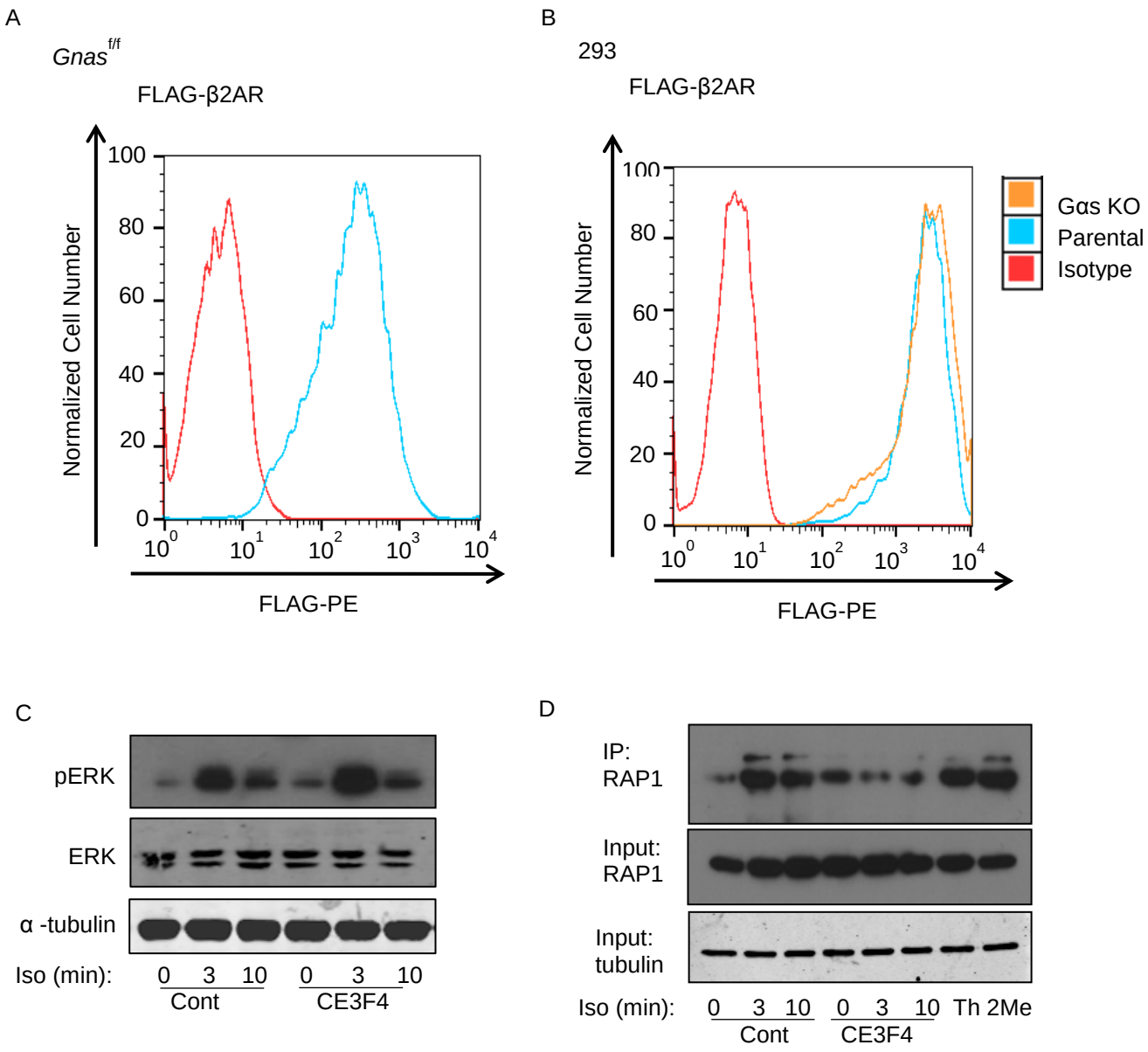

Fig. S7. Stable expression of FLAG-β_2AR on *Gnas* f/f MEFs and Gα_s KO cells and effect of EPAC inhibition on isoproterenol-mediated ERK phosphorylation and RAP1A activation. A) Flow cytometry analysis of stably-expressed FLAG-β_2AR relative to isotype control in *Gnas* f/f MEFs. Representative of three independent experiments. B) Flow cytometry analysis of FLAG-β_2AR in Gα_s KO and parental cells relative to isotype control. Representative of three independent experiments. C) Western blot showing the amounts of pERK in 293 FLAG-β_2AR cells pretreated with DMSO control or CE3F4 (10 µM), unstimulated or stimulated for 3 min or 10 min with Iso (10 µM). Representative of three independent experiments. D) Western blot showing active RAP1A pull down (IP) and control input levels of RAP1A and α-tubulin at 3 min and 10 min Iso stimulations (10 µM) following pretreatment with DMSO control (Cont) or the EPAC inhibitor CE3F4 (10 µM). Stimulations with the EPAC analog 8-pCPT-2'-O-Me-cAMP (2Me) at 100 µM and thrombin (Th) at 0.1 Units/ml were used as positive controls for pull down of active RAP1A. Representative of two independent experiments.

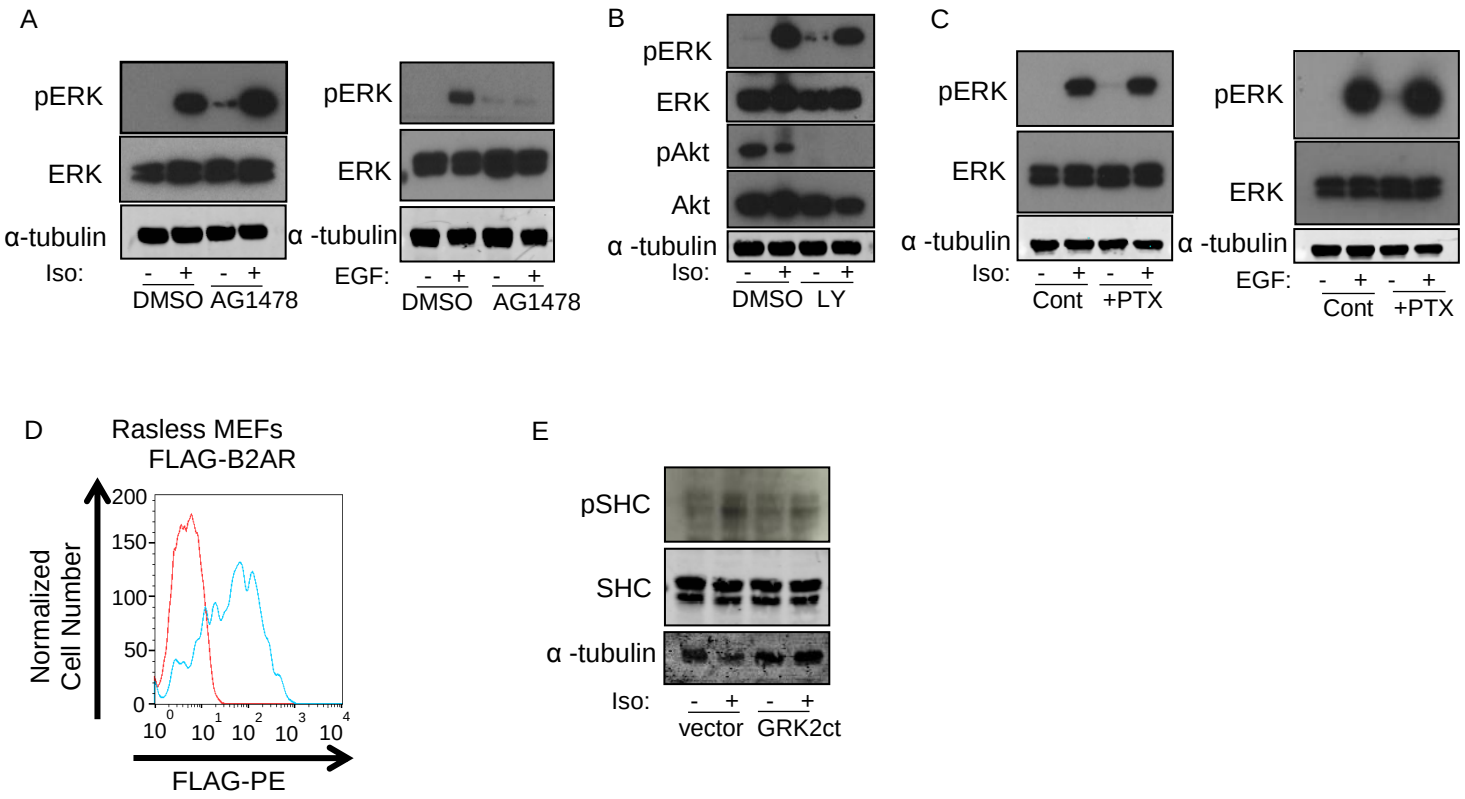

Fig. S8. EGFR, PI3K, and Gα$_i$ signaling are dispensable for activation of ERK by β$_2$AR, but Gβγ signaling is important. (A) Western blot showing the amounts of pERK and total ERK and α-tubulin as loading controls in 293 FLAG-β$_2$AR cells pretreated with DMSO control or the EGFR inhibitor AG1478 (10 μM), unstimulated (-) or stimulated (+) for 3 min with Iso (10 μM, left) or EGF (10 ng/mL, right). Representative of three independent experiments. B) Western blot showing the amounts of pERK and pAkt (S473) relative to total protein controls and α-tubulin in 293 FLAG-β$_2$AR cells pretreated with DMSO control or the PI3K inhibitor LY294002 (25 μM), unstimulated (-) or stimulated (+) for 3 min with Iso (10 μM). Representative of three independent experiments. C) Western blot showing the amounts of pERK and total ERK and α-tubulin as loading controls in 293 FLAG-β$_2$AR control cells or cells pretreated with pertussis toxin (PTX) to block Gi signaling (50 ng/mL), unstimulated (-) or stimulated (+) for 3 min with Iso (10 μM, left) or EGF (10 ng/mL, right). Representative of three independent experiments. D) Flow cytometry profile of stably-expressed FLAG-β$_2$AR relative to isotype control in Rasless MEFs. Representative of three independent experiments. E) Western blot showing the amounts of pSHC, and total SHC and α-tubulin as loading controls, in 293 FLAG-β$_2$AR cells transfected with control plasmid or GRK2ct plasmid, unstimulated (-) or stimulated (+) for 3 min with Iso (10 μM). Representative of three independent experiments.