Supporting Information

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SI Methods

Reagents. Polyubiquitin chains with Lys-48 or Lys-63 linkages were from Boston Biochem. Endogenous USP33 in HEK-293 cells was detected with an antibody purchased from Bethyl Laboratories. FK2 antibody was from Biomol. DNA and siRNA transfections were performed with the Lipofectamine2000 reagent (Invitrogen); siRNA transfections were also done with Genesilencer reagent (Genlantis). USP33 gene (IMAGE: 3491447) was cloned into pCDNA3-HA, pEGFP-N1, pDsRED-C1 vectors by standard cloning protocols, and the DNA constructs were verified by sequencing. C. Nanoff (Medical University of Vienna) kindly provided the USP4 plasmid.

RNA Interference. Chemically synthesized, double-stranded siRNAs, with 19-nt duplex RNA and 2-nt 3′ dTdT overhangs were purchased from Dharmacon in deprotected and desalted form. The siRNA sequences (sense, 5′ to 3′) that target respective human mRNA are listed below:

Control: Nontargeting sequence used was UUCUCCGAACGUGUCACGU
β-arrestin2: GGACCGCAAAGUGUUUGUG
Mdm2: GCCAUUGCUUUUGAAGUUA
USP33–1 CAAUGUUAAUUCAGGAUGA
USP33–2 GGCUUGGAUCUUCAGCCAU
USP33–3 GAUCAUGUGGCGAAGCAUA

For experiments in Figs. 3 *C* and *D*, and 4 *A* and *C*, early-passage HEK-293 cells that were 40–50% confluent on 100-mm dishes were transfected with siRNA, using the Genesilencer transfection reagent. The cells were assayed 48–60 hr after transfection. For immunostaining experiments (Fig. 4*B*) 70–80% confluent cells were transfected with Lipofectamine 2000 and cells were assayed 48–60 hr after transfection.

Immunoprecipitation and Immunoblotting. Cells were solubilized in a lysis buffer (LB) containing 50 mM Hepes (pH 7.5), 0.5% Nonidet P-40, 250 mM NaCl, 2 mM EDTA, 10% (vol/vol) glycerol, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, leupeptin (5 μg/ml), aprotinin (5 μg/ml), pepstatin A (1 μg/ml), and benzaminidine (100 μM). Soluble extracts were mixed with FLAG M2 affinity beads and rotated at 4 °C overnight. For experiments that involved stabilization of ubiquitinated β-arrestin (Figs. 3*C*, 4*A*, 5*A*, and Fig. S6), 10 mM NEM was included in the lysis buffers. Nonspecific binding was eliminated by repeated washes with LB and bound protein was eluted with SDS sample buffer. The proteins were separated on a polyacrylamide gradient gel (4–20%, Invitrogen) and transferred to nitrocellulose membrane for Western blotting. Chemiluminescence detection was performed by using SuperSignal West Pico reagent (Pierce). Mdm2 detection at endogenous levels required West Femto reagent (Pierce). Protein bands were quantified by densitometry and analyzed with Genetools software (SynGene).

USP33 Purification. COS-7 cells grown in 150-mm dishes were transfected with either pCDNA3-HA or pCDNA3-HA-USP33. Cells were harvested 24–30 hr after transfection in an EDTA-free lysis buffer (LB) containing 50 mM Hepes (pH 7.5), 0.5% Nonidet P-40, 250 mM NaCl, 10% (vol/vol) glycerol, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, leupeptin (5 μg/ml), aprotinin (5 μg/ml), pepstatin A (1 μg/ml), and benzaminidine (100 μM). Clarified supernatant was mixed with anti-HA affinity agarose beads and rotated at 4 °C. Repeated steps of centrifugation and washes eliminated nonspecific binding, and bound HA-USP33 was eluted by competing with HA peptide (60–100 μg of peptide per mL). Eluted protein was concentrated with Vivaspin ultrafiltration spin columns (50,000 molecular weight cutoff; Vivascience). About 600 μg of HA-USP33 enzyme was obtained from monolayer COS-7 cells collected from 6–8 150-mm dishes; 5 μg was used for testing purity on Coomassie-stained gels (Fig. S2*A*); and 0.5–2.0 μg was used in various enzymatic assays.

Ub-VS Labeling. We assessed the accessibility of active site of HA-USP33 by incubating 5- to 10-fold excess Ub-vinyl sulfone over HA-USP33 for 30 min at 37 °C in 50 mM Tris·HCl (pH 7.5) and 1 mM DTT. The reactions were stopped by addition of 3× SDS sample buffer and boiling. The samples were separated on a Tris-glycine 6% polyacrylamid gel (Invitrogen) and protein bands were detected by SYPRO-Ruby staining.

In Vitro Deubiquitination Assays. One microgram of polyubiquitin chain (Lys-48 or Lys-63) was incubated at 37 °C with buffer (50 mM Tris·HCl, pH 8.0), 0.5 μg of purified USP33 or 0.1 μg of Isopeptidase T in the presence of 1 mM DTT and 5 mM $MgCl_2$. The reactions were terminated by the addition of SDS sample buffer and samples were analyzed by SDS/PAGE and Coomassie blue staining.

In Vitro USP33 Binding. Purified β-arrestin2-His_6 was mixed in a volume of 50 μL with 3–5 μg of HA-USP33 alone or with 5-fold excess (mole:mole) of receptor C-terminal peptide (either V2R nonphosphopeptide: ARGRTPPSLGPQDESCTTASSSLAKDTSS, V2R phosphopeptide: ARGRTPPSLGPQDESCTTASSSLAKDTSS (phosphorylated at the underlined residues), β_2AR-nonphosphopeptide: QGTVPSDNIDSQGRNCSTNDSLL, or β_2AR -phosphopeptide: QGTVPSDNIDSQGRNCSTNDSLL) and incubated for 30 min, after which Talon beads were added, volume was increased to 500 μL and samples were rotated at 4 °C. Nonspecific binding was eliminated by repeated centrifugation and wash steps. Bound proteins were analyzed by Western blotting for USP33 and β-arrestin2 levels.

Confocal Microscopy. Confocal images were obtained on a Zeiss LSM510 laser scanning microscope (LSM) using a 100× oil-immersion objective and the pinhole set to 1.0 Airy units for single fluorophore imaging. For multichannel acquisition, multitrack sequential excitation (488, 568, 633 nm) and emission (515–540 nm GFP; 585–615 nm, Texas Red; 650 nm, Alexa633) filter sets were used and the pinholes were adjusted so that each channel has the same optical slice (= 1 μm). Fixed cells were scanned at room temperature and live cell images were acquired on a 37 °C heated stage. Images were imported into Adobe Photoshop 7.0, and brightness and contrast were adjusted in Photoshop for the entire LSM image.

HEK-293 cells expressing HA-V2R (Fig. 2 *B* and *C*) or Flag-β_2AR (Fig. 4*B*) plated on 10-cm dishes were transiently transfected with pcDNA3 or USP33 (Fig. 2 *B* and *C*) or with control or USP33 siRNA (Fig. 4*B*). Cells were plated on collagen-coated 35-mm glass-bottom plates 36–40 hr after transfection; 12–15 hr later, cells were starved for at least 2 hr in serum-free medium before stimulation. After stimulation, cells were fixed with 5% formaldehyde diluted in PBS containing calcium and magnesium. Fixed cells were permeabilized with 0.01% Triton X-100 in PBS containing 2% BSA for 60 min and

then incubated at 4 °C with appropriate primary antibodies. The secondary antibody incubations were for 1 hr at room temperature followed by repeated washes with PBS. Although USP33 is detected across membrane network, vesicle, and perinuclear compartments (Fig. S3*A*), when 2 or more fluorophores are analyzed, the apparent distribution is seen only in the perinuclear region and/or vesicles. This results from the lower efficacy of rabbit Alexa633 and some attenuation of signals during the multichannel signal acquisition.

Limited Tryptic Proteolysis. Rat β-arrestin2 was purified as previously described [Xiao K, Shenoy SK, Nobles K, Lefkowitz RJ (2004) Activation-dependent conformational changes in β-arrestin 2. *J Biol Chem* 279:55744–55753]. A 5:1 molar ratio of receptor peptides (see above) to β-arrestin2 (0.5–1 mg/mL) was used to reveal the effects of peptide on limited tryptic proteolysis of β-arrestin2. Before proteolysis, β-arrestin2, in the absence or presence of peptide, was incubated at room temperature for 30 min. A 1:5,000 (trypsin/β-arrestin2) wt/wt ratio of TPCK-treated trypsin was added to the mixture. The samples were incubated at 37 °C for indicated times. At each time point, 5 μL (2.5–5 μg of β-arrestin2) was removed from each reaction. Each aliquot was transferred to a new microcentrifuge tube containing 5 μL of 2× SDS sample buffer and boiled for 5 min to quench the tryptic digestion. The samples were run on 4–20% gradient gels (Invitrogen) to determine the effects of ligands on the digestion pattern of β-arrestin2.

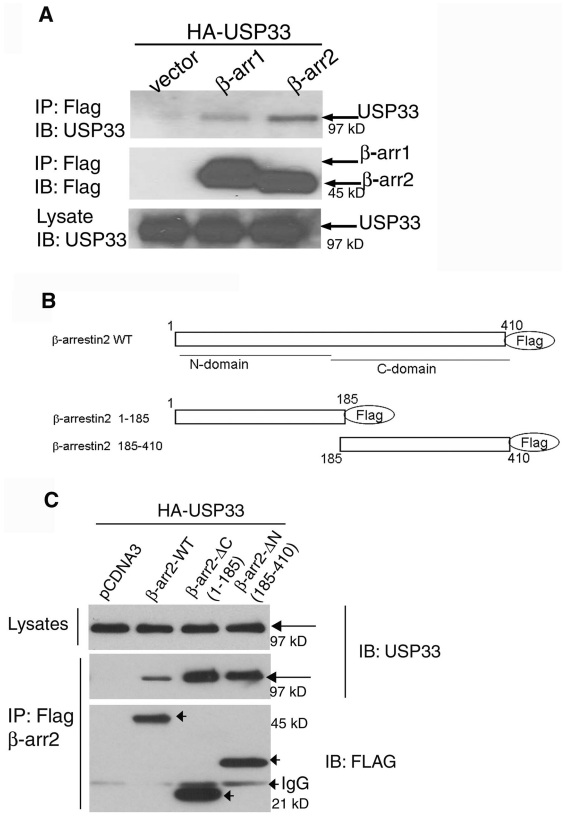

Fig. S1. (*A*) Western analyses of Flag-β-arrestin1 and -2 immunoprecipitates isolated from COS-7 cells with an USP33 antiserum (*Top*). The blot was reprobed with an anti-Flag M2 antibody to determine β-arrestin levels (*Middle*). A lysate blot for HA-USP33 levels is also displayed (*Bottom*). (*B*) A diagrammatic representation of β-arrestin2 to show the N and C domains. (*C*) COS-7 cells were transfected with HA-USP33 along with vector, β-arrestin2-Flag, β-arrestin2 ΔC-Flag, or β-arrestin ΔN-Flag. Flag immunoprecipitates were probed for USP33 (*Top*) and β-arrestin2 species (*Bottom*). USP33 expression as detected in lysates is also shown (*Middle*). This experiment is representative of 3 independent experiments with identical results.

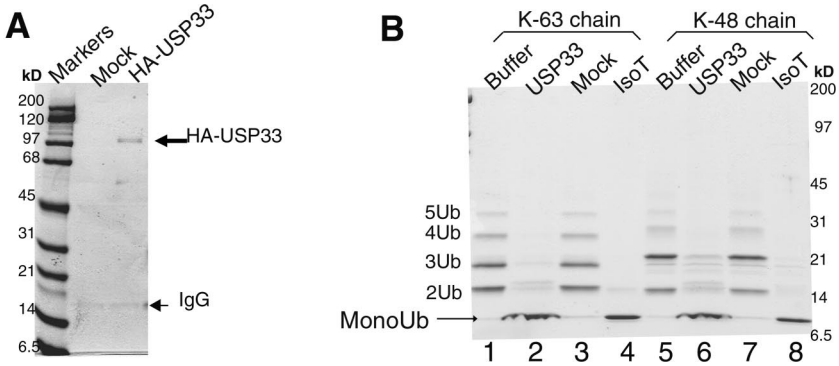

Fig. S2. (*A*) Coomassie staining of 5 μg of protein isolated from pcDNA3 (Mock) and HA-USP33-transfected cells. Arrow indicates the ≈100-kDa band corresponding to full-length USP33. (*B*) Lys-63- (lanes 1–4) and Lys-48- (lanes 5–8) linked polyubiquitin chains, which lack monoubiquitin, were incubated with buffer (lanes 1 and 5), purified USP33 (lanes 2 and 6), mock purification (lanes 3 and 7), and isopeptidase T (lanes 4 and 8). The appearance of a monoubiquitin band corresponds to the depolymerizing activity of USP33. Protein bands were separated on a 4–20% gradient Tris-glycine gel and visualized by Coomassie blue staining.

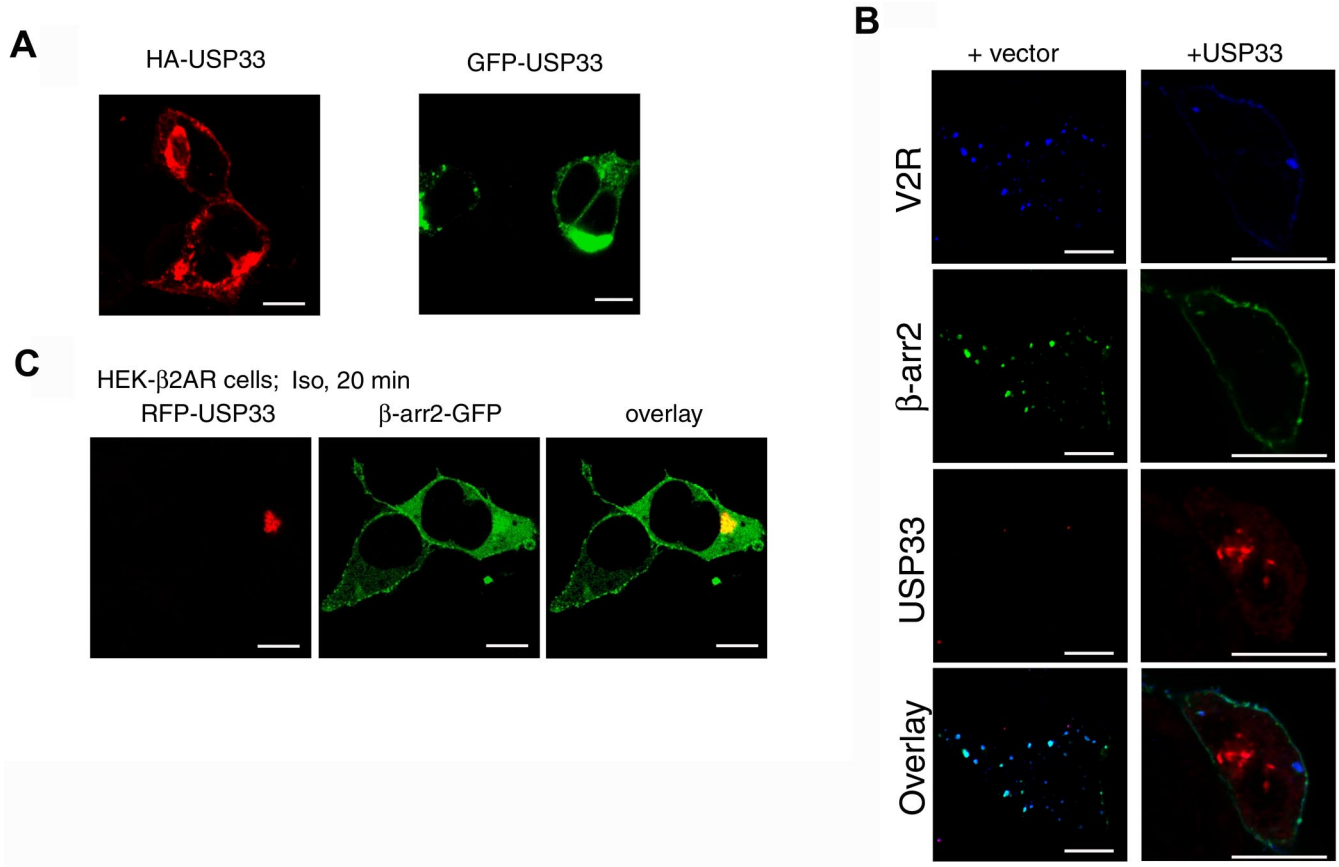

Fig. S3. (*A*) HEK-293 cells showing distribution of HA-USP33 (*Left*) and GFP-USP33 (*Right*). (*B*) Confocal micrographs depict HEK-293 cells expressing HA-V2R (blue), β-arrestin2-GFP (green), and USP33 (red). Displayed are the subcellular distributions of each protein in cells that have been stimulated with 1 μM AVP for 10 min. (*C*) HEK-293 cells with stable Flag-β_2AR transiently transfected with β-arrestin2-GFP and RFP-USP33, stimulated with 1 μM isoproterenol for 20 min. (*C*) shows 2 cells, one with no detectable RFP-USP33 and the other cell that expresses USP33, both with plasma membrane recruitment of β-arrestin. (Scale bars, 10 μm.)

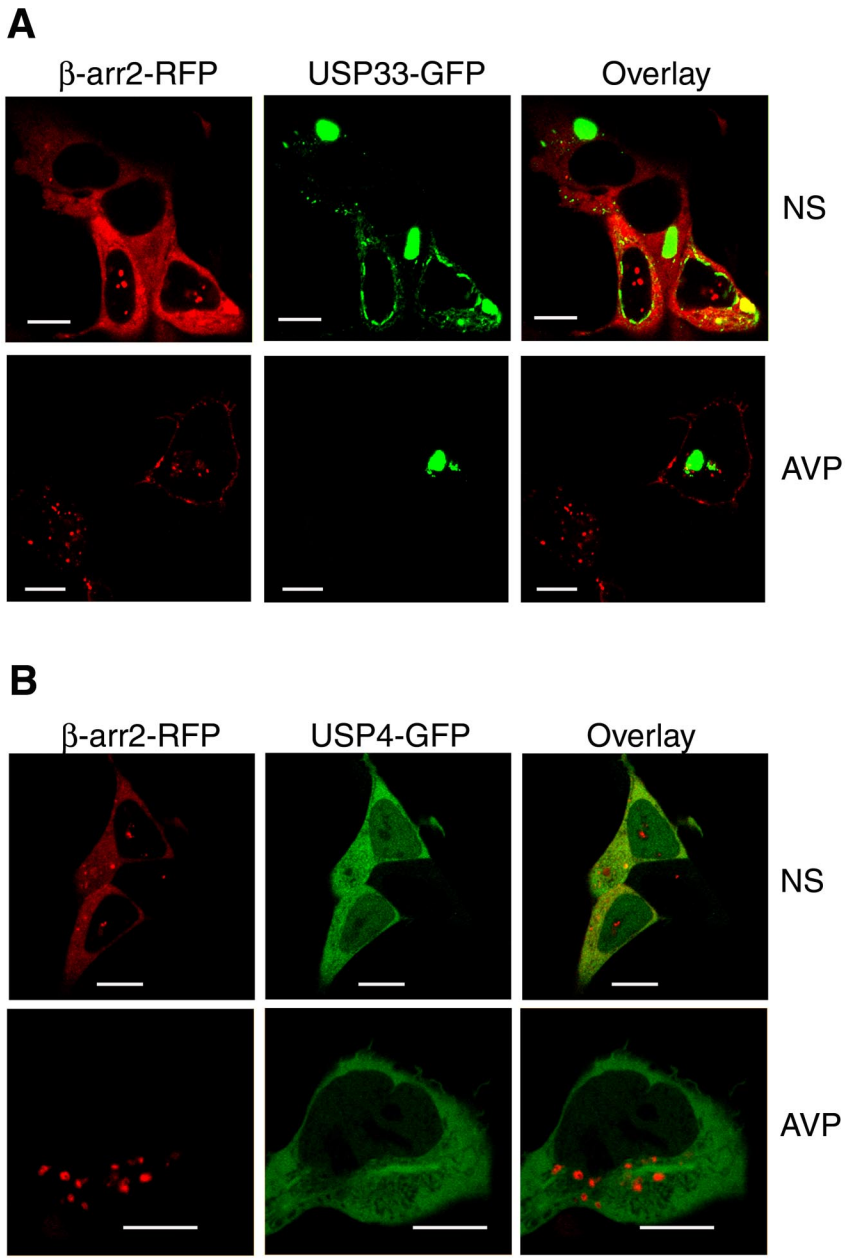

Fig. S4. (*A*) Confocal micrographs displaying β-arrestin2-RFP (red), USP33-GFP (green), and HA-V2R in unstimulated cells (*Upper*) and AVP-stimulated cells (*Lower*). (*B*) Confocal micrographs displaying β-arrestin2-RFP (red), USP4-GFP (green), and HA-V2R in unstimulated cells (*Upper*) and AVP-stimulated cells (*Lower*). (Scale bars, 10 μm.)

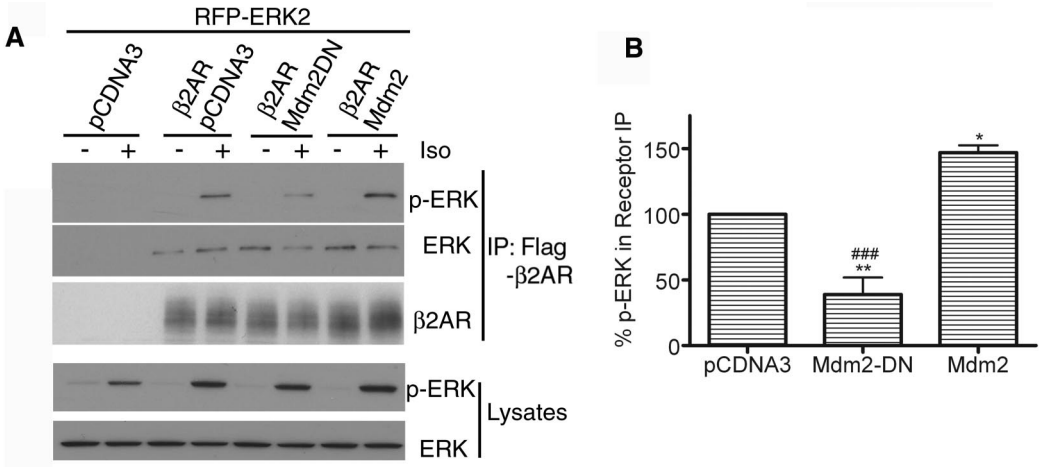

Fig. S5. (*A*) COS-7 cells were transfected with RFP-ERK2 and FLAG-β_2AR along with either vector Mdm2$^{1-400}$ or Mdm2-WT. After 5-min agonist stimulation, receptors were immunoprecipitated, separated on gels, and probed for the p-ERK content. The blots were reprobed for total ERK (second panel) followed by a second reprobe for the β_2AR (third panel). The levels of RFP-pERK2 and RFP-ERK2 in the cell extracts are also shown (Lysates). (*B*) Bar graph depicts the quantification of p-ERK coprecipitated with the agonist-occupied receptor. Data represent mean value ± SE from 3 independent experiments. One-way ANOVA; *, $P < 0.05$, vector versus Mdm2; **, $P < 0.01$, vector versus Mdm2 1–400; ###, $P < 0.001$, Mdm2 versus Mdm2$^{1-400}$.

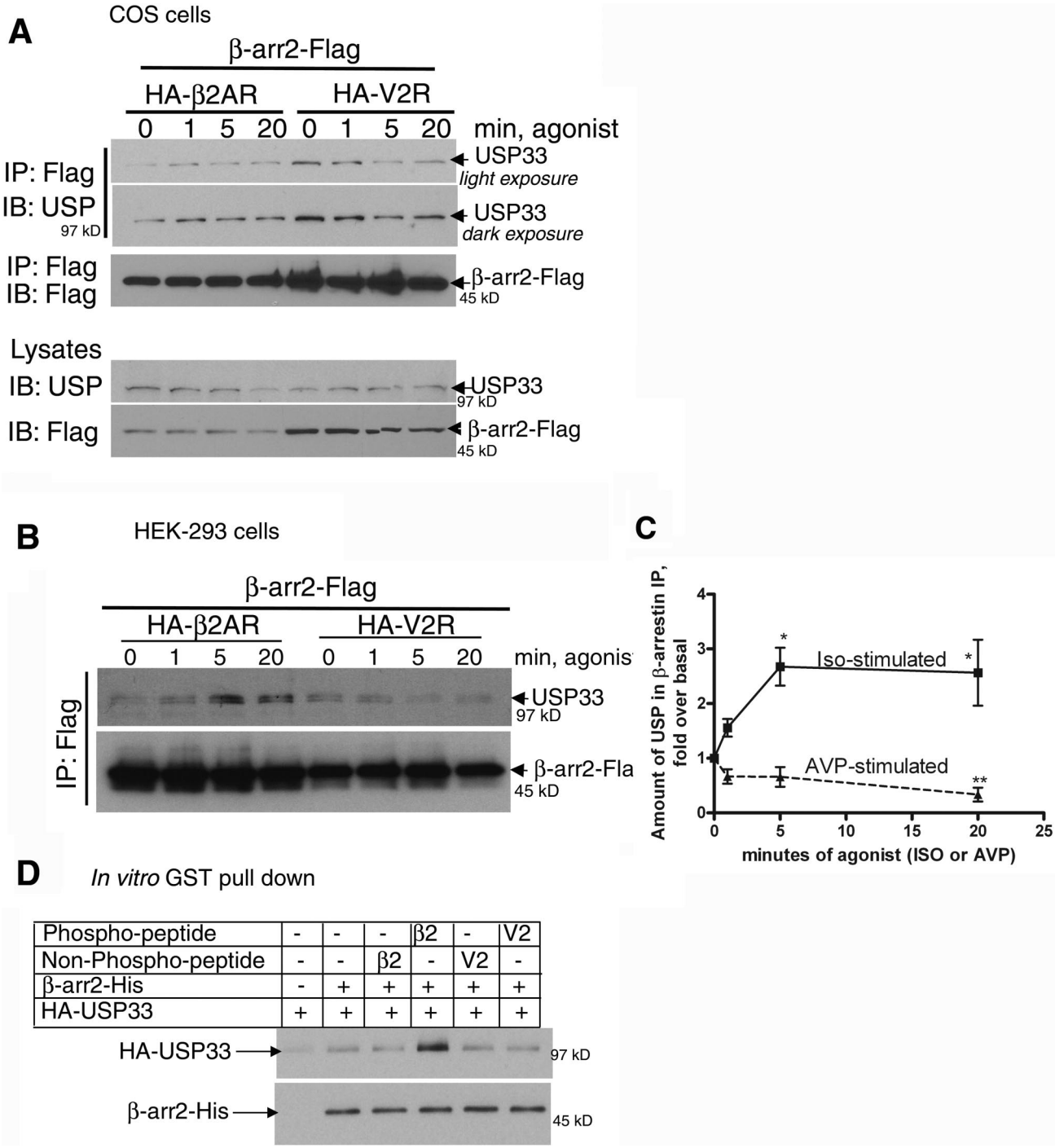

Fig. S6. (*A*) COS-7 cells transiently expressing either HA-β_2AR R or HA-V2R along with β-arrestin2-Flag were stimulated with respective agonists for the indicated times, β-arrestins were immunoprecipitated, and the immunoprecipitate was probed with USP33 antisera (first and second gels) and for β-arrestin in the immunoprecipitate sample as detected by an anti-Flag M2 antibody (third gel). The levels of USP33 and β-arrestin in whole-cell extracts as detected by the respective antibodies are also displayed in the 2 lower gels. (*B*) HEK-293 cells transiently expressing either HA-β_2AR or HA-V2R along with β-arrestin2-Flag were stimulated with respective agonists for the indicated times, β-arrestins were immunoprecipitated, and the immunoprecipitate was probed with anti-USP33 antibody (*Upper*) and for β-arrestin in the immunoprecipitate sample as detected by an anti-Flag M2 antibody (*Lower*). (*C*) Quantification of USP33 in β-arrestin precipitates obtained from 3 independent experiments. The 4 time points within each binding curve are analyzed by 1-way ANOVA. In each case, stimulated samples are significantly different from unstimulated samples; *, $P < 0.05$; **, $P < 0.01$. (*D*) HA-USP33 was incubated alone or with β-arrestin2-His_6 without or with indicated receptor peptides (β-arrestin:USP33:peptide 1:1:5 molar ratio). The protein mixture was mixed with Talon affinity beads and bound proteins were separated from unbound sample by repeated washing and analyzed by Western blotting for USP33 (*Upper*) and β-arrestin (*Lower*) in each sample.