The deubiquitinases USP33 and USP20 coordinate β2 adrenergic receptor recycling and resensitization

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Supplementary Information

Materials and Methods:

Cell lines: COS-7 and HEK-293 cells were obtained from the American Type Culture Collection and were maintained respectively in Dulbecco's modified Eagle's medium or in Minimal Essential Medium (Sigma) supplemented with 10% foetal bovine serum and 1% penicillin/streptomycin. Transfections were performed using Lipofectamine 2000 reagent (Invitrogen) or FuGENE 6 Transfection Reagent (Roche Applied Science).

Synthesis of Small Interfering RNAs (siRNAs). Double-stranded siRNAs, with 19-nucleotide duplex RNA and 2-nucleotide 3'-dTdT overhangs, were chemically synthesized, in deprotected and desalted form (DHARMACON). Sequences of siRNA oligonucleotides are:

USP33-1 GAUCAUGUGGCGAAGCAUA

USP33-2 GGCUUGGAUCUUCAGCCAU

USP20-1 CGUCGUACGUGCUCAAGAA

USP20-2 GGACAAUGAUGCUCACCUA

A non-silencing RNA duplex (5'-AAUUCUCCGAACGUGUCACGU-3'), as the manufacturer indicated, was used as a control. Gene Silencer (GenLantis) reagent was used for transfection of siRNA except for immunostaining experiments, where Lipofectamine 2000 was used.

USP enzymatic activity measured by in vitro assay. COS-7 cells transfected transiently with the HA-USP33 WT or mutants were solubilized in lysis buffer containing 50 mM HEPES (pH 7.5), 0.5% Nonidet P-40, 250 mM NaCl, 10% (v/v) glycerol and anti-proteases (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), benzaminidine (100 μM); Sigma). After centrifugation, soluble extracts were mixed with EZview™ Red Anti-HA Affinity Gel (Sigma) and rotated at 4 °C overnight. Nonspecific binding was eliminated by repeated washes with lysis buffer. By competition with a HA-peptide, bound enzymes were eluted and concentrated with Vivaspsin ultrafiltration spin columns (50000 MWCO; Vivascience). Purified enzymes (0.2 – 0.5 μg) were incubated with the polyubiquitin chain (K48 or K63) at 37 °C. SDS buffer was then added and the cleaved fragments of ubiquitin chains were separated on a gradient gel (Invitrogen) and stained with Coomassie Blue.

Supplementary Figure legends

Figure S1: USP33 inhibits β_2AR ubiquitination in COS-7 cells

A. COS-7 cells were transfected transiently with pcDNA3 or HA-USP33 and stimulated with Iso (10 μM) for 15 min. The Flag-β_2AR was immunoprecipitated with M2 Flag affinity beads and ubiquitinated receptor detected with an anti-ubiquitin antibody (upper panel). The amounts of Flag-tagged β_2AR, and phosphorylated receptors in the IP were detected with specific antibodies (M2 anti-Flag and an anti-phosphoβ_2AR (serines 355, 356)). Expression of USP33 is detected in the lowest panel. **B**. The bar graphs represent the ratio between the β_2AR ubiquitination signal obtained after Iso stimulation and the ubiquitination signal obtained in nonstimulated condition. The result is the mean ± SEM of 6 independent experiments. Statistical analyses were performed by a paired t test *** $p<0.001$.

Figure S2: Cellular distribution of HA-USP33 and β2ARs

A. HA-USP33 was transiently expressed in HEK-293 cells and immunostained with an anti-USP33 antibody followed by a secondary anti-rabbit Alexa594 (left panel, red) or with 12CA5 anti-HA antibody followed by a secondary anti-mouse Alexa 633 (right panel, red). Scale bar =10 μm. **B.** Confocal micrographs display β_2AR-YFP (green) and HA-USP33 (red) in the merged panels in HEK-293 cells that have been stimulated with Iso for indicated times. Individual protein distribution is shown in black and white. A small region is enlarged for detailed viewing. Arrows indicate regions of colocalization between HA-USP33 and β_2AR.

Figure S3: USP33 mutants bind the β_2AR

HA-USP33 (WT, HIS, CYS or HIS.CYS) were transiently expressed in HEK-293 cells stably transfected with Flag-β_2AR. Flag receptor immunoprecipitates were isolated from unstimulated and Iso-stimulated cells and analyzed for USP33 (top panel) and receptor

(middle panel) amounts by Western blotting. The expression levels of the USP33 constructs are shown in the lysate panel.

Figure S4: Amino acid sequence alignment of human USP33 (hUSP33) and USP20 (hUSP20).

USP33 and USP20 are identical in approximately 59% of the amino acids with strong homology in the N-terminus and C-terminus and a weaker similarity in the middle region (conserved residues are boxed). Conserved cysteine and histidine are indicated by *arrows*. Cys- and His-boxes are underlined.

Figure S5: Agonist-stimulated β_2AR trafficking in control and USP 20 and 33 depleted cells

HEK-293 cells expressing Flag-β_2AR-mYFP are transfected either with siRNA targeting nothing (control) or USPs 20 and 33. After Iso (10 μM) stimulation for the indicated times, cells were fixed and stained with anti-LAMP2 antibodies. Merged images of representative confocal scans are shown with the β_2AR in green and LAMP2 in red (Alexa 594). Yellow indicates colocalization of receptor and LAMP2.

Figure S1

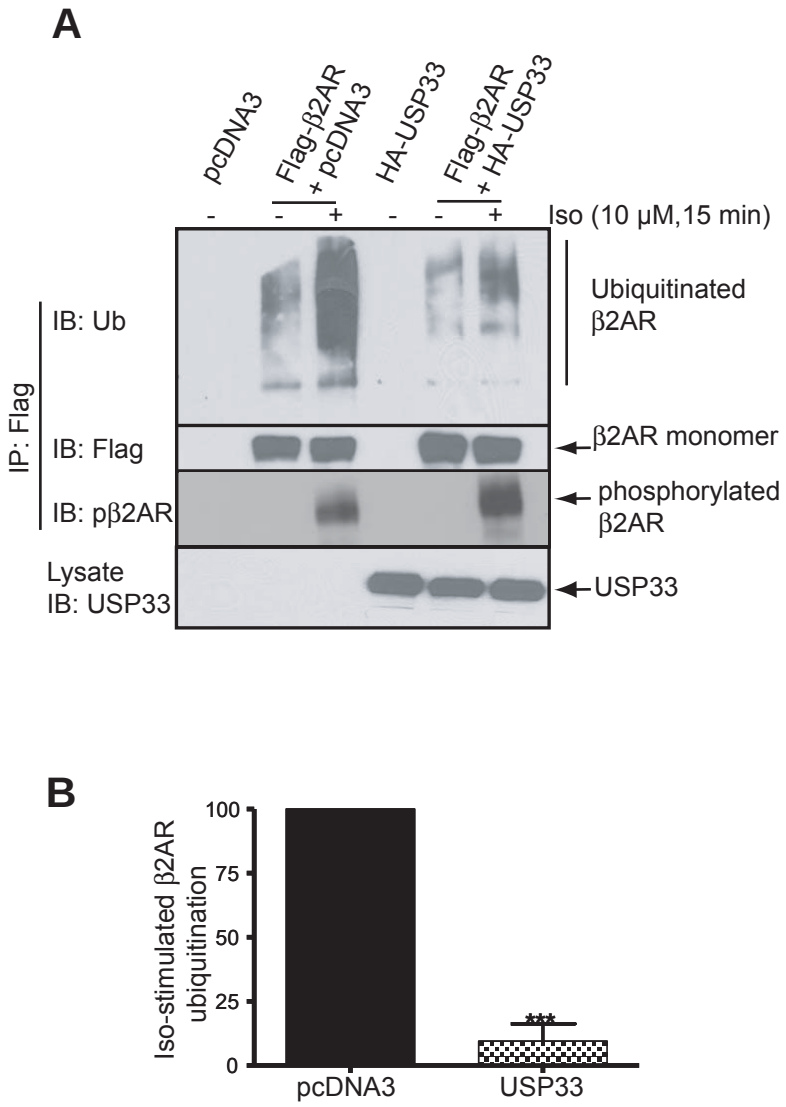
A
pcDNA3
Flag-β2AR + pcDNA3
HA-USP33
Flag-β2AR + HA-USP33
Iso (10 μM,15 min)
IP: Flag
IB: Ub
IB: Flag
IB: pβ2AR
Lysate IB: USP33
Ubiquitinated β2AR
β2AR monomer
phosphorylated β2AR
USP33
B
Iso-stimulated β2AR ubiquitination
100
75
50
25
0
***
pcDNA3
USP33

Figure S2

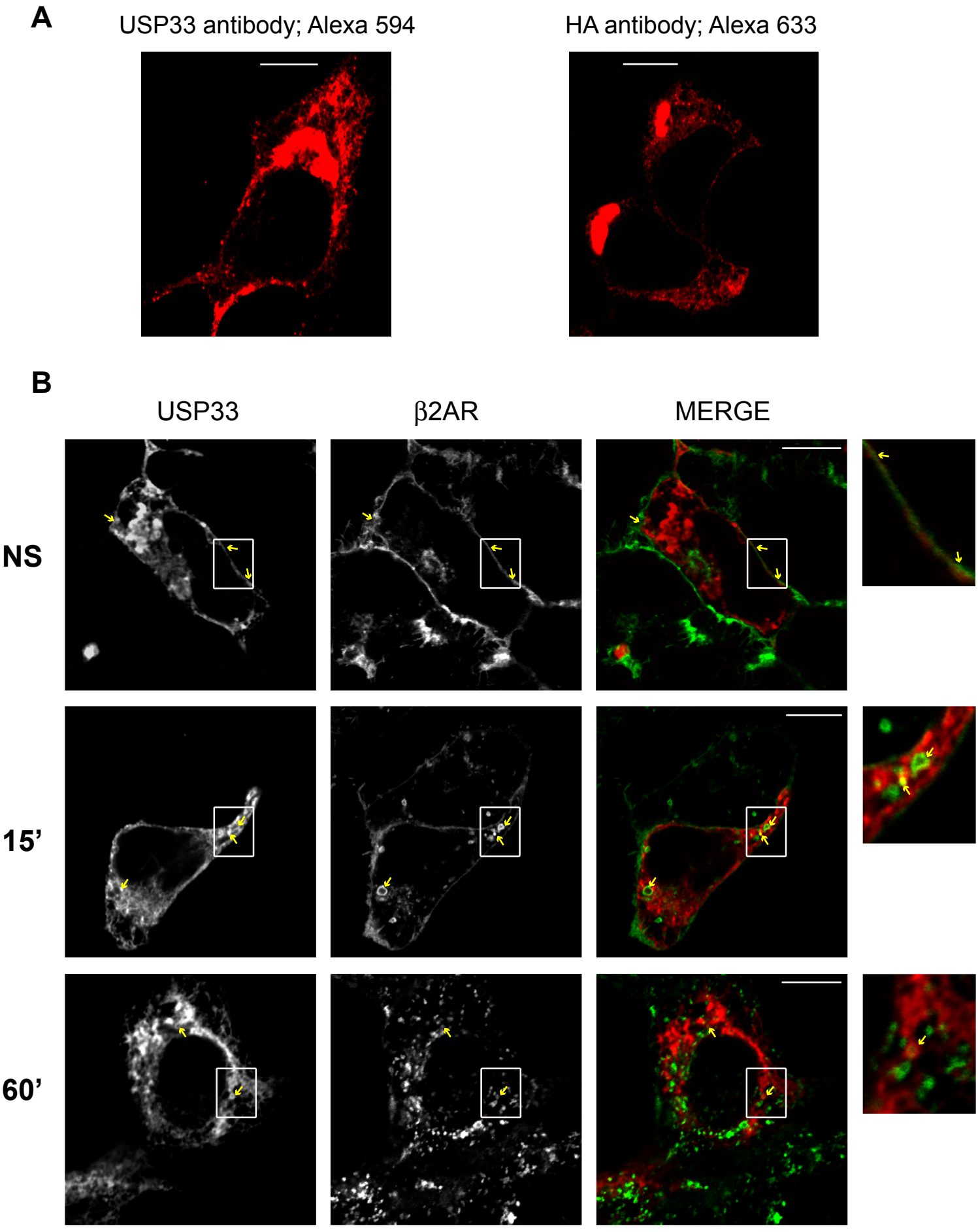
A
USP33 antibody; Alexa 594
HA antibody; Alexa 633
B
USP33
β2AR
MERGE
NS
15'
60'

Figure S3

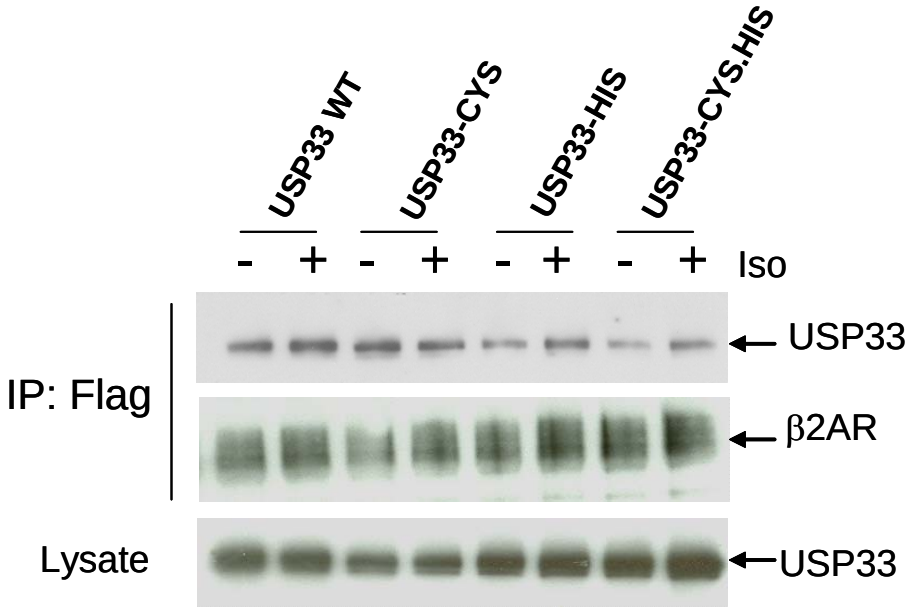
USP33 WT
USP33-CYS
USP33-HIS
USP33-CYS.HIS
- + - + - + - +
Iso
IP: Flag
USP33
β2AR
Lysate
USP33

Figure S4


hUSP33 MSAFRNHCPH LDSVGEITKE DLIQKSLGTC QDCKVQGPNL WACLENRCSY VGCGESQVDH 60
hUSP20 MGDSRDLCPH LDSIGEVTKE DLLLKSKGTC QSCGVTGPNL WACLQVACPY VGCGESFADH 60
hUSP33 STIHSQETKH YLTVNLTTLR VWCYACSKEV FLDRKLGTQP SLPHVRQPHQ IQENSVQDFK 120
hUSP20 STIHAQAKKH NLTVNLTTFR LWCYACEKEV FLEQRLAA-- --PLLGSSSK FSE---QDSP 113
hUSP33 IPSNTTLKTP LVAVFDDLDI EADEEDELRA RGLTGLKNIG NTCYMNAALQ ALSNCPPLTQ 180
hUSP20 PPSHP-LKAV PIAVADEGES ES-EDDDLKP RGLTGMKNLG NSCYMNAALQ ALSNCPPLTQ 171
hUSP33 FFLDCGGLAR TDKKPAICKS YLKLMTELWH KSRPGSVVPT TLFQGIKTVN PTFRGYSQQD 240
hUSP20 FFLECGGLVR TDKKPALCKS YQKLVSEVWH KKRPSYVVPT SLSHGIKLVN PMFRGYAQQD 231
hUSP33 AQEFLRCLMD LLHEELKEQV MEV-----EE DPQTITTEET MEEDKSQSDV DFQSCESCSN 295
hUSP20 TQEFLRCLMD QLHEELKEPV VATVALTEAR DSDSSDTDEK REGDRSPSED EFLSCDS--S 289
hUSP33 SDRAENENGS RCFSEDNNET TMLIQDDENN SEMSKDWQKE KMCN-KINKV NSEGEFDKDR 354
hUSP20 SDRGEGDGQG RGGGSSQAET ELLIPDEAGR AISEKERMKD RKFSWGQQRT NSE-QVDEDA 348
hUSP33 DSISETVDLN NQETVKVQIH SRAS------ EYITDVHSND LSTP-QILPS NEGVNPRLSA 407
hUSP20 DVDTAMAALD DQPAEAQPPS PRSSSPCRTP EPDNDAHLRS SSRPCSPVHH HEG-HAKLSS 407
hUSP33 SPPKSGNLWP GLAPPH--KK AQ--SASPKR KKQHKKYRSV ISDIFDGTII SSVQCLTCDR 463
hUSP20 SPPRASPV-- RMAPSYVLKK AQVLSAGSRR RKE-QRYRSV ISDIFDGSIL SLVQCLTCDR 464
hUSP33 VSVTLETFQD LSLPIPGKED LAKLHSSSHP TSIVKAGSCG EAYAPQGWIA FFMEYVKRFV 523
hUSP20 VSTTVETFQD LSLPIPGKED LAKLHSAIYQ NVPAKPGACG DSYAAQGWLA FIVEYIRRFV 524
hUSP33 VSCVPSWFWG PVVTLQDCLA AFFARDELKG DNMYSCEKCK KLRNGVKFCK VQNFPEILCI 583
hUSP20 VSCTPSWFWG PVVTLEDCLA AFFAADELKG DNMYSCERCK KLRNGVKYCK VLRLPEILCI 584
hUSP33 HLKRFRHELM FSTKISTHVS FPLEGLDLQP FLAKDSPAQI VTYDLLSVIC HHGTASSGHY 643
hUSP20 HLKRFRHEVM YSFKINSHVS FPLEGLDLRP FLAKECTSQI TTYDLLSVIC HHGTAGSGHY 644
hUSP33 IAYCRNNLNN LWYEFDDQSV TEVSESTVQN AEAYVLFYRK SSEEAQKERR RISNLLNIME 703
hUSP20 IAYCQNVING QWYEFDDQYV TEVHETVVQN AEGYVLFYRK SSEEAMRERQ QVVSLAAMRE 704
hUSP33 PSLLQFYISR QWLNKFKTFA EPGPISNNDF LCIHGGVPPR KAGYIEDLVL MLPQNIWDNL 763
hUSP20 PSLLRFYVSR EWLNKFNTFA EPGPITNQTF LCSHGGIPPH KYHYIDDLVV ILPQNVWEHL 764
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hUSP20 YNRFGGGPAV NHLYVCSICQ VEIEALAKRR RIEIDTFIKL NKAFQAEESP GVIYCISMQW 824
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hUSP20 FREWEAFVKG KDNEPPGPID NSRIAQVKGS GHVQLKQGAD YGQISEETWT YLNSLYGGGP 884
hUSP33 EVILRPPVVH -VDPDILQAE EKIEVETRSL 911
hUSP20 EIAIRQSVAQ PLGPENLHGE QKIEAETRAV 914

Figure S5