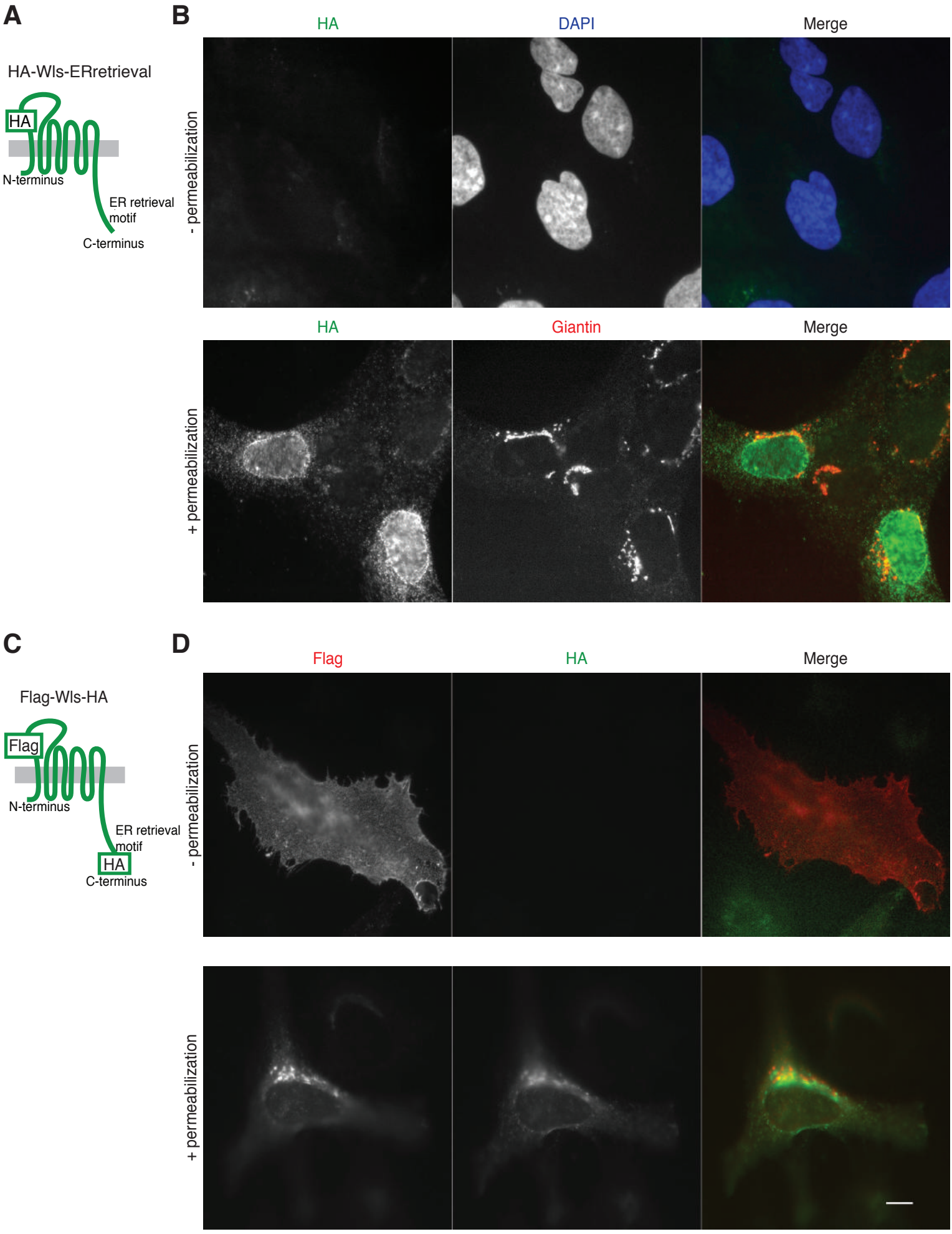

A
HA-Wls-ERretrieval
HA
N-terminus
ER retrieval
motif
C-terminus
B
HA
DAPI
Merge
- permeabilization
HA
Giantin
Merge
+ permeabilization
C
Flag-Wls-HA
Flag
N-terminus
ER retrieval
motif
HA
C-terminus
D
Flag
HA
Merge
- permeabilization
+ permeabilization

**Figure S1**

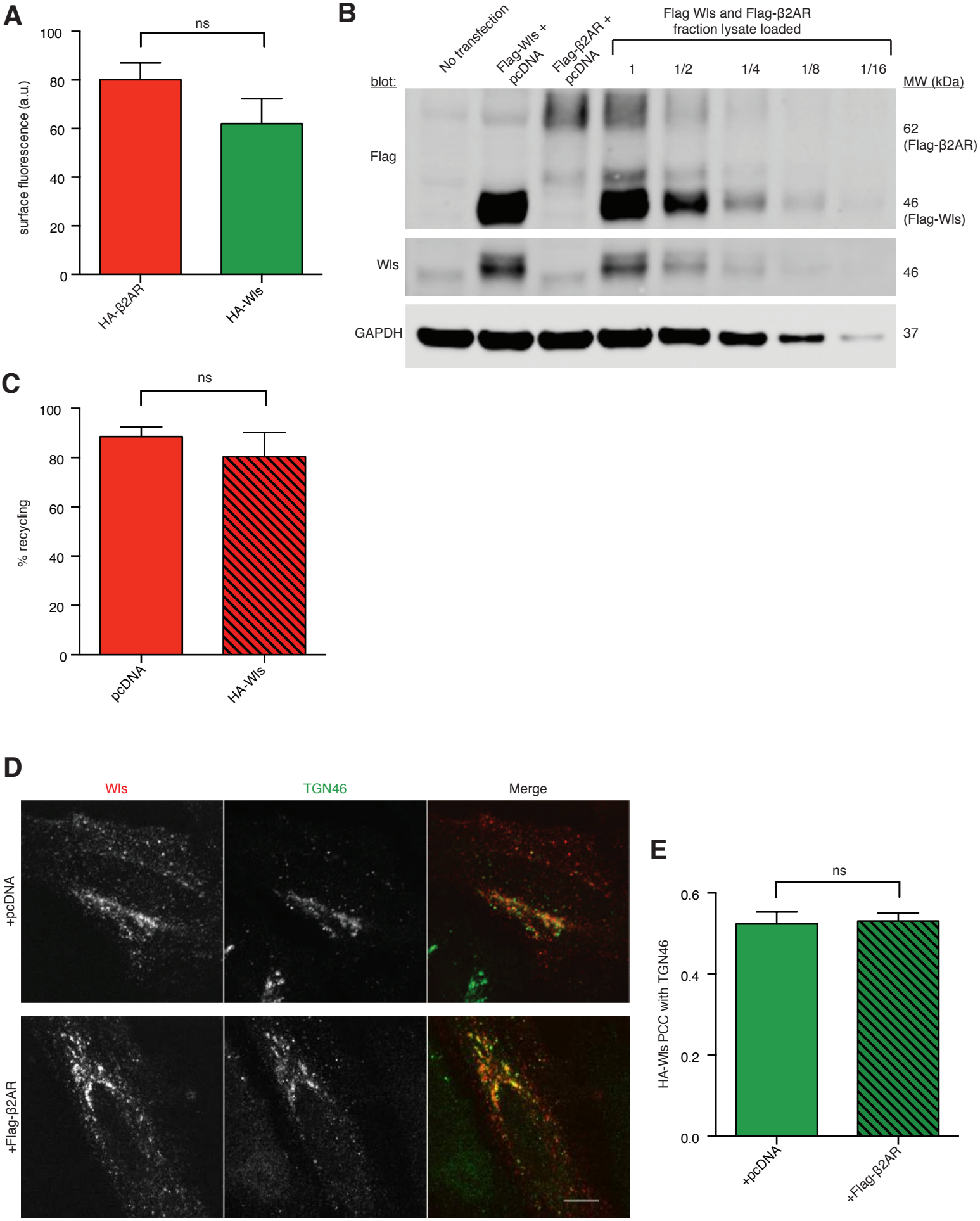
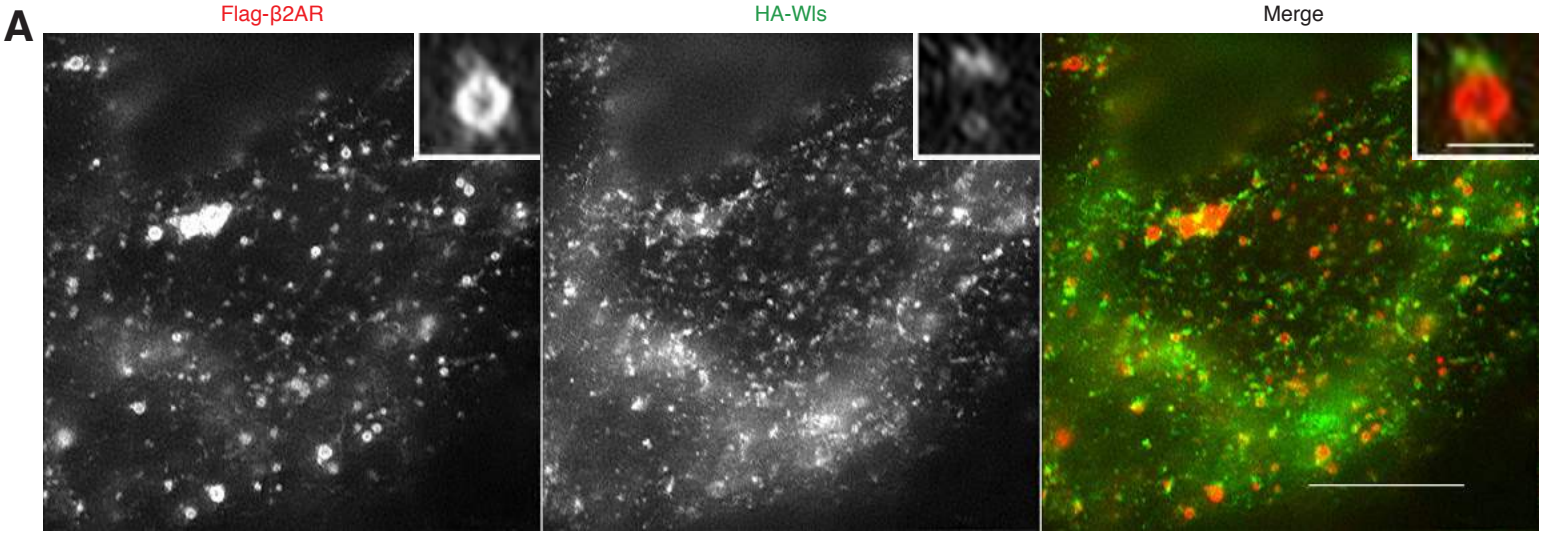

A
ns
surface fluorescence (a.u.)
100
80
60
40
20
0
HA-β2AR
HA-Wls
B
No transfection
Flag-Wls + pcDNA
Flag-β2AR + pcDNA
Flag Wls and Flag-β2AR fraction lysate loaded
1
1/2
1/4
1/8
1/16
blot:
MW (kDa)
Flag
62 (Flag-β2AR)
46 (Flag-Wls)
Wls
46
GAPDH
37
C
ns
% recycling
100
80
60
40
20
0
pcDNA
HA-Wls
D
Wls
TGN46
Merge
+pcDNA
+Flag-β2AR
E
ns
HA-Wls PCC with TGN46
0.6
0.4
0.2
0.0
+pcDNA
+Flag-β2AR

**Figure S2**


A
Flag-β2AR
HA-Wls
Merge

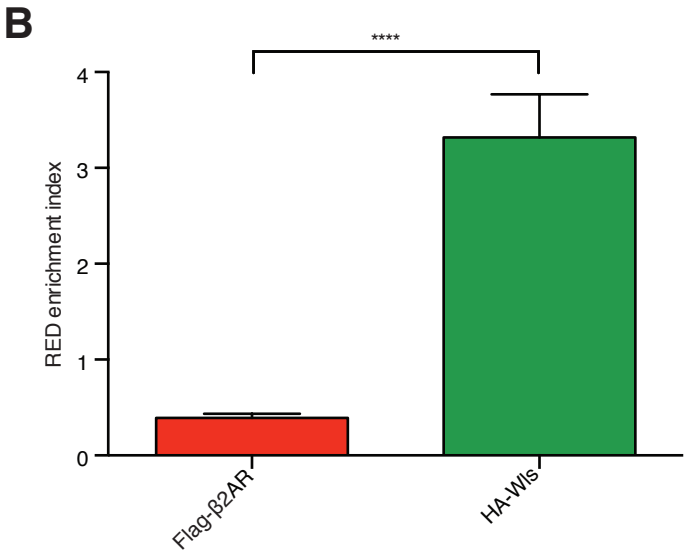

B
****
RED enrichment index
4
3
2
1
0
Flag-β2AR
HA-Wls

**Figure S3**

**A**

i. wild type β2AR cytoplasmic tail sequence:

PDFRIAFQELLCLRRSSLKAYGNGYSSNGNTGEQSGYHVEQEKENKLLCEDLPGTEDFVGHQGTVPSDNIDSQGRNCSTNDSLL

ii. wild type human Wls cytoplasmic tail sequence:

APSHKNYGEDQSNGDLGVHSGEELQLTTTITHVDGPTEIYKLTRKEAQE

iii. WlsΔ35 cytoplasmic tail sequence:

APSHKNYGEDQSNG

iv. WlsΔ518-541 cytoplasmic tail sequence:

APSHKNYGEDQSNGDLGVHSGEELQL

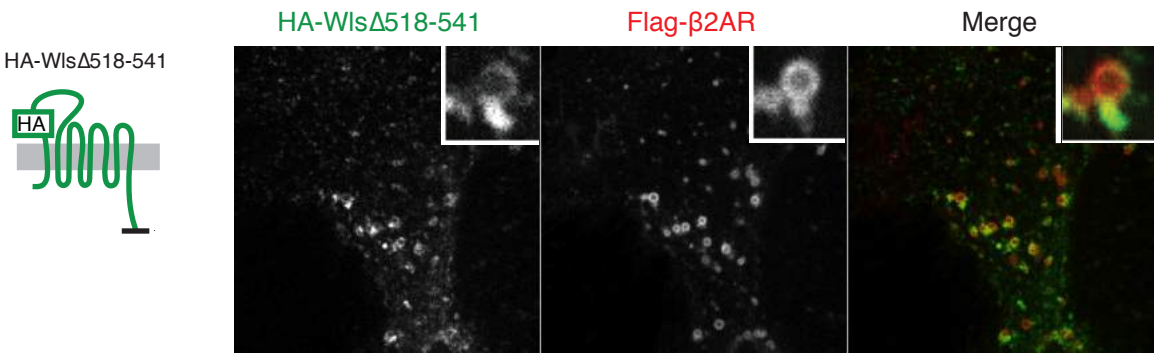
v. WlsΔ506-518 cytoplasmic tail sequence:

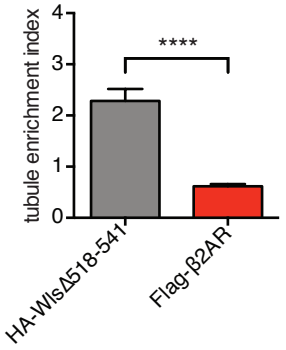
APSHKNYGEDQSN|———|TTTITHVDGPTEIYKLTRKEAQE

vi. Wls(LQL+YKL>AAA) cytoplasmic tail sequence:

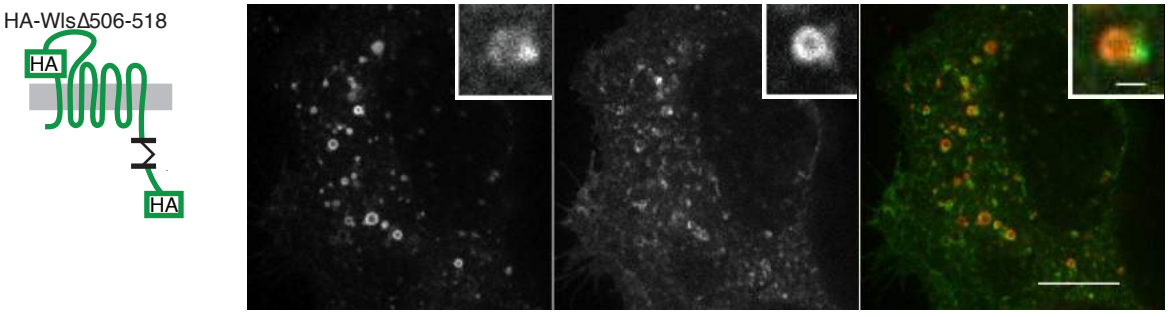
APSHKNYGEDQSNGDLGVHSGEEAAATTTITHVDGPTEIAAATRKEAQE

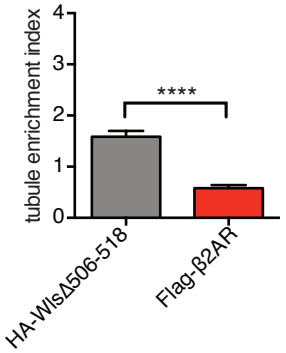
**B**



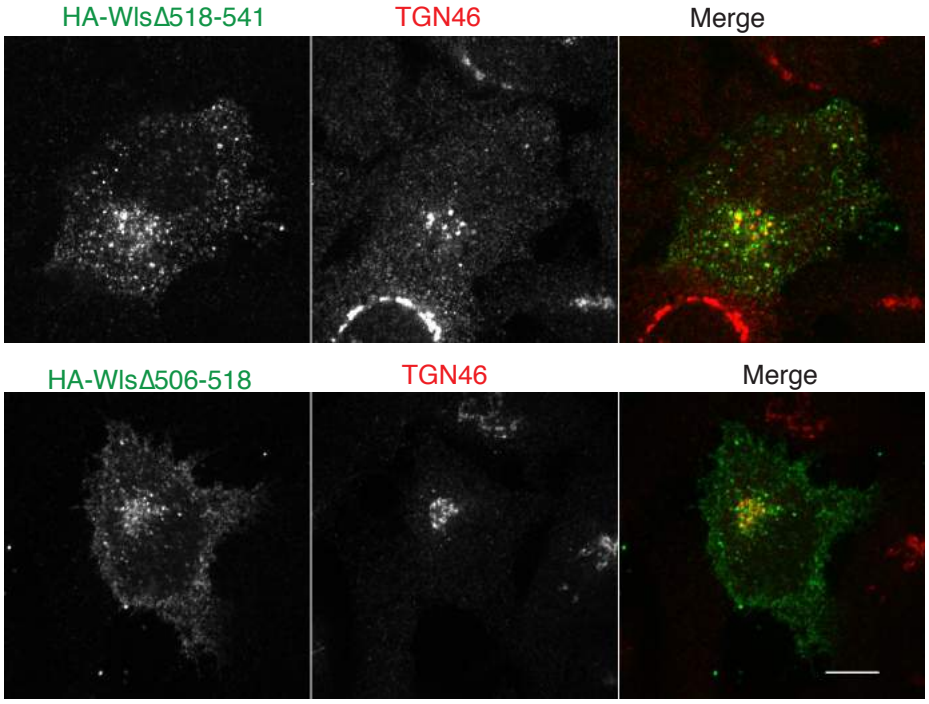




**C**




**D**




**E**

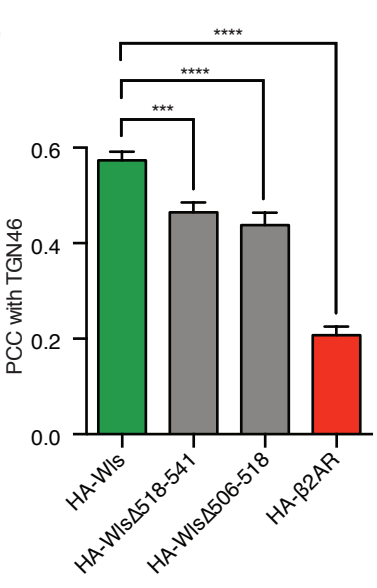



**Figure S4**

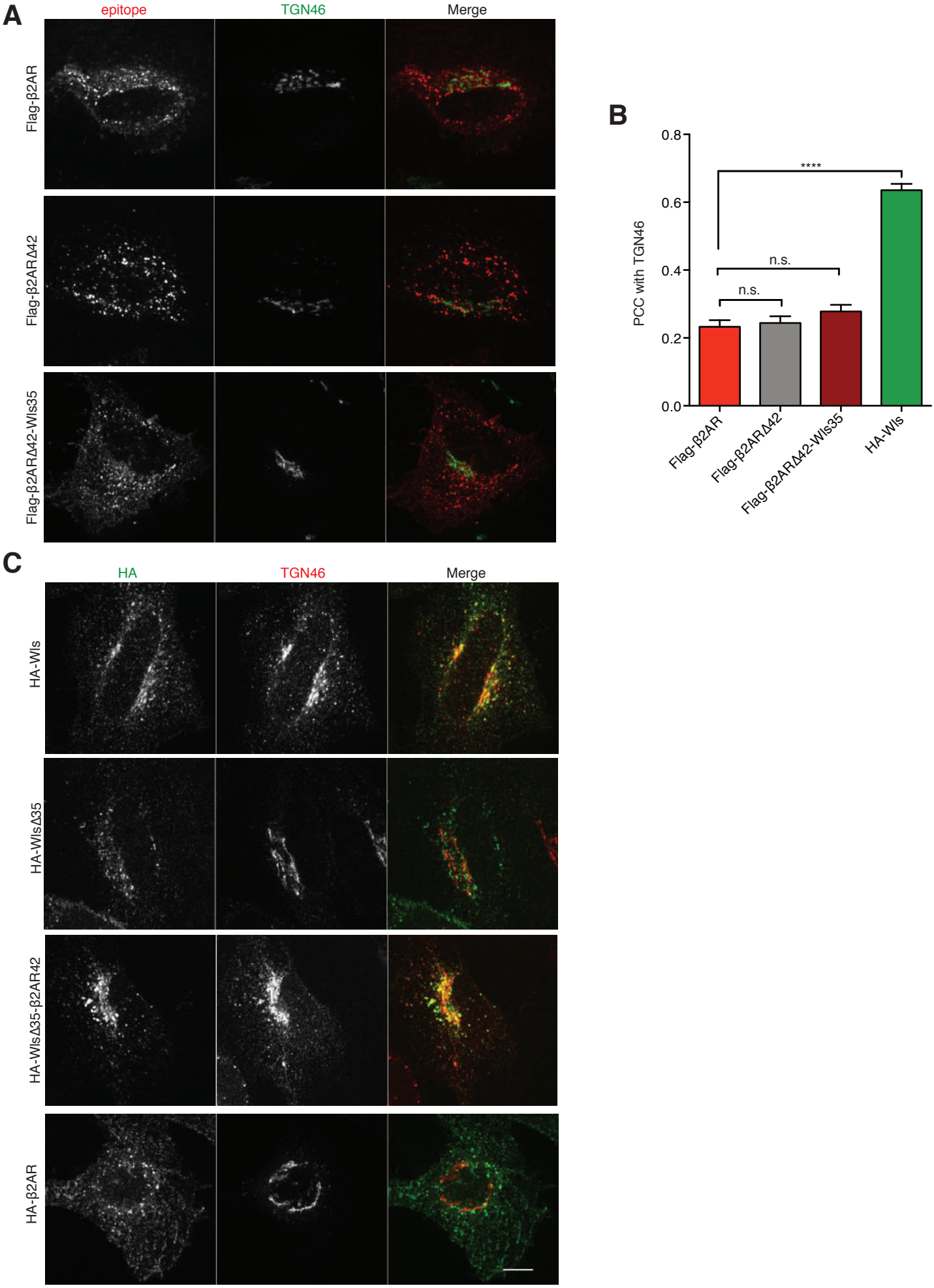

A
epitope
TGN46
Merge
Flag-β2AR
Flag-β2ARΔ42
Flag-β2ARΔ42-Wls35
B
PCC with TGN46
0.8
0.6
0.4
0.2
0.0
****
n.s.
n.s.
Flag-β2AR
Flag-β2ARΔ42
Flag-β2ARΔ42-Wls35
HA-Wls
C
HA
TGN46
Merge
HA-Wls
HA-WlsΔ35
HA-WlsΔ35-β2AR42
HA-β2AR

**Figure S5**

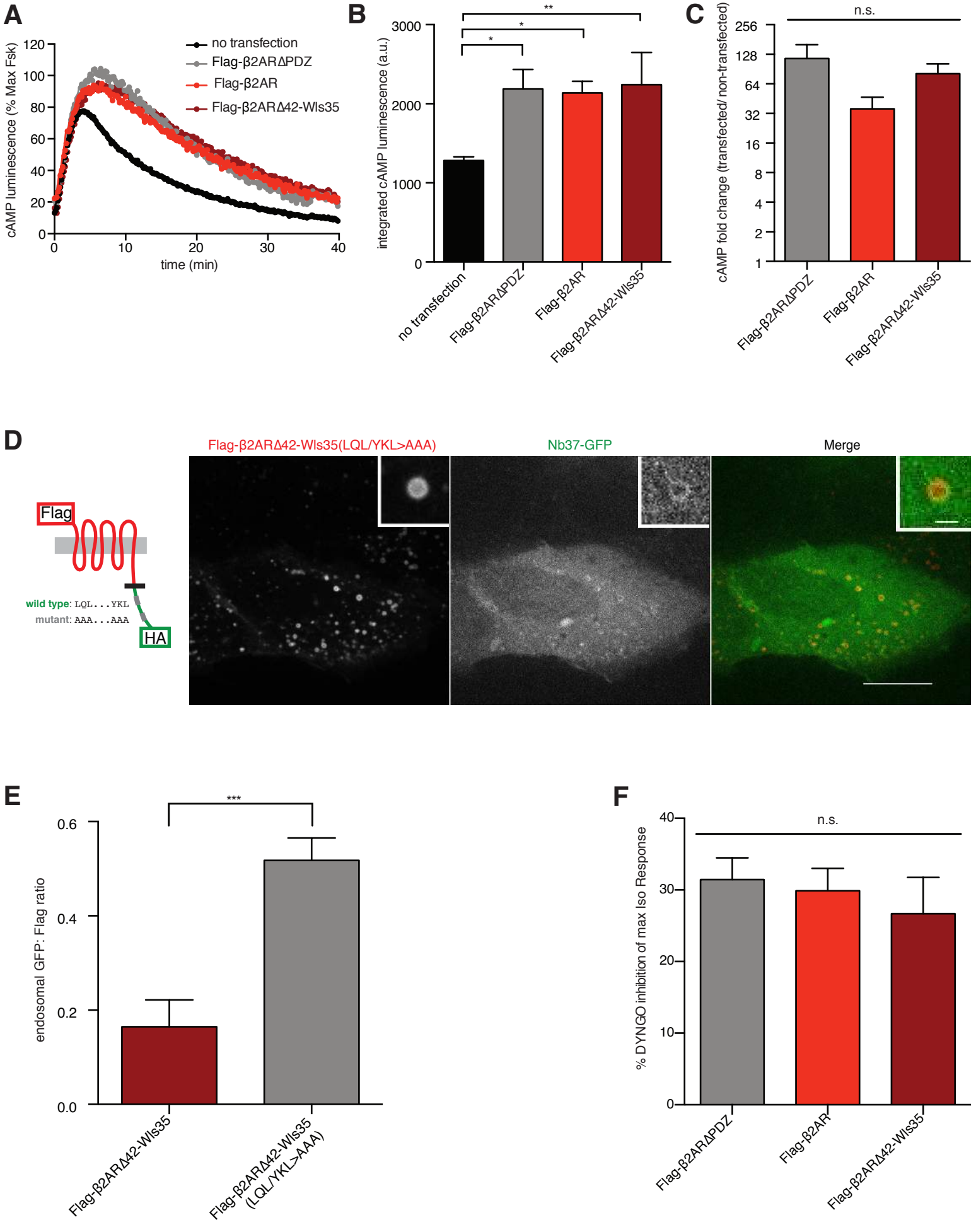

A
cAMP luminescence (% Max Fsk)
time (min)
no transfection
Flag-β2ARΔPDZ
Flag-β2AR
Flag-β2ARΔ42-Wls35
B
integrated cAMP luminescence (a.u.)
no transfection
Flag-β2ARΔPDZ
Flag-β2AR
Flag-β2ARΔ42-Wls35
C
cAMP fold change (transfected/ non-transfected)
n.s.
Flag-β2ARΔPDZ
Flag-β2AR
Flag-β2ARΔ42-Wls35
D
Flag-β2ARΔ42-Wls35(LQL/YKL>AAA)
Nb37-GFP
Merge
Flag
wild type: LQL...YKL
mutant: AAA...AAA
HA
E
endosomal GFP: Flag ratio
Flag-β2ARΔ42-Wls35
Flag-β2ARΔ42-Wls35 (LQL/YKL>AAA)
F
% DYNGO inhibition of max Iso Response
n.s.
Flag-β2ARΔPDZ
Flag-β2AR
Flag-β2ARΔ42-Wls35

**Figure S6**

**Supplemental Figure Legends**

**Figure S1. Topology and localization of additional epitope-tagged Wls constructs. Related to Figure 1.**
(**A**) Schematic representation of HA-Wls-ERretrieval, which does not contain the C-terminal HA epitope to block the endogenous ER retrieval motif, showing topology and epitope tagging. (**B**) Representative epifluorescence images of fixed HEK-293 cells expressing HA-Wls-ER retrieval, without (top) or with (bottom) permeabilization, showing that the recombinant protein is not strongly localized to the plasma membrane and instead accumulates in a juxtanuclear region that does not colocalize with Golgi-resident protein Giantin, indicative of ER localization. (**C**) Schematic representation of Flag-Wls-HA showing topology and epitope tagging. (**D**) Representative epifluorescence images of fixed HEK-293 cells expressing Flag-Wls-HA, without (top) or with (bottom) permeabilization, showing that recombinant protein can be detected at the plasma membrane with only Flag antibody and that both epitopes colocalize almost perfectly at a perinuclear region, indicative of TGN localization.

**Figure S2. Relative expression levels of epitope tagged β2AR and Wls and assessment of co-expression on membrane trafficking. Related to Figure 1.**
(**A**) Relative surface expression of HA-β2AR and HA-Wls measured by fluorescence flow cytometry (*n*=3 independent experiments). (**B**) Representative immunoblot showing relative levels of (1) Flag-β2AR to Flag-Wls and (2) Flag-Wls to endogenous Wls. HeLa cells were transfected with constructs as indicated above each lane and cell lysates expressing both Flag-β2AR and Flag-Wls were loaded in a dilution series to quantify relative expression levels. Top image shows representative blot probed with anti-Flag antibody, showing relative protein expression of Flag-β2AR and Flag-Wls expression; relative expression was quantified at 6.5 Wls: 1 β2AR in this example and overexpression averaged 12.5 ± 5.1 Wls: 1 β2AR (*n=4*). Center image shows blot probed with anti-Wls antibody, showing relative protein expression of endogenous Wls and overexpressed Flag-Wls. Relative expression was quantified at 5.3 Flag-Wls: 1 endogenous Wls in this example and overexpression averaged 4.3 ± 1.8 Flag-Wls: endogenous Wls (*n=4*). Lower image shows blot probed with anti-GAPDH, showing relative loading. Molecular weights and corresponding epitope-tagged proteins are denoted on right. (**C**) Average percent recycled Flag-β2AR upon co-expression of either empty vector (pcDNA) or HA-Wls (*n*=3). (**D**) Representative confocal images of fixed HeLa cells expressing HA-Wls and co-expressing either empty vector (pcDNA) or Flag-β2AR, surface labeled with anti-HA antibodies and chased for 60 minutes. Scale bar, 10um. (**E**) Accumulation of HA-Wls in the TGN when co-expressed with either empty vector (pcDNA) or Flag-β2AR after a 60-minute chase incubation (n=20 cells from 2 independent experiments). Error bars represent s.e.m.

**Figure S3. Structured illumination microscopy (SIM) confirms β2AR and Wls have distinct abilities to enrich in REDs. Related to Figure 3.**
(**A**) Representative SIM reconstruction images of a live HEK-293 cell expressing HA-Wls and Flag-β2AR and surface labeled with anti- Flag and HA antibodies after chase incubation. Inset images show representative endosomes at higher magnification. Large image scale bars, 10μm; inset scale bars, 1μm. (**B**) Average RED enrichment indices of Flag-β2AR and HA-Wls. (*n*=30 REDs pooled from 2 independent experiments).

**Figure S4. Bipartite nature of the retromer sorting determinant in the Wls cytoplasmic tail. Related to Figure 4.**
(**A**) Amino acid sequences of the cytoplasmic tails of: (i) wild type human β2AR, (ii) wild type human Wls, (iii) WlsΔ35, (iv) WlsΔ518-541, (v) WlsΔ506-518, and (vi) Wls(LQL+YKL>AAA). In the β2AR sequence, the PDZ motif is underlined and in the Wls sequences, the ϕ-X-[L/M] motifs are in cyan. In the Wls(LQL+YKL>AAA) sequence, mutated residues are shown in grey. (**B, C**) Left, schematic representations of HA-WlsΔ518-541 (B) and HA-WlsΔ506-518 (C) showing topology, epitope tagging, and mutations. Center, representative confocal images of live HEK-293 cells expressing HA-WlsΔ518-541 (B) or HA-WlsΔ506-518 (C) and Flag-β2AR after surface Flag and HA labeling and subsequent chase incubations. Inset images show representative endosomes with REDs at higher magnification. Right, average RED enrichment indices of HA-WlsΔ518-541 (B) or HA-WlsΔ506-518 (C) compared to Flag-β2AR in the same REDs (*n*=30 REDs pooled from 3 independent experiments). (**D**) Representative confocal images of HeLa cells expressing HA-WlsΔ518-541 or HA-WlsΔ506-518 surface labeled with anti-HA antibodies and chased for 60 minutes (**E**) Accumulation of each cargo in the TGN, quantified by average Pearson's correlation coefficient with TGN46 after a 60-minute chase incubation (*n*=30 cells pooled from 3 independent experiments). Large image scale bars, 10μm; inset scale bars, 1μm. Error bars correspond to s.e.m.

**Figure S5. Cytoplasmic tail swapping between β2AR and Wls does not change downstream destination. Related to Figure 5.**
(**A**) Representative confocal images of fixed HeLa cells expressing Flag-β2AR, Flag-β2ARΔ42, or Flag-β2ARΔ42-Wls35 surface labeled with anti-Flag antibody and chased for 60 minutes. (**B**) Accumulation of each cargo in the TGN after 60-minute chase incubation, compared to HA-Wls, quantified by average Pearson's correlation coefficient with TGN46 (*n*=30 cells pooled from 3 independent experiments). Error bars correspond to s.e.m. (**C**) Representative confocal images of fixed HeLa cells expressing HA-Wls, HA-WlsΔ35, HA-WlsΔ35-β2AR42, or HA-β2AR surface labeled with anti-HA antibodies and chase incubated for 60 minutes. Scale bars, 10μm.

**Figure S6. B2AR mutant constructs are functional in signaling assays and do not significantly alter the fraction cAMP produced from endosomes. Related to Figure 7.**
(**A**) Isoproterenol-induced cAMP luminescence traces, normalized to forskolin maxima, from live HEK-293 cells stably expressing indicated constructs (*n*=2). (**B**) Integrated cAMP luminescence from plot shown in A. (**C**) Fold change of cAMP in cells stably expressing the indicated constructs lysed after 30 minutes of isoproterenol treatment compared to non-transfected cells measured by Elisa (*n*= 3). (**D**) Left, schematic representations of Flag-β2ARΔ42-Wls35(LQL+YKL>AAA) showing corresponding mutations, topology, and epitope tagging. Right, representative confocal image of a live HEK-293 cell expressing Flag-β2ARΔ42-Wls35(LQL+YKL>AAA) and Nb37-GFP after Flag surface labeling and subsequent chase incubation. Inset images show representative endosome at higher magnification. Large image scale bar, 10μm; inset scale bar, 1μm. (**E**) Average Nb37-GFP to Flag fluorescence enrichment ratio in endosomes, (*n*=10 cells from 1 experiment). (**F**) Percent inhibition of maximal isoproterenol-induced cAMP luminescence by endocytic inhibitor Dyngo-4a in cells stably expressing the indicated constructs (*n*=4).

**Supplemental Experimental Procedures**

**DNA constructs**

Flag-β2AR [S1], HA-β2AR [S2], VPS29-mCherry [S3], Nb80-GFP and Nb37-GFP [S4], and Flag-SpH-β2AR [S5] were described previously. HA-Wls, HA-Wls-ERretrieval, and Flag-Wls-HA were created by PCR of human Wntless cDNA (Open Biosystems) to add an internal HA or Flag epitope and restriction sites followed by ligation into pIRESneo3 with or without C-terminal HA tag. HA-Wls deletion constructs (HA-WlsΔ35, HA-WlsΔ518-541, and HA-WlsΔ506-518) and Flag-β2AR deletion constructs (Flag-β2ARΔPDZ and Flag-β2ARΔ42) were created by Phusion PCR amplification and blunt-end ligation (Phusion Site-Directed Mutagenesis Kit, Thermo Scientific). Chimeric constructs (HA-WlsΔ35-β2ARtail and Flag-β2ARΔ42-Wls35), HA-SpH-Wls, Flag-B2ARΔ42-Wls35(LQL/YKL>AAA), and HA-Wls(LQL/YKL>AAA) were created using homology directed ligation (In-Fusion HD Cloning kit, Clontech), and for HA-Wls(LQL/YKL>AAA) a gBlock (Integrated DNA technologies) with desired mutations was used for the insertion fragment.

**siRNAs**

siRNAs were purchased from Qiagen for control (1027281), VPS35 (SI04287605), and FAM21 (custom sequence 5′- GAACAAAACCCAAGGCAAA -3′).

**Antibodies**

Commercial primary antibodies used were: mouse anti-Flag M1 (Sigma), rabbit anti-Flag (Sigma), mouse anti-HA (Covance), rat anti-HA (Roche applied science), rabbit anti-Giantin (Covance), sheep anti-TGN46 (AbD Serotec), rabbit anti-WASH complex subunit FAM21-C (EMD Millipore), and mouse anti-GAPDH (EMD Millipore). Chicken anti-Wls was a generous gift from Dr. Robert Levenson. Commercial secondary antibodies used were: Alexa Fluor 555 or 488 donkey anti-mouse, Alexa Fluor 555 or 488 donkey anti-rabbit , Alexa Fluor 488 or 555 goat anti-rat, or Alexa Fluor 488 donkey anti-sheep (Life Technologies). Conjugated primary antibodies used for live cell imaging and flow cytometry, with dilutions used in parentheses, were mouse anti-Flag M1 antibody conjugated to Alexa Fluor 555 or 647 (Alexa Fluor protein labeling kits, Invitrogen; 1:1000), mouse anti-HA epitope 488 conjugate (Invitrogen; 1:100), and mouse anti-HA epitope 647 conjugate (Thermo Scientific; 1:100). LI-COR secondary antibodies used for immunoblotting were donkey anti-mouse IR800, donkey anti-rabbit IR680 , and goat anti-chicken IR800 (LI-COR biosciences).

**SIM imaging**

SIM images were acquired using a Nikon Ti-E inverted microscope through an Apo TIRF 100X 1.49 NA objective (Nikon) and 3D EX V-R SIM grating (Nikon) illuminated by sapphire lasers of 488 and 561 nm (Coherent) with an Andor iXon DU897 EMCCD camera. NIS-Elements 4.12 software was used to control hardware and reconstruct images.

**Image analysis**

Fluorescence intensity plots were measured using ImageJ software. For data plotted as % max fluorescence, % max fluorescence was calculated for each image as follows: % max fluorescence= mean fluorescence- min fluorescence/ max fluorescence. $\Delta F/F_0$ for TIR-FM insertion event time series was calculated as: (mean fluorescence- $F_0$ )/ $F_0$, where $F_0$ is defined as the average fluorescence in the ten frames prior to the insertion event.

To measure RED enrichment index, regions of interest (ROIs) were drawn as freehand lines 5 pixels wide around the endosome limiting membrane and down the RED. To correct for background fluorescence, the ROIs were moved to adjacent regions within the cell and the mean background fluorescence values were subtracted from the endosome and RED fluorescence values. RED enrichment index was calculated as the background subtracted RED fluorescence per pixel divided by endosome fluorescence per pixel. Exiting vesicle: endosome ratio was calculated in a similar manner, but with freehand ROIs around the endosome and exiting vesicle.

Pearson's correlation coefficient of cargo and TGN46 was measured using the Coloc2 plugin in Fiji software (http://fiji.sc/Fiji). ROIs were drawn around the cell of interest in the cargo channel and PCC was calculated only within cellular ROIs.

Nanobody enrichment on endosomes was calculated by drawing: (1) circular ROIs around 5 endosomes per cell in the Flag-receptor channel, (2) circular ROIs adjacent to each endosome to correct for local background fluorescence in the Flag-receptor channel, and (3) a freehand ROI around the cell in the nanobody channel. To

normalize for expression levels between cells, the mean endosome enrichment in each channel was calculated using the formula: mean endosome enrichment= (mean endosome fluorescence-local endosome background)/(mean cell fluorescence-local endosome background). The nanobody: Flag receptor ratio for each cell was calculated as the average ratio of mean endosome enrichment in the nanobody channel divided by that of the Flag channel for the 5 endosomes measured per cell. Values reported are the mean for *n* cells.

**Immunoblotting**

Cells were transfected with siRNAs and/ or DNA constructs as indicated, grown to confluency, and lysed in a buffer of 0.2% Triton-X100, 150mM NaCl, 25mM KCl, 10mM Tris pH7.4, 1 mM EDTA supplemented with protease inhibitor cocktail (Roche). Lysates were cleared by centrifugation and protein concentration was determined by Coomassie protein assay (Thermo scientific.) Lysates were denatured by SDS sample buffer (Novex) + 0.2M Dithiothreitol. Equal total protein amounts were loaded and run on 4-12% Bis-Tris gels (NuPAGE, Invitrogen), transferred to nitrocellulose membranes, and probed by immunoblotting, first with primary antibodies as indicated, and then with secondary antibodies conjugated to infrared dyes (LI-COR). Blots were imaged on a LI-COR scanner and images were analyzed with Image Studio Lite software (https://www.licor.com/bio/products/software/image_studio_lite/).

**cAMP Assays**

Cellular cAMP was detected by two methods. The first method utilizes a luminescence-based biosensor (pGlo-20F Promega) in live HEK-293 cells treated with saturating concentrations of either isoproterenol (10μM) or Forskolin (2.5μM), as described in Irannejad, et al., 2013. The second method measured the concentration of cAMP in HEK-293 cell lysates treated for 30 minutes with saturating isoproterenol (10μM) using a cAMP enzyme immunoassay kit (ENZO life sciences) per mg protein, quantified by Coomassie protein assay (Thermo scientific). To assay for cAMP produced from endocytosed receptors, cells were pre-treated in serum-free media for 15 minutes with either 30uM Dyngo-4a (Abcam) to block endocytosis or 0.1% DMSO (Sigma-Aldrich). The 5 maximum cAMP luminescence values were averaged for each condition, and reported as percent change of Dyngo-4a treated compared to DMSO-treated.

**Supplemental References:**


S1. Tang Y, Hu LA, Miller WE, Ringstad N, Hall RA, Pitcher JA, DeCamilli P, Lefkowitz RJ. (1999). Identification of the endophilins (SH3p4/p8/p13) as novel binding partners for the beta 1-adrenergic receptor. Proceedings of the National Academy of Sciences *96*, 12559–64.

S2. von Zastrow M, Kobilka BK. (1992). Ligand-regulated internalization and recycling of human beta 2-adrenergic receptors between the plasma membrane and endosomes containing transferrin receptors. J. Biol. Chem. *267*, 3530–8.

S3. Temkin P, Paul T, Ben L, Stefanie J, Peter C, Krogan NJ, von Zastrow M. (2011). SNX27 mediates retromer tubule entry and endosome-to-plasma membrane trafficking of signalling receptors. Nat. Cell Biol. *13*, 717–23.

S4. Irannejad R, Tomshine JC, Tomshine JR, Chevalier M, Mahoney JP, Steyaert J, Rasmussen SGF, Sunahara RK, El-Samad H, Huang B, et al. (2013). Conformational biosensors reveal GPCR signalling from endosomes. Nature *495*, 534–8.

S5. Yudowski GA, Puthenveedu MA, von Zastrow M. (2006). Distinct modes of regulated receptor insertion to the somatodendritic plasma membrane. Nat. Neurosci. *9*, 622–7.