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SNX27 mediates retromer tubule entry and endosome-to-plasma membrane trafficking of signaling receptors

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Abstract

Endocytic sorting of signaling receptors between recycling and degradative pathways is a key cellular process controlling the surface complement of receptors and, accordingly, the cell's ability to respond to specific extracellular stimuli. The beta-2 adrenergic receptor (β 2AR) is a prototypical seven-transmembrane signaling receptor that recycles rapidly and efficiently to the plasma membrane after ligand-induced endocytosis. β 2AR recycling is dependent on the receptor's C-terminal PDZ ligand and Rab4^{1,2}. This active sorting process is required for functional resensitization of β 2AR-mediated signaling^{3,4}. Here we show that sequence-directed sorting occurs at the level of entry into retromer tubules and that retromer tubules are associated with Rab4. Further, we show that sorting nexin 27 (SNX27) serves as an essential adapter protein linking β 2ARs to the retromer tubule. SNX27 does not appear to directly interact with the retromer core complex, but does interact with the retromer associated Wiskott-Aldrich Syndrome Protein and SCAR Homolog (WASH) complex. The present results identify a role for retromer in endocytic trafficking of signaling receptors, in regulating a receptor-linked signaling pathway, and in mediating direct endosome-to-plasma membrane traffic.

After treatment with agonist such as isoproterenol, β 2ARs trigger a signaling cascade and undergo clathrin mediated endocytosis. β 2ARs are then rapidly recycled from the early endosome antigen 1 (EEA1) compartment (Fig 1a, b, 4a) to the plasma membrane (Fig 2c), resensitizing the cell⁵. Internalized transmembrane proteins are generally thought to leave

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Author Contributions

PT and MvZ conceived the project and wrote the manuscript. PT performed most of the experiments with additional contributions from BL. Mass-Spectrometry was performed by SJ, analyzed by PC, in the lab of NJK.

Competing Interest Statement

The authors of this paper declare no competing financial interests.

the endosome through tubules⁶. In the case of β 2AR, receptor-containing tubular endosomal protrusions can be visualized in living cells (Fig 1a, c)⁷. β 2AR recycling is sequence-dependent, requiring a C-terminal PDZ ligand². When this ligand is occluded by a HA tag (β 2AR-HA), mutant receptors fail to recycle efficiently and are not seen in endosomal tubules (Fig 1c)². Therefore, these tubules likely represent the structure responsible for sequence-dependent recycling of β 2AR.

Cargo that are capable of recycling efficiently with bulk membrane flux, such as the transferrin receptor (TFR), exit endosomes via multiple dynamic tubules⁸. The β 2AR was previously shown to enter a specific subset of these tubules⁷. To investigate the hypothesis that β 2AR-containing tubules are biochemically distinct, we took a candidate-based approach to identify specific tubule components.

Because both recycling (β 2AR) and non-recycling (β 2AR-HA) receptors were localized to similar endosomes but appeared to differ in lateral distribution near the highly curved neck region of endosomal tubules (Fig 1c), we asked whether curvature sensing/inducing Bin–Amphiphysin–Rvs (BAR) domain-containing proteins might be localized there. We first considered sorting nexins, because several contain a BAR domain and an endosome-associating PX domain^{9,10}. We looked at four of these BAR domain-containing sorting nexins (SNX1, SNX4, SNX5, and SNX9). We noticed striking, and nearly complete, overlap of SNX1 and SNX5 with β 2AR-containing tubules (Fig 1c; data not shown for SNX5). Quantification across multiple cells verified that the vast majority (92.5%; n = 40 tubules) of endosomal tubules containing internalized β 2ARs co-localized with a concentrated region of GFP-SNX1⁸.

SNX1 and SNX5 have been previously associated with retromer. Retromer is composed of two distinct multi-protein subcomplexes, one containing sorting nexins and the other containing vacuolar protein sorting (VPS) proteins VPS26, VPS29, and VPS35^{11,12}. Recruitment of the VPS26/29/35 subcomplex to the endosome requires previous recruitment of the SNX-containing subcomplex and Rab7¹³. Therefore, we next visualized a component of the VPS26/29/35 retromer subcomplex to determine whether the complete retromer complex was forming on these tubules.

VPS29-GFP was also concentrated on β 2AR-containing tubules (Fig 1c, Supplementary Movie); quantification across multiple examples indicated that 96.5% (n=57 tubules) of β 2AR-containing endosome tubules were decorated with VPS29-GFP. These VPS29 foci extended off the edge of the endosome limiting membrane and co-localized with tubular β 2AR staining. In rapid (2Hz) image series, β 2AR could be seen extending distally beyond the SNX1 or VPS29 marked portion of the tubule (Fig 1c, d, Supplementary Movie). Essentially all VPS29-GFP puncta co-localized with β 2AR on vesicles (95.4%, n=177 spots) (Fig 1a). Additionally, 31.1% of these VPS29-GFP spots were tubular protrusions from an endosome with a resolvable lumen. Consistent with this co-localization, biochemical purification of EEA1 endosomes co-purified β 2AR and retromer component VPS35 (Fig 1b; uncut blots for all figures are in Supplementary Figure 2). Together, this data suggests that β 2ARs access the retromer marked tubular endosomal network¹⁴.

In contrast to β 2AR, recycling-defective β 2AR-HA did not extend into retromer tubules (Fig 1c). This distinction between β 2AR and β 2AR-HA localization was quantified by circumferential line scan analysis around the endosome limiting membrane. Whereas β 2AR was enriched ~50% at the base of VPS29-GFP coated tubules relative to the rest of the endosome membrane, β 2AR-HA was not (Fig 1d, e). These results indicate that β 2ARs specifically enter retromer-associated tubules following endocytosis, and suggest that tubule entry is the primary sorting step required for β 2AR recycling to the plasma membrane.

To investigate if retromer is required for β 2AR recycling, we depleted retromer components by RNAi. We first assessed trafficking effects using fluorescence microscopy to visualize ligand-dependent redistribution of surface-labeled receptors between the plasma membrane and intracellular membranes. The β 2AR agonist isoproterenol induced pronounced redistribution of antibody-labeled β 2ARs from the plasma membrane to intracellular puncta, indicative of ligand-induced endocytosis. This process was not detectably inhibited by siRNA-mediated knockdown of VPS35 (Fig 2a). Subsequent recycling of receptors to the plasma membrane after agonist removal was obviously reduced in VPS35-depleted cells (Fig 2a).

We then quantified this effect of retromer depletion using a flow cytometry assay measuring changes in the internalized pool of receptors. β 2AR recycling, as measured by antibody efflux following agonist removal, was strongly inhibited by multiple siRNA sequences targeting either VPS26 or VPS35 (Fig 2b). Further, retromer depletion inhibited β 2AR recycling at all time points examined (Fig 2c). Consistent with this, VPS35 depletion, which prevents endosome association of VPS29-GFP, resulted in endosomes devoid of β 2AR tubules (Fig 2d). Additionally, we verified that retromer is required for β 2AR recycling in physiologically relevant A10 cells, a rat atrial-derived vascular smooth muscle cell line (Supplementary Figure 1).

Retromer depletion leads β 2ARs to be misrouted to the lysosome and degraded (Fig 2e-g). This effect of retromer depletion on receptor degradation is fully consistent with the observed inhibition of β 2AR recycling, and similar to that of disrupting the receptor's C-terminal PDZ ligand².

To examine the specificity of retromer depletion effects, we tested another protein that traffics through the same endosome as β 2AR. For this we used TFR, a constitutively endocytosed nutrient receptor that can recycle with bulk membrane¹⁵. When TFR-GFP or labeled Transferrin was imaged alongside internalized β 2AR in living cells, β 2AR only appeared in a subset of the TFR containing tubules projecting from the limiting membrane of individual early endosomes (Fig 3a, b), as shown previously⁷. Additionally, only a subset of TFR tubules labeled with VPS29-mCherry (Fig 3a). As TFR can access non-retromer recycling tubules, it should be able to exit the endosome in the absence of retromer tubules. Consistent with this, retromer depletion had no effect on degradation of endogenous TFR (Fig 3c, d) and produced only a small kinetic delay in the recycling of TFR back to the plasma membrane (Fig 3e). This second observation is consistent with the ability of a visible fraction of TFR to enter retromer tubules and further supports the ability of these tubules to mediate rapid plasma membrane recycling.

As retromer complex has not previously been implicated in recycling from endosomes to the plasma membrane, we next sought to compare β 2AR localization and trafficking to that of a membrane cargo which exhibits retromer-dependent trafficking to the TGN. For this purpose we focused on the cation-independent mannose phosphate receptor (CIMPR)^{16,17}. We first evaluated the localization of β 2AR relative to CIMPR in fixed cells. β 2AR was induced to reach a steady-state of internalization and recycling so that it populated various intermediates in endocytic and recycling pathways. Under this condition, β 2AR was localized primarily in EEA1 marked endosomes and at the plasma membrane (Fig 1b, 4a)^{18,19}. CIMPR co-localized with β 2AR in a large fraction of early endosomes but was also prominently localized in perinuclear endomembranes not containing β 2AR (Fig 4a). This is consistent with steady state localization of CIMPR in the TGN and late endosomes²⁰. Supporting this, the localization of perinuclear CIMPR was similar to, or closely adjacent to, that of the trans-Golgi/TGN marker galactosyltransferase-GFP (GalT-GFP)^{21,22}. We also note that retromer cargo that traverse the TGN on the way to indirect plasma membrane delivery have shown significant colocalization with markers of the TGN region²³. Importantly, and in marked contrast, the β 2AR localization observed in these cells was clearly distinct. β 2ARs localized to peripheral early endosomes but not in the perinuclear distribution characteristic of CIMPRs (Fig 4a).

Given that β 2AR and CIMPR achieve different steady state localization patterns, we next used live imaging to ask if these distinct membrane cargoes localize to the same endosomes and, if so, if they localize to the same retromer tubules. Confocal microscopy revealed that both FITC-conjugated anti-CIMPR antibody (CIMPR AB), used to detect endogenous receptor, and a GFP-tagged CIMPR construct (cCIMPR-GFP) clearly co-localized with β 2AR in the same endosomes and in the same tubules (Fig 4b, f). This overlap was extensive, as >90% of the β 2AR-positive tubules resolved also contained GFP-tagged CIMPR.

We next asked how the retromer tubule could support rapid endosome-to- plasma membrane traffic, evidently independent of traversing the TGN. Knowing that the small GTPase Rab4A had previously been implicated in this process we tested if it associated with β 2AR containing endosomes. GFP-Rab4A localized to these compartments and was visibly enriched on the β 2AR-containing retromer tubules (Fig 4c)⁷. Further, as expected, depletion of Rab4A by siRNA inhibited recycling of β 2AR (data not shown)¹. In contrast, depletion of several components that function in endosome-to-TGN trafficking of CIMPR (Rab6A', Rab7b (a distinct gene from Rab7), and GCC185) failed to significantly affect β 2AR recycling²⁴⁻²⁸ (Fig 4d). As a control for depletion, we established in parallel experiments that these siRNAs caused a pronounced increase in cell surface expression of CIMPR, consistent with net disruption of normal endosome-TGN cycling of CIMPRs established previously (Fig 4e)¹⁶.

Despite the co-occurrence of both membrane cargoes in a large fraction of endosomal tubules, β 2AR and CIMPR were not identically localized in individual tubules imaged at high temporal resolution. Specifically, CIMPR was typically enriched near the distal end of tubules or in protrusions that extended beyond the β 2AR labeled areas (Fig 4f). Apparent separation of cargo was visualized for both endogenous CIMPR and the GFP CIMPR

transgenic protein (Fig 4f). These results support the previously proposed idea that an elaborated tubular endosomal network, capable of mediating the trafficking of cargoes to multiple destinations, is fed by the retromer tubule¹⁴. Further work will be required to investigate this, as there is also evidence that a significant fraction of CIMPRs can traffic to the plasma membrane following endosome exit²⁰.

We next investigated the mechanistic basis for the role of retromer in β 2AR recycling. For CIMPR, it is proposed that a direct interaction between the cytoplasmic tail and retromer complex is required for proper trafficking²⁹. β 2AR trafficking is dependent on a PDZ motif present in the cytoplasmic tail that is both necessary and sufficient to mediate its plasma membrane recycling, but the core retromer complex is devoid of any recognizable PDZ domain. SNX27 contains a PDZ domain that binds the β 2AR tail, and has recently been shown to be essential for PDZ-directed recycling of the β 2AR³⁰. Verifying this, knockdown of SNX27 robustly inhibited recycling of β 2ARs (Fig 5a). Despite this pronounced decrease in β 2AR recycling, CIMPR distribution in the same cells appeared unaffected. Furthermore, SNX27 depletion did not alter CIMPR surface expression (Fig 5b) or CIMPR turnover in the presence of cyclohexamide (data not shown).

Live imaging of SNX27 depleted cells revealed that VPS29-GFP puncta still associate with endosomes but that β 2ARs no longer appear in these tubular structures (Fig 5c). Depletion of SNX27 also decreased the aggregation of β 2ARs at the retromer tubule. In control siRNA treated cells there was a relative receptor density of 1.51 ± 0.061 ($n=16$ endosomes) at the base of the VPS29 tubule, while in SNX27 depleted cells this dropped significantly to 1.15 ± 0.055 ($n=19$ endosomes, $p=.0002$), phenocopying disruption of the PDZ motif (β 2AR-HA). Together, this data suggests that SNX27 acts as a cargo adaptor to retromer, but is not a core component.

To investigate further how SNX27 was serving as a cargo adaptor to the retromer tubule, we used an affinity tagging/purification-mass spectrometry approach to determine which other proteins physically associate with it. In all 5 independent SNX27 purifications, components of the WASH actin nucleation complex were predominant hits (Supplementary Table). However, WASH complex was not detected in control purifications or in those of other bait molecules run at the same time. WASH has been shown to physically link to the retromer complex³¹. Therefore this association with SNX27 supports the adaptor hypothesis by establishing protein connectivity.

Association of SNX27 with the retromer tubule was further supported by extensive colocalization between SNX27 and VPS29-GFP in fixed cells (Fig 5d). Moreover, SNX27 co-immunoprecipitated endogenous VPS35 but not EEA1, an abundant protein that localizes to the same endosomes. Interestingly, VPS35 co-immunoprecipitated with SNX27 only under crosslinking conditions, in contrast to WASH complex components that co-purified with SNX27 in the absence of crosslinking (Fig 5e). These observations suggest that SNX27 does not interact with the core retromer complex directly, but that its association with the retromer tubule is mediated by other interaction(s) including through the WASH complex. Together, these results indicate that SNX27 acts, through novel connectivity involving the

WASH complex, as a specific adapter to promote PDZ-directed plasma membrane sorting through the retromer tubule (Fig 5f).

To probe the generality of the SNX27/retromer-mediated recycling pathway we next examined two other GPCRs that are known to recycle rapidly to the plasma membrane after endocytosis. Efficient recycling of the beta-1 adrenergic receptor (β 1AR) requires a class 1 PDZ motif present in its distal cytoplasmic tail, but this motif differs in primary structure and binding properties from the PDZ motif present in the β 2AR tail^{32,33}. The D1 dopamine receptor (D1R) recycles efficiently after endocytosis but does not contain a C-terminal PDZ motif, and truncation of its distal cytoplasmic tail does not disrupt receptor recycling³⁴. We observed a significant inhibition of β 1AR recycling following knockdown of either VPS35 (33.7%±0.1 inhibition, n=9, p= 0.0013) or SNX27 (19.5%±0.1 inhibition, n=9, p= 0.0293). Recycling of FLAG-D1Rs, in contrast, was not significantly inhibited by either manipulation. Thus, retromer-dependent recycling is not unique to the β 2AR and can be specified by distinct PDZ motifs. This suggests that many other membrane cargoes which contain C-terminal PDZ ligands, including a large group of G-protein coupled receptors (GPCRs), may traffic via this SNX27/retromer pathway³⁵.

Retromer has been shown to function in trafficking of various cargo from endosomes to the TGN, and to function in transcytosis in polarized cells^{12,36}. Recently several retromer cargo have been identified that traffic to the plasma membrane, though it is unclear whether they do so directly from the endosome or via the TGN³⁷⁻³⁹. In this paper we identify an essential role of retromer in mediating rapid recycling of a prototypical GPCR apparently directly to the plasma membrane. We also establish that β 2ARs can enter the same retromer tubules as a canonical TGN-directed cargo, and identify a distinct adaptor protein that links signaling receptors specifically to the retromer-dependent plasma membrane recycling pathway. Our results support a revised view of retromer tubules as a multi-functional exit port supporting diverse membrane itineraries, including direct endosome-to-plasma membrane trafficking.

Our results have fundamental implications in the field of cell signaling because they indicate that retromer plays a critical role in mediating the sorting of a prototypical GPCR between the functionally opposing pathways of resensitization and down-regulation, the distinct physiological consequences of which are well established^{4,40}. Consistent with this, depletion of VPS35 significantly decreased isoproterenol mediated signaling from the β 2AR, as measured by cellular cAMP accumulation (Fig 5g). Interestingly, retromer depletion also affected receptor-independent activation of adenylyl cyclase by forskolin, suggesting that retromer tubules have other role(s) in cell signaling.

Methods

Cell Culture, cDNA constructs, and Transfection

HEK 293 and A10 cells were grown in Dulbecco's modified Eagles medium supplemented with 10% fetal bovine serum (UCSF Cell Culture Facility, San Francisco, CA). Stably transfected HEK 293 cell clones expressing the indicated receptor constructs were created using the previously described FLAG-tagged β 2AR and β 2AR-HA and G418 selection at 500 μ g/ml (Invitrogen)².

GFP-SNX1 and VPS29-GFP were generous gifts from J. Bonifacino²⁵. Vps29-mCherry was created by PCR-mediated amplification of VPS29 from the VPS29-GFP construct, inserting it into pENTR using the pENTR directional cloning kit (Invitrogen), and then into a pcDNA3 Dest53 mCherry vector using the LR clonase kit (Invitrogen). Galt-GFP was a gift from J. Lippincott-Schwartz²². cCIMPR-GFP was created by amplifying the transmembrane and cytoplasmic domains of CIMPR from a cDNA library with the primers TATACAAAACCGGTCTGTCAGAACGGAGCCAGGCAGTCGGC and TTTGTATAGGATATCCCCCTGCAGGCACTGCGGAGTCAGATG and cloning this fragment into a N-terminal signal sequence and GFP-containing pcDNA3 vector using AgeI and EcoRV. GFP-Rab4A was a gift from S. Ferguson⁴¹. Snx27-HA³⁰, FLAG- β 1AR⁴², and FLAG-D1R³⁴ constructs were previously described.

For transient expression of constructs, cells were transfected using Lipofectamine 2000 (Invitrogen) according to manufacturers' instructions. Cells expressing FLAG-tagged receptors were harvested with PBS EDTA and plated in 12 well plates at 80% confluence before transfection with plasmid DNA. Cells were cultured for a further 48 h before experimentation. Target sequences for knockdown were hVPS26 (1: CTGCATAATGTTGATTATAAAA, 2: CACCAAGGAATTAGAATTGAA), hVPS35 (1: CTGGACATATTTATCAATATA, 3: CAGGAAATGCATAATTAT), RnVPS35 (ACCAGGTAGATTCCATAATGA), hRab4A (AATGCAGGAACTGGCAAATCT), hRab6A (CCCACTTATTGTCACCTTGTA), hRab6A' (AACAGCTGTAGTAGTTTACGA)²⁸, hRab7B (AAGTAGCTCAAGGCTGGTGTA)²⁶, hGCC185 (AAGGAGTTGGAACAATCACAT)²⁷, hSnx27-4³⁰, and control (1027281, Qiagen). 25 picomoles of duplex RNA (Qiagen) were transfected into 30% confluent cells in a 12 well dish with Lipofectamine RNAi Max (Invitrogen) 36 h prior to experimentation.

Live Receptor Imaging and Quantification

Live imaging of FLAG-tagged receptors and the indicated GFP-labeled protein was performed using a previously described antibody feeding method⁷. Briefly, cells expressing both constructs were plated onto glass coverslips and surface receptors were labeled by the addition of M1 anti-FLAG antibody (Sigma) conjugated to Alexa 555 (A10470, Invitrogen) to the media for 30 min. Isoproterenol was added (Sigma) and cells were imaged on a Nikon TE-2000E inverted microscope with a 100 \times 1.49 NA TIRF objective (Nikon) and a Yokagawa CSU22 Spinning Disk confocal head (Solamere, Salt Lake City, UT). A 488nm Ar laser and a 568nm Ar/Kr laser (Melles Griot) were used as light sources for imaging GFP and FLAG signals, respectively. Movies of endosomes were taken between 5 and 30 min after agonist addition and exported as TIF files. Each frame corresponds to 450 ms.

Analysis of receptor co-enrichment with VPS29-GFP was performed using the ImageJ (<http://rsb.info.nih.gov/ij/>) and the "Oval Profile" plugin. Endosomes with single VPS29-GFP spots were outlined using the oval tool. Oval Profile was used to perform a linear fluorescence intensity scan in 60 subsections of both VPS29-GFP and β 2AR signals as well as background signals from an endosome free region. The data was then exported to Excel (Microsoft) where the background was subtracted and the top four consecutive points of VPS29-GFP fluorescence were determined. To determine the enrichment of receptor at the

tubule as compared to the rest of the endosome, the average intensity of the four β 2AR points corresponding to the four peak VPS29 points was divided by the average receptor intensity of the rest of the endosome except for the 1200 centered at the VPS29 peak.

Recycling Assays

Visualization of FLAG receptors was carried out using fluorescence microscopy of stably transfected HEK 293 cells or transiently transfected A10 cells that had been plated on glass coverslips. Surface receptors were labeled by exposing intact cells to M1 anti-FLAG antibody conjugated to Alexa 555 (Sigma) for 25 min at 37 °C in the presence of 10 μ M isoproterenol to promote endocytosis of labeled receptors. Cells were then either fixed or washed and further incubated in media for 45 min in the presence of the antagonist 10 μ M alprenolol (Sigma) to allow for recycling. Cells were fixed using 4% formaldehyde freshly dissolved in PBS and imaged on the aforementioned spinning disk microscope.

In the flow cytometry based recycling assay, cells were plated in 12 well plates and treated as in the visual assay except that instead of fixation, HEK 293 cells were lifted in PBS EDTA, which strips the calcium dependent M1 antibody from the cell surface. When surface CIMPR was measured, CD222-FITC (BioLegend) was added to the PBS-EDTA at 1:250. Lifting of A10 cells required addition of trypsin. Fluorescence intensity profiles of cell populations (5,000 cells/sample for HEK 293 and >1500 cells/sample for A10) were measured using a FACS-Calibur instrument (BD Biosciences).

Transferrin recycling experiments involved washing cells 3 times with PBS, applying Alexa-488 labeled transferrin (Invitrogen) in serum free media for 25 minutes, and then rapid transferrin efflux occurred by switching cells to media that contained serum and no labeled transferrin. 10% serum was added to the PBS-EDTA when cells were lifted to strip all surface transferrin.

In each experiment, triplicate treatments were analyzed for each condition. All experiments were carried out on at least three separate days (number indicated in figure legends), and values reported were derived from the mean determination across experiments. The percentage of antibody recycled was calculated from internal fluorescence values as follows: % recycling = $1 - (\text{"agonist"} \rightarrow \text{"antagonist signal"} / \text{"agonist signal"})$

Fixed Cell Imaging

HEK 293 cells stably expressing β 2AR plated onto cover slips were treated with the agonist isoproterenol for 30 min prior to fixation with 4% formaldehyde in PBS and processed for immunocytochemical staining. Staining of β 2AR was done using Rabbit anti-FLAG (Sigma) and the secondary goat anti-rabbit Alexa 594 (Invitrogen). Mouse antibodies against EEA1 (BD Biosciences) and CIMPR (BioLegend) were used in combination with donkey anti-mouse Alexa 488 (Invitrogen). Fixed cells were imaged using the aforementioned spinning disk microscope.

Degradation Assays and Western Blotting

Briefly, stably transfected HEK 293 cells expressing FLAG- β 2AR were transfected with siRNA VPS35-1 or VPS35-3 as described above, and stimulated with 10 μ M isoproterenol for four h before washing three times in ice-cold PBS and lysed in extraction buffer (0.2% Triton X-100, 50 mM NaCl, 5 mM EDTA, 50 mM Tris pH 7.4, Complete EDTA free protease inhibitor cocktail (Roche)). Extracts were clarified by centrifugation (21,000 \times g for 10 min), and then mixed with SDS sample buffer for denaturation. The proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and probed for FLAG-tagged β 2AR receptor (M1 antibody, Sigma), VPS35 (generously provided by C. Haft and J. Bonafacino), or GAPDH (Chemicon) by immunoblotting using horseradish peroxidase-conjugated sheep anti-mouse IgG or donkey anti-rabbit IgG (Amersham Biosciences), and SuperSignal extended duration detection reagent (Pierce). Band intensities of unsaturated immunoblots were analyzed and quantified by densitometry using a 12-bit cooled CCD camera and FluorChem 2.0 software (AlphaInnotech Corp.). The amount of FLAG- β 2AR remaining at each time point was first expressed as a percentage of the amount of FLAG- β 2AR in the identically transfected unstimulated cells. The lysosomal protease inhibitor ZPAD (Bachem) or the proteosomal inhibitor epoxomicin (Sigma) were added to cells 40 minutes prior to addition of isoproterenol when used at 200 μ M and 2 μ M respectively.

To compare degradation of endogenous TFR to FLAG- β 2AR, stably transfected HEK 293 cells were grown in 6 well dishes, washed with ice-cold PBS and incubated with 300 μ g/ml sulfo-N-hydroxysuccinimide-biotin (Pierce) in PBS for 30 min at 4 $^{\circ}$ C to biotinylate surface proteins. Following washing with Tris buffered saline to quench unreacted biotin, cells were returned to 37 $^{\circ}$ C for incubation in media, with or without agonist, before extraction as described above. Biotinylated proteins were isolated from cell extract by immobilization on streptavidin-conjugated sepharose beads (Amersham) and washed three times with extraction buffer. Washed beads were eluted with SDS sample buffer before resolving by SDS-PAGE as above. Blotting was performed for β 2AR and TFR (13–6800, Invitrogen). In each experiment, triplicate treatments were analyzed for each condition. All experiments were carried out at least in triplicate (number indicated in figure legends), and values reported were derived from the mean determination across experiments.

Immunopurification of SNX27-HA was done using HEK 293 cells three days after transient transfection. Cells were cross-linked using 0.3mM DSP (Pierce) for 30 min prior to lysis in extraction buffer and addition of mouse HA11 antibody (Covance). SNX27-HA complexes were isolated using protein A/G beads (Pierce), washed four times with extraction buffer, and eluted with Nupage LDS buffer (Invitrogen).

Mass Spectrometry

Immunopurification and mass spectrometry of SNX27-3 \times FLAG was performed as previously described⁴³.

Endosome Purification

Endosome purification was performed as previously published⁴⁴. In addition to previously mentioned antibodies, Calnexin (ab22595, Abcam) and LAMP1 (H4A3, Santa Cruz) antibodies were used.

Signaling

HEK 293 cells stably expressing β 2AR were treated with 10 μ m isoproterenol or 1 μ m forskolin (Sigma) for 20 minutes before signaling assays were performed according to the manual of the Direct cAMP EIA Kit (Enzo Life Sciences, Plymouth Meeting, PA). cAMP levels were normalized to protein level using Coomassie Plus Protein Quantification Reagent (Thermo Scientific).

Statistics

Results are presented as mean \pm SEM. based on data averaged across multiple independent experiments. The n value of an experiment represents experiments done on different days unless otherwise noted. To assign significance, results were compared to control experiments with an unpaired t-test using Prism (v4.03, GraphPad, La Jolla, CA). Only p-values below 0.05 are shown. Statistical comparison of knockdown effect on CD222 surface labeling (Fig 4e and 5d) and of knockdown effect on cAMP accumulation (Fig 5f) was performed using the one sampled t-test against the normalized value of one or 100%. Two-way ANOVA with Bonferonni posttest was performed on the recycling time-courses of β 2AR and TFR. In these graphs * represents a $p < 0.05$, ** represents $p < 0.01$, and *** represents $p < 0.001$ as no value is given by the program.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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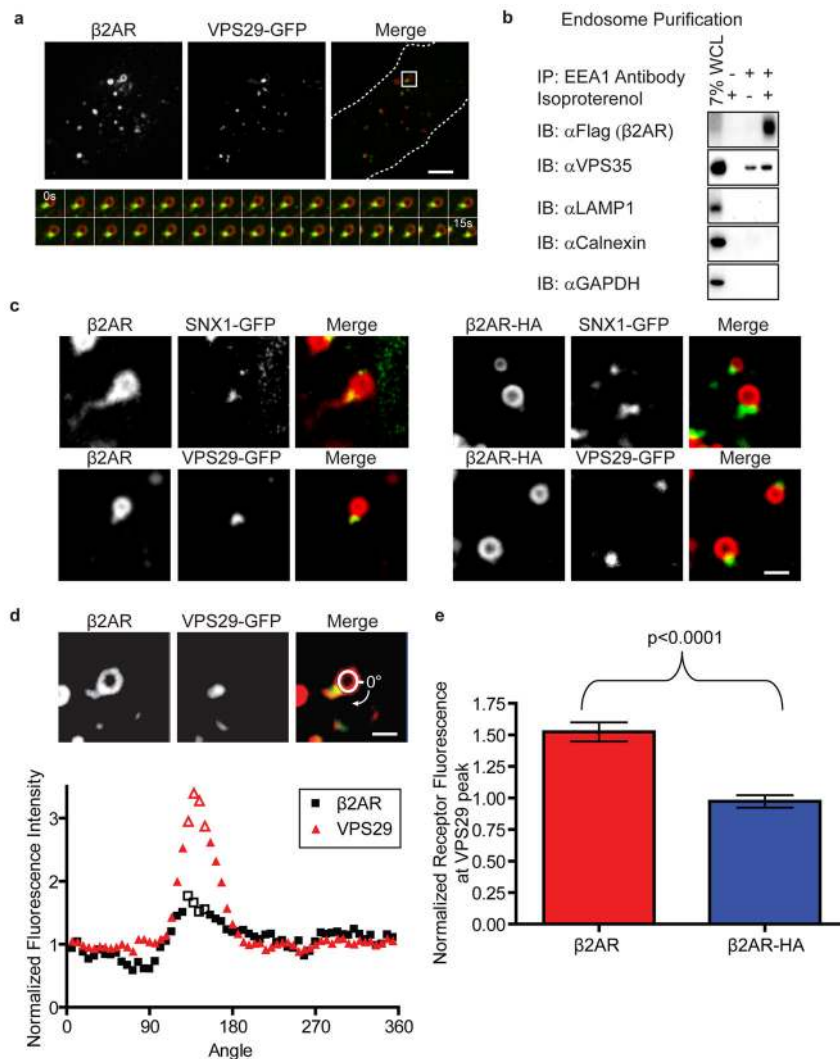


Figure 1. Rapid recycling β 2ARs selectively enter retromer-associated endosomal tubules
(a) Representative images from a movie of a cell expressing β 2AR (red) and VPS29-GFP (green). Below, the inset box is shown across multiple frames of the movie. The edges of the cell are shown as dotted lines in the merged image. The scale bar represents 4 μ m. **(b)** Representative immunoblots are shown from endosomes that were immuno-purified using antibody against the early endosome component EEA1 (n=3). **(c)** Representative images of endosomes containing wild type β 2AR or recycling-defective β 2AR-HA receptors (red) and SNX1-GFP or VPS29-GFP (green). Images were acquired by confocal microscopy of living cells after stimulating receptor endocytosis with isoproterenol. Wild type β 2ARs, but not recycling-defective β 2AR-HA mutant receptors, were visible in the VPS29-associated tubule extending from the endosome body. The scale bar represents 1 μ m. **(d)** Fluorescence intensity tracing of labeled β 2AR (black squares) and VPS29 (red triangles) around the edge of the endosome. Each point represents average fluorescence over a six degree arc of the endosome circumference. The four points of greatest VPS29-GFP fluorescence (open triangles) were used to mark the tubule. β 2AR fluorescence values were background-corrected and normalized to the average fluorescence of a portion (240 $^\circ$) of the endosome,

excluding the 1200 of circumference centered at the tubule base. The scale bar represents 1 μm . (e) Relative receptor enrichment (average of open squares in panel b) of β2AR (red bar) or $\beta\text{2AR-HA}$ (blue bar) at the tubule base. 20 endosomes (4 independent experiments), extending a single retromer-associated tubule, per receptor type were analyzed. Data points are the mean \pm standard error of the mean (SEM).

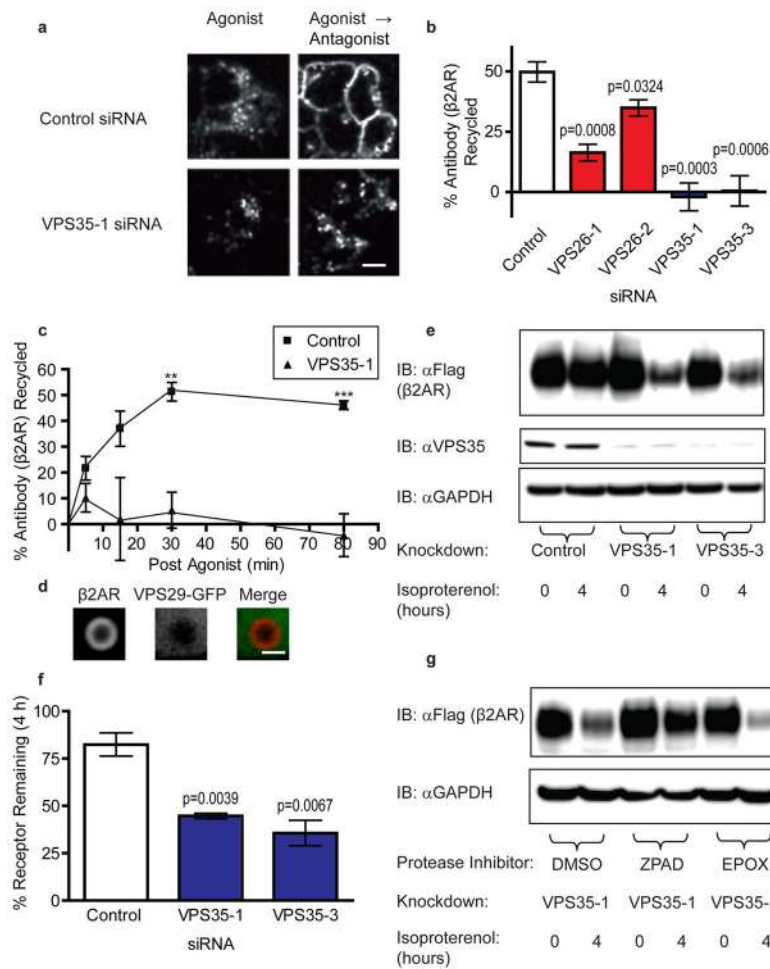


Figure 2. Knockdown of retromer by RNAi inhibits β2AR recycling and misroutes internalized β2ARs to lysosomes

(a) Representative images from a visual assay for β2AR trafficking are shown. Stably transfected HEK 293 cells expressing FLAG-β2AR were transfected with either control siRNA or siRNA targeting the retromer component VPS35. In the “Agonist” condition, cells were incubated in the presence of the β2AR agonist isoproterenol (10 μM) and Alexa-conjugated M1 anti-FLAG for 25 min. In the “Agonist → Antagonist” condition, cells were incubated with isoproterenol for 25 min and then for an additional 45 min in the absence of isoproterenol (and in the presence of 10 μM of the β2AR antagonist alprenelol to prevent effects of any residual agonist). The scale bar represents 20 μm. (b) Flow cytometric analysis of β2AR recycling by uptake and efflux of bound M1 anti-FLAG antibody (n=4). (c) A time-course of recycling is shown for β2AR. The experiment was performed as in (b) but the duration after agonist washout was varied (n=4). (d) Representative confocal image from live cell imaging showing an endosome from a VPS35-1 siRNA treated cell expressing FLAG-β2AR (red) and VPS29-GFP (green). The scale bar represents 1 μm. (e) A representative immunoblot assay of agonist induced FLAG-β2AR degradation. Detergent extracts were prepared from HEK 293 cells expressing FLAG-β2AR incubated in the absence of agonist or in the continuous presence of 10 μM isoproterenol for 4 h. Knockdown was verified by VPS35 immunoblot of the same lysates (middle panel), and equal loading

was verified by immunoblotting for GAPDH (bottom panel). (f) FLAG- β 2AR immunoblots were quantified by scanning densitometry across multiple experiments (n=3), and the percent receptor remaining after 4 h isoproterenol exposure was calculated. (g) Representative immunoblot showing isoproterenol induced β 2AR degradation in cells depleted of VPS35 and treated with the vehicle dimethyl sulfoxide (DMSO), the lysosomal cathepsin inhibitor *N*-CBZ-L-phenylalanyl-L-alanine-diazomethylketone (ZPAD), or the proteosomal inhibitor epoxomicin (EPOX) (n=3). Data points are the mean \pm SEM.

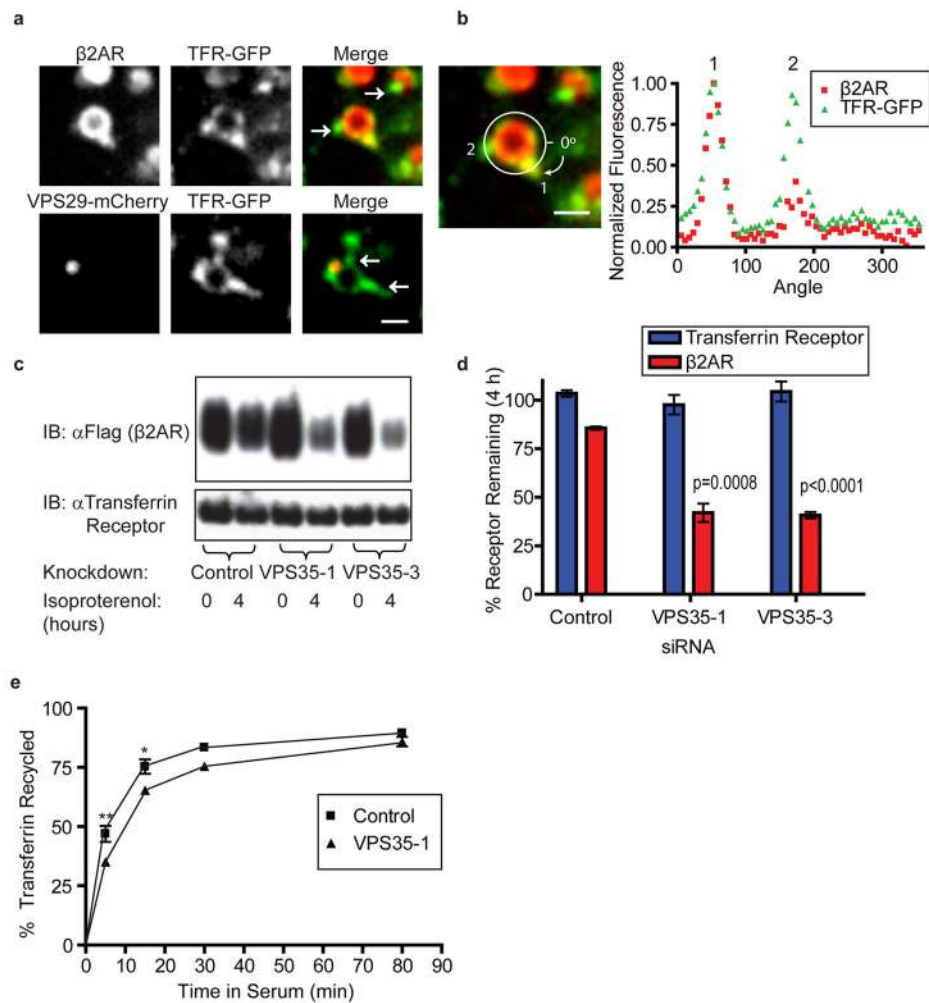


Figure 3. Retromer depletion preferentially affects β 2ARs over TFRs traversing the same endosomes

(a) Top panels show representative images of endosomes containing β 2AR (red) and TFR-GFP (green). Bottom panels show localization of VPS29-mCherry (red) relative to TFR-GFP (green). Arrows point to TFR tubules lacking β 2AR in the top image and lacking VPS29 labeling in the bottom image. The scale bar represents 1 μ m.

(b) Fluorescence intensity scan of labeled β 2AR (red squares) and TFR (green triangles) on a circumferential path set outside of the representative endosome shown in panel (a) but crossing both TFR-labeled tubules projecting from the endosome body, as indicated on the detail merged image. Positions of the tubules are marked by the numbers 1 and 2. The scale bar represents 1 μ m.

(c) Biochemical analysis comparing degradation of surface-biotinylated β 2AR (top panel) and TFR (bottom panel) in cells maintained in the absence or continuous presence of isoproterenol for 4 h. A representative immunoblot is shown. Knockdown of VPS35 using two independent siRNA targets selectively destabilized β 2AR.

(d) Receptor immunoreactivity from the experiment shown in panel (c) was quantified by scanning densitometry and the percent receptor remaining after 4 h was calculated by dividing the 4 h agonist treatment by the untreated control. Recovery of TFR (blue bars) and β 2AR (red bars) was averaged across experiments (n=3).

(e) The graph shows efflux of labeled transferrin in

HEK 293 cells treated with siRNA against a control sequence or VPS35 (n=3). Percent transferrin recycled was calculated from these flow cytometry experiments as in Fig 2b. Data points are the mean \pm SEM.

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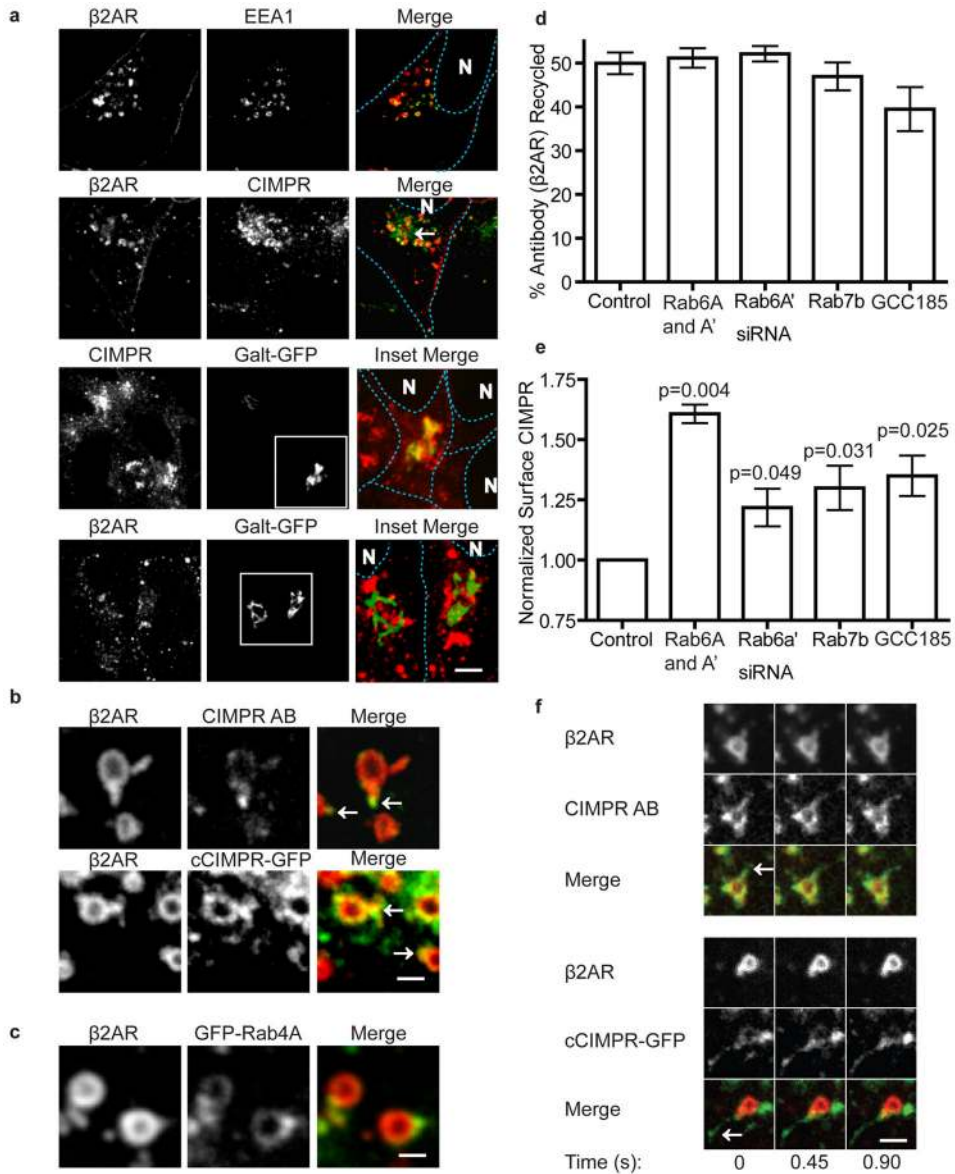


Figure 4. β 2AR and CIMPR follow divergent trafficking paths upon exit from the same retromer-associated tubule

(a) Comparative localization of CIMPR and β 2AR (red) with early endosome marker EEA1 or trans-Golgi marker GalT-GFP (green). Cells were fixed and imaged following incubation with isoproterenol for 30 min to drive β 2AR to steady state. Plasma membrane and nuclei (N) have been outlined with dotted lines. The arrow indicates an example of a trans-Golgi region labeled for CIMPR but not β 2AR. The scale bar represents 6 μ m.

(b) Representative endosome images from live cell confocal microscopy showing β 2AR (red) localization in tubules with endogenous (top panels) or recombinant (bottom panels) CIMPR (green). Arrows indicate tubules containing both β 2AR and CIMPR. The scale bar represents 1 μ m.

(c) Images, as in (b), of β 2AR (red) and GFP-Rab4A (green) localization on the endosome.

(d and e) Various components of the endosome to TGN transport pathway were depleted and β 2AR recycling (panel d) or CIMPR surface immunoreactivity (panel e) was quantified

by flow cytometry (n=4 experiments) to assess integrity of β 2AR and CIMPR trafficking. (f) Representative images from live confocal imaging showing differential enrichment of CIMPR (green) relative to β 2AR (red) localization on distal regions of the same endosomal tubules. Each column represents a frame from a continuous time lapse movie (450 ms/frame). Arrows indicate distal regions of the indicated tubules on which CIMPR labeling was visibly enriched relative to β 2AR. Multiple frames are shown to demonstrate that the observed difference in lateral enrichment did not occur as an artifact of tubule movement. The scale bar represents 1 μ m. Data points are the mean \pm SEM.

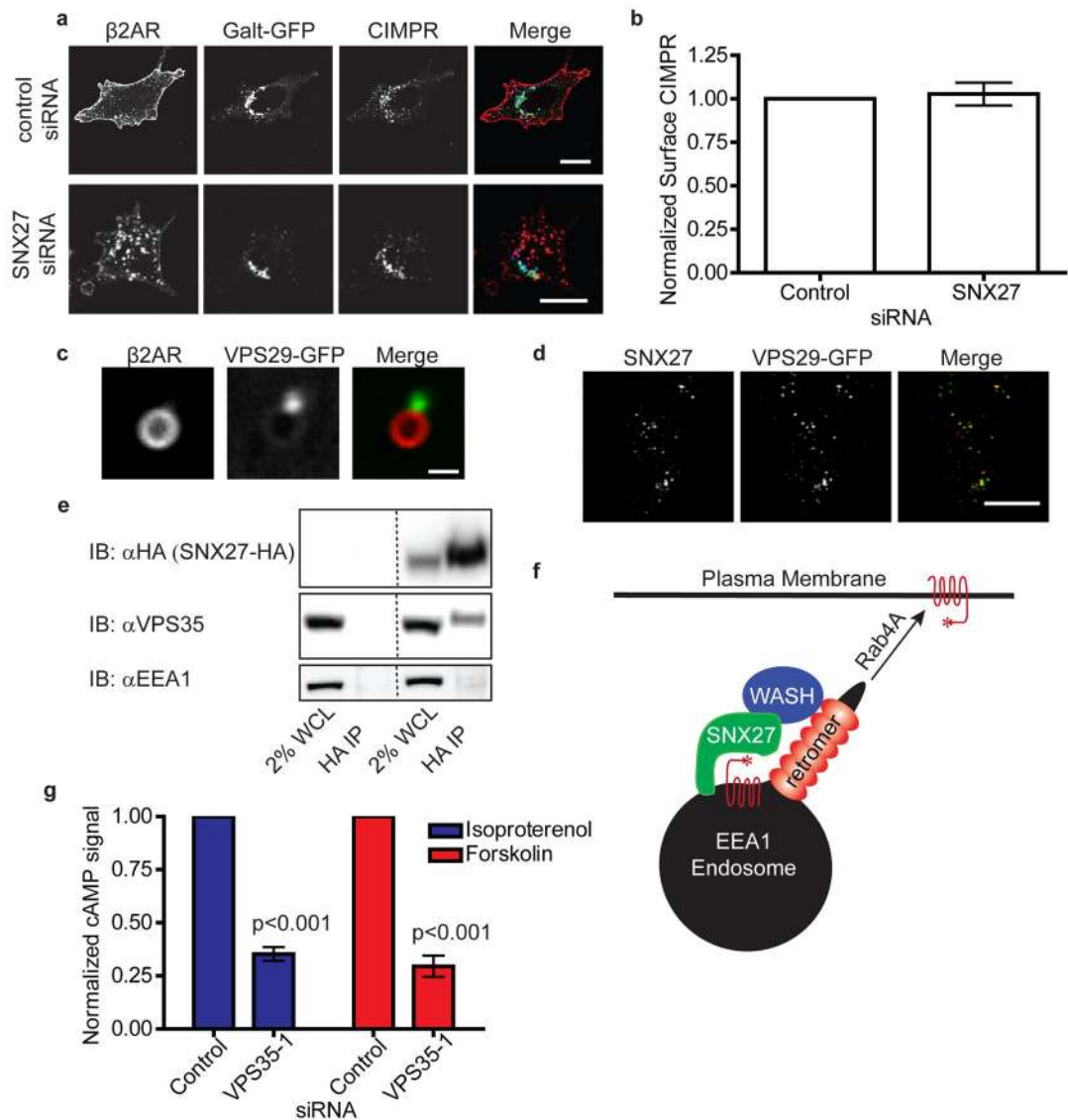


Figure 5. SNX27 serves as an adapter for β 2AR, sorting it into the retromer tubule
(a) Comparative localization of β 2AR (red) with the trans-Golgi marker GalT-GFP (green) and CIMPR (blue). Cells were fed anti-FLAG antibody in the presence of isoproterenol for 25 minutes prior to agonist washout with alprenelol for 45 minutes. Successful knockdown of SNX27 was judged by failure of β 2AR to recycle to the plasma membrane. The scale bar represents 20 μ m. **(b)** CIMPR surface immunoreactivity was quantified by fluorescence flow cytometry (n=6) to assess the integrity of CIMPR trafficking when SNX27 is depleted with siRNA. Successful depletion of SNX27 was confirmed by looking at visual recycling assays of β 2AR as in (a). **(c)** Representative confocal image from live cell imaging showing an endosome from a SNX27-4 siRNA treated cell expressing FLAG- β 2AR (red) and VPS29-GFP (green). The scale bar represents 1 μ m. **(d)** Confocal image of transiently transfected SNX27-HA (red) and VPS29-GFP (green) in fixed cells. The scale bar represents 20 μ m. **(e)** Representative immunoblot showing co-immunoprecipitation of endogenous VPS35 with transiently transfected SNX27-HA. Lanes 1 and 3 show 2% whole cell lysate for the mock

IP at left and the experiment at right. (f) SNX27 mediates plasma membrane recycling of PDZ motif containing cargo by linking to the retromer through an interaction with the WASH complex. SNX27 also interacts with the endosome directly through its lipid binding PX domain. (g) Isoproterenol or forskolin induced cAMP formation was measured in HEK 293 cells stably expressing β 2AR, in the absence of IBMX, 20 minutes after agonist addition (n=6). Data points are the mean \pm SEM.

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