Lauffer et al., http://www.jcb.org/cgi/content/full/jcb.201004060/DC1

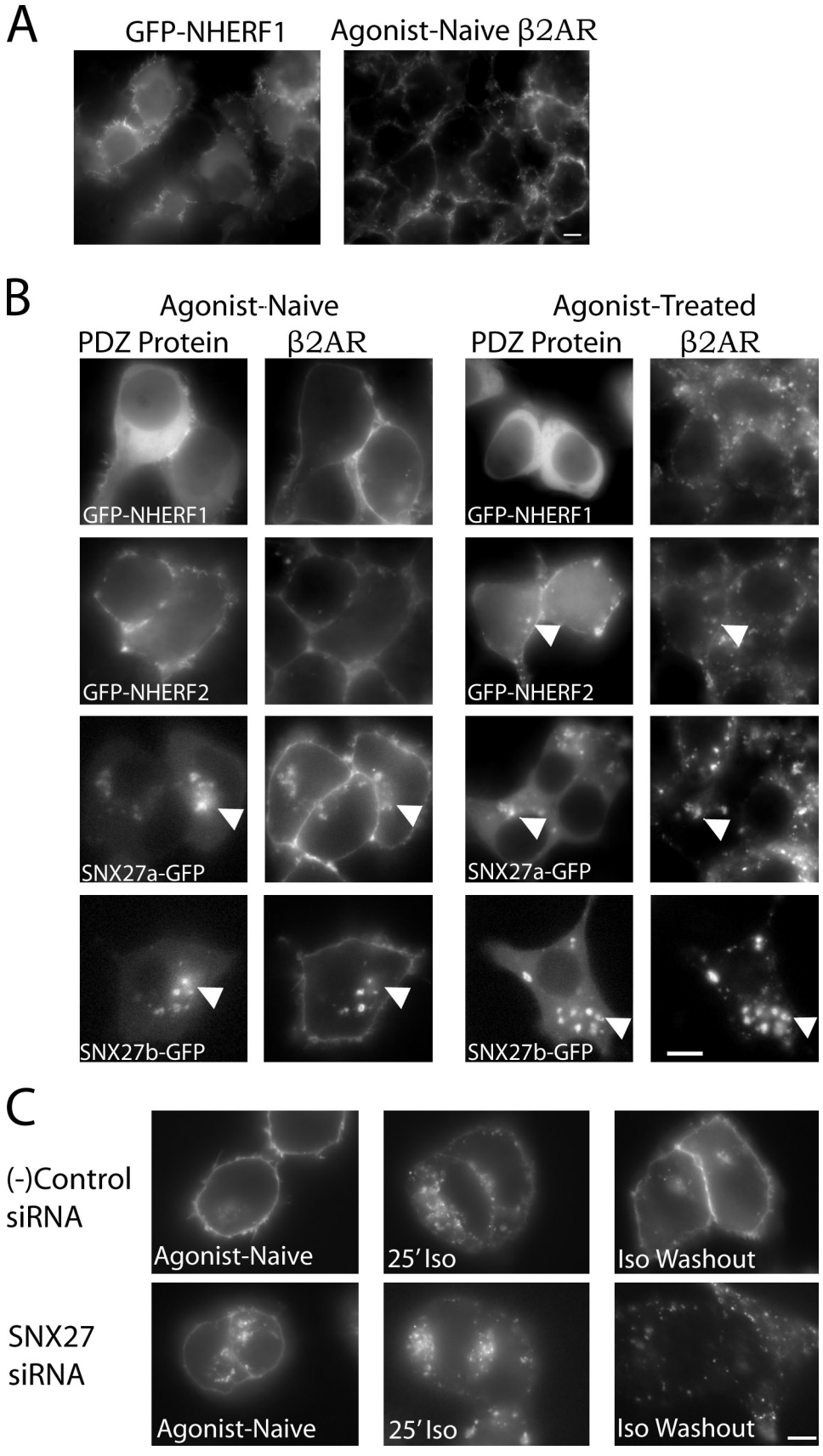
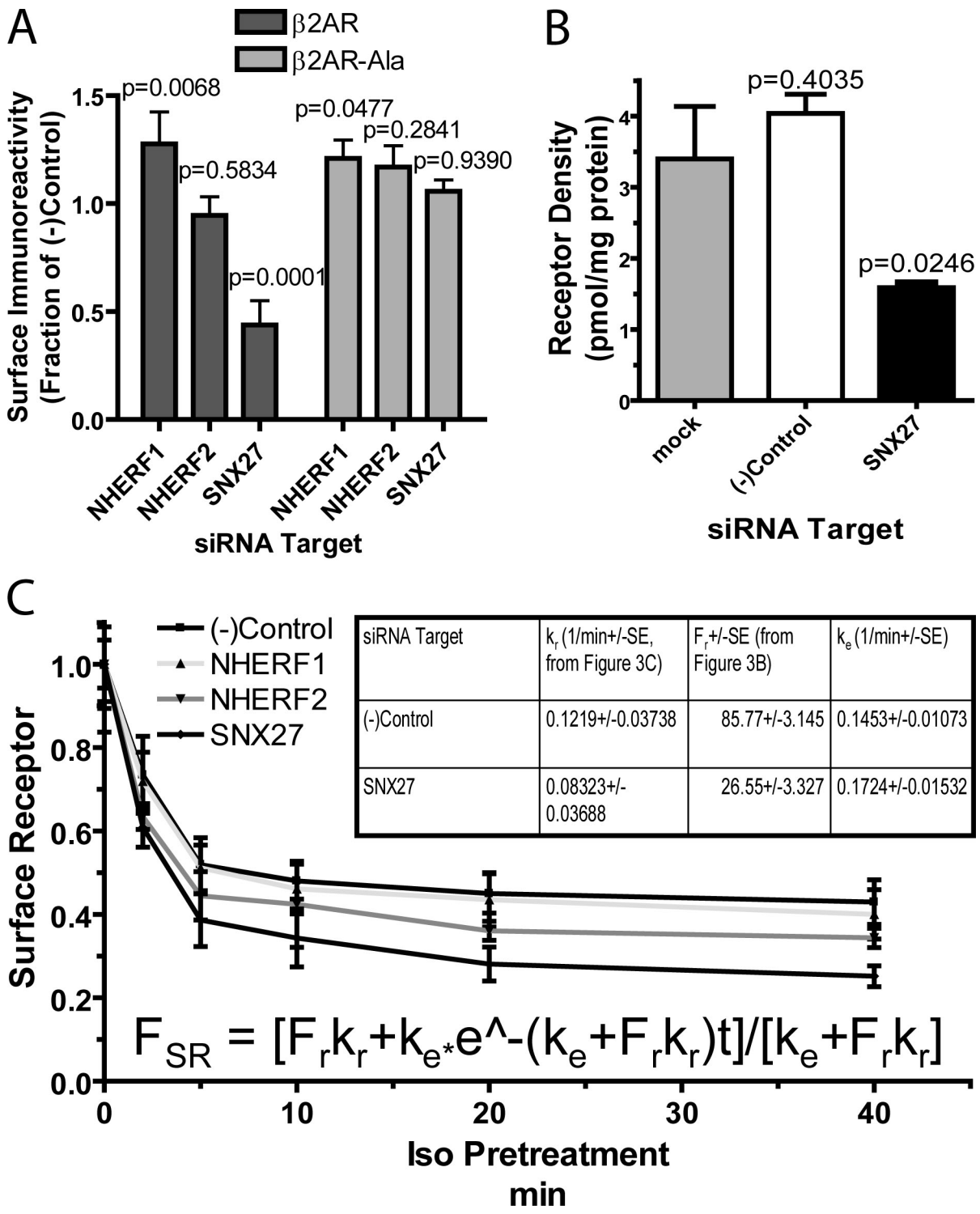


Figure S1. **Subcellular localization of FLAG-β_2AR and PDZ-interacting proteins in agonist-naive HEK293 cells, and the recycling defect of SNX27 depletion as visualized by fluorescence microscopy.** (A) Representative dual-channel epifluorescence micrographs showing the localization of GFP-NHERF1 and FLAG-β_2AR in a focal plane near the base of the cell. (B) Representative epifluorescence micrographs showing localization of the indicated GFP-tagged

PDZ proteins relative to agonist-naive (left) and agonist-internalized (right) FLAG-β_2AR observed in a focal plane near the middle of cells. Arrowheads indicate examples of receptor-containing endosomes. (C) Stably transfected HEK293 cells expressing FLAG-β_2AR were transfected with nonsilencing negative control or SNX27-silencing siRNA. FLAG-β_2AR localization in representative cells is shown, as visualized by epifluorescence microscopy using a mid-focal plane. (C, left) SNX27 depletion produced a partial but clearly visible increase in internal FLAG-β_2AR immunoreactivity in agonist-naive cells (compare top and bottom images), which is consistent with a low level of constitutive (agonist-independent) endocytosis of receptors and impaired recycling in SNX27-depleted cells. (C, middle) SNX27 depletion did not detectably impair isoproterenol-induced internalization of FLAG-β_2AR. (C, right) SNX27 depletion produced a visually obvious reduction in FLAG-β_2AR recycling after agonist washout, effectively preventing the internalized receptor pool from returning to the plasma membrane and thereby further verifying the primary recycling defect established quantitatively by flow cytometry., Bars, 10 μm.



siRNA Target	k_r (1/min+/-SE, from Figure 3C)	F_r+/-SE (from Figure 3B)	k_e (1/min+/-SE)
(-)Control	0.1219+/-0.03738	85.77+/-3.145	0.1453+/-0.01073
SNX27	0.08323+/-0.03688	26.55+/-3.327	0.1724+/-0.01532

Figure S2. **Effects of PDZ protein knockdown on steady-state receptor expression and internalization.** (A) Effect of the indicated PDZ protein knockdown on steady-state surface expression of FLAG-β_2AR (bars on the left) and FLAG-β_2AR-Ala (bars on the right) was assessed by fluorescence flow cytometry. HEK293 cells expressing the indicated receptor construct were transfected with the indicated siRNA and assessed for surface receptor immunoreactivity by anti-FLAG surface staining and flow cytometry 72 h later. Data are normalized to the surface receptor measured in the same cell clone transfected with nonsilencing negative control RNA duplex. Error bars reflect the SEM of at least four experiments, and p-values of a Student's *t* test between the surface receptor to negative control-treated cells are shown. (B) The effect of SNX27 knockdown on total cellular receptor density of FLAG-β_2AR measured by radioligand binding. FLAG-β_2AR–expressing HEK293 cells were mock-transfected (left), transfected with nonsilencing negative control siRNA (middle), or transfected with SNX27 siRNA (right), and total cellular receptor expression was estimated by B_{max} determination using the membrane-permeant radioligand dihydroalprenolol, as described in Materials and methods, 72 h later. Error bars indicate SEM (n = 6), and p-values shown are results of a Student's *t* test relative to the mock-transfected condition. (C) Flow cytometric analysis of isoproterenol-induced FLAG-β_2AR internalization in cells depleted of the indicated PDZ protein. Data were incorporated into a two-compartment model reflecting simultaneous endocytosis and fractional recycling, assuming first order kinetics for each process, as specified as an equation in the figure and further described in Materials and methods. The recycling rate constant (k_r) and maximum fraction of recycled receptor (F_r) were derived from experiments shown in Fig. 3, in which recycling was measured in the absence of receptor endocytosis. Endocytic rate constants (k_e) were calculated by iterative best-fit analysis performed using GraphPad Prism software. The inset table shows a summary of the kinetic parameters determined for each condition. Error bars indicate SEM of four experiments. This analysis indicates that SNX27 depletion primarily affected F_r.

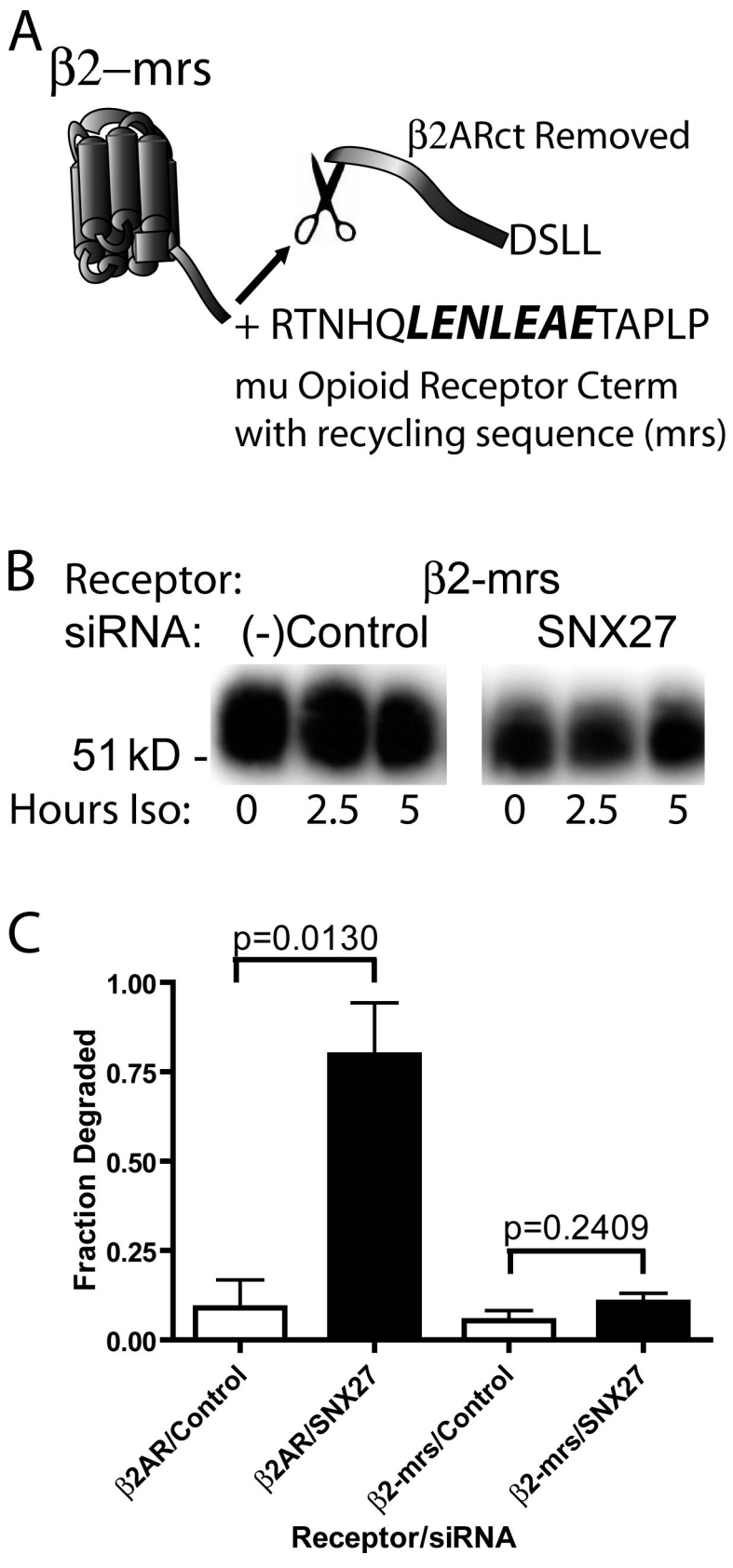


Figure S3. **Schematic and relative degradation of the chimeric, FLAG-β_2AR-mrs.** (A) Schematic showing detail of the FLAG-β2-mrs, which is a previously characterized mutant receptor construct (Tanowitz and von Zastrow, 2003) possessing a non-PDZ recycling sequence in place of the PDZ motif present in the β_2AR. (B) The effect of SNX27 depletion on turnover of surface-biotinylated FLAG-β2-mrs after incubation of cells for the indicated time period with 10 μM isoproterenol. A representative immunoblot is shown. (C) Quantification of the effect of SNX27 knockdown on anti-FLAG immunoreactivity representing biotinylated FLAG-tagged receptors, detected after exposing cells to 10 μM isoproterenol for 5 h. Error bars reflect the SEM of three experiments; p-values were calculated by a Student's *t* test. The data for SNX27 effect on FLAG-β_2AR are replotted from Fig. 3 for comparison.

Reference

Tanowitz, M., and M. von Zastrow. 2003. A novel endocytic recycling signal that distinguishes the membrane trafficking of naturally occurring opioid receptors. *J. Biol. Chem.* 278:45978–45986. doi:10.1074/jbc.M304504200