Supplemental Figure 1. (**A**) A GFP-tagged version of Rab11 (S25N) was expressed in HEK293 cells by transient transfection. 72 hours after transfection, cells were pre-incubated with Alexa594-conjugated diferric transferrin and washed as described in Materials and Methods. Cells were then fixed using 4% formaldehyde examined by epifluorescence microscopy. Representative widefield images of GFP (green) and Alexa (red) fluorescence are shown immediately after Alexa-Tf loading (0 min) and 30 minutes after incubation of cells in the absence of labeled transferrin and presence of excess unlabeled transferrin in the culture medium (30 min). (**B**) Cells were selected at random from the same microscopic fields with visible GFP fluorescence (Rab11 S25N) or not expressing detectable GFP fluorescence (NT), and residual cell-associated Alexa-Tf fluorescence was quantified by integrated intensity analysis at the 30 minute washout time point. Each integrated intensity was normalized to that measured in the same cell population immediately after Alexa-Tf loading (i.e., the '0 min' condition in panel A). Bars represent mean cell-associated Alexa-Tf fluorescence and error bars represent SEM (n = 21 cells co-expressing mutant Rab11 and 19 cells not expressing detectable mutant Rab 11).

Supplemental Figure 2. (**A**) The same population of SpH-B2AR –expressing HEK293 cells as studied in Figure 3 were pre-incubated with 10 μM isoproterenol for 10 minutes, and sequential 60 second image series were collected in the continued presence of isoproterenol either before (ISO) or after (ISO + H-89) addition of the PKA inhibitor H-89 (1 μM). Event frequency measured after H-89 addition was normalized to that measured in the preceding 1 minute interval (n = 8 cells, 297 events). (**B**) Quantification of insertion event frequency measured in multiple cells expressing SpH-B2AR or SpH-B2AR with alanine substitution of the first PKA site (corresponding to S261,262 in the wild type B2AR sequence), as indicated. Insertion events were detected by TIR-FM in n = 10 cells (381 events), and the frequency of events observed in a 60 sec interval after agonist washout (Wash) was normalized to that observed in the presence of 10 μM isoproterenol (ISO). Mutating the first PKA site did not detectably affect agonist-dependent regulation of insertion event frequency. (**C**) HEK293 cells transfected with SpH-TfR in the absence of recombinant B2AR construct were imaged by rapid TIR-FM and exocytic event frequency was scored in three 1 minute intervals from n = 19 cells (726 events): In the absence of isoproterenol (Pre), after incubation in the presence of 10 μM isoproterenol for 1 min (ISO 1), and after incubation in the presence of 10 μM isoproterenol for 5 min (ISO 5). Event frequency was normalized to that observed in the 'Pre' condition. Bars represent mean and error bars represent SEM. P values of the indicated comparison were determined using Student's t-test. (**D**) HEK293 cells co-expressing SpH-TfR and FLAG-B2AR were imaged by rapid TIR-FM and exocytic event frequency was scored in three 1 minute intervals from n = 6 cells as follows: In the absence of isoproterenol (BASAL), after incubation in the presence of 10 μM isoproterenol for 1 min (ISO), and after incubation in the presence of 10 μM isoproterenol for 10 min followed by medium change into agonist-free medium (WASH). Event frequency was normalized to that observed in the basal condition.

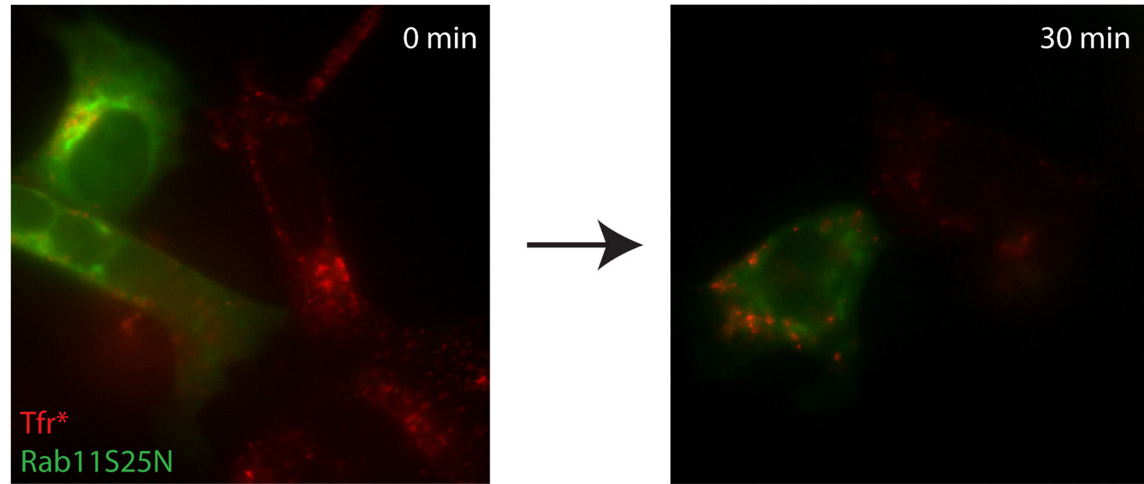
Supplemental Movie 1. TIR-FM image series collected at 10 frames / sec, showing the appearance of SpH-B2AR recycling events imaged following pre-incubation with 10 μM isoproterenol for 10 min followed by agonist washout. The plot below the fluorescence image shows the maximum intensity trace of the ROI indicated by box. The movie is presented in real time. A time stamp (in sec) is included in the bottom right corner.

Supplemental Movie 2. SpH-B2AR events measured in a cell pre-incubated for 10 minutes in the presence of 10 μM isoproterenol, showing the 1 min interval of image collection and maximum intensity trace determined from the indicated ROI. The ROI shown is deliberately chosen to include a portion of the plasma membrane that is relatively 'quiet' with respect to insertion events, in order to emphasize the increased activity produced by agonist removal (as shown in Supplemental Movie 3).

Supplemental Movie 3. The same cell as in movie #2 except that data were collected in the minute interval immediately following washout of isoproterenol. Increased event frequency is visually evident throughout the imaged plasma membrane, and is particularly clear in the indicated ROI (and corresponding maximum intensity plot). The time stamps in Supplemental Movies 2 and 3 are contiguous, but do not include the non-imaged interval (~15 sec) during which isoproterenol was washed out by medium change.

Supplemental Movie 4. A segment from a time series of SpH-TfR imaging, showing a typical exocytic event followed by lateral dispersion.

A



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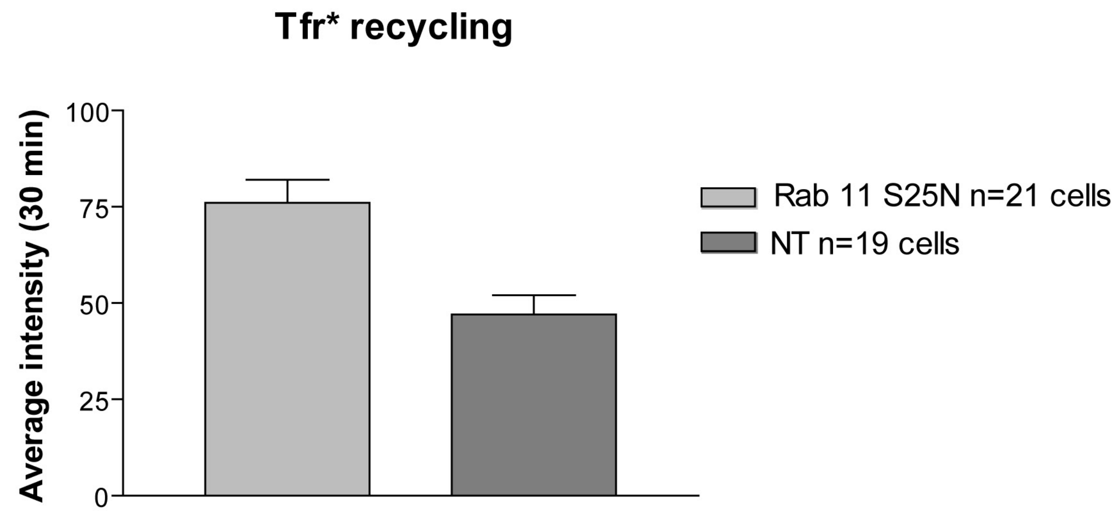

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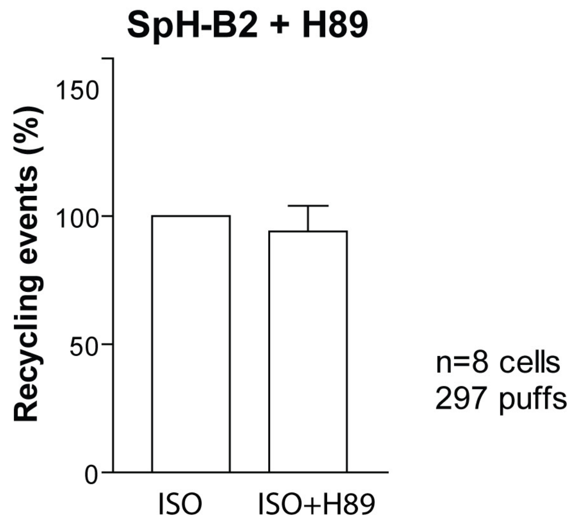

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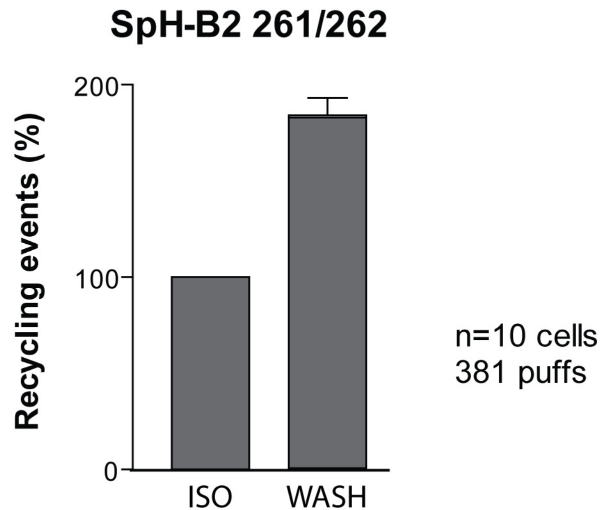

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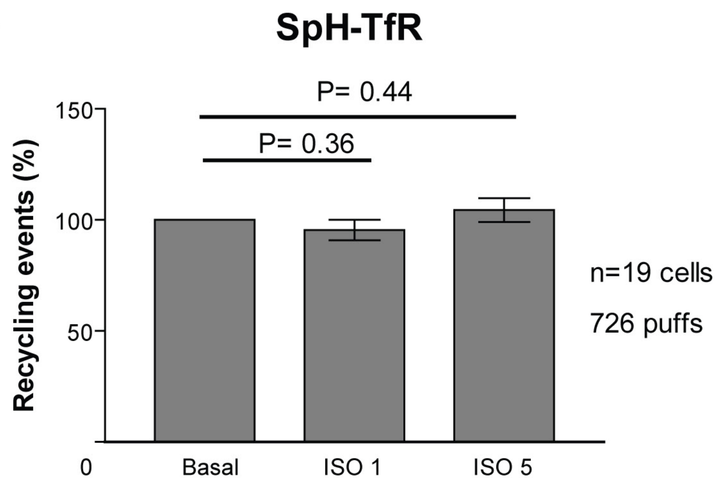

D