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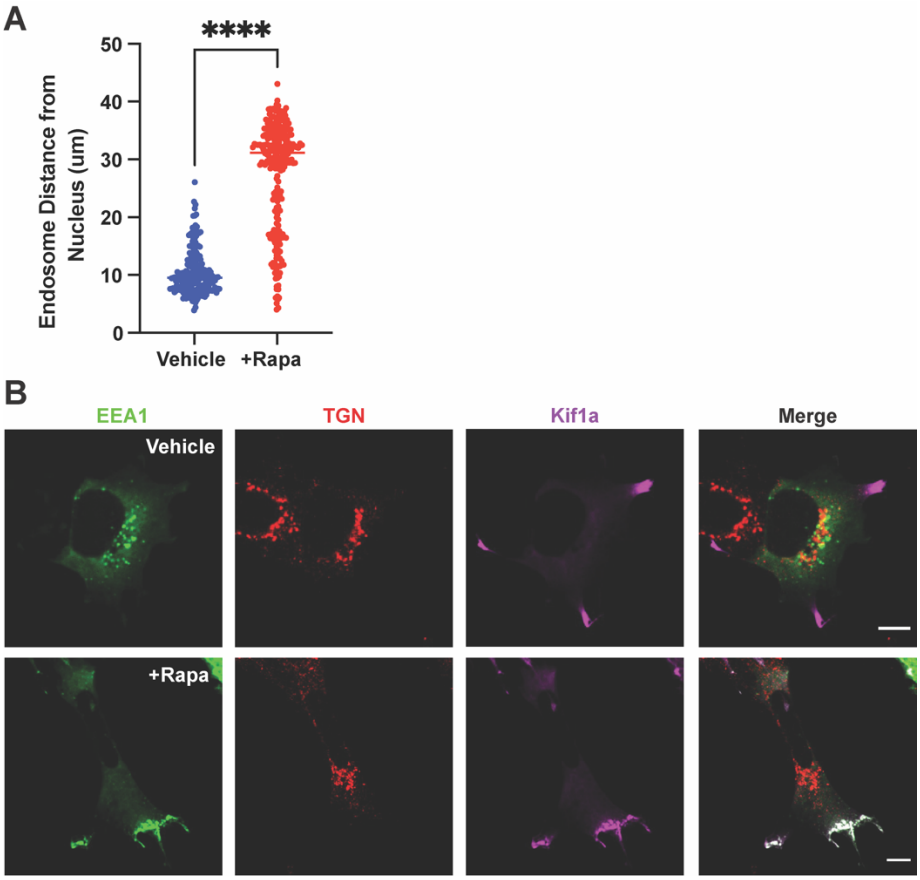
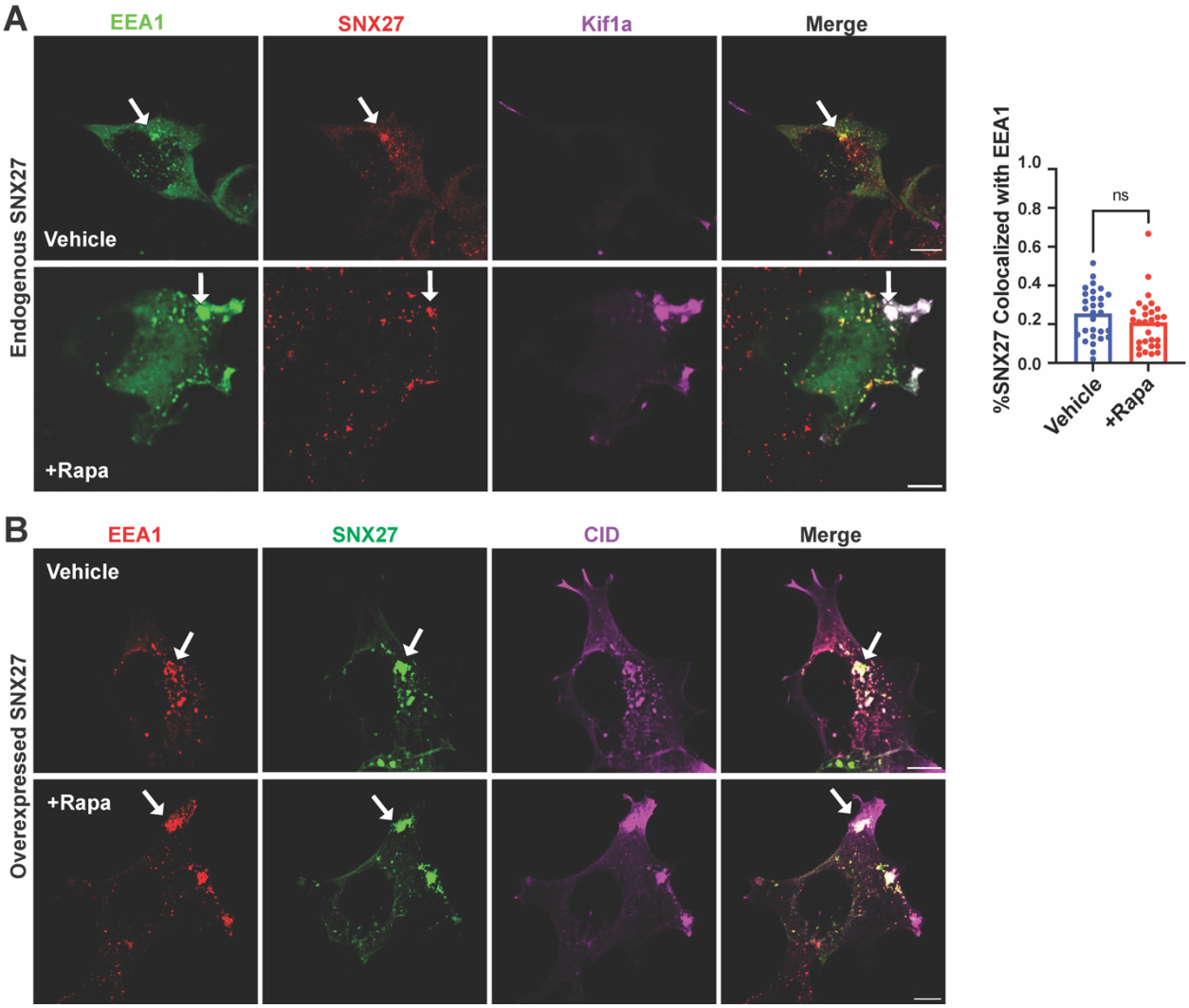
Supplementary Figures

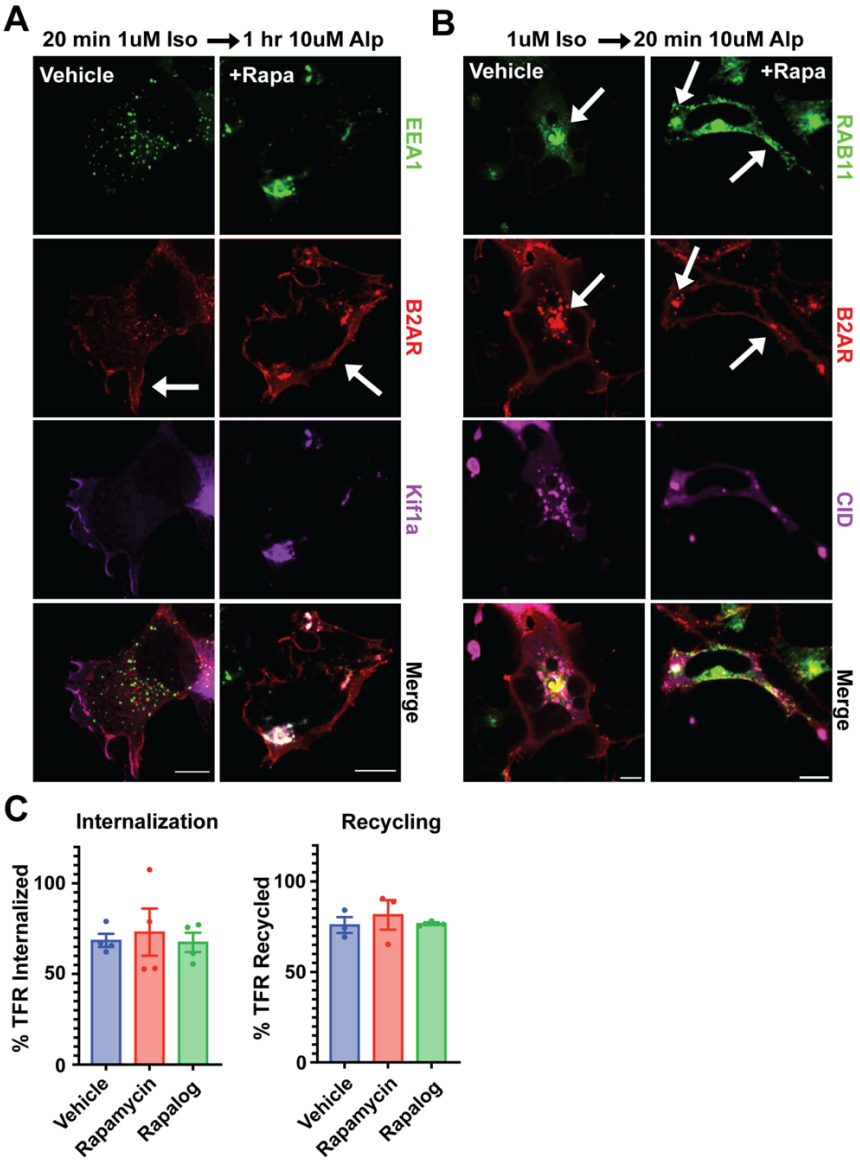
Supplementary Figures



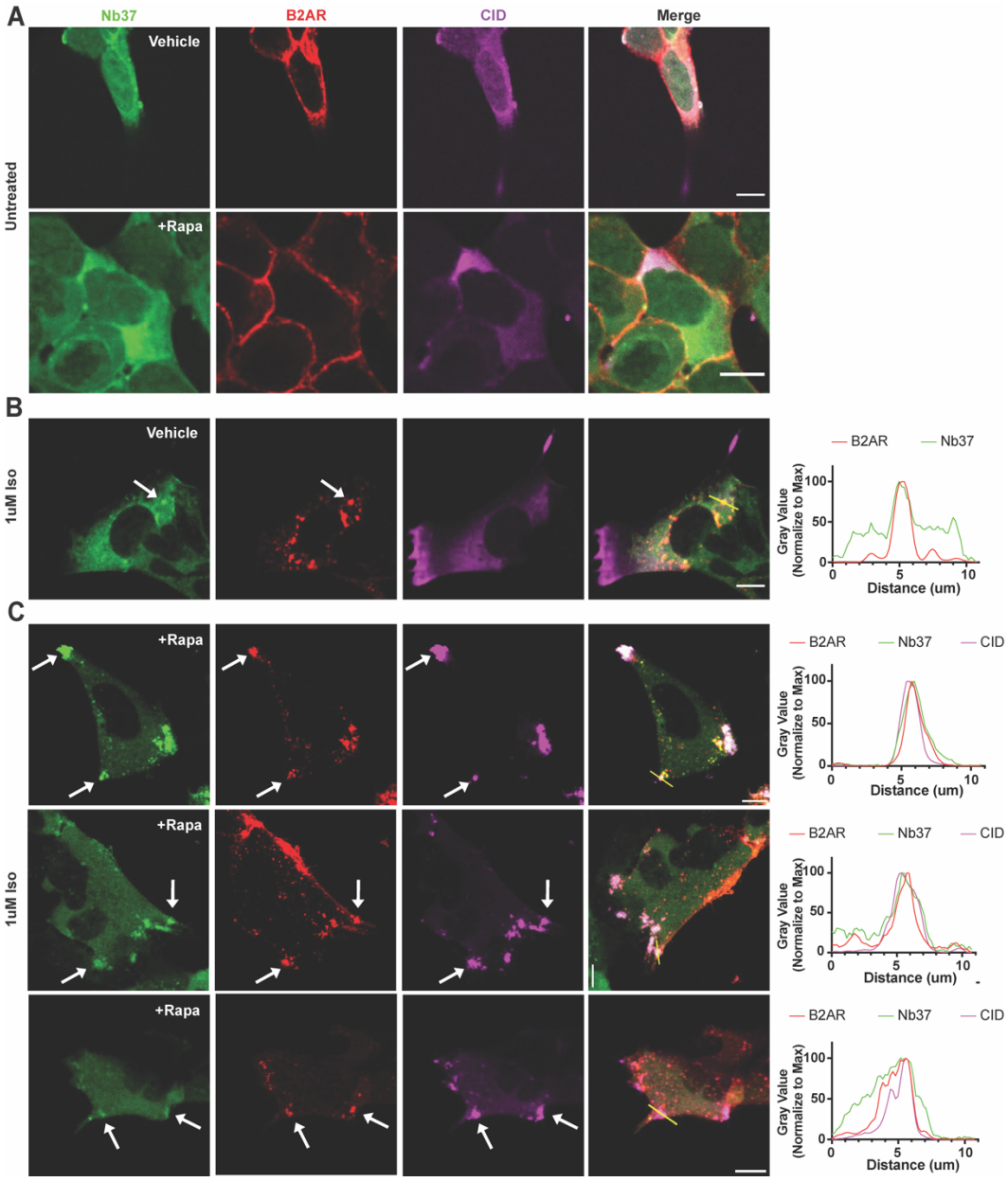
Supplementary Figure 1. The CID approach effectively relocalizes endosomes but does not impact Golgi localization. A-B. HEK293 cells expressing EGFP-FRB-EEA1 (green) and Kif1a-FKBP (magenta) were pre-treated with ethanol ("Vehicle") or 1μM rapamycin ("+Rapa") for 30 min. Cells were fixed and imaged by immunofluorescence microscopy. **A.** Quantification of the distance (μm) of individual endosomes to the center of the nucleus from five representative cells per condition from **Fig. 1C**. **** = $p < 0.0001$ by unpaired two-tailed Student's *t*-test. **B.** Representative images of EGFP-FRB-EEA1 (green), Kif1a-FKBP (magenta), and TGN marker (red) visualized by immunofluorescence microscopy in fixed cells. Scale bar = 10μm.



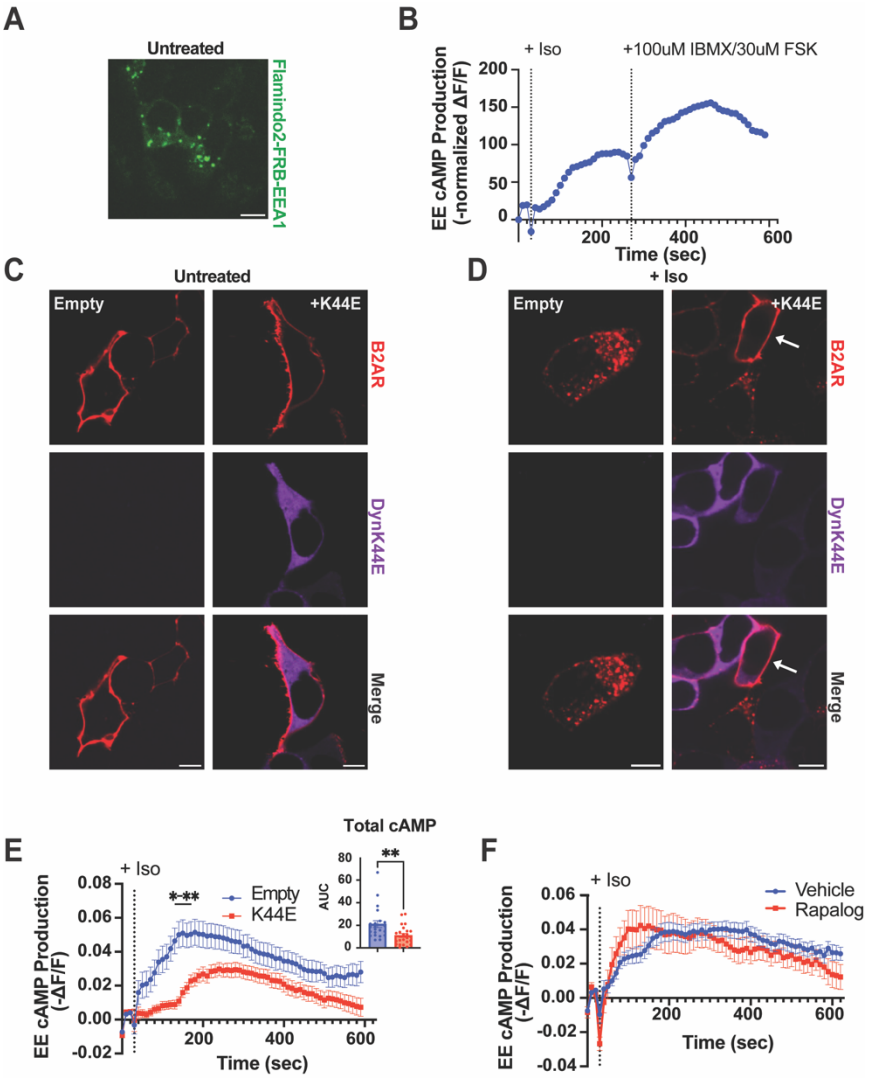
Supplementary Figure 2. The CID approach does not interfere with endosomal components of the recycling machinery. A-B. HEK293 cells were pre-treated with ethanol ("Vehicle") or 1μM rapamycin ("+Rapa") for 30 min. Cells were fixed and imaged by immunofluorescence microscopy. **A.** Endogenous SNX27 (red) co-localizes with early endosomes (green) in both vehicle- and rapamycin-treated cells. Object-based colocalization analysis was used to calculate SNX27/EEA1 colocalization as the ratio of [number of SNX27 puncta colocalized with EEA1]/[total SNX27 puncta]. Data are mean from n = 2 biologically independent replicates; 29 cells total/condition; ns by unpaired two-tailed Student's *t*-test. **B.** Overexpressed SNX27 (green) co-localizes with anti-EEA1 antibody (red) and the chemical inducible dimerization (CID) machinery (magenta). White arrows indicate representative co-localization between EEA1 and SNX27. Scale bar = 10μm.



Supplementary Figure 3. The CID approach does not impact cellular trafficking. A-B. HEK293 cells were pre-treated with ethanol ("Vehicle") or 1μM rapamycin ("+Rapa") for 30 min, fixed and imaged by immunofluorescence microscopy. **A.** Fixed-cell images of Flag-β2-AR (red) recycling after 20 min of 1μM isoproterenol-stimulated internalization into early endosomes followed by 1 h of 10μM alprenolol treatment. White arrows highlight β2-AR localization. **B.** Overexpressed Rab11 (green) with Flag-β2-AR (red) and the CID system (EEA1 and Kif1a in magenta). Cells were treated with 1μM isoproterenol followed by 20 min of 10μM alprenolol treatment and imaged live. White arrows indicate examples of colocalization between RAB11 with β2-AR. **C.** Pre-treatment for 30 min with 1μM rapamycin or 1μM of rapalog does not impede trafficking of the transferrin receptor (TFR) compared to ethanol ("Vehicle"). TFR expression was measured by flow cytometry using Alexa-488-conjugated transferrin. Single cells were gated as shown in **Supplementary Fig. 6A** (top, left). Data are mean from n = 4 ("Internalization") and n = 3 ("Recycling") biologically independent replicates ± s.e.m. Scale bar = 10μm.

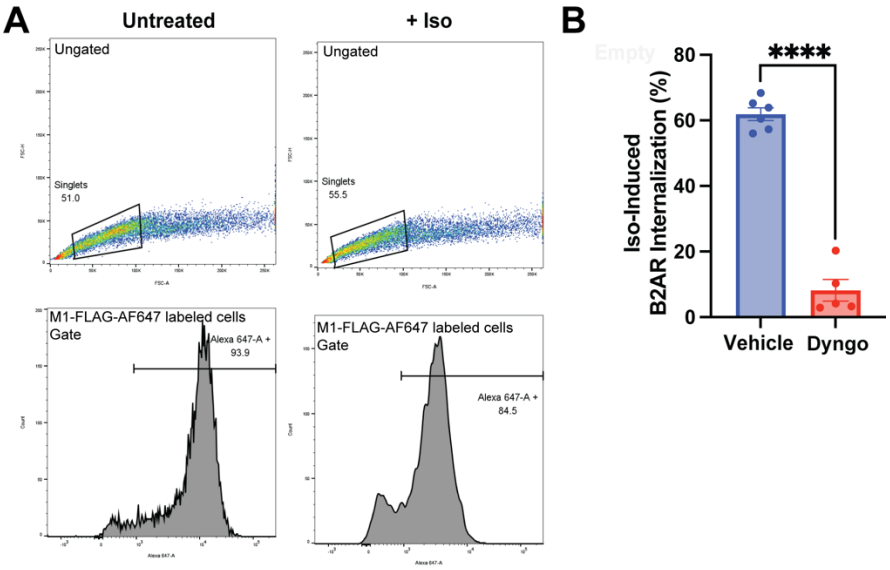


Supplementary Figure 4. Intact β2-AR signaling in repositioned endosomes. A-C. HEK293 cells were pre-treated with ethanol ("Vehicle") or 1μM rapalog ("+Rapa") for 30 min. Flag-β2-AR (red) and Nb37 (green) localization in untreated (**A**) and agonist-stimulated CID-expressing cells (**B-C**). **A.** Nb37 is cytosolic in the absence of β2-AR stimulation. **B-C.** Natively localized (**B**) and repositioned (**C**) endosomes containing Flag-β2-AR colocalize with Nb37 following 1μM isoproterenol treatment for 30 min. Yellow line: line scan analysis of gray value in each channel. Fluorescence intensities were normalized to max within each channel and plotted next to each representative image. White arrows indicate examples of colocalization between Nb37 with β2-AR. Scale bar = 10μm.

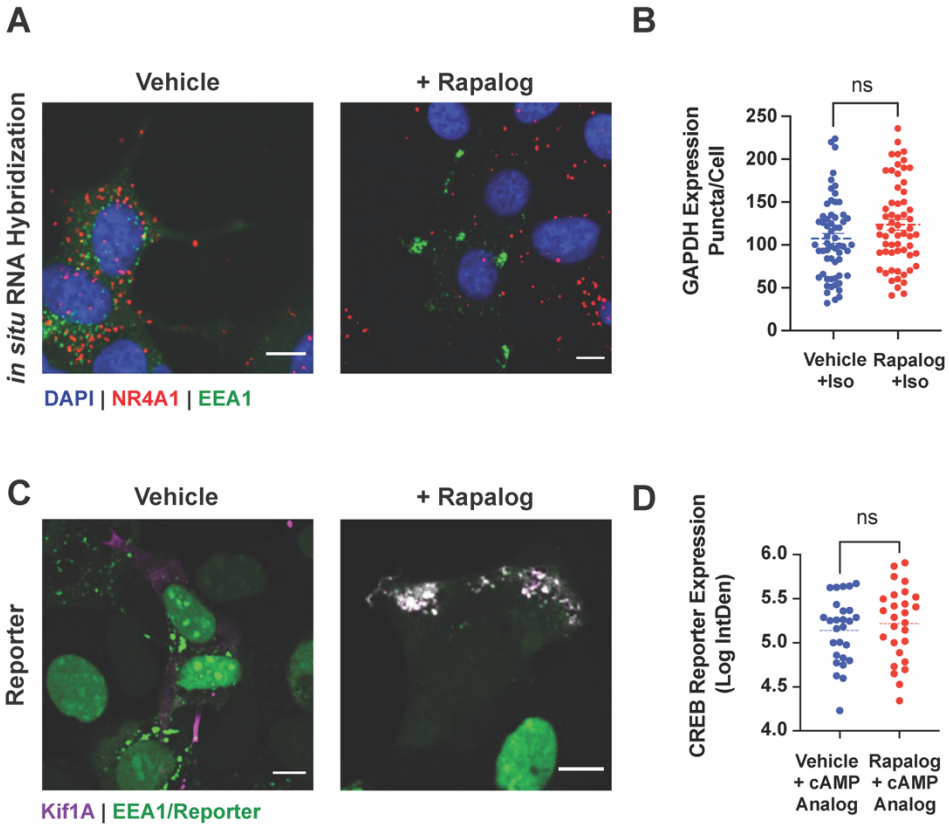


Supplementary Figure 5. Characterization of the endosomal cAMP sensor. A-B, E-F. cAMP produced on endosomes of HEK293 cells transiently expressing Flamindo2-FRB-EEA1 measured following 1μM isoproterenol treatment. The delta F/F (ΔF/F) ratio was measured every 10 sec. **A.** Representative image of Flamindo2-FRB-EEA1 (green) localization in the absence of β2-AR stimulation ("Untreated"). Scale bar = 10μm. **B.** Following 3 min of 1μM isoproterenol treatment, cells were treated with 100μM IBMX and 30μM forskolin for 7 min. The delta F/F (ΔF/F) ratio multiples to -1 is shown as percent of the maximum ratio in the isoproterenol-treated condition. Data are mean from n = 2 biologically independent replicates; 14 cells total. **C-D.** HEK293 cells expressing Flag-β2-AR (red) and either DynK44E-mCherry ("+K44E", magenta) or empty pcDNA3.0 vector ("Empty") were visualized by fluorescence microscopy. Representative images of β2-AR localization in the absence of stimulation ("Untreated" in **C**) or following 1μM isoproterenol stimulation for 20 min ("+Iso" in **D**). White arrow highlights β2-AR on the cell surface after agonist stimulation. Scale bar = 10μm. **E.** Cells were transfected with DynK44E ("K44E") or empty pcDNA3.0 vector ("Empty") and Flamindo2-FRB-EEA1 and treated with 1μM isoproterenol. Data are mean from n = 6 ("Empty") and n = 5 ("K44E") biologically independent replicates ±

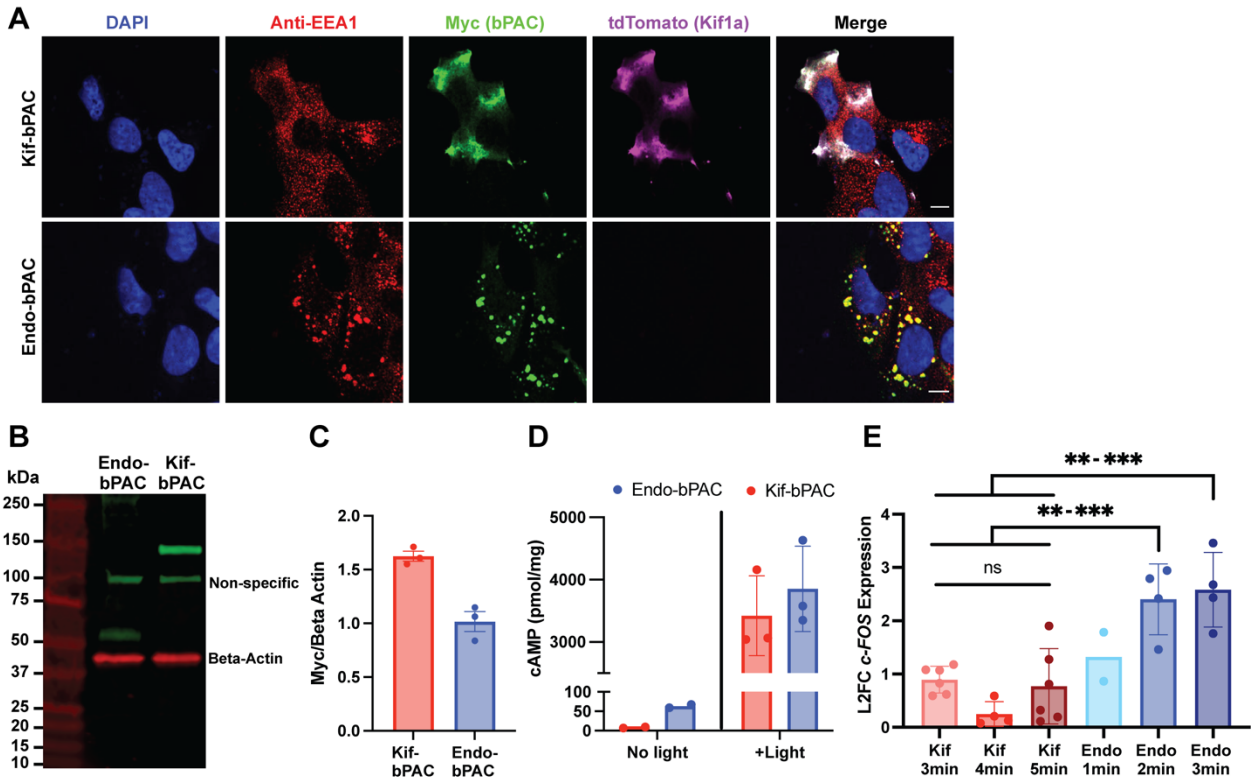
s.e.m.; 22 cells total/condition. * = $p < 0.05$, ** = $p < 0.01$ by two-way ANOVA-repeated measure test with Bonferroni multiple comparisons at each highlighted time point. Total EE cAMP production was calculated as area under the curve (AUC, top right). ** = $p = 0.0082$ by unpaired two-tailed Student's *t*-test. **F.** Cells transfected with Flamindo2-FRB-EEA1 and empty pcDNA3.0 vector in the absence of Kif1a-tdTomato-FKBP were pre-treated with ethanol ("Vehicle") or 1μM AP21967 ("Rapalog") for 30 min before isoproterenol stimulation. Data are mean from n = 3 biologically independent replicates ± s.e.m.; 10-16 cells total/condition.



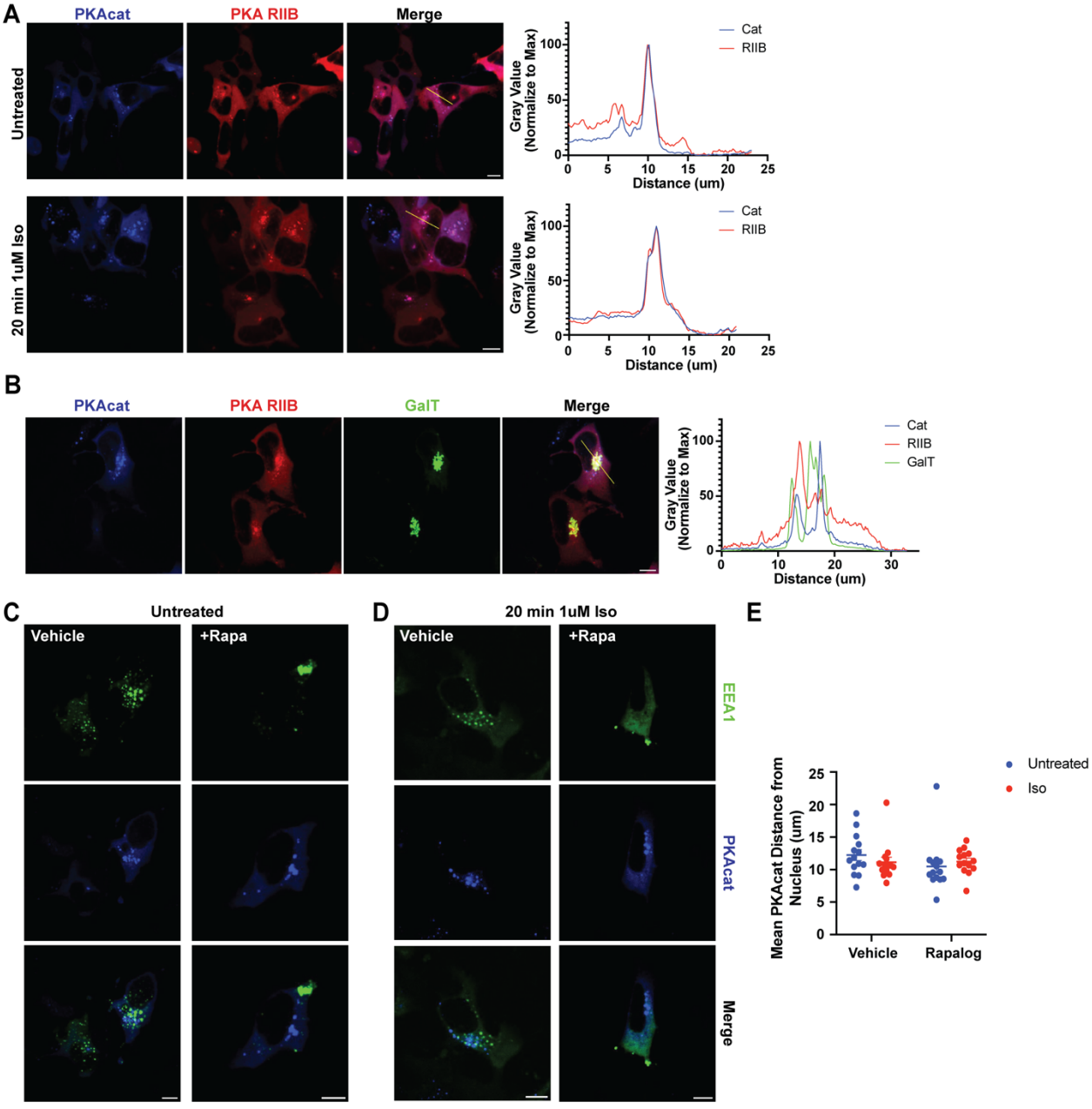
Supplementary Figure 6. Pharmacological approach to inhibit agonist-induced β2-AR internalization. A-B. HEK293 cells stably expressing Flag-β2-AR were treated with 1μM isoproterenol stimulation for 30 min and quantified by flow cytometry. **A.** Gating strategy for singlets in the 647-channel in untreated and agonist-stimulated conditions. **B.** Cells were pre-treated with DMSO ("Vehicle") or 30μM Dyngo-4a ("Dyngo") for 20 min, followed by 1μM isoproterenol stimulation for 30 min. Surface β2-AR expression was measured by Alexa 647-conjugated Flag antibody and quantified by flow cytometry. Data are mean from n = 6 ("Vehicle") and n = 5 ("Dyngo") biologically independent replicates ± s.e.m. **** = $p < 0.0001$ by unpaired two-tailed Student's *t*-test.



Supplementary Figure 7. Analysis of transcriptional signaling upon endosome redistribution. A-D. Cells expressing the CID system were pre-treated with ethanol ("Vehicle") or 1μM AP21967 ("Rapalog) for 30 min before agonist stimulation. **A.** Representative images of *NR4A1* expression (red puncta) upon β2-AR activation with 1μM isoproterenol for 2 h measured by PLISH analysis. **B.** Quantification of *GAPDH* expression by PLISH analysis in cells stimulated with 1μM isoproterenol for 2 h. Data are mean from n = 3 biologically independent replicates ± s.e.m.; 60 cells total/condition; ns by unpaired two-tailed Student's *t*-test. **C.** Representative images of CREB nuclear reporter expression (green) upon β2-AR activation with 1μM isoproterenol/1μM Shield for 4 h visualized by fixed-cell immunofluorescence microscopy. **D.** Quantified CREB reporter expression following application of 150μM 8-CPT-cAMP/1μM Shield for 4 h. Data are mean from n = 2 biologically independent replicates; 27 cells total/condition; ns by unpaired two-tailed Student's *t*-test. Scale bar = 10μm.

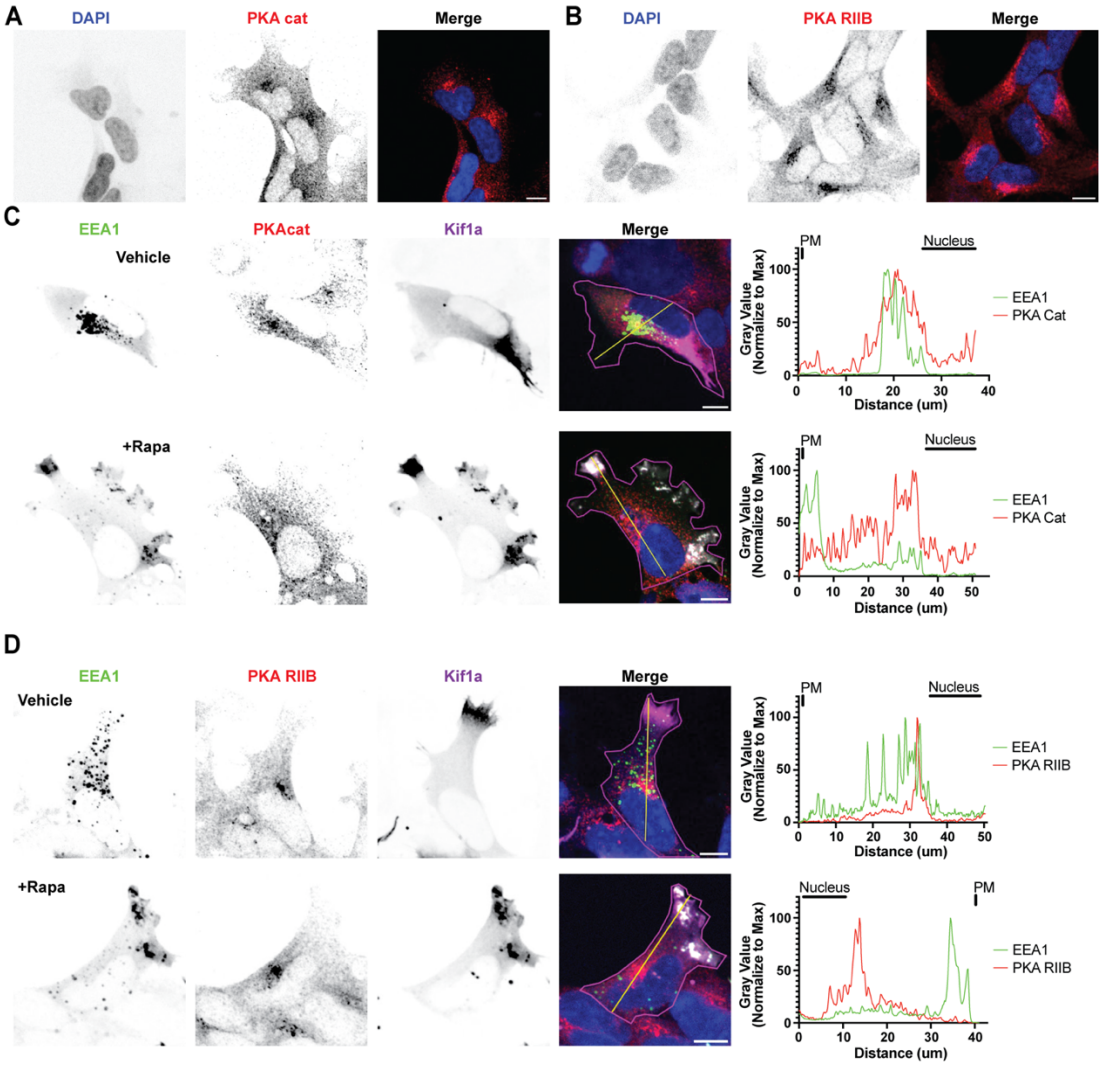


Supplementary Figure 8. Analysis of bPAC localization, expression and signaling. A. Representative images of bPACs (green) localized to the kinesin motor ("Kif-bPAC", top) or early endosome ("Endo-bPAC", bottom) visualized by immunofluorescence microscopy using Alexa 647-conjugated anti-myc antibody. Relevant compartment markers (EEA1 in red, Kif1a in magenta) are shown. **B.** Representative Western blot analysis of bPAC expression (anti-myc, green) relative to beta actin control (red). A non-specific band is detected by this antibody at 100kDa (indicated). The uncropped Western blot image is provided on p. 18 of the Supplementary Materials. **C.** Western blot quantification. bPAC expression was normalized to beta actin levels. Data are mean from n = 3 biologically independent replicates ± s.e.m. **D.** cAMP levels from Kif-bPAC and Endo-bPAC after photostimulation for 3 min of light were measured using a colorimetric immunoassay. Data were normalized to total protein concentrations. Data are mean from n = 2 ("No Light") and n = 3 ("+Light") biologically independent replicates ± s.e.m. **E.** *c-FOS* induction measured by RT-qPCR analysis after bPAC photostimulation with indicated light doses. Data are mean from n = 6 ("Kif, 3 min"), n = 4 ("Kif, 4 min"), n = 6 (("Kif, 5 min"), n = 2 ("Endo, 1 min"), n = 4 ("Endo, 2 min"), and n = 4 ("Endo, 3 min") biologically independent replicates ± s.e.m. *** = $p < 0.001$, ** = $p < 0.01$ by one-way ANOVA test with Tukey. Scale bar = 10μm.

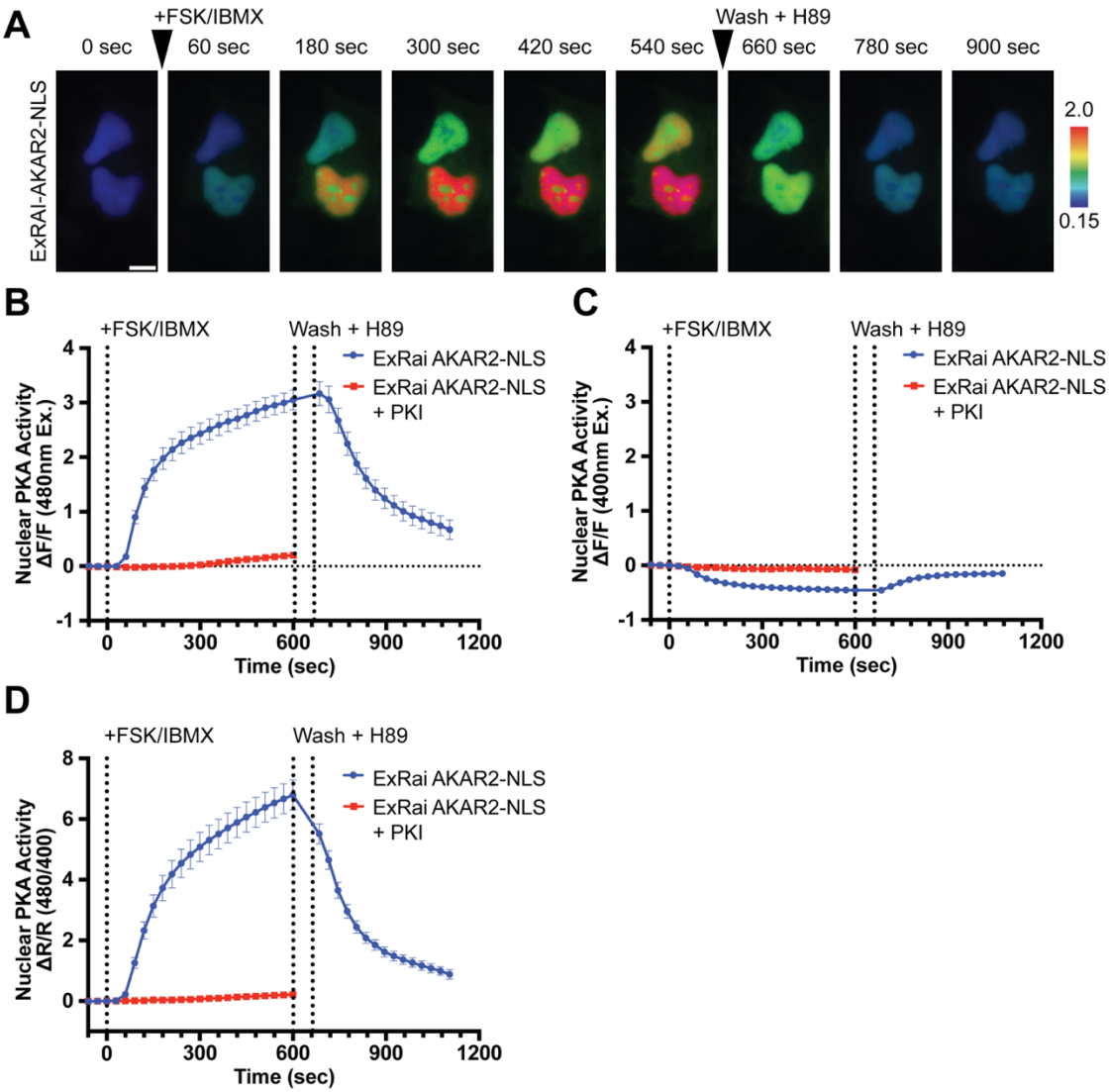


Supplementary Figure 9. Characterization of overexpressed PKA localization. A-B. Left: Cells expressing mTagBFP2-PKA$_{cat\alpha}$ (blue) and PKA-$_{RIIB}$-mCherry (red) were imaged in the absence of β2-AR stimulation ("Untreated", top) and following 1μM isoproterenol ("Iso") treatment for 20 min (bottom). Right: Line scan analysis of colocalization between PKA$_{cat\alpha}$ and PKA$_{RIIB}$. **B.** Left: Cells expressing mTagBFP2-PKA$_{cat\alpha}$ (blue), PKA-$_{RIIB}$-mCherry (red), and GalT-eGFP (green) were imaged. Right: Line scan analysis of colocalization between PKA$_{cat\alpha}$, PKA$_{RIIB}$, and GalT is shown. Yellow line: line scan analysis of gray value in each channel. Fluorescence intensities were normalized to max within each channel and plotted next to each representative image. **C-E.** Cells expressing mTagBFP2-PKA$_{cat\alpha}$ (blue), PKA-$_{RIIB}$-mCherry (red), Kif1a-tdTomato-FKBP and EGFP-FRB-EEA1 (green) were pre-treated with ethanol ("Vehicle") or 1μM AP21967 ("+Rapa")

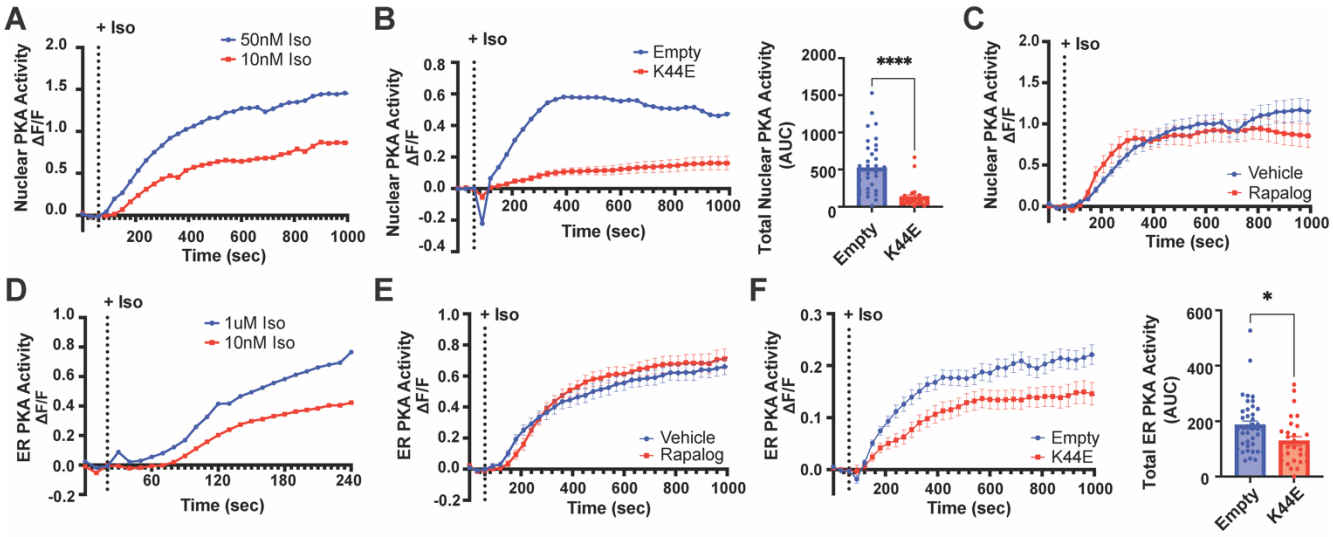
for 30 min before agonist stimulation, as indicated. Because multiple fluorescently tagged constructs had to be co-expressed and with limited availability of fluorescent colors for tagging, PKA_{RIIB} and Kif1a-FKBP were both tagged with a red fluorescent protein, and the red fluorescent channel is not shown. **C-D.** Representative images of endosome and PKA_{cat} localization in the absence of β2-AR stimulation ("Untreated", (**C**) and following 1μM isoproterenol ("Iso") treatment for 20 min (**D**). **E.** Quantification of the distance of $PKA_{cat\alpha}$ from the nucleus in response to ethanol ("Vehicle", left) or 1μM AP21967 ("Rapalog", right) in untreated (blue) or isoproterenol-treated ("Iso", red) cells. Mean $PKA_{cat\alpha}$ distance per cell = 12.23μm ± 0.83 (Vehicle, Untreated), 11.14μm ± 0.77 (Vehicle, Isoproterenol), 10.48μm ± 1.05 (Rapalog, Untreated), 11.24μm ± 0.51 (Rapalog, Isoproterenol). Data are mean from n = 2 biologically independent replicates; 14 cells total/condition. Scale bar = 10μm.



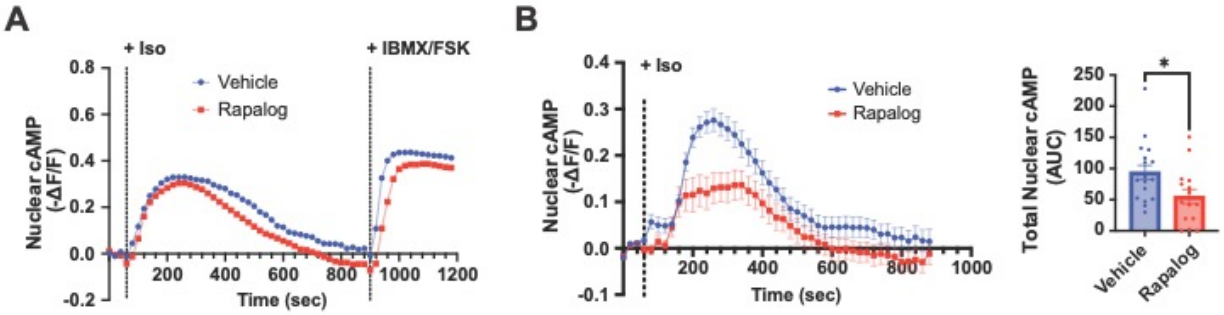
Supplementary Figure 10. Endogenous PKA localization with CID system. A-B. Representative images of fixed HEK293 cells stained with antibodies for endogenous $PKA_{cat\alpha}$ (red, **A**) and PKA_{RIIB} (red, **B**) localization compared to the DAPI-stained nucleus (blue). **C-D.** Cells expressing EGFP-FRB-EEA1 and Kif1a-tdTomato-FKBP (purple) were treated with ethanol ("Vehicle") or 1μM AP21967 ("+Rapa") for 30 min, fixed and stained for endogenous $PKA_{cat\alpha}$ (red, **C**) and PKA_{RIIB} (red, **D**). Line scan analysis of colocalization between EEA1 and either $PKA_{cat\alpha}$ (**C**) or PKA_{RIIB} (**D**) in the absence (top) or presence (bottom) of endosome redistribution. Yellow line: line scan analysis of gray value in each channel. Fluorescence intensities were normalized to max within each channel and plotted next to each representative image. Magenta line: cell outline determined based on the kinesin fluorescent channel. Individual channels were inverted with a grayscale to aid visualization. Scale bar = 10μm.



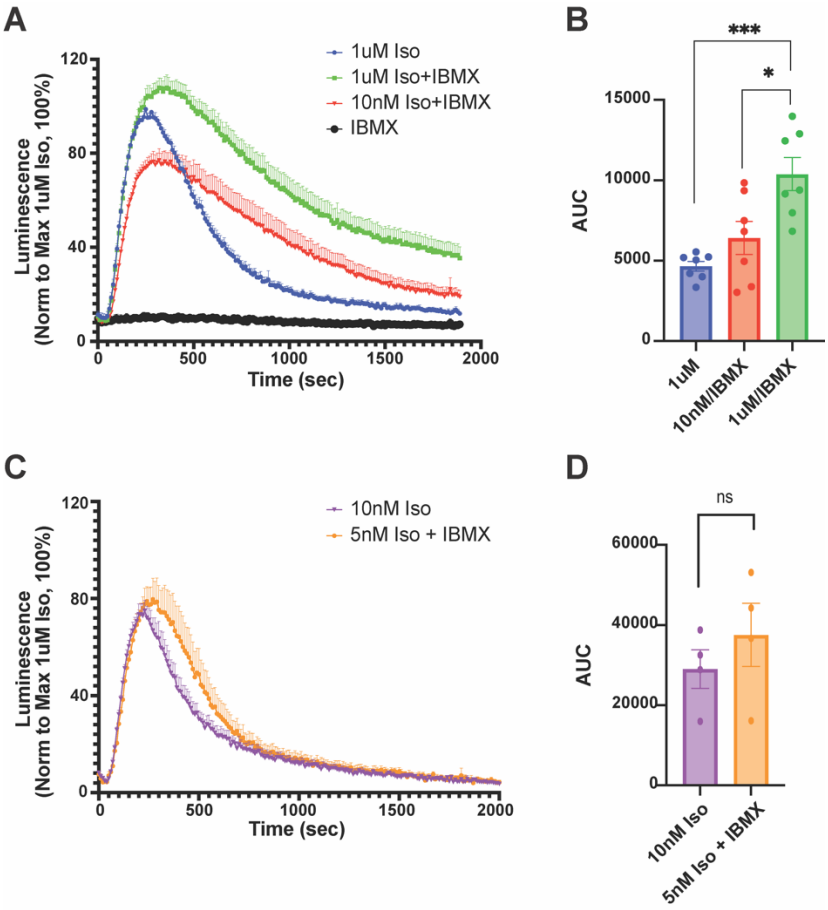
Supplementary Figure 11. Characterization of ExRai-AKAR2-NLS. A-D. HeLa cells expressing ExRai-AKAR2-NLS were treated with 50μM Forskolin and 100μM IBMX ("+FSK/IBMX) for 10 min then washed out and treated with 20μM H89 ("Wash + H89"). **A.** Representative pseudocolor images of the 480nm/400nm emission ratio showing ExRai-AKAR2-NLS activity following treatment. Arrows indicate drug treatment timepoint. Scale bar = 10μm. **B-D.** Average time course of cells expressing ExRai-AKAR2-NLS in the absence (blue) or presence of co-expressed PKA inhibitory construct, PKI-NLS-mCherry ("ExRai-AKAR2-NLS + PKI", red). Every 30 sec, the delta F/F (ΔF/F) ratio following 480nm excitation (**B**), delta F/F (ΔF/F) ratio following 400nm excitation (**C**), and the ratio of 480/400 responses ("ΔR/R", **D**) were measured. Data in panels **B-D** are mean from n = 4 (no inhibitor) and n = 3 (+PKI) biologically independent replicates ± s.e.m; 15-17 cells total/condition.



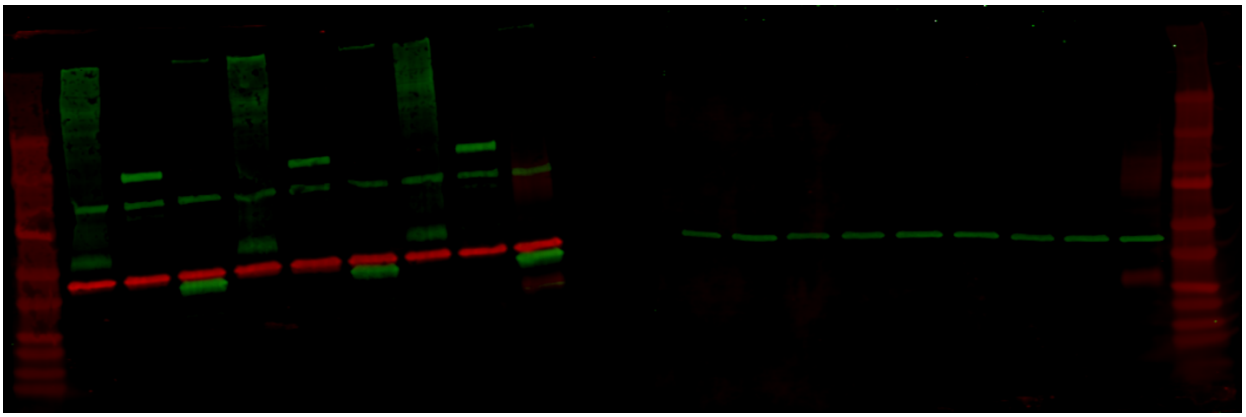
Supplementary Figure 12. Analysis of subcellular ExRai-AKAR2 activity. A-C. HEK293 cells transiently expressing ExRai-AKAR2-NLS following isoproterenol treatment by live-cell fluorescence microscopy. The delta F/F (ΔF/F) ratio was measured every 30 sec. **A.** Nuclear PKA activity following a dose-response treatment with isoproterenol ("Iso"). Data are mean from n = 2 biologically independent replicates; 19-20 cells total/condition. **B.** Cells were transfected with DynK44E ("K44E") or empty pcDNA3.0 vector ("Empty") and treated with 10nM isoproterenol. Total nuclear PKA activity was calculated as area under the curve (AUC, right). Data are mean from n = 2 ("Empty") and n = 3 ("K44E") biologically independent replicates ± s.e.m.; 26-36 cells total/condition. **** = $p < 0.0001$ by unpaired two-tailed Student's *t*-test. **C.** Cells were pre-treated with ethanol ("Vehicle") or 1μM AP21967 ("Rapalog") for 30 min followed by 10nM isoproterenol stimulation. These experiments were carried out in the absence of CID transfection. Data are mean from n = 3 biologically independent replicates ± s.e.m.; 16-29 cells total/condition. **D-F.** HEK293 cells transiently expressing erExRai-AKAR2 following isoproterenol treatment were imaged by live-cell fluorescence microscopy. The delta F/F (ΔF/F) ratio was measured every 30 sec. **D.** ER-proximal PKA activity following a dose-response treatment with isoproterenol ("Iso"). Data are mean from n = 2 biologically independent replicates; 16-20 cells total/condition. **E.** Cells were pre-treated with ethanol ("Vehicle") or 1μM AP21967 ("Rapalog") for 30 min before 10nM isoproterenol stimulation. These experiments were carried out in the absence of CID transfection. Data are mean from n = 4 ("Vehicle") and n = 3 ("Rapalog") biologically independent replicates ± s.e.m.; 27-31 cells total/condition. **F.** Cells were transfected with DynK44E ("K44E") or empty pcDNA3.0 vector ("Empty") and treated with 10nM isoproterenol. Total ER-proximal PKA activity was calculated as area under the curve (AUC, right). Data are mean from n = 3 biologically independent replicates ± s.e.m.; 28-41 cells total/condition. * = $p = 0.0168$ by unpaired two-tailed Student's *t*-test.



Supplementary Figure 13. Analysis of nuclear cAMP accumulation. A-B. HEK293 cells transiently expressing nlsFlamindo2 were pre-treated with ethanol ("Vehicle") or 1μM AP21967 ("Rapalog") for 30 min followed by 10nM isoproterenol stimulation ("Iso"). nlsFlamindo2 fluorescence was analyzed by live-cell microscopy. The delta F/F (ΔF/F) ratio was measured every 20 sec. **A.** Nuclear cAMP accumulation following treatment with isoproterenol for 15 min, and treatment with 100μM IBMX/10μM forskolin for 5 min. These experiments were carried out in the absence of CID transfection. Data are mean from n = 2 biologically independent replicates; 29-30 cells total/condition. **B.** Nuclear cAMP accumulation in cells co-transfected with nlsFlamindo2 and CID constructs. Total nuclear cAMP was calculated as area under the curve (AUC, right). Data are mean from n = 3 biologically independent replicates ± s.e.m.; 15-18 cells total/condition. * = p = 0.023 by unpaired two-tailed Student's t-test.



Supplementary Figure 14. cAMP production following isoproterenol stimulation with or without PDE inhibition. A-B. HEK293 cells stably expressing the CREB reporter (pCRE-NLS-DD-zsGreen1) and a luminescence-based cAMP sensor were treated with 1μM or 10nM isoproterenol with or without 100μM IBMX. Data are mean from n=7 biologically independent replicates ± s.e.m. Kinetics and magnitude of cAMP production were recorded every 10 sec following agonist addition. **B.** Quantification of total cAMP as area under the curve (AUC) for stimulated cells. **C-D.** HEK293 cells transiently expressing a luminescence-based cAMP sensor were treated with 10nM isoproterenol or 5nM isoproterenol and 100μM IBMX. Data are mean from n=4 biologically independent replicates ± s.e.m. **C.** Kinetics and magnitude of cAMP production were recorded every 10 sec following agonist addition. **D.** Quantification of total cAMP as area under the curve (AUC) for stimulated cells. *** = p = 0.0005, * = p = 0.0112 by one-way ANOVA test with Tukey.



Uncropped Western blot for Supplementary Figure 8C. Lanes 1-3 are shown in panel **C**.