

SUPPLEMENTARY INFORMATION for**Retromer terminates the generation of cAMP by internalized
parathyroid hormone receptors**

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Supp. Methods p. 2

Supp. Results p. 6

Supplementary Methods

Cell culture

Human embryonic kidney (HEK-293) and osteoblastic ROS 17/2.1 cell lines, were cultured at 37°C in a humidified atmosphere containing 5% CO₂. For transient expression, cells were transfected with the appropriate cDNAs using Effectene (QiagenTM) or FuGENE (Roche) according to the manufacturer's instructions. HA-PTHR was stably expressed from HEK-293 cells maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf and 400 mg/ml G418 (Merck).

RNAi and plasmid transfections

We depleted β -arrestins 1 and 2 from HEK293 cells using a 21-nt sequence that is common to both mRNAs, 5'-GCGCCTTCCGCTATG-3'¹. Vps35 was depleted using a custom-designed pool of four oligonucleotide duplexes (siGENOME SmartPool, Thermo Scientific). Control transfections were done with a scrambled siRNA (OriGene). Briefly, 24 h after plating cells were transferred to serum- and antibiotic-free medium and transfected with X-TremeGene reagent (Roche, Basel, Switzerland) according to manufacturer's directions. Medium was supplemented with serum and antibiotics 24 h after transfection, and experiments were performed between 72 h and 96 h after transfection. Plasmid transfections were done using FuGene (Roche) according to manufacturer's directions.

Quantitative Real-Time PCR

Total RNA for qRT-PCR was extracted from cultured HEK293 cells and human kidney at 24–64 years of age using Trizol reagents (Invitrogen) according to the manufacturer's protocol. For qRT-PCR, cDNA was prepared with random primer using the SuperScript First-Strand Synthesis System (Invitrogen) and analyzed with the SYBR GreenMaster Mix (Qiagen) in the thermal cycler with two sets of primers specific for each targeted gene. Pre-validated primers for human Vps35, β -arrestin1 and β -arrestin2 were purchased from OriGene (cat# HK207887, HP207416 and HP207646, respectively).

Primers for GAPDH were described previously². Relative expression was calculated for each gene by the 2-XXCT method with GAPDH for normalization.

Intracellular calcium ion measurement

To measure changes in intracellular calcium $[Ca^{2+}]_i$, we loaded HEK293 cells with the live cell calcium sensor Fluo4³ according to manufacturer's directions (Molecular Probes, Carlsbad, CA). Cells were imaged at 2 s intervals using an inverted epifluorescence microscope as described above. In order to determine whether PTH-dependent calcium signaling was a result of Gs-coupled cAMP signaling, or else a consequence of PTHR coupling with a separate G protein such as G_i or G_q , we measured intracellular calcium ion concentrations in the absence of extracellular calcium and in the presence of 25 μ M MDL12,330A (Enzo Life Sciences, Farmingdale, NY), a fast and effective inhibitor of adenylyl cyclase. In both cases a robust calcium response was observed.

Western blot and immunoprecipitation

Western blot analyses were performed according to standard procedures. Briefly, HEK293 cells were transfected and harvested 48 h after transfection. The total protein extract was run on an SDS-PAGE and blotted onto 0.45 mm PVDF membranes (Bio-Rad, Hercules, CA). Immuno-detection of HA-tagged PTHR and retromer subunit Vps26^{YFP} was carried out using monoclonal anti-HA (HA.11, Covance) and a polyclonal anti-GFP (A-11122, Invitrogen), respectively. Horseradish peroxidase-conjugated donkey anti-mouse and donkey anti-rabbit antibodies (Jackson Immuno Research) were used for the detection of primary antibody. Antibody binding was visualized with Immobilon ECL reagent (Millipore). Immunoprecipitations were performed as described previously⁴. Briefly, HEK293 cells co-transfected with HA-PTHr and either Vps29^{YFP} or an empty vector control challenged with 100 nM PTH(1-34) as indicated. At designated time points after a brief ligand challenge, medium was washed once with PBS and cells were transferred to ice-cold immunoprecipitation buffer (50 mM Tris at pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 and a protease inhibitor mix) and incubated for 20 min. Cells were lysed by scraping and a post-nuclear supernatant was prepared by brief centrifugation (5 min at 5,000 x g). A 10 μ l aliquot was collected and frozen in Laemmli

sample buffer (Bio-Rad; “2% loaded”) and then lysates were incubated overnight at 4 °C with 20 µl of anti-HA beads (Covance). Beads were washed 4-time in TBS. Dried beads were boiled for 5 min in 40 µl Laemmli sample buffer and then loaded onto a 12% SDS-PAGE gel for analysis.

MAP kinase assays

Osteoblastic-like ROS 17/2.8 cells were transfected with cDNA encoding β -arrestin1[IV-AA] using FuGENE® 6 transfection reagent (Roche). After 48 hours, cells seeded onto 12-well plates were starved for 5 hours in serum-free medium before the stimulation. Then cells were stimulated for various time with 100 nM PTH (1–34). After stimulation, cells were washed with ice-cold phosphate buffered saline and lysed for 20 min on ice in a buffer containing 50 mM Tris, 150 mM NaCl, 5 mM EDTA 0.5% Nonidet P40, supplemented with protease inhibitor mixture I. Equal volumes of cell extracts were separated on 10 % Tris-glycine polyacrylamide gels and transferred to nitrocellulose membranes for Western Blot analysis. Phosphorylated ERK1/2 were detected by incubating membranes overnight with anti-phospho p44/42 MAPK antibodies (1:1000) (Cell signaling). In order to monitor loading differences, we used anti-ERK antibodies (1:3000) (Cell signaling) as reference. The secondary antibodies were HRP-coupled and visualized using Luminol with ECL™ Western Blotting Detection Reagents (GE Healthcare). The densitometry of signals was analyzed by the program Image Quant®. Intensities of ERK1/2 phosphorylation were normalized to total ERK1/2 expression. Statistics were evaluated by ANOVA analysis and Bonferroni post test.

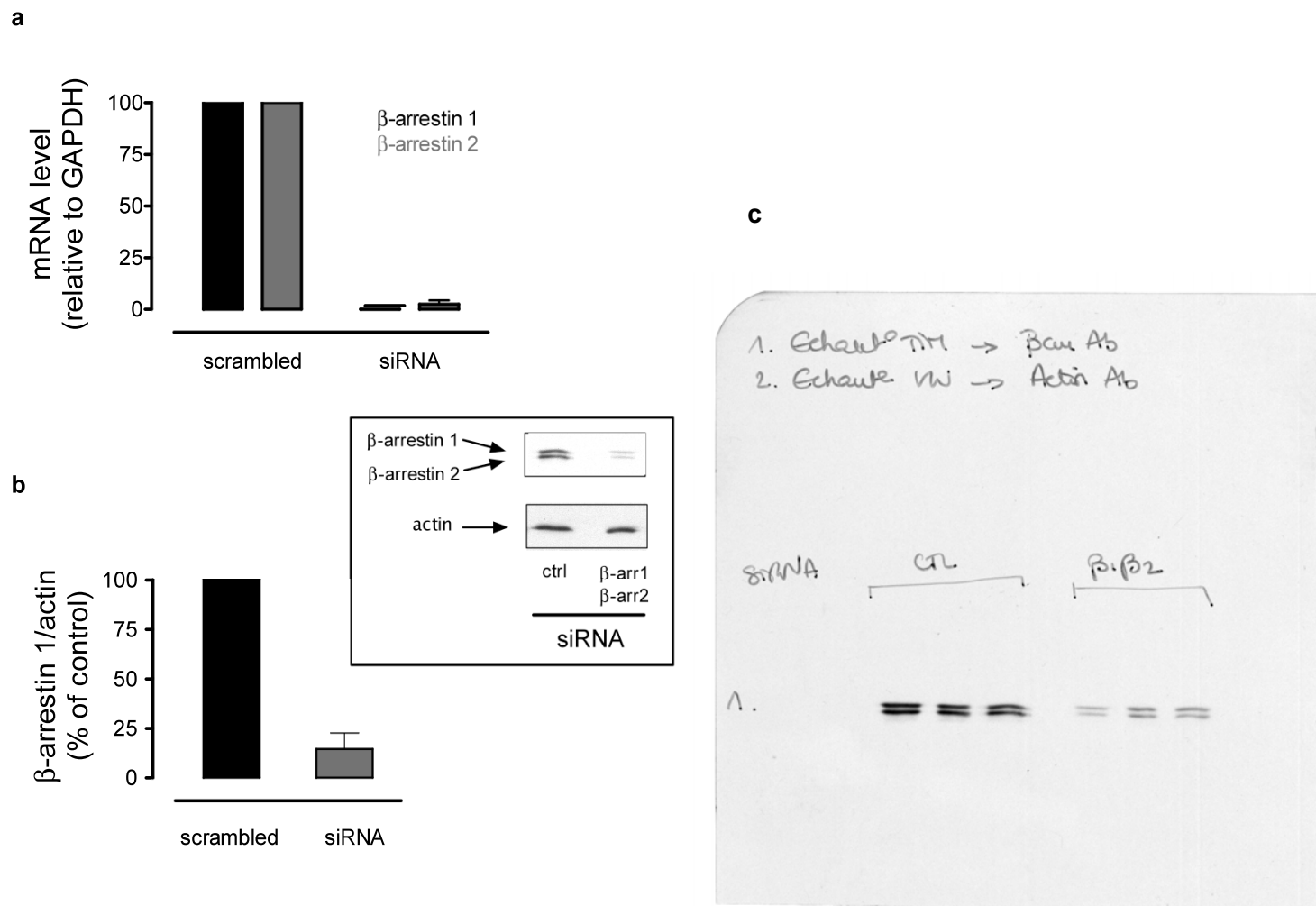
Competition binding assay

As described previously⁵, ligand binding studies were performed on stably transfected cells grown in 24-well plates. Cells were incubated in DHB buffer (serum-free DMEM containing 20 mM HEPES, pH 7.4, and 1 % BSA) for 1 h at 0 °C followed by a 1.5 h incubation with the same buffer containing [¹²⁵I]-PTH(1–34) (100,000 cpm per well) with or without varying concentrations of unlabeled peptides. Cells were washed three-times with iced-cold PBS buffer prior to extraction with 0.8 M NaOH. Cell-associated [¹²⁵I]-PTH(1–34) was then counted.

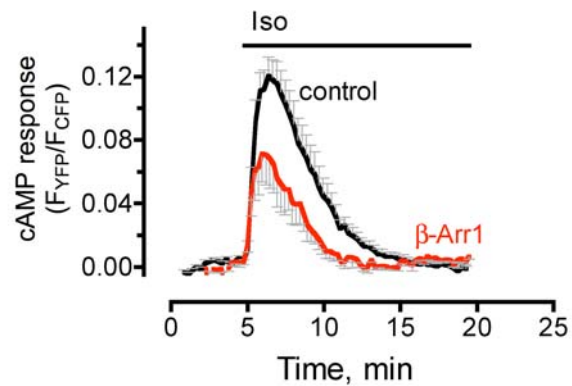
Supplementary References

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2. Aaboe, M. *et al.* SOX4 expression in bladder carcinoma: clinical aspects and in vitro functional characterization. *Cancer Res* **66**, 3434-42 (2006).
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5. Dean, T., Vilardaga, J.P., Potts, J.T., Jr. & Gardella, T.J. Altered selectivity of parathyroid hormone (PTH) and PTH-related protein (PTHrP) for distinct conformations of the PTH/PTHrP receptor. *Mol Endocrinol* **22**, 156-66 (2008).

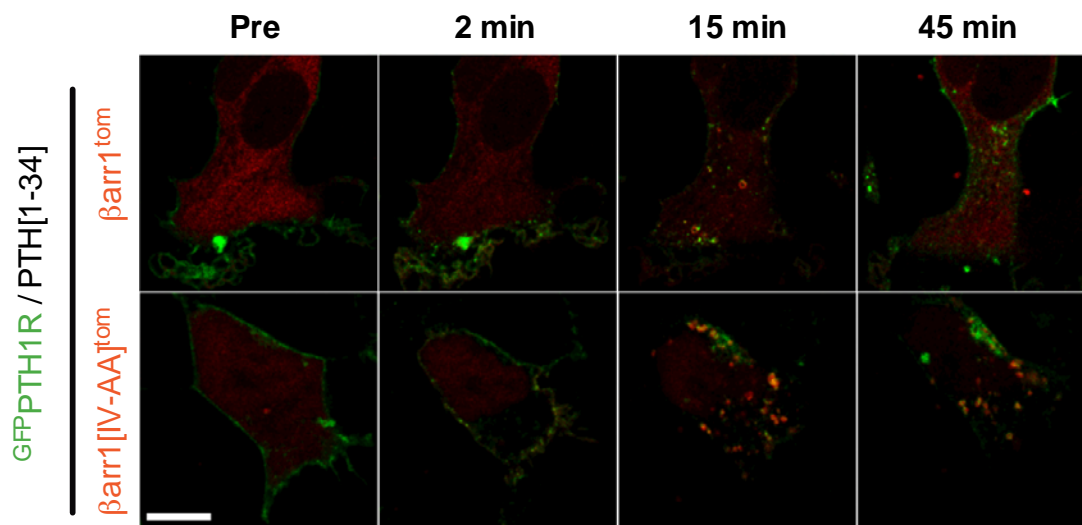
Supplementary Results



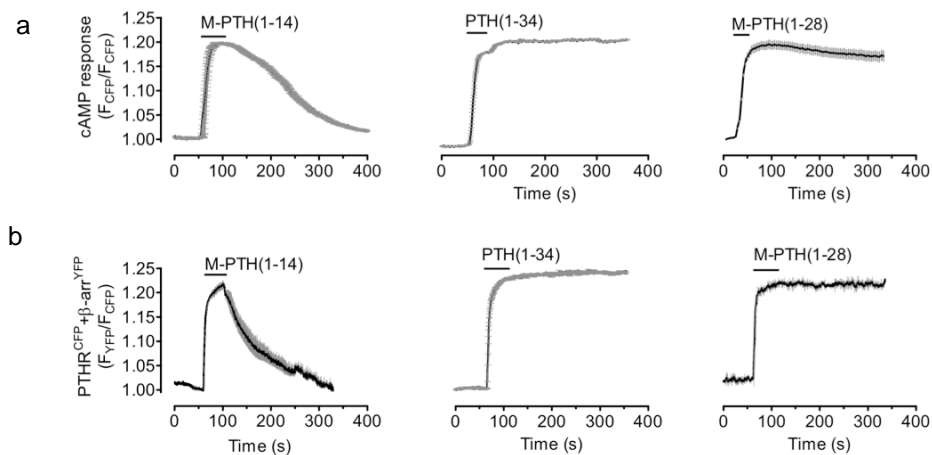
Supplementary Figure 1 Depletion of β -arrestins from HEK293 cells using siRNA. **a**, Quantitation of mRNA for β -arrestin 1 (black bars) and β -arrestin 2 (grey bars), normalized to GAPDH, by qPCR performed as described in Methods. Bars represent the mean \pm s.e.m. of $n = 5$. **b**, Example of depletion of β -arrestin1 measured by Western blot. Inset, representative graph showing the depletion of β -arrestin1 and 2. Bars represent the mean \pm s.e.m. of $n = 3$ separate experiments shown in c. **c**, Full gel of experiments shown in panel b.



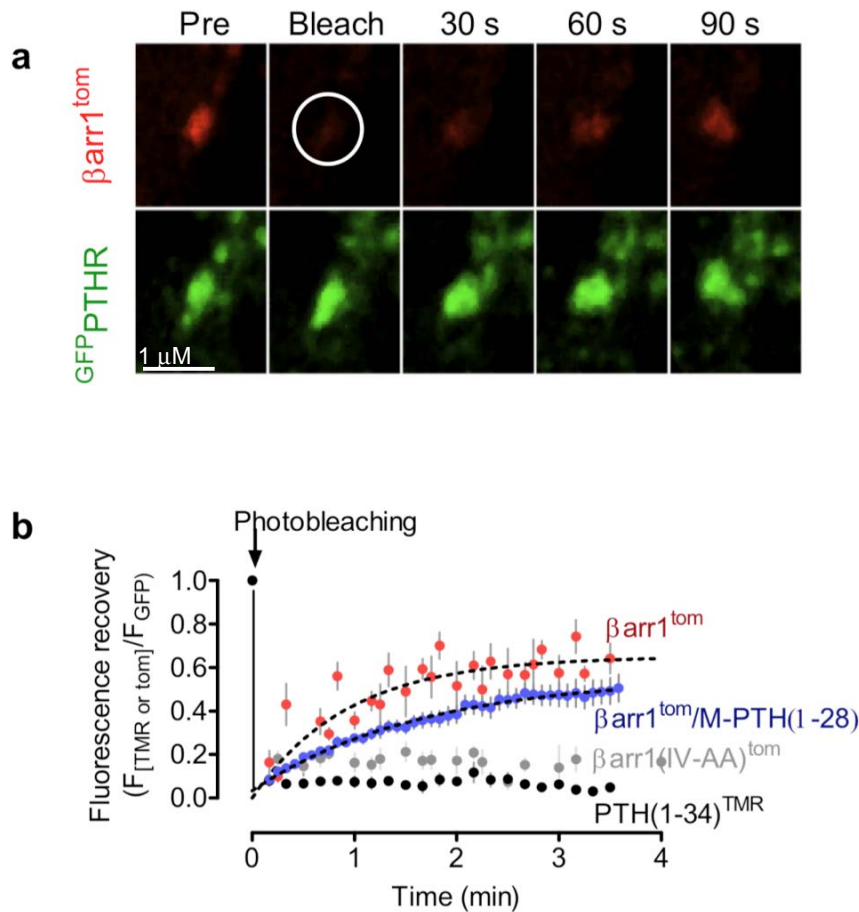
Supplementary Figure 2 cAMP response mediated by the β_2 -adrenergic receptor. Averaged cAMP response time courses measured by FRET changes from HEK293 cells expressing β_2 -AR and transiently expressing the cAMP reporter Epac^{CFP/YFP} with or without β -arrestin1. Cells were continuously perfused with control buffer or 10 μ M isoproterenol (horizontal bar). Data represent the mean \pm s.e.m. of $N = 3$ separate experiments and $n = 10$ cells.



Supplementary Figure 3 PTHR-arrestin dynamics induced by PTH(1–34). HEK293 cells were co-transfected with ^{GFP}N-PTH1R and β-arrestin 1[IV-AA]^{tom} and imaged at 1 min intervals for 1 h after a brief challenge with 100 nM PTH(1–34). The horizontal white bar represent 10 μM.



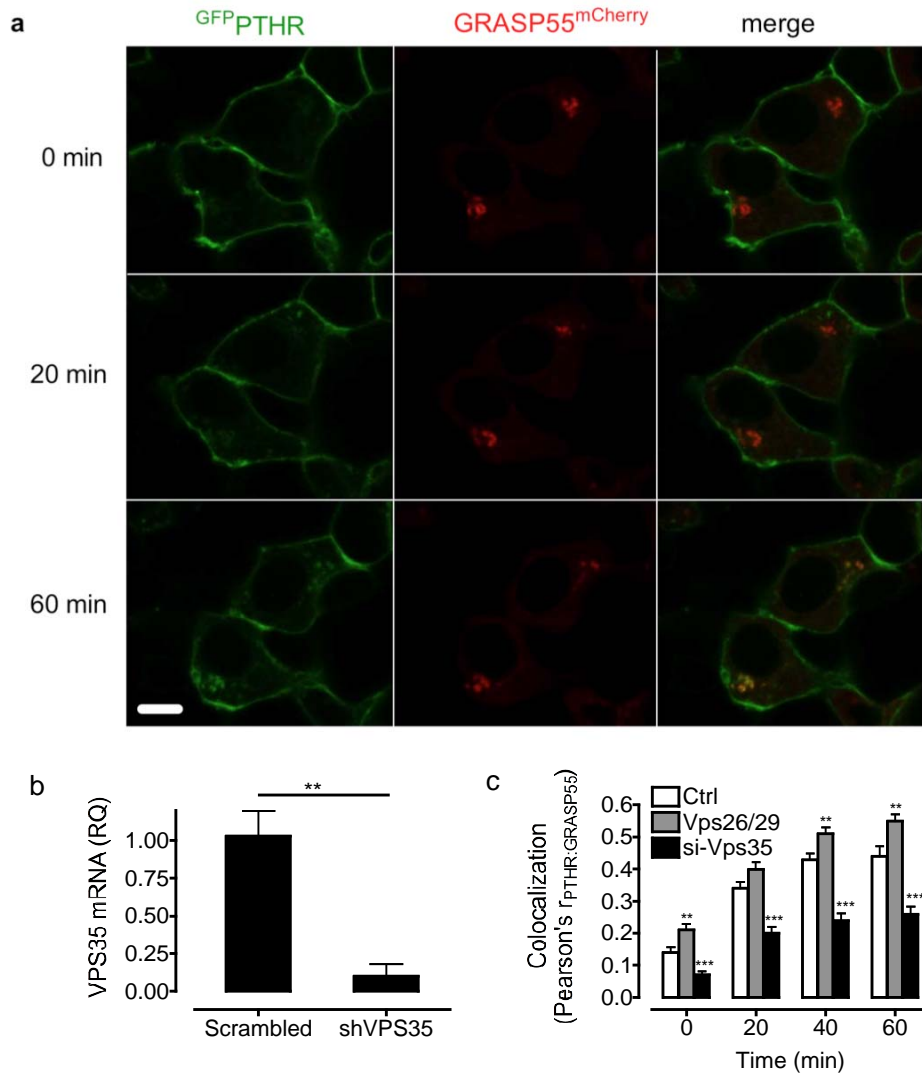
Supplementary Figure 4 Action of M-PTH(1–14), M-PTH(1–28) and PTH(1–34). FRET time-courses of cAMP responses (a), and PTHR–βarrestin1 interactions (b) in HEK-293 cells stably expressing PTHR and transiently expressing the cAMP biosensor, EpacCFP/YFP (a), or PTHR^{CFP} and β-arrestin1^{YFP} (b). Individual cells were continuously perfused with buffer or with 100 nM of a ligand for the time indicated by the horizontal bar. Traces represent the calculated FRET ratio corrected according to equation 1 with the initial value at $t = 0$ set to 1. Data represent the mean \pm s.e.m. of $N = n > 6$.



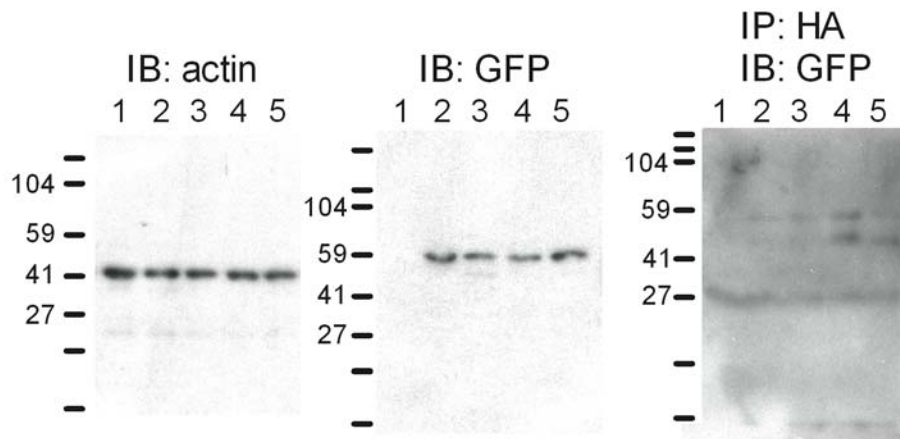
Supplementary Figure 5 Dynamics of Arrestin-PTHR interaction on endosomes.

a, Measuring the stability of arrestin-endosome binding using FRAP. HEK293 cells were co-transfected with GFP^N-PTHR and either wild-type β -arr1^{tom} or β -arr1[IV-AA]^{tom} and challenged with a brief pulse of PTH (100 nM). 10-20 min after ligand addition, endosomes were identified and β -arr1^{tom} fluorescence was bleached using a laser focused on one selected endosome (white circle, 1 μ m diameter). GFP^N-PTHR was used as a control to normalize arrestin recovery and account for endosome motion in X, Y and Z planes. For a non-recovering control, red fluorescent PTH(1-34)^{TMR} was bleached instead of β -arr1^{tom}.

b, Time course of fluorescence recovery after photobleaching of endosome-localized β -arr1 or PTH(1-34). Fluorescence values (Y-axis) represent the fluorescence intensity of background-subtracted endosomes after normalization to GFP fluorescence values. All values were subsequently scaled to range from zero to one, with one representing the average normalized fluorescence of three images taken before bleaching. Experiments were discarded if endosomes left the imaging plane or merged with other endosomes before at least 45 s of post-bleach imaging, as determined by GFP fluorescence. Data represent the mean \pm s.e.m of $N = 3$ independent experiments and cell number $n = 6$ for β arr1/PTH(1-34), $n = 12$ for β arr1/M-PTH(1-28), $n = 9$ for β arr1[IV-AA], and $n = 6$ for PTH(1-34)^{TMR}.

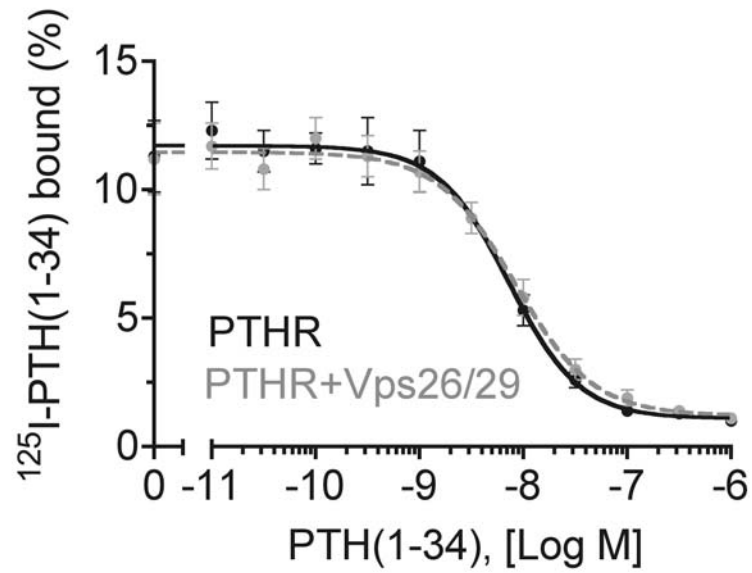


Supplementary Figure 6 Time dependence distribution of PTHR in the Golgi apparatus. **(a)** HEK-293 cells stably expressing GFP^{PTHR} were transfected with cDNA encoding $\text{GRASP55}^{\text{mCherry}}$. Cells were continuously perfused with control buffer or with 100 nM PTH(1–34) for ≈ 20 s. Images are representative of $n = 15$ experiments. **(b)** Depletion of the retromer subunit Vps35, as measured by quantitative RT-PCR. Graphs represent mean \pm s.e.m. of $n = 4$ experiments. **(c)** Quantitative measurement of PTHR-GRASP55 colocalization. Pearson's colocalization values were determined for GFP^{PTHR} and $\text{GRASP55}^{\text{mCherry}}$ at designated time points after challenge with PTH(1–34); data represent mean \pm s.e.m. of $N = 4$ experiments and $n = 15$ cells. Statistical comparison with the control (Ctrl) was performed by a t-Test (*, $P < 0.05$; ***, $P < 0.001$).

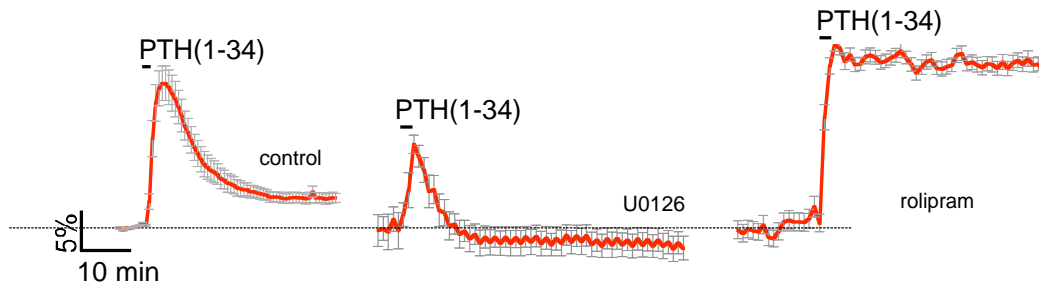


- 1: pCDNA3.1 empty vector
- 2: Vps26-myc/Vps29^{YFP}
- 3: Vps26-myc/Vps29^{YFP}; PTH(1-34) 5 min.
- 4: Vps26-myc/Vps29^{YFP}; PTH(1-34) 15 min.
- 5: Vps26-myc/Vps29^{YFP}; PTH(1-34) 25 min.

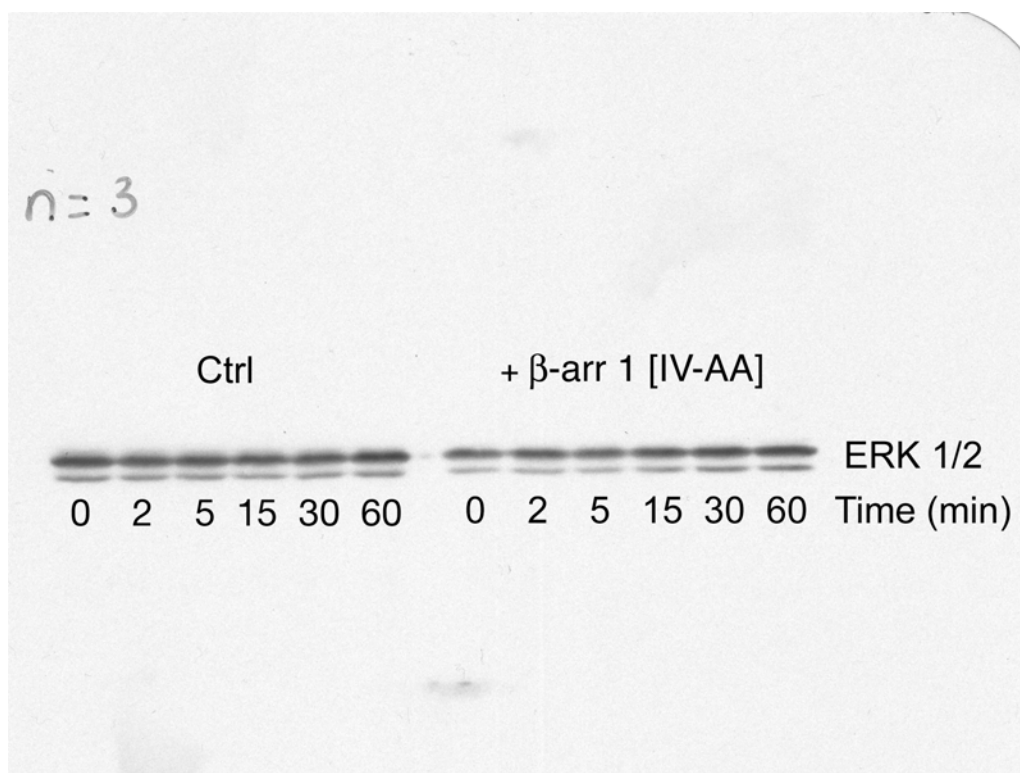
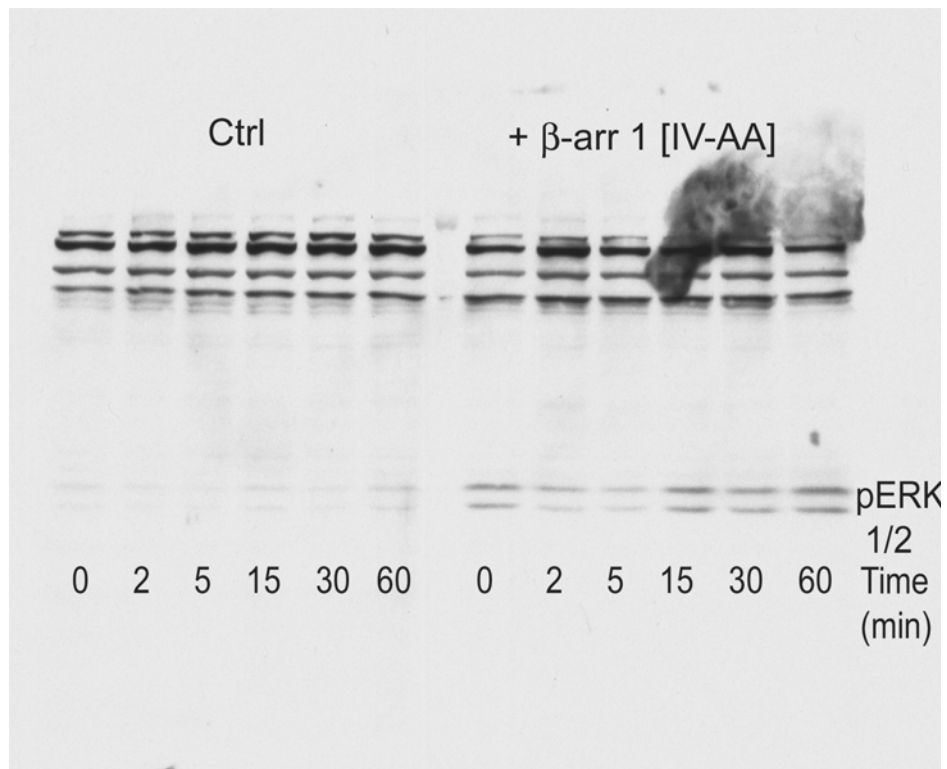
Supplementary Figure 7 Full gels for those in Figure 3c.



Supplementary Figure 8 Competition binding assays between unlabeled and labeled PTH(1-34) for PTHR stably expressed in HEK-293 cells with or without overexpression of retromer subunits Vsp26 and Vsp29. Data are the means \pm s.e.m. of $N = 4$ experiments each performed in triplicate. Curves were fitted to the data by non-linear regression analysis.



Supplementary Figure 9 Action of MEK1, and PDE4 inhibitors on PTH mediated cAMP. FRET time-courses of cAMP responses in ROS 17/2.8 cells transiently expressing the cAMP biosensor in the absence (left) or presence of 10 μ M U0126 (selective inhibitor of MEK-1 activation, central), or 10 μ M rolipram (selective inhibitor of PDE4 activation, right). Individual cells were continuously perfused with buffer or with 100 nM of PTH(1-34) for the time indicated by the horizontal bar. Traces represent the calculated FRET ratio corrected according to equation 2 with the initial value at $t = 0$ set to 1. Traces represent the mean \pm s.e.m. of $N = 4$ experiments and $n = 25$ (control), $n = 14$ (U0126), and $n = 15$ (rolipram) cells.



Supplementary Fig. 10 Full gels for those in Figure 5d.

Supplementary Video 1 A 3-dimensional reconstruction of a representative endosome labeled with Vps29^{YFP} (blue), ^{GFP}PTHR (green) and β -arr1^{tom} (red). The movie represents a 360° rotation about the vertical axis in three colors and then three more rotations with each combination of two colors. This endosome was imaged at 22 minutes and shows characteristic mixed colocalization of PTHR with both arrestin and retromer endosomal domains. Imaging, spectral deconvolution and spatial deconvolution were done as described in Methods. The endosome measures approximately 1 μ m x 0.5 μ m in the long and short dimensions.