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ANNALS OF THE NEW YORK ACADEMY OF SCIENCES

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The orphan receptor GPR125 (ADGRA3) belongs to subgroup III of the adhesion G protein−coupled receptor (aGPCR) family. aGPCRs, also known as class B2 GPCRs, share basic structural and functional properties with other GPCRs. Many of them couple to G proteins and activate G protein−dependent and −independent signaling pathways, but little is known about aGPCR internalization and β-arrestin recruitment. GPR125 was originally described as a spermatogonial stem cell marker and studied for its role in Wnt signaling and cell polarity. Here, using cell-based assays and confocal microscopy, we show that GPR125 is expressed on the cell surface and undergoes constitutive endocytosis in a β-arrestin−independent, but clathrin-dependent manner, as indicated by colocalization with transferrin receptor 1, an early endosome marker. These data support that the constitutive internalization of GPR125 contributes to its biological functions by controlling receptor surface expression and accessibility for ligands. Our study sheds light on a new property of aGPCRs, namely internalization; a property described to be important for signal propagation, signal termination, and desensitization of class A (rhodopsin-like) and B1 (VIP/secretin) GPCRs.

Keywords: adhesion GPCR; GPR125; ADGRA3; internalization; endocytosis; β-arrestin

Introduction

G protein−coupled receptors (GPCRs) are versatile, seven-transmembrane domain (7TM) proteins that regulate a variety of intracellular signaling cascades in response to extracellular stimuli, such as hormones, neurotransmitters, ions, photons, and odorants.[1] They are among the most intensively studied drug targets due to their substantial involvement in human pathophysiology and pharmacological tractability[2] with up to 35% of all marketed drugs acting via GPCRs.[3] Ligand-mediated activation of GPCRs initiates signaling through heterotrimeric G proteins, as well as through G protein−independent pathways. Moreover, some GPCRs also show constitutive activity that can be further modulated by ligands of different efficacies.[1]

Class B2 of the superfamily of GPCRs consists of 33 adhesion GPCRs (aGPCRs) in humans. Like other GPCRs, aGPCRs are membrane-located proteins that possess the signature 7TM domain, with a structural hallmark containing a large extracellular N-terminus with adhesion properties. Many aGPCRs are autoproteolytically cleaved at the GPCR proteolytic site (GPS), which lies within the GPCR autoproteolysis−inducing domain. This results in the N-terminal fragment (NTF) that is noncovalently linked to the C-terminal fragment (CTF) containing the 7TM domain.[4,5] Multiple molecular mechanisms of G protein activation have been proposed for aGPCRs, involving the positioning of the NTF relative to the CTF, which enables the Stachel (also called stalk) located downstream of the GPS motif to act as tethered agonist.[6,7]
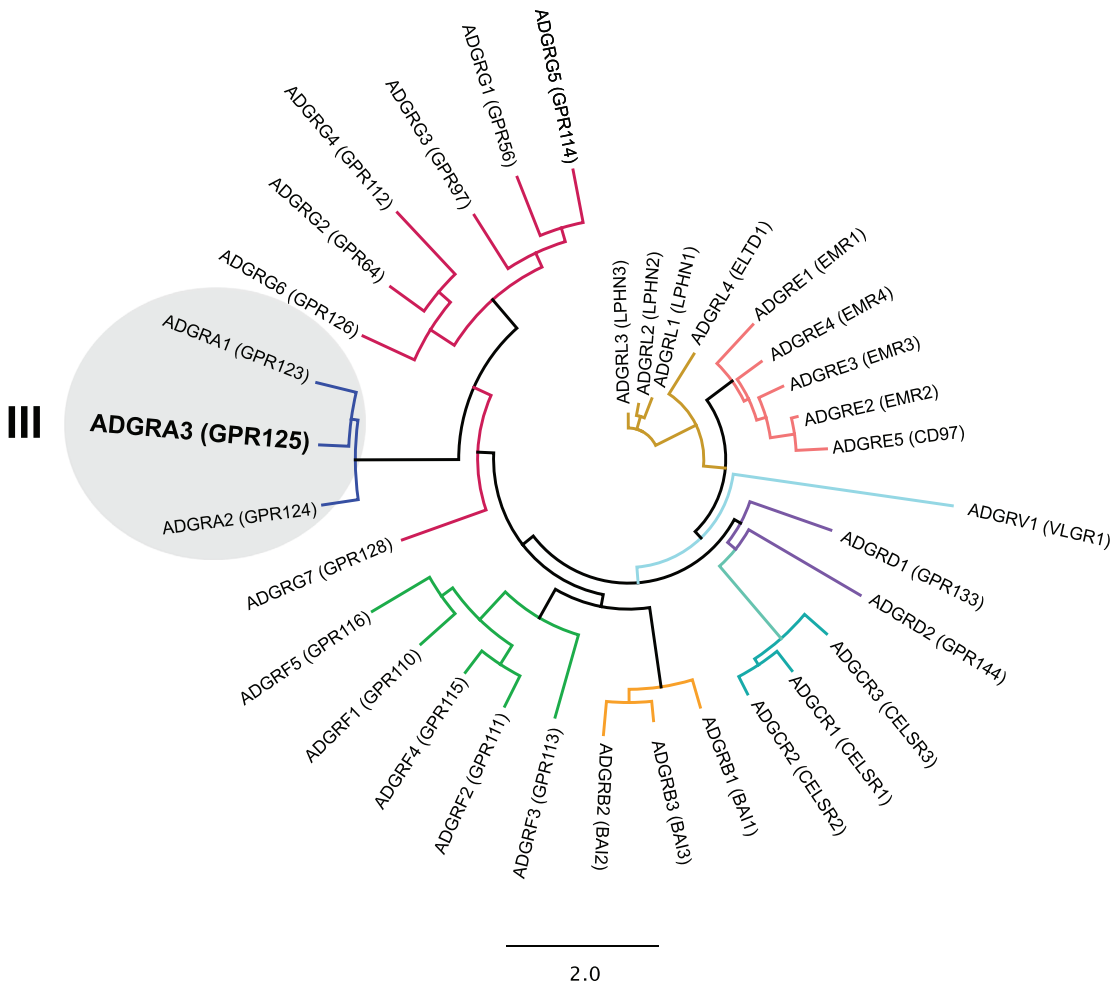
Figure 1. Cladogram of class B2 adhesion GPCRs (aGPCRs). aGPCR amino acid sequences were aligned using MAFFT and the cladogram was constructed using PhyML; Likelihood-joining. Class B2 receptors are divided into nine different subfamilies marked by the different colors of the branches. GPR125 (ADGRA3) belongs to subfamily III, together with GPR123 (ADGRA1) and GPR124 (ADGRA2).

In contrast to class A rhodopsin−like receptors, where several G protein−recognizing motifs have been identified,[8] there is so far no conserved aGPCR domain that would determine whether the receptor interacts with G proteins. Despite this, ligand-mediated[9–15] and constitutive[16] G protein signaling have been described for several aGPCRs involving activation of all four major subclasses of G proteins ($G_{\alpha s}$, $G_{\alpha i}$, $G_{\alpha q}$, and $G_{\alpha 12}$) and a variety of intracellular signaling second messengers leading to many different functional endpoints.[16–24]

After internalization, the receptors are either targeted for degradation or recycled back to the cell surface where they can be stimulated again by their ligands.[25] To our knowledge, little is known about intracellular trafficking of aGPCRs compared with class A, B1, and C receptors, apart from certain aGPCR's interaction with β-arrestin and endosomal proteins.[17,18,26–28] The arrestin family consists of four members: arrestin-1 and arrestin-4, also known as visual arrestins, are exclusively expressed in the retina,[29] whereas the two β-arrestin isoforms, β-arrestin-1 and β-arrestin-2 (also named arrestin-2 and arrestin-3, respectively), are ubiquitously expressed in most tissues.[30]

GPR125 (ADGRA3) belongs to subfamily III of aGPCRs (Fig. 1), also denoted as ADGRA,[31] and was initially identified as a spermatogonial stem

cell marker.[32] The receptor is broadly expressed in various tissues with a particularly high expression reported in the murine choroid plexus, where it is upregulated after brain injury.[33] GPR125 has been suggested to be essential during development as a modulator of planar cell polarity (PCP) in zebrafish.[34] Using genetically manipulated zebrafish, it has been shown that GPR125 regulates noncanonical Wnt signaling by recruiting Dishevelled (Dvl) proteins to the cell surface, and thereby directing cellular orientation during convergence and extension movements in gastrulating zebrafish.[32,34] GPR125 has also been studied in the context of cancer. Specifically, its ability to modulate Wnt signaling leads to increased survival of patients with colorectal cancer,[35] thereby suggesting that GPR125 is acting as a tumor suppressor in it. However, in myeloid sarcoma, GPR125 seems to have an oncogenic effect as its upregulation contributes to tumor formation.[36]

Inspired by the regulation of GPR125 expression in cancer, we decided to investigate the cellular distribution of this receptor and how β-arrestin and clathrin are involved in the regulation of GPR125 expression on the cellular surface.

Materials and methods

Phylogenetic analysis
For phylogenetic analysis of the 33 human aGPCRs (Table S1, online only), amino acids were aligned using MAFFT plug-in Geneious Pro Version 10.2.6 (Biomatters Ltd). The phylogenetic tree was constructed with the neighbor-joining module, and the reliability of the tree was analyzed by bootstrap analysis (100-fold resampling).

Constructs and cloning
GPR125 (codon-optimized for mammalian cell expression) with N-terminal flag-tag in pcDNA3.1(+) vector was obtained from GenScript.

Cells and culture conditions and transfection
HEK293 cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS), 180 units/mL penicillin, and 45 μg/mL streptomycin. The clustered regularly interspaced short palindromic repeats (CRISPR)-modified β-arrestin-1/2 gene knockout (KO) and parental HEK293 cells were grown as previously described.[37] The stable clone of inducible GPR125 HEK293

cells was generated as previously described[38] and grown the same as parental HEK293 cells. GPR125 knockdown (KD) HEK293 cells (see below for the generation of these cells) were grown the same as parental HEK293 cells in the medium supplemented with 5 μg/mL puromycin. CHO-K1 EA-arrestin2 cells were grown in Ham's F-12 medium containing 10% FBS, 2 mM glutamine, 180 units/mL penicillin, 45 μg/mL streptomycin, and 250 μg/mL hygromycin. HEK293 cells were transfected using Lipofectamine® 2000 (Invitrogen) according to the manufacturer's recommendation for the enzyme-linked immunosorbent assay (ELISA) and the β-arrestin-1 and β-arrestin-2 recruitment bioluminescence resonance energy transfer (BRET) experiments. The calcium phosphate method for transfection was used in the β-arrestin-1 recruitment BRET assay in GPR125 KD cells, as previously described.[37] For immunohistochemistry and PathHunter® GPCR β-arrestin-2 experiments, HEK293 and CHO-K1 EA cells were transfected using the FuGENE® transfection reagent (Promega) according to the manufacturer's recommendation. All experiments were performed 24 h after transfection, unless otherwise stated.

Generation of GPR125 KD HEK293 cells
HEK293 cells were seeded in a 96-well plate. The next day, the media was changed and hexadimethrine bromide (at a final concentration of 8 μg/mL) was added to each well (100 μL total volume). The cells were transduced with lentivirus transduction particles—shRNA human GPR125 (clone ID NM-145290.2_3261s1c1; Sigma) according to the manufacturer's recommendation. Treatment with puromycin (5 μg/mL) was initiated 2 days post transduction and continued until control cells (nontransduced HEK293 cells) were dead. The receptor KD was confirmed by real-time qPCR. To reintroduce GPR125 expression, stable GPR125 KD cells were transfected with codon-optimized GPR125 plasmid DNA that could not be targeted by the shRNA due to an altered nucleotide sequence.

Real-time qPCR on cell lysates
RNA was extracted using the RNeasy® Micro Kit (Qiagen). Approximately 1 μg of total RNA was reverse transcribed with the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). qPCR was performed using

the QuantStudio™ 6 Flex Real-Time PCR System (Thermo Fisher Scientific) and the PowerUP SYBR® Green Master Mix (Thermo Fisher Scientific). Cycle threshold (Ct) values were obtained using the QuantStudio Real-time PCR Software, and the $\Delta-\Delta Ct$ method was used to calculate the relative fold change of cDNA levels compared with a reference gene (glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)). Sequences of the forward and reverse GPR125 primers were CTGGCACATGCTTGT-GAACT and GGCAAGGGTGGAATAGTGAA, respectively.

Surface expression determined by ELISA

Transiently transfected HEK293 cells were fixed for 10 min with 3.7% formaldehyde, washed with TBS/CaCl$_2$, and blocked for 30 min at room temperature (RT) with 2% BSA/TBS/CaCl$_2$. For total GPR125 cell content, cells were permeabilized with 0.2% saponin (Sigma) after fixation. Cells were incubated with primary antibody (mouse M1 anti-flag 1:2250, Sigma) in 1% BSA/TBS/CaCl2 for 1 h at RT, washed with TBS/CaCl$_2$, and incubated with secondary antibody (goat anti-mouse HRP 1:1000, Thermo Fisher Scientific) in 1% BSA/TBS/CaCl$_2$ for 1 h at RT. They were subsequently exposed to 3,3′,5,5′-tetramethylbenzidine for 5 min, and the reaction stopped with 0.2M H$_2$SO$_4$. Absorbance at 450 nm was measured with the EnVision® plate reader (PerkinElmer). The experiment was performed in triplicate at least three times independently. Data were analyzed using GraphPad Prism (GraphPad Software, San Diego, CA).

Internalization

HEK293 cells were seeded and transiently transfected. Twenty-four hours posttransfection, cells were incubated in cold DMEM containing primary antibody (mouse M1 anti-flag 1:2250, Sigma) for 1 h at 4 °C. After three washes with cold DMEM, cells were either immediately fixed ($t = 0$) or incubated with prewarmed DMEM at 37 °C for various periods of time ($t = 10, 20, 30, 40,$ and 50 min) to induce internalization before being fixed at the given time points. From there on, the procedure followed that of the ELISA described above. The experiment was performed in quadruplicate at least three times independently. Data were analyzed in Graph-Pad Prism.

Western blots using HEK293 cell lysates

HEK293 cells were lysed using 150 μL RIPA lysis buffer (Milipore), containing cOmplete® Mini 20 protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail 3 (Sigma) and 50 μL 4× Laemmli's sample buffer containing 50 mg/mL dithiothreitol. The cell lysates were sonicated and centrifuged at $13,000 \times g$ for 10 min at 4 °C.

The samples were incubated at 30 °C for 30 min before running on an SDS gel (4−15% TGX Stain-Free Precast Gels (Bio-Rad)). The proteins were blotted on a polyvinylidene difluoride membrane using the Trans-Blot turbo transfer system (Bio-Rad). For the antibody staining, the membrane was first blocked with 2× Odyssey blocking buffer (Li-Cor) for 30 min, incubated with primary antibody (mouse anti-FLAG M1 1:2000, Sigma) in 1× blocking buffer/PBS/CaCl$_2$ for 1 h at RT, washed with PBS + 0.01% Tween 20 (Sigma), and incubated with secondary antibody (goat anti-mouse IRDye 800CW Li-COR) in 1× blocking buffer/PBS/CaCl$_2$ for 1 h at RT. After washing twice with PBS + 0.01% Tween 20 (Sigma) and once with PBS, the membrane was imaged using a Li-Cor scanner and analyzed with the Image Studio™ software. The experiment was performed at least three times independently.

Immunocytochemistry and confocal microscopy

Inducible HEK293 cells were cultured on glass coverslips placed in 24-well plates, and receptor expression was induced with tetracycline (0.25 μg/mL). Cells were fixed 24 h after tetracycline induction with 3.7% formaldehyde, washed with PBS, permeabilized in 0.1% Triton™ X-100 for 10 min at RT, blocked in PBS with 0.1% Tween 20 and 2% bovine serum albumin for 1 h at RT, and then incubated with primary antibody (mouse M1 anti-FLAG, 1:2250, Sigma) for 1 h at RT. After washing with PBS, the cells were incubated with secondary antibody (donkey anti-mouse conjugated to Alexa Fluor 568, 1:500, Life Technologies) for 1 h at RT. Cells were nuclear stained with 4′,6-diamidino-2-phenylindole (DAPI) and mounted in Mowiol® (Sigma) before imaging on a confocal microscope (LSM 700, Zeiss) with a 63× oil immersion plan-apochromat objective.

Antibody feeding experiments

Fibronectin-coated glass slides were placed in 24-well plates and HEK293 cells or β-arrestin-1 and

β-arrestin-2 KO cells were allowed to adhere before transfection the next day. Twenty-four hours post-transfection, the cells were incubated with primary antibody (mouse M1 anti-FLAG, 1:2250, Sigma) in media at 4 °C for 1 hour. They were then fixed immediately ($t = 0$) or incubated in normal media for 30 min at 37 °C to induce internalization ($t = 30$) before fixation. All samples were blocked in 2% BSA before incubation with Alexa Fluor 488−conjugated goat anti-mouse antibody (1:500, Invitrogen) for 1 h at RT. Cells were then permeabilized with 0.2% saponin (Sigma) and incubated with Alexa Fluor 568−conjugated donkey anti-mouse antibody (1:500, Life Technologies) for 1 h at RT. The glass slides were removed from the 24-well plate and mounted on slides, sealed, and imaged as described above. To study colocalization, GPR125 was cotransfected with GFP-tagged plasmids containing either transferrin receptor 1 (TfR1) or lysosomal-associated membrane protein 1 (LAMP1). Here, GPR125 was detected by Alexa Fluor 568−conjugated antibodies but otherwise, the procedure was the same as described above. CoLocalizer Pro 5.4.3 (CoLocalization Research Software) was used to detect colocalization of the red and green fluorescence emitted by GPR125 (red) and TfR1 (green) or LAMP1 (green), and to calculate Pearson's correlation coefficients (Pr). To quantify the amount of internalized receptor in parental HEK and in β-arrestin-1 and β-arrestin-2 KO cells after 30 min, 10 images were taken, respectively, using a confocal microscope (LSM 710, Zeiss) and a plan apochromat $63\times/1.40$ Oil DIC M27 objective. For the red fluorescence signal quantification, the following settings were used: 8.21 airy units, optical section 7-micron, 561 nm laser at 2%, and averaging four times (fixed for all quantified images). For the green fluorescence signal quantification, the following settings were used: 9.59 airy units, optical section 7-micron, 488 nm laser at 1%, and averaging four times (fixed for all quantified images). Data analysis was performed using the Zen software (Zeiss).

Real-time β-arrestin-1 and β-arrestin-2 recruitment experiments

The BRET experiments were carried out as previously described.[29,39] Briefly, 1 day after seeding cells in a 6-well plate, 60–80% confluent parental HEK293 cells and stably transduced GPR125 KD cells were transiently transfected with the following plasmids: GPR125, the human glucagon-like peptide-1 (GLP-1) receptor (GLP-1R, positive control), or empty pcDNA3.1(+) vector (negative control), in combination with the BRET donors *Renilla* luciferase−fused arrestins RLuc8–arrestin-2–Sp1 or RLuc8–arrestin-3–Sp1, the BRET acceptor mem-linker-citrine-SH3, and GPCR kinases 2 or 6 (GRK2 or GRK6) to facilitate β-arrestin-1 and β-arrestin-2 recruitment. One day (for Lipofectamine-transfected cells) or 2 days (for cells transfected with the calcium-phoshate method, i.e., GPR125 KD cells) later, the cells were washed with PBS and resuspended in PBS with 5 mmol/L glucose. We added 85 μL of cell suspension to each well on a 96-well isoplate followed by the addition of PBS with 5 μmol/L coelenterazine h (final concentration). After 10 min at RT, increasing concentrations of GLP-1 were added to the positive control cells. Luminescence was measured by EnVision plate reader (PerkinElmer) (RLuc8 at 485 ± 40 nm and YFP at 530 ± 25 nm). The experiment was performed in triplicate at least three times independently.

PathHunter GPCR β-arrestin-2 experiments

The recruitment of β-arrestin-2 was measured using the PathHunter β-arrestin assay as described previously.[29] Briefly, cDNA encoding GPR125 and US28 (positive control) was fused to the ProLink[TM] (PK) C-terminal protein tag and the small fragment of β-galactose (β-gal) and cloned into the PK2 vector. The experiments were performed using the CHO-K1 EA arrestin cell line with the stable expression of β-arrestin coupled to the β-gal large fragment. One day after seeding the cells (96-well plate), they were transfected at a cell density of 60–80% with FuGENE reagent using different amounts of cDNA (GPR125, US28, and PK2 (vector control)). β-Arrestin-2 recruitment was detected as β-gal activity using the PathHunter detection kit (DiscoverX). Chemiluminescent substrate composed of Galacton-Star[TM] substrate, Emerald-II[TM] solution, and PathHunter cell assay buffer in a ratio of 1:5:19, respectively, was added to the cells (50 μL/well). The luminescent signal was determined after 60 min incubation using the EnVision Multilabel Plate Reader (PerkinElmer).
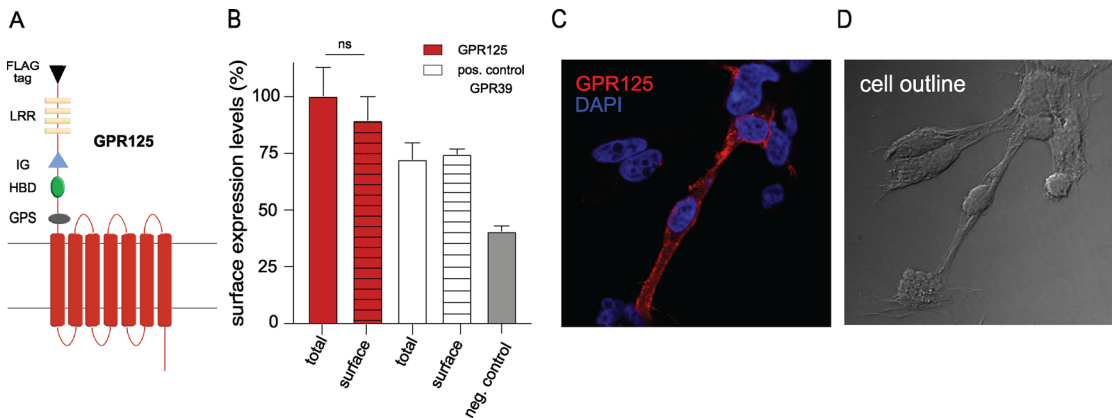
Figure 2. Cell surface expression of GPR125. (A) Diagram of the GPR125 receptor construct with the indicated motifs: GPS, 7TM proteolytic site; HBD, hormone-binding domain; IG, immunoglobulin domain; and LRR, leucine-rich region. (B) Cell surface expression of GPR125 (red bar) in transiently transfected HEK293 cells determined by ELISA against the N-terminal flag-tag, using pcDNA vector (gray bar) as baseline. The total amount of the receptors was measured by permeabilizing the cell membrane (striped bars). GPR39 was included as a positive control (white bar). (C) GPR125 is mainly expressed on the cell surface (red) of stably transfected HEK293 cells (pool clones) 24 h after induction with tetracycline. The nucleus is stained with DAPI (blue) and the cell outline is shown in D. Error bars in B indicate SEM for at least three independent biological replicates.

Statistical analysis

Data were analyzed using a paired Student's *t*-test or a one-way ANOVA (multiple comparisons). P values < 0.05 were considered statistically significant.

Results

GPR125 is expressed on the cell surface and undergoes constitutive internalization

To determine the localization pattern of GPR125, we used an N-terminally flag-tagged construct (Fig. 2A). GPR125 surface expression was determined by ELISA in transiently transfected HEK293 cells (Fig. 2B) and the overall cellular localization was observed in inducible GPR125 HEK293 cells by microscopy (Fig. 2C and D). We found that GPR125 is mainly expressed on the cell surface of HEK293 cells, as the total amount of GPR125 expressed in these cells was comparable with surface-expressed GPR125 (Fig. 2B). This was also the case for the positive control, GPR39[40] (Fig. 2B). The internalization of GPR125 was determined using an anti-body feeding approach, in which primary antibodies were directed against the N-terminal flag-tag of GPR125. Differently labeled secondary antibodies distinguished surface expression (green) and intracellularly located receptors (red). This is done by application before (green, $t = 0$ min) and after (red, $t = 30$ min) a temperature shift that induced the

internalization event, followed by cell permeabilization to allow intracellular staining (Fig. 3A and B). Consistent with the surface expression determined by ELISA and microscopy (Fig. 2B and C), GPR125 was initially detected on the cell surface at 4 °C (Fig. 3A and B). After 30 min at 37 °C to allow internalization, GPR125 was mainly located in intracellular vesicles (Fig. 3C and D). These data were corroborated in transiently transfected nonpermeabilized HEK293 cells, by measuring GPR125 cell surface expression over time after a temperature shift from 4 to 37 °C. After 30 min, the cell surface expression of GPR125 had decreased to 48% of the initial level, and after 1 h only 29% GPR125 remained on the cell surface (Fig. 3E). The class A receptor GPR183 (also known as EBI-2) was included as a positive control. This receptor is predominantly cell surface−expressed[41] and internalizes constitutively as well as ligand dependently (after addition of the endogenous agonist 7α,25-dihydroxycholesterol (7α,25-OHC)).[42,43] Consistent with a previous report,[43] 23.5% of surface-expressed GPR183 was constitutively internalized after 30 min (Fig. 3F), whereas the addition of 7α,25-OHC enhanced the internalization to 43% (Fig. 3G), that is, to comparable levels with the constitutive internalization of GPR125 (Fig. 3E). In summary, GPR125 is mainly expressed on the cell surface and undergoes constitutive
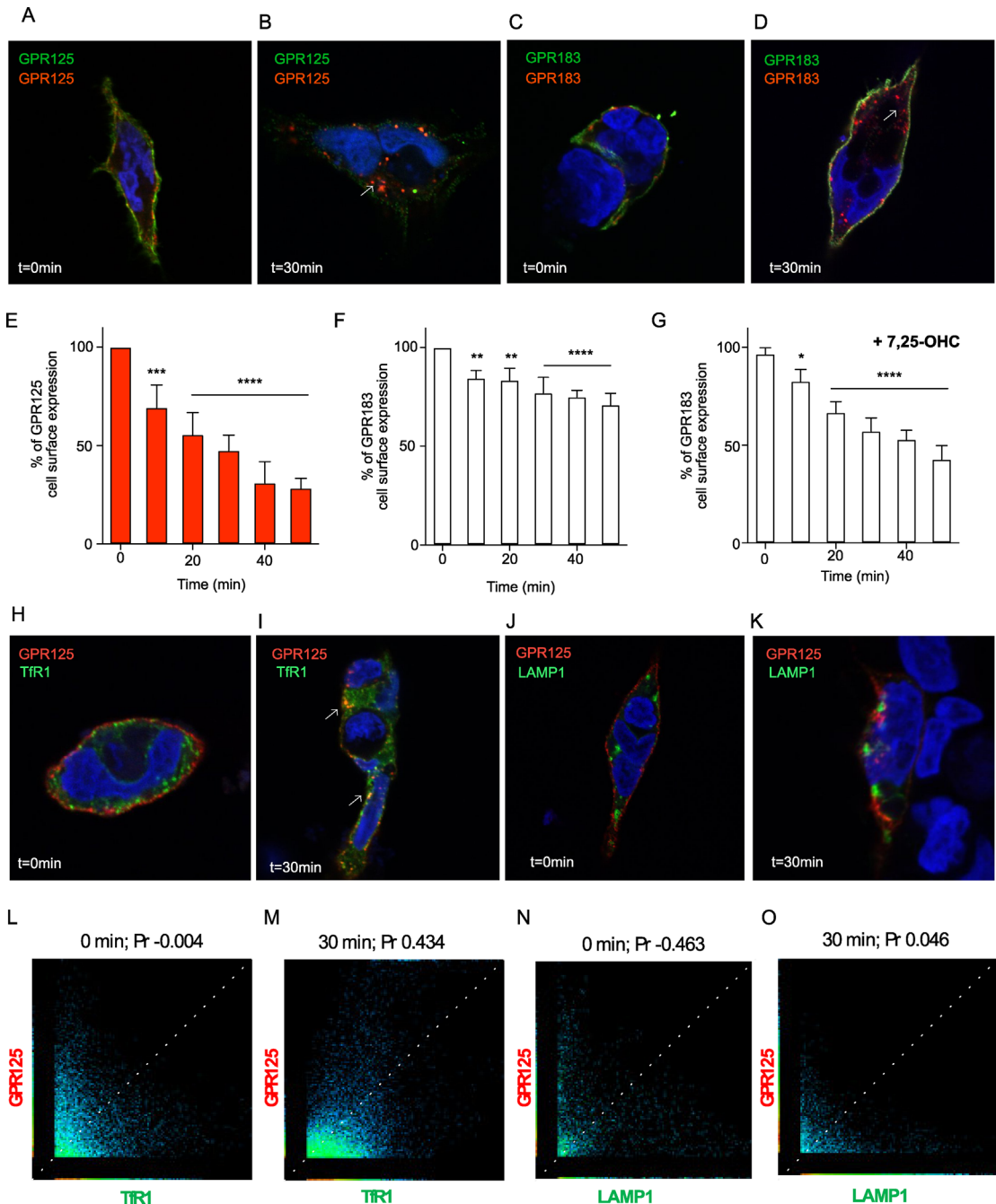
Figure 3. Constitutive endocytosis of GPR125 and colocalization with early and late endosomal markers. (A) Cell surface localization of GPR125 before (green) and (B) after induction of internalization, (red). (C and D) GPR183 included as a positive control. (E) Cell surface expression of GPR125 and GPR183 (F and G) after inducing receptor internalization (+/−ligand). The expression levels were normalized to each receptor at $t = 0$ minute. (H and I) Colocalization of TfR1 fused to GFP expressed together with GPR125 detected before, H, and after, I, inducing receptor endocytosis (white arrows). (J and K) Colocalization of LAMP1 fused to GFP expressed together with GPR125 detected before and after induction of endocytosis. (L−P) Scatterplots of the correlation between GPR125 and TfR1 or LAMP1 in panels E−H. (M and N) Determination of Pearson's correlation coefficient (Pr) between GPR125 and TfR1. (O and P) Pr of GPR125 and LAMP1. Error bars in E−G indicate SEM for three independent biological replicates. $^{*}P < 0.05$; $^{**}P < 0.01$ $^{***}P < 0.001$; and $^{****}P < 0.0001$, using one-way ANOVA analyses.

internalization under physiological temperatures to the same degree as the agonist-induced GPR183.

GPR125 colocalizes with the early endosome marker TfR1 but not with the late endosome marker LAMP1

Having established that GPR125 is constitutively internalized, we cotransfected GPR125 with GFP-tagged marker of early endosomes (TfR1) and late endosomes/early lysosomes (LAMP1) to determine the route taken by internalized receptors. Using this approach, we would thus be able to describe whether GPR125 is internalized via clathrin-mediated endocytosis as TfR1[44] (colocalization of GPR125 with TfR1) or following lysosomal protein degradation pathways (colocalization of GPR125 with LAMP1[39]). Consistent with the surface expression of GPR125, the colocalization between GPR125 and the endosomal markers TfR1 and LAMP1 was weak at 4 °C (Fig. 3H and J, respectively). For TfR1, a Pearson's correlation coefficient (Pr) of −0.043 was obtained (Fig. 3L), whereas the Pr of LAMP1 was −0.463 (Fig. 3N). After 30 min at 37 °C, GPR125 moderately colocalized with TfR1 (Fig. 3I) with a Pr of 0.463 (Fig. 3M), but only weakly with LAMP1 (Fig. 3K) with a Pr of 0.046 (Fig. 3O). In conclusion, GPR125 colocalized with TfR1 after internalization, indicating that GPR125 is predominantly endocytozed in a clathrin-mediated manner and possibly recycled similarly to what is observed with TfR1,[45] as the receptor does not follow protein degradation pathways within the time frames analyzed in these experiments.

β-Arrestin-1 and β-arrestin-2 recruitment to GPR125

Given the robust internalization of GPR125 (Fig. 3), we wanted to determine if GPR125 recruits β-arrestin to initiate internalization. To this aim, we used a BRET-based real-time β-arrestin recruitment assay, which gives a luminescence signal, when RLuc8-fused β-arrestin (RLuc8–arrestin-2–Sp1 or RLuc8–arrestin-3–Sp1, BRET donors) is recruited to the cell membrane in proximity to the membrane-anchored citrine (mem-linker-citrine-SH3, BRET acceptor).[39] Following cotransfection with GPR125, we did not observe any increase in β-arrestin-1 and β-arrestin-2 recruitment to the cell membrane (Fig. 4A and B). In fact, a decreased recruitment signal was observed in both β-arrestin-1 (Fig. 4C) and β-arrestin-2 (Fig. 4D)

upon cotransfection with GRK2 or GRK6 kinases that are both ubiquitously expressed in human cells. GPR125 expression in cells was confirmed by western blotting and its surface expression by ELISA (Fig. 4G and H). Given that endogenous expression of GPR125 in HEK293 cells (Fig. S1, online only) may be affecting this experiment, we wanted to confirm the effect observed upon reintroduction of GPR125 in GPR125 KD cells. We used a stable lentivirus-transduced HEK293 cell line, where endogenous GPR125 expression was knocked down by GPR125-specific short-hairpin RNA (shRNA). The KD of GPR125 was confirmed by real-time qPCR (Fig. S1A, online only). GPR125 was reintroduced in a concentration-dependent manner by transfection with engineered GPR125 plasmid DNA untargetable by the shRNA (Fig. S1B, online only). Under these settings, the lack of β-arrestin-1 recruitment was confirmed and again a decrease in β-arrestin-1 was detected upon cotransfection with GRK2 and GRK6, although these results did not reach statistical significance (Fig. S1C, online only). As a positive control, we included the GLP-1R stimulated with increasing concentrations of the endogenous agonist GLP-1 and observed a ligand-dependent β-arrestin recruitment in parental HEK293 and GPR125 KD cells (Fig. 4E and F and Fig. S1D, online only). To further determine if there is a direct interaction of β-arrestin with GPR125, we used transiently transfected cells, coexpressing the PK-tagged GPR125 and the enzyme acceptor (EA)-tagged β-arrestin-2. In the case of β-arrestin-2 interacting with the receptor, the β-galactosidase enzyme fragments (EA and PK) will reconstitute a functional enzyme and a chemiluminescent signal can be detected. We determined a moderate interaction of GPR125 with β-arrestin-2 at 60 min, but this recruitment of β-arrestin-2 was not comparable with the interaction observed with our positive control US28 (Fig. S1E, online only).

To confirm that GPR125 internalization was independent of β-arrestins, the antibody feeding experiments were performed in parental HEK293 and β-arrestin-1 and β-arrestin-2 KO cells. GPR125 got internalized in both cell lines (Fig. 5A−D). Red fluorescence intensities were measured to quantify the amount of internalized receptors; no difference was found in the amount of internalized GPR125, comparing parental HEK293 and
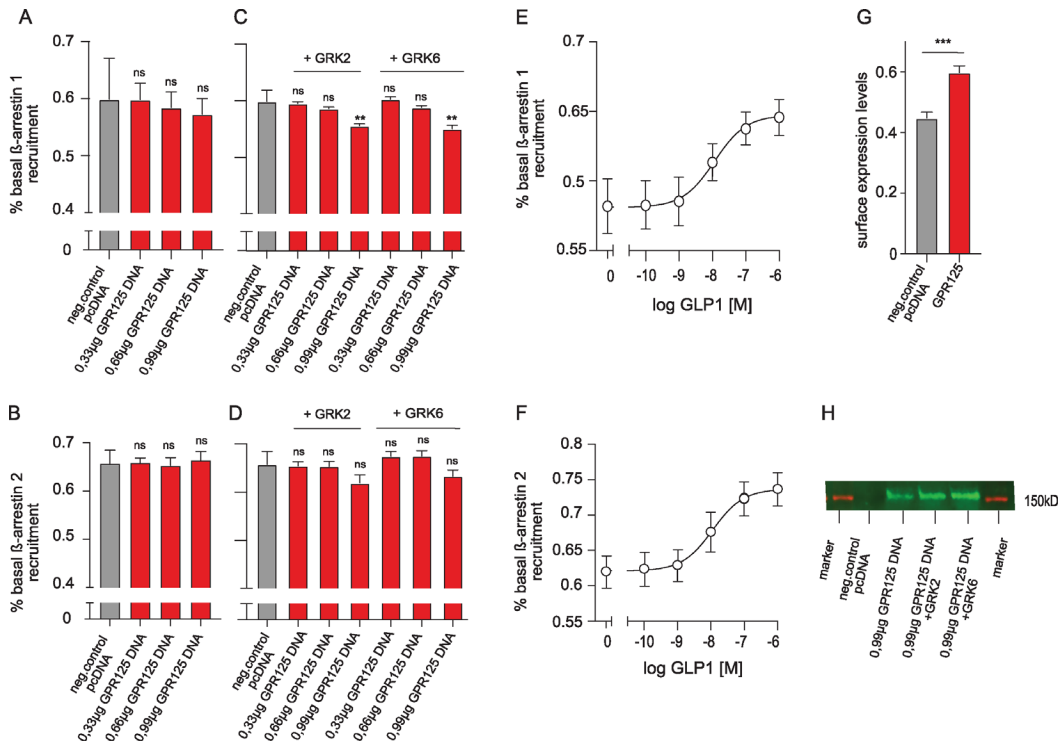
Figure 4. β-Arrestin recruitment by GPR125. (A and B) β-Arrestin-1 and β-arrestin-2 recruitment was determined by performing BRET experiments in HEK293 cells. (C and D) β-Arrestin-1 and β-arrestin-2 recruitment of GPR125 after cotransfection with GRK2 and GRK6 in HEK293 cells. (E and F) GLP-1R−mediated β-arrestin-1 and β-arrestin-2 recruitment in a ligand-dependent manner was included as a positive control. (G and H) GPR125 expression was confirmed by western blotting and ELISA. Error bars indicate SEM for at least three independent biological replicates. **$P < 0.01$ and ***$P < 0.001$, using one-way ANOVA analyses.

β-arrestin-1 and β-arrestin-2 KO cells (Fig. 5E). Moreover, the amount of remaining surface-expressed GPR125 (green fluorescence signal) was also equivalent in both cell lines (Fig. 5E).

Discussion

In the present study, we show that GPR125 is expressed on the cell surface and that it is internalized in a constitutive manner at a speed corresponding to that of an agonist-induced class A receptor (GPR183). Moreover, we show that the internalization of GPR125 happens in a β-arrestin−independent, but TfR1 colocalizing/clathrin-dependent manner. These findings are important amendments to the current knowledge of the biology of GPR125. No ligands have yet been described for GPR125, and it, therefore, still remains an orphan, like most aGPCRs.[46] Moreover, its cleavage status is not known and based on its atypical GPS-motif (SLS) and not the usual (HLS/HLT), it is possibly not cleaved via an auto-

proteolytic process. Finally, no G protein−mediated signaling has been described for GPR125. In this manner, it resembles the two other adhesion receptors in the subfamily III (GPR123 and GPR124), with which it also currently shares the status of being an orphan[47] (Fig. 1).

Receptor endocytosis is a common mechanism to regulate receptor function

β-arrestin−mediated endocytosis is a common and well-described desensitization mechanism for class A and B1 receptors.[37,48] Also, β-arrestin−independent internalization, as observed here for GPR125 (Fig. 4), has been described before as not all receptors rely on arrestins for their internalization. Clathrin-mediated endocytosis is the major pathway for uptake of molecules and is essentially regulated by the GTPase dynamin.[45] There is a growing evidence that GPCRs use clathrin-mediated internalization as an alternative route, independent of
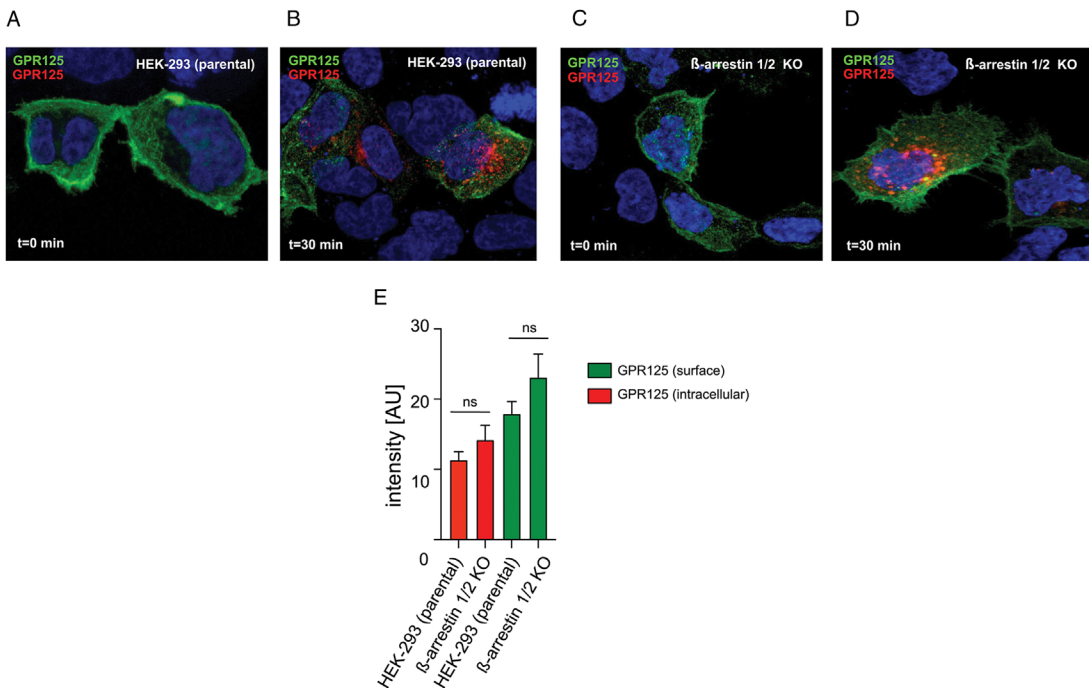
Figure 5. Constitutive endocytosis of GPR125 in parental HEK293 and β-arrestin-1 and β-arrestin-2 KO cells. (A) Cell surface localization of GPR125 before (green) and (B) after induction of internalization, (red) in parental HEK293 cells and (C and D) β-arrestin-1 and β-arrestin-2 KO cells. (E) Quantification of internalized GPR125 (red fluorescence signal) and remaining cell surface GPR125 (green fluorescence signal) in parental HEK293 and β-arrestin-1 and β-arrestin-2 KO cells. Error bars indicate SEM.

phosphorylation and arrestin recruitement.[48] For instance, this has been described for the class A melanocortin-4 receptor (MC4R)[49] and the two chemokine receptors, CXCR4 (a human CXC-chemokine receptor)[50] and US28[51] (encoded by the human cytomegalovirus, HCMV). For CXCR4, it has been suggested that the constitutive and agonist-induced receptor endocytosis are defined by distinct endocytic machineries: the constitutive internalization initiated by CXCR4 phosphorylation by protein kinase C, leading to dynamin-dependent internalization, whereas the ligand-induced internalization relies on arrestin recruitment.[50,52,53] For US28, the internalization can take place in the absence of arrestins, yet US28 also recruits arrestins and is able to use them as an internalization route.[51] Of particular note, US28 internalization is an intrinsic function of this receptor, as it scavenges chemokines as an immune escape mechanism for HCMV.[54]

We observed a relatively fast constitutive internalization of GPR125 (Fig. 3E). In contrast to the comprehensive literature on ligand-mediated recep-

tor endocytosis, less is known about constitutive internalization and how it regulates GPCR function and biology,[55] except for those cases where it functions as a ligand scavenger (like for US28[54] and atypical chemokine receptors[56]) or where constitutive internalization (and recycling) ensures sufficient receptor expression during prolonged exposure to the agonist (as for GPRC6A).[57] The constitutive endocytosis of GPR125 could be a consequence of basal receptor activity, as described for the MC4R[49] and since no G protein signaling has been described for GPR125, this remains to be determined in the future. Moreover, emerging evidence suggests that GPCRs are capable of signaling intracellularly from endosomes, as shown for the vasopressin receptor 2 (V2R; class A) GLP-1 (class B1).[58,59] It is, therefore, possible that internalized GPR125 located in early endosomes could signal intracellularly. Since it does not colocalize with the late endosome marker LAMP1 (Fig. 3K), it is likely that GPR125 gets recycled back to the cell surface as it has been described for the β2-adrenergic receptor[60] and the incretin receptors.[37]

Arrestin recruitment and the following downstream signaling cascade

Interaction of β-arrestins with activated GPCRs was initially considered to be the stop signal of receptor activation and subsequent initiation of receptor endocytosis.[61] Recruitment of arrestins to initiate endocytosis has been vastly documented for class A and B1 receptors and most GPCRs seem to get internalized in an arrestin-dependent manner.[25] However, the role of arrestins solely as signaling stoppers and receptor endocytosis initiators has expanded to include the capacity to act as signaling molecules on their own, independently of G proteins.[62] There have been examples of arrestin-initiated signaling cascades, that is, induced by ERK1/2 MAP-kinases.[63] Recent results though showed for a broad panel of class A GPCRs that β-arrestins are nonessential for driving ERK/MAPK signaling.[64,65] The acknowledgement of G protein—independent signaling has fostered another phenomenon among GPCRs, namely biased signaling, describing pathway-dependent signaling.[66] In class A receptors, it is most often seen that endogenous ligands are unbiased with similar potencies for G protein activation and β-arrestin recruitment, although exceptions exist, such as CCR7.[67] By contrast, in class B1 receptors (VIP/secretin receptors), there is a general bias toward higher potency for G protein signaling than for arrestin recruitment.[37] However, for aGPCRs, very little is known about their use of arrestins and here, signaling bias remains to be described. Some aGPCRs (BAI1/ADGRB1, BAI3/ADGRB3, GPR56/ADGRG1, and GPR64/ADGRG2) have been shown to interact with β-arrestins.[17,26,27,68] For BAI1, BAI3, and GPR56, only receptor interaction with β-arrestin was detected,[17,68] whereas the interaction of β-arrestin-1 with GPR64/$G_{\alpha q}$/CFTR (an ion channel) was shown to initiate signaling.[26] For GPR125, where only the modulation of Wnt signaling has been described as the sole signaling,[35] we observed a suppression of β-arrestin recruitment to the receptor when measured in real time; the effect that was increased upon cotransfecting with GRK2 and GRK6.

Is the endocytosis of GPR125 a part of its biological function?

Receptor-mediated endocytosis enables cells to take up molecular complexes, such as ligand—receptor complexes. Uptake of transferrin by TfR1 defines clathrin-dependent internalization.[61] We show that GPR125 gets internalized in a clathrin-dependent manner, determined by its colocalization with the transferrin receptor in our confocal microscopy studies. It is still unclear how endocytosis and Wnt signaling are intertwined, but it is known that clathrin-dependent endocytosis promotes PCP.[61] GPR125 recruits a subset of PCP components into the membrane subdomains, which suggests that GPR125 modulates the composition of the Wnt/PCP membrane complexes.[34] Therefore, GPR125-induced clathrin-dependent endocytosis could be important for the modulation of Wnt signaling and subsequently, PCP. Another receptor of the same subgroup III, GPR124 has also been suggested to be a potential candidate for stabilization of ligand–receptor interactions and thereby essential for Wnt-induced internalization.[51] It will be of interest to investigate whether internalization of GPR125 (and of its sibling GPR124) is directly linked to the modulation of Wnt signaling. Our study paves the way to a better understanding of aGPCR internalization and contributes to the knowledge about their biological function.

Acknowledgments

Author contributions

K.S. designed the research study, conducted the experiments, analyzed the data, and wrote the manuscript. S.O.B and T.L.J. designed the research study, conducted the experiments, analyzed the data, and revised the manuscript. K.R., W.A.L., K.J.M., V.D., J.K.H.R., M.S.K., and G.M.H contributed to the experimental work, analyzed the data, and revised the manuscript. M.M.R. designed the research study, analyzed the data, and wrote the manuscript. All authors commented on and approved the final manuscript.

Supporting information

Additional supporting information may be found in the online version of this article.

Figure S1. β-Arrestin recruitment by GPR125. (A) Real-time qPCR analysis of GPR125 in HEK293 and GPR125 knockdown (KD) HEK293 cells relative to the expression of the control gene, *GAPDH*. (B) β-Arrestin-1 recruitment was determined by performing BRET experiments in HEK293 GPR125 KD cells, (C) after cotransfection with GRK2 and GRK6 as in the parental HEK293 cells. GLP-1 receptor—mediated β-arrestin-1 recruitment in a ligand-dependent manner was included as a positive control in HEK293 GPR125 KD cells. (D) β-Arrestin-2 recruitment to GPR125 using the PathHunter DiscoverX system. Error bars indicate SEM for at least three independent biological replicates. *$P < 0.05$; **$P < 0.01$ using Student's *t*-test (E).

Table S1. aGPCR genes and corresponding NCBI accession numbers.

Competing interests

The authors declare no competing interests.

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